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Partial purification and characterization of soluble cyclic nucleotide phosphodiesterases in human and murine tissues

Robinson, Marion Frances

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displaying very high affinity for cAMP, however, have since been reported.

A disconcerting problem at that time was the apparent disparity in molecular mass estimations of the type IV cAMP PDE between different authors. Estimations were based on partial purification of the soluble enzyme, usually attained through elution on DEAE-cellulose columns followed by various methods for molecular mass determination. The particulate forms were partially purified usually by first separating out the membranes of interest, solubilizing the enzyme, and then applying techniques for molecular mass determination. Russel and associates (1973) reported a size of 400000 daltons for the enzymes in rat liver. Another group demonstrated a value of 200000 to 250000 daltons (Thompson *et al.* 1971) while three different values of 55000, 110000 and 240000 for the enzyme in the soluble portion of human lung were reported (Bergstrand and Lundquist 1976). In rat adipocytes, the membrane-bound form, when released by DTT, gave an apparent molecular mass of 300000 to 360000 which was almost half the value of 720000 reported for the holoenzyme solubilized by detergents (Makino *et al.* 1980).

The first group to purify to apparent homogeneity a type IV cAMP PDE was Thompson and his associates (1979(a)) and they based their studies on the soluble form of the enzyme in dog kidney. Since then, the enzyme, both soluble and particulate, has been purified either to homogeneity or extensively so from: the soluble extract of human lung (Moore and Schroedter 1982);

plasma membranes of rat liver (Marchmont *et al.* 1981(a)); rat skeletal myoblasts (Narindrasorasak et al. 1982); cytosol of rat platelets (Grant and Colman 1984); human myeloid leukaemic cells (two soluble forms) (Onali *et al.* 1985); cytosol of human and bovine platelets (Macphee et al. 1986; rat adipocytes (hormone-sensitive particulate form) (Saltiel and Steigerwalt 1986; Saltiel 1987); rat heart (Fougier *et al.* 1986); and lastly, a form from hypotonic extracts of bovine heart (Harrison *et al.* 1986). These various forms often differ with respect to size, kinetic behaviour, hormone sensitivity and response to effectors and inhibitors.

The Thompson team subsequently characterized the type IV enzyme from dog kidney further (Epstein *et al.* 1982 (b)) which was later reviewed (Thompson *et al.* 1984). The soluble, dog kidney high-affinity type IV cAMP PDE has an apparent native and molecular mass of about 60000 daltons although a 57000 dalton form, which is immunologically related, also co-purifies with the enzyme, in addition to some low molecular mass fragments. The Km values for cAMP and cGMP are 2 uM and 530 uM respectively with a similar Vmax of 0.3 umol/min/mg protein for both substrates. The enzyme exhibits linear kinetics, cannot be phosphorylated by protein kinases in the presence of ATP and there is no direct evidence of hormonal sensitivity. The enzyme has a hydrophobic domain and inhibition studies indicate that both cAMP and cGMP are hydrolyzed at the same catalytic site. The enzyme is also highly labile, requires protease inhibitors, is inactivated by DTT in dilute solution and requires ethylene

glycol for stability during purification and storage. Of interest, was the finding by the group that there was cross-reaction of the soluble dog kidney type I cPDE with a polyclonal antibody developed against the purified type IV cAMP PDE suggesting at least some shared homology in structure (Sarada *et al.* 1982).

The Heyworth, Houslay and Marchmont group have, since 1980, purified to apparent homogeneity and extensively characterized one of two membrane-bound forms of type IV cAMP PDE in rat liver plasma membranes (Marchmont *et al.* 1980(a),(b)). The one membrane-bound form is a high-affinity enzyme that is peripherally bound to the plasma membrane. This type of membrane-bound enzyme can be solubilized by high ionic strength or chelating agents whereas the other form which is a low-affinity integral, membrane-bound enzyme (Km for cAMP > 2 uM) can only be solubilized by detergent. The peripheral type IV cAMP PDE has been purified to apparent homogeneity (Marchmont *et al.* 1981(a,b)) and exhibits an apparent subunit and native size of 58000 daltons with Km values for cAMP and cGMP of 0.7 uM and 120 uM respectively. The respective Vmax values for cAMP and cGMP are 9.1 umol/min/mg protein and 0.4 umol/min/mg protein and the Vmax for cAMP is 30 fold greater than the enzyme reported in dog kidney (Thompson *et al.* 1984). The enzyme exhibits negative co-operative kinetics which is ascribed to a mnemonic reaction mechanism (review Houslay *et al.* 1984). It is worth noting that the two membrane-bound forms face the inner plasma membrane and are therefore intracellular

and not ectoenzymes (Smoake *et al.* 1981(b)). The Marchmont group have claimed the findings of Smoake and co-workers to be erroneous because of possible membrane contamination of the peripheral, high-affinity type IV cAMP PDE (Marchmont *et al.* 1981(a)). Treatment of isolated rat liver plasma membranes by the Marchmont team with insulin showed activation of the peripheral, high affinity type IV cAMP PDE. This activation effect was found to be elicited through phosphorylation of the enzyme (Marchmont *et al.* 1981(a)). The same group demonstrated the same effect in intact hepatocytes (Heyworth *et al.* 1983(b)). The mechanism of insulin's action is proposed to be mediated via a specific guanine regulatory protein termed, Nins, with a possible size of 25000 daltons (Heyworth *et al.* 1983(a,b)). Other membrane-bound type IV cAMP PDE forms in dense-vesicles of rat liver have also been described by this group (Heyworth *et al.* 1986). Two membrane-bound forms were found in rat liver mitochondria (Cercek and Houslay 1982) and two or more in the endoplasmic reticulum of the same tissue (Wilson and Houslay 1983(a),(b)). The dense-vesicle form is considered to be a distinct enzyme since it appears to be activated by insulin via a different mechanism and, in addition, glucagon, IBMX and dibutryl cAMP were found to stimulate the dense vesicle enzyme but not the one in plasma membranes (Heyworth *et al.* 1983(b)). In fact, glucagon prevents the high-affinity type IV cAMP PDE from being activated by insulin (Wallace *et al.* 1984). The two distinct enzymes bound to the inner and outer membranes of rat liver mitochondrion have not been purified although it appears

that both forms are peripheral. The outer membrane form is possibly a discrete enzyme since it exhibits a low affinity for cAMP (Km >2 uM), not previously reported for a peripheral type IV enzyme, and also displays linear kinetics. The inner membrane form is, at present, indistinguishable from the high affinity plasma membrane type IV enzyme. The endoplasmic reticulum forms need to be further purified and characterized, although a peripheral form has been demonstrated to be activated and solubilized by DTT, in agreement with findings on the type IV form by the other workers mentioned at the beginning of this discussion (Sakai *et al.* 1978; Loten *et al.* 1980). It appears, therefore, that there are at least four distinct membrane-bound type IV cAMP PDE enzymes in rat liver identified by the Marchmont team. The cell separation techniques used by these authors have been very thorough but proteolytic inhibitors have not been used nor even proteolysis tested during the purification procedures (Marchmont and Houslay 1981(b)). The lack of use of protease inhibitors by the Marchmont group has prompted others to question the reported molecular size of the rat liver pripheral type IV enzyme (Major *et al.* 1983). More recently, the Marchmont group, using the novel methodology of "irradiation inactivation of rat liver plasma membranes" have reported that the integral low-affinity type IV cAMP PDE, although not fully purified, yields an estimated molecular mass of 120000. They report, however, a range of 54000 to 140000 daltons for the peripheral form depending on the presence or absence of insulin prior to

irradiation (Houslay *et al.* 1984).

The Appleman and Whitson group have identified two high-affinity type IV cAMP PDE forms in freeze-thawed rat liver preparations (Whitson and Appleman 1982). Their results differ from the findings of the Marchmont team with regard to the high affinity for cGMP exhibited by both forms (no Km values provided) in addition to a much higher affinity for cAMP. The two forms, however, did show negative co-operativity. The molecular mass values for the forms varied according to the method of partial purification adopted. If the tissue was detergent solubilized a type IV cAMP PDE yielded an apparent molecular mass of 200000 suggesting that it is the integral membrane-bound enzyme. The two high-affinity type IV cAMP PDE forms, termed A and B, solubilized by freeze-thawing, gave apparent molecular sizes of 100000 and 85000 respectively and appear to be variants of the peripheral forms described by the Marchmont group. Lengthy storage has shown a conversion of the A to the B form and antisera raised against the B form cross-reacts with the A form (Whitson and Appleman 1982; review Appleman *et al.* 1984). Two high-affinity forms have also been identified in adipose tissue extracts and only one of them is insulin dependent (Appleman *et al.* 1984). One of the high-affinity forms in intact rat adipocytes has been shown to be activated by papaverine and MIX (Wong and Ooi 1985) which is analagous in behaviour to that of the dense-vesicle enzyme in rat liver (Heyworth *et al.* 1983(b)). Recent work has shown that the insulin activation of insulin-sensitive type IV cPDE does

not involve the components of adenyl cyclase regulation (Weber *et al.* 1987). Furthermore, Saltiel (1987) has demonstrated that the acute actions of insulin on the activities of cPDE, adenyl cyclase and pyruvate dehydrogenase might be mediated by an identical intracellular carbohydrate complex composed mainly of inositol phosphate and glucosamine.

Additional purification studies of type IV enzymes in other tissues such as the soluble form in human lung indicate this enzyme to be very similar to the soluble dog kidney form (Moore and Schroedter 1982) although these authors speculate that the form is possibly a catalytic subunit of a larger native enzyme. The high-affinity cAMP PDE hormone-dependent enzyme in rat adipocytes has been recently purified to homogeneity and found to have a subunit molecular mass of 60000 daltons but there was no definitive data on the native structure (Saltiel and Stiegerwalt 1986).

Recently there has been the suggestion of classifying a subgroup of the type IV cAMP PDE enzymes as being "cGMP inhibited low Km cAMP PDE" because these forms are markedly inhibited by low concentrations of cGMP (Harrison *et al.* 1986). These activities have been reported in mutant S49 mouse lymphoma cells (Brothers *et al.* 1982); human platelets (Hidaka and Asano 1976; Grant and Colman 1984); bovine heart (Donnelly 1978; Harrison *et al.* 1986); and rat liver (Russel *et al.* 1973). Within this subgroup there is disparity between molecular mass estimations and substrate affinities. For example the human platelet soluble enzyme has been reported to

be a dimer of 140000 daltons with a subunit of 61000 daltons. The Km values for both cAMP and cGMP are very low, and are 0.18 uM and 0.02 uM respectively, with a very low Vmax for cGMP. Of interest, was the lack of cross-reactivity between this form and the polyclonal antibody raised to soluble dog kidney enzyme. In contrast, the subunit for high-affinity bovine heart enzyme, purified from hypotonic extracts, is estimated to be 110000 daltons and can be phosphorylated. A polyclonal antibody developed against this enzyme does not cross-react with either bovine lung nor dog kidney type IV cAMP PDE.

Finally, a type IV cPDE, partially purified by Umekawa and associates (1984) in human platelets, appears to hydrolyze both cAMP and cGMP with high affinity and almost equal preference. The apparent molecular mass is about 175000 daltons and either substrate acts as a competitive inhibitor of the other. Yet another team has purified a soluble type IV cPDE isoenzyme (apparently exhibiting the majority of soluble cPDE activity in human platelets) and the isoenzyme has a reported molecular mass of 110000 daltons (Macphee *et al.* 1986)

In conclusion, this class of enzymes has proven, by far, to be the most difficult to which to assign general properties, apart from the trend of a higher affinity displayed for cAMP and lack of calcium/calmodulin activation. The reports of distinct enzymes are numerous and the substrate specificities and estimated molecular sizes vary considerably. Whether the discrepancies in molecular mass estimations may be explained by either aggregation of a 60000 dalton species, or whether the

lower values represent proteolytic fragments of a larger holoenzyme incurred during processing remains to be ascertained. If it proves to be the latter case it is possible that the variation in substrate affinities and kinetics may, in part, be the result of changes in conformational structure resulting in modified action at the catalytic and binding sites of the proteolytic fragments.

Evidently there are a large number of cPDE enzymes and some isoenzymes both within tissues of the same species and between species. The aim of this thesis was to examine cPDE forms in human mammary and uterine tissues, hitherto rather neglected. A murine line exhibiting spontaneous mammary tumours was also studied and the forms of cPDE between selected tissues of this syngeneic mammalian model have been compared. Some of the forms observed have been partially purified and characterized. The techniques used have either followed methods described in the literature or have been modified to adapt to conditions of small tissue samples. The latter being an obvious consequence of studying human tissues with the exception of human leiomyoma (benign tumours) of the uterus. The ensuing chapters will therefore cover the stated topics of interest in much greater detail and the findings will be compared and contrasted closely with the classifications of cPDE enzymes outlined above.

CHAPTER TWO

CHARACTERIZATION OF SOLUBLE CPDE ACTIVITIES IN MURINE TISSUES.

Objectives

My first attempts to characterize and partly purify cPDE enzymes in neoplastic mammary tissues were performed in murine tissues. I had available, by kind permission of Dr Kundig (Department of Pharmacology, Medical School, University of the Witwatersrand), a female, murine line which exhibited spontaneous tumours in multiparous females at an incidence of greater than 90%. These multiparous mice were the female F1 generation of a cross between female Balb/cfC3H and male DBA/8 animals. These hybrids have been described in the literature (Stolfi *et al.* 1971). The spontaneous mammary tumours apparently result from infection of the genetic material with a virus that is passed on or inherited by the offspring. During my studies, based on electron-microscopy of the hybrid tumour tissue, I noted the presence of budding viruses in some of the ultrasections. The advantages of working with this animal model were:

1. The neoplastic mammary tissue was derived from practically syngeneic animals and thus the F1 hybrids were a source of uniform tumour tissue. Indeed, oestrogen and progesterone

receptor assays performed in over 30 separate tumours yielded consistently negative findings.

2. Many workers have noted similarities between spontaneously occurring primary murine mammary tumours and human breast neoplasms especially with respect to spreading and morphology, familial inheritance, hormonal factors and spontaneous regression (see Stolfi *et al.* 1971).

3. Large quantities of similar tumours could be accumulated enabling purification procedures to be tested.

4. A constant supply of fresh tissue could be made available by planned breeding schedules.

5. Comparative studies of cPDE enzymes in the neoplastic tissue could be made with the other murine tissues such as liver, kidneys, heart and brain.

The one disadvantage, however, was that a comparison between the murine mammary tumour and the normal mammary tissue in the same animal was not feasible on account of the lack of sufficient quantities of tissue for cPDE enzyme detection in the latter. In later chapters, working on human mammary and uterine tissues, it was possible to achieve this comparative work within each patient although the problem of tumour heterogeneity between patients, especially in human mammary tissues, remained.

Initially, the soluble cPDE forms were studied in murine mammary tissues by means of non-denaturing polyacrylamide disc gel-electrophesis coupled to a specific enzyme activity stain for cPDE enzymes. Partial purification of cPDE (using cAMP as a

substrate for detection) was achieved by means of continuous or batch elution of the crude cytosol on DE-52 cellulose anionic-exchange chromatography followed by chromatofocusing and gel exclusion chromatography. Finally, a polyacrylamide-slab-gel electrophoresis system was adopted and the specific enzyme stain technique slightly modified so that comparative studies of soluble cPDE forms in the crude cytosols of murine tissues of brain, kidney, heart, liver and neoplastic mammary tissue could be made. Much of the experimentation performed in this animal model enabled the development of more optimal procedures for studying cPDE enzymes in human tissues.

Introduction

It would seem that present day evidence precludes a generalized causal effect of either, the levels of cAMP, cGMP and specific metabolic enzymes or the ratio of the two substrates, in the proliferation or tranformation of all cells. That any or most of these factors might be causal in certain specific tumours or transformed cells cannot be discounted. For good reviews on the subject of cyclic nucleotides and their metabolism in relation to growth and proliferation of normal and neoplastic cells see (Ryan and Heidrick 1974; Hunt and Martin 1980; Prasad 1981; Boynton and Whitfield 1983).

Many of the early studies which proclaimed cAMP to be a negative regulator of cell growth were based on cell line studies (Heidrick and Ryan 1970; 1971). There were, however, a

number of significant problems associated with cell line models which could and have, in some cases, given rise to false conclusions concerning the effect of cyclic nucleotides on cell growth, some of which include:

1. Studies which were based on asynchronously dividing cells where cAMP has been shown to surge in most, but not all cells, during G1 and also in late S or early G2.
2. The degradation products of cPDE enzyme action such as 5'AMP and other further breakdown products like adenosine have also been shown to act as inhibitors of cell growth.
3. Problems with comparing cells during different growth stages i.e. exponential phases of growth with quiescent monolayers.
4. External problems such as serum depletion of the culture media leading to inhibition of cell growth.
5. Experiments by some workers based soley on the use of the cAMP analogue, dibutyryl cAMP, where the breakdown product, n-butyric acid has been implicated in causing growth inhibition.

Obviously these potential sources of error could also extend to observations based on adenyl cyclase, guanyl cyclase and cPDE enzymes, especially since there is evidence that cPDE activity is induced by its own substrate (Bourne *et al.* 1973). It appears, therefore, that in well-controlled experiments on cell lines that either cAMP or dibutyryl cAMP has been shown to be either negative regulators of growth in HeLa, neuroblastoma and murine sarcoma cell lines respectively (Friedman 1976; Prasad 1979; Ridgway *et al.* 1988) or positive regulators in many other

cells (review Boynton and Whitfield 1983).

There have also been problems related to the measurement of cyclic purine nucleotides *in vivo* which may be summarized:-

1. Too long a delay between excision of the tissue and processing leading to catabolism of the substrates by endogenous cPDE enzymes.
2. Processing the tissue with a cPDE inhibitor such as theophylline which only inhibits some of the many cPDE enzymes present.
3. Problems in accurately measuring cGMP levels which are often present at only one tenth the concentration of cAMP in many tissues (Coffey *et al.* 1978).
4. Expression of the levels of substrate on the basis of tissue wet weight, per unit protein or in terms of DNA yield conflicting data.

It appears that increased cAMP levels in tumours versus normal tissue have been reported in aggressive mammary tumours in humans (Minton *et al.* 1976; Guerinot *et al.* 1977; Kung *et al.* 1977) in rats (Chatterjee and Kim 1975; Rillema *et al.* 1978) and also in rat cells (Cohen 1976). Also, relatively higher levels of cAMP have been measured in Morris hepatomas (Hickie *et al.* 1977) and in induced hepatomas (DeRubertis and Craven 1980). By contrast, lower levels of cAMP in comparison to normal tissue counterparts have been described in a specific Morris hepatoma (Hickie *et al.* 1975) in human colon (DeRubertis *et al.* 1976) and in leukaemic lymphocytes (Monahan *et al.* 1975). It can be seen, therefore, that there are substantial

differences between tumours and cancer cells compared to normal cells in the reported levels of cAMP (also cGMP) which might reflect true differences or be the result of one or more of the above outlined methodological problems. Since the methodological errors are difficult to assess, the measurement of adenyl and guanyl cyclase and cPDE levels in tumours (*in vivo*) probably provide more meaningful results. Nonetheless, errors would still arise if the enzymes were: 1. highly labile and 2. the problem of expressing enzyme activity according to DNA content or on the basis of protein would still remain.

It should also be noted that in many of the earlier reports (in the Seventies) before the cPDE enzymes had been tentatively classified there was a tendency to report on low affinity cPDE (detected at 100 uM substrate concentration) and high affinity cPDE (detected at 1 uM substrate concentration) in crude cytosols as if these were two discrete cPDE forms. Whether these do, in fact, represent separate enzymes as was originally supposed or different binding sites on the same enzyme remains to be determined. To avoid added confusion in the following summary of cPDE levels in tumours, I have summarized the high affinity cPDE measurements (1 uM cAMP or 1 uM cGMP) since this reflects a closer approximation to the normal physiological level of the substrates within cells. This, of course, was an oversimplification in view of the possibility of compartmentalization leading to cAMP and cGMP gradients within the cell (Fell 1980).

A survey of the literature has yielded a wide variation of cPDE

levels in different tumours or tumour cells (see Hunt and Martin 1980) and even conflicting results within the same tumour type. For example, in comparative studies between lymphocytes and leukaemic lymphocytes (review Epstein and Hachisu 1984), some authors report that only one cAMP-specific PDE (type IV) is present in lymphocytes (Takemoto *et al.* 1978) while others have shown cGMP hydrolysis (Wedner *et al.* 1979). It generally appears, though, that leukaemic cells are reported to have higher levels of type IV cAMP PDE than normal lymphocytes (Monahan *et al.* 1975; Epstein *et al.* 1977; Hait and Weiss 1979; Epstein and Hachisu 1984).

There have been several studies based on cPDE levels in mammary tumours but the heterogeneous character of human mammary carcinomas has raised difficulties in comparative studies of cyclic nucleotide metabolism. High tumour concentrations of cAMP (and sometimes cGMP) compared to normal mammary tissues when expressed on a per protein basis have been detected in human breast tumours (Minton *et al.* 1976; Guerinot *et al.* 1977; Kung *et al.* 1977), in rat mammary tumours (Chatterjee and Kim 1975), and rat mammary cells (Cohen *et al.* 1976). Higher levels than normal of soluble cPDE (usually only testing cAMP as substrate) have been reported in crude cytosols of human mammary carcinoma (Singer *et al.* 1976; Kung *et al.* 1977), in murine mammary tissues (Sheth *et al.* 1980) and in rat mammary tissues (Rillema *et al.* 1980). An *in vitro* experiment, however, based on cultured rat mammary cells showed lower levels of cPDE (only cAMP tested) in the homogenates of neoplastic cells

compared to normal cells (Cohen *et al.* 1976) and these findings were similar to those reported for hepatomas versus normal liver (Rhoads *et al.* 1972; Clark *et al.* 1973). Nevertheless, when the results were compared in rat mammary cell supernatants (soluble cPDE) the cPDE activity was higher in the neoplastic cells (Cohen *et al.* 1976).

Where normal mammary tissues were not available in sufficient quantities, such as in murine and rat mammary tumour-bearing lines, work has been reported in comparative studies of the components of cAMP/cGMP metabolism in tumours and cell lines of different growth rates. Reports have been described by workers where cPDE levels in microsections were higher in human benign mammary tumours (Larner and Rutherford 1982) and higher activities were reported in non-metastasizing rat tumours versus metastasizing tumours (Chatterjee and Kim 1975). The Larner and Rutherford team were able to choose malignant and benign human mammary tumours of similar cellular densities (confirmed by microscopic analysis) for comparative enzyme studies in microsections but when their results were expressed in supernatants on a per protein basis there were either no significant differences in cPDE levels between the two tissues or the tumours had higher levels (Larner and Rutherford 1982). Work by Cho-Chung and associates has shown that dibutyryl cAMP caused *in vivo* regression of oestrogen-dependent rat mammary tumours induced by 7,12-dimethylbenzanthracene (DMBA) and also *in vitro* suppression of human breast cancer cells (MCF7) (Cho-Chung *et al.* 1980; Cho-Chung 1986). They have also found,

as well as other workers, that in DMBA-induced rat mammary tumours, cAMP levels were greatest in regressing tumours of ovariectomized animals (Matusik and Hilf 1976; Cho-Chung *et al.* 1978).

Conflicting evidence has been reported, however, in uterine cells which are also oestrogen and progesterone-dependent, where an initial acute cAMP surge has been measured following injection of oestradial 17-Beta (Szego and Davis 1967; Rosenfeld and O'Malley 1970). Also, in a rat mammary adenocarcinoma cell line there was evidence that dibutyryl cAMP positively controlled the growth rate of the cell line (Klein and Loizzi 1977).

There have also been opposing reports on the levels of cPDE activity. For example, Cho-Chung has reported increased cPDE activity (only cAMP tested) in regressing DMBA-induced rat mammary tumours (Cho-Chung *et al.* 1978) whereas others using the same rat model, have shown higher enzyme levels in growing tumours (Ip and Dao 1980). Higher levels of cPDE in growing mammary tumours have also been described in humans (Singer *et al.* 1976). A more recent finding has been that physiological concentrations of oestradiol inhibit cPDE activity in human and rat uterine tissues and that there appears to be a functional relationship between oestrogen receptors and cPDE activity (Etingof *et al.* 1984).

All these studies have been based on measurements of cPDE levels in either crude homogenates or cytosols. Clearly, some of the opposing data are the result of the tumour model

studied, the methodology used and the method of expression of cPDE enzyme activity. My findings, based on comparisons between the different murine tissues will be discussed.

Materials and Methods

Reagents ^{3}H-17-Beta-oestradiol (specific activity 95 Ci/mmol), cyclic (8-^{3}H) AMP (specific activity 26 Ci/mmol), cyclic (8-^{3}H) GMP (specific activity 35 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, UK). Tritiated 17Beta-methyl-promogestone (specific activity 87 Ci/mmol) and cold promogestone were purchased from New England Nuclear (NEN, Boston, MA, USA). *Crotalus atrox* 5'-nucleotidase (Grade IV) cAMP, cGMP, cCMP, 5'AMP, adenosine, bovine heart calmodulin-insensitive phosphodiesterase, calmodulin -stimulated phosphodiesterase, theophylline, diethylstilbesterol and 1-methyl-3-isobutylxanthine (MIX) were obtained from Sigma (Sigma, St Louis. Mo, USA). Alkaline phosphatase (AP) calmodulin, calmidizolium and trifluoroperazine were purchased from Boehringer (Boehringer, Lewes, UK). Molecular mass markers for sodium dodecylsulphate (SDS) electrophoresis and for gel filtration; cyanogen bromide-activated Sepharose for affinity chromatography; Sephacryl S-200 for gel filtration; reagents for isoelectrofocusing; chromatofocusing gel and ampholyte Polybuffer 74 for chromatofocusing were all obtained from Pharmacia (Pharmacia, Uppsala, Sweden). Dowex AG1-X2(200-400

mesh) and the Bio-Rad protein kit were purchased from Bio-Rad (Bio-Rad Laboratories, Calif., USA). DEAE-52 for anion-exchange chromatography; acrylamide, N',N'-bismethylene acrylamide, ammonium persulphate, mercaptoethanol, bromophenol blue and glycine for non-denaturing polyacrylamide-gel electrophoresis (slab or disc) were obtained from BDH (BDH Chemicals, Poole, UK). All other reagents used were of analytical grade and were purchased from Merck (Merck, Darmstadt, FRG) or from BDH (BDH Chemicals, Poole, UK).

Storage of Radiochemicals.

The tritiated solutions of cAMP and cGMP have a certain percentage contamination with tritiated water even when fresh. To remove this source of contamination, the radiolabelled cyclic nucleotides were either purified using a suitable column on a Waters HPLC system or another procedure, which I modified, was used. In the literature a method of removing tritiated water is described whereby the radioactive ligand (200 ul) is applied to a Dowex AG 2x8 (200-400mesh) resin column (Bio-Rad), washed with 10 ml of 40 mM Tris buffer and eluted with 5 ml of 0.3 N HCl in 0.5 ml fractions. The pH is then adjusted to 7.5 with 1 N NaOH and stored at -20° C in 40% ethanol. I, however, considered this to be a rather excessive dilution of the radioligand which could result in non-specific breakdown and thus developed another method. The radioligand (250 ul) was aliquoted (20 ul) into sterile vacutainer tubes and stored at -70° C for no longer than 2 months. On the day of the assay, the

tube was placed on ice and the solution evaporated under a steady stream of pure, filtered nitrogen to remove tritiated water as gas. The tube was then equilibrated in the assay buffer for ten minutes on ice. The specific activity of the ligand was calculated for each assay by placing a 50 ul aliquot into scintillation cocktail (Packard) and counting the disintegrations per minute (dpm) of the "total counts" vial. The solution (ligand plus buffer) was always adjusted so that about 200000 dpm was present in a 50 ul aliquot.

Preparation of Dowex AG 1-X2 resin for cPDE assays.

The resin (50 g) was washed with 250 ml 0.5 N HCl made up with deionized, distilled water. The resin was left to stand for 30 minutes and the supernatant gently discarded. A 250 ml volume of 0.5 N NaOH was then added and allowed to equilibrate for 30 minutes. The first step was then repeated using 0.5 N HCl. Thereafter, the resin was repeatedly rinsed in deionized, distilled water and allowed to settle until the supernatant registered a pH reading of about pH=5. The settled volume of resin (approximately 70 ml) was then resuspended in two volumes (140 ml) of 3 mM glacial acetic acid and stored at 4° C for no more than three weeks or if blank values were unacceptably high.

Theophylline-Sepharose affinity matrix preparation.

This affinity medium was prepared from the method of Marchmont

et al. (1981).

Preparation and storage of DE-52 cellulose.

The gel was washed successively (by settling and decanting) with 0.5 N NaOH, several changes of water. Then washed in 0.5 N HCl and several changes of water until a pH of 4 to 5 was attained. Prior to experimentation, the gel was equilibrated in 4 volumes of freshly reconstituted buffer. The gel was stored in buffer plus 0.5 N NaCl at 4° C when not in use.

Tissue preparation and storage.

The mammary tumour tissue, brain, kidney, liver and heart tissues from the F1 multiparous females were supplied by Dr Kundig (Deparment of Pharmacology). The tissues were excised and either immediately placed in liquid nitrogen for a period not exceeding three weeks or placed in ice cold buffer for processing that day.

Fresh tissue preparation for soluble cPDE enzyme analysis.

The tissues were trimmed of fat, minced and homogenized with an ultraturrax homogenizer for 5 second bursts in 4 parts (w/v) of various ice-cold buffers depending on the experimental method to be used. These were (A) 0.01 M Tris/HCl, pH 7.5, 0.3 mM phenylmethylsulphonylfluoride (PMSF), 3.75 mM mercaptoethanol, 1.1 M glycerol, 1 mM $CaCl_2$, 5 mM $MgCl_2$ (B) 0.025M imidazole/HCl,

pH 7.5, 0.3 mM PMSF, 3.75 mM mercaptoethanol, 1.1 M glycerol (C) 0.0625 M Tris/HCl, pH 6.8, 0.3 mM PMSF, 3.75 mM mercaptoethanol, 1.1 M glycerol. The homogenates were then centrifuged in a Beckman Ultracentrifuge at 100000xg for 1 hour at 4° C. The supernatants were either further purified, or precipitated with ammonium sulphate (AS), or stored in aliquots at -70° C. The pellets were stored at -70° C.

Frozen tissue preparation for soluble cPDE enzyme analysis.

The tissues were pulverized under liquid nitrogen. The powders were then reconstituted in ice-cold buffers A, B or C and homogenized briefly with the ultraturrax prior to ultracentrifugation at 100000xg for 1hr at 4° C. The supernatants and pellets were treated as described above for fresh tissue.

Oestrogen and progesterone receptor assays of murine mammary tumour tissue.

These assays were routinely performed in the Department of Nuclear Medicine by myself with technical assistance and were assayed according to previously published methods (Collings *et al.* 1980; Sadan *et al.* 1987). Essentially, the method involved a multiple point dextran-coated charcoal radioassay technique followed by Scatchard plot analysis (Scatchard 1949) for quantification and characterization of the steroid receptors.

Ammonium sulphate precipitation of murine tissue cytosols either crude or partially purified.

EXAMPLE.

1. Add 4.0 ml ice-cold ammonium sulphate (AS) saturated solution to 6.0 ml murine supernatant (cytosol) to make up a 40% A.S. solution.
2. Stir gently and stand on ice for 20 minutes.
3. Centrifuge for 30 minutes at 25000xg at 4° C.
4. Transfer supernatant and store 40% pellet for later reconstitution in buffer of choice followed by dialysis.
5. Add X ml ice-cold AS saturated solution to supernatant to make up a 75% solution by solving for X :-

$$(0.4x10+X)/(10+X) = 0.75$$

hence X = 14 ml

6. Stir gently and stand on ice for 20 minutes and then centrifuge at 25000xg at 4° C for 30 minutes.
7. Discard supernatant and store pellet at -70° C for later reconstitution in buffer of choice followed by dialysis.

Preparation of dialysis tubing.

Suitable lengths of dialysis tubing were excised and placed in a solution containing 1 mM EDTA and enough $NaHCO_3$ such that the pH of the solution was greater than a pH of 8. The tubing was boiled for 10 minutes and then was thoroughly rinsed in distilled, deionized water. The tubing was then boiled again for 10 minutes in distilled, deionized water to remove all

impurities and stored in sterile water at 4° C for no longer than two weeks. It was deemed better not to add preservatives since cPDE enzymes are, in addition to being very labile, sensitive to and inhibited by a large range of reagents.

Analysis of the soluble cPDE enzymes of murine cytosols by non-denaturing polyacrylamide-gel electrophoresis.

The electrophoresis of the murine cytosols was routinely run at a 7.5% polyacrylamide gel concentration under non-denaturing conditions using either an LKB Disc Electrophoresis system or an LKB Vertical Electrophoresis system.

The methods used were adapted from those of Davis (1964) and Laemmli (1970). The following modifications were: no SDS was used, 3.75 mM mercaptoethanol instead of 0.72 M and the tank or electrode buffer (pH 8.3) contained 2.5 mM Tris and 19.2 mM glycine. Crude tissue cytosols or purified extracts were loaded in 50 ul aliquots (<5 mg protein/ml) and run at 2 mA per disc gel or 20 mA per slab (constant current) for 5 hours at 4° C. Cytosols which had not been homogenized nor equilibrated in buffer C, had an equal volume of gel sample buffer added (0.5 M Tris/HCl, pH 6.8, 1.1 M glycerol, 0.025% bromophenol blue) prior to electrophoresis. Gels were stained for enzyme activity from the method of Goren *et al.* (1971), with the following modifications. The gels, discs or slabs, were placed in filtered (Whatman No.2) buffer (300 ml) of 0.1 M Tris-maleate, pH 7.0, and gently rinsed 2 or 3 times. The final reaction

mixture was then added to the slabs and contained 0.1 M Tris-maleate, pH 7.0, 2.5 mM magnesium sulphate, 1.5 mM lead nitrate, alkaline phosphatase (1 unit/ml) and cAMP or cGMP at 300 uM concentration unless otherwise stated. The slabs were incubated for 30 minutes to 1 hour at 37° C or overnight at room temperature with gentle agitation in a waterbath shaker. Then the gels were washed with constant agitation in several changes of distilled, deionized water for 2 hours and developed for 2 minutes in 0.01 M ammonium sulphide solution. The gels were stored in distilled water until photographed or analyzed by densitometry scanning using a Beckman Densitometer CDS 200. Preparative gels used for isolation of enzyme bands for cPDE activity analysis or SDS polyacrylamide gel-electrophoresis were prepared as above except that a blank comb was used and a 2 cm vertical strip of the central portion of the gel was developed for activity in the minimum time (2 hours) while the rest of the gel was kept at 0-4° C. When the bands in the central portion were developed the area of undeveloped gel corresponding with the same Rf as that of the aligned activity bands was excised. The strips (2-3 mm) were placed in labelled glass test tubes and homogenized with a glass homogenizer and eluted for 24 hours at 4° C in 0.25-0.5 ml buffer A. The mixture was filtered through a Millipore 0.45 um pore filter under pressure and stored at -70° C until a sufficient quantity had been accumulated for enzyme analysis.

Non denaturing polyacrylamide electrophoresis for slab gels.

Acrylamide stock solution:-

29.2 g acrylamide

0.8 g bis acrylamide

water to make up a volume of 100 ml

The dry chemicals were added to a volumetric flask and water (distilled, deionized) was added to make up a volume of 100 ml. The mixture was stirred using a magnetic-stirrer until it formed a clear solution. The solution was filtered through a millipore filter (45 micron pore size) attached to a 50 ml syringe. The filtered solution was stored at 4° C.

Lower Tris stock solution (4X):-

1.5 M Tris/HCl pH 8.8

The titrated solution was made up to a volume of 100 ml with the remaining water (distilled, deionized), filtered and stored as above.

Stack buffer stock solution (4X):-

0.5 M Tris/HCl pH 6.8

The titrated solution was filtered as described above and stored at 4° C.

Sample buffer:-

0.5 M Tris/HCl pH 6.8

5 ul bromophenol blue (1%)

1.1 M glycerol

If crude or purified cytosols had not been equilibrated in buffer C then an equal volume of sample buffer was added. Otherwise 5 ul of 1% bromophenol blue was added to the sample

Ammonium persulphate solution (APS):-
A 1% solution (w/v) was prepared freshly for each experiment and stored on ice until ready for use.

Tank buffer (pH 8.3):-
2.5 mM Tris
19.2 mM glycine
This solution was always prepared fresh on the day of the experiment in a 5 litre volumetric flask with water (at 4° C) and discarded after use.

Lower gel solution for 4 slabs (7.5%):-

Lower Tris (stock)	33.75 ml
Acrylamide (stock)	33.75 ml
Water	66.75 ml
APS	0.675 ml
Temed	45 ul

Stack gel solution for 4 slabs (5%):-

Stack buffer (stock)	10 ml
Acrylamide (stock)	5.32 ml
Water	24.40 ml
APS	200 ul
Temed	20 ul

SDS-polyacrylamide gel-electrophoresis.

A 10% solution of sodium dodecylsulphate (SDS) was prepared fresh on the day of the experiment in a 60 ml volume.

Lower gel solution for 4 slabs:-

Lower Tris (stock)	22.5 ml
Acrylamide (stock)	30.0 ml
Water	36.1 ml
APS	450 ul
Temed	30 ul
SDS	0.9 ml

Stack gel solution for 4 slabs:-

Stack buffer (stock)	10 ml
Acrylamide (stock)	5.32 ml
Water	24.80 ml
APS	200 ul
Temed	40 ul
SDS (stock)	400 ul

Tank solution for 4 slabs (SDS):-

glycine	72 g
Tris	15 g
SDS (stock)	50 ml

The above were reconstituted in 5 litres of water (distilled, deionized) at 10° C. This solution was discarded after use.

Preparation of sample for SDS-polyacrylamide electrophoresis.

Sample SDS buffer 2X:-

Stack buffer (stock)	2.5 ml
SDS (10% solution)	4.0 ml
Glycerol	2.0 ml
Mercaptoethanol	1.0 ml
Water	0.5 ml

Divide into 1 ml aliquots and freeze at -70° C.

Treatment of samples:-

1. Trichloroacetic acid (TCA) 20% (w/v).
2. Make up above solution and store until ice-cold.
3. Add an equal volume of 20% TCA solution to crude or purified fraction.
4. Place in Eppendorf tubes and incubate on ice for 15 minutes.
5. Spin in Eppendorf mini-centrifuge at maximum speed for 5 minutes.
6. Discard supernatant.
7. Add 0.5 ml ethanol (absolute) to tube and vortex vigorously.

8. Spin in Eppendorf mini-centrifuge at maximum speed for 5 minutes.

9. Discard ethanol and repeat steps 7 and 8.

10. Discard ethanol and leave samples in tubes at room temperature until completely dry.

11. Add suitable volume of sample buffer (SDS 2X, see above) with an equal volume of water and store in freezer at -70° C until ready for use.

12. On day of experiment, take out tubes and place on a polystyrene float and boil samples for 5 minutes.

13. Remove the tubes and cool.

14. Load and run on SDS-polyacrylamide gels.

Coomassie blue staining of SDS gels.

Fixative:-

methanol	50%
acetic acid	10%
water (v/v/v)	40%

The gels were fixed in the above solution for a minimum of 1 hour with gentle agitation.

Staining solution:-

Coomassie blue R-250	0.1%
methanol	25%
acetic acid	10%
water (v/v/v)	65%

The solution was filtered before use and the gels stained with gentle agitation for 2 hours or longer if necessary.

Destaining solution:-

methanol	7%
water (v/v)	93%

The gels were placed in the destaining solution for 24 to 48 hours and then photographed.

Silver staining of SDS gels.

This was done according to the method of Merril and Harrington (1984).

Prepare following solutions:-

1. 0.36% (w/v) NaOH
2. 19.4% $AgNO_3$
3. 1% (w/v) citric acid

Store solutions at 4° C. The gels were fixed for one hour and then placed in the above solutions for the times stipulated.

Gradient-gel non-denaturing polyacrylamide electrophoresis.

Example: 5 to 15% non-denaturing polyacrylamide gradient electrophoresis:-

Dense acrylamide stock:-

acrylamide	73 g
bisacrylamide	4 g
75% glycerol	250 ml

Light acrylamide stock:-

acrylamide	73 g
bisacrylamide	4 g
water	250 ml

Stir solutions very well and store in dark at 4° C.

Solutions adequate for 2 slabs:-

Dense gel:-

dense acrylamide	22.5 ml
lower Tris (stock)	7.5 ml
APS	43 ul
Temed	14.4 ul

Light gel:-

light acrylamide	5.0 ml
lower Tris (stock)	7.5 ml
water	17.5 ml
APS	7.2 ul
Temed	14.4 ul

The de-aerated solutions were placed in the correct mixing chambers of the LKB-gradients gel former. Under gentle stirring

and a flow rate of 3-5 ml/minute the linear gradient solution was carefully pumped into the prepared slab plates of the LKB-Vertical system. The gels were overlayed with 2 ml water and allowed to set for 4 hours. The overlay was then decanted and the usual stacking gel applied (see previous directions). The cytosols were in 50 ul aliquots (<5 mg/ml) protein and the electrophoresis was run at 20 mA per slab for 18 hours at 4° C.

Photography of polyacrylamide gels.

Technical Pan 135 mm (ASA 50) film was used. The settings on the Nikon camera usually were: f stop = 3.5, shutter speed 1/8th second. The gel to be photographed was placed on a glass sheet and then placed on an upturned X-ray film viewer. All excess light was blotted out by placing dark cardboard pieces around the gel. The exposure time was dependent on the intensity of the cPDE activity bands and the background of the gel and varied from 21 to 10 seconds. Occasionally a green filter was used.

Radioassay for cPDE activity in crude and partially purified fractions.

This was a two-step radioassay of (Thompson and Appleman 1971; Thompson *et al.*1974) and further modified by Boudreaux and Drummond (1975) and Azhar and Menon (1977). For routine assays, a 200 ul final incubation mixture included: buffer A, ^{3}H-cAMP (200000 d.p.m.), unlabelled cAMP at 1 and 100 uM concentrations

and suitably diluted cytosols. After incubation for 10 minutes at 30° C, the tubes were placed for 2 minutes in a boiling water bath and then allowed to cool. 5'-Nucleotidase was added to each tube (1 unit/tube) in a 50 ul aliquot and the reaction mixture incubated for 10 minutes at 37° C. The reaction was terminated by addition of 750 ul of a slurry (1:3) of Bio-Rad AG 1-X2 resin in 3 mM acetic acid. After vortexing, the tubes were centrifuged at 5000xg for 10 minutes and the radioactivity in a 500 ul aliquot of the supernatant was measured by liquid scintillation.

Calculation of the results

Formula steps:-

1. $$\frac{2x(\text{dpm (total)}-\text{dpm(blank)})}{2220 \text{ dpm (where 1 nCi = 2220 dpm)}} = \text{nCi}$$

2. Divide nCi by the calculated specific activity of ^{3}H-cAMP + cold cAMP or ^{3}H-cGMP + cold cGMP (in Ci/mmole) to give picomoles cAMP or cGMP hydrolyzed.

3. Divide this value by the soluble protein content of the sample (mg/ml) to give picomoles cAMP or cGMP hydrolyzed/mg protein.

4. Divide this value by the incubation time (usually 10 minutes) to give final specific activity. Thus the cPDE units = cAMP or cGMP hydrolyzed/mg protein/min.

For rapid calculation of cPDE specific activities, data was entered into a formula written in a BASIC programme run on an

HP 85 computer.

Notes

Assays which yielded blank values greater than 8% of counts were discarded and the assays rerun with either fresh label (^{3}H-cAMP or ^{3}H-cGMP) or freshly prepared Dowex Resin.

Schematic outline of the two-step radioassay batch method.

^{3}H-cAMP/ ^{3}H-cGMP cAMP/cGMP ---endogenous cPDE---> ^{3}H-5'AMP/ ^{3}H-5'GMP 5'AMP/5'cGMP ---exogenous 5'nucleotidase--->

^{3}H-adenosine and adenosine/ ^{3}H-guanosine and guanosine + Pi

The basic assumptions in this assay are:-

1. The cytosols do not contain high levels of non-specific phosphodiesterases which have been described in the literature (see Introduction).

2. That the charged products of cPDE hydrolysis namely, 5'AMP and 5'GMP, are not converted to other by-products during the first incubation step. For example, Rutten and associates (1973) outlined a scheme that showed the enzyme 5'AMP deaminase acts on 5'AMP to form 5'IMP. The latter product is converted by 5'nucleotidase to form inosine + Pi.

3. The uncharged or neutral products of adenosine or guanosine (tritiated or cold) formed in the second incubation step by excess, exogenous 5'-nucleotidase remain in the supernatant. Conversely, all the charged species such as cAMP, ^{3}H-cAMP, 5'GMP or ^{3}H-5'GMP are absorbed by the anionic-exchange resin. The

latter situation, if it did not occur, would result in "blank" values (that is blanks with no cPDE activity) with unacceptably high counts (>8%). It is less easy, however, to determine whether adenosine or guanosine have been absorbed to the anionic resin thus leading to an underestimation of cPDE activities (see Rutten *et al.* 1973, Lynch and Cheung 1975, Thompson *et al.* 1979). For this reason, the modified method of Azhar ard Menon (1977) was adopted whereby the Dowex resin when equilibrated in 3 mM acetic acid resulted in optimum recovery of ^{3}H-adenosine and when equilibrated in 0.75 M methanol gave optimum recovery of ^{3}H-guanosine.

Protein Determination.

Protein was measured in cytosols or partially purified fractiors using the Bio-Rad protein assay kit modified from the method of Bradford (1976) using bovine serum albumin as a standard.

Characterization of the soluble cPDE enzymes of murine mammary tumours by anion exchange chromatography, chromatofocusing and gel exclusion.

Pulverized murine tumours (20 g) were homogenized at 4° C in buffer B (4 parts) and centrifuged at 150000xg for one hour. About 100 ml of clear supernatant (A) was adjusted to 0.1 M NaCl and run on a DE-52 anionic-exchange column which had previously been equilibrated with buffer B. This was washed in

the adjusted buffer until no more protein was detected. The 0.1 M NaCl fraction (B) was pelleted with 70% ammonium sulphate and stored at -70° C. The DE-52 column was then eluted with a gradient of 0.1 to 0.4 M NaCl in buffer B. The pooled protein peak (C) was then dialyzed overnight in buffer B (since this was to be the starting buffer for the chromatofocusing column) to give fraction (D). Fraction (D), still in the dialysis bag was then, placed in dry solid sucrose at 4° C and concentrated to about 10% of the original volume to give fraction (E). Fraction (E) was then applied to the chromatofocusing column previously equilibrated with buffer B. The column was then washed with buffer B until no more protein was detected. The unabsorbed fraction (F) was either tested immediately for cPDE activity or pelleted with 70% ammonium sulphate and stored at -70° C. The chromatofocusing column was then eluted with Polybuffer 74-HCl (pH range from 7 to 4) and 4 ml fractions were collected until the pH measured 4 (about 120 to 150 ml total eluent volume). The fractions were then titrated to a pH of 7.5 with 1 M imidazole to prevent loss of cPDE activity and then the fractions were analyzed for both cPDE activity at 1 and 100 uM cAMP concentrations and protein concentration. The cPDE activity peaks were pooled to give fraction (G). For gel filtration, fraction (G) was dialyzed overnight in buffer B with 5 mM magnesium chloride and 1 mM calcium chloride. The dialyzed sample was centrifuged at 40000xg for 30 minutes at 4 ° C to remove precipitate and then concentrated in sucrose (about 10 to 20% of the original volume) to give fraction (H).

Fraction (H) (about 2 ml) was then applied to a Sephacryl S-200 column (molecular mass determination range 5000 to 250000 daltons) which had been previously equilibrated with buffer B. The molecular mass standards used to standardize the column prior to the application of fraction (H) were ovalbumin 43000, albumin 67000, aldolase 158000 and catalase 232000. The eluted 1 ml fractions from the S-200 gel were tested for cPDE activity and protein concentration.

Schematic outline of the characterization procedure:

TUMOUR TISSUE

1. fresh or pulverized 20 g

2. homogenize in 4 parts buffer B

CRUDE HOMOGENATE

3. spin at 100000xg for 1 hour

SUPERNATANT A PELLET
store at -70° C

4. apply A to equilibrated DE-52 resin (anionic exchange)

BOUND PROTEIN UNABSORBED FRACTION B

5. run 0.1 M to 0.4 M NaCl gradient in buffer B through DE-52

6. collect gradient NaCl fractions

POOLED PROTEIN PEAK C

7. dialyze C overnight in buffer B

DIALYLSATE D

8. concentrate D 10x in sucrose

CONCENTRATE E

9. apply E to equilibrated chromatofocusing column

BOUND PROTEIN THROUGH FRACTION F

10. elute CF column with PB74-HCl (pH 7.4)

11. collect 4 ml fractions

12. measure pH and titrate to pH 7

13. measure enzyme activity and protein concentration

POOLED FRACTIONS G

14. dialyze, centrifuge and concentrate

FRACTION H

15. apply to S-200 for mass determination

Results

Physical characterization of soluble cPDE activity in cytosols of mammary tumours.

The pH optimum of the soluble cPDE activity was pH = 7.5-8.0 (Table 2:1) and thus buffer A was used in all subsequant cPDE activity determinations. Experiments showed that when exogenous calmodulin (30 units) was added in the presence of calcium (present in buffer A) there was no significant change in enzyme activity for either cAMP or cGMP ($p>0.5$) (see Table 2:2). In the presence of EDTA (50 uM), a chelating agent of magnesium and also calcium ions, there was a decrease in cPDE activity observed for both cAMP and cGMP (see Table 2:2). The optimal protein concentration for measurement of cPDE activity by radioassay (as described in materials and methods) in crude mammary supernatant was found to be 2 mg/ml (Table 2:3).

Table 2:1. The soluble cPDE activities of crude murine mammary supernatant assayed at different pH values. Results are the mean of two experiments.

Assay pH	cAMP nmol/mg/min 100 uM	1uM
6.5	0.92	0.027
7.0	1.55	0.053
7.5	1.98	0.097
8.0	2.02	0.120
8.5	1.62	0.057
9.0	1.02	0.029

Table 2:2. The soluble cPDE activities in crude fractions of murine mammary tumour. The results represent the mean of two experiments

Fraction	Protein conc. mg/ml	cAMP nmol/mg/min 100 uM	1 uM	cGMP nmol/mg/min 100 uM	1 uM
Crude homogenate	16	0.64	0.019	-	-
Crude supernatant A	2	1.88	0.127	5.59	0.104
Crude supernatant A +calmodulin/Ca	2	2.09	0.118	4.30	0.106
Crude supernatant A +EDTA	2	0.94	0.056	2.23	0.096

Table 2:3. The cPDE activities when measured by radioassay at different protein concentrations of crude mammary tumour supernatant. The results represent the mean of two experiments.

Tissue	Protein conc. mg/ml	cAMP nmol/mg/min 100 uM	1 uM
Murine Tumour	15	0.92	0.019
	7	1.11	0.056
	2	1.88	0.127
	1	1.67	0.119

Oestrogen and progesterone receptor levels in murine mammary tumours.

Of 32 murine mammary tumours analyzed for steroid receptor content, none of the tumours had levels of cytosolic unbound oestrogen or progesterone receptors that exceeded 10 fmol/mg protein. Most tumours, 29/32, had undetectable receptors according to Scatchard plots and all the tumours were classed as oestrogen receptor deficient and progesterone receptor deficient tissues. This was in accordance with accepted classification where oestrogen receptor rich and progesterone receptor rich tissues must exceed 10 fm/mg bound receptor protein and exhibit Scatchard analysis with statistically acceptable linear plots. The murine mammary tumours were

therefore similar in growth dependence and appeared to be oestrogen and progesterone independent.

Non-denaturing polyacrylamide gel-electrophoresis of murine mammary cytosols with specific cPDE activity staining.

Initially, electrophoresis runs were performed on disc gels at 7.5% acrylamide concentrations for both cAMP and cGMP at 100 uM concentrations (Fig. 2:1a, 2:1b). Control incubations in the presence of 5 mM theophylline showed differential inhibition of the cPDE enzyme activity bands for cAMP and cGMP respectively (see right hand gels of Figs. 2:1a and 2:1b). At concentrations greater than 10 mM theophylline there was little activity staining observed but, by that stage, there were problems in trying to solubilize fully the theophylline in the incubation medium and the cloudy precipitate in the medium might have prevented band formation mechanically through the precipitate settling on the gel rather than by chemical action.

Control incubations in the absence of cAMP or cGMP or alkaline phosphatase gave no cPDE activity bands (Fig. 2:2). There was no staining observed when 5'AMP, adenosine or ATP were substituted for either cAMP or cGMP.

Fig. 2:1a. Non-denaturing polyacrylamide disc gel-electrophoresis followed by specific cPDE activity staining of crude cytosol of murine mammary tumours. The left gel shows four bands (arrowed) in the presence of cAMP (100 uM). The right gel shows a control incubation of cAMP (100 uM) in the presence of theophylline (5 mM). Soluble protein = 2 mg/ml in 50 ul aliquots.

Fig. 2:1b. The same experiment as for 1a except that the incubations were performed in the presence of cGMP (100 uM). It was noted that the resolution of the cPDE activity bands were clearer when cGMP was used.



Fig. 2:2. Controls of the cPDE forms on non-denaturing polyacrylamide disc gel-electrophoresis in crude murine mammary cytosol. Soluble protein = 2 mg/ml in 50 ul aliquots. Control incubation from L to R: without cAMP, without cGMP, without alkaline phosphatase. No activity bands were observed under the above conditions in over three separate experiments performed.



Adaptation of cPDE activity stain to slab gel electrophoresis.

After the technique was perfected so that clear gels were possible, two commercially available forms of bovine heart cPDE (from Sigma) one activator deficient (AD) and the other activator insensitive (AI) were tested (Figs. 2:3a, 2:3b). The ADcPDE showed increased activity with added calmodulin (20 to 50 units) whereas the AIcPDE form becams more diffuse. The relative mobilities (Rf) of the ADcPDE and AIcPDE bands were very similar and were closest to the band 2 form observed in murine mammary tumour using 7.5% polyacrylamide concentration (Table 2:4).

Comparative study of soluble cPDE activity forms in various murine tissues.

The soluble cPDE forms showed similarities and differences in the activity patterns of the following murine tissues: brain, kidney, liver, heart and mammary tumour (Figs. 2:4a and 2:4b). The relative mobility (Rf) range for the various bands for the different tissues using 7.5% polyacrylamide gel concentrations are summarized (Table 2:4). One band appeared to be common to all tissues including the commercially obtained bovine heart cPDE forms.

Fig. 2:3a. Non-denaturing polyacrylamide slab gel-electrophoresis of commercially obtained bovine heart cPDE. Soluble protein = < 100 ug/ml in 50 ul aliquots. Activity band of activator deficient (AD) cPDE of commercial bovine heart. Lane 1=ADcPDE, lane 2=ADcPDE + 10 units calmodulin, lane 3=ADcPDE + 20 units calmodulin, lane 4=ADcPDE + 50 units calmodulin.

Fig. 2:3b. Activity band of activator insensitive (AI)cPDE of bovine heart. Lane 1=AIcPDE, lane 2 = AIcPDE +50 units calmodulin (7.5%).



a

Fig. 2:4a. Non-denaturing polyacrylamide slab gel-electrophoresis at 7.5 % acrylamide concentration. Soluble protein = 1 mg/ml in 50 ul aliquots for brain and kidney and 2 mg protein/ml in 50 ul aliquots for the other tissue cytosols. Soluble cPDE activity bands for the murine tissues (in duplicate from left to right) brain (B), kidney (K), liver (L), heart (H), and tumour (T). The activity bands, numbered consecutively from the top of the gel for each tissue, were the same for both cAMP (above) and cGMP (below).

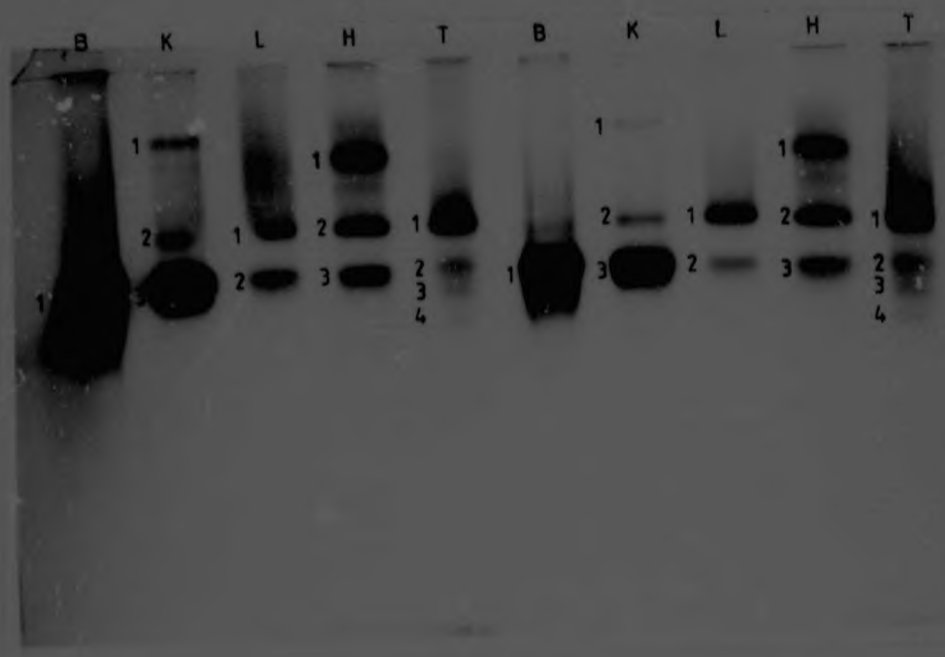
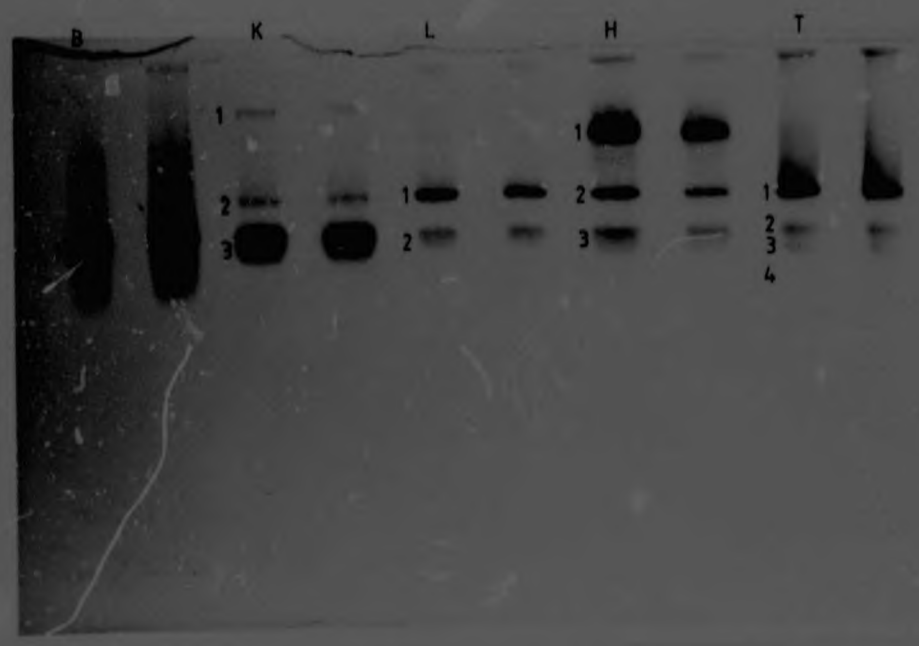

B K L H T B K L H T

Fig. 2:4b. Non-denaturing polyacrylamide slab gel-electrophoresis at 7.5% acrylamide gel concentration. Soluble protein loaded was 1 mg/ml in 50 ul aliquots for brain and kidney and 2 mg protein/ml in 50 ul aliquots for the other tissue cytosols. The cPDE activity bands observed in brain (B), kidney (K), liver (L), heart (H), and mammary tumour (T) when incubated in 100 uM cAMP (top left), 50uM cAMP, 25 uM cAMP and 10 uM cAMP (bottom right).


B
K
L
H
T
1
2
3
1
2
1
2
3
1
2
3
4

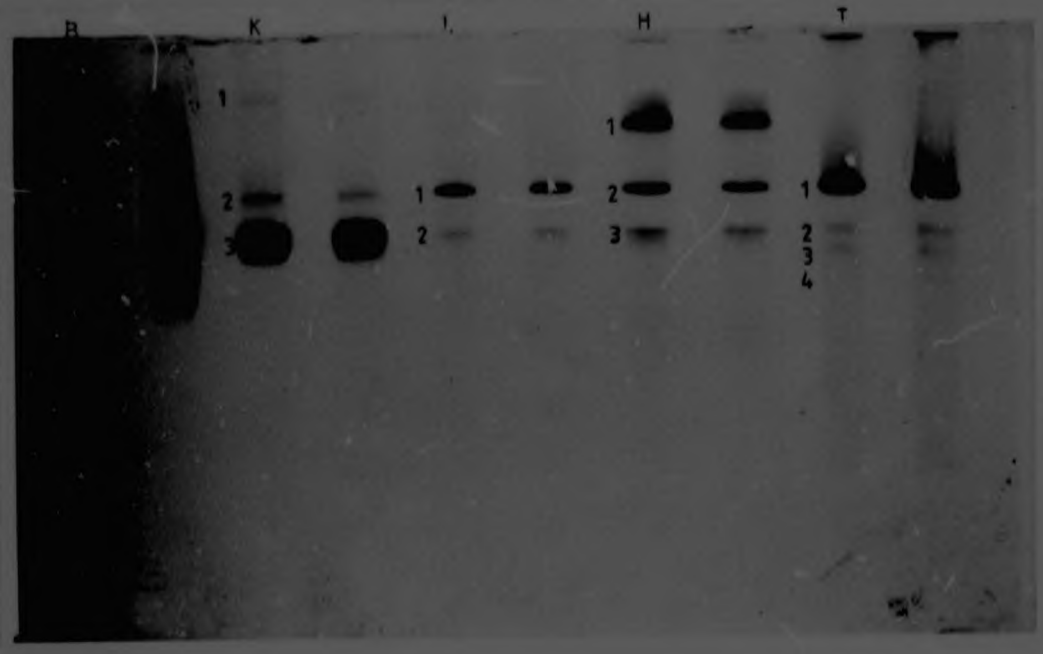

B
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Table 2:4. Relative mobility (Rf range) of cPDE activity bands observed in 7.5% gels as shown in Fig. 2:4a for both cAMP and cGMP.

	Rf range from top of gel	Tissue and band position (Fig. 2:4)
A.	0.095-0.11	kidney (1)
B.	0.12-0.14	heart (1)
C.	0.24-0.28	tumour (1), heart (2), liver (1), kidney (2),
D.	0.29-0.35	bovine heart forms, heart (3), brain (1), liver(2), kidney (3), tumour (2)
E.	0.34-0.36	tumour (3)
F.	0.37-0 41	tumour (4)

The Rf positions C and D had fairly wide Rf ranges since the activity bands in brain and kidney were very dense and spread out (see Fig. 2:4a). This observation was to lead to further experiments to establish whether these dense, apparently single bands were, in fact, more than one cPDE form. At this stage, however, with 7.5% gels, there appeared to be only one activity form of cPDE in the two commercial sources of bovine heart enzymes and murine brain. There appeared to be two forms in murine liver, three in murine heart and kidney and four bands in the murine mammary tumours.

When lower substrate concentrations of either cAMP or cGMP were

used it was noted that the upper bands particularly in the Rf range of 0.24-0.28 (position C from top of gel) in murine tumour and liver became more prominent at lower substrate concentrations (Fig. 2:4b). This seemed to suggest that these forms had a higher affinity for either cAMP or cGMP.

Densitometry scans of the murine tissue cytosols when run on nd PAGE coupled to the cPDE activity stain.

Densitometry scans were performed on 7.5 % acrylamide gels to give the cPDE activity profiles of the various murine tissue cytosols (loaded with either 5 mg protein/ml or 2 mg protein/ml or 1 mg protein/ml in 50 ul aliquots). This enabled a quantitative assessment of the cPDE activity in each band (calculated as the % area under the peak) of a specific tissue cytosol (Figs. 2:5a, 2:5b, 2:5c, and 2:5d). The more densely staining cytosols such as murine brain (Fig. 2:5a) and murine kidney (Fig. 2:5b) had to be traced at a lower sensitivity setting (that is at 1 as averse to 2, 3 or 4) in addition to the fact that they were loaded at lower soluble protein concentrations (1 mg protein/ml). Therefore quantitative comparisons, except in a relative sense, were not made between those tissue cytosols that were traced at both different sensitivity settings and had been loaded at different soluble protein concentrations. The densitometry scans of murine heart cytosol at 50 and 100 uM cAMP (sensitivity setting = 2) (Fig. 2:5e) when compared to those at either 10 uM cAMP (sensitivity

setting = 4) (left scan of Fig. 5:2e) or 10 uM cGMP (sensitivity = 4) (left scan of Fig. 5:2f) showed that the lower subtrate concentrations had relatively increased cPDE activity in band 1. This effect was particularly pronounced for the densitometry scan with 10 uM cAMP (left scan of Fig. 2:5e). This suggests that the band 1 of murine heart cytosol is a higher affinity enzyme compared to bands 2 and 3. When comparisons were made between the cPDE activity profiles of murine heart cytosol at 10 uM cAMP (left scan of Fig. 2:5e) with that of 10 uM cAMP + 1 uM cGMP (right scan of Fig. 2:5e) there was slightly increased cPDE activity in band 1 of the mixed substrate profile. Whereas, all three bands showed increased activity in the cPDE activity profile of murine heart cytosol at 10 uM cGMP + 1 uM cAMP (right scan of Fig. 2:5f) with that of 10 uM cGMP (left scan of Fig. 2:5f). Thus a soluble cGMP-stimulated cPDE (type II cPDE) may have been detected in the band 1 of murine heart cytosol. Nonetheless, low concentrations of cAMP seemed to stimulate all the murine heart soluble cPDE forms. Also, there was more cPDE activity in all the murine cPDE bands at 10 uM cGMP compared to 10 uM cAMP (this effect was not observed at higher substrate concentrations). This suggests that there may be a higher affinity binding site for cGMP.

Fig. 2:5a. Densitometry scans of murine brain cytosol when run on nd PAGE coupled to the cPDE activity stain at 50 uM cAMP (left scan) and 100 uM cAMP (right scan. The sensitivity setting = 1. Soluble protein loaded was 1 mg/ml in 50 ul aliquots. The relative cPDE activity for each band as a % of the total cPDE activity for each scan was: background of brain 8% at 50 uM and 10% at 100 uM; band 1 of brain 92% at 50 uM and 90% at 100 uM.

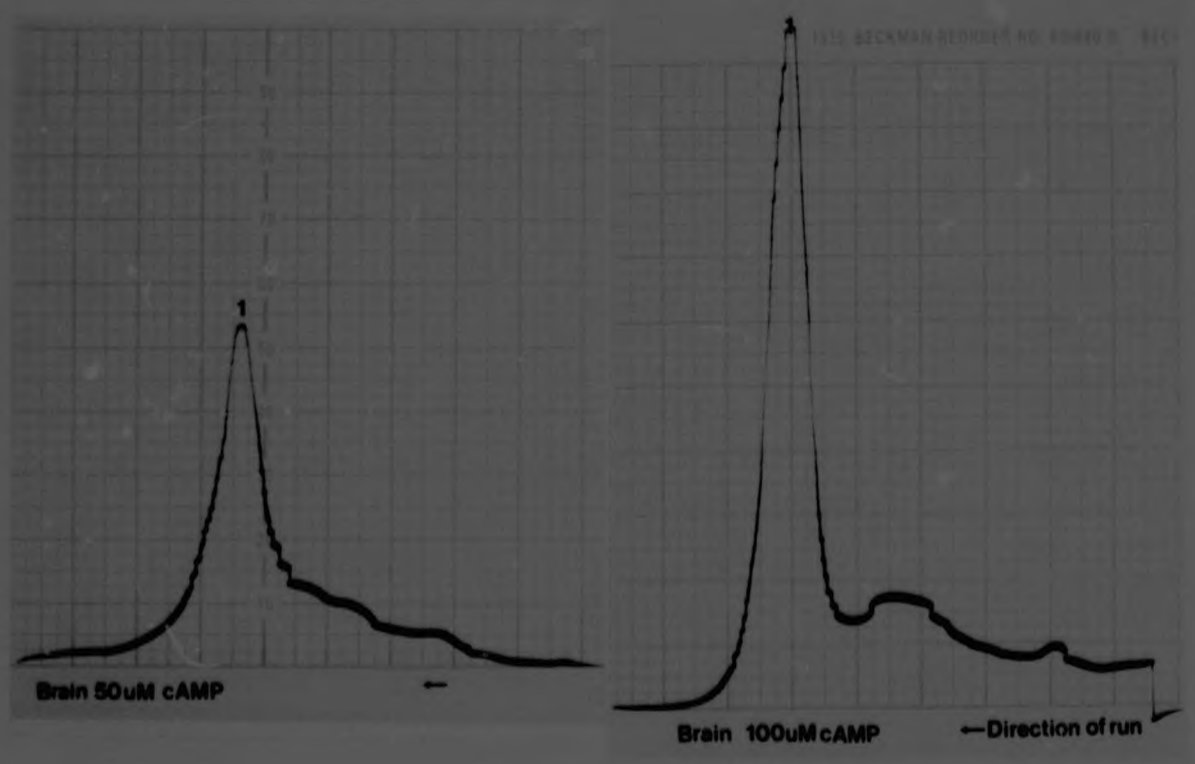


Fig. 2:5b. Densitometry scans of murine kidney cytosol when run on nd PAGE coupled to the cPDE activity stain at 50 uM cAMP (left scan) and 100 uM cAMP (right scan). The sensitivity setting = 1. Soluble protein loaded was 1 mg/ml in 50 ul aliquots. The relative cPDE activity for each band as a % of the total cPDE activity for each scan was: background of kidney 2% at 50 uM and 1% at 100 uM; band 1 of kidney 11% at 50 uM and 13% at 100 uM; band 2 of kidney 39% at 50 uM and 39% at 100 uM; band 3 of kidney 46% at 50 uM and 48% at 100 uM.

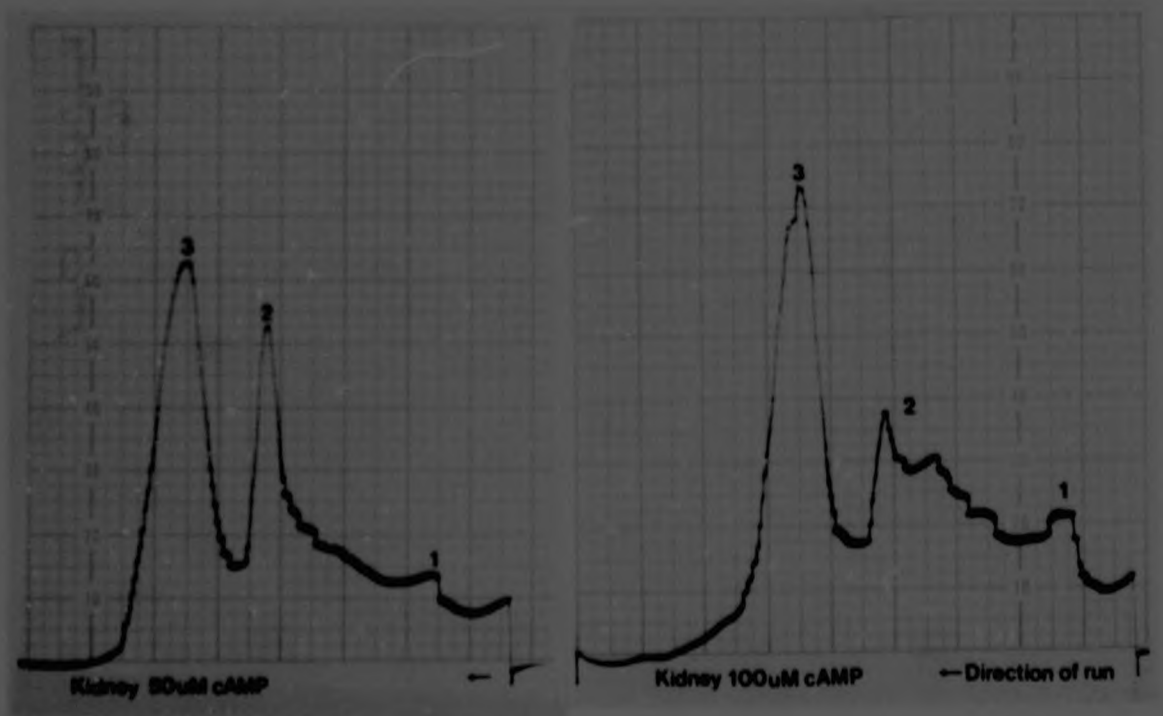


Fig. 2:5c. Densitometry scans of murine liver cytosol (left scan at 50 uM cAMP) and murine mammary tumour cytosol (right scan at 100 uM cAMP) when run on nd PAGE coupled to the cPDE activity stain. Sensitivity settings = 2 for liver and 3 for tumour. Soluble protein loaded was 2 mg/ml in 50 ul aliquots. The relative cPDE activity for each band as a % of the total cPDE activity for each scan was: background of liver 9%; band 1 of liver 38%; band 2 of liver 53%; background of tumour 1%; band 1 of tumour 46%; and bands 2 to 4 of tumour 53%.

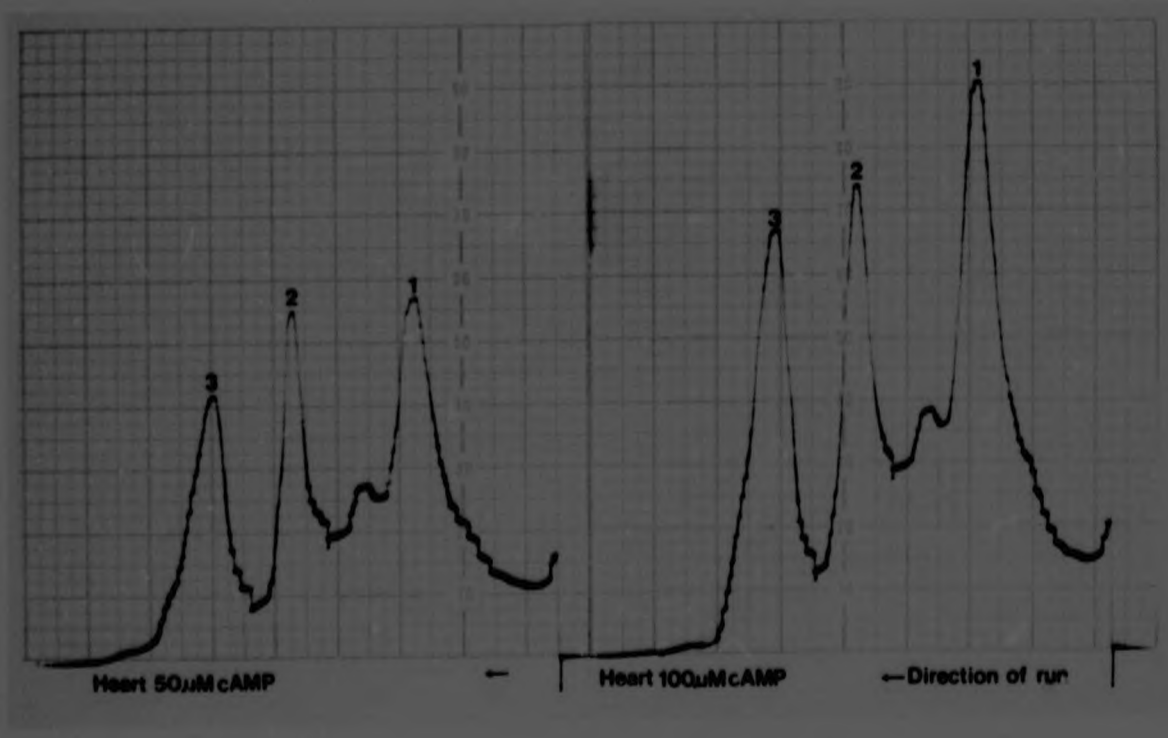


Fig. 2:5d. Densitometry scans of murine heart cytosol when run on nd PAGE coupled to the cPDE activity stain at 50 uM cAMP (left scan) and 100 uM cAMP (right scan). The sensitivity setting was = 2. Soluble protein loaded was 2 mg/ml in 50 ul aliquots. The relative cPDE activity for each band as a % of the total cPDE activity for each scan was: background of heart 10% at 50 uM and 11% at 100 uM; band 1 of heart 45% at 50 uM and 44% at 100 uM; band 2 of heart 21% at 50 uM and 20% at 100 uM; band 3 of heart 24% at 50 uM and 24% at 100 uM.

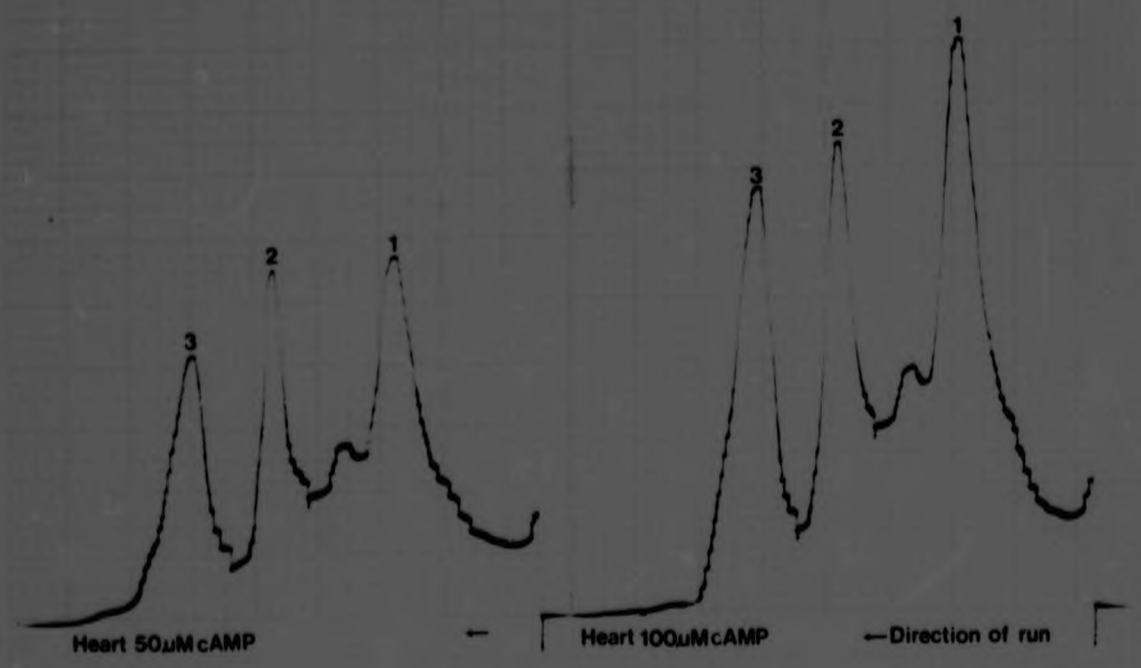


Fig. 2:5e. Densitometry scans of murine heart cytosol when run on nd PAGE coupled to the cPDE activity stain at 10 uM cAMP (left scan) and 10 uM cAMP + 1 uM cGMP (right scan). Sensitivity setting for each scan = 4. Soluble protein loaded was 5 mg/ml in 50 ul aliquots. The relative cPDE activity for each band as a % of the total cPDE activity of the 10 uM cAMP scan was: background of heart 6%; band 1 of heart 76%; band 2 of heart 7.5%; band 3 of heart 10.5%. For the mixed substrate scan on the right the values were: background of heart 6%; band 1 of heart 79%; band 2 of heart 6.5 %; band 3 of heart 8.5%.

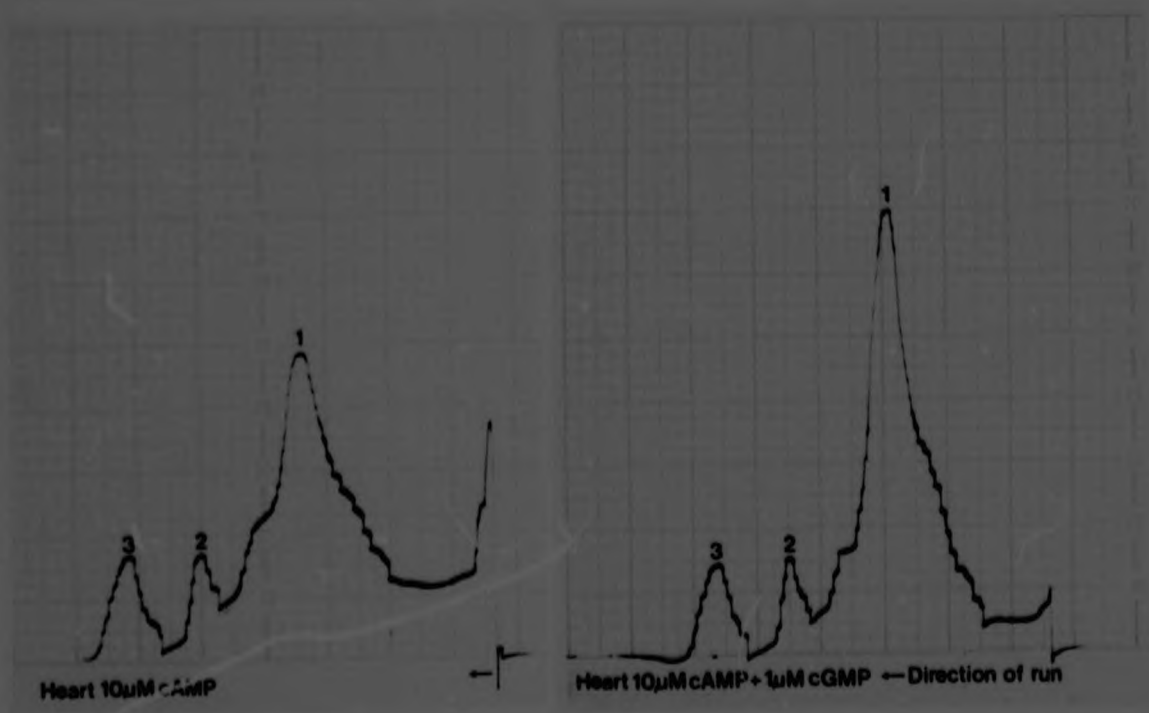
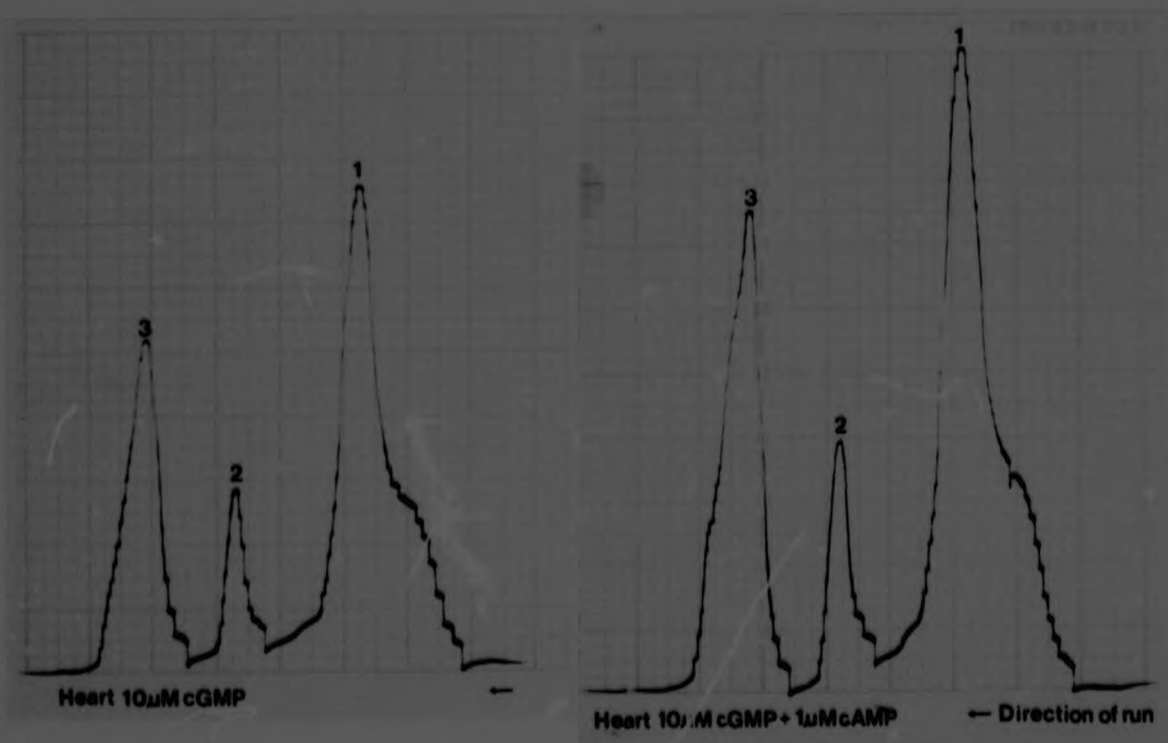


Fig. 2:5f. Densitometry scans of murine heart cytosol when run on nd PAGE coupled to the cPDE activity stain at 10 uM cGMP (left scan) and 10 uM cGMP + 1 uM cAMP (right scan). Sensitivity setting for each scan = 4. Soluble protein loaded was 5 mg/ml in 50 ul aliquots. The relative cPDE activity for each band as a % of the total cPDE activity of the 10 uM cGMP scan was: background of heart 1%; band 1 of heart 61%; band 2 of heart 10%; band 3 of heart 29%. For the mixed substrate scan on the right the values were: background of heart 1%; band 1 of heart 58%; band 2 of heart 10%; band 3 of heart 31%.



Control experiments of the soluble cPDE activities of murine tissues using either the gel method or the radiometric assay.

Controls using nd PAGE coupled to the cPDE activity stain were run with the addition of a. calmodulin, b. 5 mM trifluoroperazine, and c. EDTA (50 uM) (Fig. 2:6). Trifluoroperazine, which is a potent inhibitor of type I cPDE (calmodulin dependent cPDE), was found to inhibit completely all the cPDE activity bands at a 5 mM concentration although it was shown to inhibit differentially the more rapidly moving forms at lower inhibitor concentrations. Added calmodulin (50 units) did not appear to have an effect on the intensity of the cPDE activity bands in murine tissues (Fig. 2:6).

The cPDE activities in crude cytosols of murine tissues by radioassay, using both cAMP and cGMP, are summarized in Tables 2:5, 2:6, 2:7 and 2:8.

The cPDE activities in murine tissue cytosols were also tested in the presence of calmidazolium (5 uM), which is a potent inhibitor of type I cPDE, and also in the presence of exogenous calmodulin.

Fig 2:6. Non-denaturing polyacrylamide slab gel-electrophoresis at 7.5% and 8% acrylamide concentrations. Controls were run with brain (0.5 mg protein/ml), kidney (1 mg protein, liver (1 mg protein/ml), heart (2 mg protein/ml) and tumour (2 mg protein/ml) in 50 ul aliquots and stained for cPDE activity. Top left (7.5% gel) was a complete incubation in 100 uM cAMP. Top right (7.5% gel) shows post-electrophoresis incubation of gel in 5 mM trifluoroperazine. Lower left (8.0% gel) shows calmodulin added to 100 uM cGMP. Lower right (8.0% gel) shows incubation in 100 uM EDTA following electrophoresis. The two lower migrating cPDE bands observed in murine brain cytosol in the presence of 100 uM EDTA are arrowed.

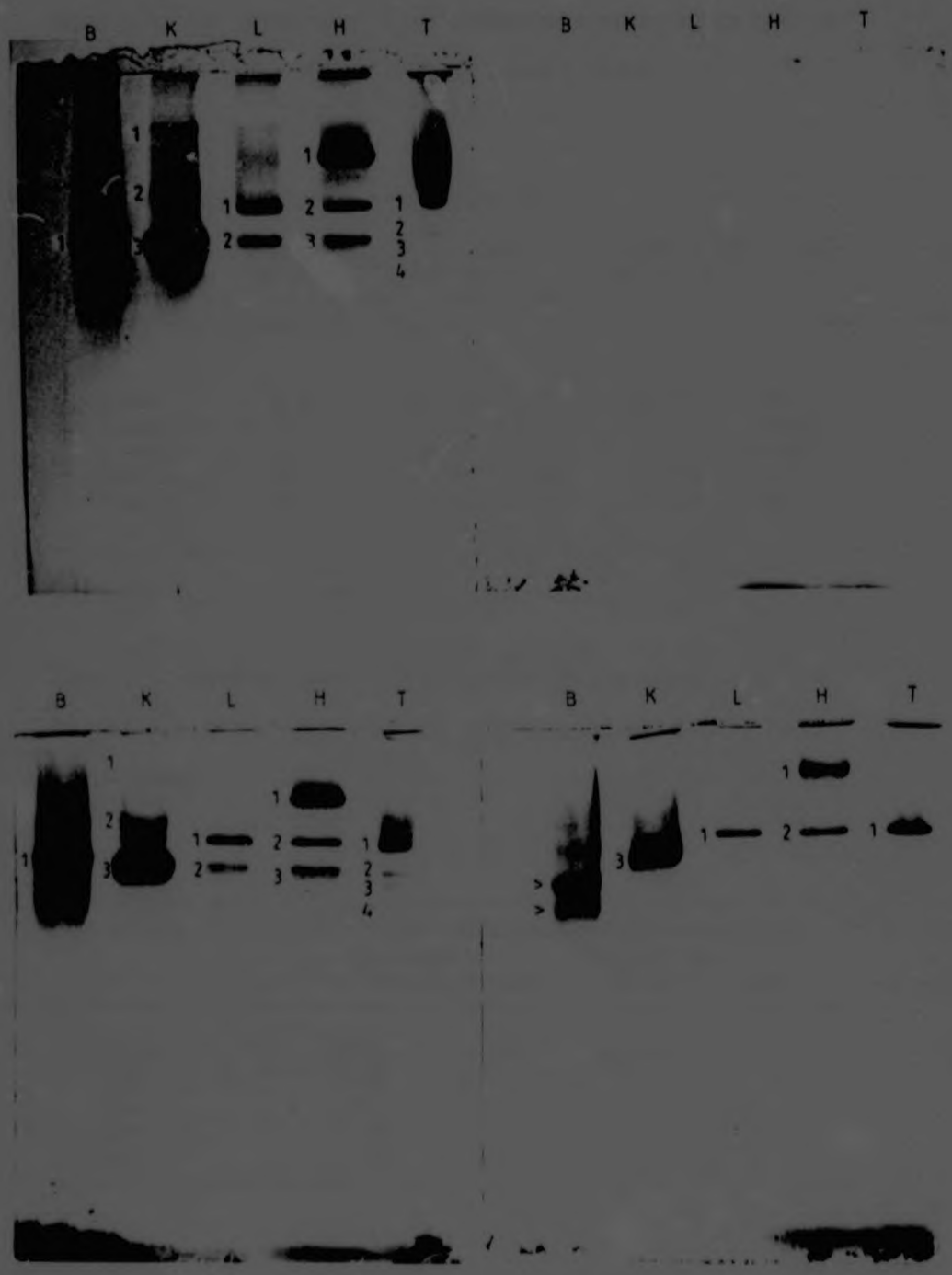

B K L H T
B K L H T
B K L H T
B K L H T

Table 2:5. The cPDE activities in murine tissues using cAMP as substrate at either 100 uM or 1 uM concentrations.

Tissue	Protein mg/ml	cAMP nmol/mg/min		cAMP + TFP (1 mM) nmol/mg/min	
		100 uM	1 uM	100 uM	1 uM
Brain	0.5	27.04	0.163	0.62	nd
Kidney	1	6.35	0.332	0.22	0.008
Liver	1	8.51	0.145	0.156	0.005
Heart	2	0.86	0.088	0.199	0.016
Tumour	2	1.80	0.127	0.171	nd

Table 2:6. Parallel control experiments in the presence of the inhibitor calmidazolium (500 uM) or exogenous calmodulin (20 units/assay).

Tissue	cAMP+inhibitor nmol/mg/min		cAMP+calmodulin nmol/mg/min	
	100 uM	1 uM	100 uM	1 uM
Brain	16.24	0.356	26.18	0.512
Kidney	4.98	0.295	5.76	0.309
Liver	2.69	0.102	8.41	0.154
Heart	0.94	0.056	0.94	0.078
Tumour	1.90	0.108	2.09	0.118

Table 2:7. The cPDE activities in murine tissues using cGMP as substrate at either 100 uM or 1 uM concentrations.

Tissue	Protein mg/ml		cGMP nmol/mg/min		cGMP + TFP (1mM) nmol/mg/min	
	100 uM	1 uM	100uM	1uM		
Brain	1		26.28	0.526	1.173	0.065
Kidney	1		7.51	0.266	0.4	0.005
Liver	1		8.7	0.203	0.31	0.005
Heart	2		1.64	0.098	0.205	0.010
Tumour	2		2.59	0.104	0.152	0.004

Table 2.8. Parallel control experiments in the presence of the inhibitor calmidazolium (500 uM) or exogenous calmodulin (20 units/assay).

Tissue	cGMP+inhibitor nmol/mg/min		cGMP+calmodulin nmol/mg/min	
	100 uM	1 uM	100 uM	1 uM
Brain	27.54	0.267	28.18	0.437
Kidney	6.12	0.272	6.91	0.261
Liver	3.31	0.184	6.80	0.205
Heart	1.44	0.037	1.27	0.115
Tumour	2.23	0.096	2.30	0.104

The inhibitor calmidazolium at a concentration of 500 um appeared to cause some inhibition of cPDE activity in the crude tissue cytosols especially when cAMP was used as substrate. The inhibitor, trifluoroperazine at 1 mM concentration, had a marked inhibitory effect on all the soluble cPDE activities in the murine tissues examined. The addition of calmodulin, however, had little effect on the cPDE specific activities and this was in agreement with the gel electrophoresis findings.

Table 2:9. The specific activities of cPDE (nmol/mg protein/min) in the extracts of particulate fractions of murine brain and kidney.

Tissue	100 uM cAMP	1 uM cAMP	100 uM cGMP	1 uM cGMP
Brain	14.3	0.160	13.8	0.167
Kidney	1.6	0.029	1.71	0.028

These cPDE activities associated with the membrane components of murine brain and kidney were obtained by ultrasonication of the tissue pellets. The pellets were well rinsed in buffer B to remove cytoplasmic contamination prior to ultrasonication and cPDE activity determination.

Elution of cPDE forms from the excised gel bands of murine tissue cytosols run on preparative non-denaturing polyacrylamide gel electrophoresis (5%).

This was achieved by using a blank comb on the slabs and layering volumes of 1 ml of the cytosol of either murine brain, kidney, liver, heart, and tumour. The elution technique was as described in Materials and Methods.

Table 2:10. The purification of the different activity bands was greatest for the brain tissue but there was poor recovery in other tissues. The specific activities for the various bands are summarized below.

Tissue	Band No. (Fig 2:4)	cAMP 100 uM nmol/mg/min	cAMP 1 uM pmol/mg/min	cGMP 100 uM nmol/mg/min	cGMP 1 uM pmol/mg/min
Brain	1	391	23,500	250	28,889
Kidney	1	-	-	-	-
	2	28.3	316	19.6	1,327
	3	19.5	611	23.3	2,236
Liver	1	2.5	195	4.3	232
	2	1.5	55	nd	366
Heart	1	3.9	232	2.5	88
	2	0.98	70	1.25	89
	3	nd	29	nd	74
Tumour	1	0.46	126	1.7	57
	2	1.7	57	0.45	131
	3	3.3	113	0.603	156
	4	nd	88	-	349

Table 2:10 shows that there was cPDE activity in all the eluted bands studied although the low affinity binding (100 uM cAMP or 100 uM cGMP) was not always detectable (nd). Clearly, there was loss of cPDE enzyme activity, apart from that in murine brain tissue, and this was probab' due to the fact that cPDE enzymes are labile and activity loss was incurred in the overnight elution step. It appeared that in the eluted bands there was significantly higher affinity for cGMP compared to cAMP in some of the bands, particularly in bands 2 and 3 of kidney. Whether this was due to: the very much lower protein concentrations (ug protein/ml that the cPDE enzymes were exposed to during the elution stage) which is known to cause loss of cPDE activity (Epstein *et al.* 1982(b)); or the result of a conformational change in the catalytic sites for cAMP or cGMP hydrolysis (differently affected depending on the tissue studied, namely, kidney) occurring during the overnight elution step could not be determined.

Non-denaturing polyacrylamide slab gel-electrophoresis, using linear acrylamide gradients, of murine crude cytosols coupled to the cPDE activity stain.

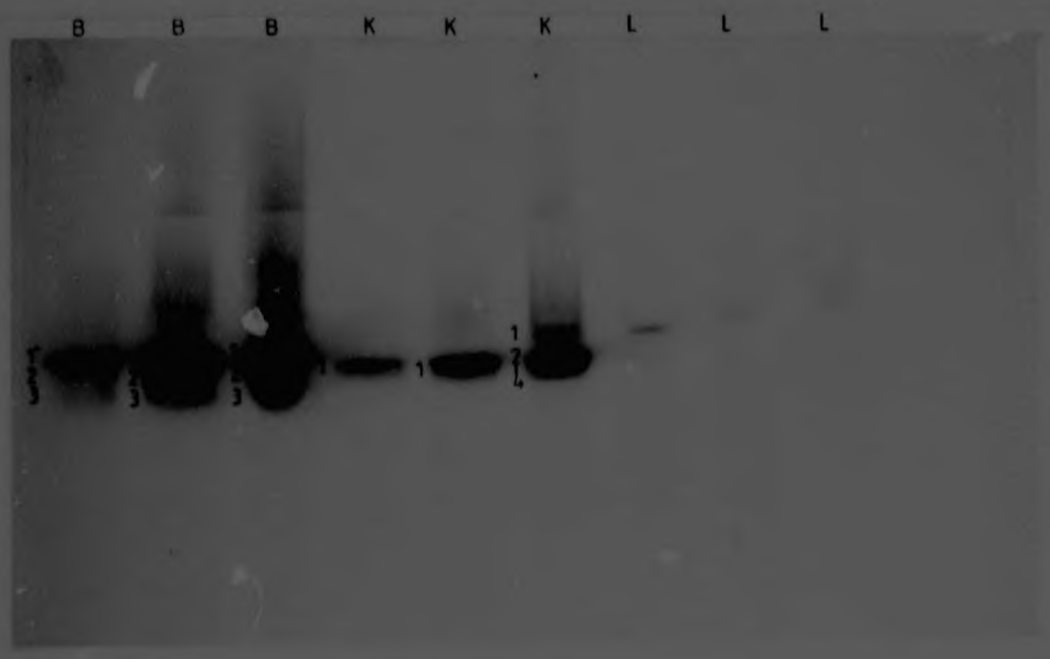
Initially, gradients between 5 and 15% concentrations were used but this was changed to a range from 5 to 22.5% since this range gave a better resolution of the cPDE forms. Greater protein concentrations of the crude cytosols had to be loaded since this method seemed to lead to loss of cPDE activity in

that only faint bands developed. For this reason, best results were obtained for the murine brain and kidney cytosols (Fig. 2:7). Three cPDE activity bands were observed in brain and four in kidney although the fourth band was only just discernable in kidney cytosol at a higher protein concentration (Fig. 2:7). An estimation of the molecular masses of these forms was achieved by comparing the Rf of the activity bands to a graph of the slope of the Rf values of molecular mass standards ve[illegible]g molecular mass. The results are summarized below in Table 2:11. The molecular mass standards used were :- albumin (A) 68000, lactate dehydrogenase (L) 140000, catalase (C) 232000, ferritin (F) 440000, thyroglobulin (T) 669000.

Table 2:11. Molecular mass determinations of the soluble cPDE enzymes of mur ne brain and kidney by non-denaturing polyacrylamide gradient gel-electrophoresis. The findings are the result of three separate experiments.

Murine Tissue	Band No. from top	Molecular Mass	+/- SD
Brain	1	209000	8100
	2	186000	7300
	3	169000	7400
Kidney	1	426000	11000
	2	229000	8400
	3	199000	8600
	4	182000	8600

Fig. 2:7. Non-denaturing polyacrylamide gel slab gel-electrophoresis run on a 5 to 22.5% acrylamide gradient. From left to right are shown three increasing protein concentrations (1, 3 and 5 mg protein/ml in 100 ul aliquots) of murine brain (B), kidney (K) and liver (L). Note the resolution of three activity bands in murine brain. Below are shown the native protein bands of the standards (on the left lane) and crude cytosols B, K, L, H and T stained with Coomassie blue.The faint bands observed for murine liver, heart, and tumour all corresponded to a molecular mass ranging between 180000 to 220000 daltons


B
B
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L

Molecular mass determination of cPDE activity forms by Ferguson plot analysis.

This was achieved by measuring the Rf values of the different cPDE activity bands at different gel concentrations and applying Ferguson plot analysis (Ferguson 1964). The molecular mass standards used were: albumin 67000, lactate dehydrogenase 140000 catalase 232000, ferritin 440000. The polyacrylamide concentration range was from 5% to 8% (Fig 2:8). The calculated molecular masses for the cPDE forms in the murine tissue cytosols are summarized below.

Table 2:12. Molecular mass determinations of the various murine tissue forms of cPDE by Ferguson Plot. The results represent the mean of two experiments.

Tissue	Band No. (Fig. 2:4)	Molecular Mass	+/-SD
Brain	1	280000	12000
Kidney	1	395000	15000
	2	282000	13500
	3	245000	11300
Liver	1	233000	12000
	2	226000	10600
Heart	1	246000	9600
	2	225000	10700
	3	227000	9300
Tumour	1	219000	10100
	2	226000	7500
	3	234000	11000
	4	229000	10100

The values, using the Ferguson plot technique, yielded higher molecular masses (of the order of 7-19%) for the cPDE forms in murine brain and kidney compared to the values determined by gradient gels. It was not established why there was this difference but may reflect the degree of error associated with the two techniques. Nonetheless, the results showed similar molecular masses for the cPDE forms in all the tissue cytosols examined except for the band 1 form of murine brain, band 1 and band 2 forms of kidney and band 1 of heart. The band 1 of the kidney form could represent an aggregation of the band 3 form of cPDE. The band 2 kidney form appeared to be a dimer at higher gel concentrations (top left 8% gel Fig. 2:8).

Fig. 2:8 Non-denaturing polyacrylamide slab-electrophoresis at different acrylamide concentrations coupled to the cPDE activity stain of murine tissue cytosols: brain (B), kidney (K), liver (L), heart (H) and tumour (T). Soluble protein loaded was 1 mg/ml in 50 ul aliquots of brain and kidney and 2 mg protein/ml in 50 ul aliquots of the other tissue cytosols. From top left, are gels of 8% and 6.5%. Lower left is a 5% gel. Lower right shows a 6.5% non-denaturing gel of standard proteins stained with Coomassie blue.


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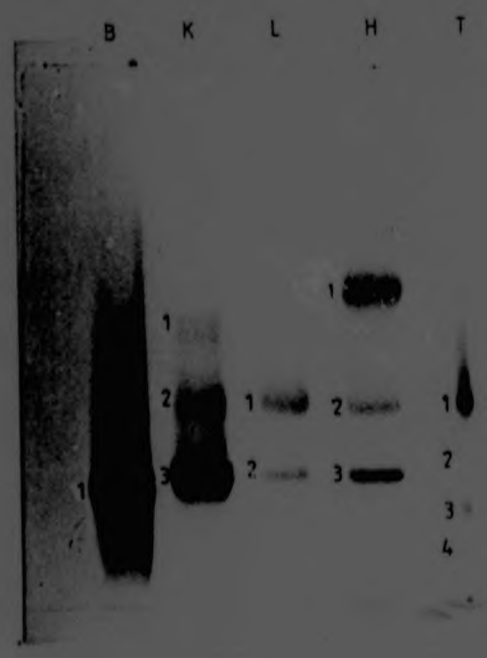
B K L H T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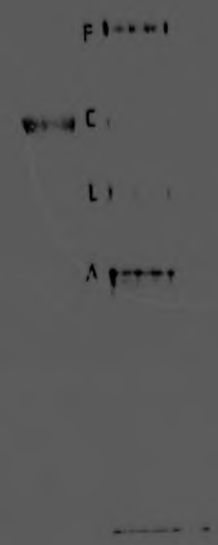

F
C
L
A

Attempted purification of murine mammary tumour soluble cPDE enzymes.

This was done according to the purification scheme as outlined in materials and methods. A typical representaion of the cPDE activities during purification stages is represented in Table 2:13.

Table 2:13. A typical representation of the partial purification steps of soluble cPDE in murine mammary tumour as outlined in the methods of Chapter Two. All cytosols or fractions were adjusted to a protein concentration of approximately 2 mg/ml for the cPDE activity determinations (only cAMP tested). Units for specific activity (S.A.) were in nmol/mg protein/ min for low affinity measurements and in pmol/mg protein/min for high affinity measurements

Fraction	Total mg	Low affinity S.A.	Low affinity Total	High affinity S.A.	High affinity Total
A	359	2.35	343.65	69.40	24,915
B	144	0.17	10.4	4.25	254
C	198	1.79	357.35	97.76	19,356
E	195	1.32	257.4	68.20	13,299
F	68	0.83	56.8	52.10	3,543
G	91	-	-	59.60	5,424

There was a large loss of cPDE activity (53% at 1 uM cAMP concentration) in the dialysis and concentration stage (E) of the pooled DEAE fractions. The final recovery for cPDE after elution from the chromatofocusing column followed by titration and pooling of cPDE fractions was 22% at 1 uM cAMP concentration. There was total loss of activity at 100 uM cAMP at the G stage.

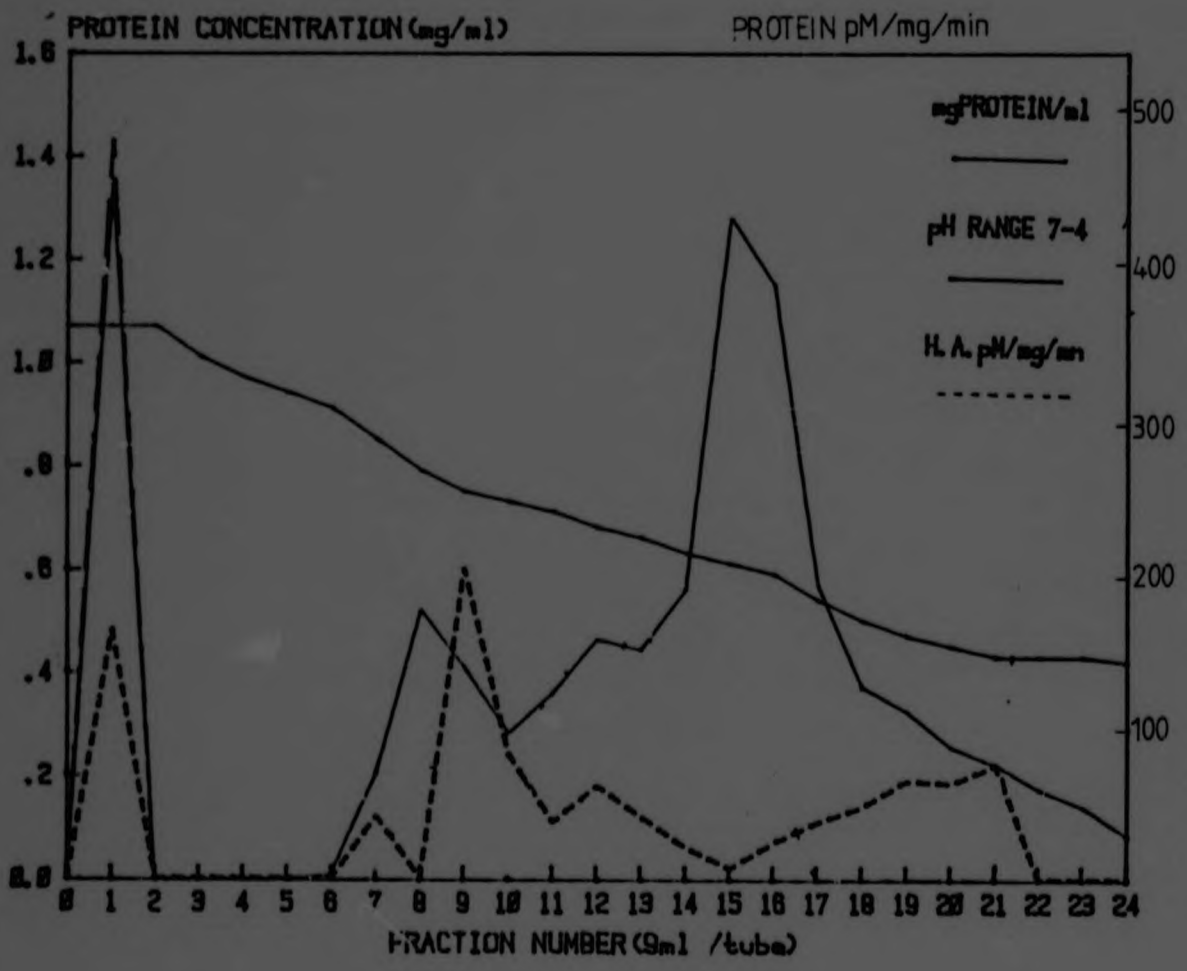
It was also suspected that the enzyme activity was being affected by the low pH of the Polybuffer 74-HCl (pH range 4 to 7). An experiment was performed whereby different protein concentrations of crude cytosol of murine mammary tumour were placed in Polybuffer 74-HCl for 2 hours at 4° C (pH=4). The cPDE activity was tested and after titration results are shown below in Table 2:14.

Table 2:14. Test of cPDE activity after incubation in Polybuffer 74.

Fraction	Protein conc. mg/ml	100 uM cAMP nmol/mg/min	1 uM cAMP pmol/min/mg
cytosol with buffer	7.34	1.3	29
	5.02	0.8	34
	1.39	0.41	13
	1. 5	0.00	7
	0.97	0.00	7.5
control without buffer	5.02	1.6	35
	1.15	1.85	62

The results showed that at lower protein concentrations the cPDE enzyme activities at both 1 uM cAMP and 100 uM cAMP were severely affected. The purification experiments were adjusted such that, after measurement of the pH, the fractions eluted through the chromatofocusing column were immediately titrated to a pH of 7.5 with 1 M imidazole. In addition, total protein concentrations of greater than 100 mg were loaded on the chromatofocusing column. The fractions eluted from the chromatofocusing column gave three main peaks for cPDE at 1 uM cAMP concentrations (Fig. 2:9). The specific activity ranges for 1 uM cAMP were 140-170, 180-210 and 52-70 pmol cAMP hydrolyzed/min/mg protein. The pI values of the three peaks were respectively pH>7, pH 5.2-5.6 and pH 4.1-4.2 (Fig. 2:9).

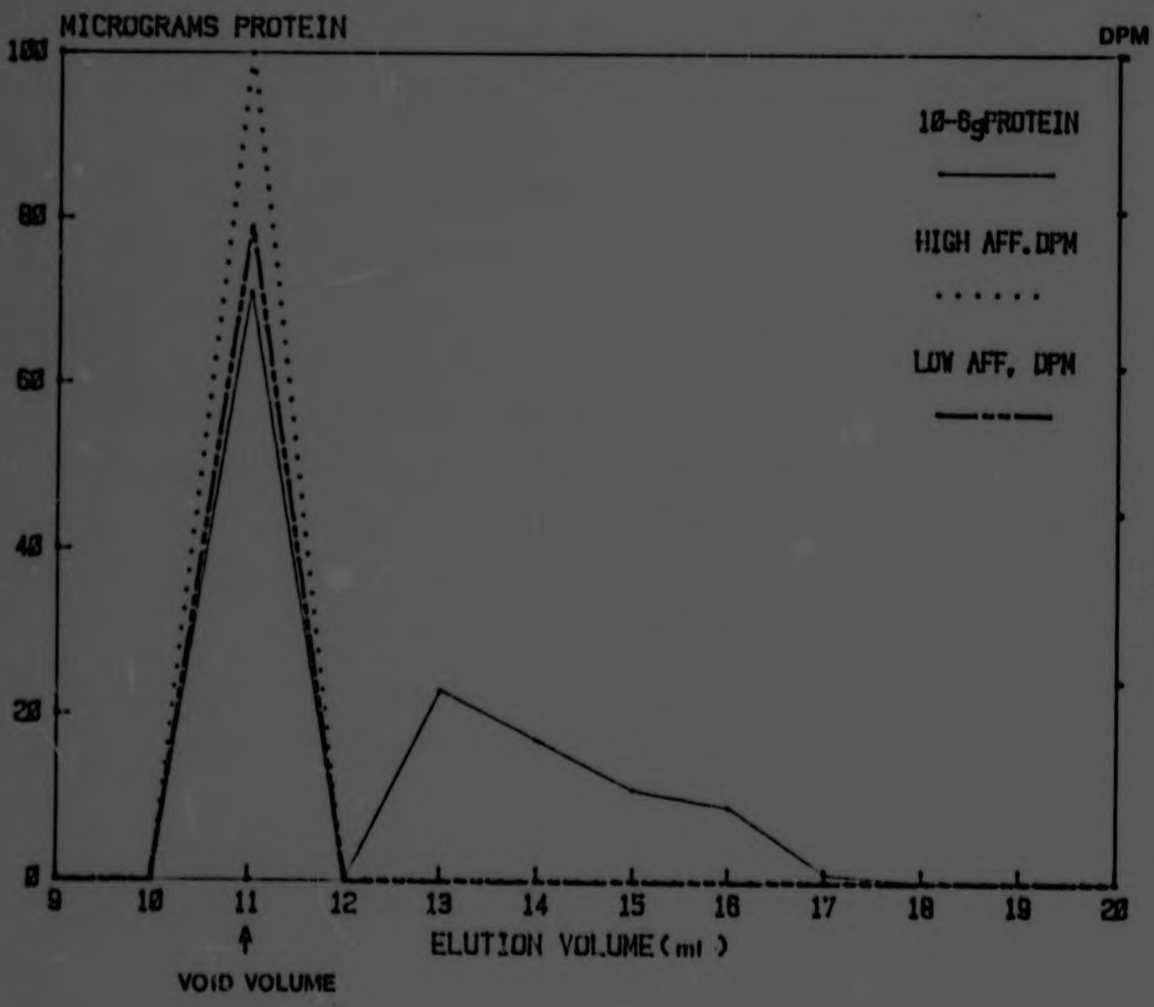
Fig 2:9. Separation of high affinity cPDE activity by chromatofocusing of cytosol of mouse tumour. The cPDE activity (on 1 cAMP tested) was expressed as pmol cAMP hydrolyzed/mg protein/min and the PI ranges are numbered 1 to 3.



Molecular mass determination by gel filtration.

The (G) fraction (see Table 2:13) was pooled and dialyzed overnight according to the technique described in materials and methods to give the (H) fraction. The gel filtration medium was Sephacryl S-200 (molecular mass range 5000-250000 daltons) and the molecular mass standards used were ovalbumin 43000, albumin 67000, aldolase 158000 and catalase 232000. The cPDE activity all eluted just at or after the void volume which indicated that the molecular mass of the partially purified cPDE enzymes of murine mammary tumour were greater than 200000.

Fig. 2:10. Profile of the molecular mass of cPDE in the H fraction when run on Sephacryl S-200. The cPDE activity (only cAMP tested) was expressed as dpm of cAMP hydrolyzed/min.



Discussion

Since all the murine tumours were shown to be oestrogen and progesterone-independent and there was insufficient normal mammary tissue, comparisons of cPDE activity between either tumour tissue versus normal counterpart or hormone-dependent tissue versus hormone-independent tumour tissue, was not possible. Nevertheless, some interesting comparisons were made between certain tissues of the syngeneic murine model namely, brain, kidney, liver, heart and mammary tumour. Also, by means of the adapted non-denaturing polyacrylamide-slab-gel electrophoresis technique coupled to the specific cPDE enzyme activity stain, it was possible to characterize the different tissue soluble cPDE forms simultaneously. This is an aspect which has not previously been reported to my knowledge.

In the soluble murine tissue fractions, a total of six migrating cPDE forms were detected with characteristic Rf ranges (see Table 2:4). One of the observed cPDE activity forms was common to all the tissues with the Rf range of 0.29-0.35 (Table 2:4) whereas some of the other cPDE forms were either tissue-specific (the murine mammary tumour bands 5 and 6 with respective Rf ranges of 0.34-0.36 and 0.37-0.41) or common to some but not all of the tissues. For example, the cPDE band with Rf range 0.24-0.28 was present in all tissues except the murine mammary tumour cPDE activity profile. All the soluble forms of cPDE activity that were detected in the tissues, using the gel method, hydrolyzed both cAMP and cGMP. The band

patterns were identical when either substrate was used and under the experimental conditions used, the band intensities appeared to be similar for either substrate (Fig. 2:4a). Although, my results do not disprove the theory that the cPDE forms I observed, which consistently hydrolyzed both cAMP and cGMP, are discrete enzymes the very similar cPDE profiles with either substrate concurs better with the theory that there are two separate catalytic sites on the same enzyme (Wells *et al.* 1975; Sullivan *et al.* 1987).

The experiment, based on the test for cPDE activity in the eluted enzyme bands from the non-denaturing polyacrylamide gels (Table 2:10) showed that, despite the poor recovery of soluble enzyme activity, the relative activities of the soluble cPDE enzymes in the five murine tissues did not vary. That is, by far the highest activity was found in brain followed by kidney then liver and the remaining tissues, heart and mammary tumour were similar in levels of cytosolic cPDE activities. Of note, though, band 2 in kidney (Rf range 0.24-0.28 see Fig. 2:4b) when eluted from polyacrylamide gels and tested for cPDE activity (Table 2:10) showed a higher activity for cPDE at either 100 uM cAMP or 100 uM cGMP concentrations (low affinity) compared to band 3 (Rf range 0.29-0.35). From observing the stained gel (Fig. 2:4b) the opposite result would have been expected since band 3 of kidney exhibited denser staining. The unexpected finding might be attributed to either one or both of the following: 1. The enzyme in band 3 of kidney was more labile than band 2 and thus incurred a greater loss of cPDE

activity in the overnight extraction step.

2. The lead ions (1.5 mM) present in the incubation mixture might have differentially inhibited the bands such that kidney band 2 was more inhibited than band 3 giving rise to stained gels depicting denser staining for band three (Fig. 2:4b).

Also, noteworthy, was the observation that the loss of cPDE enzyme activity after the overnight elution step appeared to be greater for the hydrolysis of cAMP at 1 uM concentration compared to that for cGMP (1 uM) (Table 2:10). This differential loss of activity for the hydrolysis of cAMP compared to that for cGMP, following a 16 hour elution step at 4° C, implies an important *caveat* in assessing the cPDE kinetic parameters reported for either substrate when experimental methods involve long periods of exposure of cPDE at low protein concentrations during the processing steps. For example, following a rather lengthy purification procedure using bovine brain a type I cPDE high affinity enzyme (Km of 2 uM) has been characterized which preferentially hydrolyzes cGMP (Shenolikar et al. 1985). This cGMP hydrolyzing type I form has not been described by any other workers despite the fact that they have studied the same tissue (see Introduction section on type I cPDE enzymes).

The soluble cPDE forms in the murine tissues were also tested for susceptibility to inhibition to four inhibitors namely: isobutylmethylxanthine (MIX 100 uM) and theophylline (5 mM) which are non-specific inhibitors and trifluoroperazine (1 mM) and calmidazolium (5 uM) which are reversible inhibitors

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