

IN VITRO EFFECTS OF THREE
ORGANIC CALCIUM CHANNEL BLOCKERS
ON THE RAT PINEAL GLAND

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Drawing by Leonardo da Vinci (1452 to 1519)

ABSTRACT

The calcium signal has emerged as an important component of intracellular regulation. Pineal function was thought to be slowed by the prominent calcification seen with increasing age, but recently it has been shown that calcium plays a crucial role in the adrenergic regulation of the gland. Beta-adrenoceptor stimulation increases melatonin (aMT) synthesis by increasing the activity of cyclic 3'-5' adenosine monophosphate (cAMP). Cyclic-AMP regulates the production of the pineal hormone, melatonin, from serotonin via the rate-limiting enzyme N-acetyltransferase (NAT). Increased intracellular cAMP is essential to the adrenergic induction of NAT. Noradrenaline (NA) also elevates pinealocyte cyclic guanosine monophosphate (cGMP). Adrenergic regulation of these cyclic nucleotides involves both α_1 - and β -adrenoceptors. Beta-adrenoceptor stimulation is an absolute requirement. Alpha₁-adrenoceptor activation, which is ineffective alone, serves to amplify the β -stimulated cAMP and cGMP responses via a positive effect on a Ca^{2+} -phospholipid-dependent protein kinase (Protein kinase-C) and a net influx of Ca^{2+} into the pinealocyte.

Previous studies suggest the use of organic calcium channel blockers (CCBs) as probes of calcium-mediated processes. Applying this concept, the study set out to investigate the influence of a representative of each of the structurally diverse groups of calcium channel blockers viz. verapamil, diltiazem and nifedipine, and to examine their effect on β -adrenoceptor stimulation. It used the β -agonist isoprenaline (ISO) and the mixed [α_1/β] agonist noradrenaline (NA), for its combined [α_1/β] adrenoceptor stimulation, on agonist-induced increases in the production of radio-labelled aMT and N-acetylserotonin (aHT) – measured as the sum of N-acetylated product – from [^{14}C] serotonin. This was done using organ cultures of rat pineal glands. It was speciously assumed that this drug paradigm would allow the determination of Ca^{2+} influx and/or the blocking thereof in the reported potentiation by using ISO as a non Ca^{2+} -entry stimulating agonist, compared with NA and its Ca^{2+} -entry stimulating properties. Surprisingly, all 3 CCB's potentiated the effect of NA. Only diltiazem was found not to potentiate the effect of ISO.

In an attempt to uncover the reason for these results, the study moved toward a mechanistic approach, focusing in an antecedent manner on the various steps in the indole metabolic pathway to identify the point at which the change occurred, and hence possibly elucidate the mechanism responsible for the paradoxical increase.

Experiments which assayed the levels of NAT, under the same drug conditions, showed the paradoxical increase to be already evident at this stage. Secondary experiments confirmed that NA stimulation of the pineal is dependent on Ca^{2+} , both in organ culture and with NAT: the Ca^{2+} chelator EGTA abolished adrenergically-induced stimulation, while Ca^{2+} added after EGTA, restored the enzyme activity. The ionophore A23187 (which is able to transport Ca^{2+} directly into the pinealocyte via a mechanism which differs from the α_1 -mechanism) when used in conjunction with ISO or NA, was able to potentiate the responses of these two agonists relative to control values (agonist-alone), but by itself had no effect.

With the enzyme NAT critically dependent upon cAMP for its induction, it was decided to determine the levels of cAMP and then those of its regulator, cAMP-phosphodiesterase (cAMP-PDE). This

reasoning was prompted by reports of anti-calmodulin activity shown by the CCBs, in addition to their channel blocking effects. By binding to calmodulin (CaM), the CCBs are reportedly able to inhibit the CaM-dependent activation of cAMP-PDE. Following NA stimulation, verapamil caused a significant decrease in cAMP-PDE levels and an increase in cAMP. The other CCBs showed a similar trend. Glands stimulated with ISO in the presence of verapamil and nifedipine showed no significant differences in cAMP or cAMP-PDE levels. Diltiazem, however, was found to decrease the effect of ISO on cAMP while causing a concomitant increase in cAMP-PDE. This i) supported a possible hypothesis that the observed enhancement is a result of cAMP levels remaining elevated due to an inhibition of cAMP-PDE by the CCBs and ii) pointed to the possible presence of a CaM-sensitive PDE within the rat pineal gland.

To test this hypothesis, two drugs which are more specific in their actions on CaM effects were chosen to see if the earlier results could be mimicked and thereby confirmed. Glands stimulated with NA in the presence of the specific CaM inhibitor R 24571 showed increased NAT activity and [¹⁴C]-aMT production. cAMP-PDE levels were clearly down, thus corroborating the possibility of cAMP-PDE inhibition. Glands incubated in the presence of M&B 22948, a CaM-sensitive PDE inhibitor, showed similar increases in NAT activity and [¹⁴C]-aMT. These findings therefore support the initial results and although indirect, confirm the hypothesis that the paradoxical increase following predominantly NA stimulation could be a result of cAMP levels remaining elevated, due to inhibition by the CCBs of the CaM-dependent activation of its regulator cAMP-PDE.

In summary, data presented herein concur with proposals that:

- i) the CCBs are not specific enough to be used as tools to research Ca²⁺-mediated events, as they appear to have sites of action other than the voltage operated channel (VOC); eg. binding to calmodulin,
- ii) there are functional differences between the CCBs as shown by diltiazem in this series of experiments,
- iii) there is a CaM-sensitive-PDE present in the pineal.

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List of Abbreviations

α	alpha
A23187	Ca ²⁺ - ionophore
A	angstrom
AA	arachidonic acid
AC	adenylyl cyclase
aHT	<i>N</i> -acetylserotonin
aMT	melatonin
ANOVA	analysis of variance
ATP	adenosine triphosphate
AVP	arginine vasopresin
β	beta
b. wt	body weight
Bq	becquerel
BSA	bovine serum albumin
BZP	benzodiazepine
c.f.	refer to
ca	approximately
Ca ²⁺	calcium
[Ca ²⁺] _i	calcium ion intracellular concentration (free cytosolic Calcium concentration)
[Ca ²⁺] _o	calcium ion extracellular concentration
CaM	calmodulin
cAMP	cyclic 3',5' - adenosine monophosphate
CCB(s)	organic calcium channel blocker(s)
CCS	calcium calmodulin system
CDR	calcium dependent regulator
[¹⁴ C]-	carbon-14 radiolabel
cGMP	cyclic 3',5' - guanosine monophosphate
cpm	counts per minute
Cu	Curie
D600	methoxyverapamil
DG	<i>sn</i> -1,2-diacylglycerol
DIL	diltiazem
DMSO	dimethyl sulphoxide
dpm	disintegrations per minute
DSIP	delta-sleep inducing peptide
EGTA	Ethyleneglycol-bis(β -aminoethylether)- <i>N,N,N',N'</i> -tetraacetic acid
F	femto 10 ⁻¹²
γ	gamma
g	gram
G-protein	guanine nucleotide binding protein
GABA	gamma aminobutyric acid
(G _s / G _i)	α regulatory (stimulatory and inhibitory) subunits of GTP-like proteins
GTP	guanosine triphosphate
h	hour
HA	5-hydroxyindoleacetic acid
HIOMT	hydroxyindole- <i>O</i> -methyltransferase
HL	5-hydroxytryptophol
HT	serotonin
IP ₃	inositol-1,4,5-triphosphate
IP ₄	inositol- tetraphosphate
ISO	isoprenaline
K ⁺	potasium ion

K_d	equilibrium constant
K_m	Michaelis-Menten constant
KOH	potassium hydroxide
l	litre
L channel	long lasting calcium channel
LD 12:12	light to dark cycle of 12 hours
LO	lipoxygenase,
m	milli 10^{-6}
M	molar
M&B 22948	2- <i>o</i> -propoxyphenyl-8-azapurin-6-one propane
MA	5-methoxyindoleacetic acid
MAO	monoamine oxidase
$\mu W/cm^2$	micro Watts / centimeter cubed
mCi	milli Curie
mg	milligram
min	minute(s)
ML	5-methoxytryptophol
ml	millilitre(s)
mol	mole
mol wt.	molecular weight
$N_i N_s$	inhibitory and stimulatory regulatory guanidine nucleotide binding protein.
n	nano 10^{-9}
N channel	Neuronal calcium channel
NA	noradrenaline
NaOH	sodium hydroxide
NaP	<i>N</i> -acetylated product
NAT	<i>N</i> -acetyltransferase
NIF	nifedipine
NPY	neuropeptide Y
OAG	1-oleoyl-2-acetyl- <i>sn</i> -G
p	pica 10^{-12}
PDE	phosphodiesterase
PE	phenylephrine
PG	prostaglandin
PHI	peptide N-terminal histidine C-terminal isoleucine
PI	phosphatidylinositol
PK-C	protein kinase C,
PKA	protein kinase A,
PLA ₂	phospholipase A ₂ ,
PLC	phospholipase C,
PMA	4 β -phorbol 12-myristate 13 acetate
PPT	precipitate
praz	prazosin
prop	propranolol
R24571	calmidazolium (1-[bis(<i>p</i> -chlorophenyl)methyl]- 3-[2,4-dichloro- β -(2,4-dichlorobenzoyloxy)phenethyl] imidazolium chloride
ROC	receptor-operated calcium channels
SCG	superior cervical ganglia
SCG	suprachiasmatic nuclei
SEM	standard error of the mean
SP	substance P
T channel	transient calcium channel
[³ H]-	tritium radiolabel
TCA	trichloroacetic acid
TFP	trifluoperazine

TLC	thin layer chromatography
U/mg	units per milligram
VER	verapamil
VIP	vasoactive intestinal (poly)peptide
VOC	voltage-dependent / voltage-operated Ca ²⁺ channels

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One must learn by doing the thing,

For though you think you know it

You have no certainty until you try

Sophocles (495 to 406 B.C.)

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CHAPTER 1

LITERATURE REVIEW

1 INTRODUCTION

1.1 History: The pineal and calcium

The discovery of the human pineal has generally been ascribed to Herophilos (325 to 280 B.C.), the "father of anatomy". During the medieval period circa 400 B.C., when Galen and Hippocrates were the "medical authorities", Galen coined the term "konareion" ("conarium" in Latin) for the pineal gland, because of its 'pinecone'-like shape in man (c.f. Ariens Kappers, 1981). He also made the first reference to the calcareous concretions often found in this gland (Galen c.f. Simon, 1906). This formed the first tentative link between calcium and the pineal, a link that would stimulate much research in the succeeding years. The function of the acervuli, and for that matter the function of the pineal, remained enigmatic for the next twenty centuries.

Interest in the pineal was again revived in the latter nineteenth century, after the discovery of endocrine glands by Claude Bernard (1813 to 1878) and Brown-Sequard (1817 to 1894). The medical profession continued to believe that the pineal was of no functional significance, and that its only medical value was to the neuro-radiologists who made use of the radio-opaqueness of the acervuli as a welcome reference point (c.f. Ariens Kappers). In this same period Sydney Ringer made the serendipitous discovery that calcium was needed to maintain cardiac contraction, after mistakenly making up saline with tap water rather than distilled water (Ringer, 1883).

In 1963, the year when the first congress on the pineal gland was held in Amsterdam, Albert Fleckenstein was able to demonstrate that a newly-synthesized compound, viz. verapamil, could mimic the effects of calcium withdrawal from the medium, and thus effectively inhibit the cardiac contractility of his isolated frog heart (Fleckenstein, 1964). Verapamil was the first of a new group of compounds, the organic calcium channel blockers, which could be used as probes for calcium mediated effects. A host of research was to follow.

Since the initial link of calcium to hormone secretion by Douglas and his co-workers (Douglas and Rubin, 1963; Douglas and Poisner, 1964), the crucial role of Ca^{2+} ions in the organ, and particularly in excitable cells, has become well-known (Campbell, 1983). The original term "stimulus-secretion coupling" was used to describe an event in which the inward flux of calcium was an inevitable and concomitant result of the stimulus and a necessary determinant of the hormone response. This was changed by Rasmussen to "stimulus-response coupling", a term denoting any specific cellular response to a physiological stimulus which is wholly or partly

determined by calcium. A specific mechanism is not implied thereby (Millar and Struthers, 1984). The pinealocyte, considered to be a paraneuron or excitable cell (Fujita, 1981; Ueck and Wake, 1977), is responsible for the transformation of nerve stimuli into a chemical response i.e. (by Wurtman and Axelrod's definition, 1965) a neuroendocrine transducer.

The presence of acervuli in the pineal makes it one of the rare tissues in the body actually containing calcifications under normal conditions (Krstic, 1985). It is the presence of these acervuli, and the growing evidence that Ca^{2+} is involved in the physiological regulation of pineal function by noradrenaline (NA), that has sparked the renewed interest in Ca^{2+} metabolism within the pineal gland (Zatz and Romero, 1978; O'Dea and Zatz, 1976; Vanecek *et al.*, 1986; Sugden *et al.*, 1986; Sugden *et al.*, 1987). The pineal gland has in fact a higher Ca^{2+} concentration than any other soft tissue in the body; this is indicative of its intense metabolic activity, since Ca^{2+} is involved whenever and wherever hormones are produced and secreted (Krstic, 1985; Meyer and Theron, 1988).

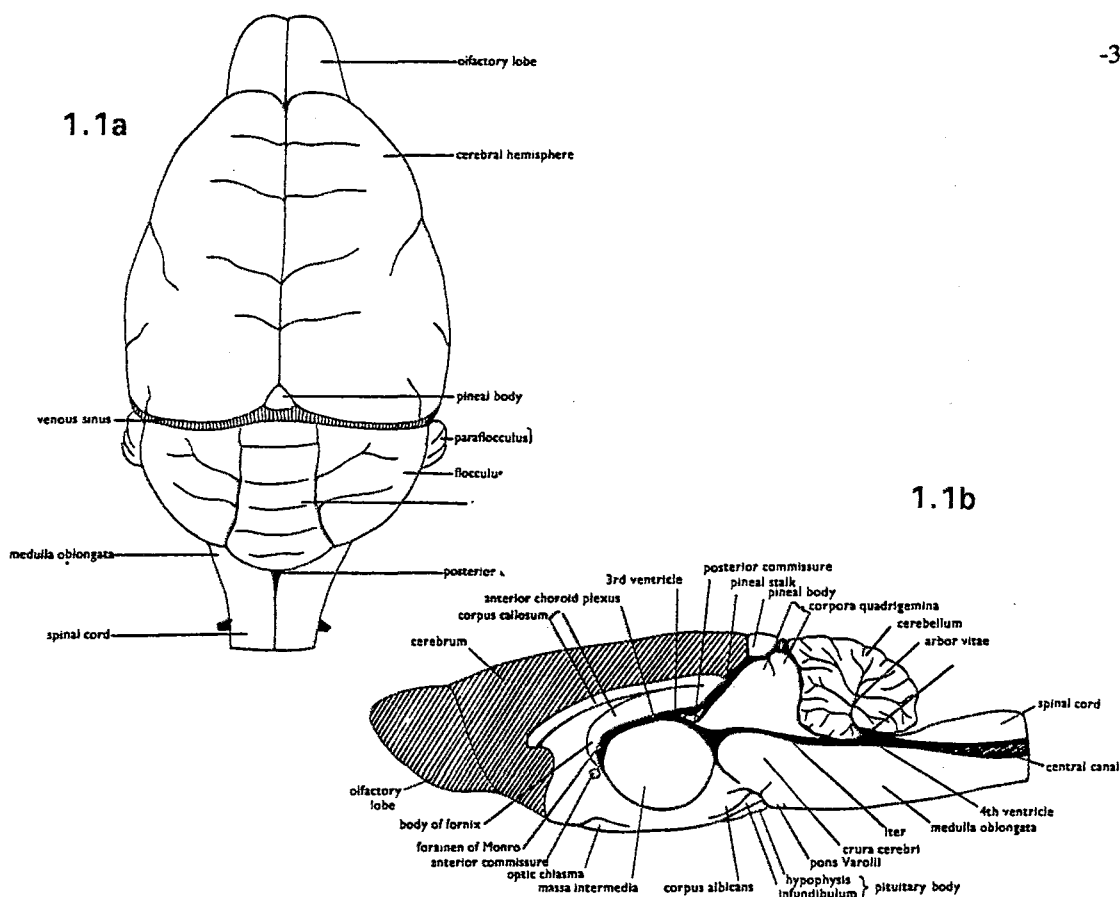
1.2 Anatomy and Physiology

1.2.1 Location and characteristics

The pineal body of the rat is rounded and visible in a dorsal view of the brain, lying rostral to the cerebellum, and between the occiput poles of the cerebral hemispheres (Figure 1.1a). It is connected to the commissural region of the brain via the pineal stalk (Figure 1.1b). It is generally agreed that pineal glands form as evaginations (diverticuli) from the dorsal wall of the diencephalon, but that their histology varies among the mature forms of the various vertebrate groups (Allen *et al.*, 1981).

The central projections of the pineal complex of the silver lamprey have been studied. They show that the tract courses caudally along the left side of the habenular commissure, and while a few fibres penetrate the brain through the caudalmost portion of this commissure, most continue caudally to enter the brain through the posterior commissure, in a similar fashion to those reported for other anamniote vertebrates (Puzdrowski and Northcutt, 1989).

The pineal gland is considered to lie outside the blood-brain barrier, with its own arterial blood supply of up to four branches of the posterior cerebral artery, and venous drainage consisting of some sixteen veins into the great cerebral vein which leads to the systemic circulation via the confluens sinuum. The pineal is thus sensitive to stimuli from the periphery, including hormones and those drugs not entering the brain (Axelrod, 1974; Tamarkin *et al.*, 1985).



Figures 1.1a and 1.1b: *Dorsal view (1.1a) and vertical section (1.1b) of the rat brain showing the position of the pineal gland and its stalk (adapted from Rowett, 1962)*

1.2.2 Histology

The cell types within the pineal gland are generally divided into parenchymal and interstitial cells. The parenchymal cell is the pinealocyte, showing the characteristics of both a paraneuron (Ueck and Wake, 1971) and a cell belonging to the amino-acid precursor uptake and decarboxylation - APUD- series (Ralph, 1983). The classification of the interstitial cells is uncertain but are thought to be of glial origin (Erlich and Apuzzo, 1985).

By definition, paraneurons are derived from neuroectoderm, produce substances identical- or related to real neurotransmitters, and are capable of receptor-modulated responses. Paraneurons also contain synaptic vesicle-like or neurosecretory granules from which they release products in response to stimuli. Parenchymal cells of the pineal gland fit all aspects of this definition (Walker and Aloyo, 1987). Pinealocytes metabolise and secrete serotonin (Saavedra, 1977), receive adrenergic innervation (Klein *et al.*, 1971) and resemble serotonergic neurons.

The pineal contains various types of ultrastructural features involved in intercellular

communication, such as synapses, gap junctions and tight junctions. Further features indicative of active secretory processes include dense-core, clear, granular and agranular vesicles, in addition to the normal organelles found in other cells (Samarasinghe *et al.*, 1983). Pineal cells also have synaptic ribbons (considered by some to be the stimuli-transmitting organs facilitating pineal secretory function), synaptic ribbon fields and myeloid bodies which make them distinctive (Samarasinghe *et al.*, 1983). It is interesting to note that these processes also show a circadian rhythm, coupled to the secretion and metabolic phases of the pineal (Booth, 1987).

Nuclear and cytoplasmic areas of pinealocytes and the area of condensed chromatin have been shown to exhibit inversely-related circadian rhythms; the former two increased, while the latter decreased during the light period. Pericapillary and wide intercellular spaces increase during the light period, and decrease during the dark period. The number of fenestrae also increase during the light period, and decrease at night. It is thought these changes reflect the increased secretory functions at night (Matsushima *et al.*, 1989).

1.2.3 Innervation

1.2.3.1 Neuroanatomical and neurophysical connections

The first detailed studies of the innervation of the rat pineal gland were carried out by Kappers (1960, 1965). The gland is innervated by pinealopetal orthosympathetic and pinealopetal parasympathetic fibres. The parasympathetic fibres come to the pineal by way of the facial and greater superficial petrosal nerves but their function is unclear (Booth, 1987). While there is convincing evidence for the existence of central pinealopetal fibres, which enter the gland via its stalk (Korf and Moller, 1984), their function also remains an enigma (Reiter, 1989). What is certain though, is that if the sympathetic innervation is destroyed, the pineal is rendered non-functional by all currently measured parameters (Reiter, 1989). A dense innervation by vasoactive intestinal peptide (VIP) immunoreactive fibres has also been found in the peripheral superficial part of the organ, with the fibres penetrating into its central portion. There they mainly terminate in the vicinity of the capillaries. This suggests a possible role for VIP- influence in this gland as well (Mikkelsen, 1989).

The effects of light on the pineal are mediated by a monosynaptic pathway, leaving at the level of the optic chiasma and terminating at the suprachiasmatic nuclei (SCN). Using radio-labelled amino acids, Moore (1973) was able to prove unequivocally the existence of this retinohypothalamic pathway. The SCN contain the circadian timing system which controls the pineal. They can continue to oscillate on an ca 24 h cycle, even in animals kept in the dark; the "clock" is autonomous, although environmental lighting has the effect of resetting and keeping the clock

entrained to the light cycle (Reiter, 1991b).

From the SCN the fibres continue as follows:

SCN → paraventricular nuclei → intermediolateral cell column → superior cervical ganglia (SCG) → nervii conarii → pineal gland.

The postsynaptic fibres from the SCG follow the internal carotid, to arrive caudally in the form of the paired nervii conarii (Booth, 1987). There is general agreement that the fibres do not form distinct synapses with the pinealocytes (Kappers, 1976), but end in the perivascular spaces and that the neurotransmitter simply diffuses to the surface of the pineal cells. A few of the processes may terminate between the perikarya of the pinealocytes (Matsushima *et al.*, 1981). Apart from the delivery of NA, the sympathetic nerves can also act as a sponge, taking up and concentrating catecholamines from the perivascular space (Kvetnansky *et al.*, 1979). This protective effect has been demonstrated when stressful treatments, known to elevate catecholamine levels have had little effect on the pineal / plasma melatonin levels (Vaughan, 1982). This is true, provided that the uptake process is not blocked (Parfitt and Klein, 1976).

1.2.3.2 The third eye

Considered the vestigial remains of the third eye, the pinealocytes of mammals are derived from the same progenitor cells. Occasionally they contain rudimentary structures reminiscent of the photoreceptor elements still found in lower mammals (Pevet, 1981). Although the mammalian pineal gland no longer responds directly to light, it has evolved a neural pathway to the eye, and is thus still influenced by light cycles.

Innervated by the peripheral autonomic system, the postganglionic sympathetic nerve fibres show dramatic biphasic activity, which in turn is reflected in the biphasic pattern of melatonin production and secretion (Kappers, 1960; 1965). Influenced by light received on the retina, the neural input is converted to an endocrine output, resulting in the pineal gland being termed a neuroendocrine transducer (Booth, 1987).

This gland therefore functions as a biological clock converting information about environmental lighting into a specific hormonal output (Brown *et al.*, 1981). This specific hormonal output in the form of melatonin, is able to provide information about absolute day / nightlength to responsive organs. Thus, internal organs are provided with photoperiodic information by the circulating melatonin, enabling them to be aware of increasing daylength, e.g. the seasonal change from spring into summer, and allowing them to begin physiological adjustments in advance of the

time that a change in function will be required (Kennaway, 1984; Reiter, 1991b).

The opposite is true for pinealectomised mammals. They become either totally aseasonal, or their natural annual cycle free-runs; in either case, their physiology becomes inappropriate to the current season (Reiter, 1991b).

1.2.3.3 Light

Light is the *Zeitgeber* (entraining agent) regulating melatonin production (Erlich and Apuzzo, 1985). It has two apparent effects on the melatonin rhythm: acute reduction of amplitude (synthesis and output), and entrainment of the underlying pacemaker as revealed by changes in period or phase.

The acute exposure of animals or humans to light at night leads to a precipitous decline in pineal melatonin synthesis and release. The reduction to daytime levels requires only 20 to 30 min. Light exposure at night suppresses pineal melatonin production in all animals tested, but the brightness (irradiance) of light required to achieve this result varies greatly among mammals (Reiter, 1985). To illustrate how these species may differ: the albino rat pineal gland can respond to as little as $0.0005 \mu\text{W}/\text{cm}^2$ of white light at night, whereas the Richardson's ground squirrel requires a light irradiance of about $1850 \mu\text{W}/\text{cm}^2$. The human pineal is intermediate between these two extremes, responding to about $150 \mu\text{W}/\text{cm}^2$ of white light at night. Normal room light averages 50 to $100 \mu\text{W}/\text{cm}^2$, while on a clear day the sunlight at noon can be as bright as 25 000 to $50\,000 \mu\text{W}/\text{cm}^2$ (Reiter, 1988a).

Besides its brightness, the wavelength of the light also determines its ability to suppress melatonin synthesis. Light in the blue range, 500 to 510 Å, seems to be most suppressive of pineal melatonin production (Brainard, 1985). This includes light in the yellow-green range reported by Wurtman and Moskowitz (1977).

Of interest with regard to light and the pineal gland, is information from autoradiographic studies which indicates that vitamin D3 (solatriol) is a somatotrophic activator and modulator. It is regulated by the amount of sunshine and the endocrine status of the individual, and its purpose is the promotion of development, reproduction, and the maintenance of life. Regulation of Ca^{2+} homeostasis is only one of its many functions. A close link to the pineal hormone system is apparent. Evidence supports the concept that the skin-derived hormone of sunlight and the pineal hormone(s) of darkness are messengers with comprehensive actions on endocrine-, autonomic-, sensory-, skeletal-, and motor functions. Thus both hormone systems complement each other, and

appear to correlate biological activities with the daily and seasonal changes of our solar environment (Stumpf, 1988).

1.2.3.4 Circadian Rhythms

Conceptually, circadian systems consist of a clock-like pacemaker (Pittendrigh, 1981), *from* which there is at least one pathway driving an overt rhythmic function, and *to* which there is at least one pathway from a photoreceptor that can entrain (Aschoff, 1981) the pacemaker. The overt hormonal and biochemical rhythm which has been studied most intensively is that of melatonin production (Binkley, 1981; Klein, 1985). In the rat (and other mammals), the components of this circadian system are anatomically distinct; the photoreceptors are in the retina, the pacemaker is in the suprachiasmatic nucleus of the hypothalamus (Moore, 1983), and melatonin is synthesized in the pineal gland. Multisynaptic pathways connect these components. **Figure 1.2** shows the neural connections from the eyes to the pineal and the associated rhythms. *In vitro*, the rat pineal is neither rhythmic nor photosensitive, but can be stimulated to make melatonin (Zatz, 1982; Klein, 1985).

In contrast to the rat pineal, the chick pineal *in vitro* is both rhythmic and photosensitive. Circadian pacemakers (Deguchi, 1979), photoreceptors (Deguchi, 1981), and the mechanisms that regulate melatonin production (Binkley, 1981) and release, continue to cycle up at night and down in the day 'spontaneously' in culture, a rhythm which persists for several cycles even in constant darkness (Robertson and Takahashi, 1988).

Calcium influx appears to play an important role in the induction of melatonin synthesis (Zatz and Romero, 1978; Sugden *et al.*, 1986). The results of Zatz and Mullen (1988), however, fail to support a role for Ca^{2+} influx in the regulation of the pacemaker. They suggest rather a role distal to the pacemaker, probably at the level of the melatonin-synthesizing apparatus.

Calcium may even play a role in the secretion of melatonin (Morton *et al.*, 1989), but this is not certain. As melatonin is produced in the pineal gland, blood levels rapidly increase in parallel (Wilkinson *et al.*, 1977; Reiter, 1990). Besides the circadian release of melatonin from the pineal, an ultradian release has also been reported by Chen and co-workers (1990). The pulsatile release of melatonin is especially apparent when levels are measured in pineal venous effluent (Reiter and Vaughan, 1990). Because of its high lipophilicity and the ease with which melatonin passed through cell membranes, it was always thought that it diffused out of the pinealocytes and into the circulation. However, now that episodic release has been demonstrated, the existence of at least short-term storage and an active release mechanism for melatonin must be assumed (Reiter,

1991a).

Whatever the function of the episodic melatonin release, changes in the circadian production and release remain important for the execution of its role as a chemical expression of darkness (Stetson and Tay, 1983; Reiter, 1991b). Melatonin's high lipophilicity presumably still permits its rapid transfer into other fluids. All body fluids tested to date have proven positive for melatonin. These include cerebrospinal fluid (Hedlund *et al.*, 1976), milk (Reppert *et al.*, 1979), saliva (Vakkuri, 1985), ovarian follicular fluid (Brzezinski *et al.*, 1987), male seminal fluid (Bornman *et al.*, 1989), amniotic fluid (Kivela *et al.*, 1989) and the fluid of the anterior chamber of the eye (Yu *et al.*, 1990). In each of these fluids a circadian melatonin rhythm was found which paralleled that found in blood. Thus essentially all cells are exposed to the melatonin rhythm. Because of the lipophilicity of melatonin, the cell's organelles too, are exposed to melatonin and thus a signal of the photoperiodic state of the organism, provided of course that the signal can be received e.g. via receptors (Reiter, 1991b).

1.3 Melatonin biosynthesis

1.3.1 Tryptophan

The production of melatonin within the pinealocyte requires the uptake of the amino acid tryptophan from the plasma, by the pineal. Tryptophan within the blood is derived from two sources: ingested protein, and free tryptophan pools. Some of the tryptophan is used in protein synthesis, while the rest goes into the indole pathways shown in **Figure 1.3**. Once taken up into the pinealocyte, tryptophan is oxidised at the 5-position to form the amino acid 5-hydroxytryptophan. This conversion depends on the activity of the enzyme, tryptophan hydroxylase and a reduced pteridine co-factor (Lovenberg *et al.*, 1968). The high substrate K_m for tryptophan hydroxylase relative to the low levels of free tryptophan, suggest that the enzyme is normally unsaturated, and thus the rate of hydroxylation would depend on the availability of the amino acid.

1.3.2 Serotonin

Once formed, 5-hydroxytryptophan is converted to 5-hydroxytryptamine (serotonin) in the presence of the pyridoxine-dependent enzyme aromatic *L*-amino acid decarboxylase (dopa-decarboxylase) (Lovenberg *et al.*, 1982). Serotonin concentrations and turnover are very high in the pineal, exceeding those of any other organ in the body (Quay, 1974).

Walker and Aloyo (1987) have shown that the secretion of serotonin from rat pineal glands

appears to be a NA-mediated event acting via α_1 -adrenoceptors. The intracellular second messenger involved in the serotonin-release process, is a Ca^{2+} -phospholipid-dependent protein kinase (the effects of the phorbol ester, PMA, simulated those of NA).

1.3.2.1 Monoamine oxidase

Three routes exist for the metabolism of serotonin (HT) in the pineal.

The major enzyme for the metabolism of HT in other organs, monoamine oxidase (MAO), is also present in the pineal. Some pineal serotonin is metabolised by MAO to 5-hydroxyindole acetaldehyde, an unstable intermediate that is either oxidised to 5-hydroxyindoleacetic acid (HA) or reduced to 5-hydroxytryptophol (HL).

Two different isozymes of MAO have been identified in pineal tissue: one is confined to the pinealocyte (MAO-B), and one to the sympathetic nerve terminals (MAO-A) within the gland (Goridis and Neff, 1972). As serotonin (HT) is only deaminated by MAO-A, the association of one isozyme with each cell type implies that two cellular compartments are required in the pineal gland for the synthesis of 5-methoxytryptophol (ML) and 5-methoxyindoleacetic acid (MA), while melatonin (aMT) and 5-methoxytryptamine (MT) require only one, the pinealocyte (Masson-P'evet and P'evet, 1989). The indoleamines are abbreviated according to the system of nomenclature introduced by Smith (1981). A full listing is presented in **Figure 1.3** .

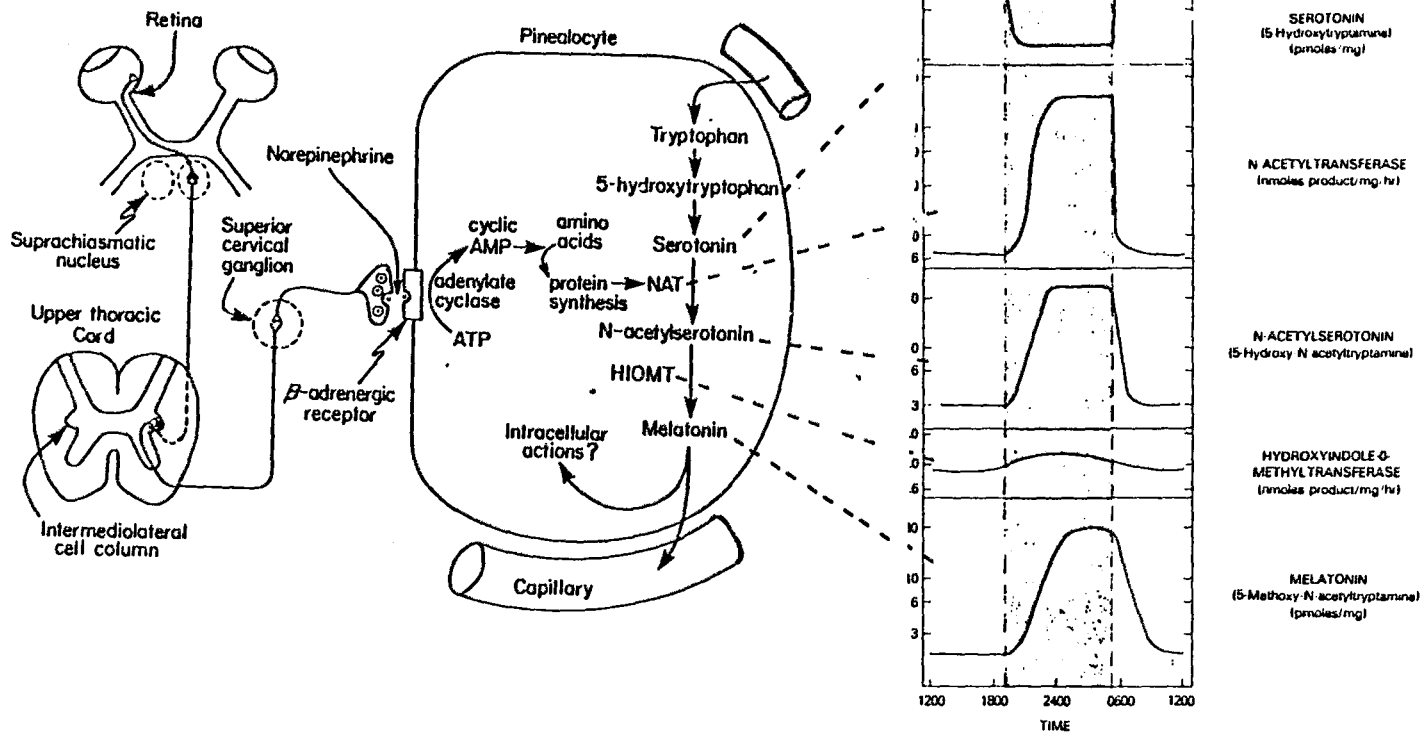


Figure 1.2:

Schematic representation of a pinealocyte, showing the neural connections between the eyes and the pineal gland (left), the conversion of tryptophan to melatonin (middle) and the day/night fluctuations of the various enzymes and substrates involved in melatonin biosynthesis (right), Shaded portion indicates the dark phase of the light period (adapted from Reiter, 1982).

1.3.3 Hydroxyindole-*O*-methyltransferase

5-Hydroxyindoleacetic acid and 5-hydroxytryptophol are substrates for hydroxyindole-*O*-methyltransferase (HIOMT), and are metabolised to 5-methoxyindoleacetic acid or reduced to 5-methoxytryptophol respectively. 5-Methoxytryptophol may be released into the blood in a circadian manner (Wilson *et al.*, 1978). It reportedly possesses some endocrine effects, although these have been sparingly investigated (Reiter, 1980). HIOMT may act directly on serotonin, leading to the formation of 5-methoxytryptamine (Prozialeck *et al.*, 1978). Although this compound has been promoted as a pineal hormone (Pevet, 1983), its activity is only one tenth that of melatonin, measured relative to effects on the reproductive system (Rollag, 1982). The secretion of 5-methoxytryptamine has not yet been proven, although it may be converted peripherally to melatonin.

Several forms of HIOMT exist in pineal tissue (Jackson and Lovenberg, 1971). Unlike the enzyme *N*-acetyltransferase (NAT), it is restricted to only a few other organs. It is found in the retina (Cardinali and Rosner, 1971), the harderian gland (Vlahakes and Wurtman, 1972) and in erythrocytes (Rosengarten *et al.*, 1972). Monoclonal antibodies to bovine HIOMT, which shows a high degree of structural similarity with HIOMT from other species, have allowed the immunocytochemical cytosolic localization of this enzyme (Deguchi *et al.*, 1987).

1.3.4 *N*-acetyltransferase

Certainly the major pathway for the metabolism of serotonin in the pineal gland is its conversion to melatonin via *N*-acetylation by the enzyme *N*-acetyltransferase (NAT) to yield *N*-acetylserotonin. Next, a methyl group is transferred from *S*-adenosylmethionine to the 5-hydroxy position of *N*-acetylserotonin, yielding melatonin (Weissback *et al.*, 1960; Axelrod and Weissback, 1961). This conversion is catalysed by HIOMT.

NAT activity is generally considered to be rate limiting in the production of melatonin (Klein, 1985). Thus the pineal melatonin content typically rises in parallel to NAT. There are however notable exceptions, where a large melatonin peak is present, without a commensurate large increase in NAT activity (Menendez-Pelaez *et al.*, 1990). The nightly increase in NAT activity varies between species (Rudeen *et al.*, 1975), from as little as two-fold to the 100-fold increase found in the rat (Klein, 1985; Reiter, 1991b).

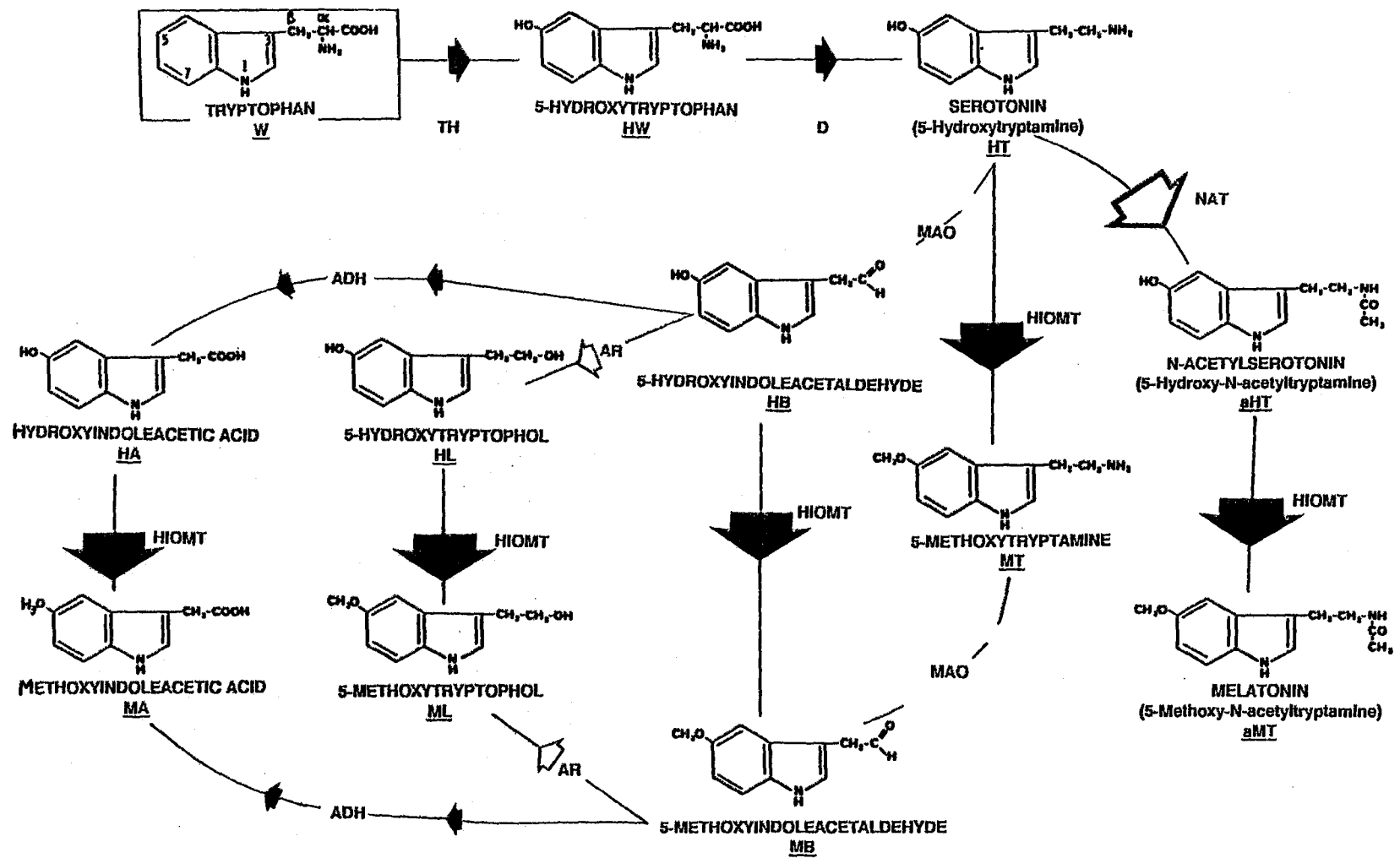


Figure 1.3: Schematic representation showing the chemical and metabolic pathways derived from tryptophan, and the accompanying nomenclature published by Smith in 1981.

1.3.5 Melatonin

Melatonin is also synthesized in the other organs where HIOMT is found e.g. the retina (Cardinali and Rosner, 1971), the harderian gland (Vlahakes and Wurtman, 1972) and in erythrocytes (Rosengarten *et al.*, 1972). Their contribution to the circulating levels is questionable (Reiter, 1989), although Ralph, (1981) has suggested that the retina in some mammals may in fact be the major producer.

Once synthesized, melatonin is released into the circulation [§ 1.2.3.4]. Plasma levels can thus give an accurate reflection of melatonin production (Wilkinson *et al.*, 1977). From the circulation, melatonin can be taken up by all organs (Erlich and Apuzzo, 1985) to be used if required. Once in the liver it is metabolised to 6-hydroxymelatonin.

1.4 Possible roles for melatonin

Since its discovery some 30 years ago, melatonin has been the subject of much investigation with regard to its synthesis, the distinct rhythms which it has and its possible endocrine consequences. In spite of this, no singular true role can be ascribed to the hormone. In fact, it is not even certain if all the attention has been rightly focussed on melatonin, and not rather on one of the other indoles synthesized in the pineal. With the widespread distribution of melatonin, the question of which organ system is influenced, has become rather, which system is not? The 24 h cycle is found in most mammals, with the night-time peak common to all mammals, indeed all vertebrates, regardless of their locomotor activity pattern (Gern and Nervina, 1986), even when there are variations in photoperiod. The magnitude of the rise varies fairly dramatically between species, but the level of change (peak:trough ratio) needed for differences in interpretation by the target receptor, is not known.

The major use of the transducing of light/photoc signals into a hormonal response seems to be seasonal physiological adjustments, which would control breeding (Arendt, 1986; Reiter, 1988b). There are three main hypotheses regarding the interpretation of the hormonal rhythm; the **duration hypothesis**, the **coincidence hypothesis** and the **amplitude hypothesis** (Reiter, 1987). The **duration hypothesis** takes into account the length of the night-time high in melatonin, which would give seasonal information, e.g. longer nights in winter and shorter nights in summer. This hypothesis has now been extended to take into account the direction of change of elevated nocturnal melatonin i.e. whether the period of elevated levels is becoming longer as the season changes to winter, or shorter, as summer approaches (Hoffamn *et al.*, 1986; Reiter, 1991b).

The coincidence hypothesis takes into account the increase in melatonin at night, but also has a so-called "window of sensitivity": although the level of melatonin is raised, it will mean nothing to the particular organ unless that organ is sensitive to a signal at that point in time (Carter and Goldman, 1983; Stetson and Watson-Whitmyre, 1986).

The third hypothesis looks at the **amplitude** of the nocturnal rise in melatonin, and although this is seldom used in animal studies, as most species have comparable nighttime blood concentrations (Reiter, 1991a), it is often used in human studies (Berga *et al.*, 1988). The latter group often show altered melatonin levels in the presence of certain disease states.

While melatonin has an obvious role in controlling hibernation and reproduction in seasonal breeders, its role in humans is more debatable .

In terms of reproductive influences, it has been reported to have a role in sperm motility and hence fertility (Oosthuizen *et al.*, 1986) and in oligospermia or aspermia (Karasek *et al.*, 1990). It is however more famous in medical history for the association of pineal tumours and precocious puberty, first described by Heubner in 1898. The tumours resulted in reduced pineal function, leading Marburg (1909) to suggest that the pineal has an inhibitory role on sexual function. Age-related reductions in nighttime melatonin levels associated with puberty have been reported by Waldhauser and Dietzel, 1985. Unusual melatonin rhythms have also been found in females with hypothalamic amenorrhoea (Berga *et al.*, 1988).

As for hibernation, the role of light could well play a determining role in another disease linked to the pineal, namely seasonal affective disorder syndrome (SADS). Described by Ebadi and Govitrapong (1986), SADS is characterised by patients exhibiting a depressive period from December through to February (in the northern hemisphere). Increased levels of light (to supposedly reduce plasma melatonin levels) are of benefit to these patients.

Among the more diverse range of reported effects/involvements are:

1) hormonally dependent tumours: patients dying of breast cancer often have enlarged pineals (Tapp, 1982; Blask, 1984), while those dying of disseminated forms of cancer invariably have high HIOMT levels, and less Ca^{2+} deposits in their pineals (Kerenyi *et al.*, 1977). In rats pinealectomy generally promotes the growth of cancer tissue, while melatonin administration does the opposite (Reiter, 1989);

- 2) immunocompetence (Pierpaoli and Maestroni, 1987);
- 3) stress (Lynch and Deng, 1986), and the effects of stress e.g. stress related ulcers (Khan *et al.*, 1990);
- 4) Sudden infant death syndrome or "cot deaths" (Sparks, 1988);
- 5) phase shifts or "jet-lag", resulting from changes in the environmental light input into the "clock", and which reportedly can be readjusted by the administration of triazolam (Turek, 1987). (This effect may be blocked with the specific benzodiazopine antagonist flumazenil, which is useful since it could shorten the time of re-entrainment to the circadian rhythm, and thus readjust the sleep-wake cycle shift.); and
- 6) part of the "diseases of the aged" syndrome. Ageing has been related to a relative melatonin deficiency, a result of the gradual failure of the pineal gland. The hypothesis proposes that the persistence of serotonin in the midst of the melatonin decline results in the chain of neuro-endocrine and peripheral events which promote ageing (Rozencwaig *et al.*, 1987). Of interest here is a study by Hasegawa *et al.*, (1987). Pineal decline and the presence of calcifications in the pineal have often been described. This study, however, found that although there existed a correlation between pineal weight and the degree of calcification, some pineals in patients over 90 years old showed no calcification and were indistinguishable from the ones of younger subjects. This suggested that the pineals in humans do not necessarily degenerate after involution.

The finding of a specific melatonin receptor antagonist, luzindole (N-0774), and the advances with radio-labelling of melatonin analogues, may well be the long-needed tools with which to clarify and elucidate the mechanisms through which melatonin operates (Dubocovich, 1988).

Despite initial reports documenting the location and function of melatonin receptors a decade ago, reliable characterizations have only come more recently (Stankov, 1990). The major breakthrough came in 1984 with the introduction of 2-iodomelatonin, a potent melatonin analogue (Vakkuri *et al.*, 1984).

Not surprisingly most studies have centered on the central nervous system. Within the medial basal hypothalamus, melatonin binding sites have been found in the SCN (Reppert *et al.*, 1988), where it is usually tacitly implied that such receptors, may in part mediate the effects of melatonin on the neuroendocrine-reproductive axis (Reiter, 1991a). Autoradiographic studies using radioactive melatonin have also shown sites in the pars tuberalis of the pituitary gland (Morgan

and Williams, 1989). Sites have also been described in nonhypothalamic neurons such as the hippocampus, the septum and reticular formation (Zisapel, 1988, Stankov and Reiter, 1990). Two interesting peculiarities are noted: 1) wide differences in melatonin receptor distribution have been reported, even between closely related species, and 2) many of the binding sites show changes in receptor density over a 24 h period, (Zisapel, 1988).

In the final analysis, there may well be not a single organ system that escapes the influence of this hormone (Klein, 1988); certainly the wide range of data being collected from both animal and human sources indicates much interest in the pineal gland and its possible roles, very different from the previous lack of enthusiasm for this gland.

1.4.1 Influence of age on the pineal and melatonin biosynthesis

There are reports of reduced serum melatonin levels with old age in several species. In rodents, the pineal melatonin rhythm is not present at birth, but appears before the pups are weaned (Vanecek and Illnerova, 1979; Rollag and Stetson, 1981). A pre-pubertal decline in melatonin secretion is noted however, and thought to play a possible role in the development of their reproductive systems (Tang and Pang, 1988).

In humans, although the rhythm is not fully developed at birth, it is fully functional by the end of the first year (Arendt, 1985). The rhythm continues through adulthood, but then the nocturnal rise declines in advanced age (Reiter *et al.*, 1980). It is felt that the decline is not as a result of the deposition of Ca^{2+} in certain individuals (Kitay and Altschule, 1954), as a similar reduction is found in certain species which do not exhibit acervuli (Reiter, 1989). Despite these changes in the melatonin rhythm through life, their significance to either endocrine or physiological events remains unproven (Reiter, 1989).

One peptide does hold some interest with regard to ageing: Arginine vasotocin (AVT) release by melatonin appears to play a role in sleep patterns (Arendt *et al.*, 1982). Administration causes the rapid onset of slow-wave sleep, the period during which most of the body's restorative and reparative events take place. Slow-wave sleep has been shown to decline with age, which has been linked to the pineals decreasing metabolic activity (Rozencwaig *et al.*, 1987).

In the Wistar rat, the capacity of the β -adrenoceptors to develop supersensitivity during the light period is lost during maturation (ca 6 months), rather than in old age. However, neither β -nor α_1 -receptor density declines with age. HIOMT activity does drop in rats over 18 months, but NAT

activity is not altered. This suggests that the reported lowered serum levels of aMT result from a reduced capacity of the pineal to synthesize aMT, rather than from an altered responsiveness of the gland to neural stimulation (Dax and Sugden, 1988).

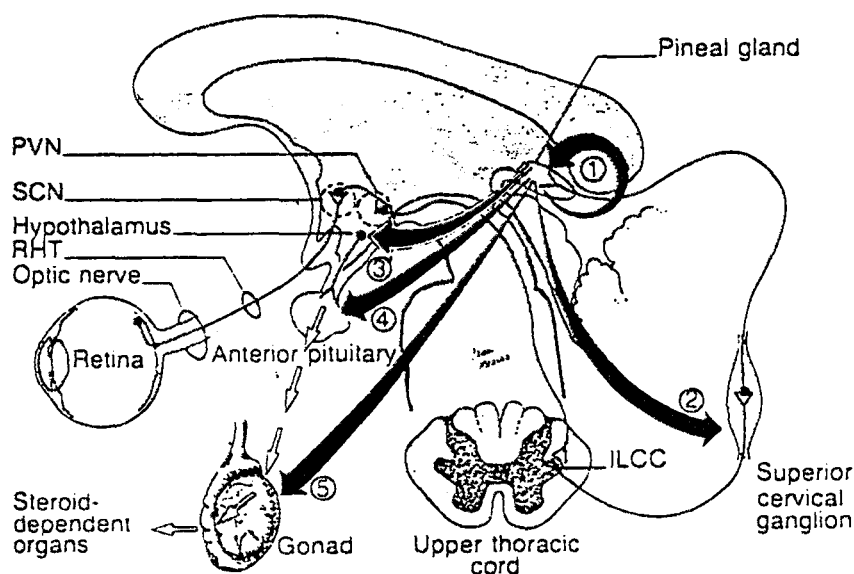


Figure 1.4:

Summary of some of the relationships of the visual system to the pineal and possible sites of action for the pineal hormones.

Some potential sites that have been proposed include the pineal itself (1), the superior cervical ganglia (2), the brain, especially the hypothalamus and brain stem (3), the anterior pituitary gland (4), and the gonads (5), and other organ systems which are not necessarily on the neuro-reproductive axis (adapted from Reiter, 1989).

KEY: ILC = Intermediolateral cell column, PVN = paraventricular nuclei, RHT = retinohypothalamic tract, SCN = suprachiasmatic nuclei.

1.4.2 Pathways possibly modulating melatonin biosynthesis

As stated earlier, there are three neural inputs into the pineal gland, and while the sympathetic fibres and their transmitter are fairly lucid, the remaining circuits are not. Some of the following putative transmitters / peptides have been localized in central pinealopetal connections (for references see Korf and Moller, 1984; Shiotani *et al.*, 1986; Cardinali and Vacas, 1988), although their exact significance is not clear (Morgan *et al.*, 1987). These include vasoactive

intestinal peptide (VIP) (Shiotani *et al.*, 1986), arginine vasopresin (AVP), oxytocin (OT) (Numburger and Kolf, 1981) and lutenizing hormone-releasing hormone (Matsuura *et al.*, 1983). The distribution and origins of neuropeptide Y (NPY) calcitonin gene-related peptide, VIP and substance P (SP) containing nerve fibres innervating the pineal gland of the gerbil have also been described (Shiotani *et al.*, 1986). Gauquelin *et al.* (1988), have demonstrated the presence of AVP and OT in the harderian gland as well.

Physiological roles for both VIP and NPY are likely, as VIP has been shown to affect both cAMP levels and NAT activity in rats (Kaneko *et al.*, 1980), and more recently NPY has also been shown to affect NAT activity (Reuss and Schroder, 1987).

1.4.2.1 Vasoactive intestinal peptide

The presence of VIP-immunoreactive fibres in the pineal has been mentioned. It exhibits a potent stimulating activity on rat pineal cAMP and melatonin synthesis (Kaku *et al.*, 1985, 1986), and an effect on cAMP accumulation in the ovine pineal has also been shown (Morgan *et al.*, 1988). These effects are mediated via interaction with specific receptor sites in pinealocyte membranes (Korf and Moller, 1984, Kaku *et al.*, 1985). This is suggested by the fact that combined stimulation with isoprenaline and VIP is additive, and not synergistic (Morgan *et al.*, 1988). The probable mechanism involved will be discussed presently [§ 1.11.12], once the relevant background has been covered. There is a daily rhythm in pineal VIP (Kaku *et al.*, 1986), and the density of VIP binding sites found in the pineal, is amongst the highest in the central nervous system (Besson *et al.*, 1986). This suggests some role in pineal function.

1.4.2.2 Neuropeptide Y

In the pineal gland NPY is detectable by immunohistochemical techniques (Schon *et al.*, 1985; Schroder, 1986). NPY appears to exert a dual effect in the pineal gland by enhancing melatonin synthesis and NA post-synaptic effects at low concentrations, and inhibiting sympathetic transmissions at high concentrations. It may even play a role in the transfer of light information, as administration of NPY to the SCN area causes phase shifts in the circadian rhythm (Harrington *et al.*, 1985).

1.4.2.3 Substance P

Substance P has been detected in the pineal gland of several mammals. Receptors for substance P

have been characterised in bovine pineals, but not in rat pineals (Govitrapong and Ebadi, 1986).

1.4.2.4 Delta-sleep inducing peptide

Delta-sleep inducing peptide (DSIP) is a presumptive peptide transmitter found in discrete populations of central synapses (Graf and Kastin, 1986). Various effects on sleep as well as other physiological functions have been reported for this peptide (Schoenenberger, 1984; Graf and Kastin, 1984). DSIP has been proposed as a "programming substance for circadian rhythms" (Graf *et al.*, 1982). It reduces the nocturnal increase in rat pineal NAT and also stimulation of NAT following α - and β -adrenoceptor agonists, both *in vivo* and *in vitro* (Graf and Schoenenberger, 1986). DSIP, however, enhances the effect of NA on NAT through an α_1 -adrenoceptor mechanism which will be discussed (Graf and Schoenenberger, 1987).

1.4.2.5 Opioids

Opioid synapses probably participate in pathways controlling the nocturnal maximum of melatonin synthesis. This is revealed by the blocking effect of naloxone on the night-time peak melatonin production (Lowenstein *et al.*, 1984) and its stimulatory effects on melatonin release in rats (Mouren *et al.*, 1986).

Figure 1.5 shows some of the transmitters (many of them released from central pinealopetal fibres) which may play a role in the fine-tuning of melatonin synthesis.

1.4.3 Radioligand binding sites identified

Employing radioligand binding techniques, several nonadrenergic neurotransmitter- and neuromodulator acceptor sites have been described in the mammalian pineal gland. $\text{Cl}^-/\text{Ca}^{2+}$ -dependent glutamate binding (Govitrapong *et al.*, 1986), gamma-aminobutyric acid (GABA) binding (Ebadi and Chan, 1980), benzodiazepine (BZP) binding (Lowenstein and Cardinali, 1983, Matthew *et al.*, 1984; Basile *et al.*, 1986), serotonin (5₂) (Govitrapong *et al.*, 1984) and muscarinic binding (Taylor *et al.*, 1980), VIP binding (Korf and Moller, 1984), and the binding of substance P (Govitrapong and Ebadi, 1986) and peptide N-terminal histidine C-terminal isoleucine (PHI) (Tsuchiya *et al.*, 1987) are known to occur in pineal subcellular fractions. Additionally, dopaminergic binding sites of the D2 subtype are detectable in bovine pineal membranes (Govitrapong *et al.*, 1984). For several of these sites a formal demonstration of their receptor nature is still lacking. Some of the putative transmitters mentioned can be localized in

the sympathetic nerves. There is a decreased release of NA from the terminals during the light period, a consequence of the concomitant low activity of the adrenergic innervation. Sympathetic activity and NA release are increased during the dark phase (Taylor and Wilson, 1970; Brownstein and Axelrod, 1974). This is shown diagrammatically in Figure 1.6.

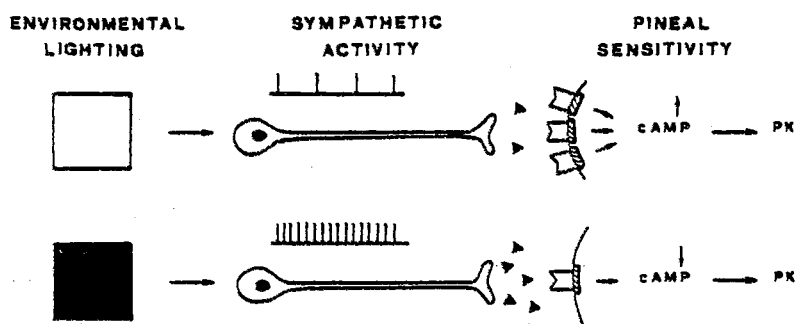


Figure 1.6: Schematic diagram of the light-induced changes in sympathetic activity and compensatory biochemical mechanisms in the pineal gland (Adopted from Benfanati et al., 1986).

Because of the reduced sympathetic activity, constant light induces a supersensitive state of the β -receptors, while a sub-sensitivity is observed for intact animals kept in constant darkness. Glands taken from animals exposed to light for 24 h have 70% more β -adrenergic binding sites than glands removed from animals at the end of their 12 h dark period (Kebabian *et al.*, 1975).

Similar alterations in the sensitivity of β -receptors to adrenergic stimulation can be achieved by pharmacological manipulations and lesion studies.

NA is a mixed α - and β -agonist and can act via α_1 -, α_2 -, β_1 -, and β_2 - receptors, although it is suggested that it may have more selectivity than adrenaline for the α_1 - and β_1 -adrenoceptors (Ariens *et al.*, 1979; Fain and Garcia-Sainz, 1980). The α_1 - and β_1 -adrenoceptors on pinealocytes have been fully characterized (Auerbach *et al.*, 1981; Sugden and Klein, 1983). Both are important to pineal functions, including cAMP-, cGMP-, NAT- and melatonin synthesis (Klein *et al.*, 1983b; Vanecek, *et al.*, 1985).

1.5.1 Pineal β -Adrenoceptor-Mediated Activation

The pharmacological characteristics of the rat pineal β -receptors, as established by agonist-potency ratios and the use of selective antagonists, indicated a dominant β_1 -subclass (Dickenson *et al.*, 1986). They resembled β_1 -adrenoceptors found in the heart and brain in having a high affinity for

NA; they differed from β_2 -adrenoceptors, which mediate catecholamine metabolic effects in several tissues, and which exhibit a low affinity for NA (Weiner, 1980). A single peptide of M_r 42,000 was detected after covalent labelling of rat pineal β_1 -adrenoceptors using a photoaffinity probe; this differs from the M_r 60,000 peptide found in various other tissues (Dickenson *et al.*, 1986).

β_1 -Adrenoceptor-mediated activation of pineal adenylyl cyclase enhances cAMP synthesis, the binding of cAMP to protein kinase A, phosphorylation of pineal proteins, protein synthesis and activation, and the *de novo* synthesis of enzymes (NAT and HIOMT) in the melatonin pathway (Axelrod, 1983; Ebadi, 1984).

In contrast to that of the rat, the ovine pineal contains a mixed population of β_1 - and β_2 - receptor subtypes. This conclusion is supported by data from experiments where practolol inhibited NA-stimulated cAMP accumulation by about 75%, indicating a slight predominance of β_1 -receptors (Morgan *et al.*, 1988).

More recent data have shown that the commonly accepted view that the pineal gland is controlled exclusively by β -adrenoceptors is not valid, and that α -adrenoceptors are also present.

1.5.2 The α -Adrenergic System.

Originally demonstrated by Vacas *et al.* (1980) using biochemical means, the presence of α_1 -adrenoceptors has been confirmed in the rat pineal gland by Sugden and Klein (1984). Using the high-affinity ligand [125]iodo-2- $[\beta$ -(4-hydroxy-phenyl)ethylalminomethyl] tetralone ([125 I]-HEAT) to label these receptors, Sugden and Klein (1985) found that, unlike the β_1 -adrenergic receptors, the binding sites labelled by this ligand do not show a circadian rhythm. One difference from the β -adrenoceptors is that acute or chronic administration of α_1 -adrenoceptor agonists failed to change the number of specific binding sites. They also demonstrated that these α_1 -adrenoceptors are indeed under neuronal control, since an increase in the number of [125 I]HEAT binding sites was observed after ganglionectomy or three weeks constant light exposure (which blocks central stimulation of the gland).

It would appear that the density of pineal α_1 -adrenoceptors (\cong 400 fmol/ mg protein) is only slightly less than the density of β -adrenoceptors in the gland (\cong 600 fmol/ mg protein) (Sugden and Klein, 1984). An exact comparison is difficult in view of the diurnal fluctuation in β -adrenoceptor density (Romero *et al.*, 1975). The rapid nature of the elevation in pineal β -

adrenoceptor density at the end of the dark period (Romero *et al.*, 1975), coupled to the inability of cycloheximide to block receptor loss after ISO treatment (Kebabian *et al.*, 1975), suggest that the number of β -adrenoceptors may be regulated by an activation and inactivation process possibly involving internalization and recycling (Stadel *et al.*, 1983), rather than the slower processes of synthesis and degradation. In contrast, the lack of a diurnal rhythm in pineal α_1 -adrenoceptor density and the slow increase after interruption of neural input suggest that the number of these receptors may be regulated via synthesis and/or degradation (Sladeczek and Bockaert, 1983; Sugden and Klein, 1985).

Further evidence suggests that these α_1 -adrenoceptors are located postsynaptically, as their presence was still demonstrable after bilateral superior cervical ganglionectomy. This is in agreement with the work of Smith *et al.*, (1979) which also showed the existence of postsynaptic α -adrenoceptors following treatment with 6-hydroxydopamine.

1.5.3 α_2 -Adrenoceptor

It should be noted that presynaptic α_2 -receptors are also detectable in rat pineals by pharmacological means (Pelayo *et al.*, 1977). NA release has been shown to be regulated via a negative feedback mechanism mediated via these receptors. Further work by O'Dea and Zatz (1976) suggests that increased cGMP levels may also be involved in this reduction in NA release. Of further interest is that NA has been shown to inhibit cAMP levels in chick pineal glands which have a high percentage of postsynaptic α_2 -receptors (Naylor *et al.*, 1982). Similarly, Bylund *et al.*, (1988) have shown a NA-induced inhibition of NAT activity in chick pineal cells: the receptors responsible were identified as belonging to the α_{2A} subtype.

Pratt and Takahashi (1988) have shown that pertussis toxin-sensitive guanine nucleotide-binding proteins (G-proteins) were involved in the transduction of this postsynaptic α_2 -adrenergic signal, and that pertussis toxin can in fact block the NA-mediated inhibition in this gland.

1.5.4 Alpha-receptors and phosphatidylinositol turnover

Ligand binding and pharmacological studies by Fain and Garcia-Sainz (1980) indicate that α_1 -adrenergic receptors can be divided into α_{1A} and α_{1B} . α_1 -Receptors mediate those metabolic effects of α catecholamines which involve phosphatidylinositol (PI) turnover and the release of bound intracellular Ca^{2+} as well as the entry of extracellular Ca^{2+} . The α_2 effects of catecholamines are due to non-specific inhibition of adenylyl cyclase through a mechanism

independent of Ca^{2+} .

There is a significant correlation between hormone effects on PI turnover and elevation of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$). Available data suggest that the turnover in membrane-bound PI is involved in Ca^{2+} gating in rat hepatocytes, rat and hamster adipocytes and blowfly salivary glands. This effect has also been demonstrated in the rat pineal gland (Smith *et al.*, 1979), but not in the ovine pineal gland (Morgan *et al.*, 1988).

α_1 - Receptors mediate effects secondary to an elevation of $[\text{Ca}^{2+}]_i$ and involve increased turnover of PI. In contrast, the α_2 receptors mediate effects which are due to inhibition of adenylyl cyclase. There is an additional complexity, since elevation of Ca^{2+} due to activation of α_1 -receptors can increase cAMP accumulation if cells contain an adenylyl cyclase which is activated by Ca^{2+} .

The hypothesis that α_1 effects are due to elevation of cytosol Ca^{2+} and that α_2 effects are due to adenylyl cyclase inhibition has also been suggested by Wikberg (1979), and supported by Sabol and Nirenberg (1979). Jones and Michell (1978) suggested that α_1 -receptors are involved in elevation of cytosol Ca^{2+} and PI turnover. They ignored α_2 effects and referred to postsynaptic α_1 responses, as being α effects.

The differentiation between α_1 and α_2 receptors is functional rather than anatomic. The difference between the two receptors is no more pre- versus postsynaptic than it is excitatory versus inhibitory. Rather, the classification reflects the difference between elevation of Ca^{2+} (via α_1) versus inhibition of adenylyl cyclase (via α_2) (Fain and Garcia-Sainz, 1980).

The role of PI turnover and Ca^{2+} in the pineal gland is fully discussed [§ 1.11].

1.6 Calcium, the cyclic nucleotides, phosphoinositidyl turnover and the pineal gland

The occupation of the cell-surface receptors leads to the generation of intracellular second messengers, e.g. cyclic nucleotides, Ca^{2+} and diacylglycerol (DG), which act as allosteric effectors to activate a variety of protein kinases (Shenolikar, 1987).

Although Ca^{2+} levels in human pineal glands vary widely, and remain poorly understood, experimental biochemical studies are revealing multiple contributions by Ca^{2+} to molecular control mechanisms within pineal tissue. These involvements of calcium pertain to: 1) closure of

pineal canaliculi (Allen *et al.*, 1981), 2) sensitivity of pineal β -receptors (Zatz and Romero, 1978; Wilkinson, 1978) and 3) the activation or induction of enzymes participating in pineal synthesis of catechol- and indoleamines. Such enzymes include adenylyl cyclase, phosphodiesterase (PDE) (Cheung, 1980), the CaM-activated tryptophan hydroxylase (Kuhn and Lovenberg, 1982), and *N*-acetyltransferase (Klein and Weller, 1970).

1.6.1 Cyclic-AMP

The pineal possesses a relatively high activity adenylyl cyclase, which catalyses the conversion of ATP to adenosine 3', 5'-monophosphate (cAMP). Activated by NA (released from the sympathetic nerve endings in the pineal gland) the activity of this enzyme system is influenced by the age and sex of the animal as well as by environmental lighting and neural activity (c.f. Strada *et al.*, 1972 and references therein).

Activation of the β_1 -adrenergic receptor elevates cAMP levels ca 10-fold within 10 min of stimulation, and the effect gradually decreases as a result of desensitization mechanisms (Vanecek *et al.*, 1985). NA however is active via both β - and α_1 -adrenoceptors, and when combined stimulation of both the β - and α_1 -receptors by NA occurs, the rat pineal gland response measured in terms of cAMP is greatly potentiated, resulting in increases of between 30- and 100-fold (Klein, 1985; Reiter, 1991b). Thus α_1 -adrenoceptor stimulation is able to potentiate β -adrenoceptor stimulation of pineal cAMP, when the two are combined. α_1 -Adrenergic stimulation alone, however, has no effect on cAMP production in the pineal gland (Klein, 1985).

1.6.1.1 Cyclic nucleotide-dependent protein phosphorylation

The cAMP-dependent protein kinase from the rat pineal gland has been purified and characterized by Fontana and Lovenberg (1971). The gland is a rich source of this enzyme, which has an activity higher than that reported in brain tissue. Its role in pineal function is that of NAT induction (Benfenati *et al.*, 1986). Time taken between the activation of the cAMP-dependent kinase, transcription of mRNA, and the eventual rise in NAT varies between species. It is unclear whether new mRNA induces the *de novo* synthesis of NAT molecules or just stimulates a NAT activator protein. The former seem more likely (Reiter, 1991a; 1991b).

Other phosphorylations have been reported, namely that of a specific nuclear protein (34 000 mol wt.) during NAT induction (Winters *et al.*, 1977), and a diurnal variation in the content of pineal synapsin I (Nestler *et al.*, 1982), an endogenous substrate for both cAMP- and Ca^{2+} / CaM-

dependent protein kinases (Ueda and Greengard, 1977; Krueger *et al.*, 1977).

1.6.1.2 Functional correlates

NAT is controlled by the pineal sympathetic innervation and its activity also shows a light:dark cycle with maximal activity in the scotophase when β -adrenergic stimulation is increased (Klein and Weller, 1970).

Melatonin synthesis in the rat is critically dependent upon the production of cAMP as an intracellular second messenger (Morgan *et al.*, 1988). As cAMP rises, so must NAT activity, resulting in a decrease in HT and HT-oxidation products and an increase in aHT and ultimately aMT by mass-action effects (Klein and Weller, 1973; Klein *et al.*, 1978; Klein, 1985).

Of interest is the inter-species variation in time taken from cAMP activation to the rise in NAT and the eventual melatonin peak. In the Djungarian hamster, peak pineal NAT activity and melatonin levels occur 1-2 h after the onset of darkness. However, in the pineal gland of the Syrian hamster, both cAMP and mRNA transcription have already peaked by 4 h (Gonzalez-Brito *et al.*, 1990; Santana *et al.*, 1990), while NAT activity and melatonin only peak after ca 8 h of darkness have elapsed (Panke *et al.*, 1979).

Another inter-species difference is the magnitude of the nocturnal rise in pineal NAT. Variations are seen from a low 2-fold increase to the high 50- to 100-fold increase found each night in the rat pineal gland (Rudeen *et al.*, 1975).

These differences presumably account for the different patterns of melatonin biosynthesis seen between species, as shown in Figure 1.7.

In vitro studies show that the addition of NA or ISO to pineals in organ culture causes an increase in NAT activity, while the addition of propranolol would antagonise the effect (Klein and Berg, 1970; Deguchi and Axelrod, 1972b; Deguchi, 1973). These data have also been confirmed in *in vivo* studies (Deguchi and Axelrod, 1972a). Cholera toxin, an irreversible activator of adenylyl cyclase, and dibutyryl-cAMP are also able to increase the enzyme activity. The response to adrenergic stimulation is reduced *in vitro* when Ca^{2+} is removed from the medium (Zatz and Romero, 1978). Stimulation of NAT activity *in vitro* by forskolin, an agent which directly activates adenylyl cyclase, is found to be significantly reduced in pineals removed from rats that have been exposed to alcohol *in utero* (Rudeen *et al.*, 1987).

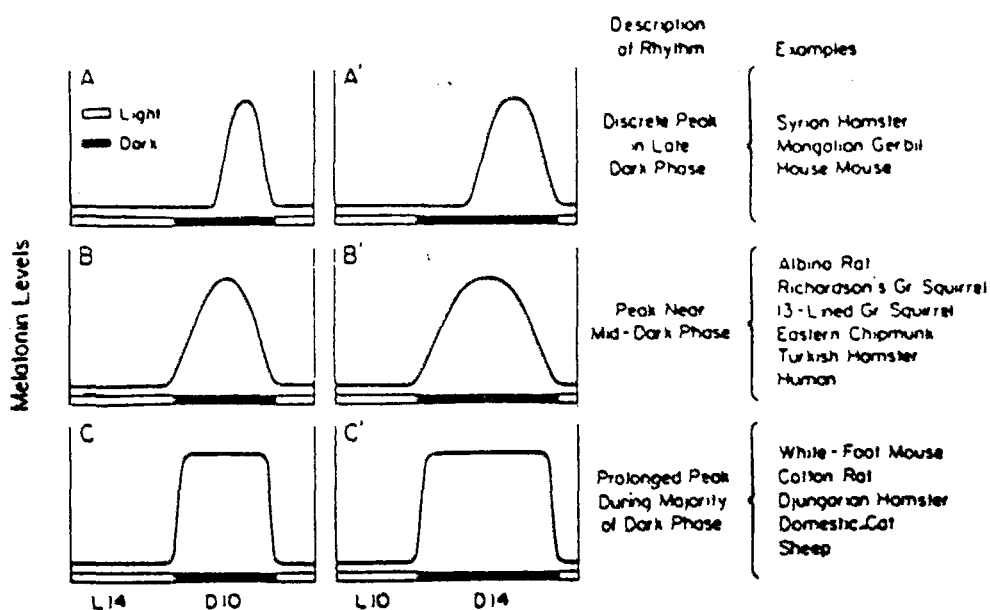


Figure 1.7: *Provisional classification (A,B, and C) of the melatonin rhythm in various mammalian species. The significance, if any, of the rhythms remains unknown. Regardless of the type of rhythm a species displays, if the daily dark period is increased the duration of elevated melatonin is likewise prolonged (A', B' and C'). Adapted from Reiter (1987).*

1.6.2 Cyclic-GMP

Cyclic-GMP, whose concentration is elevated 400-fold in pinealocytes by NA, is regulated in a manner somewhat similar to cAMP (Vanecek *et al.*, 1985). β -Adrenergic activation is again a requirement, while α_1 -adrenergic stimulation, by itself without significant effect, potentiates β -adrenergic stimulation (Klein, 1985). There are some marked differences though. Firstly, whereas β -adrenergic stimulation produces 10-fold increases in cAMP levels, it produces only a twofold increase in those of cGMP. Secondly, whereas α_1 -adrenergic potentiation produces a seven to 10-fold increase in cAMP concentrations, it produces a 200-fold increase in those of cGMP, showing cGMP to be more dependent on α_1 -adrenergic activation.

1.6.2.1 Functional correlates

The role of cGMP in pineal functions has not yet been entirely elucidated. It is responsive to stimulation of the rat pineal e.g. *in vitro* exposure of the glands to NA, or to depolarizing concentrations of potassium (K^+), increases cGMP levels (O'Dea and Zatz, 1976).

Some weak evidence associates cGMP with specific pineal functions: because cGMP is thought to

be involved in the regulation of the release of granules in other tissues (Kapoor and Krishna, 1977), it is possible that it plays a similar role in pinealocytes. Granulated vesicles, an enigmatic feature of pinealocytes, are concentrated in stumpy projections called polar processes (Pevet, 1979). The content of these vesicles is a mystery, but the commonly held view is that they contain peptides. It is of interest that the number of these granules appears to vary as a function of stimulation (Pevet, 1979).

Secondly, a cGMP-dependent protein kinase does exist in the pineal and is active on the same protein substrates of the cAMP-dependent protein kinase. However, due to the narrow substrate specificity of the cGMP-dependent protein kinase, there is no evidence in the literature for the existence of endogenous substrates for both cAMP- and cGMP-dependent protein kinases in the brain, with the exception of histones and some peptides (Nestler and Greengard, 1984; Nairn *et al.*, 1985).

1.6.3 Subsensitivity and supersensitivity in cyclic nucleotide responses: see-saw signal processing.

A fascinating feature of the pineal gland is that there are reciprocal changes in the magnitudes of cAMP and cGMP responses produced by changes in chronic neural stimulation (Deguchi and Axelrod, 1973; Klein *et al.*, 1981b). Following long periods of stimulus deprivation, produced by keeping animals in constant lighting or denervating the pineal gland, the cAMP response increases two-fold, an example of denervation supersensitivity. This is similar to what is generally seen in neural and hormonal regulation. In contrast, the pineal cGMP response exhibits the opposite, a denervation subsensitivity. Following constant light, superior cervical ganglionectomy or decentralization, the cGMP response to NA falls from a nearly 80-fold increase in concentration to less than a four-fold increase. These effects of a supersensitive cAMP response and a subsensitive cGMP response are termed "see-saw" signal processing. The importance of this has not been established, but it may have important implications because it shifts the pineal gland between a mono- and a bi-cyclic nucleotide second messenger system. Assuming that each regulates different processes, one can imagine that the two cyclic nucleotides might be responsible for turning "on" and "off" different hormone systems e.g. while cAMP controls aMT production, cGMP might regulate another hormone.

1.6.4 Cyclic-nucleotides: protein regulation and phosphorylation

Experiments designed by Benfenati and co-workers (1986) to study protein phosphorylation in

different physiological and pharmacological conditions affecting the adrenergic system, showed that changes in pineal function could be reflected by changes in the state of phosphorylation of substrate proteins.

They further showed that the sensitivity of the pineal gland to β -adrenergic stimulation seemed to be modulated at different levels of the recognition and transduction mechanisms. The observed results prove that the variations in cAMP-dependent protein kinase activity are mediated by β -adrenergic receptors, since the stimulatory effect induced by ISO can be blocked by the β -receptor antagonist propranolol. On the contrary, Ca^{2+} -dependent protein kinase activity seems to be unaffected by environmental lighting and β -adrenergic receptor systems. Thus the Ca^{2+} -dependent phosphorylating processes may be involved in different aspects of pineal function.

As is customary in cell systems, the phosphorylation cannot continue unabated, but is curbed by the termination of its responsible cyclic-nucleotide. It is well-known that the physiological regulator of cAMP is cAMP-PDE. Work from Sugden and Klein (1987), suggests that the regulation of cGMP levels in the pineal gland involves an N_s -like GTP-binding regulatory protein. This is of interest because it is the first indication that cGMP is regulated by such a GTP-binding protein in nonretinal tissue. Although N_s has been implicated in both receptor-mediated α_1 -adrenergic and opiate stimulation of cGMP in hybrid NG108-15 cells (Kurose and Ui, 1985), it remains to be seen if the mechanisms involved in the transmembrane regulation of cAMP and cGMP in any other tissues are similar.

With seemingly only a single type of β -adrenoreceptor (β_1) present on the rat pinealocyte membrane [§ 1.5.1], the stimulation of both cAMP and cGMP could be mediated by the same receptor. Such a receptor may be coupled not only to adenylyl cyclase, but also to a membrane-bound guanylyl cyclase, via a common or related GTP-binding protein.

The alternative to a GTP-binding protein, is the possible involvement of a CaM-sensitive cGMP-PDE regulating pineal cGMP, as is the case in the retina. Support for this comes from the finding that the mammalian retina and pineal share a number of unusual proteins (Axelrod and Weissbach, 1962; Somers and Klein, 1984), some of which e.g. rhodopsin kinase (Somers and Klein, 1984) and S-antigen (Van Veen *et al.*, 1986a) are involved in transmembrane signalling (Stryer, 1986).

Rhodopsin activates the G-protein transducin, which in turn stimulates cGMP-PDE and although an immunoreactive transducin does not appear to be present in the rat pineal gland (Van Veen *et al.*, 1986b), a related N-protein controlling a specific cGMP-PDE could be present (Sugden and

Klein, 1987). Graziano and Gilman, (1987) have reported the existence of a pathway consisting of rhodopsin, transducin and a cGMP-PDE.

1.7 Calcium entry

The major route of entry for Ca^{2+} ions is through the Ca^{2+} channel. In nearly all eukaryotic cells the Ca^{2+} permeability of the plasma membrane is low at negative membrane potentials. This permits the 10 000-fold concentration gradient, which is sustained by energy-consuming mechanisms.

Depolarization of the plasma membrane leads to an increase in Ca^{2+} permeability by activation of voltage-dependent / voltage-operated Ca^{2+} channels (VOC). Besides regulation by the membrane potential, many types of ion channels are regulated by phosphorylation. The sheer diversity thus altered suggests that regulation of ion channel function by phosphorylation is a common and physiologically important property of many ion channels (Nestler *et al.*, 1984).

Electrophysiological studies of voltage-activated Ca^{2+} currents have demonstrated that the activity of Ca^{2+} channels can be modulated *in vivo* by cAMP-dependent phosphorylation; the precise nature of Ca^{2+} channels thus modulated cannot, however be established. β -Adrenergic receptor activation of cAMP-dependent protein kinase can increase voltage-dependent Ca^{2+} influx 3- to 4-fold, while calcineurin, a Ca^{2+} and CaM-dependent phosphatase, accelerates Ca^{2+} -dependent inactivation. The question that remains is whether the response to phosphorylating agents reflects a direct action of the kinase on Ca^{2+} channels, or an indirect action mediated by other regulatory molecules whose own activity depends on phosphorylation (Armstrong and Eckert, 1987).

The data of Rosenthal and Schultz, (1987) suggest that besides intracellular messengers, and protein kinases stimulated by intracellular messengers, G proteins constitute a third group of regulatory components capable of modifying the activity of VOCs. Thus extracellular signals acting via G protein-coupled receptors can also influence / modulate the activity of VOCs.

This mechanism may be restricted to the membrane, for there is no evidence for the participation of protein kinases stimulated by cytosolic signal molecules, such as cAMP. So far, the only identified components of this signal transduction chain are receptors for extracellular signals and G proteins. In the heart, a G_s -protein which is activated by cholera toxin, stimulates cardiac Ca^{2+} channels without the involvement of a cAMP-dependent intermediate step (Rosenthal *et al.*,

1988).

Until recently, the effects of guanine nucleotides on agonist binding were the only indications that a G protein may be involved in the action of Ca^{2+} -mobilizing receptors (Taylor and Merritt, 1986), now G proteins appear to participate in the modulation of transmembranous Ca^{2+} fluxes.

1.7.1 G proteins

G Proteins act as transducers functionally coupling membrane receptors activated by extracellular signals and enzymatic effectors controlling the concentrations of cytosolic signal molecules such as cAMP, cGMP, IP and Ca^{2+} . The enzymatic effectors include adenylyl cyclase, the retinal light-sensitive cGMP-PDE, and hormone-sensitive phospholipases C and A_2 .

G proteins are divided into 2 major groups (G_s and G_i), which differ in their α subunits (α_s and α_i) and thus mediate the effects of receptors that stimulate or inhibit adenylyl cyclase (Taylor and Merritt, 1986).

With retinal cGMP-PDE, stimulation of enzyme activity has been shown to be caused by direct interaction of G protein α subunits with the respective enzyme (for a review see Casey and Gilman, 1988). Similarly, β -adrenergic stimulation of the pinealocyte is known to activate adenylyl cyclase via a stimulatory guanine nucleotide-binding regulatory protein (G_s) (Spiegel, 1989). In the rat pineal gland, the outcome is a rapid and large increase, up to 60-fold, in pineal cAMP (Reiter, 1991a).

Ribosylation of the G-protein by cholera toxin can substitute for β -adrenergic receptor stimulation (Sugden, 1989a), and α_1 -receptor stimulation can augment the stimulatory effect of cholera toxin (Ho *et al.*, 1987). Since α_1 -receptor stimulation can also augment forskolin induced cAMP production, the interactive site for the α_1 - and β -adrenergic mechanisms is clearly distal to the β -adrenergic receptor (Chik and Ho, 1989).

1.7.1.1 Modulation of ion channel activity by G proteins

It has been demonstrated that G proteins mediate hormonal regulations of ion channel activity, apparently independently of intracellular receptors (cAMP, cGMP and Ca^{2+}). Therefore, G proteins may interact directly with ion channels; alternately, there may be regulatory target molecules of G proteins, which once activated may interact directly with the VOC, either

stimulating or inhibiting it (Rosenthal and Schultz, 1987).

In intact cells, the VOCs are presumably opened by the transient rise of cytosolic Ca^{2+} that follows receptor-mediated activation of PLC; activation of these VOCs may contribute to a maximal and sustained elevation of cytosolic Ca^{2+} , resulting in full activation. Moreover, in a variety of cells, protein kinase C mimics, i.e. phorbol esters or synthetic diacylglycerols, increase or decrease transmitter release or the activity of transmembraneous ion currents, including Ca^{2+} and K^{+} .

By analogy with their role in receptor-mediated regulation of enzymes, G proteins may act as transducers between cell surface receptors and VOCs, mediating not only hormonal stimulation but also inhibition of ion channel activity (Rosenthal and Schultz, 1987; Rosenthal *et al.*, 1988).

1.8 Calcium channels

Calcium channels play important roles in excitability, excitation-contraction coupling, excitation-secretion coupling and other cellular functions (Hamilton and Perez, 1987). In fact, there is hardly a tissue where Ca^{2+} channels have not been postulated as an important part of the signal transduction mechanism. Voltage-operated calcium channels are a key component of all excitable cells which transduce electrical signals into biochemical events. In contrast, receptor-operated calcium channels (ROC) have not been detected by electrophysiological techniques to date (Hoffman *et al.*, 1987).

A major distinction between excitable and non-excitable cells in terms of their modes of Ca^{2+} regulation is the presence in excitable cells (and absence in non-excitable cells) of voltage-activated Ca^{2+} channels. Many (but not necessarily all) of these channels are potently inhibited by Ca^{2+} -channel blocking drugs, such as verapamil, diltiazem and nifedipine. Such channels are not present in non-excitable cells, and in these cells, Ca^{2+} -entry is activated by Ca^{2+} -mobilizing agonists, but not by membrane depolarization (Putney, 1987). Thus, in non-excitable cells, receptor regulation of Ca^{2+} mobilization is insensitive to inhibition by the CCBs.

Because channel activity is altered by β -adrenergic agonists, 8-bromo-cAMP, intracellular injection of cAMP, or by injection of the α_1 -catalytic subunit of protein kinase (for references see Hamilton and Perez, 1987), a cAMP-dependent phosphorylation reaction may well modify channel function. Supporting this, Gray and Johnston, (1987) reported that NA increased the activity of VOCs in granule cells of the hippocampal dentate gyrus. The action appeared to be

mediated by β -adrenoreceptors and could be mimicked by cAMP. Results from Armstrong and Eckert, (1987) demonstrated that the dihydropyridine-sensitive class of VOC, or molecules closely associated with the channels in the membrane, had to be phosphorylated for the channels to open when the membrane was depolarized. Thus while phosphorylation through the cAMP-dependent protein kinase plays a role in the control of VOCs (Livitan, 1989), more recent evidence [§ 1.7] indicates that guanine nucleotide-binding (G) proteins are involved in both turning on and turning off Ca^{2+} channels (Stevens, 1986; Holz *et al.*, 1986). One possible explanation is that the G proteins directly couple channels and receptors. Such membrane organization would have important implications to both Ca^{2+} channel organization and the definition of structure-activity relationships for ligand modulation of Ca^{2+} channels.

The link between G proteins and VOCs was further strengthened by the work of Scott and Dolphin, (1987a) which demonstrated that the activation of a guanine binding (G) protein is an essential step in coupling certain receptors to the inhibition of VOCs. A residual sustained L-type component of the Ca^{2+} -channel current was resistant to inhibition by internal guanosine 5'-O-3-thiotriphosphate (GTP- γ -S) (Dolphin and Scott, 1987b). Because Ca^{2+} -channel antagonists such as D600, nifedipine and diltiazem inhibit L currents (Nowychy *et al.*, 1985; Rogart *et al.*, 1985), they examined their effect on GTP- γ -S-modified currents. The results represented the first electrophysiological evidence that guanine nucleotides are able to influence the cellular responses to Ca^{2+} -channel ligands.

1.8.1 Classification of channels

In an attempt to classify the different types of Ca^{2+} -channels, Hamilton and Perez (1987), made use of specific toxins. However, a new classification was recently adopted which brings into focus many earlier anomalies (Spedding, 1987). Voltage-operated calcium channels have been differentiated into T (transient), N (neuronal) and L (long lasting) channels.

A small transient current is carried by the T channel which is activated from quite negative membrane potentials. A larger current is carried by N- channels, found only in certain neuronal cells (controlling neuro-transmitter release) T- and L- channels have been identified in most cells tested (L- channels being found predominantly on cell bodies).

The third class of channel –the L-channel– corresponds to the channel described by Reuter in 1967. This slowly inactivating channel, which is only activated from relatively depolarized membranes, is the site of action of verapamil and diltiazem analogues and of the dihydropyridines.

The new classification partially explains the anomaly that although there were many L-type

channels and dihydropyridine binding sites in the brain, dihydropyridines had little effect on neurotransmitter release.

There are at least four binding sites for the drugs in the L-channel. Binding to these is critically dependent on the state of the channel and hence on membrane potential.

The cardiac L-type calcium channel was the first channel known to be modulated by hormones (Reuter, 1984; Tsien *et al.*, 1986). β -Adrenergic receptors which activate the cAMP-dependent protein kinase can increase by 3- to 4-fold, voltage-dependent Ca^{2+} influx via the VOC while cGMP lowers the cAMP levels via the activation of a cGMP stimulated cAMP-PDE (Hartzell and Fischmeister, 1986). Both mechanisms result in a dephosphorylation of the channel and thereby decrease the open state probability. The dihydropyridines are highly specific for L-type calcium channels (Miller, 1987; Triggle and Janis, 1987), and the dihydropyridine Ca^{2+} -agonist Bay 8644 strongly increases the probability of opening of L-, but not N- or T-type channels (Nowycky *et al.*, 1985).

Activators of protein kinase C increase L-type calcium channel current in both Swiss mouse 3T3 and human fibroblasts. Inositol-1,4,5-triphosphate (IP_3) and the inositol- tetraphosphate derivative (IP_4) activate directly a plasma membrane localized calcium channel in T lymphocytes and possibly in other tissues. It is possible that IP_4 is the true and direct activator of the channel since many cells can phosphorylate IP_3 to IP_4 .

1.8.1.1 Biochemistry of L-type calcium channels

The L-type calcium channel has been identified *in vitro* by three of its drug binding sites which are specific for dihydropyridines (e.g. nifedipine), phenylalkylamines (e.g. verapamil) and benzothiazepines (e.g. diltiazem)(Ruth *et al.*, 1985; Triggle and Janis, 1987). These stereospecific sites have a high affinity for each respective group. The binding to one site is regulated allosterically by i) the occupation of the other sites, ii) by divalent cations and iii) by temperature. An example of the three distinct receptor sites and the allosteric reciprocal interaction between them and divalent cation binding sites is shown in **Figure 1.8**.

In addition to these high affinity sites, most tissues contain low affinity, high capacity binding sites for calcium channel blockers which are not located on the L-channels (Oeken *et al.*, 1986; Hofman *et al.*, 1987.)

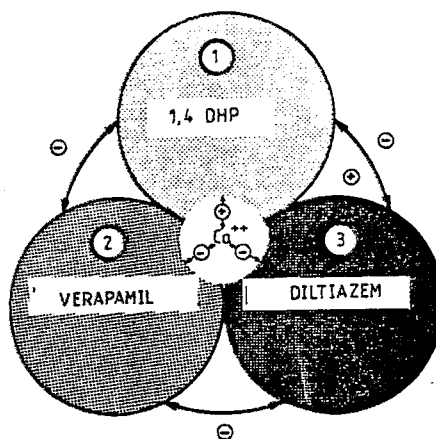


Figure 1.8: *Calcium channels three distinct receptor sites. The allosteric reciprocal interaction (arrows) between the receptor sites and divalent cation binding sites of the channel (adapted from Glossman et al., 1985).*

1.8.2 The L-channel, the CCBs and the pinealocyte

G proteins are involved not only in the hormonal modulation of Ca²⁺-channel activity, but can apparently also modify the effects of Ca²⁺-channel ligands such as D600, nifedipine, and diltiazem on Ca²⁺ currents (Scott and Dolphin, 1987).

These findings raise the question of whether CCBs interact exclusively with the channel protein or if, in addition, they interact with G proteins or other components that participate in the modulation of VOCs. In spite of this uncertainty, the CCBs have been used previously in studies involving melatonin and the pineal gland.

Meyer *et al.* (1986), studied the effects of two dihydropyridines on night-time melatonin serum levels, while more recently Zatz and Mullen, (1988) used CCBs to study the nocturnal release of [¹⁴C]-aMT by chick pineal cells cultured in a (LD 12:12) cycle. At 1 μM the dihydropyridines, nitrendipine and nifedipine, were most effective (virtually abolishing the nocturnal rise in melatonin output). Verapamil inhibited the increase about 50%, and diltiazem had little effect. At higher concentrations [10 μM], diltiazem did inhibit the nocturnal rise (about 50%), whereas verapamil and the dihydropyridines lowered melatonin output to well below daytime levels. However the simplest test of a role for Ca²⁺-influx remains the omission of Ca²⁺ from the

medium. The result of this test was the prevention of most of the nocturnal rise in [^{14}C]-aMT output.

The results of these experiments strongly suggest that influx of Ca^{2+} through VOCs is important in the regulation of melatonin production by chick pineal cells. Again it should be noted that melatonin output reflects synthesis of the lipophilic hormone, rather than exocytotic release of stored material (Zatz, 1982; Klein, 1985).

Omission of Ca^{2+} from the medium, or addition of inorganic Ca^{2+} channel blockers inhibited the melatonin output. This suggests a requirement for extracellular Ca^{2+} .

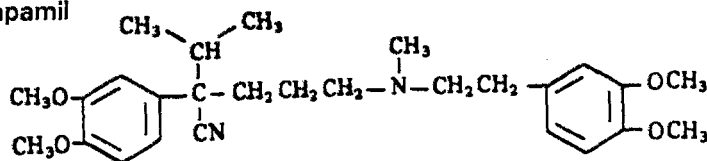
Electrophysiological studies have demonstrated the presence of sustained (L-type) and transient (N-type) Ca^{2+} currents in these cells (Harrison *et al.*, 1987). Nitrendipine, a potent and specific blocker of L-type Ca^{2+} channels (Miller, 1987; Triggle and Janis, 1987), was potent and effective in reducing melatonin output, whereas Bay K 8644, a potent and specific 'agonist' for L-type Ca^{2+} channels, was potent and effective in increasing melatonin output. These results suggest that it is the L-type Ca^{2+} channels that mediate most of the effects of Ca^{2+} on melatonin output. Since nitrendipine and Bay K 8644 were effective at all phase points, it appears that Ca^{2+} -channels in the chick pineal cells are neither maximally 'open' nor maximally 'closed' at any time in the melatonin cycle (Zatz and Mullen, 1988).

1.9 Calcium ion antagonists

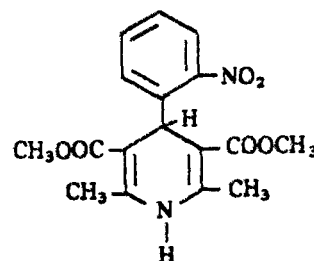
A number of phenomenological criteria are used in identifying Ca^{2+} currents. Amongst these are: that the action potential should be able to be blocked by the metal cations like cobalt (Co^{2+}), lanthanum (La^{3+}) and manganese (Mn^{2+}), or organic Ca^{2+} channel blockers such as verapamil, diltiazem and nifedipine at reasonable concentrations (Hagiwara and Byerly, 1981). According to Fleckenstein, (1977) a calcium antagonist is a drug whose predominant action is the inhibition of Ca^{2+} current in a dose dependent manner.

Since the selective inhibition of the inward Ca^{2+} current is most characteristic of the organic members of the groups of calcium antagonists, and is a better description of their role than the mere antagonism of calcium ions, the terms calcium entry blockers and calcium channel blockers (CCB) are more favoured for this subgroup (Van Zwieten and Timmermans, 1983). The structures of some groups of calcium active drugs and examples thereof discussed in this chapter and used later are shown in Figure 1.9a-h.

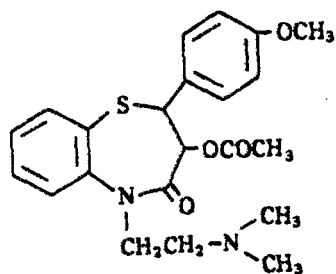
1.9a Verapamil



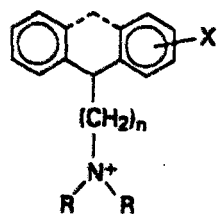
1.9c Nifedipine



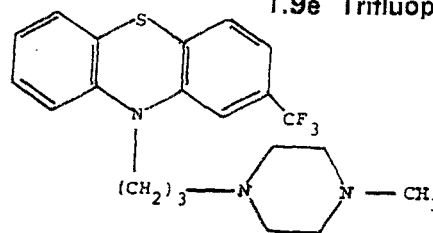
1.9b Diltiazem



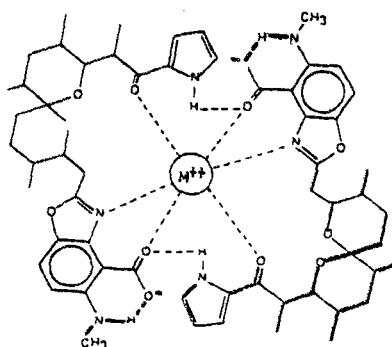
1.9d



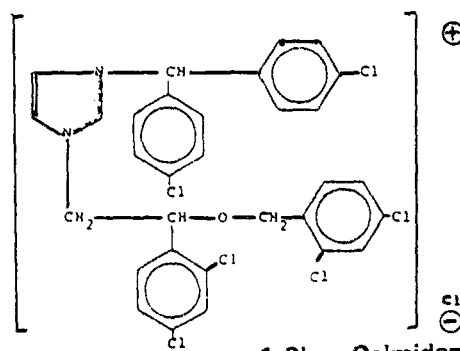
1.9e Trifluoperazine



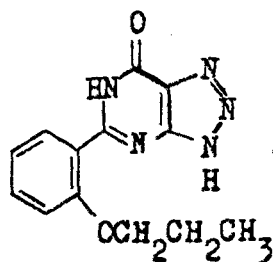
Generalized structural characteristics of calmodulin inhibitors.
Note the similarity to trifluoperazine (1.9e).



1.9f A23187



1.9h Calmidazolium



1.9g M&B 22948

Figure 1.9a-h:

The structures of some groups of calcium active drugs and examples thereof.

1.9.1 Inorganic Ca^{2+} -antagonists

Metal cations like cobalt (Co^{2+}), lanthanum (La^{3+}), manganese (Mn^{2+}) and nickel (Ni^{2+}) have the ability to block several Ca^{2+} -dependent processes, including the Ca^{2+} channel (Triggle, 1981). This appears to be as a result of their ability to substitute on Ca^{2+} binding sites (Triggle and Swamy, 1983).

1.9.2 Organic Ca^{2+} -antagonists

The subgroup of compounds collectively known as calcium channel blockers (CCBs) blocks voltage-dependent calcium channels in a variety of cell types. Of these compounds, the 1,4-dihydropyridines, such as nifedipine and nitrendipine, have become widely used tools to study calcium channels both functionally and biochemically (Brush *et al.*, 1987). Thus while the VOCs have been reclassified, the concept of calcium antagonists blocking Ca^{2+} entry into cells by interacting with voltage-operated Ca^{2+} channels (VOCs or L channels) is still valid.

Following on from the work of Albert Fleckenstein, several groups of compounds were developed that acted reasonably specifically to block Ca^{2+} -channels. These compounds have become known as Ca^{2+} -channel blockers although certain of them (e.g. diphenylalkylamines, such as prenylamine) can exert additional pharmacological effects (Stanfield, 1986 and references therein).

There are three primary groups. These are the phenylalkylamines, such as verapamil (Figure 1.9a) (and its analogues D600 or methoxyverapamil), the benzodiazepines such as diltiazem (Figure 1.9b), and the dihydropyridines, such as nifedipine (Figure 1.9c) and nitrendipine.

1.9.2.1 Binding Sites

Debate continues as to whether the first two groups act at the same site or at a different site in the Ca^{2+} -channel, even though verapamil competitively inhibits diltiazem binding in certain tissues (Murphy *et al.*, 1983). The differences to pH, temperature, thiol-reagents, and 1,4-dihydropyridines in the sensitivity of binding of these two ligand classes make the suggestion of a common site unlikely (Triggle and Janis, 1987). Several studies on purified membranes from skeletal and cardiac muscle (Garcia *et al.*, 1986; Erlich *et al.*, 1985) reveal three distinguishable sites for Ca^{2+} -channel ligands, one each for 1,4-dihydropyridine, verapamil, and diltiazem analogs, and indicate the ratio of these binding sites to each other as being 1:1:1. Less-pure membranes however yield different stoichiometries (Glossman and Ferry, 1985; Reynolds *et al.*, 1986). Both ligands show a substantial use-dependence in their action. Both affect the binding of the third group to the VOC, verapamil reducing and diltiazem enhancing the binding of

nitrendipine (Murphy *et al.*, 1983). The inhibitory effect of verapamil on nitrendipine binding is non-competitive and is believed to involve allosteric effects within the channel protein, the binding site of nitrendipine being separate from that of the two other drugs.

1.9.2.2 Use dependence and allosteric inhibition

The actions of verapamil and diltiazem are use-dependent. On application of the drug there is little or no blockage of Ca^{2+} -current until stimulation has opened channels (Pelzer *et al.*, 1982; Lee and Tsien 1983). The onset of blockage then increases in rate with increasing frequency of stimulation.

The hypothesis of a modified receptor, where the affinity of the channel for a drug depends on the state (open, closed or inactivated) of the channel, is also necessary to account for the action of dihydropyridines, which are lipid soluble compounds acting primarily on L-type channels. Antagonists among this group appear to bind preferentially to the inactivated state, and the difference in affinity between inactivated and closed (that are ready to open) Ca^{2+} -channels is very large (Stanfield, 1986 and references therein).

The binding sites are allosterically coupled such that at 37°C 1,4 dihydropyridine antagonists and diltiazem reciprocally stimulate binding. Verapamil (and phenylalkylamines) and 1,4 dihydropyridines are mutually inhibitory, independent of temperature.

Diltiazem and verapamil also show mutual inhibition of binding. In contrast to the effects of 1,4 dihydropyridine antagonists, activators such as Bay K 8644 inhibit diltiazem binding in heart membranes (Garcia *et al.*, 1986) and stimulate it in brain membranes, but only at high concentrations (Triggle and Janis, 1987 and references therein).

1.10 Intracellular Calcium receptors

The possibility that the intracellular effects of Ca^{2+} might require the presence of a binding protein was first mooted by Meyer *et al.*, (1964). The discovery of more calceproteins (other than troponin C) i.e. proteins that bind Ca^{2+} with an affinity constant with the role of intracellular receptor, shows the wide range of events triggered by Ca^{2+} (Stoclet, 1981). Some of these proteins included parvalbumin, calsequestrin and vitamin D-dependent calcium binding protein.

The low intracellular $[\text{Ca}^{2+}]_i$ is maintained by intracellular buffers as well as the plasmamembrane. Several intracellular structures can take up and bind Ca^{2+} . The mitochondria can take up large amounts of Ca^{2+} and are considered to be buffers of Ca^{2+} , but while this buffer

may act in the short term, proper balancing of extrusion versus Ca^{2+} -entry must still occur (Godfraid, 1980).

1.10.1 Calbindin-27 kDa

This calcium binding protein was first reported by Wasserman and Taylor in 1966. The exact role of calbindin-27 kDa or vitamin D-dependent calcium binding protein is not clearly established, but the present view is that it acts as a buffer to take up the increased Ca^{2+} levels occurring in response to physiological stimuli. Its presence has been demonstrated in both the pineal organ and retina of humans (Roman *et al.*, 1988).

A study by Krstic (1988) showed a lack of calbindin-immunoreactivity in the pineal bodies of the Mongolian gerbil (known to form acervuli), but a considerable amount of calbindin-reactive cells in the pineal bodies of rats (which do not form acervuli). He suggested that calbindin might have a special role in the pineal gland, namely the removal of Ca^{2+} which enters during stimulation.

1.10.2 Calmodulin

While the above proteins appear as specialized molecules, one calciprotein has retained its structure and is found in both the plant and animal kingdoms. Calmodulin is an ubiquitously occurring, thermally stable, Ca^{2+} -binding protein of 16,500 daltons, containing 148 amino acid residues in a single polypeptide chain. It has now been implicated in conferring calcium sensitivity (i.e. Ca^{2+} -dependent activation) to ca 30 different target proteins (for reviews see Means and Dedman, 1980; Cheung, 1980; Stoclet, 1981; Manalan and Klee, 1984), and thus plays the role of multifunctional intracellular Ca^{2+} receptor.

In purification procedures, use is made of its thermal stability in the presence of Ca^{2+} and its acidic nature. It is in fact these properties which lead to its discovery [§ 5.1.3].

It is well established that calmodulin contains four calcium binding sites (Dedman *et al.*, 1978) with a dissociation constant within the range of 10^{-6}M (Klee, 1977; Wolff *et al.*, 1977).

On calcium binding (two Ca^{2+} ions per mole) to the four Ca^{2+} -binding sites, large structural changes, which include increases in α -helix and intrinsic tyrosine fluorescence, occur. This results in the ^{exposure/}formation of hydrophobic ^{drug/}protein-binding sites (LaPorte *et al.*, 1980; Johnson and Wittenauer, 1983), probably the most significant calcium-dependent event to occur on the surface of calmodulin.

These resultant interfacial sites are thought to be the sites where CaM interacts with its target proteins and with inhibitory drugs (calmodulin antagonists).

1.10.2.1 Allosteric control

The work by Johnson (1983) was the first to show that CaM was an allosteric protein with respect to its drug/protein binding site. Using felodipine, which fluoresces on binding to the sites, he was able to show other drugs e.g. R24571 and prenylamine binding to- and enhancing felodipine. In the case of CaM drug-induced potentiation of felodipine binding, the potentiating drugs bind to CaM only in the presence of Ca^{2+} , and produce their potentiated state by binding to sites which are distinct from the calcium-binding sites on CaM (Johnson, 1983; Mills and Johnson, 1985).

The two Ca^{2+} -dependent hydrophobic domains exert allosteric control over one another. Consistent with these findings, Inagaki and Hidaka (1984) have suggested that prenylamine and butaclamol bind to one class of binding sites on calmodulin, while trifluoperazine binds preferentially to a distinct site. It is apparent that some, but not all, of the drug binding sites on calmodulin and its target protein binding sites are mutually exclusive. Although the relationship between the interaction site(s) is unclear (Johnson and Mills, 1986), it is thought that allosteric control may provide one mechanism by which CaM can control so many other enzymes.

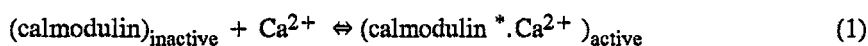
Mills and Johnson, (1985) have also done an interesting study on capital (C) and auxiliary (A) sites. For the binding of the 1,4-dihydropyridine derivative - felodipine - to occur, the Ca^{2+} -binding (C) sites must be filled, leading to the formation of the allosteric conformer. A further requirement is that the A sites are also occupied. Occupancy of A sites in the resting cell (empty C sites) induces subtle conformational changes which could abolish certain hydrophobic interactions of CaM (Milos *et al.*, 1987). In the presence of Ca^{2+} (filled C sites) occupancy of the A sites seems to modify the mode of interaction of CaM with drugs and enzymes (Mills and Johnson, 1985). This therefore suggests a further means of control (Cox, 1988).

An intriguing possibility is that these interactions stabilize a preformed complex between CaM, an adjacent enzyme, and its substrate, and would facilitate rapid activation in response to increased Ca^{2+} concentration. Most models accounting for modulation of the Ca^{2+} signal by CaM, involve the implicit assumption that free CaM is present in some tissues in a limited amount. Despite this, estimates of the total levels of CaM-binding proteins in these tissues are high. There is additional evidence suggesting that a redistribution of CaM or its target proteins from one cellular compartment to another may play an important role in mediation of the Ca^{2+} signal (Nehmad *et al.*, 1982; Saitoh and Schwartz, 1983). This difficulty in knowing exactly where the CaM is present, makes any inferences concerning local concentrations rather difficult (Manalan

and Klee, 1984).

1.10.2.2 Ordered calcium binding hypothesis for calmodulin

The mode of action of CaM was first established with the phosphodiesterase system. Calmodulin itself is not active; its active form is the CaM-Ca²⁺ complex. Once bound to Ca²⁺, CaM assumes a more helical conformation to become the active species, which binds reversibly to the apoenzyme of PDE, resulting in the formation of an active holoenzyme (Haiech *et al.*, 1981). The sequence of events associated with the stimulation of PDE by calmodulin may be depicted as follows:



where E stands for the apoenzyme and the asterisk (*) for a new conformation. According to this scheme, the cellular flux of Ca²⁺ plays a crucial role in regulating PDE activity.

1.10.2.3 Calmodulin as dual regulator of adenylyl cyclase and cAMP-PDE

Amongst the many enzymes activated by CaM, are both adenylyl cyclase and cAMP-PDE. It appears odd that the same activator can be required for both activation and destruction within one system. It appears however, that the activation of adenylyl cyclase takes place at much lower concentrations of Ca²⁺ than those required for the activation of cAMP-PDE. Thus once the optimal Ca²⁺ concentration for PDE activation is reached, adenylyl cyclase is already being inhibited. As shown in Figure 1.10, there is also a partly sequential effect. Adenylyl cyclase is located in the plasma membrane and is thus activated as the first Ca²⁺ influx occurs. The Ca²⁺ must then penetrate to the cytoplasm to reach the PDE found there (Marme, 1981; Boehringer, 1983).

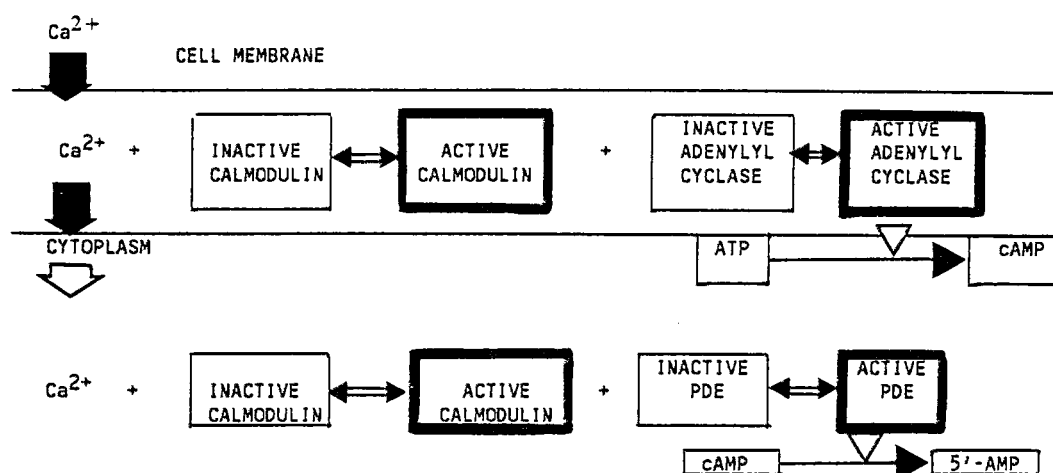


Figure 1.10: *The sequential activation of adenylyl cyclase and cAMP-PDE in response to Ca^{2+} influx through the plasma membrane and into the cytoplasm. (Adapted from Cheung, 1982).*

1.10.2.4 Calmodulin antagonists

Because of the increasing interest in CaM and the enzymes it controlled, several approaches were used to prove its control, with varying degrees of success.

Cheung (1980), proposed several criteria to prove that a process is CaM-regulated: 1) the tissue or cell must possess sufficient CaM in the appropriate locale, 2) depletion of endogenous CaM should render the experimental system susceptible to exogenous CaM, 3) sequestering Ca^{2+} in the reaction system should return the CaM-induced activity to basal level, 4) addition of trifluoperazine should return the CaM-dependent activity to the steady-state level, and 5) the effect of CaM should be reversed by its antibody (Kranias *et al.*, 1980).

However, because of the nature of the binding sites, a number of different types of pharmaca have been found to bind to CaM in a Ca^{2+} -dependent manner. These include phenothiazines, tricyclic antidepressants and naphthalene.

1.10.2.4.1 Cation binding and antagonism

In addition to drug-binding sites, CaM has metal-binding sites. Not only calcium but lanthanum (La^{3+}), terbium (Tb^{3+}), lead (Pb^{2+}), samarium (Sm^{3+}), strontium (Sr^{2+}), mercury (Hg^{2+}), cadmium (Cd^{2+}), zinc (Zn^{2+}), and manganese (Mn^{2+}) have been shown to interact with calmodulin (Wallace *et al.*, 1982; Wang *et al.*, 1984; Chao *et al.*, 1984). Some of these metals can compete for the Ca^{2+} binding sites on CaM while others may have effects on CaM's

conformation beyond those produced by Ca^{2+} alone e.g. magnesium (Mg^{2+}), nickel (Ni^{2+}), Co^{2+} and barium (Ba^{2+}) do not activate CaM, but La^{3+} , Sm^{3+} and Tb^{3+} are effective activators (Luchowski *et al.*, 1984).

The ability of metal cations to inhibit Ca^{2+} binding is related to their ionic radii. In general, the closer the radius of a metal cation was to that of Ca^{2+} , the more effective was that cation as a substitute for Ca^{2+} . The range of effective ionic radii was approximately $1 \pm 0.2 \text{ \AA}$. CaM-stimulated PDE activity by the cations can be reversed by trifluoperazine (TFP), an antagonist of CaM (Chao *et al.*, 1984).

1.10.2.4.2 Trifluoperazine

Since the initial observation by Weiss *et al.* (1974) on the interaction of TFP with CaM, this drug has been used frequently to prove the role of CaM in cell processes. The weakness of such evidence is that high concentrations [10^{-5}]M are needed, and these are several orders of magnitude greater than the drugs affinity for dopamine and serotonergic receptors (Landry *et al.*, 1980; Van Belle, 1981).

The development of specific inhibitors of either CaM or PK-C activity is needed to elucidate the physiological role of each pathway in response to different external stimuli.

Although the interaction of neuroleptic agents such as TFP with CaM is unrelated to the clinical efficacy of these agents (Norman *et al.*, 1979), this observation has provided investigators with a probe for CaM function in isolated cell systems. However, when Schatzman *et al.*, (1981) reported that TFP as well as other neuroleptic agents are inhibitors of PK-C activity, its use declined (Norman *et al.*, 1986).

1.11 Introduction to the Potentiation and Ca^{2+} phenomenon

Interest in Ca^{2+} and its growing role in the physiological regulation of pineal function by NA resulted in much new research (Vanecek *et al.*, 1985; 1986, Sugden *et al.*, 1985; 1986), especially because of the unusual synergistic mechanism involving α_1 - and β - adrenoceptors, through which NA regulates pinealocyte cAMP and cGMP (Klein *et al.*, 1983; Vanecek *et al.*, 1985). β -Adrenergic stimulation is an absolute requirement in both cases, alone producing a 10-fold increase in cAMP and a 3-fold increase in cGMP. α_1 -Adrenergic activation alone produces no detectable change in either cyclic nucleotide. It potentiates β -adrenergic stimulation of cAMP about 10-fold, apparently through a Ca^{2+} - / phospholipid-dependent protein kinase - PK-C -

(Sugden *et al.*, 1985), and that of cGMP, about 100-fold.

Both *in vivo* and *in vitro* experiments with the α_1 -agonist phenylephrine (PE) in the rat pineal gland demonstrated potentiation and prolongation of the stimulating effect of the β -agonist ISO on NAT activity (Klein *et al.*, 1983; Alphas and Lovenberg, 1984), cAMP (Vanecek *et al.*, 1985) and cGMP accumulation (Vanecek *et al.*, 1986), and even low K_m cAMP-PDE (Vacas *et al.*, 1985). Two other α_1 -adrenoceptor agonists were tested to see if other more specific α_1 -agonists (apart from PE) would also potentiate β -adrenergic stimulation. 6-Fluoronorepinephrine and cirazoline were chosen. Results obtained with these two drugs confirmed that NA-regulated indole metabolism could be mimicked by other α_1 -adrenoceptor agonists (Sugden *et al.*, 1984).

This potentiation was found even after degeneration of the pineal sympathetic nerves, thus indicating it's essentially postsynaptic nature.

1.11.1 Essential role of Ca^{2+} influx in the pineal

Work by Sugden and co-workers, (1986; 1987) showed an essential role for Ca^{2+} influx in the adrenergic regulation of both cAMP and cGMP. Chelation of external Ca^{2+} with EGTA, or inhibition of Ca^{2+} influx with inorganic Ca^{2+} channel blockers (La^{3+} , Co^{2+} or Mn^{2+}) reduced the cyclic nucleotide response to NA, but not to ISO, a β -adrenergic agonist. α_1 -Adrenergic effects could be mimicked by agents which elevated cytosolic Ca^{2+} levels, e.g. A23187, when used together with β -adrenoceptor stimulation, while these agents had no effect alone.

They concluded that $[\text{Ca}^{2+}]_i$ was raised, not from internal Ca^{2+} supplies, but that external Ca^{2+} supplies ($[\text{Ca}^{2+}]_o$) were required. They demonstrated unequivocally that pinealocyte $[\text{Ca}^{2+}]_i$ was increased 7-fold as a result of a net influx of Ca^{2+} , brought about by a mechanism involving NA acting through α_1 -adrenoceptors: prazosin was able to block the α_1 effect, while yohimbine did not influence the effect.

Initial support for a role of Ca^{2+} in pineal adrenergic regulation had come from observations in *in vitro* glands which showed that i) the removal of Ca^{2+} from the culture medium resulted in the inhibition of the NAT and cAMP increases which would normally follow adrenergic stimulation (O'Dea and Zatz, 1976; Zatz and Romero, 1978), ii) the calcium ionophore A23187 was able to mimic the effects of α_1 -adrenergic stimulation on the cyclic nucleotides in rat pinealocytes stimulated with ISO (Vanecek *et al.*, 1986), and iii) the potentiation of cAMP was dependent on the activation of the Ca^{2+} -regulated PK-C (Sugden *et al.*, 1985).

Sugden *et al.* (1987), suggested the Ca^{2+} concentrations in rat pinealocytes were influenced by two NA-refractory mechanisms, namely, voltage-dependent calcium channels and the $\text{Na}^+/\text{Ca}^{2+}$ exchange. Previously, Meyer *et al.*, (1986) had demonstrated that the *in vivo* injection of dihydropyridine calcium antagonists was able to decrease the amplitude of the nighttime melatonin rise in baboons, thus supporting the possible role of a VOC.

In experiments to examine whether the effects of A23187 and K^+ were dependent upon Ca^{2+} influx, $[\text{Ca}^{2+}]_o$ was reduced by adding EGTA. This blocked the effects of A23187 on PK-C redistribution, as well as cAMP and cGMP accumulation in ISO-treated cells. Secondly, voltage-sensitive Ca^{2+} channels were blocked with nifedipine, which has been found to block the stimulatory effects of K^+ on $[\text{Ca}^{2+}]_i$ in pinealocytes (Sugden *et al.*, 1987) and to inhibit the amplifying effects of K^+ on cAMP and cGMP levels in ISO-treated pinealocytes (Sugden *et al.*, 1986). Nifedipine was able to block the K^+ -mediated redistribution of PK-C. EGTA and nifedipine were more potent in inhibiting the cGMP response than the cAMP response, reflecting the greater Ca^{2+} dependency of the cGMP response, which is probably not related to PK-C (Ho *et al.*, 1988).

Although it appeared that PK-C was involved in the α_1 -adrenergic potentiation of β -stimulated cAMP, the same did not seem to apply to cGMP (Sugden *et al.*, 1985). Ho *et al.*, (1987), investigated this and found that cGMP levels could be potentiated in β -stimulated pinealocytes, with the PK-C activator 4 β -phorbol 12-myristate 13 acetate (PMA), provided that Ca^{2+} levels had been raised with e.g. A23187. Similarly PMA stimulated cGMP levels in cells treated with cholera toxin and A23187, but not in cells treated with just cholera toxin. Thus it seemed that PK-C mediated (at least in part) the α_1 -mediated potentiation, and that Ca^{2+} was also necessary for cGMP potentiation.

Arachidonic acid (AA) production also appears necessary for cGMP stimulation, as the phospholipase A_2 inhibitor, mepacrine, blocks the adrenergic stimulation of cGMP, but not that of cAMP (Vanecek *et al.*, 1986). Of further interest is i) that stimulation of AA production by NA is Ca^{2+} -dependent (Gilman, 1984) while ii) the metabolism of AA in the pineal is unusual in that it generates lipoxygenase products at 20-fold higher rates than other tissues (Yoshimoto *et al.*, 1984). Although roles have been suggested for prostaglandins in NA-induced melatonin synthesis, the exact role of the lipoxygenase pathway is not that clear (Vacas *et al.*, 1987). Inhibition with the lipogenase inhibitor NDGA, suggest that it is involved. However this may also inhibit other systems e.g. cyclooxygenase. As indomethacin has been shown to impair pineal activation (Cardinali *et al.*, 1982), this may only be a partial effect. Rozengurt *et al.*, (1983) have shown indomethacin to inhibit the conversion of AA to prostaglandin E in 3T3 cells, hence

inhibiting the normal increase in cAMP in these cells.

The fact that PK-C activators could potentiate the effects of cholera toxin stimulation of cGMP suggests that the PK-C acts with the cGMP system at a point distal to the adrenoceptor, bringing in the possibility of the GTP-binding protein having a role in cGMP levels. β -Adrenergic stimulation of pinealocyte cAMP is known to involve *Ns* (Minneman and Iverson, 1976; Vanecek *et al.*, 1986), the stimulatory GTP-binding regulatory protein which couples receptor occupation to the activation of adenylyl cyclase (Hurley *et al.*, 1984). Cyclic-GMP is probably regulated by the α subunit of a related GTP binding protein, $G_s\alpha$ (Sugden and Klein, 1987).

1.11.2 The role of phosphoinositides in signal transduction

1.11.2.1 Introduction

A compelling amount of evidence indicates that the ubiquitous inositol lipid signalling pathway has a key role in neuro-transmission and neuro-modulation initiated by α_1 -adrenoceptor with the cell membrane (Exton, 1985).

Bob Michell (1975) first called attention to two points of circumstantial evidence that suggested a causal role for the inositol lipids and the mobilization of cellular Ca^{2+} : 1) whenever receptors were linked to PI turnover, these same receptors were also known to activate Ca^{2+} mobilization – PI turnover never, for example, occurred when receptors activated adenylyl cyclase; and 2) activation of PI turnover itself did not appear to be a consequence of Ca^{2+} mobilization, as evidenced by the relative insensitivity of the process to experimental regimens which depleted cellular Ca^{2+} , and by the inability of the Ca^{2+} ionophores to stimulate turnover. A characteristic feature of this inositol lipid receptor mechanism is that the transduction process has a bifurcation point leading to the generation of two intracellular second messengers. The widely-accepted cascade is thus: the precursor lipid (phosphatidylinositol 4,5-biphosphate IP_3), located within the inner leaflet of the plasma membrane, is cleaved in response to α_1 -agonists (by the receptor-activated phospholipase C [PLC] in the plasma membrane which hydrolyses the PI) to yield the second messenger couple of *sn*-1 2-diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP_3).

The latter IP_3 has been identified as a signal for intracellular Ca^{2+} mobilization (Berridge, 1984)(probably from within the endoplasmic reticulum), whereas DG is known to activate PK-C (Nishizuka, 1984). Additionally, increases in Ca^{2+} influx, either through the opening of a Ca^{2+} "gate" in the cell membrane (as seen in smooth muscle) or through an ouabain-sensitive Na^+/Ca^{2+} exchange mechanism, have been described in most tissues after α_1 -adrenergic stimulation (Exton, 1985).

The IP₃ and DG branches of the phosphoinositide cascade appear to act synergistically to phosphorylate proteins, the former by elevating Ca²⁺ to activate calmodulin-dependent protein phosphorylation and the latter to activate PK-C, which phosphorylates a different set of proteins (Seiler *et al.*, 1987). The elevated Ca²⁺ may also activate reactions not dependent on calmodulin (Sekar and Hokin, 1986). The predominant event; the rises in IP₃ and DG appear to be necessary for maximal responses.

1.11.2.2 Events in the pineal

Thus, while this gives an outline of the events, there are several reviews dealing with the various aspects of polyphosphoinositide metabolism and its significance *vis-a-vis* receptor activation (for references refer to Sekar and Hokin, 1986). It would however, still seem pertinent to briefly cover those aspects relevant to the pineal gland, and the possible mechanisms involved in its observed potentiation.

Briefly, α₁-adrenergic stimulation has been shown to potentiate β-adrenoceptor stimulation by a variety of complex mechanisms. Some of those implicated (and which are present in the pineal) are an increase in intracellular Ca²⁺ levels which is associated with, or may even be essential to, i) the activity of pineal phospholipase C (Smith and Hauser, 1981), ii) the activity of phospholipase A₂ (Ho and Klein, 1987), and iii) the translocation and activation of PK-C (Sugden *et al.*, 1985). Figure 1.11 shows a diagrammatic representation of the presumed signal transduction mechanisms.

Reports that the activation of the α₁-adrenergic receptors induced changes in PI turnover originated some time ago (Hauser *et al.*, 1974; Nijjar *et al.*, 1980). The mechanism through which the α₁-adrenoceptor induced potentiation occurred appeared to involve a net Ca²⁺ influx and the phospholipids.

The net influx of Ca²⁺ into the pinealocyte has already received much attention. α₁-Adrenoceptor stimulation also leads to PI turnover, an increase in the activity of PLC, and the generation of the intracellular messengers DG (Smith *et al.*, 1979; Zatz, 1985) and inositol phosphates (Berridge, 1986).

The direct demonstration of receptor-mediated increases in inositol phosphates is facilitated by the use of the anti-manic ion, lithium. This blocks the breakdown of inositol monophosphate and subsequent recycling, permitting direct measurement consequent to phospholipid breakdown (Hallcher and Sherman, 1980; Zatz, 1985).

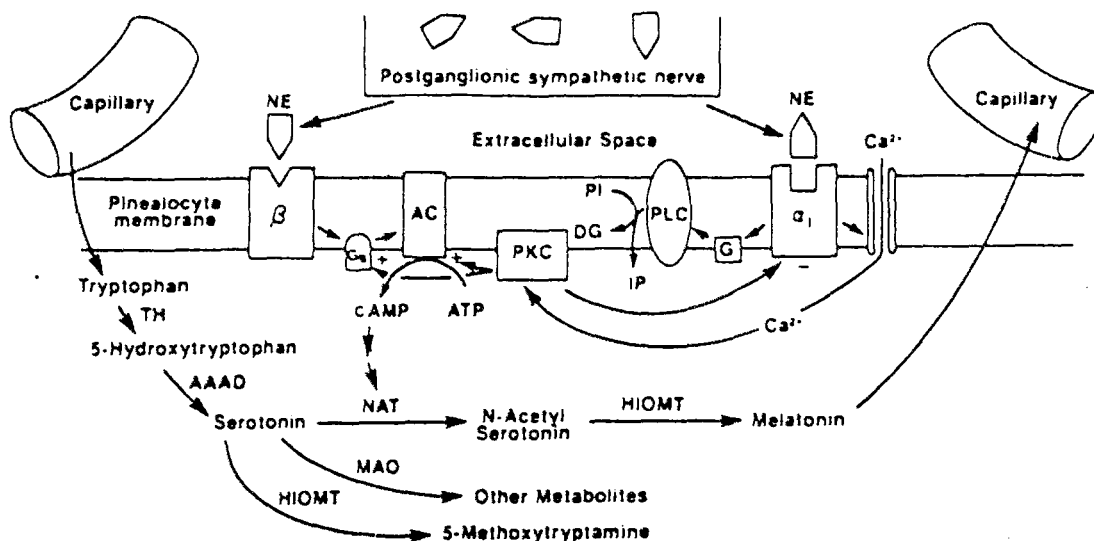


Figure 1.11:

Diagrammatic representation of the presumed signal transduction mechanisms between the postganglionic sympathetic neurons and the mammalian pinealocyte.

KEY: AAAD = *l*-aromatic amino acid decarboxylase, AC = adenylyl cyclase, ATP = adenosine triphosphate, cAMP = adenosine 3':5'-monophosphate, DG = diacylglycerol, G = guanine nucleotide-binding protein, G_s = stimulatory G, HIOMT = hydroxyindole-O-methyltransferase, IP = inositol phosphate, MAO = monoamine oxidase, NAT = N-acetyltransferase, NE = noradrenaline, PI = phosphatidylinositol, PK-C = protein kinase-C, PLA_2 = phospholipase A_2 , PLC = phospholipase C, TH = tryptophan hydroxylase, α_1 - = α -adrenoceptor and β - = β -adrenoceptor. (Adapted from Reiter, 1991a).

In the pineal the increase in $[Ca^{2+}]_i$ and DG are thought to translocate and activate PK-C (Nishizuka, 1984). Sugden and co-workers, (1985) proposed that DG might be the second messenger mediating this effect, their reason being that a synthetic DG, 1-oleoyl-2-acetyl-glycerol (OAG), was able to mimic the α_1 -adrenergic stimulation. The site of potentiation appeared to be after β -adrenoceptor activation, as a similar potentiation could be produced by PE in cells stimulated with cholera toxin. Phorbol esters are also able to potentiate the effects of the cholera toxin (Klein, 1985). The phorbol ester, PMA, which can directly activate PK-C, is able to mimic the potentiating effects of α_1 -agonists (Sugden and Klein, 1988).

1.11.3 Translocation

Following the demonstration of translocation / redistribution it became of interest to determine the subtype of adrenoceptor involved in the adrenergic translocation (increased membrane association) of PK-C. Four adrenergic agonists were tested, including the mixed α_1 -, β -adrenergic agonist NA and the α_1 -adrenergic agonist phenylephrine (PE). The findings were consistent with the interpretation that α_1 -adrenoceptors were involved (Sugden *et al.*, 1985).

The mechanism involved in translocation by NA, K^+ , and A23187 does not appear to be precisely the same. In the case of α_1 -adrenoceptor-mediated translocation, it appears that translocation may occur as a result of both the increase in Ca^{2+} influx and in DG production.

There are two lines of evidence that suggest DG is involved. Firstly, in prior studies α_1 -adrenergic activation of the pineal gland has been found to produce a gradual increase in PLC activity (Zatz, 1985; Ho *et al.*, 1987) and secondly, it was found that NA caused a gradual increase in [3H]-diacylglycerol kinase. Thus it appears reasonable to suspect that both the increases in Ca^{2+} and in DG accumulation could be important for translocation of PK-C by adrenergic agonists, and that Ca^{2+} and DG act synergistically to regulate PK-C, as seen in cell-free systems.

From the above data, it seems most conservative to assume that at least two classes of PK-C translocating agents exist. One acts through an increase in Ca^{2+} influx and is characterised by A23187 and K^+ . The second depends upon both an increase in Ca^{2+} influx and in DG levels and could include NA and PE.

The fact that Ca^{2+} influx alone can trigger redistribution of PK-C in the intact cell, is of fundamental importance because it emphasizes that PK-C may be activated in many systems by transmitters, hormones, or other agents which elevate Ca^{2+} influx, but which may have no other direct effects. Thus, Ca^{2+} influx might be the critical element regulating the degree of membrane association of PK-C. Accordingly, results obtained from studies in which cells are treated with agents that increase Ca^{2+} influx must be interpreted with this in mind (Ho *et al.*, 1988).

1.11.4 The individual participants in the cascade

1.11.4.1 Protein kinase C

Protein kinase C is the enzyme most linked to the potentiation. The reasons for suspecting this

come from the fact that i) PE has been shown to translocate PK-C (Sugden *et al.*, 1985), ii) activators of PK-C mimic the potentiating effects of PE (Ho *et al.*, 1987), and iii) that an inhibitor of PK-C inhibits the potentiation (Ho *et al.*, 1988).

When PK-C was first found, in 1977, as a proteolytically activated protein kinase in many tissues, it had no obvious role in signal transduction (Inoue *et al.*, 1977). Later, it was shown to be a Ca^{2+} -activated, phospholipid-dependent enzyme (Takai *et al.*, 1979), and was firmly linked to signal transduction. This was demonstrated by the fact that DG, one of the earliest products of signal-induced inositol phospholipid breakdown, greatly increased the affinity of PK-C for Ca^{2+} , thereby rendering it fully active (Nishizuka, 1984), without a net increase in Ca^{2+} . Thus the activation is biochemically dependent on Ca^{2+} , but physiologically independent of Ca^{2+} concentration (Kikkawa and Nishizuka, 1986).

During cell stimulation, PK-C is transiently activated by the DG produced in the membrane during signal-induced PI turnover. (Similarly, PMA and other tumour-promoting phorbol esters may directly stimulate PK-C). During such activation PK-C, which is mainly a cytosolic enzyme, is translocated to the cell membrane (Sekar and Hokin, 1986).

Sugden (1989b), studied the developmental appearance of PK-C in the rat pineal gland. He found it to be already detectable before birth in both cytosol and membrane fractions, reaching full activity ca 30 days post nately. His study further indicated i) that the PK-C was located in the pinealocyte and not the presynaptic adrenergic terminals, and ii) that adrenergic stimulation was not required to maintain high levels of PK-C activity within the pineal.

Phenylephrine is known to stimulate the translocation of PK-C in rat pinealocytes (Sugden *et al.*, 1985).

Activation of the latter receptor is also known to produce a sustained increase in $[\text{Ca}^{2+}]_i$ by increasing net influx (Sugden *et al.*, 1985), pointing to the possible importance of Ca^{2+} influx in the subcellular redistribution (activation) of PK-C in intact cells. Ho *et al.*, (1988), in fact established that an increase in Ca^{2+} influx was sufficient to trigger translocation of PK-C. In addition, a very close correlation existed between the translocation of PK-C by PE, K^+ , and A23187 and their ability to potentiate β -adrenergic stimulation of cAMP and cGMP accumulation.

This adds further support to the proposal that the translocation of PK-C is required for the potentiation of β -adrenergic stimulation of pinealocyte cAMP and cGMP accumulation.

1.11.4.2 Diacylglycerol

The neutral lipid diacylglycerol is normally absent from membranes. On receptor stimulation, it appears transiently and disappears within seconds, due to its conversion back to inositol phospholipids and its degradation to arachidonic acid (AA), which in turn can generate other messengers e.g. the prostaglandins.

Synthetic DGs having the 2,3-*sn* configuration are not active in intact cell systems (Nomura *et al.*, 1986). Tumour-promoting phorbol esters have a 1,2-*sn* configuration, similar to DG, and are thus able to activate PK-C on a 1:1 basis (Kikkawa *et al.*, 1983).

The DG formation resultant from PI breakdown, following α_1 -adrenergic stimulation, and the subsequent effects on cyclic nucleotides (Sugden *et al.*, 1985b) and NAT activation (Zatz 1985b) have been adequately demonstrated in rats. Both cyclic nucleotide-dependent and Ca^{2+} -dependent protein phosphorylation takes place in rat pineal glands (Cimino *et al.*, 1987).

1.11.4.3 Phorbol esters

Studies from Nishizuka (1984) and Castagna *et al.*, (1982), have shown the PK-C is a target for phorbol esters because the tumour promoters can substitute for DG and directly activate PK-C.

It appears that tumour-promoting phorbol esters e.g. PMA, as well as 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), can activate the DG site of PK-C in intact cells. This has facilitated the probing of PK-C function (Sekar and Hokin, 1986). These esters have been shown to activate PK-C in the pineal gland as well.

1.11.4.4 Phospholipase A₂

Another well-studied consequence of increased $[\text{Ca}^{2+}]_i$ levels is the activation of phospholipase A₂ (Exton, 1985). Several of the arachidonic acid (AA) metabolites produced by this enzyme after the cleavage of inositol phospholipids, e.g. prostaglandins (PGs), thromboxanes, and leukotrienes, may participate in mediation or modulation of the final response of the pineal cells to the α_1 -adrenergic transmitter. For example, inhibition of PLA₂ by mepacrine blocked NA stimulation of cGMP but enhanced NA stimulation of cAMP in rat pinealocytes (Vanecek *et al.*, 1986).

An interesting possibility is that while both α_1 -adrenergic agonists and PK-C activators stimulate

PLA₂ and AA generation, only α_1 -adrenergic agonists (but not PK-C activators) can stimulate the conversion of AA to an active metabolite through a Ca²⁺-dependent mechanism. This is especially attractive because Ca²⁺-dependent processes appear to play a larger role in the pineal cGMP response to NA than the cAMP response (Sugden *et al.*, 1986). If this explanation is correct, it should be possible to experimentally potentiate the cGMP response to PK-C activators in β -adrenergically treated pinealocytes with agents that elevate [Ca²⁺]_i.

In addition to these PK-C-dependent mechanisms, it is possible that NA stimulates PLA₂ through mechanisms which do not involve PK-C. One possible such mechanism involves an increase in [Ca²⁺]_i. Pinealocyte [Ca²⁺]_i is increased by NA acting through α_1 -adrenoreceptors (Sugden *et al.*, 1987). The studies reviewed above clearly indicate that the NA stimulation of pineal PLA₂ activity is Ca²⁺-dependent and can be elicited by a Ca²⁺ ionophore. Phospholipase A₂ is known to be a Ca²⁺-dependent enzyme (Van den Bosch, 1980), and it is thus not impossible that an increase in [Ca²⁺]_i is involved in the NA stimulation of PLA₂ activity.

In summary, the evidence presented indicates that NA stimulates the pineal PLA₂. The sequence of events appears to be α_1 -receptor elevation of Ca²⁺ influx and PLC activation, leading to an elevation of [Ca²⁺]_i and DG. These events together seem to activate PK-C, which could activate PLA₂ by phosphorylating lipocortin (Touqui *et al.*, 1986), and thus removing the inhibitory effect of this PLA₂ regulator, or by stimulating Na⁺/H⁺ exchange (Besterman *et al.*, 1985), or a combination of these effects. In addition, the increase in [Ca²⁺]_i produced by NA may be involved in the stimulation of PLA₂ through PK-C-independent mechanisms (Ho and Klein, 1987).

1.11.4.5 Phospholipase-C

Another enzyme associated with phospholipid metabolism and transmitter receptors is phospholipase-C (PLC) (Berridge, 1984).

α_1 -Adrenergic stimulation increases PI turnover (mainly inositol monophosphate) in rat pinealocytes (Zatz, 1985a). Ho *et al.*, (1987) in work with rat pinealocytes have shown this to be mediated via the Ca²⁺-dependent activation of PLC.

Stimulation of this Ca²⁺-dependent enzyme catalyses the conversion of PIP₂ to IP₃ and DG. As with the cyclic nucleotide system, the receptor-mediated stimulation of PLC is probably associated with a guanine nucleotide binding protein (Wallace and Fain, 1985).

Once formed, IP_3 and DG serve as second messengers, with the former liberating intercellular stores of calcium ion and the latter stimulating the calcium-activated, phospholipid-dependent enzyme, PK-C. Like the cAMP-dependent protein kinase, PK-C modifies cellular activity by catalysing the phosphorylation of various substrates.

Studies by Ho *et al.*, (1987), studied the effect of ouabain on PLC activity in dispersed pinealocytes, because of the reported increase in Ca^{2+} influx and PLC activity. Smith *et al.*, (1978) have previously reported that NA can stimulate PLC via an action on the α_1 -adrenoceptor, hence ouabain was compared to NA, but proved about 10-fold less potent. Prazosin however blocked NA effects, but not those of ouabain, showing that different mechanisms were involved. EGTA prevented both treatments working, confirming the Ca^{2+} dependence of PLC. One implication of this study is that if high levels of ouabain are employed experimentally, activation of PLC could occur, with subsequent generation of the second messengers DG and the inositol phosphates, and possibly even PK-C.

1.11.5 Interaction with other signalling systems

1.11.5.1 Classes of signalling systems

Signalling systems can be sub-divided. One class depends only on cAMP generation as its second messenger, while the other induces PI turnover and Ca^{2+} mobilization. This then leads to the release of arachidonate (AA) and increases in cGMP. Thus PK-C activation, Ca^{2+} mobilization, AA release and cGMP formation appear to be integrated into a single receptor cascade (Kikkawa and Nishizuka, 1986).

In *bidirectional* control systems the two classes of receptor system counteract each other, whereas in *monodirectional* systems, the one class of receptors may potentiate the other. The pineal is considered to belong to the latter group of control systems. This is supported by work from Zatz (1985), which showed that the phorbol esters could mimic α_1 -adrenergic stimulation in the pineal, supporting the role of PI turnover and PK-C in the α_1 -adrenoceptor stimulation. He favours a feedback mechanism to β -adrenoceptors, as PE and PMA potentiate rather than stimulate, and under conditions of maximal stimulation, they fail to potentiate, suggesting a convergent system.

The PK-C presumably potentiates the adenylyl cyclase system, or acts co-operatively with protein kinase A. Arachidonate is derived from phospholipids, initially through a PLC mechanism and subsequently a PLA_2 mechanism.

1.11.5.2 Phospholipase A₂ and Protein kinase C

Enna and Karbon, (1987) suggest that PLA₂ and PK-C may be linked, especially because of the finding that α₁-adrenergic agonists stimulate both PI turnover and augment β-adrenergically mediated cAMP in rat pinealocytes. A variety of fatty acids including AA are capable of directly stimulating PK-C (McPhail *et al.*, 1985; Murakami and Routtenberg, 1985). If this occurred *in vivo*, then stimulation of PLA₂, and the liberation of AA, could activate PK-C, which in turn may mediate the cAMP augmenting phenomenon. PK-C is thus the common link to cAMP augmentation; its activation resulting either from stimulation of PLC leading to DG and IP₃, or stimulation of PLA₂, and the release of AA.

PK-C can regulate cAMP accumulation via the G protein (Figure 1.12) Firstly it could phosphorylate G_s, thereby increasing its activity and hence that of adenylyl cyclase. Alternately, PK-C may catalyse the phosphorylation of G_i, thus reducing the latter's inhibitory capacity on adenylyl cyclase, and increasing the system's response to G_s.

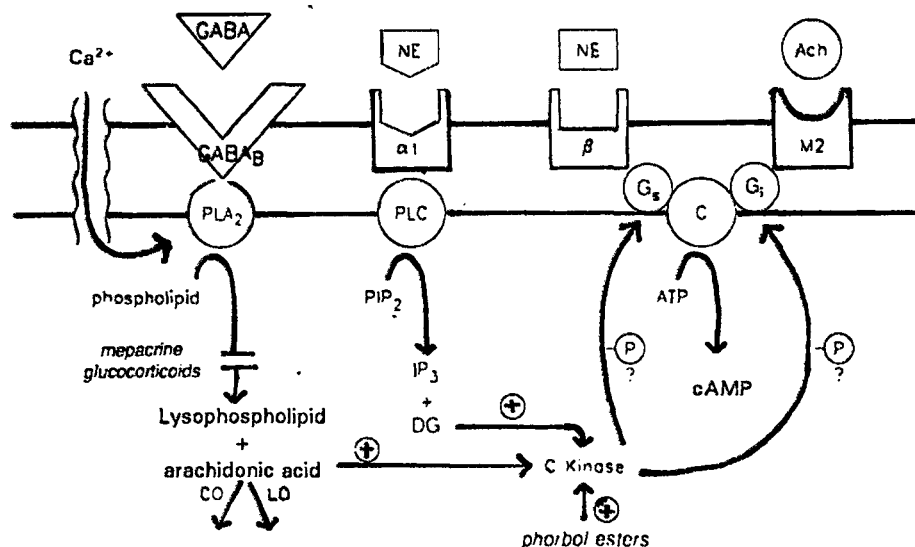


Figure 1.12:

A model describing the possible interaction between phospholipid metabolism and the cAMP generating system in the brain.

KEY: GABA = γ-aminobutyric acid, NE = noradrenaline, Ach = acetylcholine, PLA₂ = phospholipase A₂, PLC = phospholipase C, G_s and G_i = the stimulatory and inhibitory guanine nucleotide binding proteins, respectively, C = the catalytic unit of adenylyl cyclase, CO = cyclooxygenase, LO = lipoxygenase, PIP₂ = phosphatidylinositol 4,5-bisphosphate, IP₃ = inositol triphosphate, DG = diacylglycerol, ATP = adenosine 5'-triphosphate, cAMP = adenosine 3':5'-monophosphate, P = phosphate transfer catalysed by protein kinase C, + = activation (adapted from Enna and Karbon, 1987).

Whatever the end result, the data indicate that activation of PK-C, through stimulation of PI turnover or an increase in the availability of AA, may be the critical step that subserves the α_1 -adrenergic and GABA_B receptor regulation of cAMP responses in brain and rat pineals (Enna and Karbon, 1987). Reiter, (1991b) suggests that the resultant activation of PK-C increases the efficiency of activation of adenylyl cyclase most probably through phosphorylation of the cyclase.

1.12 Summary of the potentiation

In summary, the amplification mechanism appears to involve a positive effect of Ca^{2+} - / phospholipid-dependent protein kinase (PK-C) [§ 1.11.4.1]. Activation of the pinealocyte α_1 -adrenoreceptor rapidly translocates this enzyme by increasing $[\text{Ca}^{2+}]_i$ [§ 1.11.3] a correspondingly rapid increase in DG generation appears to be totally dependent on extracellular Ca^{2+} [§ 1.11.3]; there is no indication that adrenergic agents alone increase the production of IP_3 (Zatz, 1985; Sugden *et al.*, 1988) or that IP_3 -dependent mobilization of Ca^{2+} occurs (Sugden *et al.*, 1987).

Protein kinase C activation appears to enhance cAMP and cGMP synthesis at a step beyond β -adrenoreceptor activation, by phosphorylating G_s or the adenylyl cyclase and guanylyl cyclase [§ 1.11.1]. In the case of cGMP, the increase in $[\text{Ca}^{2+}]_i$ also appears to be important for activation of processes independent of PK-C [§ 1.11.5.2]; these processes may involve activation of phospholipase-A₂ [§ 1.11.4.4] and the arachidonic acid cascade and are required for full potentiation of β -adrenergic stimulation (Vanecek *et al.*, 1986).

The intracellular mechanism involved in the VIP and α_1 -adrenergic interaction might resemble the mechanism involved in the interaction of α_1 - and β -adrenergic receptors which stimulates cGMP. These mechanisms are perhaps similar, as VIP and β -adrenergic receptors can activate adenylyl cyclase, probably by acting via $G_{s\alpha}$, the GTP binding protein which regulates this enzyme; additionally the effects of β -adrenergic stimulation appear to be mediated by a $G_{s\alpha}$ -like protein. Thus, it seems reasonable to entertain the hypothesis that β -adrenergic and VIP receptors activate a membrane-bound guanylyl cyclase via this protein (Ho *et al.*, 1987).

1.12.1 A desensitization mechanism is present in the pinealocyte.

As is usual in biological systems, a control system also appears to be present. Just as cAMP can not rise without the activation of its terminator cAMP-PDE, so a desensitization mechanism appears to be present here too.

One factor contributing to the differences in the time courses for these events is that stimulation by NA, A23187, and K^+ may be subject to PK-C-dependent feedback inhibition, and that the effects of PK-C activators are less sensitive to this. For example, NA activates PLC and increases Ca^{2+} influx: effects required for NA to activate PK-C. However, PK-C activation also leads to an eventual inhibition of receptor stimulation of PLC and Ca^{2+} influx (for references see Ho and Klein, 1987). As a result, the NA stimulation of PK-C would not be sustained, and would gradually diminish as a result of this feedback mechanism.

In contrast, the effects of PMA do not depend upon an increase in PLC or Ca^{2+} influx, and the effects of PMA on activation of PK-C would not be subject to this type of feedback inhibition. In addition to these effects on NA-stimulated events, PK-C has been found to inactivate voltage-gated Ca^{2+} channels (Di Virgilio *et al.*, 1986). These channels are present in the pinealocyte and are involved in the effects of K^+ (Sugden *et al.*, 1986, 1987). Accordingly, it seems reasonable that the activation of PK-C by NA and other agents that increase $[Ca^{2+}]_i$ may induce feedback inhibition, and that feedback inhibition explains in part the more transient nature of their stimulation of PLA_2 , as compared with that of PMA. The eventual decrease in PLA_2 activation seen with all activating agents might be explained by enzymatic inactivation of PK-C by a common mechanism.

Taken together, these findings indicate that a desensitization mechanism is present in the pinealocyte. The hypothetical sequence of events is: α_1 -adrenergic activation elevates influx of Ca^{2+} , which increases $[Ca^{2+}]_i$; the increase in $[Ca^{2+}]_i$ activates PK-C (Ho *et al.*, 1988). The activated enzyme potentiates β -adrenergic stimulation of cAMP and cGMP (Vanecek *et al.*, 1985; Ho *et al.*, 1987). In addition, it reduces the α_1 -adrenergic elevation of $[Ca^{2+}]_i$. As a result, activation of PK-C is attenuated, and the α_1 -adrenergic potentiation of β -adrenergic cAMP and cGMP production decreases (Sugden *et al.*, 1988).

1.13 Involvement of phosphoinositides in hypertension

1.13.1 Introduction

Although this does not relate strictly to the pineal, it does involve the CCBs in their therapeutic role, phosphoinositide metabolism, Ca^{2+} influx and the catecholamines.

1.13.2 Therapeutic use

The first CCBs were characterized as coronary vasodilators and were introduced initially in the

treatment of angina pectoris (Buhler *et al.*, 1982). Use of verapamil in hypertension therapy was first proposed in 1968 by Hagino. Use of the remaining CCBs rapidly ensued.

1.13.2.1 Hypertension

Two processes appear to be at work in hypertension; there is an initiating abnormality specific to each form of hypertension, which in turn is linked to a "slow" process capable of amplifying arterial pressure (Lever, 1986). Structural changes (e.g. atherosclerosis) occurring in the blood vessel can considerably elevate pressure by increasing arterial resistance (Takeshita *et al.*, 1982). The amplification of pressor mechanisms further raises blood pressure (Lever, 1986). Atherosclerosis is reported to change the reactivity of blood vessels to pressors (Resink *et al.*, 1987). The enhancement of the vasoconstrictor effect of serotonin was one of the first such changes reported (Henry and Yokoyama, 1980).

Studies of the pathogenesis of atherosclerosis suggest several factors are involved including enhanced arterial permeability to lipoproteins, smooth muscle cell (SMC) migration and proliferation, and platelet deposition (Ross, 1986).

Several arguments suggest that the phosphoinositide pathway is involved in hypertension (Baudouin-Legros and Meyer, 1990).

1) The phosphorylation of membrane PI has already been covered at length. Of the two products produced by PL-C activation, the water soluble product IP_3 is released into the cytoplasm inducing the release of Ca^{2+} from a nonmitochondrial vesicular pool while the lipophilic product DG activates a phospholipid and Ca^{2+} -dependent protein kinase, which controls the Na^+ / H^+ membrane-exchange system (Weinstein and Heider, 1988).

2) SMC proliferation appears to be induced by intracellular Ca^{2+} transformation triggered by phosphoinositide metabolism. Coupled to this, studies have found patients with essential hypertension showed increased free platelet Ca^{2+} and increased PL-C activity (Erne *et al.*, 1984).

3) The constrictor agents mediate their effects via PL-C activation e.g. agents such as angiotensin II, serotonin and the catecholamines acting via postsynaptic α_1 -receptors can be shown to increase free Ca^{2+} , IP_3 and DG. It is accepted that the contractile effect stems largely from the translocation of Ca^{2+} brought about by IP_3 (Owen, 1986).

The CCBs prevent the increase of a stimulated influx of Ca^{2+} through VOCs, irrespective of the

stimulus that elicits opening of the L-channels. Hence, they are capable of antagonizing vasoconstriction in those blood vessels where the activating stimulus is associated with depolarization and L-channel activation (Baudouin-Legros and Meyer, 1990).

Of further interest are the findings that the major classes of CCBs show an antiatherosclerotic activity albeit more effective prophylactically. Some evidence shows regression of lesions when administered in combination with an appropriate diet.

However, since the activity is observed with representatives of all the classes of CCBs, independent of their disparate nonspecific effects, it is tempting to consider Ca^{2+} antagonism the common denominator for the activity, as suggested by Fleckenstein and co-workers (1987).

Could it be that the target cells show differential sensitivity toward the different CCBs, e.g. the 1,4-dihydropyridine derivative isradipine is active at very low doses, while the other members require much higher doses than used for mere L-channel blockade?

The usefulness of the CCBs in hypertension and their potential to prevent or slow the development of atherosclerosis is certainly interesting, as are the basic links to Ca^{2+} handling.

1.14 Calcium and the pineal gland: a relationship?

The relationship between cations and the pineal gland remains vague, in spite of much literature suggesting such a connection. Melatonin synthesis has been shown to be dependent on calcium ions, and studies have shown a diurnal rhythm in both melatonin production and cation levels. Calcium also has strong links to the reported α_1 -receptor mediated potentiation in the pineal. The calcium channel through which calcium enters has also been the focus of attention in this field, with Morton and co-workers (1989) suggesting a possible role for the channel itself in melatonin secretion. The role of calcium in the function of the pineal gland remains an open and interesting topic which warrants further investigation.

CHAPTER 2

ORGAN CULTURE STUDIES

2

INTRODUCTION

2.1

The role of calcium in cell regulation and the pineal gland

An increase in the free calcium ion concentration, $[Ca^{2+}]_i$, within stimulated cells underlies several important biological processes e.g. muscle contraction (Ebashi and Endo, 1968; Allen and Kurihara, 1980), the regulation of enzyme activities (Cohen, 1982), the control of membrane ion permeabilities (Meech, 1978; Colquhoun *et al.*, 1981; Maruyama and Petersen, 1982) and the secretion of transmitters and hormones (Katz, 1969; Baker and Knight, 1981). The secretion of the pineal hormone, melatonin, is also influenced by Ca^{2+} levels [§ 2.5.1.2]. The $[Ca^{2+}]_i$ in resting (unstimulated) cells is in the region of $0.05\mu M$ to $0.5\mu M$, while that of the extracellular fluid is from $1\mu M$ to $10\mu M$. The calcium concentration outside the cell is some 10000 times greater than that inside the cell, providing the equivalent of sink conditions, the importance of which will be discussed later. Although the concentration of calcium seems low, it must be noted that this is free Ca^{2+} , the Ca^{2+} to which calcium-dependent and calcium controlled enzymes respond (Irvine, 1986). On stimulation, either chemical or electrical, the $[Ca^{2+}]_i$ rises some two orders of magnitude either as a result of Ca^{2+} influx across the membrane of the cell, or through the release of Ca^{2+} from intracellular stores, or both (Reuter, 1983).

It is an inescapable fact that the ultimate control of the overall Ca^{2+} levels is vested in the plasma membrane, for only this membrane has access to sink conditions, i.e. the "bottomless" supply of Ca^{2+} present in the extracellular fluid.

In view of the importance of Ca^{2+} ions in the control of cellular functions it will be of considerable interest to explore the calcium fluxes through which the cells are regulated. Both the voltage operated channel (VOC) and the Na^+/Ca^{2+} exchange have been implicated in the changes in $[Ca^{2+}]_i$ in the pinealocyte (Sugden *et al.*, 1987). The organic calcium channel blockers (CCBs) represent a class of drugs which were suggested to act by the selective inhibition of Ca^{2+} influx through cell membranes (Fleckenstein, 1977). As such, they have long been suggested as being useful probes for the study of hormone mediated effects associated with calcium fluxes (Ehlert *et al.*, 1982; Millar and Bramley, 1984; Millar and Struthers, 1984; Lamers *et al.*, 1985). The rat pineal gland lends itself to pharmacological investigation, because of the relative ease with which the gland can be removed, and its ability to survive in explant culture (Zatz, 1981) where, as with all isolated organ studies, various pharmacological manipulations can be performed

without the complexity of the *in vivo* milieu. It is therefore not surprising that several researchers have developed organ culture systems that allow them to monitor the well-characterised synthesis of indoleamines in the pineal for changes brought about by various experimental manipulations.

2.1.1 Organ culture

"Organ culture" has its origins in the Strangeways Laboratory, some 60 years ago (Strangeways and Fell, 1926; Fell and Robinson, 1929). At that time the technique was restricted to the culture of embryonic organ rudiments mainly because those were small enough to be cultured in their entirety. The technique was later modified by Trowell (1959), so that *in vitro* cultures of an organ (or part thereof) was possible i.e keeping the organ viable without either further growth or dedifferentiation thereof. He also introduced the concept of stabilizing the culture medium's alkalinity by including 5%CO₂ with the O₂. Using these modifications he was able to keep pineal glands viable in culture for a minimum of 6 days. This was later confirmed by Klein (1969).

Several pinealologists have since developed their own versions of organ culture (Axelrod *et al.*, 1969; Klein and Rowe, 1970; Parfitt *et al.*, 1976; Alphas *et al.*, 1980; Cardinali *et al.*, 1981; Yuwiler, 1987). In this laboratory Daya (1982) developed a system of culturing 4 pineal glands in culture vessels made from ampoules. Morton (1982) started culturing pineals individually in culture tubes, a technique later employed successfully by both Skene (1985) and Daya and Fata (1986), and discussed in [§ 2.2].

2.1.2 Theory of Assay

Pineal glands co-incubated with a suitable precursor (tryptophan, 5-hydroxytryptophan or serotonin) will take it up and synthesize the indole metabolites associated with pineal metabolism. These are then secreted back into the culture medium. It is usual to incubate rat pineal glands for 24 h, after which the incubation is terminated by the removal of the gland from the medium. Aliquots of the medium are then spotted onto thin layer chromatography (TLC) plates and the metabolites separated.

If a radioactive precursor is used, the labelled indole metabolites formed may be separated by TLC and quantitated by liquid scintillometry. This method provides a more quantitative picture of pineal indole metabolism and has been shown to be sensitive to pharmacological manipulations (Klein and Rowe, 1970; Balemans *et al.*, 1983b; Voisin *et al.*, 1983; Daya and Potgieter, 1985) as well as adequately reflecting changes in pineal indole metabolism (Daya and Fata, 1986).

2.1.3 Objectives

In the following section, the general technique for pineal gland organ culture, TLC separation of the pineal indoles and measurement of the [¹⁴C]-serotonin metabolites is outlined. A full 24 hour profile of pineal indole metabolism is also shown in validation of this technique. Variations from this general outline, where required for different experimental applications, are listed in the respective methods sections succeeding this.

2.2 ORGAN CULTURE TECHNIQUE

2.2.1 Materials and Methods

2.2.1.1 Chemicals, drugs and reagents

5-Hydroxy [*side-chain-2-¹⁴C] tryptamine creatinine sulphate (57 mCi/mmol) was purchased from Amersham Laboratories, England; the synthetic indoles serotonin, 5-hydroxytryptophol, 5-hydroxyindoleacetic acid, melatonin, *N*-acetylserotonin, 5-methoxytryptophol, 5-methoxyindoleacetic acid, and 5-methoxytryptamine were obtained from Sigma Chemical Co., USA and BGJ_b culture medium from Gibco, Europe. Benzylpenicillin sodium, streptomycin sulphate and amphotericin B were obtained from local sources. All other reagents used were of analytical grade, from commercial sources.*

2.2.1.2 Animals

Because of the reported variation in HIOMT sensitivity to oestradiol during the estrus cycle of female rats (Cardinali *et al.*, 1981; Daya, 1982), male Albino Wistar rats were used throughout the organ culture studies. The rats (200-250 g b. wt) were housed six per cage in a temperature controlled room (20-21°C) under a fixed lighting cycle of LD 12:12 (lights on 06:00). Cool-white fluorescent tubes provided an average light intensity of 1800 lux during the light phase. The rats had free access to food and water. They were sacrificed between 09h00 and 09h30 by neck fracture.

2.2.1.3 Pineal glands

After decapitation, the rat pineal glands were exposed by making an incision through the bone (on either side of the parietal suture) from the foramen magnum to near the orbit, and by removing the calvarium with forceps. The pineal glands were swiftly removed using sterile dissecting forceps,

cleaned and placed into organ culture tubes as described in [§ 2.2.1.4].

2.2.1.4 Organ Culture of Pineal Glands

Following decapitation, the rat pineal glands were exposed as described in [§ 2.2.1.3]. They were immediately removed, placed on a cold stainless steel spatula, and dissected free of the pineal stalk and any adhering tissue. They were transferred to sterile (borosilicate 75 x 10mm) culture tubes containing 52 μ l culture medium. The culture medium consisted of Dr Fitton-Jackson's modification of BGJ_b medium, further supplemented with benzylpenicillin sodium, streptomycin sulphate and amphotericin B. The components of the culture medium and their respective concentrations are listed in Table 2.1. Each culture tube thus contained 1 pineal which floated just below the surface of the medium. To this, 8 μ l of the substrate [¹⁴C]-serotonin (0.4 μ Ci) was added. The air in the tube was displaced with carbogen (95% O₂/5% CO₂), and once the atmosphere was saturated the tubes were tightly sealed with Parafilm[®]. The culture tubes were placed in a Forma Scientific[®] model 3028 incubator and the pineals were incubated in the dark at 37°C for 24 h.

2.2.1.5 Thin Layer Chromatography

2.2.1.5.1 Separation of the [¹⁴C]-Indoles

This TLC technique is based on the method developed by Klein and Notides (1969). After 24 h in culture the reaction was stopped by removal of the pineal glands from the culture tubes. A 10 μ l aliquot of the culture medium was spotted onto TLC plates (silica gel 60, type F₂₅₄, 0.25mm x 20cm x 20cm aluminium plates, cut to 10 x 10cm from Merck, West Germany.)

Following this, standards of all the pineal serotonin metabolites measured, were spotted on top of the culture medium spot. A total of 10 μ l of a solution containing 0.2mg/1ml of each standard was spotted. The solution containing the standards was made up as follows:- 1mg of each of the 8 standards used was dissolved together in a test tube containing 2.5ml of 95% ethanol. To this, 2.5ml of a solution of 1% ascorbic acid in 0.1 N HCl was added. The ascorbic acid was included by Klein and Notides (1969) to protect the indoles against oxidation. The standards used are listed in Figure 2.1. This reference solution was stored in a light-resistant container at -20°C, and remained stable for the six months of the study despite the warning that the ascorbic acid might increase decomposition of the indoles by catalysing aromatic hydroxylation (Udenfriend *et al.*, 1954). The plates were spotted in a room under subdued light. At all times while spotting the plates, a gentle stream of nitrogen was used to dry the spots, which were no larger than 0.5cm, as

Table 2.1: *The constituents of BGJ₆ culture medium, (Fitton-Jackson Modification)*

<u>Components</u>	<u>Conc.</u>	<u>Components</u>	<u>Conc.</u>
Inorganic Salts	mg/L	Amino Acids	mg/L
NaH ₂ PO ₄ · H ₂ O	90.00	L-Alanine	250.00
MgSO ₄ · 7H ₂ O	200.00	L-Arginine	175.00
KCl	400.00	L-Arginine HCl	—
KH ₂ PO ₄	160.00	L-Aspartic acid	150.00
NaHCO ₃	3,500.00	L-Cysteine HCl-H ₂ O	101.00
NaCl	5,300.00	L-Glutamine	200.00
		Glycine	800.00
Other Components		L-Histidine	150.00
Calcium lactate	555.00	L-Histidine HCl-H ₂ O	—
Glucose	10,000.00	L-Isoleucine	30.00
Phenol red	20.00	L-Leucine	50.00
Sodium acetate (anhyd)	50.00	L-Lysine	240.00
		L-Lysine HCl	—
Vitamins		L-Methionine	50.00
Alpha tocopherol phosphate, (disodium salt)	1.00	L-Phenylalanine	50.00
Ascorbic acid	50.00	L-Proline	400.00
Biotin	0.20	L-Serine	200.00
D-Calcium pantothenate	0.20	L-Threonine	75.00
Choline chloride	50.00	L-Tryptophan	40.00
Folic acid	0.20	L-Tyrosine	40.00
I-Inositol	0.20	DL-Valine	65.00
Nicotinamide	20.00		
Para aminobenzoic acid	2.00		
Pyridoxal phosphate	0.20		
Riboflavin	0.20		
Thiamine hydrochloride	4.00		
Vitamin B ₁₂	0.04		

This medium has a mean tonicity of 390 milliosmoles. Where appropriate the following drugs (in the listed concentrations) were added to the medium to protect it against microbial contamination.

Benzyl Penicillin	100 units/ml
Streptomycin Sulphate	0.1 mg/ml
Amphotericin B	2.5 µg/ml

well as to prevent atmospheric oxidation of the indole metabolites. Morton (1982) reported no difference between plates spotted and run in normal light, as opposed to those spotted and run in the dark (as suggested in the original method).

Once the plates were spotted, they were put in TLC tanks containing solvent A: chloroform-methanol-glacial acetic acid (93:7:1). The solvent was allowed to run up to 9cm. Once the solvent had reached this point (± 25 min), the plates were taken out of the TLC tanks and the position of the solvent front marked. They were dried under nitrogen, then placed back into the same solvent and run once again in the same direction up to the mark. Following this, the plates

were dried under nitrogen, turned 90° and placed in a second TLC tank containing solvent B (ethyl acetate). They were run in this second direction up to a height of 6cm.

The ethyl acetate was evaporated off the plates with nitrogen and the plates were sprayed with Van Urk's reagent (1g of paradimethylbenzaldehyde dissolved in 50 ml of 25% HCl followed by the addition of 50ml of 95% ethanol). The plates were then placed in an oven at 60°C for 20 min to visualize the spots.

2.2.1.5.2 Quantitation

The various spots were clearly visible after their development. The locations of the various metabolites together with the directions in which the solvents were run are shown in **Figure 2.1**. These locations have been identified by previous workers in this laboratory (Daya, 1982; Morton, 1982). Only 5-methoxytryptamine did not migrate and remained at the starting spot with serotonin. The radioactivity in each of the identified spots was determined by scraping off the spot into a scintillation vial containing 1ml absolute ethanol. After the ethanol had dissolved the scraping, 3ml of scintillation cocktail (Beckman Ready-Solv HP/b[®]) was added to the vials, and they were vortexed on a Rota-mixer Deluxe[®] for 30 seconds. Thereafter the vials were placed in a Beckman LS 2800 scintillation counter and each vial was counted for a total of 5 min.

2.2.1.6 Analysis of Data

The counts per minute (cpm) were converted to disintegrations per minute (dpm) using the *H*-Number method of quench monitoring. The scintillation counter determines the amount of quench in a given sample using the external ¹³⁷Cs standard. It then computes the *H*-Number from previously constructed quench curves.

Blanks were run in exactly the same way except that no tissue was included. Blank values, which were very low, were first subtracted from the results before these were finally expressed as dpm of product formed/ 10μl of medium/ pineal gland.

Nomenclature for the pineal indoles follows the system suggested for international adoption by Smith (1982). A list of the indoles, together with abbreviations is presented in **Figure 2.1**.

Data analysis for all studies in this thesis were performed by computer assisted analysis using either the BMDP[®] (Statistical Software, Los Angeles) programme or KWIKSTAT[®] (Mission

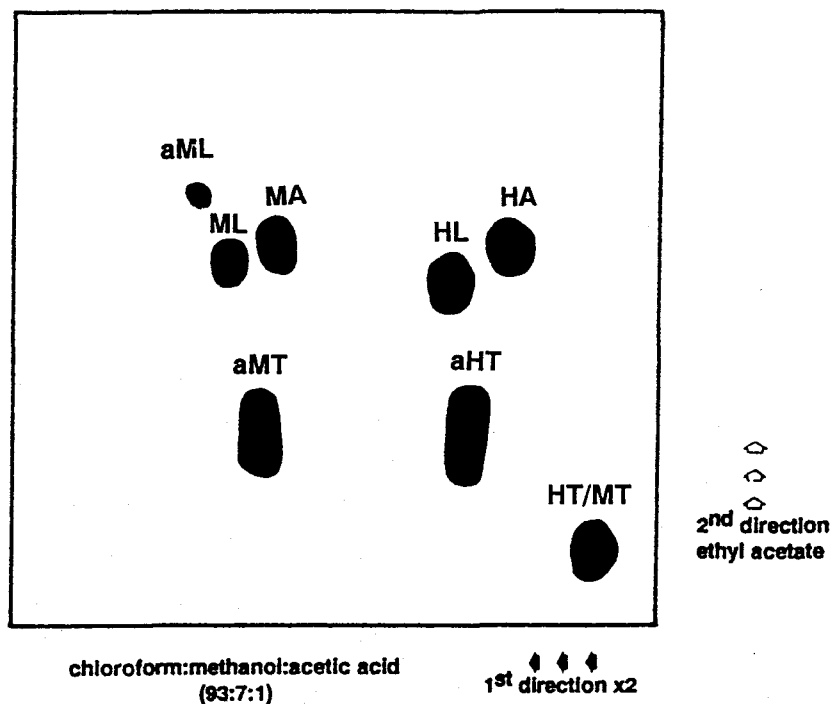


Figure 2.1: *A trace of a TLC plate, showing the direction in which the solvents were run, and the subsequent location of the pineal indole metabolites. Nomenclature as to Smith (1982).*

Key: HT/MT	Serotonin and 5-methoxytryptamine at the origin
aHT	<i>N</i> -acetylserotonin (5-hydroxy- <i>N</i> -acetyltryptamine)
HA	5-hydroxyindoleacetic acid
HL	5-hydroxytryptophol
aMT	melatonin (5-methoxy- <i>N</i> -acetyltryptamine)
MA	5-methoxyindoleacetic acid
ML	5-methoxytryptophol
aML	<i>O</i> -acetyl-5-methoxytryptophol

Technology Software). All statistical treatments carried out are listed in each individual study and were chosen after consultation with Radloff, 1988 and Van Schalkwyk, 1988.

2.2.2 Discussion

This system has recently been validated by Skene (1985). She was able to confirm that removal of aliquots of medium, did in fact present an accurate picture of the indole synthesis within the isolated pineal gland, and also that the radioactivity on the TLC plates was present within the area of the standard spots. She was able to demonstrate a recovery from the medium of expected metabolites in excess of 90%. Although Morton (1982) does not include acetic acid in solvent A, it was found that the acetic acid was essential to good separation of the ML and MA indoles. For this reason I have continued to include it in solvent A. Separation of the indole in terms of R_f

values changed according to weather conditions. However, the relative positions of the various spots always remained the same.

2.3 Experiment 1: THE EFFECT OF ISOPRENALINE STIMULATION OF [¹⁴C]-SEROTONIN METABOLISM BY ORGAN CULTURE OF RAT PINEAL GLANDS OVER A 24 HOUR PERIOD.

2.3.1 Introduction

Although several different systems of organ culture have been documented in [§ 2.1.1], and the methods of Klein and Notides (1969) are frequently referred to, only Daya and Fata (1986) have described a system which allows the progressive formation of the indole metabolites to be monitored over a 24 hour period. If the pineal gland is to be used as a test system, as has so often been suggested, then it would be of great value to know i) the relative proportions of metabolites at any one time, and ii) if 24 hours is indeed the ideal time at which to terminate the reaction. The β -agonist, isoprenaline (ISO) is often used to stimulate melatonin synthesis in the rat pineal gland (Deguchi and Axelrod, 1972a; Auerbach *et al.*, 1981). This study therefore investigates the effect of isoprenaline stimulation on [¹⁴C]-serotonin metabolism by organ cultures of rat pineal glands over a 24 hour period.

2.3.2 Materials and Methods.

2.3.2.1 Materials

l-Isoprenaline-HCl was purchased from the Sigma Chemical Co., USA. All other materials are recorded in [§ 2.2.1.1].

2.3.2.2 Animals

Adult male Albino Wistar rats (200 - 250 g b. wt) were used in this study. Their environment has been described in [§ 2.2.1.2].

2.3.2.3 Methods

Six pineal glands were rapidly collected and cultured individually in the presence of 0,4 μ Ci [¹⁴C]-serotonin and [10 μ M] isoprenaline as described in [§ 2.2.1.4]. A further six pineal glands were incubated in the absence of isoprenaline to act as a control group. For each tube containing

a pineal, a duplicate tube containing the identical constituents but without any tissue was also incubated. At two-hourly intervals, 5 μ l aliquots of culture medium were removed from each of the tubes and an equivalent volume immediately replenished from its corresponding duplicate tube. The 5 μ l aliquots were immediately spotted onto TLC plates, the metabolites were separated using the TLC methods outlined in [§ 2.2.1.5] and their radioactivity quantitated.

2.3.2.4 Data analysis and statistics.

Data are expressed as dpm/5 μ l medium, with each point representing the mean \pm SEM for 6 glands. Statistical comparisons are made using Students *t*-test.

2.3.3 Results

The production of melatonin (aMT) and its precursor *N*-acetylserotonin (aHT) rose sharply after the first two hours of culture in those pineals stimulated with isoprenaline. The rise continued progressively, reaching a peak after 16 h (Figure 2.2). This peak in the levels of aMT and aHT was significantly higher than the corresponding levels of aMT ($p < 0.001$) and aHT ($p < 0.001$) in unstimulated control pineals. The levels of aHT closely followed those of aMT in control pineals up to 16 h, whereafter the aMT levels decreased to below those of aHT ($p < 0.05$).

On the other hand, aMT levels in the isoprenaline stimulated pineals, were always significantly higher than aHT levels, a trend which remained even at 24 h. After 16 h in culture no further increases were observed in either stimulated or control pineals.

Progressive increases in the levels of the other indole metabolites, methoxyindoleacetic acid (MA), methoxytryptophol (ML), hydroxyindoleacetic acid (HA) and hydroxytryptophol (HL), occurred for up to 14-16 h, with HA > HL > ML > MA. After this period of culture there were no significant differences in the levels of these metabolites (between stimulated and control pineals).

One interesting observation was that during the first few hours of culture, pineal glands stimulated with isoprenaline showed an increase ($p < 0,05$) in hydroxyindole production (Figures 2.3 and 2.4) with a corresponding decrease ($p < 0,05$) in methoxyindole production (Figures 2.5 and 2.6).

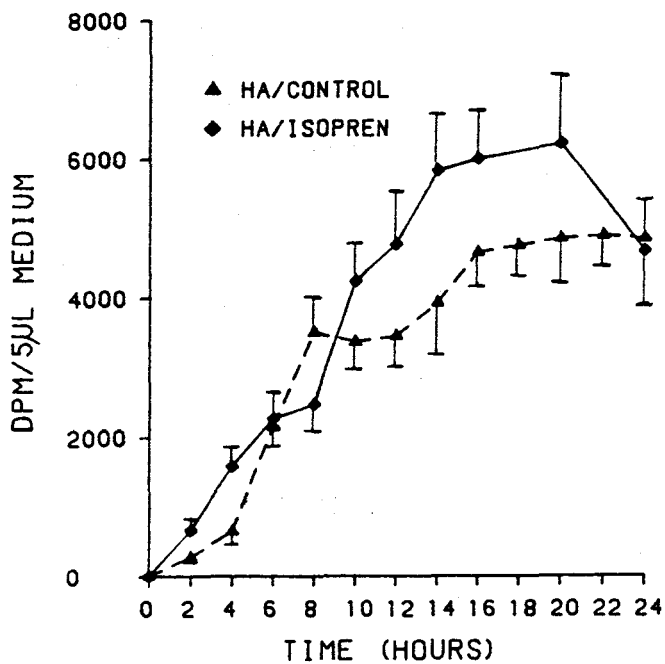
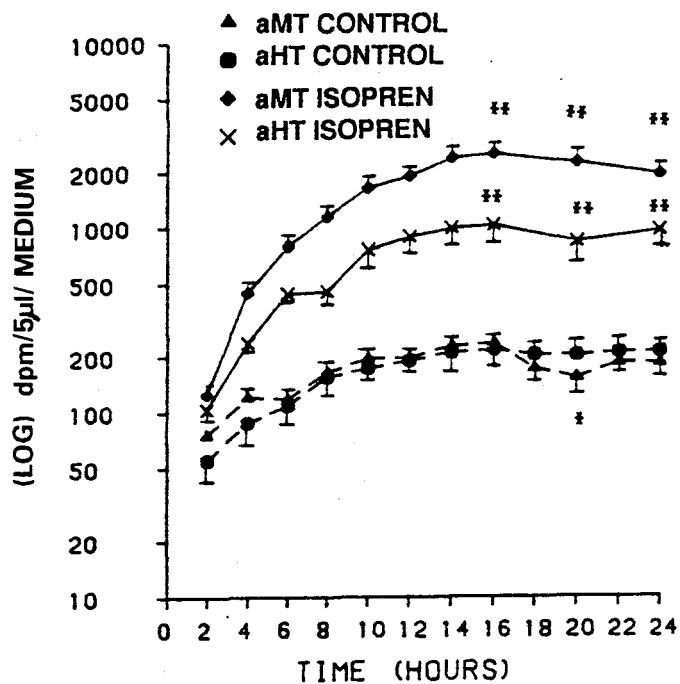


Figure 2.3:

A 24 h profile of [¹⁴C]-serotonin metabolism to 5-hydroxyindoleacetic acid (HA) in isoprenaline (ISO) stimulated and control pineal glands (means ± SEM; n = 6; p < .*0.05; Student's t test)

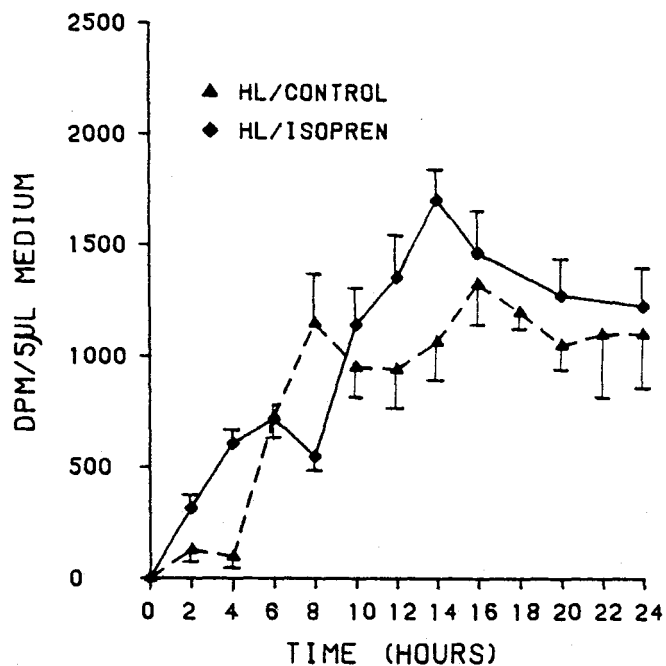


Figure 2.4: A 24 h profile of hydroxytryptophol(HL) in pineal glands (means \pm SE)

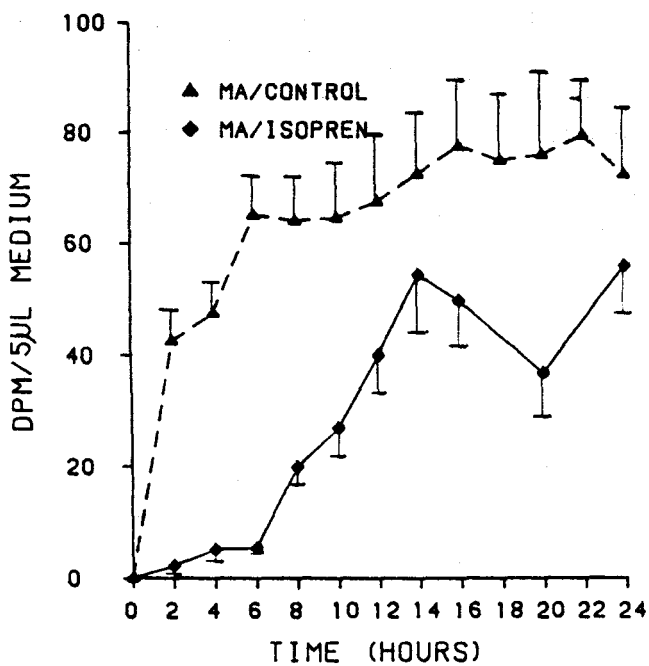


Figure 2.5: A 24 h profile of [¹⁴C]-serotonin metabolism to 5-methoxyindoleacetic acid (MA) in isoprenaline (ISO) stimulated and control pineal glands (means \pm SEM; n = 6; p < .*0.05; Student's t test)

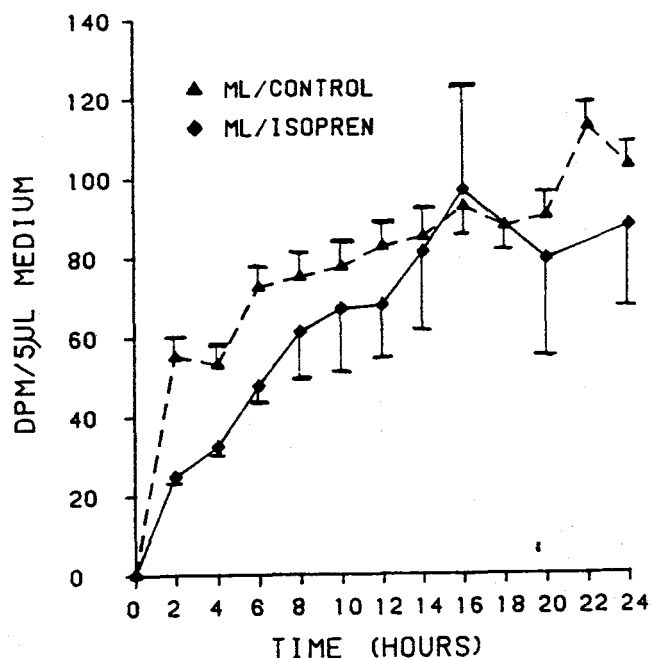


Figure 2.6: A 24 h profile of [^{14}C]-serotonin metabolism to 5-methoxytryptophol (ML) in isoprenaline (ISO) stimulated and control pineal glands (means \pm SEM; $n = 6$; $p < .^*0.05$; Student's t test)

2.3.4 Discussion

As shown in Figure 2.2, stimulation with the β -adrenoceptor agonist isoprenaline causes a marked rise in the synthesis of aMT and aHT compared with that seen in the unstimulated control pineal glands. These observations support those of other workers (Axelrod *et al.*, 1969; Deguchi, 1973) and confirm that production of the pineal hormone aMT is under β -adrenergic control. It appears that the production of these metabolites in stimulated pineals rises rapidly for the first few hours to attain maximum levels after 16 h in culture (Figure 2.2). This sharp rise can be explained by the observation that the activity of serotonin N -acetyltransferase (NAT), the enzyme responsible for the synthesis of aHT, rises markedly for the first three hours in organ cultures of pineals treated with isoprenaline, whereafter it slowly declines (personal observation).

After 16 h of culture in the presence of ISO, the aMT level is approximately ten-fold greater and the aHT level, four-fold greater compared to the unstimulated controls. Interestingly, in the control group the aMT level drops to a significantly lower level after 16 h in culture ($p < 0.05$),

whereas in the ISO stimulated group this is not observed and aMT levels are always above those of aHT levels. The rate at which aHT is converted to aMT is determined by the activity of the enzyme hydroxyindole-*O*-methyltransferase (HIOMT) (Wurtman *et al.*, 1963). From these results, it would appear that HIOMT is stimulated by isoprenaline (Banoo *et al.*, 1987). Klein and Berg, (1970) have previously shown a small increase in HIOMT activity following NA stimulation, although much controversy surrounds this point. More recently β -adrenergic receptor stimulation of HIOMT has again been found (Sugden and Klein, 1983a; 1983b).

It is therefore more likely, because HIOMT is one of the more abundant single proteins in the pineal gland (Jackson and Lovenberg, 1971) and because of the high levels of aHT present due to the ISO induced stimulation of NAT, that this conversion is occurring as a result of simple mass action (Klein and Rowe, 1970; Klein *et al.*, 1970a).

The marked reduction in the conversion of HL and HA to ML and MA observed during the first few hours of culture, in the presence of isoprenaline, is probably due to substrate competition for the methylating enzyme.

2.3.5 Conclusion

These results confirm that organ culture is both a valid and valuable technique with which to study the variation in pineal indole production, when pineal glands are subjected to various pharmacological manipulations. They further show that 16 h profiles would appear to be optimal for determining the behaviour of isolated pineal glands in the presence of various drugs (Banoo *et al.*, 1987).

2.4 Experiment 2: THE EFFECT OF ISOPRENALINE, NORADRENALINE OR COMBINED ISOPRENALINE AND PHENYLEPHRINE STIMULATION OF ORGAN CULTURES OF RAT PINEAL GLANDS.

2.4.1 Introduction

It was known that noradrenaline released from sympathetic nerves innervating the pineal (Klein *et al.*, 1971) stimulated primarily β_1 -receptors on pinealocytes, leading to a rapid increase in cAMP, followed by an elevation of NAT (Klein *et al.*, 1981). Later Sugden and Klein (1984) identified α_1 -adrenoceptors on the pinealocyte, confirming that NA was probably acting through

both α_1 and β_1 receptors (Klein *et al.*, 1983). More recently it has been shown that combined stimulation of both α_1 and β adrenergic receptors increases changes in cAMP and cGMP levels (Vanecek *et al.*, 1986) and NAT activity (Klein *et al.*, 1983) more than those following β -adrenergic stimulation alone and that α_1 stimulation by itself proves ineffective. These studies used the alpha-adrenergic agonist, phenylephrine. Since these initial studies, protein kinase C (Sugden *et al.*, 1985) as well as Ca^{2+} fluxes have been implicated in this potentiation (Sugden *et al.*, 1986, 1987).

2.4.1.2 Objective

As the intention of the current study is to investigate the Ca^{2+} fluxes following α_1 adrenoceptor stimulation in the pineal gland, it set out to examine the possible use of NA in place of the combination of ISO and PE to stimulate pineal glands in organ culture.

2.4.2 Materials and Methods

2.4.2.1 Materials

l-Isoprenaline-HCl, phenylephrine-HCL and *l*-noradrenaline bitartrate salt were purchased from Sigma Chemical Co., USA. Sources of other materials are described in [§ 2.2.1.1].

2.4.2.2 Animals

Sixteen adult male Wistar rats (210-230 g b. wt) were used for this study. They were housed as described in [§ 2.2.1.2]. The rats were sacrificed between 09h00 and 09h30 by neck fracture. Their pineal glands were swiftly removed, cleaned and transferred to tubes containing culture medium as described in [§ 2.2.1.3].

2.4.2.3 Methods

The glands were incubated in the presence of either ISO [10 μ M] or NA [10 μ M] or the combination of PE [10 μ M] + ISO [10 μ M]. These 3 groups were compared with unstimulated control glands.

Table 2.2: *Effect of isoprenaline (ISO) [10 μ M], noradrenaline (NA)[10 μ M] or the combination of isoprenaline [10 μ M] + phenylephrine (PE)[10 μ M] on [14 C]-serotonin metabolism by rat pineal glands in organ culture.*

Metabolite	Control	ISO	NA	ISO + PE
MT/HT	106505.25 \pm 5439.6	80954.50 \pm 1750.4 ^(b)	78813.75 \pm 5112.4 ^(b)	91129.50 \pm 7630.1
aHT	1070.55 \pm 163.0	3258.30 \pm 511.5 ^(b)	3905.50 \pm 597.7 ^(b)	2120.53 \pm 388.1
aMT	2136.60 \pm 168.6	3447.28 \pm 397.1 ^(b)	4442.75 \pm 731.1 ^(b)	7080.73 \pm 448.4 ^(a)
HA	19262.50 \pm 900.7	34230.40 \pm 4895.6	34060.65 \pm 7378.8	21966.2 \pm 3588.8
HL	2251.28 \pm 313.2	15658.85 \pm 2936.4	13740.38 \pm 2039.0 ^(b)	8976.85 \pm 1002.7 ^(b)
MA	219.50 \pm 79.8	381.35 \pm 102.2	187.52 \pm 26.2 ^(d)	312.46 \pm 48.8
ML	261.88 \pm 69.4	585.95 \pm 113.4	301.45 \pm 57.9 ^(d)	502.18 \pm 86.3
NaP	3208.15 \pm 199.4	6705.58 \pm 426.5 ^(b)	8348.25 \pm 1288.1 ^(b)	9201.26 \pm 467.1 ^{(b)(c)}

10 μ l aliquots of medium were chromatographed by TLC. Results are expressed as dpm/pineal gland (mean \pm SEM; n = 4 individually cultured glands per group). Significance is computed using the Neuman-Keuls multiple range test.

- (a) Significantly different from all other groups at $p < 0.05$
- (b) Significantly different from control at $p < 0.05$
- (c) Significantly different from ISO at $p < 0.05$
- (d) Significantly different from ISO and PE groups at $p < 0.05$

The incubation was terminated after a 24 h incubation period. 10 μ l aliquots of medium were removed, and spotted onto TLC plates. The radioactive metabolites were separated by two-dimensional TLC methods, and quantitated using liquid scintillometry as described in [§ 2.2.1.5].

The adrenergic agonists were dissolved in culture medium. They were protected from oxidation by the addition of ascorbic acid, 0.1mg/ml medium in which they were dissolved (Sugden and Klein, 1984).

2.4.2.4 Data analysis and statistics

The results are analysed primarily using the two indole metabolites aHT and aMT as biological markers of pineal activity. The reasons for this are discussed later in [§ 2.5.2.4]. The results are therefore discussed using these values.

In spite of the earlier findings [§ 2.3.5], that 16 h profiles would appear to be optimal, it was decided that in view of the investigative nature of these studies, they all be continued to the 24 h time point, thus allowing for easier comparison to be made with reported literature values if needed.

Data are expressed as dpm/ 10 μ l medium. Statistical analysis for multiple groups is best analysed using an analysis of variance (ANOVA) test (Modrak, 1983). The nonparametric equivalent test is the Kruskal-Wallis *H* test, and would probably be more appropriate where small groups ($n=4$) are used (Radloff, 1988). The significantly different groups were identified using the *post-hoc* Neuman-Keuls multiple range test. Groups were judged at the $p < 0.05$ level of significance.

2.4.3 Results

The results are presented in Table 2.2 and Figure 2.7

2.4.4 Discussion

Glands stimulated with the combination ISO+PE showed the greatest increase in aMT, supporting previous literature reports. This potentiation has even been observed in the Syrian hamster pineal gland (Santana *et al.*, 1988a). The observed reduction of the aHT levels in relation to the other two agonist groups is probably a result of increased substrate usage by NAT, caused by the higher

stimulation taking place in these glands. In the NA group, relative to the other stimulated groups, the methoxy products are reduced.

Previous work done by Weiss (1968) has reported a reduction in HIOMT in the presence of NA stimulation. Subsequent data from Brownstein and Heller (1968), were able to confirm these findings.

The ISO+PE group shows a reduction in the hydroxylated indoles (HT and HL) relative to the ISO and PE groups. The reasons for this are not apparent from the present work. It could be that a possible inhibition of the monoamine oxidase (MAO) responsible for the deamination of HT is occurring, or that because of the increased stimulation, the second metabolic pathway for HT is taking precedence. These two pathways can be seen in Figure 1.3.

Figure 2.7 best depicts the aim of this experiment. It clearly shows that when pineal function is measured as the sum of the *N*-acetylated products (NaP), the ISO+PE group shows the greatest metabolic activity. Although the ISO+PE group showed the greatest increase, it was not significantly different from the NA group, in terms of the statistical test employed here.

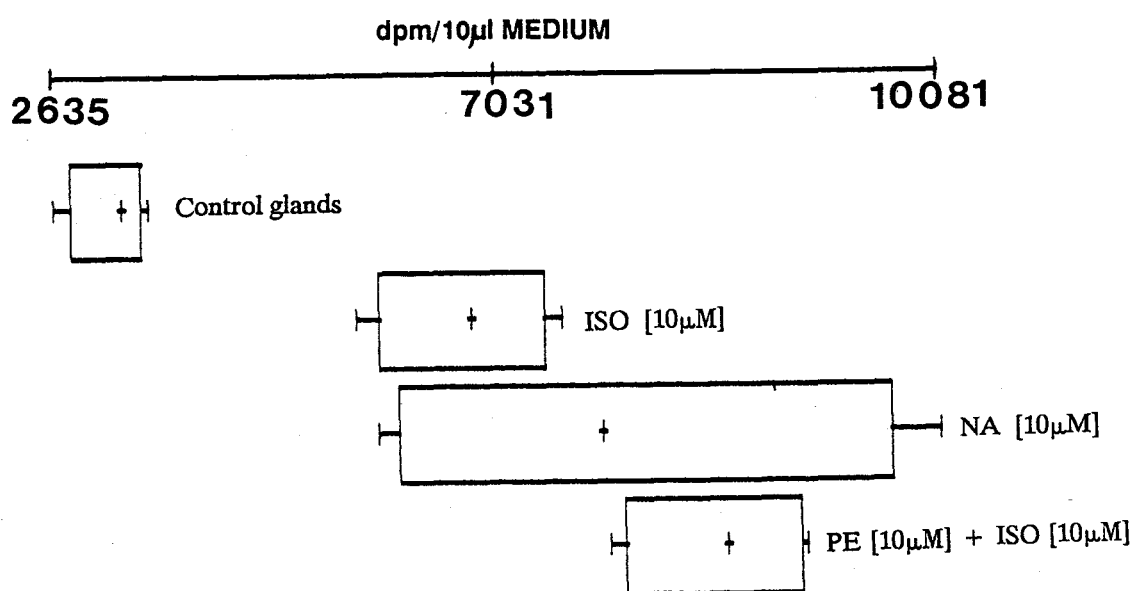


Figure 2.7: Results from experiment 2, plotted in terms of a statistical "Box and Wisker" plot of the NaP groups (means \pm SEM; $n = 4$; $p < : *0.05$; Neuman-Keuls multiple range test)

The results of this study therefore confirm that NA is able to stimulate pineal glands in organ culture, in a manner similar to ISO, and also ISO+PE. NA does not show the marked clear potentiation, consistent with α_1 and β combined stimulation, because it has both different intrinsic activity and affinity compared to the former agonists. Differences in the effects of these compounds under the same conditions, have been observed previously (Vanecek *et al.*, 1985).

It would however seem justifiable to use ISO and NA for their respective β and mixed α/β properties in the ensuing studies. Using dispersed pinealocyte preparations, Sugden *et al.*, 1987 have reported that the basal intracellular Ca^{2+} concentration (0.1nM) rose almost threefold within 1 min of exposure to NA, and that this phenomenon could be blocked, using the α_1 -receptor blocker prazosin (Cardinalli and Vacas, 1987).

2.5 Experiment 3: THE EFFECT OF THREE ORGANIC CALCIUM CHANNEL BLOCKERS ON [^{14}C]-SEROTONIN METABOLISM BY ORGAN CULTURES OF RAT PINEAL GLANDS

2.5.1 Introduction

2.5.1.1 A role for calcium in the pineal gland?

Calcium appears to play an important role in the pineal gland. Initially pineal calcium was of interest because of the increasing prominence of calcification seen in the gland with increasing age, both in man and some mammals (Diehl, 1978; Allen *et al.*, 1981; Welsh, 1984; Krstic 1985).

More recently the role of calcium in the adrenergic regulation of the rat pineal gland has come under greater scrutiny because of its link with the α_1 -adrenoceptor potentiation of the β -adrenoceptor induced increase of cAMP.

2.5.1.2 A role for calcium in the regulation of the pineal gland.

In the rat pineal gland, β -adrenoceptor stimulation increases cyclic 3'-5' adenosine monophosphate (cAMP) by activating adenylyl cyclase. Cyclic-AMP regulates the production of the pineal hormone melatonin(aMT), from serotonin, via the rate-limiting enzyme serotonin-*N*-acetyltransferase (NAT) (Axelrod, 1974). Alpha $_1$ -adrenoceptor activation, which alone is without effect, has been shown to enhance β -adrenoceptor stimulation of cAMP. This enhancement has

been linked to an increase in the entry of Ca^{2+} into the pinealocyte (Sugden *et al.*, 1986; Sugden *et al.*, 1987). Additional support came from the observations in *in vitro* glands which show that, i) the removal of calcium from the culture medium resulted in the inhibition of NAT and cAMP which would normally follow adrenergic stimulation (O'Dea and Zatz, 1976; Zatz and Romero, 1978), ii) the calcium ionophore A23187 was able to mimic the effects of α_1 -adrenergic stimulation on the cyclic nucleotides in rat pinealocytes stimulated with isoprenaline (Vanecek *et al.*, 1986), and iii) the potentiation of cAMP was dependent on the activation of a calcium-regulated phospholipid-dependent enzyme, protein kinase C (Sugden *et al.*, 1985).

2.5.1.3 The use of the pineal gland to study calcium's role

The rat pineal gland has previously been suggested as being an ideal environment in which to study the effects of calcium in cell biology (Meyer and Theron, 1988). In organ culture, the gland represents an attractive experimental tool. Various drug manipulations can be examined directly, without the complexities of *in vivo* control (Axelrod, 1974). This is especially useful in investigations into the effects of ions, e.g. Ca^{2+} , where the ionic environment can be manipulated, and toxic drugs, e.g. EGTA and A23187. The well-documented metabolic pathway through which melatonin is synthesized, with its high-amplitude circadian rhythms of enzymes and metabolites (Zatz, 1981), provides a relatively convenient way in which to monitor the gland for iatrogenic changes. It was hoped that by using the CCBs as described below, any effects of these drugs would be reflected as altered melatonin production. It would be interesting to see if the CCBs could inhibit the influx of calcium ions across the cell membrane, a phenomenon associated with the α_1 -receptor enhancement of β -receptor stimulation in the gland.

The CCBs have been used in many studies as probes for calcium-dependent processes [§ 2.1]. Since pineal Ca^{2+} levels vary diurnally (Piechowiak and Schnizer, 1976; Theron *et al.*, 1988; Pizarro *et al.*, 1988) in harmony with NAT and aMT, and the synthesis of the latter is dependent on Ca^{2+} (Zatz and Romero, 1978), it would seem that aMT could be a useful marker of calcium-mediated events in the pineal (Meyer and Theron, 1988). It is not known to what extent the calcium channel plays a role in the regulation of aMT synthesis, although it has been shown that a VOC, opened by K^+ induced depolarization and blocked by nifedipine, exists (Sugden, *et al.*, 1986).

2.5.1.4 Objectives

It was therefore decided to determine the effects of three structurally diverse CCBs, viz. verapamil,

diltiazem and nifedipine, on the conversion of [^{14}C]-serotonin to its *N*-acetylated products ([^{14}C]-aMT + [^{14}C]-aHT) using organ cultures of rat pineal glands.

The effect of these agents was assessed in the presence of i) isoprenaline (ISO), a β -specific agonist known to stimulate melatonin synthesis, but which would not influence the Ca^{2+} influx associated with α_1 -receptor stimulation in the rat pineal gland, and ii) noradrenaline (NA), a mixed $\alpha+\beta$ agonist which would stimulate both the α_1 and β receptors. It was speciously hypothesised that this drug protocol would show to what extent the synthesis of aMT was dependent on the calcium influx.

2.5.2 Materials and Methods

2.5.2.1 Materials

Verapamil-HCl, diltiazem, nifedipine, *l*-isoprenaline-HCl and *l*-noradrenaline bitartrate salt were purchased from Sigma Chemical Co., USA. Sources of other materials are described in [§ 2.2.1.1].

2.5.2.2 Animals

Adult male Wistar rats (250-280 g b. wt) were used for this series of experiments. Their environment has been discussed in [§ 2.2.1.2]. The rats were sacrificed between 09h00 and 09h30 by neck fracture. Their pineal glands were swiftly removed, cleaned and transferred to tubes containing culture medium for culture as described in [§ 2.2.1.3].

2.5.2.3 METHODS

2.5.2.3.1 Experiment A

The culture medium contained either the organic calcium channel blocker, verapamil [$10\mu\text{M}$], or vehicle. The glands were exposed to verapamil or vehicle 30 minutes prior to the addition of the adrenergic agonists ISO and NA [$10\mu\text{M}$]. Glands were thus incubated in the presence of verapamil and either ISO or NA [$10\mu\text{M}$]. These were compared with control glands incubated with vehicle, in the presence of either ISO or NA [$10\mu\text{M}$].

The incubation was terminated after a 24 h incubation period. $10\mu\text{l}$ aliquots of medium were removed, and spotted onto TLC plates. The radioactive metabolites were separated by two-dimensional TLC methods, and quantitated using liquid scintillometry as described in [§ 2.2.1.5].

Isoprenaline and noradrenaline were dissolved in culture medium. The adrenergic agonists were protected from oxidation by the addition of ascorbic acid, 0.1mg/ml medium in which they were dissolved (Sugden and Klein, 1984). Verapamil was dissolved in distilled water. It was added to the cultures, in a 10 μ l volume from a freshly prepared stock solution. Control incubations therefore contained 10 μ l distilled water.

2.5.2.3.2 Experiment B

The procedure in Experiment A [§ 2.5.2.3.1] was repeated, but verapamil was replaced by diltiazem at the same molar concentration.

Diltiazem was also dissolved in distilled water, with control incubations receiving the equivalent amount of vehicle.

2.5.2.3.3 Experiment C

The procedure in Experiment A [§ 2.5.2.3.1] was repeated, but verapamil was replaced by nifedipine at the same molar concentration. Because nifedipine was relatively insoluble in water, it was first dissolved in 70% ethanol, before further dilution in distilled water. The final concentration of ethanol did not exceed 1%. Control incubations contained the equivalent amount of vehicle.

In addition, because of nifedipine's sensitivity to light, all solutions of nifedipine were made up in dim light and glassware was covered with aluminum foil. Earlier work on the Langendorff isolated perfused rat heart preparation had shown that nifedipine solutions made up in this manner, produced their characteristic dose-dependent decline in cardiac activity, which was reversible on washout (Brown, 1985).

2.5.2.4 Data analysis and statistics

When using the pineal as an experimental tool it would seem logical to use the two indole metabolites, aHT and aMT, as biological markers of pineal activity. These two metabolites represent the products of the two primary enzymes, NAT and HIOMT, in indole metabolic pathways. Skene (1985) suggested that an overall gauge of pineal activity could be the sum of the *N*-acetylated product (NaP), i.e. (aHT and aMT). The results are therefore analysed primarily

using these values. However, it would not be fair to ignore the other indoles produced, especially the hydroxy indoles HA and HL, which are produced in such great quantity. As these two hydroxy indoles are further methylated by HIOMT, to MA and ML respectively, any correlations evident here could be used as a possible indicator of HIOMT activity.

Data are expressed as dpm/ 10 μ l medium. As there were only 4 pineal glands per group, significance was computed using the Mann-Whitney *U* test, the nonparametric equivalent of the Students *t*-test. This was suggested by Radloff (1988).

2.5.3 Results and Discussion

2.5.3.1.1 Results of glands stimulated in the presence of verapamil

Figure 2.8 shows the values for aHT, aMT and the *N*-acetylated product (NaP), the latter being defined as the sum of [¹⁴C]-aHT and [¹⁴C]-aMT. Table 2.3 shows all the metabolites separated by TLC, with the exception of the starting spot which contains both HT and MT.

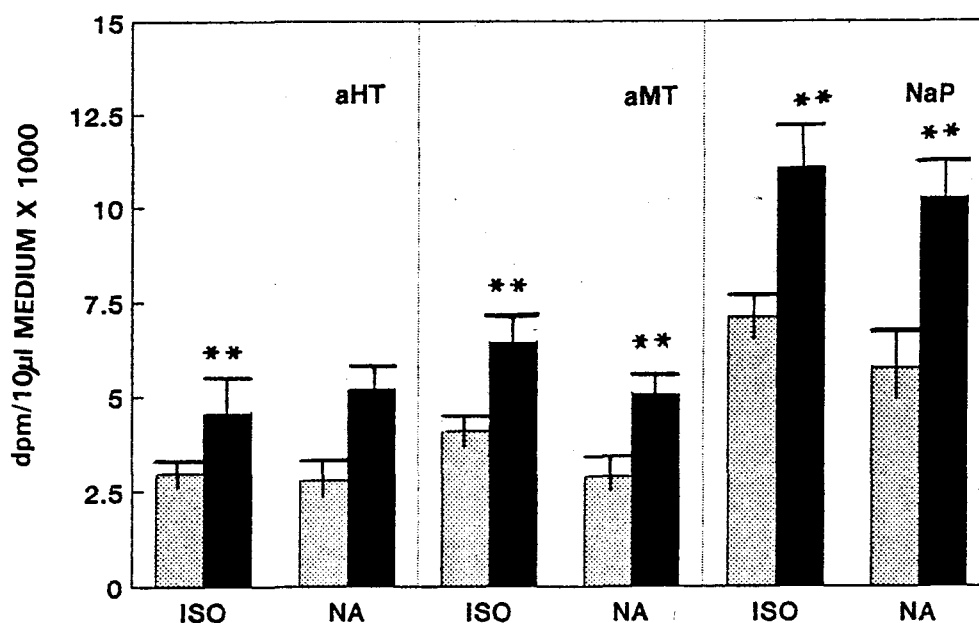


Figure 2.8:

*The effect of verapamil [10 μ M] [■] on [¹⁴C]-serotonin metabolism by rat pineal organ cultures, to [¹⁴C]-aHT, [¹⁴C]-aMT and NaP, in the presence of either ISO or NA [10 μ M], compared to control glands with vehicle [▨], in the presence of either ISO or NA [10 μ M]. (means \pm SEM; n = 4; p < : *0.01, **0.025 Mann-Whitney U test*

Table 2.3.

Effect of verapamil on [¹⁴C]-serotonin metabolism by rat pineal organ cultures, stimulated with either isoprenaline (ISO) [10 μ M] or noradrenaline (NA) [10 μ M].

Metabolite	ISO + Vec	ISO + VER	Level of significance	NA + Vec	NA + VER	Level of significance
aHT	2979.98 \pm 320.3	4583.54 \pm 924.7	p<0.100	2803.47 \pm 523.1	5189.34 \pm 616.3	p<0.025
aMT	4142.51 \pm 415.9	6439.64 \pm 682.7	p<0.025	2954.14 \pm 533.6	5066.64 \pm 495.3	p<0.025
HA	48892.60 \pm 4568.4	46696.65 \pm 2287.5	N.S	39209.41 \pm 4103.3	41560.28 \pm 4916.4	N.S
HL	12275.42 \pm 2003.6	12808.59 \pm 712.6	N.S	14249.97 \pm 1991.8	12510.03 \pm 2069.9	N.S
MA	150.83 \pm 33.7	224.46 \pm 68.5	N.S	160.06 \pm 54.3	172.47 \pm 17.8	N.S
ML	556.32 \pm 180.8	456.82 \pm 116.09	N.S	423.15 \pm 136.42	266.65 \pm 50.1	N.S
NaP	7122.49 \pm 566.3	11023.18 \pm 1188.68	p<0.025	5757.62 \pm 947.7	10255.99 \pm 1034.2	p<0.025

10 μ l aliquots of medium were chromatographed by TLC. Results are expressed as dpm/pineal gland (mean \pm SEM; n = 4 individually cultured glands per group). Significance is computed using the nonparametric Mann-Whitney U test (2 tails).

2.5.3.1.2 Discussion

Pineal glands stimulated with ISO in the presence of VER, showed an unexpected 54% increase in *N*-acetylated product ($p < 0.01$), relative to the control glands which were stimulated with ISO only. *N*-acetylserotonin was raised 53% and aMT a corresponding 55%.

Those glands stimulated with NA in the presence of VER showed an even greater (78%) increase in *N*-acetylated product ($p < 0.025$). *N*-acetylserotonin increased by 85% relative to its control, and aMT by 71%.

A further surprise is evident on examining the levels of the remaining metabolites. There is no change in the levels of HA and HL in the presence of VER after either ISO or NA stimulation. A similar pattern is seen with the MA and ML metabolites. If any trend is apparent, it is that the ML metabolites in the presence of VER are slightly decreased. The rate at which aHT is converted to aMT is dependent on the activity of HIOMT (Wurtman *et al.*, 1963). Thus, as HIOMT is responsible for the conversion of aHT to aMT as well as HL and HA to ML and MA, this suggests some possible variation in the activity of HIOMT. Either that i) HIOMT is primarily responsible for the conversion of aHT to aML (i.e. it has a greater affinity for this reaction), and the other metabolites are methylated only if there are free catalytic sites available and they are at a suitable concentration (Morton, 1982), or ii) there are two species of HIOMT, as suggested by Jackson and Lovenberg (1971) and later confirmed by Balemans *et al.*, (1980). This is further supported by Pevet *et al.*, (1980) who have reported that aMT is produced at night and in the middle of the light period by one HIOMT species, while the HIOMT enzyme responsible for ML production, is only produced during the day.

2.5.3.2.1 Results of glands stimulated in the presence of diltiazem

Figure 2.9 shows the values for aHT, aMT and the *N*-acetylated products, the latter being defined as the sum of [¹⁴C]-aHT and [¹⁴C]-aMT. Table 2.4 shows all the metabolites separated by TLC, with the exception of the starting spot which contains both HT and MT.

2.5.3.2.2 Discussion

Following ISO stimulation of glands in the presence of DIL, aHT levels are raised by 46% while Melatonin levels are decreased by 46%. The result is that indole metabolism measured as *N*-acetylated product shows no significant difference. This finding tends to suggest some effect of

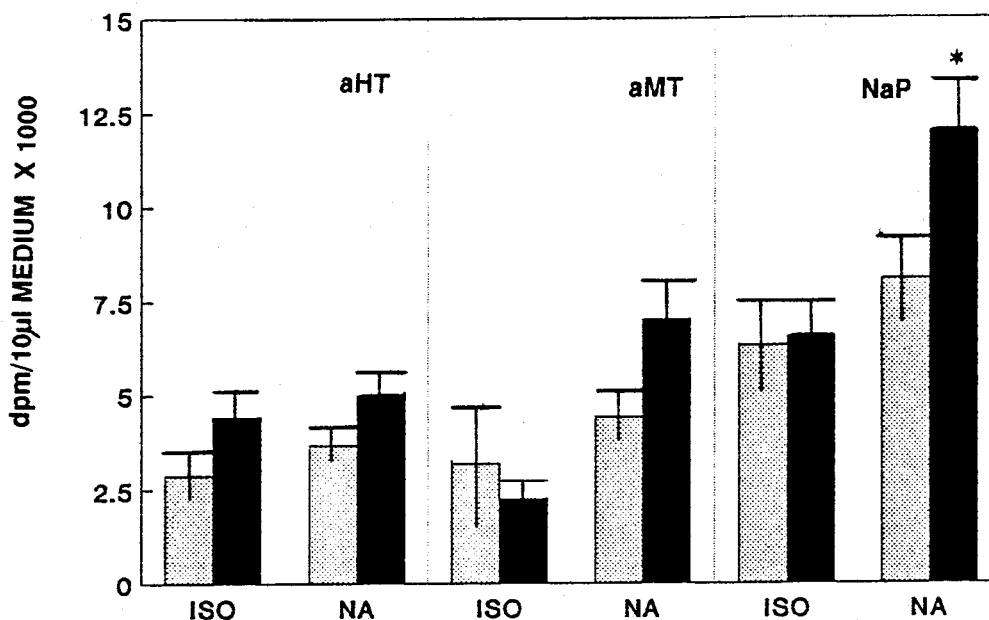


Figure 2.9:

*The effect of diltiazem [10µM] [■] on [¹⁴C]-serotonin metabolism by rat pineal organ cultures, to [¹⁴C]-aHT, [¹⁴C]-aMT and NaP, in the presence of either ISO or NA [10µM], compared to control glands with vehicle [▨], in the presence of either ISO or NA [10µM]. (means ± SEM; n = 4; p < : *0.05 Mann-Whitney U test*

DIL on HIOMT activity, as HIOMT would normally convert the aHT (which is raised) to aMT. The HL level also shows an increase, while ML is slightly decreased. This does seem to suggest that some inhibition of HIOMT activity occurs when the glands are stimulated with ISO in the presence of DIL.

After NA stimulation of glands in the presence of DIL, aHT levels are increased by 36%, and aMT levels by 59%, resulting in a nett significant ($p < 0.05$) increase of 48% in *N*-acetylated product.

The remaining indoles all show increases as well, relative to their controls. The strong correlation between aHT and aMT and the increased level of MA and ML suggests an increase in HIOMT

Table 2.4

Effect of diltiazem on [¹⁴C]-serotonin metabolism by rat pineal organ cultures, stimulated with either isoprenaline (ISO) [10 μ M] or noradrenaline (NA) [10 μ M].

Metabolite	ISO + Vec	ISO + DIL	Level of significance	NA + Vec	NA + DIL	Level of significance
aHT	3008.89 \pm 638.2	4420.34 \pm 793.6	p<0.10	3682.99 \pm 490.1	5031.72 \pm 609.3	p<0.10
aMT	3297.45 \pm 1521.9	2256.31 \pm 483.3	N.S	4410.85 \pm 701.7	7023.11 \pm 1024.1	p<0.10
HA	39230.47 \pm 5010.4	32620.34 \pm 6120.5	N.S	34060.31 \pm 4231.8	44468.76 \pm 3690.7	N.S
HL	15659.09 \pm 2936.5	27660.94 \pm 4510.2	N.S	13739.65 \pm 2038.6	20358.25 \pm 2961.5	p<0.10
MA	381.61 \pm 102.2	263.18 \pm 128.8	p<0.10	163.32 \pm 35.1	276.29 \pm 51.7	p<0.05
ML	586.26 \pm 113.1	404.89 \pm 53.2	N.S	290.74 \pm 49.8	477.94 \pm 132.8	p<0.10
SUM	6306.35 \pm 1282.1	6676.65 \pm 895.9	N.S	8093.84 \pm 1184.3	12054.84 \pm 1392.7	p<0.05

10 μ l aliquots of medium were chromatographed by TLC. Results are expressed as dpm/ pineal gland (mean \pm SEM; n = 4 individually cultured glands per group). Significance is computed using the nonparametric Mann-Whitney U test (2 tails).

activity. It would therefore seem that HIOMT activity is stimulated by NA in the presence of DIL. It could also be that monoamine oxidase activity is somehow influenced by DIL following NA stimulation, and that the increase in methoxyindoles is merely a result of mass action (Banoo *et al.*, 1987) following the increased availability of substrate. Monoamine oxidase converts serotonin to the unstable 5-hydroxyindole-3-acetaldehyde intermediate, which is rapidly converted to HT and HL by aldehyde reductase and dehydrogenase respectively (Lerner and Case, 1960). These metabolic pathways are shown in Figure 1.3.

2.5.3.3.1 Results of glands stimulated in the presence of nifedipine

Figure 2.10 shows the values for aHT, aMT and the *N*-acetylated products, the latter being defined as the sum of [14 C]-aHT and [14 C]-aMT. Table 2.5 shows all the metabolites separated by TLC, with the exception of the starting spot which contains both HT and MT, and is not included.

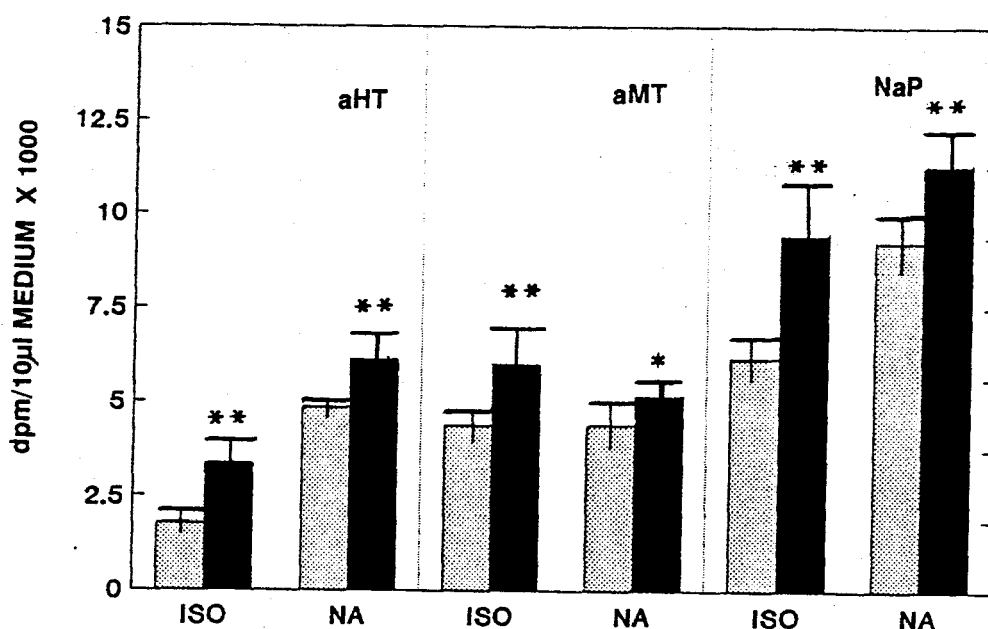


Figure 2.10:

*The effect of nifedipine [10µM] [■] on [14 C]-serotonin metabolism by rat pineal organ cultures, to [14 C]-aHT, [14 C]-aMT and NaP, in the presence of either ISO or NA [10µM], compared to control glands with vehicle [▨], in the presence of either ISO or NA [10µM]. (means \pm SEM; n = 4; p < : *0.05, **0.025 Mann-Whitney U test*

Table 2.5.

Effect of nifedipine on [¹⁴C]-serotonin metabolism by rat pineal organ cultures, stimulated with either isoprenaline (ISO) [10 μ M] or noradrenaline (NA) [[10 μ M].

Metabolite	ISO + Vec	ISO + NIF	Level of significance	NA + Vec	NA + NIF	Level of significance
aHT	1784.66 \pm 295.4	4517.50 \pm 812.8	p<0.025	4841.00 \pm 160.8	8110.50 \pm 982.1	p<0.025
aMT	4371.66 \pm 363.7	8041.25 \pm 1251.6	p<0.05	4386.33 \pm 596.1	6936.01 \pm 489.4	p<0.025
HA	34749.33 \pm 3192.8	35400.05 \pm 2001.1	N.S	38545.33 \pm 2344.6	25706.75 \pm 5113.3	p<0.10
HL	7398.38 \pm 841.5	7271.50 \pm 576.4	N.S	12508.10 \pm 621.67	6832.40 \pm 1006.1	p<0.025
MA	160.67 \pm 46.9	129.75 \pm 12.3	N.S	133.31 \pm 13.8	69.75 \pm 22.7	p<0.10
ML	289.01 \pm 96.1	159.09 \pm 25.60	N.S	187.33 \pm 28.22	33.25 \pm 5.4	p<0.025
SUM	6156.31 \pm 533.1	12558.75 \pm 1989.7	p<0.025	9227.38 \pm 727.4	15046.51 \pm 1348.7	p<0.025

10 μ l aliquots of medium were chromatographed by TLC. Results are expressed as dpm/ pineal gland (mean \pm SEM; n = 4 individually cultured glands per group). Significance is computed using the nonparametric Mann-Whitney U test (2 tails).

2.5.3.3.2 Discussion

Glands stimulated with ISO in the presence of NIF also show an increase in aHT (150%), aMT (83%) and the *N*-acetylated product shows a resultant twofold increase ($p < 0.025$). The remaining metabolites show the same trend as that seen with VER and ISO in [§ 3.5.3.1.2], i.e. the HA and HL levels do not vary significantly, while the MA and ML levels tend to decrease relative to the control levels. This again tends to suggest the view that HIOMT primarily converts aHT to aMT, the remaining indoles being methylated if a site becomes available to them.

After NA stimulation in the presence of NIF an overall increase of 63% is found in *N*-acetylated product ($p < 0.025$). *N*-acetylserotonin increases by 67%, and aMT by 50%. However a decrease in other metabolites is found: HA ($p < 0.10$), HL ($p < 0.025$), MA ($p < 0.10$), and ML ($p < 0.025$). The decreased ML and MA levels could be because of reduced substrate availability, or they could add further support to the possibility of a difference in HIOMT activity. The high levels of hydroxyindoles found in the pineal suggest that there is still sufficient substrate available and make the former explanation improbable.

2.5.4 Conclusion

In conclusion then, the presence of diltiazem did not alter the ISO-induced increase in *N*-acetylated products. This is to be expected since Ca^{2+} influx in the pineal has been associated with the α_1 -receptor stimulation. However, both verapamil and nifedipine potentiated the effect of ISO. It is difficult to speculate at this stage as to why this happens. This phenomenon could be associated with the action of VER and NIF at a site other than the calcium channel (Millar and Struthers, 1984; Lamers *et al.*, 1985; Brown *et al.*, 1989a). Alternatively, Morton (1987) has recently shown that high levels of Ca^{2+} are able to inhibit bovine HIOMT activity, which could have some bearing on these results. If the CCBs are reducing Ca^{2+} entry into the cells by some small amount, this could account for less inhibition on HIOMT activity, and hence for the increased aMT levels seen. The differences observed with diltiazem, relative to results using verapamil and nifedipine, could possibly point to a difference in the respective ability of the CCBs to inhibit Ca^{2+} entry. This however is purely speculative, and should be regarded with care in view of the large intra-species variations seen in pineal work. For instance Meyer *et al.*, (1986) have shown that 2 classes of CCB administered *in vivo* to baboons, produce different effects on circulating plasma aMT levels. Three dihydropyridine CCBs of which one was nifedipine, reduced aMT levels, while verapamil had no effect.

In the presence of NA, all three CCBs were found to potentiate the effect of NA on *N*-acetylated product formation. This was opposite to the original hypothesis that the CCBs would inhibit the Ca^{2+} influx following α_1 stimulation and thus show a decrease in aHT following NA stimulation. It would appear that the CCBs are in some way bringing about an increase in NAT levels, resulting in an increase in aHT. With the exception of those glands stimulated with ISO in the presence of DIL, HIOMT continued to methylate the increased substrate available, and melatonin rose accordingly, in tandem with its precursor aHT as could be expected (Axelrod and Weissbach, 1960; Zatz, 1978). HIOMT activity was however variable with respect to the other methoxyindoles, showing a general trend towards decreased ML levels. This suppressed production of ML suggests that a second form of HIOMT would appear to be present, and that some inhibition of the latter is possibly occurring. Continuing from the work of Morton (1987), this second species perhaps displays more sensitivity towards Ca^{2+} levels. This could possibly explain the differences observed between diltiazem and nifedipine, following NA stimulation. In the presence of diltiazem, HL, HA, MA, and ML are raised, while in glands in the presence of nifedipine, these levels are decreased. Any relationship between the Ca^{2+} levels and HIOMT activity appears to be highly complex, without further complicating factors introduced by the CCBs.

While the levels of the methoxyindoles remain variable and open to different interpretation, the salient feature of these results is the increased level of aHT observed. It would seem that the CCBs are in some way causing an increase in NAT, the enzyme responsible for the formation of aHT (Klein and Weller, 1970). It thus seems pertinent to examine the effects of these CCBs on the level of NAT in isolation. This would clarify if NAT is indeed raised as suggested by the high levels of aHT observed.

CHAPTER 3

N-ACETYLTRANSFERASE STUDIES

3 INTRODUCTION

3.1 Adrenergic stimulation and N-acetyltransferase in the rat pineal gland.

In the rat pineal gland the release of NA (from the nerve terminals innervating the gland), stimulates β -receptors, which leads to an elevation of intracellular cAMP. The elevated cAMP levels in turn stimulate a series of protein phosphorylations (Morgan *et al.*, 1988) which induce the activity of the arylalkylamine *N*-acetyltransferase (NAT; EC 2.3.1.87), the rate-limiting enzyme in the conversion of serotonin to melatonin (Klein *et al.*, 1981). The mechanism through which NA controls cAMP involves both α_1 and β -receptors. The presence of α_1 -receptors - in the pineal gland was confirmed by Sugden and Klein (1984). Noradrenaline regulates pinealocyte cAMP and cGMP through an unusual synergistic mechanism. While β -adrenoceptor stimulation increases both cAMP and cGMP, α_1 -adrenoceptor stimulation of the pineal gland alone produces no detectable change in the levels of these two nucleotides (Vanecek *et al.*, 1986). However, α_1 -adrenoceptor stimulation amplifies the β -response by potentiating the accumulation of cAMP and cGMP (Klein *et al.*, 1983; Vanecek *et al.*, 1985; Klein, 1985; Vanecek *et al.*, 1986).

β -Adrenergic activity sufficient for the induction of NAT (Klein, 1978; Zatz, 1982) has also been shown to be potentiated by α_1 -receptor stimulation (Klein *et al.*, 1983), although the effect is not as pronounced as that seen with cAMP levels (Illnerova *et al.*, 1983; Klein, 1985; Illnerova, 1985).

Although NAT was originally thought to be similar to liver arylamine *N*-acetyltransferase (EC 2.3.1.5), that enzyme has broad substrate specificity (Voisin *et al.*, 1984). Serotonin *N*-acetyltransferase (NAT; EC 2.3.1.87) found in the retina, harderian gland and pineal gland (Pang *et al.*, 1977), has been found to have narrow substrate specificity for arylalkylamines, - aromatic compounds with a primary amine on the side-chain - e.g. serotonin (Klein, 1985). This enzyme also differs from liver arylamine *N*-acetyltransferase in that it is under β -adrenergic control via cAMP (Klein and Weller, 1973; Chan and Ebadi, 1980), and it exhibits a circadian rhythm with its levels rising during the scotophase of the light:dark cycle.

3.1.2 The use of NAT as an indicator of pineal activity

Before the advent of the very sensitive radioimmunoassays for melatonin the most common indices of pineal function and especially of melatonin production, were the activities of NAT (Klein and Weller, 1970) and HIOMT (Wurtmann *et al.*, 1963). Of the two, NAT had been shown to best correlate with melatonin production (Panke *et al.*, 1978; Wilkinson *et al.*, 1977; Morton, 1982). This led to the popular belief that NAT was the rate-limiting enzyme in the formation of melatonin (Klein and Weller, 1970, 1973). Some evidence has accumulated which shows that NAT activity does not always mirror the production of its product, melatonin. For example, King *et al.*, (1984) have shown that HT may also play a role. Rats subjected to various forms of stress have also shown varied NAT vs aMT results (Seggie *et al.*, 1985; Vollrath and Welker, 1988), which could however stem from investigating the wrong end parameter. When examining the effect of stress on a rat, and the response of the pineal gland to this stress, the production of melatonin itself would probably be the best indicator; the pineal appears to modulate the body's response to stress via a mechanism involving the release of melatonin. In support of this, Tannenbaum *et al.*, (1988) working with rats exposed to cold stress, demonstrated results showing decreased NAT levels in the presence of raised aMT levels. Khan *et al.*, (1990) have demonstrated that ulcers induced by cold-stress are reduced in the presence of melatonin, while Morton *et al.*, (1989) has shown that the CCB nifedipine is able to inhibit the release of melatonin, when rats are stressed by forced swimming.

On the other hand, NAT would still appear to be a sensitive biochemical parameter when examining the effects of agents on the pineal gland.

3.1.3 Objective

In terms of the previous chapter's results, it would seem useful to evaluate the effect of the CCBs on NAT activity. As the activity of this enzyme is modulated by an adrenergic cAMP mechanism, any effects of the CCBs on the levels of cAMP should manifest themselves at this level. The theory of the NAT assay is discussed below [§ 3.1.4] while the full protocol is outlined in [§ 3.2].

3.1.4 Theory of the Assay

The principle involves the *N*-acetylation by endogenous pineal NAT of a substrate, tryptamine-HCl, using [³H]-acetyl coenzyme A as the acetyl donor. Under the conditions of the assay, NAT

is able to utilize tryptamine far more efficiently than the endogenous HT. The radiolabelled product, [³H]-*N*-acetyl tryptamine, is extracted into an organic solvent and the radioactivity is evaluated by routine liquid scintillometric techniques.

3.2 NAT ASSAY TECHNIQUE

3.2.1 Materials and Methods

3.2.1.1 Chemicals, drugs and reagents

[³H]-Acetyl-coenzyme A (250 μ Ci/ml : specific activity 4.3 Ci/mmol) was purchased from Amersham Laboratories, England. Tryptamine-HCl was purchased from the Sigma Chemical Co., USA. All reagents used were of analytical grade, from commercial sources.

3.2.1.2 Animals

Female Albino Wistar rats were used throughout the NAT studies as NAT, unlike HIOMT, has been shown not to be influenced by the various stages of the estrous cycle (Cardinali and Vacas, 1978). Illernova (1975) showed NAT in rats to be insensitive to oestradiol treatment.

The rats (200-250 g b. wt) were housed four per cage in the environment previously described in [§ 2.2.1.2]. They were sacrificed between 09h00 and 09h30 by neck fracture, at the same time as those rats used in the organ culture studies. This was done to avoid possible interference with β -receptor sensitivity changes. It is well known that the increase in NAT activity following adrenergic stimulation is dependent on the time of day (Romero and Axelrod, 1974). The β -receptors show down-regulation after the full stimulation of the night, a rhythm in receptor density which is well-documented (Reiter *et al.*, 1985; Gonzalez-Brito *et al.*, 1988; Santana *et al.*, 1988b).

3.2.1.3 Pineal glands

Following decapitation of the rats the pineal glands were exposed as described in [§ 2.2.1.3]. They were immediately removed, placed on a cold stainless steel spatula, and dissected free of the pineal stalk and any adhering tissue. They were transferred to sterile homogenation tubes, kept on ice, which contained 70 μ l cold 0.05mM phosphate buffer (pH 6.5).

3.2.1.4 Assay of *N*-acetyltransferase

The assay used is a modification of that originally described by Deguchi and Axelrod (1972). Rat pineal glands were individually placed in thick-walled, 2ml, glass tubes (Pyrex[®] 10 x 75mm, thick walled, no rim) containing 70 μ l of cold 0.05M phosphate buffer (pH 6.5) and kept on ice. Pineal glands were homogenised by 6-8 full rotations of a glass homogenation rod. Fifty microlitres of homogenate was removed and transferred to a corresponding culture tube (Kimble[®] 10mm x 75mm). To this, 10 μ l of a 10mM stock solution of tryptamine was added, followed by 10 μ l of [³H]-acetyl coenzyme A. The tubes were sealed and incubated on a waterbath at 37°C for 10 min. The final reaction volume of 70 μ l thus contained 1.4mM tryptamine and 29.1 pM [³H]-acetyl-CoA. The reaction was terminated by the addition of 500 μ l ice-cold 0.2M borate buffer (pH 10.0).

The contents of each tube was transferred to a 15ml glass stoppered tube containing 3ml toluene:isoamyl alcohol (97:3). Extraction of the radioactive product of the reaction into the organic solvent was aided by vortexing the tubes for 1 min on a Rota-Mixer Deluxe[®]. The resulting emulsion was broken by centrifuging the tubes at 3500 rpm on a Hettich[®] physiological centrifuge for 10 min. Two millilitre aliquots of the organic phase were aspirated into scintillation vials containing 3ml cocktail (Beckman Ready-Solv HP/b[®]). The vials were shaken for 20 min, allowed to stand for a further 20 min and then placed in a Beckmann LS 2800 scintillation counter, where the radioactivity in each was quantitated.

3.2.1.5 Data analysis and statistics

Blanks were run in the same way, except that they contained buffer in place of homogenate. The cpm were converted to dpm as described in [§ 2.2.1.6]. Blank values were first subtracted from the assay values before expressing the results as picomoles of [³H]-*N*-acetyl tryptamine formed per gland per hour (Deguchi and Axelrod, 1972).

Statistical significance was computed using, where appropriate, the nonparametric Mann-Whitney *U* or Kruskal-Wallis *H* tests. *Post hoc* analysis using the Neuman-Keuls multiple range test for comparison of significance among the means at the $p < 0.05$ level (Zar, 1974) was performed.

3.3 Experiment 1: DETERMINATION OF THE LARGE DIURNAL VARIATION IN NAT ACTIVITY IN RAT PINEAL GLANDS

3.3.1 Introduction

A circadian rhythm in *N*-acetyltransferase with a large variation in amplitude is known to exist (Klein and Weller, 1970). The data presented by Iuvone and Besharse, (1986) indicate that the increase in NAT requires extracellular Ca^{2+} in the micromolar range and that calcium influx through a VOC is involved. This large and well-documented rhythm, during which there is a 15 to 30 fold increase in *N*-acetyltransferase activity, allows an easy assessment of the assay's integrity to be made. This study therefore set out to determine the levels of *N*-acetyltransferase in rats' pineal glands at 09h00 and 23h00, in an elementary validation of the technique.

3.3.2 Materials and Methods.

3.3.2.1 Materials

All materials used are described in [§ 3.2.1.1].

3.3.2.2 Animals

Adult female Albino Wistar (which are suitable for use in the NAT assay [§ 3.2.1.2]) rats (250 - 280 g b.wt) were used in this study. Their environment is described in [§ 2.2.1.2].

3.3.2.3 Methods

Four rats were sacrificed at 09h00 by neck fracture. Their pineals were rapidly removed [§ 2.2.1.3.] and transferred to glass homogenation tubes and assayed as described in [§ 3.2.1.4]. A further 4 rats were sacrificed at 23h00. This was performed in the dark using protected red lighting of very low intensity to avoid the precipitous drop known to occur in NAT levels when light exposure occurs at night (Vollrath and Hueson, 1988). The pineal glands were assayed in the normal manner.

3.3.2.4 Data analysis and statistics.

Data are expressed as pmol of *N*-acetyltransferase activity/ pineal gland/ h, with each point representing the mean \pm SEM for 4 glands. Statistical comparisons are made using the

nonparametric Mann-Whitney *U* test.

3.3.3 Results

The results are presented below in Table 3.1

TABLE 3.1: *The diurnal change occurring in NAT activity in the rat pineal gland*

Time	<i>N</i> -Acetyltransferase activity (pmol/ pineal/ h)	
9h00	0.88 ± 0.15	<i>p</i> < 0.001
23h00	35.6 ± 3.41	

(*mean* ± *SEM*; *n* = 4 glands per group; Mann-Whitney *U* test)

3.3.4 Discussion

The results confirm those of previous workers (Deguchi and Axelrod, 1972). The large diurnal variation in NAT activity is seen, showing the assay to be functional.

3.4 Experiment 2: CUMULATIVE DOSE RESPONSE RELATIONSHIPS FOUND FOLLOWING ISOPRENALINE AND NORADRENALINE STIMULATION OF NAT ACTIVITY IN RAT PINEAL GLANDS

3.4.1 Materials and Methods

3.4.1.1 Materials

l-Isoprenaline-HCl and noradrenaline bitartrate was purchased from the Sigma Chemical Co., USA. All other items are described in [§ 3.2.1.1].

3.4.1.2 Animals

Adult female Albino Wistar rats (180 - 220 g b.wt) were used in this study. Their environment is described in [§ 2.2.1.2].

3.4.1.3 METHODS

3.4.1.3.1 Experiment A

Rats were sacrificed between 09h00 and 09h30. Their pineal glands were removed [§ 2.2.1.3] and transferred to sterile culture vessels consisting of the bottom part of 2 ml amber glass ampoules that had been cut ca 10mm from the base. These vessels contained BGJ_b culture medium (Fitton-Jackson modification) at the required volume. Four pineal glands were transferred to each vessel. The required volume of ISO, previously dissolved in culture medium, was added, resulting in a final incubation volume of 200 μ l. The final concentration of agonist in each of the five culture vessels was then 0 μ M (basal level), 0.3 μ M, 1 μ M, 3 μ M, and 10 μ M. These culture vessels were placed in the bottom of 30cc Poly-Top[®] glass vials and the atmosphere therein was saturated with carbogen (5%CO₂:95%O₂), before the top was firmly sealed. The vials were placed in a Forma Scientific[®] incubator, and incubated in the dark at 37°C for 3 h.

After the 3 h, the incubation was terminated by removing the glands from the vessels and placing them in individual 1ml glass homogenation tubes. They were assayed by the usual technique [§ 3.2.1.4].

3.4.1.3.2 Experiment B

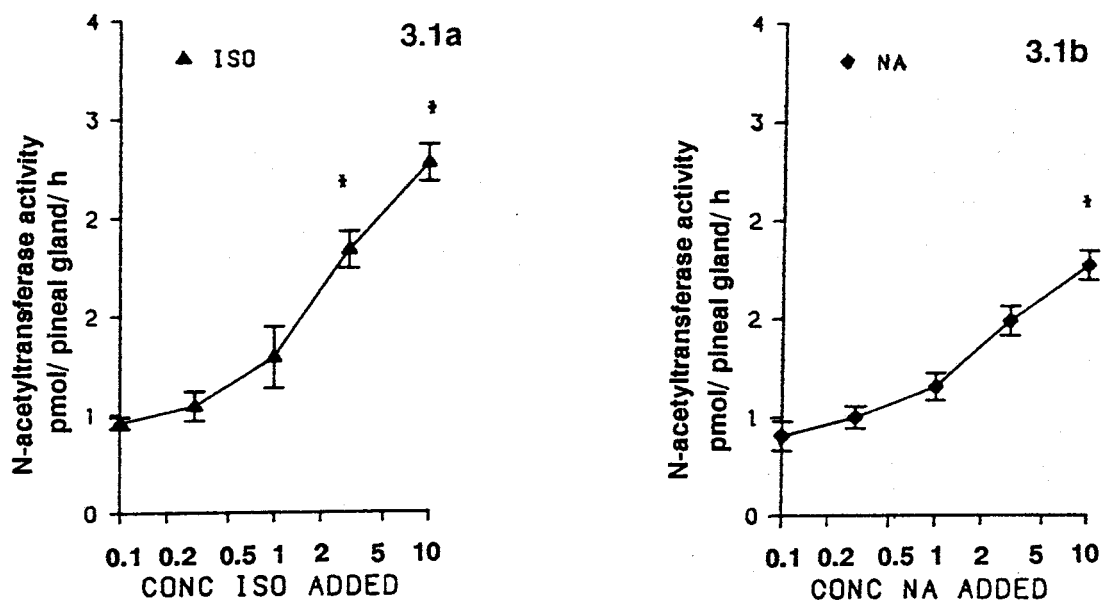
The procedure in Experiment A [§ 3.4.1.3.1] was repeated, but NA replaced ISO at the same concentrations.

3.4.1.4 Data analysis and statistics.

Data are expressed as pmol *N*-acetyltransferase activity/ pineal gland/ h, with each point representing the mean \pm SEM for 4 glands. Statistical comparisons are made using the nonparametric Kruskal-Wallis *H* test for multiple groups. *Post-hoc* analysis to identify the significantly different group means was performed using the Neuman-Keuls multiple range test at the $p < 0.05$ level.

3.4.2 Results

The results are presented in Figures 3.1a and 3.1b



Figures 3.1a and 3.1b: Cumulative dose response relationships found following isoprenaline (ISO)[Left] and noradrenaline (NA)[Right] stimulation of NAT activity in rat pineal glands (mean \pm SEM; $n = 4$; $p < .^* 0.05$; Neuman-Keuls multiple range test)

3.4.3 Discussion

Significant stimulation of NAT was only evident at concentrations of $[3\mu\text{M}]$ and $[10\mu\text{M}]$. NA showed less stimulation of NAT than ISO had done. This follows the trend first seen in Chapter 2 [§ 2.2.4], where NA showed smaller stimulation of aMT than ISO did. This provides further evidence of intrinsic activity - and/or affinity differences between the two agonists. The results also confirm the findings of other investigators, that $[10\mu\text{M}]$ appears to provide optimal stimulation of NAT (Klein *et al.*, 1983; Vanecek *et al.*, 1985), and thus it seems justifiable to use a concentration of $[10\mu\text{M}]$ in the succeeding studies to investigate the role of the CCBs in NAT stimulation.

3.5 Experiment 3: ISOPRENALINE AND NORADRENALINE STIMULATION OF NAT IN THE PRESENCE OF PROPRANOLOL AND PRAZOSIN

3.5.1 Introduction

As discussed in [§ 3.1], NAT has been shown to increase following adrenergic stimulation. Concurrent α_1 and β stimulation has been shown to produce a greater increase in NAT than does β -receptor stimulation alone. This enhancement has been associated with an influx of Ca^{2+} ions following the α_1 -receptor stimulation. NAT activation is however critically dependent on the activation of adenylyl cyclase (Klein *et al.*, 1970; Klein *et al.*, 1978) leading to increased cAMP levels. These are controlled by β -receptor stimulation.

3.5.1.1 Objective

This experiment investigates the role of the respective β and α_1 receptors in the adrenergic stimulation of NAT.

3.5.2 Materials and methods

3.5.2.1 Materials

dl-Propranolol-HCl was purchased from Sigma Chemical Co., USA. Prazosin-HCl was donated by Pfizer, Johannesburg, S.A. Sources of all other materials have been described [§ 3.2.1].

3.5.2.2 Animals

Adult female Albino Wistar rats (280 - 320 g b.wt) were used in this study. Their living environment has been described [§ 2.2.1.2].

3.5.2.3 METHODS

3.5.2.3.1 Experiment A

Rats were sacrificed between 09h00 and 09h30. Their pineal glands were removed as described in [§ 2.2.1.3] and transferred to 4 sterile culture vessels containing BGJ_b culture medium (Fitton-Jackson modification) at the required volume. Four pineal glands were transferred to each vessel. Prazosin was dispersed in 50 μ l of absolute ethanol/ mg prazosin before diluting it to volume in

distilled water (Yuwiler, 1987). The vehicle control contained an equivalent volume of ethanol. Propranolol was dissolved in culture medium.

After a 10 min pre-incubation period with glands in the presence of either vehicle, propranolol or prazosin, ISO[10 μ M] was added. The 4 vessels thus contained in a final incubation volume of 200 μ l, the following combinations of drugs, at the listed concentrations:

	Column 1	Column 2
i)	Control	+ vehicle (unstimulated control),
ii)	[10 μ M]ISO	+ vehicle,
iii)	[10 μ M]ISO	+ [10 μ M]propranolol,
iv)	[10 μ M]ISO	+ [10 μ M]prazosin.

The vessels were then placed in glass vials as described in [§ 3.4.1.3], incubated for 3 h and assayed by the usual technique [§ 3.2.1.4].

3.5.2.3.2 Experiment B

The procedure in Experiment A [§ 3.5.2.3.1] was repeated, but NA replaced ISO at the same concentrations.

3.5.2.4 Data analysis and statistics

Data are expressed as pmol *N*-acetyltransferase activity/ pineal gland/ h, with each point representing the mean \pm SEM. Statistical comparisons are made using the nonparametric Kruskal-Wallis *H* test for multiple groups. *Post-hoc* analysis to identify the significantly different group means was performed using the Neuman-Keuls multiple range test at the $p < 0.05$ level.

3.5.3 Results and Discussion

3.5.3.1.1 Glands stimulated with ISO in the presence of blockers.

The results for those glands stimulated with ISO in the presence of propranolol and prazosin are presented in Figure 3.2.

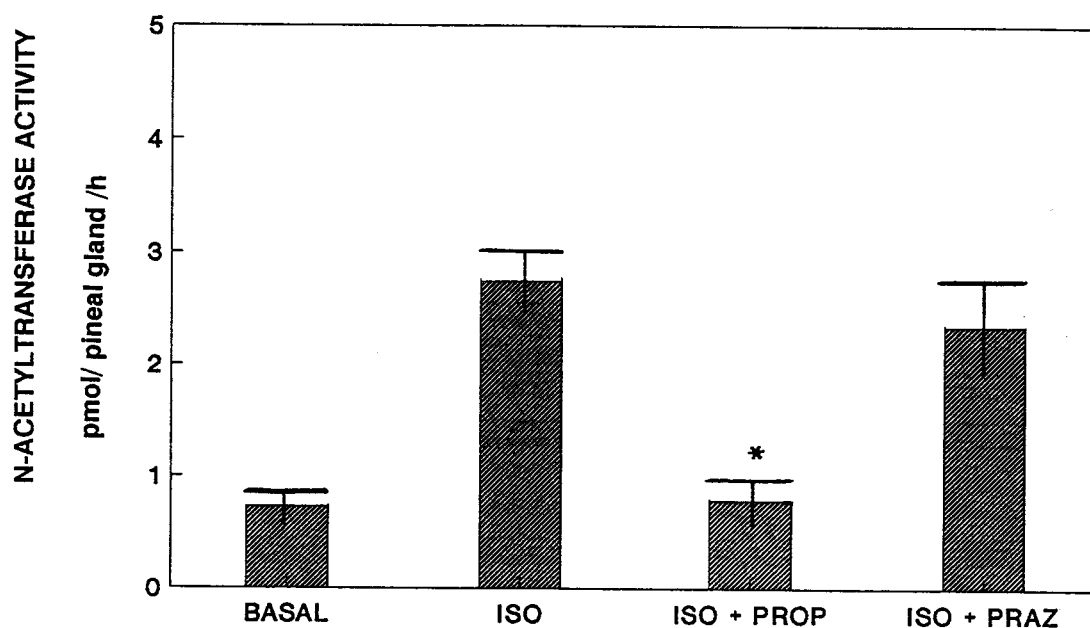


Figure 3.2: *Effects of vehicle, isoprenaline and isoprenaline plus adrenergic blockers on NAT activity in rat pineal glands (mean \pm SEM; n = 4; p < . * 0.05 Neuman-Keuls multiple range test)*

3.5.3.1.2 Discussion

ISO produced an increase in NAT as expected. This increase was however totally abolished in the presence of propranolol, the β -adrenoceptor blocker. This is in accordance with previous results (Deguchi and Axelrod, 1972b; Parfitt *et al.*, 1976) and confirms that NAT is dependent on β -adrenoceptor stimulation. Prazosin, on the other hand, had no apparent effect on ISO stimulation of NAT. This is to be expected as prazosin is a pure α_1 -adrenoceptor blocker (Cavero and Roach, 1977; Cambridge *et al.*, 1977), and ISO at a [10 μ M], should be acting primarily on β adrenergic receptors.

3.5.3.2.1 Glands stimulated with NA in the presence of blockers.

The results for those glands stimulated with NA in the presence of propranolol and prazosin are presented in **Figure 3.3**.

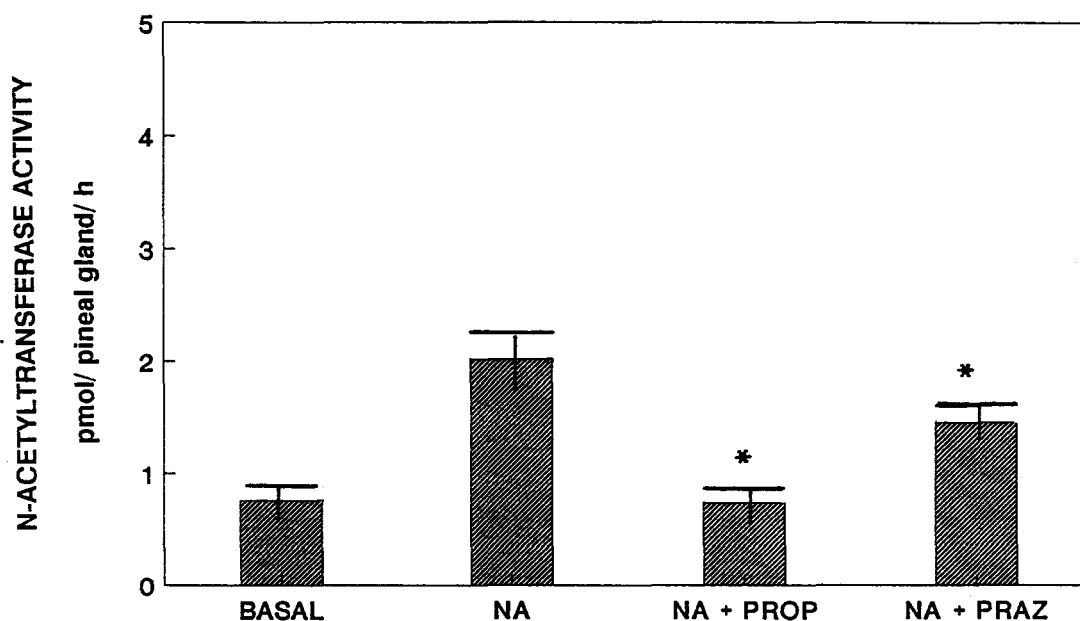


Figure 3.3: *Effects of vehicle, noradrenaline and noradrenaline plus adrenergic blockers on NAT activity in rat pineal glands (mean \pm SEM; n = 4; $p <^* 0.05$; Neuman-Keuls multiple range test)*

3.5.3.2.2 Discussion

NA produced an increase in NAT activity as expected. It was not as large as that seen with ISO, confirming the results seen in the dose response experiments [§ 3.4.2]. Propranolol again produced a complete abolition of NAT activity, confirming the dependence of the latter on β -adrenoceptor stimulation. Prazosin however was able to bring about a significant ($p < 0.05$) reduction in NAT activity, but far less acute than that found with propranolol. This is to be expected as NA should be acting through both β and α_1 -receptors (Vanecek *et al.*, 1986), and blocking the latter should still allow the β -receptors to remain functional as demonstrated [§ 3.4.3.1.2]. Similar findings were reported by Klein *et al.*, (1983).

3.5.4 Conclusion

These results therefore demonstrate that NAT is stimulated through both α_1 and β receptors, with the latter being an absolute requirement for NAT activity, and the α_1 component acting to potentiate this effect.

3.6 Experiment 4: THE EFFECT OF CALCIUM MANIPULATIONS ON NAT ACTIVITY IN RAT PINEAL GLANDS

3.6.1 Introduction

Calcium ion fluxes have been implicated in the potentiation subsequent to combined α_1/β adrenoceptor stimulation in the rat pineal gland. This is discussed in [§ 3.1].

3.6.1.1 Objective

This investigation examines the role of calcium in the adrenergic receptor control of NAT, by using several experimental manipulations which vary the concentration of calcium within the culture vessels. Ethyleneglycol-bis(β -aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) is a selective chelating agent with high selectivity for Ca^{2+} (Miller, 1984). Calcium ionophore A23187 strongly increases the permeability of the plasma membrane to bivalent cations, by combining with Ca^{2+} and transporting it through the membrane (Pressman, 1976). It can thus be used to mimic the Ca^{2+} influx that occurs with the α_1 -receptor stimulation following ISO stimulation (by by-passing the Ca^{2+} gating mechanism). This is similar to the way in which dibutyryl cAMP has been used to mimic β -adrenoceptor stimulation in the pineal gland, and hence to increase NAT (Deguchi and Axelrod, 1972; Klein *et al.*, 1970).

3.6.2 Materials and methods

3.6.2.1 Materials

Ethyleneglycol-bis(β -aminoethylether)-*N,N,N',N'*-tetraacetic acid was purchased from Sigma Chemical Co., USA. A23187 was purchased from Boehringer Mannheim, West Germany. Dimethyl sulphoxide (DMSO) was of analytical grade from commercial sources, and the sources of the other materials are recorded in [§ 3.2.1.1].

3.6.2.2 Animals

Adult female Albino Wistar rats (280 - 320 g b.wt) were used in this study. Their environment has been described in [§ 2.2.1.2].

3.6.2.3 METHODS

3.6.2.3.1 Experiment A

Rats were sacrificed between 09h00 and 09h30. Their pineal glands were removed [§ 2.2.1.3], and transferred to sterile culture vessels containing BGJ_b (Fitton-Jackson modification) culture medium at the required volume. Four pineal glands were transferred to each vessel. Stock solutions of A23187 [0.1M] were made up in DMSO and serially diluted in culture medium, while EGTA was dissolved in distilled water. These agents were added to the vessels in the required volumes. The unstimulated control received an equivalent amount of vehicle. The adrenergic agonist ISO was then added. The 7 vessels thus contained in a final incubation volume of 200 μ l, the following combinations of drugs, at the listed concentrations:

	Column 1	Column 2
i)	Control	+ vehicle (unstimulated control),
ii)	[10 μ M] ISO	+ vehicle,
iii)	[10 μ M] ISO	+ [3.3mM] CaCl ₂ ,
iv)	[10 μ M] ISO	+ [3.3mM] EGTA,
v)	[10 μ M] ISO	+ [3.3mM] EGTA + [3.3mM] CaCl ₂ ,
vi)	[0 μ M] agonist	+ [10 μ M] A23187,
vii)	[1 μ M] ISO	+ [10 μ M] A23187

The vessels were then placed in glass vials as described in [§ 3.4.1.3], incubated for 3 hours and assayed by the usual technique [§ 3.2.1.4].

Prior to this experiment, the concentration of Ca²⁺ in the culture medium was determined to be 2.38 ± 0.03 mM Ca²⁺ using a Varian AA 1275 Atomic Absorption Spectrophotometer[®], in a flame of acetylene/nitrous oxide.

A concentration of 3.3mM EGTA is commonly reported in the literature (Sugden *et al.*, 1986, 1987), and is thus deemed sufficient to chelate the Ca²⁺ present in the incubation medium.

3.6.2.3.2 Experiment B

The procedure followed in Experiment A [§ 3.6.2.3.1] was repeated, but NA replaced ISO. Two groups were not included. These were NA + CaCl₂ and A23187 alone. They were not tested as they did not show any unexpected results in [§ 3.6.3]. The 5 vessels thus contained the following combinations of drugs, at the listed concentrations in a final incubation volume of 200 μ l:

	Column 1	Column 2
i)	Control	+ vehicle (unstimulated control),
ii)	[10 μ M]NA	+ vehicle,
iii)	[10 μ M]NA	+ [3.3mM]EGTA,
iv)	[10 μ M]NA	+ [3.3mM]EGTA + [3.3mM]CaCl ₂ ,
v)	[1 μ M]NA	+ [10 μ M]A23187

3.6.2.4 Data analysis and statistics

Data was analysed as in [§ 3.5.2.4].

3.6.3 Results and Discussion

3.6.3.1.1 Results of glands stimulated with ISO

The results are presented in Figure 3.4

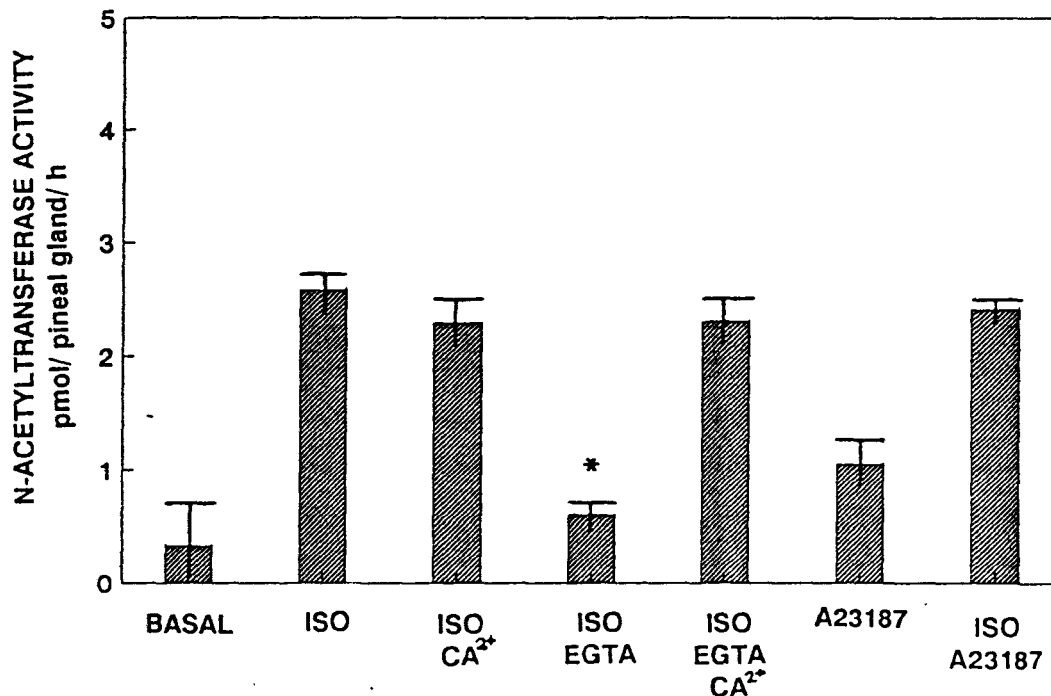


Figure 3.4: Effects of vehicle, A23187, isoprenaline and isoprenaline plus calcium manipulating agents on NAT activity in rat pineal glands (mean \pm SEM; n = 4; p <: * 0.05 Neuman-Keuls multiple range test).

3.6.3.1.2 Discussion

Glands stimulated with ISO showed an increase in NAT as can be expected. The addition of Ca^{2+} to the culture medium of glands already stimulated with ISO did not bring about any significant change in the level of NAT. This suggests that the Ca^{2+} must somehow be introduced into the cells for its effect to be seen. On the other hand, removal of calcium from the culture medium, with the calcium chelating agent EGTA, totally abolished NAT activity. This finding has been demonstrated before (Zatz and Romero, 1978; Wilkinson, 1978). Addition of [3.3mM] calcium to the medium containing EGTA, was able to restore NAT activity to a level that was not statistically different from that containing ISO alone.

The calcium ionophore A23187 was unable to stimulate NAT by itself, but when added to the vessel containing ISO [1 μ M], it was able to potentiate NAT activity to a level similar to that seen with [10 μ M] ISO. This is in agreement with other reports showing a role for calcium in the adrenergic regulation of this gland [§ 2.5.1.2]. These results also show that an influx of calcium coupled to β -receptor stimulation can produce a synergistic effect.

3.6.3.2.1 Results of glands stimulated with NA

The results are presented in Figure 3.5.

3.6.3.2.2 Discussion

These results are similar to those seen with ISO stimulation. EGTA totally abolished the stimulation of NAT, while the addition of calcium to the medium containing EGTA was able to restore NAT activity. This confirms the dependence of NAT on calcium. Addition of A23187 to glands stimulated with [1 μ M] NA brought about an even larger stimulation of NAT than that seen with ISO [1 μ M] + A23187 [10 μ M]. It is possible that increased calcium in the cell due to the ionophore, when it accompanies α_1 -adrenergic stimulation, leads to a greater enhancement of NAT activity.

3.6.4 Conclusion

These results then confirm that NAT activity is indeed sensitive to manipulations of Ca^{2+} levels.

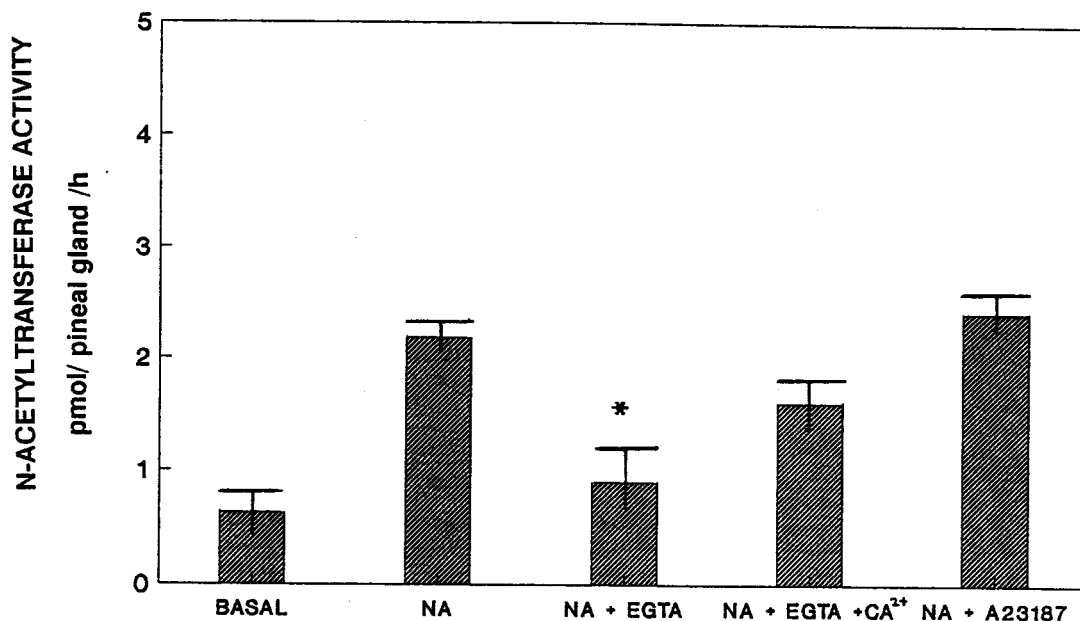


Figure 3.5: *Effects of vehicle, noradrenaline and noradrenaline plus calcium manipulating agents on NAT activity in rat pineal glands (mean \pm SEM; n = 4; p < * 0.05 Neuman-Keuls multiple range test)*

3.7 Experiment 5: THE EFFECT OF THREE ORGANIC CALCIUM CHANNEL BLOCKERS ON NAT ACTIVITY IN RAT PINEAL GLANDS.

3.7.1 Introduction

Calcium ion fluxes have been implicated in the potentiation subsequent to combined α_1/β adrenoceptor stimulation in the rat pineal gland. A VOC is reportedly present on the pinealocyte membrane. In Chapter 2 [§ 2.5] an attempt was made to investigate these fluxes, by using the CCBs to probe them. Instead of the CCBs inhibiting the Ca^{2+} entry into the pinealocyte, and thus inhibiting the enhancement discussed in [§ 2.5.1.2], a paradoxical increase in aMT production was found (Brown *et al.*, 1989). *N*-acetylserotonin, the metabolic precursor to aMT, was also found to be raised. *N*-acetyltransferase is the enzyme responsible for the conversion of serotonin to *N*-acetylserotonin. Raised levels of NAT would add credibility to the findings of Chapter 2 and possibly suggest interference at the level of cAMP, the nucleotide on which NAT activation is dependent. This dependence was shown by the abolition of NAT activity in the presence of the β -adrenergic blocker propranolol [§ 3.5.3].

3.7.1.1 Objective

It was therefore decided to investigate the effects of the three structurally diverse CCBs, verapamil, diltiazem and nifedipine, on the conversion of tryptamine to its *N*-acetylated product using the assay protocol described in [§ 3.2] and adapted to *in vitro* studies [§ 3.4.1.3]. This would serve as an indicator of NAT activity in the pineal glands.

3.7.2 Materials and methods

3.7.2.1 Materials

Verapamil-HCl, diltiazem, nifedipine, *l*-isoprenaline-HCl and *l*-noradrenaline were purchased from the Sigma Chemical Co., USA. Sources of all other materials have been recorded in [§ 3.2.1.1].

3.7.2.2 Animals

Adult female Albino Wistar rats (190 - 220 g b. wt) were used in this study. Their environment has been described in [§ 2.2.1.2].

3.7.2.3 METHODS

3.7.2.3.1 Experiment A

Rats were sacrificed between 09h00 and 09h30 by neck fracture. Their pineal glands were removed [§ 2.2.1.3] and transferred to sterile culture vessels containing BGJ_b culture medium (Fitton-Jackson modification) at the required volume. Four pineal glands were transferred to each vessel.

The culture medium contained either the CCB verapamil [10 μ M] or vehicle. The glands were exposed to verapamil or vehicle 30 min prior to the addition of the adrenergic agonists ISO and NA [10 μ M]. Glands were thus incubated in the presence of verapamil and either ISO or NA [10 μ M]. These were compared with control glands incubated with vehicle, in the presence of either ISO or NA [10 μ M]. The total incubation volume was 200 μ l. A fourth vessel contained no adrenergic agonist. This served to determine basal levels of NAT, and also acted as a system standard control in the series of experiments. The vessels were then placed in glass vials as described in [§ 3.4.1.3], and incubated as before. The reaction was terminated after a 3 h incubation period. The glands were assayed individually by the usual technique [§ 3.2.1.4].

protected from oxidation by the addition of ascorbic acid medium in which they were dissolved (Sugden and Klein, 1984). Verapamil was dissolved in distilled water. It was added to the cultures, in a 10 μ l volume from a freshly prepared stock solution. Control incubations therefore contained 10 μ l distilled water.

3.7.2.3.2 Experiment B

The procedure in Experiment A [§ 3.7.2.3.1] was repeated, but verapamil was replaced by diltiazem at the same molar concentration.

Diltiazem was also dissolved in distilled water, with control incubations receiving the equivalent amount of vehicle.

3.7.2.3.3 Experiment C

The procedure in Experiment A [§ 3.7.2.3.1] was repeated, but verapamil was replaced by nifedipine at the same molar concentration. Because nifedipine was relatively insoluble in water, it was first dissolved in 70% ethanol, before further dilution in distilled water. Final concentration of ethanol did not exceed 1%. Control incubations contained the equivalent amount of vehicle. In addition, because of nifedipine's sensitivity to light, [§ 2.5.2.3.3] all solutions of nifedipine were made up freshly just prior to use and kept in glassware protected from light.

3.7.2.4 Data analysis and statistics

Data were analysed as described in [§ 3.5.2.4], however a nonparametric Mann-Whitney *U* test was used to determine significance between each agonist/vehicle control and its corresponding agonist/CCB combination.

3.7.3 Results and Discussion

3.7.3.1.1 Results of glands stimulated in the presence of verapamil

The results of those experiments in which glands are stimulated with ISO and NA in the presence of verapamil are presented in Table 3.2 and Figure 3.6

TABLE 3.2: *N-acetyltransferase activity per rat pineal gland in the presence of vehicle or CCB, when stimulated with either isoprenaline (ISO) [10 μ M] or noradrenaline (NA) [10 μ M]. (mean \pm SEM; n=4 glands per group)*

TREATMENT	<i>N-acetyltryptamine formed (pmol/ hr/ pineal gland)</i>		
	Verapamil	Diltiazem	Nifedipine
Basal	0.83 \pm 0.17*	0.78 \pm 0.46*	0.79 \pm 0.08*
ISO + vehicle	2.38 \pm 0.53	3.19 \pm 0.86	3.06 \pm 0.25
ISO + CCB	3.44 \pm 0.18	1.62 \pm 0.35 ^(b)	2.87 \pm 0.15
NA + vehicle	2.09 \pm 0.13	1.86 \pm 0.27	2.16 \pm 0.08
NA + CCB	3.04 \pm 0.33 ^(a)	2.66 \pm 0.16	3.45 \pm 0.95 ^(a)

$p < :$ ^(a) 0.025, ^(b) 0.05, (Mann-Whitney *U* test) compared to respective vehicle control.

* Significantly different from respective stimulated groups (Kruskal Wallis *H* test $p < 0.005$).

3.7.3.1.2 Discussion

The results differ slightly from those observed in the parallel experiment in the organ culture studies [§ 3.5.2.3.1]. There is no difference between NAT activity in those glands stimulated with ISO, and those stimulated with ISO in the presence of verapamil. The organ culture studies showed an increase in aHT in the parallel experiment. The results from studies on glands stimulated with NA in the presence of verapamil are in agreement with the organ culture study, showing the paradoxical increase in the level of NAT.

3.7.3.2.1 Results of glands stimulated in the presence of diltiazem

The results of those experiments in which glands are stimulated with ISO and NA in the presence of diltiazem are presented in Table 3.2 and Figure 3.7.

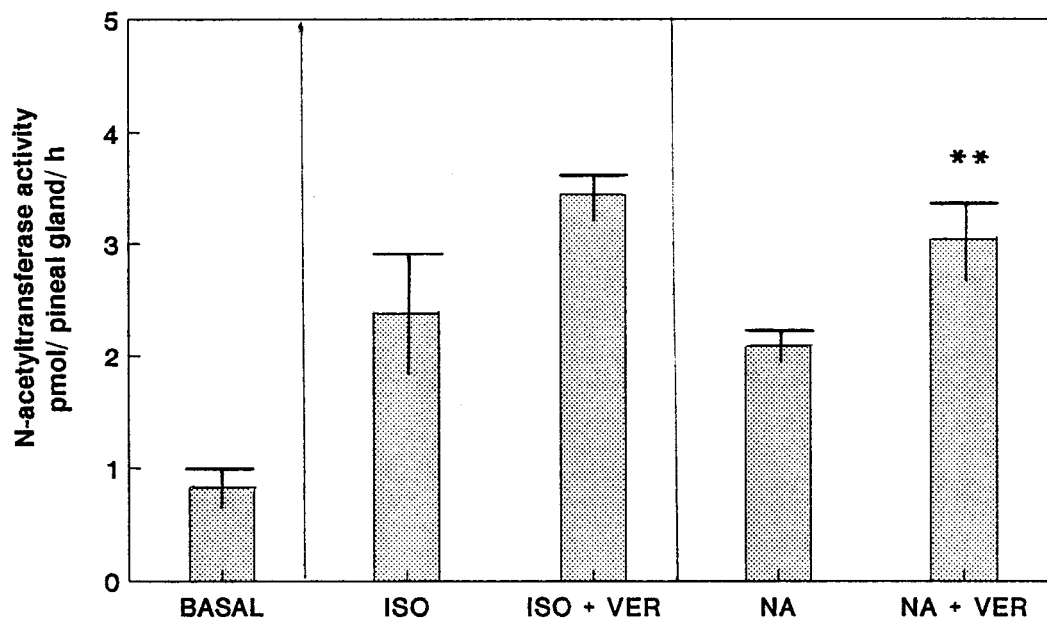


Figure 3.6: *The effect of verapamil (VER) on N-acetyltransferase activity per rat pineal gland in the presence of either ISO or NA [10 μ M], compared to control glands with vehicle, in the presence of either ISO or NA [10 μ M] (mean \pm SEM; n=4 glands per group; p < : *0.05;Mann-Whitney U test)*

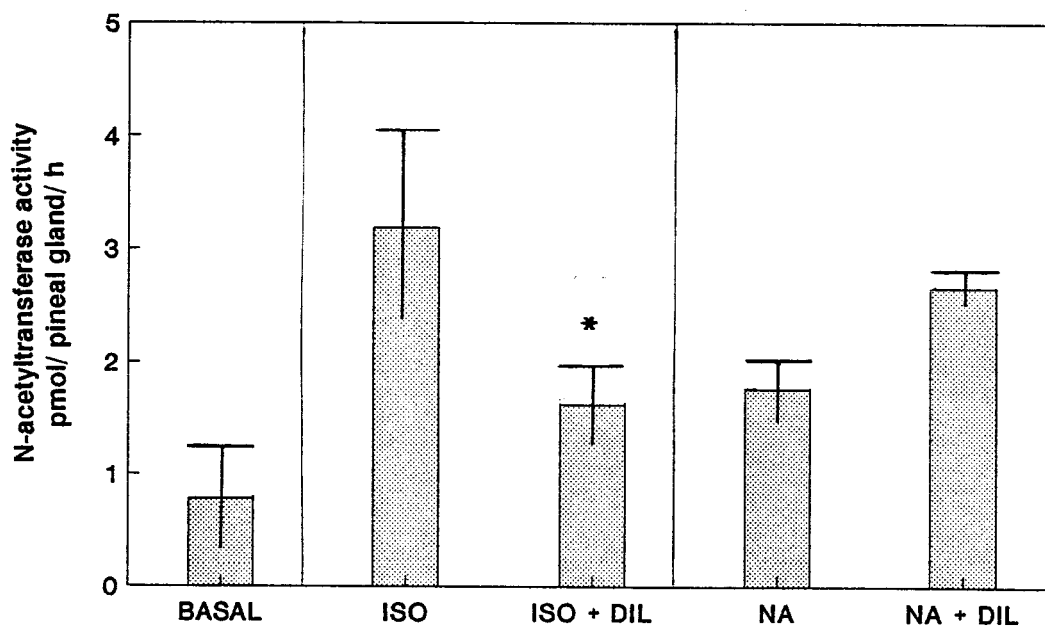


Figure 3.7: *The effect of diltiazem (DIL) on N-acetyltransferase activity per rat pineal gland in the presence of either ISO or NA [10 μ M], compared to control glands with vehicle, in the presence of either ISO or NA [10 μ M]. (mean \pm SEM; n=4 glands per group; p < : *0.05; **0.025 Mann-Whitney U test)*

3.7.3.2.2

Discussion

The paradoxical increase following NA stimulation in the presence of diltiazem is still observed, but in contrast, those glands stimulated with ISO in the presence of diltiazem show a significant decrease in NAT levels. This supports the findings of a decrease in aMT observed in this group in the organ culture studies [§2.5.3.1.1], although those did not show a decrease in aHT. This could imply that the NAT assay is a more sensitive parameter, or it could be that the organ culture study returned a false high in aHT levels, as this substrate was not being converted to aMT, because of an inhibition of HIOMT activity [§ 2.5.3.1.2]. To further complicate this matter, the recent data of Walker and Aloyo, (1988) shows an increase in the secretion of HT from the pineal following NA stimulation. This theoretically would reduce the substrate available to NAT in organ culture.

3.7.3.3.1

Results of glands stimulated in the presence of nifedipine

The results of those experiments in which glands are stimulated with ISO and NA in the presence of nifedipine are presented in Table 3.2 and Figure 3.8.

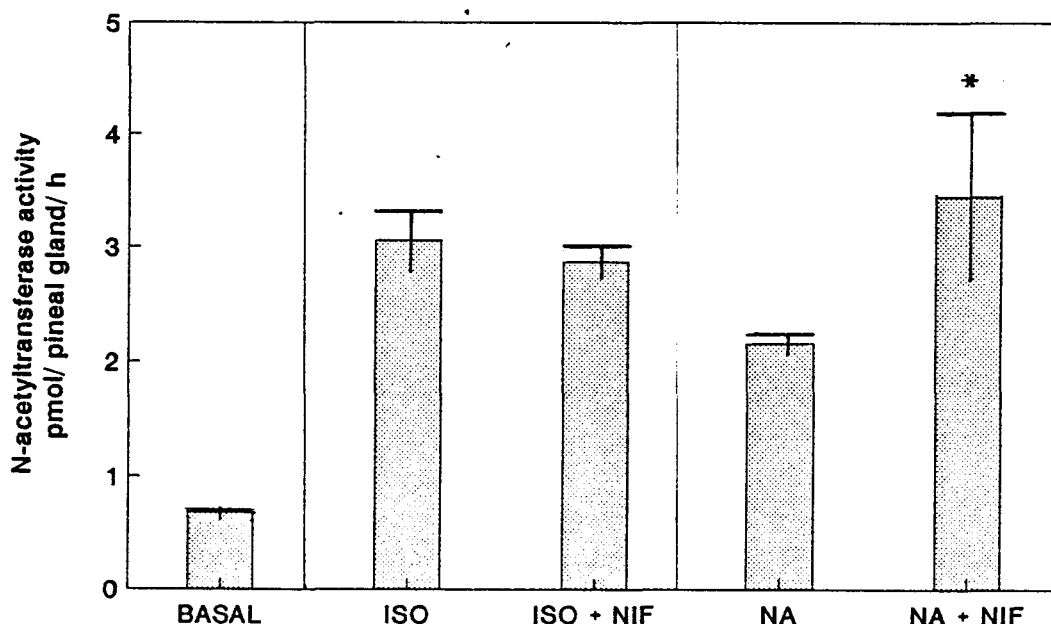


Figure 3.8:

*The effect of nifedipine (NIF) on N-acetyltransferase activity per rat pineal gland in the presence of either ISO or NA [10 μ M], compared to control glands with vehicle, in the presence of either ISO or NA [10 μ M] (mean \pm SEM; n=4 glands per group; p < *0.05; Mann-Whitney U test)*

3.7.3.3.2 Discussion

A similar trend is seen here, to that observed with the verapamil group [§ 3.7.3.1.2]. Glands stimulated with ISO are not significantly different from those stimulated with ISO in the presence of nifedipine. Those glands stimulated with NA in the presence of nifedipine again show the paradoxical increase in NAT as they did in aMT previously [§ 3.5.3.2.2].

3.7.4 Conclusion

In summary then, the following trend emerges: all glands stimulated with NA in the presence of any one of the CCBs showed an increase in NAT activity. Glands stimulated with ISO showed no significant differences in NAT activity in the presence of verapamil and nifedipine, but did show a decrease in the presence of diltiazem. Once again diltiazem shows a difference when compared with the other two CCBs [§ 2.5.4].

It would thus seem that the CCBs are in some way influencing cAMP levels and interfering with the metabolic cascade simplistically represented as:



Although purely speculative at this stage, the following mechanism may provide a plausible explanation for the observed phenomenon:

Following adrenergic stimulation in the pineal gland, the intracellular concentration of cAMP rises, coupled to a corresponding increase in its regulator cAMP-phosphodiesterase (PDE) (Oleshansky and Neff, 1975). The cellular flux of Ca^{2+} , by way of its interaction with the ubiquitous intracellular protein calmodulin (CaM), plays a crucial role in regulating PDE activity (Cheung, 1982).

Ca^{2+} must first bind to CaM in its inactive state. This causes a conformational change in the latter, resulting in the exposure of a hydrophobic surface (La Porte *et al.*, 1980) and the formation of the active conformer (CaM- Ca^{2+}). This in turn binds to PDE producing the active holoenzyme Ca^{2+} -CaM-PDE (Klee *et al.*, 1980). More specifically the CCBs have been shown to bind to the exposed hydrophobic surface of CaM- Ca^{2+} , preventing the active conformer, CaM- Ca^{2+} binding PDE (Johnson and Wittenauer, 1983; Lamers *et al.*, 1985). This proposed mechanism is shown diagrammatically in Figure 3.9. Both the CaM-sensitive and CaM-insensitive forms of cAMP-

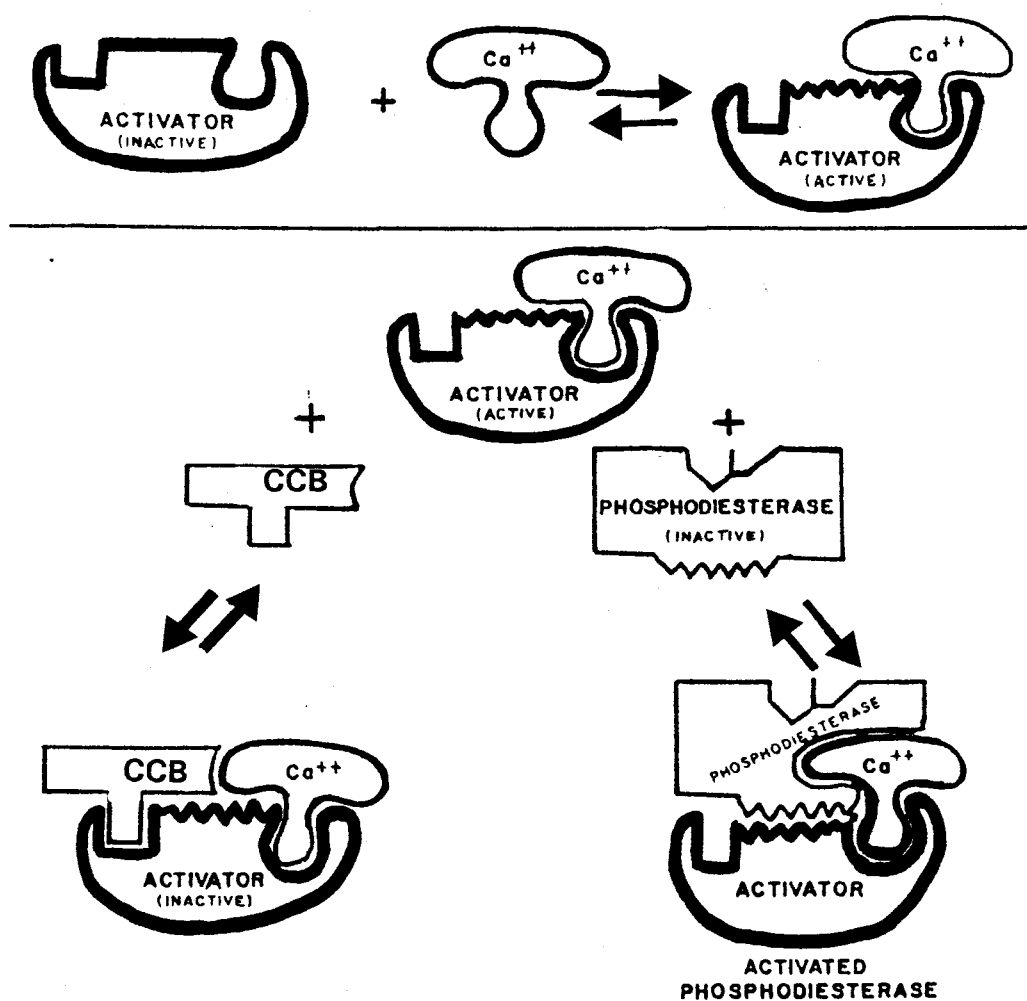


Figure 3.9:

A diagrammatic representation of the activation of calmodulin by Ca^{2+} , the conformational change which occurs, resulting in the active conformer, to which either the CCB binds (thereby preventing PDE binding) or the enzyme to be activated binds e.g PDE resulting in the active holoenzyme (adapted from Weiss and Levin, 1978).

PDE have been shown to be inhibited at the micromolar concentrations used in this study (Epstein *et al.*, 1982).

If this is the case then cAMP levels could be expected to remain high, with correspondingly higher levels of NAT, leading to an increased acetylation of HT, and ultimately to higher concentrations of aMT. Some credence would be lent to this hypothesis if increased cAMP levels were demonstrable in the presence of the CCBs.

CHAPTER 4

CYCLIC-AMP STUDIES

4.1 INTRODUCTION

4.1.1 A role for calcium in cyclic-AMP regulation ?

Sutherland and Rall (1958) were the first to demonstrate the existence and function of a cyclic nucleotide: cyclic 3',5'-adenosine monophosphate (cAMP). They showed the accumulation of cAMP in liver tissue, following adrenaline stimulation. Sutherland and his co-workers were able to demonstrate that cAMP was formed via a membrane-bound adenylyl cyclase, while its destruction occurred via an enzyme –cyclic-AMP phosphodiesterase (cAMP-PDE)– present in the cytosol. This work has been reviewed in Sutherland *et al.*, (1968). By 1968 they were able to advance the second messenger concept.

Stated simplistically this concept embodies a first messenger, an external stimulus specific for a particular cell type, interacting with the membrane-bound adenylyl cyclase. This interaction initiates a flow of information into the cell and induces that cell to generate increased cAMP levels. The generated cAMP then acts internally as the so-called second messenger, performing a variety of specific functions (Sutherland *et al.*, 1968). Kuo and Greengard (1969) extended this concept to include the stimulation of second messenger dependent protein kinases.

Second messengers are now understood to include not only cAMP, but also cGMP and Ca^{2+} , all of which play a prominent role in the regulation of protein phosphorylations (Rasmussen, 1981). They are discussed in [§ 1.6].

It was investigations into the control of cAMP in brain tissue that implicated calcium as a controller of several intracellular events. In 1970 Cheung linked Ca^{2+} levels to the control of cAMP-PDE. Teo *et al.*, (1973) found that Ca^{2+} acts via some protein, to influence cAMP-PDE. They named the protein responsible for this control, calcium dependent regulator (CDR), in the light of both its high affinity for Ca^{2+} , and the need for Ca^{2+} to be present for its activity. In 1980, Cheung renamed it calmodulin, a name, derived from its function as a calcium modulating protein. Calmodulin (CaM) is discussed in [§ 1.10.2].

The duality of Ca^{2+} and cAMP in the control of so many control functions within the universal

system of stimulus response coupling led to adoption of the term "synarchic control". This is based on an analogy to the ancient Greek *Arcons*: Heralds of special status sent out in twos, each carrying part of a message and *syn* meaning together (Rasmussen, 1981). The analogy between the arcons and the dual messengers, cAMP and calcium, is easily perceivable. An analysis of second messenger function in a variety of cells shows that calcium mediates many of the cellular responses, while cAMP acts to modulate the action of calcium (Berridge, 1984).

4.1.2 Synarchic control in the pineal gland.

In the rat pineal gland NA stimulates cAMP, cGMP and Ca^{2+} through α_1 and β_1 receptors (Cardinali and Vacas, 1987). The characterization of these receptors has been discussed in [§ 1.5.2].

β -Adrenergic stimulation of pinealocyte cAMP is known to involve *Ns* (Minneman and Iverson, 1976; Vanecek *et al.*, 1986), the stimulatory GTP-binding regulatory protein which couples receptor occupation to the activation of adenylyl cyclase (Hurley *et al.*, 1984). Cyclic-GMP is probably regulated by the α subunit of a related GTP binding protein, *Gs α* (Sugden and Klein, 1987). α_1 -Adrenergic stimulation potentiates β adrenoceptor stimulation by a variety of complex mechanisms. For example, an increase in intracellular Ca^{2+} levels is associated with, or may even be essential to, i) the activity of pineal phospholipase C (Smith and Hauser, 1981), ii) the activity of phospholipase A2 (Ho and Klein, 1987), and iii) the translocation of protein kinase C (Sugden *et al.*, 1985). In the rat pineal gland, the α_1 blocker prazosin is able to inhibit this effect, while the α_2 blocker yohimbine shows no effect, confirming that in this system, NA is acting via α_1 and β receptors (Ho *et al.*, 1988).

The activity of NAT is regulated via an adrenergically mediated stimulation of adenylyl cyclase (Klein and Berg, 1970; Klein *et al.*, 1978). β -Adrenoceptor stimulation increases cAMP, in the rat pineal gland, by activating adenylyl cyclase. Cyclic-AMP levels are critical in the production of the pineal hormone melatonin(aMT) from serotonin, via the rate-limiting enzyme *N*-acetyltransferase (NAT) (Axelrod, 1974). α_1 -Adrenoceptor activation, which alone is without effect, has been shown to enhance β -adrenoceptor stimulation of cAMP. This enhancement has been linked to an increased influx of Ca^{2+} into the pinealocyte (Sugden *et al.*, 1986). Furthermore, treatments which result in increased cAMP levels have been shown to increase NAT levels (Klein and Berg, 1970; Klein *et al.*, 1970). A similar result could be expected if cAMP-PDE (the enzyme responsible for the termination of cAMP activity) were to be inhibited.

4.1.3 Objectives

It was therefore proposed to investigate the effect of these three CCBs on rat pineal cAMP levels, to ascertain if the latter were indeed raised, as suggested by the high levels of NAT found in Chapter 3. To enable such a study to be made, a suitable cAMP assay had to be selected from the many methods currently available for this purpose.

4.1.4 Methods for the determination of cyclic-AMP

Although several very sensitive radioimmunoassays for the determination of cAMP are available, their cost was prohibitive. Since the action of cAMP in all tissues involves its binding to some type of protein (Oye, 1973), several techniques have been devised which make use of a natural binding protein coupled to a radioisotope type of dilution assay, thereby giving greater sensitivity. The methods of Gilman (1970) and Brown *et al.*, (1971) are two well known examples of these so-called saturation binding assays. While Gilman's method requires the onerous isolation of a protein from bovine muscle, that of Brown *et al.*, (1971), makes use of a crude protein, easily extracted from bovine adrenal tissue, to bind cAMP. A brief outline of this latter method follows [§ 4.1.5]

4.1.5 Summary of the assay

The assay is based on the competition between unlabelled cAMP and a fixed quantity of the tritium labelled compound for binding to a protein which has a high specificity and affinity for cAMP. The amount of [³H]-cAMP bound to this protein is inversely related to the amount of cAMP present in the assay sample. The amount of unlabelled cAMP in the sample is calculated by measurement of the protein-bound radioactivity. Separation of the protein-bound cAMP from the unbound nucleotide is achieved by centrifugation. The supernatant (which contains the protein-bound complex) is added to scintillation cocktail and its activity determined by liquid scintillometry. The concentration of unlabelled cAMP in the sample is then determined from a linear standard curve. The method used for the isolation of this cAMP binding protein and its subsequent use to investigate the effect of the CCB on pineal gland cAMP levels is described hereafter [§ 4.2].

4.2 CYCLIC-AMP DETERMINATION TECHNIQUE

4.2.1 Materials and Methods

4.2.1.1 Chemicals, drugs and reagents

[8-³H]Adenosine 3',5'-cyclic phosphate, ammonium salt (1.0 mCi/ml : specific activity 26.1 Ci/mmol) was purchased from Amersham, England, and the activated charcoal from Merck, Germany. *l*-Isoprenaline-HCl, noradrenaline bitartrate, phosphodiesterase (EC 3.1.4.17, specific activity ca 0.31 U/mg), cyclic-AMP, theophylline and bovine serum albumin were obtained from Sigma Chemical Co., USA. The chemicals used in buffers were of analytical grade from commercial sources.

4.2.1.2 Animals

Male Wistar rats (220-250 g b. wt) were housed four per cage in a temperature controlled room under a fixed lighting cycle of LD 12:12 (lights on 06h00) with food and water *ad libitum*. The rats were sacrificed between 09h00 and 09h30. This maintained uniformity with the previous studies [§ 3.2.1.2].

Male rats were chosen for the cAMP studies because they tended to be less anxious, when handled, than the female rats. It was therefore felt that their cAMP levels would more accurately reflect the drug treatment, rather than exogenous stress influences. Skene (1985) has reported higher basal cAMP levels in female rats, which she ascribed to their more anxious nature. As each rat acts as its own control [§ 4.2.1.6] any variance in basal levels should be removed.

4.2.1.3 Pineal glands

The pineal glands for assay were rapidly removed and bisected under a dissecting microscope on a cold glass sheet. The pineal hemisections were then transferred to prewarmed (37°C) BGJ_b culture medium (Fitton-Jackson modification).

4.2.1.4 Assay of cAMP in rat pineal glands

4.2.1.4.1 Collection and preparation of binding protein

Bovine adrenals were dissected out as soon as possible after slaughter and transported from the abattoir on ice. The cortices were separated from the medullas, finely chopped and homogenised

(Ultra-Turrax[®]) in 1.5 volumes of ice-cold buffer (0.25M sucrose, 50mM Tris-HCl buffer, pH 7.4, 25mM potassium chloride, 5mM magnesium chloride). The homogenate was spun (Hettich Universal 2S[®]) at 2000g for 5 minutes and the supernatant respun at 5000g for 15 minutes. Aliquots of the supernatant were stored in 1 ml Eppendorff[®] tubes at -20°C . The stored protein remained stable for the duration of this study.

4.2.1.4.2 Dilution

For each cAMP assay [§ 4.2.1.5.1], an aliquot of binding protein was thawed and diluted with an appropriate volume of cyclic-AMP buffer (50 μ M Tris-HCl buffer, pH 7.4, 8 μ M theophylline, 6 μ M mercapto-ethanol). As the concentration of isolated binding protein varied from batch to batch, it was essential to first determine the appropriate dilution of stock protein that would be suitable for a particular batch. Serial dilutions of binding protein in buffer were assayed for radioactivity, by following the zero dose column in Table 4.1 and adding increasing dilutions of stock protein.

A typical protein dilution curve obtained (cpm vs dilution used) is shown in Figure 4.1. It shows that a 1:3 dilution with buffer provides both sufficient binding (80%) and radioactive counts. This dilution factor was thus used in all subsequent cAMP assays with this batch of stock protein. Most other batches used in this laboratory required a similar dilution factor.

4.2.1.5.1 Cyclic AMP Assay

Cyclic AMP levels were measured in duplicate pineal homogenate samples by the saturation binding method of Brown *et al.* (1971). A simplified scheme adapted to pineal work is presented in Table 4.1.

The pineal hemisections were pre-incubated at 37°C for 10 min. One half was then transferred to fresh, prewarmed incubation medium containing [20 μ M] isoprenaline, the other half to control incubation medium containing vehicle. Both preparations were incubated for another 10 min at 37°C. The halves were then removed and homogenised separately in 110 μ l ice-cold cAMP buffer. Duplicate (50 μ l) aliquots of the tissue suspension were transferred to reaction tubes.

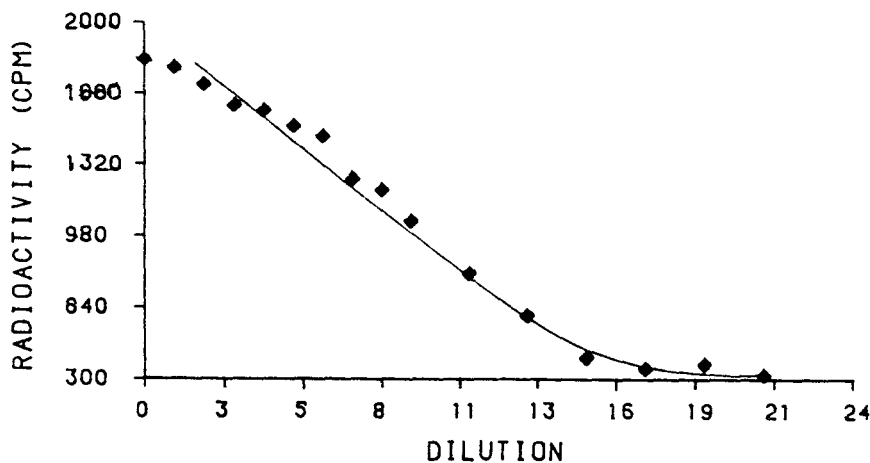


Figure 4.1: *A typical protein dilution curve obtained in these experiments*

Briefly each reaction tube contained 50 μl of sample or known amounts (0 - 8 pmol) of cAMP standard; 50 μl of [^3H] cAMP (8 nCi), cAMP buffer and 100 μl of a 1:3 dilution of binding protein. The final incubation volume was 350 μl . The incubation tubes were placed on ice and stored at 4°C. After 100 min, 100 μl of a 10% w/v suspension of charcoal in buffer containing 2% w/v bovine serum albumin, was added to each tube and the tubes were briefly vortexed on a Rota-Mixer Deluxe[®]. After centrifugation (14000g for 15 minutes at 4°C), a 100 μl aliquot of the supernatant was added to 3ml Ready Solv HP/B[®] (Beckmann). The vials were shaken (Griffin Flask Shaker[®]) for 10 min and then counted for radioactivity in a Beckmann LS 2800 liquid scintillation counter at an efficiency of 60% to 69%.

4.2.1.5.2 Quantitation

A calibration curve was set up for each assay. Calibration curves were plotted using the data obtained from the standard amounts of cAMP added to the tubes as outlined in Table 4.1 columns 2 through 8. The amounts of unknown cAMP in samples were determined by reference to such curves.

In order to linearize the standard curves a slightly different plotting procedure was used from that employed by Brown *et al.*, (1971). The terms used are derived from the resultant mean cpm as follows:

$$C_0 = \text{mean cpm}_{\text{zero standard}} - \text{mean cpm}_{\text{blank}}$$

$$C_x = \text{mean cpm}_{\text{standard}} - \text{mean cpm}_{\text{blank}} \quad \text{or for the samples, mean cpm}_{\text{sample}} - \text{mean cpm}_{\text{blank}}$$

TABLE 4.1: *Protocol used for the determination of cyclic AMP using the method of Brown et al., (1971). Quantities are in microlitre.*

	BLANK	0 DOSE	0,25	0,5	1,0	2,0	4,0	8,0	SAMPLE
μl Buffer	250	150	150	150	150	150	150	150	150
μl Std (1:10)	-	-	12,5	25	50	-	-	-	-
μl Std (as is)	-	-	-	-	-	10	20	40	-
Dist Water	50	50	37,5	25	-	40	30	10	-
^3H -cAMP	50	50	50	50	50	50	50	50	50
Homogenate for assay	-	-	-	-	-	-	-	-	50
Mix	Mix								
μl Diluted Protein	-	100	100	100	100	100	100	100	100
Mix gently	Mix gently to avoid frothing								
Incubate	in cold room at 4°C for 100 minutes								
Charcoal	100	100	100	100	100	100	100	100	100
Vortex	Vortex Briefly								
Centrifuge	at 15 000g for 15 min using precooled rotor								
Carefully remove	100 μl Supernatant and add to 3 ml cocktail, and count radioactivity								

The ratio C_0/C_x (ordinate) was calculated from the standard data, and plotted against the corresponding concentration (pmol/tube) of cAMP (abscissa).

An example of such a calibration curve is shown in **Figure 4.2**

4.2.1.6 Data analysis and statistics.

Vetulani *et al.*, (1976) expressed the cAMP response to isoprenaline administration as the difference in cAMP levels between two halves of tissue preparation, one half being exposed to isoprenaline and the other half serving as the control. The advantages of using this technique are many. Since each animal acted as its own control, animal, environmental and experimental variables could be controlled. Thus, the reported effect of decapitation on cAMP levels (Weiss

and Strada, 1972) would not affect the results. The possible effects of the oestrous cycle on adenylyl cyclase activity (Weiss and Crayton, 1970), would also be avoided.

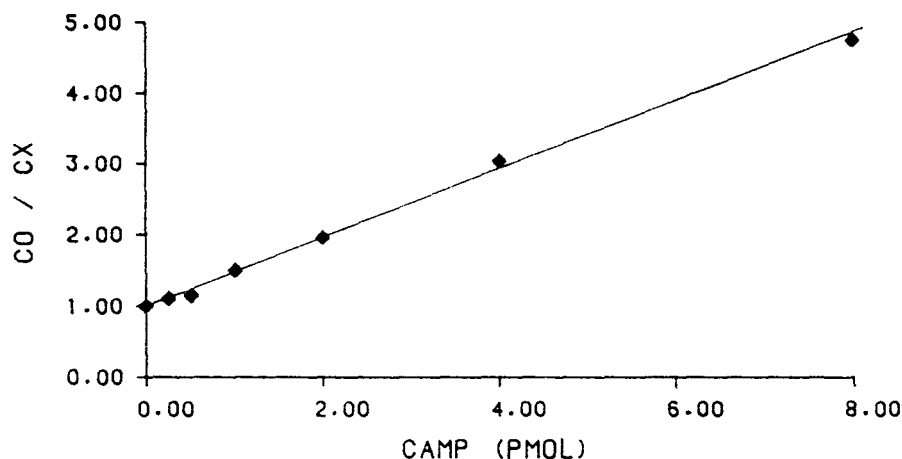


Figure 4.2: *A typical cAMP calibration curve obtained in these experiments*

This technique was adapted for use in the experiments with the CCBs [§ 4.4.0], as it allowed one half of a pineal to be incubated in the presence of an agonist and vehicle, while the corresponding half was incubated in the presence of agonist and CCB.

4.2.1.6.1 Statistical Analysis

Cyclic AMP levels –the mean of duplicate determinations– were determined from the cAMP standard curve and expressed as pmol/ pineal \pm SEM. For each drug group, the the cAMP levels in those glands stimulated with agonist and CCB were compared with those in control glands stimulated with agonist, but incubated in the presence of vehicle. The significance was evaluated using Student's paired *t*-test (2-tailed).

Pairing data for statistical analysis normalizes the data to its own control, thereby adjusting for any variations in basal activity. Warnick *et al.*, (1988) are one group of researchers using this method in their analysis.

4.2.1.7 Results and discussion

Although initial pilot studies had shown that [10 μ M] isoprenaline stimulation for 5 or 10 min was not able to stimulate cAMP in pineal gland hemisections, further investigation revealed that pineal gland hemisections incubated with [20 μ M] isoprenaline for 10 min, did show stimulation of cAMP well above basal levels.

The integrity of the assay was further validated as described in [§ 4.2.1.8].

4.2.1.8 Validation of results

Three approaches were used to test the validity of the system.

i) A stock solution of PDE 0.31 U/mg was made up. One unit should hydrolyse 1 μ M of 3', 5'-cAMP to 5'-AMP per min at pH7.5 at 30°C. Aliquots of increasing concentrations of PDE were added to some tubes, and these were incubated for 30 min. This ensured a quantitative hydrolysis of the cAMP present. Results obtained from this procedure could be used to test the sensitivity of the system.

ii) After addition of tracer quantities of [³H]-cAMP, BSA-coated activated charcoal was added to the tubes. Assay of the supernatant showed all tracer had been removed.

iii) Known quantities of standard cAMP were added to tubes containing zero added cAMP. These were placed among the normal assay tubes, and the results from these were tested to see that they agreed with the calibration curve.

4.2.1.9 Discussion

The results obtained confirm that this assay is suitable for the determination of cAMP in pineal hemisections. However, one additional test remained: to test the assumption that splitting the pineal gland would indeed produce equal halves.

4.3 Experiment 1. PROTEIN DETERMINATION IN CORRESPONDING HALVES OF PINEAL GLANDS.

4.3.1 Introduction

To vindicate the use one half of the pineal gland as the control, while the other half was stimulated with agonist in the presence of an CCB, it was important to check that equal amounts of protein were present in each of the halves.

Two methods are commonly used for the determination of protein in biological samples.

Bradford (1976) makes use of a dye-binding method, while Lowry and his workers, use a slightly more complex method. As the dye (Coomassie Brilliant Blue G-250) was not available in this laboratory, but the reagents required for the second method were available, it was decided to use the method of Lowry *et al.*, (1951). A simple protocol for this method using bovine serum albumin as standard is outlined in Table 4.2.

4.3.2 Materials and Methods.

4.3.2.2 Materials

All reagents used were of analytical grade from commercial sources.

4.3.2.1 Animals

Six adult female Wistar rats were used for this experiment. As the objective was to quantify the relative amounts of protein in each hemisection and not to assay cAMP levels, female rats could be used here, to conserve male rats for use in subsequent assays.

4.3.2.2 Pineal glands

Pineal glands were rapidly removed, placed on a cold glass sheet, and bisected under a dissecting microscope. The pairs of halves were kept together to ensure that the correct pairs were transferred to corresponding homogenization tubes (Pyrex[®], thick wall, no rim 10 x 75mm tubes).

4.3.2.3 Methods

Each hemisection was placed in a 2 ml glass homogenization tube, and homogenised in 100 μ l of cAMP buffer. A further 900 μ l of buffer was added to the tubes. Due care was taken to ensure that the homogenate was adequately mixed with this buffer. Duplicate 100 μ l aliquots of homogenate were assayed following the procedure outlined in Table 4.2.

Briefly 2ml of reagent C (2% Na₂CO₃ in 0.1N NaOH; 0.5% CuSO₄.5H₂O) in 1% sodium tartrate (50:1) was added to 400 μ l of Tris-HCl buffer (50mM,pH8.0) containing known amounts of bovine serum albumin (BSA) as standard or 100 μ l pineal homogenate. This solution was vortexed for 1 min and then allowed to stand for 15 min at room temperature (21-23°C). To each tube, 200 μ l of a 1:1 dilution of Folin-Ciocalteu phenol reagent was added and the tube immediately vortexed. After 30 mins at room temperature, the absorbance of the samples was measured at 750 nm using a spectrophotometer (Bausch & Lomb Spectronic Model 1001[®]).

4.3.2.4 Data analysis and statistics.

A calibration curve had to be set up for each assay. Calibration curves were plotted using the data obtained from the standard amounts of BSA added to the tubes as outlined in Table 4.2 rows 1 and 2. The amounts of unknown protein in samples were determined by reference to such curves. An example of such a calibration curve is shown in Figure 4.3.

For each group, the significance of the difference in protein levels between the two halves was determined. The significance was evaluated using Student's paired *t*-test (2-tailed).

4.3.3 Results

The mean protein content (μ g \pm SD) in the first half of glands was 78.4 \pm 10 μ g, while that of the corresponding halves was 82.5 \pm 8 μ g.

These results show that there is no significant difference ($p < 0.445$) between the halves if due care is taken, when splitting the halves under a dissecting microscope.

4.3.4 Discussion

It would thus seem that provided due care is taken when splitting the pineal glands into two

TABLE 4.2: *Scheme for the determination of protein (Lowry et al., 1951).*

REAGENTS (mls)	BLANK	SAMPLES (μg)					SAMPLE
		2.5	5.0	1.0	2.0	4.0	
STANDARD (0.1 mg/ml)	-	0.025	0.05	0.1	-	-	-
STANDARD (1 mg/ml)	-	-	-	-	0.02	0.04	-
SAMPLE	-	-	-	-	-	-	0.1
BUFFER *	0.4	0.375	0.35	0.3	0.38	0.36	0.3
REAGENT C **	2.0	2.0	2.0	2.0	2.0	2.0	2.0
STAND: 15 min at room temperature							
REAGENT E ***	0.2	0.2	0.2	0.2	0.2	0.2	0.2
STAND: 30 min at room temperature							
READ at 750nm.							

* Tris-HCl buffer (50mM, pH 8.0) containing 3mM MgCl₂.

** 2% Na₂CO₃ in 0.1 N NaOH: 0.5% CuSO₄.5H₂O in 1% sodium tartrate (50:1).

*** Diluted Folin-Ciocalteu phenol reagent (1:1).

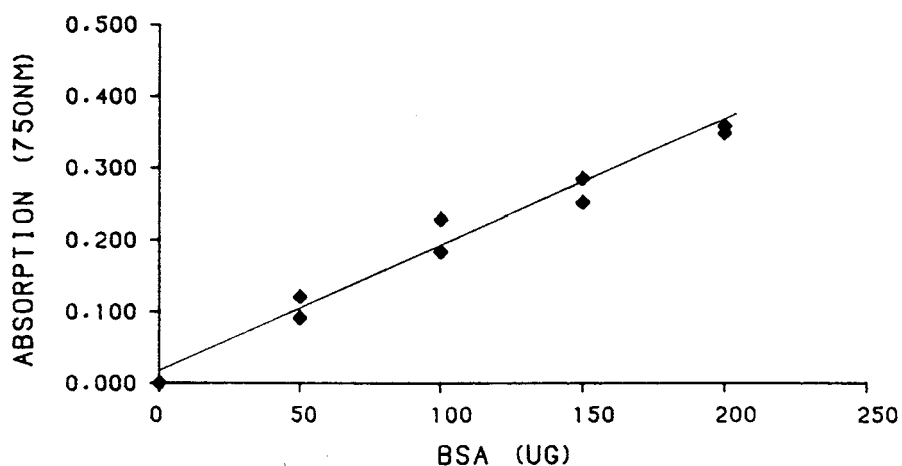


Figure 4.3: *A typical protein calibration curve obtained in these experiments*

halves, it would be justifiable to split a pineal and use one half as a control of the other half. This would then enable the determination of the effect of the CCB on pineal cAMP levels in the same gland, by using one half as a control (agonist in the presence of vehicle) while the other half was stimulated with agonist in the presence of CCB.

4.4 Experiment 2: THE EFFECT OF THREE ORGANIC CALCIUM CHANNEL BLOCKERS ON CYCLIC-AMP LEVELS IN RAT PINEAL GLANDS

4.4.1 Introduction

The findings in Chapter 3 [§ 3.7.4] suggest that the increased NAT levels seen are as a result of raised cAMP levels.

It is well known that adrenergic agonists will elevate cAMP, however the same effect may be brought about by an inhibition of cAMP-phosphodiesterase (cAMP-PDE). A few recent reports have suggested that the organic calcium channel blockers might well be able to inhibit cAMP-PDE by binding to calmodulin (CaM), a protein itself requiring activation by Ca^{2+} before it will activate cAMP-PDE (Lamers *et al.*, 1985; Johnson and Mills, 1986).

4.4.1.1 Objective

To ascertain whether this might account for the potentiation previously observed, the effect of the three CCBs on rat pineal cAMP levels was investigated, using the same drug paradigm used in Chapter 3 [§ 3.7], i.e. isoprenaline (ISO) was used for its β stimulating properties while NA was used for its dual α/β properties. It was hoped that by keeping to the original experimental design, it would be possible to pursue the original hypothesis, and to see if the CCB would alter the Ca^{2+} influx following α_1/β stimulation. At the same time an explanation of the paradoxically increased stimulation previously observed might be found.

4.4.2 Materials and Methods

4.4.2.1 Materials

Verapamil-HCl, diltiazem, nifedipine, *l*-isoprenaline-HCl and *l*-noradrenaline-bitartrate were purchased from the Sigma Chemical Co., USA. Sources of all other materials have been recorded

in [§ 4.2.1.1].

4.4.2.2 Animals

Male Wistar rats (230-260 g b. wt) were used in this study. Their living environment has been described [§ 2.2.1.2].

4.4.2.3 METHODS

4.4.2.3.1 Experiment A

The rats were killed by neck fracture between 09h00 and 09h30. This maintained uniformity with previous studies [§ 3.2.1.2].

Their pineal glands were rapidly removed, placed on a cold glass sheet and bisected with a scalpel blade under a dissecting microscope. The two halves were transferred into separate wells (Falcon[®] 18x6 rows) containing 50 μ l of BGJ_b incubating medium (Fitton-Jackson modification) and pre-incubated at 37°C for 5 min.

One half was then transferred to fresh, prewarmed incubation medium containing verapamil [10 μ M], the other half to control incubation medium containing vehicle. Both preparations were incubated for a further 10 min at 37°C, whereafter they were stimulated with either NA[10 μ M] or ISO [20 μ M] for 10 min.

The halves were then removed and homogenised separately in 110 μ l cyclic AMP buffer (50 μ M Tris-HCl buffer, pH 7.4, 8 μ M theophylline, 6 μ M mercaptoethanol). Duplicate 50 μ l aliquots of the tissue suspension were assayed for cAMP by the saturation binding method of Brown *et al.*, (1971) [§ 4.2.1.5.1].

4.4.2.3.2 Experiment B

The procedure in Experiment A [§ 4.4.2.3.1] was repeated, but verapamil was replaced by diltiazem at the same molar concentration.

Diltiazem was also dissolved in distilled water, with control incubations receiving the equivalent amount of vehicle.

4.4.2.3.3 Experiment C

The procedure in Experiment A [§ 4.4.2.3.1] was repeated, but verapamil was replaced by nifedipine at the same molar concentration. Because nifedipine was relatively insoluble in water, it was first dissolved in 70% ethanol, before further dilution in distilled water. Final concentration of ethanol did not exceed 1%. Control incubations contained the equivalent amount of vehicle. All solutions of nifedipine were made up freshly just prior to use and kept in glassware protected from direct light [§ 2.5.2.3.3].

4.4.2.4 Data analysis and statistics

Cyclic-AMP levels were determined from the cAMP standard curve and expressed as pmol/ pineal \pm SEM. For each drug group, the significance of the difference between the cyclic AMP levels stimulated with agonist in the presence of vehicle (control), and those glands stimulated with agonist in the presence of CCB was evaluated using Students paired *t*-test (2-tailed).

Nifedipine was partly dissolved in ethanol and the other CCBs were dissolved in distilled water as described. This, coupled to the fact that experiments with each CCB were performed on different days, necessitated the use of separate control groups. A nonparametric Kruskal Wallis *H* test was performed to ensure that the control groups were not significantly different.

4.4.3 Results and Discussion

4.4.3.1.1 Results of glands stimulated in the presence of verapamil

The results of those experiments in which glands are stimulated with ISO and NA in the presence of verapamil are presented in Table 4.3 and Figure 4.4.

4.4.3.1.2 Discussion

The results differ slightly from those observed in the parallel experiment in the organ culture studies [§ 2.5.3.1.2], but are in agreement with those finding in the NAT study [§ 3.7.3.1.2].

There is no significant difference between cAMP levels in those glands stimulated with ISO, and those stimulated with ISO in the presence of verapamil. The organ culture studies showed an increase in aHT in the parallel experiment.

The results from those glands stimulated with NA in the presence of verapamil are in agreement with those from the organ culture study, i.e. an increase ($p < 0.025$) in cAMP levels is seen which could account for the increased level of NAT found previously.

TABLE 4.3: Cyclic-AMP formed per rat pineal gland in the presence of vehicle or CCB, when stimulated with either isoprenaline (ISO) [20 μ M] or noradrenaline (NA) [10 μ M]. (mean \pm SEM; n = 4 glands per group)

Treatment	cAMP formed (pmol/ 10 min/ pineal gland)		
	Verapamil	Diltiazem	Nifedipine
ISO + vehicle	1.98 \pm 0.13	2.12 \pm 0.22	1.75 \pm 0.22
ISO + CCB	1.61 \pm 0.22	1.20 \pm 0.24**	1.99 \pm 0.15
NA + vehicle	3.53 \pm 0.38	3.17 \pm 0.28	4.71 \pm 0.27
NA + CCB	6.76 \pm 0.55**	7.28 \pm 1.81**	7.44 \pm 1.22*

In comparison with respective vehicle control, $p < : *0.05; **0.025$ (Student's paired t -test)

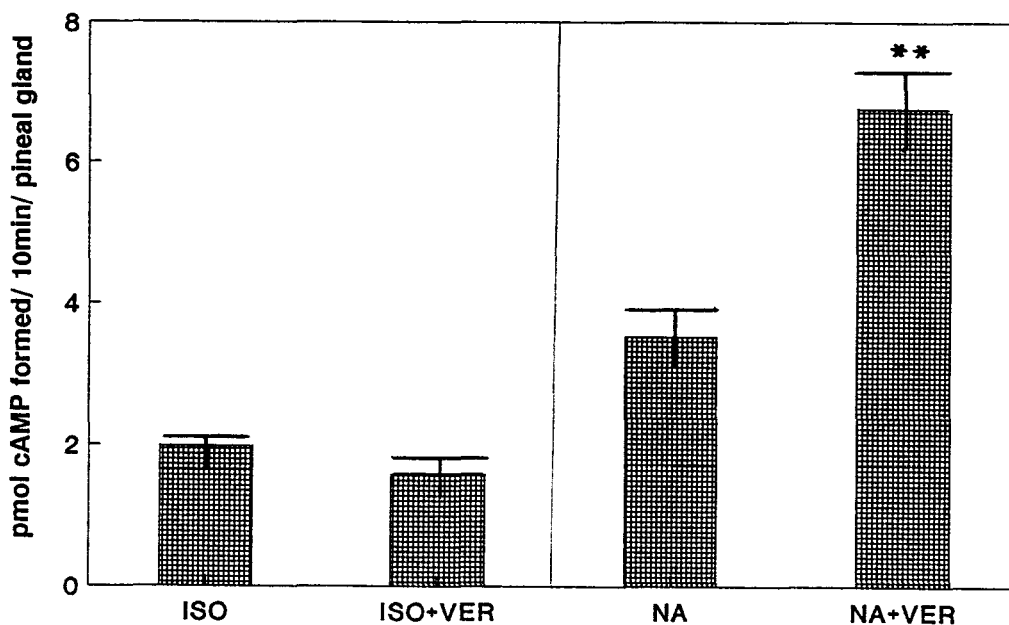


Figure 4.4: Cyclic-AMP formed per rat pineal gland in the presence of vehicle or verapamil (VER), when stimulated with either isoprenaline (ISO) [20 μ M] or noradrenaline (NA) [10 μ M] (mean \pm SEM; n = 4 glands per group; $p < : **0.025$ w.r.t NA -vehicle control-: Student's paired t test)

4.4.3.2.1 Results of glands stimulated in the presence of diltiazem

The results of those experiments in which glands are stimulated with ISO and NA in the presence of diltiazem are presented in Table 4.3 and Figure 4.5.

4.4.3.2.2 Discussion

The paradoxical increase ($p < 0.025$) following NA stimulation in the presence of diltiazem is again observed, but in contrast, those glands stimulated with ISO in the presence of diltiazem show a significant decrease ($p < 0.025$) in NAT levels. This supports the findings of a decrease in aMT observed in this group in the organ culture studies [§2.5.3.2.1], as well as the decrease found in the NAT study [§ 3.7.3.2.2].

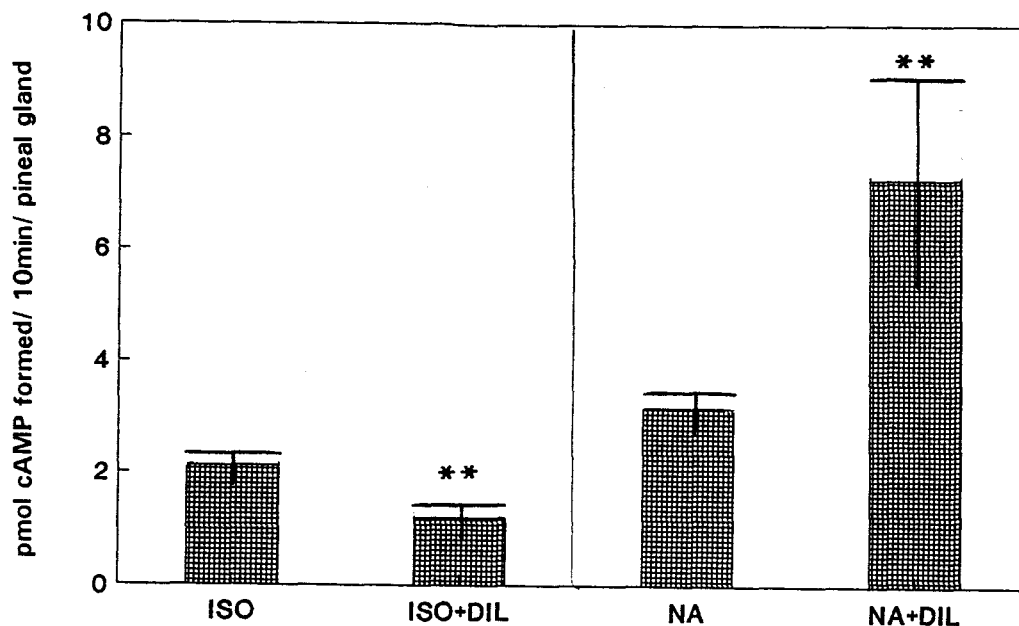


Figure 4.5: *Cyclic-AMP formed per rat pineal gland in the presence of vehicle or diltiazem (DIL), when stimulated with either isoprenaline (ISO) [20µM] or noradrenaline (NA) [10µM] (mean ± SEM; n = 4 glands per group p < :**0.025 w.r.t respective vehicle control: Student's paired t test)*

4.4.3.3.1 Results of glands stimulated in the presence of nifedipine

The results of those experiments in which glands are stimulated with ISO and NA in the

presence of nifedipine are presented in Table 4.3 and Figure 4.6.

4.4.3.3.2 Discussion

A similar trend of cAMP production in glands is seen here, to that observed with the verapamil group [§ 4.4.3.1.1]. Results from glands stimulated with ISO are not significantly different from those of glands stimulated with ISO in the presence of nifedipine, while those glands stimulated with NA in the presence of nifedipine again show a paradoxical increase ($p < 0.05$) in cAMP. The finding for this group are consonant with the previous NAT [§ 3.7.3.3.2] and organ culture studies [§ 2.5.3.3.2] for the same group.

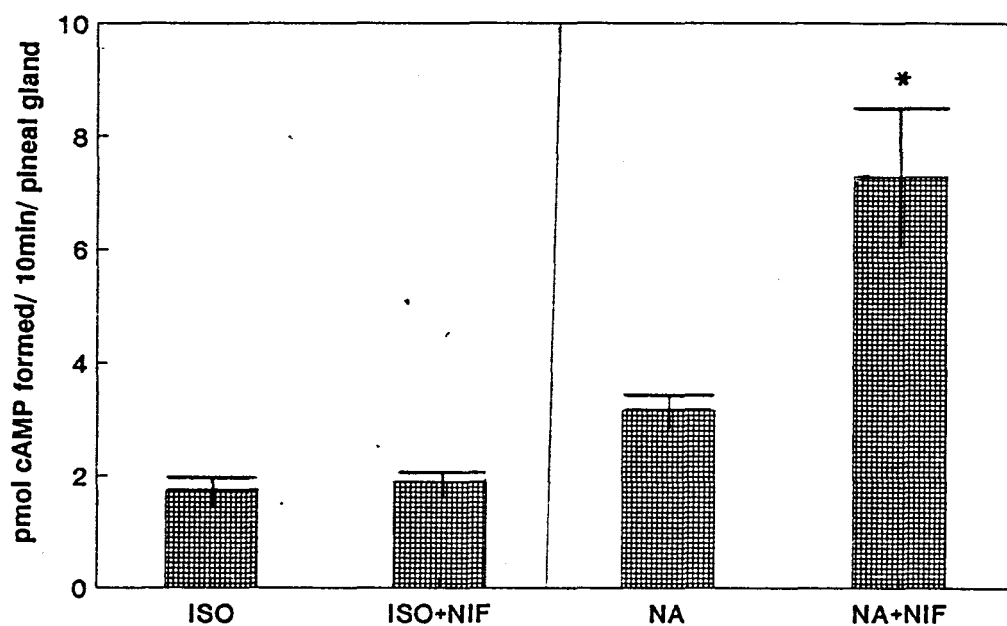


Figure 4.6: Cyclic-AMP formed per rat pineal gland in the presence of vehicle or nifedipine, when stimulated with either isoprenaline (ISO) [20 μ M] or noradrenaline (NA) [10 μ M] (mean \pm SEM; n = 4 glands per group p < :**0.05 w.r.t NA -vehicle control-: Student's paired t test)

4.4.4 Conclusion

In summary then, the results from the cAMP studies on the CCB show the following:

The control groups did not differ significantly over the three test days.

The presence of nifedipine and verapamil did not alter the ISO induced increase in cAMP . This is to be expected since Ca^{2+} influx in the pineal has been associated with α_1 -receptor stimulation.

Diltiazem, however, inhibited the effect of ISO on cAMP, showing a statistically significant ($p < 0.025$) decrease in cAMP levels.

So once again diltiazem differs from the other two CCBs [§ 2.5.4], [§ 3.7.3.2]. Why this happens is speculative, and difficult to define. For instance, although the CCBs as a group share several common properties such as antagonising Ca^{2+} entry, there exists certain distinct fundamental *in vitro* differences amongst these drugs. It could be due to differences in receptor occupation. Alternately, data probably not relating to channel function suggest that all these agents bind to a greater or lesser degree to CaM, and yet another of the studied discrepancies is that because of allosteric interaction, verapamil-like agents inhibit nitrendipine binding in several tissues whereas diltiazem facilitates its binding at these sites (Johnson, 1984). Whatever the reason, the difference persists.

In those pineal glands stimulated with NA, all three CCBs were found to potentiate the effect of NA on cAMP formation: verapamil ($p < 0.025$), diltiazem ($p < 0.025$) and nifedipine ($p < 0.05$).

In Chapter 3 [§ 3.7.4] it was suggested that that the CCBs might in some way influence cAMP levels and interfere with the metabolic cascade simplistically represented as:



Intracellular levels of cAMP reflect a balance between adenylyl cyclase and cAMP phosphodiesterase activities.

Shenolikar, (1988) suggests that when cell regulation is initially being studied, the main focus of attention is usually directed at the level of adenylyl cyclase, because this initiates a cascade of events facilitated by the intracellular second messengers and mediated in many cases by protein kinases.

It could be that adenylyl cyclase is being influenced by the use of NA in this test system, because the paradoxical increase in stimulation always occurs following NA stimulation of pineal glands incubated in the presence of CCB.

There are two basic types of α -adrenergic receptor. The first type (α_1) is located postsynaptically. Activation of these is associated with changes in cell Ca^{2+} fluxes, which are believed to be responsible for the physiological responses to α_1 -receptor stimulation, and are the focus of this work.

The second type (α_2) is located both pre- and postsynaptically. Activation of presynaptic α_2 receptors results in an inhibition of NA release from the adrenergic nerve endings, while activation of the postsynaptic α_2 receptors results in a decrease in cAMP via an inhibition of adenylyl cyclase (Exton, 1981).

NA is relatively non selective between α_1 and α_2 receptors - its selectivity on a particular tissue is determined by the relative densities of α_1 and α_2 receptors available (Exton, 1981). As such, it has been shown to inhibit cAMP levels in chick pineal glands which have postsynaptic α_2 -receptors (Nayler *et al.*, 1982). The CCB have been found to bind to the α_1 and α_2 receptors present on rat pineal, cardiac and brain tissue (Glossmann and Hornung, 1980). If they bound to the α_1 receptors in the rat pineal gland one would expect an inhibition of NA stimulation. Were they to bind to the inhibitory α_2 receptor, an increase in cAMP levels could be expected, as NA would not be able to exert its inhibitory influence via these α_2 receptors. Two lines of evidence argue against the operation of either of these mechanisms in the rat pineal gland. Firstly, an abundance of α_1 receptors is present (Ho *et al.*, 1988), yet the CCBs do not appear to have bound to these. Verapamil, the most potent α_1 blocker should have demonstrated this (Glossmann and Hornung, 1980), yet instead the potentiation is seen (Brown *et al.*, 1989a, 1989b). Secondly, the lack of effect of yohimbine on NA stimulation of cAMP (Ho *et al.*, 1988), suggests that α_2 receptors do not play a significant role in NA stimulation in the rat pineal gland, via their reported inhibitory role on adenylyl cyclase (Exton, 1981), as the concentration at which the CCBs act on α_2 receptors is higher than that at which they would influence α_1 receptors (Glossmann and Hornung, 1980).

As the adenylyl cyclase does not seem to hold the answer, it would seem justifiable to examine the role of cAMP-PDE, the counterpoise, in the control of cAMP levels.

The heterogeneous group of compounds classified as Ca^{2+} -entry-blockers, Ca^{2+} channel blockers or even calcium channel antagonists which share the common pharmacological property of inhibiting Ca^{2+} entry, has been reported to inhibit several calcium-dependant processes, including the activation of cAMP-PDE (Epstein *et al.*, 1982; Lamers *et al.*, 1985; Johnson and Mills,

1986).

Thus while the compounds as a group share several common properties such as antagonising Ca^{2+} entry, there exists certain distinct fundamental *in vitro* differences amongst these drugs, e.g. data probably not relating to channel function suggest that all these agents bind to a greater or lesser degree to CaM. The studies of Johnson and Mills, (1983) provided the first evidence that CaM was indeed an allosteric protein with regard to its drug protein binding sites. With regard to allosteric interaction, one of the best studied discrepancies is that verapamil-like agents inhibit the nifedipine analogue nitrendipine in several tissues whereas diltiazem facilitates its binding at these sites (Johnson, 1984).

Other non-channel *in vitro* differences are reported. Verapamil and diltiazem but not nifedipine have the ability to interfere with CaM activation of the Ca^{2+} -pump and ATPase. The failure of nifedipine to do this is in agreement with the studies of Lamers *et al.*, (1985) who found the same discrepancy when working with soluble dog brain CaM-activated-PDE.

The differences observed with diltiazem, relative to results using verapamil and nifedipine in the present study, could possibly point to a difference in the respective ability of the CCBs to inhibit Ca^{2+} entry. This however is purely speculative, and should be regarded with care in view of the large intra-species variations seen in pineal work. For instance Meyer *et al.*, (1986) have shown that two classes of CCB administered *in vivo* to baboons, produced different effects on circulating plasma aMT levels. Nifedipine reduced aMT levels in the plasma, while verapamil did not. On the other hand, Morton *et al.*, (1989) shows data where verapamil prevents a decrease in aMT (in rats forced to swim), while nifedipine is less effective.

CHAPTER 5

cAMP-PHOSPHODIESTERASE STUDIES

5 INTRODUCTION

5.1 Regulatory role of cAMP-PDE

Since cyclic nucleotides play important regulatory roles in many biological functions (Robison *et al.*, 1968), it is reasonable to assume that their intracellular concentrations are delicately controlled (Teo *et al.*, 1973).

In any informational system, the quenching of the signal and its generation must be considered equal. The hydrolytic cleavage of the cyclic nucleotides to their corresponding 5'-nucleotides is catalysed by one or more types of phosphodiesterase, and is the only well-established mechanism through which the biological actions of these nucleotides is terminated.

5.1.2 Nomenclature / Classification

In eucaryotic systems at least three types of PDE activities have been demonstrated:

- i) a Ca^{2+} -activator (calmodulin) dependent form hydrolysing both cAMP and cGMP
- ii) a cAMP specific or 'low- K_m ' cAMP-PDE, (Reviewed in Hardman and Wells, 1975), and
- iii) a form in which cGMP levels activate the hydrolysis of cAMP (Terasaki and Appleman, 1975; Gain and Appleman, 1978).

The characterisation of the regulatory properties of cAMP-PDE is fundamental to the delineation of how cAMP effects are controlled.

The PDE's were characterized by Strada and Thompson (1984) according to their substrate preference and regulatory properties as follows:

Type I: Calmodulin-sensitive cyclic nucleotide PDE. This enzyme hydrolyses both cAMP and cGMP, and its activity is enhanced by Ca^{2+} and calmodulin.

Type II: cGMP-sensitive cyclic nucleotide PDE. This enzyme also hydrolyses both cAMP and cGMP, but its cAMP hydrolytic activity is enhanced by cGMP.

Type III: Rhodopsin-sensitive cGMP-PDE.

Type IV: Cyclic AMP-PDE. This enzyme is relatively specific for cAMP and has been referred to as the "low- K_m " or high-affinity cAMP-PDE.

Literature however does not seem to adhere to the above but follows the more commonly used format whereby the PDE's are named in the order of their elution from a chromatographic column. Thus *Types I, II* and *IV* are more commonly designated *PDE I, PDE II* and *PDE III* respectively.

5.1.3 History

The history of phosphodiesterase is interesting. Sutherland and Rall (1958) were the first to demonstrate the presence of PDE. In 1967, Butcher and Sutherland were able to partially purify the enzyme from bovine heart. The next important event was when Kakiuchi and Yamazaki (1970), were able to show that rat brain PDE could be inhibited by EGTA.

After passing the enzyme through an anion exchange column in order to purify it and enable better characterisation, Cheung (1970) noted a precipitous drop in activity, relative to his starting product. This he found was due to dissociation of an activator protein from the enzyme. In independent studies Kakiuchi *et al.*, (1970) also demonstrated the presence of an activator.

Teo and Wang, (1973) were able to show that the activation of PDE was dependent on the presence of both the activator and Ca^{2+} . This activator protein has since been named calmodulin (Cheung, 1980).

Calmodulin (CaM) has been fully discussed [§ 1.10.2]. As shown above, its role was first demonstrated in investigations with PDE. Since then, this Ca^{2+} -dependent protein regulator has been shown to play a role in the function of ca 30 physiological processes. As such, it has been termed "a universal receptor of the Ca^{2+} signal" in cells (Klee *et al.*, 1980). Whenever the free Ca^{2+} concentration in a cell changes, the concentration of the different Ca^{2+} -CaM complexes varies, thus modulating the target enzymes that determine the cell's response. As part of its activation mechanism, hydrophobic domains on CaM are exposed during the conformational change occurring when Ca^{2+} first binds to it. It is these exposed domains which are recognised by the target enzymes and for which the CaM antagonists compete, preventing the interaction of CaM

with its target protein.

A growing volume of evidence indicates that many diverse classes of drugs can in fact modify the activity of CaM. Inasmuch as CaM can influence a wide variety of cellular events, modification of its activity by drugs might well have profound pharmacological implications. One of the several forms of PDE is activated by CaM and this activation can be inhibited by the phenothiazine group of antipsychotic drugs (Weiss *et al.*, 1974). More recently other drugs including the CCBs have been found to inhibit CaM activation of PDE.

5.1.4 Objectives

The purpose of this study was therefore to develop a suitable cAMP-PDE assay, and to use this to ascertain if the raised cAMP levels in the previous chapter could be correlated to any inhibition of cAMP-PDE levels.

5.1.5 Methods for the determination of cAMP-PDE

Several methods of measuring PDE activity are presently available. Because separation of the cyclic nucleotide from its corresponding nucleoside is generally easier than from the monophosphate, most assays incorporate the principle introduced by Butcher and Sutherland (1962). This is a two-step process in which cAMP is first hydrolysed by the cAMP-PDE present, then a 5'-nucleotidase from snake venom is used to convert the adenosine 5'-monophosphate, to adenosine. The inorganic phosphate formed is then measured chemically.

The method, although simple, is relatively insensitive (Lynch and Cheung, 1975), and so the more widely-used and sensitive methods employ isotopically labelled cyclic nucleotides. The separation of radioactive substrate from the hydrolysed radioactive product is generally achieved by means of batch- or column anion-exchange resins (Thompson and Appleman, 1971; Lynch and Cheung, 1975; Gulyassy and Farrad, 1976), paper chromatography (Nakai and Brooker, 1975), or precipitation (LeDonne and Coffee, 1979).

The batch method of Thompson and Appleman (1971) is popular because of its simplicity and economy of time. The method makes use of anionic exchange resin which presumably binds the unreacted cAMP (which carries a negative charge) but not the adenosine (which has no net charge). However Lynch and Cheung (1975) have shown that this method may underestimate the true cAMP-PDE activity, as the resin may sometimes not bind all the unreacted substrate. They

feel that column methods under certain circumstances may produce the same error.

The method of LeDonne and Coffee, (1979) which employs a precipitation (PPT) technique, has been used in this laboratory, but as the filter apparatus was being fully utilized in a binding study, it was decided to use the thin-layer chromatography (TLC) method of Neuman (1983) because both the plates and solvents were readily available. This method and a validation thereof is described below [§ 5.2]

5.1.6 Summary of the assay

In this method cAMP and tritiated cAMP (tracer) are used as substrate. Pineal tissue homogenate is then added. The phosphodiesterase present in the homogenate hydrolyses the cAMP substrate. The reaction is then terminated by the addition of trichloroacetic acid. An aliquot of the reaction mixture is spotted onto a TLC plate and the 5'-AMP formed from the hydrolysis of cAMP is separated by TLC. The [³H]-5'-AMP is then quantitated, to be used as an indicator of PDE activity.

5.2 CYCLIC-AMP PDE DETERMINATION TECHNIQUE

5.2.1 Materials and Methods

5.2.1.1 Chemicals, drugs and reagents

[8-³H]Adenosine 3',5'-cyclic phosphate, ammonium salt (1.0 mCi/ml : specific activity 26.1 Ci/mmol) was purchased from Amersham, England. Phosphodiesterase (EC 3.1.4.17, specific activity ca 0.31 U/mg), Adenosine 3',5'-cyclic phosphate and Adenosine 5'-monophosphate were obtained from Boehringer Mannheim, West Germany. TLC plates (Alugram[®] Sil G/UV254 0.025 x 20 x 20 cm) were from Macherey-Nagel, West Germany. The solvents used for TLC, and chemicals used in buffers were of analytical grade from commercial sources.

5.2.1.2 Animals

Male Albino Wistar rats (200-240 g b. wt) were housed four per cage in a temperature controlled room under a fixed lighting cycle of LD 12:12 (lights on 06h00) with food and water *ad libitum*. The rats were sacrificed by neck fracture between 09h00 and 09h30 (to maintain uniformity with the previous studies [§ 4.2.1.2])

Male rats were chosen for all the cAMP-PDE studies for the same reasons outlined in the cAMP studies [§ 4.2.1.2].

5.2.1.3 Pineal glands

Pineal glands for cAMP-PDE determinations were swiftly removed as before [§ 2.2.1.4] and placed in culture vessels containing 200 μ l BGJ₆ culture medium. Four pineal glands were transferred to each vessel.

5.2.1.4 Assay of cAMP-PDE in rat pineal glands

5.2.1.4.1 Introduction

Oleshansky and Neff, (1975) reported that the amount of PDE activity in the pineal gland changes as a function of adrenergic stimulation. Treatment of animals with ISO results in a 50% increase in the activity of the low- K_m PDE enzyme.

Since increased cAMP levels are known to induce cAMP-PDE, and stimulation of the former is relatively easy in the pineal gland, it was decided to use this as a test of the integrity of Neuman's cAMP-PDE assay, modified to suit pineal work.

5.2.1.4.2 CAMP-PDE assay

Four pineal glands were exposed to vehicle (control glands) while the remaining four glands were stimulated with the adrenergic agonist ISO. The culture medium in the one culture vessel thus contained ISO[10 μ M] while the other culture vessel contained the control pineal glands in the presence of vehicle alone.

After a 3 h incubation period at 37°C, the pineals were removed from the vessels and each pineal gland was transferred to its own homogenisation tube, for the determination of cAMP-PDE activity as described below.

The pineal glands were individually homogenised in 100 μ l of buffer (40 μ M Tris-HCl, pH 8, 5 μ M MgCl₂). A 10 μ l aliquot of pineal homogenate was removed and transferred to a reaction tube. It was incubated at 37°C with 1 μ M cAMP, 3 x 10⁵ cpm [³H]-cAMP and buffer (final volume 50 μ l). The reaction was stopped after 30 min by the addition of 30 μ l of 10% trichloroacetic acid (TCA), and the [³H]-5' -AMP formed was separated from the [³H]-cAMP remaining, on thin layer

chromatography (TLC) plates, using a solvent system containing isopropyl alcohol, 25 % water of NH_4OH and water (7:1:2).

5.2.1.4.3 Thin-layer chromatography

Duplicate $10\mu\text{l}$ aliquots of each sample were spotted 15 mm from the bottom of the plates, and in the center of 15 mm wide channels inscribed on the silica gel TLC plates. The mobility of cAMP and 5' - AMP was monitored routinely by spotting $10\mu\text{l}$ aliquots of standard solutions containing 0.2mg/ml authentic cAMP or 5' - AMP, dissolved in distilled water, in adjacent channels at one end of the plate. Following development of the plates (± 2 h), the plates were dried under a stream of nitrogen, then placed in an oven at 60°C (10 min) to remove any remaining moisture.

5.2.1.5 Quantitation

Visualization of these markers was accomplished under short wave ultraviolet light. The radioactivity in the identified spots was determined by scraping off the spot into a scintillation vial containing $400\mu\text{l}$ 0.01M KOH (Beckman Information Brochure).

After the KOH had solubilized the scraping, 3ml of scintillation cocktail (Beckman Ready-Solv HP/b[®]) was added to the vials. They were then shaken on a Griffith Flask Shaker[®] for 20 min. Thereafter the vials were placed in a Beckman LS 2800 scintillation counter and each vial was counted for a total of 5 min.

5.2.1.6 Data analysis and statistics

The counts per minute (cpm) were converted to disintegrations per minute (dpm) using the *H*-Number method of quench monitoring as discussed previously [§ 2.2.1.6]. The mean of the duplicate determinations was used as the dpm result for each sample.

Blanks were run in exactly the same way except that TCA was added to the tubes before tissue or PDE was included. Thompson *et al.*, (1974) described several factors which could influence blank values. They suggest that the method of terminating the reaction by boiling for 2 min could possibly increase blanks in tritium-based assays, apparently due to formation of tritiated water. Wells and Hardman (1977) suggest that the construction of appropriate blanks is best achieved by the addition of the "stopping mixture" prior to the addition of tissue or PDE. They report that this should equal blank values obtained if boiled PDE were added at the beginning of the

incubation.

Blank values, which were very low, were first subtracted from the results before these were finally expressed as nmol cAMP hydrolysed/ 30 min/ pineal gland.

5.2.1.6.1 Statistical analysis

Cyclic-AMP PDE levels (the mean of duplicate determinations) for the assay are expressed as nmol cAMP hydrolysed/ 30 min /pineal gland. The significance of the difference between those glands stimulated with ISO and the control glands was evaluated using the nonparametric Mann-Whitney *U* test because of the small group sizes.

5.2.1.7 Results and Discussion

The relative R_f values for the cAMP and 5'-AMP were 0.16 and 0.61 respectively. These R_f values are comparable with those reported in the literature for a similar method (Goldberg *et al.*, 1969).

The results (nmol \pm SEM) show a significant difference ($p < 0.001$) in cAMP-PDE levels between glands stimulated with ISO (1.29 ± 0.12) and the control glands (0.56 ± 0.03). This confirms reports that PDE levels are raised following ISO stimulation, and also shows that the assay is able to quantitatively assess this difference.

Although the results show a difference in PDE activity following ISO stimulation, it was still deemed necessary to ensure that i) the radioactivity being applied to the TLC plates was in fact being recovered, and ii) that the assay results were comparable with a different technique. This would safeguard against false values through possible underestimation of cAMP-PDE activity, if for instance radioactivity were to be adsorbed onto the silica gel.

5.2.1.8 Validation of results

Two methods were used to validate the assay: i) the recovery of chromatographed [^3H]-cAMP was assessed, and ii) this assay was compared with that of LeDonne and Coffee (1979), by using a standard solution of PDE.

5.2.1.8.1 Recovery of chromatographed [³H]-cAMP

In order to assess the recovery of cAMP from silica gel TLC plates in the assay system, sample tubes were made up containing the equivalent amounts of reaction mixture that they would have contained under assay conditions. The TCA was added first to inactivate any PDE in the pineal tissue homogenate used.

Aliquots (10 μ l) were then removed from these sample tubes and applied to TLC plates, either before or after development, or directly to scintillation vials. The quantitation and counting procedures were then followed as described above [§ 5.2.1.6]. The results of this study are presented in Table 5.1.

TABLE 5.1: *Recovery of [³H]-cAMP from silica gel TLC plates. Blanks were made up and aliquots from these were spotted either before or after development of TLC plates, or directly to scintillation vials. The selected regions as described were quantitated by the method of Neuman (1983).*

Procedure and/or region quantitated	cpm	%recovery
Spotted, developed, quantitated:		
	8	-
Starting spot after development	21	-
	9	-

	44	-
5'-AMP region after development	52	-
	48	-

	56105	86.6
cAMP region after development	60890	90.8
	58395	92.0

	60761	93.8
Developed, spotted, quantitated (starting spot)	57939	86.4
	58783	92.7

	64770	--
Added direct to scintillation vial	67052	--
	63445	--

The efficiency of the recovery of chromatographed [³H]-cAMP from the silica plates is shown in Table 5.1 to range from 86.4% to 93.8%. These results are comparable with previously reported results in a similar system (Goldberg *et al.*, 1969).

The radioactivity remaining at the origin or at the 5'-AMP region was negligible in the TCA

blanks, with the major radioactivity being recovered at the [^3H]-cAMP region.

5.2.1.8.2 Comparison of a standard phosphodiesterase by two methods

Once the recovery had been tested, it remained to verify that the assay results would be an accurate reflection of cAMP-PDE activity. To test this a standard solution was made up containing 1mg/ ml PDE. Aliquots (5 μl and 10 μl) of this solution were then added to assay tubes in place of homogenate, and assayed either by the TLC method of Neuman, (1983) or by the precipitation (PPT) method of LeDonne and Coffee (1979). The latter technique was chosen as a method of comparison, because it too determines the amount of 5' - AMP formed, and does not proceed on to the nucleotidase step. The results are presented in Table 5.2.

TABLE 5.2: *Comparison of a Standard phosphodiesterase solution as determined by a TLC procedure and a PPT procedure (Values are cpm recovered as hydrolysed cAMP)*

Aliquot	Assay method	
	TLC	PPT
	3455 (100)	43287 (96)
	3501 (100)	45519 (100)
	3605 (100)	46144 (98)
	7348 (100)	102063 (106)
	8152 (100)	108421 (102)
	7809 (100)	100736 (99)

The activity of a standard solution of cAMP-PDE was assayed by the TLC procedure described or by a precipitation (PPT) procedure. The numbers in parentheses are the percentage of the corresponding cpm by the TLC procedure, which is taken as 100.

5.2.1.8.3 Discussion

Although the results may look vastly different at first glance, this is because the TLC method quantitates only a 10 μl aliquot of reaction mixture, while the second method utilizes the entire volume of reaction mixture. The relative amounts of cAMP hydrolysed do in fact correlate.

The results obtained with the TLC assay compare favourably with those of the PPT assay and

therefore this technique is shown to be valid.

Using this technique, it would be possible to determine the levels of cAMP-PDE in the pineal gland in the presence of the CCB, under the same test conditions employed in the previous studies.

5.3 Experiment 1: THE EFFECT OF THREE ORGANIC CALCIUM CHANNEL BLOCKERS ON CYCLIC-AMP PDE LEVELS IN RAT PINEAL GLANDS

5.3.1 Introduction

The findings in Chapter 4 [§ 4.4.4] show that cAMP levels are indeed raised in those groups of glands where the paradoxical increase in NAT activity was noted [§ 3.7.4].

It is often inferred that the relative rates of formation and degradation of the cyclic nucleotides determine the steady-state levels of these compounds. For this reason, the concept delineated by Robison *et al.* (1971), that inhibition of cAMP-PDE activity should potentiate the effect of a hormone acting via cAMP, has often been used in attempts to demonstrate cAMP involvement in a hormone response (Wells and Kramer, 1981). A corollary of this is that the demonstration of cAMP potentiation in the presence of the hormone is due to cAMP-PDE inhibition.

In these studies the potentiation of cAMP following stimulation with NA in the presence of the CCB is very clear. It therefore seems valid to assay the level of cAMP-PDE in the pineal glands under the same conditions in which the raised levels of cAMP are found.

5.3.1.1 CAMP-PDE in the pineal gland

The PDE forms have been reviewed by Wells and Hardman (1977). They pointed out the difficulty of summarising the results, both because of the extreme diversity of methods in use and the heterogenicity of tissues.

Kinetically, the PDE forms of many tissues have been shown to have two activities with characteristic low- and high- K_m values for cAMP. The pineal is no exception. Oleshansky and Neff (1975) found the Michaelis constants (K_m) of the two pineal forms to be ca $2\mu\text{M}$ and $37\mu\text{M}$ for the low- and high- forms respectively. They also reported that the level of low- K_m PDE

increased significantly following ISO stimulation while that of the higher form showed only a small increase. Vacas *et al.*, (1984) were able to show that the low- K_m also increased following combined α_1 and β stimulation. Epplen *et al.*, (1982) have reported similar K_m values in the pineal.

Minneman and Iversen (1976) have demonstrated a diurnal rhythm (which parallels that of NAT) in both pineal cAMP-PDE and cGMP-PDE activities, with peak levels raised by some 160% three hours into the dark period (w.r.t. the equivalent time in the light photoperiod).

More recently Chons *et al.*, (1988) making use of a histochemical technique, have demonstrated the presence of cAMP-PDE along the plasma membrane of pinealocytes. The same location was evident when cGMP was used as substrate. This is an important finding because it suggests that the same PDE isozyme may be hydrolysing both forms of cyclic nucleotide in the pineal. The low- K_m form, by definition, should only hydrolyse cAMP. On the other hand *PDE I*, a calmodulin-activated form of PDE, exhibits no such substrate preference; instead it hydrolyses both cyclic nucleotides.

In the light of the findings of Chons *et al.*, (1988), the possibility that the CCB might be influencing the activation of PDE present in the pineal becomes more feasible. Pineal cAMP-PDE levels were therefore assayed using the same drug paradigm as in the cAMP studies, to ascertain if there was a relationship between the increased cAMP levels found in the previous chapter [§ 4.4.4] and the levels of cAMP-PDE.

5.3.2 Materials and methods

5.3.2.1 **Materials**

Verapamil-HCl, diltiazem, nifedipine, *l*-isoprenaline-HCl and *l*-noradrenaline bitartrate were purchased from the Sigma Chemical Co., USA. Sources of all other materials have been recorded in [§ 5.2.1.1].

5.3.2.2 **Animals**

Male Albino Wistar rats (230-260 g b. wt) were used in this study. Their living environment has been described [§ 2.2.1.2.].

5.3.2.3 METHODS

5.3.2.3.1 Experiment A

The rats were killed by neck fracture between 09h00 and 09h30. This was at the same time as in the other studies, thus maintaining uniformity with those studies, and also avoiding any variation in basal cAMP-PDE levels which might be evident in view of the reported diurnal variation (Minneman and Iversen, 1976).

Their pineal glands were swiftly removed as before [§ 2.2.1.4] and placed in culture vessels containing 200 μ l BGJ_b culture medium. Four pineal glands were transferred to each of four vessels.

The pineal glands were exposed to verapamil 20 min prior to the addition of the adrenergic agonists, NA and ISO. The culture medium for each group thus contained either ISO or NA (10 μ M), in the presence of either vehicle, or verapamil (10 μ M).

After a 3 h incubation period at 37°C, the pineals were removed from the vessels and each transferred to its own homogenisation tube containing 100 μ l "cAMP-PDE buffer". The cAMP-PDE activity was then assayed as described above [§ 5.2.1.4].

5.3.2.3.2 Experiment B

The procedure in Experiment A [§ 5.3.2.3.1] was repeated, but verapamil was replaced by diltiazem at the same molar concentration.

Diltiazem was also dissolved in distilled water, with control incubations receiving the equivalent amount of vehicle.

5.3.2.3.3 Experiment C

The procedure in Experiment A [§ 5.3.2.3.1] was repeated, but verapamil was replaced by nifedipine at the same molar concentration.

Nifedipine was dissolved as described previously [§ 4.3.2.3.3]

5.3.2.4 Data analysis and statistics

Cyclic-AMP PDE levels were assessed as described [§ 5.2.1.4] and are expressed as nmol cAMP hydrolysed/ 30 min/ pineal gland \pm SEM. For each group, the significance of the difference in cAMP-PDE levels between glands stimulated with agonist in the presence of CCB and the control glands was evaluated using the nonparametric Mann-Whitney *U* test (because of the small group sizes).

Nifedipine was partly dissolved in ethanol and the other CCBs were dissolved in distilled water as described previously [§ 4.4.2.3.3]. This, coupled to the fact that experiments with each CCB were performed on different days, necessitated the use of separate control groups. A Kruskal Wallis *H* test (nonparametric version of the ANOVA) was performed to ensure that the control groups were not significantly different.

5.3.3 Results and Discussion

5.3.3.1.1 Results of glands stimulated in the presence of verapamil

The results of those experiments in which glands are stimulated with ISO and NA in the presence of verapamil are presented in Table 5.3 and Figure 5.1.

5.3.3.1.2 Discussion

The results are in agreement with the findings of the NAT [§ 3.7.3.1.2] and cAMP studies [§ 4.3.3.1.2].

There is no significant difference between cAMP-PDE levels in glands stimulated with ISO (control), and those stimulated with ISO in the presence of verapamil. While the organ culture studies showed an increase in aHT in the parallel experiment, the NAT and cAMP studies have shown no difference, and therefore one is not expected here either.

Glands stimulated with NA in the presence of verapamil show a significant decrease ($p < 0.025$) in cAMP-PDE levels. This decrease could account for the increased levels of NAT and cAMP found previously.

TABLE 5.3: *Cyclic-AMP-phosphodiesterase activity per rat pineal gland in the presence of vehicle or CCB, when stimulated with either isoprenaline (ISO) [10 μ M] or noradrenaline (NA) [10 μ M] (mean \pm SEM; n = 4 glands per group)*

Treatment	cAMP hydrolysed (nmol/ 30 min/ pineal gland)					
	Verapamil		Diltiazem		Nifedipine	
ISO + vehicle	1.12	\pm 0.20	1.22	\pm 0.10	1.42	\pm 0.15
ISO + CCB	1.24	\pm 0.28	1.81	\pm 0.10*	1.59	\pm 0.30
NA + vehicle	2.68	\pm 0.28	2.69	\pm 0.16	2.77	\pm 0.13
NA + CCB	1.59	\pm 0.15**	1.92	\pm 0.06	1.99	\pm 0.25

*In comparison with respective vehicle control, p < *0.05; **0.025 (Mann-Whitney U test).*

5.3.3.2.1 Results of glands stimulated in the presence of diltiazem

The results of experiments in which glands are stimulated with ISO and NA in the presence of diltiazem are presented in Table 5.3 and Figure 5.2.

5.3.3.2.2 Discussion

A decrease, which although not significant, may well be real, is observed in glands stimulated with NA in the presence of diltiazem. In contrast, glands stimulated with ISO in the presence of diltiazem show a significant increase ($p < 0.05$) in cAMP-PDE levels consistent with the decreased cAMP level found in the previous chapter. This supports the findings of a decrease in aMT observed in this group in the organ culture studies [§2.5.2.2.2], as well as the decrease found in the NAT study [§ 3.7.3.2.2].

5.3.3.3.1 Results of glands stimulated in the presence of nifedipine

The results of experiments in which glands are stimulated with ISO and NA in the presence of nifedipine are presented in Table 5.3 and Figure 5.3.

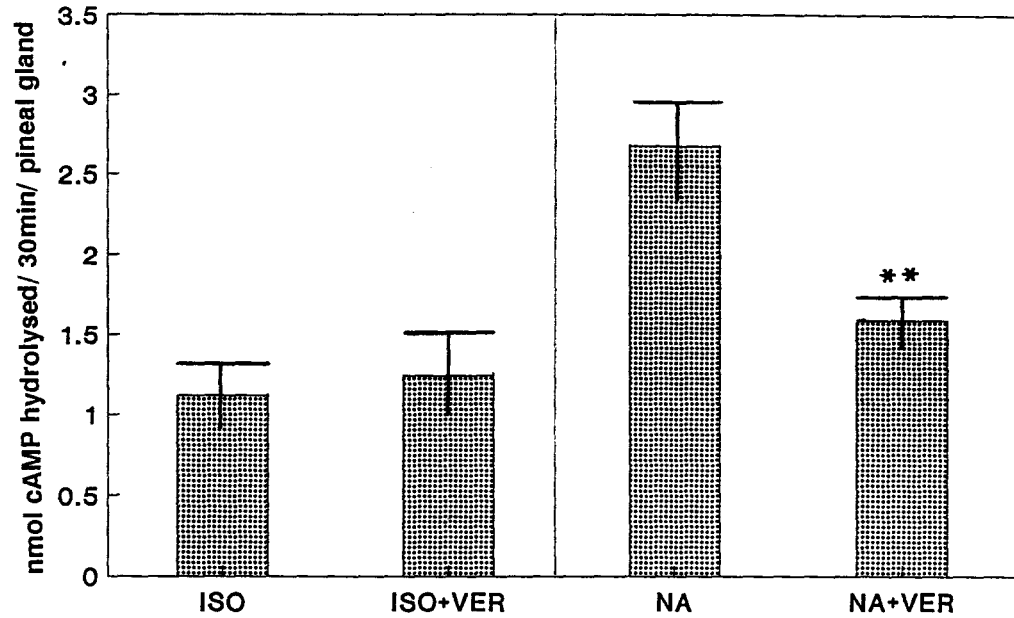


Figure 5.1: Cyclic-AMP-phosphodiesterase activity per rat pineal gland in the presence of vehicle or verapamil, when stimulated with either isoprenaline (ISO) [10µM] or noradrenaline (NA) [10µM] (mean ± SEM; n = 4 glands per group; p < .**0.025 Mann-Whitney U test)

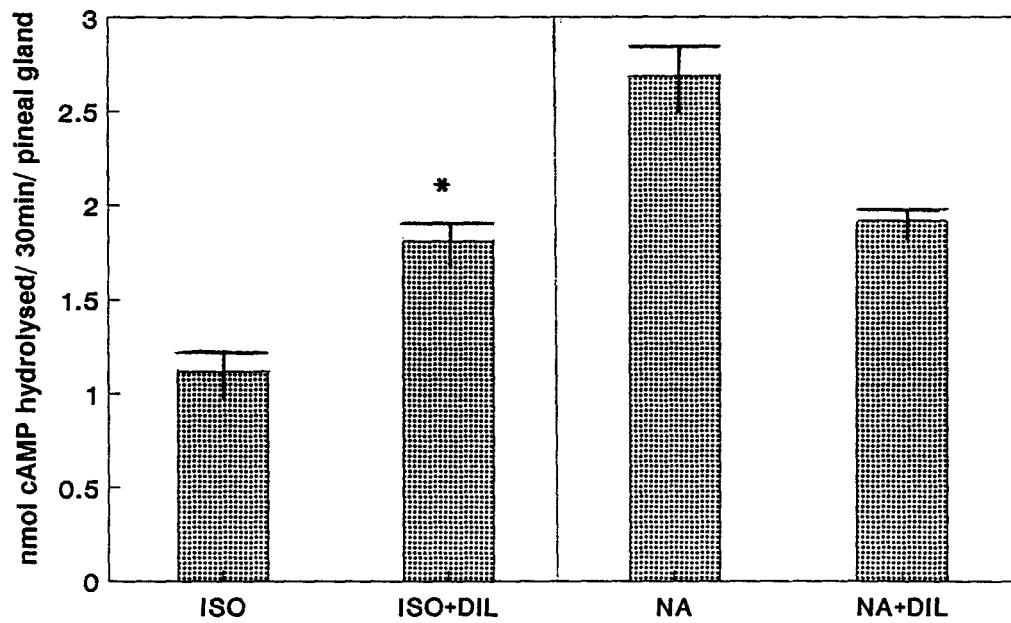


Figure 5.2: Cyclic-AMP-phosphodiesterase activity per rat pineal gland in the presence of vehicle or diltiazem, when stimulated with either isoprenaline (ISO) [10µM] or noradrenaline (NA) [10µM] (mean ± SEM; n = 4 glands per group; p < .*0.05 Mann-Whitney U test)

5.3.3.3.2

Discussion

Again the findings for this group parallel those of the verapamil group [§ 5.3.3.1.1]. Glands stimulated with ISO are not significantly different from those stimulated with ISO in the presence of nifedipine, while glands stimulated with NA in the presence of nifedipine also show a decrease in cAMP-PDE. Although this decrease does not reach significance ($p < 0.10$), the finding for this group are consonant with the previous NAT [§ 3.7.3.3.2] and organ culture studies [§ 2.5.3.3] for the same group. The trend toward a decrease in cAMP-PDE levels would support the raised cAMP levels observed in the previous chapter [§ 4.4.3.3.2].

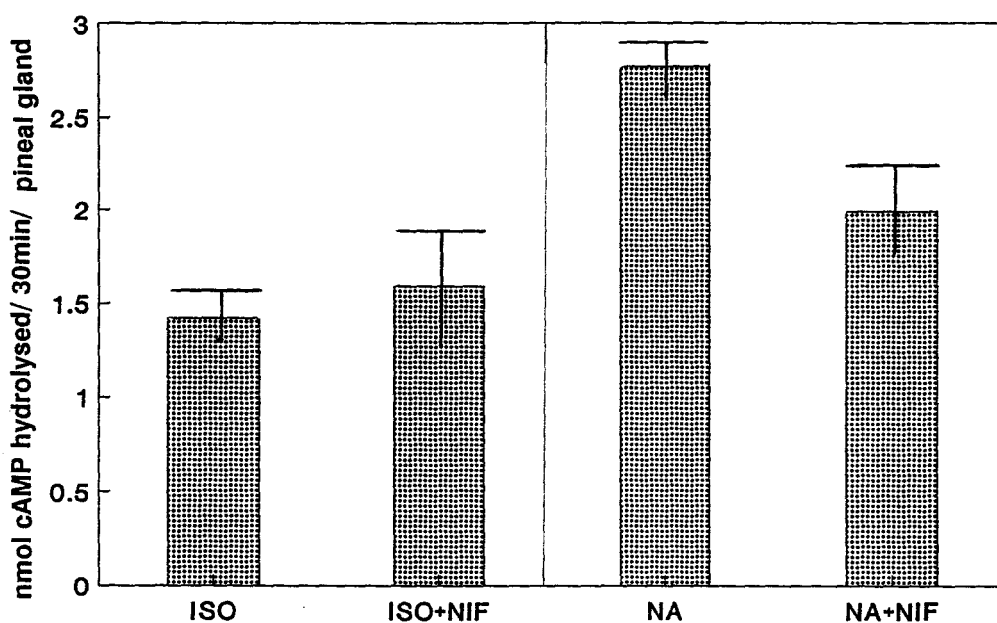


Figure 5.3: *Cyclic-AMP-phosphodiesterase activity per rat pineal gland in the presence of vehicle or nifedipine, when stimulated with either isoprenaline (ISO) [10 μ M] or noradrenaline (NA) [10 μ M] (mean \pm SEM; n = 4 glands per group;)*

5.3.4

Conclusion

In summary then, the results from the cAMP and cAMP-PDE studies on the CCB show the following:

The cAMP-PDE levels of the control groups do not differ significantly over the three test days.

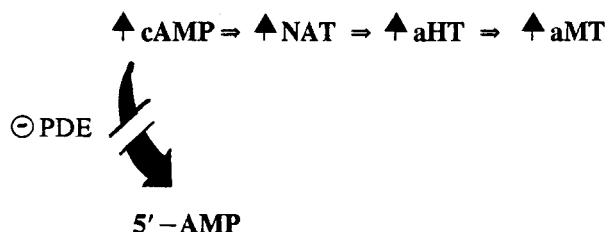
The presence of nifedipine and verapamil did not alter the ISO induced increase in cAMP or

cAMP-PDE. Diltiazem, however, inhibited the effect of ISO on cAMP, showing a statistically significant ($p < 0.025$) decrease in cAMP levels, and produced a corresponding increase ($p < 0.05$) in cAMP-PDE.

Diltiazem again shows a difference when compared with the other two CCBs [§ 2.5.4], [§ 3.7.3.2] and [§ 4.4.3.2.2].

Pineal glands stimulated with NA in the presence of the CCB showed that all three CCBs potentiated the effect of NA on cAMP formation; verapamil ($p < 0.025$), diltiazem ($p < 0.025$) and nifedipine ($p < 0.05$). The findings of this study support the above. They show a significant decrease ($p < 0.025$) in the verapamil group, with the remaining two CCBs following a similar trend, but not reaching significance.

Despite the difficulty of interpretation, a consistent pattern does emerge from this study. It suggests that the CCBs could be influencing cAMP –via an inhibition of cAMP-PDE– in the metabolic cascade represented as:



5.3.4.1 Implications of this study

The implications of this study gain importance when viewed in the light of the existing literature:

The phosphodiesterase forms have been reviewed by Wells and Hardman (1977). One of the major forms (*PDE I*) found in many tissues (i.e. brain, heart, smooth muscles) can be activated by CaM. This form has a greater maximal velocity (V_{\max}) and a higher Michaelis constant (K_m) for cyclic cAMP than for cGMP. Hence it is often described as the "high- K_m " PDE. The effect of CaM is to increase its V_{\max} on cAMP, and to a lesser extent on cGMP (Stoclet, 1981). The role of this enzyme, which is soluble, is not clear at present.

According to a theoretical analysis based on the assumption that adenylyl cyclase and low- K_m PDE would be bound to the plasma membrane whereas high- K_m PDE would be cytosolic, the

high- K_m enzyme would be ineffective in regulating the cAMP concentration near the membrane, but would be effective in regulating the cyclic nucleotide level further inside the cell. A consequence of this would be the generation of a concentration gradient of cAMP from the plasma membrane to the interior of the cell (Fell, 1980).

If this hypothesis is true, the effect of activation of the high- K_m PDE (cytoplasmic PDE) by CaM might be to oppose an increase in cAMP concentration inside the cell, without preventing a local increase in cAMP concentration at the membrane (where adenylyl cyclase is stimulated and calcium simultaneously released under the influence of NA and other hormones and neuromediators).

Association between adenylyl cyclase stimulation and elevation in intracellular Ca^{2+} level has been described in the pineal [§ 1.11.1].

5.3.4.2. Role of Ca^{2+} and calmodulin

Ca^{2+} is needed for the activation of CaM to its active conformer [§ 1.10.2]. Although the concentration of CaM in cells is generally high, so too are estimates of total CaM-binding proteins. However, because there may be nonuniform distribution within the cellular compartments, any inferences about local CaM concentrations are difficult to make (Nehmad *et al.*, 1982; Saitoh and Schwartz, 1983). Most models therefore involve the implicit assumption, that free CaM is present in a limiting amount (Kranias *et al.*, 1980), and that the influx of Ca^{2+} or its intracellular concentration becomes the critical factor in the modulation and activation of these Ca^{2+} -dependent processes (Manalan and Klee, 1984).

The activities of both adenylyl cyclase and PDE may be viewed as being regulated by the cellular flux of Ca^{2+} . According to this scheme, the influx of Ca^{2+} through the plasma membrane (or the release of membrane-bound Ca^{2+}) in response to stimuli, activates adenylyl cyclase which results in an increase of intracellular cAMP. Ca^{2+} thus made available in the cytoplasmic space stimulates the soluble cytoplasmic PDE, which then returns the elevated intracellular levels of cAMP to its prestimulated level. The sequential stimulation of adenylyl cyclase and PDE could thus allow momentary elevation of cellular cAMP in response to NA or a hormone (Cheung *et al.*, 1975).

Of further interest is that the cytoplasmic PDE also catalyses the hydrolysis of cGMP: in fact, at micromolar concentrations of substrates the rate of cGMP hydrolysis is greater than that of cAMP

(Kakiuchi *et al.*, 1973; Lin *et al.*, 1974). The influx of Ca^{2+} , therefore, could result in an increase of cAMP and a concomitant decrease of cGMP (Brostrom *et al.*, 1975). This is of interest in the pineal gland, in the light of its reported see-saw signal processing [§ 1.6.3].

The membrane enzyme is thought to maintain cAMP levels under steady state conditions, whereas the cytoplasmic enzyme becomes critical when the cell is confronted with an upsurge of cAMP. If activation of this cytoplasmic enzyme were to be inhibited, it would leave the cell in a state of perturbation.

5.4.3.3 Possible role in pineal function

Pineal PDE is characterized by having both a high- and a low- K_m . The low- K_m PDE, that is relatively specific for cAMP-PDE is generally considered (with little evidence to support the supposition) to be the relevant enzyme (Wells and Kramer, 1981). In support of this, these authors point to their earlier work (Wells *et al.*, 1975a) showing that the enzyme is inhibited *in vitro* by relatively low concentrations of cGMP.

A further bone of contention is the use of low substrate concentrations [$1\mu\text{M}$] when measurements are attempted on the amount of low- K_m PDE. This practise is based on the assumption that enzymes with a high- K_m will not catalyse the hydrolysis of low concentrations of substrate. Wells and Hardman (1976) feel that this assumption is incorrect, and that changes in the kinetic parameters of either the high- or low- K_m fractions will be detected in assays with low cAMP substrate, and could be interpreted incorrectly as changes in only the low- K_m enzyme. The data of Wells *et al.*, (1975b) lend support to this by showing that as is true of most tissue, where both PDE I and the low- K_m are present together, they will contribute equally to the hydrolysis of [$1\mu\text{M}$] cAMP. Simmons and Hartzell, (1988) presented data which adds further support to the above.

5.4.3.4 Implications for low- K_m PDE in the pineal

The results of Oleshansky and Neff (1975), as well as the results of the present study are based on low cAMP substrate levels, because that would assay the low- K_m PDE enzyme reportedly present in the pineal.

In terms of the arguments presented above, the data collected could just as well represent data from an isozyme other than the low- K_m one. Oleshansky and Neff (1975) after kinetic analysis do

in fact see a significant increase in the V_{\max} for their low- K_m enzyme, and also a small but significant increase in its K_m .

This is a characteristic of calmodulin activation of *PDE I*.

Of further interest in this argument is the recent data of Chons *et al.*, (1988). Using a histochemical technique they were able show the presence of cAMP-PDE activity along the cytoplasmic membrane of pinealocytes. However the same localization was evident when cGMP was used, which suggests that the PDE enzyme present in the pinealocyte may hydrolyze both nucleotides.

The hydrolysis of both nucleotides by the same enzyme is also a characteristic of *PDE I*. Either way it disputes the assumption that the low- K_m PDE is the dominant enzyme in the pineal.

It should be mentioned that other calmodulin-sensitive PDE forms with equal affinity for both cAMP and cGMP, have been described in some tissues (Ilien *et al.*, 1978).

The findings of the present study show a distinct trend of PDE inhibition in the presence of the CCBs. The latter group of drugs are reported to inhibit the calmodulin-activated form of PDE. **Figure 3.9** showed a diagrammatic representation of the mechanism whereby the CCBs bind to, and inhibit the activation of cAMP-PDE by CaM. As the CCBs are not specific inhibitors of calmodulin, but inhibit it because of their structural arrangement, it was decided to investigate this interesting concept further, by using CaM-inhibitors which were far more specific in their mode of action.

CHAPTER 6

CALCIUM-CALMODULIN SYSTEM STUDIES

6 Introduction

6.1 Calcium-calmodulin System Manipulations.

Calcium has long been known to be important for the maintenance or control of diverse cellular functions. This led Rasmussen to suggest that, like the cyclic nucleotides, Ca^{2+} be afforded "second messenger" status (Rasmussen, 1970). However its intracellular mechanisms of action have only recently become understood (Norman *et al.*, 1987). One such mechanism works through the class of Ca^{2+} binding proteins which can directly activate intracellular enzymes. Calmodulin (CaM) is the most abundant of these enzymes. In terms of second messenger function it has been suggested that CaM, acting as a receptor of Ca^{2+} , serves an analogous role toward Ca^{2+} , as does protein kinase towards cAMP (Klee and Haiech, 1980). An extension of this would be the naming of the collaborative system involving Ca^{2+} and CaM in the activation of many enzyme systems, the calcium-calmodulin system (CCS).

Sutherland *et al.*, (1968) have proposed that the potentiation of a hormonally stimulated cellular response by inhibition of PDE should be one of the criteria for proving that a response is mediated by an intracellular increase in cAMP. Minneman, (1977) has shown that small alterations in cAMP PDE activity could have marked effects on pineal cellular cAMP levels. In the previous chapter not only has an increase in cAMP been observed, but PDE has also been shown to be inhibited in the presence of the CCBs. Interaction with CaM may explain some of the effects of Ca^{2+} -antagonists which cannot be related to Ca^{2+} -entry blockade only, since CaM mediates many intracellular effects of Ca^{2+} . Norman *et al.*, (1983) have shown that some calcium entry blockers inhibit CaM-dependent PDE. This, coupled to the findings of the previous chapter, suggests the possibility of a CaM-sensitive PDE in the pineal gland.

6.1.1 Calmodulin-stimulated PDE's.

CaM-stimulated PDE's have been identified in a wide variety of tissue from both vertebrate and invertebrate species (for reviews see Beavo *et al.*, 1982; Klee and Vanalan, 1982). Further evidence of the ubiquity of this enzyme is provided by Manalan and Klee, (1984). Because of the several forms of PDE present in most tissues, the physiological role of the CaM-stimulated enzyme is difficult to assess. In the rat, chronic β -adrenergic stimulation results in an initial

increase in cAMP but that then declines despite the continued presence of β -adrenergic agonist. The observed decrease can be inhibited by Ca^{2+} depletion (using EGTA) or by PDE inhibition (alkylated xanthenes). An association between the decrease in cAMP content and the translocation of CaM to the membrane compartment has been suggested (Claybreger *et al.*, 1981; Stoclet, 1981).

Similarly, CaM levels have been shown to rise intracellularly in the presence of agents that mimic, or conditions that increase stress, i.e. that cause an increase in cAMP levels (Wells and Hardman, 1977 and references therein).

Several mammalian tissues have been shown to contain high concentrations of CaM (Hidaka *et al.*, 1979 and references therein). The pineal gland is no exception, the presence of CaM having been demonstrated by Zhou *et al.*, (1985).

6.1.2 Objective

As the CCBs are not PDE inhibitors by design, but appear to be doing so as a result of their spatial arrangement, at least in the view of present literature, it was decided to use more specific "tools" to elucidate these findings. It was hoped that the judicious use of such tools to manipulate the CCS, would allow the mimicking of the present results, thus adding support to those findings.

6.1.3 Selectivity of action?

Johnson and Wittenauer, (1983) made use of a dansylated CaM to characterise the interaction of hydrophobic ligands with CaM and CaM-antagonists.

The current understanding of a mode of action favours a primary action of the CCB, particularly the dihydropyridines, on a VOC with additional secondary sites of action within the cell. The CaM and CaM-regulated enzymatic systems represent one such secondary site of action.

Thus, in addition to being Ca^{2+} -antagonists as defined by Fleckenstein (1977), it should be recognised that they are also "CaM antagonists" and could alter many CaM-mediated effects. The binding of these hydrophobic ligands exhibits some specificity when binding the hydrophobic domains on CaM and competing with the binding of CaM-activated proteins.

However, the CCBs verapamil, diltiazem and nifedipine do not bind to CaM with enough affinity to be considered "powerful CaM antagonists", a term used by Asano *et al.*, (1982). Trifluoperazine (TFP) was deemed to be a specific CaM antagonist since it not only bound CaM with reasonable affinity (equilibrium constant K_D ca $1\mu\text{M}$), but it also has the classical structure of a CaM inhibitor as proposed by Weiss *et al.*, (1982).

In general, the phenothiazine group of neuroleptics (and the CCBs) have the structural features previously identified as important for binding to CaM (Prozialek and Weiss, 1982): a hydrophobic ring system with a flexible side chain containing an amino group which should be positively charged at physiological pH.

6.1.3.1 The use of the phenothiazines as CaM inhibitors

The first CaM inhibitors then, were the phenothiazines (Weiss *et al.*, 1982). Because TFP was thought to be the classical inhibitor many older experimental approaches used this drug as sole indicator of CaM activity. This approach was however fraught with problems.

Admittedly the phenothiazines bound CaM only in the presence of Ca^{2+} , and they did show "selectivity". However, the result of this assumption was that phenothiazine inhibition of a phenomenon was equated with that phenomenon being a CaM-dependent one. It is now clear that the phenothiazines are not that highly selective, as they interact not only with CaM, but also with α_1 and DA receptors as well as PK-C.

They have also been shown to have membrane stabilising effects, a result of which would be decreased Ca^{2+} flux through the membranes. This in itself would be sufficient to produce an effect independent of CaM (Landry *et al.*, 1981). Alternatively it might be argued that membrane stabilisation is due to CaM inhibition. Phospholipase A_2 , an enzyme responsible for hydrolysing membrane phospholipids and thus increasing membrane permeability (Hirata and Axelrod, 1980) is also CaM-dependent (Wong and Cheung, 1979).

Blackmore *et al.*, (1981) have shown that TFP and chlorpromazine can bind to α_1 receptors (in the rat liver) and that experimental results thus obtained are a result of α_1 inhibition and not CaM inhibition. To circumvent this problem, one experimental approach would be the use of agents that can elicit the same response as α_1 agonists active via Ca^{2+} -dependent manner but which do not interact directly with the α_1 receptor to produce the Ca^{2+} influx. An example of one such agent is A23187. Accordingly, one should not use phenothiazine agents alone, when investigating

the effects of Ca^{2+} fluxes and α_1 -receptor stimulation.

As the aim of this exercise was to enable manipulations of the CCS which would give a better representation of PDE activity in the pineal gland, and thus clarify the results obtained with the CCBs, it was important to get an agent which was not only more specific than the CCBs, but which would also have few infelicitous effects.

6.2 Experiment 1: THE EFFECT OF CCS MANIPULATIONS ON [^{14}C]-SEROTONIN METABOLISM BY ORGAN CULTURES OF RAT PINEAL GLANDS

6.2.1 Introduction

To enable specific manipulations of the CCS, the following agents were chosen for their reportedly selective modes of action. It was hoped that results thus obtained would verify the earlier findings and strengthen them.

6.2.1.1 CaM inhibition

Of particular interest then is the antimicotoc agent, calmidazolium (1-[bis(*p*-chlorophenyl)methyl]-3-[2,4-dichloro- β -(2,4-dichlorobenzyloxy)phenethyl] imidazolium chloride) more conveniently referred to by its code number R24571, a highly specific CaM antagonist.

Van Belle, (1981a,b) shows that R24571 inhibits the activation of brain PDE by CaM with a 500 times greater potency than TFP. Unlike the other agents (phenothiazines) it has no affinity for dopamine receptors at concentrations that completely inhibit CaM-regulated functions. R24571 is therefore proposed as a useful experimental tool.

6.2.1.2 Calcium depletion

Ethyleneglycol-bis(β -aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) is a selective chelating agent with high selectivity for Ca^{2+} (Miller, 1984). EGTA is widely used in biochemistry and physiology to buffer Ca^{2+} ions. It has a stability constant such that Ca^{2+} can be buffered in the micromolar range near neutral pH, as is often required to mimic physiological concentrations. It has poor cellular penetration and therefore binds predominantly the external Ca^{2+} (present in the medium) with a molar ratio of 1:1 (Bennet, 1979).

EGTA therefore provides a useful means of depleting extracellular Ca^{2+} .

6.2.1.3 CaM-sensitive PDE inhibition

Agents used widely for their ability to inhibit PDE activity are the alkylated xanthines [caffeine, theophylline and 1 methyl-3-isobutyl-xanthine]. Progressive modification of the methyl xanthines (known inhibitors of cyclic nucleotides) led to the flavonoids, a group of compounds with antiallergic properties.

Various other pharmacological effects have also been attributed to the flavonoids. Of these, the finding that they inhibited cAMP hydrolysis by PDE is one of the most interesting (Graziani Chayoth, 1977). Ruckstuhl and Landry (1981) have reported that some flavonoids are more selective for cGMP.

Bergstrand *et al.*, (1976) studied the antiallergic drug 2-*o*-propoxyphenyl-8-azapurin-6-one propane (M&B 22948) an inhibitor of cGMP and found that it has high selectivity for cGMP. Miot *et al.*, (1983) have shown that in the dog thyroid gland, a CaM-sensitive PDE is activated by a rise in $[\text{Ca}^{2+}]_i$. This becomes the dominant catalytic isozyme and M&B 22948 is able to inhibit the response.

M&B 22948 should thus inhibit the CaM-sensitive cGMP component of PDE (Wells and Kramer, 1981), were it present in the pineal gland.

6.2.1.4 Ca^{2+} Ionophore

The most currently active area of ionophore interest arises from the ability of a carboxylic antibiotic to transport the key biological control ion, Ca^{2+} , across biological membranes.

A23187 is a unique ionophore tool because it is predominantly selective for divalent over monovalent ions. (Pressman, 1976 and references therein). It is thus capable of stimulating various Ca^{2+} -dependent biological reactions without disturbing pre-existing balances of Na^+ and K^+ .

Since the ionophore appears to introduce calcium rapidly into the cell under mild experimental conditions, this reaction might be used as an effective experimental tool to study the response

pathways of Ca^{2+} -dependent effects. As in the case with the synthetic butyryl derivatives of 3',5'-cAMP which penetrate the cell and activate certain biological systems, the ionophore may serve as an experimental by-pass in some hormonal receptors. Sellinger, (1974) has made effective use of this ion bearer in a parallel system, to elucidate the role of Ca^{2+} influx following α_1 -adrenergic stimulation in the rat parotid gland.

Thus with a suitable armamentarium at hand, the modes of action of these agents could be used to manipulate the CCS in such a way as to mimic the effect of the CCBs on the conversion of [^{14}C]-serotonin to its *N*-acetylated products ($\text{NaP} = ([^{14}\text{C}]\text{-aMT} + [^{14}\text{C}]\text{-aHT})$ in organ cultures of rat pineal glands.

6.2.2 Materials and methods

6.2.2.1 Materials

Ethyleneglycol-bis(β -aminoethylether)-*N,N,N',N'*-tetraacetic acid (97% purity) was purchased from Sigma Chemical Co., USA. A23187 and R 24571 were purchased from Boehringer Mannheim, West Germany. M&B 22948 was a gift from May & Baker Ltd, Port Elizabeth, S.A.. Dimethyl sulphoxide (DMSO) was of analytical grade from commercial sources, and the sources of the other drugs and materials have been recorded [§ 2.2.1.1].

6.2.2.2 Animals

Adult male Albino Wistar rats (180 - 220 g b.wt) were used in this organ culture study for the reasons previously outlined [§ 2.2.1.2]. Their environment has been described in the same section.

6.2.2.3 METHODS

6.2.2.3.1 Experiment A

Rats were sacrificed between 09h00 and 09h30. Their pineal glands were removed [§ 2.2.1.3], and each transferred to a separate sterile culture tube containing BGJ_b (Fitton-Jackson modification) culture medium at the required volume.

Tubes were arranged in groups of four. The agents listed below in **column two** were then added to the culture medium, whereafter the glands were incubated for 30 min. After this 30 min period, the adrenergic agonist ISO was added at the concentrations listed in **column one**.

The groups of tubes thus contained in a final incubation volume of 50 μ l, the following combinations of drugs, at the listed concentrations:

	Column 1		Column 2
i)	[10 μ M]ISO	+	vehicle (Control)
ii)	[10 μ M]ISO	+	[3.3mM]EGTA
iii)	[1 μ M]ISO	+	[10 μ M]A23187
iv)	[10 μ M]ISO	+	[10 μ M]M&B 22948
iv)	[10 μ M]ISO	+	[1 μ M]R 24571

The incubation was terminated after a 24 h incubation period. 10 μ l aliquots of medium were removed, and spotted onto TLC plates. The radioactive metabolites were separated by two-dimensional TLC methods, and quantitated using liquid scintillometry as described in [§ 2.2.1.5]. Results from all groups of glands were then compared with control glands incubated with vehicle, in the presence of ISO [10 μ M].

Stock solutions [0.1M] of A23187, R 24571 and M&B 22948 were made up in DMSO and serially diluted in culture medium, while EGTA was dissolved in distilled water. These agents were added to the vessels in the required volumes. ISO and NA were dissolved in culture medium. The unstimulated control received an equivalent amount of vehicle.

DMSO (1%) solvent in control experiments produced no change in the aHT, aMT or *N*-acetylated product levels.

6.2.2.3.2 Experiment B

The procedure in Experiment A [§ 2.5.2.3.1] was repeated, but NA replaced ISO at the same molar concentrations.

The groups of tubes thus contained in a final incubation volume of 50 μ l, the following combinations of drugs, at the listed concentrations:

	Column 1		Column 2
i)	[10 μ M]NA	+	vehicle (Control)
ii)	[10 μ M]NA	+	[3.3mM]EGTA
iii)	[1 μ M]NA	+	[10 μ M]A23187
iv)	[10 μ M]NA	+	[10 μ M]M&B 22948
iv)	[10 μ M]NA	+	[1 μ M]R 24571

The incubation was terminated after a 24 h incubation period. 10 μ l aliquots of medium were removed, and spotted onto TLC plates. The radioactive metabolites were separated by two-dimensional TLC methods, and quantitated using liquid scintillometry as described in [§ 2.2.1.5]. Results from all groups of glands were then compared with control glands incubated with vehicle, in the presence of NA [10 μ M].

Agents were dissolved as described above [§ 6.2.2.3.1].

6.2.2.4 Data analysis and statistics.

Data are expressed as dpm / 10 μ l medium, with each point representing the mean \pm SEM of each group of four glands.

In keeping with the earlier convention, the results of this organ culture study were assessed using primarily the aHT and aMT metabolites as markers. *N*-acetylated product (NaP) was used as a gauge of overall pineal activity.

Statistical analysis for multiple groups is best analysed using an analysis of variance (ANOVA) test (Modrak, 1983). The Kruskal-Wallis *H* test is the nonparametric equivalent test. The significantly different groups were identified using the *post-hoc* Neuman-Keuls multiple range test. Groups were judged at the $p < 0.05$ level of significance.

6.2.3 Results and Discussion

6.2.3.1.1 Results of glands stimulated with ISO

The results are presented in Table 6.1 and Figure 6.1.

6.2.3.1.2 Discussion

Glands stimulated with ISO showed an increase in aHT, aMT and *N*-acetylated product, similar to that seen in the initial studies [§ Chapter 2]. The addition of Ca²⁺ to the culture medium of glands **already** stimulated with ISO was not attempted since initial pilot studies showed no effect here. The earlier NAT studies had also shown no effect here. This is in contrast to the findings of Morton, (1987) who shows an inhibitory role for Ca²⁺.

Table 6.1: *The effect of CCS manipulations on [¹⁴C]-serotonin metabolism in rat pineal glands.*

GROUP	Metabolite			GROUP	Metabolite		
	aHT	aMT	NaP		aHT	aMT	NaP
ISO[10 μ M] + Vehicle Control	2850 \pm 185	3044 \pm 162	5894 \pm 301	NA + Vehicle	3481 \pm 200	3283 \pm 555	6764 \pm 750
ISO[10 μ M] + [3.3mM]EGTA	1150 \pm 221 ^(a)	2044 \pm 121	3194 \pm 270 ^(a)	NA + EGTA	2029 \pm 227 ^(a)	2530 \pm 163	4559 \pm 411
ISO[1 μ M] + [10 μ M]A23187	1809 \pm 112 ^(a)	4000 \pm 276	5810 \pm 200	NA + A23187	3065 \pm 363	3448 \pm 126	6513 \pm 247
ISO[1 μ M] + [10 μ M]M&B 22948	2687 \pm 280	3695 \pm 444	6382 \pm 307	NA + M&B 22928	3275 \pm 503	7782 \pm 371 ^(b)	11057 \pm 631 ^(b)
ISO[1 μ M] + [1 μ M]R24571	2908 \pm 283	3460 \pm 206	6368 \pm 401	NA + R 24571	4311 \pm 265	6422 \pm 265 ^(b)	10734 \pm 525 ^(b)

10 μ l aliquots of medium were chromatographed by TLC. Results are expressed as dpm/ pineal gland (mean \pm SEM; n = 4 individually cultured glands per group). Significance is computed using the Neuman-Keuls multiple range test; $p < 0.05$ w.r.t respective vehicle control.

(a) significantly decreased w.r.t vehicle control
 (b) significantly increased w.r.t vehicle control | Neuman-Keuls multiple range test; $p < 0.05$

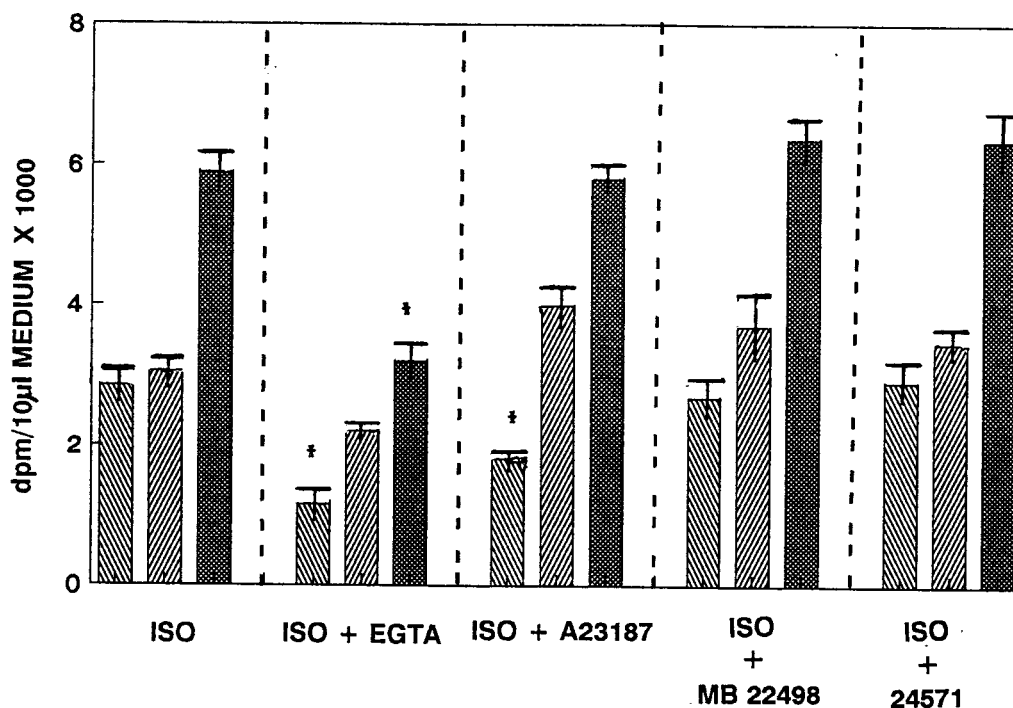


Figure 6.1: *The effect of CCS manipulations on [¹⁴C]-serotonin metabolism ([diagonal lines] = aHT, [vertical lines] = aMT, [solid black] = NaP) in rat pineal glands stimulated with ISO (mean ± SEM; n = 4 glands per group; p < *0.05 w.r.t vehicle control: Neuman-Keuls multiple range test)*

This suggests that the Ca²⁺ must somehow be introduced into the cells for its effect to be seen. On the other hand, removal of Ca²⁺ from the culture medium, with the calcium chelating agent EGTA, resulted in a decrease in aHT, aMT and *N*-acetylated product. This finding is predictable from the NAT studies [§ Chapter 3] and has been demonstrated before (Zatz and Romero, 1978; Wilkinson, 1978).

The Ca²⁺ ionophore A23187 was unable to cause any significant effects by itself in the NAT studies, and showed a small significant decrease here in aHT levels in the presence of [1µM] ISO. It potentiated [1µM] ISO stimulated levels of aMT and *N*-acetylated product, to levels similar to those found in glands stimulated with [10µM] ISO. This shows that the ionophore is able to mimic the potentiation shown to occur in glands following combined α₁- and β-adrenergic stimulation.

This is in agreement with other reports showing a role for Ca²⁺ influx in the adrenergic regulation of this gland [§ 2.5.1.2]. The results with EGTA show that there is a requirement for Ca²⁺ even in the presence of β-adrenoceptor stimulation.

There does not seem to be any significant effect on either aHT, aMT or *N*-acetylated product in the presence of M&B 22948 or R 24571. This is similar to earlier findings which measured NAT, cAMP and PDE levels in glands stimulated with ISO in the presence of verapamil or nifedipine.

6.2.3.2.1 Results of glands stimulated with NA

The results are presented in Table 6.1 and Figure 6.2.

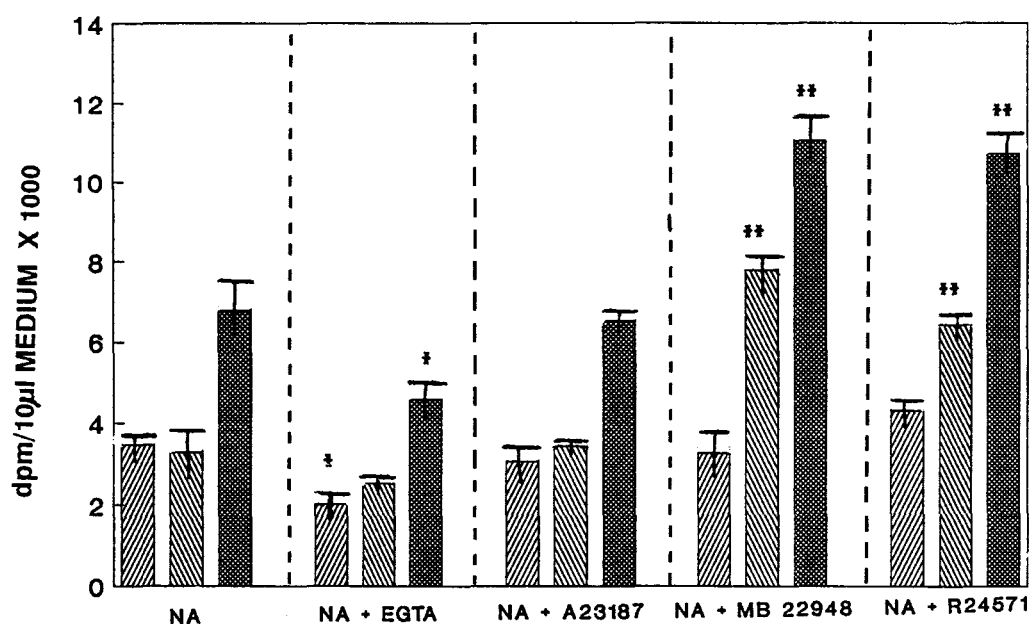


Figure 6.2: *The effect of CCS manipulations on $[^{14}\text{C}]$ -serotonin metabolism (aHT = aHT, aMT = aMT, NaP = NaP) in rat pineal glands stimulated with NA (mean \pm SEM; n = 4 glands per group; p < *0.05 decreased w.r.t vehicle control; p < ** 0.05 increased w.r.t. vehicle control: Neuman-Keuls multiple range test)*

6.2.3.2.2 Discussion

These results are similar to those observed following NA stimulation in the presence of CCB.

EGTA abolished the stimulation of NA, with the *N*-acetylated product being significantly decreased.

The combination of A23187[10µM] and [1µM] NA shows a synergistic effect similar to that seen with NA [10µM]. The dose response experiments [§ 3.4.3] found that [1µM] NA produced only a small, non-significant increase in NAT activity.

Although aHT does not seem to be increased to any extent, it could be the result of simple substrate elimination, i.e. it is being used up to produce the peaks in *N*-acetylated product seen in the presence of A23187, M&B 22948 and R 24571 and also the peak in aMT seen in the presence of the latter two agents.

These findings clearly support the earlier hypothesis that a CaM-mediated effect (the inhibition of CaM-sensitive PDE) could be responsible for the results found.

6.2.4 Conclusion

These studies highlight the critical nature of Ca^{2+} levels in the control of the CCS.

It has been shown that Ca^{2+} is necessary for the adrenergic function of the pineal gland. This necessity is far greater in the case of α_1 -receptor stimulation where it would seem more systems are reliant on the Ca^{2+} influx. One example is the potentiation seen following α_1 - and β -receptor stimulation which occurs via a Ca^{2+} -dependent protein kinase.

If the concentration of $[\text{Ca}^{2+}]_i$ is so critical, then any deviation would make less of it available, and the system more sensitive to what is available. If this hypothesis is extended further to include a fixed quantity of CaM available for Ca^{2+} to bind to and activate (Manalan and Klee, 1984) the following situation is possible: assuming that the CCBs are blocking some of the VOC available, then less Ca^{2+} can enter the cell. (This is also assuming that the CCBs are binding some to CaM present in the cell). This situation would have equal consequences in ISO- or NA-stimulated cells, provided there was no difference in the Ca^{2+} utilization of the stimulated cells. As stated above, α_1 -receptor stimulation is accompanied by a greater requirement for Ca^{2+} , thus the NA system would be more sensitive to the CCBs, were they to bind to CaM. This would allow less CaM to be available for the activation of cAMP-PDE, and the potentiation due to PDE inhibition, found thus far, would be possible.

It however remains to be seen if NAT activity and PDE activity can be shown to be altered using these CCS manipulations.

6.2.5 Objective

The next investigation examines the role of calcium and the CCS in the adrenergic receptor control of NAT and PDE, by using several experimental manipulations which vary not only the

concentration of Ca^{2+} within the culture vessels, but which also manipulate the CCS's activation of the CaM-sensitive PDE.

6.3 Experiment 2: THE EFFECT OF CCS MANIPULATIONS ON NAT AND cAMP-PDE ACTIVITY IN RAT PINEAL GLANDS

6.3.1 Introduction

In a number of tissues there appears to be an interaction between cAMP and calcium in their regulation of the effects of hormones (Zatz and Romero, 1978). Data indicate that at least part of the action of intracellular calcium is indirect and that it affects induction at a step beyond cAMP because the latter still rises after 10 min when stimulated with butyryl derivatives of cAMP.

The rise in NAT occurring at night is well documented. The data presented by Iuvone and Besharse (1986), indicate that the increase in NAT requires extracellular calcium in the micromolar range and that calcium influx through a VOC is involved. Antagonists of VOCs including nifedipine and methoxyverapamil (D600) were found to be effective inhibitors of the dark-dependent increase in NAT activity. TFP at $100\mu\text{M}$ also effectively inhibits this increase.

However data concerning the CCBs are not always in agreement e.g. Meyer *et al.*, (1986) have shown that two classes of CCB administered *in vivo* to baboons, produce different effects on circulating plasma aMT levels at night. Three dihydropyridine CCBs, one of which was nifedipine, reduced aMT levels. Verapamil had no effect.

In order to clarify the results found in the preceding chapters, and maybe to elucidate the mixed findings in the literature, it was hoped that NAT and PDE levels could be determined in the same glands, while the CCS was manipulated with the test drugs.

Quite by chance it appeared that this would be possible if the glands were homogenised in the NAT buffer but not the PDE buffer. Steinlechner *et al.*, (1984) have shown that NAT is sensitive to pH and is irreversibly blocked by pH higher than 6.9, while PDE is not that critical of pH (pH's of 7 to 8 are commonly reported in PDE assays in the literature). In addition, the phosphate present in the NAT phosphate buffer would have a beneficial effect on the PDE assay. It has been shown to help prevent the further hydrolysis of 5'-AMP by 5'-nucleotidase, under assay conditions (Goldberg *et al.*, 1969).

Pilot studies confirmed that in pineal glands stimulated with [10 μ M] ISO or [1 μ M] ISO and [3mM] aminophylline, an increase in both NAT and PDE activity was observed, and that the effect of the lower dose of ISO was potentiated by the aminophylline. Oleshansky and Neff, (1975) have reported similar results.

6.3.2 Materials and Methods.

6.3.2.1 Materials

Sources of all drugs, materials and chemicals have been recorded in previous chapters.

6.3.2.2 Animals

Adult male Albino Wistar rats (200 - 250 g b. wt) were used in this study. Their environment has been described in [§ 2.2.1.2].

6.3.2.3 METHODS

6.3.2.3.1 Experiment A

Rats were sacrificed between 09h00 and 09h30. Their pineal glands were removed [§ 2.2.1.3], and transferred to sterile culture vessels containing BGJ_b (Fitton-Jackson modification) culture medium at the required volume. Four pineal glands were transferred to each vessel.

Stock solutions [0.1M] of A23187, M&B 22948 and R 24571 were made up in DMSO and serially diluted in culture medium, while EGTA was dissolved in distilled water. These agents were added to the vessels in the volumes required to achieve the concentrations listed below for each group for glands.

The unstimulated control received an equivalent amount of vehicle. The adrenergic agonists ISO (at the concentration indicated below) and NA[10 μ M] were added after a 20 min preincubation with the test agents.

The eight vessels thus contained in a final incubation volume of 200 μ l, the following combinations of drugs, at the listed concentrations:

	Column 1		Column 2
i)	[0 μ M]agonist	+	vehicle (Basal)
ii)	[10 μ M]ISO	+	vehicle
iii)	[1 μ M]ISO	+	[10 μ M]A23187
iv)	[10 μ M]NA	+	[3.3mM]EGTA
v)	[0 μ M]agonist	+	[1 μ M] R 24571
vi)	[10 μ M]NA	+	[1 μ M] R 24571
vii)	[10 μ M]NA	+	[10 μ M] M&B 22948
viii)	[10 μ M]NA	+	vehicle

The vessels were then placed in glass vials as described in [§ 3.4.1.3], incubated for 3 hours at 37°C, whereafter the pineal glands were removed from the culture medium, and each transferred to its own homogenisation tube, containing 80 μ l of cold 0.05M phosphate buffer (pH 6.5) and kept on ice. Pineal glands were homogenised by 6-8 full rotations of a glass homogenation rod.

Fifty microlitres of homogenate was removed and transferred to a corresponding culture tube for the determination of *N*-acetyltransferase activity as described [§ 3.2.1.4].

6.3.2.3.2 Experiment B

A further 8 μ l aliquot was removed from each homogenisation tube used above [§ 6.3.2.3.1]. These aliquots were transferred to reaction tubes and assayed for cAMP-PDE activity as described previously [§ 5.2.1.4.2].

6.3.2.4 Data analysis and statistics.

Data from the NAT assay are expressed as pmol of *N*-acetyltransferase activity/ pineal gland/ h, while data resulting from the determination of cAMP-PDE levels (the mean of duplicate determinations) are expressed as nmol cAMP hydrolysed/ 30 min/ pineal gland.

Each bar represents the mean \pm SEM of 4 glands.

Statistical comparisons are made using the nonparametric Kruskal Wallis *H* test. Significantly different groups were identified using the Keuls-Neuman multiple range test at the ($p < 0.05$) level.

6.3.3 Results and Discussion

6.3.3.1.1 Results of glands assayed for NAT and cAMP-PDE activity

The results are presented in **Figure 6.3**.

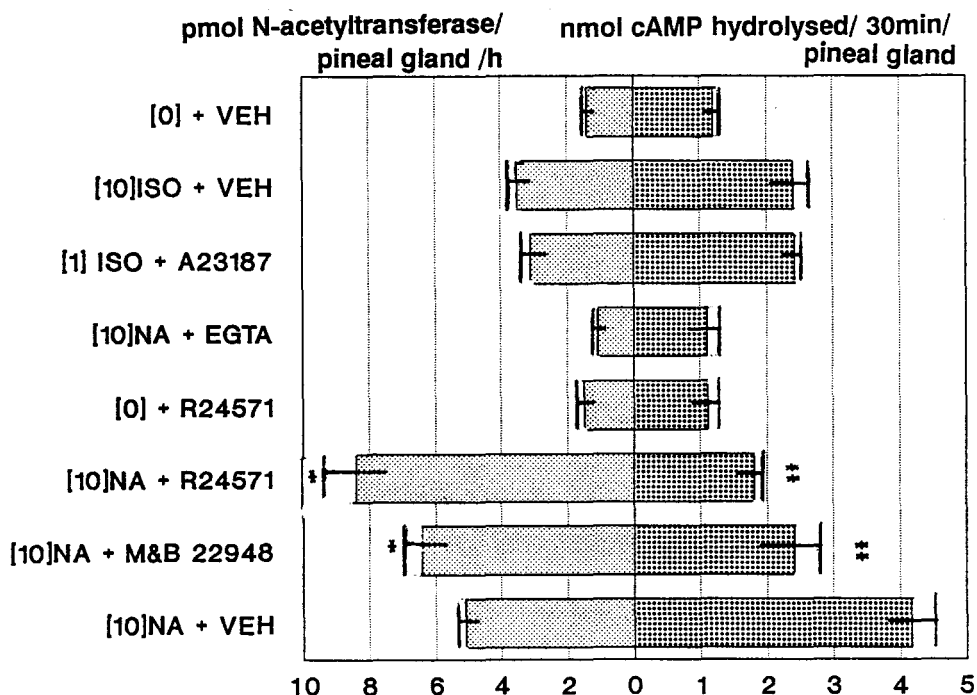


Figure 6.3: Levels of N-acetyltransferase (pmol)[] and cAMP-PDE (nmol)[] activity determined in the same pineal glands stimulated with either ISO or NA in the presence of CCS manipulations as indicated (mean \pm SEM; n = 4 glands per group; p < *0.05 increased w.r.t. vehicle control; p < ** 0.05 decreased w.r.t. vehicle control: Neuman-Keuls multiple range test)

6.3.4 Discussion

The results from both experiments will be discussed together to facilitate their interpretation.

Those glands stimulated with ISO [10 μ M] show the expected increase in NAT, as well as a concomitant increase in cAMP-PDE, as would be expected from earlier findings [§ Chapter 5].

[1 μ M] ISO + [10 μ M] A23187 show the predictable synergistic results, seen earlier in the organ culture experiment above [§ 6.2.2.2.1]. Coupled to this, the cAMP-PDE levels for this group have also risen in parallel. The reasons for the increase in PDE following adrenergic stimulation have been discussed in Chapter 5.

NA [10 μ M] + EGTA [3.3mM] shows an inhibition of the NA activation of NAT, and cAMP-PDE also does not rise above basal levels. The fact that the latter does not rise could be ascribed to two facts: i)cAMP-PDE is coupled to increases in adrenergic activity, and ii) a possible Ca²⁺-sensitive mechanism may be at work i.e. Ca²⁺ is needed for the activation of cAMP-PDE. Levin and Weiss, (1976) have presented data showing two groups of Ca²⁺-CaM-sensitive PDE inhibitors to exist. The first group (EGTA type) prevents the Ca²⁺-dependent activation of PDE by binding the Ca²⁺, while the second group of inhibitors (as exemplified by the phenothiazine group of compounds) bind to CaM itself and prevent activation thereof.

R 24571 by itself produces no effect, either on NAT or cAMP-PDE. This is to be expected from the previous experiment, where it appeared that basal activity was not influenced by this CaM inhibitor. Epstein *et al.*, (1982) have shown that the calcium antagonists are all two to five fold more potent against the CaM-sensitive PDEs in their activated state than in their resting basal state. From these findings it would appear that the activated state would be analogous to the situation during which the Ca²⁺ influx is occurring.

In support of this, in the presence of NA, a potentiation with R 24571 is seen which is greater than either ISO[10 μ M] or NA[10 μ M] alone. Of even greater interest is the fact that cAMP-PDE has not risen in a parallel manner, as would be expected following normal adrenergic activation. This suggests that some form of PDE inhibition is probably occurring.

This is by far the best proof the a CaM-dependent mechanism is at work in the potentiation following NA stimulation in the presence of the CCBs.

The CaM-sensitive PDE inhibitor causes a rise in NAT activity. The PDE activity for this group shows a rise greater than that with R 24571, but less than that found, when a reference competitive PDE inhibitor (aminophylline) was used in pilot studies.

Pure competitive inhibition resulted in large increases in available PDE. In this assay system; this is as a result of levels of PDE rising due to adrenergic triggering, but being inhibited from acting by the aminophylline in the culture medium. Thus the cAMP continues to rise (within the pineal gland) and continues to trigger an increase in its terminator (PDE) which is then detected in the assay system.

The finding implies that some mechanism, other than pure competitive inhibition is active.

Although purely speculative, it could be that M&B 22948 is inhibiting the cGMP-PDE component of *PDE I*, and in so doing is making less cAMP-PDE available (partial inhibition). This would account for the increased NAT levels found. Alternately, it could also be that the CaM-sensitive cGMP-PDE is being inhibited. It would not have been possible to determine this in the present system, but the result would be that cGMP rises. Kabovetz *et al.*, (1979) have shown that the 8-azapurine-6-one (M&B 22948) can selectively increase cGMP levels. The role of cGMP in the pineal gland is unclear, but it is well known that in many tissues, cGMP can inhibit cAMP-PDE activity.

Work from Sugden and Klein (1987), suggests that the regulation of cGMP levels in the pineal gland involves an N_s -like GTP-binding regulatory protein. This is of interest because it is the first indication that cGMP is regulated by such a GTP-binding protein in nonretinal tissue.

Continuing on from this, is the possibility that a cGMP-PDE may be involved in regulating pineal cGMP, as is the case in the retina. Support for this comes from the finding that the mammalian retina and pineal share a number of unusual proteins (Axelrod and Weissbach, 1962; Somers and Klein, 1984 and Van Veen, 1986a), some of which (rhodopsin kinase (Somers and Klein, 1984) and S-antigen (Van Veen, 1986a) are involved transmembrane signalling (Stryer, 1986). In the rod outer segment, the GTP-binding protein or G protein, transducin, couples the photon receptor rhodopsin to a specific cGMP phosphodiesterase; light sensitive activation of this enzyme cascade results in a decrease in cGMP through activation of the cGMP-PDE (Bitensky *et al.*, 1984; Rosenthal and Schultz, 1987). The resulting decrease in cytosolic cGMP concentration causes a reduced cation channel activity, and hyperpolarization of the cell (i.e. "turn off") (Rosenthal and Schultz, 1987).

Were there to be an inhibition of the activation of this cGMP-PDE, the cation channel activity would not be decreased, and activation of the VOC would contribute to a sustained elevation of Ca^{2+} , and its dependent processes. In related work by Stern *et al.*, (1986), diltiazem has been shown to inhibit the "turn off" to light response, in rod photoreceptors.

Although immunoreactive transducin does not appear to be present in the rat pineal gland (Van Veen *et al.*, 1986b), a related N protein controlling a specific cGMP phosphodiesterase could be present.

This is however purely speculative and a full biochemical characterization would be required before any firm conclusions can be drawn, as to the type of inhibition taking place, and secondly,

the specific PDE which is being inhibited. For example, competitive inhibition is best analysed with Dixon plots, and Lineweaver-Burk plots would also differentiate the types of inhibition (Dixon, 1953).

What is apparent however, is i) that a CaM-sensitive PDE seems to be present in the pineal, and ii) that the CCBs were binding to CaM, and inhibiting the function thereof, especially when the pineal was being stimulated *in vitro* with NA in the presence of the CCBs, verapamil, diltiazem and nifedipine. This conclusion is reached from the data presented above which shows that specific CaM-antagonists are able to mimic the results found with the CCBs.

6.4 COMMENTARY AND CONCLUSIONS ON THESE STUDIES

The data of Theyer and Fairhurst, (1983) suggest that the dihydropyridine and CaM-antagonists (TFP and R24571) have a common binding site, to which they bind in a Ca^{2+} -dependent manner as described by Levin and Weiss (1979). The data also rule out non-specific membrane effects but show that the binding is competitive for the hydrophobic domains exposed on CaM, when it binds Ca^{2+} . The dihydropyridines alter the ^{113}Cd NMR spectrum of CaM (Brostrom, 1981) in an analogous manner to R 24571. Johnson and Wittenauer, (1983) made use of a dansylated CaM to characterise the interaction of hydrophobic ligands (including several dihydropyridines) with CaM and CaM-antagonists. More recent data suggest that the dihydropyridines may bind at the second binding site on CaM, while TFP binds to the carboxy-terminal domain (Orosz *et al.*, 1988; Inagaki and Hidaka, 1984). Johnson and Mills (1986) have shown that the two binding domains exert allosteric control over each other, but the exact relationship to target proteins has not been clarified. In some cases the antagonists bind to CaM, and prevent further binding, while in others a non-active adduct binds to PDE e.g. a TFP-CaM (Newton *et al.*, 1985).

6.4.1 Ca^{2+} -dependent interaction with a CaM-sensitive PDE in the pineal gland

Results presented herein support a possible mechanism of action for the CCBs, verapamil, diltiazem and nifedipine, which involves Ca^{2+} -dependent interaction with CaM and consequent inhibition of Ca^{2+} -dependent enzymes e.g. cAMP-PDE. Such inhibitory effects would favour the present findings, and have been demonstrated with the dihydropyridine derivative, felodipine.

6.4.2 Differences in functional *in vitro* activity

To date several attempts have been made to sort out and to classify the various compounds exhibiting Ca^{2+} -entry-blockade activity by means of their structural properties, their binding characteristics in general (and more specifically to CaM) and their pharmacology.

Thus while the compounds as a group share several common properties such as antagonising Ca^{2+} entry, there exist certain distinct fundamental *in vitro* differences between them, e.g. data probably not relating to channel function suggest that all these agents bind to a greater or lesser degree to CaM. The studies of Johnson and Mills, (1983) provided the first evidence that CaM was indeed an allosteric protein with regard to its drug protein binding sites. With regard to allosteric interaction, one of the best studied discrepancies is that verapamil-like agents inhibit the nifedipine analogue nitrendipine in several tissues whereas diltiazem facilitates its binding at these sites (Johnson, 1984).

A further question on their mechanism of action is being raised by the presence of G proteins. With their role in receptor-mediated regulation of enzymes, G proteins may act as transducers between cell surface receptors and the VOC, mediating not only hormonal stimulation but also inhibition of ion channel activity (Rosenthal and Schultz, 1987). G proteins are not only involved in the hormonal modulation of Ca^{2+} channel activity, but apparently also modify the effects of Ca^{2+} channel ligands such as D600, nifedipine, and diltiazem on Ca^{2+} currents (Scott and Dolphin, 1987).

These findings raise the question of whether CCBs interact exclusively with the channel protein or, if in addition, they interact with G proteins or other components that participate in the modulation of voltage dependent Ca^{2+} channels (Rosenthal *et al.*, 1988).

Other non-channel *in vitro* differences are reported. Verapamil and diltiazem but not nifedipine have the ability to interfere with CaM activation of the Ca^{2+} -pump and ATPase. The failure of nifedipine to do this is in agreement with the studies of Lamers *et al.*, (1985) who found the same discrepancy when working with soluble dog brain CaM-activated-PDE.

The differences observed with diltiazem relative to results using verapamil and nifedipine in the present study, could possibly point to a difference in the respective ability of the CCBs to inhibit Ca^{2+} entry. This however is purely speculative, and should be regarded with care in view of the large intra-species variations seen in pineal work. For instance Meyer *et al.*, (1986) have shown

that two classes of CCB administered *in vivo* to baboons, produced different effects on circulating plasma aMT levels. Nifedipine reduced aMT levels in the plasma, while verapamil did not. On the other hand, Morton *et al.*, (1989) shows data where verapamil prevents a decrease in aMT (in rats forced to swim), while nifedipine is less effective.

Thus explanations for the selectivity and/or functional differences of the pharmacological action of these drugs may also include: (a) differences in the functional dependence of certain organs or blood vessels on the extracellular calcium, (b) a possibility that modulation of the Ca^{2+} channel may modify specific binding and consequently sensitivity to Ca^{2+} channel inhibitors, (c) the fact that different vascular beds are dominated to a different extent by the sympathetic nervous system, and (d) differences in the relative importance of α_1 -, α_2 - and γ -adrenoreceptors (Neild and Zelcer, 1982) for a given system (Janis and Scriabine, 1983).

It would however appear that in this system yet another functional *in vitro* difference between this heterogeneous group of compounds is apparent, as typified by the difference which diltiazem (following ISO stimulation) exhibited for most of these studies. This leaves one firm conclusion to be made; caution should be exercised in the interpretation of experiments in biological systems when these drugs are used.

6.4.3 Therapeutic relevance

Considering the concentrations used in all these studies in an attempt to bring about inhibition, it would be hard to reconcile these results with any therapeutic relevance. No clinically employed drug is presently known to exert its principal pharmacologically desired effects by binding to CaM, in spite of much research and even newer more specific agents (Van Belle, 1984; Agre *et al.*, 1984). The micromolar concentrations used herein are in general higher than typical dose ranges for verapamil or diltiazem (0.01 to $10\mu\text{M}$) and nifedipine (0.001 to $1\mu\text{M}$) (Kim and Raess, 1988). However, it is known that some of these drugs may reach concentrations in membranes that are ten to twenty times higher than their extracellular levels (Pang and Sperelakis, 1983; Keefe and Kates, 1982). Also, all the studies recorded herein are with *in vitro* studies, which could well be vastly different from the *in vivo* situation.

CHAPTER 7

CHAPTER RESULTS AND SUMMARIES

7.1 Chapter 1

Systematic anatomical and physiological studies have led to the idea of the pineal gland as a neuroendocrine transducer converting neural and environmental information into a hormonal response, namely the secretion of melatonin. In the literature, the calcium signal has emerged as an important component of intracellular regulation. In the pineal gland, the prominent calcification seen in the gland with increasing age was thought to slow pineal function, but more recently, Ca^{2+} have been found to play a crucial role in the adrenergic regulation of the gland, acting via α_1 -adrenoceptors.

NA released into the pineal perivascular space from sympathetic nerve terminals at night, increases melatonin synthesis by increasing the activity of NAT. An essential step in the adrenergic induction of NAT activity is an increase in intracellular cAMP. NA also elevates pinealocyte cGMP. Adrenergic regulation of these cyclic nucleotides involves both α_1 - and β -adrenoceptors. β -Adrenoceptor stimulation is an absolute requirement, α_1 -adrenoceptor activation amplifies the β -adrenergically stimulated cAMP and cGMP responses.

The amplification mechanism appears to involve a positive effect of a Ca^{2+} -/phospholipid-dependent protein kinase (Protein kinase-C). Activation of the pinealocyte α_1 -adrenoceptor rapidly translocates this enzyme by increasing $[\text{Ca}^{2+}]_i$; a correspondingly rapid increase in diacylglycerol (DG) generation appears to be totally dependent on extracellular Ca^{2+} .

Protein kinase-C (PK-C) activation appears to enhance cAMP and cGMP synthesis at a step beyond β -adrenoceptor activation, perhaps involving adenylyl and guanylyl cyclase. In the case of cGMP, the increase in $[\text{Ca}^{2+}]_i$ also appears to be important for activation of processes independent of PK-C; these processes may involve activation of phospholipase- A_2 and the arachidonic acid cascade and are required for full potentiation of β -adrenergic stimulation.

As a Ca^{2+} influx into the pinealocyte appears to play a major role in the regulation of the pineal gland, this study attempted to examine this influx.

In order to do so it was proposed to make use of the pineal gland's well-characterised metabolic activity and pathways, as a gauge of that gland's activity, and to use the Ca^{2+} antagonistic

activity of three heterogenous members of the CCB family to probe this phenomenon.

7.2 Chapter 2

Organ culture provides an interesting and useful way to study and monitor both the metabolic activity, and the effects of drugs thereon, in the pineal gland.

By way of a validation of the culture system, the effect of isoprenaline stimulation on [¹⁴C]-serotonin metabolism was monitored over a 24 h period, thus showing the progressive formation of the indole metabolites. This was the first study to show a full 24 h profile of isoprenaline stimulated [¹⁴C]-serotonin metabolism in organ cultures of rat pineal glands.

The results confirmed that organ culture was both a valid and valuable technique with which to study the variation in pineal indole production, when pineal glands are subjected to various pharmacological manipulations. They further showed that 16 h profiles would appear to be optimal for determining the behaviour of isolated pineal glands in the presence of various drugs. These findings were published, and received much interest (Banoo *et al.*, 1987; Daya *et al.*, 1989).

To study the effect of the Ca²⁺ influx reported to concur with combined α_1 – and β -adrenoceptor stimulation in the pineal gland, two adrenergic agonists, ISO and NA, were selected for their respective β - and α_1/β –adrenoceptor stimulating properties. It was speciously hypothesised that the use this drug paradigm would allow the Ca²⁺ influx occurring with NA stimulation to be compared with that of ISO stimulation in the pineal glands (where this influx should not be seen). An initial pilot study confirmed that NA[10 μ M] produced the same effects as the α_1 adrenergic agonist phenylephrine[10 μ M] when used in combination with ISO[10 μ M].

7.2.1 Results

The results of this study are summarised below in **Figure 7.1**.

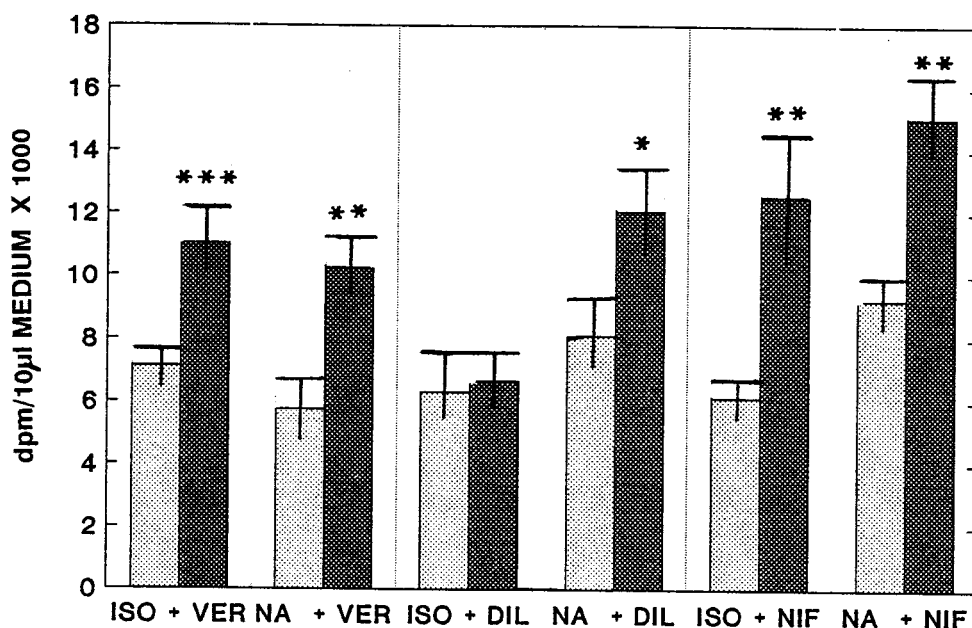


Figure 7.1: *The effect of organic calcium channel blockers [■] on the production of N-acetylated products (NaP), i.e., [sum of [¹⁴C]-N-acetylserotonin and [¹⁴C]-aMT] in the presence of either ISO or NA[10µM], compared to control glands with vehicle [□], in the presence of either ISO or NA[10µM]. Each bar represents the mean ± SD; n=4 pineal glands; p < : *0.05, **0.01 and ***0.025 (Students-t test).*

Following NA stimulation of glands incubated in the presence of verapamil, diltiazem and nifedipine a paradoxical increase in stimulation was found. A decrease in NaP production would be expected were the CCBs blocking the Ca²⁺ influx.

After ISO stimulation verapamil and nifedipine showed the same enhancement, while diltiazem did not. In all the organ culture studies, the levels of aHT were found to be increased. These findings were published (Brown *et al.*, 1989a).

7.2.2

Discussion

It thus seemed that the CCBs were in some manner causing an increase in NAT, the enzyme responsible for the formation of aHT. This made it pertinent to examine the effects of these CCBs

on the level of NAT in isolation. This would clarify if NAT levels are indeed raised, as suggested by the high levels of aHT observed.

7.3 Chapter 3

Leading on from the results of Chapter 2, the levels of NAT, the rate-limiting enzyme in the metabolic pathway to aMT synthesis, were investigated to see if they would corroborate those findings.

Three initial investigations were undertaken to confirm relationships reported in the literature.

7.3.1 Results

A dose-response study showed significant stimulation of NAT to be evident only at concentrations of [3 μ M] and [10 μ M]. NA showed less stimulation of NAT than ISO had done, providing some evidence of intrinsic activity - and/or affinity differences between the two agonists. This dose range was similar to that used in the literature.

The effects of either β - or α_1 - receptor blockade on ISO and NA stimulation of NAT was examined.

The increase in NAT produced by either ISO or NA was totally abolished in the presence of propranolol, a β -adrenoceptor blocker, confirming that NAT is dependent on β -adrenoceptor stimulation. Prazosin, on the other hand, had no apparent effect on ISO stimulation of NAT. It did produce a significant reduction in NA stimulation of NAT activity which was less acute than that found with propranolol. This confirmed that NA was active on NAT via both its β - and α_1 - receptors.

The next investigation examined the role of Ca²⁺ in the adrenergic receptor control of NAT, by using several experimental manipulations designed to vary the Ca²⁺ concentration.

EGTA was used as a selective chelating agent, while the calcium ionophore A23187 was used to mimic the Ca²⁺ influx that occurs with the α_1 -receptor stimulation following ISO stimulation.

Glands stimulated with ISO showed an increase in NAT. The addition of Ca²⁺ to the culture medium of glands already stimulated with ISO did not bring about any significant change in the level of NAT, but the removal of Ca²⁺ from the culture medium (using EGTA), totally abolished

NAT activity. Addition of Ca^{2+} to this medium restored NAT activity. A23187 was unable to stimulate NAT by itself, but when added to the vessel containing ISO [$1\mu\text{M}$], it was able to potentiate NAT activity to a level similar to that seen with [$10\mu\text{M}$] ISO, showing that an influx of Ca^{2+} coupled to β -receptor stimulation could produce a synergistic effect and the potentiation previously reported.

Results with NA were much the same, except that the A23187 produced an even greater potentiation than that seen in the equivalent ISO study.

The results in general confirmed that NAT activity was sensitive to manipulations of Ca^{2+} levels.

Continuing the original investigation, levels of NAT activity in the pineal glands stimulated with either ISO or NA in the presence of the CCBs were determined.

The following trend emerged: all glands stimulated with NA in the presence of any one of the CCBs showed an increase in NAT activity. Glands stimulated with ISO showed no significant differences in NAT activity in the presence of verapamil and nifedipine, but did show an inhibition of NAT activity in the presence of diltiazem.

7.3.2 Discussion

Looking at the metabolic pathway simplistically represented as:



the next logical point of investigation was cAMP. If cAMP levels remained high, correspondingly higher levels of NAT, leading to increased acetylation of HT and ultimately to higher concentrations of aMT, could be expected.

7.4 Chapter 4

The effect of the the three CCBs on rat pineal cAMP levels, was determined to ascertain if the latter were indeed raised, as suggested by the high levels of NAT found in Chapter 3.

The pineal glands were split into two halves to enable the determination of the effect of the CCB on pineal cAMP levels in the same gland, by using one half as a control (agonist in the presence of vehicle) while the other half was stimulated with agonist in the presence of CCB. Initial

studies showed that provided sufficient care was taken when splitting the glands, this approach was valid and the cAMP assay chosen was suitable.

7.4.1 Results

The finding for the cAMP groups are consonant with the previous NAT organ culture studies.

The presence of nifedipine and verapamil did not alter the ISO induced increase in cAMP. Diltiazem, however, inhibited the effect of ISO on cAMP, showing a statistically significant decrease in cAMP levels.

In those pineal glands stimulated with NA, all three CCBs were found to potentiate the effect of NA on cAMP formation.

7.4.2 Discussion

In Chapter 3 it was suggested that that the CCBs might in some way influence cAMP levels and interfere with the metabolic cascade:



Intracellular levels of cAMP reflect a balance between adenylyl cyclase and cAMP-PDE activities. Although α_2 - receptors are known to be present on the pineal, and have a negative effect on adenylyl cyclase, the nature of the data collected argued against an effect at that level. This left the role of cAMP-PDE (the counterpoise) in the control of cAMP levels open to question.

It is well known that adrenergic agonists will elevate cAMP, and that the same effect may be brought about by an inhibition of cAMP-PDE. A few recent reports had suggested that the organic calcium channel blockers might well be able to inhibit cAMP-PDE by binding to CaM, a protein itself requiring activation by Ca^{2+} before it can activate cAMP-PDE.

7.5 Chapter 5

Chapter 5 therefore made use of a TLC cAMP-PDE assay, coupled to the same drug paradigm, to determine whether cAMP-PDE levels were indeed being influenced.

7.5.1 Results

A decrease, although not highly significant ($p < 0.10$), was observed in those glands stimulated with NA in the presence of diltiazem. In contrast, those glands stimulated with ISO in the presence of diltiazem showed a significant increase in cAMP-PDE levels, consistent with the decreased cAMP level found above.

Glands stimulated with ISO were not significantly different from those stimulated with ISO in the presence of verapamil or nifedipine. However, those glands stimulated with NA in the presence of either verapamil or nifedipine also showed a decrease in cAMP-PDE, which could explain the raised cAMP levels observed in the previous chapter.

The results from the cAMP and cAMP-PDE studies on the CCB's are combined in Figure 7.2 and clearly show the complementary increases in cAMP, where decreases in cAMP-PDE activity occur. These results have been published (Brown *et al.*, 1989b).

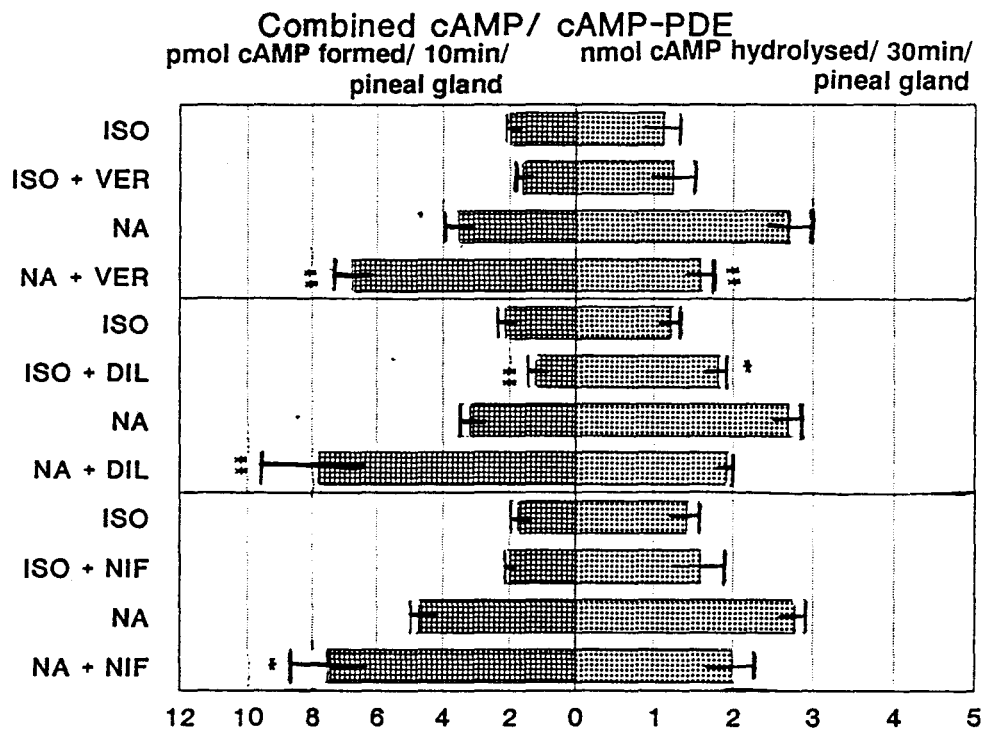
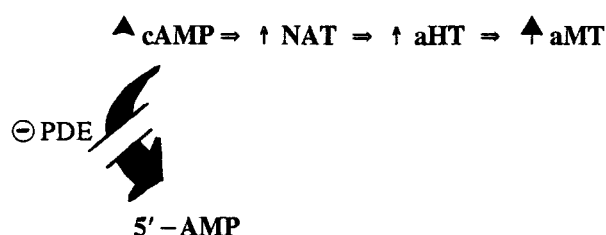


Figure 7.2: Levels of cAMP (left side) [▨] and cAMP-PDE (right side) [▧] activity determined in pineal glands stimulated with either ISO or NA in the presence of vehicle or the CCBs as indicated (mean ± SEM; n = 4 glands per group; $p <$: * 0.05; ** 0.025 w.r.t. respective vehicle control: Student's paired t -test for cAMP bars, and Mann-Whitney U test for cAMP-PDE bars

7.5.2

Discussion

The pattern emerging thus showed that the CCBs could be influencing cAMP via an inhibition of cAMP-PDE. The possible mechanism for this inhibition, and its role in the pineal gland metabolic cascade is shown in the diagrammatic representation in Figure 7.3. Simplistically the effects of cAMP-PDE inhibition are:



The findings of Chapters 2 through 5, show a distinct trend of PDE inhibition in the presence of the CCBs. The latter group of drugs are reported to inhibit the CaM-activated form of PDE. Thus although not by design, the data collected from this study had a rather important consequence: they demonstrate the possible presence of a CaM-sensitive PDE within the pineal gland, which corresponds to the *PDE I* form of PDE's, disputing the assumption that the low- K_m PDE is the dominant enzyme in the pineal.

7.6

Chapter 6

As the CCBs are not specific inhibitors of CaM, but inhibit it because of their structural (spatial) arrangement, it was decided to investigate this interesting concept further, by using CaM-inhibitors which were far more specific in their mode of action. It was hoped that the judicious use of more specific tools to manipulate the CCS would allow the results found thus far to be mimicked. If this could be done, it would add considerable support to those findings.

Experiments were aimed at two main objectives. Firstly experiments were performed at the organ culture level to see whether the earlier work involving Ca^{2+} manipulations remained valid at this level, and secondly to determine whether the results with the CCBs could be mimicked by the more specific CaM-inhibitor R 24571 and the CaM-sensitive PDE inhibitor, M&B 22948.

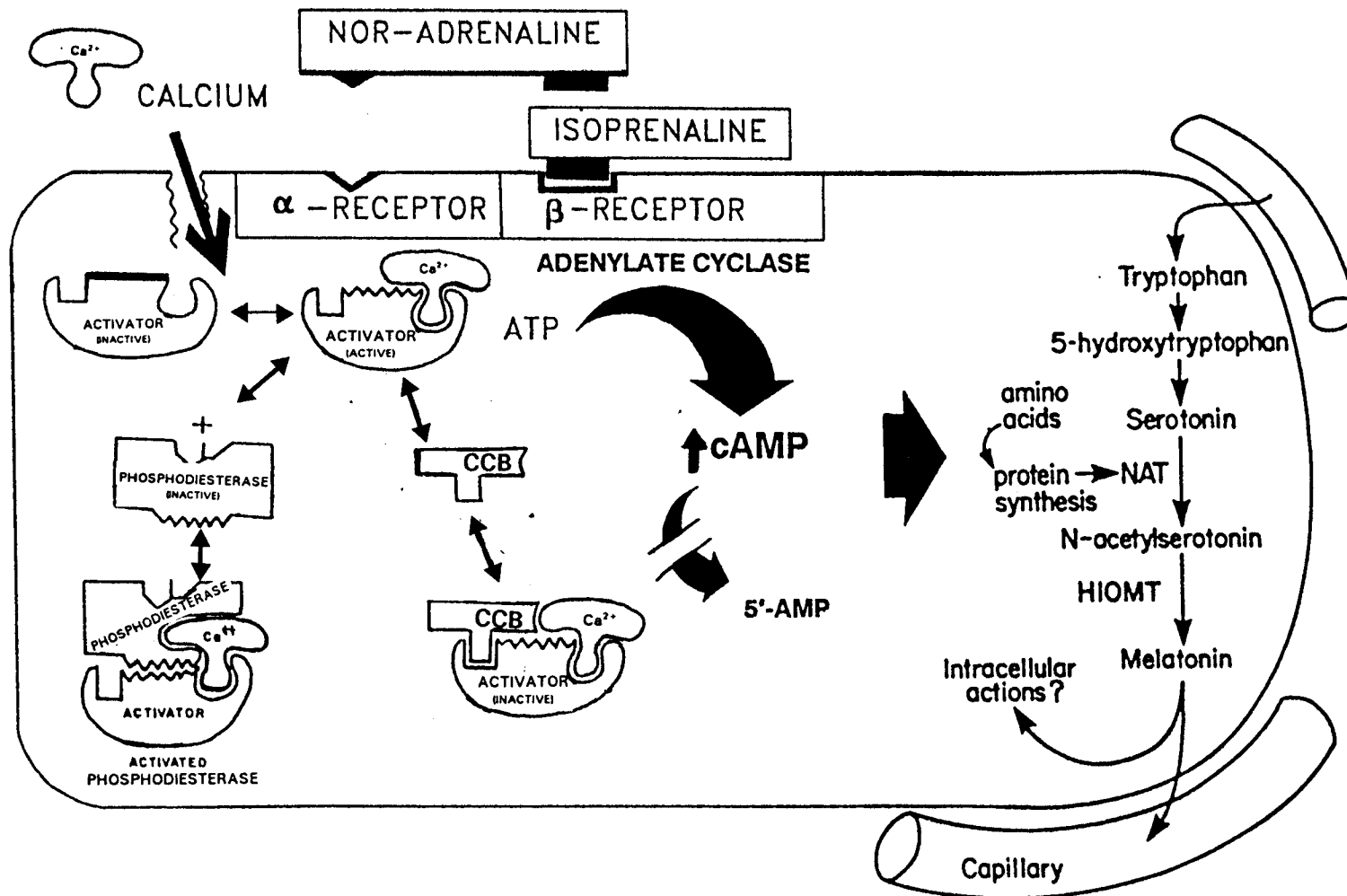


FIGURE 7.3:

A diagrammatic representation of a pinealocyte, showing the activation of CaM by Ca²⁺ and its subsequent activation of cAMP-PDE resulting in the active Ca²⁺/CaM/cAMP-PDE complex, or the blockade thereof by the CCBs, resulting in a nonactive CaM/cAMP-PDE/CCB complex, in relation to the indole metabolic pathway (adapted from Weiss and Levin, 1979 and Klein, 1981).

7.6.1 Results

Results obtained in both ISO - and NA stimulated glands in the presence of the various drug manipulations, closely resembled those of the earlier studies, showing that it was in fact possible to mimic those findings, i.e. following NA stimulation in the presence of R 24571, a peak in NaP production was evidenced similar to that seen in the presence of the CCBs following NA stimulation, while the peak did not occur following ISO stimulation.

They clearly highlighted the critical nature of Ca^{2+} levels in the control of the CCS, and supported the earlier hypothesis that a CaM-mediated effect (the inhibition of CaM-sensitive PDE) could be responsible for the results found.

The final investigation examined the role of calcium and the CCS more specifically at enzyme level, i.e. NAT and PDE activity were determined within the same gland, using experimental manipulations which varied not only the concentration of Ca^{2+} within the culture vessels, but also the CCS's activation of the CaM-sensitive PDE.

As expected, cAMP-PDE rose in unison with NA, in both ISO - and NA stimulated glands. EGTA abolished the stimulation of both NAT and cAMP-PDE, showing their Ca^{2+} dependence.

7.6.2 Discussion

R 24571 by itself produced no effect, either on NAT or cAMP-PDE. In the presence of NA, a potentiation with R 24571 was seen which was greater than that with either ISO[10 μ M] or NA[10 μ M] alone. Of even greater interest was the fact that cAMP-PDE had not risen in a parallel manner, as would have been expected following normal adrenergic activation. This suggests that some form of PDE inhibition was probably occurring, and adding support to the hypothesis that a CaM-dependent mechanism is involved. Inhibition thereof (by binding to the CaM) prevented the activation of cAMP-PDE, thereby allowing cAMP levels and therefore NAT levels to rise, leading to the potentiation seen following NA stimulation in the presence of the CCBs.

Supporting this, the CaM-sensitive PDE inhibitor (M&B 22948) brought about a rise in NAT activity. The PDE activity for this group, although greater than that observed with R 24571, remained considerably less than that found when a reference competitive PDE inhibitor (aminophylline) was used in pilot studies.

This finding implied that some mechanism other than pure competitive inhibition was active, and although purely speculative, it could be that the M&B 22948 was inhibiting the cGMP-PDE component of *PDE I*, and in doing so, making less cAMP-PDE available (partial inhibition). A full biochemical characterization would however be required before any firm conclusions could be drawn.

However, the trend is clearly seen, and parallels that seen in **Figure 7.2**, where increased cAMP levels accompany the decreased cAMP-PDE levels found in those glands stimulated with NA in the presence of verapamil and nifedipine.

7.6.3 Summary

The data thus collected tentatively suggested that a CaM-sensitive PDE would appear to be present in the pineal, and that when the pineal was stimulated *in vitro* with NA in the presence of verapamil, diltiazem and nifedipine, these drugs were possibly binding to - and inhibiting the function thereof. This conclusion was reached from the fact that the specific CaM-antagonist -R 22571-, and the CaM-sensitive PDE inhibitor -M&B 22948- were both able to mimic the results found with the CCBs.

CHAPTER 8

CONCLUSION

"A tenet of biochemical pharmacology is that the pharmacological effects of drugs are ultimately explainable by discrete biochemical actions. The caveat, of course, is that a particular biochemical action, no matter how well documented, may not necessarily be the cause of the pharmacological effect. In fact, rarely is the biochemical mechanism underlying the pharmacological effect of a drug known with certainty".

The results of the current study are a perfect example of this view expressed by Weiss *et al.*, (1982).

In the rat pineal gland, β -adrenoceptor stimulation increases cAMP by activating adenylyl cyclase. Cyclic-AMP regulates the production of the pineal hormone melatonin, from serotonin, via the enzyme NAT. α_1 -adrenoceptor activation, which alone is without effect, has been shown to enhance β -adrenoceptor stimulation of cAMP. This enhancement has been linked to an increase in the entry of Ca^{2+} into the pinealocyte. The organic calcium channel blockers have been used in many studies as probes for calcium-mediated processes. The influence of three CCBs viz verapamil, diltiazem and nifedipine, on this calcium-mediated event was investigated.

Instead of a decrease in melatonin production (which would be expected were the Ca^{2+} influx coupled to combined α_1/β -adrenergic stimulation to be inhibited by the CCBs) a paradoxical increase was found.

This study then focused on a mechanistic approach adopted to elucidate the possible mechanism responsible for the paradoxical increase in melatonin production which is seen following incubation of pineal glands when stimulated with the α_1/β -adrenergic agonist NA, in the presence of the three CCBs.

Interpretation of the data collected, leaves the following conclusions.

- 1) The concentrations used to bring about any inhibition, coupled with the *in vitro* techniques used in this study, make it hard to reconcile these results with any therapeutic relevance.
- 2) The widespread use of the organic calcium channel blockers as probes for calcium-mediated processes, is not sound practice, as they do not appear to be as specific as one would have liked. In fact, this lack of specificity complicated the present study by producing a paradoxical increase,

where a decrease was expected on pharmacological grounds.

3) The results presented herein support a possible secondary mechanism of action for the CCBs, verapamil, diltiazem and nifedipine. This involves Ca^{2+} -dependent interaction with CaM and consequent inhibition of Ca^{2+} -dependent enzymes e.g. cAMP-PDE. Such inhibitory effects would favour the present findings, which suggest i) the possibility that a CaM-sensitive PDE is present in the pineal gland and ii) that the inhibition of this enzyme was in fact responsible for the paradoxical increase observed.

4) Considering the possibility that the paradoxical increase observed is as a result of PDE inhibition, although this study can not give a clear answer as to which precise PDE it is, it does add tentative support to the findings of other authors as to the possible existence of an N_s -like GTP-binding regulatory protein which controls a specific CaM activated cGMP-PDE, similar to that involved in the retina, and which in turn has control over ion channel activation.

5) Further, this study raises the possibility of yet another functional *in vitro* difference between this heterogenous group of compounds exhibiting Ca^{2+} - entry-blockade activity, as typified by the difference which diltiazem exhibited (following ISO stimulation), relative to verapamil and nifedipine.

6) Finally, this study shows that the interpretation of experiments in biological systems can be extremely difficult when these three drugs are used, and therefore cautions that care should be exercised if they are to be used.

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