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PARTIAL PURIFICATION AND CHARACTERIZATION OF SOLUBLE CYCLIC NUCLEOTIDE PHOSPHODIESTERASES IN HUMAN AND MURINE TISSUES

by

Marion Frances Robinson

A thesis submitted to the Faculty of Medicine,
University of the Witwatersrand, Johannesburg
in fulfilment of the requirements for the degree
of Doctor of Philosophy

Johannesburg, 1988

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Marion Robinson

Marion Frances Robinson

28th day of September, 1938

DEDICATION

To my parents, grandmother
and twin, Caroline, for their
enduring support and encouragement.

Now I know in part; then I shall
understand fully , even as I have been fully
understood.

I Cor 13;12

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ABBREVIATIONS

Abbreviations used in the text:

AC	- adenyl cyclase (E.C. 4.6.1.1)
A-kinase	- cyclic-3',5'-adenosine monophosphate-specific protein kinase (E.C. 2.7.1.37)
5'-AMP	- adenosine 5'-monophosphate
AP	- alkaline phosphatase (E.C. 3.1.3.1)
APS	- ammonium persulphate
AS	- ammonium sulphate
ATP	- adenosine triphosphate
bis acrylamide	- N,N'-methylenebisacrylamide
CaM	- calmodulin
cAMP	- cyclic-3',5'-adenosine monophosphate
cCMP	- cyclic-3',5'-cytidine monophosphate
CF	- chromatofocusing
cGMP	- cyclic-3',5'-guanosine monophosphate
cIMP	- cyclic-3',5'-inosine monophosphate
cPDE	- cyclic nucleotide phosphodiesterase (E.C. 3.1.4.17)
DE-52	- diethylaminoethyl
DEAE	- diethylaminoethyl
dibutyryl cAMP	- N ,O ' -dibutyryl cyclic-3',5'-adenosine monophosphate
DMBA	- 7,12-dimethylbenzanthracene

DNA	- deoxyribonucleic acid
dpm	- disintegrations per minute
DTT	- dithiothreitol
EDTA	- ethylenediaminetetraacetic acid
EGTA	- ethylene glycol bis(beta-aminoethyl ether) -N,N',-tetraacetic acid
F1	- first generation progeny of a cross between two parental strains
FSH	- follicle stimulating hormone
GC	- guanyl cyclase (E.C. 4.6.1.1)
5'-GMP	- guanosine 5'-monophosphate
G-kinase	- cyclic-3',5'-guanosine monophosphate-specific protein kinase
G proteins	- guanine nucleotide binding proteins
IBMX	- see MIX
IPM	- interphotoreceptor matrix
Kft	- fractional turnover constant
Km	- that concentration of substrate which gives half the maximum velocity of enzyme activity
LH	- luteinizing hormone
MIX	- 1-methyl-3-isobutylxanthine
MNNG	- N-methyl-N'-nitro-N-nitroso guanidine
N	- guanine nucleotide regulatory protein of adenyl cyclase
NAD	- nicotinamide adenine dinucleotide

NAD-pyrophosphorylase	- nicotinamide adenine dinucleotide pyrophosphorylase (E.C. 2.7.1.1)
nd PAGE	- non-denaturing polacrylamide gel-electrophoresis
NMN	- nicotinamide mononucleotide
5'-nucleotidase	- 5'ribonucleotide phosphohydrolase (E.C. 3.1.3.5)
PAGE	- polyacrylamide gel-electrophoresis
Pi	- inorganic phosphate
PMSF	- phenylmethylsulphonylfluoride
PPi	- inorganic pyrophosphate
rbc	- red blood cell
Rf	- relative mobility
ROS	- rod outer segments
RS 82856	- N-cyclohexyl-N-methyl-4-((1,2,3,5-tetrahydro-2oxoimidazo(2,1-b) quinazolin-7-yl)oxy)butyramide
S	- sedimentation coefficient (Svedberg unit)
SDS	- sodium dodecylsulphate
TCA	- trichloroacetic acid
Temed	- N,N,N',N'-tetramethylethylenediamine
TFP	- trifluoroperazine
Tris/HCl	- tris(hydroxymethyl)aminomethane hydrochloride
Vmax	- maximum velocity

Abbreviations used in the figures and tables:

AD	- activator deficient
AI	- activator insensitive
B	- brain (murine)
EM	- electron microscope
H	- heart (murine)
K	- kidney (murine)
L	- liver (murine)
L	- leiomyoma of the uterus (human)
M	- myometrium (human)
Mo or Mr	- molecular mass
r	- linear regression coefficient
(s)	- substrate concentration
SD	- standard deviation
T	- tumour (murine)
U	- uterus (human)
v	- velocity
Yo	- charge density

ABSTRACT

Distribution and characterization of soluble and particulate cyclic nucleotide phosphodiesterases (cPDE) were studied in the murine tissues of brain, kidney, liver, heart and mammary tumour. Characterization and partial purification of cPDE enzymes in human mammary and uterine tissues were also achieved. The soluble forms of cPDE in the murine cytosols were examined by non-denaturing polyacrylamide gel-electrophoresis coupled to a specific cPDE activity stain which had been modified and adapted to vertical slab gels. A total of 6 migrating bands of cPDE activity (with characteristic relative mobilities - Rf's) were observed in the different murine tissues. Some of these bands were either common to all or some of the tissues whilst other bands were unique to the tissues such as the two faster migrating cPDE activity bands solely characteristic of the murine mammary tumour. All the observed cPDE activity bands hydrolyzed both cAMP and cGMP and, furthermore, the observed cPDE activity profiles for either substrate were very similar. The various cPDE activity forms in the murine tissues were tested for susceptibility to two non-specific inhibitors theophylline and isobutylmethylxanthine (MIX) and to two competitive inhibitors of calmodulin-sensitive cPDE enzymes namely trifluoroperazine (1 mM) and calmidazolium .5 uM). The inhibitor isobutylmethylxanthine (MIX 100 uM) was found to be the most potent inhibitor and trifluoroperazine

(TFP 1 mM) also caused significant inhibition suggesting the majority of the soluble cPDE activity in the murine tissues to be calcium/calmodulin-dependent. Molecular mass analysis of the forms, either by means of Ferguson plot or linear polyacrylamide gradient gels or gel filtration, yielded apparent molecular masses that ranged from 168000 to 426000 daltons. Attempts to characterize further the soluble murine mammary tumour cPDE by chromatofocusing revealed three activity peaks with respective pI ranges of >7, 5.2-5.6 and 4.1-4.2. Measurement of the unbound cytosolic oestrogen and progesterone receptor content in 32 murine mammary tumours demonstrated that there were undetectable levels of both receptor types and, therefore, the tissues exhibited hormone-independent growth. Next, using the same adapted gel technique, the soluble cPDE activities were studied in human normal and malignant mammary tissues. In contrast to the murine mammary tumours, six forms designated bands 1-6 with the most cationic migrating species being band 1, were observed in human malignant mammary tissues. Although, there appeared to be only four forms in the normal human mammary cells at low gel concentrations (<7%). These forms in either malignant or normal mammary tissues hydrolyzed both cAMP and cGMP. The calculated molecular masses and charge densities (Yo) for the respective malignant bands were: band 1 had an Mr of 168000 $\pm$ 5000 and Yo of 2.37; bands 2-6 had an Mr range of 158000-163000 $\pm$ 8000 and Yo values of 2.59, 2.71, 2.81, 2.95 and 3.21. The normal mammary tissue exhibited bands at the

same relative mobilities (Rf) of bands 1, 3, 4 and 5 evident in the malignant tissue and, in addition, the normal mammary cPDE bands had similar molecula masses and charge densities. Enzyme kinetic analysis, using cAMP as substrate, of band 1 which had been eluted after preparative native electrophoresis of the malignant cytosol, revealed a non-linear Lineweaver-Burk plot exhibiting apparent negative co-operativity and calculated approximate Km values of 3.8 uM and 50.1 uM with respective Vmax values of 4.5 and 25 nmol/mg protein/min. The effect of preincubation of cytosols with 2 mM EGTA, prior to electrophoresis, resulted in the inhibition of bands 2-6 whereas band 1 remained active and a new lower molecular mass form was also observed.

The particulate forms of cPDE in human and murine mammary tissues, either normal or malignant, were studied by using an ultracytochemical technique coupled to the cPDE activity stain. The detectable cPDE activity, which was found to be mainly localized on and within the nuclear membrane, nucleoli and chromatin, was strikingly observed in malignant cells, to a lesser extent in fibrocystic tissue and was not apparent in the parenchymal cells of normal tissue. Only cAMP was tested as substrate and the results suggested a nuclear membrane-bound form of cPDE to be significantly associated with malignant mammary tumour cells.

Finally , the soluble cPDE activities were studied in human uterine tissues. Human leiomyoma of the uterus contained seven

forms of cPDE in the crude cytosol as revealed by the specific cPDE activity stain on non-denaturing polyacrylamide gel electrophoresis. The enzymes from human myometrium and normal uterus showed an identical activity pattern although both tissues exhibited denser staining for the cPDE activity profiles in comparison to leiomyoma at identical soluble protein concentrations (5 mg protein/ml). Furthermore, the myometrium cytosols exhibited lower oestrogen and progesterone receptor content compared with the cytosols of leiomyoma. Ferguson plot analysis of the cPDE profiles of leiomyoma showed four different molecular mass species of; band 1 Mr 229000 ± 4000; band 2 Mr 186000 ± 4000; bands 3-6 Mr 174000 ± 4000; and band 7 Mr 162000 ± 4000 although this latter band was not always observed.

Partial purification of bands 3-6 was achieved using DE-52 anionic exchange chromatography at 0.1-0.4 M ionic strength followed by theophylline-Sepharose affinity chromatography and native and SDS polyacrylamide gel-electrophoresis. Four subunits of Mr 65000, 59000, 44000 and 42000 were seen on SDS polyacrylamide gel-electrophoresis. Enzyme kinetic analysis of band 1 eluted from non-denaturing polyacrylamide slab gels resulted in a linear Eadie-Hofstee plot with a Km of 5.0 ± 0.3 uM and a Vmax of 26 ± 3.5 nmol/mg protein/min. Band 2, similarly extracted, gave a non-linear plot showing apparent negative co-operativity with respective Km values of 5.8 and 36 uM and Vmax values of 12 and 64 nmol/mg protein/min.

Sucrose gradient ultracentrifugation analysis of crude leiomyoma cytosol revealed three peaks of cPDE activity (1 uM cAMP) at 3.6 ± 0.3 S, 8.1 ± 0.3 S and 11.8 ± 0.3 S. The purified theophylline-Sepharose affinity form yielded one peak at 7.8 ± 0.3 S.

CHAPTER ONE

INTRODUCTION

General Concepts

The purpose of this chapter is to introduce the past to present day perspectives of purine cyclic nucleotides with emphasis on the regulatory roles of the catabolic enzymes, classified as cyclic nucleotide phosphodiesterases, towards these moieties at the cellular and molecular level. This will progress towards a summary of the more significant findings related to cyclic nucleotide phosphodiesterases and an attempt to define the different forms of these enzymes based extensively on *in vitro* studies. The nomenclature system used will be similar to that suggested by a committee headed by Dr. M. M. Appleman on a symposium on the subject,(Volume 16: Advances in Cyclic Nucleotide and Protein Phosphorylation Research 1984) and the slight changes adopted will be the result of recent reports. In brief, a summary of the forms with abbreviations in parenthesis are as follows:

1. Type I, calmodulin-sensitive cyclic nucleotide phosphodiesterase (type I cPDE)
2. Type II, cyclic GMP-sensitive cyclic nucleotide phosphodiesterase (type II cPDE).

3. Type III, cyclic GMP-specific phosphodiesterase (type III cGMP PDE).
4. Type IV, cyclic AMP-specific phosphodiesterase (type IV cAMP PDE).

The nomenclature system is, at best, an arbitrary one based on substrate preference and general similarities of physical behaviour in the test tube. To what extent this reflects the *in vivo* enzyme(s) remains to be fully defined. Although several authors have reported purification of different enzyme forms to apparent homogeneity there has been no amino acid sequencing data achieved either wholly or in part in mammalian tissues. Advances in the development of monoclonal antibodies to mammalian cyclic nucleotide phosphodiesterase (Beavo *et al.* 1982) or calmodulin (Hansen and Beavo 1986) and definition to within 5 kilobases of DNA of a *Drosophila melanogaster* form (review Davis and Kauvar 1984), partial or complete identification of a slime mold phosphodiesterase gene (Podgorski *et al.* 1986; Lacombe *et al.* 1986) and two yeast cPDE genes (Nikawa *et al.* 1987) should be of significance in the unravelling of the complexities of the cyclic nucleotide phosphodiesterase(s). In summary, are the enzyme forms observed *in vitro* either the result of different structural genes, processing of the mRNA(s) in the cytoplasm, covalent modification of the translation product(s) or possibly artifacts arising from separation techniques or a combination of one or more of the above? The final part of this chapter will briefly outline the objectives of this thesis which have

centred, for the most part, on the cyclic nucleotide phosphodiesterases of both human and murine normal and neoplastic tissues. Since the enzyme forms are present in mammalian tissues in small amounts and are highly labile there is a paucity of data on either purification or characterization of human tissue forms. To each ensuing chapter there will be a more detailed introduction with pertinent references to the particular subject of interest.

Function of the Cyclic Nucleotide Phosphodiesterases.

Cyclic nucleotide phosphodiesterases (EC 3.1.4.17) represent the only known specific catabolic enzymes of the cyclic purine nucleotides namely cyclic adenosine 3':5'-monophosphate (cAMP) and cyclic guanosine 3':5'-monophosphate (cGMP). These activities have been identified in practically all eukaryote organisms (Arch and Newsholme 1976) and also in prokaryotes (Iwasa *et al.* 1982). A nonspecific phosphodiesterase which catalyzes the hydrolysis of cAMP, in addition to many other substrates, with a Km for cAMP of about 100 uM and Vmax of 7 umol/min/mg protein has been purified from bovine intestine (Kelly and Butler 1977). A cCMP phosphodiesterase which also hydrolyzes cAMP with a Km of about 25 uM and Vmax of 1.6 umol/min/mg protein has been described in bovine liver (Helfman *et al.* 1981). Apart from these two latter exceptions to the specific cyclic nucleotide phosphodiesterases (EC 3.1.4.17) and whether they play a major role in purine cyclic nucleotide

regulation remains to be established. The cyclic nucleotide phosphodiesterases act by hydrolyzing the 3' bond of the 3'carbon atom of the ribose rings and the reaction possibly proceeds by a displacement mechanism involving nucleophilic substitution at the phosphorus atom (Goldberg *et al.* 1984). The products are the inactive compounds 5'-AMP and 5'-GMP. The hydrolysis of the cyclic nucleotides is a highly exothermic reaction with a small change in entropy and may essentially be regarded as an irreversible thermodynamic switch. Evidence presented, however, will show that this enzyme(s) system does not merely represent an off switch but that the cyclic nucleotide phosphodiesterases probably hold a key position in the regulation of signals within the cell.

It would be useful now to digress to the subject of the purine cyclic nucleotides; their respective discoveries, general physiological data and relationship with other second messengers in both proliferation and regulation of cellular growth mechanisms. This, at best will be the briefest of surveys with only a few highlights mentioned from the immense array of literature and several references to excellent reviews will be given.

Purine Cyclic Nucleotides.

Sutherland and Rall first collected and purified a substance which activated phosphorylase and identified it later as cAMP (Sutherland and Rall 1958). During the ensuing eight years.

using as a quantitative assay the ability of cAMP to activate phosphorylase, Sutherland and his colleagues found that cAMP was widely distributed in all tissues and cells. Furthermore they demonstrated that an enzyme found in the cell membrane, adenyl cyclase (EC 4.6.1.1.), catalyzed the synthesis of cAMP and inorganic pyrophosphate (PPi) from adenosine triphosphate (ATP) (Rall and Sutherland 1962). In the same year, working closely with Butcher, Sutherland identified a specific enzyme, a cyclic nucleotide phosphodiesterase that converted or catabolized cAMP to 5'-AMP (Butcher and Sutherland 1962). It is interesting to note that at that time the apparent Km of the cAMP phosphodiesterase (about 0.1 mM) was calculated to be very much greater than the intracellular concentrations of cAMP (about 1 uM or less). This seemed to suggest that the enzyme did not have a particularly significant role in the regulation of the cyclic nucleotide and it was only several years later when Brooker and associates (1968) produced a more sensitive assay procedure based on the use of tritiated cAMP that a much higher affinity cAMP phosphodiesterase was measured. This was further confirmed by Beavo and workers (1970) who demonstrated that the bovine heart enzyme exhibited an apparent Km for cAMP of 25 to 40 uM and for cGMP of 1 to 3 uM. This finding prompted quickened interest in the enzyme and subsequent increase in publications on the subject. Prior to that, most interest was focussed on the synthetic enzyme adenyl cyclase and the importance of cAMP in cell mechanisms. Studies on this moiety accelerated exponentially and resulted in the second messenger

theory being proposed (Sutherland and Robison 1966) in which a hormone or nerve impulse is first messenger and cAMP the second intracellular messenger. This theory was further elaborated to include firstly, the hormone receptor-adenyl-cyclase complex as the transducer of the extracellular primary messenger to the intracellular second messenger cAMP and secondly, the site of action of cAMP to be cAMP-specific protein kinases (Robison *et al.* 1971). The latter two enzymes, mainly detected in the cytoplasm although also associated with cell membranes, are often referred to now as A-kinases and their only known function in eukaryotes, following activation by cAMP, is the phosphorylation of substrate proteins (review Lohmann and Walter 1984).

The structure of the receptors coupled to adenyl cyclase have still to be elucidated with the exception of the beta-adrenergic receptor. Also, the mechanisms of action of the receptors, either stimulatory or inhibitory, have been demonstrated to be complex. Later a third regulatory component of the adenyl cyclase system was ascertained through the discovery and purification of coupling proteins (Ns and Ni). It was deduced that the regulation of adenyl cyclase by hormones necessitated the simultaneous action of a guanine nucleotide and that the Ns and Ni proteins mediated the guanine nucleotide regulation of adenyl cyclase (reviews Ross and Gilman 1980; Clark 1986).

It should be stated that the most important criterion of a "second messenger" is to show that the messenger directly

stimulates a rate-limiting step of the effector (Berridge 1975). This, in fact, was to prove more difficult to show for cGMP and only recently has there been conclusive evidence that cGMP directly opens sodium channels in the retinal rod cells of the lens (review Stryer 1986). The purine cyclic nuclectide was first isolated in rat urine by Ashman and co-workers (1963) and subsequent measurements of cGMP levels, 10-100 nM, in cells and tissues indicated that it was present in lower concentrations than cAMP, about one tenth (Goldberg *et al.* 1969), which necessitated more sensitive and hence accurate methods for its detection (Coffey *et al.* 1978). The concentration was found to be somewhat higher in lung and cerebellum (Goldberg *et al.* 1973) and unusually high (70 uM) in retinal rod cells (Goridis *et al.* 1974) which, as will be seen later, is significant in the type of cyclic nucleotide phosphodiesterase expressed. Evidence for a cGMP-activated protein kinase (G-kinase) in mammalian tissues was later identified (Kuo and Greengard 1969). This enzyme is usually referred to as G-kinase and, like the A-kinases, is mostly associated with the soluble fraction of tissues (about 95%). The concentration of G-kinase is about five to ten fold less than A-kinases in tissues such as heart, lung and smooth muscle although much lower levels have been reported in liver and adipose tissue. Of interest, is the finding that G-kinase, in contrast to the A-kinases, is extremely sensitive to cGMP by about two orders of magnitude than to cAMP whereas it has been shown that fairly high levels of cGMP may activate A-kinases. The rate of substrate

phosphorylation by G-kinase is considerably slower than that of the A-kinases and the discovery of other proteins which specifically bind cGMP make it difficult to assess the actions of cGMP (review Murad *et al.* 1979; Lincoln and Corbin 1983; Walter 1984).

Other Second Messengers.

Evidence ensued that there was a definite interrelationship between the purine cyclic nucleotides and calcium ion in many stimulus-induced cellular responses. The resting concentration of free intracellular calcium ion is about 0.1 to 1 uM in most mammalian cells (Exton 1986) and an increase in the concentration by ten to one hundred fold causes the activation of many enzymes including type I cPDE. The cellular processes which arise, as a result of this increase, in conjunction with cyclic nucleotides and often calmodulin or other calcium-binding proteins include: hormone secretions such as thyroid and pituitary, cell proliferation, cell architecture, smooth muscle contraction and lysosome release. The changes in intracellular calcium ion concentration can be elicited either extracellularly, where the basal calcium concentration (1 mM) is 1000-10000 fold greater than intracellular calcium, through voltage and agonist dependent calcium channels or via intracellular sources such as mitochondria, endoplasmic reticulum and possibly the nucleus. These activation signals are deactivated by the reduction in intracellular free calcium

levels caused by energy driven ATPase calcium pumps, translocation processes and by calcium binding proteins that do not participate in activation processes (reviews Berridge 1975; Goldberg *et al.* 1975; Rasmussen and Goodman 1977; Exton 1986). The question as to how the purine cyclic nucleotides and calcium ion interact encompasses a subject of immense complexity and there is always the problem of oversimplifying the facts but some general patterns seem to have emerged. There appear to be two major ways of transmitting information within the cell; firstly via cAMP through receptors coupled either to stimulation or inhibition of adenyl cyclase, for instance in liver, adipose tissue, adrenal cortex, thyroid and skeletal muscle; secondly, via calcium ion through calcium ion coupled receptors (review Gill 1985). Recently Rassmusen and colleagues (1984; Rasmussen 1986(a),(b)) have proposed that there are two pathways of information flow from the calcium receptor system. They propose that activation of the calcium receptors leads to the production of two intracellular messengers, inositol trisphosphate (Berridge and Irvine 1984(a)) and diacylglycerol (Kishimoto 1980) and these, in turn, activate two branches of information flow. The one fork, initiated via inositol trisphosphate, operates through "amplitude modulation of calmodulin-regulated events". That is, activation by calcium ion is elicited in various ways; either directly through activating an enzyme such as mitochondrial glyceraldehyde phosphate dehydrogenase; or indirectly by association with calcium-binding proteins such as calmodulin that can then

elicit the activation of type I cᵅDE which will be discussed in greater detail later; or by activation of the plasma membrane calcium pump; or lastly by controlling the activity of A-kinases and calcium-activated/calmodulin protein kinases. This branch is responsible for either brief responses or the initial phase of sustained responses. The other branch functions in close association with diacylglycerol defined as a second messenger (Kishimoto 1980) via "sensitivity modulation" of the calcium-activated, phospholipid dependent protein kinase system (C-kinase system). In short, the C-kinase system is responsible for the sustained phase of cellular responses. For the sake of brevity, the major transducing event in this calcium system is, in essence, phosphatidylinositol turnover and will not be detailed except to emphasize that the cell surface receptors, in response to certain hormones, neurotransmitters, growth factors and several mitogens then mobilize calcium, often release arachidonate and increase cGMP but not cAMP. In contrast, the extracellular messengers which induce cAMP usually do not induce phosphatidylinositol turnover or increase cGMP concentration (reviews Nishizuka *et al.* 1978; Takai *et al.* 1984). Experimental results reported (Rasmussen *et al.* 1984) suggest that in some cells cAMP concentration influences events in the C-kinase branch. Thus, there is increasing evidence that the pathways discussed above are not independent. Examples of actions of the other defined calcium branch which often operates in conjunction with cGMP include bidirectional systems such as contraction and metabolism of

smooth muscle, secretion processes (mast cells, platelets) and alpha-1 receptors. The precise relationship between calcium and cGMP is not clear and good reviews on the subject may be found (Berridge 1975; Rasmussen *et al.* 1975; Rasmussen and Goodman 1977; Coffey *et al.* 1978). It also appears that, in monodirectional systems and bidirectional systems as defined (Berridge 1975), cAMP in the former seems to act as a positive modulator of calcium whereas in bidirectional systems it opposes the calcium signal. It thus appears that calcium mediates most cellular responses and cAMP modulates the action of calcium either by changing the processes which generate calcium or by altering the responsiveness to calcium signals (Ralph 1983; Berridge 1984(b)). Calcium, in turn, controls cyclic nucleotide levels by its regulation of adenyl cyclase, guanyl cyclase, protein kinases and type I cPDE. In conclusion, there exists a hierarchy of feedback control mechanisms governing the second messengers within the defined pathways.

Second Messengers in Growth and Proliferation of Cells.

Early work on insulin and serum induced proliferation of fibroblasts in culture showed that a decrease in cAMP concentration seemed to act as a positive signal for the induction of proliferation (Johnson *et al.* 1971). In support of this, Sheppard and Prescott (1972) reported that the level of cAMP was highest at the G1 phase (quiescent phase of cell cycle) of synchronized hamster cells in culture whereas the

lowest concentrations were exhibited during mitosis. Studies based on cultured lymphocytes suggested that increases in cGMP and calcium represented a positive signal for induction of cellular proliferation, a bidirectional mechanism, whereas an increase in cAMP and decrease in calcium concentration represented a negative signal towards growth. Similarly, in cancer cell cultures, it was observed that the unregulated growth of cancer cells might be related to depressed levels of intracellular cAMP (Heidrick and Ryan 1970; 1971; Ryan and Heidrick 1968; 1974). More recent work on cell lines, however, and the respective roles of cAMP and cGMP in the *in vivo* control of both normal cell growth and the process of malignant transformation has not indicated direct causal effects. Work by DeRubertis and Craven (1980) showed that chemical carcinogens exposed to cell structures could increase cGMP levels by activating the guanyl cyclase system. The authors could not show a direct relationship between increased cGMP levels and stimulation of DNA synthesis. By contrast, even in the Seventies, the reverse effect of cAMP was reported in tissue studies, that is, it was claimed to be a positive regulator of cell growth (review Boynton and Whitfield 1983). According to the evidence presented in this very extensive review, carcinogenesis is the result of many different processes and the most frequently observed change in early carcinogenesis is an increase in adenyl cyclase activity and an increased responsiveness to beta-adrenergic agonists. It appears though that once neoplastic cells have become established there is no

clearcut evidence of increased or decreased levels of adenyl cyclase, cPDE or A-protein kinases. The subject of the differences between normal and neoplastic cells with reference to cPDE will be covered more fully in subsequent chapters of this thesis.

Cyclic Nucleotide Phosphodiesterases and Second Messenger Homeostasis.

A prominent feature of second messenger homeostasis, that is for cAMP, calcium and cGMP, is the extremely rapid turnover of these intracellular mediators, the concentration of which, is dependent on the balance between supply and removal. For the purine cyclic nucleotides this would seem to be largely dependent on the rate of synthesis by the purine cyclases and catabolism by the purine cyclic nucleotide phosphodiesterases (cPDE). In some cells, egress or efflux of cAMP or cGMP, may also be important in removal. One group defined a parameter, Kft, which is the fractional turnover constant of cAMP in intact cells and they calculated that in a transformed fibroblast cell line, VA13, as much as 18% of cAMP elimination was due to efflux (Barber and Butcher 1981). In a later report Butcher (1984) studied cell lines and found that, with one exception, and taking efflux into consideration, the cAMP PDE activity measured from cell free extracts was adequate to account for cAMP removal in the cell lines studied. In certain cell lines, however, namely S49 lymphoma cells, which have been

shown to contain high levels of the high affinity type IV cAMP PDE, the calculations for Kft were found to be significantly different. When the Kft was measured in intact cells and the value compared to ruptured cells the results showed that the cPDE activity was considerably higher in broken cell extracts (Butcher 1984; Butcher *et al.* 1986). Less is known about cGMP efflux although the phenomenon has been studied in rat liver slices where cGMP efflux is shown to increase steeply after mutagen stimulation with MNNG in the presence of a cPDE inhibitor (Tjornhammer *et al.* 1983). *In vivo* determinations of cAMP levels in mammalian plasma samples, using EDTA as a cPDE inhibitor, indicate serum concentrations to be about 10 nM which is one hundred fold less than intracellular levels of cAMP (Broadus 1977). Once again, there is apparently poor correlation with cell culture studies versus *in vivo* measurements.

Finally, the effect of purine cyclic nucleotide binding proteins remains to be defined. These include the purine cyclic nucleotide-activated protein kinases (reviews Doskeland and Ogried 1981; Lincoln and Corbin 1983; Lohmann and Walter 1984) and the recently described specific cyclic GMP binding protein found in rat platelets (Hamet and Coquil 1978; Coquil *et al.* 1980; review Hamet *et al.* 1984), in rat lung (Francis *et al.* 1980, review Francis 1985) and a similar protein in retina has been described (Yamazaki *et al.* 1980). Apparently this unique binding protein, not yet fully purified, but exhibiting specific characteristics has both cGMP binding activity and

cPDE activity and has been termed the cGMP birding protein phosphodiesterase (cGMP BPP) although its funtion in the modulation of intracellular cGMP levels is unclear. Apart from these above considerations and the previously mentioned non-specific phosphodiesterases, all of which, certainly render a more complex light to the issue of cAMP and cGMP removal it is probably fair to assume that the cPDE system is largely responsible for the removal rate of the cyclic nucleotides in many tissues. The most conclusive *in vivo* evidence for this has emanated from studies on cAMP removal in specific *Drosophila* mutants (review Kiger *et al.* 1981). It has been found in mutant flies lacking chromomere 3D4, which contains the dunce gene, that they have two to seven fold higher levels of cAMP compared to their wildtype counterparts. The dunce gene is believed to code for a form II cPDE which resembles the type IV cAMP PDE defined in mammalian tissues and is a high-affinity cAMP-specific enzyme. Studies have shown that in flies with two or more doses of dunce gene (duplication mutants) the gene dosage is proportional to the measured level of the form II cPDE.

Endogenous Effectors and Inibitors.

The following survey will add credence to the theory that cPDE is more than a mere catabolic switch but is in fact a complex regulatory enzyme system subject to allosteric control and feedback mechanisms. It has been shown that raised

concentrations of cAMP elicited by reagents such as cholera toxin induce *de novo* synthesis of cAMP PDE in both normal cell cultures such as fibroblasts (Manganiello and Vaughan 1972) and in neoplastic lymphoma cells (Bourne *et al.* 1973). This has yet to be demonstrated for cGMP although this nucleotide is known to regulate cAMP hydrolysis by type II cPDE *in vitro* where it induces stimulation of the enzyme at low cGMP concentrations (Beavo *et al.* 1971). In contrast, cGMP at low concentrations, has been shown to inhibit markedly cAMP hydrolysis by certain isoenzymes of type IV cAMP PDE (Harrison *et al.* 1986). For an early review on the above and related cyclic nucleotides see Chasin and Harris (1976). Recent experiments by Galoyan and Gurvitz (1985) on bovine hypothalamus soluble cPDE demonstrated that the type I cGMP PDE's were activated by low levels of cAMP (0.1-3.0 uM) under conditions where thiol groups were oxidized and these same conditions rendered the forms insensitive to calcium/calmodulin. The results were reversed in the presence of sulphydryl reducing agents such as dithiothreitol (DTT) or beta-mercaptoethanol. This is the first report in the literature of a type I cPDE being under cyclic nucleotide stimulation and appearing to require an "oxidized" enzyme structure while calcium/calmodulin activation of type I cPDE requires a "reduced" enzyme structure. In addition to regulation of cPDE by cyclic nucleotides, it is now well established that the type I cPDE is under allosteric control by the effector, calmodulin, which in the presence of saturating calcium ion activates the enzyme about ten fold (review Wolff

and Brostrom 1979). Certain lipids, unsaturated fatty acids and phospholipids are also reported to stimulate the type I cPDE and the stimulation is independent of calcium (Tai and Tai 1982). One group has shown that lysophosphatidylinositol was comparable in stimulatory effect to that of calcium/calmodulin on activator deficient type I cPDE (Tai and Tai 1982). Type II cPDE has been found to be strongly inhibited by lysophosphatidylcholine and also by unsaturated fatty acids whereas other phospholipids and saturated fatty acids were without effect (Yamomoto *et al.* 1984(b)). It has been demonstrated that some phospholipids such as phosphatidylserine and phosphatidylglycerol activate a partially purified particulate type IV cAMP PDE from rat adipocytes while phosphatidylinositol-4-phosphate was inhibitory (Macaulay *et al.* 1983). In contrast, it was found in mouse pancreatic islets that phospnatidylinositol and phosphatidylserine inhibited both soluble and particulate forms of cPDE (Capito *et al.* 1986). Metabolism of phospholipids has been implicated in the regulation of insulin secretion in pancreatic islets (Best *et al.* 1984). Clearly, there are species and tissue differences with regard to the effects of fatty acids and phospholipids on cPDE activity, their interaction being dependent on the biochemical milieu of the individual cell type. It is well established that cPDE, most probably some forms of type IV cAMP PDE, have been shown to be under hormonal control. The earliest report was by Loten and Sneyd (1970) on the effect of insulin on adipose tissue which showed a stimulation of cPDE activity.

Later it was found that insulin could increase more than one type of cPDE and some reports showed that the hormone could induce *de novo* synthesis of cPDE (review Wells and Hardman 1977). Also, insulin was demonstrated *in vivo* to activate, about two fold, and phosphorylate a particulate type IV cAMP PDE form in rat liver plasma membranes (Marchmont and Houslay 1980(a),(b); Marchmont and Houslay 1981(b)). It was soon demonstrated by this same group to occur in intact hepatocytes (Heyworths *et al.* 1983(b)) and they suggested that the activation and phosphorylation might be mediated through a specific guanine nucleotide regulatory protein (Heyworth *et al.* 1983(a),(b)). Thus, based on the above evidence, it appears that insulin's actions on type IV cAMP PDE are mediated through protein(s) other than A-kinase in rat liver plasma membranes. Another group, however, also studying rat liver and using similar methodology have been unable to demonstrate the activation of type IV cAMP PDE by insulin, either *in vitro* or *in vivo*, in plasma membranes. Only in plasma membranes prepared in isotonic media, not hypotonic as used by the above, were they able to observe insulin stimulation of the specific type IV enzyme in plasma membranes and particularly in Golgi fractions (Benelli *et al.* 1986). Of interest, the type I cPDE purified from rat brain was found to be phosphorylated but not activated by A-kinase (Sharma *et al.* 1984; Shenolikar *et al.* 1985). Other researchers, using higher substrate concentrations of cAMP and cGMP (100 uM), have demonstrated that an isolated endogenous A-kinase from rat brain does phosphorylate the type

I cPDE from the same tissue (Fukunaga *et al.* 1984). To what extent this latter finding reflects the *in vivo* situation is not clear. Activation of both type III and type IV cPDE forms by cAMP-dependent phosphorylation has also been reported in rat platelets (Tremb.ey *et al.* 1985).

Other hormones, for example catecholamines, have been shown to elicit a specific cPDE form in cloned astrocytoma cells (Uzonov *et al.* 1973) and several other hormones, including: adrenocorticotropin, progesterone, oestradiol, luteinizing hormone, follicle-stimulating hormone, growth hormone, aldosterone, prostaglandins and thyroxine have been reported to modify the activity of cPDE (Holtz *et al.* 1981; Patwardhan *et al.* 1983). Of particular interest, are the increasing number of *in vivo* studies on the effects of hormones on cPDE activity. For instance, some *in vivo* studies have revealed that FSH stimulated type IV cAMP PDE in seminiferous tubules of the testes of immature rats (Conti *et al.* 1983; Rossi *et al.* 1985) and in rat granulosa cells (Conti *et al.* 1984). By contrast, in immature rat ovaries, FSH and gonadotropin releasing hormone both together maximally stimulated membrane type IV cAMP PDE in the tissue (Knecht *et al.* 1983) but FSH alone inhibited the type IV activity. Further studies on type I cPDE have revealed that parathyroid hormone caused a rapid increase in activity of the enzyme and appeared to require calcium/calmodulin for its effect (Marcus and Grant 1983). The effects of steroid hormones with respect to mammary and uterine tissues will be dealt with in detail in a subsequent chapter. The precise ways in which

different hormones act in the stimulation or inhibition of cPDE remain to be elucidated. Clearly, hormones have complex effects with respect to cPDE enzymes and the fact that different patterns of isoenzymes of soluble type IV cPDE have been reported during the development of rat testis (Eppelen *et al.* 1980), rat ovary (Schmidtke *et al.* 1980) and chick ventricular myocardium (Epstein *et al.* 1987) would emphasize this point. As well as endogenous effectors there have been many recent reports in the literature on endogenous inhibitors which appear to inhibit different forms of cPDE. In addition to several publications on heat stable inhibitors two reports on the purification of an inhibitor protein to type I cPDE in brain were published in the same year (Klee and Krinks 1978; Wallace *et al.* 1978). The protein was heat-labile and composed of two nonidentical subunits with molecular masses of 15000-18500 and 60000-61000 respectively and was later termed calcineurin (review Manalan and Klee 1984). Low molecular mass peptides of less than 15000 that inhibit brain type I cPDE have been partially characterized (Collier *et al.* 1982). In cultured human epithelial cells, however, an endogenous, partially heat-stable inhibitor, to types I and III cPDE was identified (Niles and Loewy 1981). It is interesting to note that in retinal rod cells the type III cGMP PDE has a bound inhibitory subunit designated lambda of molecular mass 11000 which inhibits the enzyme in the dark state (Hurley and Stryer 1982). This inhibitory subunit is possibly displaced by GTP binding proteins in the presence of light (Sitaramaya *et al.* 1986).

Also a glycoprotein inhibitor of 47000 daltons is bound to extracellular *Dictyostelium* cPDE, a separately classified enzyme from the mammalian forms (Franke and Kessin 1981). There seem to be a variety of endogenous proteins that inhibit cPDE, the majority of which, inhibit type I cPDE in mammalian brain tissues. Also, it has long been recognized that cPDE, from most sources, requires magnesium ion (greater than 1 mM) for full activity (Butcher 1974) and other divalent cations such as manganese, zinc, nickel, cobalt and copper can be slightly stimulatory for some cPDE forms at low concentrations (less than 0.1 mM) but generally inhibitory at high concentrations (greater than 1 mM). Whether any of the latter divalent cations attain such high levels *in vivo* remains doubtful. An important publication by Donnelly and Barron (1981), however, has demonstrated that divalent cations influence the action of several cPDE inhibitors *in vitro*. They caution the use of only adding magnesium ion to monitor the actions of inhibitors with cPDE. Also a membrane-bound type IV cAMP PDE from rat liver plasma membranes has been postulated to be a metalloenzyme containing tightly bound zinc or manganese (Londesborough 1985).

Finally, it appears that carboxymethylation of the type I cPDE weakens the activation of the enzyme by calcium/calmodulin (Billingsley *et al.* 1984).

In conclusion, the action of endogenous effectors and inhibitors are dependent on both the cPDE enzyme and the internal biochemical environment of the cell.

Exogenous Effectors and Inhibitors.

It has been shown that imidazole activates the type I cPDE (Teo and Wang 1973; Kuo *et al.* 1978) but by far the greatest preponderence of the literature concerning exogenous additives has been based on inhibitors. Early it was reported that methylxanthines were competitive inhibitors of soluble beef heart cPDE (Butcher and Sutherland 1962) and, since then, a vast spectrum of pharmacological agents have been identified as inhibitors. It soon became obvious, at that time, that a knowledge of the tissue and cellular distribution of different forms of cPDE was necessary to gain any practical benefits from using inhibitors. By the mid-Seventies it had been tentatively demonstrated that some inhibitors such as methylxanthines and papaverine were relatively non-specific in their inhibition of cPDE enzymes (Miyamoto *et al.* 1976) whereas others such as trifluoperazine differentially inhibited activator (later termed calmodulin, Cheung *et al.* 1978) sensitive cPDE. As cPDE forms had not been purified to homogeneity there were problems in the interpretation of inhibitor actions (early reviews Weiss 1975; Wells *et al.* 1975; Chasin and Harris 1976). The subsequent definition of purified cPDE enzymes and isoenzymes, as a result of improved *in vitro* techniques, led to numerous reports of the selective action of inhibitors on these respective enzyme forms. For instance the type I cPDE, in addition to inhibition by EGTA (a chelating agent that specifically binds calcium ion), has been demonstrated to be

selectively inhibited *in vitro* by trifluoroperazine, calmidazolium, DDT and penfluoridol amongst others (review Van Belle 1984). Of interest, one of the findings by Van Belle (1984) was that verapamil, which is classified as a calcium antagonist, poorly inhibited type I cPDE. In fact verapamil has been shown to inhibit calmodulin insensitive forms (Epstein *et al.* 1982(a)). Trifluoroperazine (TFP) also a calcium antagonist and calmodulin inhibitor, however, was a very potent inhibitor of type I cPDE suggesting that the action of inhibitors is complex. Futhermore, many other agents classified to be "anticalmodulins" were more potent than TFP *in vitro* such as calmidazolium but when the latter drug was tested *in vivo*, in pharmacological screening tests, it proved to have no effect. This has many implications, not the least of which is the *caveat* of inferring too much from *in vitro* studies to the *in vivo* situation. Four recent reviews on all the selective inhibitors to the different cPDE isoenzymes and pharmacologic evaluation with some apparently positive findings for about twenty "second generation inhibitors" such as; vinocetine and zaprinast (selective for type I cPDE); rolipram (selective for type III cGMP PDE); and RS-82856 (specific for type IV cAMP PDE) have been published (Hagiwara *et al.* 1984; Hidaka *et al.* 1984; Weishaar *et al.* 1985; Venuti *et al.* 1987). Finally, it is noteworthy that some methylxanthines such as IBMX, at low concentrations, have been shown to stimulate certain purified cPDE enzymes (Erneux *et al.* 1982; Wong and Ooi 1985).

Multiple Forms.

The first report of multiple forms of cyclic nucleotide phosphodiesterases performed using gel filtration, was on soluble rat brain cortex (Thompson and Appleman 1971). Since then, there have been numerous publications demonstrating *in vitro* multiple enzyme forms differing with respect to: species, tissue localization, substrate specificities, intracellular distribution (that is either membrane-bound or soluble forms), response to endogenous or exogenous effectors and inhibitors, kinetic parameters, molecular masses, tryptic peptide maps and reaction to several monoclonal antibodies. Initially, several methods to separate the forms were reported, apart from gel filtration, namely; starch gel electrophoresis where, at most, four forms were separated in different mammalian tissues (Monn and Christiansen 1971); anion exchange chromatography which yielded three forms in rat liver (Russel *et al.* 1973) or six forms in somatic cell hybrids of rat muscle cells (Ball *et al.* 1979); four or less forms from sucrose gradient centrifugation (Thompson *et al.* 1973); at least six forms have been identified using polyacrylamide-gel-electrophoresis (Goren *et al.* 1971; Campbell and Oliver 1972; Uzonov and Weiss 1972; Weiss *et al.* 1974); and finally isoelectricfocusing yielded six isoenzymes in the soluble fraction of rat brain (Pledger *et al.* 1974). A matter of some concern, was that it was often not clear how enzymes or isoenzymes isolated by one method compared with respect to those forms separated by a different technique. Indeed, it was cautioned by one group that, in order to avoid

biochemical anomalies. cPDE enzymes should be analyzed by a variety of methods (Van Inwegen *et al.* 1976). It appears that cationic cofactor requirements, kinetic parameters, number, molecular mass and net charge of the forms can be altered by several factors such as pH, sulphydral compounds, hydrophobic interactions, proteolysis either endogenous or exogenous and temperature. An early but good review concerning some of the above problems on the subject was provided by Strada and colleagues (1978). A further complication was the description by various teams that some forms of cPDE were interconvertible in cell free systems (Chassy 1972; Pichard and Cheung 1976; Epstein *et al.* 1978; Strada *et al.* 1981; Ayad and Tobia 1980). The third report (Epstein et *al.* 1978), however, was disputed later by Keravis and colleagues (1980) and they suggested that the method previously used (sucrose gradient ultracentrifugation) did not resolve all the molecular forms of cPDE in the rat uterus. The demonstration of distinct non-interconvertible forms by three groups (Erneux *et al.* 1980; Keravis *et al.* 1980; Hansen and Beavo 1982) and the development of more sophisticated *in vitro* techniques such as improved affinity columns, tryptic peptide maps and immunological methods utilizing monoclonal antibodies enabled the purification to homogeneity of the cPDE enzymes and tentative classification of four types as outlined at the beginning of this chapter. The subject will be dealt with in greater detail in the following chapters and discussion.

It is noteworthy that there is one unique viewpoint, proposed

by a team, that cPDE exists as an aggregate with other enzymes (Wombacher 1980, 1982). Recently this group purified an enzyme aggregate containing cPDE and 5'-nucleotidase activities from the soluble fraction of adrenal cortex (Boldicke *et al.* 1986). They concluded that an aggregate of cPDE and 5'-nucleotidase is important for the combined regulation of adenosine and cAMP concentrations (only cAMP tested) (Boldicke *et al.* 1986).

Good general reviews on multiple phosphodiesterases may be found (Appleman *et al.* 1973; Appleman and Terasaki 1975; Wells and Hardman 1977; Thompson *et al.* 1979(b); Beavo *et al.* 1982; Vaughan *et al.* 1983; Strada *et al.* 1984). It is worth noting, though, that since the primary studies in rat liver by Russel *et al.* (1973) using diethylaminoethyl (DEAE)-cellulose anion-exchange chromatography this has largely been the method of choice for the separation of multiple cPDE forms on the basis of net protein charge. To such an extent that a survey of the literature indicated that this technique has been almost exclusively used as the initial step prior to further purification procedures to homogeneous preparations of the four defined cPDE types. One exception is the purification of the type III cGMP PDE from rod outer segments of the lens where polyhistidine agarose was used (Yoshi *et al.* 1975). More recently, there has been renewed interest in isoelectricfocusing (Nemoz *et al.* 1981, 1983; Nagasaka *et al.* 1983) and non-denaturing polyacrylamide gel electrophoresis techniques (Robinson *et al.* 1987).

It will now be of value to define the first of the four classes

of cPDE as delineated at the beginning of the introduction and also the subgroups within the first class. For instance, there appear to be four subgroups of type I cPDE. This will be followed by a detailed survey of the respective forms with recent discoveries.

1. Type I(a) cPDE: calmodulin-sensitive cPDE with higher affinity for the substrate cGMP but essentially hydrolyzing both nucleotides.
2. Type I(b) cPDE: calmodulin-sensitive cPDE mainly specific for cAMP.
3. Type I(c) cPDE: calmodulin-sensitive cPDE with very high affinity for both cAMP and cGMP.
4. Type I(d) cPDE: calmodulin-sensitive cPDE specific for cGMP.

General properties of the type I cPDE enzymes.

The type I cPDE enzymes, in the presence of magnesium ion, appear to be regulated by calcium via the binding-protein calmodulin. In light of the previous discussion on the pathways of purine cyclic nucleotides and calcium mediated signals, type I(a,b,c,d) cPDE enzymes clearly have a vital regulatory role as a site for both the co-ordination and integration of these events. The type I cPDE forms have been identified mainly as soluble enzymes in a wide variety of tissues and although the majority of activity appears to be without strict specificity for either cAMP or cGMP (Type I(a) cPDE) the novel specific or high affinity forms (Type I(b,c,d) cPDE have recently bee

identified and will be detailed following a short review on calmodulin.

It is worth delineating the major chronological *in vitro* discoveries concerning calmodulin with reference to several in-depth reviews. The majority of discoveries concerning calmodulin were made prior to the findings of different *in vitro* enzymes of type I cPDE and, to avoid confusion, reference will be made to type I cPDE without subclassification of the different forms in this section. The protein, calmodulin, was first discovered independently by two different teams as a heat-stable, dissociable activator of brain cPDE (Cheung *et al.* 1970, Kakiuchi *et al.* 1970) and, in fact, the name calmodulin was only coined by Cheung and associates (1978) several years later. The function or mode of action of this "activator" was determined by workers (Teo and Wang 1973; Wang *et al.* 1975) who purified it from heart and reported it to be a calcium binding protein. Hence, a theory was proposed by this team that under normal resting conditions of intracellular free calcium ion (10-100 nM) there would be no significant binding between calcium and calmodulin. Once the intracellular concentrations of calcium ion were increased to saturating levels then binding could occur leading to a conformational change that would enable interaction with inactive enzyme, that is type I cPDE, to form an active complex and that this mechanism would be fully reversible. This proposal has been evidenced in many cell free systems and has been elegantly tested out, of late, in intact cells (Saitoh *et al.* 1985). They observed in intact

porcine coronary strips that type I cPDE associated with calcium/calmodulin and furthermore, the functional association was dependent on the concentration of calcium ion. In such a way that the extent of the association in the intact cells of the enzyme-calcium/calmodulin complex was considerably greater in contracted arteries (highest calcium ion). Only from 1975 onwards, was it discovered that calmodulin, in association with calcium ions, activates many other enzymes such as adenyl cyclase in certain cell types, NAD kinase, calcium-dependent protein kinase and guanyl cyclase. This accounted for the observation that calmodulin was often in excess of the type I cPDE. For example, one of the highest known concentrations (10 uM) of calmodulin has been noted in bovine brain. Also, novel calcium-binding proteins were described and one of them, troponin-C, was demonstrated to share sequence homology with calmodulin (Dedman *et al.* 1977; 1978). Since the 1980's, calmodulin has been sequenced and found to be composed of 148 amino acids with a molecular mass of about 16500 daltons with an isoelectric point of pH=4 and it has four binding sites for calcium ions (Tomlinson *et al.* 1984). It is thought that on binding calcium ion, calmodulin exposes a hydrophobic site which then removes an inhibitory site somewhere on the target enzyme. This theory is based on the observation that either limited proteolysis or oleic acid and phosphatidylserine, acting as detergents, stimulate calcium/calmodulin dependent enzymes such as type I cPDE to the same extant as calmodulin but at higher concentrations (Van Belle 1984). It appears that

calmodulin levels increase during periods of cell growth and differentiation, although the reason why is not clear, and the acidic protein has been remarkably conserved between species probably because of its unique ability to interact with many proteins and peptides (review Manalan and Klee 1984). In addition, a whole symposium on the subject may be found (1980) where, of particular interest, is the report of the isolation and molecular cloning of a portion of the calmodulin gene from the electric eel. The gene was labelled and used as a probe in comparative studies (Munjaal *et al.* 1980). The mode or mechanism of action by which the type I enzyme is activated by calcium and calmodulin remains to be fully resolved at the molecular level. It has been reported, using equilibrium binding and kinetic methods, that four calcium ions are required to bind to calmodulin for full activation of type I cPDE (Huang *et al.* 1981; Huang and King 1985). These workers claim that this is the dominant form which increases the affinity of type I cPDE for calmodulin from a dissociation constant of greater than 10 mM for calmodulin without calcium ion, to a remarkable dissociation constant of about 10 nM when calmodulin is bound to four calcium ions. Whereas, from their model, they claim that when three or less calcium ions are bound, the affinity of type I cPDE for these complexes would be almost negligible. Unfortunately there is no data given on the dissociation constants of these other species. Another group applying a different model state that calmodulin saturated with either 3 or 4 calcium ions behave respectively as activating

species (Cox *et al.* 1981). Mutus and coworkers (1984) using a fluorescence-labelled type I cPDE, which is somewhat modified from the normal type I cPDE because the degree of activation by calmodulin is about 50% less, in the presence of saturating concentrations of calcium ion, state that by fluorometric analysis of the binding of calcium ion to the protein complex it should be possible to determine the dissociation constants for all the five species. The use of Nuclear Magnetic Resonance (NMR) studies and other conformational methods should also provide further insight into this field (Manalan and Klee 1984). It is noteworthy that calmodulin is distributed in both the soluble and particulate fractions of cells and Kakiuchi and coworkers (1978) found that about 52% of calmodulin is associated with the particulate fraction of rat brain. Furthermore, they also demonstrated the presence of type 1 cPDE in the particulate fractions of rat liver, kidney, heart and, for the first time, in rat brain. They studied three particulate fractions namely nuclear, mitochondrial and microsomal and found the highest specific activity to be associated with the microsomes. Unfortunately, to date, the calcium-binding site of particulate cPDE is easily destroyed by sonication, freeze thawing (Kakiuchi *et al.* 1978), or by trypsin. The effect of trypsin would suggest that the enzyme is located on the surface of the membrane. Thus there has been no complete purification attained for any cPDE activity. There has been partial purification and characterization of a membrane-bound form in hamster liver

membranes (Smoake *et al.* 1981(a)) and partial characterization in the rat erythrocyte membrane (Clayberger *et al.* 1981). In canine thyroid slices, Miot and colleagues (1983) suggest that observed reductions in cAMP levels induced by muscarinic agonists may also involve a calcium mediated activation of type I cPDE. It does seem, however, that the majority of type I cPDE activity is associated with the soluble fraction of many different cells.The various subgroups within the type I cPDE, defined on the basis of subtrate affinities, will now be delineated.

1. Type 1(a) cPDE; Soluble calcium/calmodulin sensitive cyclic GMP with lower affinity for cAMP.

Type I(a) cPDE has been purified more than two thousand fold, often to homogeneity in: bovine or mammalian brain (Klee and Krinks 1979; Morril *et al.* 1979; Sharma *et al.* 1980; Tucker *et al.* 1981; Kincaid *et al.* 1981; Hansen and Beavo 1982); in bovine heart (Ho *et al.* 1977; LaPorte *et al.* 1979; Hansen and Beavo 1982); and in rat pancreas (Vandermeers *et al.* 1983). The enzyme has also been partially purified and characterized in male rat germ cells (Purvis *et al.* 1981; Geremia *et al.* 1982), in chicken gizzard smooth muscle (Birnbaum and Head 1983) and in human lung and bronchial tissue (Bergstrand and Lundquist 1976; 1978). There have also been partial purification and characterization reports of possibly this subgroup of type I(a) cPDE in neoplastic cells, either malignant or benign, such as Morris hepatoma (Turnbull and Hickie 1984) or in human mammary cells or human leiomyoma of the uterus (Robinson *et al.* 1987). The reported fold activation of the enzyme by calmodulin varies widely (2 to 50 fold). Calmodulin increases the Vmax of the enzyme with cAMP or cGMP as substrate but inconsistent effects on the Km values have been noted (Klee *et al.* 1979; Tucker *et al.* 1981). A frequent observation has been the disparity between authors of what constitutes a "high affinity" enzyme and a "low affinity" enzyme. For instance, in the review by Manalan and Klee (1984) which discusses the purified brain and heart Type I(a) cPDE they term the Type I(a) cPDE form as "high

Km enzymes (low affinity) without strict specificity for cAMP or cGMP." Whereas the identical enzyme form is described as being "high affinity"(low Km) for both cAMP and cGMP (Km range from 6 to 30 um)(Purvis *et al.* 1981). Yet another author defines the type I(a) cPDE as "a soluble enzyme with a high affinity (low Km) for cyclic GMP and low affinity for (high Km) for cyclic AMP" (Smoake *et al.* 1981(b)). In view of this, it is clearly better to provide the Km values whenever possible and state that, in this thesis, on the basis of recent publications, Km values of less than 2 uM will be regarded as high affinity (low Km) and those with Km values greater than 2 uM will be regarded as low affinity (high Km) forms. Thus, from the few kinetic studies performed on the purified brain and heart soluble Type I(a) cPDE it is deemed suitable to classify the enzyme as a low affinity phophodiesterase since it has an apparent Km for cAMP of 100-200 uM (Klee *et al.* 1979; Tucker *et al.* 1981). The enzyme hydrolyzes cGMP with Km values of 3 to 9 uM (Kincaid and Vaughan 1979; Tucker *et al.* 1981). The Vmax for cAMP hydrolysis (200-300 umol/min/mg protein) may either be an order of magnitude greater than the Vmax for cGMP (15-50 umol/min/mg protein)(Morrill *et al.* 1979) or exhibit similar rates of hydrolysis (Hansen and Beavo 1982). Michaelis Menten Kinetics (linear) have been observed in the presence of saturating calcium/calmodulin (Tucker *et al.* 1981) although others have reported this effect only when both cyclic nucleotides were present in supraphysical concentrations (Vandermeers *et al.* 1983). In the absence of

calcium/calmodulin, the Type I(a) cPDE exhibits negative co-operativity with both nucleotides (Klee *et al.* 1979; Vandermeers *et al.* 1983). Recently, however, type I(a) cPDE in male mouse germ cells has been shown to exhibit non-linear kinetics for cAMP (Geremia *et al.* 1984) which they attributed to three discrete catalytic sites. Others, however, have reported that cAMP and cGMP appear to be hydrolyzed by a common catalytic site on the enzyme because each substrate has been reported to be a competitive inhibitor of the other and, furthermore, the Ki values of cAMP and cGMP are similar to the respective Km values as substrates (Wells *et al.* 1975; Sullivan *et al.* 1987).

It has long been known that proteolysis of type I(a) cPDE can irreversibly activate the enzyme (probably also applicable to the lesser characterized type I(b,c,d) cPDE forms) and cause it to lose its responsiveness to calcium/calmodulin (Cheung 1971; Moss *et al.* 1978; review Kriaks et al. 1984). The degree to which limited proteolysis affects loss of calmodulin remains uncertain (Tucker *et al.* 1981; Kincaid *et al.* 1981). Limited proteolysis studies have indicated that type I(a) cPDE has a separate calcium/calmodulin binding site from the catalytic site and that the binding domain exerts an inhibitory effect on the catalytic site when not complexed to calcium/calmodulin (Stellwagen *et al.* 1984). Reversible activation of the enzyme, in the presence of proteolytic inhibitors, as previously discussed, can be effected by phosphatidylinositol and other phospholipids (see section on endogenous effectors).

Prior to 1980, the main method of purification to homogeneity of type I cPDE enzymes was usually achieved through the use of calmodulin-Sepharose affinity columns (Klee *et al.* 1979; La Porte *et al.* 1979; Sharma *et al.* 1980(b)). These columns, in addition to binding the type I cPDE forms, would also bind other calcium-binding proteins such as calcineurin that then necessitated further purification steps such as Blue-Dextran chromatography (Wallace *et al.* 1978). The development of antibodies to several cPDE enzymes was next attained (Tucker *et al.* 1981; Hansen and Beavo 1982; Mumby *et al.* 1982; Sarada *et al.* 1982) which enabled a faster and less complicated technique for purification. One team (Hansen and Beavo 1982) prepared a conformation-specific monoclonal antibody chromatography column for the immuno-purification of bovine heart and brain type I(a) cPDE complexed to calcium/calmodulin. These authors demonstrated the apparent subunit for the brain enzyme was 61000 daltons but that for heart was 59000 daltons. Proteolysis, either endogenous or exogenous, could not be entirely excluded since multiple forms of type I(a) cPDE forms from brain tissue, have been isolated depending on the presence or absence of protease inhibitors (Tucker *et al.* 1981; Stellwagen *et al.* 1984).

The question as to the native structure of type I(a) cPDE was at that time (1982) controversial whereas there seemed to be general agreement that the subunit(s) structure was 60000 $\pm$ 3000 daltons. Most groups favoured the theory that the native enzyme existed as a dimer of identical subunits in solution, with 2

molecules of calmodulin required for activation, and the molecular mass of the enzyme complex (A_2C_2) would be approximately 155000 (La Porte *et al.* 1979; Beavo *et al.* 1982; Sharma *et al.* 1980(a)). Another group suggested that the native enzyme existed as a tetrameric structure with a molecular mass of 240000 daltons for the uncomplexed enzyme (Tucker *et al.* 1981) and finally, monomer to dimer conversion during purification or ageing of the enzyme was suggested (Kincaid *et al.* 1981). They postulated that type I(a) cPDE existed as a monomer in fresh brain supernatant that would aggregate to dimers or even tetramers during purification or lengthy storage. Since 1982, the subject of the native structure of type I(a) cPDE still remains very speculative and the original theories have been further modified in the light of recent findings. It appears that Kincaid and associates (1984) have revised their theory of interconvertible monomeric and oligomeric species and favour the conclusion that the enzyme exists mainly as a homodimer of about 60000 dalton subunits in bovine heart and brain. Of interest, was their further finding, using a modified method of stepwise elutions from DEAE-agarose followed by calmodulin-Sepharose affinity chromatography, that two isoenzymes with different isoelectric points were present in both ovine and bovine brain. Another team, who previously postulated a tetrameric structure for type I(a) cPDE (Tucker *et al.* 1981) have since changed their idea, due to additional facts gleaned from proteolysis studies, on soluble brain type I(a) cPDE (Stellwagen *et al.* 1984). Briefly, they suggest that

the unproteolyzed type I(a) cPDE exists as a dimer of identical subunits of about 63000 daltons and each subunit possesses two domains namely, the calmodulin binding site and the catalytic site. It appears that unproteolyzed type I(a) cPDE can exist in two equilibrium conformations with the highest catalytic activity exhibited in the presence of saturating calmodulin. Proteolysis at the area of the calmodulin-binding domain to a 45000 dalton fragment irreversibly activates the enzyme and changes the less active conformation structure of calmodulin deficient type I(a) cPDE to the conformation structure of maximum activity normally exhibited by calcium/calmodulin bound type I(a) cPDE complex. Stellwagen and workers (1984) suggest that the model fits in with the frequent reports of variation both in size and fold activation by calmodulin of the type I(a) cPDE *in vitro*.

Finally, Sharma and co-workers (1984) using monoclonal antibodies have demonstrated that the type I(a) cPDE from bovine brain is composed of two discrete subunits of 60000 and 63000 daltons respectively. In the native state the type I(a) cPDE may therefore exist as a heterodimer or two homodimers resulting in three isoenzymes with distinct kinetic properties (Km data not provided). Furthermore, a later study showed that only the 60000 dalton subunit could be phosphorylated *in vitro* in the presence of non-saturating levels of either calcium or calmodulin by A-kinase and the phosphorylation is accompanied by a marked decrease in affinity for calmodulin (Sharma and Wang 1985). This is the first report of purified isoenzymes of

type I(a) cPDE being identified in one tissue. Peptide mapping by this group demonstrated differences between the two subunits, although one of their developed monoclonal antibodies cross-reacted with both subunits, indicating a degree of homology (Sharma *et al.* 1984). Further experiments by this group have shown that the 63000 dalton subunit homodimer can also be phosphorylated with a mixed preparation of bovine brain calmodulin-binding proteins in a calcium/camodulin dependent way (Sharma *et al.* 1986(a)). The phosphorylation event reduces the homodimer affinity for calmodulin as evidenced by an increased requirement for calcium ion concentration. The effect is reversed in the presence of calcineurin which causes dephosphorylation of the enzyme (Sharma *et al.* 1986(a),(b); review Sharma and Wang 1986(c)). The authors have not identified the endogenous protein kinase in the bovine brain mixture which effects the phophorylation mechanism. It appears that another group, who have developed an antibody against calmodulin which specifically recognizes calmodulin complexed to type I(a) cPDE, support some of the findings by Sharma and his co-workers (Hansen and Beavo 1986). Hansen and Beavo (1986) isolated two isoenzymes of type I(a) cPDE in bovine brain but only one enzyme in bovine heart. The two brain isoenzymes differ slightly from each other with respect to molecular mass, the subunit sizes being 58000 and 63000 daltons respectively. The lower molecular mass isoenzyme is preferentially phosphorylated by an A-kinase which parallels the observations of Sharma and his colleagues (1985). In addition to their work

on bovine heart and brain, the latter group have studied type I(a) cPDE in bovine lung. Their findings indicate the lung form shares immunological homology with the 60000 brain isoenzyme because it cross-reacts with the monoclonal antibody (C1) developed against the brain isoenzyme (Sharma *et al.* 1986(b)). The one interesting difference is that the native form of the lung enzyme exists as a dimer tightly bound to calmodulin. Even after successive elutions on DEAE-cellulose in the presence of EGTA the lung enzyme could still be activated in the presence of saturating calcium ion (Sharma and Wirch 1979; Sharma *et al.* 1986(b)). Another group, using the ACAP-1 hybridoma line of Beavo (Hansen and Beavo 1982), isolated only one form of soluble type I cPDE in porcine brain (Keravis *et al.* 1987) unlike the two isoenzymes demonstrated in bovine brain (Hansen and Beavo 1986).

Earlier reports have demonstrated the presence of multiple forms of soluble type I(a) cPDE within one form of tissue based on isoelectricfocusing of crude cytosols coupled to the cPDE activity stain (Nemoz *et al.* 1981). For example, soluble rat heart gave several bands of cPDE activity, some of which appeared to be calcium\calmodulin dependent as they were sensitive to EGTA inhibition (*ibid*). Whether these forms may be found within one cell type or are dispersed between the heterogenous cell populations that constitute rat heart tissue has not been satisfactorily resolved.

2. Type I(b) low affinity cAMP calmodulin-dependent phosphodiesterase.

A novel, low-affinity cAMP-specific PDE stimulated by calmodulin (Km=2.5 uM) was first identified as a membrane-bound form in hamster liver membranes (Smoake *et al.* 1981(a)). The Km for hydrolysis of cGMP was not given.

3. Type I(c) high affinity cAMP, cGMP calmodulin-dependent phosphodiesterase.

This high affinity form of type I(c) cPDE was initially partially purified and characterized as the first eluting enzyme activity in immature rat testis (Purvis *et al.* 1981), in chick hearts (Andrenyak and Epstein 1982) and chicken gizzard smooth muscle (Birnbaum and Head 1983). The latter group calculated the Km of the "1b" form to be 0.5-1.6 uM for both nucleotides with a Vmax range of 0.28-0.57 umol/min/mg protein. This form was purified more than five thousand fold by Vandermeers and co-workers (1983) and was termed the "PI"enzyme. The high-affinity type I(c) cPDE exhibited a Km of less than 0.5 uM for both nucleotides and a Vmax range of 19-27 umol/mg/min protein. More recently, another group have described two enzymes of type I cPDE, namely the (a) and (c) forms, from male mouse germ cells (Geremia *et al.* 1984). The "high salt" eluting form exhibits low Km, according to the authors, (about 5 uM)(which in this thesis is really high km)

for both cyclic nucleotides and therefore most closely resembles the type I (a) cPDE description. Bergstrand and Lundquist (1978) partially characterized a high affinity (Km<0.4 uM) type I(c) cPDE active with both nucleotides in human bronchial tissue.

4. Type I(d) cPDE high affinity cGMP-specific.

This high affinity type I(d) cGMP PDE (Km about 1 uM) was first described as one of the three forms of type I cPDE in immature rat testis (Purvis *et al.* 1981). A form resembling the description of a high-affinity cGMP-specific type I(d) cPDE was isolated from bovine brain supernatant and purified over 1000 fold (Shenolikar 1985). The apparent Km for cGMP was about 2 uM with a Vmax of 40 umol/min/mg protein which was about 50 fold higher than the Vmax calculated for cAMP. The apparent subunit molecular mass was 74000 daltons with a native dimer structure of about 150000.

Clearly there is *in vitro* evidence for enzymes and isoenzymes of soluble type I cPDE while the particulate forms remain to be fully purified. Whether these four subgroups of soluble type I cPDE are of physiological significance or represent proteolytically modified enzymes either endogenous or arising during the different "purification processing techniques" of various workers remains speculative.

Type II cGMP-sensitive cyclic nucleotide phosphodiesterase

This enzyme which is calcium/calmodulin independent was first characterized in the soluble fraction of rat liver (Beavo et al. 1970; Beavo *et al.* 1971) and particulate fractions of a few other tissues such as human platelets (Hidaka and Asano 1976) and adrenal medulla (Egrie and Siegel 1977; review Wells and Hardman 1977). The enzyme activity was shown to display positive co-operative kinetic behaviour (Terasaki and Appleman 1975) and at low concentrations of cGMP (<10 uM) the nucleotide could activate cAMP hydrolysis and cGMP appeared to be the preferred effector (Russel *et al.* 1973). Cyclic IMP was also found to be stimulatory , to a lesser extent, but not 5'-GMP and at higher concentrations (>50 uM) either IMP, 5'-GMP or cAMP and cGMP behaved as a competitive inhibitor of the enzyme. The type II cPDE was partially purified from both the supernatant and particulate fractions of rat liver (Moss *et al.* 1977) where comparable kinetic properties were observed for both forms. It was shown that only cGMP (less than 10 uM) could decrease the apparent Km for cAMP forms 89 to 25 uM while cGMP exhibited a Km of about 28 uM. Hence the enzyme hydrolyzed comparably both cAMP and cGMP. The enzyme activity stimulated by cGMP obeyed linear Michaelis Menten kinetics on Lineweaver plots. On the basis of these experiments, the concept of multiple catalytic sites was proposed. An important publication followed (Gunn and Cheung 1980) where the two authors demonstrated that, cGMP at low levels could only activate soluble liver type II cPDE at

low enzyme (low protein) concentrations in the cell free assay. They proposed that the enzyme existed in either an aggregated form (at high enzyme concentrations) or a dissociated form, where the regulatory site for cGMP (Terasaki and Appleman 1975) was masked when in the aggregated form, but exposed and thence active in the dissociated enzyme. Kinetic analysis of a partially purified soluble rat liver type II cPDE, which was immobilized on hexyl agarose by hydrophobic interaction, indicated the binding site for cGMP to be distinct from the hydrolytic and activating sites (Couchie *et al.* 1981). Further work by the same team (Erneux *et al.* 1981; Couchie *et al.* 1983) working with nucleotide analogues appeared to confirm that there were distinct catalytic sites on the enzyme.

The first report of purification to homogeneity of the type II cPDE was attained in soluble bovine adrenal and heart tissues (Martins *et al.* 1982). These workers were able to obtain pure enzyme from a procedure involving DEAE-cellulose chromatography followed by cGMP affinity chromatography. The subunit structure exhibit an apparent molecular mass of about 106000 daltons and the native structure revealed a molecular mass of about 240000 daltons, although occasionally larger aggregates were noted. The Km values for cAMP and cGMP were 30 uM and 10 uM respectively (Vmax values were not given) and the increase in activity brought about by cGMP was about 5 to 6 fold. The kinetics were similar to those previously reported and the cAMP and cGMP hydrolysis was characterized by positive homotropic

co-operativity. A later report on the same tissue (Sabatine and Coffee 1986) revealed a somewhat higher Km of 54 uM for cGMP and stimulation of the enzyme by cGMP (5 uM) was only effective at subsaturating levels of cAMP (about 1 uM). Interestingly, they were able to show a slight stimulation of enzyme activity by cAMP of 1.5 fold, previously never reported in that tissue, when cGMP labelled substrate was 40 nM. Of concern, was the apparent lack of evidence of a type I cPDE form which had previously been detected by other authors in the bovine adrenals (Egrie and Siegel 1977).

The type II cPDE has also been purified to apparent homogeneity from soluble calf liver and has been demonstrated to have a subunit structure of 102000 daltons with a native molecular mass of 201000, suggestive of a dimer of two similar or identical subunits (Yamomoto *et al.* 1983(a)). The kinetics were fairly analogous to those published on the form(s) in the bovine tissues (Martins *et al.* 1982) and Yamomoto and associates (1983(a)) reported the Vmax for cAMP and cGMP to be 170 and 200 umol/min/mg protein respectively. The authors also calculated a much higher stimulation of the enzyme by cGMP of about 35 fold in the presence of magnesium ion, but not by other divalent cations, compared to only about 6 fold stimulation by cGMP in the bovine tissues. Furthermore, the Yamomoto team also showed complex effects of divalent cations on the catalytic activity of the type II cPDE. Additional studies on this purified enzyme in calf liver, using several inhibitors, demonstrated complex effects which the authors

attributed to structural differences in both the catalytic and regulatory binding sites of type II cPDE (Yamomoto *et al.* 1983 (b); Yamomoto *et al.* 1984(a)). Recently, a team has shown that the soluble type II cPDE of calf liver when subjected to a range of pH values (pH 7.5-9.0) in the assays yielded complex effects on the cooparative behaviour and expression of catalytic activity by the enzyme (Wada *et al.* 1987(a)). Simply put, incubation at pH 9.5 to 10 appeared to activate the type II cPDE. Wada and associates, working with the presence or absence of methylxanthine analogues (competitve inhibitors for cPDE) have also proposed that there are discrete binding sites for cAMP and cGMP which are high and low affinity with the former exhibiting more stringent structural determinants (Wada *et al.* 1987(b)).

A report, on the purification of both cytosolic and particulate type II cPDE enzymes from rat liver yielded subunit structures of 67000 daltons existing as dimers of about 134000 daltons (Pyne *et al.* 1986). The Km values were found to be similar to those reported previously although the Vmax was about forty fold less compared to calf liver (4 umol/min/mg protein versus 150 umol/min/mg protein) although the degree of stimulation of the enzyme by cGMP (6 fold) was of similar magnitude to that observed in bovine tissue (Martins *et al.* 1982). Tryptic peptide maps of the particulate and soluble forms indicated a large degree of homology. Thus, there appeared to be distinct species differences in the liver type II cPDE between the calf and the rat.

The development of specific monoclonal antibodies to the soluble bovine heart type II enzyme (Humby *et al.* 1982; Hurwitz *et al.* 1984(b)) have indicated the form to represent a substantial portion of total cPDE activity in tissues such as liver, heart and adrenal tissue.

Results obtained with a newly developed tritiated cGMP binding assay (Miot *et al.* 1985) have suggested that the cGMP-binding activity is directly related to the allosteric (non-catalytic or regulatory) cGMP binding site. It had previously been noted that methylisobutylxanthine (MIX) stimulates the type II enzyme at low concentrations (Erneux *et al.* 1982). Further studies (Erneux *et al.* 1985) using analogues of cAMP and cGMP on purified enzyme still confirm the earlier postulate that catalytic (hydrolytic) and regulatory (or allosteric) sites must be separate. It also appears that the use of reversed-phase HPLC by the Erneux team (Braumann *et al.* 1986) will yield even more interesting results. Preliminary tests suggest that both a negative charge and an equatorial oxygen atom on the cyclic phosphate residue are prerequisite for the hydrolytic (catalytic) activity of type II cPDE. A discussion of the two site competitive models is reviewed (Wada et al.1987(b)).

In conclusion, the purification of the type II cPDE enzyme(s) to homogeneity from tissues has been rather limited and the forms appear similar in bovine tissues but differ considerably in comparison to the liver tissue of the rat.

Type III cGMP-specific and rhodopsin cGMP phosphodiesterase.

A few forms of this calcium/calmodulin independent cGMP-specific PDE have been identified although the rhodopsin-sensitive cGMP PDE is tissue specific for the retina. A summary of the different enzyme forms within the type III classification are as follows:-

1(a). Particulate rhodopsin-sensitive cGMP phosphodiesterase of the rod outer segment of the lens.

1(b). Soluble rhodopsin-sensitive cGMP phosphodiesterase of the interphotoreceptor matrix (IPM) of the lens.

2. Soluble cGMP-specific phosphodiesterase from mammalian tissues.

1(a). Rhodopsin-sensitive cGMP phosphodiesterase (ROS cGMP PDE).

This enzyme exists tightly bound to rod outer segment membranes (ROS) of the retina and since it is a peripheral membrane-bound protein it can be released by vigorous washing under conditions of low ionic strength in the absence of magnesium ion. Rod outer segments (ROS) of the retina were found to contain markedly high levels of cGMP (about 70 uM) compared to other tissues (Goridis *et al.* 1974) and the author and others noted that light exposure to intact ROS activated the now classified type III 1(a) ROS cGMP PDE. Miki and associates (1975) were the first to purify to homogeneity and characterize ROS cGMP PDE from the frog retina. Since the enzyme is membrane-bound it had

initially to be solubilized in EDTA, then applied to sucrose gradients for ultra-centrifugation and purified to apparent homogeneity by batch ion-exchange chromatography. The latter method, as previously discussed was not on DEAE-cellulose but, in fact, polyhistidine agarose. The two subunit molecular masses of the dimeric protein were 120000 and 110000 respectively with a native molecular mass of 240000. The activation of the enzyme in the membrane-bound state was dependent upon the presence of an allosteric effector, GTP, (review Pober and Bitensky 1979). It was also found that in all the mammalian species studied the highest levels of ROS cGMP PDE (10 to 500 fold) were associated with the ROS of the retina rather than the inner segments. A subsequent purification of the enzyme from bovine retina yielded somewhat discrepant findings, compared with the previous results by Miki and co-workers (Baehr *et al.* 1979). The latter team's method, essentially entailed the application of hypotonic supernatants of extract to DEAE-cellulose followed by gel filtration and sucrose gradient ultracentrifugation. The bovine form differed from the frog form in having subunits with apparent molecular masses of 84000 and 88000 daltons respectively and a native molecular weight of about 185000. Furthermore, Baehr and associates (1979) demonstrated the presence of a low molecular weight inhibitory component with mass of 13000 daltons which was confirmed by others and later referred to as the lambda subunit (Hurley and Stryer 1982) and the subunit primary structure has been determined (Ovchinnikov *et al.* 1986). It was

noted that trypsin, histone, protamine or excessive dilution could activate the type III 1(a) ROS cGMP PDE by removing the inhibitory constraints of the lambda subunit. Further studies with partially purified ROS PDE from many mammalian species and also the frog preparations of the enzyme indicated a major doublet of 84000 and 88000 daltons and tryptic peptide mapping demonstrated that there were at least six peptides which were conserved in all species (Takemoto *et al.* 1984(a)). The availability of pure ROS PDE enabled the production of monoclonal antibodies, of which, two were prepared (Hurwitz *et al.* 1984(a)). The one antibody (ROS1) inhibited enzyme activity upon binding. This latter monoclonal antibody was tested in a number of species to determine molecular masses of the enzyme and, in agreement with Takemoto and associates (1984(b)), there were no major differences between several species. Further work (Hurwitz *et al.* 1985) using the two monoclonal antibodies demonstrated that ROS-1 antigenically bound to both ROS and cone outer segments (COS) of the retina whereas ROS-2 only recognized a determinant present on ROS indicating possibly either enzymes or isoenzymes for the type III PDE.

1(b). Type III 1(b) rhodopsin-sensitive cGMP-specific phosphodiesterase.

The isolation and partial purification of another type III cGMP PDE, a soluble form, present in the interphotoreceptor matrix (IPM) was subsequently reported (Barbehenn *et al.* 1985).

Although the enzyme could be bound by one of the monoclonal antibodies indicating some antigenic similarity to the type III 1(a) ROS PDE it differed in its location, subunit size and substrate specificity. The novel enzyme exhibited two subunit sizes of 45000 and 47000 respectively and an inhibitory subunit of 13000 daltons with a Km for cGMP of 33 uM and for cAMP of 2.2 mM. In summary, there appear to be at least two enzymes, one soluble and one particulate, of rhodopsin-sensitive type III cGMP PDE of the lens.

2. Type III cGMP-specific PDE (Type III (2) cGMP PDE).

This form is calcium/calmodulin independent and preferentially hydrolyzes cGMP.The first apparent report of the partial purification of this type III (2) cGMP PDE enzyme was in the supernatant fraction of guinea pig lung (Davis and Kuo 1977). The Km for cGMP was about 0.6 uM and that for cAMP was 150 uM. The native molecular mass of the enzyme was estimated to be 137000 to 168000 daltons. Other reports on preparations from human enzyme (Bergstrand and Lundquist 1976) differed considerably in that the human lung enzyme displayed an affinity for cGMP some two orders of magnitude lower. Unfortunately, there have been no publications of purification to homogeneity of all the cPDE forms in lung tissue to date. It is possible that the activator could have remained tightly bound to the human enzyme during the purification process as previously discussed in rabbit lung (Sharma *et al.* 1986(b)).

Nasu and associates (1978) described a comparable type III activity to the one in human lung, in hog heart, but their calculated Km for cGMP, which displayed non-Michaelis Menten kinetics, was about 2 uM. A much more convincing report on a type III (2) cGMP PDE form stemmed from partial purification and characterization studies in rat liver and hepatoma cells (Strewler *et al.* 1983; Manganiello *et al.* 1984). This latter group isolated two forms of cPDE, E-Ia and E-Ib, the second of which, E-Ib, resembled the type III (2) form. The enzyme displayed anomalous kinetic behaviour, preferentially hydrolyzed cGMP with an apparent Km for cGMP of 1.2 uM while that for cAMP was 15.4 uM. The calculated native size was about 325000 daltons and the hepatoma cells yielded analagous findings but the hepatoma form was shown to display linear kinetics. The fact that Strewler and associates used protease inhibitors and the molecular mass demonstrated for the E-Ib form of 325000 was markedly greater than that of the type I cPDE (E-Ia of about 150000 daltons) made it highly unlikely that it was a proteolytically altered type I form. A recent report on the identification and characterization of a soluble type III (2) cGMP PDE in bovine adrenal medulla (Sabatine and Coffee 1986) has yielded results which differ somewhat to the findings of the Strewler team. The calculated native molecular mass of the enzyme in bovine adrenal medulla is about 216000 daltons with an apparent Km for cGMP of 23 uM. However, both the normal rat liver and bovine adrenal medulla displayed anomalous kinetics.

In conclusion, the type III (2) forms appear to differ substantially between species in both molecular masses and substrate affinity. It is difficult to determine whether the varied reports reflect true species differences or whether the differences may be attributed to variations in methodology used by different authors.

Type IV cAMP-specific phosphodiesterase.

This last classification describes a form of cPDE which preferentially hydrolyses cAMP and the enzyme often exhibits a rate of hydrolysis (Vmax) that is about ten fold higher compared to that for cGMP and lower Km values. There are also forms which appear to hydrolyze cAMP exclusively such as in human lung (Moore and Schroedter 1982), in human leukaemic cells (Onali *et al.* 1985) and in a human melanoma cell line (King *et al.* 1978). Other general findings indicate that there are several enzymes of this type IV class. They are calcium/calmodulin independent, either soluble or membrane bound, exhibit high or low affinity for cAMP, exhibit linear or complex kinetics and some of the forms, usually membrane-bound, are hormone sensitive. A survey of the literature has indicated there to be some agreement with regard to substrate specificities but somewhat contentious over molecular size and kinetic properties. In view of this, a summary of the findings of different groups will proceed, *ad seriatim*, the historical aspect.

A hormone-sensitive, membrane-bound, low Km type IV cAMP PDE was first described, whereby, insulin was observed to activate the enzyme at substrate concentrations of less than 10 uM cAMP, in rat adipocytes (Loten and Sneyd 1970). This was confirmed by others using the same tissue (Manganiello and Vaughan 1973; Kono *et al.* 1975). The latter authors also reported the membrane-bound enzyme to be largely associated with endoplasmic reticulum and that reducing agents such as dithiothreitol (DTT) inhibited the activating effect of insulin on the type IV cAMP PDE. This DTT effect was in contrast to the observations of Manganiello and Vaughan (1973). Later, studies based on cell free extracts of rat adipocytes, confirmed the stimulating effect of DTT on activation by insulin of type IV cAMP PDE (Makino *et al.* 1980). It was suggested that the effect of DTT was caused by the activation of an endogenous thiol protease (Makino *et al.* 1982(a)). They also showed, for the first time in rat adipocytes, that hypotonic media could also cause activation of the enzyme (Makino *et al.* 1982(b)). Previously it had been demonstrated in liver tissue, that hypotonic shock or freeze-thawing could activate and solubilize membrane-bound type IV cAMP PDE (Loten *et al.* 1978; Sakai *et al.* 1978). At that time, it was reported that the membrane-bound forms of type IV cAMP PDE tended to display high affinity for cAMP compared to the lower affinity for the substrate shown by soluble forms (Loten *et al.* 1978; Westwood *et al.* 1979; Elks and Manganiello 1985). Soluble forms in dog kidney (Thompson *et al.* 1979(a)) and human platelets (Grant and Colman 1984),

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