MODULATION OF PHOSPHODIESTERASES OF THE BRAIN

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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ABSTRACT

The rationale behind the work in this thesis has been to develop methods for the quantitation of adenosine 3',5'-cyclic monophosphate (cAMP) hydrolysis (catalysed by phosphodiesterases) in intact cells of the central nervous system. The rate of decay of cAMP after cessation of synthesis (by addition of antagonist) in previously stimulated tissue provides one way to measure cAMP elimination. However, in brain slices, the β-adrenoceptor (β-AR) stimulation of cAMP was too poor to allow thorough kinetic investigation. Although conditions were sought which would serve to enhance the stimulation, none were found that could be applicable to these studies.

Attention was then turned to using homogeneous model cell systems such as the SH-SY5Y neuroblastoma and 1321N1 astrocytoma cell lines. In the neuroblastoma cells, an unexpected difference in the efficacy of a non-selective PDE inhibitor, 1-methyl-3-isobutylxanthine (IBMX) was observed when compared to type IV PDE inhibitors. This was apparently due to another action of IBMX at a locus distinct from PDE, and whilst adenosine receptor antagonism was at least partially responsible for the disparity, the possibility that IBMX was acting at a third site could not be eliminated. Such a complication, as well as the poor cAMP radiolabelling, rendered these cells an unattractive system for measuring agonist-stimulated cAMP turnover.

In 1321N1 cells, the decay method was successfully applied to assess the relationship between cAMP accumulation and PDE activity in the intact cell. Using a range of β-adrenoceptor agonist concentrations, whole cell Michaelis-Menten-type kinetics for cAMP elimination were derived, and suggested that cAMP disposal processes (possibly dominated by Ca^{2+}/CaM-dependent PDE) were being saturated under conditions of β-AR activation. The deduced nature of the PDE population then has implications for the profile of agonist-induced cAMP accumulation. This novel technique for performing intact cell kinetics has a variety of applications.

Another technique to measure cAMP turnover utilising a dual isotopic pulse-radiolabelling regimen was also instigated, with studies focussing upon the 1321N1 cells (brain slices and SH-SY5Y cells were inadequate models). This method was compared to the decay approach under conditions of β-AR activation; in conjunction with the examination of receptor- and non-receptor-mediated increases in cAMP turnover, the pulse-labelling protocol was then deemed to be an accurate description of receptor-induced cAMP turnover, and provided another way of assessing PDE activity in the glial-type cells.
This thesis is lovingly dedicated to the memory of my late father to whom I owe so very much, and, of course, to Mertxe, with my ongoing gratitude.
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Chapter 1 Introduction

1.1 General Remarks The purpose of this introduction is to establish the context in which the work conducted is placed and will systematically review, in outline or in detail, the various elements that comprise, for the most part, the cyclic nucleotide signalling pathway, with particular emphasis upon the family of phosphodiesterases which terminate their action.

1.2 Transmembrane Signalling Overview

With the exception of the steroid and thyroid hormones, most intercellular messengers such as hormones and neurotransmitters are non-penetrative, and are recognised by target cells via some type of cell-surface receptor protein. In order to respond, therefore, the target cell requires that the extracellular signal be converted into an intracellular message, be it a change in membrane permeability, second messenger concentration, protein phosphorylation or any combination of these.

Hormone responsive adenylyl cyclase (AC) is well known to be the sole route for generation of cAMP, under the control of receptors via the stimulatory or inhibitory G proteins, $G_s$ or $G_i$ respectively [Krupinski 1991]. The function of cAMP, via activation of Protein Kinase A (cAMP-dependent protein kinase - PKA), cyclic AMP's primary function is to regulate the degree of phosphorylation of specific cellular proteins, and thereby, their activity [Nairn et al. 1985] (although cAMP is now recognised as a direct modulator of other eukaryotic proteins such as cation channels [Kaupp 1991]).

The longevity of the hormonal or neurotransmitter signal is quite variant, depending essentially upon the rate of dissociation of the receptor-ligand complex and the rapidity of the elimination of the receptor ligand from the interstitial fluid bathing the target cells. Upon removal of the extracellular stimulus, there must be a commensurate elimination of the intracellular message, irrespective of its form, in order to maintain the strict correlation
between the two. With regard to the cAMP pathway, this termination is executed by phosphodiesterases which metabolise the cyclic ribonucleotide to its corresponding 5'-monophosphate (reviewed in [Beavo 1988, Beavo & Reifsnyder 1990]). Since the catabolite is ineffective at coordinating with cAMP binding proteins, the signal no longer persists, mirroring the hormonal status. Much less attention has been paid to the importance of degradation as a potential modulator of cellular cAMP concentration, an oversight which is slowly being rectified and forms the basis of the work presented in this thesis.

Elements of the Cyclic AMP Signalling System

1.3 cAMP SYNTHESIS

1.3.1 Cell-Surface Receptors

Characterisation of ligand-binding sites in terms of density, affinity and specificity has been both the delight and burden of molecular pharmacologists for some years, ever since radiolabelled agonists and, more importantly, antagonists became available. However, whilst in many instances the correlation of such ligand binding to a functional response had been achieved, the precise spatial and physical nature of the binding protein itself, let alone the mechanism underlying its activation, was unknown. The major breakthrough came in 1986 with the sequencing of the cDNA of the β2-adrenergic receptor (AR) from hamster [Dixon et al. 1986]. Considering its centrality in the studies presented in this thesis, the β-AR will be briefly described.

The deduced amino acid sequence of the receptor was compared with other known primary structures with the most surprising result: it displayed considerable sequence homology with the rhodopsin, the visual light 'receptor', for whom, hydropathy plots and a variety of physicochemical measurements predicted the presence of seven transmembrane domains (TMs) [Applebury & Hargrave 1987]. By inference, a model of the β2-AR was proposed which
described seven membrane-spanning regions connected by intra- and extracellular loops (see figure 1). It is now apparent that what once held true for the β-adrenoceptor now seems also true for other G protein-linked receptors: analysis of the cDNA sequences of an ever increasing number of receptors leads us to conclude that the 7TM model is equally applicable to a whole superfamily of proteins (reviewed in [Dohlman et al. 1991]).

![Diagram of transmembrane receptor](image)

**Figure 1** Diagramatic representation of the essential topographical features of a typical transmembrane-spanning cell surface receptor, after [Lefkowitz 1991]

With a variety of complementary biophysical and genetic techniques (reviewed by [Tota et al. 1991]), the binding site for biogenic amines has emerged as being a deeply buried pocket within the helices of the TMs, wherein the ligand intercalates, and interacts with specific amino acid residues. The varied intracellular domains of receptors are of paramount importance with regard to their regulation of, and by, intracellular proteins. That the i3 loop is critical for G protein coupling is underscored by a wealth of evidence including those from chimaeric receptor constructs: Lefkowitz and his colleagues replaced the entire i3
of the $\alpha_2$-C10-AR with the corresponding region of the $\beta_2$-AR, creating a stable chimaera that could stimulate adenylyl cyclase whilst still exhibiting the pharmacology of an $\alpha_2$-AR [Kobilka et al. 1988].

Although 13 is intimately involved with the events downstream of receptor activation, it has been additionally implicated in the regulation of the receptor by other proteins viz providing possible substrate sites for protein kinases [Hausdorff et al. 1989][Benovic et al. 1988], and influencing receptor expression [Kubo et al. 1988]. Receptor phosphorylation by PKA (cAMP-dependent protein kinase) and $\beta$ARK ($\beta$-adrenergic receptor kinase) initiate events in the process of uncoupling the $\beta$-AR from $G_s$. Phosphorylation of the receptor by either kinase in vitro results in an impairment of its ability to interact with $G_s$ [Benovic et al. 1985, Benovic et al. 1987] and it has been proposed that this phosphorylated form of the receptor avidly binds to another protein, arrestin (or an analogous protein) preventing it from interacting with G proteins (reviewed in [Benovic et al. 1988]). From these and other studies, it has been suggested that PKA plays a major role in heterologous desensitisation, whilst $\beta$ARK and also receptor sequestration are more important in homologous desensitisation ([Benovic et al. 1988]).

1.3.2. GTP Binding Proteins (G proteins)

Many excellent reviews have been published recently which emphasise the different aspects of GTP binding proteins (amongst which are [Gilman 1987, Chabre & Deterre 1989, Birnbaumer et al. 1990, Houslay 1990, Taylor 1990, Kaziro et al. 1991, Simon et al. 1991]). Although diverse in their primary structures and tissue distribution, the mechanism of activation, and deactivation, is common to all.

The G proteins are a family of heterotrimeric proteins with subunits designated $\alpha, \beta$ and $\gamma$ (in order of decreasing size, in the ratio 1:1:1) wherein the $\alpha$ peptide binds and hydrolyses guanine nucleotides, and the $\beta\gamma$ subunits are closely
associated and function almost as one entity. Since the smaller subunits are very similar, and, as yet, functionally indistinguishable, the individual members of the family are identified by their more distinctive $\alpha$ peptides. Thus, $G_s$ is composed of $\alpha_s \beta \gamma$, and transducin ($G_t$) by $\alpha_t \beta \gamma$.

**Figure 2** Schematic Representation of the Cycle of $G$ protein Activation where $\alpha$ and $\beta \gamma$ refer to the subunits, $\text{Pi}$ to inorganic phosphate, and 'Rec' to the agonist-receptor complex.
**G Protein Activation**

Under resting conditions, the GTPase activity of the \( \alpha \) subunit dominates the guanine nucleotide exchange rate and therefore the majority of the G protein will be in the GDP-bound ('inactive') form [Gilman 1987]. Activated receptors increase the rate of GDP exchange with the resulting dissociation of the holomer into its \( \alpha \) and \( \beta\gamma \) subunits (figure 2). The dissociation occurs because the affinity of the \( \beta\gamma \) complex for \( \alpha\)-GTP is far less than that for \( \alpha\)-GDP, as has been demonstrated for transducin [Fung 1983] and the released \( \alpha \) subunit is then free to interact with its appropriate effector, which can be any of a plethora of enzymes or ion channels (see below).

The ternary complex of agonist-bound receptor and G protein (LR-G\(_{\alpha\beta\gamma}\)) [DeLean et al. 1980] is short-lived, surviving only milliseconds [May & Ross 1988] i.e. once GTP is bound, the purpose of the receptor is completed, the affinity of the LR for the G protein is reduced and they dissociate.

**G protein Diversity, Structure and Function**

**\( \alpha \) Subunit family** Each of the mammalian \( G_\alpha \)s lies within the range of 39-45KDa in size [Kaziro et al. 1991]. With now more than 30 G protein cDNAs cloned, they can be grouped into sequence-related subfamilies, apparently four in number, according to the analysis of [Simon et al. 1991]:

(a) **G\(_5\)-like family** coupled to activation of adenylyl cyclase and cardiac and skeletal muscle Ca\(^{2+}\) channels, and includes \( G_{\text{olf}} \) (\( G_5 \))

(b) **G\(_i\)-like family** linked to inhibition of adenylyl cyclase, stimulation of phospholipase C, activation of K\(^+\) and Ca\(^{2+}\) channels, inhibition of Ca\(^{2+}\) channels, stimulation of retinal cGMP phosphodiesterases. Encompasses the \( G_{5s} \), \( G_0 \), \( G_t \) and \( G_{2x}(G_x) \).

(c) **G\(_q\)-like family** coupled to phospholipase C\(_s\). Includes \( G_q \), \( G_{15} \), \( G_{16} \), \( G_{11} \) and \( G_{14} \).

(d) **G\(_{12}\) family** Function unknown. Only known members are \( G_{12} \) and \( G_{13} \).
Discussion of all G protein subtypes is beyond the scope of this overview, and therefore attention shall be centred upon those families that are directly concerned with the regulation of cyclic nucleotide metabolism i.e. G_s and G_i.

**G_sα** Although there is only one gene for the 'classical' α_s peptide, alternative splicing of the initial pre-messenger transcript results in four cDNAs being cloned from human brain, encoding two small and two large forms [Bray et al. 1986]. While it is now accepted that these two sizes of G_s have different tissue distributions [Mumby et al. 1986], what is unclear is whether or not there are any functional differences in their coupling to adenylyl cyclase (e.g. c.f. [Jones et al. 1990] and [Walseth et al. 1989]).

Recently, it was also proposed that β-AR-mediated regulation of calcium channels of the heart, and skeletal muscle was partly, but directly facilitated by G_s (reviewed in [Brown & Birnbaumer 1990]), which together with a report of β-AR-elicited modulation of cardiac tetrodotoxin-sensitive Na^+ channels through G_s [Schubert et al. 1989] implies that, in heart, G_s acts as an integrator and signal disseminator, and not merely as a transducer of incoming stimuli.

**G_iα** That the so-called inhibitory G protein, G_i, represents a family with more than one member (and at least three [Itoh et al. 1986, Jones & Reed 1987, Itoh et al. 1988]) has gained more universal recognition than its antagonistic, stimulatory equivalent. Adenylyl cyclase inhibition is, of course, its classical rôle, even though the exact molecular mechanism underlying the phenomenon remains open to conjecture (see 'Regulation of Adenylyl Cyclase'), but it has been clearly implicated in regulation of many cation channels as well, including those conducting K^+, Na^+ or Ca^{2+} [Brown and Birnbaumer 1990]. Moreover, any of the three types of α_i can mediate hormonal inhibition of
adenylyl cyclase [Bourne et al. 1990, Birnbaumer 1990a] or couple muscarinic M$_2$ receptors to K$^+$ channels in atria [Yatani et al. 1988].

**G protein-mediated Regulation of Adenylyl Cyclase**

**Stimulation**

Should the hydrophobic $\beta\gamma$ subunits be entirely responsible for membrane anchorage of the $G$ protein holomeric complex [Rodbell 1985, Sternweis 1986, Iyengar et al. 1988, Rasnas et al. 1989], then activation and dissociation could allow the more soluble $\alpha$ peptide to leave the membrane and enter the cytosol. Evidence would suggest that such a phenomenon may be artefactual [Tolkovsky & Levitzki 1978, Levitzki 1986] and given that the $\alpha_s$ subunit may be tightly associated with AC [Arad et al. 1984], Levitzki has championed the idea of a 'partial dissociation model' of AC activation where $\alpha_s$ is strongly attached to the catalytic subunit at all times, with $\beta\gamma$ shuttling to and from the $\alpha_s$.AC complex [Levitzki 1984, Levitzki 1987, Levitzki 1987a]. However, this is not necessarily universally accepted (see [Gilman 1987]).

**Inhibition**

Unlike $G_s$, $G_i$ does not form a complex with AC, and it has not been possible to demonstrate an interaction of the two in reconstitution experiments [Smigel 1986]. Functional analysis of resolved $\alpha_i$ and $\beta\gamma$ revealed that only modest inhibitory activity is associated with the activated $\alpha$ chain in platelet or wild-type S49 membranes; the major contribution to inhibition was from the $\beta\gamma$ species [Bockaert et al. 1985]. A direct effect of $\beta\gamma$ upon AC is unlikely, and its proposed mechanism of action therefore stems from its scavenging free $\alpha_s$, shifting the equilibrium between dissociated and holomeric $G_s$ in favour of the trimer, $G_\alpha\beta\gamma$ [Katada et al. 1984, Smigel 1986, Gilman 1987].

However, such a mechanism is insufficient to account for all AC inhibitory phenomena since in the S49 cys$^-$ mutant cells (that do not express $G_s$), a modest inhibition of AC was elicited under the control of somatostain and $\alpha_2$-
adrenergic receptors [Katada et al. 1984], and has subsequently been observed in other cells [Roof et al. 1985]. An \( \alpha_1 \) inhibition was demonstrated that was competitive with respect to \( \alpha_s \) [Katada et al. 1986]. Clarification of the precise nature of AC stimulation and inhibition will clearly require more work.

### 1.3.3 Adenylyl Cyclase

Synthesis of cAMP from its precursor ATP is catalysed by adenylyl cyclase (AC) which is under very stringent control, both from the external and the internal cellular faces, and the nature of which will be now outlined.

**Structure**

The information concerning AC structure has been somewhat sparse until its recent purification [Pfeuffer et al. 1985, Yeager et al. 1985, Pfeuffer et al. 1989] and cloning of various cDNAs [Krupinski et al. 1989, Bakalayer & Reed 1990, Reed et al. 1990] (reviewed in [Krupinski 1991]). Heterogeneity appears to prevail within the AC family with the apparent \( M_r \)s extending from 120-180 kDa, depending upon source [Mollner & Pfeuffer 1988, Pfeuffer et al. 1989], and a heterogeneous AC population may well be expressed within a particular tissue: in bovine brain, for instance, up to three forms may be present as a determined by immunoblotting [Mollner and Pfeuffer 1988]. From its primary sequence, brain type I AC is predicted to have a membrane-spanning topography which resembles channels and the multidrug resistance protein [Krupinski et al. 1989]. The possibility that AC was a multifunctional protein has never before been suspected, and thus the speculative transporter aspect of AC awaits substantiation.

**Regulation of Adenylyl Cyclases**

G protein modulation was primarily outlined above, but the scheme has been further complicated by a recent report [Tang & Gilman 1991]: upon expression of specific types of AC (including type I, II and IV found in brain), it was observed that \( \beta \gamma \) subunits had direct effects upon the cAMP production in
the presence of Gsα. Depending upon the isozyme, synthetic activity was stimulated, inhibited or unaffected by βγ, which suggests a possible mode of regulation that was never before appreciated.

Although its physiological relevance is less well understood, the regulation of AC from the intracellular face can be mediated by four mechanisms:

1) "P"-site-mediated inhibition
2) Ca2+/calmodulin activation
3) Ca2+ inhibition
4) Phosphorylation

**P-site-mediated Regulation** The site on the cytoplasmic face derives its name from a demonstrable affinity for purine-containing compounds [Londos & Wolff 1977]. Occupation of this site which is distinct from the catalytic centre [Johnson & Shoshani 1990] leads to a suppression of synthetic activity [Johnson 1982, Florio & Ross 1983]. Sequence data suggests the existence of two putative 'nucleotide binding-sites' on AC [Krupinski et al. 1989], one of which may prove to be the P site.

Like with Gi-mediated inhibition, the non-competitive P site-suppression [Johnson and Shoshani 1990] is best observed with stimulated AC, although it does not itself require the presence of G proteins [Florio and Ross 1983, Johnson et al. 1989]. Whilst adenosine analogues such as 2',5'-dideoxyadenosine are effective inhibitors [Johnson et al. 1989], the most potent, known natural ligands for the site are products of nucleic acid metabolism viz 3'-AMP and 2'd3'-AMP [Bushfield et al. 1990]. Interestingly, there is a variation in AC sensitivity to these molecules, depending upon the enzyme source, and brain AC is the form most sensitive to 3'-AMP (IC50~10μM) [Bushfield et al. 1990]. A possible link thus exists between fluctuations in nucleic acid metabolism and altered AC sensitivity.
**Calmodulin Activation** This is the longest established example of second messenger cross talk, where Ca\(^{2+}\)-mobilising agonists can indirectly lead to an AC activation whose magnitude varies depends upon the source [Cooper et al. 1988]. Indeed, the tissue distribution of CaM-sensitive AC has been shown, by immunological criteria, to be mainly limited to neural tissue [Rosenburg & Storm 1987, Mollner et al. 1991], which has lately been confirmed to be neuronal by mRNA hybridisation studies [Xia et al. 1991].

Brain has both a CaM-sensitive and insensitive isozyme, each of which can be stimulated by Mn\(^{2+}\), Gs\(_0\), and forskolin [Rosenburg and Storm 1987, Mollner and Pfeuffer 1988, Krupinski 1991], which implies that several agents can modulate the same locus by different means. However, in cerebellar membranes, CaM and a β-adrenoceptor agonist (or Gpp(NH)p) were non-additive in their activation of AC, suggesting that synergy need not necessarily occur between Gs-linked, and Ca\(^{2+}\)-mobilising agonists [Ahlijanian & Cooper 1987].

**Ca\(^{2+}\)-Inhibition** A growing number of reports are consonant with low (submicromolar) concentrations of Ca\(^{2+}\) \textit{per se} inhibiting membrane AC activity of pituitary-derived cells, platelets, and NCB-20 cells by 35-50% [Giannatasio et al. 1987, Narayanan et al. 1989, Boyajian & Cooper 1990, Boyajian et al. 1991]. In these studies it has been shown that it is not mediated by a pertussis toxin-sensitive G protein, phosphodiesterase or protein kinase C in membranes, and is not affected by CaM. Its precise mechanism requires further research, as does its physiological importance.

**Protein Phosphorylation** AC can be phosphorylated directly by both PKA and PKC, the former resulting in desensitisation of glucagon stimulation [Premont & Iyengar 1990], the latter in a sensitisation of AC [Yoshimasa et al. 1987]. Indirectly, phosphorylation of G\(_i\) by PKC has been suggested to impair G\(_i\)
The regulation of the rate of production of cAMP is obviously a critical step, and the elaborate mechanisms effecting this modulation underscore its importance. Although direct receptor coupling to AC offers a powerful means of regulating cAMP synthesis, it is by no means the only option adopted by the cell, as other motifs on AC are capable of interacting with factors besides $G_{\alpha}$.

Furthermore, cAMP levels need not be controlled solely from the point of synthesis, since regulation of processes which will reduce the amount of cAMP also have profound effects upon the degree of PKA (or ion channel) activation.

Elimination of cAMP may be spatial (via extrusion from the cell), or chemical, where the action of phosphodiesterases (PDEs) transforms the cyclic nucleotide into an 'inactive' species. The latter process, the only known catabolic pathway for cAMP, represents a novel target for both physiological and pharmacological manipulation that is independent of receptor-AC coupling. A more thorough understanding of PDE-mediated modulation of cAMP must be gained, not least for the more effective and proper use of PDE inhibitors in either an investigative or therapeutic context. With this in mind, the following section summarises the current view of PDE research.

1.4 cAMP ELIMINATION

1.4.1 CYCLIC NUCLEOTIDE PHOSPHODIESTERASES

The only known enzymatic route for the termination of cyclic nucleotide action is that effected by a heterogeneous group of phosphodiesterases (PDEs). The families are distinguished by their differential substrate specificity, kinetic characteristics and regulation by allosteric effectors inter alia [Beavo 1988, Beavo and Reifsnyder 1990], and are currently divided into 5 classes, within which different isoforms may be represented:
Type I: Ca\(^{2+}\)/calmodulin stimulated

II: cGMP-stimulated

III: cGMP-inhibitable

IV: cAMP-specific, low \(K_m\)

V: cGMP-specific, cGMP-binding

With this perspective, each type will be discussed in terms of its physical nature, catalysis and regulation, with a particular emphasis made on PDEs prevalent in cells of the nervous system.

**Calcium/Calmodulin-stimulated PDEs (Type I)**

Pioneering work by Kakiuchi and Yamazaki [Kakiuchi & Yamazaki 1970], and Cheung [Cheung 1967, Cheung 1970, Cheung 1971] showed that a cyclic nucleotide phosphodiesterase activity was stimulated by Ca\(^{2+}\) in a manner dependent on the small, heat-stable protein which we now call calmodulin (CaM). Its salient features will be outlined below.

**Distribution**

Brain provides the richest source of CaM-dependent PDE, (~10mg/kg in bovine tissue [Sharma et al. 1980]) where it is enriched in the cytoplasm of certain neural cell populations [Kincaid et al. 1987], and indeed, with few exceptions, most mammalian tissues express a form of CaM-PDE [Kakiuchi et al. 1975, Wells & Hardman 1977, La Porte et al. 1979, Appleman et al. 1982, Beavo et al. 1982]. The proportion of PDE that is CaM-stimulated varies from tissue to tissue [Beavo et al. 1982], for example, this form predominates in bovine brain, whereas less than half of the total cardiac PDE activity displays Ca\(^{2+}\)-dependency [Teo & Wang 1973].

Although thought of primarily as a soluble enzyme, more rigorous distribution studies have asserted that small amounts (10-20%) of brain, heart,
kidney and liver CaM-PDEs are associated with the particulate fraction [Kakiuchi et al. 1978, Grab et al. 1981]. Interestingly, CaM-PDE has been shown to be associated with synaptic densities [Kakiuchi et al. 1978] and even more intriguingly with the nuclear fraction in rat heart [Ahluwalia et al. 1984].

**Isoenzymic forms**

In bovine brain, probably three distinct forms of PDE I exist with differing subunit compositions. Existing as homodimers (~120-150kDa), each has a distinctive subunit (60-75kDa) [Sharma et al. 1980, Sharma et al. 1984, Schenolikar et al. 1985], and are capable of binding 2 mol CaM per dimer [Wang et al. 1980]. By comparison, only two subunits have been so far identified in rat brain, having similar M₆s of ~63kDa [Hansen & Beavo 1986], whilst amongst peripheral tissues, bovine heart tissue also contains two subunit forms, slightly smaller than the brain PDEs (57 and 59kDa) which also appear as dimers [Hansen & Beavo 1982, Beavo 1988]. PDE I heterogeneity is clearly apparent therefore.

**Sequence Information** In spite of their being immunologically cross reactive, the 59kDa heart and 60kDa brain have demonstrably distinct amino acid sequences, as does the brain 63kDa CaM-PDE [Charbonneau 1990]. To date, no molecular genetics regarding type I PDEs have been published.

**Kinetics**

The emerging trend that CaM-PDEs can be subdivided, kinetically speaking, into two basic groups is summarised as:

(a) those having a higher affinity for cGMP over cAMP e.g. bovine tissue PDEs (including brain), coronary artery, adrenal cortex, and one form from rat testis.

(b) those having a high affinity for both: restricted to another rat testicular isozyme [Rossi et al. 1988], and a PDE I from various sources of cardiac ventricle (e.g. Kₘ of ~1μM for both cAMP and cGMP in human tissue with similar Vₘₐₓ [Reeves et al. 1987] (and refs. therein)).
Brain PDEs are summarised in the table below (the Michaelis parameters shown are determined in the presence of Ca\(^{2+}\)/CaM).

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>(K_m) ((\mu)M) cAMP</th>
<th>(K_m) ((\mu)M) cGMP</th>
<th>(V_{max}) ratio (cAMP/cGMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Brain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60(^1)</td>
<td>32</td>
<td>2.7</td>
<td>1.8</td>
</tr>
<tr>
<td>63(^2)</td>
<td>11</td>
<td>1.2</td>
<td>0.3</td>
</tr>
<tr>
<td>75(^3)</td>
<td>2.9</td>
<td>2.7</td>
<td>0.07</td>
</tr>
<tr>
<td>Rat Brain(^4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (M_r)</td>
<td>ND</td>
<td>ND</td>
<td>1.2</td>
</tr>
<tr>
<td>High (M_r)</td>
<td>ND</td>
<td>ND</td>
<td>0.68</td>
</tr>
</tbody>
</table>

Table 1 Summary of kinetics of type I PDEs found in brain

Refs: 1) [Sharma & Wang 1986] 2) [Sharma & Wang 1986a] 3) [Schenolikar et al. 1985] 4) [Hansen and Beavo 1986]

The enzymes have different efficiencies for hydrolysing cyclic nucleotides in spite of their being within the same family, where the 63 and 75kDa forms have a greater capacity to remove cGMP (higher \(V_{max}\)). Comparing the above with heart and lung, it appears that the similar (~60kDa) species from each tissue possesses very similar kinetic properties [Sharma and Wang 1986], perhaps in keeping with some similarities in their primary structure.

**Modulation of activity**

1. Calmodulin Regulation

The activity of brain and heart CaM-PDEs is composed of a basal activity, and a higher, stimulated activity effected by allosteric regulation by calmodulin. The requirement for mM Mg\(^{2+}\) is preserved in both states, whilst the stimulation, by definition, needs elevated Ca\(^{2+}\) levels [Lin & Cheung 1980,
Sharma et al. 1988]. The degree of stimulation by CaM that has been observed is variable, but is, generally speaking, of the order of 5-15 fold, where CaM lowers the substrate's $K_m$, raises the $V_{max}$ or both [Lin and Cheung 1980]. From these latter properties it should be clear that the fold stimulation will be less at saturating concentrations of cyclic nucleotide.

**Mechanism of activation**

The small heat stable protein, CaM, binds Ca$^{2+}$ and then interacts through a combination of hydrophobic and ionic forces with amphipathic $\alpha$ helices on the PDE (and other CaM-dependent proteins) leading to its activation [O'Neill & DeGrado 1985, Sceholzer et al. 1986]. In the absence of Ca$^{2+}$, the affinity of CaM for PDE is too low for accurate measurement ($>10^{-4} \text{M}$), but upon binding of the cation, a $10^5$-$10^6$ fold increase in the affinity is evoked ($K_a$ 0.1-1nM) [Wang et al. 1980].

![Figure 3: Representation of the Activation Stoichiometries of PDE type I by Calmodulin and Calcium](image)
How can the actual binding of the Ca\(^{2+}\)/CaM complex be envisaged to affect enzyme activity? A similar scheme has been invoked to describe the regulation of protein kinases [Taylor 1989], and requires the assumption that the PDE is composed of various domains *viz* the catalytic, the inhibitory and the CaM-binding domains [Charbonneau 1990]. Thus, under basal conditions, the first two domains strongly interact to suppress the PDE activity, and it is only upon binding of the modulator that a change of conformation is produced which weakens or disrupts that interaction.

The mechanism of PDE activation may be similar in all isoforms, but the CaM sensitivity of various PDE isozymes, and target proteins in general, is not uniform. According to reports, the brain 60/63kDa enzymes both have a CaM $K_a$ of ~1nM which is 10-20 times higher than the corresponding heart enzymes [Mutus *et al.* 1985, Hansen and Beavo 1986] (and bovine lung has CaM as a subunit - hence, the highest affinity PDE [Sharma and Wang 1986]). At constant CaM concentrations therefore, the heart will be activated by lower Ca\(^{2+}\) levels than in the brain, although the higher CaM concentration in the brain may negate this difference [Hansen and Beavo 1986, Sharma *et al.* 1988].

### 2. Modulation by Phosphorylation

Not only are there differences in kinetics of type I PDE isoforms, but also in their non-CaM regulation. Both the 60kDa and the 63kDa isozymes are substrates for serine/threonine kinases, but interestingly, *different* kinases. That is, the 60kDa is a PKA substrate, the 63kDa is phosphorylated by calmodulin-dependent kinase II (CaM-K II) [Sharma and Wang 1986a, Hashimoto *et al.* 1989].

The functional correlate in each case is a reduced sensitivity to activation by Ca\(^{2+}/\)CaM, seen as a shift in the CaM concentration response curve, with the 60kDa form being more affected after maximal phosphorylation by PKA [Sharma & Wang 1985, Sharma & Wang 1986a] Notably, the phosphodiesterases' other properties, such as $K_m$ or $V_{max}$ are unaffected [Sharma & Wang 1985,
Sharma and Wang 1986a]. Under conditions of CaM saturation (μM range), this change will be manifested as a different sensitivity to Ca\(^{2+}\), where higher cation concentrations will be required to produce the same effect. Unfortunately, that these changes occur in vivo has not yet been demonstrated.

**In Vivo Regulation of Type I PDEs**

The kinetic data presented above seemed to suggest that this family of enzymes expedited the removal of cGMP more than cAMP. In bovine artery, this may well occur for application of the relatively selective PDE I inhibitor, 8-methoxymethyl-IBMX, elevated cGMP, but not cAMP (even in the presence of a β-AR agonist) [Lorenz & Wells 1983]. However, there is a wealth of evidence from other tissues and cell lines that indicates that this is not exclusively the case. Muscarinic cholinergic receptors (MACHRs), coupled to elevations in cellular Ca\(^{2+}\), can attenuate cAMP responses via activation of type I PDEs. This modulatory role has been reported in canine thyroid cells [Erneux et al. 1985], WI-38 fibroblast cells (after PGE\(_1\)) [Nemecek & Honeyman 1989], prostate gland [Shima et al. 1983], and 1321N1 astrocytoma cells [Tanner et al. 1986]. Indeed, other Ca\(^{2+}\)-mobilising receptors besides MACHRs can antagonise cAMP accumulation via this mechanism: histamine (via H\(_1\) receptors) was shown to activate PDE in the 1321N1 cells [Nakahata et al. 1986], and α\(_1\)-ARs has been suggested to induce similar changes in cAMP turnover in cardiomyocytes [Buxton & Brunton 1985], thyroid [Berman et al. 1987], and granulosa cells [Ranta et al. 1983] after AC activation.

**Pharmacological Inhibitors**

Compounds interfering with CaM-PDE activity can be subdivided into those that that inhibit PDE activity directly, and those antagonise the CaM activation.
Direct PDE Inhibitors

The non-selective PDE inhibitors such as 1-methyl-3-isobutylxanthine (IBMX), theophylline and papaverine will obviously reduce hydrolysis of cyclic nucleotides, but there are no highly selective inhibitors yet available. The most specific agents available include some substituted IBMX analogues like 8-methoxymethyl-IBMX [Wells & Miller 1988] or M&B 22948 [Ruckstuhl & Landry 1981] which show some discrimination (up to 30-50 fold), as do two inhibitors developed by Hidaka and colleagues, vinpocetine and HA 558 [Hidaka & Endo 1984a, Hidaka et al. 1984b].

Inhibitors of CaM Activation

Even this class can be further subdivided into those that:

a) inhibit calcium binding to CaM e.g. calcium chelators such as EGTA.

b) prevent CaM/PDE interaction by binding to CaM e.g. antagonists like trifluoperazine (TFP) [Levine & Weiss 1977], and the more CaM-selective, 5-iodo-C8-W7 [MacNeil et al. 1988], toxins such as mellitin and mastoparan [Comte et al. 1983, Malencik & Anderson 1983], βγ subunits of G proteins [Asano et al. 1986], and other bona fide CaM-binding proteins.

c) prevent CaM/PDE interaction by binding to PDE e.g. inactive CaM-derivatives with [Newton & Klee 1984], or without [Newton et al. 1984a] CaM-antagonists covalently bound.

Cyclic GMP-stimulated PDE (type II)

Within the scheme proposed by Beavo [Beavo and Reifsnyder 1990], this family of enzymes are directly regulated by cyclic nucleotides themselves, and as the name suggests, their activity is positively modulated.

Distribution

Cyclic GMP-stimulated PDEs (cGS-PDEs) are ubiquitous in their distribution throughout mammalian tissues and species, found as soluble
enzyme in bovine tissues (heart [Martins et al. 1982], adrenal [Martins et al. 1982, Miot et al. 1985]), or as a particulate isoform in rabbit or bovine cerebral cortex [Whalin et al. 1988, Murashima et al. 1990]. Indeed, in bovine brain, there is evidence for the existence of discrete forms of cGS-PDE, one being soluble, the other (more major) form, particulate [Murashima et al. 1990].

**Structure and Kinetics**

Like type I, cGS-PDEs exist primarily as homodimers, but whose subunits are much larger (~105kDa) [Martins et al. 1982, Yamamoto et al. 1983, Beavo 1988, Whalin et al. 1988]. An exception to this rule may be found in the rabbit brain where the PDE may be expressed as a homotetramer [Whalin et al. 1988], although it still hydrolyses cyclic nucleotides with similar characteristics.

Kinetically, all isoforms are almost identical, with the apparent $K_m$s for cAMP being $\sim 30\mu M$, and for cGMP, $\sim 15\mu M$. That there is a similarity of maximal velocity towards each substrate therefore suggests a marginal preference for cGMP [Martins et al. 1982, Yamamoto et al. 1983, Beavo 1988, Murashima et al. 1990]. By way of emphasis, the property that distinguishes this family from all the other PDEs is the cooperativity of substrate hydrolysis - type II PDEs catabolise cyclic nucleotides with positively cooperative kinetics [Martins et al. 1982, Yamamoto et al. 1983, Beavo 1988, Whalin et al. 1988]. That is, there is a sigmoid rather than a hyperbolic relationship between substrate concentration and reaction velocity, where Hill coefficients are $\sim 1.6$.

**Allosterism**

The observed deviation from Michaelis-Menten kinetics is by virtue of the enzyme possessing a so-called regulatory, allosteric site, wholly distinct from the catalytic site [Erneux et al. 1981, Couchie et al. 1983]. Although occupation by either cAMP or cGMP will result in an enhanced destruction of substrate [Moss et al. 1977a], cGMP has a higher affinity for binding and stimulation than does
cAMP: the $K_a$ for cGMP is 0.35-0.5μM [Martins et al. 1982, Yamamoto et al. 1983, Beavo 1988, Whalin et al. 1988], and in the presence of Mg$^{2+}$, can evoke up to a 30 fold increase in cAMP hydrolysis (a $K_m$ effect), at subsaturating substrate concentrations [Yamamoto et al. 1983]. By contrast, cAMP (20μM) maximally enhances cGMP hydrolysis 3-5 fold [Yamamoto et al. 1983]. Given the levels of cAMP and cGMP that exist in vivo [Gilman 1972, Goldberg & Haddox 1977], it is more likely that elevations of cGMP levels will have an effect upon cAMP hydrolysis than the reverse. Hence the nomenclature of this family.

Unlike the other PDE families, no selective competitive inhibitors are yet available for type II PDEs, which has presumably hampered attempts to implicate this isozyme in physiological regulation of cAMP levels.

**Sequence Data & Isoforms**

Beavo and colleagues directly obtained the amino acid sequence of the bovine heart enzyme and also a partial sequence of a cDNA clone from adrenal glands [Le Trong et al. 1990]. Comparison with other PDE sequences confirmed the presence of the proposed conserved region corresponding to the catalytic site [Charbonneau 1990], and a second, central region of homology with PDE V (see below) postulated to represent the cGMP allosteric site of cGS-PDEs. The full sequence of an adrenal cortical cGS-PDE has now been published and northern blots have highlighted corresponding single mRNAs present in extracts from specific brain regions (e.g. cerebral cortex, basal ganglia and hippocampus, but not cerebellum, and spinal cord) [Sonnenburg et al. 1991]. Moreover, RNase protection analysis in this study suggested that alternative splicing may selectively occur within tissues which may govern the membrane distribution of the PDE.
Physiological Regulation of Type II PDEs

No data has been published regarding the rôle of cGS-PDEs in intact tissue of the nervous system although its presence is without question; its function remains enigmatic. Some guide-lines for further research are hopefully provided by the example of other cellular systems, as will be outlined below.

1) Chronic Regulation

In a rat hepatoma cell line (HTC), the selective induction of different PDEs can be effected by chronic treatment: dexamethasone pretreatment (48-72h) apparently reduces cGS-PDE levels [Ross et al. 1977]. Similarly, exposure of the canine kidney sarcoma cell MDCKy to butyrate results in the elevation of both the soluble and particulate cGS-PDE activity [Manganiello et al. 1984].

2) Acute Regulation

Modern criteria have confirmed early evidence that type II PDE is highly localised in the zona glomerulosa of the adrenal gland [Martins et al. 1982]. Beavo envisages that cAMP-dependent processes (e.g. steroidogenesis) could be functionally antagonised by atrial natriuretic factor (which elevates cGMP, activates cGS-PDE and lowers cAMP levels) [Beavo 1988]. Somewhat by way of confirmation, PC12 pheochromocytoma cells are a model rat adrenal medullary cell line, and a system in which cAMP catabolism was enhanced by cGMP-raising agents [Whalin et al. 1991].

Work conducted in frog ventricle, where elevation of cAMP ultimately results in the PKA-mediated activation of an inward Ca^{2+} current, indicated that diminution of current resulted after introduction of cGMP (but not 8-bromo-cGMP, an activator of cGMP-dependent protein kinase (PKG)) into the system, and that this was antagonised by non-selective PDE inhibitors [Hartzell & Fischmeister 1986, Simmons & Hartzell 1988]. The conclusion was that cAMP levels were lowered by the activation of a PDE type II by cGMP.
Cyclic GMP-inhibited PDE (type III)

To date, no PDE III activity has been reported to be present in cells of the central nervous system, and therefore discussion of this family will not be dealt with in as great a depth as the more pertinent isozymes.

Distribution

Its distribution is quite varied, and the cyclic GMP-inhibitable PDE (cGIPDE) is notably found in adipocytes (particulate [Degerman et al. 1987]), liver (dense vesicle fraction [Boyes & Loten 1988]), platelets (soluble [Grant & Colman 1984]) and heart tissue (both soluble and particulate, depending on species [Weishaar et al. 1987a, Weishaar et al. 1989]).

Purification and Kinetics

Modern rapid isolation procedures (e.g. immunoaffinity columns) have overcome the severe proteolysis problems that plagued earlier studies, and the general agreement is that these enzymes have a subunit component of ~110kDa which is the holomer in rat adipose tissue [Degerman et al. 1987] or platelets [Grant et al. 1988], but associates to form a dimer in bovine heart [Harrison et al. 1986]. The variation in subunit stoichiometry in type II also prevails in this family.

Concerning their catalytic properties, most highly purified PDEs show linear Michaelis-Menten kinetics with respect to hydrolysis of substrates, cAMP or cGMP. Hydrolysing cAMP and cGMP with extraordinarily high affinity (K_m 0.1-0.8μM), and a ratio of V_max of 4-10 (cAMP/cGMP), these isoenzymes are readily distinguished by their potent competitive inhibition of cAMP hydrolysis by cGMP (in fact with the K_i=K_m for cGMP) [Beavo 1988]. Disappointingly, the physiological relevance of this interaction between nucleotides has not yet been firmly established.
Inhibitors

The observation that inhibition of PDE type III could increase positive inotropy in the heart [Weishaar et al. 1987a, Weishaar et al. 1987b] led to the development of inhibitors with an increased ability to discriminate between the two low K_m isozymic families (III and IV). Good selective competitive inhibitors for each class are available where milrinone, cilostamide (OPC-3689) and fenoximone target the type III PDEs [Beavo 1988]. By far the most selective agent is cilostamide, which has a ≥1000 fold selectivity for cGI-PDE over any other family (the next most susceptible being the type V, cGMP-specific).

Regulation of Enzyme Activity

1) Phosphorylation

A considerable amount of work has been conducted into this aspect, particularly in adipocytes, where cGI-PDE is frequently referred to as the 'hormone-sensitive PDE'. In brief, this PDE is subject to regulation by PKA in adipocytes, heart, liver [Gettys et al. 1987] and platelets [MacPhee et al. 1988], where activation of the kinase by cAMP analogues [Gettys et al. 1987], or via receptors, rapidly increases PDE activity [Smith & Manganiello 1989]. This represents a negative feedback mechanism which curtails the cAMP signal and has been further confirmed with phosphorylation studies both in vitro with purified components [Degerman 1988] (and perhaps [Gettys et al. 1988]) and also in vivo [Degerman et al. 1990].

As well as activation subsequent to β-AR occupation, cells exposed to insulin also show changes in cGI-PDE activity, although somewhat slower than with β-AR agonists. Able to stimulate PDE activity in its own right (c.f. reports of 50 and 300% [Kono et al. 1977, Smith and Manganiello 1989]), insulin also shows a marked, but transient synergy with β-ARs in increasing cAMP hydrolysis i.e. simultaneous activation of both receptors results in a ~3 fold increment in PDE
III activity [Smith and Manganiello 1989]. There is good evidence to suggest that phosphorylation of a single serine residue of cGI-PDE occurs when insulin receptors are stimulated [Degerman et al. 1990], but by which kinase and how it is regulated by insulin is unknown.

2) Chronic Regulation of PDE

Expression of type III in adipocytes is sensitive to both thyroid status [Elks & Manganiello 1985], and to glucocorticoid status, where treatment with the latter can decrease the amount of basal and hormone-stimulated PDE activity [Lai et al. 1982, Elks et al. 1983].

3) Regulation by other means

The microsomal hepatic PDE III of liver is, like the adipocyte isoform, subject to modulation by insulin, but via mechanisms in addition to phosphorylation. Reports encompass critical thiol group stabilisation by oxidation [Kono et al. 1975, Loten & Redshaw-Loten 1986], mobilisation of phospholipids [Macaulay et al. 1983], and lipid-derived mediators [Saltiel et al. 1986]. More recently, oxidation and formation of disulphide bonds was shown to perhaps result in dimerisation of PDE monomers and a consequent increase in PDE activity [Thompson et al. 1991] which reemphasises the importance of the cellular redox state for PDE activity.

Ro 20-1724-sensitive, low Km cAMP-specific PDE (type IV)

Whereas the CaM-sensitive PDE has a relatively high Km for cAMP, this family of enzymes have Kms in the micromolar or submicromolar range, and therefore efficiently dispose of substrate at lower degrees of receptor occupancy, a situation that may be more physiologically relevant.
Distribution

Brain was the first tissue in which a separable high affinity component for cAMP hydrolysis was discovered (reviewed [Strada et al. 1984]). Indeed, brain holds quite an unusual position within the tissue spectrum insofar that its distribution of total PDEs between soluble and particulate fractions is equal, whereas other tissues typically express only 3-10% particulate PDE [Strada et al. 1984]. Type IV PDE apparently exists in both the soluble [Strada et al. 1984] and the particulate fraction [De Mazancourt & Giudicelli 1988] of rat brain.

Isolation, Purification & Kinetics

Although PDE IV purification was previously thwarted by extensive proteolysis, more recently, highly resolved PDE IVs (not necessarily to homogeneity) have been reported from dog kidney, and the liver of rat. These data are summarised in table 2:

<table>
<thead>
<tr>
<th>Source</th>
<th>$K_m$ (cAMP) (µM)</th>
<th>$K_m$ (cGMP) (µM)</th>
<th>$V_{max}$ (µmol/min/mg protein)</th>
<th>Native Mr</th>
<th>SDS Mr</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog Kidney</td>
<td>2.2</td>
<td>312</td>
<td>10</td>
<td>71-74</td>
<td>82</td>
<td>[Thompson et al. 1988]</td>
</tr>
<tr>
<td>Rat Liver</td>
<td>0.7</td>
<td>120</td>
<td>0.93</td>
<td>52</td>
<td>52</td>
<td>[Marchmont et al. 1981]</td>
</tr>
<tr>
<td>Rat Brain</td>
<td>1.0</td>
<td>ND</td>
<td>ND</td>
<td>53</td>
<td>60.6</td>
<td>[Davis 1984]</td>
</tr>
</tbody>
</table>

Table 2 Resolution of several type IV PDEs PM = plasma membrane enzyme; $K_m$ quoted in µM; $V_{max}$ in µmol/min/mg protein; Mr in kDa

In table 2, it can be seen that these purer preparations are PDEs with kinetics such that their specificity and affinity for cAMP is generally high (~1µM),
but their maximum capacity is quite low. Moreover, their subunit $M_r$ are of similar values, where some PDEs, like those of liver or kidney are monomers [Marchmont et al. 1981, Thompson et al. 1988], whilst others, from Sertoli cell or lymphocytes, may exist as dimers [Thompson et al. 1976, Epstein & Hachisu 1984].

**Cloning of Mammalian type IV PDEs**

The cloning of the *Drosophila* 'dunce' gene (a low $K_m$ cAMP-PDE) [Chen et al. 1986, Chen et al. 1987] has been invaluable in stimulating the elucidation of the primary sequences of this little-appreciated family of proteins. Using low stringency cross hybridisation procedures, successful screening of rat brain and rat testis with 'dunce' cDNA has provided us with the first insight into the relationship of type IV PDEs, both to the PDE superfamily and to each other.

Four PDE transcripts were isolated from rat testis, with the evidence suggesting they were products of distinct genes (PDE1-4) [Swinnen et al. 1989, Swinnen et al. 1989a]. Suffice to say that the complete clones from testis have very similar predicted molecular masses (~65kDa), and splicing of the initial pre-mRNAs could give rise to yet more forms. Indeed, the PDE 3 transcript already illustrates this point, encoding two possibly PDEs [Swinnen et al. 1989a].

Returning again to the nervous system, generally more than one rat brain transcript cross-hybridised with the testicular gene probes [Swinnen et al. 1989] viz PDE 1 (two weak signals), PDE 2 (4.5 & 4.0 kb), PDE 4 (4.6, 4.2 & 3.9 kb), raising the possibility of a whole host of brain type IV PDEs, should these prove to be fully functional, and should the probes faithfully associate with distinct transcripts. Simultaneously, other workers screened brain libraries directly and found either three alternative transcripts from one PDE IV gene [Davis et al. 1989], or only one transcript, apparently from a different gene to Davis [Colicelli et al. 1989]. Clearly, at least two genes are expressed in the central nervous system, which may generate even more PDE isoforms (at least 4) through alternative splicing. More definitive evidence that the brain RNA transcripts encode...
functional PDEs is contained within a report which shows that the expression of cDNAs derived from the expressed brain genes in yeast mutants devoid of endogenous PDE, results in a marked increase in soluble PDE IV [Henkel-Tigges & Davis 1990].

From the above discussion, it is clear that there is overlap within these studies. A clarification of the findings was recently drawn together by [Beavo and Reifsnyder 1990], and his scheme is elaborated upon in table 3. The existence of possibly 4 distinct genes has governed its construction, where A,B,C and D refer to such genes, and the adjoined number, a possible splice variant.

**Regulation of PDE IV Activity**

1) **Phosphorylation**

Again, no demonstration of this modification has been reported in the CNS, and will therefore only be outlined as an instructive model.

Stimulation of liver phosphodiesterase activity by insulin has been recognised for about twenty years (reviewed [Smoake & Solomon 1989]), and is thought to account for part of the cAMP lowering effect of the hormone. In the intact cell, an antibody to this PDE IV was used to show that insulin does elicit a tyrosyl phosphorylation and activation of the enzyme [Pyne et al. 1989], and a more elaborate technique, radiation inactivation, suggests that the 54kDa PDE IV (and not PDE II) associates with a 90kDa protein [Wallace et al. 1990], perhaps the β subunit of the insulin receptor. However, the work of Houslay's group has been called into question by others who have been unable to reproduce some of their observations [Makino & Kono 1981, Benelli et al. 1986].

2) **Non-phosphorylating mechanisms**

Another, less widely accepted mechanism for the modulation of cAMP-PDE activity in liver and brain is that implicating G proteins. In membranes, GTP, and its non-hydrolysable analogues, stimulate PDE activity [Heyworth et al. 1983, De Mazancourt & Giudicelli 1984], and moreover, were synergistic with the activation evoked by insulin in liver [Heyworth et al. 1985] or
the adenosine analogue N\(^6\)-phenylisopropyladenosine (PIA) in brain [De Mazancourt and Giudicelli 1984]. A putative G protein ('G\(_{\text{ins}}\)') was proposed to alter PDE activity in liver [Houslay 1986, Smoake and Solomon 1989], but the data supporting G protein regulation are, however, not convincing and require elaboration.

<table>
<thead>
<tr>
<th>ISOZYME</th>
<th>(M_r) (kDa)</th>
<th>TISSUE DISTRIBUTION</th>
<th>PREVIOUS NAME</th>
<th>Confirmation of PDE activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>68</td>
<td>Brain, testis, cerebellum, lung, &amp; heart</td>
<td>RD1/PDE2</td>
<td>+</td>
</tr>
<tr>
<td>A2</td>
<td>?</td>
<td></td>
<td>RD2</td>
<td>ND</td>
</tr>
<tr>
<td>A3</td>
<td>?</td>
<td></td>
<td>RD3</td>
<td>ND</td>
</tr>
<tr>
<td>B1</td>
<td>62</td>
<td>Not examined. Brain, testis, liver, heart, kidney</td>
<td>DPD</td>
<td>+</td>
</tr>
<tr>
<td>B2</td>
<td>64</td>
<td></td>
<td>PDE4</td>
<td>+</td>
</tr>
<tr>
<td>C1</td>
<td>&gt;47</td>
<td>Brain?, testis, kidney</td>
<td>PDE1</td>
<td>+</td>
</tr>
<tr>
<td>D1</td>
<td>57</td>
<td>Testis, ND</td>
<td>PDE3.1</td>
<td>+</td>
</tr>
<tr>
<td>D2</td>
<td>67</td>
<td></td>
<td>PDE3.2</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3 Summary of familial relationship of putative PDE IV coding sequences. References and explanations are contained within the text and * in [Beavo and Reifsnyder 1990]. ND = not determined.
3) Regulation of PDE expression

The induction of PDE activity after chronic PKA activation (>3 hours) was shown some years ago in rat C6 glioma cells [Uzunov et al. 1973, Schwartz & Passonneau 1974] and also in Sertoli cells of the rat [Conti et al. 1981, Verhoeven et al. 1981]. With the advent of the cloning of the PDE, it was only a matter of routine to confirm that this regulation was due to an increase in the mRNA for PDE IV in these cells (mostly rat PDE 3 ("PDE IVd") transcript) [Swinnen et al. 1989a]; from the preliminary data of Swinnen et al., and [Schwartz & Onali 1984], it is thought to be due to enhanced transcription, rather than a stabilisation of mRNA.

4) Regulation in the Cell Cycle

The entry to and exit from the cell cycle may be regulated by cAMP [Rozengurtz 1986]. Indeed, mitosis and PDE activity are linked in fibroblasts, where replication initiated by serum is accompanied by a 3-10 fold increase in cAMP degradation by a low $K_m$ PDE [Pledger et al. 1979]. Cell meiotic capability is also subject to regulation, and insulin and IGF-1 induce activation of PDE (thought to be a low $K_m$ cAMP PDE) in oocytes, which appears to be intriguing, given that the activated protooncogene $c$-ras has also been shown to activate the oocyte PDE [Sadler & Maller 1989].

5) Regulation of PDE IV in Memory

Selective inhibitors of the type IV PDE subfamily exhibit the potential to be stimulators of the nervous system, as has been shown for rolipram, Ro 20-1724 and the alkylxanthine, denbufylline [Wachtel 1983, Nicholson et al. 1988]. The combination of the inhibitors' abilities to enhance memory or elevate mood [Nicholson et al. 1988], and the diminished learning behaviour of *Drosophila* individuals encoding the dnc− genotype [Chen et al. 1986] emphasises the
intimate relationship between PDE IV isozymes and nerve function. Moreover, PDEs of the microvasculature have the potential to play a part in controlling blood flow to brain regions since denbufylline has been shown to increase the oxygen tension in skeletal muscle [Angersbach & Ochlich 1984], and to improve blood flow properties [Jukna & Nicholson 1987], but with the current paucity of PDE distribution data, mechanisms for their actions are matters of speculation.

**Cyclic GMP Specific PDEs (Type V)**

Like type III PDEs, the cGMP-specific isozymic family has not been demonstrated in brain, but, for the sake of completeness, will be briefly discussed. As the majority of characterisation has been conducted with purified lung and rod outer segment (ROS) PDEs, they will be the models discussed in more detail.

**Lung PDE type V**

Purification of PDE V has been effected from rat and bovine lung [Francis & Corbin 1988, Thomas et al. 1988], and are notable for their avid ability to bind cGMP at a site distinct from their catalytic centre (and therefore termed the GMP-binding PDE, 'cG-BPDE', in which regard they resemble the type II cGS-PDEs). Their characteristics are summarised in table 4:

<table>
<thead>
<tr>
<th></th>
<th>Rat Lunga</th>
<th>Bovine Lungb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native Mr (kDa)</td>
<td>177</td>
<td>178</td>
</tr>
<tr>
<td>Monomeric Mass (kDa)</td>
<td>93</td>
<td>93</td>
</tr>
<tr>
<td>K_m for cGMP (μM)</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 4 Summary of physical properties of lung PDEs Refs: a [Francis et al. 1980, Francis and Corbin 1988] b [Thomas et al. 1990]
Several relatively selective PDE V inhibitors are currently available such as zaprinast (M&B 22948), dipyridamole and MY-5445 that show at least a 10-fold selectivity for type V over other PDEs [Beavo 1988], but because of such a poor selectivity, the investigator would be wise to derive a rank order of potency of PDE inhibitors to attribute effects to PDE V.

**cGMP-binding to lung PDE V**

A body of evidence suggests that there are sites accommodating cGMP-binding to the PDE (minimum of one per monomer) that are not the catalytic centre [Francis et al. 1980, Francis 1985, Francis and Corbin 1988, Francis et al. 1990], and which do not require metal ions either for binding of cGMP or the subsequent induction of the change in conformation [Francis et al. 1990, Thomas et al. 1990]. However, a definitive functional correlate has not been ascribed to this phenomenon. Some workers have suggested that there is a change in the hydrolytic site upon cGMP binding [Hamet & Coquil 1978], but the conclusive proof is still lacking. As yet, the only consequence that has been noted is an elevated susceptibility to phosphorylation, as will now be elaborated upon.

**Enzyme Phosphorylation**

In the presence of cGMP (but not in its absence), purified cG-BPDE from bovine lung is phosphorylated by PKA, and, at a ten times faster rate, byPKG which occurs on an identical, or an adjacent serine residue [Thomas et al. 1990a]. Thomas et al. have suggested that, physiologically, cGMP-dependent phosphorylation (via activation of the kinase and the substrate modification) would be the more likely to occur, but regardless, no alteration of any of the properties of the PDE was demonstrated, and the function of the modification remains obscure.
Photoreceptor PDEs

It is now well established that PDEs play a pivotal rôle in the regulation of the transduction of the light stimulus impinging upon the retina into changes in rod membrane potential (reviewed [Stryer 1986, Liebman et al. 1987, Stryer 1991]). Essentially, the model describes how cation channels in dark-adapted rods (carrying Na\(^+\) and Ca\(^{2+}\)) are kept in the open configuration by cGMP binding to it, or a closely associated element. Upon the photoisomerisation of rhodopsin, the heterotrimeric G protein, transducin (G\(_t\)) is activated, and G\(_{t\alpha}\) activates the cGMP-specific PDE. By lowering cGMP, the cation channel is closed, which has two effects: the first is cell hyperpolarisation, the second is a dramatic lowering of intracellular Ca\(^{2+}\) levels. This reduction in Ca\(^{2+}\) is detected by a small calcium binding protein termed recoverin, which, under these low Ca\(^{2+}\) conditions, then binds to and activates guanylate cyclase (GC) [Dizhoor et al. 1991]. The restoration of cGMP levels by GC inhibits the cation channel and the dark state is restored. The function, and structure of the rod outer segment (ROS) PDE of this cascade will thus be briefly described.

Structure of PDEs of the Retina

Organisation The membrane PDE is comprised of three discrete subunits (\(\alpha, \beta\) and \(\gamma\) of Mr 88, 84 & 11kDa respectively) [Baehr et al. 1979, Hurley & Stryer 1982], the largest (\(\alpha\)) can be carboxymethylated [Swanson & Applebury 1983]. The stoichiometric arrangement of these subunits is unclear since an \(\alpha\beta\gamma_2\) has been suggested [Deterre et al. 1988] as have \(\alpha_2\gamma_2\) and \(\beta_2\gamma_2\) [Hurwitz et al. 1985].

Sequencing of bovine rod cDNAs for \(\alpha\) and \(\gamma\) (and 70% of the \(\beta\)) has also been successfully performed [Ovchinnikov et al. 1986, Ovchinnikov et al. 1987].
Activation and Kinetics

Activation

Light activation of the rod membrane PDE can elevate the hydrolytic velocity for cGMP by over 200 fold [Hodgkin & Nunn 1988], which is effected in the following manner. GTP-bound-Gt stimulates PDE in a 1:1 ratio [Liebman et al. 1987], and is a fairly weak effector (K_{act} = 1 \mu M [Gillespie & Beavo 1988]), which may not matter given that the local concentration of Gt{alpha} can reach as high as 300 \mu M [Liebman et al. 1987]. Transducin (Gt{alpha}-GTP) binds to the PDE via the \gamma subunits. The small \gamma subunits inhibit the PDE activity in the holomeric complex, and the current view is that two Gt{alpha} sequentially strip \gamma subunits from the PDE, thereby alleviating the inhibition [Deterre et al. 1986, Deterre et al. 1988, Wensel & Stryer 1990, Yamazaki et al. 1990].

Kinetics

The estimated K_{m} for cGMP hydrolysis when activated by light is very high indeed (~1mM), especially considering that the local cGMP levels in ROS are of the order of 6-60\mu M [Cote et al. 1984, Cobbs & Pugh 1985], and that PDE concentration is ~30\mu M [Baehr et al. 1979]. Under these conditions the enzyme will perform well below its maximal velocity, but it must be emphasised, however, that the V_{max} is very substantial indeed, and approaches the theoretical maximum for a diffusion-limited process [Stryer 1986].

Like the lung and platelet type V isozymes, the photoreceptor PDEs also tightly associate with cGMP at two non-catalytic sites, with apparent affinities of 0.2 and 0.8\mu M in frog [Yamazaki et al. 1980, Yamazaki et al. 1982]. Once again, the function of this site is completely enigmatic, but does not apparently affect cAMP hydrolysis or transducin activation of PDE [Booth et al. 1986].
1.5 Experimental Strategy

Clearly, there has been a tremendous expansion of knowledge recently regarding the physical nature of this diverse family of proteins, but with less in the way of extrapolating such in vitro observations into the intact cell. Whilst theoretical models of the consequences of PDE activity in the intact cell have been forwarded [Erneux et al. 1980, Fell 1980, Barber et al. 1987], little has been done to experimentally confirm these hypotheses. An obvious problem is the difficulty in measuring cAMP hydrolysis in an intact cell since assessing accumulation, the net result of the synthetic and degradative processes, is inadequate.

Currently, there are three methods for quantifying cAMP turnover in intact cells: the first examines the decay in agonist-stimulated cAMP after removal of the ligand [Su et al. 1976], the second involves a radiolabelled adenine pulse-labelling protocol [Barber & Butcher 1988], and the third exploits the unique ability of PDEs to incorporate $^{18}$O from $[^{18}$O]$H_2O$ into the α-phosphoryl of nucleotides [Goldberg et al. 1983]. The third technique is a highly involved process requiring mass spectrometry and is generally untenable in the majority of laboratories examining second messenger dynamics. However, the first two regimens are appealing for their experimental simplicity, and as such, were adopted as the methods of choice.

In brain slices, work examining PDE activity in a more quantitative fashion has been restricted to a few reports regarding the effects of PDE inhibitors upon cAMP hydrolysis [Donaldson et al. 1988, Challiss & Nicholson 1990]. No attempts had previously been made to examine the nature of the PDE complement in neural cells by kinetic, rather than inhibitor studies i.e. to analyse the kinetics of hydrolysis of cAMP (in the intact cell) in order to appreciate which forms of PDE were functionally significant under conditions of agonist stimulation. However, a number of problems had to be overcome before these were possible.
By using the cAMP decay method, the first constraint is that of good agonist/antagonist pairings, which restricts studies primarily to the G\textsubscript{s}-linked β-adrenoceptor (β-AR) system. Secondly, and most importantly, the dynamic range of β-AR stimulation in brain slices is poor (maximum three fold stimulation), and the first ‘Results’ chapter of the thesis therefore concentrates on methods for enhancing this ‘window’ by examining α-adrenoceptor potentiation of the β-AR response.

In an attempt to circumvent the necessity for agonist/antagonist pairings, another method was sought to measure cAMP turnover via the pulse-labelling technique which may then have allowed the use of agonists other than β-AR agonists which give a better maximal response. It eventually became apparent that problems of cell heterogeneity would confound such studies in brain slices (chapter 5), and so simpler CNS model systems were investigated for their potential. Examination of the PDE characteristics of both SH-SY5Y neuroblastoma cells (chapter 4) and 1321N1 astrocytoma cells (chapter 5) was investigated either by studies using PDE inhibitors, or by the two kinetic techniques previously attempted in brain slices.
Chapter 2 General Methods

All general methods are detailed in this chapter and therefore will only be referred to within the "Results" chapters. Moreover, an Appendix is included which discusses the theoretical models used to analyse the kinetic data.

2.1 The Measurement of $[^3\text{H}]$cAMP Accumulation in Slices of Rat Brain

The method employed is essentially that described by Shimizu et al. (1969) and exploits the ability of the tissue to take up and utilise exogenous $[^3\text{H}]$adenine for the synthesis of cellular adenine nucleotides. Thereby, a prelabelling of ATP, the substrate for adenylate cyclase, is achieved. Clearly, any cyclic AMP subsequently produced will itself be labelled with the radioisotope and at a specific activity proportional to that of its precursor. One need only devise a method for adequately resolving the cyclic ribonucleotide from other labelled species to quantitatively assess a change in radioactive cAMP in situ under a variety of experimental conditions. Although alterations in absolute levels of the cyclic nucleotide cannot be determined, it has been shown in a number of situations (Kebabian et al. 1972) that this method compares favourably with those measuring cAMP mass.

Sensitivity of such an assay relies in part upon the degree of success with which such a separation of labelled nucleotides is achieved: ATP, rapidly turning over and constituting ~80% of the cellular adenine nucleotide pool will concentrate the majority of the $[^3\text{H}]$adenine taken up by the cell (c.f. cAMP contributes only ~1% to the nucleotide pool). Therefore, even the smallest degree of contamination of the $[^3\text{H}]$cAMP fraction by the $[^3\text{H}]$ATP will result in artefactually higher levels of the former being recorded, and thus contribute to a higher background noise in the assay. A sufficient separation of substrate and product is achieved by the sequential Dowex/alumina chromatographic technique of Saloman et al. (1974). With its speed and convenience, it was adopted as the method of choice.
2.1.1 Preparation of Brain Slices

Male Wistar rats (250 - 300g) were killed by stunning and cervical dislocation, decapitated, and the brain rapidly removed. Dissection of the appropriate brain region was performed on an ice-cooled petri dish with care being taken to excise as much of the contaminating white matter as possible. A McIlwain tissue chopper was used to prepare slices (350 x 350μm) which were then washed four times in modified Krebs Ringer buffer (KRB) (composition: see 'Appendix' at end of this chapter) in two 25ml universal tubes and placed in a shaking water bath at 37°C. Following a 60 minute pre-incubation, with buffer renewal every 15 mins, the tissue was labelled with [3H]adenine (2μCi/ml; ~25Ci/mmol) for a period of time sufficient to allow ATP to be labelled to apparent isotopic equilibrium. For cortex, a twenty minute protocol was adopted (figure 1). After prelabelling, the slices were washed three times in fresh KRB (free of [3H]adenine) then pooled and allowed to settle under gravity. Polypropylene insert vials containing 250μl of KRB (with or without agents) received 25 or 50μl aliquots of packed slices (~0.5 - 1mg protein) and were gassed and capped, and ordinarily preincubated for 15-20 mins in a shaking water bath at 37°C. Agonists and other agents were applied in 10μl volumes for appropriate times and reactions quenched by addition of 200μl of 1M HCl. To facilitate extraction of [3H]cAMP, samples were placed on ice for 15-20 mins, vortexed and diluted with 1ml of distilled water. Usually, 10μl of [14C]cAMP (0.001μCi; ~220mCi/mmol) were added to the samples to monitor recovery before being centrifuged at 3000rpm (2200g) at 4°C for 20 mins. The slight variation in slice population was accounted for by determining the radioactivity in 50μl of the resultant supernatant.
Figure 1 Effect of varying exposure time to [³H]adenine upon ATP and cAMP labelling in rat cerebral cortical slices. Slices were gassed and incubated in KRB containing 2µCi/ml [³H]adenine in sealed 50ml conical flasks and maintained in a shaking water bath at 37°C for the period indicated. After
labelling, the tissue was rapidly washed three times in label-free KRB and then dispensed in 50μl aliquots into insert vials as described. Stimulation was effected with 10μM forskolin for 10 mins, and labelled species are resolved as described below (Chromatographic Analysis). Upper panel shows progress of ATP labelling, the lower, of cAMP labelling in the presence and absence of forskolin. Results are mean±SEM of three experiments performed in triplicate.

2.1.2 Chromatographic Analysis

On the day of use, Dowex 50WX8 (H+) columns (2cm x 0.8 cm; 1ml bed volume) were washed with ~20ml of water, and the alumina columns (0.6g/col.) with 8ml of 0.1M imidazole (pH 7.3). Typical elution profiles are shown in figure 2. A 1ml aliquot of sample supernatant was loaded onto the Dowex columns and allowed to drain completely. [3H]ATP was typically eluted with 2.5ml of water (determined empirically from one batch of Dowex to the next) with this effluent being similarly discarded. Placing the Dowex columns vertically above their alumina equivalents, radiolabelled cAMP was eluted from the resin with another 3ml of water and consequently adsorbed onto the lower material. To recover the cAMP from the latter, 4ml of 0.1M imidazole (pH 7.3) was used as eluant and this was collected in a 20ml scintillation vial. 2ml of the eluate was taken for the determination of 3H and 14C using a dual label counting programme. Results were expressed as cpm or dpm [3H]cAMP per 25/50μl slices. Recovery of [14C]cAMP was routinely 65-90%. Dowex columns were washed with 10ml of 1M HCl to regenerate the resin and to remove residual radiolabelled adenine and were then stored, like the alumina, at room temperature. Occasionally, Dowex columns were washed with 1M NaOH to reduce the accumulated adenine bound to the resin which could not be entirely removed by acid washing.
Figure 2 Elution profiles of Dowex and alumina columns. Acidified KRB was spiked with ~22,000 cpm [3H]cAMP & ~20,000 cpm [32P]ATP per ml. Unlabelled cAMP (1µM) and ATP (5mM) were added as carriers. 500µl was added to the Dowex column, and eluted with water in 0.5ml aliquots. For alumina profile, Dowex columns were loaded as before, the ATP eluted with 2.5ml of water.
The former were placed over the alumina, and cAMP eluted with 3 ml of water, and allowed to drain. The labelled cAMP was then eluted from the alumina with imidazole, as detailed above. If samples derived from $[^3\text{H}]$adenine-labelled brain slices were spiked with $[^3\text{H}]$cAMP or $[^{14}\text{C}]$cAMP, then identical results were obtained, qualitatively speaking.

### 2.2 Measurement of cAMP Mass in Cell Extracts

The ability to measure absolute cAMP mass circumvents the major pitfall inherent in any prelabelling technique: under some conditions changes in the specific radioactivity of $[^3\text{H}]$cAMP may well result, such that the alteration in the radioactivity of the $[^3\text{H}]$cAMP fraction may no longer accurately reflect the true dynamics of tissue cAMP metabolism. It is worthwhile to note, however, that in spite of its obvious merits, the mass assay in reality is prone to unreliability at lower concentrations of the cyclic nucleotide (ca. < 0.1 pmol/50 μl cell extract). A more reliable evaluation of low cAMP levels can be achieved (with caution) by employing the prelabelling protocol.

Incubation conditions for slice or cell suspensions essentially followed the protocol used above. In this instance, however, after administration of pharmacological agents, acid arrest was effected by 20 μl of concentrated HCl (~10M). The samples were then neutralised with 200 μl of pretitrated ~1 M NaOH before centrifugation and their subsequent assay of extracted cAMP.

Quantification of the unlabelled species was achieved by using the binding protein assay essentially described by [Brown et al. 1971]. With this system, a fixed amount of $[^3\text{H}]$cAMP is displaced by unlabelled cAMP from the binding site of a bovine adrenal cortical preparation. The source of the cAMP was either unknown sample extracts or predetermined standards which generate a calibration curve against which the 'unknowns' are compared using a curve fitting program. Standards (0, 0.25, 0.5, 1, 2, 4, 6, 8 & 10 pmol/50 μl; non-specific binding defined by 250 pmol/50 μl) were prepared in KRB which had been treated.
with conc. HCl and NaOH in a fashion analogous to that of the samples. These were then stored at -15°C and thawed when required. Repeated freeze/thawing had no significant effect upon the calibration curve. Binding protein was prepared as below (2.2c) and also stored at -15°C, being thawed immediately prior to use.

All steps of the assay were performed at 4°C in 1.5ml microfuge tubes. To 50μl of sample/standard, 100μl of [3H]cAMP (0.4μCi/ml; 33.5 Ci/mmol) in assay buffer was added (assay buffer: 50mM Tris-HCl,4mM EDTA, pH 7.5). Finally, 150μl of diluted binding protein (~0.85 mg protein/ml buffer) was introduced to initiate the 90 minute incubation. Since this merely represented the minimum incubation period to achieve equilibrium binding, it was possible to leave the assay for longer times without compromise. Frequently, therefore, it was more convenient to leave the samples overnight in a refrigerator at 4°C. To separate bound and free [3H]cAMP, 250μl of a charcoal/BSA suspension (0.5%/0.2%(w/v)) was added for 1.5 mins and then assay tubes were centrifuged at 14,000 rpm (~14,000g) in an Eppendorf microfuge for 4 mins. To assess bound radioactivity in the supernatant, 480μl were removed and counted. Results are expressed as pmol cAMP/mg protein, and protein was determined by the method of Lowry [Lowry et al. 1951] using BSA as standard (see below).

A comparison of the methods for assessing cAMP accumulation was routinely performed in the slices or cells and some of the results are shown in figure 3.
Figure 3 Comparison of cAMP accumulations elicited by isoprenaline & forskolin measured using the prelabelling or radioreceptor technique. Slices were incubated ±2μCi/ml [3H]adenine for 30 min. In the upper panel, β-adrenoceptors were stimulated with 3μM isoprenaline for the indicated times. In the lower, forskolin stimulations were stopped after 10 mins. Data depict mean±sem of single
experiment performed in triplicate, typical of two (upper); mean±sem of 4-5 experiments performed in triplicate (lower).

Figure 3 indicates that the two methods for measuring cAMP in brain slices give essentially identical results either over extended time courses (isoprenaline - upper panel), or with higher degrees of stimulation (with forskolin-lower panel). Furthermore, the inclusion of PDE inhibitors does not change the comparison (figure 4).

Figure 4 Comparison of cAMP assay methods to measure the kinetics of isoprenaline responses in the presence or absence of PDE inhibition. Slices were labelled where indicated as previously detailed.
and preincubated with DMSO (0.8% v/v) or 100μM rolipram for 20 min. 3μM isoprenaline was added for various times, and cAMP measured as above. Figure depicts mean±SEM of 3 experiments performed in triplicate.

To assess cAMP hydrolysis in this system, the antagonist-induced decay method was initially employed (see this chapter's 'Appendix'). Figure 5 typically illustrates a problem with β-adrenoceptor stimulation in brain slices: the magnitude of the maximal stimulatory response is small. The graph shows the rate of cAMP decay using either the prelabelling or the mass assay for cAMP.

Figure 5 Comparison of rates of antagonist-induced decay in cAMP levels using two methods for the measurement of cAMP. Slices were stimulated for 10 min using 10μM isoprenaline, and then the β-AR antagonist timolol (50μM) was added (t=0 on graph), and the fall in cAMP followed with time (cpm=radiolabel assay, rra=radioreceptor assay). Timolol was added before the agonist for the ∞ point. Results are mean±range of two experiments performed in triplicate.
2.2.1 Preparation of cAMP Binding Protein

The binding protein used in the radioreceptor assay was prepared from bovine adrenal glands essentially after [Brown et al. 1971]. From a local abbatoir, 12-15 bovine adrenal glands were collected and trimmed of superficial fat, halved and demedullated at 4°C. The remaining cortex was decapsulated and finely chopped with scissors in two volumes of ice-cold buffer (50mM Tris/HCl, 4mM EDTA pH 7.5) and was homogenised using an atomix blender. The homogenate was filtered through two layers of muslin cloth and aliquoted into 50ml centrifuge tubes. Samples were centrifuged at 15,000g for 15min at 4°C. With care, the resultant supernatant was collected and the surface layer of fat, and pellet discarded. This centrifugation procedure was repeated once more, and the final supernatant dispensed in 0.5ml portions and frozen at -15°C until required. Each batch of protein that was prepared was always compared to the previously prepared binding protein stock, and the degree of dilution in the assay calculated accordingly.

2.3 Measurement of cGMP Mass

Quantitation of cGMP accumulation was performed by using a commercially available radioimmunoassay kit (Amersham; TRK 500), using an antibody prepared against acetylated cGMP. Tissue samples were generated in an identical manner to cAMP assays, including the arrest with HCl and neutralisation with NaOH.

Reagents: The antibody was diluted as per manufacturer’s instructions in 7.5ml of ice-cold water, and divided into 150 x 50μl aliquots which were frozen and stored at -80°C (stable > 2 years). On day of use, aliquots were thawed and diluted in 8 ml of 50mM sodium acetate/0.1% BSA (pH 6.2). Selectivity over cAMP >10,000 fold. For radiolabelled cGMP, 10μl of [125I]cGMP (~2000Ci/mmol) was diluted in 2 ml of the same buffer as antibody (giving ~2-
3000 dpm/25μl). By contrast, cGMP standards, prepared as a stock of 8nM in water, (800fmol/100μl) were stored at -80°C, thawed and diluted on day of use in the same buffer used to dilute the antibody to give 3, 6, 12, 25, 50, 100, 200, and 400 fmol/100μl (800 defines NSB). Bound and free was separated, like the cAMP assay, using charcoal (10mg/ml in 100mM potassium phosphate/0.25% BSA (pH 7.4)).

**Procedure** All steps performed on ice at 4°C. To 250μl of 100mM sodium acetate buffer (pH 6.2 - no BSA), 250μl of neutral sample or standard was added. Acetylation was effected by sequential addition of 10μl of triethylamine and 5μl acetic anhydride. These were then thoroughly mixed, and 100μl portions are taken, 25μl of [125I]cGMP is added to this aliquot, and the binding reaction initiated with the addition of 100μl of the antibody solution. Samples are vortexed and left for ≥ 3 hours (frequently overnight).

Once equilibrium was attained, 500μl of the charcoal suspension was incubated with the samples for 10 min, centrifuged at full speed in a microfuge, and 650μl of the supernatant removed and radioactivity determined in a γ counter.

**2.4 Determination of Protein (Method of Lowry)**

Measurement of protein in sodium hydroxide digests of tissue/cell samples was performed according to [Lowry et al. 1951]. The reference standards were prepared using bovine serum albumin (stock 1mg/ml in water, stored at -20°C). Standard curve generated using 0, 20, 40, 60, 80 μg of protein per assay.

To 200μl of standard or sample (neat or diluted), 1 ml of 'Lowry C' was added (2% Na₂CO₃/0.4% NaOH: 1% CuSO₄: 2% sodium potassium tartrate in the ratio of 100:1:1 respectively). After 10 min, the reaction was started by adding 100μl of a 1:4 dilution of Folin Ciocalteau reagent (in water). 30 min later, incubations were diluted with 1 ml of water, and the A750 measured using a Beckman spectrophotometer with on-line calibration programming.
2.5 Assay of Cyclic Nucleotide Phosphodiesterase Activity

Measurement of enzymic activity was effected by the radiochemical technique described by Arch and Newsholme (1976). Briefly, radiolabelled substrate ([³²P]cAMP or [³²P]cGMP) is converted to the appropriate 5' monophosphate ([³²P]5'-AMP or [³²P]5'-GMP) by the PDE(s) in the sample of interest and thence to labelled adenosine or guanosine by inclusion of an excess of a coupling enzyme, 5'-nucleotidase.

\[ \text{cAMP} \xrightarrow{\text{phosphodiesterase}} \text{5' AMP} \xrightarrow{\text{5' nucleotidase}} \text{Adenosine} \]

By addition of a slurry of anion exchange resin, the negatively charged moieties (e.g. [³²P]cAMP and [³²P]AMP) are bound and separated from the final, uncharged nucleoside products which remain in the supernatant after a brief centrifugation. Consequently, the amount of cyclic nucleotide hydrolysed will be directly proportional to the degree of radioactivity found in the final supernatant. Suitable controls are included as there is a degree of nonspecific nucleoside/nucleotide binding to the resin ([³²P]adenosine recovery ~95%).

Incubations were performed in microfuge tubes supported within a heat block at 37°C. For analysing activity with a final substrate concentration of 1μM, an assay 'cocktail' was prepared of incubation medium (50mM Tris-HCl, 6mM MgCl₂, 2.5mM dithiothreitol; pH 7.5) supplemented with [³²P]cAMP or [³²P]cGMP (41Ci/mmol & 18.2Ci/mmol respectively; 1.2μCi/ml), 13.7μM cAMP or 13.3μM cGMP, 5'-nucleotidase (Crotalus venom) (0.025mg/ml⁻¹), and the effectors Ca²⁺/calmodulin (100μM/75U respectively) where indicated. This cocktail (100μl) was then prewarmed along with 10μl of either a PDE inhibitor solution (or vehicle), or 1μM cGMP (or water). The reaction was initiated by
addition of 30μl of enzyme source and typically incubated for a further 15 mins 
(or whilst the assay was still in the linear reaction range - figure 6). The final 
assay volume = 140μl. A 50%(v/v) slurry of Dowex 1-X8 (200 - 400 mesh) (1.2ml) 
was added to terminate the incubation, and the tubes were centrifuged for 3-4 
mins at 14,000g. To assess radioactivity in the supernatant, 250μl portions were 
immediately removed and counted with 3ml of Optiphase X scintillation fluid. 
Additionally, two 100μl aliquots of the assay 'cocktail' were analysed for the 
amount of tritium added to each assay ("Total DPM per assay" - see below). 
Blanks were subtracted from all values, and these were obtained by incubating as 
above save that 30μl of the appropriate buffer, and not enzyme, was 
administered (blanks < 5% of total counts added). 

The data were ordinarily expressed as pmol of [3H]nucleoside 
formed/min/ml of enzyme fraction, and were calculated as follows:

\[
\frac{(\text{DPM-Blank}) \times \text{mols cNMP per assay} \times 740}{\text{Total DPM added} \times 0.03 \times 15 \times 250 \times 10^{-12}}
\]

Where: 
\( \text{DPM} \) = radioactivity in 250μl of supernatant 

'\text{mols cNMP per assay}' = absolute theoretical amount of labelled 
plus unlabelled cyclic nucleotide in assay (e.g. 1.4 \times 10^{-10} mols for 
1μM [substrate])

\text{Total DPM per assay} = \text{total radioactivity added per 100μl of} 

'cocktail'

0.03 = correction factor for volume of enzyme added

15 = 'per minute'(for a 15min assay)

740/250 = correction for volume of supernatant removed

10^{-12} = data expressed in pmols
Figure 6 Time course of PDE assay product formation. PDE source was prepared from rat cerebral cortex homogenised in 10 vols. of assay buffer, centrifuged for 15 min at 10,000g (all at 4°C). A twenty fold dilution of the resultant supernatant was used as PDE source (~1g tissue/1000ml buffer). Assay conducted with 1μM cAMP, and with ~0.6μCi [3H]cAMP per assay. Results shown as mean±SEM of single experiment performed in triplicate. Linearity was always confirmed with each new preparation.
Culture of SH-SY5Y and 1321N1 Cells

The human neuroblastoma cell line SH-SY5Y were obtained from Dr. J. Biedler, Sloane-Kettering Institute, N.Y., U.S.A., and the human astrocytoma line, 1321N1, from the Public Health Laboratory Services, Porton Down, Wiltshire.

The neuroblastoma cells (passages 70-95) were cultured in monolayer in 175cm² flasks using minimum essential medium (MEM) supplemented with 10% newborn calf serum (NCS), 100IU/ml penicillin, 100µg/ml streptomycin, 2.5µg/ml fungizone and 2mM glutamine according to [Lambert et al. 1989]. Flasks were incubated at 37°C in a humidified atmosphere containing 5% CO₂. When seeding, cells were harvested from a confluent flask with trypsin/EDTA (Gibco: in modified Puck's saline A) and divided into a maximum of 6 flasks containing 30 ml of medium. Feeding occurred every third day when old medium was completely replaced with new.

For experiments requiring a cell suspension, SH-SY5Y cells were harvested with a buffered saline/EDTA solution (10mM Hepes, 0.9%NaCl, 0.02% EDTA(w/v), pH 7.4) - after removal of medium and rinsing with harvesting buffer. 6ml of the harvesting buffer was applied to each flask and incubated for ~5 mins at 37°C. The resultant cell suspension was centrifuged at 1500 rpm (350g) in a Sterilin universal for 2 mins and the supernatant discarded. Washing the pellet twice in fresh KRB removed excess EDTA and preceded its final resuspension in KRB (4-5mg protein/ml). It was from this cell concentrate that 50µl aliquots were dispensed into insert vials and the experiments were executed essentially as per the brain slice procedure above (section 2.1). At the end of experiments, cell viability was assessed by a dye exclusion regimen, using Azur A (incubation at 0.04% for 10-15 min at room temperature) and was routinely found to be ≥ 85%.

1321N1 astrocytoma cells (passages 1-28, where number 1 is defined as the passage of the cells upon delivery from Porton Down) were maintained
and used under nearly identical conditions with the following amendments: cells were grown in Dulbecco's Minimum Essential Medium with 25mM Hepes, supplemented with 10% foetal calf serum (FCS). Cells were cultured and fed in monolayer using a similar protocol to the SH-SY5Y cells and harvesting, whether for experiments or for seeding, involved the same techniques and reagents as stated above. Cell viability was confirmed to be a similar value to that determined with SH-SY5Y cells.

2.6.1 Prelabelling Cells in Culture

An attempt to [3H]adenine label SH-SY5Y in suspension proved unsatisfactory. Although the degree of ATP labelling appeared comparable to brain slices, the fold responses elicited with forskolin were very poor when compared to the changes in the absolute levels of cAMP (figure 7). That ATP was indeed labelling was confirmed by HPLC. Cell suspensions that had been labelled with 2μCi/ml for 60 mins and incubated ±10μM forskolin (5 min) were neutralised, and samples were run on an anion exchange column; gradient (0-0.5M), eluting buffer was NaH₂PO₄ (pH 3.7). Fractions were collected every 30 sec, where the total running time was 70 min, giving a more than adequate resolution of [3H]adenine labelled species. The running time of labelled species was confirmed by the presence of unlabelled, internal nucleotide standards (at 30μM) (detected by a U.V. recorder). The resulting ratio of cAMP: AMP: ADP: ATP was approximately 1.5: 6: 28: 64 in stimulated cells. The absolute labelling of AMP, ADP or ATP was not substantially altered upon stimulation, and the poor stimulation of cAMP was again seen. It could only be assumed that the ATP that was being labelled was in a discrete compartment (mitochondria?) and less available as a substrate for adenylyl cyclase. Therefore all experiments with SH-SY5Y cells were assessed using the cAMP radioreceptor assay.
Figure 7 **Time course of ATP and cAMP labelling in SH-SY5Y cells.** Cell suspensions were preincubated ±2μCi/ml [³H]adenine in KRB for the indicated times in insert vials in a shaking water bath at 37°C. After the various times, 3μM forskolin was appropriately added for a further 5 mins, and the reaction then quenched. Ctrl=no forskolin; stim=plus forskolin. ATP and cAMP fractions were collected after Dowex/alumina chromatography, as previously detailed. Upper panel indicates kinetics of ATP labelling. Lower shows cAMP labelling, and, inset, the equivalent
stimulation of cAMP mass. Figures show mean ± s.e.m of a single experiment performed in triplicate, and repeated on two other occasions with similar results.

The prelabelling of 1321N1 cells, by contrast, proved fruitful. To determine the optimum labelling time, astrocytoma cells were seeded into six wells (r = 3.5 cm) and at confluency, medium was removed and the cells were washed twice with 1 ml of KRB. 0.5 ml fresh KRB containing 3 μCi/ml [3H]adenine was added to each well and incubated for a further 30, 60 or 90 mins at 37°C. After the appropriate interval, the labelling buffer was aspirated and the cells were washed three times with label-free KRB. To a final volume of 1 ml of KRB, 30 μl of the β-adrenergic agonist isoprenaline was added (final concentration of 3 μM) for 2 mins. The stimulation was quenched with 600 μl of 1 M HCl, the plate subsequently scraped, and a diluted supernatant subjected to the chromatographic analysis described in section 2.1. However, the ' [3H]ATP fraction' was collected and a 1 ml aliquot taken for radioactivity determination, in accordance with the need to assess the time by which equilibrium labelling was evident. In agreement with several authors [Clark et al. 1975, Meeker & Harden 1982], a 60 minute preincubation was deemed satisfactory, and was adopted for all subsequent experiments (figure 8). Labelling of cells en masse was carried out by washing confluent flasks with KRB, adding of 10 ml of the same buffer containing 3 μCi/ml [3H]adenine to each flask, and incubation at 37°C for the 60 mins. Once labelling was effected, buffer containing tritium was removed and cells were harvested in the usual way. Figure 9 shows the result of measuring agonist-stimulated cAMP responses by the two methods, and clearly indicates that efficacies, and potencies of a variety of agonist are maintained with the two techniques.
Figure 8 Time course of nucleotide radiolabelling with $[^3\text{H}]\text{adenine}$ in 1321N1 cells. Experiment conducted as described in the text. ‘Ctrl’ = unstimulated cells; ‘Stim’ = plus 1μM isoprenaline. Results represent the mean±range of a single experiment performed in duplicate, typical of two others.
Figure 9 Comparison of agonist stimulated [3H]cAMP and cAMP in 1321N1 cells. Those labelled cells were preincubated with 3μCi/ml [3H]adenine for 60 mins. All agonist additions were for 10 mins. Open symbols represent mass measurements, the closed, changes in labelled cAMP. Data depict mean±sem of 3-6 separate experiments conducted in triplicate. Basal = 1201±329; 3μM iso = 28,091±8563 (dpm/assay) (n=6). Basal = 7.29±1.19; 3μM isoprenaline = 892.97±173.01 pmol/mg protein (n=3-5).

2.6.2 Measurement of Phosphodiesterase Activity in Homogenates of SH-SY5Y Cells. Two confluent flasks of cells were harvested with HBS/EDTA in the normal way, and a cell pellet obtained by the low speed centrifugation as per above. The supernatant was discarded and the cells were resuspended in ~7mls of 50mM Tris-HCl/4mM EDTA (pH 7.5) and disrupted with a polytron at half maximal setting for 30 seconds. A low speed centrifugation (350g, 2 min) isolated unbroken cells and nuclei and the resultant supernatant was used in 30 μl aliquots (~50μg protein) as the source of 'total PDE'. The assay of PDE activity was
performed as in section 2.3, using 1μM cAMP as substrate, and a 15 minute incubation protocol (the reaction was still linear in this period - results not shown). All inhibitors were dissolved in 100% DMSO (~7% v/v final), and the results expressed as a percentage of vehicle controls.
APPENDIX

I. Assessment of cAMP Turnover in 1321N1 Astrocytoma Cells

The fractional turnover constant, $k_e$, is defined as being the fraction of a pool of metabolite that is removed per unit time [Shipley & Clark 1972]. Whether a chemical, or spatial removal is irrelevant, for both are given by the equation:

$$k_e = \frac{1}{[cAMP]} \frac{d[cAMP]}{dt}$$

Where:

- $k_e$ = fractional turnover constant
- $[cAMP]$ = concentration of cAMP
- $d[cAMP]/dt$ = turnover rate of cAMP

Experimentally, essentially two complementary techniques were employed to determine turnover in cell suspensions stimulated to a steady state cAMP accumulation - one involved examining the rate of antagonist-induced cAMP decay, and so the rate constant for decay, $k_{deg}$, is equivalent to $k_e$; the other, the relative rates of radiolabelled adenine incorporation into ATP and cAMP fractions during agonist stimulation, where $k_e$ is directly determined.

(i) **Rate of cAMP decay - Theoretical Considerations** These are essentially as detailed in [Su et al. 1976]. It will be assumed that synthesis of cAMP is zero order with respect to its concentration, and that ATP saturates adenylyl cyclase (AC) under all conditions. It is also assumed that the degree of AC activity is maintained over the period of stimulation. It is also a prerequisite that the elimination of cAMP from the cells is primarily effected by its hydrolysis, and not by egress. To analyse the decay curves generated by antagonist addition, it has been assumed that the process is first order with respect to cAMP, and that the rate constant, $k_{deg}$, of this reaction is not affected by agonists.
Thus, the rates of reaction (v) of the synthetic (s) and degradative (deg) pathways of cAMP metabolism with the zero order (k_s) and first order (k_{deg}) rate constants are given by:

\[ v_s = \frac{d\text{[cAMP]}}{dt} = k_s \]  \hspace{1cm} (1)

\[ v_{deg} = \frac{-d\text{[cAMP]}}{dt} = k_{deg} \text{[cAMP]} \]  \hspace{1cm} (2)

The net change in cAMP can therefore be expressed as the sum of the equations (1) and (2), and is shown in equation (3).

\[
\text{rate of change of cAMP} = \text{rate of synthesis} - \text{rate of degradation} \\
\text{or algebraically} : \\
\frac{d\text{[cAMP]}}{dt} = k_s - k_{deg} \text{[cAMP]} \]  \hspace{1cm} (3)

When cAMP is in a steady state, the rates of synthesis and degradation are equal, since the rate of change of cAMP is defined as zero (see equation (3)). Upon cessation of cAMP synthesis (by addition of antagonist), v_s is effectively zero, and therefore:

\[ \frac{-d\text{[cAMP]}}{dt} = k_{deg} \text{[cAMP]} \]  \hspace{1cm} (4)

Equation (4), analogous to that defining k_e, describes a first order decay in cAMP levels after application of an excess concentration of antagonist, but it is the integrated form of this equation that is applied to experimental data:
\[
\ln \frac{[cAMP]_t}{[cAMP]_0} = -k_{\text{deg}} t
\]  

(5)

Where \([cAMP]_t = \) cAMP concentration at a given time, \(t\), after antagonist addition

\([cAMP]_0 = \) cAMP concentration at \(t=0\), before antagonist is applied

\(k_{\text{deg}} = \) first order rate constant for degradative process

Thus, a plot of \(\ln \frac{[cAMP]_t}{[cAMP]_0}\) against time should result in a straight line with a gradient equal to \(-k_{\text{deg}}\). The slope of the transformed line was calculated by using the standard regression through the origin statistical analysis: for a line \(y = \beta x:\)

\[\beta = \frac{\sum x_i y_i}{\sum x_i^2}, \text{ i.e. } \beta = k_{\text{deg}}.\]

**Practical execution of experiments.** Cells were harvested and incubated in insert vials as previously described. All experiments were performed using the \(\beta\)-adrenoceptor agonist, isoprenaline, and the \(\beta\)-antagonist timolol (50\(\mu\)M, a concentration \(\sim 5000\) times its \(K_i\) at these receptors [Dickinson & Nahorski 1981]). After a ten minute stimulation with isoprenaline, and defined as \(t=0\) according to equation (5), timolol was added for various times and the ensuing decay of cAMP levels measured using the mass assay. In all experiments addition of timolol per se had no effect upon basal cAMP levels. To determine the equivalent of \([cAMP]_\infty\), isoprenaline was added to cells which had been previously exposed to timolol for at least 15 min. Again, this value was never significantly different from basal, and indicated a full blockade of \(\beta\)–ARs by this concentration of the antagonist.
(ii) Rate of cAMP Turnover - theoretical considerations  This method has been outlined previously by [Barber & Butcher 1988]. Unlike the previous protocol, turnover is measured under conditions where cAMP synthesis is fully maintained - there is no addition of antagonist. Instead, agonist is applied until cAMP accumulation is in a steady state, when radiolabelled adenine is added. The incorporation of this label into ATP is rapid and the rate labelling of cAMP is itself dependent upon ATP labelling and upon the rate of cAMP turnover. Thus, the specific radioactivity of cAMP lags behind that of ATP and it is from this delay that the turnover constant, \( k_e \) can be estimated. It should be clear that should cAMP turnover be rapid, then the lag will be small; however, when turnover is slower, this delay is more pronounced.

Derivation of Practical Equation  Upon addition of labelled adenine, the change in cAMP radioactivity will depend upon two processes: its synthesis and degradation, i.e. gain and loss of label from the pool. Thus, one can write:

\[
\frac{\text{rate of isotope change in cAMP}}{\text{rate of synthesis of cAMP}} = \frac{\text{specific activity of ATP}}{\text{specific activity of cAMP}} \times \frac{\text{rate of hydrolysis of cAMP}}{\text{specific activity of cAMP}}
\]
Which algebraically is:

\[
\frac{dq}{dt} = \frac{k_s Q}{[ATP]} - k_e \frac{[cAMP]}{[cAMP]} q
\]  

(1)

Where:
- \(k_s\) = rate of synthesis
- \(k_e\) = fractional turnover constant for cAMP
- \(q\) and \(Q\) = total radioactivities with cAMP and ATP respectively
- \([cAMP]\) and \([ATP]\) = the absolute amounts of the species

However, when cAMP accumulation is in a steady state, the concentration of both nucleotides is constant with time and therefore:

\[
\frac{dq}{dt} = [cAMP] \frac{d(q /[cAMP])}{dt}
\]  

(2)

and, since synthesis rate equals degradation:

\[k_s = k_e [cAMP]\]  

(3)

Thus, combining all three equations, and rearranging, we obtain:

\[
\frac{[cAMP]}{q} \frac{d(q /[cAMP])}{dt} = k_e \left( \frac{[cAMP]}{q [ATP]} - 1 \right)
\]  

(4)

Since specific radioactivities are defined as (amount of radioactivity/absolute concentration) i.e. \(q /[cAMP]\) and \(Q /[ATP]\) are specific radioactivities, then:

\[
\frac{1}{(SA)c} \frac{d(SA)c}{dt} = k_e \left( \frac{(SA)a}{(SA)c - 1} \right)
\]  

(5)

Where:
- \((SA)c\) = specific radioactivity of cAMP
- \((SA)a\) = specific radioactivity of ATP

To obviate the need to calculate specific radioactivities (SAs), and thereby simplify the experimental procedure, cells were prelabelled to equilibrium with
[^14C]adenine. By this manoeuvre, the cellular ATP and cAMP were thus labelled to the same SA with respect to $^{14}$C. That is:

\[
[cAMP] = k \mbox{ (cpm of } ^{14}\mbox{C in cAMP)}
\]

\[
[ATP] = k \mbox{ (cpm of } ^{14}\mbox{C in ATP)}
\]

Where $k$ is a constant.

But, $(SA)c = q /[cAMP]$, so if we substitute equation 6 into this expression we can see that $(SA)c = q / (k \mbox{ (cpm of } ^{14}\mbox{C in cAMP)}).$ The experiments are conducted such that $q$ defines the increasing amount of $^3\mbox{H}$ that is being incorporated into cAMP and therefore $q / (\mbox{cpm of } ^{14}\mbox{C in cAMP})$ represents the $^3\mbox{H}/^{14}\mbox{C}$ cpm ratio in cAMP. Therefore:

\[
(SA)c = r/k
\]

\[
(SA)a = R/k
\]

Where:

\[
r = ^3\mbox{H}/^{14}\mbox{C cpm ratio in cAMP}
\]

\[
R = ^3\mbox{H}/^{14}\mbox{C cpm ratio in ATP}
\]

Substituting these expressions back into equation 5, and rearranging, we obtain:

\[
k \frac{d}{dt} \left( \frac{r}{k} \right) = k\epsilon \left( \frac{R}{k} \frac{r}{R} - 1 \right)
\]

or

\[
\frac{1}{r} \frac{dr}{dt} = k\epsilon \left( \frac{R}{r} - 1 \right)
\]

Equation 8 can be rewritten into a more practical form, which has been the one adopted for manipulating all the data generated (eq.8):

\[
\frac{d}{dt} \ln r = k\epsilon \left( \frac{R}{r} - 1 \right)
\]
A plot of \( \frac{d \ln r}{dt} \) against \( (R/r)^{-1} \) should give a straight line passing through the origin with a slope equal to \( k_e \). In practice of course, \( \frac{d \ln r}{dt} \) can only be determined over a finite time interval, and thus \( \Delta \ln r / \Delta t \) is calculated for each adjacent pair of time points and is plotted against the corresponding mean of \( (R/r)^{-1} \). A small error (~5%) will consequently be introduced because of this point. The technique is only applicable to those systems where a turnover constant \( \leq 1 \text{ min}^{-1} \) is prevalent. If the turnover is faster than this value, then too many inaccuracies are accrued to convincingly determine the cAMP turnover constant. The carcinoma cells, HEP-2, and isolated rat adipocytes are apparently examples of cells with a profound turnover rate. However, from the literature and preliminary data, it was clear that 1321N1 astrocytoma cells would provide another cell system in which these types of experiments could be performed.

**Practical Execution of Experiments.** In those experiments where the decay and pulse labelling techniques were compared side by side, flasks of cells were washed with KRB and incubated for 60 min with KRB \( \pm 2 \mu \text{Ci/ml} \) \(^{14}\text{C}\)adenine, in a fashion comparable to the prelabelling protocol described in section 2.4. After this preincubation, cells were harvested and aliquoted into insert vials and the experiments performed in the usual way. Cells were stimulated with agonist until a steady state accumulation of cAMP was elicited, usually after 10 min. During maintained cAMP levels, \( 4 \mu \text{Ci/ml} \) \(^3\text{H}\)adenine was added in a 10\( \mu \text{l} \) volume and incubations continued for the times indicated, ordinarily for a further 7 min. Reactions were quenched with 200\( \mu \text{l} \) of 1M HCl and the samples processed as detailed in section 2.1, save that the ATP fraction was collected and not discarded. This 'ATP fraction' also will be contaminated with other nucleotides, but in S49 lymphoma cells [Barber & Goka 1981] and other systems [Plagemann & Wohlhueter 1980], a mere 90 sec exposure to \(^3\text{H}\)adenine results in the ATP consistently comprising ~80% of the labelled nucleotide pool. Therefore, the increase in radioactivity of this fraction will not
be complicated by changing a proportion of label amongst adenine nucleotides. Of the collected ATP fraction (3.5ml), 400μl were taken for determination of $^3$H and $^{14}$C using a dual label counting regimen, one wherein the dpm ratios were precalculated 'on line'. A 2ml aliquot of the cAMP fraction was similarly analysed. The ensuing transformed data were also analysed by regression through the origin, i.e. $k_e = \beta$. 
### II. Composition of modified Krebs Ringer Buffer (KRB)

<table>
<thead>
<tr>
<th>Stock Solution Composition</th>
<th>Volume (ml/L KRB) or Mass (g/L)</th>
<th>Final Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl (10mM HEPES) pH 7.45</td>
<td>778 ml</td>
<td>118 (8)</td>
</tr>
<tr>
<td>1.8% NaHCO₃</td>
<td>163</td>
<td>25</td>
</tr>
<tr>
<td>1.15% KCl</td>
<td>31</td>
<td>4.7</td>
</tr>
<tr>
<td>2.11% KH₂PO₄</td>
<td>8</td>
<td>1.2</td>
</tr>
<tr>
<td>1.62% CaCl₂</td>
<td>12</td>
<td>1.3</td>
</tr>
<tr>
<td>3.82% MgSO₄</td>
<td>8</td>
<td>1.2</td>
</tr>
<tr>
<td>10mM Glucose</td>
<td>1.8g</td>
<td>10</td>
</tr>
</tbody>
</table>
Chapter 3 Potentiation of β-adrenergic-mediated stimulation of cAMP by α-adrenergic receptors in rat cerebral cortex

Introduction

The work in this chapter represents a digression that was borne out of a need to enhance β-adrenergic stimulation of cAMP in rat cortex. Since it was of interest to examine the relationship between cAMP accumulation and PDE activity in the intact cell, it was clear from the start that the modest three-fold maximal response to isoprenaline would be insufficient to support accurate antagonist-induced cAMP decay curves over a wide range of agonist concentrations (such decay curves have been used as an index of cellular PDE activity - see Appendix, chapter 2). Consequently, conditions were sought that would enlarge the window in which such data could be gathered, including addition of forskolin or an α-adrenoceptor (α-AR) agonist. The latter enhancement has been recognised for some years [Perkins & Moore 1973, Schultz & Daly 1973], and was of sufficient effectiveness to warrant further investigation.

The very nature of a brain slice preparation makes dissection of some complex signalling mechanisms difficult, if not ultimately impossible. However, unlike homogeneous cultured cell populations, it does provide a system in which interaction of the myriad cellular compartments can be studied and modelled, if only to a degree. In terms of cAMP production, neurotransmitter receptors are positively coupled to the response in two ways: by direct activation of adenylyl cyclase (AC), and by an indirect enhancement of cAMP accumulation that necessitates the prior activation of AC.

Clearly, those receptors that are coupled to G₅, such as β-adrenergic (β-AR), H₂-histaminergic, and vasoactive intestinal peptide, require no other stimulatory input and represent members of the first class of receptors. Nevertheless, the activation of some receptors that are not coupled to AC via G₅ still elicits a cAMP response. Alone, they elicit no increase in cAMP, but act to potentiate the
response to direct (Gα-linked) receptor occupation. This second class of receptor serve to modulate rather than initiate cAMP production and are typified by the GABAB and α1-ARs [Enna & Karbon 1987].

Pharmacology of the α-AR

The synergism between β1- [Daly et al. 1981] and α-ARs in the mammalian central nervous system is an issue of some controversy. Its existence is without doubt: it is the underlying mechanism that is contentious. The pharmacology of the α-AR mediating this response is also, as yet, not clearly defined. If one bases classification upon the relative potency of classical α2- and α1-antagonists, then the receptor appears to be more α1-like [Daly et al. 1980]. Moreover, clonidine, an α2-AR agonist, does not enhance the action of other agents [Daly et al. 1981]. Although these data are consonant with the participation of an α1-AR, one group alone has suggested there is partial involvement of α2-ARs. Employment of the α1-AR antagonist prazosin incompletely blocked the α-potentiating effect (~40-50%), whereas with α2-AR-selective antagonists, the augmentation was entirely ablated [Duman et al. 1985, Pilc & Enna 1985, Pilc & Enna 1986]. Pilc et al. (1986) speculated that the complete inhibition by α2-antagonists reflected their ability to inhibit α1-ARs at higher concentrations [U'Prichard et al. 1977, Kawahara & Bylund 1985]. However, their conclusion contradicts reports from other groups [Robinson & Kendall 1989, Atkinson & Minneman 1991], and is also inconsistent with published data which demonstrate that prazosin is capable of displacing [3H]yohimbine (an α2-antagonist) binding in rat cortical membranes [Cheung et al. 1982]. Indeed, prazosin is now being used as an agent that possibly discriminates between α2-AR subtypes [Petrash & Bylund 1986]. It is pertinent to discuss the fact that α1-ARs themselves may also be subdivided, as based upon pharmacological and molecular biological criteria [Minneman 1988, Harrison et al. 1991]. There is an inherent circularity in defining receptor classes by ligand

1 It should be understood that prazosin is a non-selective alpha-1 antagonist which does not discriminate between alpha-1 subtypes, according to binding data [Morrow, 1986 #250].

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potencies, and therefore the complex data from different groups may, indeed, reflect the involvement of multiple receptors or (and?), potentially, receptor subtypes.

More recently, the issue of $\alpha_1$-adrenergic receptor subclasses has been directly addressed in rat cortex, aided by the availability of relatively selective agents for different subtypes. When measuring either the cAMP potentiating response or $\alpha_1$-AR-induced inositol phosphate (IP$_x$) generation [Brown et al. 1984], it was initially clear that the pharmacological profiles of the implicated $\alpha_1$-ARs differed [Robinson and Kendall 1989] i.e. different receptors mediated each action. This was examined further by using the selective agent niguldipine which discriminates between the so-called $\alpha_{1A}$- and $\alpha_{1B}$-ARs [Boer et al. 1989]. It was observed that this agent inhibited IP$_x$ generation in a stereoselective manner, and perhaps more potently (and certainly more efficaciously) than it inhibited cAMP potentiation by NA [Robinson & Kendall 1990]. At the same time, another study reported effects of niguldipine enantiomers upon IP$_x$ generation [Michel et al. 1990], but both reports are a little at odds with the classification based upon the original binding data.

The report of Robinson & Kendall (1990) clearly demonstrates a stereoselectivity of the niguldipine towards inhibition of inositol phospholipid hydrolysis. That is, the (+) isomer was two orders of magnitude more potent than the (-) isomer. However, whilst the displacement of $[^3H]$prazosin by niguldipine in membranes was demonstrably two-site for both enantiomers, the $K_i$s for inhibition of binding were only significantly different at the high affinity ('$\alpha_{1A}$') site [Boer et al. 1989, Michel et al. 1990]. Thus, superficially, the pattern of antagonism of IP$_x$ production would certainly seem to exclude $\alpha_{1B}$-ARs, since stereoselective inhibition is a more $\alpha_{1A}$-AR-like characteristic. It is therefore premature that [Michel et al. 1990] conclude that only ' $\alpha_{1B}$-ARs' couple to IP$_x$ production in rat cortex. The issue requires further clarification. As if to further
complicate the story, estimates of niguldipine §pKiS for IPx inhibition do not correlate well with those derived for the 'α1A-AR site' from binding experiments (8.7 and 6.7 (IP_x), versus 10.4 and 9.1 respectively (binding) [Michel et al. 1990]). Whether this discrepancy is real and precludes mediation by α1A-ARs is unknown at present. Stereoselectivity was possibly evident in the inhibition of the cAMP synergistic response (α1A-AR?), although the data are a little less convincing [Robinson and Kendall 1990].

To summarise therefore, it appears that the receptor(s) coupled to cAMP potentiation have some characteristics of α2-ARs, whilst still engendering those hallmarks of α1-adrenoceptors. The confusion is all too obvious and it is probable that multiple receptors are involved (Hill slopes of several α-AR antagonists are significantly different from unity with respect to the α-AR cAMP augmentation [Robinson and Kendall 1989]), amongst which may be undefined subtypes of α1-AR.

**Mechanism(s) Behind the Potentiation Response**

The pharmacology of the response is thus seen to be complex; what of the underlying mechanism? Unfortunately, this issue is just as embroiled. Some data have indicated that adenosine is involved, as judged by the effects of the adenosine receptor antagonist 8-phenyltheophylline (8PT) [Daly et al. 1981, Robinson and Kendall 1989], with synergy also pertaining between α1-AR and adenosine receptors in rat cortex [Johnson & Minneman 1987]. However, since a

---

5 Calculated using an estimate of the IC50s as being 0.05 and 5μM for (+)- and (-)-niguldipine respectively from the data in [Robinson, 1990 #238]. The EC50 for NA-stimulated IP_x generation was assumed to be 4μM [Robinson, 1989 #236], and the Ki was calculated using the equation:

\[ K_i = \frac{\text{IC50}}{1 + \frac{\text{[NA]}}{\text{EC50}}} \]

Where IC50 = concentration of niguldipine giving half maximal inhibition, [NA] = concentration of noradrenaline used in the assay (100μM), and EC50 = concentration of NA eliciting a half maximal response
maximal dose of 8-PT does not completely abolish $\alpha$-AR effects [Robinson and Kendall 1989], this cannot be entirely responsible for mediating the potentiating effect.

Early experiments indicated that the effect of $\alpha$-ARs relied almost exclusively on extracellular Ca$^{2+}$ ions [Schwabe & Daly 1977]. The authors were particularly careful inasmuch that the chelator was applied only some 2 min before agonists, reducing the likelihood of an extreme perturbation of intracellular Ca$^{2+}$ stores.

Upon these original studies, much of the more recent work has been based. With the recent explosion in the understanding of the regulation of cellular calcium homeostasis and its consequences, there has been ample new information available for the investigator to exploit and apply to this system. Cellular calcium is inextricably linked with inositol phospholipid turnover, either in a causative or a consequential fashion, and thus signposted the way forward in research into this system. As has been alluded to in the previous section, $\alpha_1$-AR occupation results in phospholipase C (PLC)-mediated-phosphoinositide hydrolysis and the generation of two second messengers, inositol 1,4,5-trisphosphate and diacylglycerol (DAG). The former releases Ca$^{2+}$ from intracellular stores and the latter leads to activation of protein kinase C (PKC) [Berridge & Irvine 1989]. That the $\alpha$-ARs mediating IP$_x$ production and cAMP potentiation exhibit different pharmacological profiles, strongly argues against the involvement of phosphoinositides in the synergism; at least, not in entirety, for in spite of complete elimination of the noradrenergic stimulation of IP$_x$, 60% of the cAMP augmenting phenomenon persists [Robinson and Kendall 1990]. Such a conclusion has been reached by other workers who also tentatively eliminated a role for PLC [Duman et al. 1986].

What of the other arm of the bifurcatory pathway? Although one can infer that the hydrolysis of inositol-containing lipids is not primarily responsible for the synergism, one cannot automatically exclude PKC activation as a means
of communication between adrenoceptor responses. Other parent lipids, upon hydrolysis, can release species that directly, or indirectly, lead to enhanced PKC activity. For example, phospholipase C-catalysed cleavage of other, non-inositol containing lipids can generate DAG [Billah & Anthes 1990]; hydrolysis by phospholipase D generates DAG indirectly via phosphatidic acid [Billah and Anthes 1990], and products of PLA

2

are also suggested as being PKC modulators [Shinomura et al. 1991]. It is therefore quite conceivable that activation of α-ARs activates other phospholipases which potentially converge upon PKC activation. Such a supposition is lent credence by reports of enhanced cAMP accumulation in rat cortex subsequent to phorbol ester treatment (phorbols emulate DAG) [Karbons et al. 1986]. The phorbol treatment not only mimicked the α-AR potentiation of isoprenaline responses, it appeared to enhance the response to all cAMP-raising agents applied, including maximally effective concentrations of noradrenaline itself (an activator of both α- and β-ARs). That PKC activation emulates the α-AR response is not unequivocal evidence in itself for its participation in the synergism. Thus, the activation of PKC with phorbol esters has been complemented by experiments using a variety of protein kinase inhibitors which share the ability to interfere with PKC activity. None of the agents ablated noradrenaline (NA) enhancement of the isoprenaline response, although they did reverse the phorbol ester augmentation [Robinson & Kendall 1989a]. Since other systems with demonstrated synergy between α/β-adrenoceptors display a reliance on the intervention of PKC (the pineal gland [Ho & Klein 1987] (and refs. therein), hypothalamus [Petitti & Etgen 1991]) one has a precedent to follow. In the light of such data, one can conclude that perhaps cortex differs from the other tissues but the issue of PKC involvement is not yet closed and such results demand confirmation by other workers.

Another Ca

2+-dependent enzyme, phospholipase A

2 (PLA

2

) has also been a candidate in this perplexing story. A requirement for the intermediacy of prostaglandins (PGs) was reported over ten years ago [Partington et al. 1980].
Interestingly, the CNS does not store these labile compounds like neurotransmitters, but synthesises them from arachidonic acid (AA) via prostaglandin synthase [Coceani 1974]. Since the availability of AA is the rate determining step, any modulator of its production, i.e. modulator of PLA$_2$ activity, regulates prostanoid concentration. In a paper published by Partington et al. (1980), PGs of the E, but not the F series restored the potentiation, and inhibitors of PG synthase (indomethacin, aspirin, etc.) attenuated the NA $\alpha$-AR response. Although arachidonate metabolites were further suggested to be required for synergy in other systems [Schaad et al. 1989], some recent data, examining rat cortex, have partially refuted the notion of PG involvement. The addition of cyclooxygenase or lipoxygenase inhibitors to slices had no specific effect upon $\alpha/\beta$ synergy in rat cortex [Duman et al. 1986, Robinson and Kendall 1989a], in opposition to [Partington et al. 1980]. Even though this seems to invoke a hypothesis excluding PGs, it does not eliminate PLA$_2$ activation - AA itself has been shown to be effective as a second messenger per se, regulating ion channels [Kim & Clapham 1989, Ordway et al. 1989] and possibly mobilising intracellular Ca$^{2+}$ [Chow & Jondal 1990]. Again controversy shrouds the literature inasmuch that the two groups which acknowledged that there was no association with PGs, failed to agree when it came to a rôle for PLA$_2$ (compare [Duman et al. 1986, Robinson and Kendall 1989a]). It must be said that the evidence weighs slightly in favour of the latter authors’ contention since the major evidence to the contrary relies heavily upon the action of the ‘PLA$_2$ inhibitor’, quinacrine. As Robinson & Kendall rightly point out, quinacrine is probably a competitive $\alpha$-AR antagonist based on their data and previous binding studies [Kendall 1986]. Moreover, the bee venom peptide mellitin, used by others to stimulate PLA$_2$, was not antagonised by quinacrine, and is not specific in its actions; its other known properties are as a protein kinase inhibitor, and a calcium ionophore [Robinson and Kendall 1989a]. Again it is questionable whether or not PLA$_2$ is a mediator of the augmentation of the $\beta$-AR
response, and it will require further work, and/or more selective pharmacological agents to unequivocally resolve this problem.

It is apparent that a multitude of receptors, and possibly signalling mechanisms may all contribute to varying degrees to the enhancement of β-adrenergic-stimulated cAMP responses. What has not been discussed is the possibility that they may represent elements of a cascade, activation of each one in turn interacting with the next component, i.e., they are not mutually exclusive. For instance it is conceivable that α-AR occupation leads to an increase in intracellular Ca$^{2+}$ that activates PLA$_2$, liberating AA which in turn activates PKC. However, this is only meant to be illustrative. What has been overlooked in detail is the requirement for extracellular Ca$^{2+}$. With the implication being that multiple subtypes of α-AR are somehow involved in this response, it was of interest to ascertain whether other mechanisms come into play; different α$_1$-ARs in smooth muscle and other cells couple to different effectors that ultimately raise intracellular Ca$^{2+}$. α$_{1a}$ receptors are linked to voltage sensitive calcium channels, and α$_{1b}$-ARs to inositol phosphate production [Minneman 1988]. Noradrenaline was recently shown to modulate calcium channel gating by a mechanism that seems to involve a G protein, but not any diffusible second messenger [Lipscombe et al. 1989]. Since from the introduction above it should be clear that phosphoinositides do not play a major rôle in the synergistic response, a study was undertaken to examine the possibility that modulation of Ca$^{2+}$ influx may be of some importance, by analogy with the smooth muscle system.
Results

In keeping with the work of other groups, the β-adrenergic agonist, isoprenaline (Iso) maximally elicited a three fold stimulation of cAMP at 10μM, whereas noradrenaline (NA: an α- and β-AR agonist) routinely potentiated this by three fold i.e. nine times above basal when stimulated fully with 100μM NA. In the first set of experiments, nickel and cobalt, frequently used as calcium channel blockers [Rink 1990], were added simultaneously with either Iso or NA and [³H]cAMP accumulation measured after a 12 minute stimulation (Figure 1).

In figure 1, neither transition metal, up to 100μM, had any significant effect upon Iso-stimulated cAMP; it was only 1mM Ni²⁺ that had any effect, and that was an enhancement by ~70%. By contrast, both Ni²⁺ and Co²⁺ at 100μM inhibited the NA stimulation by ~30% (figure 1b) i.e. the effect was selective for a mixed α/β-AR agonist. The inhibition by both metal ions was clearly biphasic with respect to NA, suprising in view of there being no stimulatory effect of Co²⁺ upon β-adrenergic stimulation alone.

The dose response curves generated were too superficial to ascertain if a maximal effect of, say, nickel was evident, and therefore an elaboration was undertaken. Figure 2 illustrates a more detailed dose-response relationship for nickel. The upper panel and figure 1 shows that concentrations of Ni²⁺ up to, and including 500μM have absolutely no effect upon the β response; the augmentation effected by nickel only prevails when its concentration exceeds 500μM, and thereafter proceeds in a dose-dependent fashion. The lower panel shows the effect of a range of nickel concentrations upon NA-stimulated cAMP accumulation, and it is clear that, in conjunction with figure 1, a dose of 50-100μM is adequate to sustain a maximal effect (~30-35% inhibition).
Figure 1: effect of nickel or cobalt ions upon adrenergic stimulated cAMP accumulation in rat cerebral cortical slices. Tissue was prepared as 'Methods' section. Metal chlorides were added simultaneously with agonists, and thereafter incubated for 12 mins. Upper panel displays isoprenaline-, the lower, noradrenaline-stimulated [3H]cAMP. Results are the mean±range of two experiments performed in triplicate.
Figure 2 Dose response relationship between Nickel and adrenergic-stimulated cAMP. Labelled slices were incubated for 12 mins in the presence of varying nickel chloride concentrations and either 10μM isoprenaline, or 100μM noradrenaline. Results represent the mean±sem of 3 separate experiments performed in triplicate. (•), P=0.05; (••), P=0.02; (•••), P=0.004; (••••), P<0.001 with respect to '0μM/mM (student’s t test, unpaired).
In view of the results with Iso, one can speculate that the nickel effect is selective for the $\alpha$-AR component of the NA response. Irrespective of the underlying mechanism, it should be emphasised that it is impossible to say whether or not the ‘$\alpha$-AR’, nickel-sensitive component is *maximally* inhibited at 100µM - the very biphasic nature of the dose-response curve is suggestive of another, stimulatory locus of action for the metal. Thus, when NA is driving cAMP production, higher concentrations of Ni$^{2+}$ (>100µM) are possibly exerting effects at two distinct, antagonistic loci, and the augmentation of the NA stimulation (via the $\beta$-AR component? - figure 2, upper panel) may mask the ongoing inhibition of the $\alpha$-AR component. All that can be said is that 100µM Ni$^{2+}$ is a maximally *selective* concentration.

The kinetics of the cAMP accumulation in the presence of Ni$^{2+}$ were examined in the next set of experiments. From figure 3 one can see that isoprenaline-stimulated cAMP accumulation was almost maximal by 90 sec, and was sustained for the next ten minutes. The kinetic profile was unimpaired by the inclusion of the transition metal.

By way of contrast, the NA response was attenuated by Ni$^{2+}$, and in a more complex manner than one might have envisaged. At the earliest time point measured, Ni$^{2+}$ was ineffective at altering cAMP levels. It wasn’t until times after this that the effect was seen to persist. Although the data does not allow determination of the earliest time of onset, it is prevalent by 6 mins. One can interpret the data several ways: either the early phase of the NA response is Ni$^{2+}$-insensitive; or, inefficiency of access of the metal to the site of action, be that a problem with slice penetration or a requirement for cell entry. Slice penetration is surely unlikely to present a problem in a system where a larger species like a catecholamine gains ‘immediate’ access to its receptors, which are presumably proximal to the Ni$^{2+}$-sensitive locus. Cell entry will be discussed later.
Figure 3 Time course of adrenergic-stimulated cAMP in the presence or absence of nickel chloride
Slices were challenged with maximal concentrations of isoprenaline (upper) or noradrenaline (lower panel) at t=0. Nickel (100µM) was included at the same time and the reaction was quenched at times indicated. Results are expressed as mean±sem of 3 experiments performed in triplicate. (***) $P=0.01$, student's $t$ test (unpaired) versus control at each time point.

If it is hypothesised that inorganic ions like Ni$^{2+}$ or Co$^{2+}$ are indeed exerting their effects through the blockade of Ca$^{2+}$ channels, this therefore begs the question how do the temporal aspects of Ni$^{2+}$ action compare with the
relatively selective calcium chelator, EGTA? Figure 4 addresses this question. At a concentration of 2mM, EGTA has been shown to abolish much of the response to the mixed agonist, noradrenaline, at the same time having no effect upon β-AR-mediated increases [Schwabe and Daly 1977, Duman et al. 1986]. Addition of EGTA immediately prior to NA addition resulted in a time course of cAMP accumulation not dissimilar to that observed with nickel addition.

Figure 4 Effects of EGTA and nickel upon the kinetics of noradrenaline-stimulated cAMP accumulation. Slices to be treated with EGTA were rapidly washed thrice in (and were to be incubated in) calcium-free Krebs Ringer Bicarbonate (KRB) buffer containing 2mM EGTA. Slices were aliquoted into vials containing the KRB (±Ca²⁺) and NA (±nickel- both at 100μM) at t=0. Results are from a single experiment performed in triplicate, typical of two.

In both cases, the initial phase of the increase in cAMP was relatively unaffected when compared to controls. At the later time points, the effect of EGTA or Ni²⁺
were observed, indicating that the initial rise in second messenger is not dependent on extracellular calcium, and that the kinetics observed with nickel are not incongruent with the idea that it is antagonising a calcium-dependent phenomenon. Co-addition of the two agents was not attempted because potential EGTA sequestration of the Ni\(^{2+}\) may well occur, thus making it unavailable for \(\alpha\) antagonism and rendering interpretation of any effects impossible.

What is the selectivity of the effect of nickel on cAMP concentration in rat cortex - does it have an effect on other systems besides the \(\alpha\)-AR potentiation? In the following experiments, the effect of nickel upon forskolin- and adenosine-stimulated cAMP was examined.

![Bar graph showing the effect of nickel on cAMP accumulation](image)

**Figure 5** Effect of nickel upon cAMP accumulation elicited by different agents in cerebral cortex. Adenosine (100\(\mu\)M) or forskolin (3\(\mu\)M) were added to slices simultaneously with the indicated nickel concentration and incubated for 12 mins. Results are mean±sem from 3 experiments performed in triplicate. (*) P<0.05; (**) P<0.01; (***) P < 0.001 - student's t test (unpaired) with respect to '0' mM nickel control.

Basal levels of cAMP were only modulated by higher concentrations of nickel - the concentration inhibiting the \(\alpha\) response (0.1mM) had no effect. Surprisingly, this same concentration significantly decreased both the adenosine
and forskolin responses by 35%, whilst 2mM Ni\(^{2+}\) was less effective: the adenosine response was now reduced 23%, whilst the forskolin response was no longer attenuated. Even when employing only two concentrations of nickel a biphasic effect is again suggested. As an interesting aside, it appears that the increases in cAMP at higher nickel concentrations appears to be manifest at different nickel potencies or efficacies with different agents (or different degrees of stimulation of adenylyl cyclase). The adenosine response is still significantly inhibited by 2mM Ni\(^{2+}\), whereas that of forskolin is 'unaffected' and isoprenaline is stimulated.

Up to this point, relatively non-specific antagonists have been used. The likelihood of ions such as nickel and cobolt being specific in inhibiting Ca\(^{2+}\)-channels is limited, and so to substantiate further the idea of calcium channel involvement, it was decided to assess the effectiveness of some specific, organic compounds that have reported specificities towards certain channels. Voltage-sensitive channels facilitating the permeation of calcium ions are currently subdivided by certain pharmacological and electrophysiological criteria into T, L, N and P type channels [Meldolesi & Pozzan 1987] [Tsien et al. 1991]. The L-type channel is characterised by a high voltage threshold, long duration of opening (therefore can contribute significantly to changes in \([\text{Ca}^{2+}]_i\)), and a sensitivity to certain classes of organic blocker viz dihydropyridines, benzothiazepines and phenylalkylamines [Spedding 1985]. In an attempt to discover if they were involved in the α response, the dihydropyridine blocker nitrendipine, and activator, Bay K 8644 were used.
Figure 6 Effect of dihydropyridines (DHPs) upon adrenergic-stimulated cAMP in rat cerebral cortex. Where indicated 50μM timolol (β-AR antagonist) or DHPs (1μM) (DMSO vehicle -0.01%(v/v)) were incubated for 20 mins. Isoprenaline and noradrenaline were then incubated for 12 mins at 10 and 100μM respectively. Results expressed as mean±range of 2 experiments performed in triplicate.

At concentrations shown to inhibit inositol phosphate generation in rat brain slices [Zernig et al. 1986], the DHP agonist or antagonist were found to be ineffective at altering the adrenergic stimulation of cAMP, whether β-, α-(NA plus timolol), or α/β-adrenergic (figure 6). Antagonists from other chemical classes (diltiazem, verapamil) were also without effect over a range of concentrations (≤100μM) (data not shown). This is in agreement with the finding of [Robinson and Kendall 1990] who used the more potent DHP, isradipine ((+)PN 200-110) with an equal lack of success. One can conclude that L-channels are unlikely to participate in the interaction between adrenergic receptors.

N-type channels display similar properties as the L-type, inasmuch that they are high threshold activated, and high conducting, but their lifetime of
opening is shorter and more akin to the kinetics of T channels. In addition to the antagonists for L-channels, a handful of toxins from a variety of sources have proven useful tools for the pharmacologist and electrophysiologist alike. A potent 27 amino acid polypeptide isolated from the sea snail *Conus geographus* (ω-conotoxin GVIA, ω-CgTX) persistently blocks N-type channels [Tsien *et al.* 1991], so it was pertinent to employ the toxin in this system. An ω-CgTX dose-response relationship is shown in figure 7 below.

**Figure 7** Effect of increasing concentration of ω-conotoxin upon adrenergic-stimulated cAMP in rat cortex. Slices were preincubated for 30 mins with different concentrations of toxin, and then stimulated with isoprenaline (10μM) or noradrenaline (100μM) for a further 12 mins. Results are mean ±sem of three experiments performed in triplicate. (*** P=0.003 student's t test, unpaired versus control.

Basal and isoprenaline-stimulated cAMP were wholly unaffected by any concentration of toxin used, in contrast to NA, which was sensitive to 300nM ω-CgTX (figure 7). Inhibiting 34% of the NA response, 300nM toxin is a high concentration considering its K_d for binding to synaptic membranes is 0.6nM [Jones *et al.* 1989] - a concentration of 100nM is usually supramaximal. However, one should remember that an indirect measurement of Ca^{2+} channel activity is
being monitored and the system in which these experiments are being conducted is a brain slice. In such a case, penetration and/or clearance (or metabolism) of the polypeptide may be a real problem, reducing its absolute concentration in the slice. However, one would still have to account for an estimated 200-fold discrepancy in potency, should this be mediated by conventional, fully functional ω-CgTX-sensitive channels. The previous experiments have shown that there is no involvement of L-type channels, and so by existing criteria (and with the caveats just discussed) there may be N-type channels functionally coupled to α-ARs.

Figure 8 shows that the potentiative component that is insensitive to 0.3μM ω-CgTX is also α-AR-mediated. By preincubating with the non-selective antagonist phentolamine, the toxin's effects are eliminated, showing that it is an α-related mechanism.

Figure 8 Effect of α-AR antagonism upon adrenergic-stimulated cAMP in the presence and absence of ω-CgTX. Slices were preincubated in the presence or absence of 10μM phentolamine ± 300nM ω-CgTX for 30 mins. Isoprenaline (10μM) or noradrenaline (100μM) were added for 12 mins. Results are mean±sem of three separate experiments performed in triplicate. (**) P=0.02 (unpaired student's t test versus NA ctrl).
If these phenomena demonstrated by Ni\textsuperscript{2+} and ω-CgTX are due to blockade of voltage-sensitive calcium channels, it would be ideal to show that receptor occupation (in particular α-AR occupation) results in Ca\textsuperscript{2+} influx. The ideal experiment, using Ca\textsuperscript{2+}-sensitive fluorescent dyes, is obviously unworkable in slices. Therefore, the uptake of 45Ca\textsuperscript{2+} was monitored in the slices, and any effect of agonists noted (figure 9).

Figure 9 Time course of uptake of radioactive calcium isotope in rat brain slices in the presence and absence of adrenergic receptor occupation. Iso = 10μM; NA = 100μM. 45Ca\textsuperscript{2+} was added simultaneously with agonist or its vehicle, the reaction was arrested ~10 secs before time with 3ml of ice-cold saline. The slices were immediately filtered under vacuum, rapidly washed twice with 3ml ice-cold saline and the filters with adhering slices were extracted overnight with lumasolve-spiked scintillant, and radioactivity determined. Zero time had saline added before 45Ca\textsuperscript{2+}. Results are expressed as mean±SEM of 3 experiments performed in triplicate.

Figure 9 shows that basal equilibration of the radioisotope is rapid and nearly maximal by 2 minutes. Even in the presence of adrenergic agonists, this equilibration is apparently unaffected. That is not to say that NA stimulation does not provoke Ca\textsuperscript{2+} influx, it may just be impossible to determine this effect.
in a small population of cells against such an enormous background of global, basal $^{45}\text{Ca}^{2+}$ uptake. Additional experiments could be performed wherein extracellular $\text{Ca}^{2+}$ is removed (therefore overcoming dilution of radioactive calcium), agonists added and $^{45}\text{Ca}^{2+}$ added as a 'spike' during receptor occupation. However, it is likely that these protocols would be flawed on similar grounds to the above.

Since it is likely that N-type channels are concentrated in the dendrites and cell bodies, but not the axons, of neurones [Jones et al. 1989], synaptosomes were also prepared as a relatively enriched neural source and preloaded with the dye Fura-2 AM. Whilst it was possible to demonstrate depolarisation-evoked rises in intrasynaptosomal free calcium (60mM K$^+$), it was again impossible to demonstrate NA-induced increases in fluorescence (data not shown). Such a lack of a response may be due to many factors, including the heterogeneity problem that prevailed in the $^{45}\text{Ca}^{2+}$ uptake protocol.

The next set of experiments were performed to compare, side by side, the degree of inhibition by Ni$^{2+}$ and $\omega$-CgTX, and the effect of the two together (figure 10).

Both agents alone selectively inhibited the NA response and, coincidentally, by similar amounts (~45%). However, when added together, the effect was rather surprising. A 34% inhibition of the isoprenaline stimulation was observed, and in parallel, a further decrease in the NA (presumably due to the attenuation of its $\beta$ component). The rationale behind carrying out the additivity protocol was not one underlying conventional additivity experiments (elucidation of mechanisms by using maximal doses), it was merely to see if the $\omega$-CgTX could further decrease the Ni$^{2+}$ effect - purely a phenomenological approach to ascertain if the potentiation response could be further ablated with the tools available. It was rather disappointing that selectivity was sacrificed, and disturbing that the $\beta$-AR component was modulated. One cannot, of course, infer that the two agents are acting at the same, or different loci from such data.
Figure 10  Effect of nickel and α-CgTX upon adrenergic stimulation of [3H]cAMP in cortical slices
Toxin was preincubated for 30 mins at 300nM, and 100μM nickel chloride was included in the agonist vehicle. Iso(10μM); NA(100μM) - 12 mins stimulation. Data are mean±sem of 4 separate experiments performed in triplicate. (**) P=0.003 versus iso ctrl. (••) P<0.003 versus NA α-CgTX (or Ni++).

Calcium is a prerequisite for the potentiation response and many mechanisms have been unsatisfactorily implicated. The possible involvement of calmodulin (CaM) has not been addressed in this system. An appealing mechanism for potentiation would be the α-AR-induced rise in cytosolic Ca^{2+} and the stimulation of adenylyl cyclase by Ca^{2+}/CaM [MacNeil et al. 1985]. Chemically dissimilar compounds have long been used as antagonists of CaM, and recently a derivative of the naphthalenesulphonamide W7 has been synthesised which, unlike its congenor, exhibits >300 fold selectivity for CaM-dependent processes over PKC or transglutaminase (another calcium-dependent enzyme) [MacNeil et al. 1988]. The compound is a halogenated derivative, 5-iodo-1-Cg (N-(8-aminooctyl)-5-iodonaphthalene-1-sulphonamide) which is ten times more potent than W7 and judged to be membrane permeable. Trifluoperazine (TFP) has also been used as a CaM antagonist in the past, and was thus included.
in the experiment, despite its reputation for other effects [Wingard et al. 1991]. The results of a preincubation are shown in figure 11.

No significant effect of the selective calmodulin antagonist, 5-iodo-1-C₈ was observed when compared with its vehicle control. The concentration of 30μM was shown *in vitro* to fully inhibit CaM-stimulated PDE activity [MacNeil et al. 1988], and was chosen as a modest concentration that would hopefully minimise significant effects at other sites. A twenty minute exposure to the drug was deemed sufficient access time in view of microscopy data from [MacNeil et al. 1988]. Unlike the more selective compound, TFP reduced NA-stimulated cAMP accumulation by 37%, at the same time leaving basal and the isoprenaline-induced increase untouched. An interference with CaM, or indeed with other Ca²⁺-dependent enzymes cannot be automatically inferred since phenothiazines are also α-AR antagonists in the central nervous system [Wingard et al. 1991]. In the absence of a positive control, it would be naïve to emphatically repudiate CaM-intervention solely on the data in figure 11, as accessibility problems may again prevail (the hydrophobicity of the W7 analogue may mean, for example, that it partitions into membranes and becomes less available for inhibiting the response).

**Discussion**

From the outset, it was intriguing to examine the relationship between cAMP accumulation and the commensurate phosphodiesterase activity in the intact cell. A simple index of PDE activity in agonist-stimulated cells is the rate of decay of cAMP subsequent to antagonist addition; by using different agonist concentrations, a range of cAMP accumulation levels would be generated and therefore the question posed could be addressed. However, the response to β-AR agonists in brain slices is too small to confidently permit this type of analysis. The decay curves are generated after steady-state cAMP levels are attained, and necessitates the assumption that the rate of cAMP synthesis is essentially zero.
after antagonist addition. This precludes the use of forskolin to enhance agonist responses since when receptors are occupied by the excess of antagonist, adenylyl cyclase continues to be stimulated. Another method was sought, and thought to be found in the α-potentiating effect. However, in hindsight, these conditions also rendered decay curves uninterpretable - the processes underlying the α-AR mediated augmentation also would be diminishing with time after antagonist addition, and the fall in cAMP levels might reflect a more complex, composite rate of decay. Nonetheless, the mechanism behind the potentiation was initially examined to assess its potential in this respect.

![Figure 11 Effect of Calmodulin Antagonism Upon Adrenergic Stimulation of cAMP in Rat Cortex](image)

Antagonists (30μM), or their vehicle (DMSO, 1% (v/v) final) were preincubated with labelled slices for 20mins prior to their challenge with 10μM iso or 100μM NA. Reactions were stopped 12mins later. Results shown from 3 experiments performed in triplicate (mean±sem). (**) P = 0.02, Student's t test (unpaired).

The cellular localisation of β-adrenoceptors has been studied by autoradiographic [Burgess et al. 1985], immunocytochemical [Ventimiglia et al. 1991].
1987] and functional means [Atkinson and Minneman 1991]. Although the earlier autoradiography workers concluded that β-ARs were restricted to glial cell populations, the current scheme, drawn from the later papers, supposes that they are also found on neurons in culture. The distribution throughout the cell population is uneven, as is the expression throughout areas of the plasmalemma of a given cell [Ventimiglia et al. 1987, Wang et al. 1989a, Wang 1989b]. Dialogue between adrenergic receptor subtypes has been extensively examined in brain slices as we have seen, and also in dissociated primary cultures of rat cerebral cortical cells from neural or glial origins [Atkinson and Minneman 1991]. The overwhelming conclusion is that enriched populations display differential adrenergic regulation of cAMP levels. In neurons, the response to NA and isoprenaline is indistinguishable, while in glia, the β-adrenergic stimulation is far greater than the mixed α/β challenge [Atkinson and Minneman 1991]. The implication is that β-AR responses predominate in neural cells, whereas in the glial populations there is a balance between β- and α2-AR function. It should be noted that in all these preparations, the typical α1-AR response (inositol phosphate generation) and α2-AR response (inhibition of forskolin-stimulated cAMP) can be observed. Disappointingly, in no type of primary culture cells can an α/β synergy be demonstrated, not even when neural and glial populations are co-cultured [Atkinson and Minneman 1991]. The reasons for this are not obvious, but may lie in the in vitro conditions used to prepare and maintain the cells. Such studies have not shed new light upon the mechanisms behind potentiative phenomena, but they do afford an excellent system to examine the factors that may be involved. In particular, such primary cultures of cells from rat cortex have shown that production of IPx resulting from challenge with NA is possibly mediated by an α1a-AR and may be subsequent to pertussis toxin-sensitive calcium entry - the response was unaffected by nifedipine (a DHP, L channel blocker), but ablated by the inorganic blocker cadmium [Wilson & Minneman 1990].
In the present study, the $\alpha$-AR, $\text{Ca}^{2+}$-dependent potentiation of the $\beta$-AR cAMP response was examined in rat cerebral cortical slices. Since the $\alpha$-ARs can be subdivided on the basis of coupling mechanisms, where one of those mechanisms may be a positive regulation of $\text{Ca}^{2+}$ entry, it was decided to examine whether such an effect could be implicated in the enhancement of the $\beta$-AR response. The working hypothesis was that $\alpha$-ARs may be linked to calcium channels, and that their activation underlies the potentiation of the $\beta$-AR effect.

Calcium channel activity can be modified by application of other cations that bind to, and block $\text{Ca}^{2+}$ entry, and this property was therefore exploited. Nickel has an IC$_{50}$ of 1.5mM for blocking receptor-mediated calcium entry in neutrophils, platelets or endothelial cells [Rink 1990]. However, the sensitivity of calcium channels to ions shows a remarkable variation [Rink 1990], and there are examples of greater sensitivity to nickel from other electrophysiological studies: for example, in mouse N1E-115 cells, conductances with the characteristics of T and L channels were blocked with apparent $k_d$s of 47 and 280$\mu$M respectively [Narahashi et al. 1987]. Contrastingly, 100$\mu$M nickel ions in dorsal root ganglion cells strongly reduced T currents, while N and L were little changed. With such variations notwithstanding, it could be demonstrated that the $\alpha$-AR potentiation component was substantially reduced (~50%) in the presence of 50-100$\mu$M nickel. Although the IC$_{50}$ for nickel in rat cortex cannot be properly defined because of its biphasic effect on cAMP levels (but it may be <1.5mM), it should also be born in mind that an indirect, downstream index of (putative) channel activity is being measured - that of cAMP accumulation - in a system where the concentration of agent at its site of action is indeterminate. That aside, the nickel concentration that is effective here is, generally speaking, within the range of those inhibiting $\text{Ca}^{2+}$ influx, as assessed by more direct measurements.

Although the above is somewhat reassuring, there are a great deal of points to consider before invoking $\text{Ca}^{2+}$-channel antagonism as a viable
hypothesis. For instance, the inorganic ions, Ni\textsuperscript{2+} and Co\textsuperscript{2+}, are indiscriminate in their actions, and the data accrued in this study could be alternatively interpreted: for instance, the 'selectivity' of 100\mu M Ni\textsuperscript{2+} for the \(\alpha\)-AR component of the potentiation could reflect nothing more than a 'non-competitive' type of inhibition of adenylyl cyclase activity: nickel's inhibition may only be manifest upon greater degrees of stimulation of cyclase and the extent of isoprenaline-induced AC stimulation may be insufficient to observe any inhibition. This hypothesis is not inconsistent with the data in figure 5, where both adenosine and forskolin responses were attenuated by lower nickel concentrations. These agents, purported to be 'direct activators' of AC (as defined in this chapter's Introduction), raise cAMP by a similar amount to NA (~10 fold), and are likewise (and consequently?) inhibited. Although it is possible that Ca\textsuperscript{2+}-influx may partly augment the cAMP responses of some agents, it is noteworthy that extracellular Ca\textsuperscript{2+} chelation either enhances or has little effect upon adenosine-stimulated cAMP in rat brain [Schwabe and Daly 1977].

The temporal aspects of the inorganic blockade are comfortingly consonant with their acting at a calcium-dependent locus (figures 3 & 4) and supports the idea that the initial phase of cAMP accumulation is indeed nickel-insensitive. The kinetic profiles with nickel and EGTA may be superimposable, but this again is not out of keeping with nickel having an effect distinct from channel blockade. It ought to be clear that should nickel be able to permeate the cell membrane, then a plethora of inhibition sites become available; potentially, any of the implicated Ca\textsuperscript{2+}-dependent mechanisms. It is therefore crucial to any hypothesis other than the working one that nickel effects entry into the cell. If it does not, then this is strongly suggestive of its affecting calcium permeation by interacting with the extracellular facing channel or transporter. Studies exploiting the quenching properties of Ca\textsuperscript{2+}-sensitive dyes have shown that in some cells e.g. SH-SY5Y neuroblastoma cells (D.G. Lambert - personal communication), platelets [Rink 1990], and lacrimal acinar cells [Kwan & Putney,
Jr., 1990], Ni^{2+} does not measurably penetrate the cytoplasm upon agonist stimulation. This strongly suggests that the effect of nickel in brain slices will be 'extracellular', although the confirmatory evidence is lacking.

Referring back to the debate concerning the intra- or extracellular site of action for nickel: AC is probably a membrane-spanning protein (see Introduction chapter) and therefore Ni^{2+} would not necessarily need to enter the cell to directly interfere with AC function. Such an exofacial site is thought to be the point of interaction and activation by forskolin, since its less membrane-permeant analogues are effective activators [Laurenza et al. 1987] - whether this, or another site affords a reasonable explanation of nickel action is open to conjecture.

Non-competitive inhibition of adenylyl cyclase could initially be addressed by conducting dose-response curves to NA and forskolin, for instance, and seeing if the lower concentrations of the agents were just as sensitive to nickel inhibition. Such results in themselves would be insufficient to prove the point, but would strengthen the supposition, should the effect be manifest only at degrees of stimulation greater than with isoprenaline alone.

More specific pharmacological tools were employed in an attempt to eradicate the side-effects that are inevitable with inorganic ions. The L channel blockers from a variety of chemical classes were without effect, and in conjunction with the negative result with the channel agonist, Bay K 8644, it appeared that the long lasting channels could be eliminated as candidates. Although there was no positive control in these experiments, the concentrations of DHPs applied were known to be efficacious at ablating L channel-mediated phenomena in brain slices [Zernig et al. 1986] and were in agreement with published data [Robinson and Kendall 1990].

N channels display a sensitivity to the peptide ω-conotoxin, and since these channels can contribute to significant changes in Ca^{2+}_i, its application seemed the next logical step. As was seen in figure 7, ω-CgTX did exert an effect
upon adrenergic-stimulated cAMP, albeit at high concentrations. The inhibition was apparently selective for the α-AR component (figures 7 & 8), having no effect upon basal or β-adrenergic-stimulated cAMP levels, but its maximum could not be determined through constraints of availability and stock concentration. How can this result be interpreted? Can one feel confident in assigning the effect to N channel inhibition when a ~200 fold discrepancy can be explained only by an indeterminate concentration at the site of action and/or the indirectness of the assessment of channel activity (see “Results”? This penetration/clearance problem could be clarified somewhat by conducting neurotransmitter release studies in slices (an equally indirect index) and examining the potency of ω-CgTX (a known inhibitor of release [Herdon & Nahorski 1989]). There are no other known loci of action for ω-CgTX, but that is not to say that none exist, and one should be cautious in interpreting the data as being indicative of N channel involvement. To probe the possibility of voltage-dependent calcium channel (VDCC) intervention, it would be prudent to attempt to pharmacologically manipulate plasma membrane potential. By use of potassium channel activators, such as cromakalim [Hamilton & Weston 1989], cell hyperpolarisation could result, which may make the opening of VDCCs less likely. This may well result in an inhibition of the NA, but not the isoprenaline response.

The most unexpected result stemmed from the ‘additivity’ experiments with nickel and ω-CgTX. Apparently, β-AR responses alone could be significantly reduced by the inclusion of the two ‘channel blockers’ together (figure 10). Does β-AR stimulation of the second messenger partly require calcium influx? A subtype of β-AR has been shown to link to Ca^{2+} channels in heart tissue [Brown & Birnbaumer 1990], although EGTA has been shown to have little or no effect upon cAMP accumulation subsequent to β-AR occupation in rat cortex [Schwabe and Daly 1977, Duman et al. 1986]; this makes the result unlikely to reflect calcium channel blockade. Such a conclusion is cause for concern, as one had
hoped that the actions of these two agents were restricted to calcium-dependent mechanisms. By their very having an effect upon β-adrenergic cAMP levels, it implies that the previously gathered data may not in fact be due to antagonism of calcium influx at all. Instead, it may indicate that their modulation of cAMP levels occurs at some site distinct from calcium channels which is more sensitive under conditions of NA, but not isoprenaline stimulation.

To summarise, the opening remarks that signalling mechanisms are less amenable to study in brain slices is excellently borne out by the discussion and data generated in this chapter. The attempts to implicate Ca\(^{2+}\) influx were thwarted by the lack of specific tools and by the nature of the system analysed. By adopting a phenomenological approach, the work may have generated some data that merits further investigation viz the effects of transition metal ions on neural cAMP levels and the implications for possible neurotoxicity of the species. Moreover, the α-AR potentiation of cAMP levels for whole cell PDE analysis is shown to be too complex a phenomenon to be a suitable tool.
Chapter 4 Comparison of modulation of cAMP levels in the human neuroblastoma cell line SH-SY5Y by isobutylmethylxanthine and phosphodiesterase-selective inhibitors

Introduction

Since any given level of cyclic nucleotide accumulation reflects the balance between its synthesis and degradation, it is clear that perturbation of the latter process may lead to alterations in cellular cAMP or cGMP. The potential to manipulate cAMP levels independently of receptor activation has been realised by the development of inhibitors of phosphodiesterases (PDEs), and an array of such agents are now available to selectively inhibit specific PDE isozymes [Beavo 1988] [Nicholson et al. 1991]. However, although attention has recently been focussed upon the improvement of isozyme-selective agents, non-selective PDE inhibition is still usually effected by using some of the earliest developed compounds such as IBMX (1-methyl-3-isobutylxanthine). The methylxanthines are notoriously non-specific with regard to their locus of action, reflected by an increasing number of reports of interactions with sites distinct from PDEs. For example, IBMX is a competitive adenosine receptor antagonist [Smellie et al. 1979, Daly et al. 1981, Nicholson et al. 1989] which displays a $K_i$ in the low micromolar range. Moreover, some studies have shown that methylxanthines also inhibit the adipocyte glucose transporter [Kashiwagi et al. 1983], inhibit rat brain 5'-nucleotidase [Tsuzuki & Newburgh 1975], stimulate adenylyl cyclase by inhibiting $G_i$ [Parsons et al. 1988], inhibit nucleotide diphosphokinases [Lam et al. 1982] and interfere with the extrusion of cAMP from certain cell types [Nemecek et al. 1980]. With such a plethora of activities, it is perhaps surprising that IBMX is still routinely used as a PDE inhibitor, particularly when its other actions often have a direct bearing upon cyclic nucleotide metabolism. The following study compared the degree of cAMP elevation in SH-SY5Y cells after application of IBMX or the low $K_m$ cAMP PDE (type IV) - selective PDE inhibitors such as Ro 20-
1724 or rolipram, initially with a view to ascertaining the functionally significant PDE isozymic forms.

**Results**

Figure 1 shows the effect of increasing concentrations of phosphodiesterase inhibitors on cAMP accumulation in SH-SY5Y cells in the absence (upper panel) and the presence (lower panel) of forskolin. The most striking observation is the difference in effectiveness of the 'non-selective' agent IBMX and the other inhibitors, whether non-selective (papaverine) or selective for the low $K_m$ cAMP PDE: whereas 1mM IBMX raises basal levels of cAMP by four-fold, the type IV inhibitors at maximally, or near maximally effective concentrations, elicit a much larger 21-69 fold increase. Papaverine is too weak an agent to observe its maximum, but at 300μM it raises cAMP some 16 fold. Such a substantial increase in cAMP in the unstimulated cells was unexpected and indicates that the turnover of the messenger is rapid, even under basal conditions.

Forskolin itself enhances accumulation of cAMP by ~20 fold, but nevertheless the disparity between IBMX and the other inhibitors is still evident in its presence, whilst being qualitatively different. The low plateau seen with denbufylline under basal conditions is no longer present and thus it becomes as 'efficacious' as the other selective inhibitors. Papaverine, moreover, is also notably more effective with forskolin treatment and increases the cAMP a further 7 fold over the AC activator.

The rank order of potency was the same for the chemically disparate type IV inhibitors, whether under basal or stimulated conditions (rolipram = denbufylline > Ro 20 1724). Such a large discrepancy in the effects of IBMX relative to the other compounds was interesting, and was thus pursued.
Figure 1 Effect of selective and non-selective PDE inhibition upon cAMP levels in basal and forskolin-treated cells. Various PDE inhibitors, or their DMSO vehicle (0.8% v/v) were
preincubated with cells for 20 mins. Forskolin (1μM) was then added to half the samples for a further 10 mins and acid arrested. Cyclic AMP was determined by the mass assay of Brown (Methods). Roli = rolipram; Ro 20 = Ro 20 1724; DBF= denbufylline; IBMX = isobutylmethylxanthine; Fk= forskolin. Basal cAMP = 11.59±2.34; 1μM forskolin = 255.51±647.96; 1mM IBMX = 52.85±19.99; IBMX/Fk = 531.93±194.04 pmol/mg protein. Results are mean±sem for 3-5 experiments performed in triplicate.

It was first necessary to ensure that the 20 minute exposure to inhibitors was sufficient to permit a maximal effect of each concentration to be reached - it was conceivable, although unlikely, that IBMX was somewhat delayed in its action. By varying the length of the preincubation period (figure 2), it was observed that the enhancing effect of either IBMX or rolipram was maximal by 10-20 minutes, be it under basal or stimulated conditions, and therefore the reason for the differential was not kinetic.

What explanation could be forwarded to explain the observation? An initial thought was that the PDEs present in the neuroblastoma cells were relatively insensitive to IBMX compared to the other inhibitors. A recent report suggested that a cAMP-PDE could be isolated that was insensitive to IBMX [Lavan et al. 1989], but it was later shown that its properties were more akin to a cCMP-hydrolysing PDE [Worby et al. 1991]. Therefore, this would have to be a property unique to the PDEs of this system, and despite its lack of appeal, the conjecture had to be eliminated. Therefore, to clarify that the PDEs present displayed a normal sensitivity to inhibition, a cell free preparation was examined (figure 3).
Figure 2 Effect of varying the time of exposure to PDE inhibitors upon cAMP levels. A cell suspension was dispensed into vials (at t=0) containing buffer ± inhibitors (or vehicle) and incubated for the times indicated. Forskolin (1μM) was then added to the appropriate samples for only 2 mins. Rolipram=100μM; IBMX=1mM. Basal cAMP = 7.03±1.89; IBMX/Fk = 214.65±23.89 pmol/mg protein. Data represent mean±sem of 3 experiments performed in triplicate.
Figure 3 Inhibition of phosphodiesterase activity in a homogenate of SH-SY5Y cells by a variety of selective and non-selective inhibitors. Homogenate was prepared as in the 'Methods' and represents...
total cellular PDE activity (soluble plus particulate). Assay was conducted with 1μM cAMP as substrate for 15mins at 37°C. All drugs were dissolved in DMSO (~7% v/v final). All data are mean±sem of 3 experiments performed in triplicate except for the theophylline data set which was semirange of 2 experiments performed in triplicate. Control values were 1412±180 pmol adenosine produced/15 min/mg protein (n=15 observations in triplicate).

As predicted, the PDE activity of SH-SY5Y cells was indeed susceptible to inhibition by a variety of pharmacological agents. Both IBMX and papaverine, non-selective PDE inhibitors, ablated the activity by ≥95% and with concentration-response curves which had slope factors approximating to unity. Theophylline, another xanthine-derived, non-selective inhibitor also affected hydrolysis rates, but less potently than IBMX. By contrast, the isozyme-selective inhibitors only partially diminished the PDE activity, by some 70%, and with shallow curves. This is suggestive of the low K_m cellular PDE activity predominating at low cAMP concentrations, while another 30% of the activity is attributable to indeterminate species. This is probably not the cGI-PDE (type III) since milrinone did not inhibit cAMP hydrolysis except at concentrations ~300 times its IC_{50} for this enzyme (with 1μM cAMP, IC_{50} for cGI: 0.3μM [Beavo 1988]) where its specificity is questionable (IC_{50} for type IV PDE 40μM (ibid.). Comparing the whole cell data with this cell free analysis, one can see that the order of potency of the agents is preserved: rolipram<denbufylline> Ro 20 1724>>IBMX (see table I)

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>EC_{50}(basal cell)</th>
<th>EC_{50}(forskolin cell)</th>
<th>IC_{50}(Homogenate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBMX</td>
<td>&gt; 100</td>
<td>&gt;100</td>
<td>29.4±8.2</td>
</tr>
<tr>
<td>ROLIPRAM</td>
<td>8.74±2.78</td>
<td>4.23±1.3</td>
<td>0.53±0.06</td>
</tr>
<tr>
<td>Ro-20 1724</td>
<td>30.0± 10.8</td>
<td>24.2±4.5</td>
<td>4.69±0.69</td>
</tr>
<tr>
<td>DENBUFYLLINE</td>
<td>0.78±0.21</td>
<td>1.50±0.16</td>
<td>0.21±0.04</td>
</tr>
</tbody>
</table>
Table 1 Comparison of PDE inhibition data from intact cell and cell-free homogenate. Values are presented as the EC\textsubscript{50} or IC\textsubscript{50} (in \textmu M) summarising the experimental data in figs. 1 and 3; as calculated by the iterative curve fitting program 'Allfit', fitting for one site with no fixed parameters.

Moreover, the potency of the inhibitors is greater in the \textit{in vitro} assay by about an order of magnitude for the selective compounds, whilst for IBMX the disparity is impossible to ascertain. Certainly, the overwhelming conclusion is that IBMX is fully capable of inhibiting the majority of PDEs at low substrate concentration, and therefore its lack of effectiveness must lie in some other property of the drug.

A most obvious difference between IBMX and the type IV inhibitors lies in its ability to inhibit cGMP as well as cAMP hydrolysis. During the early days of cGMP research, a hypothesis was in vogue that cAMP and cGMP were antagonistic inasmuch that elevation of one led to the diminution of the other-the so called "Ying Yang" hypothesis [Goldberg & Haddox 1977]. While this rationalisation of the existence of cGMP has fallen from favour, the fact remains that in some systems this antagonism may prevail [Goldberg and Haddox 1977]. Such opposition by cGMP may explain the results obtained here. The fact that papaverine, another non-selective inhibitor augments cAMP levels by a similar degree to the type IV inhibitors argues against this; however, the whole cell data for both non-selective agents do not attain a maximum effect and so this possibility cannot be totally excluded. Thus, a study of the effects of IBMX and rolipram on cGMP levels was undertaken. As can be seen in figure 4, neither compound had any profound effect upon cGMP levels, with certainly little difference between them. One can infer that the basal turnover level of cGMP is very low, in contrast to that of cAMP, and that the poor effect of IBMX on cAMP is not attributable to potentially antagonistic rises in the other cyclic nucleotide.
Figure 4 Effect of PDE inhibitors upon cGMP mass levels in SH-SY5Y cells. Cells were preincubated with DMSO or inhibitors for 20 mins prior to a 10 mins stimulation with 1 μM forskolin (Fk). Rolipram = 100 μM. Samples were acid quenched and assayed for cGMP as in ‘Methods’. Results expressed as mean±SEM of 3 experiments performed in triplicate.

Now that the conventional PDE behaviour of IBMX was more fully tested, it was time to turn attention to the effects of methylxanthines other than PDE inhibition. Adenosine receptors in the central nervous system can be positively coupled to adenylyl cyclase through Gs and lead to an augmented production of cAMP [Reddington et al. 1983]. Should such receptors be present upon this cell line then feasibly, any adenosine released by the cells could have an autocrine effect and stimulate second messenger production. The presence of a cAMP PDE inhibitor would clearly potentiate this apparently ‘basal’ turnover and explain the large increases in cAMP that we observe. IBMX is also a potent adenosine receptor antagonist [Smellie et al. 1979] and therefore the effects of AC
activation will be antagonised and the ‘true’ basal turnover would be unmasked. Hence, adenosine was administered to cells under a variety of conditions to verify or refute the presence of positively coupled receptors (figure 5).

Figure 5 Effect of adenosine upon cAMP accumulation under sensitizing conditions DMSO or 10μM rolipram (roli) were preincubated with cells for 20 mins. Forskolin (1μM - 'Fk'), when appropriate, was added simultaneously with the differing adenosine concentrations and all agonist additions were stopped after 13 mins. The inset represents data from forskolin plus rolipram. Basal cAMP = 3.93±1.13; roli/fk = 1667±168 pmol/mg protein. Data presented are means±sem of 3-4 experiments performed in triplicate.

Adenosine alone caused no significant rise in cAMP over basal up to 100μM. Forskolin was included in some incubations since it is known to greatly potentiate the response to Gs-linked agonists [Seamon & Daly 1986], and thus any response would be greatly magnified. That too was ineffective at
highlighting an adenosine response. In the presence of a modest, submaximal concentration of rolipram (not an adenosine receptor antagonist) no increase was noted unless forskolin was included, and under such extreme conditions a very small 60% increment was observed at the highest adenosine concentration. In the light of these results it was concluded that the cells were unlikely to express functionally coupled adenosine receptors. This apparently contradicts the literature wherein one group reports of adenosine-stimulated cAMP in these cells [Mei et al. 1989a]; however, these data do not stand up to closer scrutiny as the experiments were performed in the presence of 0.5mM IBMX, a concentration about 50 times its $K_i$ for adenosine receptors, and one that should substantially eliminate adenosine occupation of the receptor.

Although the elimination of cAMP is primarily effected by PDE activity, cells are also capable of reducing messenger concentration by actively extruding the molecule from the cytoplasm. The degree by which egress contributes to a reduction in intracellular cAMP varies from cell to cell [Brunton & Mayer 1979, Barber & Butcher 1983], and in some systems, methylxanthines can inhibit this process [Nemecek et al. 1980]. In these neuroblastoma cells, application of PDE inhibitors results in a very large increase in cAMP, but its localisation has to date not been established. The experiments are normally conducted in such a way that no attempt is made to discriminate between intra- or extracellular cyclic nucleotide. If IBMX (and not the other inhibitors) was to inhibit a significant efflux of cAMP, then the messenger would be confined to the compartment in which PDEs are located - hydrolysis is never completely ablated by PDE inhibitors, and so cAMP catabolism could still be maintained, and in view of the enormous degree of flux, possibly build up to concentrations that are capable of overcoming the competitive blockade by IBMX. With the other compounds, cAMP would be free to escape from the cell and consequently be inaccessible to the intracellular PDEs. It was therefore of importance to ascertain
the compartment in which the product resides. Such an experiment is shown in figure 6.

Figure 6: Assessment of Localisation of cAMP After PDE Inhibition

Experiments were performed in microfuge tubes, otherwise the protocol essentially followed that for earlier experiments i.e. preincubation for 20 mins with DMSO (1.6% v/v) or inhibitor (rolipram=100μM; IBMX=1mM). 1μM forskolin was added for 2 mins where indicated. For intracellular cAMP determination ('Intracell'), samples were centrifuged for 15 secs at 11,000g, the supernatant aspirated, and acid added to the cell pellet. For 'total' samples (intra- plus extracellular cAMP), tubes were centrifuged as above, but the supernatant was not removed when acid was applied. Basal cAMP: total = 4.48±1.63, intra = 3.65±1.77; IBMX: total = 13.46±5.26, intra = 11.99±3.83 pmol/mg protein. Data are means±sem of 4 experiments performed in duplicate or triplicate.

Figure 6 clearly indicates that the majority of the cAMP measured in the previous experiments was contained within the cell. Whether the increase in cAMP was elicited by PDE inhibition, forskolin or a combination of both, over
80% was found to be intracellular. The apparently large difference between treatments for rolipram plus forskolin (roli/fk) is exaggerated by the axis break on the graph, and the proportions are in fact about the same as the other conditions. In spite of a small discrepancy between total and intracellular measurements, the data convincingly demonstrate that regulation of egress cannot underlie the IBMX phenomenon (e.g. compare IBMX and Roli). The most interesting result stems from the simultaneous incubation of the inhibitors: when IBMX is included with rolipram, with or without forskolin (IBMX/roli & IBMX/Fk/roli), the large enhancement by the selective inhibitor is lost, almost to that observed with IBMX alone. This was elaborated upon in the next set of experiments.

One feels fairly confident in ascribing the disparity of inhibitor responses to a non-PDE effect of IBMX. If it is indeed acting at a locus distinct from phosphodiesterase inhibition, then what would its effect be upon cAMP levels pre-elevated by rolipram? Additivity experiments were performed to address this question (figure 7).
Figure 7 Effect of IBMX on rolipram-elicited increases in cAMP. Cells were incubated for 20 mins with 100µM rolipram plus various concentrations of IBMX. Forskolin 1µM was then added for another 10 mins where indicated. For ‘IBMX alone’, a 1mM dose was used. Rolipram alone = 77.3±41.95, rolifk = 1057±653 pmol cAMP/mg protein. Results are expressed as mean±sem of 3-4 experiments performed in triplicate.

IBMX thus dose-dependently antagonised the increases in cAMP produced by the type IV inhibitor, rolipram. Under unstimulated and forskolin-stimulated conditions, IBMX was more potent at inhibiting the rolipram response than it was at increasing cAMP itself, with IC50s lying in the 4-10µM range. The curve was biphasic when forskolin was absent, lower IBMX concentrations inhibiting the response, and higher concentrations of IBMX once again raising cAMP levels - at 30 or 100µM, the cAMP accumulation was not significantly different from that with IBMX alone (P=0.21 and 0.13 respectively, Student's t test), whereas by 1mM, the upward concavity was significant.
The increase of cAMP seen with higher IBMX concentrations is probably a result of its PDE-inhibiting properties starting to dominate. However, this biphasic effect was not observed under conditions of AC activation - a maximum inhibition was reached and maintained by 30μM that was statistically different from the IBMX plus forskolin (P=0.006 for 300μM).

The data in figure 7 illustrate that the methylxanthine decreases cAMP responses resulting from selective PDE inhibition, but does not address the issue of whether a change in rolipram potency or effectiveness is the cause. Therefore, keeping IBMX concentration constant, rolipram concentration-response curves were generated to assess this (figure 8).

The inclusion of 10μM IBMX dramatically reduced the rolipram response as predicted, but in a manner that diminished the maximum effect, and not the potency (as judged by no apparent change in the EC$_{50}$ for rolipram upon IBMX addition). Therefore, the accessibility of the active site of the type IV PDE to rolipram is not impaired by IBMX. Moreover, the difference in the IC$_{50}$ of IBMX for inhibiting stimulated as opposed to unstimulated cAMP responses (figure 7) is borne out by the data in figure 8 - 10μM IBMX obviously inhibits the forskolin-treated cells less effectively than the untreated cells.
Figure 8 Effect of IBMX on rolipram concentration-response relationship. Cells were incubated with varying inhibitor combinations for 20 mins. IBMX (10μM) or DMSO vehicle were co-incubated with
varying rolipram concentrations, and stimulated cells (lower panel) were exposed to 1μM forskolin for 10 mins. Upper panel represents unstimulated cells, the lower, forskolin treated. Data in each panel are expressed as mean±sem of 3-4 separate experiments performed in triplicate.

The experimental protocols adopted thus far have included IBMX and rolipram together for 20 minutes, and this gives no indication of the rate of onset of the IBMX inhibition. This begs the question, how long an exposure to IBMX do the cells require to see this inhibitory effect? Cells were therefore pretreated with rolipram, and IBMX added for varying periods of time thereafter (figure 9).

Figure 9 Time course of IBMX-induced decay of cAMP levels elevated by rolipram. 100μM rolipram was incubated with cells for 20 mins, after which ±1μM forskolin was applied for exactly 10 mins. Following stimulation time, IBMX was introduced at t=0 on the graph and the subsequent decay followed for the times indicated. For unstimulated cells, IBMX=10μM; for forskolin-stimulated
Almost immediately after IBMX addition (within the first minute), cAMP levels were seen to decrease, irrespective of the presence or absence of forskolin. Nonetheless, the full IBMX effect was still not realised after 10 minutes, if compared to samples in which IBMX had been present from the start of the rolipram preincubation period. Such a slow decay is perhaps not surprising considering that the maximal concentration of inhibitor (rolipram) substantially reduces cellular PDE activity; even if IBMX were to instantaneously exert its effect, the rate of cAMP elimination would be relatively slow, which is commensurate with the data presented here.

Thus far we can see that IBMX is not as capable of raising cAMP levels in SH-SY5Y cells as other inhibitors. This does not stem from an inability of IBMX to inhibit the cAMP PDEs or from its potential to increase cellular cGMP, but rather from another distinct effect of the methylxanthine. The secondary locus of action appears to antagonise cAMP synthesis in a complex fashion, and is not reminiscent of adenosine receptor antagonism. Nor is it a consequence of the modulation of cAMP egress, as the vast majority of cyclic nucleotide is restricted to the cell interior. That another locus is involved is clearly illustrated by the series of additivity experiments - IBMX potently and rapidly inhibits cAMP accumulation elicited by the type IV inhibitor, rolipram. At this stage, it seemed that another, perhaps novel site of action of IBMX was mediating the effect, since its reported ability to antagonise a Gi coupled to AC would enhance, and not diminish responses. Accumulation levels in the presence of IBMX may in fact reflect a balance between the two antagonistic effects - that of PDE inhibition and the unknown, 'restraining' effect.
Therefore, the next question that was posed was how unique was IBMX in this regard? Denbufylline, a type IV selective agent, effectively increased cAMP levels (figure 1) despite the fact that it too is a substituted alkylxanthine (1,3-di-n-butyl-7-(2'-oxopropyl)-xanthine). The analysis of PDE activity in cell homogenates indicated that theophylline (1,3-dimethylxanthine) was a weak inhibitor of cAMP hydrolysis, making it an obvious candidate for whole cell studies. Figure 10 shows the effect of increasing theophylline concentration upon basal and forskolin-stimulated cAMP accumulation.

In a dose-dependent fashion, theophylline attenuated cAMP accumulation under basal, and more clearly, under forskolin-stimulated conditions. No PDE IV inhibitors were present like in figure 7; the inhibition is manifest in cells with relatively low cAMP levels. This agent therefore probably represents one that acts primarily at the inhibitory, methylxanthine locus with little compensatory PDE inhibition - hence the decrease in cAMP, and not the steady plateau that we observe with lower IBMX concentrations (figure 1). An effect on basal cAMP levels is noisy, but relatively clear at maximal concentrations, while the larger window provided by AC stimulation affords a clearer, monophasic curve (IC\textsubscript{50} = 17.5\textmu M, Graphpad Program). The maxima apparent in both cases needn’t necessarily reflect a saturation of this unknown locus as PDE inhibition may prevail at higher concentrations (see biphasic effect of IBMX in figure 7).
Figure 10 Concentration-response relationship between theophylline and cAMP accumulation
Preincubation for 20 mins with theophylline/DMSO, or IBMX reference (1mM). Forskolin (1μM)
was added thereafter for 10 mins. Basal cAMP = 3.34±0.45, 300μM theo = 1.96±0.38; forskolin = 30.89±4.59, forskolin/300μM theophylline = 9.40±2.21 pmol/mg protein. Data represent mean±sem of 3 experiments performed in triplicate.

That theophylline affected cAMP levels in this way gave great cause for concern - in isolation, the data would be highly suggestive of adenosine receptor antagonism, since theophylline is also a reasonable competitive antagonist (Kᵢ at A₂ receptors of 24μM [Nicholson et al. 1989], similar to the IC₅₀ obtained here). Before suggesting that a novel effect of IBMX was responsible, it was pertinent to reexamine the involvement of adenosine. A manipulation that had not yet been tested was inclusion of the adenosine-eliminating enzyme, adenosine deaminase which converts the nucleoside to inosine, a poor receptor agonist. Additionally, the effects of the adenosine receptor agonist 2-chloro-adenosine (2-CA) were examined (figure 11 and inset).

A range of ADA concentrations had no measurable effect upon basal or IBMX-modulated cAMP levels. However, the increases in cAMP seen with forskolin or rolipram exposure displayed a sensitivity to the enzyme treatment, where the AC stimulator was seemingly maximally inhibited by ~35%, and rolipram by ~50%. An attempt to raise basal cAMP levels with the adenosine analogue proved fruitless over a concentration range known to significantly activate receptors [Bazil & Minneman 1986]. Superficially, the results implicate an adenosine receptor-mediated mechanism, contrary to the conclusion drawn from data in figure 5. However, there are several points to clear up: firstly, the maximal ADA ablation did not completely reduce the rolipram response to that of IBMX alone - does this suggest that while adenosine may have a hand in the disparity, it is by no means the only underlying factor? Secondly, if adenosine receptor activation is involved, why was the metabolically resistant analogue 2-CA ineffective at stimulating cAMP production, like the
natural congenor? These will be clarified in the following experiments and in the discussion.

Figure 11 Effect of adenosine deaminase (ADA) and 2-Cl-adenosine upon cAMP levels following stimulation or PDE inhibition. Cells were preincubated for 20 mins in the presence of varying ADA concentrations or its vehicle, glycerol (1.6%, v/v); PDE inhibitors or their DMSO vehicle were likewise included (rolipram=100μM, IBMX=1mM). Forskolin (1μM) was added for 10 mins. Inset: dose-response relationship between 2-Cl-adenosine and cAMP levels - abscissal units as per main figure. Roli = 79.56±36.84, roli/ADA = 31.37±11.88 pmol cAMP/mg protein. Data represent means±sem of 3-4 experiments performed in triplicate.

If ADA incompletely blocks the rolipram response, then is the ADA-insensitive component still susceptible to IBMX inhibition? Or put another way, how does ADA affect IBMX inhibition of rolipram-elevated cAMP levels?
Figure 12 Effect of adenosine deaminase (ADA) upon the IBMX inhibition response in the presence of rolipram. Cells were preincubated for 20 mins with or without ADA (2U/ml - or glycerol),
rolipram (100µM), varying IBMX concentrations (or DMSO). Forskolin (1µM) was applied for 10 mins. Roli alone = 184.06±58.72, roli/fk = 1393±417 pmol cAMP/mg protein. Data presented as means±sem of 3-4 experiments performed in triplicate.

In the upper panel of figure 12, IBMX effectively removed the ADA-insensitive component of the rolipram response, and with a potency not dissimilar to that in the absence of ADA - however, the window for the data is smaller and should, therefore, be cautiously interpreted. The lower panel demonstrates that 2U/ml of ADA is not as effective at reducing the response in the presence of AC activation and commensurately higher cAMP levels. Whether this reflects a sub-maximal activity of ADA being used (data in previous figure did not address combinations of forskolin and PDE inhibitors) or a real difference in the proportion of an ADA-insensitive component is not certain. That aside, the potency and effectiveness of IBMX was unchanged by ADA treatment. This is consistent with either (i) there are two loci which IBMX affects (the second one independent of adenosine, but inhibited equipotently) or (ii) that enzymic elimination is only partially effective whilst antagonist addition is completely capable of removing the adenosine component.

The above experiment examined the ADA-insensitive component, but what of the ADA sensitive component - in order to say with a degree of certainty that adenosine (and not some other species) is being eliminated or made unavailable, then one should first endeavour to reconstitute the full response with an adenosine analogue that is ADA resistant e.g. 2-chloro-adenosine. Figure 13 shows the results of such an experiment.
Figure 13 Effect of 2-chloro-adenosine upon basal and rolipram-elevated cAMP levels in the presence and absence of adenosine deaminase. After a 20 minute preincubation ± 2U/ml ADA,
±100μM rolipram, cells were stimulated for 10 mins with a range of 2-CA concentrations. 2-CA was dissolved in 50% DMSO (1.6% v/v final). Basal control = 15.62±6.86, basal/ADA = 6.79±0.02; roli = 180.70±51.59, roli/ADA = 104.85±4.57 pmol cAMP/mg protein (mean±range). Data represent means of 2 experiments performed in triplicate.

In agreement with previous data, the upper panel indicates that 2-CA concentrations up to 10μM have no significant effect upon cAMP production. However, by the inclusion of ADA, basal levels are apparently lowered in these experiments (unlike figure 1), thus unmasking a stimulatory effect. Moreover, higher adenosine analogue concentrations do elicit a stimulation of production, even in the absence of ADA. Rolipram responses apparently display a variable sensitivity to ADA across experiments, in these, an approximate 40% reduction was observed. But the overwhelming message from the lower panel is that 2-CA realises its potential to stimulate cAMP after PDE inhibition, irrespective of ADA treatment.

Discussion

The effect of selective and non-selective PDE inhibitors on cAMP accumulation in SH-SY5Y cells was initially examined to gain some insight into the isozymes of PDE that were functionally significant in these neuroblastoma cells. However, from the first simple experiments, it became clear that there was a surprising difference between IBMX and other potential PDE inhibitors (figure 1). The elevation of cAMP by IBMX was much lower than that elicited with the other agents. If any difference had been predicted, it would have been that IBMX (inhibiting all PDEs) would be more effective than the type IV inhibitors (inhibiting a presumably smaller enzyme population). This disparity formed the basis of further study.

Elimination of the more obvious causes for the lack of efficacy of IBMX was the rationale behind the next set of experiments. The first confirmed
that there were no temporal differences in the actions of rolipram and IBMX (figure 2), and that indeed, both were effective inhibitors of cAMP-PDE activity in vitro (figure 3). Given that IBMX, as a non-selective PDE inhibitor, could also enhance cGMP accumulation, it was feasible that elevation of this second messenger could diminish an increase in cAMP. However, no increases were observed in cGMP levels (figure 4), and once this possibility was dismissed, attention was then turned to effects of IBMX other than PDE inhibition. The primary initial candidate was adenosine receptor antagonism. Application of the ligand under a variety of conditions designed to expose a significant adenosine response proved unsuccessful, and therefore its intermediacy was discounted.

With the supposed dismissal of the more well known effects of methylxanthines, the study assumed a far more interesting aspect. It was therefore possible that IBMX was exerting its effect on cAMP metabolism at a locus distinct from those previously reported. The challenge to dissect out the point of interaction was readily taken on, and a more exact description of IBMX effects began to slowly emerge. It potently and rapidly reduced the maximum effect of the non-xanthine PDE inhibitor, rolipram, supporting its action at a non-PDE locus. A point was reached during this study when it was decided to begin to analyse structure/function relationships. Since a variety of differently substituted xanthines were available to the laboratory, the study could be quite thoroughly performed. However, the first result with a simple xanthine, theophylline, was a surprise. The fact that it lowered cAMP in naïve or forskolin-stimulated cells was reminiscent of adenosine receptor antagonism. The issue of the involvement of adenosine had to be reexamined.

Upon inspection, the subsequent data generated to address the adenosine issue were confusing, and not easily explained. The initial experiment precluded adenosine intermediacy on the grounds of a lack of effect of the natural ligand on cAMP levels under a variety of conditions designed to highlight its ability to stimulate (figure 5). This conclusion was also strongly
supported by the fact that the selective inhibitor, denbufylline, is an alkylxanthine which also acts as an adenosine receptor antagonist [Nicholson et al. 1989]; its $K_i$ is only ~4 fold greater than that of IBMX at $A_2$ receptors (46 and 10µM respectively [Nicholson et al. 1989], and yet it effectively augmented forskolin-stimulated cAMP accumulation, even at 10-100µM (figure1). Hence, one felt reasonably confident in eliminating this effect until the theophylline data were generated.

Primary experiments with the enzyme adenosine deaminase confused the issue (figure 11), to the extent that perhaps a rôle for adenosine did prevail after all, but whether the complications arising from its action were the whole story was unclear: ADA did not completely reduce the large rolipram response to that of IBMX, even at the apparently maximally effective activity of 5U/ml. Hence, it could be proposed that a small, but significant activation of adenosine receptors occurred that was magnified by the presence of forskolin or rolipram (explaining the small change in basal cAMP levels with ADA). The maximal activity of the exogenous ADA is quite considerable, and therefore, the equilibrium between receptor-bound and 'free' adenosine would be drawn towards the latter in the presence of the enzyme since the receptor affinity for its ligand is relatively low; the levels of the nucleoside after ADA treatment would be predicted to fall to submicromolar, which would not significantly activate receptors. This alone led to the conclusion that only a partial rôle for an autocrine effect of adenosine complicates the IBMX results.

There have been reports of IBMX and other xanthines actually stimulating PDE activity (around two fold) at low concentrations (by activation of the cGS-PDE [Erneux et al. 1982, Yamamoto et al. 1983a]). Could this

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if a unit of enzyme activity is defined as 'µmols substrate utilised/minute', then the 5U/ml adopted in these experiments would be capable of maximally eliminating the equivalent of 5mM adenosine every minute
phenomenon contribute to the lack of effectiveness of IBMX? It is unlikely for several reasons: first, at higher IBMX levels, the normal inhibitory actions of the xanthine predominate [Erneux et al. 1982, Yamamoto et al. 1983a], and even at 1mM IBMX, its effect is poor in these cells. Second, theophylline efficiently diminished basal and forskolin-stimulated cAMP, and theophylline is a weak activator of cGS-PDE [Yamamoto et al. 1983a]. Third, this activation by xanthines is only observed with low substrate concentrations (~1μM) [Erneux et al. 1982, Yamamoto et al. 1983a], whilst in SH-SY5Y cells, IBMX dramatically inhibits the elevation of cAMP after 100μM rolipram (which raises cAMP about 50 fold).

To reiterate, the fact that denbufylline, also an adenosine receptor antagonist, was capable of greatly elevating cAMP levels whereas IBMX could not, further mandated for another locus of action for IBMX distinct from adenosine receptors. Moreover, both compounds have a poor affinity for the adenosine uptake site (K_i 200-700μM) [Nicholson et al. 1989] which eliminated potential differences derived from dissimilar nucleoside clearance.

However, not all the data are consonant with this simplistic scheme of a partial adenosine contribution. For example, figure 5 revealed no effect of adenosine up to 100μM, and thus it would be expected that additional ligand addition would elicit a further response. By contrast, it was later found that 2-CA was indeed an agonist, more notably under conditions of PDE inhibition by rolipram (c.f. figures 5 and 13). The fact that 10μM rolipram was employed in the earlier experiment and 100μM in the later is unlikely to explain the difference - 10μM is a concentration that substantially inhibits cAMP hydrolysis (figure 3) and receptor activation should thus be reflected by a rise in cAMP levels. Since adenosine is the natural ligand, one could suggest that, upon addition, its uptake and/or metabolism was sufficiently great (to cope with 100μM) that it was unavailable for receptor occupation. If that was the case, then surely the endogenous adenosine released would likewise be removed and therefore be incapable of mediating the effect in the first place.
Can these agonist differences be explained in the light of the previous ADA results with the contention that adenosine is a complication, but is not completely involved? Unless adenosine and its congenor interact with different receptors, it cannot (2-CA shows only a modest 7 fold difference in affinity for \( \text{A}_1 \) versus \( \text{A}_2 \) receptors [Bruns et al. 1986]), which necessitates a more critical appraisal of the data as a whole. It can be proposed that adenosine receptor antagonism is the major factor, provided one envisages that the amount of adenosine released is sufficiently great that the receptors are always fully occupied. Hence, addition of exogenous adenosine cannot elicit a further response. Furthermore, should the extrusion rate be so great that, when added, ADA cannot efficiently dispose of the ligand, then the incompleteness of the effect of the enzyme at 5U/ml is explicable. Indeed, it has been previously implied that ADA at 10U/ml cannot eliminate adenosine completely from membrane preparations [Linden 1989]. Another assumption required for this new model is that the analogue 2-chloro-adenosine is a full agonist, whilst adenosine is not. Such a suggestion has been previously forwarded by others [Daly 1982, Bazil & Minneman 1986], and although not proven, is an attractive explanation for the difference in agonist effectiveness. Thus, in control cells, addition of 2-CA, resulted in a proportion of receptors being reoccupied by the full agonist, as it exchanges with the bound adenosine, and a response can be elicited. These naive cells require a significantly larger fraction of receptors to be occupied by 2-CA to observe a stimulation, and thus only at higher doses (≥100\( \mu \)M to compete with high adenosine levels - figure 13) will this be sufficient. In the presence of rolipram which magnifies responses, smaller fractional occupation by 2-CA is necessary for a stimulation to be manifest.

With this model of complete adenosine dependency, it is still difficult to reconcile the effectiveness of denbufylline at raising cAMP when compared to IBMX. This doubt is based upon the authenticity of the receptor-binding characterisation of denbufylline [Nicholson et al. 1989]. Unfortunately,
the radioligand displacement curves with denbufylline were not presented, with only the $K_i$ value being quoted, and so its true profile cannot be fully appreciated. Moreover, if it is considered that the receptors are fully occupied by adenosine (normally equivalent to $\geq100\mu M$), then the $IC_{50}$ of the theophylline inhibition would be expected to be of the order of $250\mu M^*$, more than ten fold greater than that actually observed.

To conclude, it seems that most of the data are congruent with the notion of endogenous adenosine potentiating the accumulation of cAMP elicited by the selective PDE inhibitors, whilst IBMX is a less effective agent because of its intrinsic receptor antagonist properties. This conclusion is not proven, and is admittedly at odds with some results, but seems the most viable hypothesis at this stage. Ligand binding studies in membranes prepared from these cells would unequivocally verify the presence and nature of adenosine receptors and, importantly, the affinity of the xanthines, and an assessment of extracellular nucleoside would then lend support to the conjecture that full occupation of receptors prevails. If such studies indicate that adenosine is not fully responsible then the more interesting possibility of IBMX acting at yet another locus remains.

* using the equation $K_i = \frac{IC_{50}}{[\text{ado}]}$, where the $K_i$ of theophylline $= 24\mu M$ [Nicholson, 1989 #274], $IC_{50}$ for adenosine-stimulated cAMP is estimated as $10\mu M$

[ado] = concentration of adenosine (assumed to be $100\mu M$), and the $EC_{50}$ for adenosine-stimulated cAMP is estimated as $10\mu M$. 128
Chapter 5 Assessment of cAMP Turnover in Nervous Tissue

Introduction

While a great deal of attention has been given to the investigation of PDE activities in vitro, less work has focused upon their contribution to modulating cAMP levels in the whole cell. The intact cell naturally affords the most complex system, with compartmentation and desensitisation being some of the processes that complicate predictions of cAMP accumulation following agonist challenge. Thus, in order to accurately describe the dynamics of the second messenger, a more rigorous examination of cAMP flux must be undertaken.

Simply measuring cAMP accumulation in a stimulated cell is clearly insufficient to assess its turnover. Accumulation merely reflects the net result of synthesis, degradation and sometimes escape from the cell, with the latter two processes accounting for elimination of the intracellular message. By examining the rate and extent of turnover, one can then more clearly quantify the contribution of each process to the kinetic profile of cAMP accumulation, and thereby, any regulation of adenylyl cyclase (AC) desensitisation, PDE activity, and cAMP egress after exposure to hormone. The vast majority of work addressing this question has been conducted by Butcher, Barber and colleagues, but disappointingly, has seldom been emulated by others. With so little in the way of corroborative work in other cells and by other groups, it was decided to examine cAMP turnover in the systems under study in our laboratory.

The models more extensively studied by Barber and Butcher have been the S49 lymphoma mutants [Barber & Goka 1981] and human diploid lung WI-38 fibroblasts [Barber et al. 1980], and it is only from these cell lines that the body of the information regarding cAMP turnover has been gathered. To quantitatively assess cAMP degradation in stimulated cells, essentially two techniques can be employed, the first being the rate of cAMP decay following
cessation of synthesis (either by addition of antagonist, or by washout or destruction of agonist) [Su et al. 1976]. This model is described in detail in the 'Methods' chapter, where the hydrolysis of cAMP is regarded as a first order process. Thus, the classical first order semi-logarithmic transformation of the decay data yields a constant for degradation (k_{deg}). The second technique, pioneered and refined by Barber & Butcher, relied upon the rapid incorporation of radiolabelled adenine into ATP, and thence cAMP under conditions of agonist stimulation. The delay in the cAMP labelling is indicative of the rate of its turnover, and the mathematical analysis converts this 'lag' into a fractional turnover rate constant (k_e) [Barber & Butcher 1988], which itself is defined as a first order constant [Shipley & Clark 1972].

The two techniques are quite complementary having been shown to give essentially identical values for cAMP turnover constants in a variety of S49 mutants [Barber and Goka 1981]. This elegant work strongly suggests that the methods are viable and do not generate artefactual results, since the assumptions upon which each relies are quite different. However, by way of contrast, WI-38 cells stimulated by PGE_1 did give substantially different values for turnover according to the technique used: k_{deg} was 2-3 times larger than k_e [Barber et al. 1980]. One of the major differences between the two techniques concerns receptor occupation by agonist - measuring the rate of cAMP decay after the cessation of synthesis, by definition, is carried out in the absence of agonist, whereas the pulse labelling technique determines the turnover in its continuing presence. This disparity in derived rate constants suggested to the authors that agonist occupation of prostaglandin receptors was somehow leading to an inhibition of cellular PDE. The mechanism behind this phenomenon has not been determined, but the authors suggest it is manifest almost immediately after receptor occupation; however, that the k_{deg} measured after 2 or 40min of PGE_1 was identical does not necessarily imply that the k_e will likewise be the same. This conclusion is further corroborated by PDE activities measured in
homogenates from naïve and PGE₁-stimulated cells [Nemecek et al. 1979], and by another kinetic model which attempts to describe the time course of cAMP accumulation in terms of several rate constants for the contributory processes (i.e. synthesis, adenylyl cyclase desensitisation, elimination) [Barber et al. 1980]. These analyses of cAMP flux have therefore been instrumental in elucidating, or confirming important regulatory mechanisms.

After briefly comparing the two methods, the next most obvious question to pose is how does this intact cell analysis compare with the cell free determination of PDE activity? Such a comparison was undertaken in human diploid WI-38 and VA-13 cells, the latter being an SV 40 transformant of the former. The PDE activity of the normal cell is 4 fold greater than the mutant in vitro and this trend was preserved in the intact cell, with the k₆s also differing by ~4 fold [Butcher 1984]. However, there was a poor correlation between the absolute values for the homogenate and whole cell determinations (the broken cells gave higher turnover values). Similar measurements of k₆ and cell free fractional turnover constants in S49 cells are also proportional [Butcher 1984], validating the assertion that the rate constants are a reasonable index of PDE activity. Attention was therefore directed towards turnover determinations in brain slices and later human astrocytoma cells.

**Results**

**Turnover Determinations in Rat Cerebral Cortical Slices**

The brain slice was the system operative at the time that these studies were first initiated, and therefore in which they were first attempted. They seemed to provide a unique opportunity to analyse cAMP flux subsequent to stimulation by a variety of agonists, ranging from small amines, to peptide hormones. Unlike the induction of cAMP decay in brain slices, the pulse labelling technique obviated the need for good agonist/antagonist pairs, and allowed one to examine flux under many different conditions, including after
sizeable increases in cAMP elicited by PDE inhibitors alone. Hence, the labelling protocol was initially applied during noradrenaline stimulation, since it gave a robust nine fold increase in cAMP over basal. A preliminary experiment generated data that appeared most promising, wherein the lag in cAMP labelling appeared. With a reasonable turnover constant, $k_e$, of $\sim 0.36$ min$^{-1}$ ($t_{1/2} \approx 1.9$ min) during noradrenergic stimulation being calculated, the technique seemed to be employable in slices. An estimate of $k_{\text{deg}}$ had previously been attempted (with the caveats mentioned in chapter 2) and calculated to be $\sim 0.24$ min$^{-1}$ ($t_{1/2} \approx 2.9$ min), which was somewhat similar to the value obtained here.

However, a series of subsequent experiments, attempting to assess $k_e$ under adrenergic stimulation ($\pm$ PDE inhibitors), or basal cAMP turnover proved disheartening: the initial success with NA could not be reproduced, and the attempt to elucidate the magnitude of basal flux was thwarted by a low signal:noise ratio. The irreproducibility of adrenergic flux was due to the apparent specific activity of cAMP exceeding that of its precursor, ATP, as judged by the ratio of $^3$H and $^{14}$C (figure 1). This is clearly an impossibility, and the transformation of the data resulted in highly scattered, non-linear pattern of points which were to be found in the second, and not the first quadrant, graphically speaking.

The most obvious interpretation of the data was that the excess $[^3]$H adenine added to the incubations was contaminating the cAMP fraction and resulting in erroneously high $^3$H counts, and thereby, an overestimated label ratio. This was examined empirically by a more strict adherence to the experimental protocol of [Barber and Butcher 1988]. That is, an attempt was made to drastically reduce the amount of extracellular $[^3]$H adenine carried through the chromatography procedure by centrifuging the slices and removing the supernatant after the appropriate incubation (figure 2).
Figure 1 Time course of $[^3H]$adenine incorporation into ATP and cAMP, where the dual label ratio for cAMP exceeded that for ATP. Slices were labelled with $[^{14}C]$adenine (0.5μCi/ml) as previously described. Slices were then stimulated for 10 minutes with 100μM NA, after which $[^3H]$adenine (2μCi/ml) was added for the times indicated. Results are mean of a single experiment performed in triplicate, typical of three.

From figure 2 one can glean the following facts: first, that aspiration of the excess label has little effect upon apparent cAMP ratios, but does appear to significantly reduce the ATP ratio. In other words, $[^3H]$adenine contaminates the ATP fraction to a small degree, but is not carried through to the cAMP fraction to any great extent. Secondly, the overwhelming conclusion is that the removal of the contaminant does not restore the expected, normal pattern of labelling, but in fact reinforces the aberrant one. By comparing the left (ATP) and right hand (cAMP) panels of figure 2, one can see that the labelling ratio that the cyclic nucleotide attains is again greater than its precursor.
Figure 2 Effect of [3H]adenine removal upon kinetics of nucleotide labelling in rat brain slices

Slices, prelabelled with [14C]adenine, were buoyed in 15ml KRB in a 20ml glass beaker by magnetic stirring, with a Pasteur pipette delivering a stream of O2/CO2 to the surface of the buffer. The beaker sat inside another vessel through which water at 37°C was constantly being pumped. Over the course of the experiment, a constant temperature of 37°C was maintained. NA(100μM) was added for 10 min before 2μCi/ml [3H]adenine was applied. 300μl aliquots of the slice suspension were withdrawn 30-45sec before termination, added to a microfuge tube (in which was 1 ml of KRB), and centrifuged for 3 sec in an Eppendorf bench centrifuge. The supernatant was aspirated ('asp' samples) and the reaction terminated by addition of HCl, with vigorous vortexing of slice pellet. Immediately after the above, aliquots of slice suspension were removed from the main vessel, and added to centrifuge tubes containing HCl ('ctrl' samples) at the designated time. Results are presented as the mean±range of a single experiment performed in duplicate. Essentially similar results were obtained on another occasion.

More careful consideration of the basic assumptions underlying the methodology needed to be invoked in order to fully appreciate the lack of success in brain slices. Again, the experiments were bedevilled by the heterogeneous nature of the brain slice preparation. The assumption implicit in the pulse labelling technique is that the ATP pool that is being labelled by [3H]adenine is
one that is fully available as a substrate for adenylyl cyclase, and importantly, an AC that is being stimulated by the hormone [Barber and Butcher 1988]. In the brain slice preparation, ATP is being labelled in all viable cells; cAMP production is stimulated in a small proportion of these cells. Therefore, interpretation of the data is, unfortunately, clearly impossible, and upon reflection, it was probably quite naïve to attempt these types of experiments in this system. That notwithstanding, it was an instructive period, and without it a deeper understanding of the method and its drawbacks might have been overlooked.

Not deterred by the lack of success in slices, it was decided to find a simpler model system that would circumvent the problems of cell heterogeneity. Cultured cell lines provide such an alternative, and with the availability of glial- and neural-derived cells, these were examined for their potential to continue this work. As was indicated in chapter 2 (Methods), the neuroblastoma cell line SH-SY5Y cells gave apparently poor cAMP responses with the prelabelling method, and attention was then turned to astrocyte-derived cells. The glioma cell line, 1321N1, derived from a brain tumour [Clark et al. 1975] expresses several receptors coupled positively to AC viz β-ARs, PGE$_1$ and adenosine (A$_2$) receptors. To the best of the author’s knowledge, a rigorous examination of second messenger turnover has not been undertaken in these cells, and thus provided a novel test system. With such a complement of G$_s$-coupled receptors, the types of comparative experiments that originally were to be attempted in a brain slice could be performed in this homogeneous system. Moreover, the cell’s possession of a β-AR conveniently supplied an agonist/antagonist pairing that allowed comparison of the methods for determining cAMP turnover.
Comparison of the methodologies for measuring cAMP turnover in 1321N1 astrocytoma cells

Initially simple experiments were performed to justify, and define the system's responsiveness. Agonist concentration response curves are shown for a variety of agents in figure 3:

Figure 3 Concentration response relationship for various stimulators of adenylyl cyclase in 1321N1 cells. Cells were prelabelled with 3μCi/ml [3H]adenine for 60 min as per 'Methods'. Incubations were terminated after a 10 min stimulation period. Iso=isoprenaline, PGE1 = prostaglandin E1, 2-CA= 2-chloroadenosine, ado=adenosine, and Fk=forskolin. Results expressed as the mean±sem of 3-6 experiments performed in triplicate. Basal = 1201±329; 3μM iso = 28,091±8563 (dpm/assay) (n=6)

By far the most potent and efficacious agonist was isoprenaline, with the other receptor-mediated increases being ~50% of its maximum. Of the two adenosine
receptor agonists that were employed, the analogue 2-chloroadenosine was the more efficacious, although it shared a similar potency with its congenor, adenosine. The prostanoid response was also robust, and indeed the elevation of cAMP by all agents was sufficiently great to make comparison of cAMP turnover with different stimuli a real possibility; a contrast to the more modest responses in slices. Forskolin, a direct activator of adenylyl cyclase (AC), elicited responses that were greater than isoprenaline at 30μM, but for the sake of clarity, the graph is restricted to the lower degrees of stimulation. Identical relationships existed between agonists if cAMP mass was measured, although forskolin was not included in these analyses (see “Methods”).

The time course of cAMP accumulation is described for the various agents in figure 4. In the upper panel, concentrations of agonist were chosen which elicited approximately the same increase in cAMP levels: In the lower, a modest concentration of forskolin was used. Both receptor-mediated and non-receptor mediated increases in cAMP reached a steady state during the time frame examined. In the case of agonist stimulation, maximal increases were observed after 10 min of stimulation and were maintained for a further 10 min at least. With forskolin, however, the plateau was attained only after a 15-20 min exposure, and was likewise maintained for a minimum of a further 10 min.

With the variables now defined, the comparison of methods to measure cAMP turnover in these cells could be attempted. The first requirement was to confirm that the pulse labelling protocol was not prone to the same problems that were manifest in brain slices i.e. that the ‘specific activity’ (³H/¹⁴C ratio) of cAMP never exceeded that of ATP. This was demonstrated in figure 5. The data in figure 5 give a value of the turnover constant, kₜ, as being 0.25 min⁻¹. The graph show that over a protracted time course, the ratio of nuclides in cAMP does not exceed that of ATP unlike the brain slice results. Moreover, the subsequent data that were gathered using this method never showed any likelihood of this being a problem.
Figure 4 Time course of cAMP accumulation after stimulation of adenylyl cyclase by various agents
Isoprenaline = 10nM, PGE$_1$=10μM, Adenosine (Ado)=100μM, Forskolin (lower panel) = 3μM. Data depicted are means±sem of 3 experiments performed in triplicate.

The mathematical transformation is such that the points furthest from the origin (in the type of plot inset figure 5) are equivalent to the earliest time points of the raw data, and those clumped around the origin represent the later points. Since there is a tendency for an unequal distribution of points over a longer time course, it was decided to adopt shorter pulsing times: this should
result in a more even spread of points along the transformed line, and hopefully a more accurate determination of its gradient.

Figure 5 Validation of pulse labelling technique to measure cAMP turnover in 1321N1 cells after β-adrenergic receptor stimulation. Cells were labelled for 60 min with 2μCi/ml [14C]adenine, washed and then stimulated for 10 min with 3μM isoprenaline. 4μCi/ml [3H]adenine was then added and the incorporation of label into nucleotides followed over time. Data are handled as per ‘Methods’, and represent a single experiment performed in triplicate, typical of two.

Thus, in the comparative studies, isoprenaline was used as the agonist (timolol as the antagonist) and the experimental protocol followed according to that described in the Methods section. In summary again, a maximal concentration of the β-AR agonist was used to raise cAMP to a new steady state.

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During this plateau in cAMP levels, either the antagonist or radiolabelled adenine was added; the decline in cAMP, or the increase in its labelling was followed with time, and the data transformed as detailed previously in chapter 2 Appendix. Figures 7a and 7b show the results of such an experiment.

The data in the main figure of 7a shows the time-dependent incorporation of labelled adenine into ATP and cAMP under conditions of β-AR stimulation. As theory predicted, there was a delay in the labelling of cAMP, and, over a time which had seen uninterpretable labelling in brain slices (see figure 2), there was seemingly no problem. That cAMP levels are at steady state is 'internally' demonstrated within experiments by measuring $[^{14}\text{C}]c\text{AMP}$ accumulation. Across all five experiments, this was unchanging over the 7 min exposure to $[^{3}\text{H}]\text{adenine}$ (not shown). The inset shows the linear transformation of the plot and yields a turnover constant of $0.15\pm0.01 \text{ min}^{-1}$ ($r=0.98$).

Figure 7b, on the other hand, depicts the decay in cAMP accumulation after cessation of synthesis induced by timolol addition. Note the overlapping time window with the pulse labelling method. Upon transformation of these data however, $k_{\text{deg}}$ was calculated as being $0.23\pm0.02 \text{ min}^{-1}$ ($r=0.99$). This value of the turnover constant was consistently and significantly higher ($P=0.002$ - Student’s t test) than the pulse labelling derivate. What can be inferred from such an observation?

A more cautious interpretation would be a small difference attributable to methodology. Indeed, a primary difference between the two protocols lies in the method for measurement of cAMP: in the decay method cAMP mass is being measured whereas labelled cAMP is assessed within the pulse labelling protocol. Although cAMP pools may seem to present a problem, previous data published using these cells have suggested that selective nucleotide pooling does not appear to occur [Clark et al. 1975]. The assumptions made in the two techniques' modelling are quite different and one could also argue that their descriptions of the cAMP eliminatory processes do not entirely
Figure 7a Assessment of cAMP turnover by the pulse labelling method. Cells were prelabelled with [14C]adenine (2μCi/ml) for 60 min. Washed cells were stimulated for 10 min with 1μM isoprenaline. 4μCi/ml [3H]adenine was added, and incubations stopped at the indicated times. Radioactivity in nucleotides determined as 'Methods'. Results are means±sem of 5 experiments performed in triplicate.

Figure 7b Assessment of cAMP turnover by antagonist-induced decay method. In experiments performed in parallel with the pulse labelling, cells were stimulated for 10 min with 1μM...
isoprenaline. 50µM timolol was then added and the decline in cAMP followed using a radioreceptor assay. Figure depicts mean±sem of 5 experiments performed in triplicate.

overlap. That does not appear to be the case as S49 lymphoma cells have been calculated as having identical turnover constant values using the complementary techniques [Barber and Goka 1981, Butcher et al. 1983]. This strongly suggests that the methodologies are indeed comparable, at least in S49 cells.

Although the difference is small (~60% greater in decay experiments), it cannot be dismissed as being insignificant. A difference in turnover determinations has been reported for WI-38 fibroblasts, as previously alluded to [Barber et al. 1980]. A precedent therefore exists, and has been interpreted as reflecting an inhibition of PDE activity due to receptor occupation. Could β-AR occupation also lead to changes in PDE activity in 1321N1 cells? Under these conditions, cAMP turnover was deemed to be faster using the decay method (and therefore in the absence of agonist-receptor complexes) as opposed to the pulse labelling technique, performed in the continual presence of agonist. It would be tempting to speculate that β-AR occupation results in PDE inhibition, but thoroughly premature, on the basis of these data alone. As has been shown for brain slices, a small contamination of the ATP fraction by [3H]adenine would make its labelling ratio appear faster and greater. Since the labelling of cAMP is measured relative to ATP, such a small contamination may prove significant. Indeed, should the contamination of the ATP lead to inaccuracies in its labelling ratio of 20% (a figure similar to figure 2), then the turnover constant would become 0.20 min⁻¹; if the error was as much as 30%, then the resultant calculations give a ke = 0.25 min⁻¹. It is likely that the ATP fraction will be contaminated by [3H]adenine, and as such will result in an erroneously low value being ascribed to the turnover constant. The value of ke is therefore likely
to be more comparable to the value of $k_{\text{deg}}$, substantiating the use of the methods.

How then can possible receptor modulation of PDE be further examined? To circumvent comparing methodologies, one can compare turnover using receptor mediated, and non-receptor mediated increases in cAMP. Comparison of cAMP turnover after stimulation by forskolin (which directly stimulates adenylyl cyclase) as opposed to agonists serves as a useful model, and the result of such an experiment is shown in figure 7. Cyclic AMP turnover was determined after stimulation of cAMP synthesis by either a maximal concentration of isoprenaline (1μM), or by 10μM forskolin (a concentration that had previously been shown to raise cAMP to approximately the same extent as 1μM isoprenaline - figure 3).

Figure 7 Comparison of rates of cAMP turnover in 1321N1 cells after stimulation by isoprenaline or forskolin. Cells prelabelled with 2μCi/ml $[^{14}\text{C}]$adenine were challenged with 1μM isoprenaline for 10 min, or with 10μM forskolin for 20 min prior to addition of 4μCi/ml $[^{3}\text{H}]$adenine. The main figure depicts incorporation of label into ATP and cAMP, whereas the inset shows that transformation of the data. Results expressed as mean±range of two experiments performed in triplicate.
The accumulation of $[^{14}\text{C}]$cAMP elicited by either isoprenaline or forskolin was approximately the same under these conditions (20,670±5577 and 29,778±9012 dpm/assay respectively). Thus, by attaining an apparently identical accumulation level in both instances, it was hoped that complications in turnover arising from changes in cAMP levels (see below) would be overcome, thereby simplifying the comparison. Upon examination of the raw and the transformed data, it could be appreciated that the turnover of cAMP was deemed to be insignificantly different under both conditions ($k_e$ values of 0.31±0.12 and 0.23±0.10 min$^{-1}$ for isoprenaline and forskolin respectively (mean±range)). Such data strongly argue for there being no real difference between the two methods for determining cAMP turnover in 1321N1 cells, as was initially suggested from figure 6.

Experiments of this nature can be extended further: are there agonist-specific differences in turnover? Because the pulse-labelling method obviates the need for good antagonists, and furthermore, maintains receptor occupation, comparisons of turnover with different agonists can be effected. Such studies were attempted in 1321N1 cells, where similar accumulation levels were evoked with a high concentration of 2-chloroadenosine, and PGE$_1$, but with relatively lower concentrations of isoprenaline or forskolin (c.f. figure 4). Such preliminary studies gave consistently poor data because of the decidedly lower degree of stimulation: the amount of radioactivity in the 'constant', stimulated $[^{14}\text{C}]$cAMP fraction was too low to give meaningful $^3$H/$^{14}$C ratios (only hundreds as opposed to thousands of dpm were collected per fraction). These studies could be more successfully repeated after prelabelling with far greater amounts of $[^{14}\text{C}]$adenine.
Relationship between cAMP accumulation levels and whole cell PDE activity

As was intimated at the start of chapter 2, the relationship between the degree of cAMP accumulation and its enzymic elimination was always an issue of some considerable interest. Whilst brain slice responses to β-AR agonists were insufficient to support antagonist-induced decay curves over a range of agonist concentrations, cultured cell lines such as the astrocytoma cells used above finally provided an adequate system wherein these types of experiments could be performed.

In the 1321N1 astrocytoma cells, ~90% of PDE activity is located in the soluble fraction of homogenates [Tanner et al. 1986] and, like most cell types so far examined, is a composite of more than one type of isoenzyme [Tanner et al. 1986]. Following the pattern of other tissues [Wells & Hardman 1977], these glioma cells possess both a low and a high $K_m$ soluble PDE activity with respect to cAMP [Tanner et al. 1986]: the former activity isolated after sucrose-density centrifugation appears to have a $K_m$ of the order of 0.6-5μM, and is sensitive to Ro 20-1724 (type IV isozyme). The second activity, resolved upon a DEAE-cellulose column, is stimulated by Ca$^{2+}$/calmodulin (type I) and has a $K_m$ of 200μM in the absence, and 70μM in the presence of Ca$^{2+}$.

Theoretical Models of the Relationship between cAMP concentration and PDE activity If one conducts enzyme kinetic experiments in crude homogenates of cells, it should hardly be surprising that the substrate/velocity relationship for cAMP PDEs deviates significantly from that governed by simple Michaelis-Menten kinetics. The kinetics ordinarily display negative cooperativity (in its purely phenomenological sense i.e. that the reaction velocity does not increase with substrate as much as would be predicted from simple enzyme kinetics), probably a consequence of the multiple forms of PDE that have differing kinetic properties [Russell et al. 1973]. Changes in enzyme monomer/polymer ratios due to substrate binding [Pichard & Cheung 1976] or
sequential negative cooperativity [Russell et al. 1972] also could contribute to this observed negative cooperativity. It must be stated, however, that true 'negative cooperativity' exhibited by a single enzyme activity is described by a set of equations that can also model the cooperativity resulting from a complex enzyme population [Boeynaems et al. 1974].

Dumont and colleagues derived a theoretical model of cooperativity of cAMP elimination (which could include PDE activities and/or egress processes) based upon its effect upon cellular cAMP levels after hormonal challenge [Erneux et al. 1980]. They suggested that the total elimination of cAMP should first be described by a Hill-type equation shown below:

\[
v_{\text{deg}} = \frac{V_{\text{max}} [\text{cAMP}]^{h_d}}{K_d + [\text{cAMP}]^{h_d}}
\]

(1)

Where

- \(v_{\text{deg}}\) = rate of degradation
- \(V_{\text{max}}\) = maximal rate of degradation
- \(h_d\) = Hill coefficient for degradation
- \(K_d\) = apparent dissociation constant for cAMP

Clearly, should \(h_d\) be equal to unity, then the equation becomes analogous to the classical Michaelis-Menten equation. By contrast, if \(h_d < 1\), then the kinetics become negatively cooperative (velocity increases less with an increase in substrate than predicted from simple kinetics), whereas if \(h_d > 1\), then positive cooperativity results. This equation merely represents an empirical relationship, and does not suggest any mechanism; neither will the Hill-type coefficient represent any particular kinetic parameter.

Given that the synthetic process could also be described by an analogous equation (but one wherein [Hormone] replaces [cAMP]), and that during cAMP steady state, the rate of synthesis numerically balances its
degradation, the authors derived the relationship pertaining between cAMP accumulation, hormone concentration and their respective Hill indices. A simplified format is shown here (derived from their equation (5) in [Erneux et al. 1980]):

\[
[cAMP] = K^{1/h_d} \left( \frac{[\text{Hormone}]^{h_s}}{C+[\text{Hormone}]^{h_s}} \right)^{1/h_d}  \tag{2}
\]

Where:
- \( h_s \) = Hill coefficient for synthesis
- \( h_d \) = Hill coefficient for degradation
- \([cAMP]\) = concentration at steady state
- \([\text{Hormone}]\) = concentration of challenging agonist
- \( K \) & \( C \) = composite constants (see original paper)

From this equation one can easily show that an inverse relationship exists between the cooperativity of PDEs and the cooperativity of cAMP accumulation. If cAMP elimination is catalysed by a single, classically Michaelian enzyme, then \( h_d \) is equal to unity (no cooperativity, and hence can be ignored in equation (2); therefore cAMP accumulation will only be governed by the synthesis coefficient, \( h_s \), the only Hill coefficient operative. In other words, the cooperativity of accumulation will equal the cooperativity of synthesis.

If, instead \( h_d < 1 \) (negative cooperativity of PDEs), then \( h_d \) can no longer be disregarded and both Hill coefficients influence the cAMP accumulation profile: effectively, each hormone concentration expression can be considered as \([\text{Hormone}]^{h_s}/h_d\), and it should now be clear that as \( h_d \) falls below unity, the cooperativity between hormone and cAMP is enhanced. This simply means that cAMP accumulation will increase more per increment in synthesis than if \( h_s/h_d = 1 \) i.e. accumulation becomes more positively cooperative when disposal is negatively cooperative. The converse will be true if the PDEs display
positively cooperative kinetics ($h_d > 1$). To reiterate, there is an inverse relationship between the cooperativity of cAMP elimination and its accumulation.

Another, more simple analysis of cAMP degradation has been presented by [Barber et al. 1987] which describes how multiple PDE forms contribute to cAMP removal. The elimination of cAMP can essentially be modelled as:

$$\frac{-d[cAMP]}{dt} = \frac{V_L [cAMP]}{K_L + [cAMP]} + \frac{V_H [cAMP]}{K_H + [cAMP]} + \frac{V_E [cAMP]}{K_E + [cAMP]}$$ (I)

Where

- L = low $K_m$ enzyme
- H = high $K_m$ enzyme
- E = escape process

When $[cAMP]$ is much lower than the $K_m$ for the high $K_m$ enzyme, or escape process, it follows that in their denominators, $[cAMP]$ can effectively be ignored, and thus:

$$\frac{-d[cAMP]}{dt} = \frac{V_L [cAMP]}{K_L + [cAMP]} + k_h [cAMP]$$ (II)

Where

$$k_h = \frac{V_H}{K_H} + \frac{V_E}{K_E}$$

The fractional turnover rate is defined as being:

$$\frac{-d[cAMP]}{dt} = k_e [cAMP]$$ (III)

therefore...
\[ k_e = \frac{V_L}{K_L + [cAMP] + k_h} \] (IV)

Equation IV illustrates a most important feature: the fractional turnover constant, \( k_e \), varies inversely with cAMP concentration when there are multiple processes for removing the cyclic nucleotide (and in this case when cAMP levels are substantially lower than the \( K_m \) for the low affinity enzyme or escape process). Even during the circumstance of [cAMP] being a significant fraction of, or exceeding the ‘\( K_m \)’ of the escape or low affinity processes, this inverse proportionality will clearly still persist.

The two theoretical models do not quite describe the same things. Dumont’s model analyses the relationship between PDE cooperativity and the resultant cAMP accumulation. It does not necessarily set out to explain why the PDE activity is, or is not cooperative, but predicts the outcome of such a property. As was suggested earlier, this property could be observed for a variety of reasons. Butcher and Barber’s model, on the other hand, specifically attempts to answer the question “What is the consequence of a kinetically mixed PDE population for a cell?”. The two models are nonetheless complementary, and the consequences of PDE properties should be examined in the light of them both.

These models have seldom been tested practically in intact cells, and therefore it appeared pertinent to examine their applicability to 1321N1 cells, which contain multiple PDEs and therefore would be predicted to display an apparent negative cooperativity with respect to cAMP hydrolysis.

In a cell-free system, one could easily examine the cooperativity of cAMP hydrolysis by adding different concentrations of substrate and measuring the reaction velocity that ensued. In an intact cell, the supply of substrate (cAMP) can only be manipulated by indirect means such as activation of cell surface receptors. Therefore, the experimental approach adopted was one of stimulating cAMP levels up to a variety of steady states with increasing concentrations of the
β-AR agonist, isoprenaline and examining the decay curves generated after application of a β-AR antagonist.

Figure 8 shows the initial decay in cAMP concentration after antagonism of the actions of a range of isoprenaline concentrations.

Figure 8 Antagonist-induced decay of cAMP levels after stimulation of cAMP production with increasing concentrations of isoprenaline Cells were stimulated for 10 min with the indicated range of isoprenaline concentrations. After this period (effectively $t=0$ on the figure), 50μM timolol was applied and the fall in cAMP followed with time (left panel). Transformed data shown in right figure. Isoprenaline concentrations are shown as (‘3-9’ = $3 \times 10^{-9}$ M, ‘-8’ = $10^{-8}$ M etc.). Results are expressed as means of 4-7 experiments performed in triplicate. The error bars are omitted for clarity.

At $t=0$, the concentration response relationship between isoprenaline concentration and cAMP accumulation is clearly evident. Even from a cursory inspection of the raw data, several trends emerge: first, that the absolute rate of cAMP hydrolysis (pmol/mg protein/min) increases with increasing hormone (and therefore cAMP) concentration. Second, the time taken
for the cAMP levels to fall to half their original value ($t_{1/2}$) likewise increases with hormone concentration. Such conclusions are borne out by transformation of the data (right hand panel). The slope of the lines correspond to the decay constant, $k_{deg}$, and this decreases commensurately with an increase in the agonist concentration, as can be more clearly seen in figure 9.

![Graphs showing relationship between decay constant, cAMP half-life, and isoprenaline concentration](image)

**Figure 9** Relationship between decay constant, cAMP half-life, and isoprenaline concentration in 1321N1 cells. $K_{deg}$ values are calculated from transformation of the data in figure 8. Since decay is pseudo first order, $t_{1/2} = \ln 2 / k_{deg}$, and this equation was used to derive the right hand panel. Data are shown as means±SEM of 4-7 separate experiments performed in triplicate.

As was predicted by simple examination of the raw decay data, the time taken for cAMP to decay to half its original value rises with isoprenaline concentration, in fact, by over two fold (from 2.2 to 4.9 mins; $k_{deg}$ decreases from 0.31 to 0.15 min$^{-1}$). Another point gleaned from the initial decay curves was that there was an increase in the absolute rate of cAMP elimination, and yet we observe a decrease in the value of $k_{deg}$ as agonist is increased. It is important to note that the decay rate and the decay constant are not equivalent, and the former is only a function of the latter (remember that rate = $-d[cAMP]/dt$ =
Intuitively, one would expect the rate of disposal to increase, and by substituting the appropriate values for the constant and [cAMP] into this equation, that point is confirmed in figure 10. This graph also depicts the rate of elimination as a function of the steady state cAMP concentration i.e. reaction rate versus substrate concentration - a remarkable approximation to performing conventional enzyme kinetics in an intact cell! Indeed, a Hanes' plot of the data is shown as an inset, and was apparently linear (r = 0.96) with a K_m of 610 pmol/mg protein and a V_max of 236 pmol/mg protein/min. (The errors at higher cAMP concentrations are apparently large, but the data are presented as a summary of all seven experiments. Although the inter-experimental errors were large, the intra-experimental standard errors were small, reflecting the variability of cultured cell responses on a day to day basis).

The units of pmol/mg protein may be approximated as μM by making a few assumptions: first, that there is ~100mg of protein per g of cells, and second that per g of cells, the water content is equivalent to 0.7ml. With these estimates it can be easily calculated that the K_m of 610pmol/mg protein is equivalent to 87μM. Clearly, there is an inverse relationship between the water content and the derived K_m in μM (e.g. a 90% water content results in the K_m being 68μM), whereas there is a direct proportionality between this constant and the protein content. Regardless of the details, these estimates will not vary so much as to detract from the important feature that the enzyme(s) hydrolyse cAMP with a low affinity.

The fact that k_deg decreases inversely with agonist concentration has a certain dampening effect upon the increase in the absolute rate of cAMP hydrolysis. Consider this: if the k_deg was invariant with the cAMP response, it follows that, according to the equation 'Rate = k_deg*[cAMP]', the rate of removal would increase linearly with cAMP. The reality is that although steady state cAMP rises, the hydrolysis rate does not parallel this, but falls off with a greater stimulatory input. This would not be an altogether unexpected conclusion.
provided one envisages that the substrate (cAMP) concentration approaches the maximal velocity of the elimination systems. It can therefore be suggested that, in these cells and under these conditions, cAMP attains a sufficiently high concentration to begin to saturate the removal processes. This could not otherwise be appreciated by examining agonist stimulation alone.

![Graph](image)

**Figure 10** Relationship between cAMP concentration and its degradation in intact 1321N1 cells. The figure is derived from manipulation of the original data and shows the mean±sem of 4-7 experiments performed in triplicate. Inset is a Hanes kinetic plot to determine apparent Michaelis-Menten parameters.

A most ingenious manipulation of the velocity/substrate plot illustrates a fundamental regulation of cellular cAMP dynamics. It is already known that the cAMP accumulation after a 10 min stimulation by isoprenaline has reached a steady state, during which the rate of synthesis equals the rate of degradation, by definition. Hence, as the rate of elimination has been calculated, it follows that the same velocity values can be ascribed to the synthetic reaction.
A new plot of the steady state cAMP concentration against this quantitation of the adenylyl cyclase activity yields new information (figure 11).

The most striking fact to emerge from such a plot is the non-linear relationship between cAMP synthesis and accumulation. It can be seen that as the hormone-driven AC activity increases, the cAMP accumulation level also rises - that is no surprise - but what is remarkable is that when synthesis increases beyond a certain level, the ensuing cAMP steady state increases disproportionately. In other words, for a given enhancement of cAMP production (say by a factor of ‘α’), the level at which cAMP eventually plateaus is ‘n’ times α (where n > 1).

![Figure 11: Relationship between cAMP synthetic rate and cAMP accumulation](image)

Since rate of synthesis = rate of degradation during cAMP steady state, the rates of hydrolysis previously calculated are now expressed as the rate of cAMP synthesis by adenylyl cyclase (x axis). The y axis represents the steady state cAMP accumulation levels that correspond to each velocity value.
Discussion

In order to more thoroughly describe the dynamics of second messengers, the contribution of synthetic and eliminatory processes must be quantitated. A quantitation of cAMP flux after agonist stimulation was therefore the ultimate goal, and was modelled in rat cerebral cortical slices and human astrocytoma cells.

By use of a pulse labelling technique, it was hoped to systematically probe cAMP turnover in brain slices after application of a variety of agents. After a preliminary investigation, it eventually became clear that such an undertaking was not feasible given the heterogeneous nature of the preparation. Attention was turned to a simpler system, the homogeneous cell line, 1321N1 astrocytoma cells.

These cells express several receptors positively coupled to adenylyl cyclase, including β-ARs, and were deemed an appropriate system in which to study cAMP turnover. Gratifyingly, they did not appear to be prone to problems with nucleotide labelling, and a more in depth analysis of cyclic nucleotide flux could be implemented. Once the responses to various agonists and forskolin were properly defined, the stage was set to firstly compare the techniques available for measuring cAMP turnover.

After challenge with a maximal concentration of the β-adrenoceptor agonist, isoprenaline, the value of the fractional turnover constant as calculated by the two methods, appeared to differ. In the absence of occupied receptors (decay method), it was calculated to be 0.23 min⁻¹; using the pulse labelling method (receptor occupation maintained), the data transformation gave a constant of 0.15 min⁻¹. A 60% difference was an unexpected find, and on the surface, was consonant with some change in cAMP PDE activity related to receptor occupation by agonist. A modification of the disposal by 60% would have substantial differences in the agonist-elicited cAMP response. Considering that steady state accumulation is defined as \([cAMP] = \frac{k_s}{k_e}\) (see equation 3,
section 2.5(i), 'Methods'), it can readily be appreciated that a 60% increase in the
turnover constant will be reflected by an ~60% decrease in the cAMP steady state.
That is, changes in turnover are reflected by large changes in accumulation.
Therefore, although the disparity was not a sizeable one, it could not be
overlooked as being insignificant.

However, previous data from the brain slice experiments indicated
that a small, but nonetheless significant contamination of the ATP fraction by
$[^3]$Hadenine occurred with the experimental regimen adopted. Such a
contamination would theoretically diminish the cAMP turnover constant - it
remained a possibility that the small difference in the two flux measurements
was merely a consequence of the way the experiments were conducted. The
extent of contamination can be addressed empirically using HPLC, and overcome
by changing the experimental protocol.

To assess whether or not receptor occupation was truly modulating
cAMP elimination required a complementary approach, and one that would
circumvent artefacts of comparing the two methods. By using the pulse labelling
technique only, it was decided to examine the turnover of cAMP either after
agonist challenge, or after a non-receptor mediated stimulation of adenylyl
cyclase by forskolin. At apparently similar stimulated cAMP levels, the calculated
turnover constants, whether with isoprenaline or forskolin, were not
significantly different. This suggested that receptor occupation was not indeed
altering cAMP flux, and that the previously observed, small difference between
methodologies was more apparent than real. However, this assumes that the
only action of forskolin was to stimulate cAMP synthesis and it is now known
that its specificity in this regard is questionable ([McHugh & McGee 1986,
Nevertheless, more evidence needs to be collected before a firm conclusion can
be formed (although it would be highly coincidental that the diterpene affects
cAMP turnover by the same degree as isoprenaline).
The relationship between cAMP accumulation and its disposal was also examined in 1321N1 cells, under conditions of β-AR stimulation. It has been shown that for an increase in cAMP levels, the absolute rate of hydrolysis of the second messenger increases accordingly. From such an analysis it became clear that the systems for cAMP elimination were becoming substantially saturated over the degree of agonist stimulation, a familiar hallmark of enzyme catalysis. Indeed, the data were transformed into a pseudo-linear Michaelis-Menten plot, with a $K_m$ of $\sim 600$ pmol/mg protein ($\sim 85\mu M$), and a $V_{\text{max}}$ of $\sim 200$ pmol/mg protein/min.

The calculated Michaelis constants are almost certainly likely to be overestimates since the 'free' cAMP concentration available to PDEs is indeterminate - cAMP may be compartmentalised [Hayes et al. 1980, Livesey et al. 1982, Barsony & Marx 1990] or is likely to be sequestered to some extent by intracellular binding proteins (the extent to which this occurs will vary between cell types, but has been suggested to be highly significant in some [Cheung & Patrick 1974, Coffino et al. 1976, Barber et al. 1987]). If the proportion of cAMP sequestered is equal at all degrees of stimulation, then there will be no error incurred in the $V_{\text{max}}$ determination, but there will be an over-estimate of the $K_m$ value (and by the same proportional error that plagues the 'free' cAMP evaluation i.e. if there is a 25% overestimate of free cAMP, then there will also be a 25% error in the $K_m$ determination).

However, the sites of sequestration are probably themselves saturable (being predominantly protein kinase A), and their apparent capacity to 'buffer' changes in free cAMP may vary with cAMP concentration. This would have a very different effect upon the observed kinetics than would the simpler 'constant percentage' sequestration scheme. Should these sites tend to become saturated at moderate cAMP levels, then the buffering effect will be more pronounced (percentage-wise) under smaller stimulation conditions; when cAMP attains a much higher steady state level, the proportion bound will be far
less, and consequently the error in estimating the free cAMP concentration will
be smaller. It can easily be calculated that this could make a ‘two-site’, non-linear
Hanes’ plot appear linear, as it would obscure the elimination effected by a high
affinity system (of course, depending on the affinity and capacity of the
sequestration site(s)). It therefore follows that the transformed plot may be linear
in nature for at least four reasons:

(i) the majority of hydrolysis is performed by only one species - this
raises questions about the rôles, or the nature of the PDEs isolated in vitro

(ii) the cAMP steady state concentrations do not extend to a low
enough level to observe another, higher affinity component predominating i.e.
the low K_m species is already saturated and/or its contribution to PDE
elimination is minor over the substrate range generated

(iii) significant buffering of cAMP by a system with an affinity
comparable to a ‘low K_m’ PDE could introduce large errors into the free cAMP
assessment at lower levels of cAMP production

(iv) agonist stimulation results in a change in phosphodiesterase
kinetics that is preserved after receptor occupation ceases.

An inverse relationship between cAMP concentration and the
turnover constant was theoretically predicted by Barber and Butcher’s model, and
is also substantiated by the above data. However, their model may be somewhat
prone to overinterpretation by them. Although their experiments were not
performed in the above manner, they too noted that the decay constant, k_{deg}
varied inversely with cAMP [Barber et al. 1987]. They inferred that this indicated
the presence of multiple PDE types and particularly, of a significant amount of
high affinity PDE. But consider their own equation (IV): even if there was only
one route of cAMP catabolism (ignoring the term k_h), the turnover constant
would still be inversely proportional to the steady state cAMP concentration.
Common sense dictates that there will never be a linear relationship between
removal rate and cAMP over a larger range of concentrations - a hyperbolic
relationship is a basic consequence of enzyme catalysis. Although the decrease in $k_{\text{deg}}$ may suggest recruitment of multiple PDEs to Barber and Butcher, it would be naïve to invoke such a hypothesis in 1321N1 cells given the above provisos. That the Hanes’ plot is linear goes some way towards implicating only one major PDE activity, but that too may be a falacious argument as was discussed earlier.

The non-linear relationship between synthesis and accumulation (figure 11) has fundamental implications for cellular regulation of cAMP levels, inasmuch that:

(a) The positive cooperativity of accumulation increases the sensitivity of the cell to changes in hormone concentration
(b) It serves to economically amplify the synthetic signal
(c) Since PDE activity is central to amplification of hormonal stimuli, it thus affords the cell another site at which to modulate its sensitivity to agonists

Only by possessing this particular complement of PDEs can the cell confer a greater sensitivity to a variation in the hormonal stimulus. Should the PDEs present have had a greater capacity to remove cAMP, then a more linear relationship would exist. Similarly, accounting for second messenger sequestration would serve to slightly reduce this apparent amplification of accumulation (if buffering is saturable); the points on the figure 11 corresponding to lower synthetic rates would be lower down the y axis (lower cAMP ‘free’) making the upward inflexion less marked.

Whilst cell-free PDE activity would undoubtedly display negative cooperativity (which is easily determinable), what situation actually pertains in the intact cell? This ‘sensitisation’ of the agonist response has no bearing on the cooperativity of PDEs in 1321N1 cells - amplification is merely a result of the saturable nature of its PDE population, and would occur if there was only one Michaelian enzyme. Attempts to approximate the Hill coefficients for accumulation, synthesis and ultimately degradation have proven difficult given the data generated. Can it be concluded that there is no cooperativity of PDEs,
given that the substrate/velocity relationship apparently obeys Michaelis-Menten kinetics (consonant with $h_d = 1$)? It surely cannot, unequivocally, since the doubts about sequestration of substrate, substrate range etc. have not yet been dispelled. As these have a direct bearing upon the kinetic profile, the value of the Hill coefficient is open to conjecture. Sequestration may be somewhat circumvented by inclusion of a cell permeable cAMP analogue with a high affinity for PKA (the major cAMP binding protein) and a low one for PDEs - these experiments are fraught with difficulties, not least for the design of suitable and adequate controls (e.g. experiments may be subject to misinterpretation if PKA activation leads to an alteration in PDE or AC activity). A cAMP antagonist may therefore be a more suitable tool.

To conclude, the PDE activity in 1321N1 cells has profound effects upon the cAMP accumulation profile after β-adrenoceptor stimulation, and presumably after other stimulatory inputs. Once again, it has been shown that its very presence contributes substantially to modulation of cAMP levels, and asserts that an exacting examination of cAMP dynamics cannot exclude their detailed study.
Chapter 6 Discussion

The underlying theme of the studies described in this thesis has been the modulation of cellular cAMP levels. Again and again it has been reiterated that the net accumulation of cAMP is governed by the outcome of the summation of its production and elimination. All too frequently the measurement of cAMP accumulation is interpreted entirely in terms of synthesis, overlooking the crucial rôle of phosphodiesterases, the only known catabolic pathway for cyclic nucleotides. The major body of work was directed towards elucidating the contribution of PDEs to regulation of cAMP levels, be that in apparently stimulated or unstimulated cells. In cultured cells, the use of PDE inhibitors or more complex kinetic analyses formed the foundation of part of the study. Whilst primarily focussing upon the degradative pathway as a means of manipulating second messenger concentration, a good deal of work (by default) also examined the complex result of multiple receptor activation upon cAMP synthesis in brain slices.

Since accumulation is a balance between synthesis and degradation, then interference with the latter pathway will have substantial effects upon cAMP levels. PDE inhibitors thus provide a novel method for elevating cyclic nucleotide levels which circumvents receptor-mediated increases in adenylyl cyclase activity. When it became obvious that PDE activity was multiform, much headway was made into the development of inhibitors that would be selective for one particular isoenzyme over another. At the present time, the availability of inhibitors that really do distinguish PDE isozymes is still limited to those that are selective for type IV (cAMP-specific), type III (cGMP-inhibitable), and type V (cGMP-specific). Although selective inhibitors exist for these isozymes, inhibitors of other families are not as entirely convincing in their discriminatory abilities, and it remains frustrating that no good inhibitors of type I (Ca^{2+}/calmodulin-activated) PDEs exist, nor any 'selective' inhibitors of the type
II, cGMP-stimulated PDE. With such a limited array of tools available to dissect out various PDE contributions, it is not perhaps surprising that non-selective inhibitors, such as papaverine and IBMX are still frequently used. Indeed, 'indefinable' PDE activities are often inferred from the the difference in the effectiveness of selective, versus non-selective inhibition. Therefore, the susceptibility of the SH-SY5Y neuroblastoma cells to a variety of inhibitors was examined in an attempt to distinguish the functionally significant PDE isoforms in these cells.

It was out of this simple approach that the apparent disparity between the effectiveness of IBMX and the other inhibitors became clear. After dismissal of the more obvious reasons for the difference, such as kinetics, cGMP elevation, PDE susceptibility, and adenosine receptor antagonism, the study assumed a considerably greater interest. It was possible that IBMX, used so often as a PDE inhibitor, was affecting cAMP metabolism at a site hitherto unrecognised. After a fuller characterisation of the IBMX phenomenon was achieved, another alkylxanthine, theophylline, was examined and with its inhibition of basal and forskolin-stimulated cAMP accumulation, adenosine receptor mediation had to be reassessed.

Further experiments involving the removal of adenosine with ADA were indicative of a partial, if not a full requirement for the nucleoside in potentiating the response with the selective (non-adenosine receptor antagonist) PDE inhibitors. Because of its intrinsic receptor-antagonist property, IBMX did not permit the full stimulatory effect of endogenous ligand to be realised, and consequently gave a truer indication of basal turnover of cAMP. The substantial increase in 'basal' cAMP levels after application of type IV selective inhibitors should have been taken as a warning at an earlier stage; but of course, that is with the benefit of hindsight. It is still uncertain whether or not adenosine receptor antagonism is the only factor complicating IBMX action in the
neuroblastoma cells because of the doubts raised by the effectiveness of denbufylline, another adenosine-receptor antagonist, and the data using ADA.

Nevertheless, one is left to conclude that the elevation of cellular cyclic nucleotide with PDE inhibitors is by no means an incontestible index of the hydrolytic capacity of the cell. Obviously, in cells with a low basal cAMP production rate, then the presence of PDE inhibitors will not result in great increases in the second messenger. However, when cAMP synthesis is more substantial (especially in cells stimulated by agonist), an accumulation will be manifest. Also, one must be aware of other consequences of PDE inhibitor exposure. Of the non-PDE effects of other inhibitors, the most well documented are again, adenosine antagonism, inhibition of adenosine uptake, and sensitisation of cells to calcium [Nicholson et al. 1991]. Such effects must always be borne in mind before an accurate interpretation of results may be gained. With the lack of therapeutic potential, it is perhaps not surprising that little effort has been put into developing "cleaner", non-selective inhibitors - however, they would find a good deal of usage in the laboratories where cyclic nucleotide modulation is an issue of importance. But until that point, "dirty" agents such as IBMX and papaverine will continue to be employed, hopefully with more discernment than currently prevails.

A fundamental question that was deemed one of the more intriguing at the outset of this project, and the one that was pursued with varying degrees of success over that time, was to what extent does PDE activity govern cAMP accumulation in the intact cell? Much of the literature regarding PDE activity has drawn upon the in vitro analysis of enzymes in broken cell preparations. A substantial amount of effort has gone into their characterisation (with purification, and more recently, with cloning strategies), and while these studies are of tremendous importance, more detailed work needs to be conducted into the extrapolation of such cell free activities to the intact cell. Artefactual activities generated upon cell lysis have long plagued biochemists, and coupled
with compartmentation of cAMP and/or PDEs, this surely demands a more rigorous examination of the roles of PDEs in situ. Although some aspects are hampered by the lack of specific pharmacological tools, there is still a considerable amount of analysis to be performed. With this in mind, steps were taken in this direction.

Unfortunately, accurate measurement of PDE isoenzymic activity in the whole cell is no easy thing. In cell homogenates, there are several assays of enzyme activity which are notable for their simplicity, but which are clearly untenable in intact cells - addition of say, radiolabelled substrate to whole cells will not provide the investigator with much information about intracellular PDE kinetics. There must be a change in approach, therefore. In this thesis, two methods were used as a semi-quantitative index of cellular PDE activity, both of which relied upon assessing turnover of cAMP within the cell. The first, as detailed before, involves the stimulation of cAMP production by agonist, after which, addition of an effective excess of antagonist instantly arrests synthesis; the ensuing rate of cAMP decline is indicative of the cellular PDE activity. Of the drawbacks inherent to this technique, a major one is that decay in cAMP levels is measured in the absence of receptor occupation (also, basal rates of cAMP turnover cannot be determined by this method). Nevertheless, its ease of execution makes it applicable to any system, provided that the stimulation is sufficiently robust to enable accurate kinetics to be generated and suitably selective agonist/antagonist pairings are available. With this approach available, it initially seemed possible to examine the relationship between cAMP accumulation and the commensurate PDE activity by using different agonist concentrations to generate a range of cAMP steady state levels. It was this issue that was addressed in subsequent experiments.

However, the system was not as flexible as it first seemed. The best agonist/antagonist pairing available (coupled to adenylyl cyclase (AC) activation) was that appropriate to the β-adrenoceptor. In rat cerebral cortical slices, which
was the tissue used at the time, the β-AR-mediated response was maximally only about three-fold. Clearly, such a small "window" was not amenable to performing the types of experiments mentioned earlier, which then necessitated an enlargement of the maximal fold stimulation. Various mechanisms to potentiate the β-AR response were then analysed in some detail. An initial idea was to use forskolin, long known to greatly enhance hormonal stimulation of adenylyl cyclase [Seamon & Daly 1986]. This suggestion was soon discarded since the decay curve approach requires that the rate of synthesis approximates to zero in the cells previously stimulated with agonist. The continual stimulation by forskolin obviously contravenes such a requirement. A more subtle problem further arises from the proposed mechanism of action of forskolin: its stimulation of cAMP production is enhanced by the presence of Gs, and although a definitive explanation has yet to be proffered for this observation, there have been some suggestions that forskolin reduces the reassociation of αs with βγ [Seamon and Daly 1986]. Whatever the details, that forskolin may affect G protein coupling forewarns against using it in experiments where the instantaneous arrest of hormone-stimulated adenylyl cyclase activity is a prerequisite. Other conditions were then sought which would serve to enhance β-AR mediated responses.

The potentiation of β-AR cAMP responses by simultaneous activation of receptors not directly coupled to AC was another approach that seemed promising. When rat brain slice α-ARs are co-stimulated with β-ARs, up to a three fold enhancement of the Gs-mediated effect is seen [Daly et al. 1981]. Could such an observation assist in the analysis of cAMP/PDE activity relationships? Somewhat naively, the mechanism underlying this effect was subjected to considerable examination, with the intent of assessing its applicability to enhancing cAMP responses. It must be stated that this rationale was somewhat overlooked as the study evolved, becoming as it did, an exercise in dissecting mechanistic details. A Ca²⁺-dependency of the enhancement was
interpreted as implicating adrenergic gating of Ca\(^{2+}\) channels, or at least, that was the working hypothesis. By using the limited number of pharmacological tools available viz metal ions, L channel organic blockers, and \(\omega\)-conotoxin, it eventually became apparent that such a demonstration would remain elusive. Certainly, some of these agents significantly and substantially inhibited the \(\alpha\)-AR-mediated enhancement, but their locus of action was not unequivocally shown to be calcium channel(s). Since these studies were completed, one compound SK&F 96365 has been developed which can block receptor-operated calcium channels (as well as voltage-dependent Ca\(^{2+}\) entry)[Merritt et al. 1990]. Clearly, it would be of interest to examine the effects of this agent in brain slices which may perhaps shed more light upon the mobilisation of Ca\(^{2+}\) in the potentiation response.

But what of the original question? Could this potentiation be useful for examining accumulation versus cellular PDE activity? In hindsight, the answer must be no - the Ca\(^{2+}\)-dependent effect appears too multifarious to apply it to such a simple model. The decay curves that would be generated by using e.g. noradrenaline as a non-selective adrenergic agonist (activating both \(\alpha\)- and \(\beta\)-ARs) could be fraught with several problems of interpretation. Amongst which are first, that the decay following application of adrenergic antagonists could be sensitive to \(\alpha\)-adrenergic-induced alterations in \(\beta\)-AR-effector coupling (like with forskolin above). Indeed, activation of \(\alpha\)-ARs (100\(\mu\)M noradrenaline (NA) in the presence of timolol) had no effect upon 1\(\mu\)M forskolin-stimulation of cAMP in brain slices (unreported data and c.f. [Daly et al. 1982a]), implying that the potentiation does not directly affect AC, or G\(_{\alpha}\)-AC coupling, but may somehow affect receptor-G protein coupling. However, the problems of cell heterogeneity prevail as ever, and thus not too much emphasis should be put on this one observation. Second, the half time for the \(\alpha\)-AR augmentation (as judged by a fall in cAMP after addition of phentolamine to 100\(\mu\)M NA stimulated slices) is approximately 2 mins. The decay in cAMP after complete cessation of synthesis
evoked by NA might conceivably comprise multiple phenomena, rendering the
curves open to dubious interpretation. Third, α-AR stimulation itself could have
an effect upon PDEs, but this would be difficult to ascertain if such an effect is
small (as it must be) compared to the stimulation - moreover, inclusion of PDE
inhibitors such as rolipram and Ro 20-1724 serves only to magnify adrenergic
responses in brain slices (preliminary data), suggesting that type IV PDEs are not
substantially affected. The enhancement elicited by IBMX (preliminary data) is
complicated by its ability to antagonise adenosine receptors, which appear to
have a part to play in the synergism between adrenergic receptors.

In order to answer the posed question, it was clear that a
considerable change in the approach was necessary. To that end, the larger
responses prevalent in cultured cells were deemed to be the solution to the
problem, and indeed they proved to be so. Simple models of the cell types which
are prevalent in the CNS (i.e. neurons and glia) were sought, and consistent with
the predominant β-AR expression on glial cells in the brain, the human
astrocytoma cell line, 1321N1, conveniently express β-ARs which evoke
considerable cAMP responses (the maximum being >20 fold over basal),
affording a large window in which to conduct experiments. Using a range of
isoprenaline (a β-agonist) concentrations, a sufficiently broad spectrum of cAMP
levels were produced to permit the desired analysis. Even the lowest
concentration of agonist, 3nM, resulted in a ~7 fold elevation of cAMP in those
particular experiments. Decay curves were produced for each concentration of
agonist (and therefore the PDE substrate, cAMP), whose examination brought
several points to light.

Theory predicted that the fractional turnover constant varies
inversely with cAMP concentration, and this was confirmed by the findings of
this study. In other words, as cAMP levels increase, there is a parallel increment
in the half time for cAMP decay. From these data, an approximation of the
absolute rate of cAMP decline was calculated by substitution of the appropriate
figures into the first order rate equation\(^\text{1}\). From this, the relationship between cAMP accumulation and degradation could clearly be observed - a pseudo-hyperbolic curve was seen on a plot of cAMP versus rate of elimination, which could be transformed using a standard Michaelis-Menten kinetic plot, and apparent Michaelis constants calculated. After this considerable effort, enzyme kinetics had been performed in the whole cell, with all the doubts about homogenisation artefacts consequently dispelled.

As was thoroughly discussed in chapter 5, the values of cAMP judged to have accumulated may not have been identical with those seen by the PDEs, for reasons of compartmentation or sequestration. This could introduce significant errors into the interpretation of the kinetic profiles. That is, whilst the Hanes plot appeared linear, it may have done so only because of incorrect assumptions about the free cAMP concentration, or because of the range of substrate used. Nonetheless, it probably does not invalidate the conclusion that the PDE(s) present in 1321N1 cells are close to saturation under the extremes of agonist stimulation. The PDE types known to exist in these astrocytoma cells are the Ca\(^{2+}\)/calmodulin (type I) and the rolipram-sensitive (type IV) isozyme. How do the data generated in the whole cell compare with the cell-free situation?

The complement of PDEs that a cell typically possesses, broadly speaking, can be divided into the low and high Km types. The human glioma cells fit into this category with the expression of the type I (high Km) and the type IV (low Km). What advantage does this mixed PDE population confer upon the

\(^1\) These figures compare favourably with rates calculated from assuming that the initial fall in cAMP over the first minute after antagonist addition is still linear (by analogy with in vitro kinetics studies). The difference in rates calculated by the two methods is only ~15%, with the calculation from the first order equation being the slightly greater. This difference is consistently observed across all agonist concentrations, and does not alter the kinetics, qualitatively speaking, or the conclusions.
cell? The presence of several kinetically disparate PDE activities, including a low affinity, high capacity PDE activity, allows the cell to overcome unlimited increases in the second messenger that would occur with only a low capacity, high affinity system. At low cAMP levels, its disposal will predominantly be effected by the high affinity system, but as levels increase still further, type I isozyme will be recruited as the substrate levels approach the $K_m$ of the low affinity process.

The whole cell kinetic studies in 1321N1 cells were consistent with only one type of PDE activity (with caveats discussed), which was low affinity ($K_m$ estimated as $85\mu M$). In the cell free analysis of [Tanner et al. 1986], type I PDE was reported to have a $K_m$ 70$\mu M$ in the presence and 200$\mu M$ in the absence of calcium. It is assumed that Ca$^{2+}$ does not significantly increase in these cells after $\beta$-AR activation (at least, not sufficiently to activate PDE as muscarinic receptor activation is presumed to activate type I PDE in the presence of isoprenaline [Meeker & Harden 1982]). Therefore, there is a discrepancy between the calculated $K_m$s of 85$\mu M$ and the 200$\mu M$ for the intact, and broken cell preparations respectively, but some points need stressing. Firstly, it is being assumed that the high $K_m$ activity is really type I with little evidence save its affinity, but additional experiments can affirm this (see below). Secondly, the precise cell water content and percentage protein of these cells has not been empirically determined, and some laxity should thus be accorded the $K_m$ in the intact cell. Thirdly, the kinetic analyses of [Tanner et al. 1986] were conducted in homogenates in which no protease inhibitors were included, and PDEs are renowned for their susceptibility to proteolysis. This may conceivably alter the kinetic properties of the isozyme from that found in situ.

To clarify that it is indeed PDE type I that predominates on the Hanes plot requires further work, as was alluded to above. In order to support the proposal that the calculated high $K_m$ describes the Ca$^{2+}$/CaM-sensitive isozyme, it would be instructive to generate more Hanes plots under different
conditions. For instance, in the presence of 'selective' inhibitors of either type IV (rolipram, Ro 20-1724) or type I (8-methoxymethyl IBMX), where it would be predicted that the apparent $K_m$ of the activity would only increase with inclusion of the latter, provided it was sufficiently PDE I/IV selective. As a more diagnostic test, activation of the calcium-mobilising receptors (mACHR or H$_1$-histamine) in these cells leads to significant activation of a type I PDE and the lowering of $\beta$-AR-stimulated cAMP levels [Tanner et al. 1986]. The expected effects of this treatment upon the $K_m$ and $V_{\text{max}}$ would be the first demonstration of a change in these parameters in an intact cell. Regarding the proportion of the PDEs, perturbation of the relative levels of each PDE would be effected by a change in gene expression, which has been successfully performed in other cells. In C6 glioma cells, chronic activation of PKA with either cAMP analogues or prolonged $\beta$-AR activation, resulted in the up-regulation of PDE IV [Schwartz & Onali 1984, Swinnen et al. 1989a]. An alternative approach would be to treat cells with glucocorticoids which can alter expression of other PDE isoforms [Ross et al. 1977, Lai et al. 1982, Elks et al. 1983]. With these few protocols, the nature of the functional PDE complement during $\beta$-AR activation might be more well defined.

One of the primary objectives of this study was to see whether or not the PDEs of 1321N1 cells displayed negative cooperativity in the intact cell. Should they do, then this has further consequences regarding the relationship between synthesis and accumulation (see chapter 5: e.g. when no, or negative cooperativity of PDEs pertains, then the $K_a$ for agonist for stimulating accumulation is greater than that for stimulating adenyl cyclase [Erneux et al. 1980]). However, such an undertaking proved fruitless. That the Hanes kinetic plot appeared linear was suggestive of the cellular PDE activity not exhibiting such a property. Such a conclusion may not be justified if the extent of cAMP accumulation was large enough to swamp the high affinity enzyme(s), meaning that the predominant activity was attributable to the low affinity form(s). This, it
must be said, would be the more likely explanation given the estimations of cellular cAMP concentration (up to ~100\mu M), and even the lowest level of stimulated cAMP being ~12\mu M (~90% saturated if K_m=1\mu M). This could be confirmed by generating decay curves with lower isoprenaline concentrations, which is feasible for even 3nM agonist evoked a seven-fold response. These are important experiments because a non-linear Hanes plot would be anticipated (should the higher agonist levels also be included). The negative cooperativity that was expected from the outset may then be demonstrated.

Another important confirmation of theory that emerged was that the cAMP accumulation profile after agonist challenge in cells is strongly governed by the PDE population: amplification of the hormonal input can be manifest and attributed to the nature of the PDE complement [Erneux et al. 1980]. This was demonstrated in the 1321N1 cells inasmuch that it could be shown that for given increases in its synthetic rate, the cAMP accumulation that resulted was unexpectedly large. This conferred a sensitivity to small changes in hormonal stimulation over a range predetermined by the PDE type, and moreover, amplified the degree of accumulation elicited - presumably, minimising ATP consumption over prolonged agonist exposures.

With regard to the types of PDE present in 1321N1 cells, there is some difficulty in attributing a great functional significance to the high affinity PDE in 1321N1 cells under conditions of \beta-AR activation. In addition to the above, addition of selective inhibitors like rolipram to cells produces only marginal increases in agonist-stimulated cAMP responses compared to non-selective inhibitors such as IBMX (preliminary data and [Tanner et al. 1986]). This may be confirmed by performing experiments along the same lines as [Butcher et al. 1983], wherein they reasoned that if a high affinity system predominates, then this should easily be saturated and any subsequent increase in synthesis will result in a larger than expected increase in accumulation. The net result: an upward concavity in the concentration response curve (plotted linearly - akin to
figure 12 chapter 5) over lower agonist concentrations. In 1321N1 cells, the range of isoprenaline concentrations required to demonstrate this inflexion should be lower than those used in the decay experiments because it is over the lower range that the high affinity PDE is most likely to be operative (see above). With respect to the cell-free PDE work, upon partial resolution of 1321N1 PDE activities using DEAE cellulose chromatography and sucrose density centrifugation, the ratio of apparent $V_{\text{max}}$ of the type I and IV was high [Tanner et al. 1986]. Whilst dilution of the enzymes will inevitably occur during these procedures, it does at least suggest that the calcium-sensitive PDE predominates in these glioma cells.

That is not to say that type IV PDE will not contribute to modulation of cAMP, since compartmentation may prevail: although a substantial amount of PDE is found (apparently) in the soluble fraction of homogenates, it does not follow that in situ a high affinity form may not be membrane-associated. It has been previously envisaged that a gradient of cAMP concentration could be less pronounced in the cell if a high affinity form was found near the site of synthesis (plasmalemmal location?), while the soluble, low affinity form would control the rate of approach towards the new steady state [Swillens et al. 1974, Fell 1980]. Such suppositions cannot be validated without e.g. immunocytological analysis [Barsony & Marx 1990], or cAMP image analysis [Adams et al. 1991] but do mandate for a more careful consideration of cAMP dynamics.

The turnover of cAMP was further assessed in these cells by exploiting a pulse labelling technique. Its elegance lies in the fact that receptor occupation is maintained and therefore obviates the need for good antagonists to generate decay curves. Such a protocol did not give interpretable results in cerebral cortical slices, but proved to be more successful in these cultured cells. Initially, the two methods were compared under conditions of $\beta$-adrenergic stimulation and found to give statistically different values of the cAMP turnover
constant. This initially gave some indications of following the scheme proposed for WI-38 fibroblasts where PGE₁ receptor occupation led to an inhibition of PDE. However, a more exacting criticism of the experimental protocol left one to conclude that the small difference was merely methodological. Moreover, experiments comparing forskolin with the agonist deemed that the turnover of cAMP was probably not affected by receptor occupation. This in itself was gratifying since it confirmed the validity of using either technique to measure cAMP turnover in these cells and opened up application to comparisons of turnover after stimulation by any of a number of agonists. The cells express PGE₁ and adenosine receptors which elicit sizeable increases in cAMP, and the types of experiments that were to be originally performed in the brain slice preparation (viz examining agonist-specific differences in turnover) could be conducted in a homogeneous cell population. Unfortunately, preliminary experiments comparing flux rates with all types of cAMP stimulants were unsuccessful because of the relatively low stimulation levels that have to be used (the degree of accumulation evoked must be similar with each agent). That notwithstanding, it is envisaged that the experiments could be adequately performed if the initial prelabelling with [¹⁴C]adenine was increased. The results with adenosine agonists may well prove the most interesting since the receptor complement on these cells is not restricted to just A₂ receptors (positively coupled to adenylyl cyclase). Receptors whose agonist profile is similar to A₁ are also present and these can couple both to the suppression of cAMP accumulation, and possibly to the inhibition of phosphoinositide generation [Nakahata et al. 1991]. That adenosine agonists have also been reported to modulate PDE activity in brain [De Mazancourt & Giudicelli 1988] strengthens further that it offers an interesting avenue of research.

What makes the studies of cAMP flux all the more interesting at the present time is that there is evidence to suggest that enhanced turnover may per se serve a signalling rôle which was not previously appreciated. For example,
the exacting studies from Goldberg's laboratory show that in parotid cells, isoprenaline is capable of stimulating amylase secretion by a mechanism not involving a change in accumulated cAMP, but with a considerable enhancement of its turnover [Deeg et al. 1988]. That is, synthesis and degradation of cAMP are greatly, and importantly, equally enhanced such that there is no change in steady state cAMP levels. Measurements of augmented flux of cAMP have also been performed in platelets [Walseth et al. 1983] and also cGMP turnover was quantified in neuroblastoma cells and rod outer segments [Graeff et al. 1987, Dawis et al. 1988]. The underlying mechanism in the parotid is intriguing, let alone its consequences, and interestingly, it was recently proposed that activation of PKA was greater when there was simultaneous production and degradation of cAMP [Leiser et al. 1986]. Other models excluding PKA activation encompass a local generation of protons upon hydrolysis of cAMP, or alternatively, the provision of energy upon PDE-catalysed cleavage of the energy-rich 3′ bond of cAMP [Deeg et al. 1988]. The idea that cAMP may, like Ca^{2+} [Berridge & Irvine 1989], be a frequency encoded, as well as an amplitude encoded, signal is still in its infancy, but is clearly an issue of some considerable importance.

PDE IV, the cAMP-specific low K_m isozyme, is perhaps one of the more widely appreciated forms, predominantly because of the availability of the good selective inhibitors, rolipram and Ro 20-1724. One of the more remarkable properties of this enzyme is its apparent lack of short term regulation in cells of the CNS (although its relative expression can be modulated). With the cell's usual economy and efficiency, it might have been expected that this enzyme would provide an excellent point to regulate cAMP accumulation. After all, it specifically catabolises cAMP within a substrate range that may be more physiologically relevant, and therefore slight alterations of its activity could result in dramatic changes in cAMP accumulation. Protein kinase activation may offer one route of modulation: it would be of interest to examine the effect upon [^{3}H]cAMP accumulation of short term preincubation with the cAMP analogue 8-
\( p \)-chlorophenylthio-cAMP (which activates PDE III via PKA in peripheral tissues [Gettys et al. 1987]) in 1321N1 cells. Furthermore, any possible effects of PKA activation upon the CaM-mediated stimulation of PDE I [Sharma & Wang 1986a, Hashimoto et al. 1989] may also be thoroughly examined \textit{in situ} in this cell line.

In conclusion, a substantial contribution has been made to the understanding of regulation of cAMP levels in a variety of systems \textit{viz} brain slices, SH-SY5Y neuroblastoma, and 1321N1 astrocytoma cells. Kinetic analyses of this type have been seldom applied to quantify cAMP hydrolysis in cellular systems, despite the discussion of theoretical models, and it as been shown that they can greatly assist determining the nature of the PDEs present. The experiments conducted in glioma cells represent the starting point for further studies into negative cooperativity, or changes in PDE kinetics after pharmacological or more physiological manipulations. Since it has been shown that cAMP turnover can be measured in two relatively simple ways in these cells, results can be confirmed by two complementary techniques (e.g. the change in \( k_e \) with cAMP concentration could be reassessed with the pulse-labelling method). Moreover, the derivation of pseudo-Michaelis-Menten constants by this method could be applied to other systems such as analysing the kinetics of expressed clones of PDEs in the intact cell, or the change in PDE expression. Although more elaborate protocols such as the \( \alpha \)-phosphoryl labelling may gives estimates for functional PDE kinetic parameters [Goldberg et al. 1983], or acceleration of cAMP turnover [Deeg et al. 1988], it is clear that the two techniques used here can potentially address the same issues but with a far simpler approach. The assessment of cAMP turnover has thus been demonstrated to be an invaluable tool for those wishing to study PDEs in a more physiological context.
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