

THE HORMONAL CONTROL OF cAMP SYNTHESIS IN NORMAL,
MALIGNANT AND HYBRID CELL LINES

by

Anne Marie Winskill

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*This thesis is dedicated to my husband and
my parents for their steadfast support and
encouragement.*

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other University or Institute of learning.

The author graduated from the University of Leeds with an Upper Second Class Honours degree in Biochemistry in 1980. The work presented in this thesis has been undertaken at the University of Manchester under the supervision of Dr. S.R. Ayad.

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ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CHO	Chinese hamster ovary cells
cpm	Counts per minute
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
FSH	Follicle stimulating hormone
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
Gpp (NH)p	Guanylyl imido diphosphate
GTP	Guanosine triphosphate
GTPase	GTP phosphohydrolase
GTP γ S	Guanosine 5'-O-(3-thiotriphosphate)
IBMX	Isobutyl methylxanthine
ID ₅₀	Concentration required to produce 50% inhibition
LH	Luteinizing hormone
NAD ⁺	Oxidised nicotinamide adenine dinucleotide
N _i	Inhibitory guanine nucleotide regulatory protein
N _s	Stimulatory guanine nucleotide regulatory protein
PGE ₁	Prostaglandin E ₁
RNA	Ribonucleic Acid
RSV	Rous Sarcoma Virus
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethyl-ethylene-diamine
Tris	tris(hydroxymethyl) aminomethane

ABSTRACT

Three cell lines were used in this study; a normal Chinese hamster fibroblast, CH23, a malignant mouse lymphoma, P388, and a hybrid of these two cell lines, PCM3. Intact PCM3 have a greatly enhanced response to PGE_1 than either of the parental cell lines. CH23 are more responsive than P388. The adenylate cyclase activity of broken cells was reduced in PCM3 and CH23, whereas that of P388 was increased.

The low response of intact P388 to hormone can be partially explained by low intracellular ATP levels. Exogenous ATP (0-3mM) produced enhanced cAMP accumulation. Lowering of intracellular ATP levels (2mM iodoacetate, 2mM KCN) followed by addition of extracellular ATP resulted in uptake by P388. Other nucleotides were without effect. Exogenous GTP (10^{-5} - 10^{-3} M) enhanced PGE_1 (2.5 μ M)-stimulated cAMP production. Gpp(NH)p acted similarly but GMP, GDP and guanosine were without effect.

Indomethacin pretreatment of intact cells reduced PGE_1 -stimulated cAMP accumulation to basal levels at 0.3mM for PCM3 and 1mM for P388. For CH23, 0-0.2mM indomethacin produced enhanced PGE_1 -stimulated cAMP accumulation. Synthesis of endogenous prostaglandins may render CH23 less sensitive to exogenous PGE_1 .

Intact cells treated with cholera toxin (0.1ng/ml) had enhanced cAMP production in the presence and absence of PGE_1 . The enhancement was greatest for CH23, where cAMP production approached that of PCM3. The hybrid appears to have inherited its regulatory component of adenylate cyclase from CH23. Direct measurements of the GTPase activity in plasma membranes showed CH23 activity similar to that of P388 but lower than that of PCM3. For all cell lines cytosolic factor(s) were found to be necessary for inhibition of GTPase activity of plasma membranes by cholera toxin (0.1 μ g/ml).

Colchicine and nocodazole (10^{-12} - 10^{-6} M) produced elevated levels of PGE_1 -stimulated cAMP accumulation, but not of basal levels, of all three cell lines. The effect was greatest with P388, illustrating the constraint on adenylate cyclase activity in malignant cells. No enhancement was seen in broken cells, and no effect was observed on the PGE_1 dose-response curve. Therefore action appears to be distal to the hormone receptor but not directly on adenylate cyclase.

Trypsin and chymotrypsin (0-0.05%) produced latent activation of adenylate cyclase activity of all cell lines, both basal and PGE_1 -stimulated. Little enhancement occurred in intact cells with either protease and trypsin (0-500 μ g/ml) resulted in decreased adenylate cyclase activity of homogenates. The mechanism of the latent activation is unclear.

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- I. Materials
- II. Future work.

C H A P T E R I

INTRODUCTION

I. GENERAL INTRODUCTION

Cyclic adenosine 3',5'-monophosphate (cAMP) was first isolated by Sutherland and Rall in 1958 while studying the effect of hormones, e.g. epinephrine and glucagon, on glycogenolysis in liver in the presence of Mg^{2+} and adenosine triphosphate (ATP). The compound isolated was analysed by hydrolysis and found to be identical to a product isolated previously (Cook *et al.*, 1957). An identical ribonucleotide was produced in particulate fractions of heart, skeletal muscle and brain.

Since these early days cAMP has been found in every tissue from which it was sought from bacteria, plants and mammals. Its ubiquitous nature suggests an involvement in a variety of biological functions. Sutherland and co-workers first described in detail the enzymatic conversion of ATP to cAMP in 1962 (Sutherland *et al.*, 1962; Rall and Sutherland, 1962). It was shown that the enzyme was particulate bound, required ATP and Mg^{2+} for its activity and was present in tissues from at least four phyla. In every case tested, the enzyme, named adeny cyclase, was stimulated in a tissue-specific manner by a variety of hormones. This led to the initial suggestion that the action of many hormones might be mediated by cAMP as a result of stimulation of adeny cyclase (now more correctly named adeny late cyclase) activity. This idea has since been confirmed and the subject reviewed extensively and has further led to the concept of cAMP being a second messenger for hormone action via adeny late cyclase (Sutherland and Robison, 1966).

II. METHODS USED TO STUDY THE REGULATION OF cAMP SYNTHESIS

Although *in vivo* studies are the ideal system it is often difficult to interpret the results obtained for a specific case because of the vast quantity of genetic material within the cell. The enormous complexity of regulatory mechanisms as well as their interactions also make interpretation difficult. Suitable *in vitro* systems produced for the study of the regulation of cAMP synthesis include tissue culture, somatic cell hybridisation and cells containing mutations of the cAMP system.

(a) Tissue Culture

Tissue culture is an *in vitro* system which has been developed using individual cells in culture. These cells are isolated from fragments of tissue and supplanted into medium containing the necessary nutrients for maintenance of the tissue. This leads to the formation of a primary cell line which is only capable of surviving a finite number of passages. For some cells a spontaneous change occurs which results in a culture that is capable of surviving an indefinite number of passages. Such a culture is known as an established cell line. The choice of cells that will form an established cell line is limited since the cell must be capable of growing and adhering to a surface such as glass or plastic. In addition the cell must be able to survive the subculturing techniques involving trypsin or ethylenediamine tetracetic acid (EDTA) treatment and dilution into fresh medium. These techniques are described well by Paul (1975).

The most common cell types in culture include fibroblasts, epithelial-like cells, macrophages and lymphocytes. More recently adipocytes and some nerve cells have been cultured although nerve cells are particularly difficult to grow in culture as they naturally have a very slow growth rate. Fibroblastic cells are probably the most popular in culture and are of mesenchymal origin. They tend to be spindle-shaped with cytoplasmic processes. Epithelial-like cells tend to be more polygonal and align as a cell sheet.

The use of cell culture allows direct comparison between transformed and non-transformed cells as they can be grown under the same conditions. Viral transformation is a useful tool for the comparison of transformed and normal cells (Dulbecco, 1969). Most usually it is the RNA tumour promoting viruses e.g. Rous Sarcoma virus (Roth *et al.*, 1982) and the DNA promoting polyoma virus and Simian Virus 40. Carcinogens and tumour promoting agents e.g. phorbol ester, 12-O-tetradecanolphorbol-13-acetate are also used.

(b) Somatic Cell Hybridisation

This technique also has particular application for elucidation of the properties of transformed and non-transformed cells. Cell fusion between cells from different species is performed and stable hybrids are isolated subsequently. The cells are most frequently fused using inactivated Sendai virus (Harris and Watkins, 1965),

but a number of other viruses can bring about cell fusion (Poste, 1972). Recently it has been shown that there is a critical ratio of glycoproteins of Sendai virus required for fusion (Nakanishi *et al.*, 1982). Since the fusion process is a property of the viral envelope, this necessitates the inactivation of the virus prior to use, usually by ultra-violet irradiation. Cell hybrids are also produced by polyethylene glycol treatment (Pontecorvo, 1975). Prior to 1974, the hybrids were isolated from parent cells by selective resistance but an alternative method was described for hybrid selection which did not rely on selective pressure (Ayad and Delinassios, 1974). More recently a technique involving two-colour flow sorting has been utilised (Junker and Pederson, 1981). Hybrid cells formed by fusion of malignant and non-malignant cells enable a study of gene control in malignancy. The chromosomes present in the hybrid depend on the degree of hypermethylation of the DNA (Drahovsky *et al.*, 1981). In this laboratory a hybrid PCM3 was formed by fusion between a normal Chinese Hamster fibroblast, CH23 and a malignant mouse lymphoma, P388. This hybrid does not appear malignant and this is consistent with the suggestion that in hybridization between normal and malignant cells the hybrids so produced contain suppression of malignancy (Willecke and Schafer, 1982) although others have found that the hybrid produced is malignant (Ozer *et al.*, 1977).

(c) Mutations in cAMP Synthesis

(i) S49 lymphoma cells

This is a permanent cell line derived from a Balb/c mouse T cell lymphoma. These cells have a unique property in that cAMP is cytotoxic. Thus mutants of cAMP synthesis in these cells can be isolated by selection of resistant clones, followed by cloning. Several different mutants have been isolated from these cells. Below are listed the four most common ones used to elucidate the regulation of cAMP synthesis. These mutants have led to a further understanding of the mode of action of adenylate cyclase.

cyc⁻

Adenylate cyclase in membranes from these cells fails to respond to five effectors that stimulate adenylate cyclase activity in the parental (wild-type) S49 cell line; β -adrenergic agonists, prostaglandin E_1 (PGE_1), cholera toxin, guanine nucleotides e.g. Gpp(NH)p and $GTP\gamma S$, and F^- . Cyc^- membranes have a normal number of β -adrenergic receptors and contain Mn^{2+} -stimulatable adenylate cyclase activity similar to that found in wild type membranes (Ross *et al.*, 1978; Naya-Vigne *et al.*, 1978). It has been suggested that the cyc^- mutant is lacking the guanine nucleotide regulatory protein (Farfel *et al.*, 1981).

unc

This mutant isolated by Haga *et al.* (1977) has hormone receptors which are uncoupled from the guanine nucleotide regulatory protein and adenylate cyclase. Unc membranes exhibit a normal (wild-type) number of β -adrenergic receptors and an adenylate cyclase activity that can be stimulated by cholera toxin, guanine nucleotides and F^- . However β -adrenergic agonists and PGE_1 produce very little stimulation of cAMP synthesis in intact cells or membranes. It has been suggested that the unc lesion is intrinsic to the guanine nucleotide regulatory protein (Farfel *et al.*, 1981).

 β_d

Adenylate cyclase activity in β_d membranes responds normally to stimulation by PGE_1 , cholera toxin, guanine nucleotides and F^- . However, β_d respond little if at all to β -adrenergic agents. β_d membranes exhibit an 80-90% decrease in the number of β -adrenergic receptors. (Farfel *et al.*, 1981).

H21a

This mutant fails to synthesise cAMP in response to hormones, cholera toxin, guanine nucleotides and F^- (Bourne *et al.*, 1981). This mutant appears to have a guanine nucleotide regulatory component that is incapable of interaction with the catalytic component of adenylate cyclase (Bourne *et al.*, 1982).

Recently it has been observed that the mutations, *cyc⁻*, *unc*, *H21a* arise by different mutations of a common gene (Bourne *et al.*, 1982).

(ii) Temperature-sensitive mutants

A mutant line of 3T3 having temperature sensitive cAMP metabolism exists (Willingham *et al.*, 1973). Although the mutant is identical in behaviour to the parent cell at constant temperature, a decrease or increase in temperature produces a fall in intracellular cAMP levels within seconds. This decrease in cAMP concentration is accompanied by a change in cellular characteristics i.e. the cells lose adherence and retract cell processes. This mutant allows the study of the importance of cAMP in cellular recognition.

III. CYCLIC NUCLEOTIDES AND CELL GROWTH

(a) General Aspects on Growth and morphology

(i) Growth

The first indications that cyclic nucleotides might play a role in regulating cellular growth came from the work of Burk (1968) and Ryan and Heidrick (1968). In the former case phosphodiesterase inhibitors were used to elevate intracellular cAMP levels whereas Ryan and Heidrick used exogenous cAMP. For both sets of experiments the rate of cellular growth was found to be slowed. Agents that raise the intracellular concentration of cAMP, e.g. PGE_1 , resulted in reduced cell growth (Johnson and Pastan, 1971). Analogues

of cAMP that mimic its action e.g. dibutyryl cAMP were also found to decrease cell growth (Pastan and Johnson, 1974).

Since these early days there have been numerous reports of the lower levels of cAMP in rapidly proliferating cells and higher levels in quiescent cells and several reviews have been published on the subject (Pastan *et al.*, 1975; Friedman, 1976). However, more recently the situation has become controversial. Some workers still find that an increase in intracellular cAMP levels produces a decreased growth rate. This has been demonstrated for various cell types including hepatoma (Van Meeteran *et al.*, 1982; Hargrove and Granner, 1982), adrenal chromaffin cells (Unsicker and Ziegler, 1982), carcinoma (Tisdale, 1982), pituitary cells (Martin and Ronning, 1981) and a variety of cultured cell lines (Martin and Kowalchuk, 1981). In contrast it has also been demonstrated that cAMP can act as a mitogenic signal for Swiss 3T3 cells (Rozengurt *et al.*, 1981) and epidermal keratinocytes but not dermal fibroblasts (Kuroki *et al.*, 1982). Dibutyryl cAMP stimulates the biosynthesis of DNA and RNA in carcinoma cells (Simkhovich and Selina, 1980) but decreases the proliferative activity in human aorta (Tertov *et al.*, 1982). Furthermore, even workers who have demonstrated that cAMP decreases cellular growth suggest that this may not necessarily be due to increased intracellular cAMP levels but to the formation of toxic metabolites (Hargrove and Granner, 1982) or inhibition of methylation by 5-adenosyl-L-homocysteine (Tisdale, 1982). Van Meeteran *et al.* (1982) have suggested that the short-term effect on proliferation by cAMP is due to the nucleotide itself but that the long-term effect is due to degradation

products of the nucleotide. Thus, although the bulk of the evidence indicates a role for cAMP in the control of cell growth, there is some contradictory evidence that cannot be ignored. Interpretation of dibutyryl cAMP results are difficult because of the interfering effects of butyrate.

cAMP has also been implicated in the phenomenon known as density dependent inhibition of growth. The process was previously termed contact inhibition of growth but not all of the changes are a result of cell contact (Stoker, 1967). This phenomenon refers to the restriction of the growth of normal cells at confluency. This property has been attributed to all cells studied except for chick embryo fibroblasts (Pastan *et al.*, 1975; Strada and Pledger, 1975; Andersen *et al.*, 1973) and occasionally for 3T3 fibroblasts (Oey *et al.*, 1974; Burstin *et al.*, 1974). Density-dependent inhibition of growth is associated with increased levels of cAMP compared to levels in logarithmic growth (Otten *et al.*, 1971; Anderson *et al.*, 1973; Pastan *et al.*, 1975; Strada and Pledger, 1975; Rechler *et al.*, 1977 and Ahn *et al.*, 1978). However, Haslam and Goldstein (1974) found no increase in intracellular cAMP during density dependent inhibition of growth in fibroblasts.

The evidence for cGMP having a role in cell growth regulation is not as well defined as that for cAMP. This could be due to the greater difficulty in measuring cellular cGMP levels. Prior to 1975 it was proposed that cGMP opposed the effects of cAMP in that increased levels of cGMP stimulated cellular growth (Hadden *et al.*, 1972; Siefert

and Rudland, 1974). This led to the Ying-Yang hypothesis of cell growth (Hogan and Shields, 1973) and several reports support this idea of low levels of cAMP, high levels of cGMP stimulating cell growth and high cAMP, low cGMP inhibiting growth (Seifert and Paul, 1972; Seifert and Rudland, 1974). This idea is best illustrated using insulin, a mitogenic agent, which was shown to increase levels of cGMP and decrease levels of cAMP (Iliano *et al.*, 1973). More recent work has also produced controversial results concerning the involvement of cGMP in cellular growth. Measurement of cGMP, guanylate cyclase and cGMP phosphodiesterase levels in the number of cell lines have shown little correlation between cell growth rate and the levels of cGMP in the cell (Pardee *et al.*, 1978). Boynton *et al.* (1978) have found that stimulation of growth is independent of fluctuations in cGMP or cAMP levels, while other workers still report that cGMP promotes cell growth (Miller *et al.*, 1975; Seifert, 1976). It appears likely that cGMP and cAMP have a function in growth control but the extent and the mechanism is unknown.

(ii) Morphology

Cyclic nucleotides are also thought to be involved in the control of cellular morphology. Most of the earliest work considering the effects of cAMP on morphology used the analogue of cAMP, dibutyryl cAMP (Hsie and Puck, 1971; Johnson *et al.*, 1971). Hsie and Puck observed that treatment of CHO cells *in vitro* with dibutyryl cAMP converts the culture from one of compact, randomly orientated cells that grow in multilayers to a monolayer of elongated

fibroblast-like cells growing parallel to one another. Morphological changes were inhibited by colchicine suggesting the involvement of the cytoskeleton in morphological changes (Porter *et al.*, 1974; Willingham and Pastan, 1975). Much of the work implicating cyclic nucleotides in the control of cellular morphology has been on transformed cells. However, some effects have been observed on normal cells. Tardy *et al.* (1981) have studied the effects of prostaglandins and dibutyryl cAMP on morphological changes in astrocytes. The changes produced by the two compounds were very similar. Davison and Karasek (1981) observed that endothelial cells grown in the absence of agents elevating the intracellular concentration of cAMP grew in a disorganized array, whereas cells grown in the presence of exogenous cAMP retained endothelial morphology. Spruill *et al.* (1981) have followed the temporal sequence of cell shape changes in Sertoli cells following experimental elevation of cAMP concentration. A variable number of cells were converted from flat epithelial-like morphology into the satellite morphology following exposure. However, no morphological change was observed in human fibroblasts (Dubpernell and Gavurin, 1978). Thus, it appears that cAMP has some involvement in cellular morphology but the extent appears to be variable.

(b) Malignant cells; General Properties and involvement of cyclic nucleotides

Transformed cells have properties distinguishing them from their normal counterparts (Todaro and Huebner, 1972). In general their intracellular levels of cAMP are reduced

(Sheppard, 1972; Shima *et al.*, 1976; Tisdale and Phillips, 1976). This leads to accelerated growth rates and higher cell densities since density-dependent inhibition of growth is lost. They have abnormal morphological characteristics in that normal cells tend to be elongated whereas transformed cells are spherical. The ability to adhere to substrata is completely or partially lost, and consequently malignant cells are found growing in suspension rather than as monolayers (Hsie and Puck, 1971; Ryan and Heidrick, 1974; Strada and Pledger, 1975; Friedman, 1976). This change in morphology is best demonstrated by the temperature sensitive mutant of 3T3 (Willingham *et al.*, 1973). At constant temperature the cell has a normal phenotype and grows as a monolayer exhibiting density-dependent inhibition of growth. A change in temperature produces a transformed phenotype and the mutant shows reduced intracellular cAMP levels followed by a loss of adherence and retraction of cellular processes. It is not known whether the alterations in properties of action of cyclic nucleotides are a primary or a secondary lesion in the development of malignancy.

At least some of the changes in the properties of transformed cells are considered to be due to the decreased levels of intracellular cAMP and the loss of density-dependent inhibition of growth. This has been shown by raising the intracellular cAMP concentrations of malignant cells experimentally either directly by the addition of cAMP analogues or indirectly by addition of agents that elevate cAMP levels e.g. hormones and phosphodiesterase inhibitors. Such treatment was found to restore normal properties to transformed cells. These include; slower growth rates

(Barker and Isles, 1977; Coffino and Gray, 1978; Hargrove and Granner, 1982; Cho-Chung *et al.*, 1983) and apparent or partial restoration of density-dependent inhibition of growth to some workers (Sheppard, 1971; Otten *et al.*, 1972; Smets, 1972) but not for others (Johnson and Pastan, 1971). Recently (Roth *et al.*, 1982) have shown that RSV-transformed cells are resistant to exogenous cAMP in altering cell shape or growth. Other workers have demonstrated that changes in morphology are not accompanied by changes in the concentration of cAMP (Malluci and Wells, 1976). It has also been suggested that a defect in the microfibrillar/microtubule system is the basic lesion responsible for the changes in morphology rather than alterations in cAMP levels (Puck, 1980). Thus, it appears from the majority of the findings that cAMP participates in the properties of malignant cells but the extent of this involvement is unclear.

It is believed that the low levels of cAMP in cancer cells, allowing rapid growth, are a result of alterations in cAMP metabolism. Various groups of workers have reported changes in either adenylate cyclase activity, phosphodiesterase or protein kinase activity. However, the variations observed depend on the laboratory and the cell type. Although some workers have found an increase in phosphodiesterase levels and a decrease in adenylate cyclase activity (Braun and Shigawa, 1973; Ayad and Foster, 1974; Strada and Pledger, 1975; Friedman, 1976), others find little or no difference in adenylate cyclase activity (Hickie *et al.*, 1975) or phosphodiesterase levels (Lincoln and Vaughan, 1975). Gidwitz *et al.* (1976) have suggested

that the decreased levels of adenylate cyclase activity in transformed cells are due to alterations in the microenvironment of adenylate cyclase. It has also been proposed that it is the effective level of the nucleotide and not the actual level that is important in the determination of malignancy (Goldberg *et al.*, 1975) and no direct correlation has been identified between cyclic nucleotide content and tumour growth rate (Peytremann and Engl, 1973; Hickie *et al.*, 1977).

It is generally accepted that cGMP levels are usually higher in malignant cells (Hickie *et al.*, 1977; Carpentieri *et al.*, 1980) but less work has been performed in this field and it has been found that tumours with high cGMP levels were particularly fast-growing (Goldberg *et al.*, 1975).

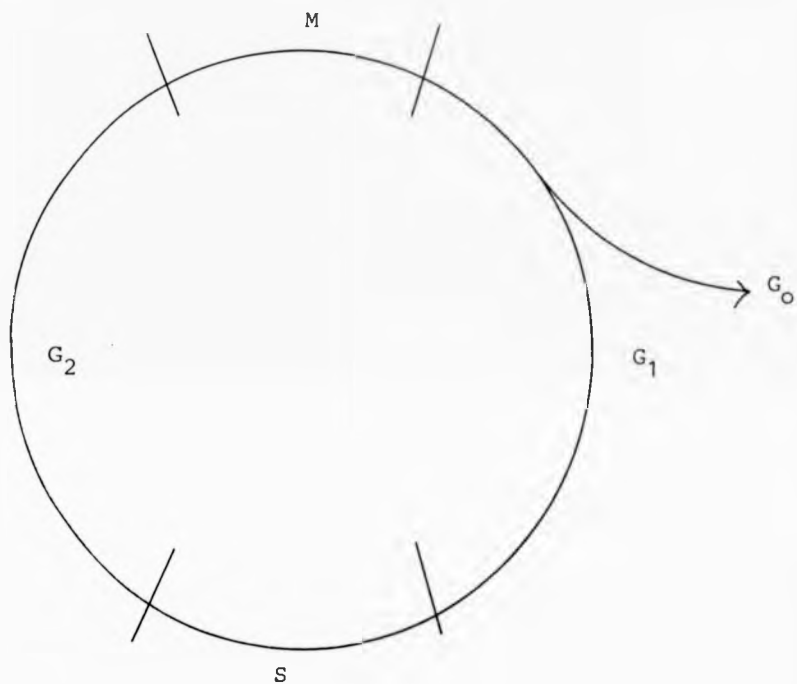
Cho-Chung (1980) has presented an hypothesis to elucidate the action of cAMP in the regulation of tumour growth via a cAMP receptor protein on the cell surface. The binding of cAMP to its receptor protein and translocation to the nucleus are thought to be responsible for tumour regression. This mechanism was originally suggested by Jungmann and co-workers (Jungman *et al.*, 1974; Jungman *et al.*, 1975). Three types of receptor proteins have been identified in mammary tumours as having molecular weights of 39 000, 48 000 and 56 000 daltons. The 48 000 and 56 000 dalton proteins appear to be regulatory subunits of cAMP dependent protein kinase and the 39 000 dalton protein a proteolytic fragment of the 56 000 protein (Cho-Chung *et al.*, 1981). cAMP receptor proteins have also been

demonstrated in HeLa cells (Friedman and Strittholt, 1981). Indeed, a 50 000 dalton cAMP binding protein has been isolated from both HeLa cells and human tumours and identified as a regulatory subunit of cAMP dependent protein kinase II (Weber *et al.*, 1982). It would thus appear that protein kinase activity is involved in this cyclic nucleotide control of properties of malignant cells.

(c) Cyclic nucleotides and the cell cycle (see figure).

The cell cycle contains four stages of growth; M, G_1 , S and G_2 . M is the mitotic phase of the cell cycle where two daughter cells are produced. The S phase is one of DNA synthesis, and G_1 and G_2 are preparatory events for these phases. The length of time spent in each phase is a characteristic of the cell type. The quiescent state, G_0 , is a state of non-growth and can be induced by serum restriction or density-dependent inhibition of growth. This phase is not necessarily entered every round of the cell cycle. One of the major problems in a survey of the properties of the cell cycle is the wide variety of systems employed and the variety of cell synchronisation procedures. This makes direct comparison of results difficult.

The bulk of the evidence for cAMP levels playing a role in the cell cycle have come from studying cells released from quiescence, usually by readdition of serum or nutrients which promote the cells into DNA synthesis. With few exceptions, stimulation of cells in G_0 to divide is accompanied by a decrease in cAMP levels (Siefert and Rudland,



The Cell Cycle

M = mitotic phase.

S = DNA synthesis.

G₁, G₂ = preparatory phases

G₀ = Quiescent state.

1974; Matsumoto *et al.*, 1975; Anderson *et al.*, 1981) and an increase in cGMP has been indicated (Rudland *et al.*, 1974; Anderson *et al.*, 1981). The decrease in cAMP levels demonstrated following release from quiescence are further indicated since increasing the cAMP levels when cells in G_0 are reprovided with nutrients potentially inhibits entry into DNA synthesis (Franks, 1972; Kurtz *et al.*, 1974). This has also been shown more recently by Leof *et al.* (1982). Agents that elevate the intracellular concentrations of cAMP e.g. cholera toxin or IBMX, inhibited serum-stimulated BALB/C-3T3 cells in mid G_1 . Others have shown that the step essential for DNA synthesis, which occurs in late G_1 , is inhibited by high concentrations of cAMP (Rechler *et al.*, 1976). However, it has also been demonstrated that removal from quiescence produces short bursts of cAMP followed immediately by DNA synthesis (Boynton *et al.*, 1981) and this was shown to involve cAMP-dependent protein kinase activity. Anderson *et al.* (1981) have also proposed the involvement of protein kinase activity in control of the cell cycle by cAMP.

For the majority of cell types, cAMP levels are reduced during mitosis (Friedman *et al.*, 1976; Howard and Sheppard, 1981) and high levels of cAMP are found on the G_1/S border (Friedman *et al.*, 1976). Thus it is conceivable that the variations observed in cAMP levels following stimulation of quiescent cells to divide arises from the differences in lengths of G_1 or S in varying cell types.

Several groups of workers have attempted to correlate the changes in cAMP levels during the cell cycle with the activities of the enzymes of cAMP metabolism. Decaestecker and Blanquer (1981) have shown that adenylate cyclase activity was highest throughout G_1 , decreased in S and markedly dropped as the cells traversed G_2 and was very low or absent in M. These findings are consistent with the high levels of cAMP present in G_1 /S border, low levels in S and lower levels in M. Phosphodiesterase activity had an oscillating pattern throughout the cell cycle but was highest in S. Anderson *et al.* (1981) have demonstrated that cAMP-phosphodiesterase was reduced when the cells multiplied rapidly, but that early changes in cAMP and cGMP levels following stimulation to divide from quiescence (1-2 minutes) were not accompanied by alteration in cAMP or cGMP phosphodiesterase activity. Penit *et al.* (1977) have shown that adenylate cyclase responsiveness to PGE_1 throughout the cell cycle closely follows the cAMP changes. It has been proposed for C_6 glial cells (Howard and Sheppard, 1981) that the intrinsic catalytic capacity of the adenylate cyclase complex is reduced during mitosis and contributes to the mitotic cell's inability to accumulate and maintain the cAMP concentration at the interphase level. Thus it appears that adenylate cyclase activity is more important than the activity of phosphodiesterase in controlling the levels of cAMP during the cell cycle.

Although, from the evidence presented above it appears that cyclic nucleotides participate in the cell cycle several groups have suggested that this is not the case. Dell'orco *et al.* (1977) have proposed that the mitotic inhibition induced by decreasing the serum concentration of the incubation medium was not mediated by increased intracellular cAMP concentrations. It has also been shown by Coffino *et al.* (1975) that cAMP is a non-essential regulator of the cell cycle since mutants of S49 cells lacking a cAMP metabolic component can progress through the cell cycle.

PCM3, a somatic cell hybrid isolated in this laboratory, shows ~~typical~~ growth characteristics in that 80% of the cells exist as a monolayer, PCM3-M and 20% as suspension, PCM3-S. PCM3-S cells were found in the mitotic phase of the cell cycle. Increasing the levels of cAMP using PGE₁ and IBMX reduced the amount of floating cells. This provides further support for the idea that cAMP levels are low during mitosis (White and Ayad, 1980).

IV. FACTORS INFLUENCING INTRACELLULAR cAMP LEVELS

(a) General Outlook

The level of cAMP that exists inside the cell at any one time represents a fine balance between the activities of enzymes of cAMP synthesis and degradation. cAMP is produced from ATP in response to hormones and other agents acting on the adenylate cyclase molecule via receptors. It

elicits its effect on cellular events via phosphorylation. Protein kinase activity is present in the cytosol. There are two isoenzymes of cAMP dependent protein kinase, I and II (Miller, 1981). Each is composed of regulatory cAMP binding subunits and catalytic subunits. When protein kinase is activated by cAMP it catalyses the phosphorylation of cell proteins e.g. histones. The enzyme responsible for degradation of cAMP in the cell is cAMP-phosphodiesterase. It too is found in the cytosol as interconvertible forms (Ayad and Tobia, 1980) but particulate phosphodiesterases also exist (Prigent *et al.*, 1981). Insulin and glucagon have been shown to regulate a low K_m phosphodiesterase activity (Loten *et al.*, 1978). Therefore the level of cAMP intracellularly, depends upon the balance between these three enzymes and their regulation by effector molecules.

(b) Phosphodiesterase Inhibitors

As stated above, the cAMP levels present in the cell at any one time are dependent on the balance between adenylate cyclase activity, protein kinase activity and phosphodiesterase activity. Methyl xanthines are well known as inhibitors of cyclic nucleotide phosphodiesterases and among them 1-methyl 3-isobutyl xanthine (IBMX) is the most potent (Reavo *et al.*, 1970). In detailed kinetic studies IBMX has been shown to be a competitive inhibitor of the calmodulin-activated phosphodiesterase from porcine coronary arteries (Wells *et al.*, 1975). IBMX is now very commonly used in intact cells to inhibit degradation of cyclic nucleotides and raise their intracellular concentrations (Wells and Kramer, 1981). Care should be taken when

choosing the concentration of IBMX to be used since very low concentrations have been shown to stimulate cAMP-phosphodiesterase activity (Erneux *et al.*, 1982) and very high concentrations have been shown to inhibit adenylate cyclase activity (Pohl *et al.*, 1971).

(c) The Effect of Hormones

The concept that cAMP acts as a second messenger in hormone action has been held for many years (Sutherland *et al.*, 1968) and many hormones have been shown to act via elevation of cAMP levels including; catecholamines, prostaglandins, glucagon, parathyroid hormone, adrenocorticotrophic hormone (ACTH), thyrotropic hormone and follicle stimulating hormone.

It was first established that prostaglandins activate adenylate cyclase to result in an increased formation of cAMP in 1972 by Kuehl *et al.* Since then the effects of prostaglandins (E_1 , E_2 , $F_{2\alpha}$, $F_{1\alpha}$, A_1) have been studied in a variety of systems including liver (Yamashita and Sweat, 1976; Gaginello *et al.*, 1978), gastro-intestinal tract (Kimberg *et al.*, 1974; Simon *et al.*, 1978), sarcoma (Atkins and Martin, 1977; Crawford *et al.*, 1978), mouse LM cells (Engelhard *et al.*, 1978), kidney (Zenser and Davis, 1977; Schlondorff *et al.*, 1978), platelets (McDonald and Stoart, 1973; Longenecker *et al.*, 1980), lung (Powell and Soloman, 1980), fibroblasts (O'Neill and Hsie, 1975; Ayad and Burns, 1977; Ayad and Foster, 1977), brain

(Berti *et al.*, 1972; Wellman and Schwabe, 1973). Recently the effect of prostaglandins has been studied for other diverse systems e.g. parathyroid cells (Gardner *et al.*, 1981), mastocytoma (Yatsunami, 1981), neuroblastoma x glioma hybrid (Moylan and Brooker, 1981) and chick embryo myoblasts (Hausman and Velleman, 1981). For nearly all of the systems studied, PGE_1 and PGE_2 had more potent effects than the other prostaglandins in elevation of cAMP levels. The activation of adenylate cyclase activity is usually in a dose-dependent manner.

Similar results have been obtained for catecholamines in β -adrenergic responsive tissues and for other hormones acting via adenylate cyclase i.e. action of the hormones produces an elevation of cAMP levels. These elevated levels have been correlated with an increased protein kinase activity (Spaulding and Burrow, 1975; Coquil *et al.*, 1977).

It is important to note that several hormones act by decreasing cAMP levels. It was shown in 1968 by Butcher and Baird that in isolated fat cells PGE_1 produced no increase in cAMP levels and PGE_1 acted as an antagonist of epinephrine action. The effects of α -adrenergic agents are typical of those acting via the inhibition of cAMP accumulation (Jakobs *et al.*, 1981)

(d) Effect of nucleotides in intact cells

Many exogenous agents apart from hormones are known to affect the intracellular levels of cAMP. These include adenosine and some nucleotide triphosphates e.g. ATP.

Adenosine has been shown to activate adenylate cyclase activity in a variety of tissues especially, malignant cells (Blume *et al.*, 1973; Green and Stanberry, 1977), epidermis (Iizuka *et al.*, 1976; Fain and Malbon, 1979), platelets (Jakobs *et al.*, 1979), coronary tissue (Anand-Srivastava *et al.*, 1982a; Avdonin *et al.*, 1982; Collis, 1983). However, adenosine has also been shown to inhibit adenylate cyclase activity in fat cells (Londes *et al.*, 1978; Kather and Simon, 1980), cerebral cortex (Cooper *et al.*, 1980) and adrenocortex (Glynn and Cooper, 1978) and liver (Johnson, 1982). It has therefore been proposed that there are two distinct adenosine-sensitive sites on adenylate cyclase (Wolff *et al.*, 1981).

(i) One locus, designated as the 'P' site on the basis of the analogue requirements for an unaltered purine moiety, invariably inhibits adenylate cyclase. This site has been tentatively assigned to the cytoplasmic surface of the plasma membrane.

(ii) Another locus, originally designated as 'R' because of its requirement for an unmodified ribose moiety. This site has all the properties of hormone receptor-mediated responses of adenylate cyclase of membranes and therefore it is generally known as the adenosine receptor.

The receptor itself can be further subdivided into two classes, one of which is stimulatory (R_s) and the other inhibitory (R_i) towards adenylate cyclase. The 'R' site is therefore thought to exist on the exterior of the membrane.

The stimulatory effect of adenosine has been demonstrated to be antagonized competitively by methyl xanthine phosphodiesterase inhibitors (Haslam *et al.*, 1978; Cooper and Londos, 1979) and this property has been useful to distinguish R and P sites (Wolff *et al.*, 1981). The potency series is identical to that seen for other methyl xanthine sensitive reactions i.e. IBMX > theophylline > caffeine. The P site can also be distinguished from the R_i site since its expression is enhanced by divalent cations (Wolff *et al.*, 1981).

It seems likely that fat cells contain only a P site attached to the adenylate cyclase whereas biphasic responses could be explained by the presence of both loci. Adenosine receptors have been shown to be important physiologically in the regulation of coronary flow (Kukovetz *et al.*, 1978).

ATP has been found to stimulate cAMP levels particularly in transformed cells (Rozengurt *et al.*, 1977; Rozengurt and Heppel, 1979; Ayad and Hughes, 1980; DeCastro and Ayad, 1982). This effect is specific for ATP and transformed cells and is readily reversible and it has been suggested that ATP enters the cell directly across the plasma membrane to elicit this effect. It is thought that

this is due to the low substrate pool of ATP in transformed cells and that ATP enters down the concentration gradient. Recently, ATP has been shown to selectively enhance plasma membrane permeability especially to monovalent cations (Van Zoelen *et al.*, 1982) and the plasma membranes of several lines of intact tumour cells are permeable to low levels of intact ADP and ATP (Rapaport, 1983). ATP in combination with mitochondrial inhibitors have been shown to cause permeability changes in the plasma membrane of transformed but not untransformed cells (Kitigawa and Akamatsu, 1981). Crooke *et al.* (1980) have demonstrated that exogenous ATP can enhance the formation of cAMP by hepatocytes and adipocytes. This was enhanced by fluoride and hormones and led the authors to suggest that some catalytic subunits of adenylate cyclase are exposed on the outer surface of the cell membrane. However, this has not been confirmed by any other workers.

(e) The Effect of Proteases

Some workers have observed that treatment of intact cells with trypsin produces enhanced levels of cAMP (Koji and Terayama, 1980; Knopp *et al.*, 1983) and it has been observed that treatment of intact cells with trypsin followed by homogenisation produces higher levels of adenylate cyclase activity in homogenates (Ryan *et al.*, 1975; Hughes and Ayad, 1980) a phenomenon termed 'latent activation' by Hughes and Ayad. Trypsin has also been shown to activate adenylate cyclase activity in membranes produced from fibroblasts (Anderson *et al.*, 1978; Wallach

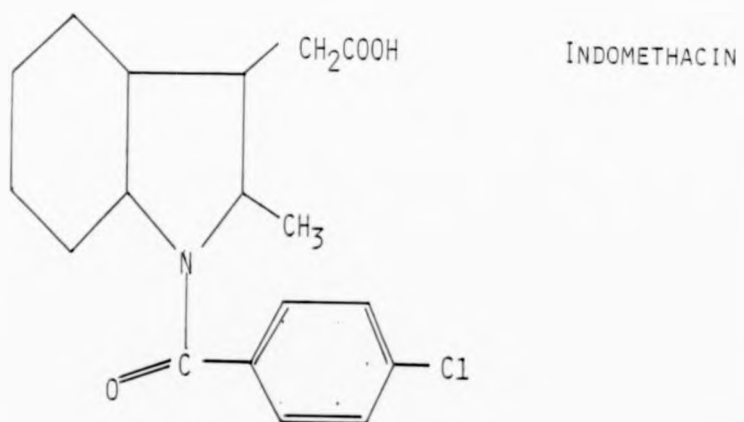
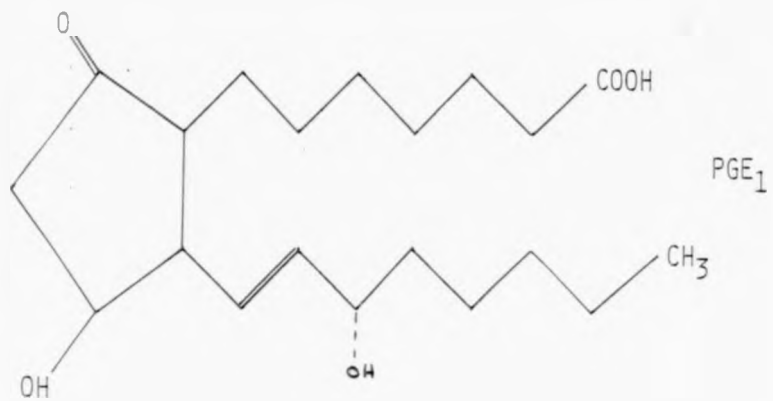
et al., 1978, Pinkett and Anderson, 1980), liver (Lacombe *et al.*, 1977), ovaries (Richert and Ryan, 1977) and thyroid (Friedman *et al.*, 1983). Other proteases e.g. chymotrypsin, elastase and papain (Lacombe *et al.*, 1977; Anderson *et al.*, 1978) have been shown to activate adenylate cyclase activity. The stimulation in this case was biphasic; activation at low concentrations and inhibition at high concentrations. The activation by proteases in some cells is more marked or uniquely observed in the presence of GTP (Yamamura *et al.*, 1977; Anderson *et al.*, 1978; Wallach *et al.*, 1978; Anderson *et al.*, 1979). Despite this finding there is some controversy about whether the activation of adenylate cyclase mediated by proteases is dependent on the presence of the guanine nucleotide regulatory protein. Stengel *et al.* (1980), using rat liver membranes and *cyc⁻* mutant of S49 lymphoma have demonstrated that proteolytic activation can occur independently of the regulatory protein, whereas Stiles and Lefkowitz (1982) have suggested that trypsin exerts its effect via an 'inhibition specific' guanine nucleotide regulatory unit and the catalytic moiety of adenylate cyclase. Other workers have suggested that trypsin exerts its effect by degradation of cell membrane proteins that modulate activity of adenylate cyclase activity (Wallach *et al.*, 1978; Pinkett and Anderson, 1980). Pinkett and Anderson have localised a 46 000 dalton membrane protein that is decreased after trypsin activation. Geynet *et al.* (1980) have shown that limited proteolysis of liver membranes results in modifications of binding characteristics of catecholamines, the receptor and regulation by guanine nucleotides but Hughes

and Ayad, (1980) have shown that the PGE_1 receptor is not affected by limited proteolysis. McIlroy and Ryan (1983) have suggested that proteolysis could activate adenylate cyclase either by direct action on the catalytic unit or by an action somewhere in the pathway of hormonal stimulation. It is unlikely that activation of adenylate cyclase by limited proteolysis is dependent on new protein synthesis because the time required is too short. Inhibition of serine proteases by Na-tosyl-L-lysine chloromethyl ketone (TLCK) at low concentrations potentiates the response of follicular cells to stimulating agents e.g. cholera toxin and FSH. This suggests that proteases are responsible for governing the responses in intact cells (La Barbera *et al.*, 1982) and this is suggested to be via a cytoplasmic origin.

V. PROSTAGLANDINS

(a) General Aspects

Van Euler first coined the term 'prostaglandin' to describe the pharmacologically active principles of seminal fluid in 1937. It is now known that prostaglandins are oxygenated derivatives of polyunsaturated fatty acids with a wide range of biological effects (Samuelsson *et al.*, 1978). The role of prostaglandins in stimulating cAMP formation has been discussed above. Prostaglandins also modulate cell growth and immune responses (Pastan *et al.*, 1975; Plescia *et al.*, 1975; Droller *et al.*, 1978a; Droller *et al.*, 1978b). Prostaglandins produced by tumour cells can inhibit cytotoxicity of lymphocytes involved in host-defence



PROSTAGLANDINS AND RELATED COMPOUNDS

PGE₁ = hormone used in studies

Arachidonic acid = parent compound

Indomethacin = inhibitor of prostaglandin synthesis

mechanisms against cancer cells (Droller *et al.*, 1978b). Cells also produce more PGE_1 when they are transformed (Hammarstrom, 1982). Prostaglandins are inhibitors of platelet aggregation, prostacyclin (PGI_2) being 30-40 times more potent than PGE_1 (Moncada and Vane, 1977), by virtue of cAMP formation (Weissman, 1982). Prostacyclin has been demonstrated to be an antimetastatic agent, inhibiting the spread of primary tumours (Honn *et al.*, 1983) and PGE_1 has shown to be capable of inhibiting tumour cell growth (Santoro *et al.*, 1976). Prostaglandins are also thought to be the molecules responsible for producing fever (Milton, 1982) in response to viral agents, bacteria etc. PGE_2 and PGE_1 are similarly active in acting as pyrogens whereas $\text{PGF}_{1\alpha}$, $\text{PGF}_{2\alpha}$, PGA_1 are almost devoid of activity. PGE_2 is one of the most potent substances known to increase deep body temperature and it is thought to act at the thermoregulatory region of the anterior hypothalamus. Prostaglandins play an important role in disease as mediators of inflammation (Weissman, 1982). Thus it appears that prostaglandins play an important role in the defence mechanism to invading organisms since they are involved in pain, inflammation and fever (Weissman, 1982; Milton, 1982).

(b) Structure of prostaglandins

Prostaglandins are cyclopentane derivatives formed from polyunsaturated fatty acids. PGE_1 , the hormone used in this laboratory is 11 α -15-dihydroxy-9-ketoprost-13-enoic acid. Prostaglandins are derived from arachidonic acid. Prostaglandins A, D₂, E₁, E₂, F_{1 α} , F_{2 α} differ in the

saturation of carbon chains and substituents of the ring structure and are formed by isomerisation and/or reduction reactions from prostaglandin endoperoxides.

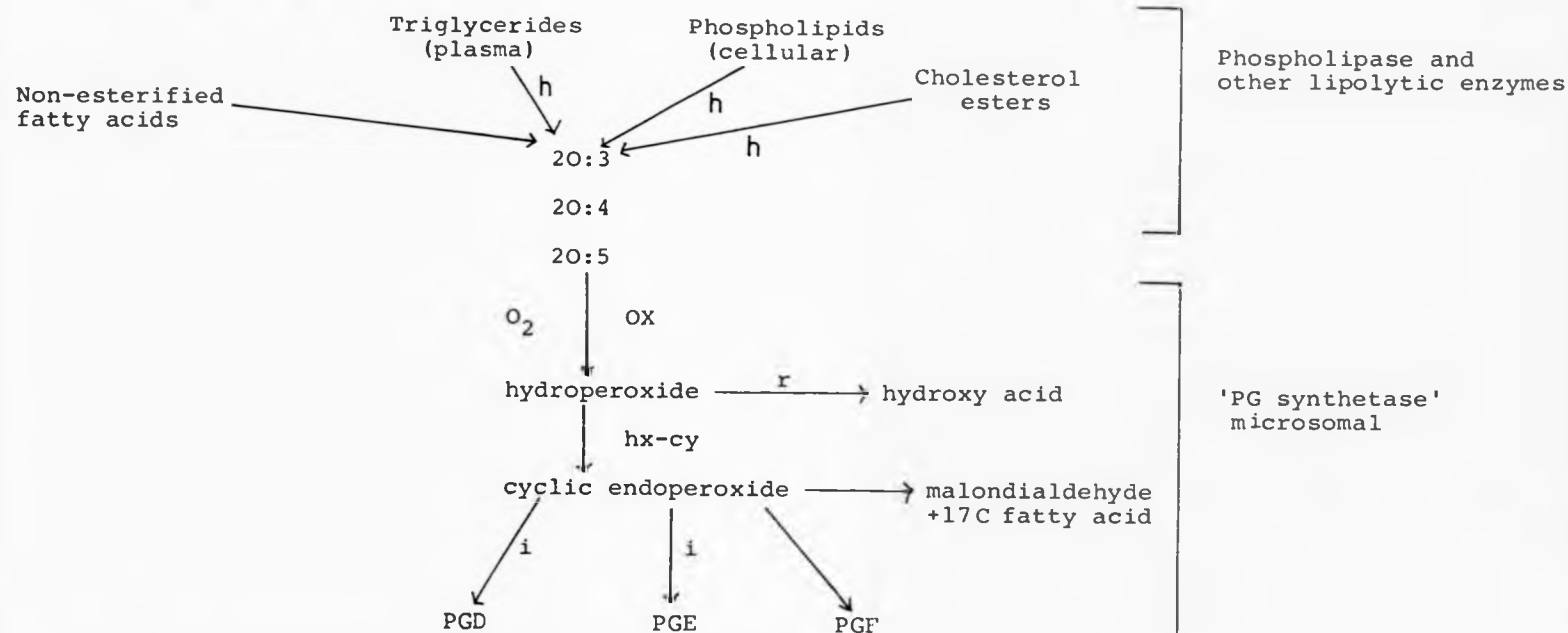
(c) Inhibitors of Prostaglandin Synthesis

In order to describe inhibitors of the synthesis process it is necessary to give a description of the synthesis. The simplest description is that of Flower and Vane (1974) (Fig. 1) although the process has been illustrated more elegantly since (Samuelsson, 1978).

Inhibitors of prostaglandin synthesis are usually anti-inflammatory and antipyretic. Nonsteroidal anti-inflammatory drugs e.g. aspirin and indomethacin inhibited the cyclooxygenase reaction (Samuelsson, 1978) via inhibition of prostaglandin synthetase. Evidence has been presented that these non-steroidal drugs react with two sites of cyclooxygenase (Humes *et al.*, 1981). Although the degree of interaction with the catalytic site determines the potency of such compounds, interaction with the supplementary site is also obligatory for efficacy as inhibitors. The ID_{50} values for inhibition of prostaglandin synthesis by indomethacin ($0.75\mu M$) and aspirin ($35\mu M$) give an idea of the relative potencies (Vane, 1971). All non-steroidal anti-inflammatory drugs possess a lipophilic group, generally as substituted phenyl or heteroaryl group and an acetic acid side chain or acidic moiety (Bayer and Beaven, 1981). The drug is largely ionized at plasma pH but sufficient non-ionized

FIG. 1

SYNTHESIS OF PROSTAGLANDINS



where h = hydrolysis, ox = oxidation, r = reduction, hx = hydroxylation, cy = cyclisation, i = isomerisation.

drug is present to enter the cells. Thus a small decrease in pH could lead to greater amounts of indomethacin within the cell. The action of indomethacin in inhibiting prostoglandin synthesis is not antagonized by exogenous prostaglandins or arachidonic acid (deMello *et al.*, 1980). There are almost thirty different systems for which aspirin and indomethacin inhibit prostaglandin synthesis (Flower and Vane, 1974).

VI. ADENYLATE CYCLASE ACTIVITY

The enzyme adenylyate cyclase whose systemic name is ATP pyrophosphate-lyase (cyclising) EC 4.6.1.11 catalyses the formation of cAMP and inorganic phosphate from ATP.

Several approaches have led to the elucidation of the hormonal regulation of adenylyate cyclase activity (Stadel *et al.*, 1982).

- (i) Cell fusion.
- (ii) Solubilisation and reconstitution.
- (iii) Genetic studies.

Cell fusion was first performed by Orly and Schramm (1976). They produced hybrid cells by virus-promoted fusion of donor cells containing complementary components of the adenylate cyclase system. It was possible to combine the β -adrenergic receptor of one cell with the cyclase enzyme of another cell that was devoid of β -receptors to produce a β -adrenergic responsive hybrid cell. Since this fusion experiment could be reproduced in the presence of protein synthesis inhibitors, the isoproterenol stimulation of the enzyme must involve an association of pre-existing components from the donor cells. This method of using fusion techniques in establishing the components of adenylate cyclase activity has since been confirmed in other laboratories (Schwarzmeier and Gilman, 1977; Pike *et al.*, 1979).

Solubilisation and reconstitution studies have been undertaken extensively over the past few years. Pfeuffer (1977) has been able to reconstitute nucleotide- and fluoride-dependent adenylate cyclase activities in detergent solution by combining distinct and partially purified components. Since then numerous laboratories have used the technique of solubilising the components in detergents e.g. Lubrol or digitonin followed by separation of the components by column chromatography and reconstitution to show the requirements for hormonal regulation of adenylate cyclase (Neer and Salter, 1981; Keenen *et al.*, 1982; Peterson and Ross, 1983). The techniques of cell fusion and solubilisation have also been used to implant a solubilised receptor into a donor membrane (Eimerl *et al.*, 1980).

The genetic approach utilises the mutants of S49 lymphoma cells described previously. Genetics was combined with the reconstitution approach to establish the nature of the alterations in the mutants (Stadel *et al.*, 1982).

These three approaches have led to the concept that the adenylate cyclase molecule is composed of at least three molecular entities; the hormone receptor, the guanine nucleotide regulatory component and the catalytic moiety.

(a) Hormone Receptors

It has become obvious that hormone receptors and adenylate cyclase are separate entities. This has been shown by solubilisation and reconstitution studies, genetic studies with S49 β_d mutants, and also by inactivating the adenylate cyclase activity with N-ethylmaleimide. Inactivation of cyclase with N-ethylmaleimide showed that hormone receptors and ligands were still capable of binding (Oye and Sutherland, 1966; Schramm and Nairn, 1970). Many hormones that act via stimulation of adenylate cyclase have receptors capable of binding to adenylate cyclase e.g. prostaglandins, β -adrenergic agents, glucagon, parathyroid hormone, FSH, luteinising hormone, ACTH as well as non-hormone e.g. adenosine, histamine. In some tissues adenosine receptors are permanently coupled to adenylate cyclase (Braun and Levitzki, 1979).

More recently it has become established that in certain systems hormones that produce decreased amounts of cAMP act by inhibition of adenylate cyclase (Londos *et al.*, 1981). Some examples of hormone receptors that appear to be coupled to attenuation of adenylate cyclase activity include muscarinic receptors of heart, opiate receptors in neuroblastoma-glioma hybrid cells, adenosine receptors in adipose and neural tissue, somatostatin receptors of liver and pituitary adenoma cells, dopamine receptors of pituitary and α_2 -adrenergic receptors of platelets, kidney or pancreas and fat cells (Jakobs *et al.*, 1981).

Most of the available information concerning structural and other characteristics of receptors has come from the β -adrenergic receptor because of the availability of various radiolabelled agonists and antagonists. The β -adrenergic receptor is an integral membrane protein that requires treatment with detergent to extract it from the membrane. Detergent-solubilised preparations of LH and β -adrenergic receptors have been characterised with respect to their hydrodynamic properties and both appear to be rather asymmetric proteins that bind fairly large amounts of detergent in solution (Dufau *et al.*, 1973; Dufau *et al.*, 1974; Haga *et al.*, 1977). In order to identify the polypeptide(s) that constitute the hormone receptor the receptor has been labelled using a radioactive ligand analogue. The covalently labelled polypeptides are then visualised on SDS polyacrylamide gels. Atlas and Levitzki (1978) have observed labelling of two polypeptides of molecular weights 41,000 and 37,000 daltons in turkey

erythrocytes and L6P with the tritiated β -antagonist. Rashidbaigi and Ruoho (1981) showed incorporation of label into two polypeptides of molecular weights 45,000 and 48,500 daltons in duck erythrocytes. Turkey erythrocytes are shown to label receptor bands of proteins of 30,000, 33,000 and 60,000 daltons and for frog of 26,000-30,000 and 58,000 daltons (Strosberg *et al.*, 1982). The 58,000 dalton polypeptide contains hormone binding activity and the 26,000-30,000 dalton fragment is either a proteolytic fragment or serves another function which is unknown at present. Benovic *et al.* (1983), have identified labelled peptides in rat lung of 64,000, 53,000 and 44,000 daltons. The reasons for the differences in molecular weights found is unknown but may reflect differences between species or β -receptor subtypes. Recently the β_2 -adrenergic receptor from canine lung has been purified. A single class of binding sites with an affinity identical to the membrane-bound receptor were isolated (Homcy *et al.*, 1983). A major band of 52-53,000 daltons was visualised on SDS gel electrophoresis. It is to be hoped that these purification procedures will soon be applied to other hormone receptors attached to adenylate cyclase.

The preparation of β -receptors has allowed some of the general properties to be characterised. Properties of the β -receptors include, the rapidity of binding to hormone, saturability, specificity for agonists and antagonists, stereospecificity, (-) isomers being more potent than (+) isomers (Lefkowitz and Williams, 1977). Some lack of strict specificity for the (-) isomer for

β -adrenergic systems has been demonstrated in undifferentiated cultures (Wahrmann and Winand, 1978). The affinity of the receptor for agonist is modulated by GTP via the guanine nucleotide regulatory component. Interaction of the agonist with the β -receptor in the absence of guanine nucleotides appears to induce or stabilise a high-affinity binding state which is very slowly reversible when a competing ligand is added (Briggs and Lefkowitz, 1982). The ability of a given agonist to form this high-affinity binding state can be correlated with the intrinsic activity or efficacy of that drug. Guanine nucleotides which are required for agonist activity of adenylate cyclase, cause a transition of this high-affinity state of the receptor to a lower affinity-state causing a rapid dissociation of agonistbound to receptor (Kent *et al.*, 1980). No such decrease in affinity by antagonists has been observed except by Wolfe and Harden (1981) who noted that guanine nucleotides increase the affinity for antagonists. Recently it has been shown that guanine nucleotides modulate the α_2 -mediated inhibition of adenylate cyclase (Pecquery and Giudicelli, 1982).

The hormone appears to act as a trigger for the receptor; indeed other specific ligands e.g. anti-receptor antibodies can play the same role both in terms of binding and in leading to adenylate cyclase activation (Strosberg *et al.*, 1982).

Desensitisation

The phenomenon of desensitisation also known as refractoriness appears to have a role in regulating the responsiveness of cells to hormonal stimulation. Prolonged incubation of cells or tissues with a hormone or drug causes a dramatic decrease in the response to that hormone (Lefkowitz *et al.*, 1980). For the hormone-responsive adenylate cyclase systems, desensitisation results in attenuation of cAMP production. This phenomenon has been observed in a wide variety of cells and with different hormones. These include prostaglandins (Ayad and Burns, 1979; Kassis and Fishman, 1982), β -adrenergic agonists (Anderson and Jaworski, 1979; Fishman *et al.*, 1981), luteinizing hormone (Lamprecht *et al.*, 1977; Hunzicker-Dunn and Birnbaumer, 1981) and glucagon (Iyengar *et al.*, 1980).

There are several types of desensitisation. For homologous desensitisation only the response to the specific desensitising hormone is altered whereas stimulation of adenylate cyclase activity by other hormones or by NaF is unchanged. Loss or down regulation of the receptors is often observed, possibly arising from internalisation of the receptors (Chuang and Costa, 1979).

Heterologous desensitisation is characterised by a general loss of responsiveness of adenylate cyclase to all activators and no down-regulation of receptors (Briggs and Lefkowitz, 1982). In homologous desensitisation the decrease in receptor-mediated stimulation of adenylate cyclase can be accelerated with a reduction of affinity of

agonist binding to receptor and for the frog erythrocyte (Kent *et al.*, 1980) it has been shown that formation of high affinity binding state was lost after desensitisation. In other systems this uncoupling of the β -receptor from adenylate cyclase can be separated temporally from down regulation of the receptors (Su *et al.*, 1980). Kinetic analyses of the β -receptor desensitisation by Harden *et al.* (1979) and Su *et al.* (1979) suggests that the phenomenon of desensitisation involves two consecutive steps (a) a rapid uncoupling of receptors from cyclase (b) loss of receptors from the cell surface.

From studies using S49 wild type cells and mutant clones (Iyengar *et al.*, 1981) it appears that the receptor is the major locus of alteration in homologous desensitisation and alterations in the β -receptor have been shown in desensitisation (Stadel *et al.*, 1982).

Heterologous desensitisation has been observed for a number of cell lines e.g. turkey (Stadel *et al.*, 1981) and pigeon erythrocytes (Simpson and Pfeuffer, 1980). Since all activators become less effective in this type of desensitisation it is suggested that this type is distal to the receptor and might involve changes in guanine nucleotide regulatory protein (Briggs and Lefkowitz, 1982). This is illustrated by Kassir and Fishman (1982) for human fibroblasts. Isoproterenol in these cells mediates homologous desensitisation which involves uncoupling of the receptor from a functional guanine nucleotide regulatory component - catalytic component complex. In contrast PGE_1

in these cells mediates heterologous desensitisation involving an alteration of the guanine nucleotide regulatory component which results in a more uncoupled and a less efficient regulatory component - catalytic component complex.

(b) Guanine Nucleotide Regulatory Protein

It has long been realised that guanine nucleotides have an obligatory role in the regulation of adenylate cyclase activity and it has been recognised that guanine nucleotides are required for stimulation of cyclase by virtually all hormones and drugs investigated (Cryer *et al.*, 1969; Rodbell *et al.*, 1971a; 1971b; Rodbell *et al.*, 1975). The specificity of action of the various nucleotides was established as $\text{Gpp(NH)p} > \text{GTP} > \text{GDP} \gg \text{GMP}$. Adenine nucleotides and pyridine nucleotides did not have significant effect. Non-hydrolysable analogues of GTP e.g. Gpp(NH)p and $\text{GTP}\gamma\text{S}$ activate the enzyme independently and in the presence of hormone to a persistently active state (Londos *et al.*, 1974; Londos *et al.*, 1977; Spiegel *et al.*, 1977). This is in contrast to activation of adenylate cyclase by hormone and GTP which is readily reversible. It seems apparent that although triphosphate is necessary for stimulation of adenylate cyclase activity the hydrolysis of the terminal phosphate is not. The activation of adenylate cyclase activity by these analogues was characterised by the presence of a lag period before the onset of activation (Londos *et al.*, 1974) and the length

of the lag period could be shortened or eliminated by increasing the concentration of hormone added. There are two main possible explanations for the presence of this lag period.

(i) A slow isomerisation may exist between these two enzyme states and that addition of hormone increases the rate of isomerisation.

(ii) The lag due to the dissociation of endogenous nucleotides from the guanine nucleotide binding site and addition of hormone might stimulate this release process.

The study of the latter possibility led Cassel and Selinger (1975) to suggest a GTPase activity associated with the guanine nucleotide regulatory site in turkey erythrocytes. This GTPase activity could be inhibited by Gpp(NH)p or GTP γ S and the inhibition correlated with the ability of these nucleotide analogues to activate adenylate cyclase (Cassel and Selinger, 1977). This suggested that the GTPase activity was a common site for adenylate cyclase activation. This GTPase activity would be responsible for 'turning off' adenylate cyclase activity.

A major focus of investigations of guanine nucleotide regulation of adenylate cyclase has centred on whether the guanine nucleotide binding site resides on an independent component or is an allosteric site of the catalytic unit. Pfeuffer and Helmreich (1975) suggested that this activity might reside on a separate subunit of the adenylate cyclase

complex. In 1977, Pfeuffer reported the development of a GTP photoaffinity probe that labelled several proteins in membranes. After partial purification of adenylate cyclase a specifically labelled 42,000 dalton protein was associated with the activity. Subsequently it was demonstrated that this protein was the site of covalent modification by cholera toxin (Cassel and Pfeuffer, 1978), and could reconstitute adenylate cyclase activity with the catalytic component in adenylate cyclase deprived of guanyl nucleotide binding proteins so that activation by Gpp(NH)p is restored (Pfeuffer, 1977; 1979). Furthermore, Pfeuffer has found that the guanyl nucleotide binding protein isolated from pigeon erythrocytes is also capable of activating rabbit myocardial adenylate cyclase that has been depleted of GTP-binding proteins (Pfeuffer and Helmreich, 1975; Pfeuffer, 1977). This finding suggests that this regulatory protein is a universal unit of the adenylate cyclase system and can therefore couple with a catalytic unit from many species. The simultaneous presence of unlabelled guanine nucleotides enhances the hormone-induced release of labelled nucleotides from the regulatory protein (Cassel and Selinger, 1977a; Cassel and Selinger, 1978). This suggests that the regulatory component of the cyclase system has either more than one nucleotide binding site or is multimeric. This has also been postulated by Rodbell, 1980.

The guanine nucleotide regulatory protein has been shown to have a total molecular weight of 130,000 (Howlett and Gilman, 1980). The regulatory component from rabbit liver has also been purported to have three putative subunits of

52,000, 45,000 and 35,000 daltons (Northup *et al.*, 1980). The 52,000 molecular weight component appears to aid in reconstitution. The regulatory protein was found to have an approximate molecular weight of 70,000. However, if the component is preactivated with GTP analogues its apparent molecular weight is decreased to 50,000 daltons (Sternweis *et al.*, 1981). The 45,000 and 52,000 dalton components are ADP-ribosylated by cholera toxin (Northup *et al.*, 1980). There appears to be some disagreement between workers since Kaslow *et al.* (1980) report a molecular weight of 126,000 and Hanski *et al.* (1981), a molecular weight of 81,000 which is reduced to 50,000 in the presence of activating ligands. In turkey erythrocyte membranes subunits of 45,000 and 35,000 have been characterised (Northup *et al.*, 1980). Thus it appears that the 52,000 subunit is not essential for activity. The role of the 35,000 subunit is less clearly understood but is an integral component of the regulatory protein (Limbird, 1981). The regulatory protein appears to be located on the inner surface of the plasma membrane (Farfel *et al.*, 1980).

Two toxins have been used to further probe the structure of adenylate cyclase.

(i) Cholera Toxin

This toxin has played an important part in determining the hormonal regulation of adenylate cyclase since it has been found to permanently activate adenylate cyclase in intact cells and homogenates via inhibition of GTPase activity. Cholera toxin is responsible for various

effects on the adenylate cyclase system including; reduced GTPase activity, increased GTP-dependent adenylate cyclase activity, increased hormone-dependent adenylate cyclase activity and abolition of the inhibitory action of GTP (Gill, 1982). Cholera toxin (choleragen) is an enterotoxin of *Vibrio Cholerae* that is responsible for the watery diarrhoea characteristic of clinical cholera (Moss and Vaughan, 1979). The toxin is an oligomeric protein with molecular weight approximately 84,000 daltons and is composed of three dissimilar peptides, A₁, A₂, and B. A₁ (23,500 daltons) is linked through a single disulphide bond to A₂ (5,500 daltons). A₁ and A₂ are also known as α and γ subunits (Duffy *et al.*, 1981) and the amino acid sequence of γ has been determined (Duffy *et al.*, 1981a). 5 B subunits are present in the oligomer and the structure of the oligomer is predicted as 5 B subunits arranged radially about an elongated A subunit which extends well above the plane of the B subunits (Dwyer and Bloomfield, 1982). Choleragen is believed to exert its effects via activation of adenylate cyclase. Activation of adenylate cyclase activity in intact cells is characterised by a lag period and this is thought to represent the generation of the active A₁ peptide (Gill, 1982; Kassis *et al.*, 1982). The initial event is the binding of the B subunits to the membrane via ganglioside GM₁ receptors on the cell surface (Moss and Vaughan, 1979; Van Heyningen, 1982). It is not known exactly how the active A subunit enters the cell but it has been proposed that it becomes available at the cytoplasmic surface of the plasma membrane by virtue of the toxin being endocytosed, processed in the lysosomes and recycled back to the plasma

membrane (Houslay and Elliott, 1981). Support comes from the observation of cholera toxin undergoing endocytosis in neuroblastoma and it has also been shown that after endocytosis cholera toxin was associated with the golgi or GERL complex (Joseph *et al.*, 1979). However, Gill *et al.* (1981) can demonstrate no such involvement.

The cholera toxin-induced activation of adenylate cyclase is most probably due to ADP-ribosylation (Cassel and Selinger, 1977b; Moss and Vaughan, 1977; Gill, 1982) of the 42,000 molecular weight component of the guanine nucleotide regulatory protein. In addition, various groups have noted the ADP-ribosylation of other peptides by cholera toxin including the 47,000 (Berthillier *et al.*, 1982; Ward and Van Heyningen, 1982), 48,000 (Enomoto and Asakawa, 1983) and 53,000 molecular weight bands (Gill, 1982). Cell homogenates are activated by the toxin but show no lag period, and require the presence of dithiothreitol, NAD^+ , nucleotide triphosphate (Moss and Vaughan, 1979; Gill, 1982) and a cytosolic protein factor of apparent size 20,000 daltons (Enomoto and Gill, 1980). In addition a plasma membrane-associated component has been found to aid in the process (Pinkett and Anderson, 1982; Schleifer *et al.*, 1982). Thus ADP-ribosylation of the guanine nucleotide regulatory protein catalysed by cholera toxin is responsible for the inhibition of GTPase activity which appears to regulate adenylate cyclase activity.

(ii) Pertussis toxins

These toxins have recently been responsible for the proposal of the concept that adenylate cyclase activity is regulated by two forms of regulatory protein, N_s mediating stimulation by hormones and N_i mediating inhibition. In many cells adenylate cyclase activity is inhibited and stimulated by hormones (Michel *et al.*, 1982) and this led to the idea of dual regulation of the cyclase. This is particularly well illustrated by α and β adrenergic receptors which often have opposing actions in the same tissue (Williams and Lefkowitz, 1978). The observation that GTP is required for hormonal inhibition of adenylate cyclase (Jakobs *et al.*, 1981) led to the question of whether the mechanisms of inhibition and stimulation may be similar. Whereas hydrolysis resistant GTP analogues elicit persistent activation of adenylate cyclase they prevent hormonal attenuation of basal- or stimulated cyclase activity (Limbird, 1983). The apparent requirement for a labile terminal phosphate for hormonal inhibition of adenylate cyclase implies that a phosphorylation-dephosphorylation mechanism of hormone-stimulated GTP hydrolysis might account for attenuation. Stimulation of a low K_m GTPase by hormones coupled to inhibition of cyclase has been demonstrated by inhibitory agents in adipocytes (Aktories *et al.*, 1982). The requirement for GTP and not Gpp(NH)p or GTP γ s to elicit hormone-mediated inhibition of cyclase suggested that different mechanisms account for GTP-mediated activation and inhibition of adenylate cyclase (Limbird, 1983). The differential sensitivities of cyclase

activation and inhibition to proteases, radiation inactivation, sulphydryl reagents as well as the 10-fold greater GTP concentrations required to elicit half-maximal effects in inhibitory as compared to stimulatory systems has suggested that the two processes might involve different guanine nucleotide binding proteins (Limbird, 1983).

The use of the Bordetella pertussis toxin, islet-activating protein (IAP) has allowed further investigation of this suggestion. Recently this toxin has been shown to ADP-ribosylate a peptide that is not part of N_s and this coincides with attenuation of hormonal inhibition of cyclase (Katada and Ui, 1982; Burns *et al.*, 1983; Murayama *et al.*, 1983; Hildebrandt *et al.*, 1983). These findings lend further support to the concept that there is a separate nucleotide binding protein mediating hormonal inhibition of adenylate cyclase activity (N_i). Even more evidence has come from studies of cyc^- mutants of S49 lymphoma which are lacking in N_s . Somatomedin has been found to lower both basal and F^- -stimulated cAMP levels and cause a concomitant increase in a high affinity GTPase activity (Jakobs *et al.*, 1983). These effects are interpreted by the presence of N_i . Studies of guanine nucleotide inhibition of cyclase activity in these cells also led to the idea of the presence of N_i (Hildebrandt *et al.*, 1982). Evidence has also come from platelets of pseudohypoparathyroid patients (Motulsky *et al.*, 1982) where N_i is expressed normally in platelets from patients genetically deficient in N_s . Pertussis toxin ADP-ribosylated a protein of molecular weight 41,000 (Murayama

et al., 1983) and cholera toxin does not have any effect on N_i (Murayama and Ui, 1983). Thus IAP has been useful in proposing distinct regulatory sites mediating inhibition and stimulation of adenylate cyclase. Evidence that cholera toxin and IAP mediate their effects by different molecular events comes from C_6 glioma cells. In these cells IAP catalyses the ADP-ribosylation of 41,000 molecular weight peptide whereas cholera toxin ribosylates 45,000 and 48,000/49,000 doublet.

(c) Catalytic Unit

Adenylate cyclase demonstrates a broad pH optimum pH 7.0-8.5 (Stadel *et al.*, 1982) and although the ionic strength of the assay medium does not appear critical, specific ions are required for activity. Stimulatory adenylate cyclase systems differ from inhibitory systems in their requirements for metal ions. For stimulatory systems the physiologically relevant cation appears to be Mg^{2+} and it must be present in excess of ATP, suggesting that the specific substrate of the enzyme is a Mg-ATP complex (Stadel *et al.*, 1982). The chelation complex between Mg^{2+} and ATP is important since $ATP H^{3-}$ is a potent competitive inhibitor (Lin *et al.*, 1975). An additional role for Mg^{2+} is postulated since Mg^{2+} can stimulate cyclase activity at concentrations beyond that necessary to bind to ATP (Birnbaumer *et al.*, 1969; Drummond and Duncan, 1970). Cech *et al.* (1980) have suggested that Mg^{2+} reacts with specific receptor-adenylate cyclase sites to modulate adenylate cyclase mechanism.

More recently, this has led to the suggestion that there are two independent metal binding sites on the receptor-cyclase complex, one site on the catalytic unit mediating enzyme activity and one site on the regulatory protein mediating agonist affinity for the receptor. These experiments used Mn^{2+} and Mg^{2+} in combination (Cech and Maguire, 1982). Chatelain *et al.* (1982) have also suggested that there may be an allosteric site for Mg^{2+} on the regulatory protein. Studies with other cations have shown that Ca^{2+} is usually inhibitory (Drummond and Duncan, 1970; Steer and Levitzki, 1975) but exceptions have been noted (Bar and Hechter, 1969; Bradham, 1972). The requirement for a divalent cation can also be satisfied by Mn^{2+} at low concentrations (1-5mM (Stadel *et al.*, 1982). However, at greater concentrations Mn^{2+} uncouples hormonal activation (Birnbaumer *et al.*, 1971; Limbird *et al.*, 1979).

There are optimum conditions for adenylyl cyclase activity; a temperature of 30-40°C, 1-2mM ATP, 5-10mM Mg^{2+} and pH 7.5-8.5 (Cooper, 1976). The use of chemical reagents to modify specific amino acid side chains has demonstrated that adenylyl cyclase activity depends on a highly reactive free sulphydryl group (Storm and Dolginow, 1973; Stadel and Lefkowitz, 1979; Drummond, 1981). Studies have also shown that the catalytic activity can also be inhibited by phenylglyoxal which reacts selectively with the guanido side chain of arginine (Varima and Londesborough, 1979).

For inhibitory systems there is an absolute requirement for Na^+ (Londos *et al.*, 1981; Limbird, 1983). The potency order of other monovalent cations was found to be $\text{Na}^+ > \text{Li}^+ > \text{K}^+ > \text{cholate}^+$. Half-maximal inhibition of cyclase by hormones was observed at 30mM Na^+ . The differences in cation requirement between inhibitory and stimulatory phases is illustrated well by the action of PGE_2 on human fat cell cyclase. PGE_2 has biphasic effects on this enzyme, inhibition at low concentrations and stimulation at high concentrations. Na^+ was found to augment the inhibitory phase but interferes with the stimulatory phase (Kather, 1982).

The enzyme from most sources appears to be responsive to NaF in broken cell preparations but not in intact cells (Robison *et al.*, 1971). It has been known from the earliest work by Sutherland and his colleagues that this is not due to fluoride inhibition of ATPase (Rall and Sutherland, 1958; Sutherland *et al.*, 1962). The magnitude of F^- stimulation above basal levels is variable between systems. Perkins (1973) has suggested that the fluoride stimulation of particulate adenylate cyclase preparations may be an artifact of the homogenate preparation since NaF does not stimulate the enzyme in intact cells even though it probably crosses the membrane. Fluoride stimulation is dependent upon the presence of the guanine nucleotide regulatory protein and the presence of the regulatory protein has been shown to be required for the reconstitution of F^- -stimulated activity (Howlett *et al.*, 1979).

Forskolin, a diterpene, has also recently been shown to activate adenylate cyclase activity in a number of cells (Insel *et al.*, 1982; Stengel *et al.*, 1982). Although it was originally thought to act solely by the catalytic site this is now thought to be untrue and that the regulatory protein is also involved (Stengel *et al.*, 1982; Morris and Bilezikian, 1983). However, a recent report does not rule out the possibility that forskolin acts directly with the catalytic site (Sano *et al.*, 1983).

The structure of the catalytic unit is less well determined than that of the receptor. This is because of the lability of the preparation and that measured molecular weights also have varying contributions from the guanine nucleotide regulatory component and/or detergent. Values reported to date have varied from 50,000 - 1,000,000 (Briggs and Lefkowitz, 1982). Recently the catalytic unit and the regulatory unit have been separately solubilised (Ross, 1981). The catalytic unit thus prepared is responsive to Mn^{2+} more than Mg^{2+} but had a complete lack of sensitivity to F^- or guanine nucleotides. It is hoped that this procedure will allow further purification of the catalytic protein. Bender and Neer (1983) have also separated the catalytic unit from the guanine nucleotide regulatory unit in caudate nucleus. This catalytic activity was also more active in the presence of Mn^{2+} than Mg^{2+} . This separated catalytic protein can also be activated by forskolin both alone and in combination with the regulatory protein. Thus it is hoped that with the advent of these

solubilisation techniques to prepare the catalytic unit, that the component can be further purified to give more information about the exact structure and properties of the catalytic component.

VII REGULATION OF ADENYLATE CYCLASE ACTIVITY BY ITS ENVIRONMENT

Adenylate cyclase activity is also controlled by its environment including the cytoskeleton, lipids and certain cytosolic factors.

(a) The Cytoskeleton

The first indications that the cytoskeleton was involved in adenylate cyclase activity was the finding that addition of microtubule inhibitors, e.g. colchicine, to intact cells caused activation of hormone-stimulated cAMP accumulation (Gemsal *et al.*, 1977; Rudolph *et al.*, 1977; Insel and Kennedy, 1978; Kennedy and Insel, 1979; Hagmann and Fishman, 1980). Conversely for a number of cell types it has been shown that colchicine has either no effect or inhibits hormone-stimulated cAMP production (Kalinin, 1977; Zor *et al.*, 1978) and Simantov and Sachs (1978) noted differing responses according to the cell type. Microtubule inhibitors e.g. cytochalasin B have also been demonstrated to increase cAMP accumulation in some cells (Insel and Coachman, 1982). Some stimulation of adenylate cyclase by cytoskeleton disrupting drugs has been observed in broken cells (Whetton and Houslay, 1980; Rasenick *et al.*,

1981). It is not known exactly whether the action of the cytoskeleton disrupting agents is due to a general effect on membrane fluidity or to a direct effect on one of the components of adenylate cyclase. However, the action of colchicine is thought to be distal to the receptor (Insel and Kennedy, 1978). Disruption of microtubules increases the localised mobility of membrane proteins in a manner similar to that of unsaturated fatty acids (Klausner *et al.*, 1980). Although some workers have suggested a direct action on one of the components of adenylate cyclase activity (Whetton and Houslay, 1980), it has also been suggested that the action of cytoskeleton disrupting agents is to alter the attachment of the regulatory component to the plasma membrane (Rasenick *et al.*, 1981; Insel and Coachman, 1982). Although colchicine is also known to inhibit phosphodiesterase activity (Ewart, 1982) and tubulin itself activates adenylate cyclase (Simonin *et al.*, 1981) these are not thought to be major factors in activation of adenylate cyclase by colchicine especially since phosphodiesterase inhibitors are included in the studies. The cytoskeleton has been shown to associate with plasma membranes (Sahyoun *et al.*, 1981; Ishikawa *et al.*, 1982) and the regulatory protein is known to have its site on the inner surface of the plasma membrane it therefore seems reasonable to propose that cytoskeleton disrupting drugs might affect adenylate cyclase via interactions with the regulatory protein. Although it has been suggested that the activation could be due to localised high concentrations of GTP produced due to the lack of GTPase activity associated with polymerisation (colchicine acts by

inhibition of tubulin polymerisation) no evidence has been found to date. Indeed it has been demonstrated that colchicine can stimulate GTPase activity (Lin and Hamel, 1981).

(b) Lipid Environment

This topic has been reviewed recently by Houslay and Gordon (1983). The three components of adenylate cyclase are able to undergo independent, free, lateral diffusion within the bilayer and interact only in the presence of the necessary activating ligands or hormones (Martin *et al.*, 1979; Houslay *et al.*, 1981). All of the components are asymmetrically disposed with the bilayer such that the receptor has its binding site at the external face whereas both the catalytic and regulatory proteins have substrate sites at the internal face of the membrane (Houslay *et al.* 1980). Although all components are integral membrane proteins the catalytic and regulatory components bind relatively small amounts of detergent (Ross and Gilman, 1980). This indicates that only a small amount of those proteins is actually incorporated into the bilayer. Thus the predominant effects on adenylate cyclase activity come from the lipids on the internal side of the bilayer. Adenylate cyclase can be modulated by lipids either by direct involvement of the chemical nature of the lipids surrounding it or by alteration of membrane fluidity.

Membrane fluidity can be altered in several ways, particularly by alterations in temperature or by addition

of local anaesthetics or fatty acids. The local anaesthetic, benzyl alcohol, when inserted into a membrane increases the fluidity (Gordon *et al.*, 1980). At low benzyl alcohol concentrations the activity of adenylate cyclase is increased. The F^- , guanine nucleotide- and hormone-stimulated activities are all increased whereas basal activity is only slightly affected. At higher concentrations benzyl alcohol becomes inhibitory. The effects due to low concentrations are thought to involve alterations in membrane fluidity whereas effects due to higher concentrations are due to removal of annular lipids surrounding adenylate cyclase. The action of benzyl alcohol in increasing cyclase activity has been observed for the enzyme derived from several sources (Houslay and Gordon, 1983). Other local anaesthetics have also been demonstrated to affect adenylate cyclase activity, the effects varying depending on the charge of the anaesthetic and therefore which half of the bilayer it affects. Activation of cyclase by the positively charged local anaesthetics prilocaine, nupercaine and carbocaine occurred (Gordon *et al.*, 1980), and this is thought to be due to changes in membrane fluidity. Inhibitory effects occurred at higher concentrations and these were thought to be due to removal of annular lipid. Anionic drugs phenobarbital, pentobarbital and salicylic acid inhibited enzyme activity (Gordon *et al.*, 1980) and this was thought to be due to interaction with the lipid bilayer. Anionic agents are considered to act on the outer half of the bilayer while cationic agents act on the inner half (Houslay and Gordon, 1983). An effect of the local anaesthetic tetracaine on adenylate cyclase activity has been observed (Ayad and White, 1977) but in this case it

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is unclear whether the drug acts on acidic phospholipids or the cytoskeleton. Cholesterol has proved interesting in the control of adenylate cyclase activity. It appears that a critical cholesterol concentration in membranes is responsible for the control of adenylate cyclase activity since decreasing (Whetton *et al.*, 1983a) or increasing (Whetton *et al.*, 1983b) the concentration inhibit glucagon-stimulated activity.

Phospholipids are also implicated in the control of adenylate cyclase. They appear to play an important role in the coupling process particularly for dopamine-dependent activation of adenylate cyclase (Anand-Srivastava and Johnson, 1982) and phosphatidylcholine has been demonstrated to promote the interaction of catalytic and regulatory subunits (Ross, 1982). Specific phospholipids have been shown to be required for the reconstitution of cyclase activity from rat brain (Hebdon *et al.*, 1981). Although it has been demonstrated that β -adrenergic agonists increase phospholipid methylation (Hirata *et al.*, 1979) other workers have not found any evidence for a requirement for phospholipid methylation for adenylate cyclase activity (Schanche *et al.*, 1982; Padel *et al.*, 1982).

Thus it appears that the lipid and phospholipid environment is important for the control of adenylate cyclase activity particularly with respect to the control of membrane fluidity.

(c) Cytosolic Factors

Several groups of workers have found evidence to show that certain cytosolic factors mediate the activity of adenylate cyclase. Soluble factors from human, rat and rabbit erythroid cell lysates are capable of stimulating basal and hormone-stimulated cyclase activity from erythroid cell membranes from homologous sources (Beaumont *et al.*, 1980). Supernatants from 300,000g sedimentation of homogenates of rat liver, heart and skeletal muscle, dog liver and rabbit liver contain activity that restores Mg^{2+} -dependent, F^- and guanine nucleotide-dependent activity to adenylate cyclase in S49 cyc^- membranes (Bhatet *et al.*, 1980). This factor was trypsin-sensitive and could be protected against heat inactivation by guanine nucleotides and had a sedimentation coefficient ($S_{20,w}^0$) of 5.3. It appears that this cytosol contains a protein having guanine nucleotide regulatory component-like activity. Rat liver plasma membranes have also been reported to have adenylate cyclase activity that can be activated by a cytoplasmic protein factor (Doberska and Martin, 1977). A factor prepared from the cytosol of rat epididymal fat cells, sedimented at 100,000g, and was found to enhance basal and epinephrine-sensitive adenylate cyclase of rat fat cell ghosts (Ganguly and Greenough, 1975). In rat lungs the supernatant was found to contain factors which enhance adenylate cyclase activity in membranes (Nijjar *et al.*, 1981). These factors were separated into two less active components (peaks 1 and 2) by DEAE-cellulose chromatography. However, their recombination restored the full activation of

adenylate cyclase activity. Further purification of these factors revealed that the activator in peak I contained two proteins of low (14,500) and high (65,000) molecular weight whereas activator in peak II contained only one protein of 65,000. Calmodulin was not involved in these activators. Pecker and Hanoune (1977) found that uncoupled β -adrenergic receptors and adenylate cyclase could be recoupled by a GTP-dependent cytosolic factor. There is also interspecies activation. Johnson *et al.* (1983) have illustrated that brain adenylate cyclase can be activated by a factor derived from bovine sperm particles. Conversely workers have shown that adenylate cyclase can be inhibited by a factor in the cytosol. This factor was inactivated by heating at 60°C and by incubation with trypsin. The inhibitor had a sedimentation coefficient of 4S (Sano and Drummond, 1981). Thus there appears to be a variety of soluble factors regulating the activity of adenylate cyclase but their physiological role is unclear.

VIII. MECHANISMS OF ACTION OF ADENYLATE CYCLASE

Early models for the activation of adenylate cyclase by hormones considered the receptor to be an integral part or allosteric site of the enzyme (Robison *et al.*, 1966). Birnbaumer *et al.* (1970) suggested a three component model consisting of a receptor (hormone discriminator), transducer, and a catalytic unit (amplifier). The transducer was used to denote 'that element which couples events occurring at the receptor to events at the catalytic unit' (Rodbell, 1972). Following the discovery that GTP was necessary for hormone action these workers modified their model to include

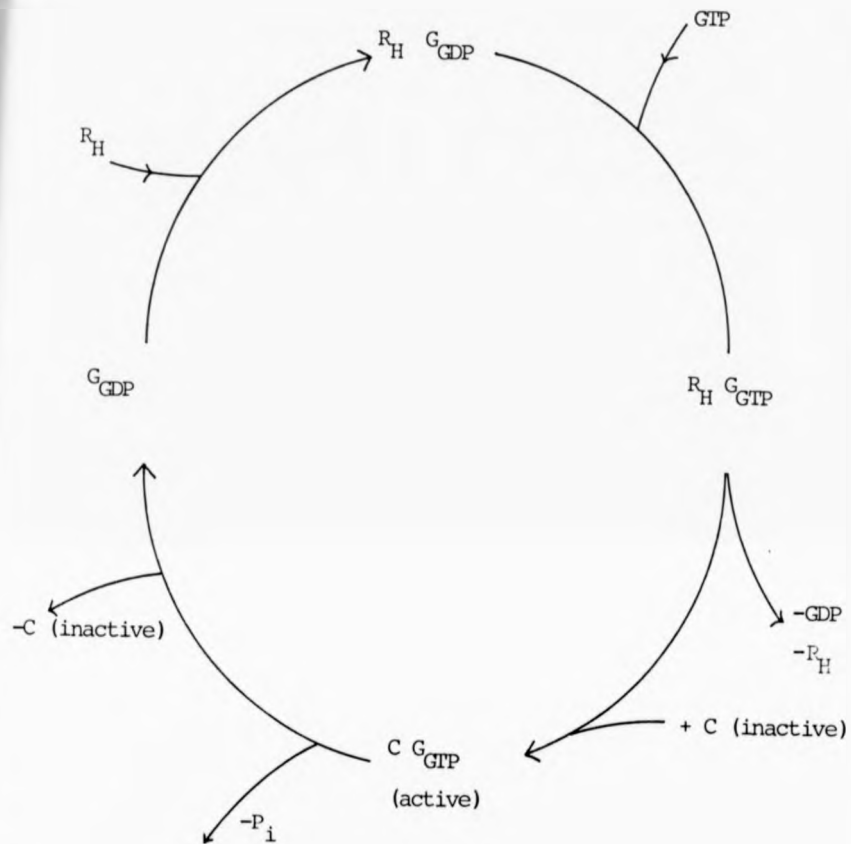
GTP. It was suggested that the transducer site contained a regulatory unit for GTP (Rodbell *et al.*, 1974). Several years later a 'floating receptor' model was proposed to explain the differences in agonist-binding properties from those described by the simple law of mass action (Boeynaems and Dumont, 1975; Jacobs and Cuatrecasas, 1976). This model suggests the concept that the mobility of individual proteins in the membrane was important to receptor-effector coupling. The 'collision coupling' model was derived from detailed kinetic analysis to describe the rate-limiting step in activation of the enzyme as the diffusion-controlled, transient collision of mobile receptors and enzyme units (Tolkovsky and Levitzki, 1978). Although these models were useful in elucidating the interaction of receptor, hormone and enzyme they did not include the guanine nucleotide regulatory unit, the GTPase activity associated with it or the interactions of this regulatory unit with the receptor and catalytic moiety. The collision coupling model has been suggested to describe adequately the molecular events occurring during adenylate cyclase activation by β -agonists in C₆ glioma cells (Homburger *et al.*, 1982) as well as for the β -receptor of turkey erythrocytes (Tolkovsky and Levitzki, 1978). Radiation-inactivation studies have been used to study the interaction of components of adenylate cyclase activity (Houslay *et al.*, 1981). In the absence of hormone, the catalytic and receptor proteins were inactivated as single targets. In the presence of hormone they were inactivated as a single target which suggests the 'mobile receptor' model. Radiation inactivation studies have also been used for the collision

coupling model in the presence of both GTP and hormone. However, evidence has been found both for (Martin *et al.*, 1979) and against (Rodbell, 1980; Schleger *et al.*, 1979). Therefore no conclusive experimental evidence exists for this mechanism.

In 1979 Pfeuffer proposed a model to account both for the collision coupling model, according to which the β -receptor activates adenylate cyclase by forming transient complexes, and the conclusions of Cassel and Selinger (1978), that the GTP-binding protein oscillates between a GTP- and a GDP-form without leaving the nucleotide binding site empty (Fig. 2).

The collision-coupling model has been analysed mathematically (MacFarlane, 1982). The model is extended into a bidirectional regulation of adenylate cyclase regulation via the allozyme hypothesis for receptor function. The analysis assumes that adenylate cyclase is switched on by a brief interaction with the agonist-occupied activating receptor. It remains on until it is switched off either by a brief interaction with an agonist occupied deactivating receptor or with a postulated basal deactivating system. Only one species of agonist-receptor complex accumulates. Both the activation and deactivation step are regarded as being irreversible and the complete on/off cycle requires a driving energy from the cells. The receptor has a catalytic role which is regulated by the agonist and switches adenylate cyclase activity between its two stable states. MacFarlane illustrates that a scheme derived from Cassel and Selinger (1977) satisfies the postulates of this mathematical model (Fig. 3).

FIG. 2



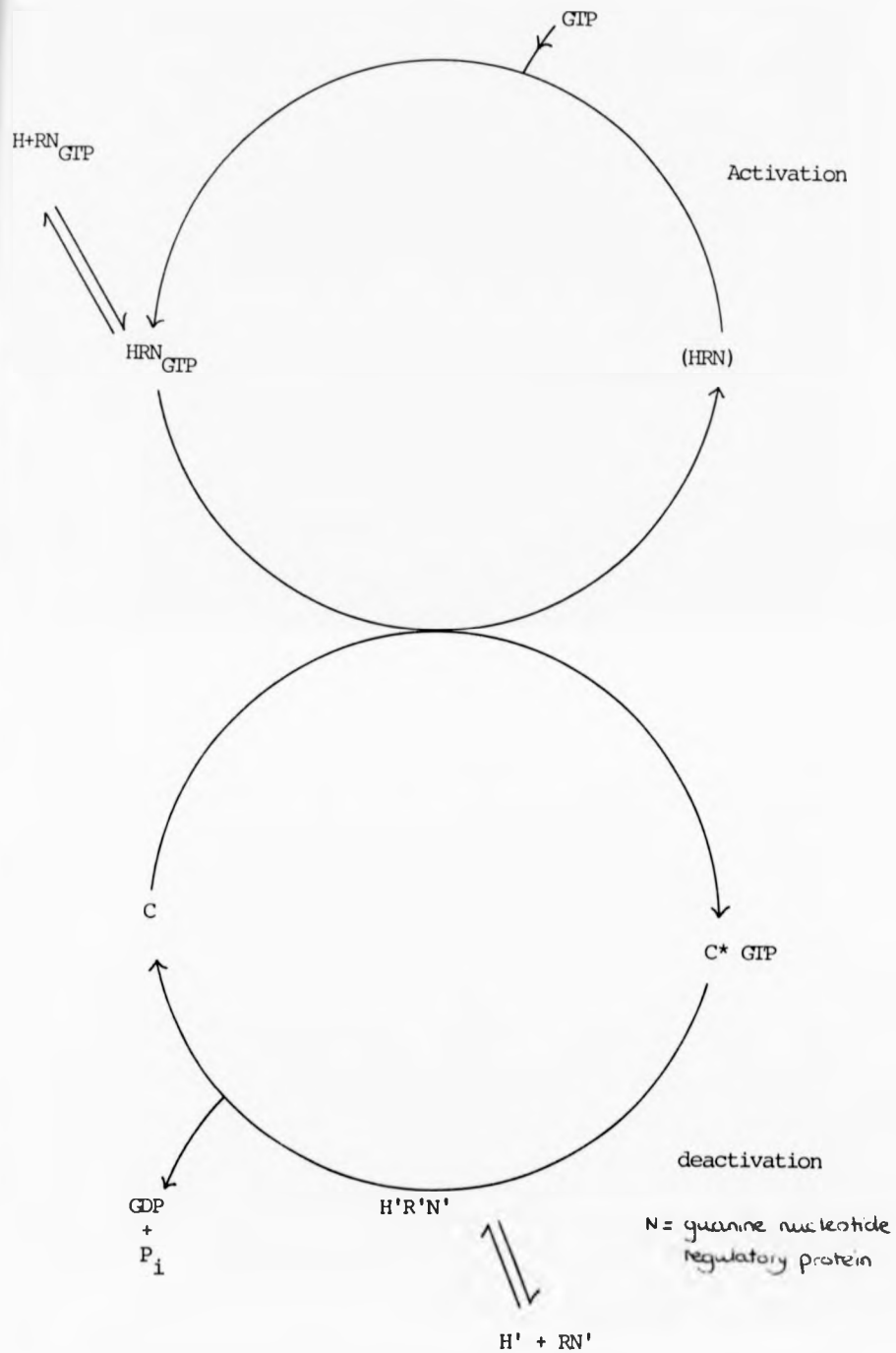
R_H = Hormone-Receptor complex

G = GTP-Binding Protein

C = Catalytic Moiety.

Model Proposed by Pfeuffer (1979)

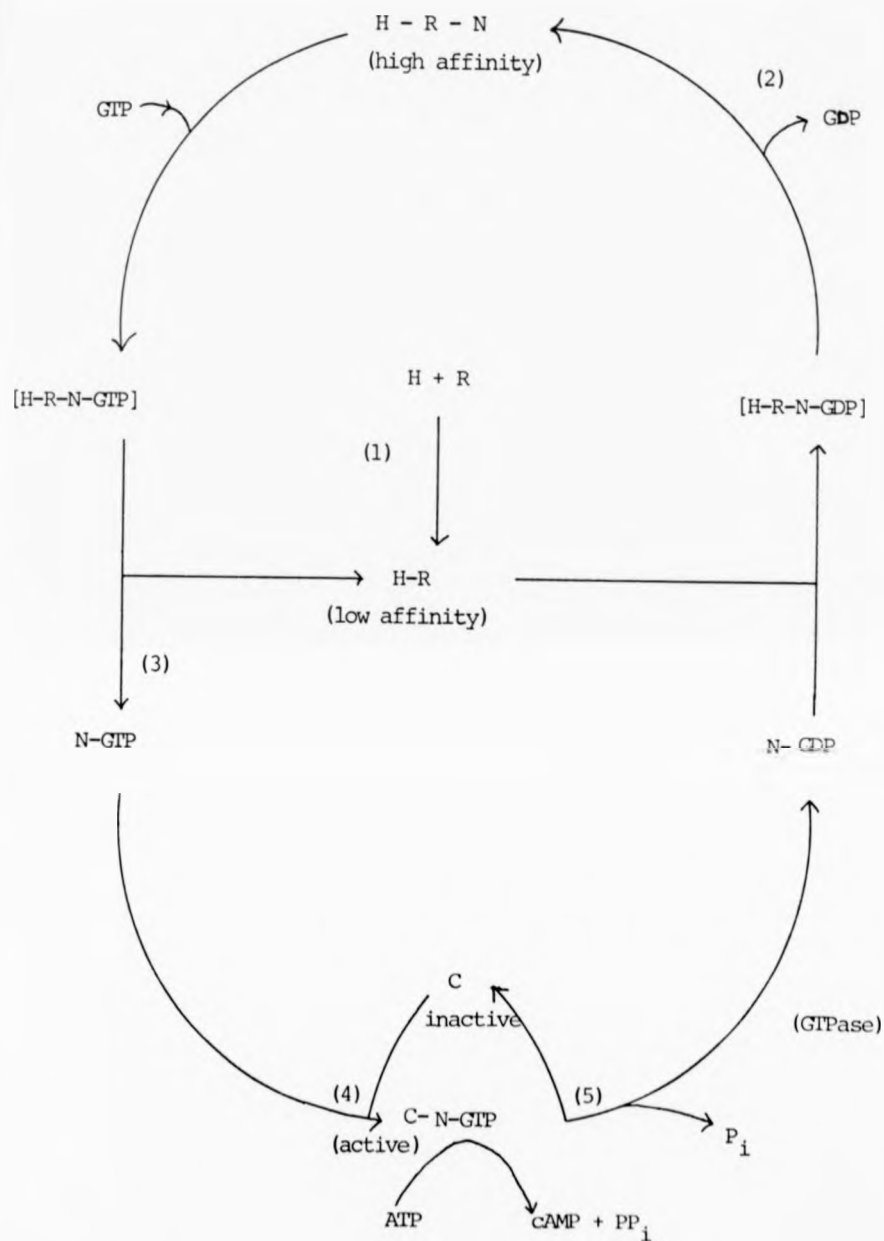
FIG. 3.



A collision-coupling model postulated by MacFarlane (1982) derived from Cassel and Selinger (1977).

DeLean *et al.* (1980) proposed a similar model implying the presence of only one nucleotide binding protein. These authors suggested that the high-affinity state of the receptor is a ternary complex $RHN(N, \text{nucleotide binding protein})$ in which N is occupied by GDP (RHN_{GDP}). In the presence of GTP and hormone this complex rapidly dissociates and the N unit loaded with GTP activates the catalytic unit of adenylate cyclase. The dissociation of N also leads to the formation of an RH complex of low affinity. This model implies that only one nucleotide binding protein is responsible for both hormonal stimulation and decrease in agonist affinity for the receptor. A similar model is also favoured by Limbird (1981). In the model illustrated by Limbird, occupancy of the receptor by agonist promotes or stabilises an interaction between the receptor and the regulatory protein. This interaction represents the high-affinity state of the receptor for agonists and provides a molecular basis for release of GDP from the regulatory protein. Binding of presumably ambient GTP simultaneously dissociates the R-G protein complex which results in a homogeneous population of dissociated receptors possessing a lower affinity for agonist and promotes or stabilises association of the G protein with the catalytic protein. Synthesis of cAMP continues until GTP is hydrolysed by the GTPase 'turn-off' reaction thus leaving GDP on the G protein and reducing affinity of G protein-catalytic protein interaction. A similar system has been described in detail (Stadel *et al.*, 1982; Briggs and Lefkowitz, 1982) - Fig. 4.

FIG. 4



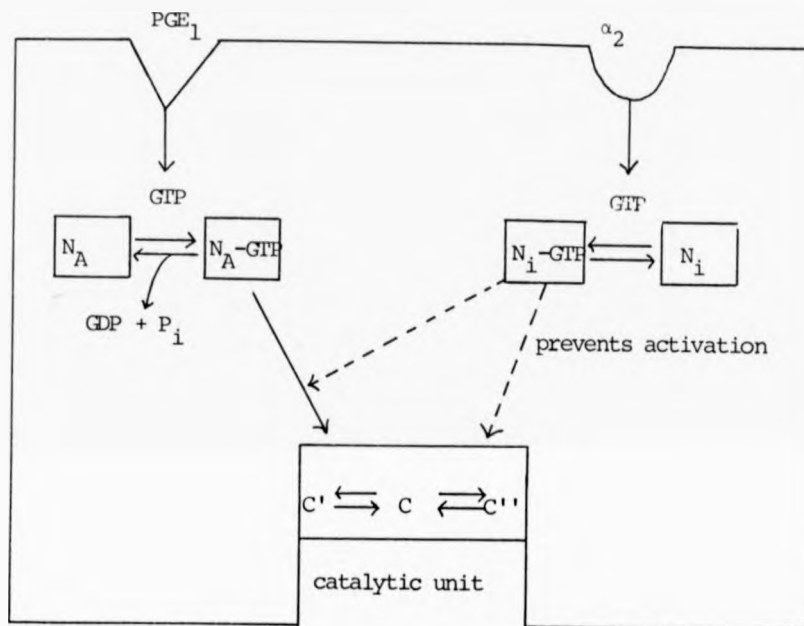
1. Hormone binding
2. Formation of high-affinity state
3. Breakdown of high-affinity state
4. Enzyme activation
5. Enzyme deactivation

R_2H_2C as in fig 2 N as in fig 3
Model proposed by Stadel *et al.* (1982) and Briggs and Lefkowitz (1982)

Rodbell (1980) has proposed a theoretical 'disaggregation-coupling' model involving oligomers of permanently coupled receptor nucleotide site complexes, RN. Hormone binding induces a conformational transition favourable to the subsequent interaction of guanine nucleotides, resulting in a disaggregation of the oligomer into activated monomers, RN, that activate the catalytic unit of the enzyme. This model incorporates the presence of N_s and N_i as well as R_s and R_i . N and R normally exist separately from C as oligomers. In RN complex, R inhibits interaction of N with GTP. Hormone binding to R triggers release of the inhibitory constraints imposed on N with resultant enhanced reaction with GTP followed by breakdown of oligomers to a monomeric RN complex. The latter reacts with catalytic units to form holoenzyme structure. Depending on the type of R and N unit attached to the catalytic unit the holoenzyme exhibits either increased or decreased production of cAMP. This theory is totally different from most of the others proposed and has been criticised (Stadel *et al.*, 1982). It is suggested that this model, like the basic collision coupling model, fails to predict the agonist-specific binding properties. It also fails to accommodate the observation of the presence of the nucleotide regulatory protein exclusively in agonist-prelabelled and not in antagonist-prelabelled soluble receptor preparations.

A more generalised model has been introduced with the definite finding of N_i (Stear *et al.*, 1982) for the platelet membrane adenylate cyclase which is stimulated by PGE_1 but inhibited by the α_2 adrenergic agent, epinephrine (Fig. 5).

FIG. 5



Model proposed by Stear *et al.* (1982).

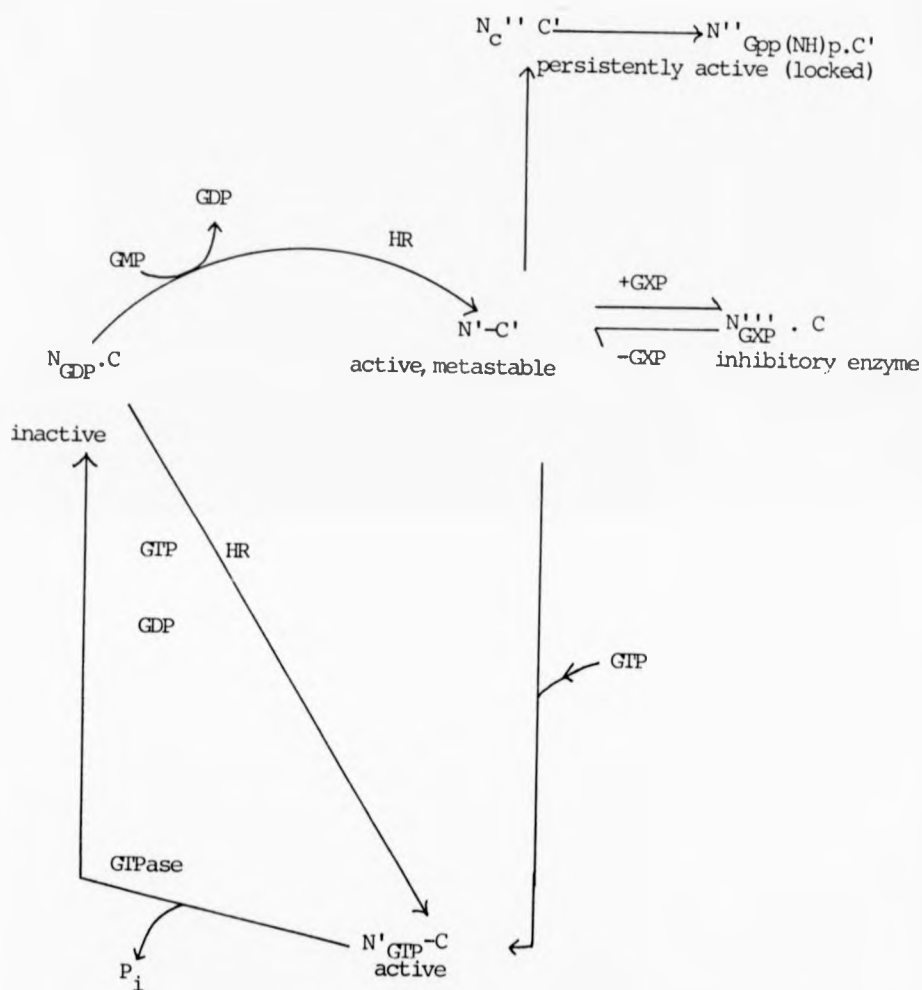
α_2 = α_2 adrenergic receptor for epinephrine

This model suggests that both stimulatory and inhibitory nucleotide states can exist in free and GTP-liganded states, and equilibria between these states is regulated by hormone occupancy of the receptor. Reversion of N_A -GTP to N_A is regulated by PGE_1 -stimulated GTPase. The mechanism by which the reversion of N_I -GTP to N_I occurs has not been properly defined. The catalytic unit (C) can be activated to its cAMP producing state (C') by N_A -GTP. Inhibition-mediated by N_I -GTP results from one or two possible events: either N_I -GTP interferes with conversion of C to C' by N_A -GTP without altering the cycle of $N_A + GTP \rightarrow N_A + GDP + P_i$. A recent study suggests that N_I also has a GTPase associated with it (Jakobs *et al.*, 1982) so that N_I might function in a similar manner to N_A .

Of all the different models suggested, none have been definitely proved for all systems. Therefore further elucidation is necessary. Several of the intermediates have been identified experimentally. HRN complexes have been identified (Rashibaigi and Ruoho, 1981; Stadel *et al.*, 1982). CN complexes have been observed by a variety of techniques such as sucrose density gradient centrifugation (Pfeuffer, 1979) and gel filtration chromatography (Near *et al.*, 1980). Thus it seems that the preliminary outlines of most of these models appear to be correct. However, many different aspects of the system remain unsolved.

A minimum model for the β -adrenergic responsive turkey erythrocyte adenylate cyclase has been proposed to explain the role of non-hydrolysable GTP analogues e.g. Gpp(NH)p (Braun *et al.*, 1982) - Fig. 6 .

FIG. 6.



Minimum model proposed by Braun *et al.* (1982) to explain the role of nonhydrolysable GTP analogues in adenylate cyclase activation.

In this model, the resting state of the regulatory protein, N_{GDP} , is inactive and confers no cyclase activity on the system. The simultaneous presence of a β -agonist bound receptor, [HR], and of GMP induces full activation of N to N'.C'. This state expresses activity only when after treatment with GMP and β -agonist it is washed free of excess GMP. It is not known whether at this stage no GMP remains at the guanyl nucleotide binding site. The GMP/epinephrine-activated species is depicted as N'.C' but may also be depicted as $N'_{GMP} C$. This state can be stabilised by GPP(NH)p such that $N'_{Gpp(NH)p}.C'$ is not readily reverted to basal inactive state unless both β -agonist and excess GTP are present. N'.C' state is fully active in the absence of excess guanyl nucleotide, GXP (GXP = GMP, GDP or GTP), which is inhibitory. This inhibition or expression of catalytic activity is fully relieved upon dilution of GXP from the 'loose' site.

Questions still remain to be solved about the role of multiple receptors and also desensitisation. Several lines of evidence also suggest that other factors may be involved in adenylate cyclase regulation. Protein phosphorylation appears to be important (Ehrlich *et al.*, 1982), possibly of the catalytic component (Akiyama *et al.*, 1983) as does the role of divalent cations (Cech *et al.*, 1980; Minocherhomjee and Roufogalis, 1982).

C H A P T E R I I

MATERIALS AND METHODS

CELL LINESChinese Hamster Fibroblasts CH23

Chinese hamster fibroblasts are a continuous cell line which was first established by Tjio and Puck in 1958 after a biopsy of an inbred Chinese hamster (*Cricetulus griseus*). The cells show typical fibroblastic characteristics; long, thin cells which grow at 37°C as a monolayer adhering to clean glass or plastic surfaces. When confluent the cells show characteristic 'swirls', and at this stage they stop dividing and become quiescent due to density dependent regulation of growth (Stoker and Rubin, 1967; Smith and Martin, 1973; Todaro *et al.*, 1974). Chromosomal pattern indicates that the chromosomal number of CH23 is 21-26 for diploid and from 44-110 for polydiploid cells (~ 8% of the total cell population). The dominant karyotyping is 23 metacentric chromosomes (Ayad and Wright, 1977). The mean generation time is 21 hours. Karyotype analysis has shown the presence of X and Y chromosomes indicating that the cell line originates from a male hamster.

Mouse Lymphoma P388-F36

The P388 lymphoma cell line was originally isolated as described by Fox and Gilbert (1966). P388-F36 was cloned from P388 in this laboratory in 1974 by Ayad and Delinassios. The mean generation time is 13-14 hours to high cell densities. The cells are round and typically lymphoid in nature with large nuclei. The chromosomal composition is

32-46 in the diploid state, but the dominant chromosome number is 36. In the polyploid state which is 27% of the total culture, the composition can vary from 64 to greater than 600. Of the 36 chromosomes in the diploid state, 33 are acrocentric and 3 are metacentric.

Hybrid Cells, PCM3

Fusion of CH23 and P388-F36 by inactivated Sendai virus produced a series of hybrids isolated by a non-selective pressure procedure (Ayad and Delinassios, 1974). Cloning of the hybrids designated PCM produced several clones which could be characterised by their isozyme pattern of esterase activity and their cyclic nucleotide phosphodiesterase content (Ayad and Wright, 1977). One of these clones, PCM3, exhibited unusual growth characteristics in that 80% exist as monolayer PCM3(M) and 20% as suspension PCM3(S). The mean generation time of the hybrid is the same as that for the parent, CH23. The adherent cells are much more regular in shape than CH23. They do not grow to such high cell densities or show tight packing characteristics of CH23 (Ayad and Wright, 1977). Chromosomal stability was attained when 4-5 mouse chromosomes co-existed with no less than 19 hamster chromosomes.

CULTURE METHODS

NCS Medium

764ml	double distilled water
100ml	Eagle's minimal essential medium

100ml	Newborn calf serum
10ml	100mM glutamine
25ml	4.4% sodium bicarbonate sterilised by filtration
0.2ml	0.5g/l streptomycin sulphate BP
0.4ml	250,000 units/ml benzyl penicillin

The pH of the solution was maintained at 7.4 by the presence of sodium bicarbonate and CO₂ by gassing the medium with a mixture of 5% CO₂ in air.. Every 2-3 months the antibiotic content of the medium was changed to gentamycin (10mg/l) and amphotericin (2.5mg/l) or kanomycin (1mg/l) plus amphotericin to avoid the possibility of drug resistance of any contaminating organisms. Medium containing 100ml Dulbecco's minimal essential medium instead of Eagle's minimal essential medium was also used. In this case 25ml of 10% sodium bicarbonate was required to maintain the correct pH. The cells showed the same growth characteristics in either medium.

The cells were cultured in the above medium in:-

100ml	Medical flat bottles
200ml	Medical flat bottles
1000ml	Roux bottles
2000ml	Bellco spinner flask
2000ml	Carrel flasks

At 37°C. The total content of the bottles was kept to a maximum of 10% to facilitate gaseous exchange.

All glassware was washed using RBS-25 or Lipsol detergent, rinsed extensively with tap water and rinsed three times with double distilled water. Sterilisation was achieved by autoclaving for 15 minutes at 20lb/square inch in an R.S.I. series autoclave. Pipettes were soaked in Lysol after use, rinsed in double distilled water and sterilised by heating at 180°C for 3-4 hours in an oven.

Passage of Cells

(a) Suspension Cells

Suspension cells, P388, were subcultured by placing an appropriate aliquot of cells from a confluent culture into a sterilised vessel containing fresh medium. Suspension cells were subcultured when they attained high cell density as determined by cell number. In the case of the spinner flask the culture was started by placing an aliquot of cells from a confluent culture (100ml) in the flask. Medium was added at about 100ml a time, the culture attaining high cell density before further aliquots of fresh medium added. The spinner flask culture was then maintained at 500ml. When cells were required they were removed under sterile conditions using one of the side arms of the flask and the volume maintained by adding fresh medium. Suspension cells were usually passaged at a ratio of 1:10.

(b) Monolayer Cells

CH23 and PCM3(M) were subcultured by removing the medium and washing the cell sheet three times with

trypsin salt solution.

	<u>g/l</u>
NaCl	6.85
KCl	0.54
D-Glucose	8.11
Tris-HCl	6.04
Phenol red	Trace

The pH was maintained at pH 7.7 using 1M HCl. The solution was sterilised by autoclaving and was stored at 4°C.

The cell sheet was removed from the surface by treatment with 0.05% trypsin (2ml per Roux bottle) for 2 minutes at 37°C. After this time the bottle was shaken vigorously until the cell sheet was visible as an opaque layer in the trypsin solution. At this point a small amount of fresh medium was added to inhibit the action of trypsin in preventing cell adhesion. The cells were dispersed by gentle pipetting and an aliquot transferred to a sterile bottle containing fresh medium. Monolayer cells were usually passaged at a ratio of 1:4 or 1:6.

Prevention and Detection of Contamination

Contamination by fungi and bacteria was prevented mainly by use of a good aseptic technique. This involved sterilisation of all glassware and solutions. All operations were performed in a sterile laminar flow hood. The surface of the hood was swabbed with 70% ethanol and flamed before use,

similarly for necks of bottles, pipettes and syringes. The presence of antibiotics in the growth medium also aided the prevention of contamination.

Contamination by fungi and bacteria was detected by routine microscopical examination. The presence of bacteria and fungi was readily detected by staining and light microscopy since the growth of fungi and bacteria exceeded that of cultured cells and for monolayers the medium went cloudy with a characteristic smell. Contamination by mycoplasma and viruses was detected using incorporation of [^3H]thymidine and autoradiography by Dr. Ayad by the method of Nardone *et al.* (1965). This technique allowed the fate of [^3H]thymidine in the cells to be determined. The cells were grown for 6 generations in the presence of [^3H]thymidine on the surface of microscope slides. Fixing of the cells followed by autoradiography for non-infected cells showed [^3H]thymidine to be concentrated in the nuclei whereas in the case of infected cells [^3H]thymidine was distributed throughout the cytoplasm.

Following detection of contamination it was necessary to autoclave all bottles before opening. The bottles were then soaked in Lysol or sodium hypochlorite solution, followed by extensive washing, rinsing and further autoclaving. Fresh supplies of cell lines were then obtained from the freezer.

Storage of Cell Lines

Cells may be stored indefinitely under liquid nitrogen in a biological freezer at -170°C without any noticeable change in viability or morphology provided dimethylsulphoxide (DMSO) is present. DMSO prevents cell damage due to crystal formation.

DMSO is toxic to cells not in a frozen state so it was important that all operations involving the addition of DMSO were performed as rapidly as possible.

One Roux bottle of monolayer cells in mid-log growth phase were treated with 1ml of trypsin in the same manner as for passaging and 8ml of foetal calf serum was added. The cells were dispersed by gentle pipetting and 1ml of DMSO was added. The total 10ml was rapidly divided into sterile plastic cryotubes on ice. The cryotubes were cooled in the vapour phase of liquid nitrogen overnight and then were transferred to liquid nitrogen.

One Roux bottle of suspension cells were centrifuged at 700g (1500 rpm, MSE Major) under sterile conditions. The medium was removed and the cells resuspended in foetal calf serum to a final volume of 9ml and 1ml of DMSO was added on ice. The procedure was then identical to that for monolayer cells.

When the cells were required, thawing was performed as quickly as possible due to the toxicity of DMSO and the contents of 1ml cryotube placed in 100ml of fresh medium in a sterile Roux bottle. For monolayer cells, the medium was removed after initial cell attachment (3-4 hours) and replaced by fresh medium. This allowed removal of any dead cells from the medium.

Viability Estimation and Cell Number

Viability of the cells was measured using the dye exclusion technique and the reagent 1% trypan blue in Hank's Balanced Salt Solution. The method involved mixing cells in suspension with dye (1:1) and leaving for 10 minutes at room temperature. The viable cells excluded the dye whereas the non-viable cells stained deep blue. The absolute viability of monolayer layers could only be measured when the cells were grown on the surface of glass coverslips. However, a small aliquot of cells obtained by trypsinization could be examined in an identical manner to suspension cells.

The cell number was obtained using a haemocytometer. A drop of the suspension was placed in the chamber giving a known volume. The number of cells was obtained by examining the haemocytometer microscopically and counting the number of cells in each square of the grid. From the volume contained in each square it is possible to calculate the number of cells per ml. Usually about 100 squares were counted and an average obtained. Viability studies were expressed as a percentage. The percentage viability of cells used for experiments always exceeded 96%.

PROTEIN DETERMINATION

Protein Concentration was determined by two methods.

(a) The Method of Lowry et al., 1951

This method was used for samples from intact cell incubations where the protein was already in solution.

(b) The Method of Peterson, 1977, a modified method of Lowry

This method was used for particulate samples which were solubilised using sodium dodecyl sulphate (SDS) and NaOH to avoid lipid oxidation and possible interference from boiling samples in order to solubilise.

Method of Lowry

REAGENTS

- A. 2% Na_2CO_3 in 0.1M NaOH.
- B. 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.
- C. 1% NaK Tartrate
- D. 50ml A + 0.5ml B + 0.5ml C freshly prepared.
- E. Diluted Folin-Ciocalteu phenol reagent (1:2 in double distilled water).
- F. Standard protein solution - bovine serum albumin (BSA) fraction V.

A stock solution of 1mg/ml was prepared and appropriate dilutions made to give a final concentration of 0-300 $\mu\text{g}/\text{ml}$ in 200 μl .

PROCEDURE

Standards and samples were made to a volume of 200 μl with double distilled water. A buffer blank was also included. 2.5ml of solution D was added, the solutions mixed and left for 10 minutes. After this time 0.25ml of solution E was added with rapid mixing and the tubes left for 40-60 minutes

at room temperature for colour development. The optical density at 750nm was measured using a Beckman 35 spectrophotometer.

A standard curve was obtained by plotting the absorbance at 750nm against the standard protein concentration ($\mu\text{g/ml}$) or using a (log-log) plot on a computer programme. The sample concentrations were calculated from the linear portion of the curve.

Method of Peterson

REAGENTS

- A₁. A solution of 0.1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.2% NaK Tartrate, 10% Na_2CO_3 .
- A₂. 10% SDS.
- A₃. 0.8M NaOH.
- B. Diluted Folin-Ciocalteu reagent (1:5 in double distilled water).
- C. Standard protein solution
500 $\mu\text{g/ml}$ BSA Fraction V with 1mg/ml sodium azide as non-interfering bacteriocide.
- D₁. 0.1% sodium deoxycholate
- D₂. 70% trichloroacetic acid (TCA)

Reagent A was prepared by mixing equal volumes of A₁, A₂, A₃ and double distilled water.

The standards were made to the appropriate final concentration (0-200 μ g/ml) in a total volume of 1ml with double distilled water. 1ml of reagent A was added and the solutions left for 10 minutes. 0.5ml of reagent B was then added with rapid mixing and the solutions left for 30-60 minutes at room temperature for colour development. The optical density at 750nm was then measured.

Samples were treated to remove any interfering substances. An appropriate volume of sample was brought to 1ml with double distilled water and 0.1ml of reagent D₁ was added. After 10 minutes at room temperature 0.1ml of reagent D₂ was added, the solutions mixed and left for a further 10 minutes prior to centrifugation for 2 minutes in an Eppendorf microfuge. The supernatant was discarded and the pellet solubilised in 1ml of 1:1 mixture of A₂ and A₃. A buffer control was treated identically to the samples. The samples were then treated in the same manner as the standards except that 0.5ml was added to 1ml of double distilled water and 0.5ml of 1:1 mixture of A₁ and double distilled water was added. After 10 minutes 0.5ml of B was added and the samples and buffer control treated identically to standards.

MEASUREMENT OF cAMP LEVELS IN INTACT CELLS

I. Incubation with hormones and other agents

(a) Monolayer cells

In order to examine the effect of various agents on the cAMP levels of intact cells growing as a monolayer, CH23 and PCM3(M), it was necessary to grow the cells on the bottom of

glass scintillation vials. The vials were washed and sterilised as previously described for culture bottles. Gassing of large numbers of these vials would have been impractical, so the biological buffer, N-2-hydroxy ethyl piperazine-N'-2-ethane sulphonic acid (HEPES) was added to maintain the pH. A concentration of 20mM was added to the medium before addition to the vials.

A Roux bottle containing a confluent cell sheet was passaged as normal and an aliquot of trypsin solution containing cells was transferred to the appropriate volume of medium containing 20mM HEPES. 2ml of this solution were transferred into each vial and the cells were grown at 37°C. When the cells reached the appropriate cell density the medium was removed gently using a vacuum line and the cell sheet was washed twice with 1ml of Hank's Balanced Salt Solution (HBSS⁻).

	<u>g/l</u>
NaCl	8.34
KCl	0.40
Na ₂ HPO ₄ · 2H ₂ O	0.06
KH ₂ PO ₄	0.06
D-glucose	1.00
Phenol red	trace

The pH was maintained at 7.6 at 20°C using 1M HCl. The solution was stored at 4°C.

The appropriate volume of HBSS⁻⁻⁻ was added to each vial to give a final volume of 1ml when 3-isobutylmethyl xanthine (IBMX), hormone and agents had been added. IBMX was used as a phosphodiesterase inhibitor at a final concentration of 1mM. The cells were preincubated for 10 minutes at 37°C in a water-bath with IBMX. Following this time addition of agents e.g. colchicine, ATP were added to initiate the incubation. The incubation time was generally 10 minutes. Hormone, PGE₁ was then added and the cells further incubated for 10 minutes. The reaction was stopped by transfer of the vials to a boiling-water bath for 5 minutes. The vials were allowed to cool and were sonicated for 10 seconds at an amplitude of 7 microns using an MSE Soniprep 150. The samples were then transferred to Eppendorf tubes and stored at -20°C to await cAMP assay. Immediately prior to cAMP assay the tubes were thawed and centrifuged for 2 minutes in an Eppendorf Microfuge to remove cell debris.

(b) Suspension cells

Measurement of stimulation of cAMP levels in suspension required that relatively large quantities of cells were grown to the required cell density and the medium was removed by centrifugation at 700g for 5 minutes (1500 rpm MSE major). The pellet was washed twice by centrifugation in HBSS⁻⁻⁻, and the final pellet was resuspended in the appropriate volume of buffer to give a protein concentration of approximately 200µg/vial. The resuspended material was divided into aliquots in glass scintillation vials so that the final volume when hormone, agents and IBMX were added was 1ml. The cells were then incubated in the same manner as monolayer cells.

For both monolayer and suspension cells, vials containing cells and HBSS⁻⁻ of the same volume as the incubations were treated identically to the incubated samples and aliquots taken for protein determination by the method of Lowry.

For incubations involving nucleotides, HBSS⁻⁻ was replaced by incubation medium.

	<u>g/l</u>
NaCl	8.0
KCl	0.4
D-Glucose	5.0
MgCl ₂	1.0
Na-HEPES	6.0

The pH was maintained at 7.7 at 37°C and the solutions stored at 4°C.

This buffer is essentially identical to HBSS⁻⁻ but provides the necessary Mg²⁺ for the cell to utilise ATP and also contains the biological buffer HEPES which protects the cell from osmotic damage due to the presence of large ionic structures.

For incubations requiring the simultaneous presence of two agents e.g. KCN and iodoacetate, the two agents were mixed in appropriate concentrations before addition of volume to give final concentration to triplicate vials.

II. cAMP Assay

The assay used had to be sensitive to levels of cAMP less than 1 pmole and capable of determining the amount of cAMP in a large number of samples per assay, and also relatively cheap. The method chosen was a saturation binding assay of Brown *et al.*, 1971 as modified by Tovey *et al.*, 1974.

REAGENTS

(i) cAMP standards:- standards were prepared by serial dilution to give a final concentration of 0.3, 1, 3, 6, 10 pmoles/20 μ l in double distilled water. The solutions were stored at -20°C.

(ii) Tritiated cAMP: [8-³H]cAMP (30-50 Ci/mmol) was diluted 1:1500 times for the assay in double distilled water. 1ml aliquots were stored in disposable Eppendorf tubes at -20°C.

(iii) Two buffers were used.

(a) Tris-EDTA buffer was used with binding protein isolated from bovine adrenals.

50mM Tris-HCl pH 7.4 at 20°C

4mM EDTA

6mM β -mercaptoethanol.

(b) For the binding protein isolated from bovine cardiac muscle 50mM phosphate, 4mM β -mercaptoethanol pH 7.0 was used. The pH was maintained using 1M NaOH.

(iv) Albumin-charcoal.

1g BSA fraction V

2g Charcoal Norit SX-1

50ml of the appropriate buffer.

The solution was stirred to give a well dispersed solution at 0°C.

(v) Scintillator - three types were used

(a) 4g PPO (2,5-diphenyloxazole)

0.2g POPOP (1,4-di-2(5-phenyloxazolyl benzene)

667ml Scintillation grade toluene, sulphur free

333ml Triton X-100 scintillation grade

3.5ml was used/0.5ml sample.

(b) PCS 2ml scintillator/0.5ml sample

(c) Readysolv. EP 3.5ml scintillator/0.5ml sample.

(vi) Binding Protein.

PROCEDURE

The assay was performed in duplicate in plastic Eppendorf tubes arranged in rows of 12 in a grid over a tray of ice. The solutions were added according to the following scheme.

	20μl	100μl		20μl		200μl	
	[8- ³ H]cAMP	Sample	Buffer	Standard	Distilled water	Buffer	Binding protein
Blank	+	-	+	-	+	+	-
S ₀	+	-	+	-	+	-	+
Sample	+	+	-	-	+	-	+
Standard	+	-	+	+	-	-	+

+ indicates an addition

The assay was initiated by addition of binding protein to successive rows of 12 at 5-minute intervals. The solutions were incubated for 90 minutes at 4°C. The reaction was stopped by addition of 0.5ml charcoal-albumin to the tubes in each row, again in five minute intervals. Each tube was immediately vortexed and centrifuged in an eppendorf microfuge for 2 minutes. The tubes were removed from the centrifuge and replaced in the ice tray until the adjacent row of tubes was centrifuged. 0.5ml of the supernatant was then removed and placed in plastic inserts with the appropriate volume of scintillator. The solution was mixed thoroughly and the vials were counted (usually for 10,000 cpm or 20 minutes) using an Intertechnique SP4000 scintillation counter and the results were calculated using a computer programme.

The theory of the assay is described by Brown *et al.* (1972). The binding protein from both sources is a protein kinase which has a high affinity for cAMP. The charcoal-albumin mixture binds the free nucleotides so that binding protein plus bound nucleotides remains in the supernatant. Thus the S_0 gives maximal counts since all of the $[8-^3H]cAMP$ exists bound to the protein. When unlabelled cAMP is added as standard, or sample, the radioactive nucleotide is displaced from the soluble binding protein and the counts decrease with increasing amounts of unlabelled cAMP added. A blank value is obtained when no binding protein is present and virtually all of the radioactive cAMP is bound by charcoal.

$$\frac{S_o}{S} = \frac{S_o \text{ cpm} - \text{Blank cpm}}{\text{Standard (or sample) cpm} - \text{Blank cpm}}$$

A plot of S_o/S against concentration of standard is used to prepare a standard curve. The values for the samples are obtained using the same ratio. The plot is linear up to 10 pmoles/20 μ l. (see Fig. 7) Volume corrections and protein concentration give pmoles cAMP produced/mg protein/10 minutes.

Preparation of cAMP Binding Protein

I. From Bovine Adrenals

Bovine adrenals were collected as soon as possible after slaughter, kept on ice, and dissected at 4°C. All fatty tissue was removed, along with the medullae, and the cortices were chopped and homogenised with 1.5 volumes of an ice-cold medium of:

0.25M Sucrose

50mM Tris-HCl pH 7.4 at 20°C

25mM KCl

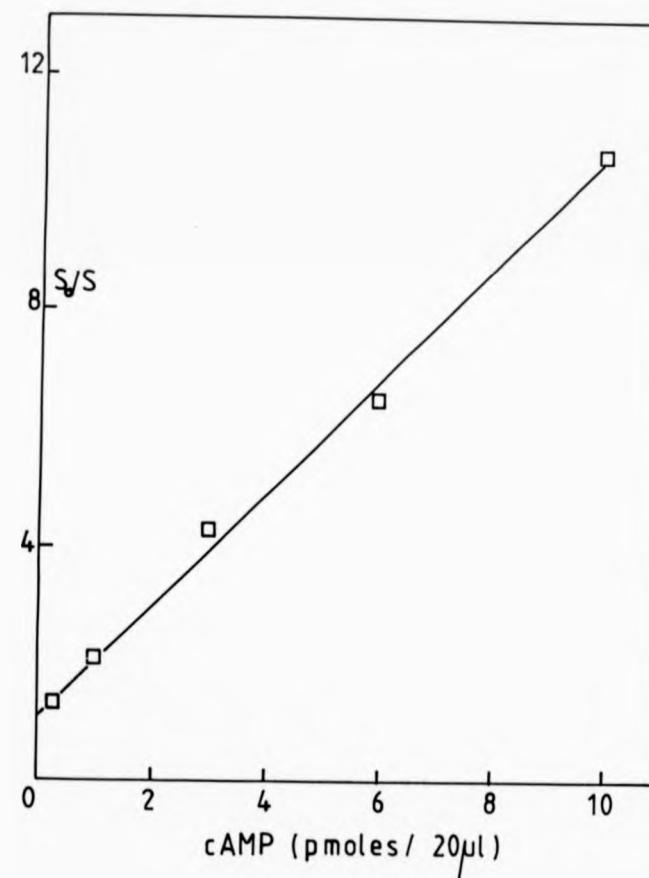
5mM MgCl₂

The homogenate was centrifuged at 2000g for 5 minutes at 5°C. The pellet was discarded and the supernatant centrifuged at 5000g for 15 minutes. The resultant supernatant was stored in 1ml aliquots at -20°C. This method is similar to that described by Brown *et al.* (1971).

FIG. 7. cAMP Standard Curve

A typical standard curve obtained using 1/100 dilution of binding protein obtained from bovine cardiac muscle.

$$\frac{S_0}{S} = \frac{\text{total(c.p.m.)} - \text{blank(c.p.m.)}}{\text{sample(c.p.m.)} - \text{blank(c.p.m.)}}$$



II. From Bovine Cardiac Muscle

This protein was prepared essentially as described by Rubin *et al.* (1975). All operations were performed at 4°C and all buffers contained 4mM mercaptoethanol. The procedure involved four stages of purification.

(1) Initial Preparations

(a) Fresh beef heart was obtained from the abattoir and the pericardium and fat tissue were removed. The heart was chopped into small pieces and then minced as finely as possible.

(b) The minced material, about 2kg in wet weight, was mixed with 4L of 40mM potassium phosphate buffer pH 6.1, containing 2mM EDTA, and homogenized in small batches at high speed for 1 minute in a Waring Blender.

(c) The homogenate was centrifuged at 10,000g for 10 minutes and the supernatant fluid was collected and passed through Whatman number 54 filter paper. The pellet was extracted twice more with 1L of the same buffer and the extracts were combined.

(2) Ammonium Sulphate Precipitation

(a) The pooled extracts were brought to 55% saturation by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ (320g/l) and the pH was maintained between 7 and 8 by addition of NH_4OH (5ml of concentrated $\text{NH}_4\text{OH/L}$).

(b) The protein was allowed to precipitate for 2.5-3 hours and then collected by centrifugation at 10,000g for 10 minutes. The supernatant was discarded and the precipitate was dissolved in 500ml of 50mM Tris buffer pH 7.6, containing 10mM NaCl. This solution was dialysed overnight against 5L of the same buffer to remove NH_4OH .

All Tris buffers were adjusted to pH 7.6 by the addition of concentrated HCl.

(3) DEAE-Sephadex Purification

(a) The dialysed enzyme preparation was stirred for 1 hour with 800ml of DEAE-Sephadex that had previously been equilibrated with 50mM Tris buffer, 10mM NaCl, pH 7.6. The kinase was absorbed by the resin under these conditions and was collected by filtration on a Buchner funnel with Whatman number 54 paper.

(b) The gel was washed with 3L of Tris-NaCl buffer or until the filtrate became colourless. The gel was then suspended in 200ml of 50 mM Tris buffer, pH 7.6 containing 0.3M NaCl and stirred for 45 minutes.

(c) The DEAE-Sephadex was collected by filtration and was washed twice with 400ml of 50mM Tris pH 7.6, 0.3M NaCl. The combined filtrates containing the protein kinase activity were brought to 35% saturation by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ (119 g/l). The pH was maintained between 7 and 8.

(d) After 1 hour, the precipitate was collected by centrifugation at 10,000g for 10 minutes and was discarded. The supernatant was brought to 75% saturation by addition of $(\text{NH}_4)_2\text{SO}_4$ (258 g/l). The precipitate formed after 1 hour was collected at 10,000g. The pellet was suspended in a minimal volume of 50mM phosphate buffer pH 7.0 and was dialysed against 2L of the same buffer overnight.

At this stage the enzyme preparation could be stored at -20°C .

(4) Alumina Cy Purification

(a) The dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction was adsorbed to alumina Cy by mixing the protein solution with the gel (2.2g of alumina Cy per mg of protein) that had previously been equilibrated with 2 x 500ml portions of 50mM phosphate buffer pH 7.0.

(b) After stirring for 45 minutes the gel was collected by centrifugation at 3000g for 5 minutes and the supernatant discarded.

(c) The gel was resuspended and washed twice with 250ml of the same buffer. Protein kinase was eluted by suspending the alumina Cy gel in 0.15M phosphate buffer, pH 7.0, stirring for 15 minutes, and removing the gel by centrifugation. Elution was carried out once with 400ml of buffer and twice with 250ml buffer. The supernatants were pooled and concentrated by addition of $(\text{NH}_4)_2\text{SO}_4$ to 80% saturation (569g/l). After centrifugation the pellet was suspended in 100ml of 0.05M Tris buffer pH 7.6 containing 10mM NaCl and dialysed overnight against the same buffer, 2 x 1L changes.

The resulting enzyme was stored in 0.5ml aliquots at -20°C prior to use and diluted to the necessary concentration immediately before use in cAMP assay.

Studies on Broken Cell Preparations

Preparation of Crude Homogenates

(a) MONOLAYER CELLS: CH23 and PCM3(M) were grown in bulk in either Roux bottles or in 1 l roller bottles until confluent. The medium was poured off and the cell sheet covered with a small volume of HBSS⁻⁻⁻. The cell sheet was removed into the buffer at room temperature by gentle scraping with a rubber policeman. The cells obtained in this manner were washed twice by centrifugation at 700g for 5 minutes in HBSS⁻⁻⁻. The final pellet was redissolved in 3ml homogenisation buffer.

50mM Tris-HCl pH 7.4 at 20°C

5mM MgCl_2

1mM Dithiothreitol

0.25M Sucrose

All steps subsequent to this were performed at 4°C . The cell pellet was homogenised using a glass hand homogeniser. CH23 were subjected to ten up and down strokes, PCM3 to 5 up and down strokes. Samples for protein determination were removed into Eppendorf tubes and stored at -20°C . The remainder was divided into aliquots and stored in the biological freezer. Immediately prior to use the homogenate was thawed and diluted with homogenisation buffer to give the appropriate protein concentration.

(b) SUSPENSION CELLS: P388 were grown in bulk to the required density in Carrel flasks or Bellco Spinner flasks. The medium was removed by centrifugation at 700g for 5 minutes at room temperature. The pellet was washed twice by centrifugation with HBSS⁻⁻⁻. All steps subsequent to this were performed at 4°C. The pellet was resuspended in 3ml homogenisation buffer and homogenised in a loose-fitting teflon homogeniser by ten up and down strokes with a Tri-R Stirrer set at speed 4. The homogenate was treated identically to that obtained from monolayer cells.

Preparation of Purified Membranes

Purified plasma membranes were obtained essentially according to the methods of Pike and Lefkowitz, 1980 and Hughes, 1980. All steps were performed at 0°C. The crude homogenate was prepared as described above.

(a) Crude homogenate was centrifuged at 700g for 10 minutes and the pellet was discarded.

(b) The homogenate supernatant was centrifuged at 40,000g (23,500 rpm Beckman L565B, SW 65L Ti). The supernatant after this centrifugation step was stored in the biological freezer and referred to as SI.

(c) The pellet was resuspended in 10ml of a buffer containing 75mM Tris-HCl pH 6.5, 25mM MgCl₂. 5ml of this solution was layered carefully onto 10ml of 35% sucrose and centrifuged for 8 minutes at 1200g. The upper layer was collected carefully using a long-form pasteur pipette.

(d) The upper layer so obtained was centrifuged for 10 minutes at 40,000g. The supernatant after this

centrifugation step was stored in the biological freezer and referred to as SII.

(e) The pellet was resuspended in 1ml buffer

(i) Homogenisation buffer if the membranes were to be used for assay of adenylate cyclase activity.

(ii) 25mM $MgCl_2$, 75mM Tris, 6mM β -mercaptoethanol pH 7.6 if the membranes were to be used for assay of GTPase activity.

Adenylate Cyclase Assay

Reagents

1. 0.1M Dithiothreitol.
2. Homogenisation buffer (see above)
3. Tris HCl 0.5M, pH 7.6.

25ml of 2M Tris and 25ml of double distilled water were mixed and the pH adjusted to 7.6 with 1M NaOH at 20°C. Volume was adjusted to 100ml with double distilled water.

4. 1% Albumin
5. 4mM IBMX
6. 100mM ATP pH 7.0

324mg of ATP were dissolved in 4ml of ice-cold double distilled water. 2M Tris was added in 20 μ l aliquots until the pH was 7.0 at 20°C. The volume was then adjusted to 5ml with double distilled water. ATP solutions were stored in aliquots at -20°C.

7. 0.3M HCl.
8. 1M Tris HCl pH 7.8

Procedure

The procedure was based on that of Salomon (1979). An assay cocktail was prepared to give the following final concentrations in the assay. Due account was taken for the presence of dithiothreitol, MgCl_2 and Tris-HCl in homogenisation buffer.

Creatine phosphate	5mM
Creatine phosphokinase	50 units/ml
Tris-HCl pH 7.8	50mM
MgCl_2	5mM
IBMX	1mM
ATP	2mM
Albumin	0.055%
Dithiothreitol	1mM

80 μ l of assay cocktail and 20 μ l of hormone or agent (10x desired final concentration) were preincubated in a water bath at 30°C for 10 minutes. After this time the incubation was initiated by the addition of 100 μ l of membrane preparation containing approximately 50 μ g of protein to duplicate tubes at 30 second intervals. The incubation time was 10 minutes. After this time 100 μ l of 0.3M HCl was added and the tubes transferred to an 85°C water bath for 45 minutes. The tubes were allowed to cool and 100 μ l of 0.5M Tris, pH 7.6 was added. The supernatant from centrifugation for 2 minutes in an Eppendorf microfuge was stored at -20°C to await cAMP assay.

Controls were set up in which the acid was added prior to membrane preparation to account for cAMP formed prior to the incubation. This value was subtracted from all other values. Standards were set up in which the membrane preparation was replaced by homogenisation buffer. The standards were then treated identically to incubation and were used to establish cAMP standard curve for cAMP assay.

For experiments studying the effect of various concentrations of an agent on a fixed concentration of PGE_1 , the hormone was included in the 80 μl assay buffer and the agent added at 10x final concentration in 20 μl .

Treatment with Cholera Toxin

(a) INTACT CELLS

Cholera toxin was added to cells to give a final concentration of $1.2 \times 10^{-9}\text{M}$ and the cells were incubated for 3-4 hours at 37°C .

(b) HOMOGENATE OR MEMBRANE PREPARATION

The homogenate and membranes were treated according to the method of Johnson *et al.* (1978). The membranous fraction was preactivated with 20mM dithiothreitol and 2.5mM NAD^+ for 10 minutes at 30°C . The fraction was then treated with cholera toxin at a concentration of $1.2 \times 10^{-6}\text{M}$ for a further 10 minutes.

Treatment of Membranes with N-Ethylmaleimide

Membranes were pretreated with N-Ethylmaleimide before assay for GTPase, according to the procedure employed by Lester *et al.*, 1982).

1ml membranes in 75mM Tris 25mM MgCl₂ 6mM β-mercaptoethanol
pH 7.6,

(0.5-1mg protein/ml)

40,000g, 10'
0°C

Pellet resuspended in 1ml of 30mM Tris 10mM MgCl₂ pH 7.4

N-Ethylmaleimide final
concentration 10mM
0°C, 30'

Add 15mM β-mercaptoethanol to quench reaction

0°C, 5'

40,000g, 10'

Pellet resuspended in 1ml 30mM Tris, 10mM MgCl₂, 2mM
β-mercaptoethanol, pH 7.4

GTPase Assay

GTPase activity was measured by the release of $[^{32}\text{PO}_4^{3-}]$ from guanosine-5'- $[-\gamma\text{-}^{32}\text{P}]$ triphosphate essentially as described by Cassel and Selinger (1976) with modifications by Pike and Lefkowitz (1980).

Reagents1. Assay Buffer - 20 μ l per incubation

An assay buffer was prepared to give the following final concentrations in the assay.

$[^{32}\text{P}]\text{GTP}$	0.25 μ M
App[NH]p	0.5mM
ATP	0.1mM
Phosphocreatine	3.0mM
Creatine Phosphokinase	75 U/ml
MgCl_2	10mM
Tris-HCl, pH 7.4	30mM
β -mercaptoethanol	2mM

Account was taken for the decay of $[^{32}\text{P}]\text{GTP}$ and concentration was calculated daily.

2. Hormone

10 μ l of 5 x the desired concentration.

3. Plasma Membrane.

20 μ l (20-50 μ g protein), that had previously been treated with N-ethylmaleimide, in 10mM MgCl₂, 30mM Tris, 2mM β -mercaptoethanol, pH 7.4 containing 0.1mM EGTA.

Procedure

(a) Assay buffer was distributed in 20 μ l aliquots into Eppendorf tubes held in a grid in an ice-tray.

(b) 10 μ l of drug with 5x the desired final concentration was added to each tube.

(c) All tubes were pre-equilibrated at 37°C for 5 minutes.

(d) Incubation was initiated by addition of suitably diluted plasma membrane^(20 μ g) to duplicate tubes, at 30 second intervals.

(e) The reaction was stopped by addition of 0.55ml of a 5% suspension of charcoal Norit A in 20mM phosphate pH 7.0 to adsorb free nucleotide.

(f) Assay tubes were centrifuged for 2 minutes at 10,000g in Eppendorf microfuge.

(g) 200 μ l of supernatant were added to 2.4ml of Readyso^lv. EP scintillator fluid for counting [³²P]

Four control tubes were prepared containing buffer instead of membranes. After incubation in an identical manner to samples, 0.55ml of 5% charcoal suspension was added to two of them and labelled 'blanks'. The remaining two tubes were treated with 0.55ml of 20mM phosphate pH 7.0 without charcoal and labelled as S₀.

GTPase activity calculated

$$\frac{\text{pmoles } [^{32}\text{P}]}{\text{mg protein} \times \text{min}} = \frac{\text{pmoles GTP hydrolysed}}{\text{mg protein} \times \text{min}} = \frac{(S-B) \times 12.5 \times 1000}{(S_0 - B) \cdot P_{\mu\text{g}} \cdot T_{\text{min}}}$$

where S = cpm sample
 S_0 = total cpm added/tube
 B = blank cpm
 P = protein concentration in μg
 T = time in minutes

SDS-Polyacrylamide Gel Electrophoresis

Essentially the method of Laemmli (1970) and Laemmli and Favre (1973).

I. Preparation of Gels

The gel system employed was a 3% stacking gel, followed by 8% and 13% running gels.

Reagents

- (a) 30% acrylamide
 0.8% N,N'-bis methylene acrylamide
- (b) 1.5M Tris-HCl pH 8.8
- (c) 0.5M Tris-HCl, pH 6.8
- (d) TEMED, N,N,N',N'-tetra-methyl-ethylene-diamine
- (e) 10% ammonium persulphate, freshly prepared.
- (f) 10% SDS

Preparation of Cassettes

Cassettes were made using apparatus from Biorad. A sandwich was made of two glass plates (16cm x 18cm) separated by PVC spacer strips positioned along the shorter edges and the plates clamped together with polycarbonate clamps. Care was taken to ensure that the spacers reached completely to the bottom of the plates so that no space remained for the gel solution to leak through. This process was aided by preparing the cassettes on the underside of the Biorad base thus ensuring that the end of the gel plates and the end of the spacers were level. The cassettes so prepared were slotted into the positions in the Biorad base and secured using locking nuts. This ensured a close fit at the bottom due to the presence of a rubber seal, reducing the risk of the solution leaking out.

Preparation of Gels

The gel system employed was a discontinuous one consisting of 13% and 8% running solutions and a 3% stacking gel. Two gels were prepared simultaneously, 50ml final volume of each solution was required.

13% Gel

12.5ml	Tris-HCl 1.5M, pH 8.8, 20°C
21.6ml	30% acrylamide, 0.8% bis acrylamide
0.5ml	10% SDS
14.95ml	H ₂ O

8% Gel

12.5ml	Tris-HCl 1.5M, pH 8.8, 20°C
13.3ml	30% acrylamide, 0.8% bis acrylamide
0.5ml	10% SDS
23.25ml	H ₂ O

3% Stacking Gel

12.5ml	Tris-HCl 0.5M, pH 6.8 20°C
5ml	30% acrylamide, 0.8% bis acrylamide
0.3ml	10% SDS
31.55ml	H ₂ O

Each solution was prepared as above and degassed in a conical Buchner flask. Following degassing the flask was covered in aluminium foil. Polymerisation was initiated by the addition of 0.05ml TEMED and 0.4ml ammonium persulphate. The mixture was placed in the gel cassettes using a long form pasteur pipette. 13% gel was added first to a depth of 6cm and a small layer of 95% ethanol was placed on top. The gels were allowed to polymerise at room temperature for at least 30 minutes. After this time the ethanol was poured off and the surface of the gel washed with 8% solution. The degassed 8% gel solution plus TEMED and ammonium persulphate was placed on top of the polymerised 13% gel using a pasteur pipette to depth of a further 6cm. Once again a small layer of 95% ethanol was layered on top and the gel allowed to polymerise for 30 minutes. The ethanol was then poured off and the surface of the gel washed with 3% gel solution. The stacking gel solution was placed on top of the polymerised 8% gel,

using a pasteur pipette, to the top of the cassettes (4cm). Teflon combs which formed the sample wells were inserted carefully to avoid bubbles. The stacking gel was allowed to polymerise for 30 minutes. Following this time the combs were removed and the wells filled with water. The gels were stored as cassettes surrounded by moist tissues in a plastic tupperware container.

Running of Gels

Reagents

(a) Electrophoresis buffer:

0.025M Tris-HCl pH 8.3, 20°C

1.192M Glycine

0.1% SDS

0.1% β -mercaptoethanol.

(b) Sample buffer:

0.0625M Tris-HCl, pH 6.8, 20°C

4mM SDS

10% Glycerol

100mM Dithiothreitol

Bromophenol blue, trace.

(c) Standards:

Ovalbumin 1mg/ml

BSA 0.5mg/ml

Ferritin 5mg/ml

Trypsin inhibitor 1mg/ml

The above were prepared in sample buffer.

Procedure

Samples were diluted into sample buffer to give the appropriate concentration of protein (50 μ g per well) in 40 μ l samples. For plasma membrane preparations the protein concentration was very low. The sample had to be used undiluted so dithiothreitol, glycerol, bromophenol blue were added as solid to the sample. The samples were boiled for two minutes, for treatment with SDS. The water was removed from the wells of the gel and the wells washed with electrophoresis buffer. The wells were then carefully filled with electrophoresis buffer to avoid bubbles. Samples were underlayered using a 100 μ l Hamilton syringe. 10 μ l of the standard solution was added using a 25 μ l Hamilton syringe.

The gel cassette was slotted into the position in the upper reservoir of the Biorad Protean tank, and secured in position using locking nuts. When only one gel was used a blank was placed into the vacant position i.e. a gel cassette without gel. The gel cassettes attached to the upper reservoir were lowered into the electrophoresis tank containing electrophoresis buffer. The upper reservoir was filled gently with buffer to prevent the samples from being washed out of the wells. The electrophoresis tank was placed on a magnetic stirrer. The electrophoresis tank was attached to a power pack and run at 25mA until the bands had stacked and then at 50mA until the bromophenol blue band was 1cm from the gel end. The cassette was removed from the tank and the gel removed from the cassettes by removing the clamps and gently prising the plates apart.

Staining of Gels

Reagents

(a) Staining solution

0.115%	Coomassie Blue
8%	Acetic Acid
20%	Ethanol

(b) Destaining solution

8%	Acetic Acid
20%	Ethanol

The gel was removed into a dish containing staining solution and was shaken gently for 1 hr. at 37°C. The stain was removed and replaced with destaining solution. The gel was shaken gently for 1 hr. at 37°C. The destaining solution was removed and the gel was left overnight.

After this time the gel was photographed by Mr. Ian Geller and the gel was scanned at 632.8nm on an LKB, Ultrascan, Laser densitometer.

Preparation of Trypsin-Sepharose

Trypsin was coupled to Sepharose 6B by Dr. S.R. Ayad.

C H A P T E R I I I

RESULTS AND DISCUSSION

I. PREPARATION OF cAMP BINDING PROTEIN

cAMP binding protein isolated from bovine cardiac muscle is essentially a cAMP-dependent protein kinase. Unlike the protein prepared from bovine adrenal glands the isolation procedure produces a protein kinase possessing both catalytic and regulatory moieties (Rubin *et al.*, 1975). The presence of the catalytic unit explains some of the differences in properties of the two binding proteins. Preliminary experiments using Tris-EDTA pH 7.6 containing 6mM β -mercaptoethanol, the buffer system employed for the binding protein isolated from adrenals, showed that this buffer would be unsuitable for the protein obtained from skeletal muscle. The S_0 values obtained over a range of concentrations of binding protein were lower than expected. It was therefore decided to use the conditions found to be optimal for the binding protein prepared from skeletal muscle according to the method of Gilman and Murad (1975) i.e. 50mM phosphate pH 7.0, 4mM β -mercaptoethanol. An essential requirement of the cAMP assay system used in this laboratory is that the regulatory unit be separate from the catalytic unit in order that cAMP can be bound. Therefore it appears that different ionic conditions are required for the binding proteins from two different sources.

Low dilutions, high concentrations of the binding protein produced flattened, non-linear standard curves (Fig. 8). However, the S_0 values obtained were high. Lowering the concentration, increasing the dilution of the

Fig. 8. Preparation of binding protein from cardiac muscle; Effect of 1/25, 1/40 and 1/60 dilution of protein on cAMP Standard Curve

cAMP assay was performed as in Materials and Methods Section. The components of the system were identical for each curve except for the concentration of binding protein. The buffer system employed was 50mM phosphate pH 7.0. Results shown are the means of duplicate determinations.

○—○ 1/25

□—□ 1/40

●—● 1/60

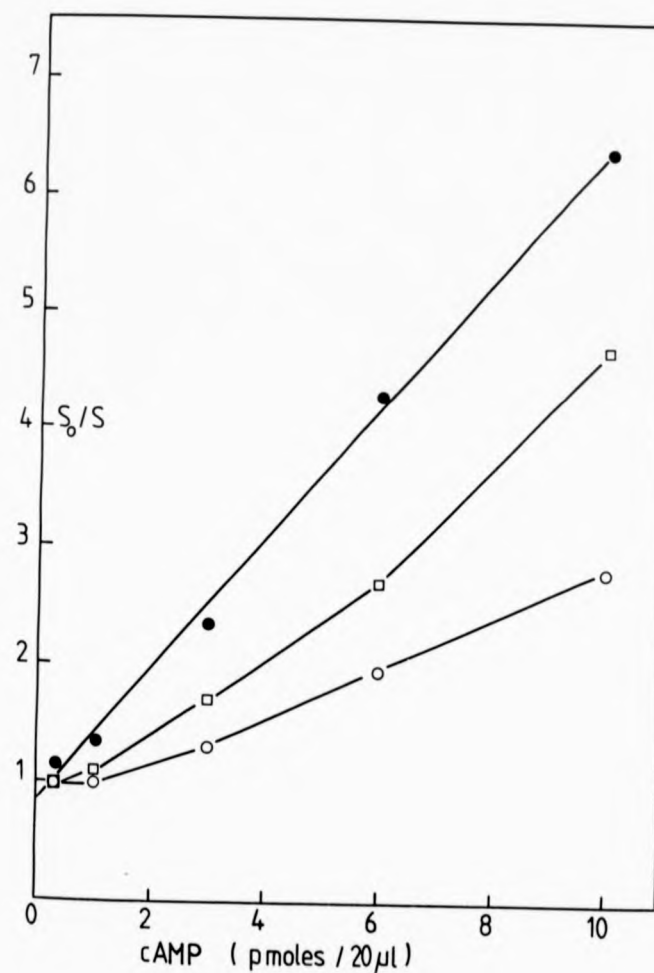


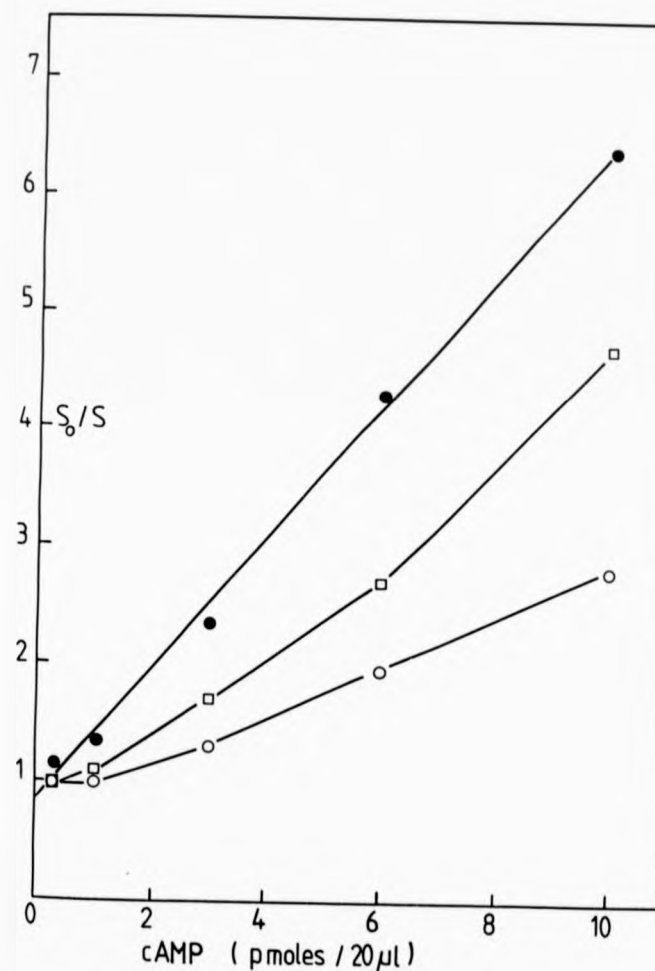
Fig. 8. Preparation of binding protein from cardiac muscle; Effect of 1/25, 1/40 and 1/60 dilution of protein on cAMP Standard Curve

cAMP assay was performed as in Materials and Methods Section. The components of the system were identical for each curve except for the concentration of binding protein. The buffer system employed was 50mM phosphate pH 7.0. Results shown are the means of duplicate determinations.

○—○ 1/25

□—□ 1/40

●—● 1/60



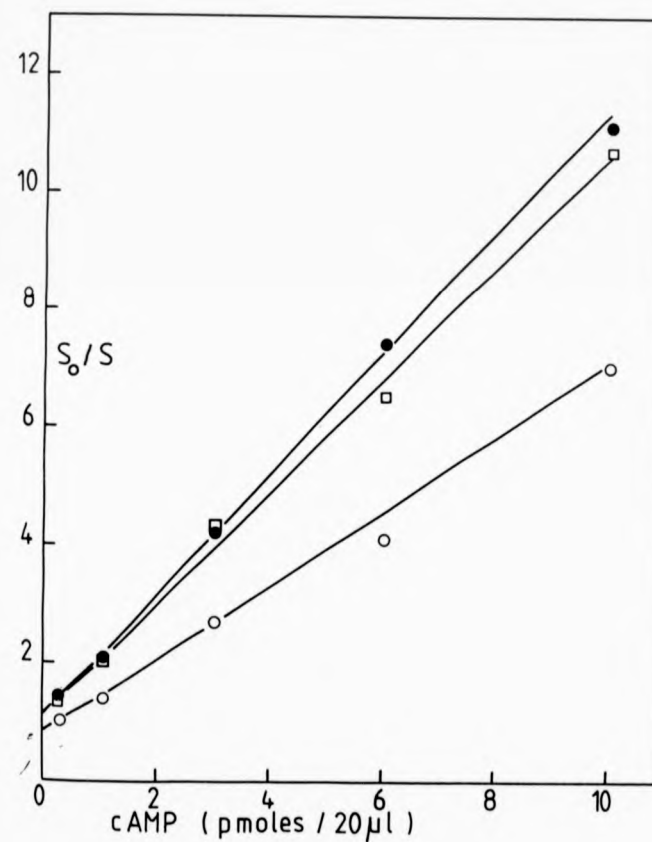
binding protein resulted in linear standard curves with a higher gradient (Fig. 8 and 9) but S_0 values were approximately the same as when high concentrations, low dilutions of binding proteins were used. This indicates that when low dilutions of binding protein were used the total binding was less than expected since the amount of [3 H]cAMP bound/mg protein for the low dilution would be smaller than that for the high dilution since the S_0 values were the same in both cases but the protein concentration was changed. The displacement of labelled cAMP by unlabelled standard cAMP for low dilutions of binding protein was inefficient since the gradient of the standard curve was small. Thus a possible explanation for the effect of varying the dilution of the binding protein is that at high concentrations of binding protein the molecules of the protein aggregate making some of the cAMP binding sites inaccessible. The curves only become linear at dilutions greater than $1/60$ (Figs. 8 and 9) and these linear curves have an intercept of 1. A dilution of $1/100$ was chosen as optimal for the cAMP assay.

The effect of Mg^{2+} on the cAMP standard curve was also studied. Gilman and Murad (1975) observed an essential requirement of Mg^{2+} for activity of the protein kinase from skeletal muscle. No such requirement was observed for the protein isolated in this study. Possibly this protein had Mg^{2+} bound when isolated.

Fig. 9. Preparation of binding protein from cardiac muscle; Effect of 1/80, 1/100 and 1/120 dilution of protein on cAMP standard curve

Method as described in Fig. 8. Results shown are the means of duplicate determinations.

- 1/80
- 1/100
- 1/120



II. FACTORS CONTROLLING cAMP LEVELS IN INTACT CELLS

(a) Dose Response to PGE₁

From Fig. 10 it can be seen that the hormonal responsiveness in terms of cAMP production is considerably greater for the hybrid PCM3 than for either of the two parent cell lines P388 or CH23. This phenomenon was first observed by Ayad and Foster (1974). In contrast to the β -adrenergic response (Gilman and Minna, 1973) the PGE₁ responsiveness is always much enhanced in hybrids from responsive (CH23) and unresponsive (P388) cells. P388 have a low response to the hormone, PGE₁, which is a characteristic of malignant cells (Oey *et al.*, 1974; Dicker *et al.*, 1980). There are several possibilities to account for the enhanced response of PCM3. It is feasible that the number and type of hormone receptors present in PCM3 could in some way explain this enhanced response of PCM3. A modification of the PGE₁ receptor of PCM3 was first suggested by Ayad and Foster (1974) and was further investigated by Ayad and Burns (1977). It was found that although PCM3 contains only one class of receptor in comparison to both the parent cell lines this was unlikely to account for the vast difference in responsiveness of PCM3. The different PGE₁ response for the three cell lines can be partially explained by their relative cAMP-phosphodiesterase activities. cAMP phosphodiesterase activity is greatest for P388 cells and least in PCM3 cells (Ayad and Wright, 1977), i.e. reciprocally related to the PGE₁ responsiveness. The values obtained for CH23 and P388 (Fig. 10) in the presence of 1mM IBMX are higher than

Fig. 10. Dose Response to PGE_1 for all three cell lines

Cells were washed twice with HBSS⁻⁻⁻. The final volume of the incubation was 1ml, the volume was adjusted with HBSS⁻⁻⁻. The cells were pre-equilibrated with 1mM IBMX for 10 minutes at 37°C followed by incubation with hormone for 10 minutes at 37°C.

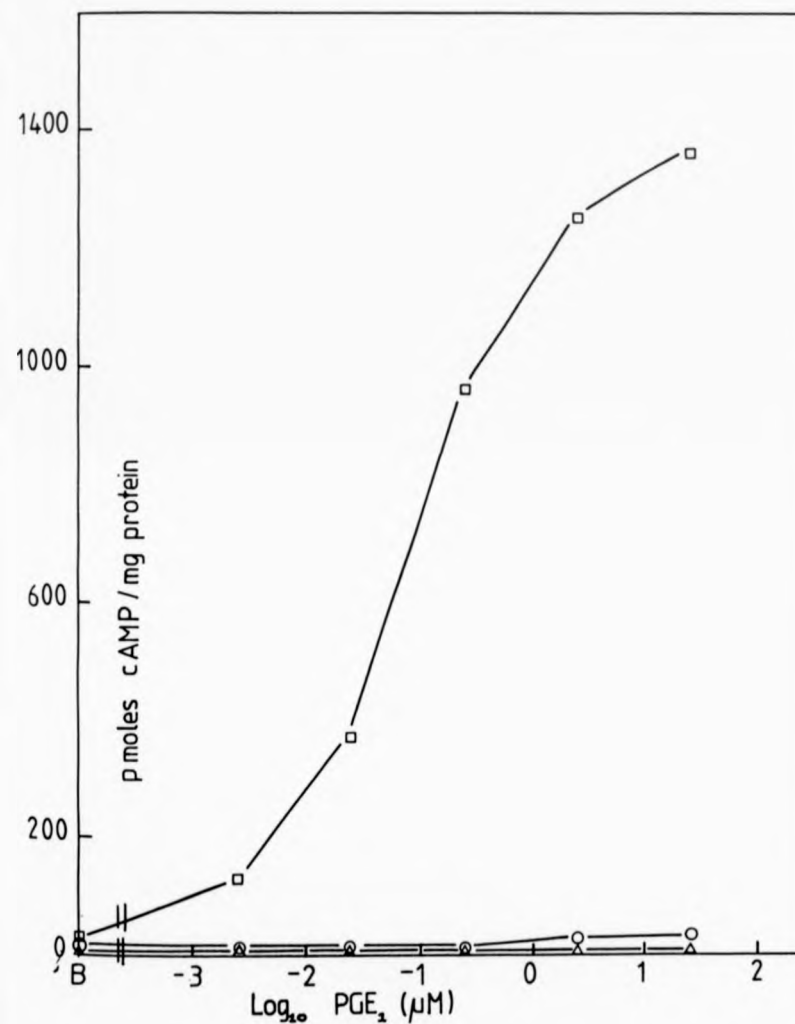
Results shown are the means of duplicate incubations and duplicate cAMP determinations of each incubation.

○—○ CH23

△—△ P388

□—□ PCM3

B indicates basal levels of cAMP in the absence of the hormone.



the values for these parental cell lines obtained in the presence of 0.2mM IBMX (Ayad and Hughes, 1980). Whereas the values for PCM3 are similar at 1mM and 0.2mM IBMX. Thus the phosphodiesterase activity of the parental cell lines may not be totally inhibited allowing some breakdown of cAMP to AMP. However, this explanation also seems insufficient to account for the vast difference in response between PCM3 and the parental cell lines. It is also possible that there are other unconsidered factors responsible e.g. a defect in receptor-cyclase coupling or a modification of one of the components of adenylate cyclase activity.

(b) The Effect of ATP on cAMP Accumulation in Intact Cells.

From fig. 11 it can be seen that ATP increases P388 cell cyclic AMP levels in a dose-dependent manner (0-3mM) both in the presence and absence of PGE_1 . This is in agreement with previous experiments in this laboratory (Ayad and Hughes, 1980; DeCastro and Ayad, 1982). Little enhancement of cAMP levels in CH23 are seen before 1.5mM ATP (Fig. 12) but after this there is an elevation of cAMP levels. There is also a slight increase in cAMP levels of PCM3 at these concentrations (Fig. 13). The results obtained for CH23 and PCM3 are dissimilar in some respects to those obtained by Ayad and Hughes (1980). These workers found a smaller increase in the levels of cAMP in response to ATP for CH23 although the increase obtained is still several orders of magnitude lower than that obtained for P388. However, it is possible that the batch of ATP used was contaminated with a small amount of GTP,

Fig. 11. Effect of ATP on P388 Intact Cells

Cells were washed twice with incubation medium. The final volume of the incubation was 1ml and the volume was adjusted with incubation medium. The cells were pre-equilibrated with 1mM IBMX for 10 minutes at 37°C followed by incubation with the required concentration of ATP for 10 minutes at 37°C and PGE_1 for a further 10 minutes at 37°C. ATP was dissolved in incubation medium to 20X the desired concentration and 50 μ l added per incubation. 20 μ l of hormone in 50% ethanol at 50X the desired concentration were added per incubation. At this concentration ethanol was without effect on cell viability. Results shown are the means of duplicate incubations and duplicate cAMP assays of each sample.

○—○ - PGE_1
●—● + PGE_1 (2.5 μ M)

B represents the basal value.

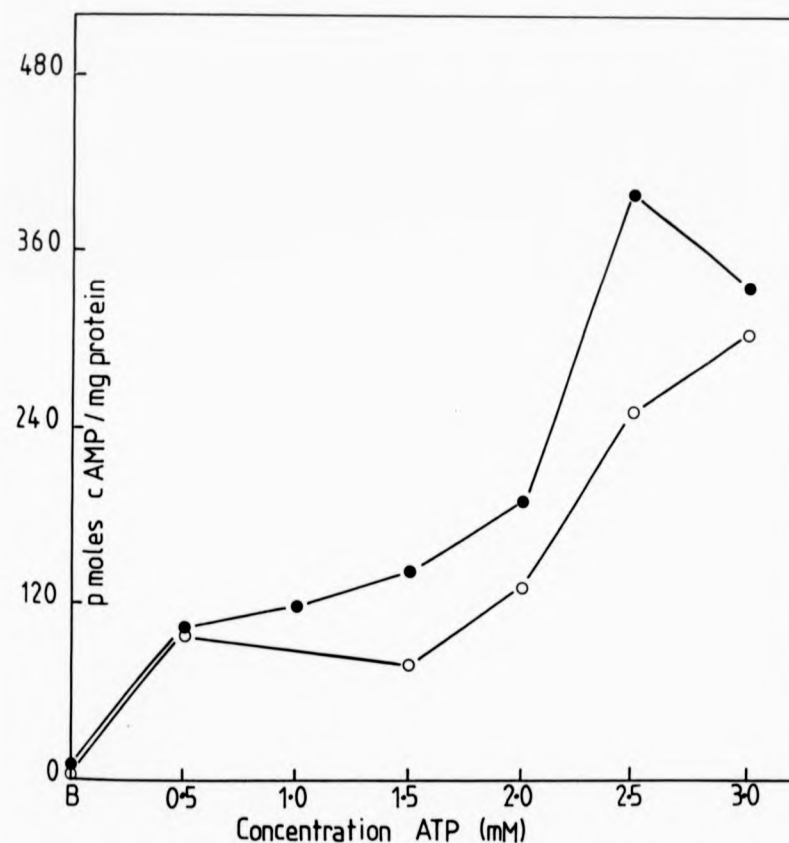


Fig. 12. Effect of ATP on CH23 Intact Cells

Method as described in Fig. 11. Results shown are the means of duplicate incubations and duplicate cAMP determinations of each sample.

○—○ $-PGE_1$

●—● $+PGE_1$ (2.5 μ M)

B represents the basal value.

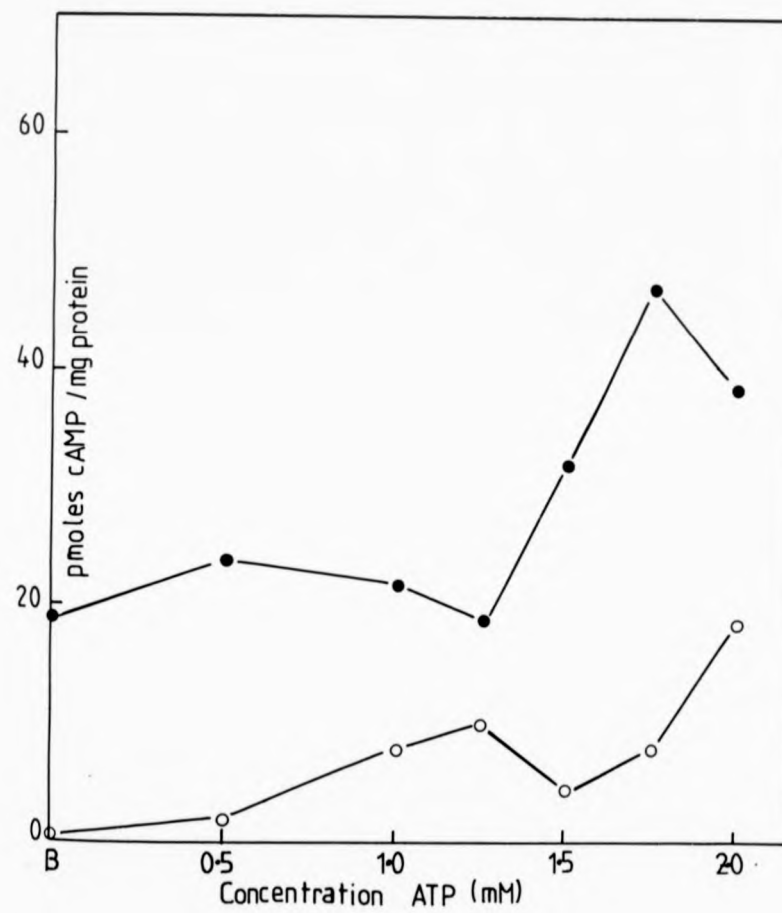


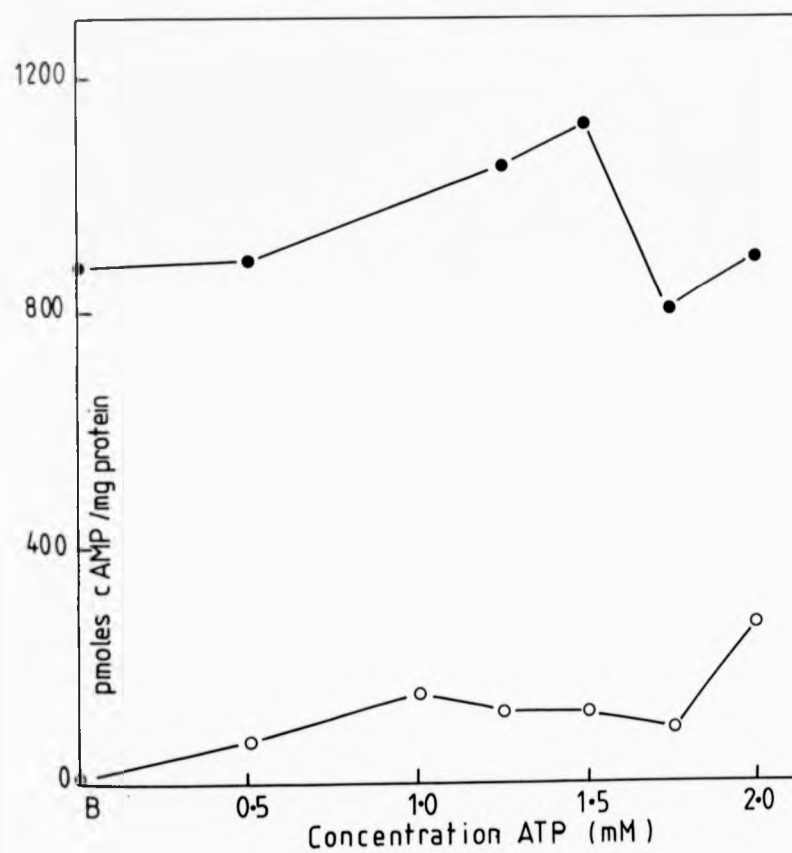
Fig. 13. Effect of ATP on PCM3 Intact Cells

Method as described in Fig. 11. Results shown are the means of duplicate incubations and duplicate cAMP determinations of each sample.

○—○ $-PGE_1$

●—● $+PGE_1$ (2.5 μ M)

B represents the basal value.



sufficient to produce an enhancement in CH23 but not to enhance the other cell lines (see below). For PCM3 these workers found a decrease in the levels of cAMP but the effect was inconsistent and varied between experiments from 0-50% inhibition (Ayad and Hughes, 1980). The overall effect on PCM3 from Fig. 13 is a 0% inhibition except at 1-1.5mM ATP where there is a slight increase in cAMP levels. This is probably explained by the inconsistent response to ATP for PCM3.

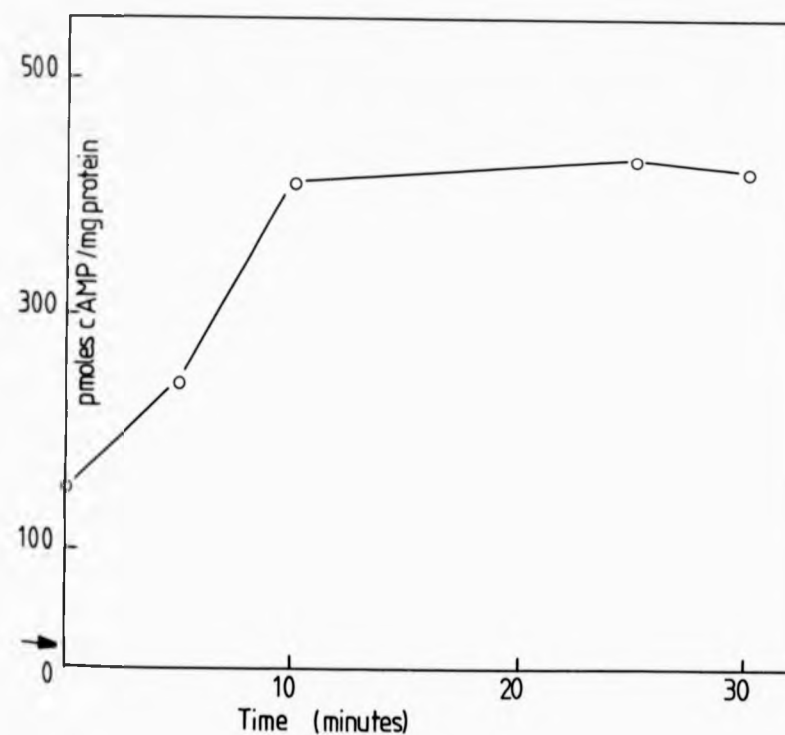
A possible explanation for the effect of ATP on elevating P388 cell cAMP levels is that ATP is dephosphorylated to adenosine which then acts as an extracellular messenger to activate adenylate cyclase. However, adenosine has not been shown to affect the cAMP levels of any of the cell lines under the conditions used for the incubation of the cells with ATP (Ayad and Hughes, 1980). IBMX included in the assay as a phosphodiesterase inhibitor has also been shown to antagonise the adenosine activation of adenylate cyclase (Sattin and Rall, 1970; Fredholm *et al.*, 1978). In the absence of IBMX, adenosine has been demonstrated to increase the cAMP accumulation in P388 cells (Ayad and Hughes, 1980). The non-hydrolysable analogue of ATP, adenylyl imidodiphosphate App(NH)p has been shown to have similar effects to ATP in P388 cells (DeCastro and Ayad, 1982). Therefore the enhancement of the cellular cAMP levels in P388 by ATP appears to be due to the ATP molecule itself and not to its degradation products. Other groups of workers have also demonstrated that ATP produces enhanced

cAMP accumulation (Rozengurt *et al.*, 1977; Rozengurt and Heppel, 1979). If ATP is not acting via degradation products it must either be crossing the plasma membrane or the cells are rather leaky and behave in the way of an homogenate. Ayad and Hughes (1980) have demonstrated that this latter possibility is unlikely because the cells appear intact by several criteria. Several other workers have shown that membranes can be permeable to ATP including the kidney (Chaudry *et al.*, 1976; Maxild, 1978) and intact rat soleus muscles of haemodiaphragms (Chaudry and Baue, 1980). It has been shown that P388 has considerably lower intracellular ATP levels than either CH23 or PCM3 (Ayad and Hughes, 1980). Therefore it seems reasonable to predict that ATP may enter the cell down the concentration gradient to supplement the pool of ATP around the adenylate cyclase molecule. The time course for the accumulation of cAMP in response to ATP for P388 (Fig. 14) shows a maximum at 10 minutes and a plateau until 30 minutes. There is no indication of a desensitisation reaction which might be expected if ATP were acting as a hormone. These results do not preclude the possibility that treatment of cells with ATP results in an increased concentration of extracellular cAMP since the cAMP measured is a total value.

Further evidence for ATP crossing the membrane comes from studies using potassium cyanide and iodoacetate as ATP depleting agents. These agents have been shown to decrease the intracellular levels of ATP in P388 in this laboratory (De Castro and Ayad, 1982). In the absence of ATP, these agents reduced the PGE_1 -stimulated cAMP levels as well as

Fig. 14. Time Course of cAMP Accumulation in Response to ATP in Intact P388 Cells

Cells were washed twice with incubation medium. Final volume of the incubation was 1ml and the volume was adjusted with incubation medium. Cells were preincubated for 10 minutes at 37°C with 1mM IBMX. Cells were incubated with 2mM ATP for the desired time, followed by addition of PGE₁ to a final concentration of 2.5μM. The cells were incubated for a further 10 minutes at 37°C. Time 0 was obtained by the addition of PGE₁ and ATP simultaneously followed by 10 minutes incubation at 37°C. Results shown are the means of duplicate incubations and duplicate cAMP determinations of each sample. Arrow indicates the basal value.



basal levels (Fig. 15). Addition of ATP to the incubation medium following preincubation with potassium cyanide and iodoacetate allowed elevation of cAMP levels in P388 cells. However, the elevation that occurred in the presence of PGE_1 ($2.5\mu\text{M}$) was less than that occurring in the absence of PGE_1 . This data appears to indicate that either the PGE_1 receptor or receptor-cyclase coupling is damaged or modified following treatment with potassium cyanide and iodoacetate. Thus it appears that for P388, depletion of intracellular cAMP levels allows the uptake of ATP to produce more cAMP following reduction of levels.

(c) The Effect of GTP on cAMP Accumulation in Intact Cells

From Fig. 16 it can be observed that for P388 there is an increase in cAMP accumulation in response to GTP. However, this increase is very small in numerical values and appears to be biphasic. Previous results in this laboratory (DeCastro and Ayad, 1982) have shown that incubation of P388 cells with 0.1mM GTP had no significant effect on cAMP accumulation. From Fig. 16 it can be seen that very little stimulation was found to occur in this range. It is not known whether the stimulation of cAMP accumulation that occurred at 10^{-10}M and 10^{-6}M is significant since the values were smaller than normally expected for P388 in the presence of PGE_1 ($2.5\mu\text{M}$).

GTP does not appear to have any effect on cAMP accumulation in PCM3 cells at any concentration (Fig. 17). For CH23 there is stimulation of cAMP accumulation at all

Fig. 15. Effect of Preincubation with KCN and Iodoacetate on ATP uptake by Intact P388 Cells

Cells were washed twice with incubation medium. The final volume of the incubation was 1ml and the volume was adjusted with incubation medium. The cells were pre-equilibrated with 1mM IBMX for 10 minutes at 37°C as usual. KCN and Iodoacetate were dissolved in incubation medium to give a final concentration of 2mM KCN and 2mM iodoacetate when added to the incubation. The cells were preincubated at 37°C for 10 minutes. After this time the required concentration of ATP was added and the cells were further incubated for 10 minutes at 37°C followed by addition of 2.5μM PGE₁ and further incubation for 10 minutes at 37°C.

Results shown are the means of triplicate incubations and duplicate cAMP determinations of each sample. Standard deviations were always less than 10%.

- | | | | |
|----|-------------------|------|---------------|
| a. | -PGE ₁ | -KCN | -Iodoacetate |
| b. | -PGE ₁ | +KCN | +Iodoacetate |
| c. | +PGE ₁ | -KCN | -Iodoacetate |
| d. | +PGE ₁ | +KCN | +Iodoacetate. |

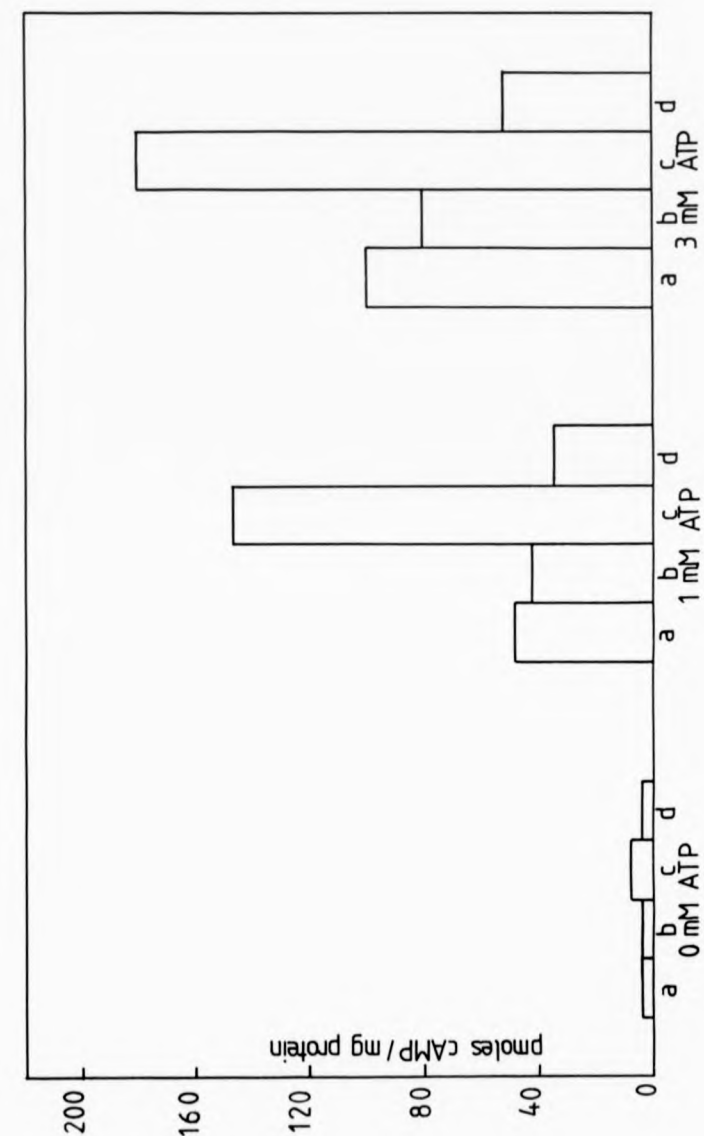


Fig. 16. Effect of GTP on PGE₁ (2.5 μ M)-Stimulated P388 Intact Cells

Cells were washed twice with incubation medium. The final volume of the incubation was 1ml and the volume was adjusted with incubation medium. Cells were pre-equilibrated with 1mM IBMX for 10 minutes at 37°C. GTP was dissolved in incubation medium and added to the incubation to give the required final concentration and the cells incubated for 10 minutes at 37°C. PGE₁ was added to give a final concentration of 2.5 μ M and the cells were further incubated for 10 minutes at 37°C. Results shown are the mean of triplicate incubations with duplicate cAMP determinations of each sample. Standard deviations were always less than 10%.

B represents the basal value.

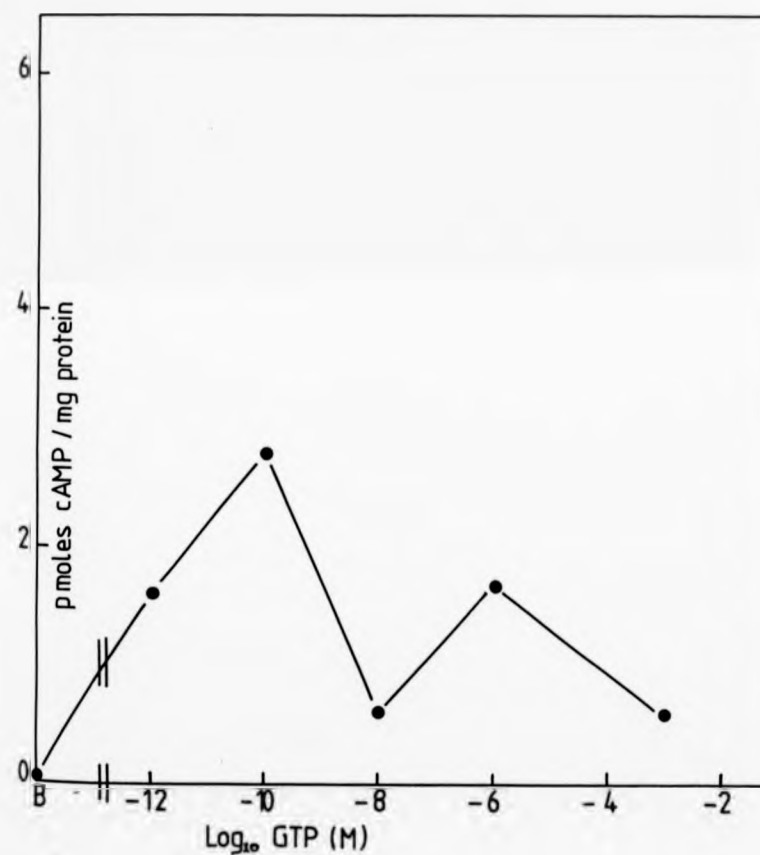
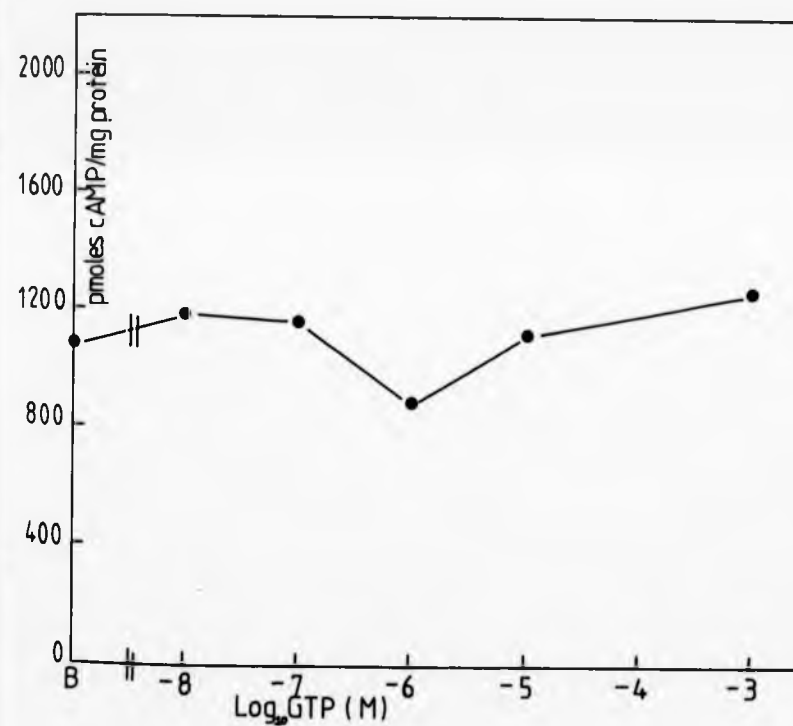


Fig. 17. Effect of GTP on PGE₁ (2.5 μ M)-Stimulated PCM3 Intact Cells.

Method as described in Fig. 16. Results shown are the means of triplicate incubations with duplicate cAMP determinations of each sample. Standard deviations were always less than 10%.

B represents the basal value.



concentrations studied with the response being dose-dependent until 10^{-6} M (Fig. 18). It was decided to further investigate this dose-dependent increase of cAMP accumulation in response to GTP in intact CH23 cells.

The most obvious explanation for the enhancement of PGE_1 -stimulated cAMP accumulation by GTP in CH23 cells is that a hydrolysis product is responsible. The phosphatase and pyrophosphatase resistant analogue of GTP, Gpp(NH)p was shown to enhance the hormone-stimulated cAMP accumulation in CH23 irrespective of the order of addition of PGE_1 and Gpp(NH)p (Figs. 19 and 20). The activation was biphasic, maximal activation occurring at 10^{-12} M and 10^{-6} M. Thus it appears that the effect of guanine nucleotide on CH23 is affecting a number of processes. The activation occurring at 10^{-6} M could be due to guanine nucleotide stimulation of adenylate cyclase since the concentration at which 50% activation is known to occur is at 10^{-8} - 10^{-7} M and there is a usual specificity Gpp(NH)p > GTP (Limbird 1981). When Gpp(NH)p is added before PGE_1 (Fig. 19) there is greater enhancement at 10^{-12} M than 10^{-6} M. However, when PGE_1 was added before the Gpp(NH)p (Fig. 20) this situation was reversed. Gpp(NH)p had little effect on PCM3 (Fig. 21) although a slight stimulation occurred. This is in keeping with the finding that GTP had little effect on PCM3 (Fig. 17). GMP (Fig. 22), GDP (Fig. 23) or guanosine (Fig. 24) had slight stimulatory effects on cAMP accumulation in CH23 intact cells but not sufficient to account for the great enhancement of GTP and Gpp(NH)p. Thus some of the enhancement due to GTP could be due to

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Fig. 18. Effect of GTP on PGE_1 ($2.5\mu\text{M}$)-stimulated
CH23 Intact Cells

Method as described in Fig. 16. Results shown
are the means of triplicate incubations with
duplicate cAMP determinations of each sample.
Standard deviations were always less than 10%.

B represents the basal value.

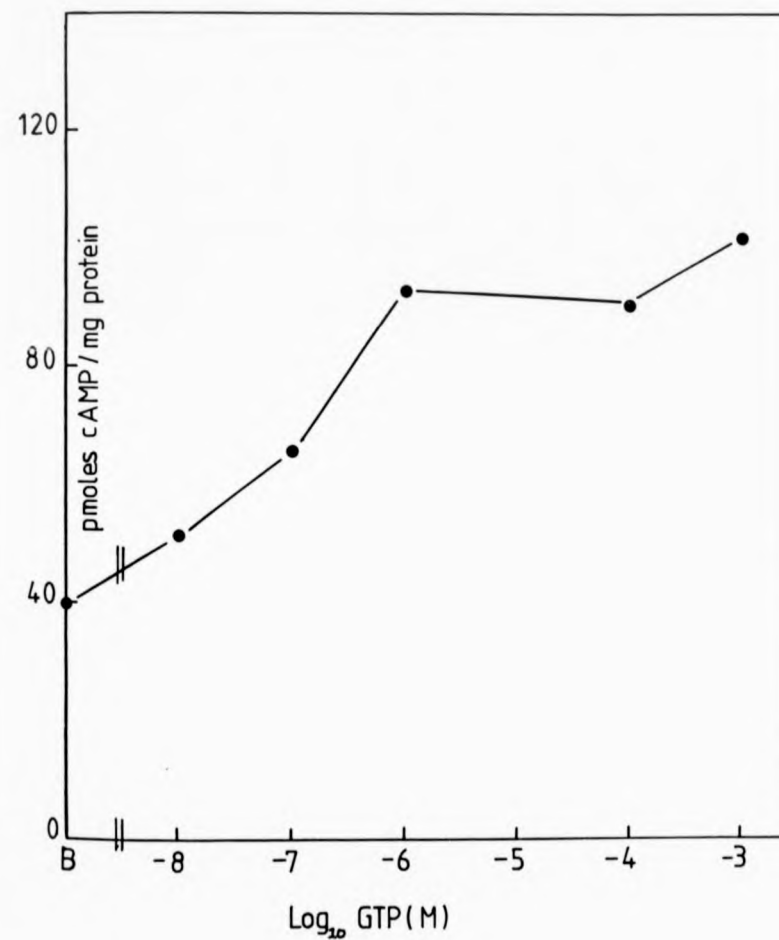


Fig. 19. Effect of Gpp(NH)p on CH23 Intact Cells

The cells were washed twice with incubation medium. The final volume of the incubation was 1ml and the volume was adjusted with incubation medium. The cells were pre-equilibrated with 1mM IBMX for 10 minutes at 37°C. Gpp(NH)p was dissolved in incubation medium and added to the incubation to give the desired final concentration and the cells were incubated for 10 minutes at 37°C. PGE₁ was added and the cells were further incubated for 10 minutes at 37°C. Results shown are means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -PGE₁
●—● +PGE₁ (2.5μM)

B represents the basal value.

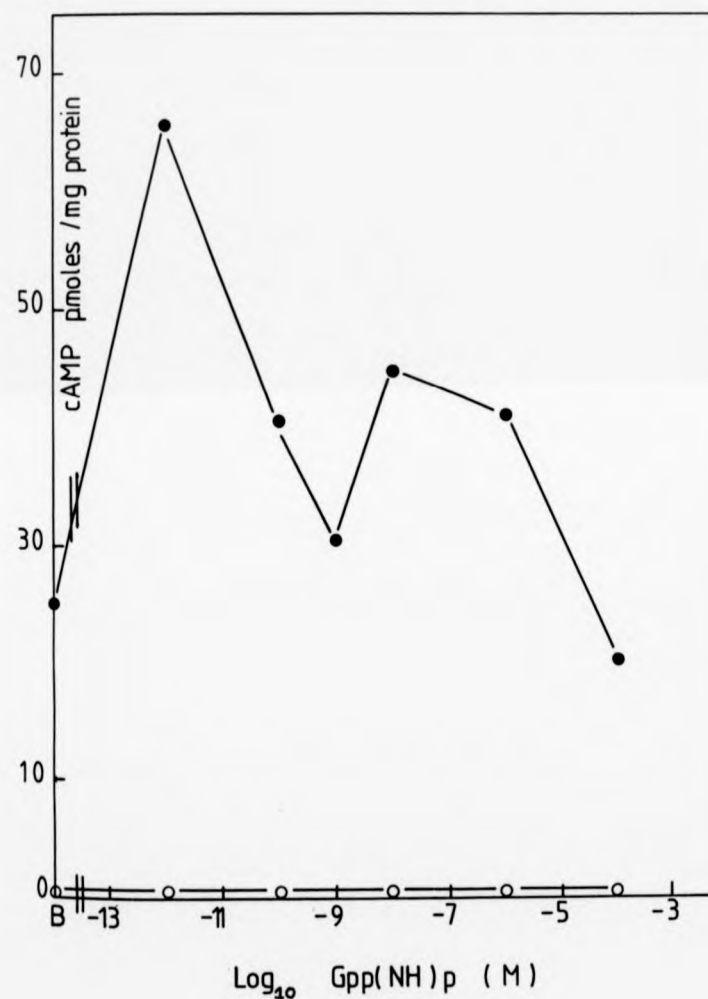


Fig. 20. Effect of Gpp(NH)p on CH23 Intact Cells

The cells were washed twice with incubation medium. The method was as described in Figure 19 with the exception that PGE_1 was added after preincubation with IBMX followed after 10 minutes at 37°C by the addition of Gpp(NH)p for a further 10 minutes. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ $-\text{PGE}_1$
●—● $+\text{PGE}_1$ (2.5 μM)

B represents basal level

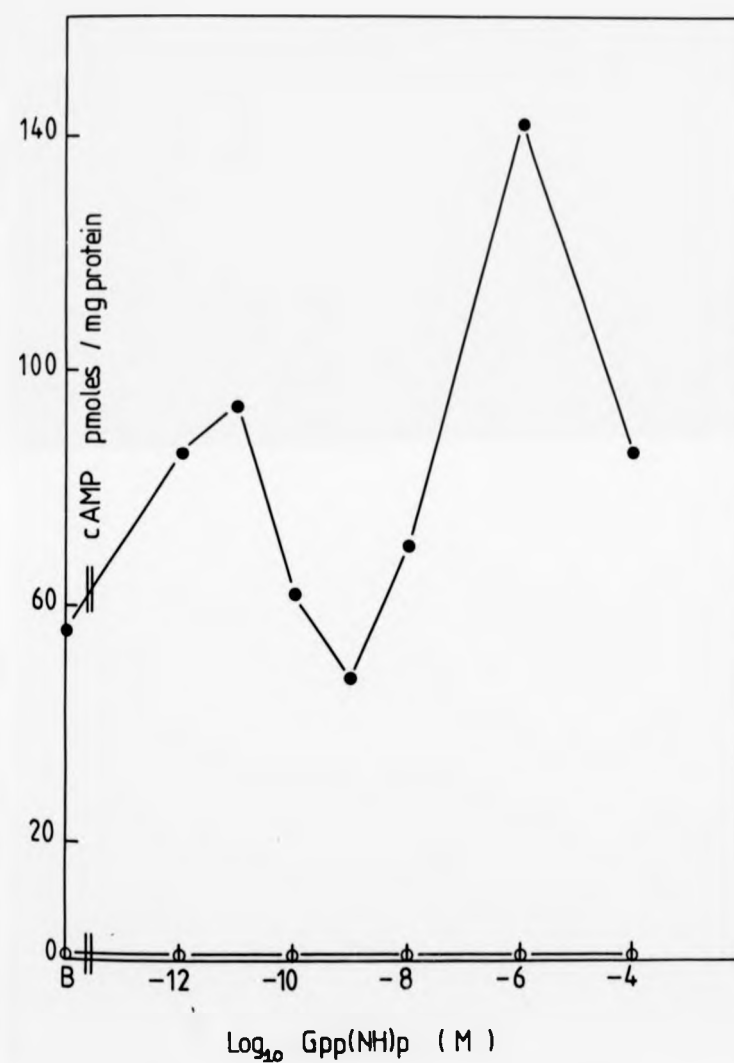


Fig. 21. Effect of Gpp(NH)p on PCM3 Intact Cells

Method as described in figure 19. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ $-PGE_1$

●—● $+PGE_1$ (2.5 μ M)

B represents the basal value.

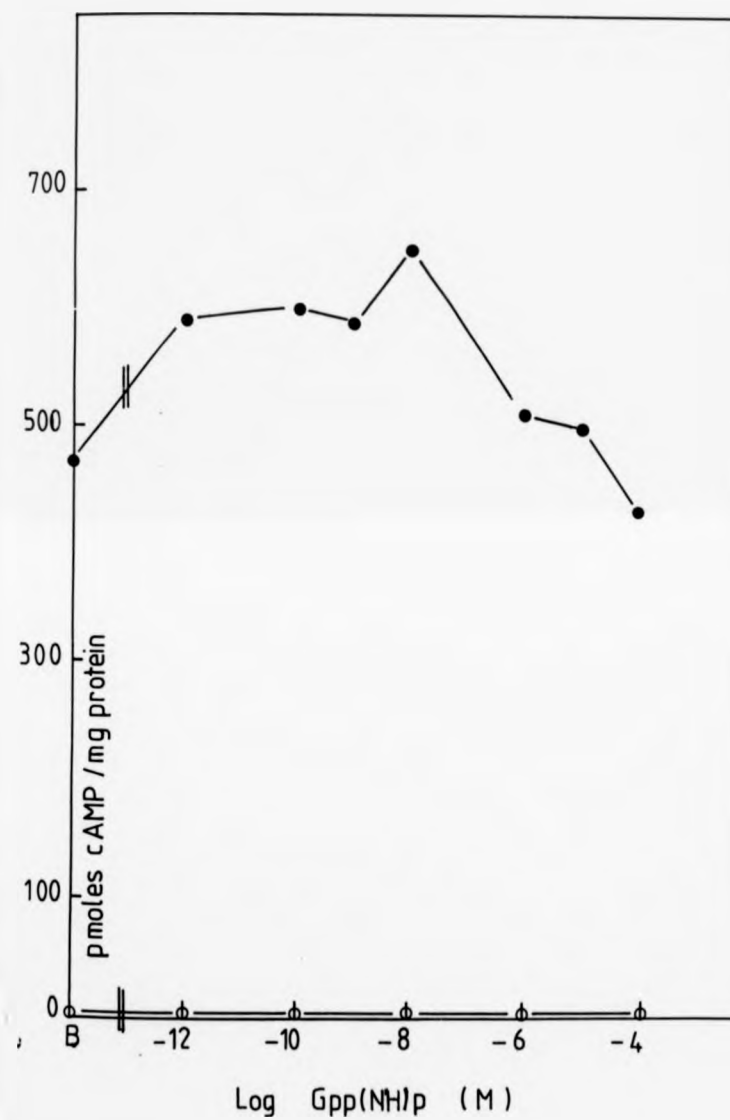


Fig. 22. Effect of GMP on CH23 Intact Cells

Cells were washed twice with incubation medium. The final volume of the incubation was 1ml and the volume was adjusted with incubation medium. The cells were pre-equilibrated with 1mM IBMX for 10 minutes at 37°C. GMP was dissolved in incubation medium and was added to the incubation to give the required final concentration. The cells were further incubated at 37°C for 10 minutes followed by the addition of PGE₁ for a further 10 minutes. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -PGE₁

●—● +PGE₁ (2.5μM)

B represents the basal level.

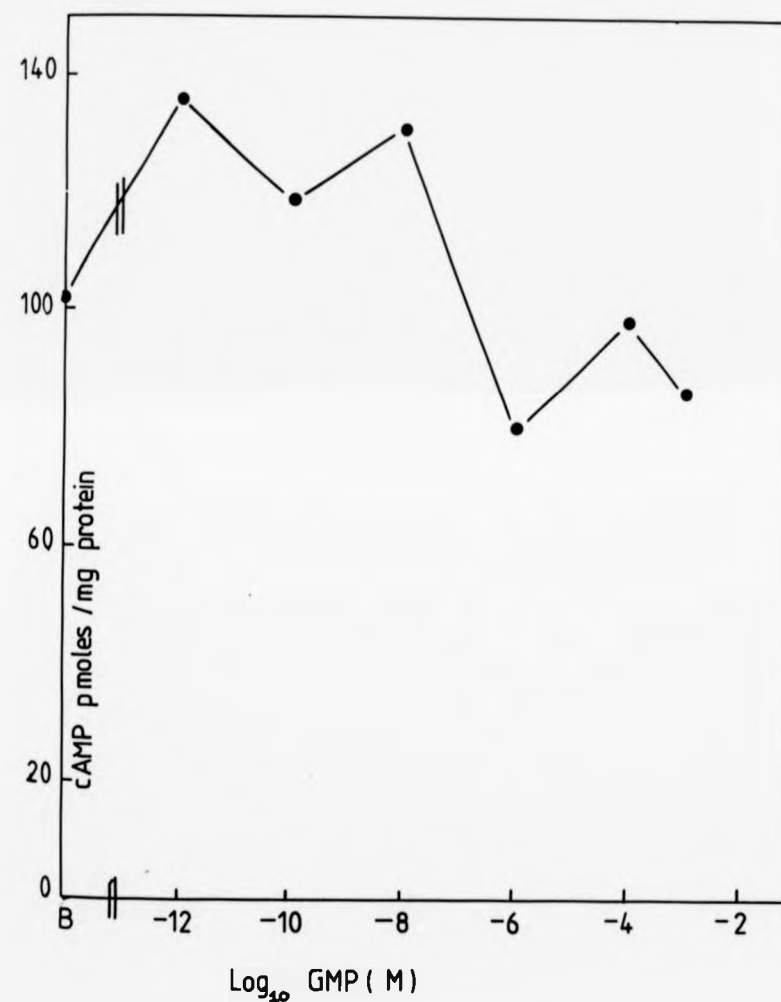


Fig. 23. Effect of GDP on CH23 Intact Cells

Method as described in figure 22 but GDP was substituted for GMP. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○ — ○ $-PGE_1$

● — ● $+PGE_1$ (+ 2.5 μ M)

B represents the basal level value.

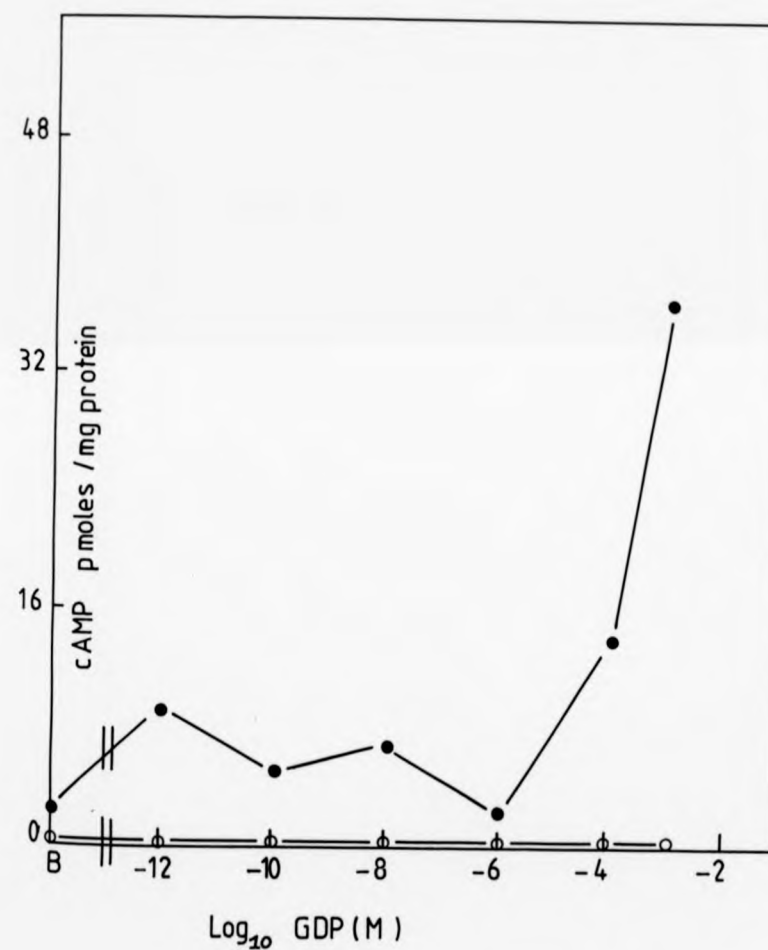


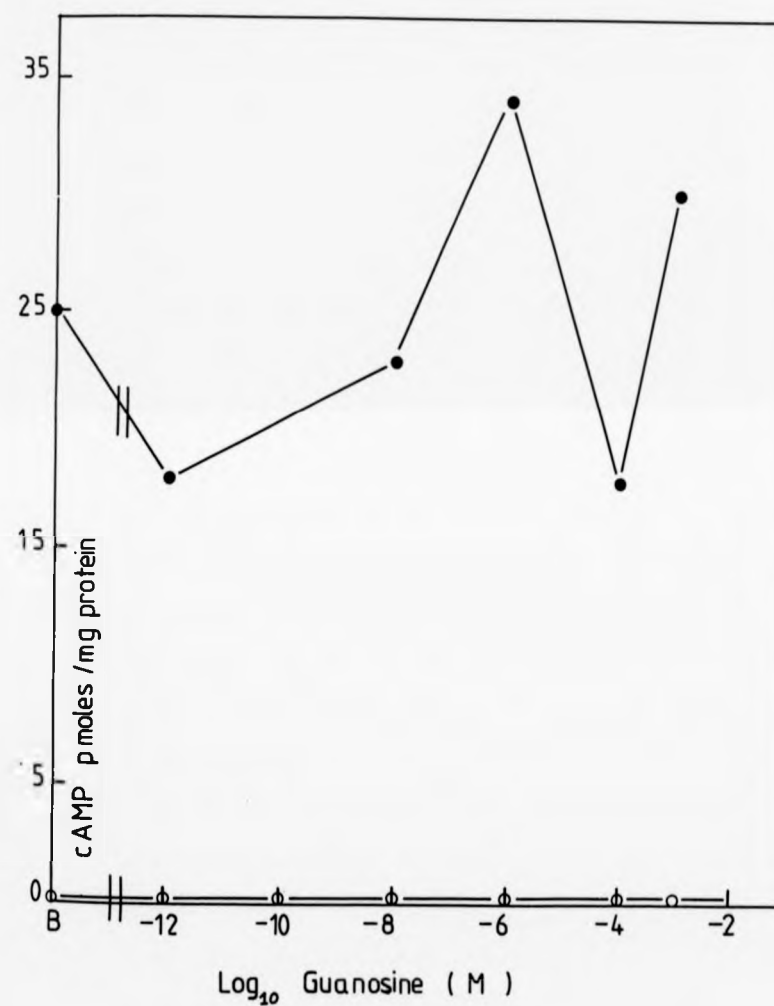
Fig. 24. Effect of Guanosine on CH23 Intact Cells

Method as described in figure 22 but guanosine was substituted for GMP. One drop of 1M NaOH was required in order to dissolve guanosine in incubation medium. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

O—O -PGE₁

●—● +PGE₁ (2.5μM)

B represents the basal value.



impurities in the GTP preparation. It does not therefore appear that the enhancement of PGE_1 -stimulated cAMP accumulation by GTP is due to hydrolysis of the triphosphate.

A further possibility for the enhancement of PGE_1 -stimulated cAMP accumulation is that the CH23 cells themselves are 'leaky' and that the cells behave in a similar manner to an homogenate. In CH23 and PCM3 homogenates the PGE_1 -stimulated levels of adenylate cyclase have been shown to be stimulated above the levels observed in the presence of optimum concentration of hormone by GTP and Gpp(NH)p whereas no effect was seen with P388 (Ayad and Foster, 1977). Thus CH23 is responsive to guanine nucleotides in the broken state. However, these results do not agree with the fact that intact PCM3 show no stimulation by GTP whereas both CH23 and P388 do, unless only CH23 cells are leaky. The intact cells of CH23 were judged to be intact morphologically by trypan blue exclusion. It is unknown whether the GTP enters the cells in order to activate adenylate cyclase by binding to the guanine nucleotide regulatory protein. Further experiments are required to verify this. If GTP is entering the cell down a concentration gradient in an analogous manner to ATP and P388, then it would also be expected that guanosine should enhance cAMP levels. The GTP content of C_6 glioma cells (Franklin and Twose, 1977) and NRK cells (Johnson and Mukku, 1979) can be reduced by addition of mycophenolic acid and resulted in a concomitant reduction in responsiveness to hormones. Addition of guanosine restored the GTP concentration and hence hormonal responsiveness. However, for CH23 (Fig. 24) guanosine

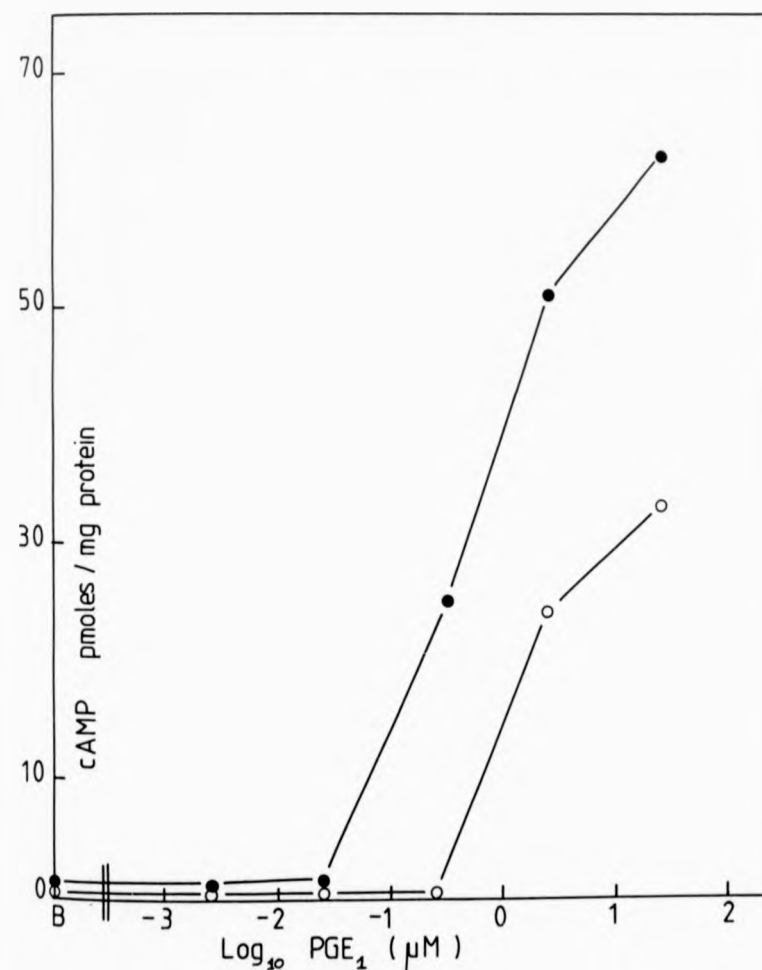
treatment produced only a slight increase in cAMP accumulation in response to PGE_1 . These results do not preclude the possibility that the addition of GTP to CH23 cells produces a loss of cAMP into the extracellular medium since the assay method in these studies measured total cAMP.

It can be seen from Fig. 25 that GTP increases the affinity for PGE_1 since the dose-response curve is shifted to the left. In addition GTP enhances the levels of cAMP produced in response to all concentrations of PGE_1 tested ($0.0025\mu\text{M}$ - $25\mu\text{M}$). A shift to the left of the hormone dose-response curve effected by guanine nucleotides has been illustrated before and has been predicted by the collision coupling model (Houslay, 1981). It is difficult to predict the mechanism by which GTP enhances the levels of hormone-stimulated cAMP accumulation in CH23 and to a lesser extent in P388 since it has not been determined whether the nucleotide enters the cell. There is an absolute requirement for guanine nucleotides in the stimulation of adenylate cyclase by hormones and drugs (Rodbell *et al.*, 1975; Limbird, 1981) but this would usually be provided by intracellular GTP. Thus is it possible that intracellular GTP levels are lower in P388 and especially CH23. The important factor would be to determine whether GTP actually enters the cell or simply facilitates the action of the hormone at the cell surface. This could be a characteristic of the cell type. These results also illustrate the similarity of PCM3 hybrid to P388 parent cells since both show little response to GTP.

Fig. 25. Effect of GTP on CH23 dose-response curve to PGE_1

The cells were washed twice with incubation medium. The final volume of the incubation was 1ml and the volume was adjusted with incubation medium. The cells were pre-equilibrated with 1mM IBMX for 10 minutes. GTP was dissolved in incubation medium and added to the incubation to a final concentration of 10^{-4}M . After 10 minutes incubation at 37°C PGE_1 was added and the cells further incubated for 10 minutes at 37°C . Results shown are the mean of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -GTP
●—● +GTP (10^{-4}M)



(d) Effect of Indomethacin on cAMP Accumulation in Intact Cells

The prostaglandin synthesis inhibitor, indomethacin was used to study the effect of endogenous prostaglandins on cAMP accumulation for the three cell lines. Indomethacin was employed to investigate the low levels of cAMP produced in response to exogenous PGE_1 addition for CH23 and P388 as compared to PCM3. It was hoped to establish whether CH23 and/or P388 synthesised prostaglandins endogenously rendering them insensitive to exogenous addition.

Following pretreatment of P388 and PCM3 with indomethacin, the PGE_1 -stimulated levels of cAMP accumulation were decreased both in the presence and absence of ATP (Fig. 26 and 28). In contrast, for the effect of indomethacin on CH23, the cAMP levels appeared to depend on the concentration of inhibitor used and the presence of ATP (Fig. 27). High concentrations of indomethacin ($>0.4\text{mM}$) inhibit the PGE_1 -stimulated levels both in the presence and absence of ATP. However, low concentrations ($0-0.2\text{mM}$) of indomethacin in the presence of ATP produce stimulation of cAMP levels in response to exogenous PGE_1 . It is therefore possible that the low response of CH23 could be due to synthesis of endogenous prostaglandins. The effect of indomethacin further illustrates the differences in properties of the three cell lines since the inhibition of PGE_1 -stimulated cAMP levels varies in extent between the cell lines. For PCM3, the PGE_1 -stimulated levels are reduced to basal levels by 0.3mM indomethacin both in the presence and absence of ATP, whereas P388 and CH23 cAMP

FIG. 26. Effect of Indomethacin on Intact P388 Cells

The cells were washed twice with incubation medium. The final volume of the incubation was 1ml and the volume was adjusted with incubation medium. Indomethacin solid was dissolved in a small amount of absolute ethanol and then diluted with incubation medium to give the required final concentration. The cells were pre-equilibrated for 10 minutes at 37°C in the presence of indomethacin and 1mM IBMX. ATP (2mM) was then added and the cells were further incubated for 10 minutes. PGE₁ (2.5μM) was then added and the cells incubated for another 10 minutes. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -ATP

●—● + ATP (2mM)

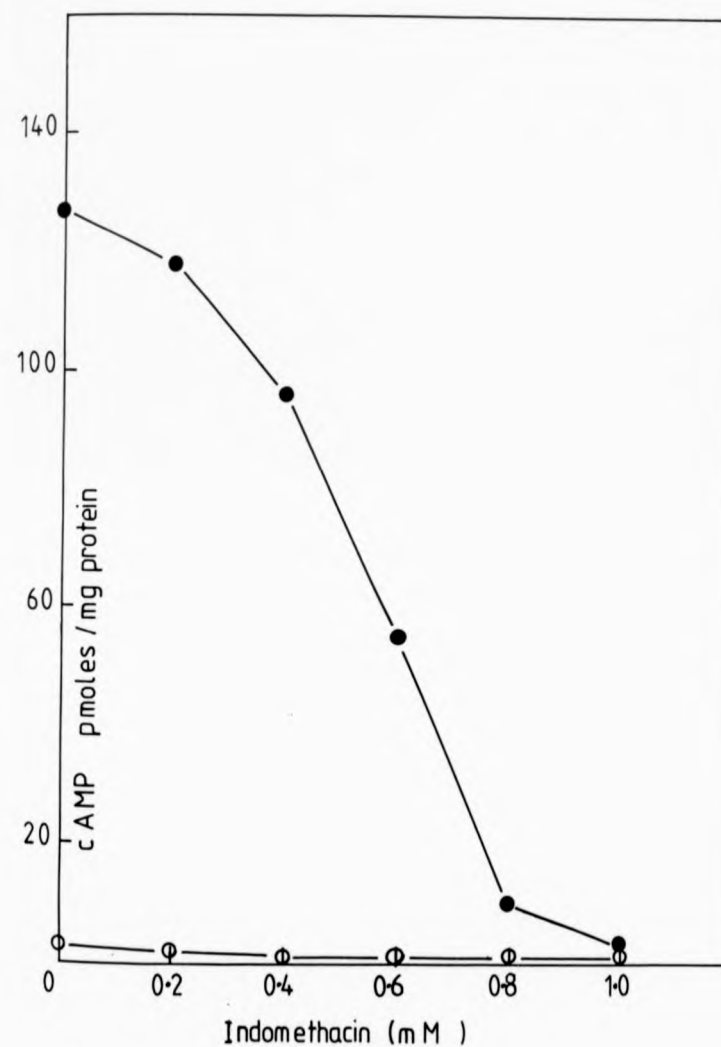


Fig. 27. Effect of Indomethacin on Intact CH23 Cells

Method as described in Fig. 26. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -ATP

●—● +ATP (2mM).

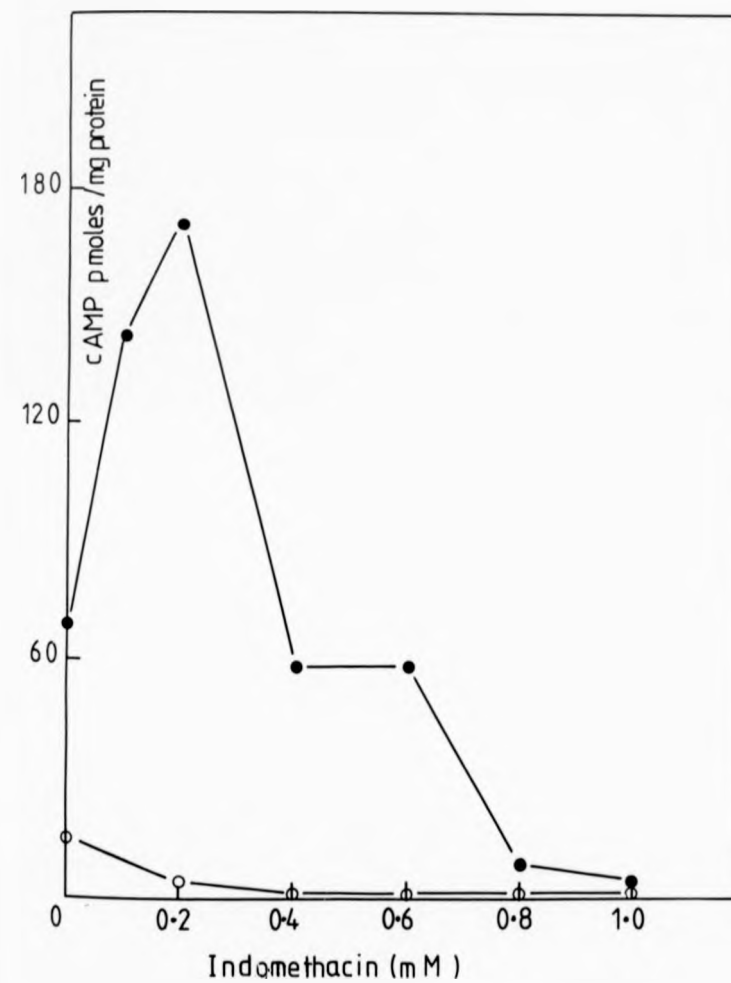
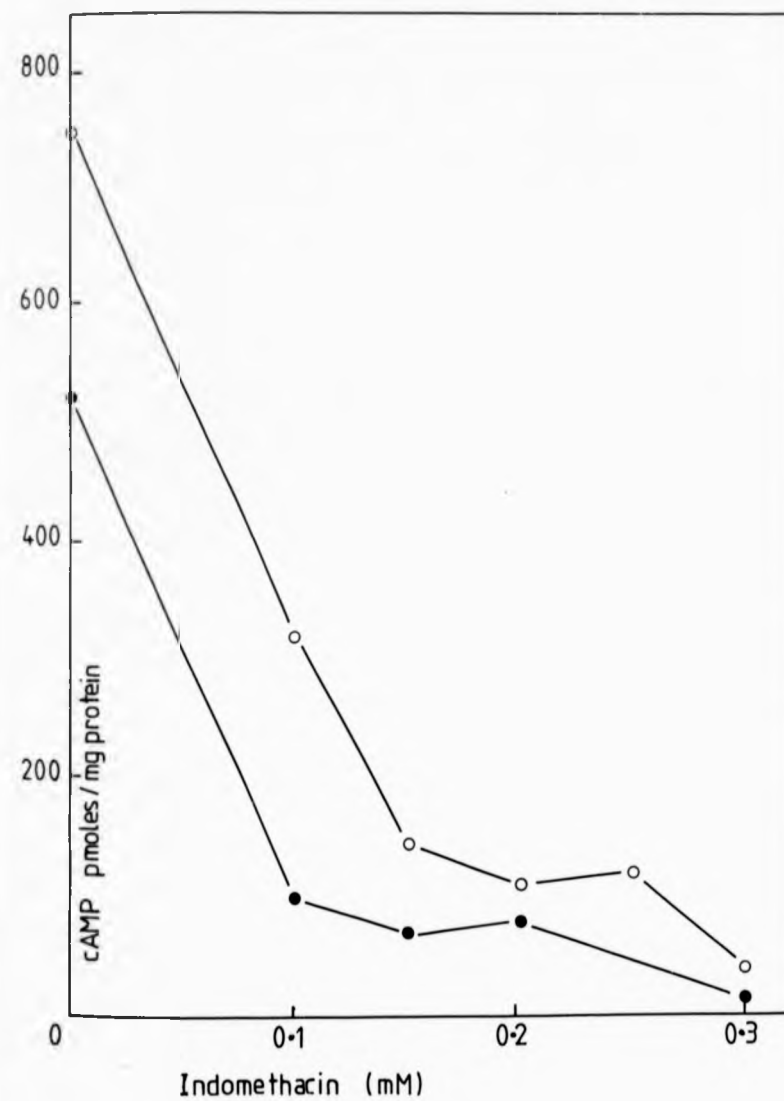


Fig. 28. Effect of Indomethacin on Intact PCM3 Cells

Method as described in Fig. 26. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -ATP

●—● +ATP (2mM)



levels are only reduced to basal levels by the presence of 1mM indomethacin. This also illustrates the properties of the hybrid cell as being different from either of the parental cell lines. From Fig. 28 it can be seen that cAMP accumulation in PCM3 cells untreated with indomethacin is lower in cells treated with ATP. This is in agreement with previous results from this laboratory (Ayad and Hughes, 1980), and illustrates the inconsistency of the effect of ATP on PCM3 intact cells as discussed earlier.

Indomethacin, in addition to being a prostaglandin synthesis inhibitor, has been shown to have various effects on cAMP metabolism. Indomethacin has been shown to increase the number of β -receptors in leucocytes (Gullner *et al.*, 1980) while basal and PGE_1 -stimulated cAMP production were unaffected. However, the cells were exposed to the drug for long periods (7 days, 2mg/day/kg man). Other workers have demonstrated an increase in the number of β -adrenoceptors and a possible interference by indomethacin of β -receptor-mediated effects (Feurstein *et al.*, 1980). Conversely, indomethacin has been shown to reversibly inhibit the growth of rat hepatoma cells and stops the cells in G_1 phase of the cell cycle (Bayer *et al.*, 1979). This finding is not in keeping with the results presented here in that indomethacin produces diminished cAMP levels and low levels of cAMP are associated with high rates of growth and high levels of cAMP are found on the G_1/S border (Friedman *et al.*, 1976). Other workers have shown that

treatment of tumour cells with indomethacin produces an increase in proliferation (Santoro *et al.*, 1976) which is consistent with the results found in P388.

Indomethacin has been demonstrated to inhibit the high-affinity binding of PGE_1 to rat thymocytes (Grunnet and Bojeson, 1976). Since in the experiments presented here the cAMP accumulation was measured following the addition of PGE_1 ($2.5\mu\text{M}$) to indomethacin pretreated cells it is possible that this action of indomethacin is an important factor in the reduction of cAMP levels in our cells. Grunner and Objeson (1976) found 50% inhibition of high affinity PGE_1 binding was obtained at $3.6 \times 10^{-5}\text{M}$. At the concentrations used in these experiments a considerable proportion of the high-affinity sites will be inhibited by indomethacin and consequently a substantial reduction in cAMP levels are expected. This possibility might also explain the differences observed for PCM3 since this cell type has been shown to have only two types of receptor of which only one type is expressed (Ayad and Burns, 1977).

It is unlikely that the effect of indomethacin is due to depletion of adenylate cyclase substrate, ATP. For P388, where ATP has been demonstrated to be taken up by the cells (DeCastro and Ayad, 1982) the effect of indomethacin was similar in the presence and absence of ATP, similarly for the other two cell lines. Pretreatment of human synoviocytes with indomethacin ($10\mu\text{M}$, 6 hours) has been shown to enhance PGE_1 -stimulation about two-fold (Ciosek *et al.*, 1975). This

also is in direct contrast to our results, however the incubation conditions employed are different. Little effect on PGE_1 ($2.5\mu\text{M}$)-stimulated cAMP levels were seen in any of our cells at concentrations less than $100\mu\text{M}$ (results not shown). High concentrations of indomethacin have been shown to inhibit cAMP-phosphodiesterase activity (Ciosek *et al.*, 1974) with a concentration of indomethacin producing 50% inhibition of $1.35 \times 10^{-4}\text{M}$. The inclusion of the phosphodiesterase inhibitor, IBMX, in all incubations should negate these effects of indomethacin. However, for P388, which has a particularly high cAMP phosphodiesterase level, this effect may be significant in explaining the lower rate of inhibition of PGE_1 -stimulated cAMP levels compared to PCM3. If a fraction of the phosphodiesterase was not inhibited by IBMX, indomethacin would have a dual effect in elevating the cAMP levels slightly by further inhibiting ~~phospho-~~ ^{phospho-} ~~diesterases~~ and decreasing the cAMP levels by a mechanism as yet unknown. The net result would be a slower rate of decline in cAMP levels produced by indomethacin in P388 compared to PCM3 whose phosphodiesterase activity is thought to be totally inhibited by 1mM IBMX as discussed previously.

Indomethacin in picomolar concentrations has been shown to inhibit partially purified protein kinase (Kantor and Hampton, 1978) and protein kinase of ileal mucosa ~~was~~ inhibited by 10^{-3}M indomethacin (Goueli and Ahmed, 1980). It has also been found by these authors that on a molar basis indomethacin was more potent as a cAMP dependent protein kinase inhibitor than an inhibitor of prostaglandin

biosynthesis. It would appear that the possible effect of indomethacin on protein kinase activity is responsible for the reduction in PGE_1 -stimulated cAMP levels observed since an inhibition of protein kinase activity would be expected to increase intracellular cAMP concentrations because less would be bound to the regulatory subunits. It appears that the precise nature of the effect of indomethacin on components of cAMP metabolism vary with the cell type and conditions used. However, none of the known effects of indomethacin on cAMP metabolism sufficiently explain the results obtained, i.e. the decrease of PGE_1 -stimulated cAMP levels to basal levels. It is possible, therefore, that these effects are due to the prevention of synthesis of endogenous prostaglandins. For CH23 it seems plausible to suggest that in the intact state the synthesis of endogenous prostaglandins plays some role in the low response of the cells to PGE_1 since indomethacin pretreatment at some concentrations does result in enhanced levels of response especially in the presence of exogenous ATP. It is also possible that synthesis of endogenous prostaglandins in some way mediates the response to exogenous prostaglandins since in indomethacin-treated cells the response is considerably diminished. The various effects of indomethacin are often controversial and depend on the laboratory studying them as well as the conditions employed. Lindgren *et al.* (1979) have demonstrated that $1\mu\text{M}$ indomethacin lowered the cellular content of cAMP and stimulated the growth of polyoma virus-transformed 3T3 fibroblasts (PV-3T3). However, no change in cAMP content was observed in normal 3T3 fibroblasts. Lower concentrations

of indomethacin (nM) also decreased the cAMP levels in transformed PV-3T3 but to a lesser extent and growth was not stimulated. Thus these findings also show the importance of the cell type and incubation conditions with indomethacin in determining the effect on intracellular cAMP levels. Therefore caution must be taken in interpreting the results obtained.

(e) The Effect of Cholera Toxin on cAMP Accumulation in Intact Cells

Time courses of the effect of cholera toxin on CH23 (Fig. 29) and PCM3 (Fig. 30) show typical responses of intact cells to the effects of this agent. Cells are still responsive to PGE_1 ($2.5\mu M$) following pretreatment with cholera toxin since the curves in the presence of PGE_1 are greater in magnitude than in the absence of hormone. In other studies membranes treated with cholera toxin show greater maximal activity with hormones (Gill, 1977) and this has been explained by Gill (1982) as being due to the longer persistence of active $N_{(GTP)}-Cu^*$. Both cell lines show a maximum and plateau in cAMP accumulation in response to cholera toxin treatment both in the presence and absence of PGE_1 . For both cell lines the plateau occurs at 180 minutes although this is a property of the cell type. For both cell types the initial lag period after treatment with $0.1\mu g/ml$ cholera toxin is most distinguishable in the absence of PGE_1 . In the presence of

N = guanine nucleotide regulatory component,

Cu = catalytic component,

* indicates active state.

Fig. 29. Time Course of Cholera Toxin-Mediated
Increase in cAMP Accumulation in Intact CH23

The cells were washed twice with incubation medium. The final volume of the incubation was 1ml and the volume was adjusted with incubation medium. Cholera toxin was added to give a final concentration of 0.1 μ g/ml. The cells were incubated for the required time at 37°C. After this time PGE₁ (2.5 μ M) was added and the cells were further incubated for 10 minutes at 37°C. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -PGE₁
●—● +PGE₁ (2.5 μ M)

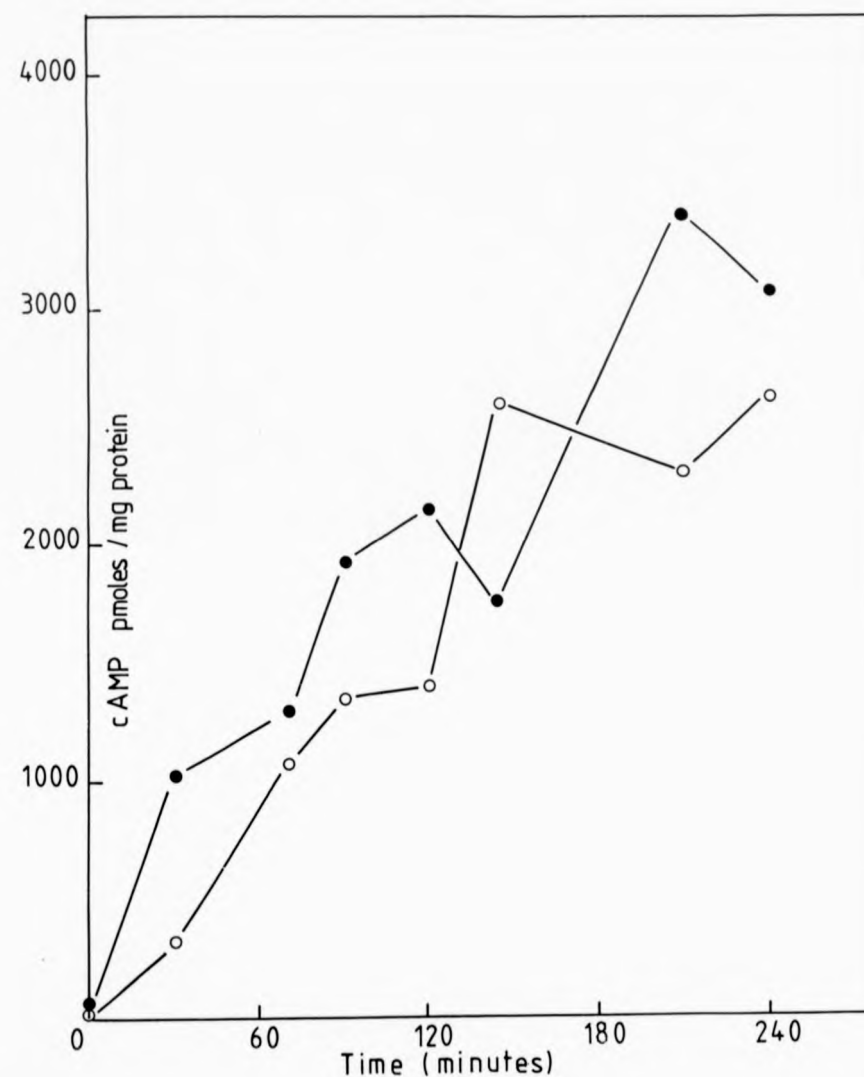
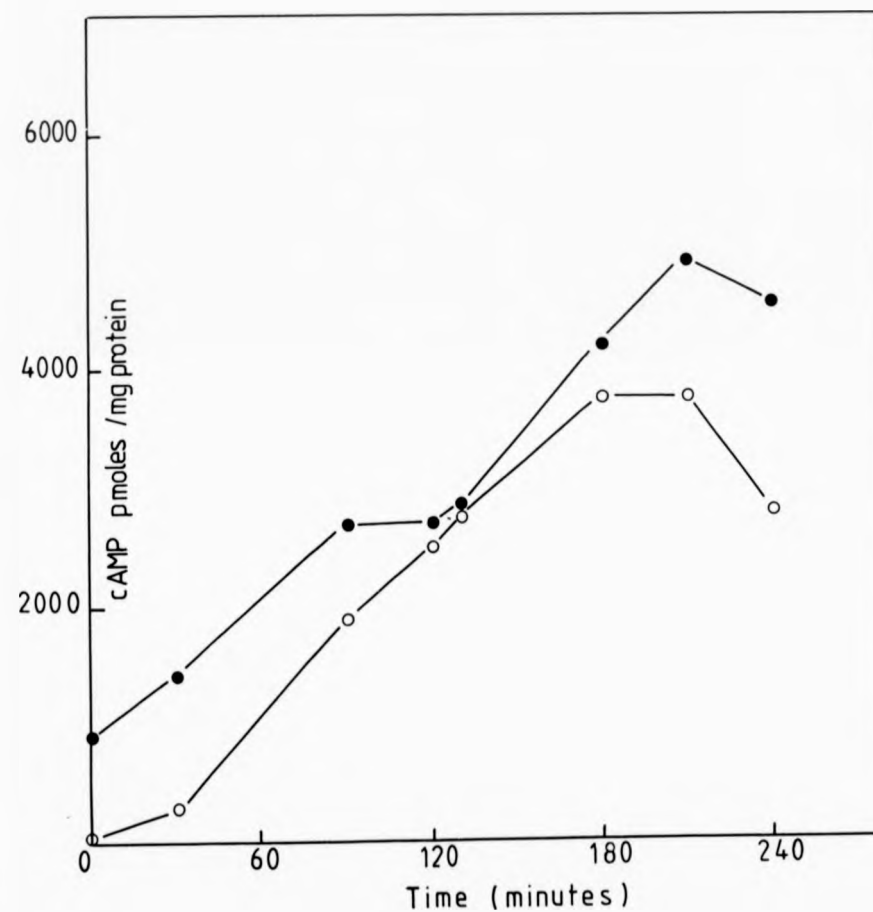


FIGURE 30. Time course of cholera toxin-mediated increase in cAMP accumulation for PCM3 intact cells

Method as described in Fig. 29. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -PGE₁
●—● +PGE₁ (2.5 μM)



hormone the lag period is indistinguishable possibly because it is less than thirty minutes. The lag period is characteristic of cholera toxin activation of intact cells and is thought to represent the time taken for toxin to bind to the cell membrane, the A and B subunits to separate and activation of adenylate cyclase by A (Gill, 1982). This time course has also been studied for P388 in this laboratory (Mujica, 1982). A similar profile was obtained with a maximum and plateau occurring at 180 minutes. Cholera toxin produces a maximum value of cAMP accumulation for P388 of the order of 100 pmoles cAMP/mg protein.

One of the most interesting observations arising from these studies is the high values of cAMP accumulated in CH23 in the presence of cholera toxin. The amounts of cAMP obtained following treatment with cholera toxin are similar for CH23 and PCM3 and both are considerably greater than for P388. Thus in this respect it appears that PCM3 resembles parent CH23. Cholera toxin is known to inhibit the GTPase activity associated with the guanine nucleotide regulatory component of adenylate cyclase (Cassel and Selinger, 1977b) by ADP ribosylation of proteins associated with the regulatory unit (Gill, 1982). Such inhibition has been shown to decrease the GTPase activity associated with the hormone-dependent stimulation of adenylate cyclase (Cassel and Selinger, 1977b). This inhibition of GTPase activity results in a persistent activation of adenylate cyclase activity. Since this inhibition is never 100%, hormones e.g. PGE_1 are still capable of raising the cyclase activity as demonstrated by both CH23 (Fig. 29) PCM3 (Fig. 30)

and P388 (Mujica, 1982). It should be noted that methyl xanthines e.g. IBMX have been demonstrated to inhibit ADP-ribosylation of guanine nucleotide regulatory proteins by cholera toxin (Gill, 1982). So although phosphodiesterase inhibitor is usually included in all incubations to reduce the phosphodiesterase hydrolysis of cAMP it was omitted from the incubation medium when cells were preincubated with cholera toxin. An effect of IBMX on the cholera toxin activation of P388 cells has been observed previously in this laboratory (Mujica, 1982).

The high maximum values of cAMP accumulation obtained with CH23 normal cells and PCM3 hybrid cells as compared to P388 malignant cells suggests the possibility that PCM3 has inherited its regulatory component of adenylate cyclase from CH23. It also demonstrates that in intact state CH23 has the capacity to respond to activating ligands to the same extent as PCM3 but is in some way constrained possibly by the presence of high GTPase activity.

III. THE ROLE OF THE CYTOSKELETON ON ADENYLATE CYCLASE ACTIVITY

Colchicine can be seen to enhance the PGE_1 (2.5 μM) - stimulated cAMP levels for P388 (Fig. 31), CH23 (Fig. 32) and PCM3 (Fig. 33) but there is no noticeable effect on the levels in the absence of hormone. The extent of this enhancement varies with the cell line, P388 > CH23 > PCM3. An effect of colchicine on these cAMP levels can be seen at

FIGURE 31. Effect of Colchicine on P388 Intact Cells

The cells were washed twice with incubation medium. The final volume of the incubation was 1ml and the volume was adjusted with incubation medium. The cells were pre-equilibrated with 1mM IBMX for 10 minutes at 37°C. Colchicine was dissolved in incubation medium and added to give the required final concentration. The cells were incubated for 10 minutes at 37°C. After this time PGE₁ was added and the cells were further incubated for 10 minutes at 37°C. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -PGE₁

●—● +PGE₁ (2.5μM)

B represents the basal value.

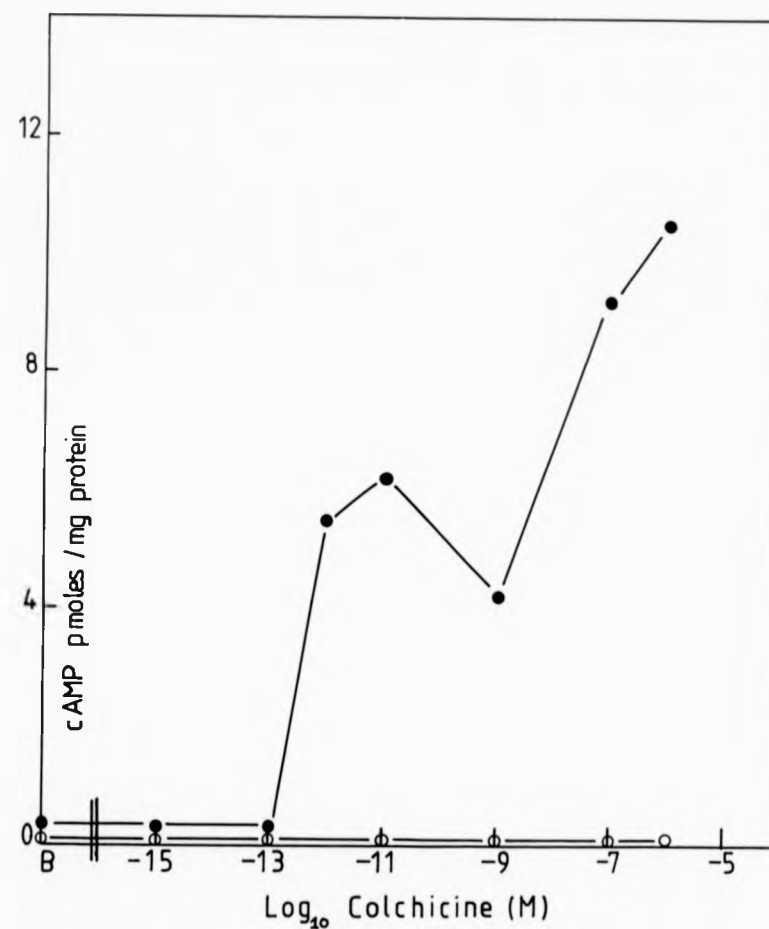


FIGURE 32. Effect of colchicine on CH23 intact cells

Method as described in Fig. 30. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -PGE₁

●—● -PGE₁ (2.5μM)

B indicates the basal value.

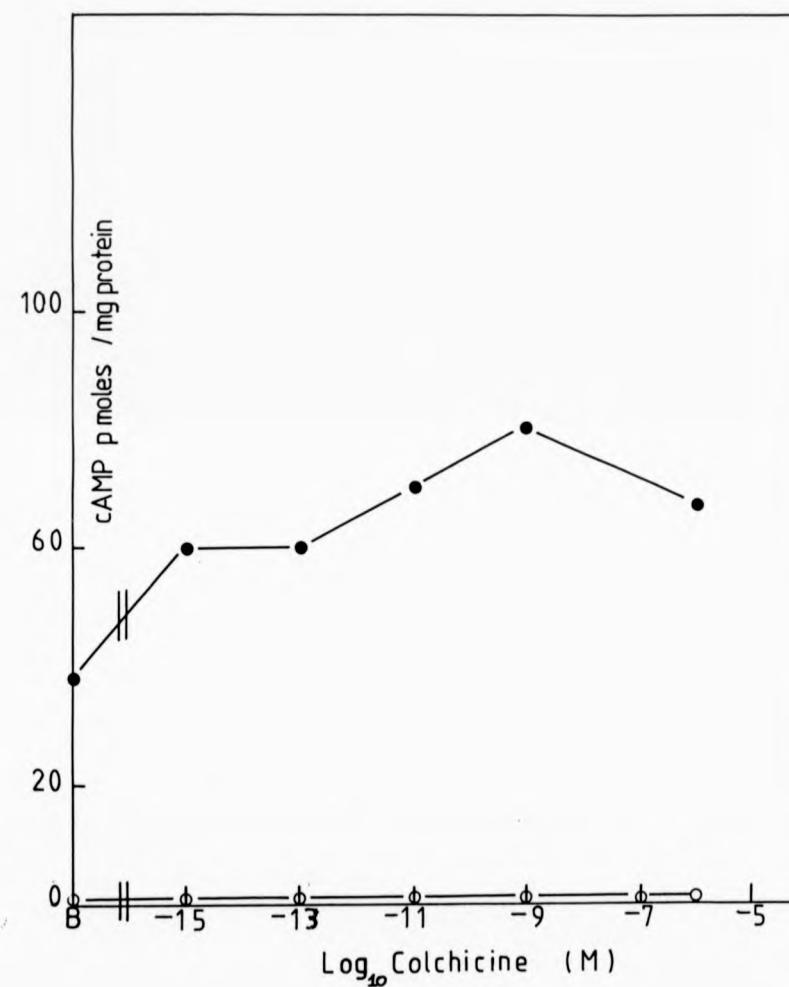


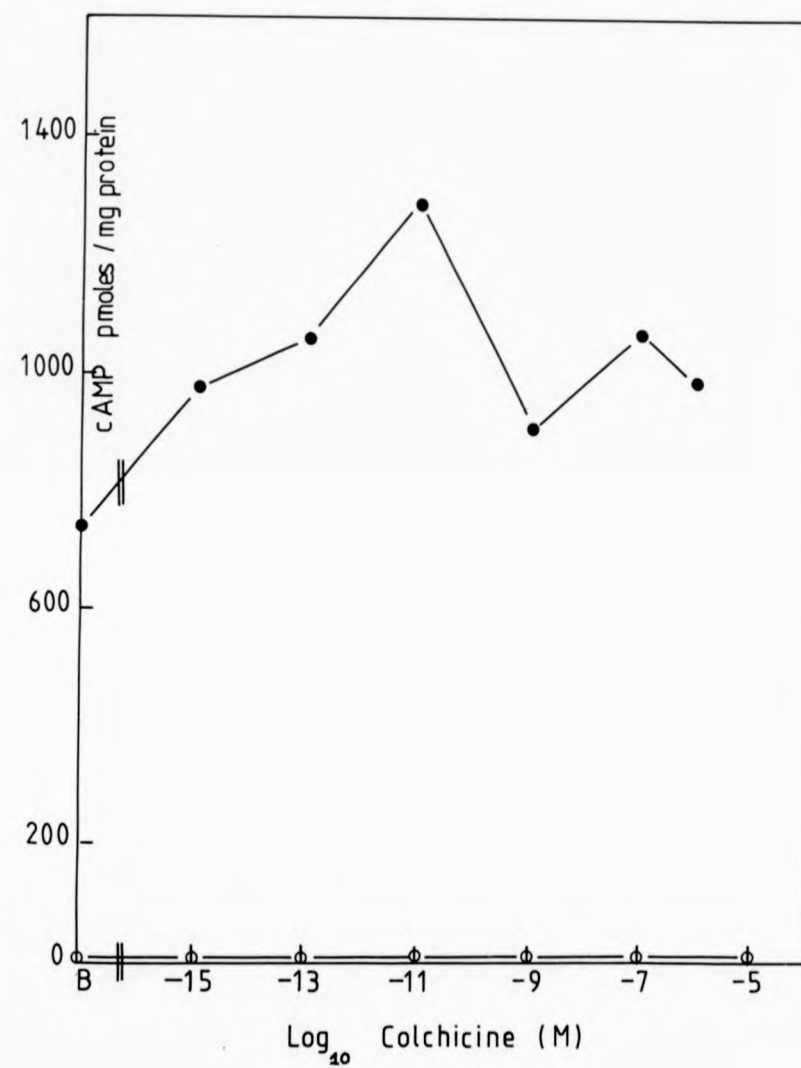
FIGURE 33. Effect of Colchicine on PCM3 Intact Cells

Method as described in Fig. 30. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -PGE₁

●—● +PGE₁ (2.5μM)

B indicates the basal value.



concentrations as low as 10^{-15} M for both CH23 and PCM3 but only as low as 10^{-12} M for P388. This is yet a further illustration of the similarity in properties seen with CH23 and PCM3. Colchicine has been shown to have a number of effects on the cell that are independent of its binding to tubulin and preventing polymerisation of tubulin into microtubules (Beebe *et al.*, 1979) including inhibition of nucleoside transport (Mizel and Wilson, 1972). Therefore it was decided to use nocodazole, a potent microtubule inhibitor which lacks at least some of these side effects of colchicine (Beebe *et al.*, 1979). The concentrations at which 50% inhibition of tubulin polymerisation occurs for colchicine [12.88×10^{-6} M] and for nocodazole [1.97×10^{-6} M] (Friedman and Platzner, 1978) indicate the greater potency of nocodazole.

Nocodazole also is shown to enhance the PGE_1 ($2.5\mu\text{M}$)-stimulated levels of cAMP accumulation for P388 (Fig. 34), CH23 (Fig. 35) and PCM3 (Fig. 36) whilst having no observable effect on levels in the absence of hormone. For all cell lines the more specific inhibitor had observable enhancing activity at concentrations as low as 10^{-15} M. Therefore it seems unlikely that the activation occurring at such low concentrations is due to non-specific activities of colchicine. The extent of the stimulation is variable between the cell lines, CH23 > P388 > PCM3. This order of enhancement is slightly different to that obtained with colchicine and could be due to the non-specific activity of colchicine. The degree of enhancement of the three cell types should reflect the amount of functional microtubular organisation. Simantov and Sachs (1978)

FIGURE 34. Effect of Nocodazole on P388 Intact Cells

The cells were washed twice with incubation medium. The final volume of the incubation was 1ml and the volume was adjusted with incubation medium. The cells were pre-equilibrated for 10 minutes at 37°C with 1mM IBMX. Nocodazole was dissolved in DMSO to the required final concentration and added to the incubation to give a final concentration of DMSO of less than 1%. At this concentration no effect on cell viability was observed. The cells were further incubated at 37°C for 10 minutes. PGE₁ was then added and the cells were further incubated for 10 minutes at 37°C. The results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -PGE₁

●—● +PGE₁ (2.5μM)

B indicates the basal value.

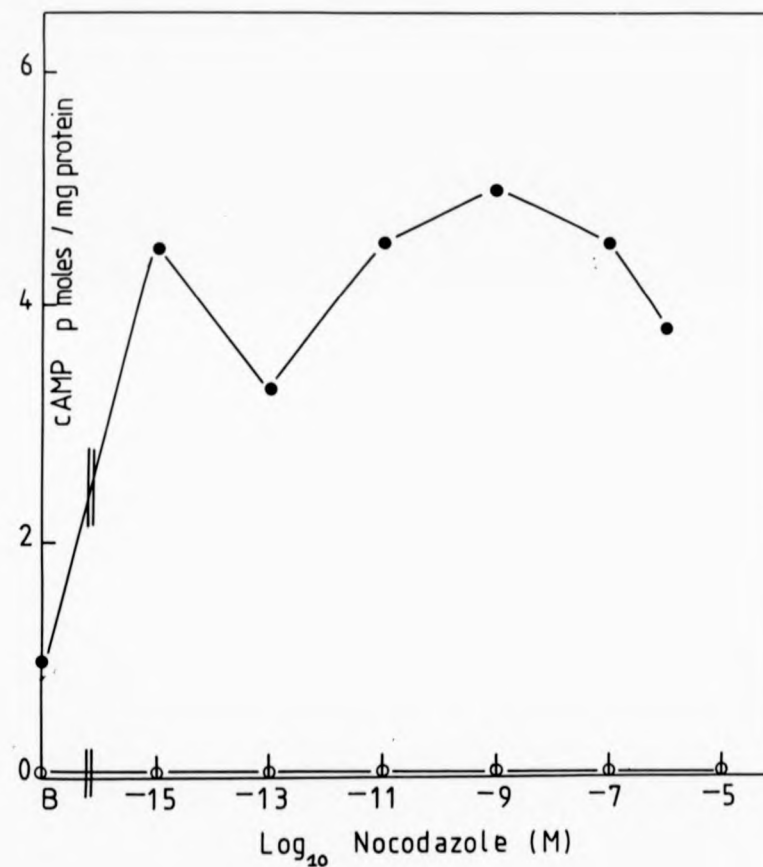


FIGURE 35. Effect of Nocodazole on CH23 Intact Cells

Method as described in Fig. 34. The results shown are means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -PGE₁

●—● +PGE₁ (2.5μM)

B indicates the basal level.

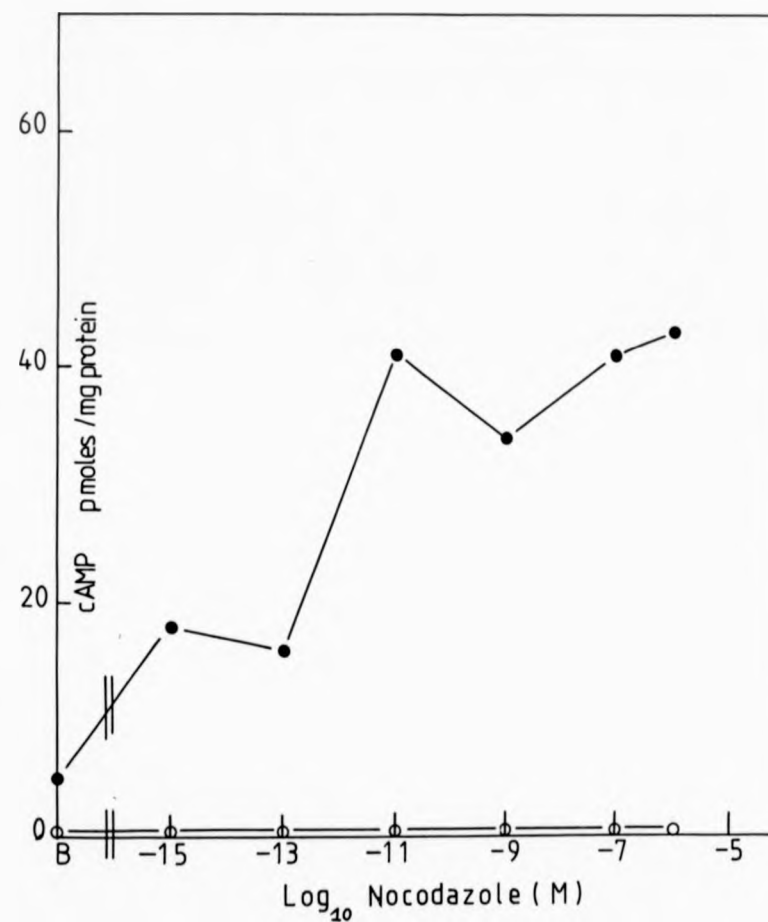


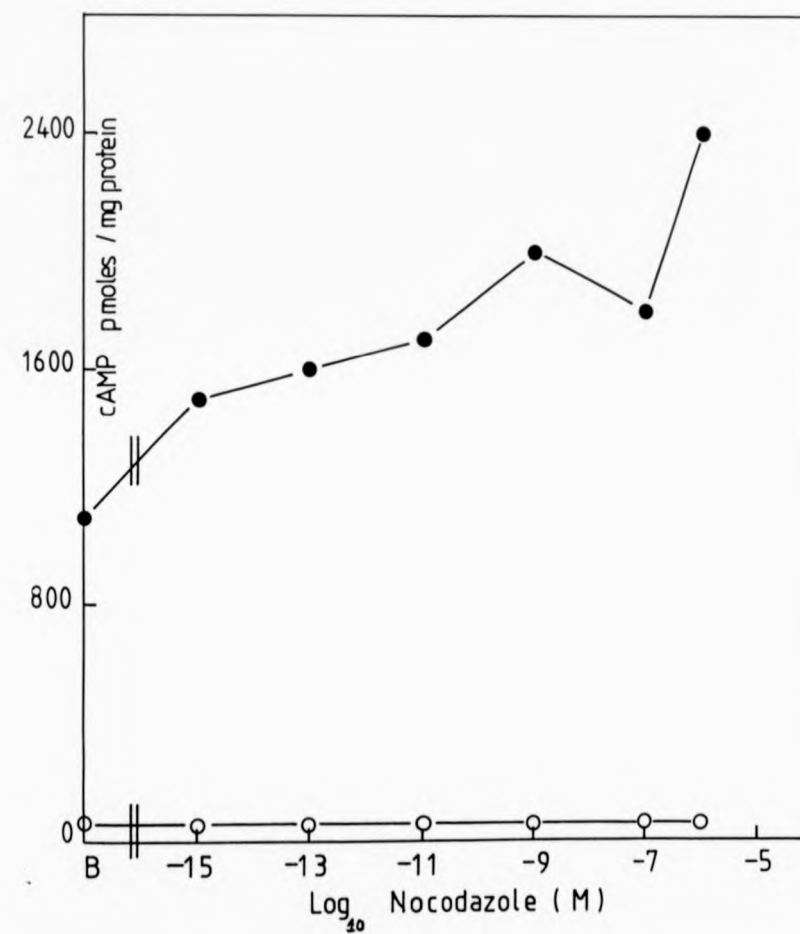
FIGURE 36. Effect of Nocodazole on PCM3 Intact Cells

Method as described in Fig. 34. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ $-PGE_1$

●—● $+PGE_1$ (2.5 μ M)

B indicates the basal value



suggested that a higher level of functional surface microtubules can explain the enhanced hormonal stimulation of cAMP when microtubules are disrupted. From the results obtained with colchicine and nocodazole, PCM3 hybrid cells therefore appear to have the smallest amount of functional microtubules since these cells have consistently lower enhancement of hormone-stimulated cAMP levels than either of the parental cell lines. This might to some extent explain the high response of PCM3 to PGE_1 in the intact state as being due to a lack of constraint by the cytoskeletal system.

Several other workers have shown enhancement of PGE_1 -stimulated cAMP levels (Gemsal *et al.*, 1977; Rudolph *et al.*, 1977; Insel and Kennedy, 1978; Kennedy and Insel, 1979; Hagmann and Fishman, 1980). Conversely for a number of cell types colchicine has either no effect or inhibits hormonal production of cAMP levels (Kalinin, 1977; Zor *et al.*, 1978) and differing response has been observed with cell type (Simantov and Sachs, 1978). Clearly cell origin plays a critical role in determining the activation of cAMP levels by microtubule-disrupting agents.

Following the suggestion that colchicine exerts its action on adenylate cyclase activity via destruction of the association of the guanine nucleotide regulatory unit of cyclase with the plasma membrane (Rasnick *et al.*, 1981) it was decided to study the effect of cholera toxin on the process of colchicine activation of cAMP levels in intact cells. Following preincubation with cholera toxin (0.1 $\mu\text{g/ml}$)

the effect of colchicine on cAMP accumulation was studied in the presence and absence of PGE_1 for P388 (Fig. 37), CH23 (Fig. 38) and PCM3 (Fig. 39). For all three cell lines cAMP levels in the absence of colchicine treatment were similar in the presence or absence of PGE_1 ($2.5\mu\text{M}$) thus indicating that cholera toxin had exerted nearly maximal activation of cyclase activity via inhibition of GTPase activity associated with 'turn-off' of adenylate cyclase activity. Following preincubation of the cells with cholera toxin there was a further enhancement of cAMP levels by colchicine as low as 10^{-15}M for P388 and PCM3 (Fig. 37 and 39) but 10^{-13}M for CH23. The enhancement of cAMP levels by colchicine in cholera toxin pretreated cells was greater in the presence of PGE_1 . For any particular concentration of colchicine studied the effects of colchicine and cholera toxin were synergistic, suggesting that the two agents act at distinct sites. Therefore it is unlikely that colchicine exerts its effects by a direct action on the guanine nucleotide regulatory component itself.

It was decided to determine whether colchicine was exerting its action on adenylate cyclase activity via disruption of plasma membrane microtubules or cytosolic microtubules by studying the effect of colchicine on crude homogenate adenylate cyclase activity. From Fig. 40 it can be seen that no enhancement of adenylate cyclase activity by colchicine occurred either in the presence or absence of PGE_1 ($2.5\mu\text{M}$) at any of the concentrations studied. Therefore it appears that colchicine exerts its action in the intact cell by disruption of cytosolic microtubule organisation since no effect is observed in broken cells or in

FIGURE 37. Effect of Cholera Toxin and Colchicine
on P388 Intact Cells

The cells were incubated with 0.1 μ g/ml cholera toxin for 3-4 hours. After this time the cells were washed to remove serum and resuspended in incubation medium. The final volume of the incubation was 1ml. The volume was adjusted with incubation medium. The cells were then incubated for 10 minutes at 37°C with 1mM IBMX. Colchicine was added and the cells further incubated for 10 minutes at 37°C. After this time PGE₁ was added and the cells were further incubated for 10 minutes at 37°C. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -PGE₁

●—● +PGE₁ (2.5 μ M)

B indicates the basal value.

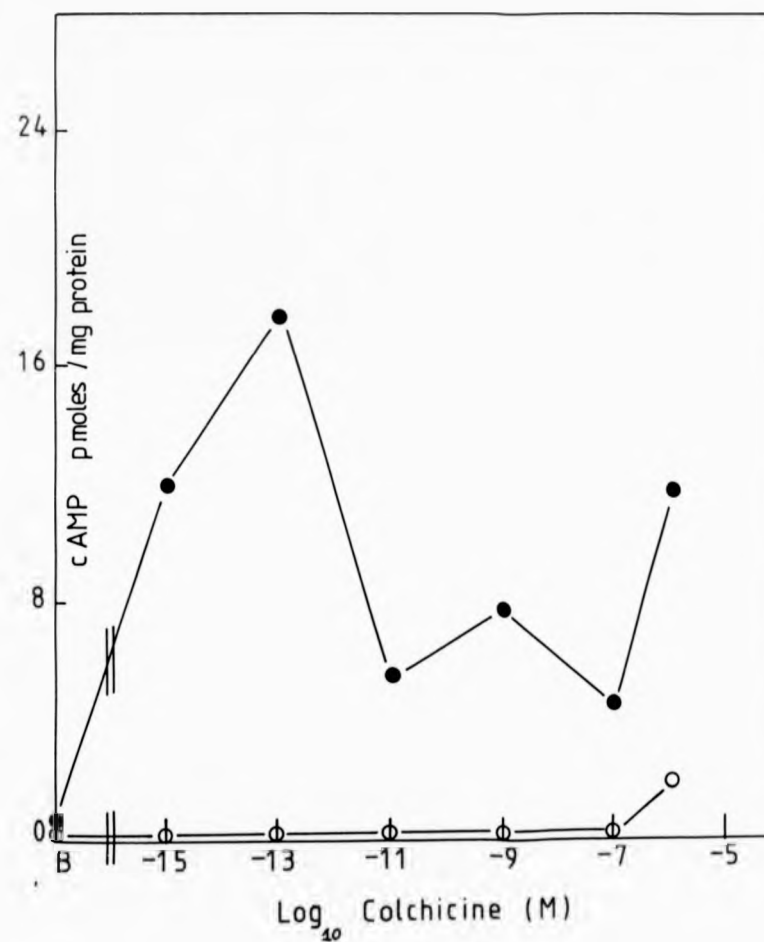


FIGURE 38. Effect of Cholera Toxin and Colchicine
on CH23 Intact Cells

Method as described in Fig. 37. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -PGE₁

●—● +PGE₁ (2.5μM)

B indicates the basal value.

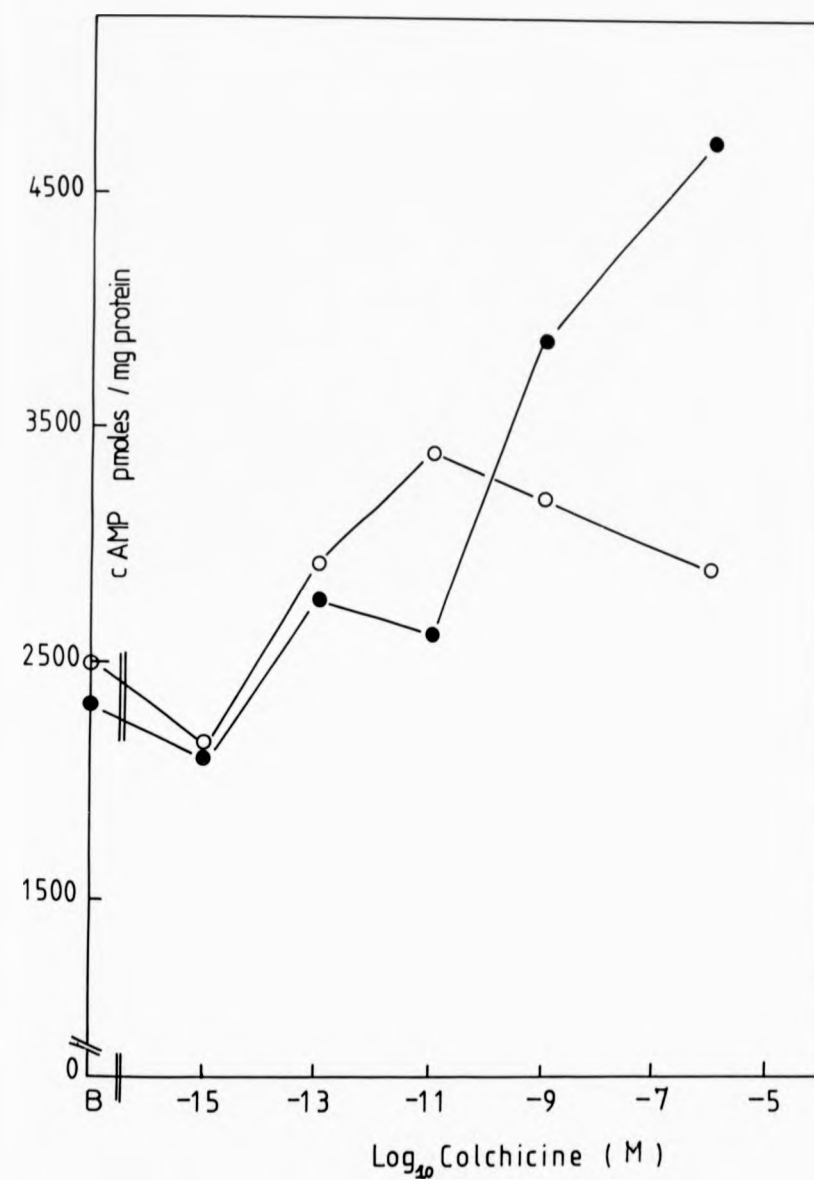


FIGURE 39. Effect of Cholera Toxin and Colchicine
on PCM3 Intact Cells

Method as described in Fig. 37. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -PGE₁

●—● +PGE₁ (2.5μM)

B indicates the basal value

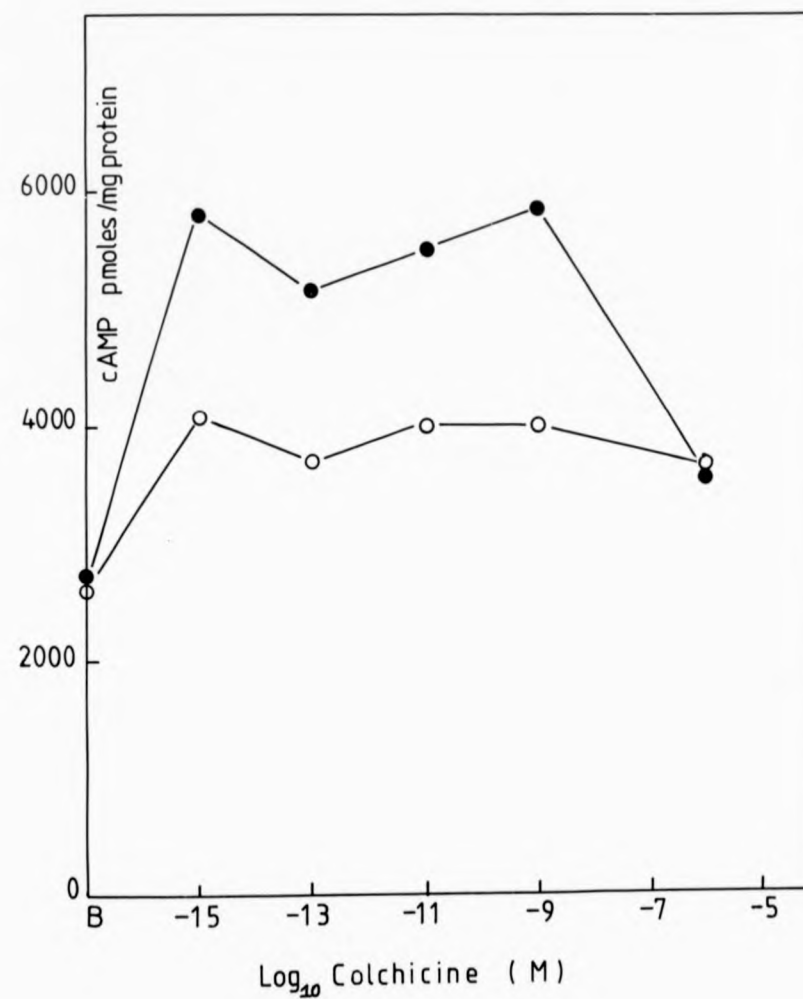
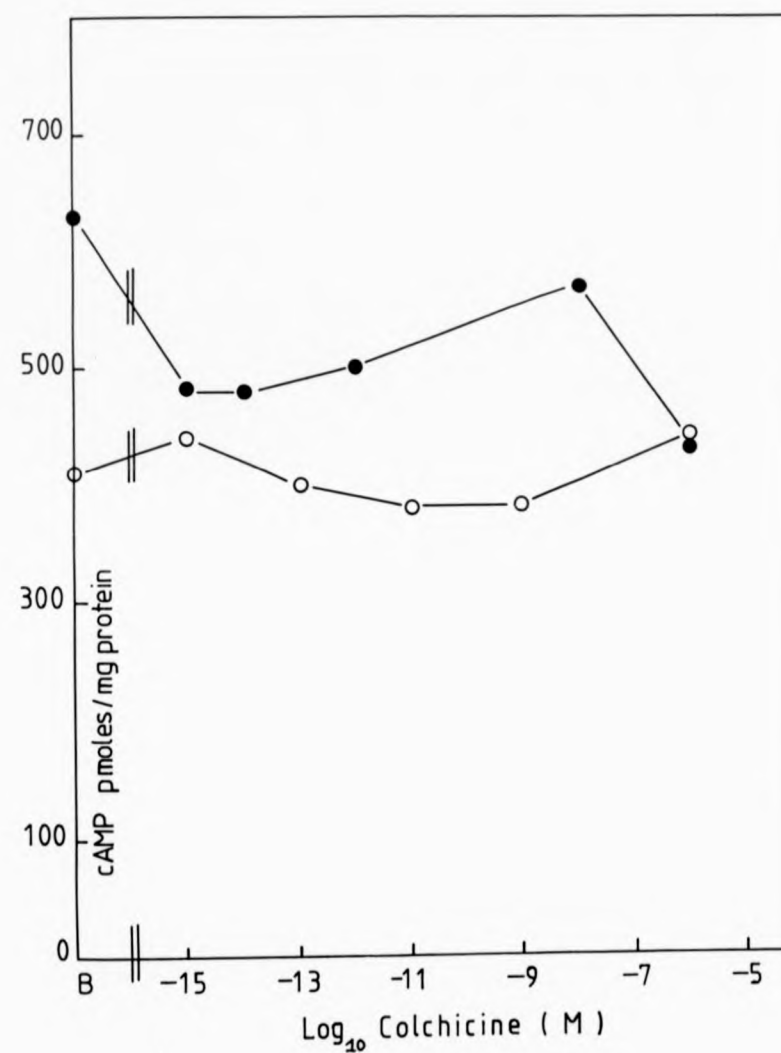


FIGURE 40. Effect of Colchicine on CH23 Homogenate

Components of adenylate cyclase assay plus colchicine and/or PGE_1 were pre-equilibrated at 30°C for 10 minutes. The incubation was initiated by addition of homogenate preparation containing 50-100 μg protein. Incubation was for 10 minutes at 30°C followed by the addition of 0.3M HCl and incubation at 85°C for 45 minutes. Results shown are the means of duplicate incubations and duplicate cAMP determinations of each sample

○—○ - PGE_1

●—● + PGE_1 (2.5 μM)



membrane preparations (data not shown). This has also been observed by others (Rudolph *et al.*, 1977).

From previous results with colchicine no effect of the drug was seen on basal levels of cAMP accumulation (Fig. 31-33), similarly for nocodazole (Fig. 34-36) for any of the cell lines. This suggested that the action of colchicine was distal to the hormone receptor. Colchicine was shown to enhance cholera toxin-stimulated levels of cAMP accumulation both in the presence and absence of PGE_1 (Figs. 37-39). Cholera toxin requires the presence of both the guanine nucleotide regulatory unit and the catalytic component of cyclase for its activity but does not require a hormone receptor. These two observations in combination suggest that microtubule-disrupting agents produce activation of adenylate cyclase activity by an action distal to the hormone receptor. This suggestion has been confirmed by others (Insel and Kennedy, 1978). To further investigate this proposal it was decided to study the effect of treatment with a fixed concentration of colchicine (10^{-11}M) on the PGE_1 dose-response curve for the two cell lines; CH23 (Fig. 41) and PCM3 (Fig. 42). For neither of these two cell lines was there any noticeable effect on the concentration of hormone required for half-maximal activation of adenylate cyclase. The values of cAMP accumulation obtained were greater in the presence of colchicine due to the enhancing effect of the drug on PGE_1 -stimulated cAMP levels. The concentration of PGE_1 appears to be important in this process since little enhancement occurs at low hormone concentrations. These results add further support for the idea that colchicine acts at a site distal to the hormone receptor.

FIGURE 41. Effect of Colchicine on CH23 Intact

Dose Response Curve to PGE₁

The cells were washed twice with incubation medium. The final volume was 1ml and the volume was adjusted with incubation medium. The cells were pre-equilibrated for 10 minutes at 37°C with 1mM IBMX. The cells were then incubated with 10⁻¹¹M colchicine for 10 minutes at 37°C, followed by incubation for 10 minutes at 37°C with PGE₁ of the appropriate final concentration. Results shown are the means of triplicate incubations and cuplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -Colchicine
●—● +Colchicine (10⁻¹¹M)

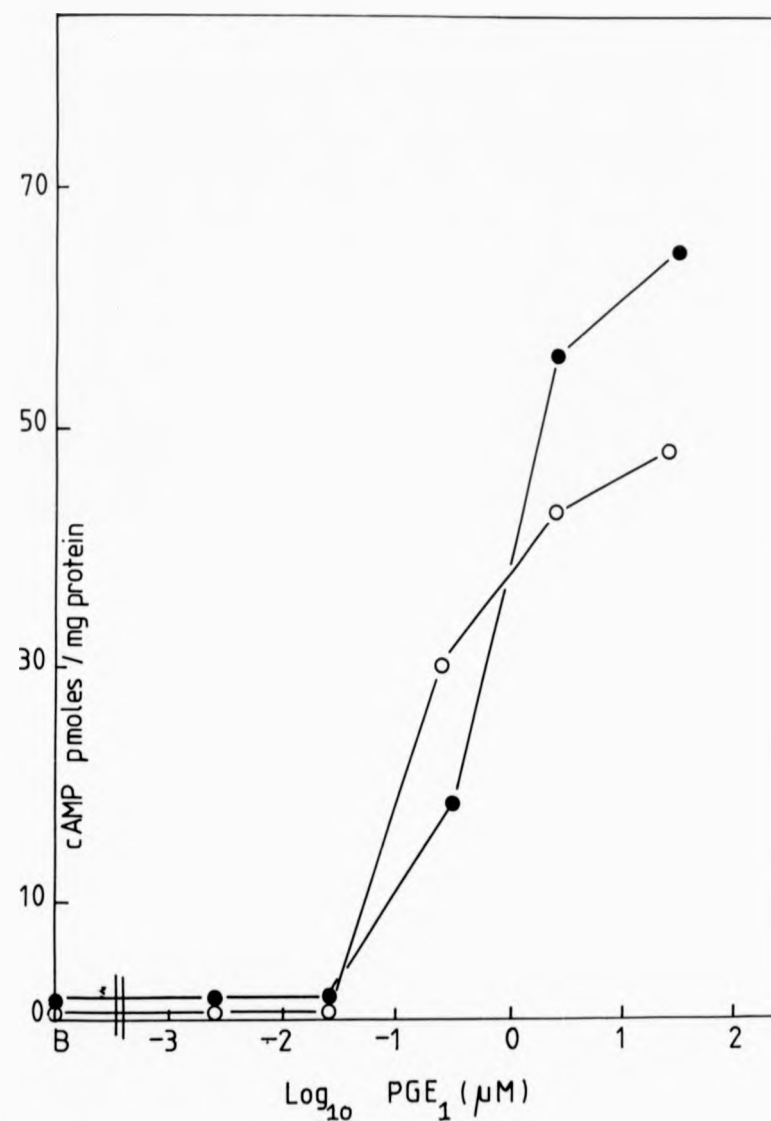
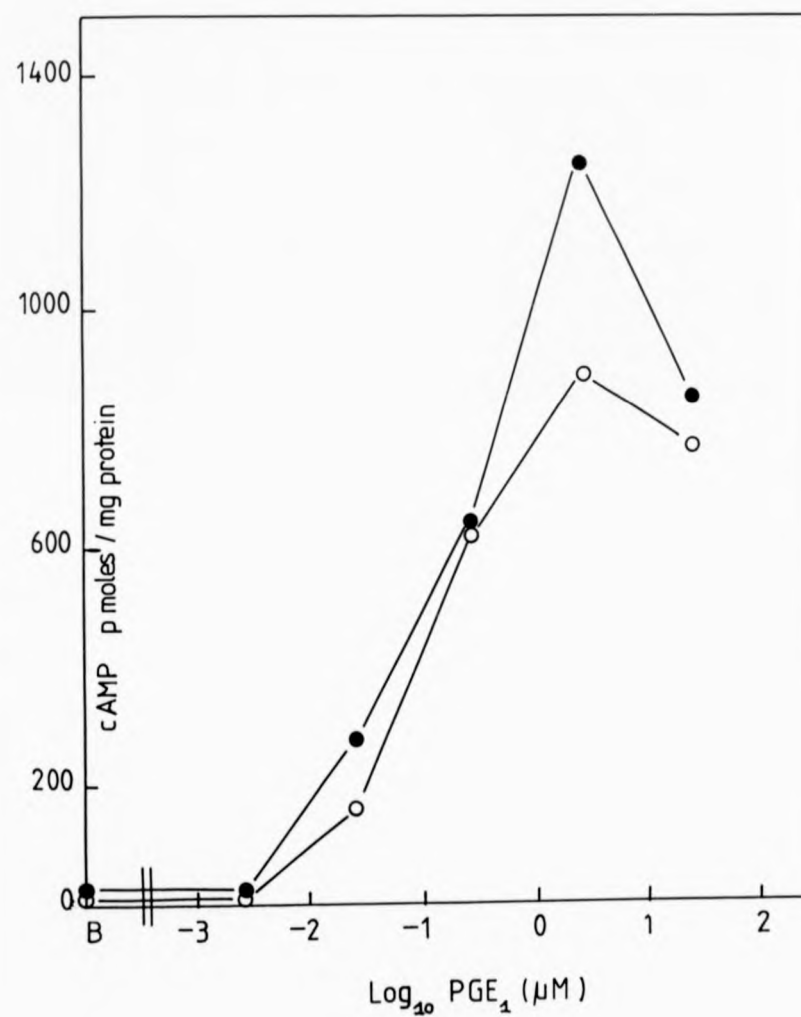


FIGURE 42. Effect of Colchicine on PCM3 Intact
Dose Response Curve to PGE₁

Method as described in Fig. 41. The results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -Colchicine
●—● +Colchicine (10^{-11} M)



There are two general mechanisms whereby colchicine and other microtubule-disrupting agents could exert their effect on hormone- and cholera toxin-stimulated adenylate cyclase activity.

(a) A direct effect on one of the components of the adenylate cyclase molecule distal to the hormone receptor i.e. guanine nucleotide regulatory component or catalytic component.

(b) A general effect on membrane fluidity by acting in the way of an unsaturated fatty acid (Klausner *et al.*, 1980). In this way the localised movement of membrane proteins would be increased and consequently the number of associations between components of adenylate cyclase would be increased.

The first possibility appears to be unlikely since there is no effect of microtubule-disrupting agents on basal levels of cAMP accumulation at any of the concentrations studied and no effect was seen of colchicine on adenylate cyclase activity in broken preparations. However, other workers have suggested that microtubule-disrupting agents act via a direct mechanism on adenylate cyclase (Whetton and Houslay, 1980).

Therefore the other possibility seems more feasible namely that colchicine acts via increasing membrane fluidity. However, the concentrations of colchicine and nocodazole that are found to enhance hormone-stimulated

cAMP levels preclude the possibility of a generalised effect on membrane fluidity. Although maximum enhancement is observed at 10^{-6} M effects are seen at concentrations as low as 10^{-15} M. For both drugs the binding constants for the binding to tubulin molecules are of the order of 10^6 M. At concentrations below this only a small fraction of tubulin molecules would be bound to colchicine and hence incapable of polymerisation to form microtubules. However, it is possible that colchicine and nocodazole act by disruption of specific microtubules involved in the control of adenylate cyclase activity. It has been suggested that it is the microtubules involved in anchoring the guanine nucleotide regulatory component that are directly affected by colchicine (Rasenick *et al.*, 1981). Our results are consistent with this proposal but not conclusive.

Other factors have been implicated in the enhancement of cAMP accumulation in response to hormones by microtubule disrupting agents. Colchicine and vinblastine have both been demonstrated to inhibit cAMP phosphodiesterase activity (Ewart, 1982). To eliminate this complication in interpretation of the results the phosphodiesterase inhibitor, IBMX was included in the incubation medium. Since there are two possible ways that cAMP levels can be increased in the cell (a) increased synthesis i.e. adenylate cyclase activation.

(b) decreased degradation i.e. phosphodiesterase inhibition, the inclusion of the phosphodiesterase inhibitor ensured that only the former phenomenon was important.

Tubulin itself has been demonstrated to activate adenylate cyclase activity (Simonin *et al.*, 1981). Since both nocodazole and colchicine exert their effects on microtubule disruption by binding to tubulin monomers and preventing polymerisation there will be an increased amount of free tubulin. However, no effect was seen on basal levels of adenylate cyclase or on membrane preparations therefore it seems unlikely that microtubule-disrupting drugs act by this mechanism. A further possibility is that by decreasing the polymerisation of tubulin an increased localised concentration of GTP is found since the GTPase activity associated with polymerisation is also diminished. However, for preliminary experiments with CH23, where GTP appears to be able to enter the cell, no enhanced effect of colchicine was seen in the presence of GTP. Therefore, it seems unlikely that the increased localised concentration of GTP is responsible for the enhanced adenylate cyclase activity. However, the GTP could be compartmentalised within the cell. However, colchicine has been shown to enhance the GTPase activity (Lin and Hamel, 1981).

These results appear to suggest that colchicine and nocodazole exert their effects on adenylate cyclase activity at a site distal to the hormone receptor and that cytosolic tubulin is involved in its action possibly at a specific location anchoring either the catalytic or regulatory component to the membrane. Other workers have shown the cytoskeleton to be attached to the membrane (Ishikawa *et al.*, 1982) and the regulatory protein is known to have its major display on

the cytosolic face of the plasma membrane. Therefore the specific microtubules affected by colchicine and nocodazole could be those anchoring the regulatory protein to the membrane as suggested by Rasenick *et al.* (1981).

IV. EFFECT OF PROTEASES ON INTACT CELLS

The effects of various concentrations of trypsin on cAMP accumulation was studied for all three cell lines, CH23 (Fig. 43a,b), PCM3 (Fig. 44a,b) and P388 (Fig. 45). Trypsin is routinely used as a prelude to passaging of monolayer cells so it is important to determine the effect of this enzyme on cellular cAMP levels. For all cell lines studied there was little noticeable effect on cAMP levels in the absence of PGE_1 . The fact that there is still activity in the presence of PGE_1 following trypsin treatment indicates that the PGE_1 receptor was unaffected by trypsin. Both P388 and PCM3 showed an increase in accumulation of cAMP in response to trypsin treatment especially at low concentrations of trypsin ($<50\mu\text{g/ml}$). This slight increase was reproducible. On the other hand CH23 exhibited a reproducible biphasic response to trypsin over the 0- $100\mu\text{g/ml}$ concentration range (Fig. 43b). Above $100\mu\text{g/ml}$ there was a consistent decrease in cAMP levels. Since the levels of cAMP measured were total levels (intracellular plus extracellular) and the phosphodiesterase levels were inhibited by the inclusion of IBMX, the values of cAMP obtained should reflect the levels of adenylate cyclase activity.

FIGURE 43. Effect of Trypsin on CH23 Intact Cells

(a) 0-500 μ g/ml

(b) 0-100 μ g/ml

The cells were washed twice with incubation medium. The final volume of the incubation was 1ml and the volume was adjusted with incubation medium. The cells were pre-equilibrated at 37°C for 10 minutes with 1mM IBMX. PGE₁ was then added and the cells incubated for 10 minutes at 37°C. Trypsin was then added and the cells were further incubated for 5 minutes at 37°C. The reaction was stopped by boiling. The results shown are the means of triplicate incubations and duplicate cAMP assays. Standard deviations were always less than 10%.

○ — ○ -PGE₁

● — ● +PGE₁ (2.5 μ M)

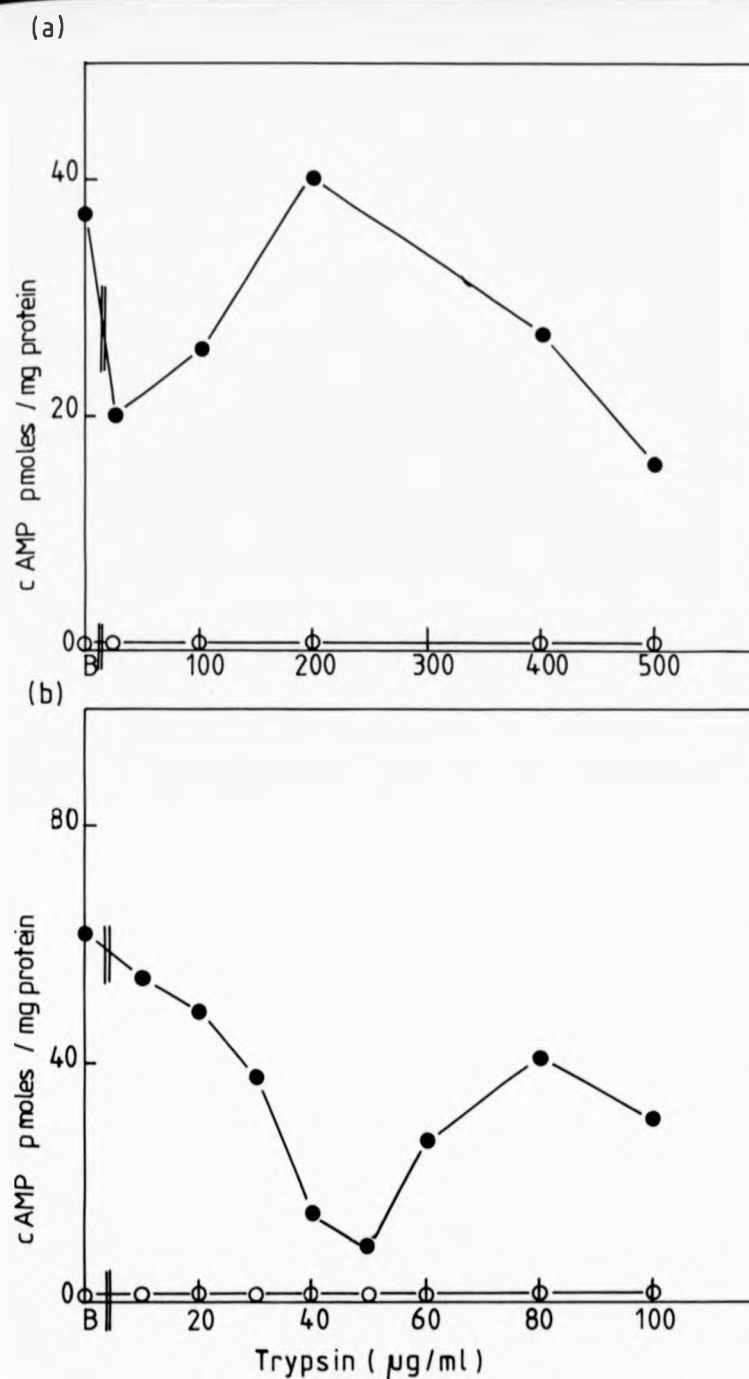


FIGURE 44. Effect of Trypsin on PCM3 Intact Cells

(a) 0-500 $\mu\text{g/ml}$

(b) 0-100 $\mu\text{g/ml}$

Method as described in Fig. 43. The results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○ — ○ - PGE_1
● — ● + PGE_1 (2.5 μM)

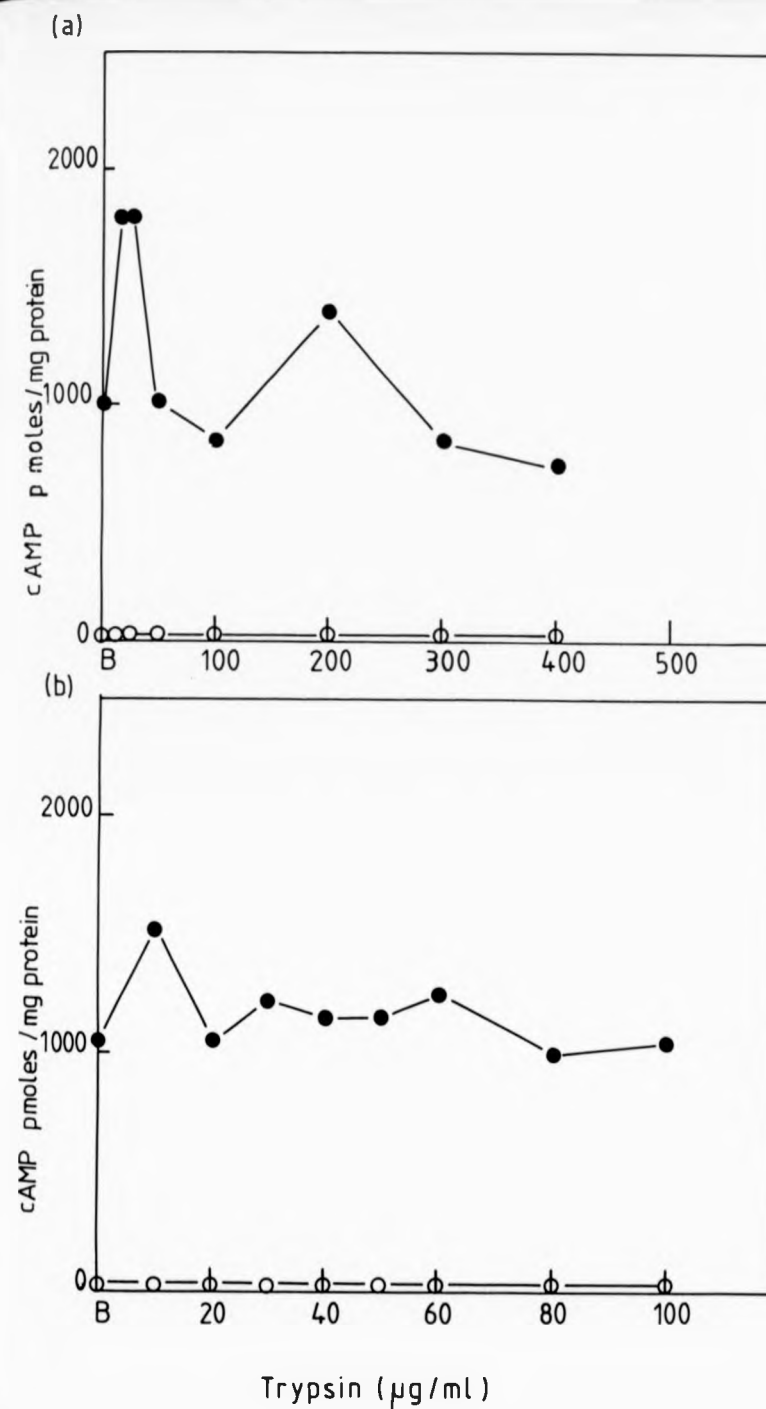
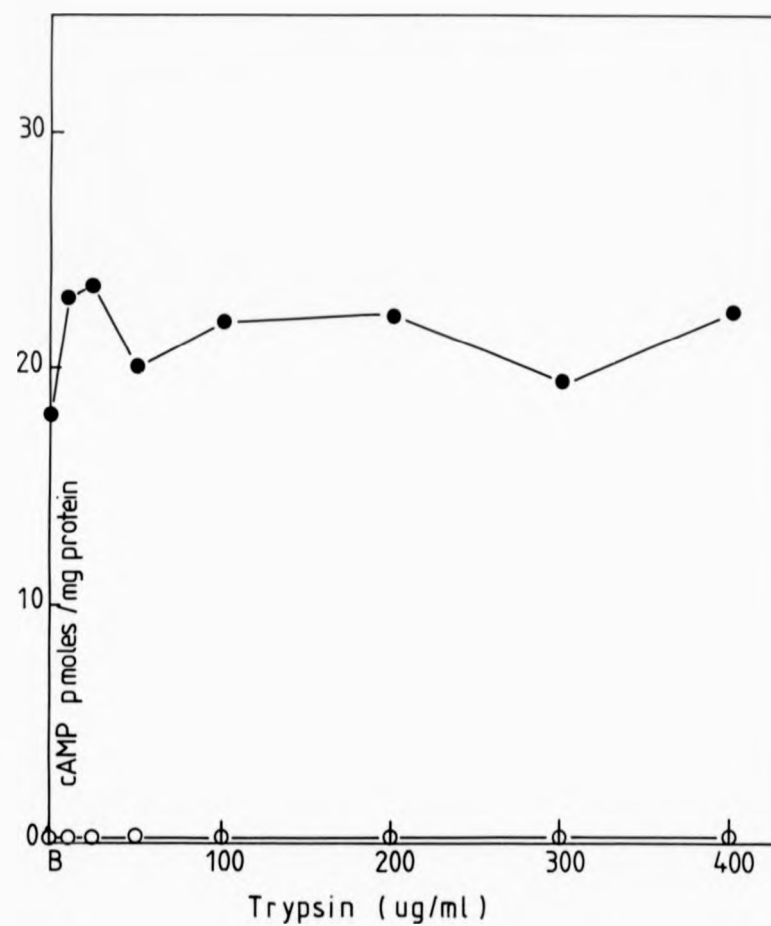


FIGURE 45. The Effect of Trypsin on P388 Intact Cells

Method as described in Fig. 43. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ $-PGE_1$
●—● $+PGE_1$ (2.5 μ g).



One possible explanation for the effect of trypsin on intact cells is that the protease is degrading the cell surface glycoproteins and/or proteins and thereby altering the environment of the adenylate cyclase molecule. This would provide a possible explanation for the variation in effect of this protease between the cell lines since the cell surface protein and glycoprotein composition has been shown to vary with cell type (Aston, 1982). It remains to be established whether trypsin can have a direct action on one of the components of adenylate cyclase.

In order to determine the specificity of action of proteases, chymotrypsin was also used to study the effects on cAMP accumulation. The same concentration range of chymotrypsin (0-500 μ g/ml) was used to measure changes in cAMP accumulation in CH23 (Fig. 46a,b) and PCM3 (Fig. 47) cells. Chymotrypsin showed a similar effect on CH23 to trypsin i.e. a biphasic effect between 0-100 μ g/ml followed by a consistent decline in cAMP levels over the 100-500 μ g/ml range. Slightly similar profiles were obtained for PCM3 in the presence of chymotrypsin as was found in the presence of trypsin i.e. a slight increase at low concentrations (0-50 μ g/ml) but this was followed by a decrease in cAMP levels at higher chymotrypsin concentrations as compared to the constant levels with trypsin (Fig. 41a). Thus it appears that the action of these two proteases is approximately comparable.

The response of the three cell lines to trypsin illustrates that PCM3 has inherited some of the characteristics of the malignant parent P388 since its response to trypsin is very similar to that of P388.

FIGURE 46. The Effect of Chymotrypsin on CH23

Intact Cells. (a) 0-500 μ g/ml

(b) 0-100 μ g/ml

The cells were washed twice with incubation medium. The final volume of the incubation was 1ml and the volume was adjusted with incubation medium. The cells were pre-equilibrated for 10 minutes at 37°C with 1mM IBMX. PGE₁ was added and the cells were incubated for 10 minutes at 37°C. Chymotrypsin was then added and the cells were incubated for 5 minutes at 37°C. The reaction was stopped by boiling. Results shown are the means of triplicate incubations and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -PGE₁

●—● +PGE₁ (2.5 μ M).

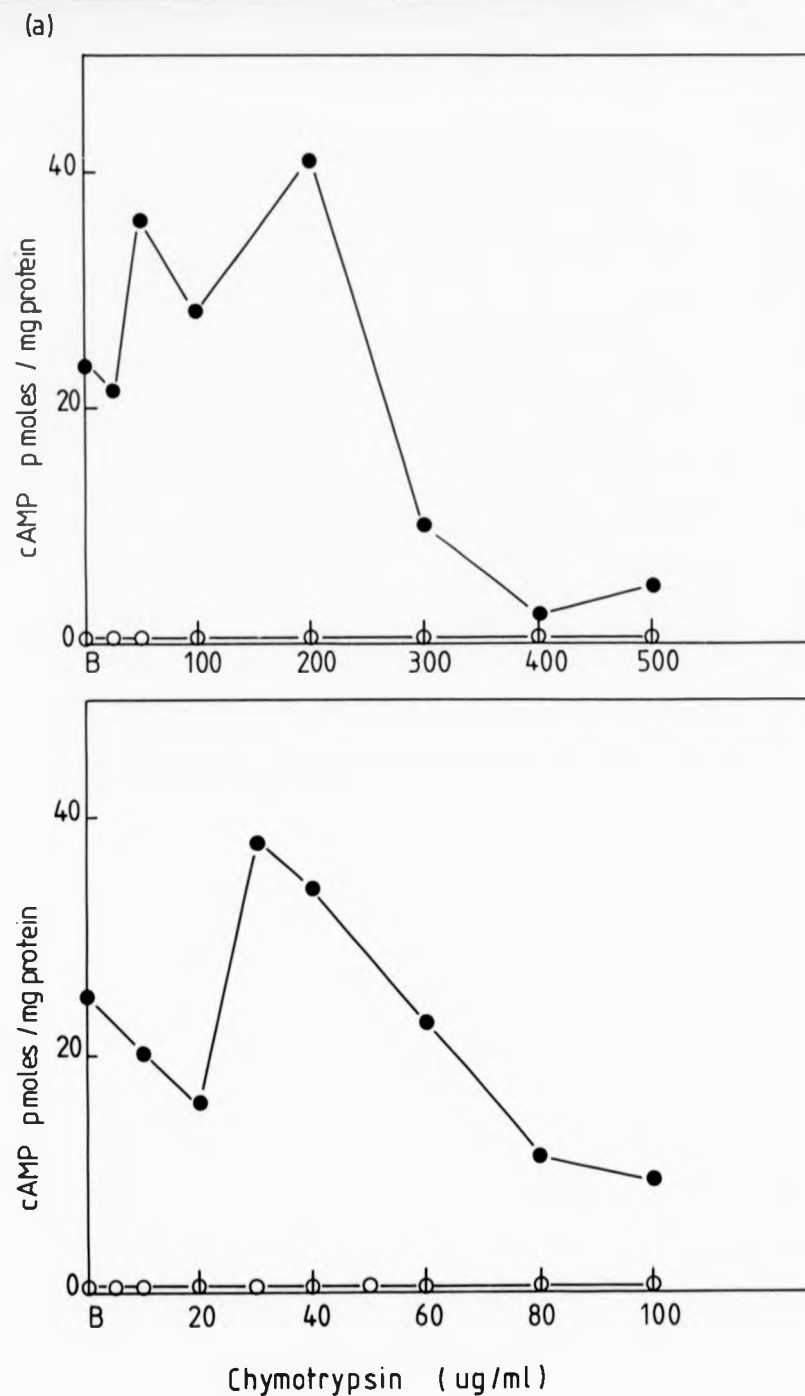
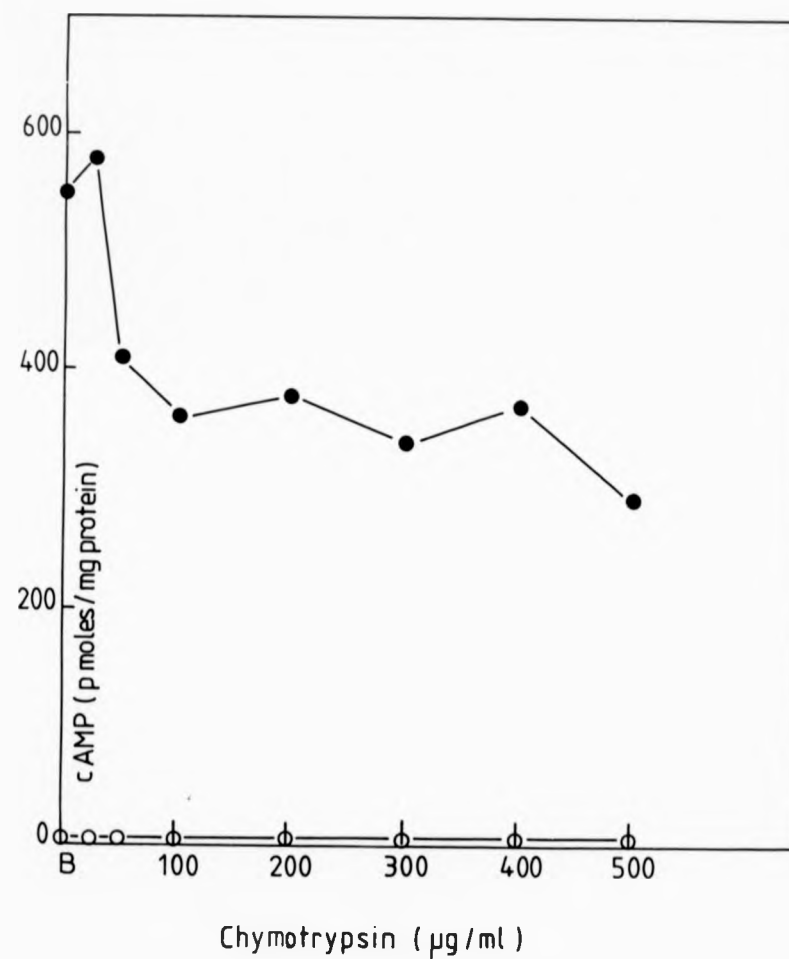


FIGURE 47. The Effect of Chymotrypsin on PCM3
Intact Cells

Method as described for Fig. 46. The results shown are the means of triplicate and duplicate cAMP assays of each sample. Standard deviations were always less than 10%.

○—○ -PGE₁
●—● +PGE₁ (2.5 μM).



Trypsin has been previously reported to reduce cellular cAMP levels (Willingham, 1976; Tomasi, 1977) whereas others have found enhanced levels of cellular cAMP (Koji and Terayama, 1980; Knopp *et al.*, 1983). It would appear that the action of proteases on cAMP levels in intact cells is therefore either a property of cell type or of experimental procedure employed. The effect of proteases in inhibiting CH23 cAMP accumulation has been noted before in this laboratory (Hughes and Ayad, 1980) and was attributed to an effect at the cell surface. The possible effects of proteases on the adenylate cyclase molecule is considered later under protease actions on broken cells.

BROKEN CELL STUDIES

I. PROPERTIES OF HOMOGENATES FROM THREE CELL LINES

(a) Response to PGE₁

The dose-response curves to PGE₁ in crude homogenate preparations of P388 (Fig. 48), PCM3 (Fig. 49) and CH23 (Fig. 50) can be seen. The order of activation is PCM3 > P388 > CH23. Similar results have been obtained in this laboratory previously (Ayad and Foster, 1977; Ayad and Burns, 1977). This order of responsiveness to PGE₁ is totally different from that observed in intact cells where PCM3 is enhanced about 40-fold and CH23 shows slight response and P388 very little response. Both PCM3 and CH23 exhibit diminished response to hormone following homogenisation. This phenomenon has been observed previously i.e. that for the

FIGURE 48. PGE_1 Dose Response Curve of P388 Homogenate
Adenylate Cyclase Activity

Adenylate cyclase assay components plus PGE_1 were pre-incubated at 30°C for 10 minutes. After this time the incubation was initiated by the addition of homogenate (50-100 μg protein). Adenylate cyclase activity was measured for 10 minutes at 30°C . After this time 0.3M HCl was added and the tubes transferred to an 85°C water-bath for 45 minutes. Results shown are the means of duplicate incubations and duplicate cAMP determinations of each sample.

B represents the basal value.

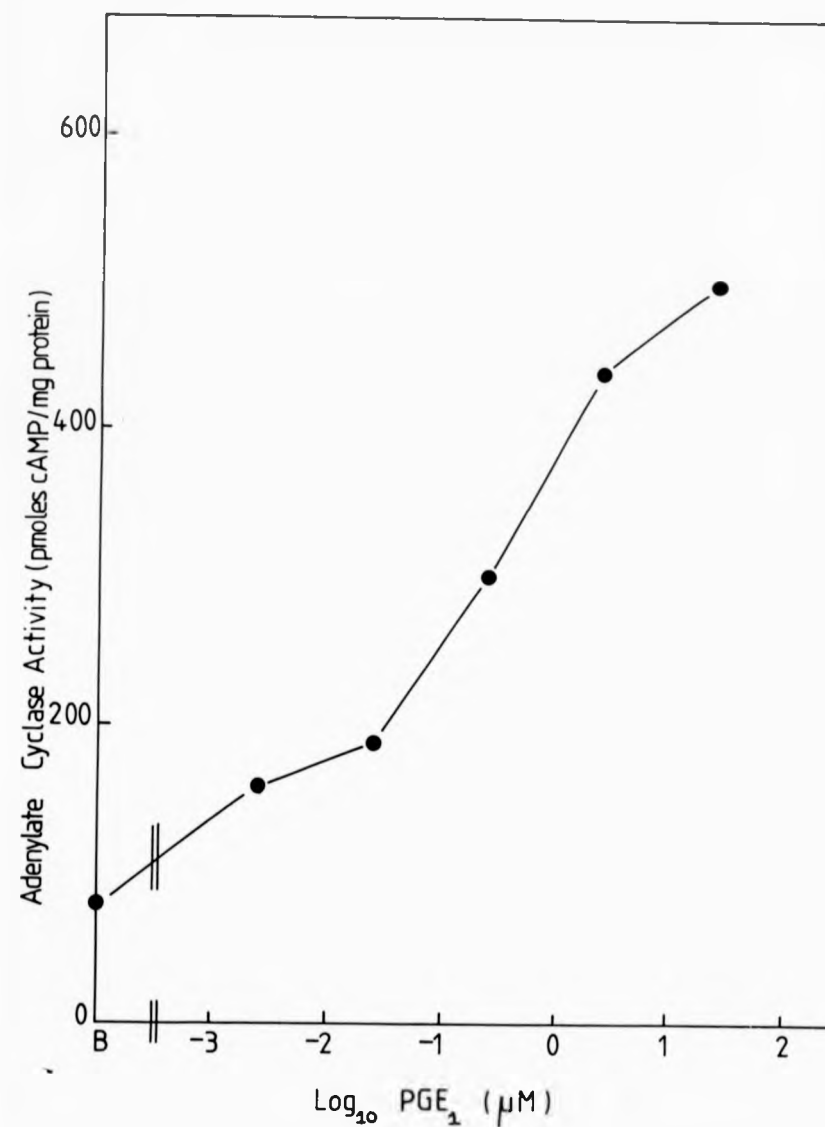


FIGURE 49. PGE_1 Dose Response Curve of PCM3
Homogenate Adenylate Cyclase Activity

Method as described in Fig. 48. Results shown are the means of duplicate incubations and duplicate cAMP determinations of each sample.

B represents the basal value.

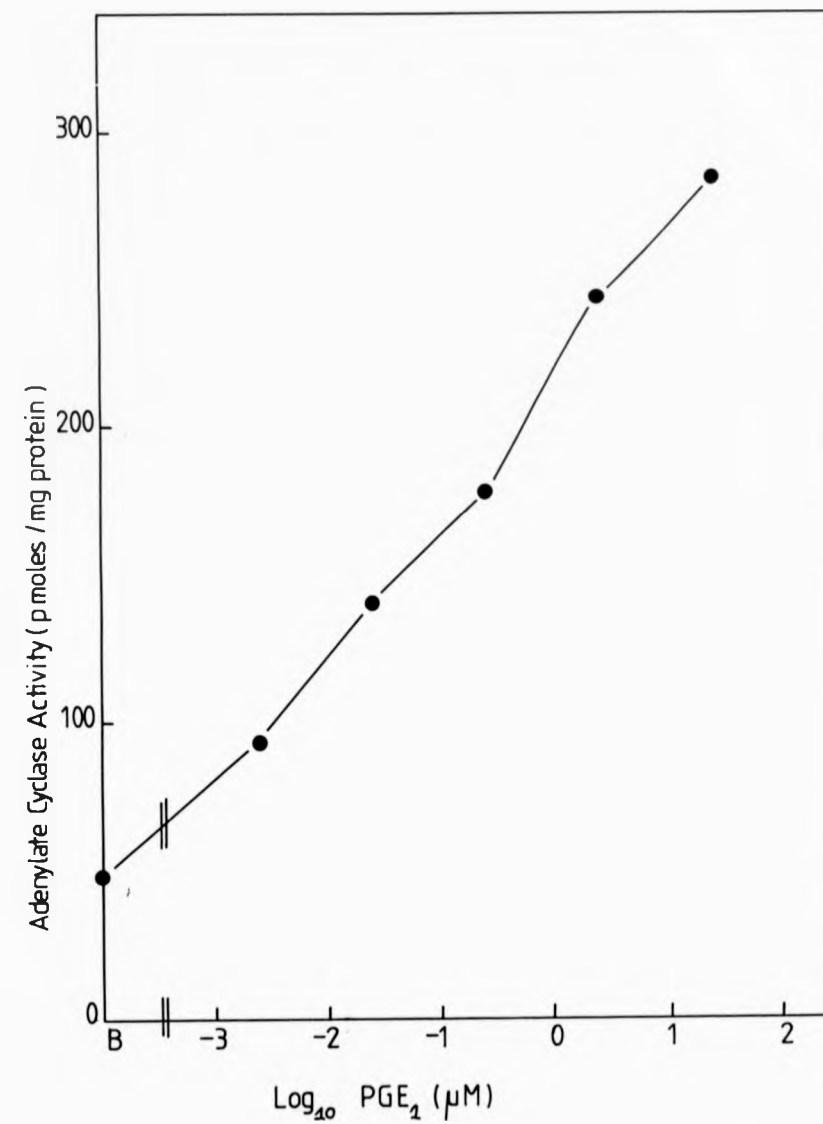
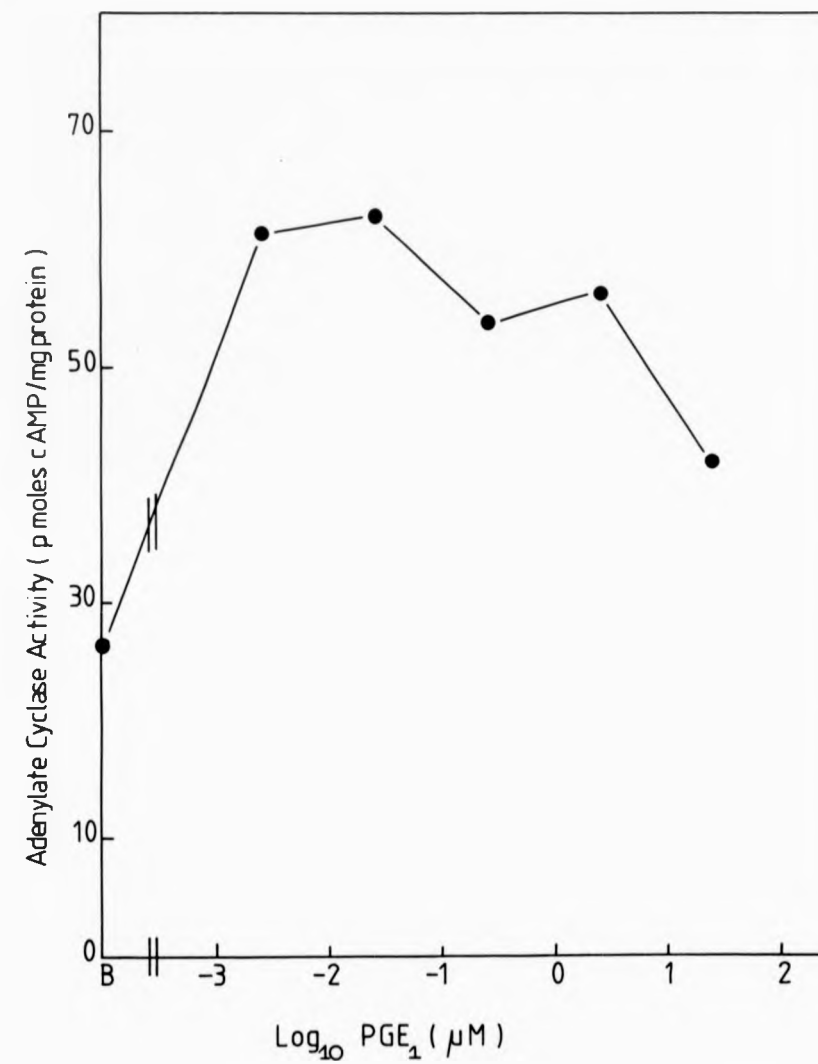


FIGURE 50. PGE_1 Dose Response Curve of CH23
Homogenate Adenylate Cyclase Activity

Method as described in Fig. 48. Results shown
are the means of duplicate incubations and duplicate
cAMP determinations of each sample.

B represents the basal value.



majority of cells the process of homogenisation results in a diminished response to hormones (Insel and Stoolman, 1978). For both PCM3 and CH23 the activation by PGE_1 of adenylate cyclase activity is reduced approximately by a factor of 10 compared to that in the intact cell. There are several possible explanations for the reduced responsiveness to hormones that is customarily found in homogenates.

(a) Homogenisation has been demonstrated to break cells into closed vesicles similar in number to the number of hormone receptors (Sahyoun *et al.*, 1977). Evidence suggests that receptors and enzyme components of adenylate cyclase are not randomly distributed in the membrane but are asymmetrically arranged allowing separation of the components of cyclase during vesicle formation (Sahyoun *et al.*, 1977). According to the mobile receptor theory of adenylate cyclase activation receptor-enzyme coupling occurs only when the receptor is occupied (Houslay *et al.*, 1977). For activation of adenylate cyclase to occur it is necessary that the hormone receptor is capable of interaction with hormones and that the hormone-receptor complex can couple both with the regulatory component and the catalytic protein. Therefore the homogenisation process in which vesicles are formed results in a decreased number of interactions between these various components.

(b) An alternative explanation for the reduced activity that occurs following homogenisation is the loss from the cell during the washing and homogenisation procedure of a cytosolic factor responsible for effective

activation of adenylate cyclase activity by hormones (Ganguly and Greenough, 1975; Beaumont *et al.*, 1980; Bhatet *et al.*, 1980). These factors all appear to have different molecular sizes so the precise nature of the cytosolic factor removed during homogenisation that could account for the reduced activity in response to PGE_1 for both PCM3 and CH23 remains to be resolved.

It is feasible that in most systems a combination of both explanations occurs and this accounts for the significantly reduced sensitivity to hormones that is seen following homogenisation.

In contrast homogenisation of P388 cells produces an homogenate having a significantly enhanced response to PGE_1 (Fig. 46) as compared to the extremely low activation seen for intact cells. This situation is far more difficult to explain. From previous results studying the effects of microtubule-disrupting agents on the cAMP accumulation in response to hormones for intact cells it was observed that colchicine had a greater effect in enhancing hormone-stimulated cAMP accumulation in P388 intact cells than either CH23 or PCM3. Therefore it is possible that in the intact state the P388 malignant cell has a greater degree of constraint imposed on the cyclase molecule compared to CH23 and PCM3 cells. Therefore following homogenisation this constraint is removed and activity is enhanced by a factor similar in magnitude to that found at optimum concentration of colchicine. However, although CH23 and PCM3 had a lower response in the presence of colchicine than

P388, CH23 had a higher response to nocodazole and activation still occurred for CH23 and PCM3 by antimicrotubule agents. It would be expected that an increase in response to PGE₁ for CH23 and PCM3 would occur following homogenisation if this was the sole explanation. Therefore although the cytoskeleton has been implicated in the low response of malignant cells to hormones (Edelman and Yahara, 1976; Boschet et al., 1981) it does not appear that it is the sole reason for the unusual increase in activity following homogenisation of P388 transformed cells.

A further possibility is that homogenisation of P388 cells and the associated washing procedures lead to the loss of an inhibitory cytosolic protein factor. It has been shown that certain cytosolic factors are responsible for inhibition of adenylate cyclase activity (Sano and Drummond, 1981).

A further possible explanation for the discrepancy observed in the behaviour of P388 homogenate arises from the assay conditions utilised for the homogenate. In the intact state the levels of ATP in malignant cells is low and hence the response to hormones in this state is measured in a situation where the adenylate cyclase molecule is in a pool low in ATP. Measurements in this laboratory have indicated that intracellular ATP levels of P388 cells are considerably lower than those of either CH23 or PCM3 (Ayad and Hughes, 1980; DeCastro and Ayad, 1982). Conversely

in the homogenate the response to hormones is measured in the presence of 2mM ATP in combination with a regenerating system. From the results previously discussed it is observed that the addition of ATP to intact P388 cells produces greatly enhanced cAMP accumulation in response to PGE_1 particularly at 2mM ATP whereas little effect is seen with CH23 or PCM3. Thus the enhanced activity of P388 homogenate can be explained by the presence of exogenous nucleotides in the incubation medium for the cyclase assay system. However at 2mM ATP and 2.5 μ M PGE_1 in the intact state (Fig. 11) the levels of cAMP are 200 pmoles/mg protein whereas in the broken state the levels are 450 pmoles/mg protein. Therefore it does not appear that the presence of ATP in the incubation medium is the sole factor governing the enhanced activation of adenylate cyclase activity in the homogenate. It is possible that it is a combination of all the factors discussed. Another possibility to account for the difference is the disruption of the hormone receptors. It is feasible that in CH23 and particularly PCM3 intact cells that the PGE_1 receptors are readily available for interaction with hormone and P388 receptors are somehow 'masked'. Following homogenisation the P388 receptor may become readily available. No evidence is available for this suggestion. This possibility was discussed by Ayad and Foster (1974) to account for the greater response of PCM3 to PGE_1 compared to either parent and temperature dependence studies by these workers led to the concept of the PGE_1 receptor of PCM3 membrane is in a different state to that in the parent cells.

From these results concerning the homogenate responsiveness it can be seen that PCM3 acts in a similar manner to CH23 but in a totally dissimilar manner to P388. This further shows the similarity of the hybrid to the CH23 parent cell.

It should be noted from Fig. 50 that CH23 does not demonstrate a typical dose-response curve to PGE_1 in the broken state. Both P388 (Fig. 48) and PCM3 (Fig. 49) exhibit typical hormone response curves with 50% activation of both P388 and PCM3 occurring in the region of 10^{-7}M . A value is unable to be measured for CH23. Ayad and Foster (1977) observed a shift to the right for the dose response of CH23 indicating that there is a reduced affinity for PGE_1 for CH23. No plateau was obtained. The results presented here differ from these. However, unlike the other two cell lines the dose response for CH23 often varied with the homogenate preparation studied although the homogenates were prepared identically each time.

(b) Response to NaF

The response of P388 (Fig. 51) and PCM3 (Fig. 52) to various concentrations of NaF are shown. Both of these cell lines show an optimal response to NaF at 5mM. These results are typical of those seen with NaF for all cells. The NaF exhibits an optimal concentration after which it becomes inhibitory possibly due to the chelation of essential Mg^{2+} by F^- as NaF concentration is increased. However, this inhibition is not overcome by increasing the Mg^{2+} concentration to 50mM (Ayad and Foster, 1977). No

FIGURE 51. The Effect of NaF on P388 Homogenate
Adenylate Cyclase Activity

Adenylate cyclase assay components plus NaF and PGE_1 were preincubated for 10 minutes at 30°C . After this time incubation was initiated by addition of homogenate (50-100 μg protein) and adenylate cyclase activity was measured at 30°C for 10 minutes. 0.3M HCl was then added and the samples were hydrolysed at 85°C for 45 minutes. Results shown are the means of duplicate incubations and duplicate cAMP determinations of each sample.

○—○ - PGE_1
●—● + PGE_1 (2.5 μM)

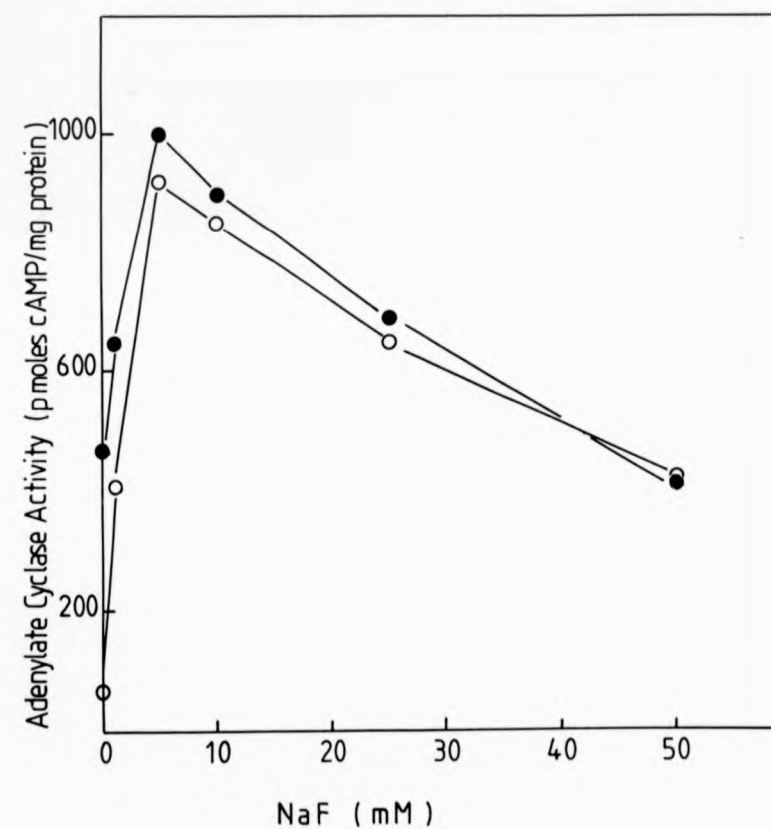
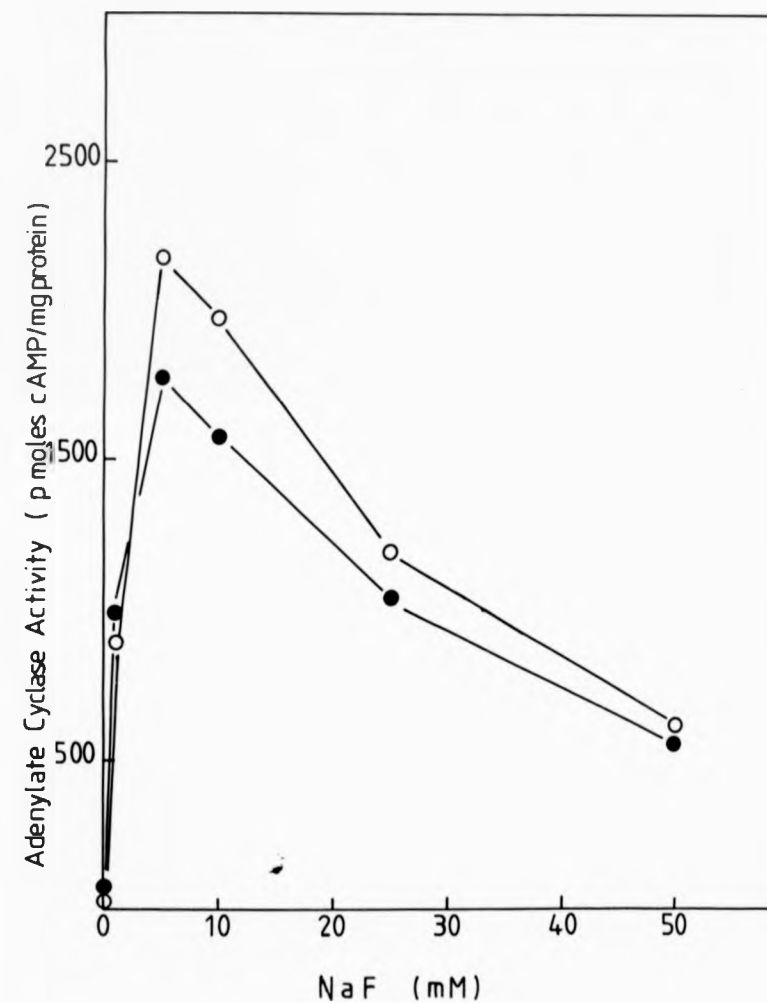


FIGURE 52. The Effect of NaF on PCM3 Homogenate
Adenylate Cyclase Activity

Method as described in Fig. 51. Results shown
are the means of duplicate incubations and duplicate
cAMP determinations of each sample.

○ — ○ -PGE₁
● — ● +PGE₁ (2.5 μM).



response of intact cells to F^- is seen although the agent is ubiquitous in its activation of broken cells, but the extent varies according to cellular origin.

There is little difference in the curves in the presence and absence of hormone since fluoride acts on the catalytic site although the guanine nucleotide regulatory component has been shown to be necessary for the activation (Howlett *et al.*, 1979). Since F^- stimulation is dependent on the presence of Mg^{2+} (Birnbaumer *et al.*, 1977) this is also consistent with the involvement of the catalytic unit in this activation.

From Figs. 51 and 52 it can be seen that PCM3 homogenate is far more responsive than P388. These results are in agreement with previous observations in this laboratory (Ayad and Foster, 1977) where the order of enhancement by NaF was established as $PCM3 > P388 > CH23$. For both PGE_1 - and NaF-dose response curves the hybrid cells appear to have enhanced adenylate cyclase activity compared to either parent although the PGE_1 -stimulated activity is very similar to that seen with P388. The greater response of the hybrid cells to NaF might suggest that the hybrid cells contain a greater amount of catalytic component of cyclase since NaF appears to act at this site. Since the regulatory component has also been shown to be involved in this process the hybrid cells might also illustrate improved catalytic component-regulatory component coupling than either of the parental cell lines.

Ayad and Foster (1977) reported the three cell types as having a different NaF optimum concentration for activity. The results presented do not agree with this since the optimum for both PCM3 and P388 appears to be the same occurring at 5mM NaF. This optimum may depend upon the conditions of preparation of the homogenates which varies slightly with each batch of homogenate prepared.

(c) The Effect of GTP on Homogenate Activity

The effect of a wide range of GTP concentrations on P388 (Fig. 53), CH23 (Fig. 54) and PCM3 (Fig. 55) homogenate adenylate cyclase activity in the presence and absence of an optimal concentration of PGE_1 ($2.5\mu\text{M}$) can be seen. These results are in agreement with those of others in this laboratory (Ayad and Foster, 1977). P388 (Fig. 53) adenylate cyclase activity shows little enhancement in response to GTP (10^{-8} - 10^{-3}M) either in the presence or absence of PGE_1 . Both CH23 (Fig. 54) and PCM3 (Fig. 55) adenylate cyclase activities show an enhancement by GTP of basal levels. The enhancement is particularly great for CH23 where levels are enhanced 5-fold at 10^{-6} - 10^{-3}M . The enhancement for PCM3 in the absence of hormone is only about 2-fold. For both cell types the response to GTP appears dose-dependent. In the presence of an optimal concentration of PGE_1 ($2.5\mu\text{M}$) there is a greater than additive i.e. synergistic effect of PGE_1 and GTP at higher GTP concentrations; 10^{-5} - 10^{-3}M for CH23 and 10^{-6} - 10^{-3}M for PCM3. It would normally be expected that the greater availability of GTP promotes the activation of adenylate cyclase activity since binding of

FIGURE 53. The Effect of GTP on P388 Homogenate
Adenylate Cyclase Activity

Adenylate cyclase assay components plus GTP and PGE_1 were pre-incubated for 10 minutes at 30°C . After this time the incubation was initiated by addition of homogenate (50-100 μg protein) and adenylate cyclase activity was measured at 30°C for 10 minutes. 0.3M HCl was then added and the samples were hydrolysed at 85°C for 45 minutes. Results shown are the means of duplicate incubations and duplicate cAMP determinations of each sample.

○—○ $-\text{PGE}_1$
●—● $+\text{PGE}_1$ (2.5 μM)

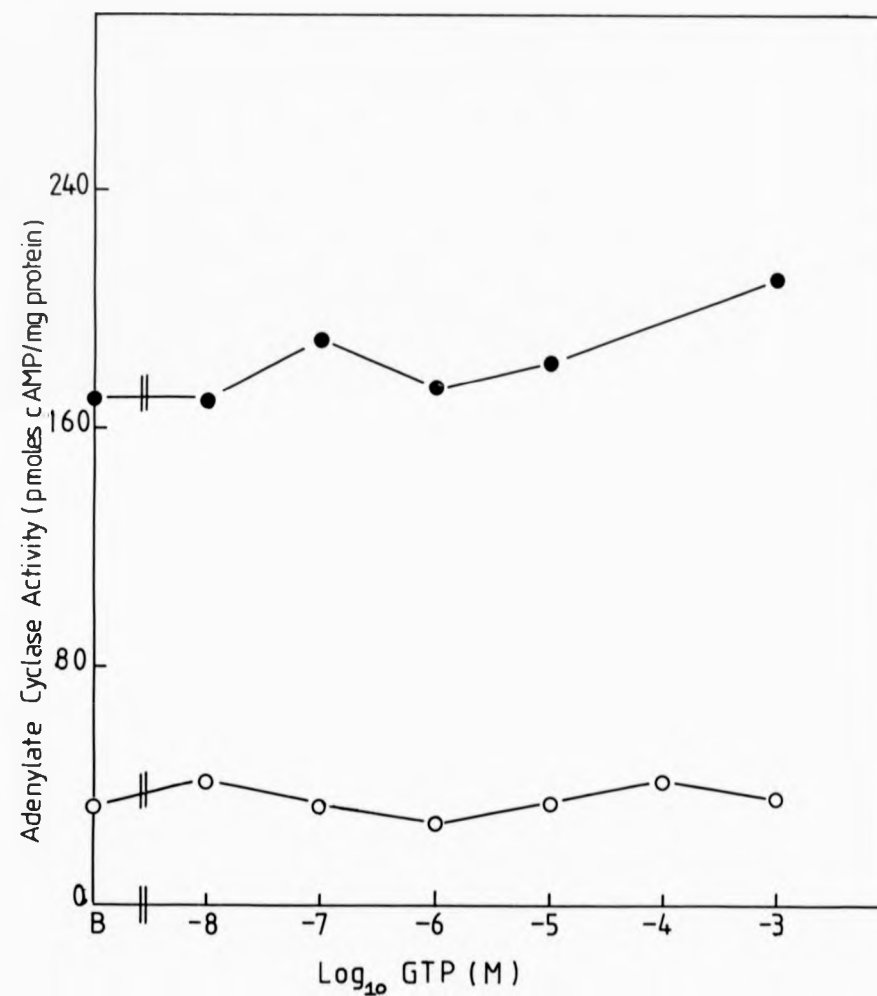


FIGURE 54. The Effect of GTP on CH23 homogenate
Adenylate Cyclase Activity

Method as described in Fig. 53. Results shown are the means of duplicate incubations and duplicate cAMP determinations of each sample.

O—O $-PGE_1$
●—● $+PGE_1$ (2.5 μ M)

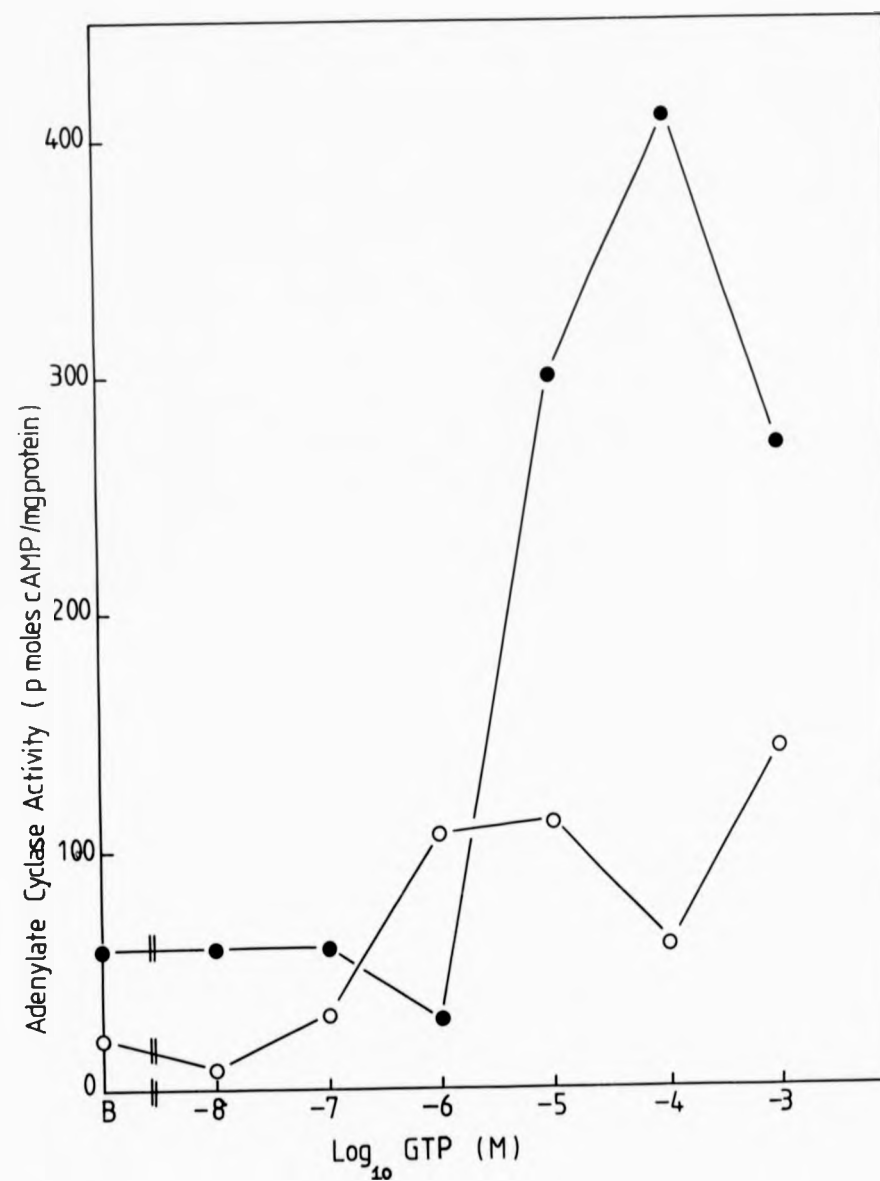
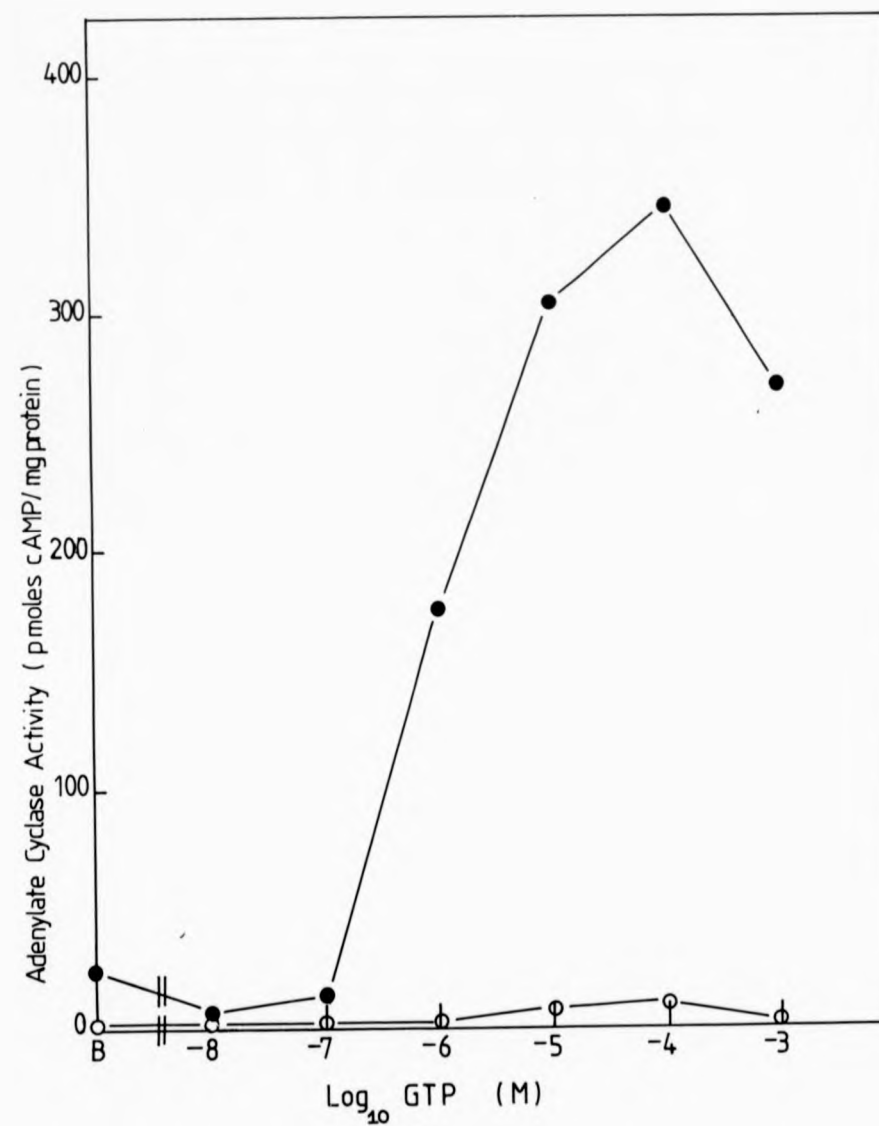


FIGURE 55. The Effect of GTP on PCM3 Homogenate
Adenylate Cyclase Activity

Method as described in Fig. 53. Results shown are the means of duplicate incubations and duplicate cAMP determinations of each sample.

○ — ○ -PGE₁
● — ● +PGE₁ (2.5 μM)



GTP to the hormone-receptor complex produces an active state capable of binding to the catalytic unit and producing greater frequency of interactions of components of adenylate cyclase. GTP has also been demonstrated to decrease the affinity of the receptor for hormones (Limbird, 1981) for most systems and Grandt *et al.* (1982) have found an increased affinity of PGE_1 receptors in hamster adipocytes and Ayad and Foster (1977) found no significant alteration of PGE_1 -dose response curves by GTP for any of the cell lines therefore little change in hormone-receptor affinity.

The difference in response of P388 adenylate cyclase may be explained by its malignant state with possibly some regulatory control being absent in transformed cells. Malignant cells have a high degree of hydrolysing enzymes and thus GTP is probably hydrolysed to GDP and therefore is incapable of activating adenylate cyclase. GDP has been shown to be incapable of supporting coupling of receptor-hormone complex to catalytic component of adenylate cyclase (Totsuka *et al.*, 1982). When exogenous GTP is added to the homogenates of PCM3 and CH23 there is an increased chance of GTP being the nucleotide occupying the binding site of the regulatory component. This ensures that the cyclase molecule is maintained in an active conformation (Limbird, 1981). Activation of adenylate cyclase by hormones has been shown to be associated with an increased GTPase activity associated with the nucleotide regulatory protein (Cassel and Selinger, 1976; Lester *et al.*, 1982). Thus addition of exogenous GTP facilitates this process. When GDP finally replaces GTP at the nucleotide binding site

the enzyme is 'turned off' and is returned to an inactive state. The GTPase activity associated with hormone stimulation of adenylate cyclase activity also is shown to be activated by GTP but K_m for the GTPase is 10 times higher than that for adenylate cyclase (Lester *et al.*, 1982).

These results also indicate the difference between malignant and normal cells as well as the similarity in behaviour between PCM3 and CH23 parent. The fact that a dose-response relationship exists with GTP and adenylate cyclase activity suggests that only one process is being affected.

Experiments with human astrocytoma cell line 132-1N1 also show no enhancement of epinephrine stimulation of adenylate cyclase activity by addition of exogenous GTP (Clark, 1978). However, for these cells a washed preparation free of nucleotides was unresponsive to epinephrine but addition of GTP and/or cytosol to this membrane preparation produced a responsive membrane preparation. The products acting synergistically with hormones in this preparation were found to be GDP and GTP. Therefore it is possible for the transformed systems that although adenylate cyclase system is sensitive to guanine nucleotides there is a sufficiently high concentration present endogenously to supply the system and hence no response to exogenous additions. It has also been shown in other cells that GTP can be formed from GDP (Totsuka *et al.*, 1982) and possibly this process of transphosphorylation is absent in P388 cells.

Recent observations (Kimura and Shimada, 1983) have suggested a possible role of membrane-associated nucleoside diphosphate kinase in determining the GTP and GDP levels at or near their binding site with adenylate cyclase. It is therefore possible that in transformed systems this regulation is in some way altered since these authors have also shown GDP to inhibit the hormonal signal to adenylate cyclase. If, as previously discussed, high levels of hydrolysing enzymes present in malignant cells lead to an increased cellular concentration of GDP compared to GTP this according to the suggestions of these authors would lead to the action of GDP as an inhibitor of cyclase activity.

Since the action of GTP in regulating adenylate cyclase activity both in the presence and absence of hormone is known to involve the regulatory component, the similarity in results with CH23 and PCM3 indicate that the hybrid cell regulatory protein is similar to that of CH23 parent. The anomalous results obtained with P388 indicate that the regulatory component of P388, at least with respect to GTP binding, must differ from CH23 and PCM3.

II. CHOLERA TOXIN TREATMENT OF HOMOGENATES

The effects of cholera toxin pre-treatment of crude homogenates of P388 (Fig. 56), CH23 (Fig. 57) and PCM3 (Fig. 58) on the PGE_1 dose-response curves can be seen. The homogenates were treated with 20mM dithiothreitol, and 2.5mM NAD^+ for 10 minutes at 30°C prior to treatment with $100\mu\text{g/ml}$ cholera toxin according to the method of Johnson

FIGURE 56. The Effect of Cholera Toxin on P388

Homogenate Adenylate Cyclase Activity

The homogenate (0.5-lmg protein/ml) was preincubated with 2.5mM NAD^+ and 20mM dithiothreitol for 10 minutes at 30°C . The homogenate was then divided into two aliquots. One aliquot was treated with 100 $\mu\text{g}/\text{ml}$ of cholera toxin for 10 minutes at 30°C . The other aliquot was treated with homogenisation buffer for 10 minutes at 30°C . Adenylate cyclase assay components plus PGE_1 (2.5 μM) were preincubated for 10 minutes at 30°C . Incubation was initiated by the addition of homogenate (50-100 μg protein) and adenylate cyclase activity was measured for 10 minutes at 30°C . 0.3M HCl was added and the samples were hydrolysed at 85°C for 45 minutes. Results shown are the means of duplicate incubations and duplicate cAMP determinations of each sample.

- -cholera toxin
- +cholera toxin (100 $\mu\text{g}/\text{ml}$)

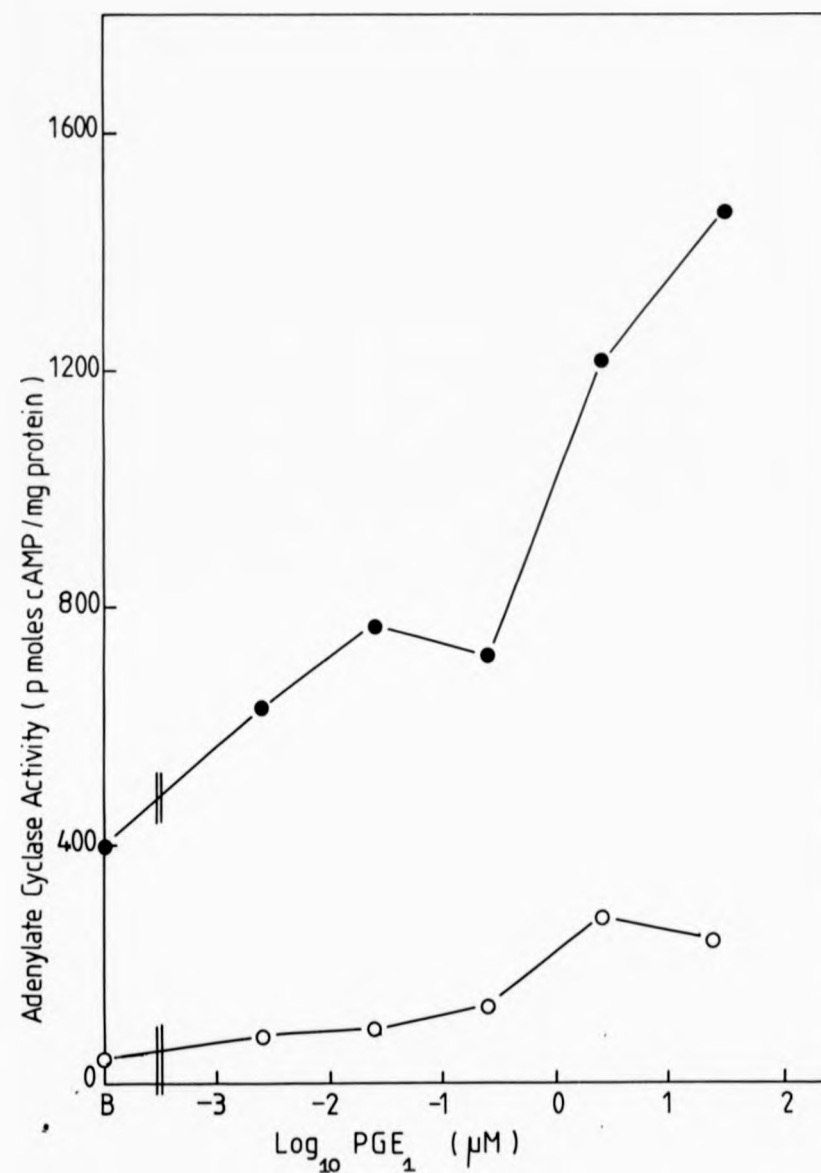


FIGURE 57. The Effect of Cholera Toxin on CH23

Homogenate Adenylate Cyclase Activity

Method as described in Fig. 56. Results shown are the means of duplicate incubations and duplicate cAMP determinations of each sample.

O—O -cholera toxin

●—● +cholera toxin (100 μ g/ml)

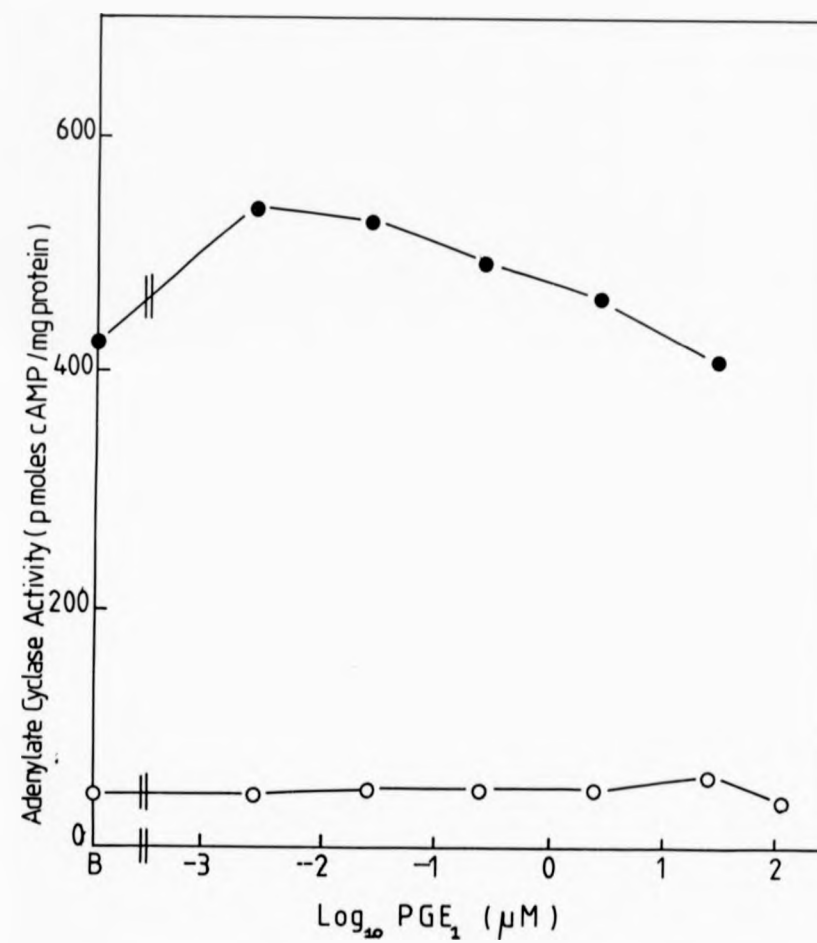
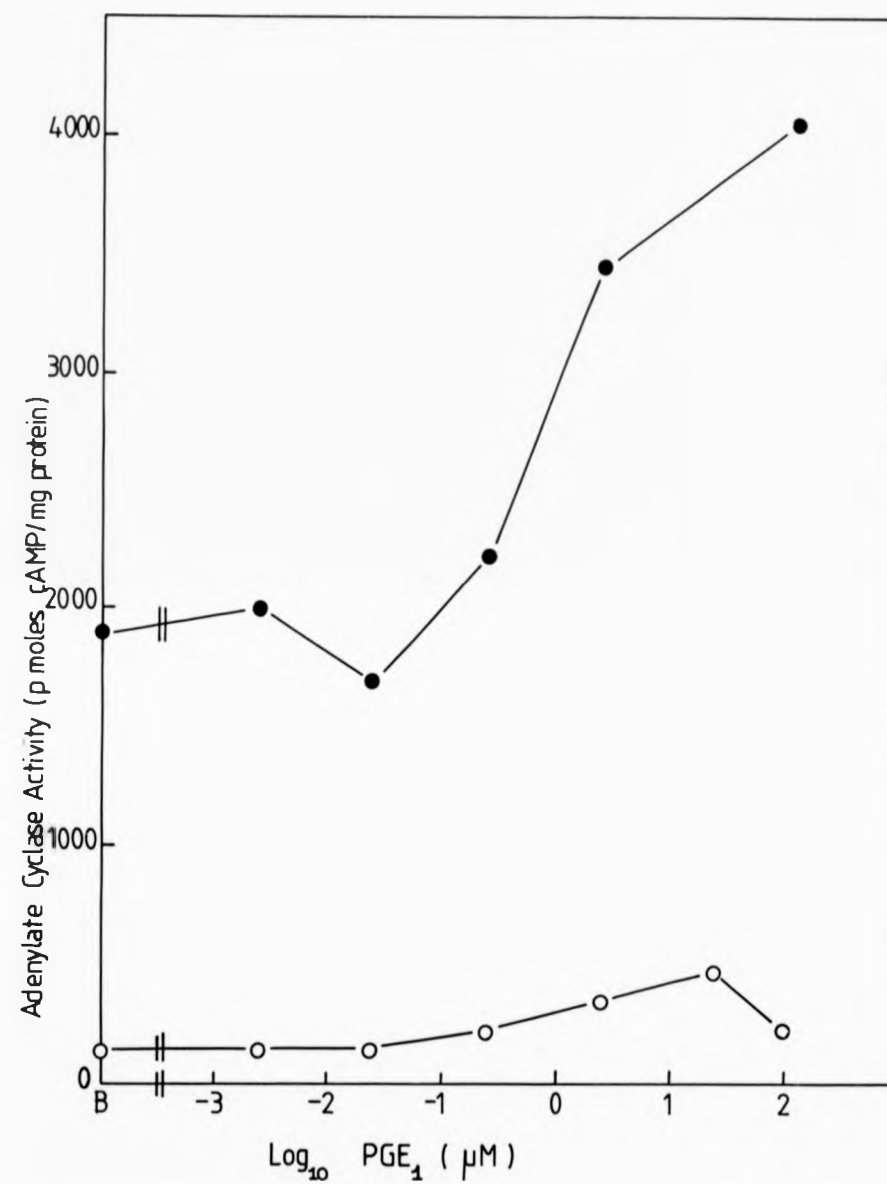


FIGURE 58. The Effect of Cholera Toxin on PCM3

Homogenate Adenylate Cyclase Activity

Method as described in Fig. 56. Results shown are the means of duplicate incubations and duplicate cAMP determinations of each sample.

- -cholera toxin
- +cholera toxin (100 μ g/ml)



et al. (1978). For all these cell lines it can be observed that pretreatment with cholera toxin produces an elevated basal level of adenylate cyclase activity as well as an increased level of activity for all concentrations of PGE_1 studied. The inconsistency of the PGE_1 dose-response for CH23 can be seen since following cholera toxin pretreatment a more discernible dose-response curve is obtained. NAD^+ has been shown to be required for the process of ADP-ribosylation of the proteins of the regulatory unit and found to have a K_m for activation in the mM range (Gill, 1982). Therefore the concentration of NAD^+ chosen was optimal. Dithiothreitol was used at 25mM since this concentration has been shown to be effective for ADP-ribosylation whereas low concentrations of sulphydryl reagents have been shown to inhibit the process (Gill, 1982). Cholera toxin is known to act by ADP-ribosylation of a site on the guanine nucleotide regulatory protein such that the associated GTPase reaction is inhibited (Cassel and Selinger, 1977b). This results in a permanent activation of the cyclase molecule. The conditions chosen for the treatment of homogenate appear to be suitable for the inhibition of the GTPase activity; although the inhibition is far from 100% for both P388 (Fig. 56) and PCM3 (Fig. 58) since considerable further activation by PGE_1 at all concentrations is seen to occur. From the results of CH23 (Fig. 57) there appears to be an anomalous situation occurring in the presence of PGE_1 and cholera toxin. However, this probably reflects the unusual dose-response curves obtained for CH23 discussed previously.

Basal levels of CH23 are stimulated approximately 10-fold by cholera toxin treatment whereas those of P388 are enhanced 8-fold and those of PCM3 15-fold. These levels are surprisingly similar between the cell lines compared to the difference observed in the intact state where the enhancement of cAMP accumulation for CH23 by cholera toxin was considerably greater than that seen for PCM3 or P388. Thus for CH23 it appears that the intact cell is required for the total activity of the adenylate cyclase molecule to approach that obtained with PCM3 following cholera toxin treatment. Thus the low levels of activity seen in intact CH23 compared to PCM3 cannot be solely explained by the presence of a high GTPase activity as was first thought. The situation occurring in the CH23 adenylate cyclase is far more complex than first realised.

For both P388 (Fig. 56) and PCM3 (Fig. 58) the concentration of PGE_1 required to produce 50% activation of adenylate cyclase in the presence of cholera toxin was in the region of 10^{-6}M . This is a factor of 10 higher than that required when cholera toxin is not present (10^{-7}M). Thus it appears that the affinity of the receptor for hormone is increased following cholera toxin treatment. A similar increase in affinity of receptors for hormones following toxin treatment has been observed by others (Fisher and Sharp, 1978). Following treatment of homogenates with cholera toxin there is a greater maximum activity of adenylate cyclase to hormones and also a greater response to moderate levels of hormone. This occurs for all three cell lines but is best seen with P388 and PCM3. This phenomenon has been seen extensively for

other systems and the subject has been reviewed (Gill, 1977). Both increases are thought to result from the longer persistence of the active $N_{(GTP)} \cdot Cu^*$ without changing the rate of activation by GTP and hormone (Cassel and Selinger, 1977b).

III. GTPase MEASUREMENTS OF PLASMA MEMBRANES

(a) Dose-Response to PGE_1

It can be seen for P388 (Fig. 59), CH23 (Fig. 60) and PCM3 (Fig. 61) that a dose-response relationship with respect to the amount of GTP hydrolysed exists in response to PGE_1 . Plasma membranes were pretreated with N-ethylmaleimide to reduce the background GTP hydrolysis as has previously been shown for platelet membranes (Lester *et al.*, 1982). Measurement of GTPase in membranes for all three cell lines in the absence of N-ethylmaleimide treatment produced values approximately 8-10 times higher than in the presence of N-ethylmaleimide but because of the high background levels of non-specific GTPase the levels in the presence of PGE_1 were not significantly higher. Therefore treatment with N-ethylmaleimide markedly decreases basal GTP hydrolysis but apparently not hormone-stimulated GTPase associated with adenylate cyclase activity (Cassel and Selinger, 1976). A dose response relationship of GTP hydrolysis associated with the adenylate cyclase in human platelet membranes has previously been observed (Lester *et al.*, 1982). These authors used the following criterion to indicate that GTP is hydrolysed by a component of

FIGURE 59. GTPase Activity of P388 Plasma Membranes

Membranes were isolated and treated with N-ethylmaleimide. GTPase assay components plus PGE_1 were pre-equilibrated at 30°C for 5 minutes. The assay was initiated by the addition of membranes (20-50 μg protein). GTPase activity was measured for 5 minutes at 30°C . Results shown are the means of triplicate incubations. Standard deviations always less than 10%.

B indicates the basal value.

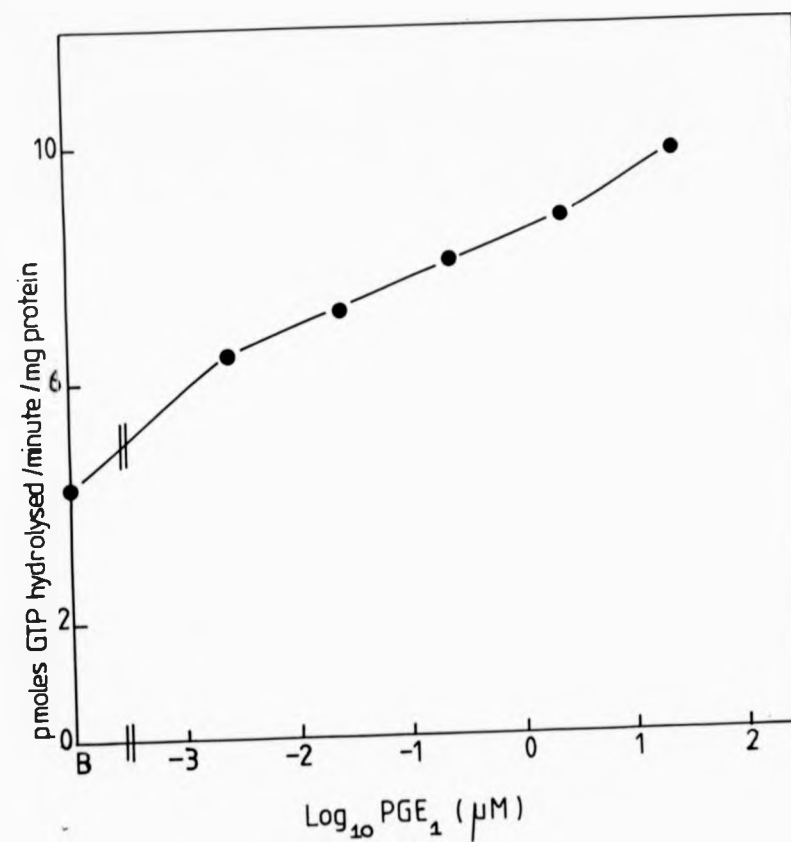


FIGURE 60. GTPase Activity of CH23 Plasma Membranes

Method as described in Fig. 59. Results shown are means of triplicate incubations. Standard deviations less than 10%.

B represents the basal value.

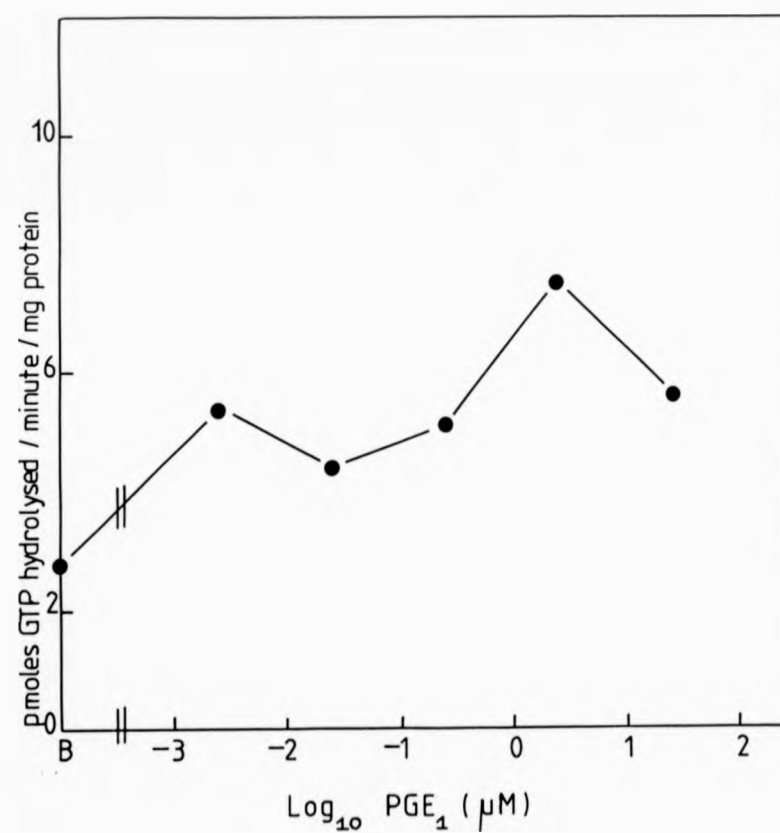
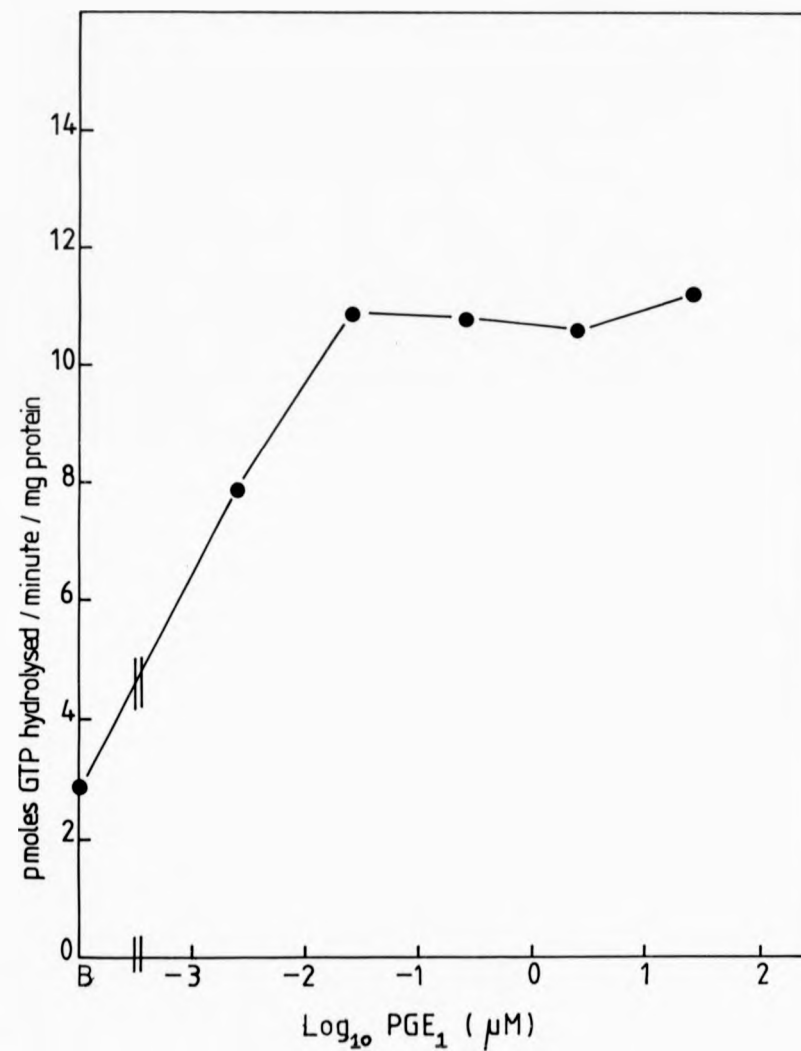


FIGURE 61. GTPase Activity of PCM3 Plasma Membranes

Method as described in Fig. 54. Results shown are means of triplicate incubations. Standard deviations less than 10%.

B represents the basal value.



adenylate cyclase in response to stimulation by hormones.

- (i) The dose-response relationship for GTPase activity should be similar to that for adenylate cyclase activity.
- (ii) The GTPase should be active at low concentrations of GTP with a K_m of less than $1 \times 10^{-6}M$.
- (iii) The GTPase activity should be blocked by cholera toxin.

From the dose-response relationships seen in Figs. 59-61 the GTPase dose-response curves are similar to those observed with adenylate cyclase activity. The curve for P388 (Fig. 59) does not attain a plateau value so it is impossible to obtain a value for K_{act} . From Fig. 60 it can be observed that the concentration of PGE_1 where 50% activation of GTPase for CH23 occurs is in the region of $10^{-6} - 10^{-7}M$ PGE_1 whereas that for PCM3 (Fig. 61) occurs at around $10^{-9}M$. From adenylate cyclase measurements the affinity for the PGE_1 receptor was similar for both PCM3 and P388 occurring at $10^{-7}M$ whereas that of CH23 was indeterminable. Following comparison of the GTPase and adenylate cyclase dose-response curves it appears that the affinity for the PGE_1 receptor is less in P388 and greater in PCM3 when GTPase activity is studied. The reason for this difference is not immediately apparent. However, the general trend of the dose-response curves measuring GTPase are similar to those attained for adenylate cyclase so it appears that the first criterion is satisfied.

The basal levels of GTPase activity in the absence of PGE₁ appear to be similar for all three cell lines. The values obtained in the presence of PGE₁ also appear to be roughly similar although the values for PCMB are slightly higher. Thus, for hybrid cells the turnover rate of the enzyme may be faster explaining, if only partially, the higher levels of cAMP obtained in PCMB intact cells compared to either of the parent cells. This in combination with the postulated greater number of catalytic units present in PCMB membranes, as indicated by the greater activity in the presence of NaF, could explain the greater adenylate cyclase activity in PCMB cells.

One surprising result is the GTPase values for CH23. The values for CH23 are no greater than for F388 and PCMB thus ruling out the possibility of high GTPase levels in CH23 to account for the low levels of cAMP response seen in intact cells as suggested by the result with cholera toxin and intact CH23.

An alternative explanation must be sought to account for the enhanced levels of cAMP produced in CH23 cells in response to cholera toxin. It is possible that a non-specific GTPase is inhibited by cholera toxin in the intact state allowing greater concentrations of GTP to activate adenylate cyclase. Alternatively other factors, possibly cytosolic might be responsible for the total expression of GTPase activity as revealed by the action of cholera toxin that are not present when the GTPase activity is measured in plasma membranes. The GTPase activity is measured at

0.25 μ M [32 P]GTP. Thus the second criterion would appear to be satisfied.

A GTPase activity associated with hormonal stimulation has been observed in a number of cell types for activation of adenylate cyclase by pancreozymin (Lambert *et al.*, 1979), glucagon (Kimura and Shimada, 1980), α -agonists (Cassel and Selinger, 1976; Pike and Lefkowitz, 1980) as well as by prostaglandins (Bitonti *et al.*, 1980; Lester *et al.*, 1982). GTPase has also been implicated in hormonal inhibition of adenylate cyclase (Aktories and Jakobs, 1981; Aktories *et al.*, 1982).

N-ethylmaleimide is known to inactivate adenylate cyclase but not shown to affect GTPase in response to PGE₁ (Lester *et al.*, 1982). However, from the results with GTPase and PGE₁ dose-response curves it is possible that the altered PGE₁ receptor affinity is due to N-ethylmaleimide. Adenylate cyclase activity has been shown to be inactivated by N-ethylmaleimide (Drummond, 1981) and was shown to have a complex effect on both F⁻- and Gpp(NH)p-stimulated activity. Therefore it is possible that pretreatment with N-ethylmaleimide, while allowing measurement of PGE₁-stimulated GTPase above basal levels, alters the adenylate cyclase system so that the affinity for the receptor is increased. Although no such effect was observed for the PGE₁ receptor in human platelet membranes (Lester *et al.*, 1982) it is feasible that the adenylate cyclase system of the hybrid cell for which the greatest change in PGE₁ receptor affinity was observed is more susceptible to treatment by N-ethylmaleimide.

N-ethylmaleimide has been shown to uncouple the α -adrenoceptor-mediated inhibition of human platelet adenylate cyclase activity whilst having no effect on PGE_1 -stimulated adenylate cyclase. It appears that this uncoupling does not occur at the level of the receptor although its site of action may be at the interaction of the receptor with the regulatory protein. The use of N-ethylmaleimide has further importance in distinguishing the GTPase associated with inhibitory and stimulatory regulatory components since the GTPase activity associated with the inhibitory site is abolished (Jakobs et al., 1982).

(b) The Importance of Cytosolic Factors in the Study of Cholera Toxin Effects on GTPase Activity

From Fig. 62 (P388), Fig. 63 (CH23), Fig. 64 (PCM3) the effect of cholera toxin treatment of membranes on GTPase activity of the three cell lines is observed. The results illustrate the importance of the presence of cytosolic factors in treatment with cholera toxin. Fig. 62a, 63a and 64a show the treatment of purified membranes with cholera toxin in the presence of NAD^+ and DTT for P388, CH23 and PCM3 cells respectively. For all cell lines a stimulation of GTPase activity is observed both of basal levels and of levels in the presence of PGE_1 ($2.5\mu\text{M}$). These results were highly unexpected since cholera toxin is a known inhibitor of GTPase activity associated with the guanine nucleotide regulatory site (Cassel and Selinger, 1976). This left two

FIGURE 62. Effect of Cholera Toxin Treatment on GTPase Activity of P388 Plasma Membranes

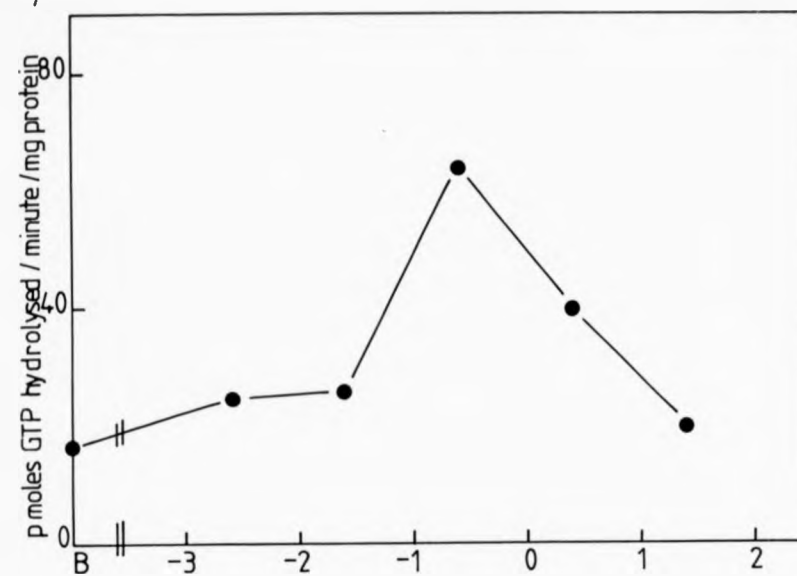
(a) N-ethylmaleimide treated membranes were further treated with 2.5mM NAD^+ and 20mM dithiothreitol for 10 minutes at 30°C , followed by 100 $\mu\text{g}/\text{ml}$ cholera toxin for 10 minutes at 30°C , GTPase.

(b) Cells were treated with 0.1 $\mu\text{g}/\text{ml}$ cholera toxin for 3 hours at 37°C and membranes were prepared from these cells. The membranes were pre-treated with N-ethylmaleimide.

For both (a) and (b) GTPase assay components were pre-equilibrated at 30°C for 5 minutes. The assay was initiated by addition of membranes (20-50 μg protein). GTPase activity was measured for 5 minutes at 30°C . Results shown are the means of triplicate incubations. Standard deviations were always less than 10%.

B represents basal value.

(a)



(b)

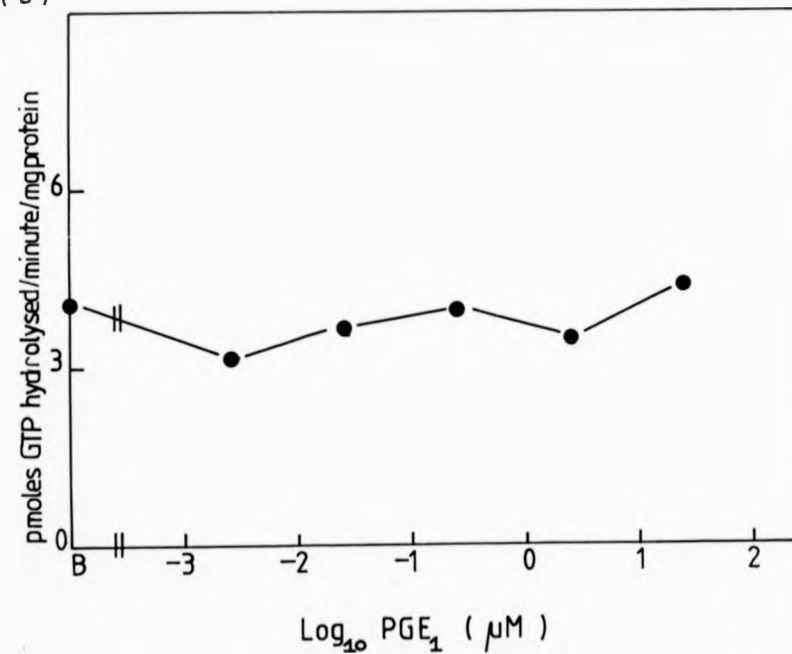


FIGURE 63. Effect of Cholera Toxin on GTPase Activity of CH23 Plasma Membranes (Method identical to Fig. 62)

(a) Membranes pretreated with 2.5mM NAD^+ and 20mM dithiothreitol and then cholera toxin.

(b) Membranes prepared from cells treated with cholera toxin (0.1 $\mu\text{g/ml}$). Results shown are the means of triplicate incubations. Standard deviations were always less than 10%. B represents the basal value. C indicates the level of hydrolysis in the presence of NAD^+ and dithiothreitol but in the absence of cholera toxin and PGE_1 .

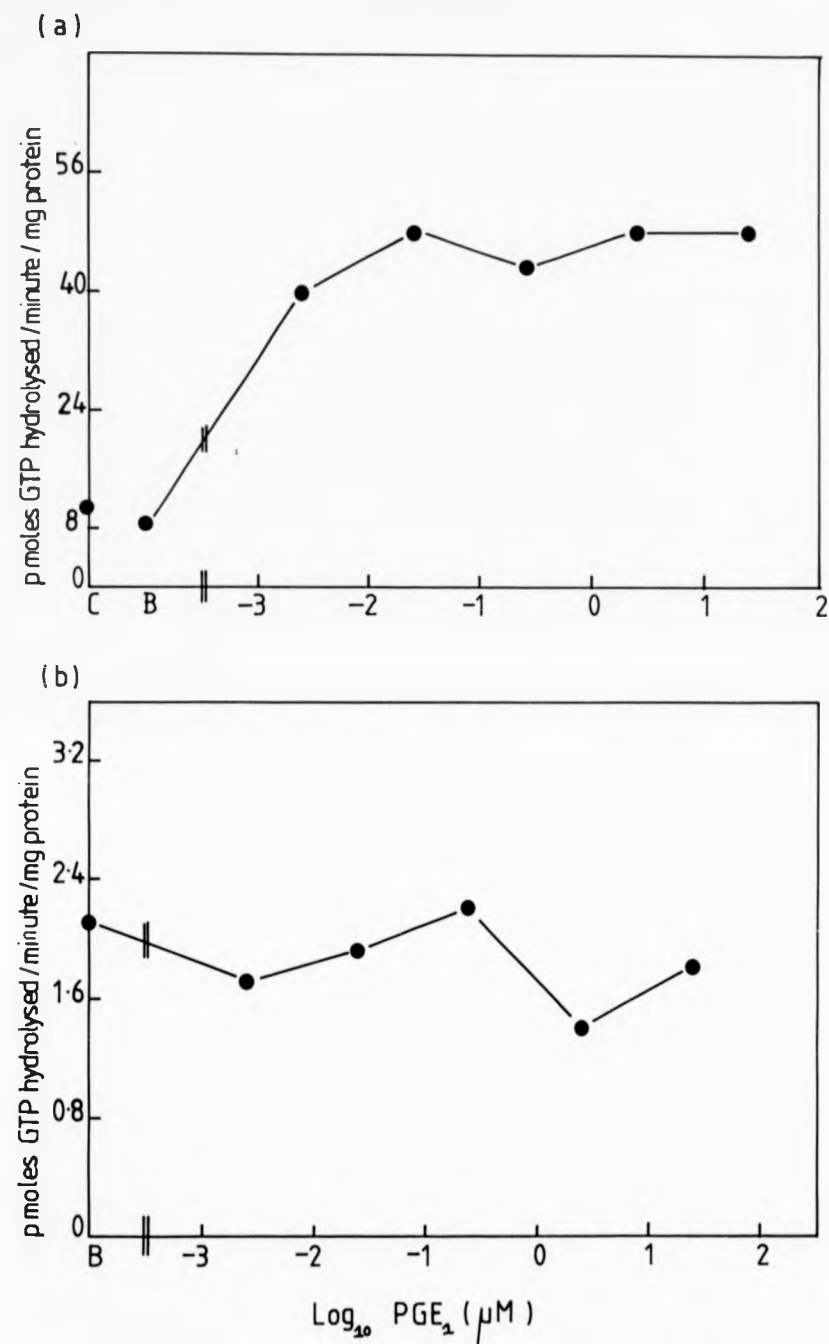


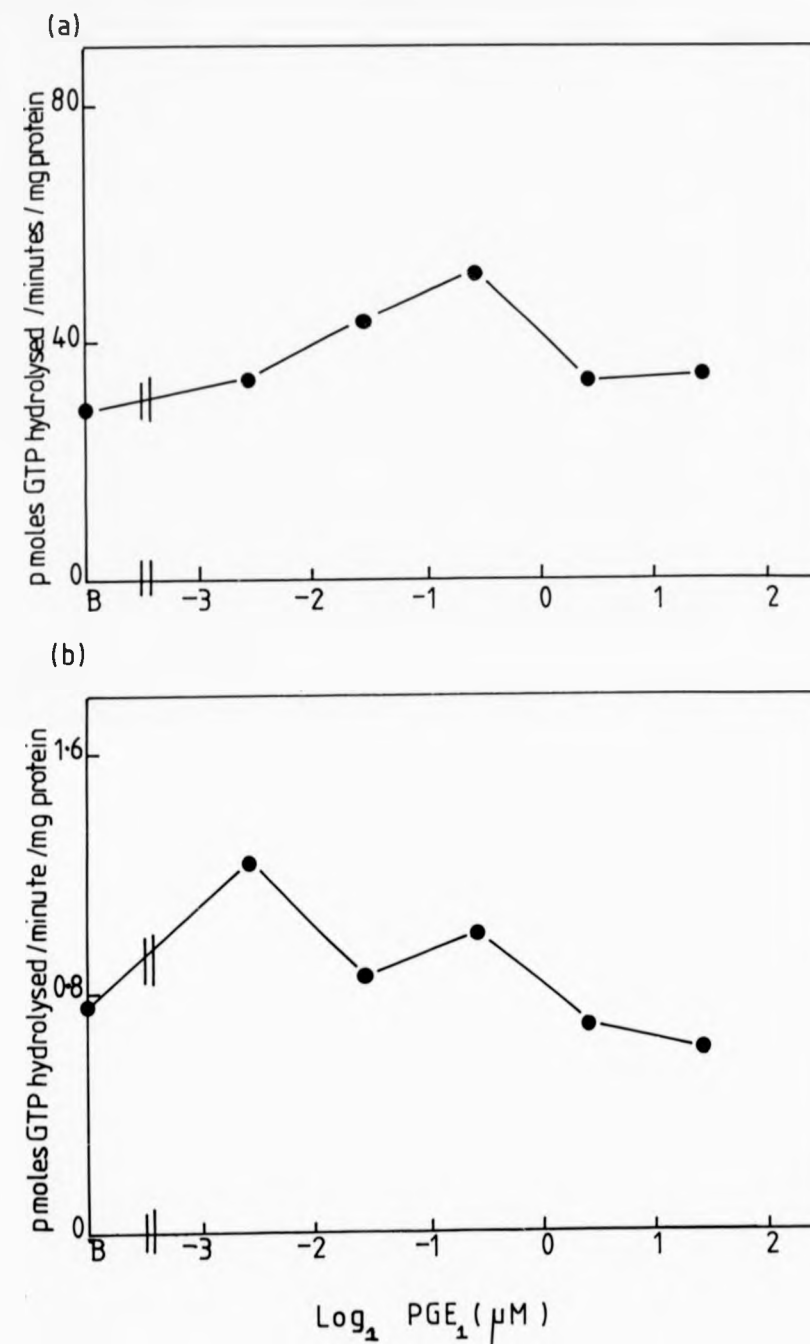
FIGURE 64. Effect of Cholera Toxin in GTPase Activity of PCM3 Plasma Membranes

Method as described in Fig. 62.

(a) Membranes pretreated with 2.5mM NAD^+ and 20mM dithiothreitol and then cholera toxin (100 $\mu\text{g/ml}$).

(b) Membranes prepared from cells treated with cholera toxin (0.1 $\mu\text{g/ml}$).

Results shown are the means of triplicate incubations. Standard deviations were always less than 10%. B represents the basal value.



possible explanations for the increase in GTPase activity observed

(a) The GTPase system measured in this series of experiments was not the one associated with the cyclase system.

(b) The conditions for treatment of the membranes with cholera toxin were unsuitable and that a true effect was not seen.

The former possibility seemed the least likely in view of the reasonable correlation occurring with adenylate cyclase activity for all the three cell lines and the low specific concentrations of GTP used in the measurements. It was therefore decided to investigate the second possibility further.

Following observations of the requirements for cytosolic factors in cholera toxin action (Gill, 1982) it was decided to study the effect on GTPase levels of treatment of intact cells with cholera toxin followed by preparation of purified plasma membranes and measurement of GTPase activity. From Figs. 62b, 63b and 64b, for P388, CH23 and PCM3 respectively treated in this way it can be seen that the basal GTPase activity of CH23 but not of PCM3 and P388 is decreased. The hormone-stimulated levels were decreased in all three cell lines, totally for CH23 and P388 and nearly completely for PCM3. This indicated that for PCM3 cell line the GTPase was not inhibited 100% by cholera toxin treatment and therefore a slight stimulation could occur in the presence of hormone. It thus appeared that the

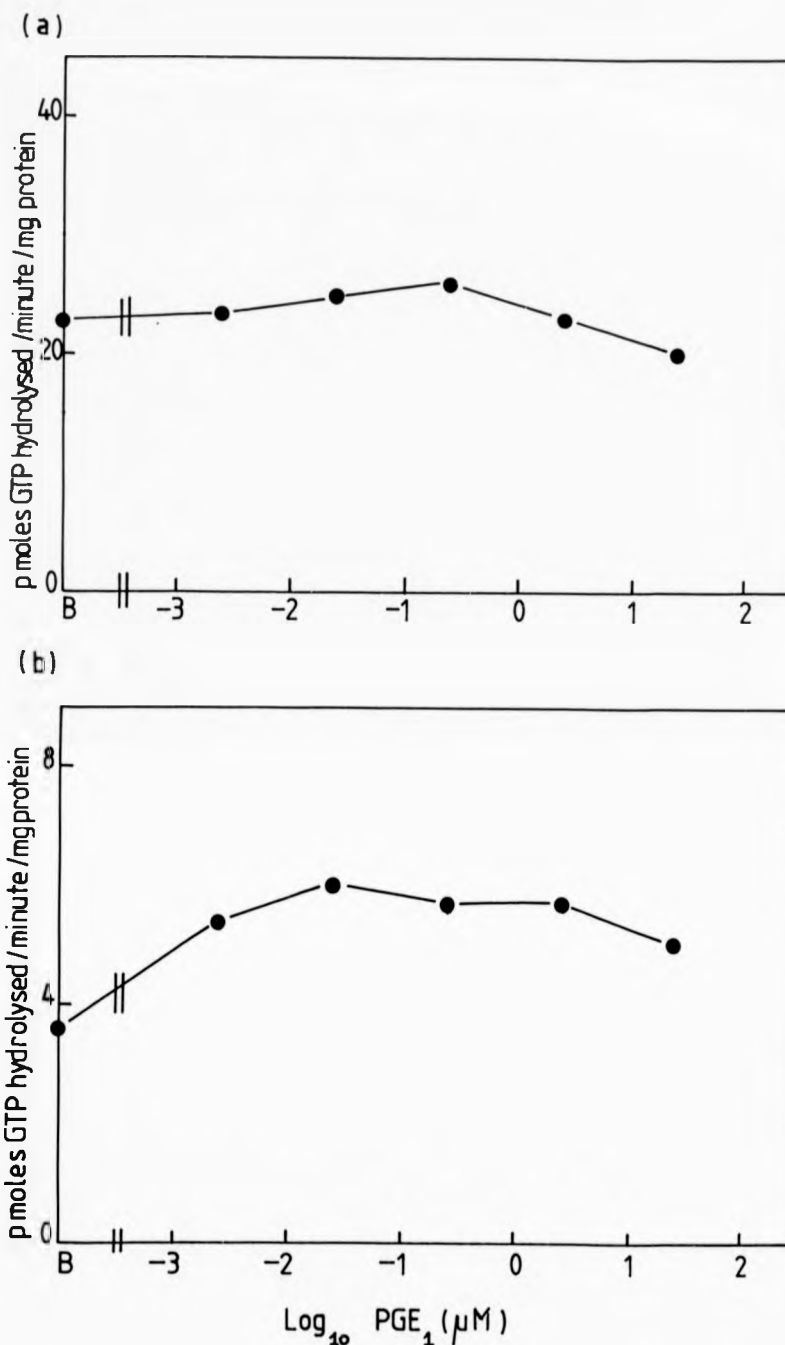
GTPase activity measured in these studies was indeed the GTPase activity associated with the regulatory component since all of the criteria established by Lester *et al.* (1982) have been fulfilled.

It was decided to further investigate the nature of the cytosolic factor believed to be involved in the cholera toxin inhibition of GTPase activity. During the preparation of purified plasma membranes employed there are two major stages of the preparation where cytoplasmic factors are lost. These are at the initial 40,000g centrifugation step (S-I) and the 40,000g centrifugation step following isolation on 35% sucrose (SII). Supernatants from these stages in the preparation of membranes from untreated P388 cells were prepared and stored at -180°C prior to use. The membranes were prepared and treated with N-ethylmaleimide as usual. The membranes were then pretreated with either SI or SII and NAD^{+} and dithiethreitol prior to treatment with cholera toxin. From Fig. 65a it can be seen that SI produces an inhibition of GTPase activity since further stimulation by PGE_1 is impossible. The enhanced basal levels would appear to be due to the protein present in SI that is untreated with N-ethylmaleimide and therefore contributes to non-specific GTPase activity. SII (Fig. 65b) has little effect on the cholera toxin treatment of P388 membranes since levels in the presence of hormone are still significantly enhanced. However, the protein concentration of SII is considerably lower than for SI and the actual enhancement of GTPase activity with PGE_1 is less in the presence of SII. Therefore both cytoplasmic

FIGURE 65. Effect of Addition of Cytosol on GTPase Activity of P388 Plasma Membranes Treated with Cholera Toxin

Plasma membranes prepared from P388 were pretreated with N-ethylmaleimide. The membranes were further treated with 2.5mM NAD^+ and 20mM dithiothreitol for 10 minutes at 30°C in the presence of (a) 40 μl of supernatant from the first 40,000g centrifugation in the plasma membrane preparation (SI) containing 350 μg protein or (b) 40 μl of supernatant from the second 40,000g centrifugation in the plasma membrane preparation (SII) containing 15 μg protein. For both cases the membranes were then treated with 100 $\mu\text{g}/\text{ml}$ cholera toxin for 10 minutes at 30°C and assayed for GTPase activity. Results shown are the means of triplicate assays. Standard deviations were always less than 10%.

B represents the basal value.



fractions aid in the action of cholera toxin on plasma membranes but it appears that the effect with SI is greater. Fig. 66 illustrates the effect of a combination of SI and SII on the cholera toxin treatment of P388 membranes. In combination SI and SII appear to have a greater effect in enhancing the effect of cholera toxin on inhibition of GTPase activity. This could imply that two factors are required for the expression of cholera toxin inhibition of GTPase or that one factor enhances the activity of the other. Fig. 67 shows the effect of varying concentrations of SI. A maximum effect appears to be observed at the lowest concentration of SI (44 μ g of protein). Obviously only a small amount of cytosol is required to enhance the cholera toxin inhibition of GTPase activity.

GTPase activities appear to be inhibited by different amounts in different tissues. In rat brain synaptic membranes the low K_m GTPase was inhibited 24% by cholera toxin (Enomoto and Asakawa, 1983) whereas for human platelet membranes (Lester *et al.*, 1982) and turkey erythrocyte membranes (Cassel and Selinger, 1977b) the GTPase activity is only inhibited by cholera toxin when stimulated by hormones. For the cell lines studied only CH23 have reduced levels of GTPase activity when intact cells treated with cholera toxin and membranes are prepared whereas all three cell lines have reduced PGE₁-stimulated GTPase. Thus, P388 and PCM3 appear to act in a similar manner to the original system measured by Cassel and Selinger (1977b) for turkey erythrocytes. These results also show the similarity between PCM3 and P388. This difference might also explain the

FIGURE 66. Effect of Addition of Cytosol on GTPase Activity of P388 Membranes Treated with Cholera Toxin

Membranes prepared from P388 were pretreated with N-ethylmaleimide. The membranes were then further treated with 2.5mM NAD^+ and 20mM dithiothreitol in the presence of 175 μg of SI plus 7.5 μg of SI for 10 minutes at 30°C. Membranes were then treated with 100 $\mu\text{g}/\text{ml}$ cholera toxin for 10 minutes at 30°C. The samples were then assayed for GTPase activity. Results shown are the means of triplicate incubations. Standard deviations were always less than 10%.

B represents the basal value.

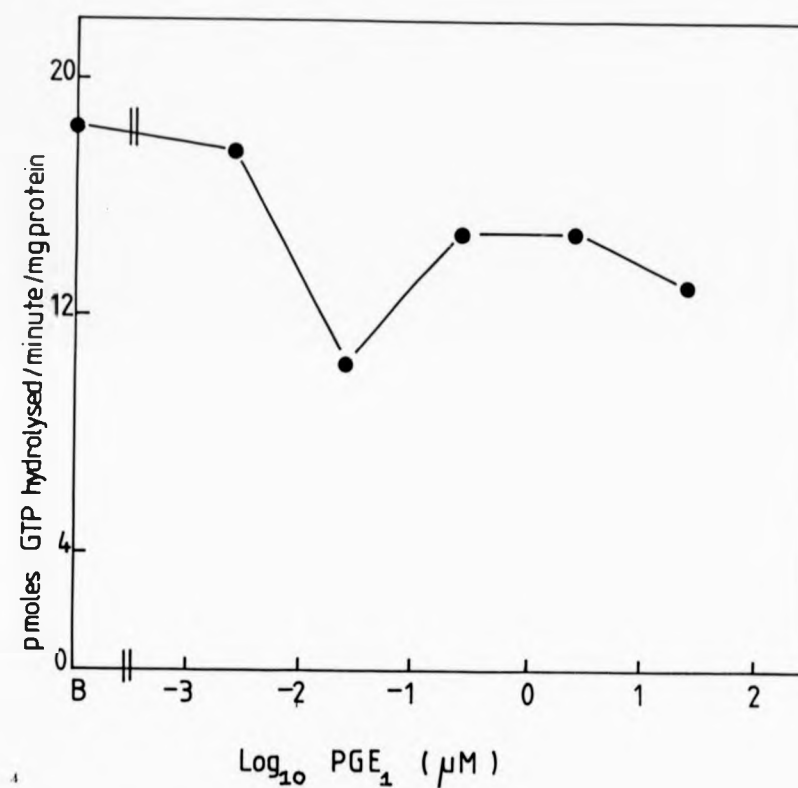
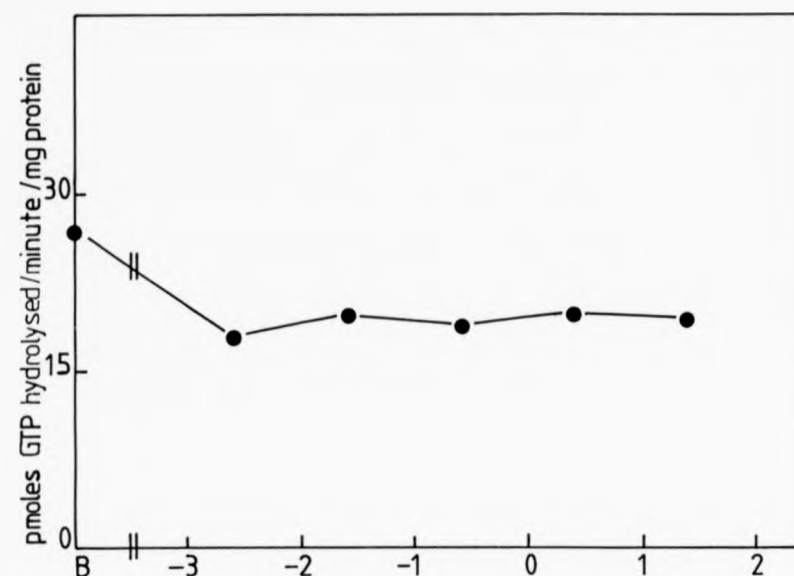


FIGURE 67. Effect of Varying Amounts of Cytosol on P388 Membrane GTPase Treated with Cholera Toxin

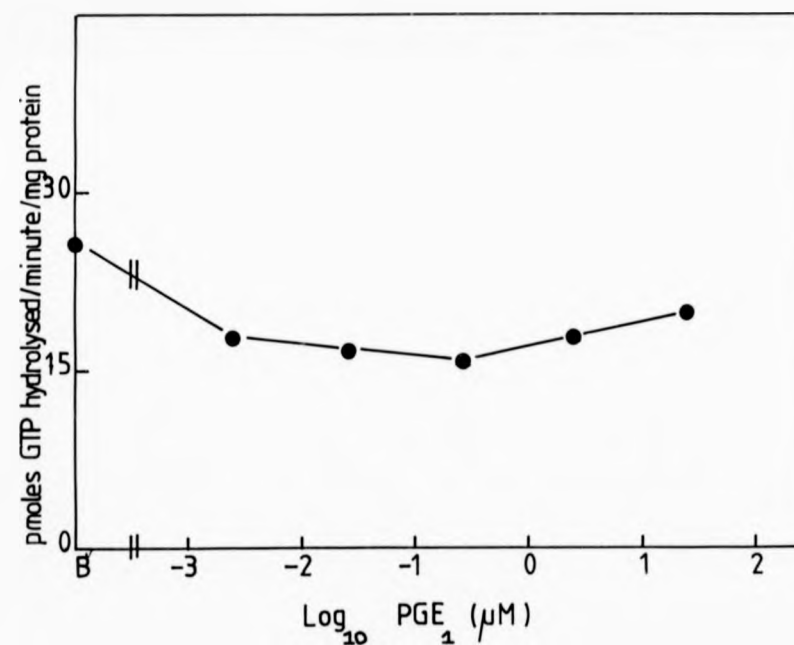
Membranes prepared from P388 were pretreated with N-ethylmaleimide. The membranes were then further treated with 2.5mM NAD^+ and 20mM dithiothreitol in the presence of either (a) 44 μg of SI or (b) 87.5 μg of SI for 10 minutes at 30°C. Membranes were then treated with 100 $\mu\text{g}/\text{ml}$ cholera toxin for 10 minutes at 30°C. The samples were then assayed for GTPase activity. Results shown are the means of triplicate incubations. Standard deviations were always less than 10%.

B represents the basal value.

(a)



(b)



difference in activation of CH23 by cholera toxin in the intact state as compared to either PCM3 and P388 since both basal and hormone-stimulated levels are inhibited as opposed to just the hormone-stimulated levels.

IV. Effects of Proteases on Broken Cells

(a) Treatment of Intact Cells Followed by Homogenisation

Figs. 68, 69 and 70 show the effect of treatment of intact cells with various concentrations of trypsin followed by homogenisation on the adenylate cyclase activity of P388, CH23 and PCM3 respectively. In all cases the basal as well as prostaglandin-stimulated levels were measured. This series of experiments were performed to examine the effects of the 0.05% trypsin used in subculturing of monolayer cells on the adenylate cyclase system. From Fig. 68 for P388 it can be seen that all concentrations of trypsin (0-0.025%) used produce enhanced basal levels of cyclase activity as well as enhanced levels of PGE_1 -stimulated activity. None of the trypsin concentrations used appears to significantly alter the K_{act} . Thus the affinity of the PGE_1 receptor would appear to be unaltered. At 0.025% trypsin the maximal value of PGE_1 -stimulated adenylate cyclase obtained is approximately 5-fold greater than that of homogenates made in the absence of trypsin. For CH23 (Fig. 69) all concentrations of trypsin used (0-0.05%) enhanced the basal levels of adenylate cyclase activity as well as PGE_1 -stimulated levels. It should be observed that even following preparation of homogenates from trypsin-treated cells there is still only a relatively small

FIGURE 68. Effect of Trypsin on the Preparation of P388 Homogenates

The cells were washed twice with trypsin salt and treated with the required concentration of trypsin for 2 minutes at 37°C. Following this time the reaction was quenched by the addition of growth medium and the cells were washed twice with HBSS⁻⁻⁻. The cells were homogenised and the homogenate was assayed for adenylate cyclase activity in the presence of PGE₁ for 10 minutes at 30°C. Results shown are the means of duplicate assays and duplicate cAMP determinations of each sample. B represents the basal value.

- (a) ○—○ control - absence of trypsin treatment
●—● 0.0025% trypsin
- (b) △—△ 0.005% trypsin
▲—▲ 0.025% trypsin

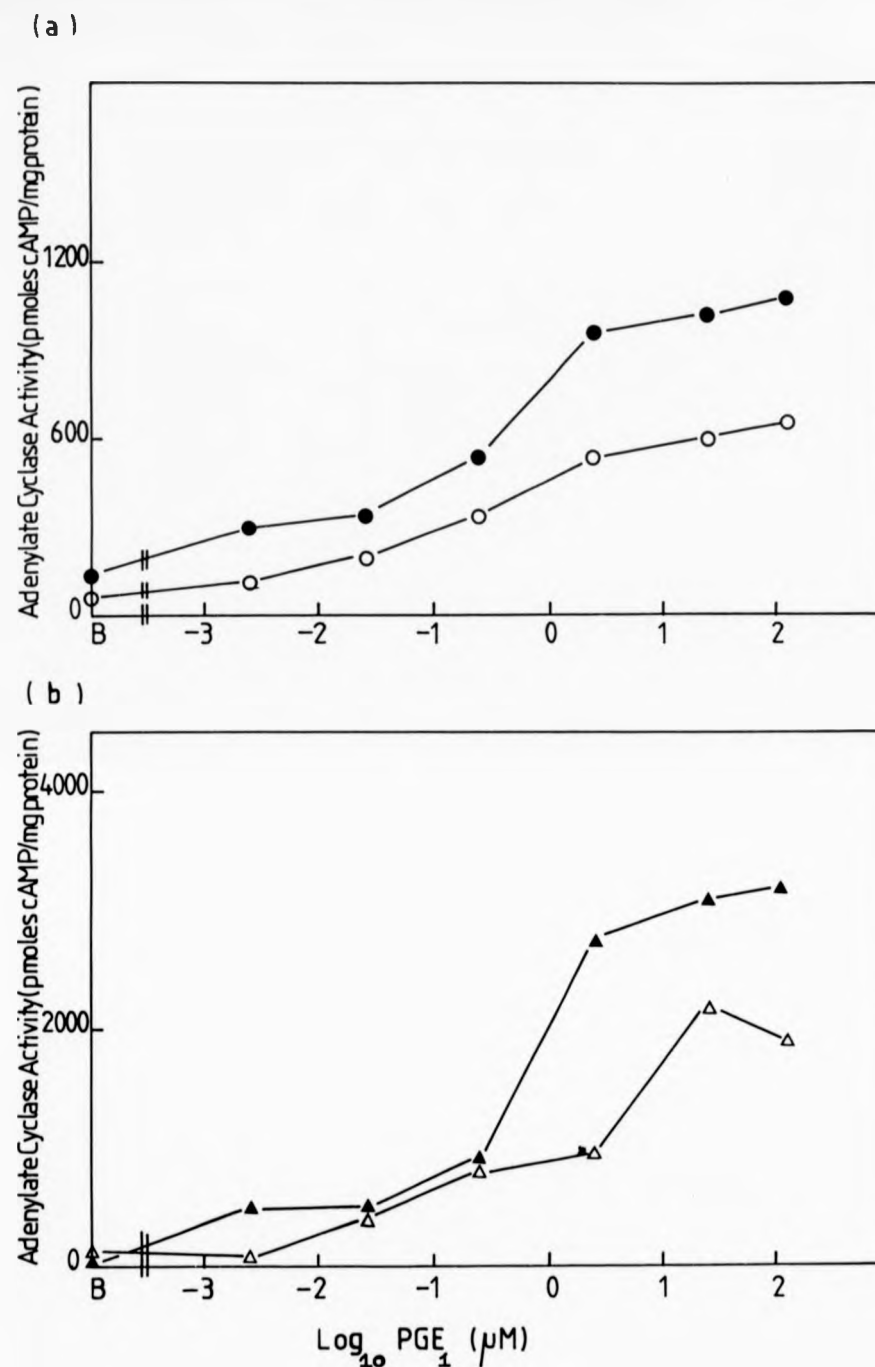


FIGURE 69. Effect of Trypsin on the Preparation of CH23 Homogenates

Method as described in Fig. 68.

Results shown are the means of duplicate assays and duplicate cAMP determinations of each sample.

B represents the basal value.

- (a) ○—○ control - absence of trypsin treatment
 ●—● 0.0025% trypsin
 △—△ 0.005% trypsin
- (b) ○—○ 0.025% trypsin
 ●—● 0.05% trypsin

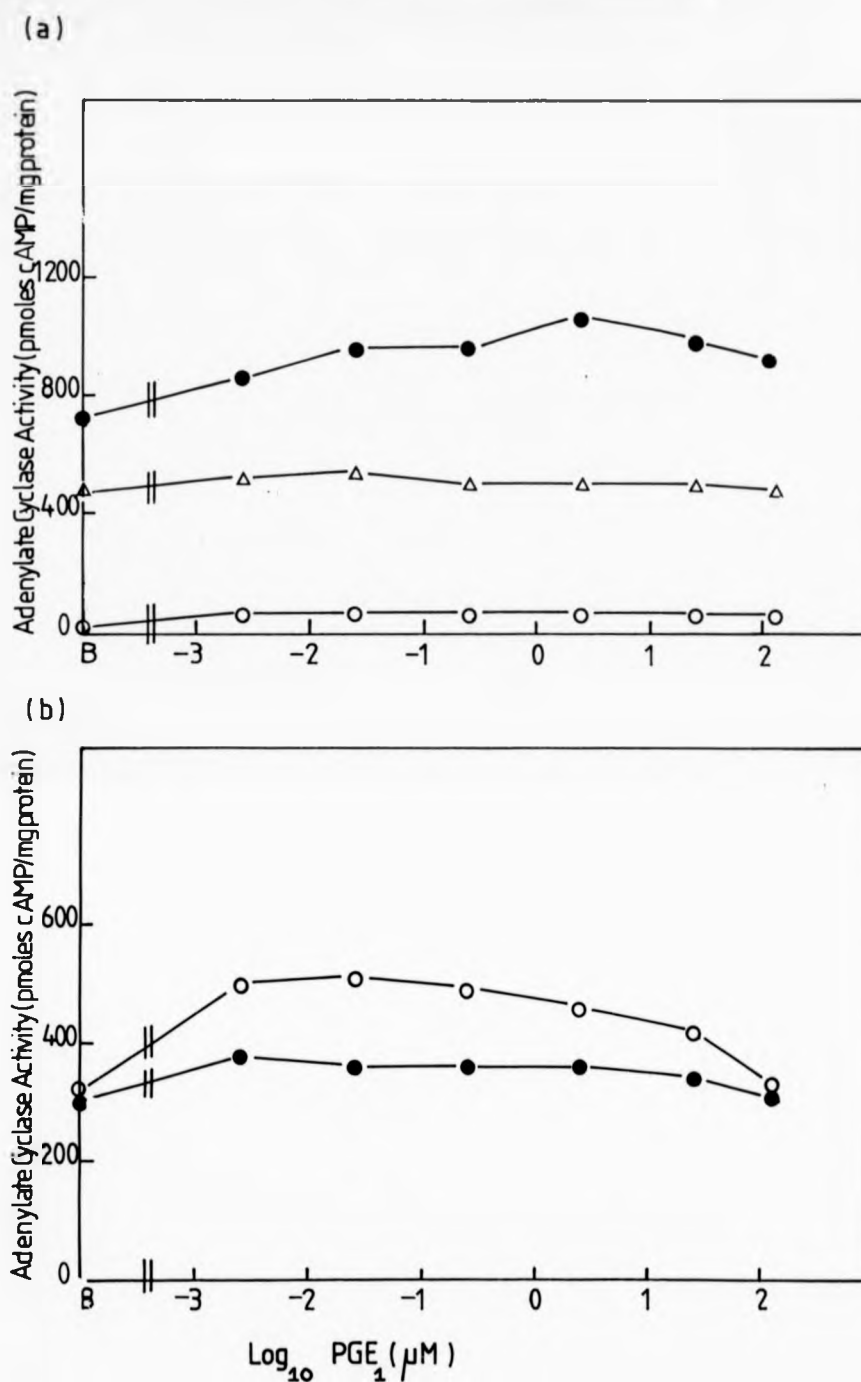
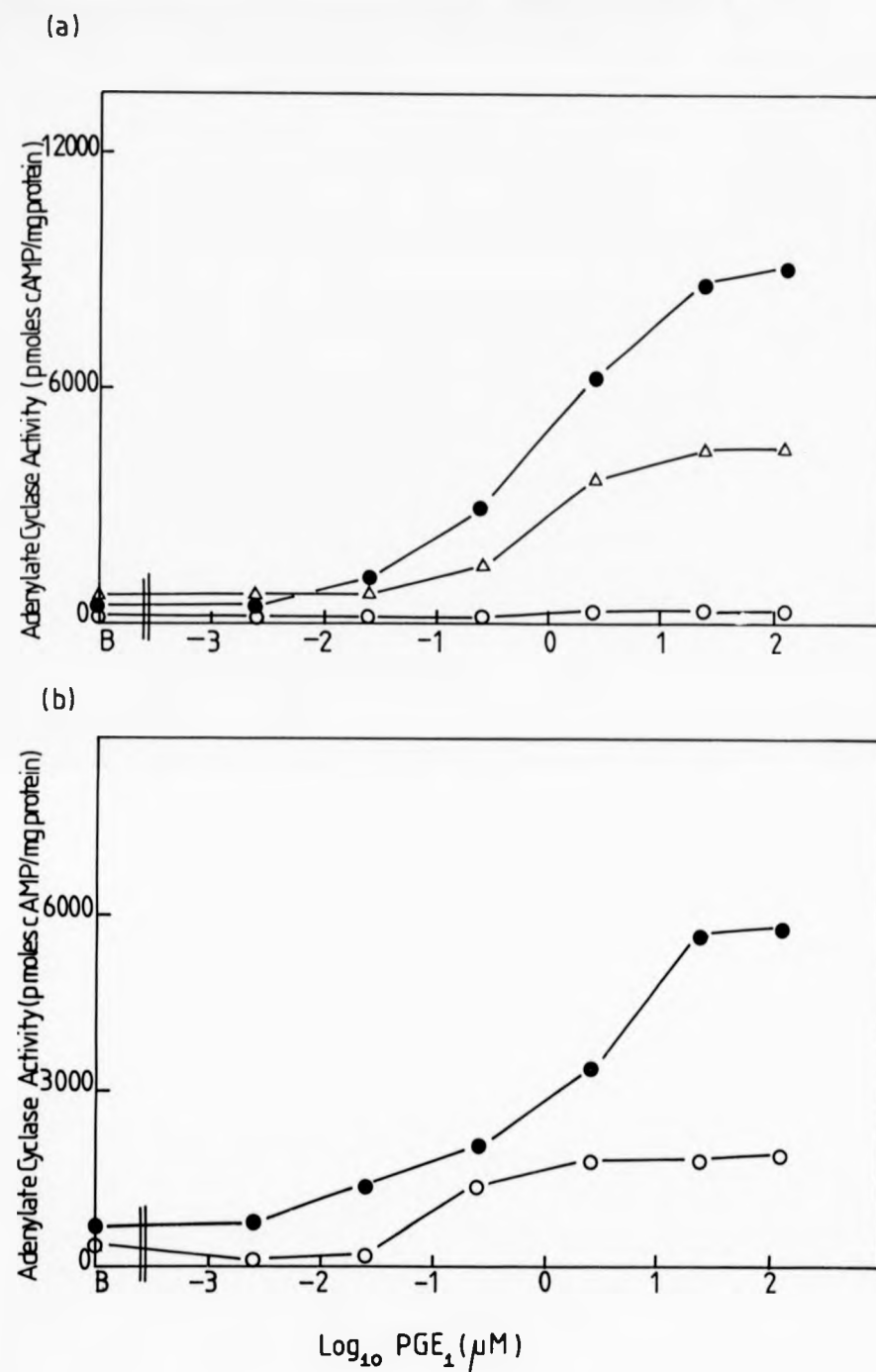


FIGURE 70. Effect of Trypsin on Preparation of
PCM3 Homogenates

Method as described in Fig. 68. Results shown are the means of duplicate assays and duplicate cAMP determinations of each sample. B represents the basal value.

- (a) O—O control - absence of trypsin treatment
 ●—● 0.0025% trypsin
 Δ—Δ 0.005% trypsin
- (b) O—O 0.025% trypsin
 ●—● 0.05% trypsin



increase when PGE_1 is added showing that CH23 adenylate cyclase is less responsive than the other cell lines. Maximum activation of adenylate cyclase activity occurs at 0.0025% trypsin where the enhancement over control values in the presence of PGE_1 is about 10-fold. For PCM3 (Fig. 70) all concentrations used (0-0.05%) enhance the basal levels of adenylate cyclase activity as well as the PGE_1 -stimulated levels. The maximum enhancement which occurs is at 0.0025% trypsin and approximately 30-fold enhancement of adenylate cyclase activity occurs at this concentration. These results illustrate the similarity in the properties of CH23 and PCM3 since they illustrate a similar concentration dependence of sensitivity to trypsin since maximum activation occurs at 0.0025% whereas for P388 enhancement is seen at 0.025% trypsin. This phenomenon of treatment of intact cells with trypsin followed by homogenisation of the cells producing enhanced adenylate cyclase activity has been observed before in this laboratory (Hughes and Ayad, 1980). The phenomenon was termed 'latent activation' by these authors since no such activation by trypsin is observed in intact cells. This phenomenon has also been observed by other workers (Ryan *et al.*, 1975; Wallach *et al.*, 1978). Several possibilities occur to explain the activation of adenylate cyclase following treatment of intact cells by trypsin and homogenisation.

(a) Trypsin could have degraded a proportion of the protein or glycoprotein on the cell surface so that when adenylate cyclase activity expressed per mg of protein values would be artificially high. However, no significant reduction in

protein concentration of homogenates from trypsin treated cells were observed.

(b) Trypsin may not have been removed during the preparation of the homogenate and the washing procedures. This possibility cannot be excluded but the trypsin would have to be bound very tightly.

(c) The activation is 'latent' i.e. only expressed when the cells are homogenised.

The fact that PGE_1 -stimulated cyclase activity is also enhanced suggests that the receptor is not altered by protease treatment. To further investigate the enhancement observed it was decided to study the specificity of this activation by studying the effect of chymotrypsin on preparation of homogenates. In addition the effect of trypsin directly on homogenates and purified plasma membranes was studied. To investigate whether trypsin was required to enter the cell in order to elicit its effect the molecule was linked to an inert support Sepharose 6B to determine if activation could still take place.

(b) Chymotrypsin Treatment of Intact Cells Followed by Homogenisation

Figs. 71 and 72 show the effect of various concentrations of chymotrypsin on the preparation of homogenates from CH23 and PCM3 respectively. For CH23 (Fig. 71) it can be seen that treatment of CH23 intact with chymotrypsin under the same conditions employed for trypsin produces both enhancement of basal levels of adenylate cyclase and prostaglandin-stimulated levels. The

FIGURE 71. Effect of Chymotrypsin on Preparation of CH23 Homogenates

Cells were washed twice with trypsin salt solution and treated with the required concentration of chymotrypsin for 2 minutes at 37°C. Following this time the reaction was quenched by the addition of growth medium. The cells were washed twice with HBSS⁺⁺. The cells were homogenised and the homogenate assayed for adenylate cyclase activity for 10 minutes at 30°C in the presence of PGE₁. Results shown are the means of duplicate assays and duplicate cAMP determinations of each sample. B represents the basal value.

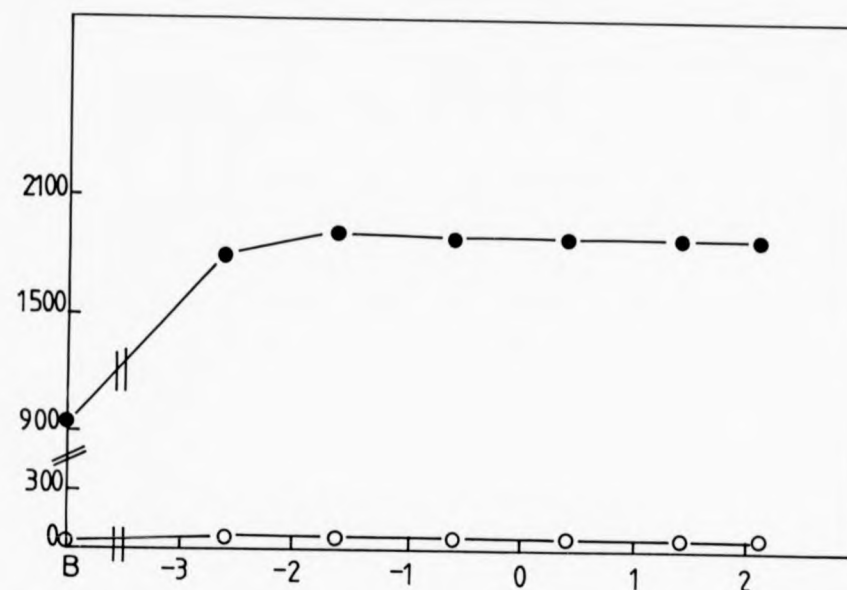
(a) ○—○ control - absence of chymotrypsin treatment.

●—● 0.0025% chymotrypsin

(b) ○—○ 0.005% chymotrypsin

●—● 0.05% chymotrypsin

(a)



(b)

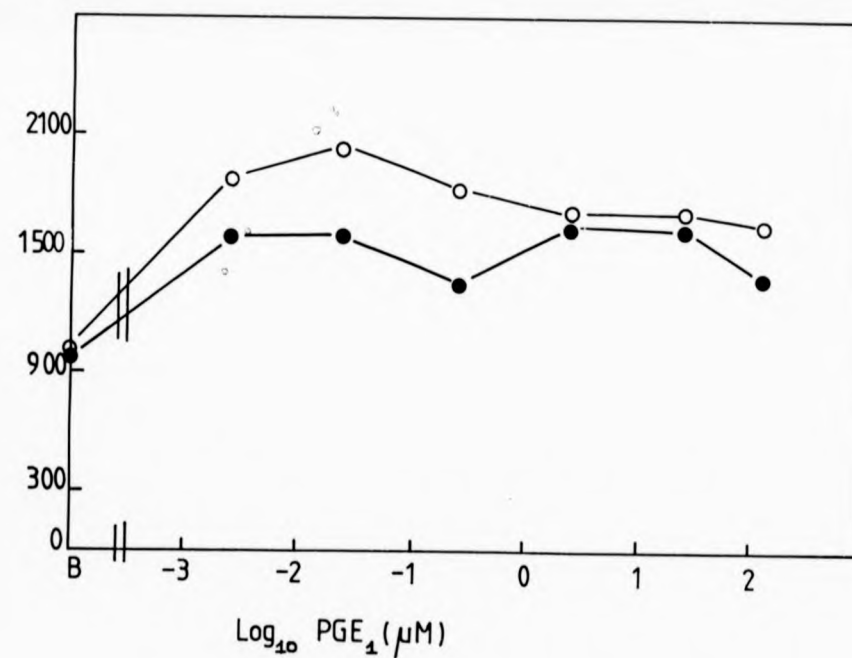
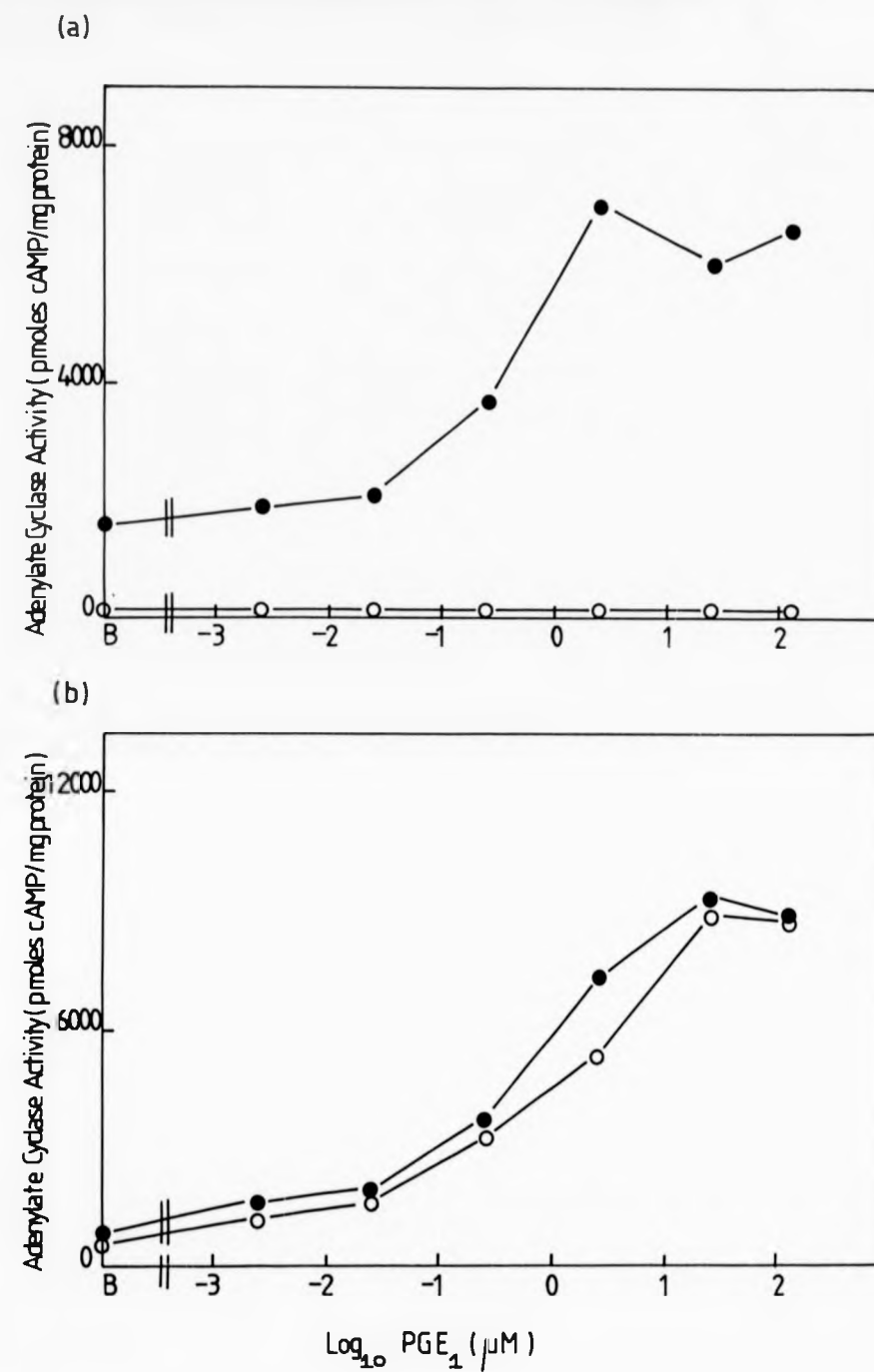


FIGURE 72. Effect of Chymotrypsin on Preparation of
PCM3 Homogenate

Method as described in Fig. 71. Results shown are the means of duplicate assays and duplicate cAMP determinations of each sample. B represents the basal value.

- (a) ○—○ control - absence of chymotrypsin treatment
●—● 0.0025% chymotrypsin
- (b) ○—○ 0.005% chymotrypsin
●—● 0.05% chymotrypsin



maximal effect is seen at 0.0025-0.005% chymotrypsin illustrating the similarity to trypsin treatment. The maximal response elicited by this concentration of chymotrypsin in combination with PGE_1 is about 20-fold. This enhancement is greater than that seen with trypsin. Once again in the presence of chymotrypsin the adenylate cyclase activity of CH23 does not show a true dose-response curve to PGE_1 . For PCM3 (Fig. 72) chymotrypsin also produces an elevation in basal levels of adenylate cyclase activity as well as PGE_1 -stimulated levels. The maximal activation occurs at 0.005% chymotrypsin again indicating a remarkable similarity to trypsin treatment. The enhancement obtained at this chymotrypsin concentration in the presence of PGE_1 is approximately 100-fold. This is greater than that seen with trypsin.

It appears that chymotrypsin has a greater enhancing effect on homogenates when intact cells are treated with protease followed by homogenisation than does trypsin. Chymotrypsin also has little effect on cAMP levels in intact cells and also enhances PGE_1 -stimulatable activity following homogenisation indicating that the PGE_1 -receptor is unaffected by chymotrypsin treatment. The processes occurring for trypsin and chymotrypsin appear to be similar although the effect is greater with chymotrypsin. No documentation for such 'latent' activation of adenylate cyclase by chymotrypsin can be found in the literature. Both chymotrypsin and trypsin are serine proteases and therefore might act in a similar manner on adenylate cyclase

activity. A sequence homology between serine proteases and the B chain of cholera toxin exists (Kurosky *et al.*, 1977) so it is possible that trypsin and chymotrypsin act in a similar manner to cholera toxin in activating adenylate cyclase. However, it has been shown in S49 lymphoma cells that cholera toxin and chymotrypsin act by independent mechanisms (Stengel *et al.*, 1980). Therefore it seems unlikely that this is the explanation for the latent activation of adenylate cyclase produced by trypsin and chymotrypsin.

Since the PGE_1 receptor has been shown to be unaffected by treatment with protease and there is little change in protein concentration of homogenates following treatment with protease there seems two further possibilities for the mode of action of proteases.

- (a) An action on the catalytic subunit
- (b) An action on either the stimulatory or inhibitory guanine nucleotide regulatory subunit. To further investigate these possibilities it was decided to study the effect of trypsin-Sepharose 6B to see if the protease must enter the cell in order to elicit its effect and the direct effect on homogenates and purified plasma membranes.

(c) The Effect of Trypsin-Sepharose on Preparation of CH23 and PCM3 Homogenates

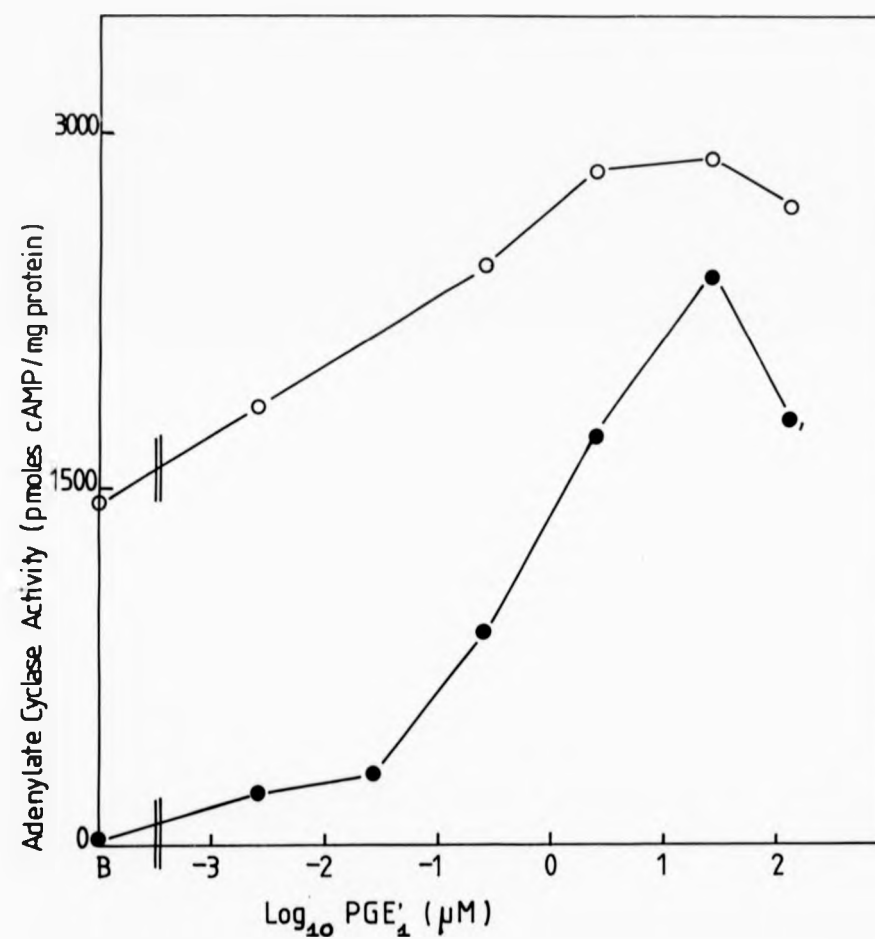
Fig. 73 illustrates the effect of trypsin-Sepharose on latent activation. From the Fig. it can be seen that for both CH23 and PCM3 there is an activation of PGE_1 -stimulated

FIGURE 73. Effect of Trypsin-Sepharose on Preparation of PCM3 and CH23 Homogenates

Cells were washed twice with trypsin salt. The cells were then treated with trypsin-Sepharose, 0.005% trypsin, for 2 minutes at 37°C. After this time the gel solution was poured off and the action of trypsin quenched by the addition of growth medium. The cells were washed with HBSS twice and then homogenised. The homogenate was assayed for adenylate cyclase activity in the presence of PGE_1 for 10 minutes at 30°C. Results shown are the means of duplicate assays and duplicate cAMP determinations of each sample. B represents the basal value.

○—○ CH23

●—● PCM3



activity but for PCM3 there appears to be little activation of basal levels. For PCM3 the level of enhancement of PGE_1 -stimulated levels of adenylate cyclase activity is similar, if slightly lower, to that obtained with trypsin alone. However, for CH23 the enhancement of both basal levels and PGE_1 -stimulated adenylate cyclase are greater than for trypsin alone. The exact concentration of trypsin attached to Sepharose was not available although an approximate measurement was obtained from the value of trypsin concentration remaining in the gel buffer. Therefore the slight discrepancy in extent of enhancement could be due to the unknown protein concentration. However, latent activation of adenylate cyclase still occurs in the presence of trypsin-Sepharose suggesting that trypsin does not need to enter the cells for its action. However, the possibility that trypsin has come uncoupled from the beads cannot be excluded. If trypsin can exert its action without entering the cell, the interaction of the protease with either the guanine nucleotide regulatory protein or the catalytic unit is unlikely since both components exhibit a major display on the cytosolic side of the plasma membrane.

(d) The Direct Effect of Trypsin on Homogenates and Plasma Membranes

The effect of various concentrations of trypsin (25-500 $\mu\text{g}/\text{ml}$) on the adenylate cyclase activity of P388 (Fig. 74) CH23 (Fig. 75) and PCM3 (Fig. 76) homogenates. Both in the presence and absence of PGE_1 (2.5 μM) trypsin decreases the adenylate cyclase activity of all the cell lines. These results confirm others from this laboratory

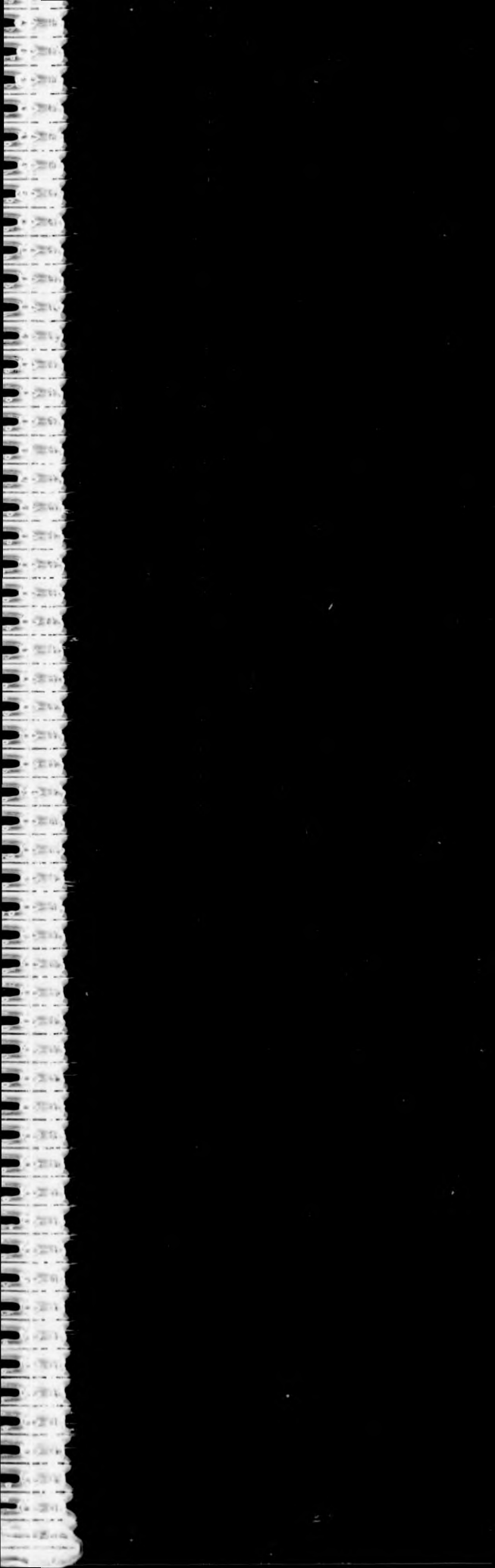


FIGURE 74. Effect of Trypsin on P388 Homogenate
Adenylate Cyclase Activity

Adenylate cyclase assay components plus PGE_1 and trypsin were preincubated at 30°C for 10 minutes. The reaction was initiated by addition of homogenate (50-100 μg protein). The adenylate cyclase activity was measured for 10 minutes at 30°C . Results shown are the means of duplicate assays and duplicate cAMP determinations of each sample. B represents the basal value.

○ — ○ $-\text{PGE}_1$

● — ● $+\text{PGE}_1$ (2.5 μM)

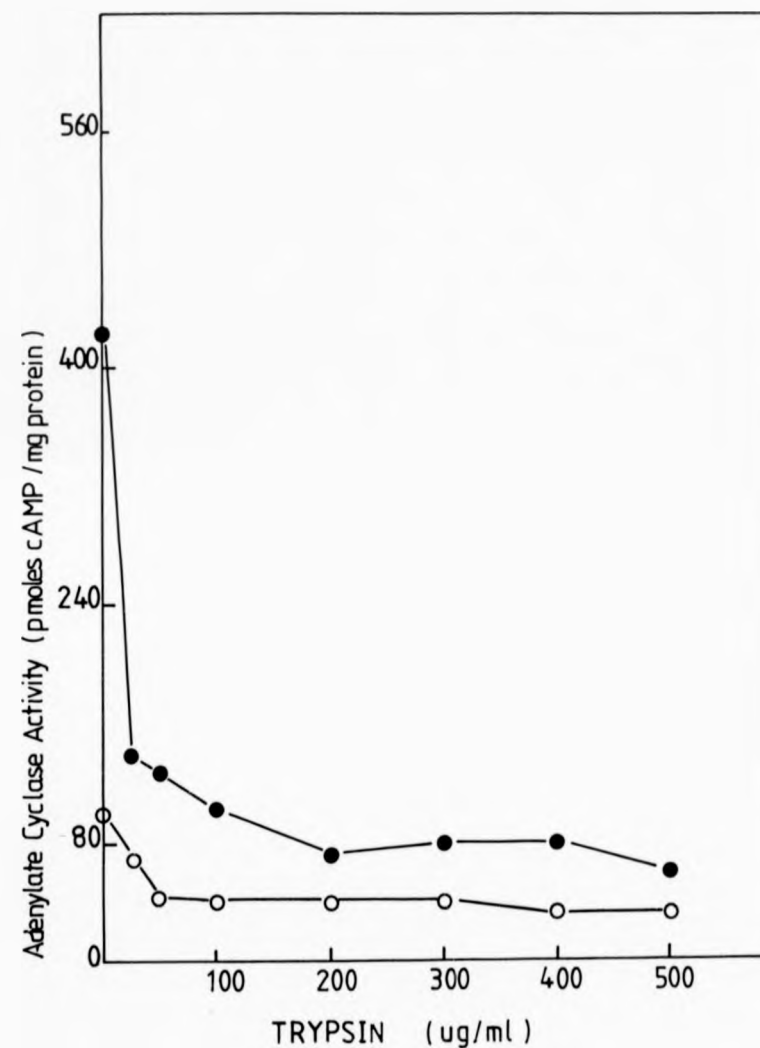


FIGURE 75. Effect of Trypsin on CH23 Homogenate
Adenylate Cyclase Activity

Method as described in Fig. 74. Results shown are the means of duplicate assays and duplicate cAMP determinations of each sample. B represents the basal value.

○—○ -PGE₁
●—● +PGE₁ (2.5μM)

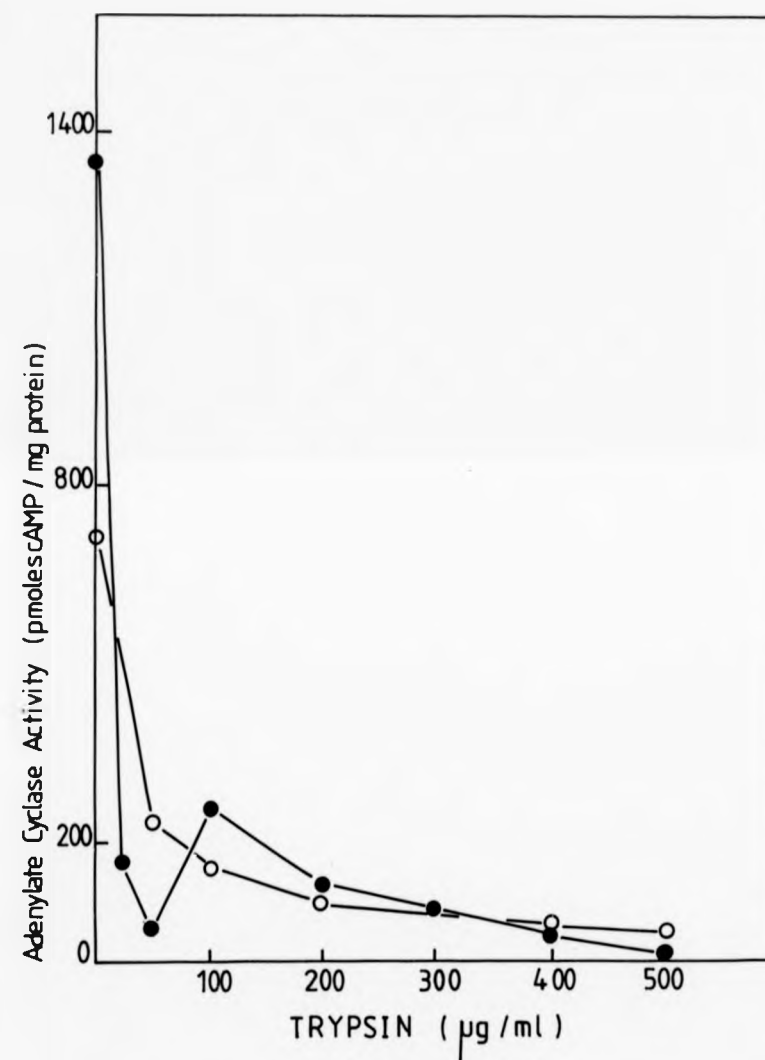
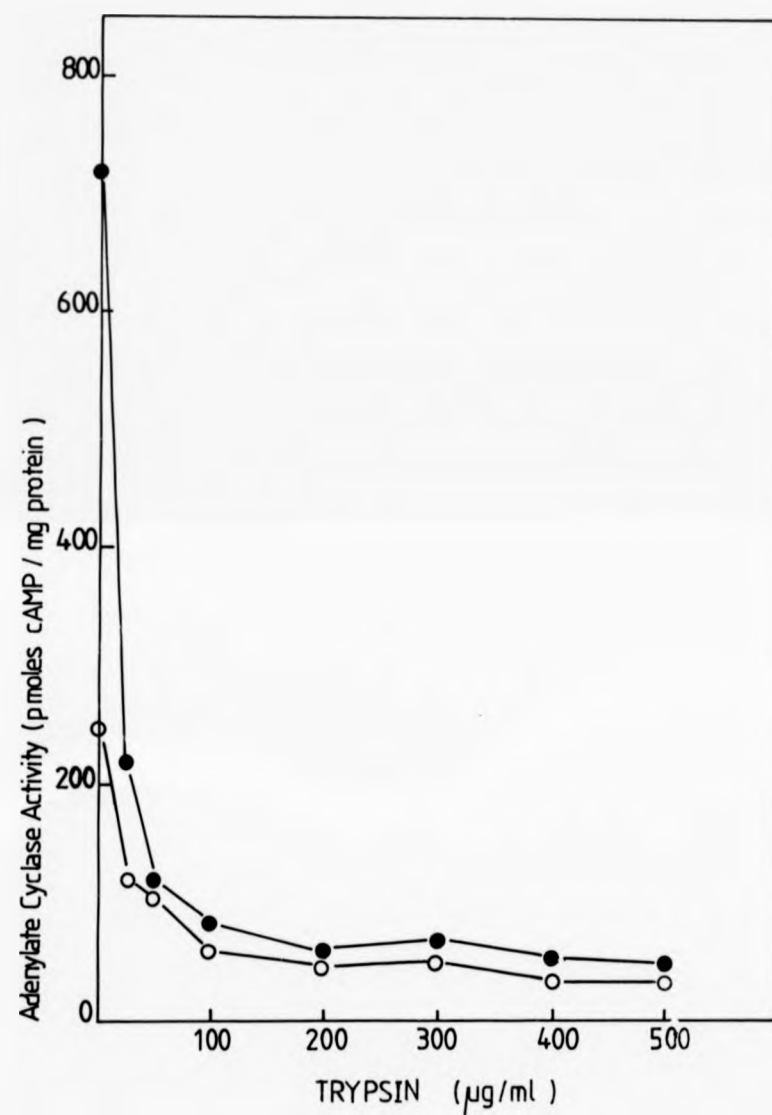


FIGURE 76. Effect of Trypsin on PCM3 Homogenate
Adenylate Cyclase Activity

Method as described in Fig. 74. Results shown are the means of duplicate assays and duplicate cAMP determinations of each sample. B represents the basal value.

○—○ -PGE₁
●—● +PGE₁ (2.5μM)



(Hughes and Ayad, 1980). These authors found stimulation of adenylate cyclase activity at low concentrations of trypsin with optimum stimulation occurring at 1-10 μ g/ml. Similar profiles were obtained when trypsin was studied on the basal, PGE₁- and Gpp(NH)p-stimulated adenylate cyclase activity i.e. a biphasic response, stimulation at low concentrations and inhibition at high concentrations. When the activity of plasma membranes was studied for P388 (Fig. 77) in the absence of PGE₁ there was an increase in adenylate cyclase activity whereas in the presence of PGE₁ (2.5 μ M) slightly erroneous results ensued, but with an overall tendency to inhibition. Possibly for P388 membranes the PGE₁ receptor is affected by the trypsin treatment. For CH23 membranes (Fig. 78) there was an activation of adenylate cyclase both in the presence and absence of PGE₁ at all concentrations of PGE₁ studied.

An explanation for the effects of trypsin on both intact cells and adenylate cyclase activity remains to be sought. It is important to ascertain whether trypsin and chymotrypsin act by a similar mechanism and if so whether the site of action for intact cells is the same as for broken cells.

Trypsin and other endopeptidases elastase, papain and α -chymotrypsin have been shown to enhance adenylate cyclase activity of fibroblast membranes (Anderson *et al.*, 1978). Maximal activation was also seen with low (1-2.4 μ g/ml) trypsin while higher concentrations progressively inactivate adenylate cyclase. Biphasic responses were also seen with other endopeptidases. The activation was most pronounced when

FIGURE 77. Effect of Trypsin on P388 Plasma
Membranes Adenylate Cyclase Activity

Adenylate cyclase assay components plus PGE_1 and trypsin were preincubated at 30°C for 10 minutes. The reaction was initiated by addition of plasma membranes (20-50 μg protein). The adenylate cyclase activity was measured for 10 minutes at 30°C . Results shown are the means of duplicate assays and duplicate cAMP determinations of each sample. B represents the basal value.

○—○ - PGE_1
●—● + PGE_1 (2.5 μM)

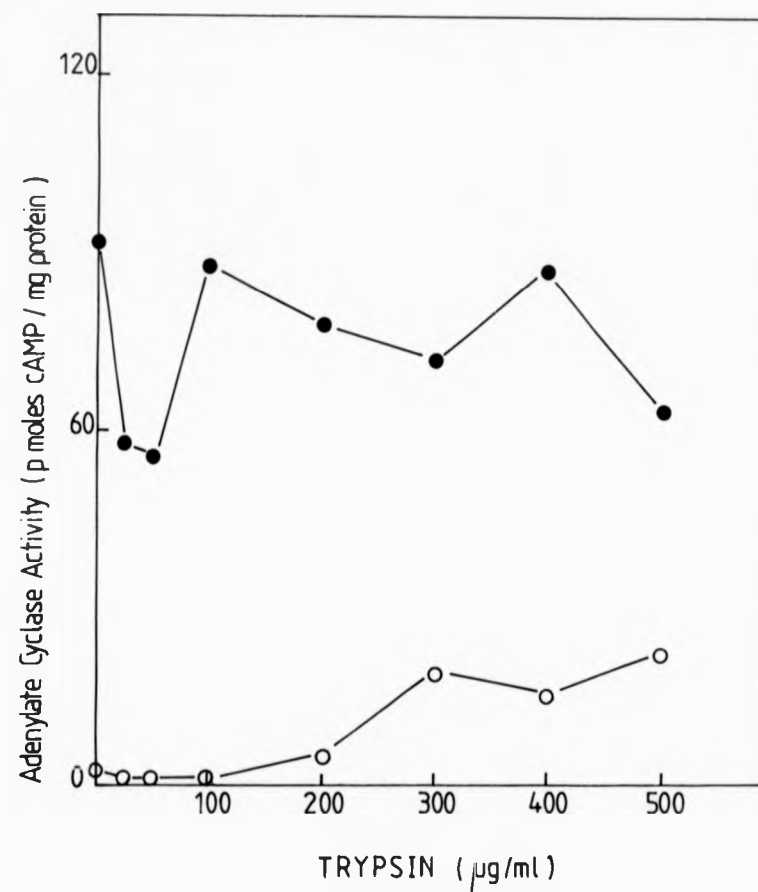
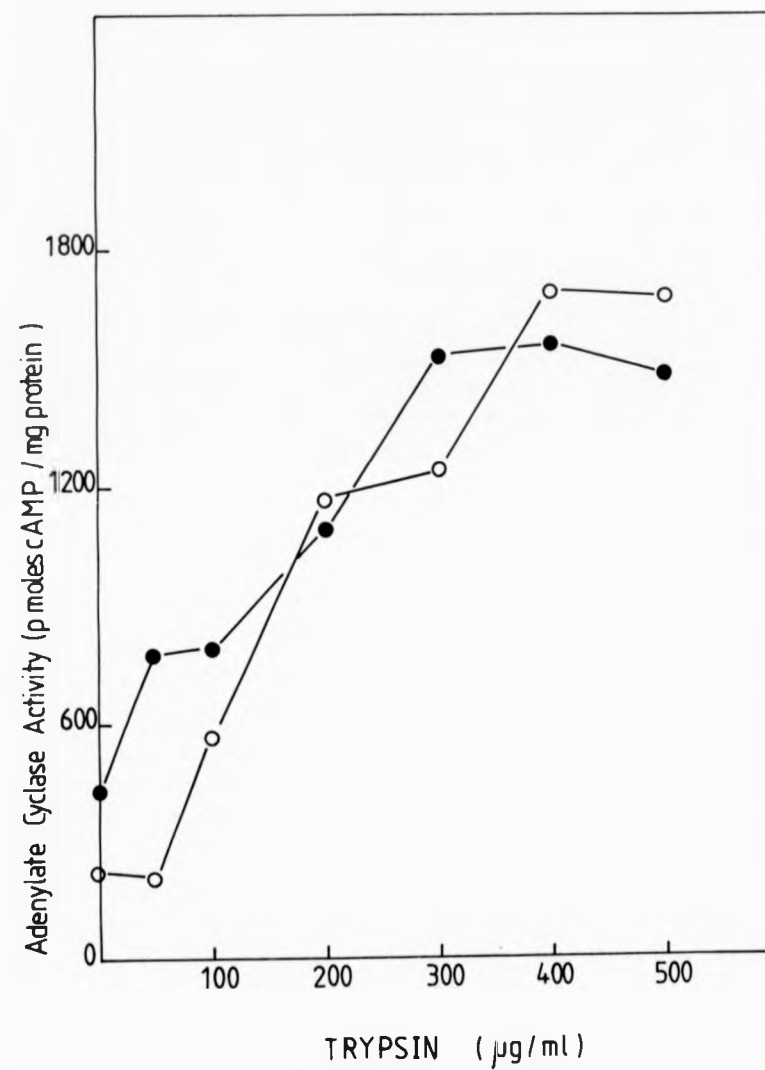


FIGURE 78. Effect of Trypsin on CH23 Plasma
Membranes Adenylate Cyclase Activity

Method as described in Fig. 77. Results shown are the means of duplicate assays and duplicate cAMP determinations of each sample. B represents the basal value.

○—○ -PGE₁
●—● +PGE₁ (2.5μM)



carried out in the presence of GTP. These authors suggest that trypsin has an effect on regulatory functions rather than by unmasking of nascent catalytic sites as suggested by Hanoune *et al.* (1977). Wallach *et al.* (1978) have observed an increase in adenylate cyclase following prior treatment of intact cells and their results implicated the degradation of regulatory components in the action of the protease whereas Ryan *et al.* (1975) suggest that trypsin appears to be acting on the catalytic unit since the effect of F^- is augmented. There appears to be a number of possible sites for protease action and it remains to be determined if there is a single site of action or multiple. Recently, both trypsin and chymotrypsin have been shown to augment both basal and hormone-stimulated adenylate cyclase of bovine thyroid in a biphasic manner (Friedman *et al.*, 1983) and the results suggested that augmentation of thyroid adenylate cyclase activity may in part result from the stimulation of endogenous proteases. Also Stengel *et al.* (1980) have demonstrated that protease stimulation occurs in cyc^- mutant of S49 lymphoma and rat liver plasma membranes independently of the stimulatory guanine nucleotide regulatory protein since effects are seen independently of cholera toxin activation and seen in the presence of Gpp(NH)p stimulation. Also cyc^- mutant is devoid in regulatory protein. The protease activation has also been shown to occur in the presence of Gpp(NH)p in this laboratory (Hughes and Ayad, 1980). Therefore it appears unlikely that trypsin exerts its activation at the level of the N_s unit of adenylate cyclase. However, for a number of systems GTP is required for protease

activation (Yamamura *et al.*, 1977; Anderson *et al.*, 1978; Wallach *et al.*, 1978; Anderson *et al.*, 1979) although activation in PCM3, CH23, P388 occurs in the absence of exogenous GTP. It seems that protease activation occurs by at least two mechanisms.

- (a) A GTP-dependent process.
- (b) A GTP-independent process.

It has been suggested by Stengel *et al.* (1979) that proteolytic activation of adenylate cyclase of rat liver is not exerted directly on the catalytic subunit of the enzyme but rather on a closely related regulatory (possibly inhibitory) component. Trypsin has been shown to obliterate the hormonal inhibition of adenylate cyclase and this has been suggested to be due to its action on the N_1 unit (Jakobs *et al.*, 1983). Therefore it is possible that trypsin exerts an action by inhibiting the N_1 component(s) of adenylate cyclase activity thus allowing a greater amount of stimulation.

Other workers have shown an alteration in amounts of membrane proteins following limited proteolysis by trypsin resulting in proteolytic activation of adenylate cyclase (Pinkett and Anderson, 1980) activity. Membranes prepared from intact NRK cells subject to trypsin treatment showed a decrease in amounts of 56,000, 46,000, 37,000, 32,000 dalton proteins. Treatment of purified plasma membranes resulted in a loss of numerous bands in the high molecular weight region ($> 150,000$ daltons) as well as decreases in

membrane proteins 56,000, 49,000 46,000 and 23,000 daltons.

It was therefore decided to treat intact CH23, PCM3 and P388 with trypsin and prepare plasma membranes. Samples were kept of the preparations at each stage and run on 13-8% gels to establish if there were any observable changes in comparison to fractions prepared from cells untreated with protease. Gel scans comparing homogenate and purified membranes from treated and untreated cells are shown for P388 (Fig. 79), CH23 (Fig. 81) and PCM3 (Fig. 83). Photographs of tracks of all samples are also shown for P388 (Fig. 80), CH23 (Fig. 82) and PCM3 (Fig. 84).

From the laser scans no proteins appear to be absolutely absent although there are some minor changes indicated by arrows. Unfortunately the bands cannot be quantified to observe relative changes in proteins since different amounts of protein were used for each sample. From the photographs it can be seen that preparation of plasma membranes results in a loss of low molecular weight material and this could be the material responsible for the enhancement of cholera toxin inhibition of GTPase when supernatant material was readded. Since the protein concentrations obtained during the procedure were so dilute the bands obtained on the gels were very faint. This led to it being impossible to determine the molecular weights of the minor components altered. It is therefore necessary to make membrane preparations from large quantities of cells and add the same amount to the gels in order to obtain the precise nature of the changes.

FIGURE 79. SDS-Polyacrylamide Gel Electrophoresis
of P388 Membrane Fractions Prepared from Cells
Treated with 0.05% Trypsin or from Untreated Cells

Samples were prepared in sample buffer to give a protein concentration of 50 μ g/well in 40 μ l samples. In the case of plasma membranes 40 μ l of the sample was used neat and solid SDS, bromophenol blue were added to the sample followed by glycerol. Samples were boiled for two minutes and were then layered on top of the gel using a Hamilton syringe. The gel was composed of 6cm depth of 13%, 6cm depth of 8% and 4cm depth of 3% stacking gel. The gels were run in electrophoresis buffer (0.025M Tris-HCl pH 8.3, 20 $^{\circ}$ C, 0.192M Glycine, 0.1% SDS and 0.1% β -mercaptoethanol) at 25mA until the proteins reached the running gel and 50mA until the bromophenol blue bond was 1cm from the gel end. The gel was stained with 0.115% Coomassie blue in 8% acetic acid, 20% ethanol for 1 hour at 37 $^{\circ}$ C and destained with 8% Acetic acid, 20% ethanol. The gel was then scanned at 632.8nm on an LKB, Ultrascan Laser densitometer.

(a) Homogenate samples prepared from control and trypsin (0.05%) treated cells (2 minutes, 37 $^{\circ}$ C). Arrows show observed differences in the profiles.

(b) Plasma membrane samples prepared from control and trypsin (0.05%)-treated cells (2 minutes, 37 $^{\circ}$ C). Arrows show observed differences in the profiles.

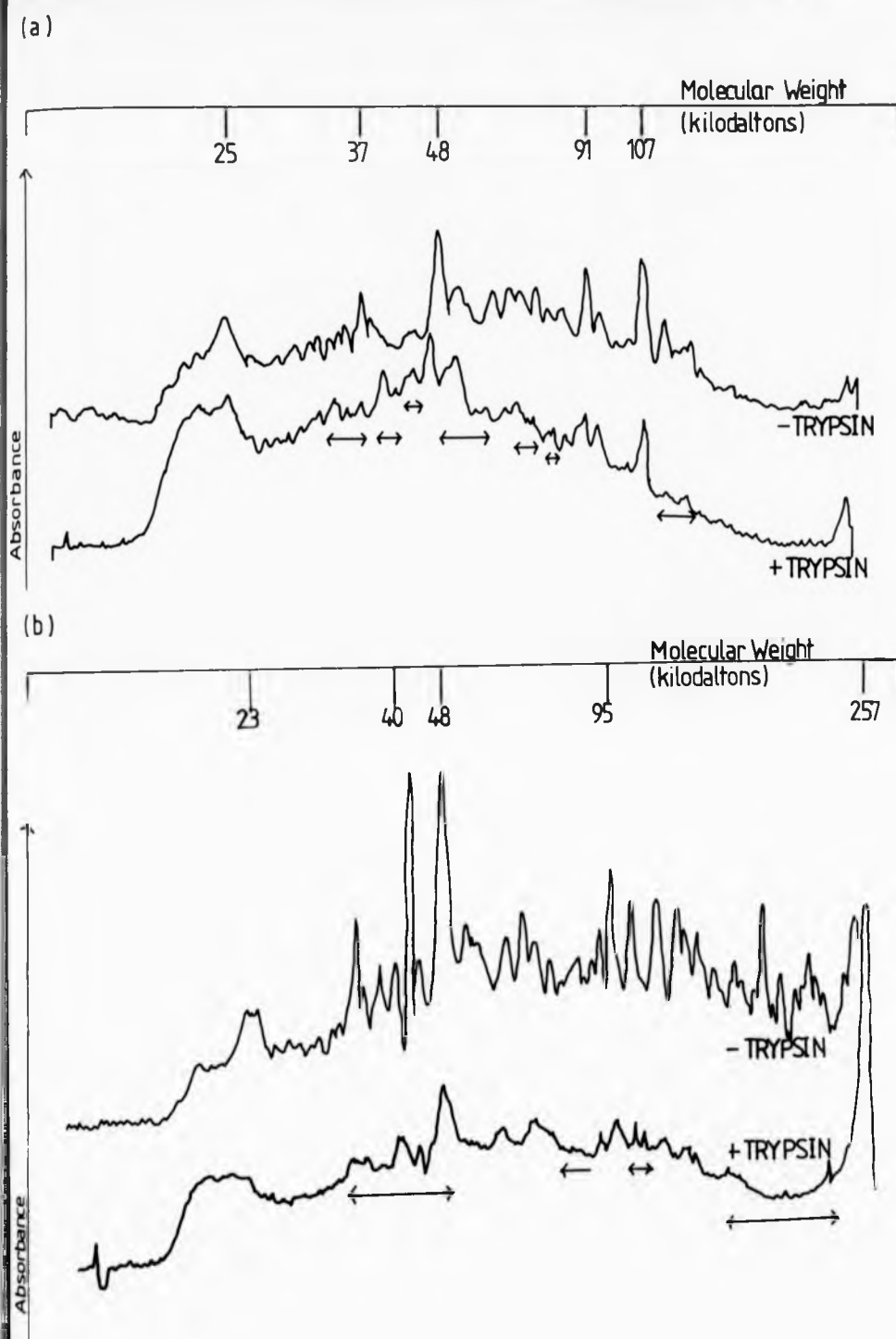


FIGURE 80. SDS-Polyacrylamide Gel Electrophoresis of
P388 Membrane Fractions Prepared from Cells Treated
with 0.05% Trypsin or from Untreated Cells

The photograph illustrates a gel obtained in the same manner as described in Fig. 79. Samples a-c and g-i were prepared in sample buffer to give a protein concentration of 50 μ g/well, in 40 μ l samples. In the case of samples d-f and j-l 40 μ l of the sample was used neat and SDS and bromophenol blue were added as solid followed by glycerol.

Tracks a-f show samples in the absence of trypsin and g-l show samples in the presence of trypsin.

- | | | |
|----|---|--|
| a, | g | Supernatant obtained after homogenate centrifuged at 700g for 10 minutes. |
| b, | h | SI, supernatant from centrifugation at 40,000g. |
| c, | i | PI, pellet obtained from centrifugation at 40,000g resuspended in 75ml Tris-HCl pH 6.5, 25mM MgCo ₂ |
| d, | j | Sample obtained from upper layer following sedimentation on 35% sucrose. |
| e, | k | SII, supernatant from second centrifugation at 40,000g. |
| f, | l | Plasma membranes. |

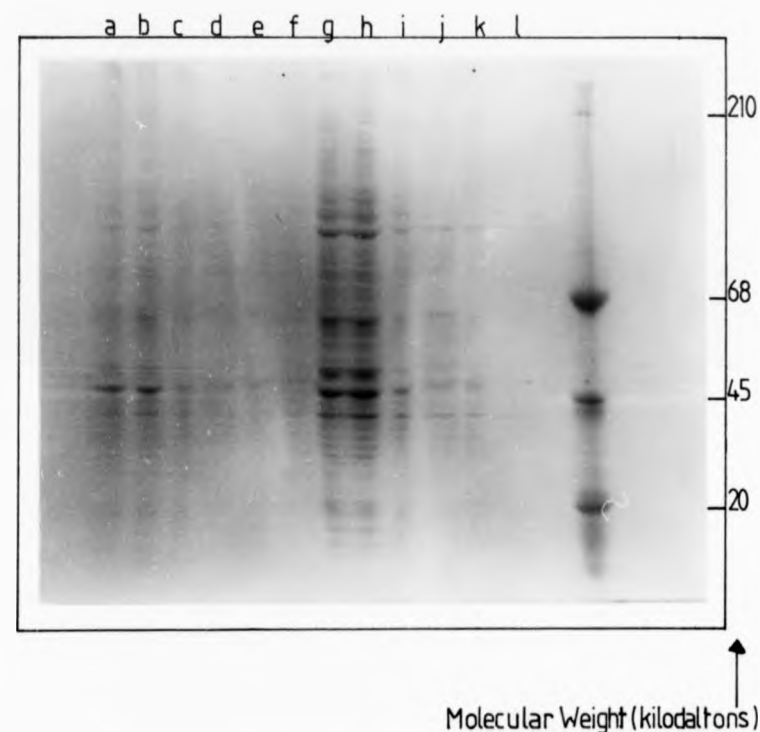


FIGURE 80. SDS-Polyacrylamide Gel Electrophoresis of
P388 Membrane Fractions Prepared from Cells Treated
with 0.05% Trypsin or from Untreated Cells

The photograph illustrates a gel obtained in the same manner as described in Fig. 79. Samples a-c and g-i were prepared in sample buffer to give a protein concentration of 50 μ g/well, in 40 μ l samples. In the case of samples d-f and j-l 40 μ l of the sample was used neat and SDS and bromophenol blue were added as solid followed by glycerol.

Tracks a-f show samples in the absence of trypsin and g-l show samples in the presence of trypsin.

- a, g Supernatant obtained after homogenate centrifuged at 700g for 10 minutes.
- b, h SI, supernatant from centrifugation at 40,000g.
- c, i PI, pellet obtained from centrifugation at 40,000g resuspended in 75ml Tris-HCl pH 6.5, 25mM MgCo₂
- d, j Sample obtained from upper layer following sedimentation on 35% sucrose.
- e, k SII, supernatant from second centrifugation at 40,000g.
- f, l Plasma membranes.

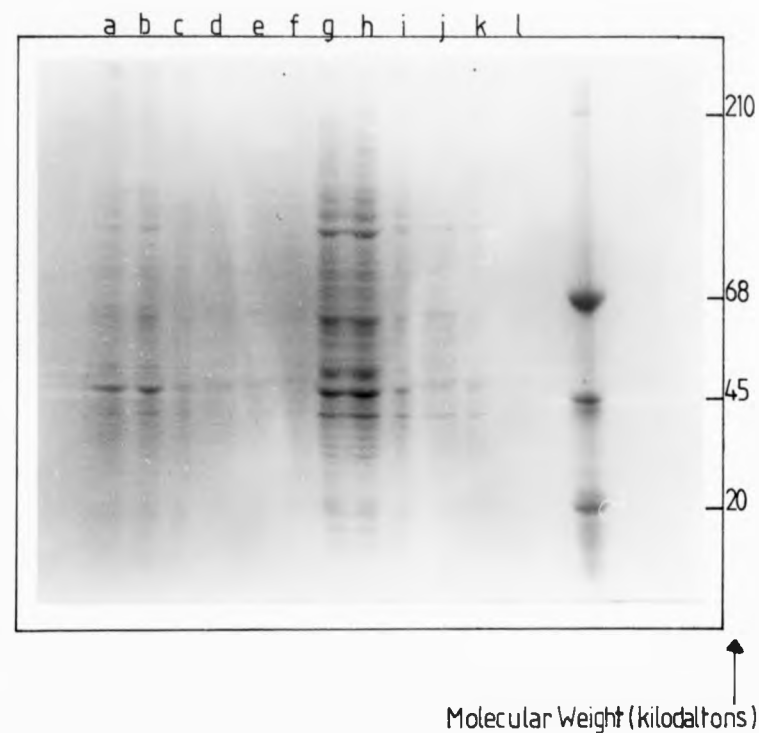


FIGURE 81. SDS-Polyacrylamide Gel Electrophoresis of CH23 Membrane Fractions Prepared from Cells Treated with 0.05% Trypsin or from Untreated Cells.

Method as described in Fig. 79.

(a) Homogenate samples prepared from control and trypsin (0.05%)-treated cells (2 minutes at 37°C).

(b) Plasma membrane samples prepared from control and trypsin (0.05%)-treated cells (2 minutes at 37°C).

Arrows show observed differences in the profiles.

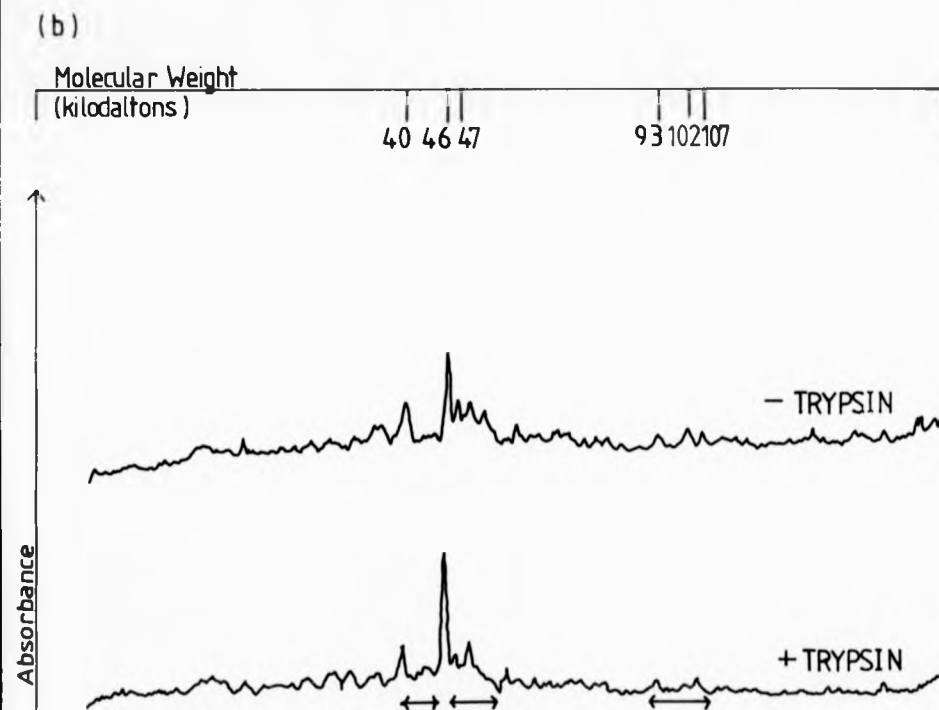
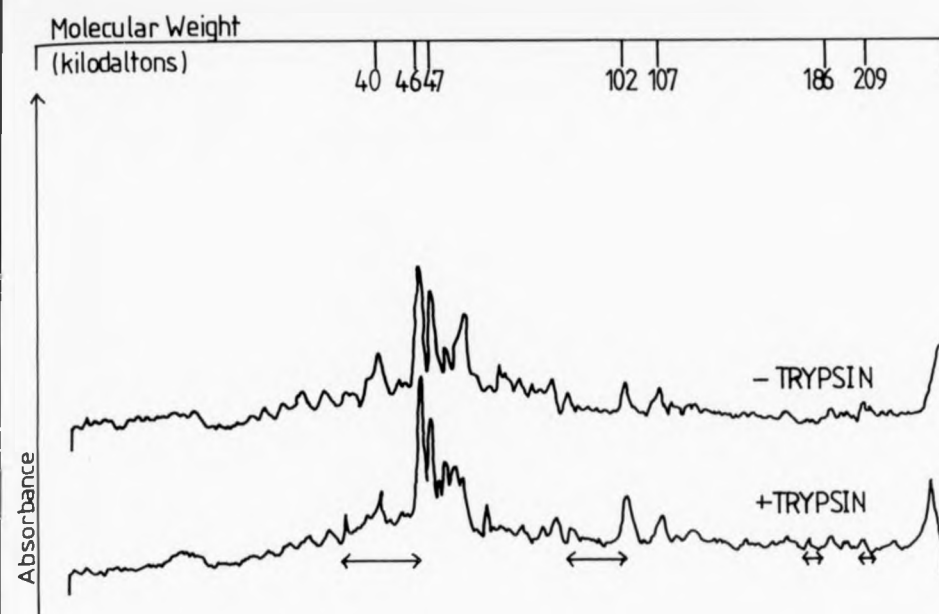


FIGURE 82. SDS-Polyacrylamide Gel Electrophoresis of
CH23 Membrane Fractions Prepared from Cells Treated
with 0.05% Trypsin or from Untreated Cells

Method as described in Figure 80.

Tracks a-f show samples in the absence of trypsin
 and g-l show samples in the presence of trypsin.

- | | |
|------|--|
| a, g | Supernatant obtained after homogenate
centrifuged at 700g for 10 minutes. |
| b, h | SI |
| c, i | PI |
| d, j | Sample obtained from upper layer
following sedimentation on 35% sucrose. |
| e, k | SII |
| f, l | Plasma membranes |

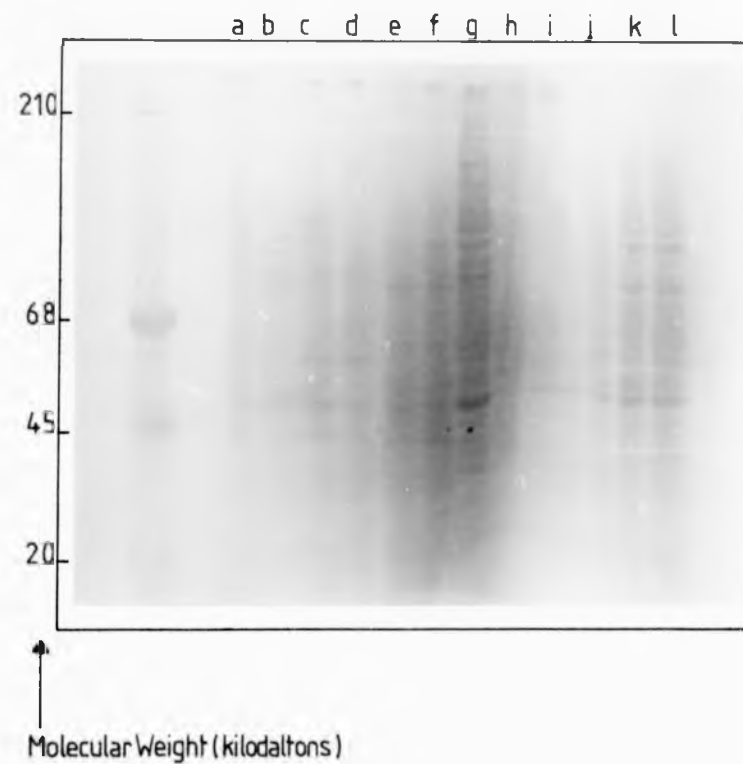


FIGURE 82. SDS-Polyacrylamide Gel Electrophoresis of
CH23 Membrane Fractions Prepared from Cells Treated
with 0.05% Trypsin or from Untreated Cells

Method as described in Figure 80.

Tracks a-f show samples in the absence of trypsin
 and g-l show samples in the presence of trypsin.

- | | | |
|----|---|--|
| a, | g | Supernatant obtained after homogenate
centrifuged at 700g for 10 minutes. |
| b, | h | SI |
| c, | i | PI |
| d, | j | Sample obtained from upper layer
following sedimentation on 35% sucrose. |
| e, | k | SII |
| f, | l | Plasma membranes |

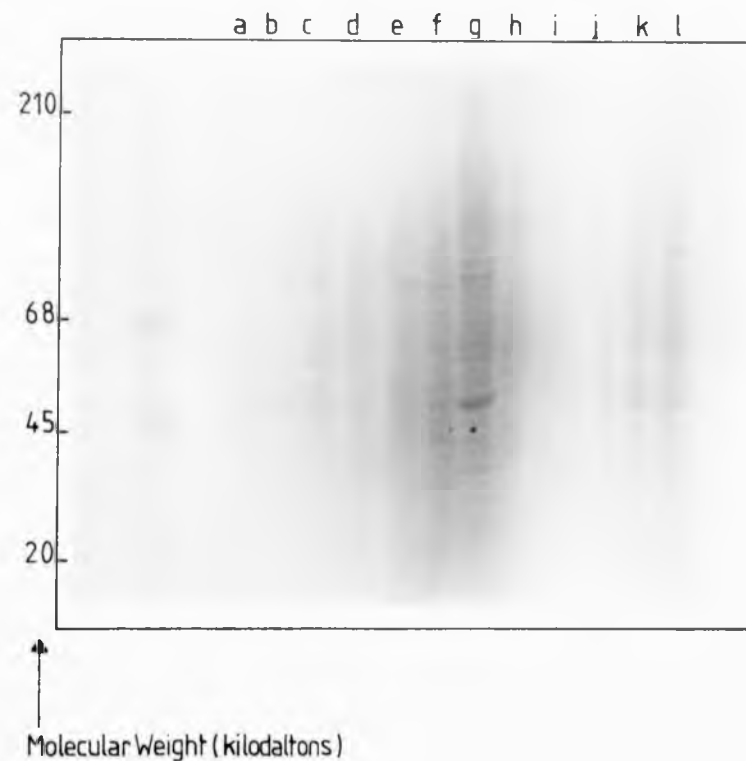


FIGURE 83. SDS-Polyacrylamide Gel Electrophoresis
of PCM3 Membrane Fractions Prepared from Cells Treated
with 0.05% Trypsin or from Untreated Cells

Method as described in Fig. 79.

- (a) Homogenate samples prepared from control and
 trypsin (0.05%)-treated cells (2 minutes at 37°C).
- (b) Plasma membrane samples prepared from control and
 trypsin (0.05%)-treated cells (2 minutes at 37°C).

Arrows show observed differences in the profiles.

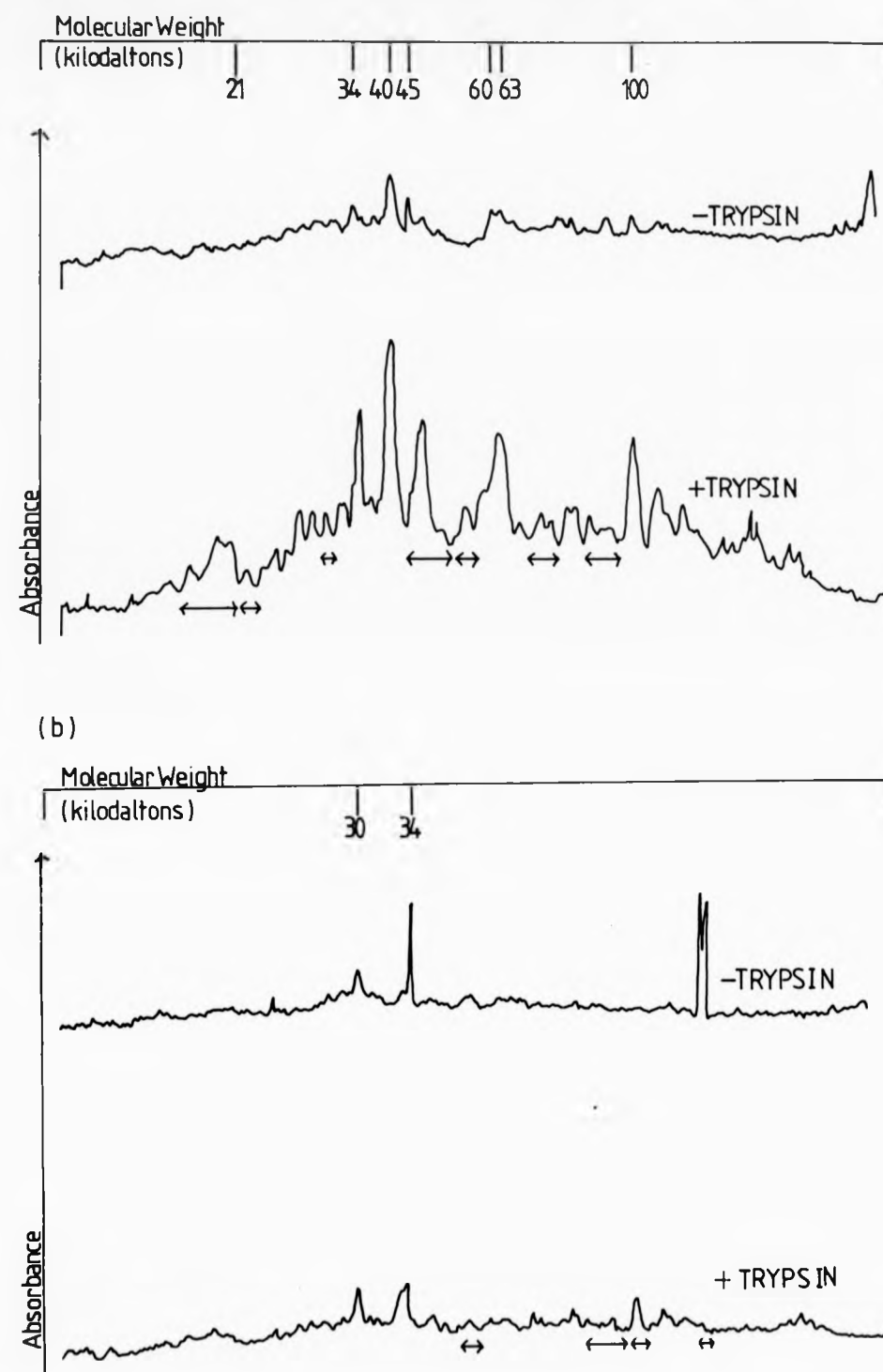


FIGURE 84. SDS-Polyacrylamide Gel Electrophoresis
of PCM3 Membrane Fractions Prepared from Cells Treated
with 0.05% Trypsin or from Untreated Cells

Method as described in Fig. 80.

Tracks a-f show samples in the absence of
 trypsin and g-l show samples in the presence of
 trypsin.

- | | |
|------|--|
| a, g | Supernatant obtained after homogenate
centrifuged at 700g for 10 minutes. |
| b, h | SI |
| c, i | PI |
| d, j | Sample obtained from upper layer following
sedimentation on 35% sucrose |
| e, k | SII |
| f, l | Plasma membranes. |

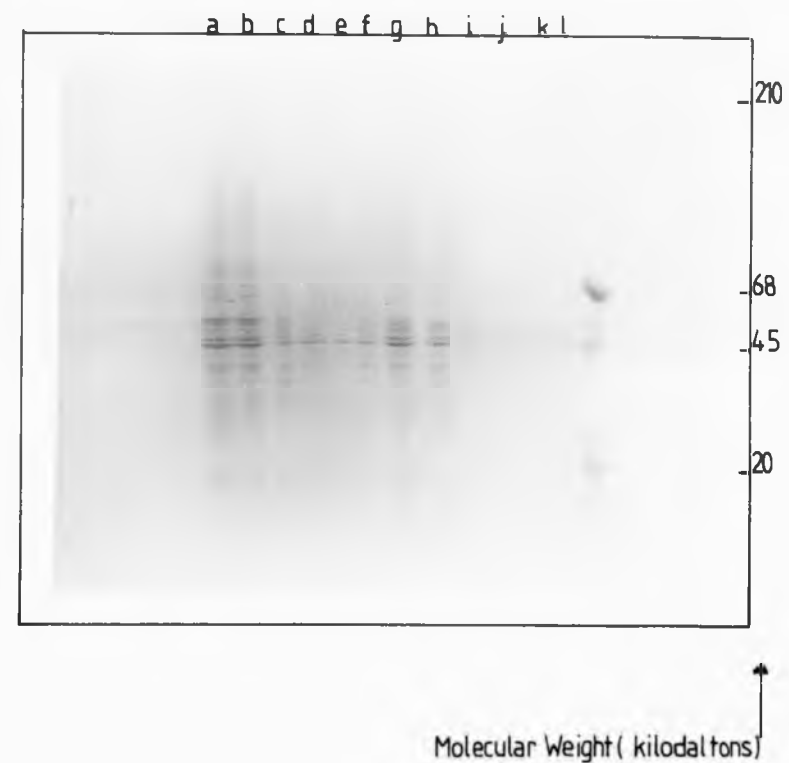
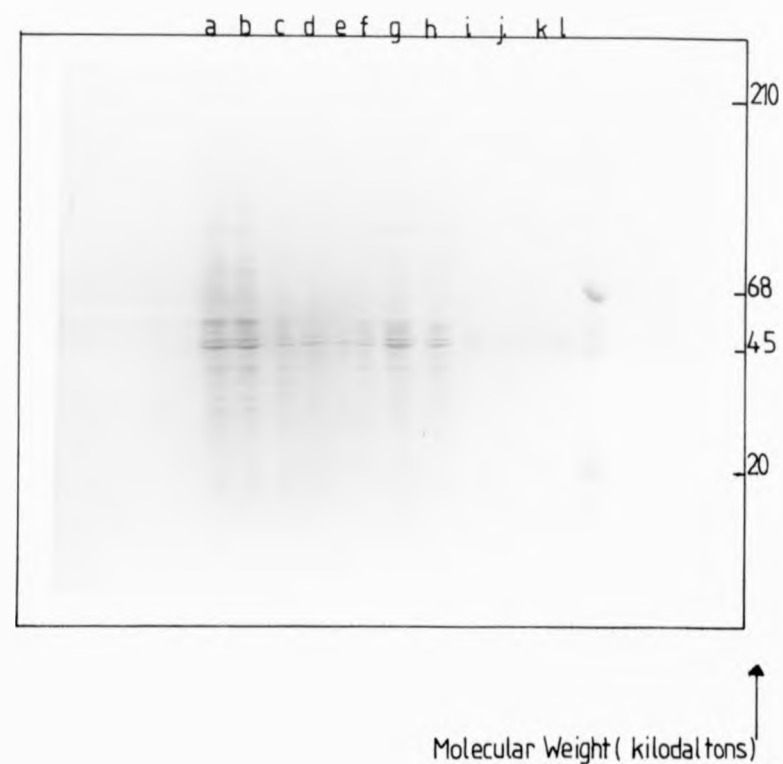


FIGURE 84. SDS-Polyacrylamide Gel Electrophoresis
of PCM3 Membrane Fractions Prepared from Cells Treated
with 0.05% Trypsin or from Untreated Cells

Method as described in Fig. 80.

Tracks a-f show samples in the absence of
 trypsin and g-l show samples in the presence of
 trypsin.

- | | |
|------|--|
| a, g | Supernatant obtained after homogenate
centrifuged at 700g for 10 minutes. |
| b, h | SI |
| c, i | PI |
| d, j | Sample obtained from upper layer following
sedimentation on 35% sucrose |
| e, k | SII |
| f, l | Plasma membranes. |



Although there appears to be several different sites possible for the action of proteases it is not possible to determine which if any, is operating in our cells. The action of proteases on adenylate cyclase is thought to be important physiologically since inhibition with protease inhibitors produces inhibited adenylate cyclase activity (McIlroy *et al.*, 1980; Friedman *et al.*, 1983). The higher basal levels of adenylate cyclase activity following homogenisation also appear to be due to the release of proteases since protease action on intact cells produces enhanced basal levels of adenylate cyclase activity.

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MATERIALS

APPENDIX I

Acrylamide	Bio-Rad
Acrylamide, bis	Bio-Rad
Adenosine triphosphate	Sigma
Adenylyl-imidodiphosphate	Boehringer Mannheim
Ammonium persulphate	Bio-Rad
Amphotericin B as fungizone	Squibb Ltd.
Benzyl Penicillin	Glaxo
Bovine Serum Albumin, fraction V	Sigma
Charcoal Norit SX-1	BDH
Creatine phosphokinase	Sigma
[8- ³ H]-cyclic adenosine mono-phosphate, 30-50 Ci/mmol	Amersham International
Dithiothreitol	Sigma
Dulbecco's minimal essential medium	Gibco
Eagle's minimal essential medium	Gibco
Ferritin	Sigma
Foetal Calf Serum	Gibco
Gentamycin	Sigma and Hope Hospital
Glutamine	Gibco
[γ - ³² P]Guanosine triphosphate, 35.9 Ci/mol	Amersham International
Guanylyl-imidotriphosphate	Boehringer Mannheim
N-2-hydroxy ethyl piperazine-N'-2-ethane sulphonic acid (HEPES) as free acid or sodium salt	Sigma
3-isobutyl-1-methyl xanthine	Sigma
Kanamycin	Sigma
β -mercaptoethanol	Koch-Light Laboratories
Newborn Calf Serum	Gibco

Ovalbumin	Sigma
PCS	BDH
Phosphocreatine	Sigma
Prostaglandin E ₁	Sigma
Readysolv EP	Beckman
Sodium deoxycholate	Sigma
Streptomycin BP	Glaxo
Sucrose	Sigma
N,N,N,N'-tetra-methyl-ethylene- diamine (TEMED)	Bio-Rad
Tris-HCl	Sigma
Trizma Base	Sigma
Trypsin	Difco
Trypsin inhibitor	Sigma

All other reagents used were of analytical grade and solutions were made in glass double distilled water.

A P P E N D I X I I

FUTURE WORK

1. Further investigation of the mechanism of action of GTP in producing enhanced cAMP accumulation of intact CH23 cells. It is important to determine whether the nucleotide actually enters the cell to exert its effect. This would be possible by incubating with [^{32}P]GTP followed by washing of the cells to remove any ^{32}P remaining in the extracellular medium. The cells would then be sonicated and a sample run on thin layer chromatography (tlc) plates to determine if [^{32}P] had entered the cell.

2. Determination of the nature of the cytosolic factor required in order for cholera toxin to inhibit GTPase activity of plasma membranes. Further purification of SI and SII supernatants by centrifugation, gel chromatography followed by SDS-polyacrylamide electrophoresis. In order for this to be feasible larger quantities of these supernatant fractions would be necessary. It would be more practicable to use P388 suspension cells for this purpose and then a greater number of cells could be obtained using large Spinner flasks.

3. Elucidation of the mode of action of proteases on the latent activation of adenylate cyclase activity.

(a) To quantify the protein bands on SDS-polyacrylamide gel electrophoresis obtained from membrane samples prepared in the presence and absence of trypsin and chymotrypsin. Large quantities of cells would need to be obtained and fractions obtained from the preparation of membranes. The same protein concentration of samples plus and minus trypsin and chymotrypsin could be run on SDS gel

electrophoresis and using the laser gel scanner to integrate the areas of the peaks obtained after Coomassie Blue staining the amount of various molecular weight components could be assessed. It would therefore be possible to assess the protein component(s) that may be altered following treatment of the cells with protease and membrane preparations.

(b) The effect of other groups of proteases on the formation of homogenates following treatment of the intact cells with protease should give precise information on the nature of protein cleavage required for latent activation to occur.

(c) To determine if trypsin action proceeds via suppression of the inhibitory guanine nucleotide regulatory component of adenylate cyclase (N_i) the effect of pertussis toxin (islet activating protein) on the action of trypsin could be studied. Pertussis toxin exerts its action via N_i therefore it would be interesting to see whether trypsin can exert its action in the presence of pertussis toxin and if so whether the effect is additive or not.

4. To study the effect of pertussis toxin on both cAMP accumulation of intact cells and adenylate cyclase activity of broken preparations of all three cell lines in the presence and absence of PGE_1 . This should give information on whether the inhibitory regulatory component of adenylate cyclase activity plays any role in the differences in enhancement of cAMP levels by PGE_1 of the three cell lines.