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PROTEIN KINASE C (PKC) AMPLIFIES THE HORMONAL STIMULATION OF
ADENYLATE CYCLASE IN T51B RAT LIVER CELLS.

by

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Thesis submitted to the Department of Biochemistry in partial fulfilment of the
requirements for the degree of Masters of Science.

University of Ottawa
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ABSTRACT

Membrane signalling is a process that is fundamental to the response of cells to extracellular stimuli. Two major pathways are used to generate intracellular "second messengers" in response to hormonal stimulation. They are the production of cAMP and hydrolysis of membrane phospholipids to produce inositol-1,4,5-trisphosphate and diacylglycerol. Recent evidence indicates that these two membrane signalling systems interact with one another.

Activation of PKC by phorbol esters (TPA) results in a modification of the cAMP system leading to either attenuation or amplification of the cyclic AMP signal. It has been shown that TPA inhibits glucagon-stimulated adenylate cyclase activity in rat hepatocytes. In the non-neoplastic T51B rat liver cell line, TPA, when added to intact cells, had no effect on the basal level of cyclic AMP synthesis but caused a 1.5 fold amplification of the stimulation induced by β -adrenergic agents, cholera toxin and forskolin. The same concentration of TPA also caused translocation of PKC from the cytosolic fraction. The time course for the translocation is longer than the time course found for the enhancement of adenylate cyclase activity. The effect of TPA treatment seen in this tissue culture system appears to be activation of membrane associated PKC rather than translocation. Activation of PKC already present in the membrane is likely to cause a change in adenylate cyclase activity.

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ABBREVIATIONS.

ARF	-	ADP-ribosylation factor.
ATP	-	adenosine triphosphate.
BCS	-	bovine calf serum.
C	-	the catalytic subunit of adenylate cyclase.
cAMP	-	adenosine 3',5'-monophosphate.
C.T.	-	cholera toxin.
DEAE	-	diethylaminoethyl.
DiC8	-	1,2-dioctanoylglycerol.
DMEM	-	Dulbecco's minimal essential medium.
DMSO	-	dimethylsulfoxide.
EDTA	-	ethylenediaminetetraacetic acid.
EGF	-	epidermal growth factor.
EGTA	-	ethylenebis(oxyethylenitrilo)-tetraacetic acid.
EPI	-	epinephrine.
FSK	-	forskolin.
G _i	-	inhibitory G-protein of the adenylate cyclase system.
G _s	-	stimulatory G-protein of the adenylate cyclase system.
GABA	-	neurotransmitter /gamma-amino-butyrac acid/.
GDP	-	guanosine diphosphate.
GTP	-	guanosine triphosphate.
GTP _γ S	-	guanosine 5'-O-(3-thiotriphosphate).
HEPES	-	N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid.

IP ₃	-	inositol 1,4,5-trisphosphate.
IPR	-	isoproterenol.
MIX	-	1-methyl-3-isobutyl-xanthine.
NAD	-	nicotinamide adenine dinucleotide.
NRK	-	a normal rat kidney cell line.
NOREPI	-	norepinephrine.
NP40	-	Nonidet P-40.
OAG	-	oleyl-acetyl-glycerol.
PBS	-	phosphate buffered saline solution.
PDBu	-	phorbol dibutyrate.
4 α PDD	-	4- α -phorbol-12,13-didecanoate.
PDE	-	cyclic nucleotide phosphodiesterase.
PGE ₁	-	prostaglandin E ₁ .
PHENEPI	-	phenylephrine
PI	-	phosphoinositide
PIP ₂	-	phosphatidylinositol 4,5-bisphosphate.
PKC	-	protein kinase C.
PLC	-	phospholipase C.
PMSF	-	phenylmethylsulfonyl fluoride
PRO	-	propranolol.
PS	-	phosphatidylserine.
R _i	-	inhibitory receptor.
R _s	-	stimulatory receptor.
S49	-	a lymphoma cell line.

- SDS - sodium dodecyl sulfate.
- T51B - a rat liver epithelial cell line.
- TCA - trichloroacetic acid
- TPA - 12-O-tetradecanoyl-phorbol-13-acetate.

TABLE OF CONTENTS.

Abstract	i
Acknowledgement	ii
Abbreviations	iii
Table of contents	vi
List of figures and tables	x
Objective	xii
1. Introduction.	1
1.1 Overview.	1
1.1.1 Discovery of G-proteins.	2
1.1.2 Adenylate cyclase system.	3
1.1.2.1 G-proteins controlling adenylate cyclase.	3
1.1.2.2 Stimulatory G-protein (G_s). The α - subunit.	4
1.1.2.3 Inhibitory G-protein (G_i). The α -subunit.	5
1.1.2.4 The β -subunit of G-proteins.	5
1.1.2.5 The γ -subunit of G-proteins.	6
1.1.3 Regulation of adenylate cyclase activity	6
1.1.3.1 Stimulation of adenylate cyclase through G_s	6
1.1.3.2 Inhibition of adenylate cyclase through G_i	8
1.1.3.3 Deactivation of the G-proteins.	8

1.1.4 The S49 lymphoma cell line, a useful tool. . . .	9
1.1.5 The catalytic subunit of adenylate cyclase.	9
1.1.5.1 Direct activation of adenylate cyclase.	10
1.1.6 The dissociation model for hormonal stimulation of adenylate cyclase.	11
1.1.7 An alternative model for adenylate cyclase activation.	11
1.1.8 Cross reaction between cyclic AMP and PI systems.	12
1.1.9 Activation of phospholipase C.	13
1.1.10 Protein kinase C.	13
1.1.10.1 Activation of protein kinase C.	14
1.1.10.2 Translocation of protein kinase C.	15
1.2 Cross-talk between the two second messenger systems.	16
1.2.1 Effect of cAMP levels on PKC.	16
1.2.2 Effect of PKC on the adenylate cyclase system.	16
1.3 The T51B cell line.	17
1.4 The tsK-NRK and n-NRK cell lines.	18
2. Methods	19
2.1 Cell culture.	19
2.2 Adenylate cyclase assay.	19
2.3 Cyclic AMP synthesis.	20
2.4 Pertussis toxin treatment.	21

2.5 Protein kinase C assay.	22
2.6 <i>In situ</i> gel assay of protein kinase C activity.	23
2.7 PDBu binding assay.	23
2.8 Statistical analysis	24
2.8.1 Adenylate cyclase assay (<i>in vitro</i>)	24
2.8.2 Cyclic AMP synthesis (<i>in vivo</i>)	25
2.8.3 Protein kinase C assay	26
3. Results	27
3.1 Adenylate cyclase assay (<i>in vitro</i>).	27
3.1.1 Initial characterization using n-NRK cells.	27
3.1.2 Effect of <i>in vitro</i> TPA treatment on adenylate cyclase activity.	29
3.1.3 T51B cell/ adenylate cyclase activity (<i>in vitro</i>).	37
3.2 Effect of TPA treatment on cyclic AMP synthesis.	39
3.2.1 Effect of TPA treatment on β -adrenergic stimulation (<i>in vivo</i>).	39
3.2.2 Phorbol ester specificity.	47
3.2.3 Effect of TPA treatment on forskolin and cholera toxin stimulation.	49
3.3 Effect of pertussis toxin treatment on adenylate cyclase activity.	51
3.4 Effect of TPA on PKC activity.	54
3.4.1 Soluble PKC activity.	54

3.4.2 Membrane PKC activity.	56
3.4.2.1 <i>In vitro</i> PKC assay.	56
3.4.2.2 <i>In situ</i> protein kinase gel assay.	58
3.4.3 PDBu binding assay.	58
3.5 Alternative protein kinase C assay.	58
4. Discussion.	61
4.1 Adenylate cyclase activity (<i>in vitro</i>).	61
4.1.1 Effect of <i>in vitro</i> TPA treatment on adenylate cyclase activity.	62
4.1.2 T51B cell/adenylate cyclase activity (<i>in vitro</i>).	64
4.1.3 Effect of <i>in vivo</i> treatment on cyclic AMP synthesis.	65
4.1.3.1 TPA effect on β -adrenergic stimulation (<i>in vivo</i>).	67
4.1.3.2 Various effects of TPA.	67
4.1.3.3 Phorbol ester specificity.	70
4.1.3.4 TPA effect on PKC.	71
4.1.3.5 Membrane PKC activity.	72
4.2 Conclusion and future directions.	73
5. Reference	75
Curriculum vitae	86

LIST OF FIGURES AND TABLES.

Fig. 1:	The adenylate cyclase system.	4
Fig. 2:	Activation of the regulatory G-proteins, G _s and G _i	7
Fig. 3:	GTP dose response in n-NRK cells in the presence of isoproterenol (IPR) <i>in vitro</i>	28
Fig. 4:	Effect of <i>in vitro</i> TPA pretreatment on adenylate cyclase dose response to GTP γ S in tsK-NRK cells at 41°C	30
Fig. 5:	Effect of <i>in vitro</i> TPA pretreatment on stimulation of adenylate cyclase by GTP γ S and forskolin.	32
Fig. 6:	Effect of different phorbol ester and diacylglycerols treatment of homogenate on adenylate cyclase activity <i>in vitro</i>	33
Fig. 7:	TPA dose response for pretreatment of homogenate followed by <i>in vitro</i> adenylate cyclase assay on membranes.	35
Fig. 8:	TPA time course for pretreatment of homogenate followed by <i>in vitro</i> adenylate cyclase assay on membranes.	36
Fig. 9:	Effect of various agents on adenylate cyclase activity in T51B cells <i>in vitro</i>	38
Fig. 10:	Time course of cyclic AMP synthesis <i>in vivo</i> in response to isoproterenol (IPR) in the absence and presence of TPA.	40
Fig. 11:	Time course for TPA stimulation of cyclic AMP synthesis <i>in vivo</i> in the absence and presence of IPR.	42

Fig. 12: Dose response to TPA in the absence or presence of isoproterenol (IPR).	44
Fig. 13: Dose response to IPR in the absence or presence of TPA.	45
Fig. 14: Effect of different phorbol esters and diacylglycerol on IPR-stimulated cyclic AMP synthesis <i>in vivo</i>	48
Fig. 15: Time course of cyclic AMP synthesis <i>in vivo</i> in response to cholera toxin.	50
Fig. 16: Effect of pertussis toxin treatment on T51B cells.	53
Fig. 17: Effect of TPA on the soluble PKC activity.	55
Table 1: Effect of various adrenergic agents on cyclic AMP synthesis <i>in vivo</i> in the absence or presence of TPA.	46
Table 2: Effect of forskolin and cholera toxin on cyclic AMP synthesis <i>in vivo</i> in the absence or presence of TPA.	52
Table 3: Effect of TPA treatment on membrane PKC activity.	57
Table 4: PDBu binding assay on different fractions of the T51B cells.. . . .	59

OBJECTIVE

Regenerating liver represents a system for studying normal cellular growth. It has been shown that Ca^{2+} and cyclic AMP play a role in regulating the regenerative process in rat liver. The T51B rat liver cell line has proven to be useful as a tissue culture model for regenerating liver (1).

Two separate cAMP surges have been found to occur in the liver remnant after partial hepatectomy (HPX). The peak of the second cAMP surge coincides with the initiation of DNA synthesis. A similar cAMP surge occurs in serum-starved T51B cells that have been stimulated to resume cycling by addition of fresh serum (1).

Parathyroidectomy (PTX) rapidly lower the rat's plasma calcium concentration causing a reduction of the hepatocytes proliferative response to HPX. Similarly T51B cells do not initiate DNA synthesis in low calcium (0.02mM) culture medium. However addition of a protein kinase C activator, the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA), enable the cells to initiate DNA synthesis.

The goal of this project was to study the relationship between PKC activation and adenylate cyclase activity. This investigation was mainly performed using the T51B rat liver cell line (2).

1. INTRODUCTION

1.1 Overview.

The regulation of cellular functions by hormonal factors acting via membrane bound receptors is triggered by specific signal transduction systems. These pathways, which include systems for neurotransmitters, peptide hormones and a large variety of other hormones, may be classified into four major groups:

- 1) Receptors which are ion channels inducing flux of specific cations or anions (examples are nicotinic acid, acetylcholine and GABA_A receptors).
- 2) Receptors which are tyrosine kinases and are assumed, at least in part, to act on the basis of this property (examples are receptors for insulin and various growth factors (EGF)).
- 3) Receptors which induce formation of inositol trisphosphate and 1,2-diacylglycerol from phosphoinositides in the plasma membrane by activation of phospholipase C (examples are α_1 -adrenergic and vasopressin receptors).
- 4) Receptors which stimulate the formation of cyclic AMP by adenylate cyclase (examples are β -adrenergic, glucagon and prostaglandin E₁ receptors).

The last two pathways mentioned above will be discussed in more detail.

1.1.1 Discovery of G-proteins.

The two major pathways generating "second messengers" are the adenylate cyclase and the phosphoinositide system. Both of these are stimulated by specific signal transduction pathways located in the plasma membrane and they both have a guanine nucleotide binding protein (G-protein) playing a central role.

The first indications of involvement of a G-protein came from studies of adenylate cyclase by Rodbell and colleagues (3,4). They demonstrated that GTP was required for glucagon activation of adenylate cyclase *in vitro* in rat hepatocytes and adipocytes. In addition, they showed that GTP enhanced the dissociation rate of radiolabelled glucagon from its receptor (5). Similar results were found with β -adrenergic receptors in erythrocytes. Using different β -adrenergic ligands it was demonstrated that GTP specifically decreases the affinity of β -adrenergic agonists for the receptor while having no effect on the affinities of antagonists (6-9). Finally it was established that the ligand binding site was different from the catalytic subunit of adenylate cyclase (10,11).

The initial reports suggested that the function of the hormone-receptor complex was to facilitate activation of adenylate cyclase by guanine nucleotides (12-14). These experiments also demonstrated a strict requirement for a divalent cation in addition to the metal-ATP substrate.

The hormonal inhibition of adenylate cyclase was studied also by Rodbell and colleagues (4,15). They reported that GTP could act bifunctionally on adenylate cyclase.

In the absence of stimulatory hormones, guanine nucleotides cause inhibition of adenylate cyclase depending upon the divalent metal ion concentrations present. Inhibitory hormones have now been identified that cause a 30-50% decrease in adenylate cyclase activity.

1.1.2 Adenylate cyclase system.

Of the membrane signalling systems, the hormone sensitive adenylate cyclase system is best understood. It consists of at least three distinct protein components: 1) the receptor; 2) the transducer (the guanine-nucleotide binding protein, G-protein); 3) the catalytic subunit, which catalyses the formation of cyclic AMP from ATP.

The cascade begins with binding of ligand to its receptor at the cell membrane surface which increases its affinity for the transducer. Depending on the receptor type (stimulatory (R_s) or inhibitory (R_i)) different G-proteins are activated (16). There is no known example where one receptor type has been shown to be both stimulatory and inhibitory (17). Figure 1 shows the sequence leading to hormonal activation of adenylate cyclase as proposed above.

1.1.2.1 G-proteins controlling adenylate cyclase.

The two G-proteins that regulate adenylate cyclase are part of a large family of GTP binding proteins which serve as transducers of external signals. These proteins are heterotrimers consisting of the larger α -subunit and the two smaller β and γ subunits (19,21).

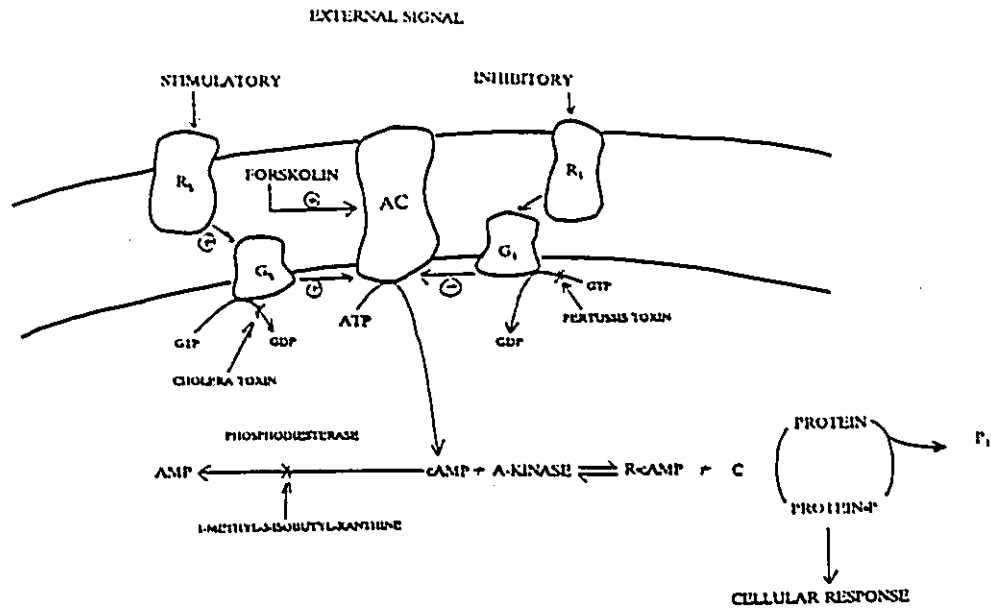


FIGURE 1: Proposed model of the cAMP pathway. Signals are transmitted from the stimulatory receptors (R_s) and the inhibitory receptors (R_i) through the stimulatory and inhibitory G-proteins (G_s and G_i) to the amplifier enzyme adenylate cyclase (AC).

1.1.2.2 Stimulatory G-protein (G_s). The α-subunit.

The stimulatory G-protein (G_s) of adenylate cyclase has two distinct forms of the α subunit (45 kDa and 52 kDa, respectively). Detailed genetic analysis has suggested that the 52 kDa α-subunit is a precursor for the 45 kDa species created by post-translational modifications (19). The α-subunit can bind both GDP and GTP with high affinity and it possesses an intrinsic GTPase activity that hydrolyses the bound GTP to GDP. The α-subunits also contain a site for NAD-dependent ADP-ribosylation catalyzed by cholera

toxin in the presence of a membrane-bound protein cofactor (ARF) (20,21). The ADP-ribosylation by cholera toxin results in inhibition of the GTPase activity of α_s and converts GTP into a superactive nucleotide, like its non-hydrolysable analogs, leaving α_s in a permanently activated state (16).

1.1.2.3 Inhibitory G-protein (G_i). The α_i -subunit.

The α_i -subunit of adenylate cyclase is very similar to α_s but smaller (41 kDa). It can be ADP-ribosylated by pertussis toxin leading to a decrease in the hormonal stimulation of the G_i -dependent hydrolysis of GTP. It is currently believed that rather than blocking the GTP to GDP conversion, ADP-ribosylation blocks the formation of the G_i -GTP complex. This is likely due to a decrease in the dissociation giving free α_i plus GDP and/or inhibition of the interaction of G_i with an R_i -type receptor (16).

1.1.2.4 The β -subunit of G-proteins.

The β -subunits of all the different G-proteins known today are very similar if not identical (16). Two distinct β -subunits exist (35 and 36 kDa respectively). They have been reported to have identical peptide maps but antibodies prepared against β -36 from transducin (the G-protein regulating retinal rod cGMP phosphodiesterase) show a marked specificity for the β -36 band of G_s and G_i and do not react at all with β -35 (19). Preliminary characterization of their biochemical activity has suggested that β -36 possesses a higher affinity than β -35 for the α_s subunit. Whether these are differences in

the primary structure or a result of post-translational modifications of the same gene product await more detailed analysis (19).

1.1.2.5 The γ -subunit of G-proteins.

The γ -subunits are the smallest of the subunits in the G- proteins (8-10 kDa). Very little is currently known about them due to difficulty in separation of β - and γ -subunits without denaturation of β (19). In other words, it has not yet been possible to obtain purified samples of β and γ separately. All that is currently known is that γ differ from G-protein to G- protein. They are hydrophilic, do not stain very well with dyes and do not fix well at acid pH (22). It has been suggested that γ could regulate the specificity of the β -subunit for α but for now this is all speculation (19).

1.1.3 Regulation of adenylate cyclase activity.

There has been some controversy about the exact mechanism of activation and inhibition of adenylate cyclase. The mechanism described in the following section is the most widely accepted today.

1.1.3.1 Stimulation of adenylate cyclase through G_s .

The stimulation of adenylate cyclase is initiated by binding of a stimulatory hormone to its receptor. This hormone-receptor complex (HR) increases the steady state levels of active G_s by accelerating the 'activation reaction'. This includes acting as a

nucleotide exchange factor by diminishing the relative affinity of G_s for GDP, allowing it to bind GTP (23). It also increases G_s 's affinity for Mg^{2+} so it becomes fully saturated by the divalent cation at physiological concentrations (16).

Studies of pure G_s in the presence of non-hydrolysable GTP- analogs have shown that activation is a multi-step event. First G_s interacts reversibly with the nucleotide. Second the G_s - nucleotide complex isomerizes in a Mg^{2+} -dependent manner to give a complex from which the nucleotide does not dissociate on dilution. Finally the G_s - nucleotide undergoes a temperature-dependent subunit dissociation reaction to give a α_s -GTP-complex and a $\beta\gamma$ -complex (24). Figure 2 shows the sequence leading to G_s activation as proposed above.

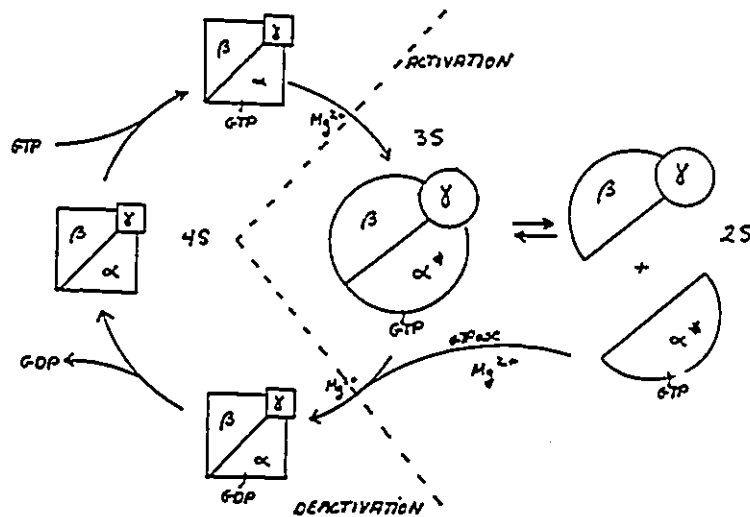


FIGURE 2: Current working hypothesis of the regulatory cycle of G_i and G_s . In this representation the cycle has been subdivided into four steps: association of GTP, activation of the G-protein, hydrolysis of GTP accompanied by deactivation of G, and dissociation of GDP. The angular shaped figures symbolizes inactive components or low affinity forms and the rounded shapes are active components or high affinity forms (revised after 16).

It is believed that the activated α_s interacts with the catalytic subunit (C) leading to increased production of cAMP.

Although it has been shown that purified G_s and G_i interact with non-hydrolysable GTP-analogs in solution and the interaction results in dissociation of α and $\beta\gamma$ subunits as proposed above, it has not yet been shown that dissociation occurs *in vivo* (16,25). It is not clear whether the subunit dissociation reaction: i) occurs with GTP; ii) is obligatory for stimulation of the catalytic subunit (C); or iii) happens at all within the normal phospholipid environment of the plasma membrane (16).

1.1.3.2 Inhibition of adenylate cyclase through G_i .

G_i resembles G_s not only structurally but also functionally. They both possess GTPase activity which is Mg^{2+} dependent. The Mg^{2+} affinity of G_i differs from that of G_s in that G_i is fully saturated under physiological conditions. The rate-limiting step of the kinetic regulatory cycle of G_i is currently thought to be the dissociation of GDP (16).

Most researchers today agree that the inhibitory and stimulatory pathways of adenylate cyclase are very similar. The inhibitory ligand binds to its receptor facilitating the exchange of GDP with GTP. The binding of GTP leads to dissociation of α_i from $\beta\gamma$ -subunit and interaction with the catalytic subunit lowering its activity. Since the concentration of G_i is about 10-fold that of G_s (19), the free $\beta\gamma$ subunits generated after the dissociation could contribute to the inhibition by binding to α_s , thereby significantly decreasing the concentration of free active α_s . Controversy exists today with respect to the contribution of the two pathways in modulating the hormonal signals (23).

1.1.3.3 Deactivation of the G-proteins.

The off-reaction of the G-proteins is thought to be due to the intrinsic GTPase activity of the α -subunit. This is often stimulated by the effector which is the catalytic subunit of adenylate cyclase. After the hydrolysis, the GDP bound α -subunit adopts a different three-dimensional structure leading to a lower affinity for its effector and a higher affinity for the $\beta\gamma$ -subunits. This leads to dissociation of GDP- α from the effector and reassociation with the $\beta\gamma$ -subunits returning the effector molecule to its inactive state (23,26).

1.1.4 The S49 lymphoma cell line, a useful tool.

The cyc- S49 cell system has been a very useful tool in the studies of adenylate cyclase. This cell type first appeared to be deficient in adenylate cyclase activity since no hormonal, GTP, cholera toxin or NaF response could be measured. However it was later shown by reconstitution experiments and by stimulation with Mn^{2+} and forskolin to contain the catalytic subunit (27). It was later shown that cyc- S49 cells lacked the substrate for ADP-ribosylation by Cholera toxin, i.e. the $G\alpha_s$ -subunit (28).

1.1.5 The catalytic subunit of adenylate cyclase.

The catalytic subunit of adenylate cyclase (C) has not been thoroughly studied. It appears to be a glycoprotein that spans the plasma membrane (17) with a molecular mass of about 150 kDa (16). The substrate for C is a metal-ATP complex, most probably

MgATP. The divalent cation is also required at an allosteric binding site for activity (17). The affinity of C for Mg^{2+} changes after association with activated G_s and G_i . While activated G_s increases the affinity of C for Mg^{2+} by up to 100- fold, hormone- or guanine nucleotide- activated G_i can completely reverse this increase in Mg^{2+} affinity of adenylate cyclase (29). The catalytic subunit can also be activated by Mn^{2+} ions in a similar fashion as with Mg^{2+} . One difference is that the binding site has about a 10- to 30-fold higher affinity for Mn^{2+} than for Mg^{2+} (29). The products formed by C are cyclic AMP and $MgPP_i$ (16,17). Kinetic studies using reconstitution of the cyc- S49 cell system have revealed that the interaction of activated G_s and activated G_i with C are of a non-competitive type (24).

1.1.5.1 Direct activation of adenylate cyclase.

The C-subunit by itself is relatively inactive but can be stimulated by activated α_s (30) or directly by Mn^{2+} or forskolin; a diterpene isolated from the plant *Coleus forskohlii* (16,17). The stimulation by forskolin has been observed in almost all hormone-sensitive adenylate cyclase systems studied so far. Its action is not dependent on active G_s as seen in the cyc- S49 cell system (16) but it is potentiated by activated G_s (17). Forskolin responsiveness appears to be dependent on a rapidly turning over protein of unknown identity (31). While it may be that forskolin acts by directly interacting with C, it seems its effect is dependent on the expression of other factors/components, G_s being one of them (32).

1.1.6 The dissociation model for hormonal stimulation of adenylate cyclase.

The model for adenylate cyclase stimulation and inhibition described above is called the dissociation model. There are a number of observations that do not support it and therefore call for modifications. One such alternative model has been put forward by A. Levitzki (25).

1.1.7 An alternative model for adenylate cyclase activation.

If all the different components R_s , R_i , G_s , G_i and C are physically separate units and dynamically interact with each other then the kinetics of activation will be complex. Experimental observations in both native membranes and in reconstituted systems have shown that activation of adenylate cyclase by hormone and guanine nucleotides follow first order kinetics. This supports the theory that adenylate cyclase is a complex of G_s and C.

Biochemical studies have also shown that it is very difficult to separate G_s from C. It requires a combination of detergent and high salt concentration. The complex is stable whether G_s is inactive (GDP-bound) or active (GMP-NH-P bound). This model can still accommodate the central point of the old G-protein activation system if it is assumed that α_s is always associated with C and that the $\beta\gamma$ subunits dissociate from the G_s -C complex leaving α_s -C behind as the active complex.

Experiments with cyc- S49 have shown that somatostatin is able to inhibit adenylate cyclase. This suggests that G_i directly interacts with C although no G_i -C complex has thus far been reported. This could be due to a weak protein-protein

interaction between G_i and C compared to the G_s -C interaction.

A new and revised model as proposed by A. Levitzki (25) for regulation of adenylate cyclase by G-proteins can be based on the following assumptions:

- 1) The G_s -protein does not dissociate as part of its mode of action.
- 2) The G_s -protein exhibits high affinity towards the catalytic subunit which it regulates, and remains associated with it at all times.
- 3) The complex G_s -C interacts with the stimulatory receptor R_s which, when bound with agonist, catalyses the GDP-GTP exchange on the G_s -C complex.
- 4) One receptor can catalyze the activation of many G_s -C units.
- 5) G_i interacts either directly with C or with G_s and confers inhibition on adenylate cyclase in a non-competitive manner with respect to G_s .

Whether this or the dissociation model describes the most correct mechanism for adenylate cyclase activation has to await future studies.

1.1.8 Cross reaction between cyclic AMP and PI systems.

There seems to be some overlap between agents mediating adenylate cyclase inhibition and those inducing phosphoinositide breakdown by activation of phospholipase C (PLC). Examples of this are vasopressin and α -adrenergic agents. (33-35). These agents increase the formation of inositolphosphates and 1,2-diacylglycerol in intact cells and inhibit adenylate cyclase in membrane preparations via activation of G_i . It has been

shown however that this dual regulation of agents is through different receptor subtypes (18).

1.1.9 Activation of phospholipase C.

In recent years it has become clear that G-protein(s) play an analogous role in hormonal stimulation of PLC as G_s and G_i do in regulating adenylate cyclase (36,37). Hormonal stimulation leads to the release of two second-messenger molecules (inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol) by the hydrolysis of membrane bound phosphatidylinositol 4,5-bisphosphate (PIP_2) by PLC. The release of water-soluble IP_3 causes a transient increase in the intracellular Ca^{2+} concentration, whereas the release of diacylglycerol activates PKC. The hormonal stimulation can be induced by GTP or by nonhydrolyzable GTP analogs and in some cases, but not all, this effect can be blocked by ADP-ribosylation with pertussis toxin. The specific G-protein involved here has been named G_p (not to be confused with the G-protein isolated from placenta and platelets of the same name). It has not yet been characterized.

1.1.10 Protein kinase C.

Protein Kinase C was discovered by Nishizuka and associates (38). This enzyme is found in virtually every tissue examined (39). It is a serine and threonine-specific Ca^{2+} -activated protein kinase that requires phospholipid for activity. The most efficient phospholipid is phosphatidylserine. Addition of diolein, an unsaturated diacyl-glycerol, increases the reaction velocity and apparent affinity for phospholipid and Ca^{2+} (40).

Recent molecular cloning analysis has shown that PKC is not a single enzyme but is composed of several isoenzymes. So far seven subspecies have been identified. They all show slightly different modes of activation, kinetic properties and substrate specificities (41). However too little is known at the moment about the specific differences of the different isozymes of PKC and in the following PKC will be taken to mean the group of isozymes unless otherwise specified.

1.1.10.1 Activation of protein kinase C.

In its resting state PKC appears to be inactive and loosely associated with the membrane. Extraction of the activity can be performed by eliminating Ca^{2+} using a media containing EDTA and EGTA. When stimulated, the kinase is translocated to the membrane where it can actively phosphorylate its substrates (42). The natural stimulation of PKC can be caused by diacylglycerol accumulation and Ca^{2+} mobilization under conditions where polyphospho-inositides are hydrolysed by phospholipase C. Diacylglycerides bind to PKC causing it to be translocated to the membrane. Phosphatidylserine (PS) present in the membrane and the physiological Ca^{2+} concentration then leads to activation of PKC. Extraction of this activated membrane bound PKC activity requires detergents.

Phorbol esters, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) have long been known to cause a wide variety of biological functions including tumor promotion and platelet aggregation. Phorbol ester derivatives showing tumor-promotion activity have been shown to activate PKC as well. Protein kinase C is now generally accepted to be a receptor of phorbol esters, although it is not known whether the activation of this

enzyme is directly related to the mechanism involved in tumor promotion. Kinetic analysis have indicated that TPA interacts at the same site as the natural activator diacylglycerol. TPA, like unsaturated diacylglycerol, greatly increases the apparent affinity of the enzyme for Ca^{2+} as well as for phospholipid. It also enhances the enzyme activation (39,40,43).

1.1.10.2 Translocation of protein kinase C.

Translocation of PKC seems to be a central regulatory mechanism. A variety of lymphocyte receptors have been shown to transduce signals via a mechanism involving translocation of PKC from the cytosol to the plasma membrane. This appears to be mediated by diacylglycerol liberation and Ca^{2+} mobilization as a result of ligand-induced polyphosphoinositide hydrolysis (44,45). Translocation of PKC to the nucleus has also been shown in B- lymphocytes. Stimulation of B cells with antibodies to B cell surface type II major histocompatibility molecules (anti-Ia antibodies) causes a rapid and transient translocation of PKC to the nucleus. It involves more than 80% of the total cellular PKC activity (45). Anti-Ia antibodies do not induce detectable changes in phosphoinositide metabolism or intracellular free calcium. It has been suggested that the translocation of PKC to the nucleus can be caused by an elevation of cellular cAMP levels (46).

A unique feature of PKC is its ability to behave as both a cytoplasmic and a membrane bound enzyme. PKC is normally not active in the cytoplasm but a specific protease-mediated cleavage can lead to an irreversible activation. The proteolytic cleavage produces two fragments. One fragment contains the catalytic fragment and the

other contains the regulatory domain. The catalytic fragment is active in the absence of the normal activators Ca^{2+} , diacylglycerol and phosphatidylserine (47).

1.2 Cross-talk between the two second-messenger systems.

Several lines of evidence suggest that the two signalling systems described above interact with one another. There have been reports both of effects of increased levels of cyclic AMP on PKC and effects of activated PKC on adenylate cyclase activity.

1.2.1 Effect of cAMP levels on PKC.

As described above, elevated cyclic AMP levels have been reported to cause translocation of PKC from the cytosolic fraction to the nucleus (45).

1.2.2 Effect of PKC on the adenylate cyclase system.

Recent studies have shown that activation of PKC by phorbol esters and diacylglycerols sensitizes the adenylate cyclase of some cells to stimulation by agents that act through G_s (48,49,50,51). However, the mechanism of this sensitization remains somewhat controversial. It has been shown that PKC phosphorylates the inhibitory guanine-nucleotide-binding regulatory component *in vitro*. This phosphorylation suppresses hormonal inhibition of adenylate cyclase and if it occurs *in vivo*, which has not yet been shown, it would switch off the inhibitory pathway of adenylate cyclase (52).

Another study, performed in frog erythrocytes, has shown that the catalytic

subunit of adenylate cyclase can be phosphorylated by PKC both *in vitro* and *in vivo* (53,54). This phosphorylation causes an enhancement of adenylate cyclase activity *in vitro*.

Phorbol ester treatment of human mononuclear leucocytes has been shown to cause β -adrenergic receptor uncoupling and a non specific desensitization of adenylate cyclase activity *in vitro* (55). The phorbol ester treatment reduces the response to agents activating the enzyme through different receptors and directly through G_s to the same degree but activation by forskolin is unaffected.

Phosphorylation of β -adrenergic receptors has been reported in both duck and turkey erythrocytes (56,57). This phosphorylation leads to adenylate cyclase desensitization in response to β -adrenergic agents.

1.3 The T51B cell line.

The main reason for the choice of the T51B cell line was that their growth seemed to be regulated by growth factors, Ca^{2+} , cyclic AMP and phospholipid signalling pathways. The T51B cell line is non-neoplastic and epitheloid. It was derived from the liver of a normal adult Fisher rat (58). Proliferation of these cells is tightly controlled by the Ca^{2+} concentration of the media. Cell proliferation in sparse cultures can be controlled simply by lowering the Ca^{2+} concentration in the media from 1.8 mM to 0.02 mM. This inhibition is shown by the inability to form colonies and the lowering of the fraction of cells actively synthesizing DNA (5-15% in 0.02 mM Ca^{2+} and 35-45% in 1.8 mM Ca^{2+}). Some of the cells are blocked at the G_1/S boundary while the remaining cells are blocked at the G_0/G_1 boundary. Adding Ca^{2+} back to the normal level will remove

both blocks allowing the cells to re-enter the cell cycle. Adding agents that elevate the intracellular cAMP level will remove only the G₁/S block. Agents that activate PKC have also been shown to remove the G₁/S block (59,60,61).

1.4 The tsK-NRK and n-NRK cell lines.

The n-NRK and tsK-NRK cell lines were generous gifts from Dr. E.M. Scolnick (Merck, Sharpe and Dhome, West Point, PA).

The n-NRK cell line is a clone of a Normal Rat Kidney cell line derived from healthy young adult Osborne-Mendel rats (62) and later further subcloned to contain only epithelioid cells (63). The tsK-NRK cells were derived by infecting n-NRK cells with a temperature-sensitive, transformation-defective mutant (ts 371) of Kirsten murine sarcoma virus (KiMSV) (64). This mutant virus produces an abnormally thermolabile p21 *Ki-ras* protein that is active at 36°C but inactive at 41°C. NRK cells infected with this virus behave as transformed cells at the permissive temperature (36°C) and proliferate in the absence of serum. At a non-permissive *Ki-ras* inactivating temperature such as 41°C these cells behave as untransformed and can not proliferate in the absence of serum. Reactivating the p21 *Ki-ras* protein by shifting the temperature from 41°C to 36°C is as effective as adding serum and permits them to start proliferation despite the lack of serum. In the following experiments the tsK-NRK cell line was used to investigate the effect of TPA on adenylate cyclase activity in whole cells. The reason for using them was that the response of adenylate cyclase to GTP and GTP analogs had already been characterized in these cells. tsK-NRK cells were only used at 41°C to eliminate the possible effects of *K-ras* on adenylate cyclase activity.

2. METHODS

2.1 Cell culture.

tsK-NRK and n-NRK cells were cultured in a medium consisting of 15% (v/v) BCS (bovine calf serum from Colorado Serum Co., Denver) and 85% (v/v) DMEM (Dulbecco's minimal essential medium) containing gentamycin and maintained at 36°C in a humid atmosphere of 95% air and 5% CO₂. Prior to experiments, cells were detached by brief exposure to 0.25% (v/v) trypsin in phosphate buffered saline (PBS). They were then plated in 60 mm or 100 mm dishes in 5ml or 10ml DMEM + 15% BCS respectively at an initial density of 0.5 - 0.75 x 10³ cells/cm² and incubated at 40°C. After 72 hours cells were arrested at G₀/G₁ by incubation at 41°C for 48 hours in DMEM/Ham's F12 (1:1) containing 10mM HEPES (pH 7.2) and 0.2% BCS.

T51B rat liver epithelial cells were cultured in a medium consisting of 15% (v/v) BCS and 85% (v/v) DMEM as described above. The cells were plated in 60mm dishes in 5ml DMEM + 15% serum and incubated at 36°C to confluency.

2.2 Adenylate cyclase assay.

Cultures were washed twice with PBS and the cells scraped off the dish in 2 ml of PBS. The cells were sedimented by centrifugation at 500 x g for 3 min., washed with a further 5 ml of PBS and centrifuged again. The pellet was frozen at -80°C. The thawed pellets were homogenized in 0.5 ml of buffer (50mM Tris/HCl [pH 7.4], 330 mM sucrose, 1mM MgCl₂, and 1 mM dithiothreitol) in a motor-driven

teflon/glass homogenizer (10 strokes, 10,000 rpm). The homogenate was centrifuged at 20,000 x g for 20 min., the supernatant fluid discarded and the pellet dispersed in a small volume of buffer. This membrane preparation contained adenylate cyclase, the activity of which was determined from the conversion of [α - 32 P]-ATP to [32 P]-cyclic AMP as described (65). The enzyme reaction was carried out in triplicate.

In vitro TPA treatment prior to *in vitro* adenylate cyclase assay was performed on whole cell homogenate. Cells were homogenized as above. To the homogenate was added leupeptin (10 μ g/ml), Ca^{2+} (0.75 mM), Mg^{2+} (10 mM), ATP (100 μ M), PS (50 μ g/ml) and TPA in the concentrations indicated. The samples were incubated for 10 minutes at 30°C. Membranes were isolated and assayed for adenylate cyclase activity as above.

2.3 Cyclic AMP synthesis.

The intracellular ATP pool was labelled by the addition of 0.125 μ Ci 2-8 3 H-adenine (from ICN, Irvin, CA) to the medium of confluent cultures for 16 hours. The culture medium was changed 2 hours before the start of each experiment into medium consisting of 49.9% (v/v) DMEM medium, 49.9% (v/v) HAM's F12 medium and 0.2% (v/v) BCS which was buffered at pH 7.2 with 10 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid). In all experiments, 1-methyl-3-isobutyl-xanthine (MIX, from Sigma Chemical Co.) was added to the cultures to prevent the degradation of newly synthesized cyclic AMP by cyclic nucleotide phosphodiesterase (PDE). Experiments were terminated by washing the cells rapidly with cold PBS and then lysing them in 0.25ml of lysis buffer containing 150mM NaCl, 10mM Na_2HPO_4 ,

1mM EDTA, 0.1mM cyclic AMP, 0.1mM MIX, 1% Nonidet P-40 and 0.001 μ Ci [14 C] cyclic AMP to monitor [3 H] cyclic AMP recovery. The culture dishes were rinsed with another 0.25 ml of lysis buffer and the combined lysate centrifuged at 1500 x g for 10 minutes to remove debris. Labelled nucleotides were separated by ion exchange chromatography as described by Franks et al. (66). The labelled ATP pool remained constant throughout the experimental period with an average radioactivity of 200000 cpm/mg protein. Replicate cultures were used to determine the cellular protein content by the method of Bradford (67).

2.4 Pertussis toxin treatment.

Treatment of whole cells with pertussis toxin was done by adding the toxin directly to the medium (25 ng/ml).

For the *in vitro* treatment, the dishes were washed twice with PBS, harvested and homogenized by vortexing in 1.5 ml NaHCO₃/MgCl₂ (1mM/5mM) buffer. Membranes were isolated by centrifugation (40000 x g, 30 minutes) and the pellet was frozen at -80°C. The membrane pellet was resuspended in 30 μ l reaction buffer (thymidine 50 mM, creatine-phosphate 25 nM /creatine phosphate kinase 3 μ g, GTP 0.3 mM, DTT 1.5 mM). Activated pertussis toxin was added and the reaction was started by addition of 32 P-NAD (5 μ Ci/tube). Samples were incubated for 30 minutes at 37°C. To stop the reaction 1 ml of the NaHCO₃/MgCl₂ buffer was added and the samples were centrifuged as above. The pellet was solubilized in 40 μ l buffer containing Tris/HCl 62.5 mM, glycerol 10%, mercaptoethanol 5%, SDS 1% and bromophenol blue 0.02%. The samples were analyzed using SDS-PAGE gel electrophoresis. The separating gel contained 12%

acrylamide and 1% SDS. Autoradiography was performed with Cronex Y-4 DC X-ray film.

2.5 Protein kinase C assay.

The membrane associated PKC activity was extracted from the membranes with a nonionic detergent (Nonidet P-40). The cells were homogenized as above and the membranes were isolated by centrifugation (100000 x g at 4°C for 30 min). The pellet was resuspended in homogenizing buffer with 1% NP-40 and the samples were left for 30 minutes on ice. Extraction of the detergent was done using 1.2 ml DEAE-Sephacel columns by eluting with 10 ml scraping buffer without detergent. The PKC enriched fraction was eluted with scraping buffer containing 75 mM NaCl. Activity eluted between 0.75-1.75 ml was used for determining the activity in the membrane fraction. The assay was performed as described below .

Soluble protein kinase C activity was extracted in a buffer containing 20 mM Tris/HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 50 mM 2-mercaptoethanol and 100 µg/ml leupeptin. The dishes were rinsed twice with cold PBS and the cells were scraped in the buffer mentioned above. The samples were centrifuged (100000 x g at 4°C for 30 minutes) and protein kinase C activity was measured in the supernatant.

Protein kinase C activity was measured as described previously (68). The assay mixture (270 µl final volume) contained 5 µmoles Tris-HCl (pH 7.5), 1.25 µmoles MgCl₂, 50 µg Histone H1 (calf thymus type V-S, Sigma Chemical Co.), 2.5 nmoles [γ -³²P]-ATP (2 x 10⁵ cpm/nmole, from ICN) and 0.27 µmole CaCl₂. The reaction was allowed to proceed in the presence or absence of 10 µg phosphatidylserine (PS) and TPA (Sigma

Chemical Co.) for 10 min. at 30°C and was stopped by adding 2 ml 20% TCA and 50 µg bovine serum albumin. The pellet was reprecipitated and counted. PKC activity was calculated as the difference between the pmoles of ³²P incorporated into Histone H1 per mg protein per minute in the presence and absence of PS and TPA.

2.6 In Situ gel assay of protein kinase C activity.

Membrane protein kinase C activity was assayed using an *in situ* gel assay as described earlier (69). PKC was extracted as described above and the samples were run on a Laemmli gel under non-denaturing conditions. Following electrophoresis, the gels were soaked on ice in a Tris/HCl buffer (50 mM, pH 7.5) for one hour with one change of buffer. For phosphorylation the gel was incubated for 30 minutes at 37°C in a buffer (Tris-acetate 30 mM pH 7.4, Mg-acetate 4 mM, NaF 10 mM, sodium phosphate 0.1 mM pH 7.4, EGTA 0.3 mM and histone II-AS 2mg/ml). After the 30 minute incubation 2 µCi/ml of γ -[³²P]ATP (1.6 µM) was added and incubation was continued for 30 minutes at 37°C. The reaction was terminated by removal of the incubation mixture and rinsing with 10% TCA/ 1% phosphoric acid. The gel was soaked overnight and rinsed twice with the TCA solution (1 1/2 hour each). After a 1 hour incubation of the gel in 30% MeOH/ 10% HAc the gel was dried and autoradiographed.

2.7 PDBu binding assay.

The PDBu binding assay was performed as described (70). Cells were washed twice in PBS buffer, scraped off and sonicated in a Tris/HCl:Mg-buffer (20 mM:1 mM).

Part of this homogenate was used as whole cell homogenate and the rest was separated by centrifugation (24500 x g, 20 min. at 4°C) into the cytosolic and the membrane fractions. The pellet was resuspended and the different fractions were incubated with ³H-PDBu in a solution containing Tris/HCl 20 mM, MgCl₂ 1 mM, leupeptin 10 µg/ml, KCl 100 mM, CaCl₂ 0.15 mM, EGTA 50 µM, 0.5% DMSO and PS 0.1 µg/µl. The samples were incubated for 20 minutes at 30°C. To stop the reaction, 0.5% cold DMSO was added and passed through glass-fibre filters presoaked in 0.3% polyethylenimine. The filters were washed three times with cold 0.5% DMSO, dried and counted. The PDBu binding was calculated as the difference between the fmols of ³H-PDBU bound per mg protein in the presence and absence of TPA.

2.8. Statistical analysis.

The statistical analysis used in this project is the two-tailed paired or independent t-test as indicated. The p-level for accepting significant difference was chosen to be p<0.05.

2.8.1 Adenylate cyclase assay (*in vitro*).

The statistical analysis used for this series of experiments was the two-tailed paired t-test comparing the mean of adenylate cyclase activity after pretreatment with or without TPA. The paired test was chosen for these experiments since each experiment was performed on one pair of dishes where one was treated with vehicle and the other was treated with TPA for the times and concentrations indicated.

2.8.2 Cyclic AMP synthesis (*in vivo*).

In this series of experiments the statistical analysis used was the two-tailed independent t-test comparing the treated with the untreated samples.

Time course and dose response experiments were performed with one determination per time point. However, in each time course and dose response experiment several different experiments were combined and a mean of the fold stimulation above basal cAMP production was determined. The conversion ratio was expressed as $10^3 \times \text{cpm } [^3\text{H}]\text{-cAMP} / \text{cpm } [^3\text{H}]\text{-ATP} / \text{mg protein}$ and the fold stimulation was calculated as (conversion ratio of stimulated sample)/(conversion ratio at basal level). The difference of the fold stimulation between treated and untreated samples was calculated and presented as a mean \pm Standard Error. The statistical analysis used was the two-tailed independent t-test comparing the means of the difference in fold stimulation. The reason for choosing the independent t-test in this case is that the mean of the difference due to TPA treatment was not paired other but contained different numbers of determinations.

Other results in this series of experiments (fig. 14 and tabl.1 and 2) are expressed as the conversion ratio ($10^3 \times \text{cpm } [^3\text{H}]\text{-cAMP} / \text{cpm } [^3\text{H}]\text{-ATP} / \text{mg protein}$). The statistical analysis performed on these experiments was the two-tailed independent t-test. The independent t-test was chosen since the experiments were performed with groups of three dishes that were not paired before the experiment.

2.8.3 Protein kinase C assay.

The statistical analysis used for this series of experiments was the two-tailed independent t-test comparing the mean of protein kinase C activity without TPA addition and mean of PKC activity after TPA treatment for the times indicated. The independent t-test was chosen for these experiments since each experiment was performed with a group of dishes where one sample was untreated and the others treated with TPA for various times as indicated. The mean \pm S.E. for each time point represent the average of several experiments and do not contain an equal number of samples.

3. RESULTS

3.1 Adenylate cyclase assay (*in vitro*).

A series of *in vitro* adenylate cyclase assays were performed to determine some of the general characteristics of the enzyme.

The n-NRK and tsK-NRK cells were chosen initially as a model for studying the effect of activated PKC on adenylate cyclase activity. It has been shown that the viral K-*ras* gene product (p21) can cause an increase in the responsiveness of adenylate cyclase to stimulation by cholera toxin, forskolin, GTP analogs and sodium fluoride (66,71-73). Recently it has been shown that the K-*ras* protein and membrane associated PKC can cooperatively increase the responsiveness of adenylate cyclase to agonists (74). The normal clone of the NRK cells (n-NRK) was used as a control and for the characterization of some general properties of adenylate cyclase stimulation by GTP.

3.1.1 Initial characterisation using n-NRK cells.

Using n-NRK cells it was demonstrated that it is possible in part to measure the different Mg^{2+} requirements of the G_s and G_i proteins. At high Mg^{2+} concentrations (eg. 10 mM) it is difficult to separate the two activities. At low Mg^{2+} levels (eg. 1 mM) that are sub-optimal for G_s but optimal for G_i (16), it is possible to detect G_i activation which will result in a lower activation of adenylate cyclase in response to guanine nucleotides. A dose response to GTP in the presence of the β -adrenergic agonist isoproterenol (0.5 mM) is shown in figure 3. At high Mg^{2+} concentrations (here 10mM)

n-NRK cells at 36°C

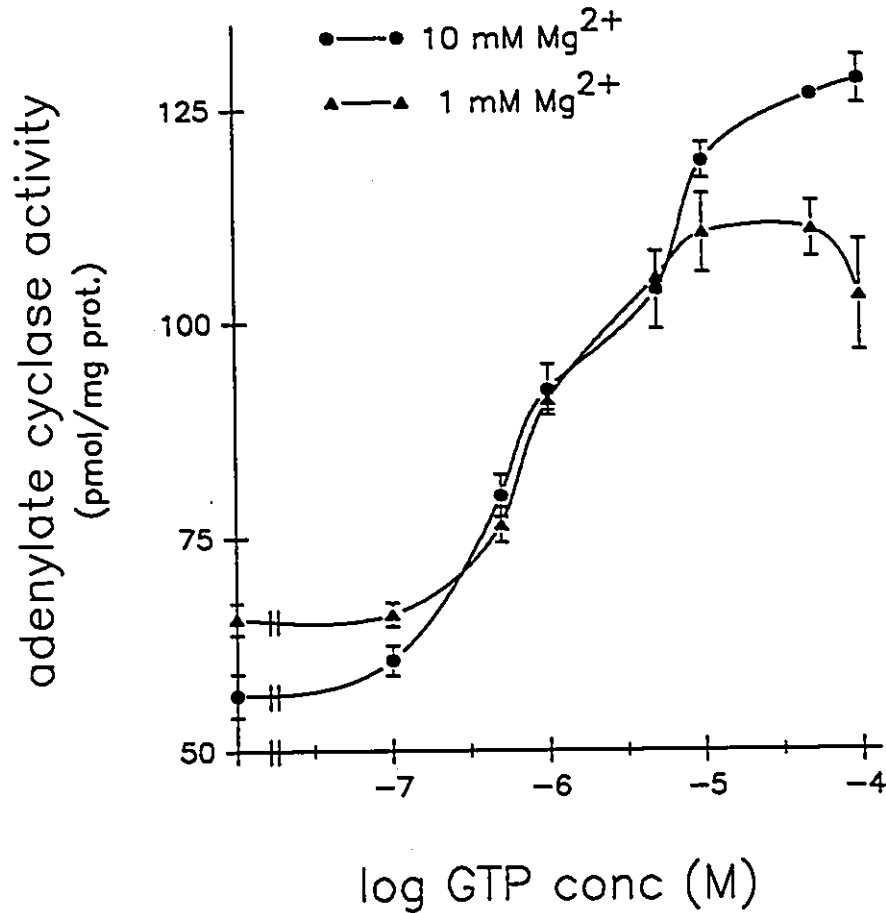


FIGURE 3: GTP DOSE RESPONSE IN n-NRK CELLS IN THE PRESENCE OF ISOPROTERENOL (IPR) *IN VITRO*

nNRK cell membranes were assayed for adenylyl cyclase activity *in vitro* at different GTP concentrations at high (10mM) and low (1mM) Mg²⁺ concentrations in the presence of IPR (0.5mM). The experiment was performed in triplicate and the results are presented as means +/- S.E.. The experiment was done several times with nonhydrolyzable GTP-analogs with similar results.

adenylate cyclase is activated at GTP concentrations above 0.10 μM and reaches a plateau at about 50 μM GTP. At low Mg^{2+} concentrations the dose response to GTP is similar to that measured at high Mg^{2+} up to 5 μM GTP. At GTP concentrations greater than 5 μM the adenylate cyclase activity levels off and a weak inhibition can be seen between 10-100 μM GTP. Similar curves were obtained using nonhydrolyzable GTP analogs such as $\text{GTP}\gamma\text{S}$ and GMPPNHP.

3.1.2 Effect of *in vitro* TPA treatment on adenylate cyclase activity.

Initially attempts were made to try to mimic the effect that K-*ras* p21 has on adenylate cyclase activity *in vivo* by incubating whole cell homogenate with TPA under conditions favouring PKC activity followed by *in vitro* adenylate cyclase assay. It was expected from these experiments that the PKC activation would cause a sensitization of adenylate cyclase to various stimulators. The cell line used for these experiments was tsK-NRK cells at the non-permissive temperature (41°C) to eliminate any effect that the active p21-*ras* protein may have on adenylate cyclase.

$\text{GTP}\gamma\text{S}$ was used as a stimulant in these experiments because it is a non-hydrolyzable GTP-analog. It cannot be hydrolysed by the intrinsic GTPase activity of the α -subunits. G_s is therefore activated to a higher extent by $\text{GTP}\gamma\text{S}$ than by GTP and the stimulation is independent of receptor activation. TPA treatment of whole cell homogenate leads to an enhancement of the $\text{GTP}\gamma\text{S}$ stimulation of adenylate cyclase activity *in vitro*. Figure 4 shows the effect on adenylate cyclase activation by $\text{GTP}\gamma\text{S}$ after treatment of whole cell homogenate with TPA. The pretreatment caused an enhancement of the activation of adenylate cyclase by $\text{GTP}\gamma\text{S}$. The effect is greatest at

tsK-NRK cells at 41°C

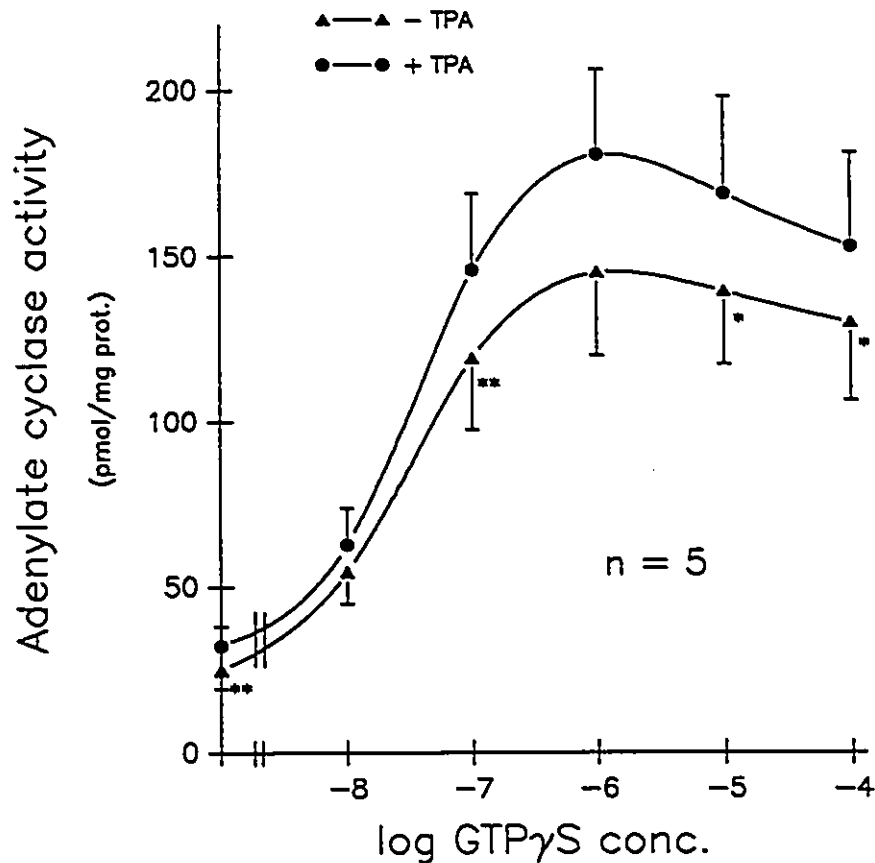


FIGURE 4: EFFECT OF *IN VITRO* TPA PRETREATMENT ON ADENYLATE CYCLASE DOSE RESPONSE TO GTP γ S IN tsK-NRK CELLS AT 41°C.

Homogenate from tsK-NRK cells grown at the non-permissible temperature (41°C) was pretreated with TPA *in vitro*. All samples were incubated with ATP (100 μ M) and PS (50 μ g/ml) at 30°C for 10 minutes. In this experiment TPA was used at 100 nM concentration. The membranes were isolated and the dose response for adenylate cyclase activity to GTP γ S at low Mg $^{2+}$ concentrations (1 mM) was measured. Results are means of five different experiments +/- S.E. A significant difference between non-treated and TPA treated samples is indicated with * for p < 0.05 and with ** for p < 0.01. Statistical analysis used in this series of experiments were the two-tailed paired t-test comparing the means of control and of TPA treated samples at the various GTP γ S concentrations. Furthermore the TPA treatment did not seem to change the inhibition seen at high GTP γ S concentrations.

GTP γ S concentrations that cause the greatest stimulation of the enzyme (10^{-7} - 10^{-5} M). Furthermore, figure 4 shows that TPA treatment does not appear to abolish the inhibition of adenylate cyclase caused by higher concentrations of GTP γ S. The curves are obtained as a mean of five different experiments. Statistical analysis (here the paired t-test) of the data showed that there is a significant enhancement of the TPA treatment. The large variability from experiment to experiment is mostly in the value of the specific activity of adenylate cyclase but the effect of TPA in each of these experiments were more consistent. However not too much emphasis should be put on these results due to the large variability.

The effects of TPA pretreatment on adenylate cyclase activity are not only seen with guanine nucleotide stimulation; figure 5 shows that TPA pretreatment also causes an enhancement of forskolin stimulated adenylate cyclase activity. Figure 5 is a combination of eight different experiments and is presented as mean \pm standard errors. Statistical analysis (here the paired t-test) of the means however showed only a small probability ($p=0.059$) of an enhanced response of adenylate cyclase activity after TPA treatment. This is due to the same problems of variability in the specific activity of adenylate cyclase as noted above.

Figure 6 shows the effect of pretreatment with both active and inactive TPA analogs on adenylate cyclase activity in tsK-NRK cells cultured at the nonpermissive temperature. The result shown in this figure is a combination of four different experiments. The effect of the phorbol esters used was as expected from their reported ability to activate protein kinase C. TPA caused an enhancement of stimulated adenylate cyclase activity and the inactive phorbol ester analog 4 α PDD showed no stimulation (if anything a slight inhibition). However, using oleyl-acetyl-glycerol (OAG), a diacylglycerol

tsK-NRK cells at 41°C

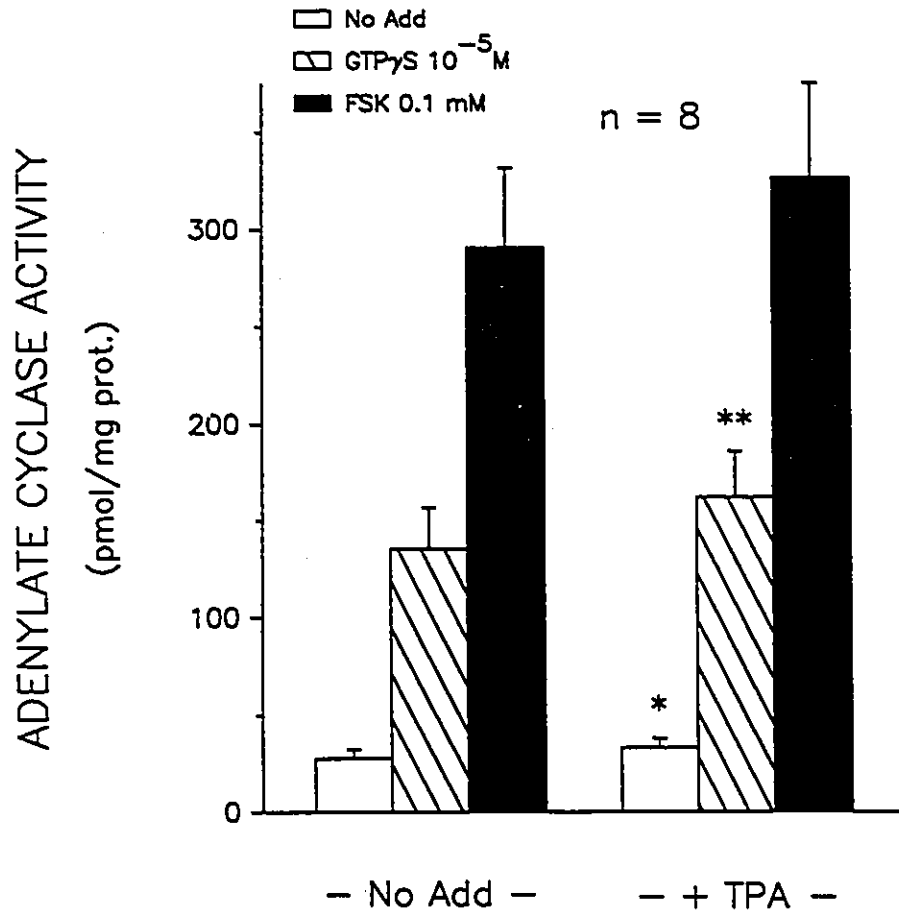


FIGURE 5: EFFECT OF *IN VITRO* TPA PRETREATMENT ON STIMULATION OF ADENYLATE CYCLASE BY GTP γ S AND FORSKOLIN.

Homogenate from tsK-NRK cells grown at the non-permissible temperature (41°C) was pretreated with TPA at 100 nM as described in fig. 4. The membranes were isolated and the stimulation by GTP γ S (1 μ M) and FSK (0.1mM) on adenylate cyclase activity *in vitro* at low Mg²⁺ concentration was measured. Results represent means of eight different experiments +/- S.E. A significant increase in adenylate cyclase activity was seen after treatment with TPA (* indicates p < 0.001 and ** p < 0.01). The two-tailed paired t-test was used as the statistical analysis comparing the means of adenylate cyclase activity with and without a TPA pretreatment.

tsK-NRK cells at 41°C

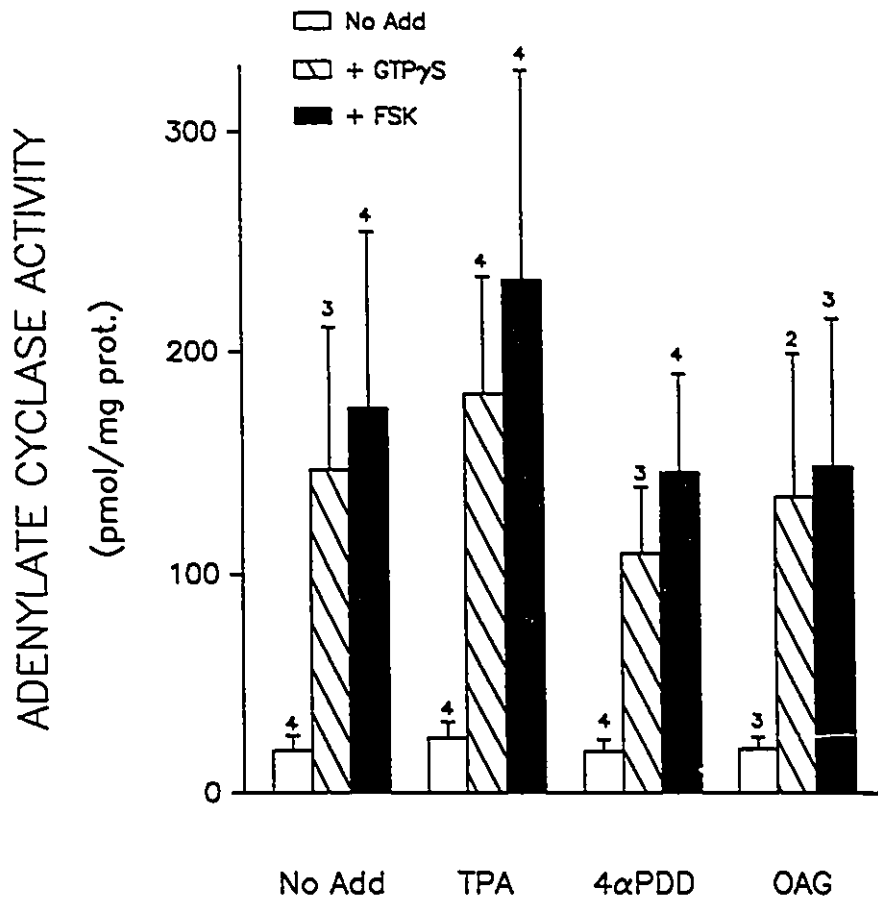


FIGURE 6: EFFECT OF DIFFERENT PHORBOL ESTERS AND DIACYLGLYCEROLS TREATMENT OF HOMOGENATE ON ADENYLATE CYCLASE ACTIVITY *IN VITRO*.

Homogenate from tsK-NRK cells grown at the non-permissible temperature (41°C) was pretreated as described in fig. 4 with the different phorbol esters and a diacylglycerol analog as indicated. The concentration of TPA and 4 α PDD was 100nM and 100 μ M for OAG. Membranes were isolated and adenylate cyclase activity at low Mg²⁺ was measured by stimulation with GTP γ S (1 μ M) and forskolin (FSK, 0.1mM). Results are presented here as mean of four different experiments +/- S.E.. The number of determinations in each value is marked by the number above the error bars. None of the treatments resulted in a significant increase in adenylate cyclase activity when analyzed by the paired t-test.

which activates PKC, gives the same effect on adenylate cyclase as with 4 α PDD. Since OAG is known to activate PKC, an activation similar to that seen with TPA was expected. Statistical analysis showed no significant effect of any of the treatments on any of the stimulating agents tested. Even though the experiments were performed in the presence of a protease inhibitor, in this case 2mM PMSF, the TPA effect was not very reproducible as can be seen by the large standard errors.

To try and determine the cause of the variability, dose response and time course experiments were performed. Figure 7 shows the effect of pretreatment with different doses of TPA. The result in this figure is a combination of four different experiments. Again each individual experiment give an indication of an enhancement of adenylate cyclase activity by TPA and that this could be dose dependent. However since no significant statistical difference was found after TPA treatment no conclusions can be made from these experiments.

The time course experiments for TPA enhancement of adenylate cyclase activity give a partial explanation to the problems of the pretreatment experiments. Figure 8 shows one experiment where various samples were incubated with different TPA concentrations and for different times. There is a great effect of pretreatment both in the absence and in the presence of phorbol ester and the effect of the incubation is more apparent than the effect of the different concentrations of TPA used. Therefore it would appear that the preincubation itself is enhancing adenylate cyclase activity independently of the PKC activating agent added. In addition to this, the pretreatment experiments may also be sensitive to an inability to control the total time necessary from homogenization of the cells to measurement of adenylate cyclase activity. After pretreatment the samples are centrifuged for 20 min. at 4°C and it is possible that

tsK-NRK cells at 41°C

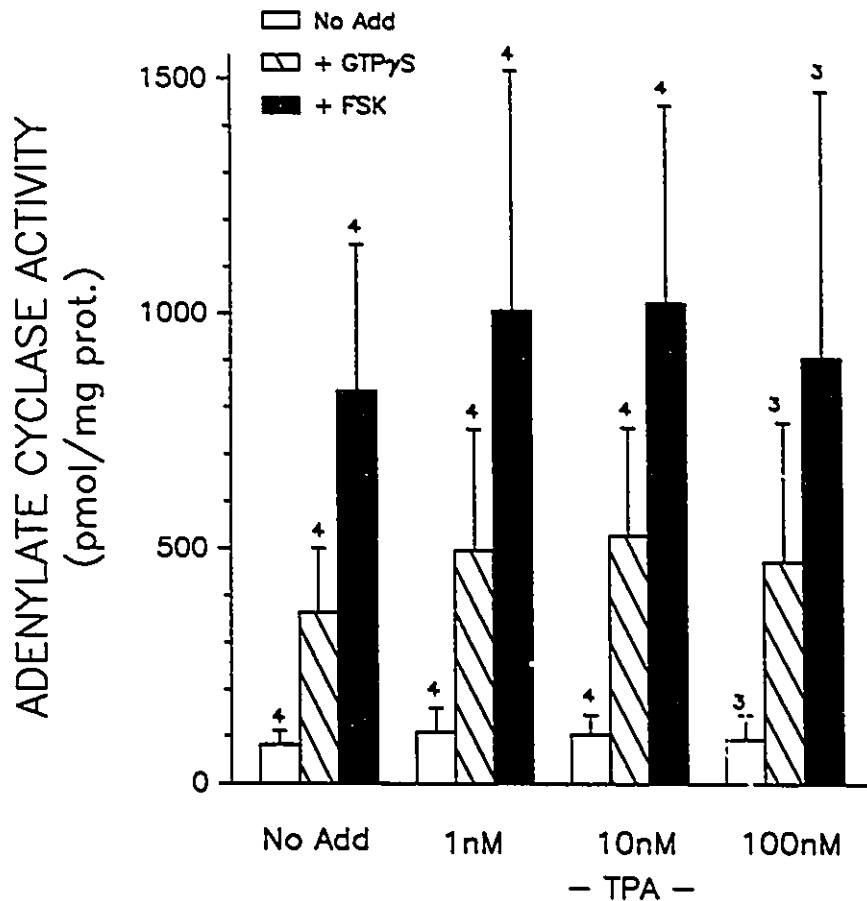


FIGURE 7: TPA DOSE RESPONSE FOR PRETREATMENT OF HOMOGENATE FOLLOWED BY *IN VITRO* ADENYLATE CYCLASE ASSAY ON MEMBRANES.

Homogenate from tsK-NRK cells grown at the non-permissible temperature (41°C) was pretreated as described in fig. 4 with different TPA concentrations. Membranes were isolated and the effect on adenylate cyclase activity was measured at low Mg²⁺ concentration (1mM) using GTP γ S (1 μ M) and forskolin (FSK, 0.1mM). Results are presented as means of four different experiments +/- S.E. The number of determinations in each value is marked by the number above the error bar. None of the treatments with TPA gave a significant increase in adenylate cyclase activity. The statistical analysis used was the paired t-test.

tsK-NRK cells at 41°C

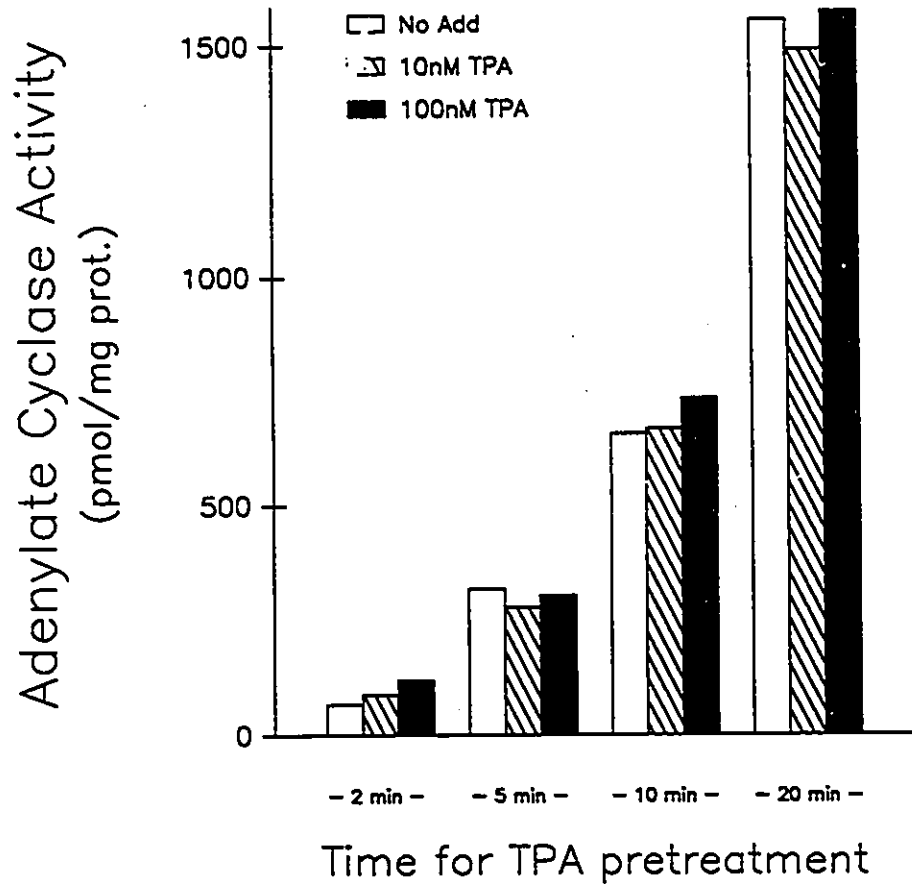


FIGURE 8: TPA TIME COURSE FOR PRETREATMENT OF HOMOGENATE FOLLOWED BY *IN VITRO* ADENYLATE CYCLASE ASSAY ON MEMBRANES.

Homogenate from tsK-NRK cells grown at the non-permissible temperature (41°C) was pretreated as described in figure 4 for different lengths of time in the absence or presence of TPA at 10 nM and 100 nM concentration as indicated. Membranes were isolated and the effect on adenylate cyclase activity *in vitro* was measured. Results are means of duplicates samples from one dish. No statistical analysis were performed on these data due to lack of other comparable experiments.

during this time PKC is still partly active and able to phosphorylate any substrates available.

Due to all of the problems encountered in the *in vitro* treatments with TPA this approach was abandoned. A different cell culture system was chosen for further study, the T51B rat liver cell, in which earlier studies had shown that phorbol esters such as TPA could stimulate DNA synthesis (60). Furthermore the proliferation of T51B cells has been shown to be controlled both by Ca^{2+} and by cyclic AMP (59). Hence the T51B rat liver cell line ought to be a better model for the study of interaction between PKC and adenylate cyclase.

3.1.3 T51B cell/adenylate cyclase activity (*in vitro*).

Plasma membrane preparations from T51B rat liver cells were examined for adenylate cyclase activity. As can be seen in figure 9, T51B cells have a stimutable adenylate cyclase system responding to agents such as forskolin which activates adenylate cyclase directly and by guanine nucleotides and NaF which activate adenylate cyclase through G_s . It can also be seen that the T51B cells respond to receptor mediated stimulation by agents such as the β -adrenergic agonist isoproterenol (IPR) and glucagon. The stimulation of adenylate cyclase activity by glucagon in the presence of 0.1mM GTP was only 3 fold above basal activity. IPR stimulated adenylate cyclase more than 10 fold above basal so this agent was chosen as the stimulant in the following experiments using whole cells.

T51B CELLS

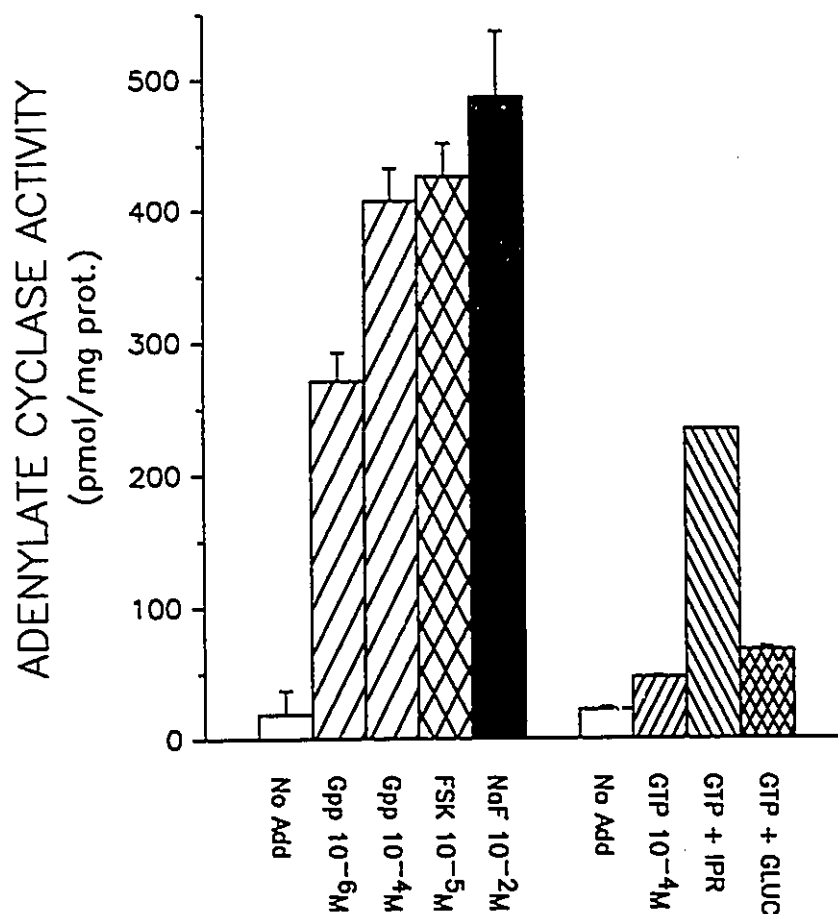


FIGURE 9: EFFECT OF VARIOUS AGENTS ON ADENYLATE CYCLASE ACTIVITY IN T51B CELLS *IN VITRO*.

T51B cell membranes were assayed for *in vitro* adenylate cyclase activity. Stimulation by different agents was measured at high Mg^{2+} concentration (10mM). In the left part of this figure is shown the stimulation of the non-hydrolysable GTP analog GMPP-NH-P (Gpp) at 10^{-6} and 10^{-4} M, forskolin (FSK) at 10^{-5} M and NaF at 10^{-2} M. The right part shows the stimulation by GTP at 10^{-4} M and by GTP in addition to glucagon (GLU) at 10^{-5} M and isoproterenol (IPR) at 10^{-5} M. The results are presented as mean of triplicates in a single experiment +/- S.E.. No statistical analysis was performed.

3.2 Effect of TPA treatment on cyclic AMP synthesis.

In the first series of experiments whole cell homogenate from T51B cells was treated with TPA as described for the NRK cells. The initial results from these experiments were quite variable, therefore a new approach was taken in which interaction of protein kinase C with the adenylate cyclase system was examined without the necessity of destroying the cellular integrity. Intact cellular adenylate cyclase activity can be measured by the ^3H -adenine prelabelling assay described in section 2.3. To clarify the difference between the two different methods by which adenylate cyclase activity is measured the prelabelling experiments are subsequently referred to as *in vivo* adenylate cyclase assays even though they are not truly '*in vivo*' since they are performed in cultured cells rather than in whole animals. The assay measuring adenylate cyclase activity in isolated membranes is referred to as an *in vitro* assay.

3.2.1 Effect of TPA treatment on β -adrenergic stimulation (*in vivo*).

The effect of TPA on the β -adrenergic agonist isoproterenol (IPR) was studied. TPA has been shown to activate PKC in the nano-molar range. Therefore in the first experiments 100 nM TPA was used. IPR stimulated cyclic AMP production within 30 seconds, reached a peak at 1 minute and then declined to almost basal level after 10 minutes of incubation. Addition of 100 nM TPA for 5 minutes caused a significant enhancement of the response to IPR (figure 10A). The actual level of cyclic AMP production expressed as a conversion ratio (10^{-3} cpm [^3H]cAMP per cpm [^3H]ATP per

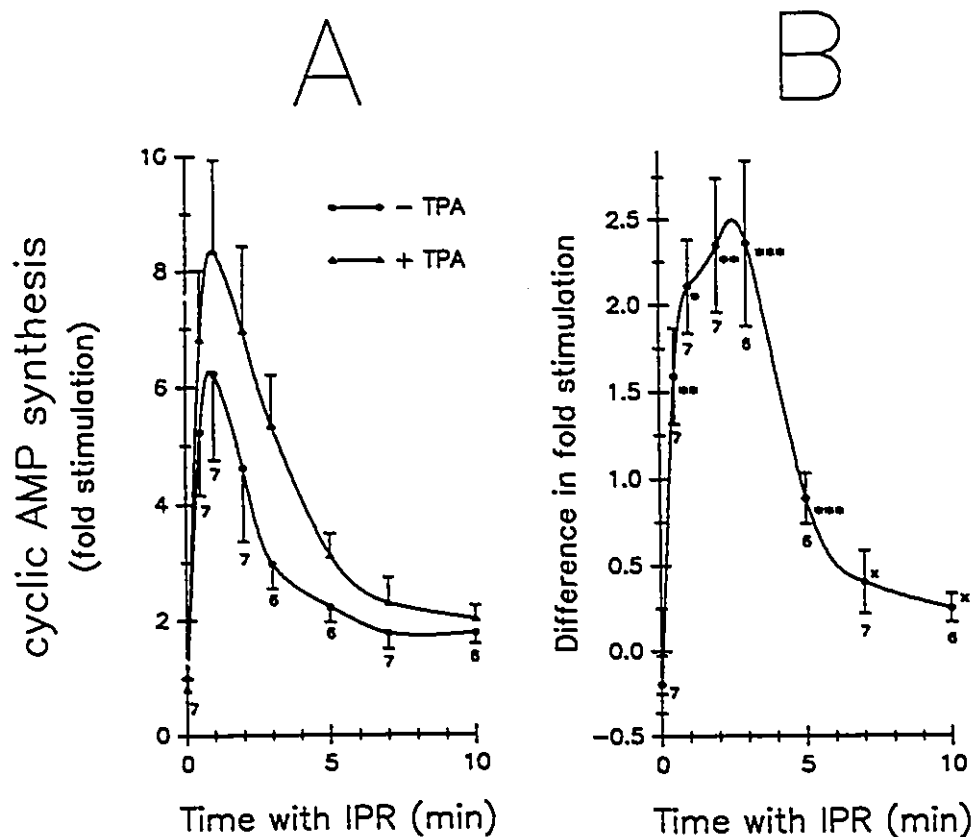


FIGURE 10: TIME COURSE OF CYCLIC AMP SYNTHESIS *IN VIVO* IN RESPONSE TO ISOPROTERENOL IN THE ABSENCE AND PRESENCE OF TPA.

A: T51B cells were treated with 1 μ M isoproterenol (IPR) in the absence or presence of 100 nM TPA for 5 minutes. The results are expressed as a fold stimulation above basal value of the conversion ratio (10^{-3} cpm [3 H]cAMP per cpm [3 H]ATP per mg protein). The time course experiment is the mean \pm S.E. of seven different experiments each with one value per timepoint. The numbers by the graph indicates the number of determinations in that value.

B: The difference in the fold stimulation of cyclic AMP synthesis between IPR stimulation and stimulation by IPR in the presence of TPA was calculated. The data shown here is the mean \pm S.E. of this difference at the various time points. A significant difference between the stimulation by TPA alone (at time 0 min.) and the stimulation by TPA and IPR at the time indicated is marked by * for $p < 0.0001$, ** for $p < 0.0005$, *** for $p < 0.005$ and x for $p < 0.05$. The statistical analysis used for this series of experiments was the two-tailed independent t-test comparing the mean of the difference in fold stimulation at time 0 min. with the means of the difference in fold stimulation at the various times with IPR.

mg protein) varied between experiments since it was dependent on the specific activity of the ^3H -ATP pool and the amount of cell protein. Therefore the results were expressed as fold stimulation above basal in each experiment and the mean is shown in the figures. Figure 10 is a combination of seven different experiments. The mean of the difference in the fold stimulation caused by TPA at the different times indicated is shown in figure 10B. As can be seen from this figure there was a significant enhancement by TPA at times from 0.5 to 10 minutes of incubation with IPR ($p < 0.0001-0.05$). The statistical analysis used in this case is the independent t-test comparing the mean of the difference in the fold stimulation caused by TPA alone (at time 0 minutes) and the mean of the difference in the fold stimulation caused by TPA on IPR stimulation at the times indicated (0.5 to 10 minutes). In each experiment there is a corresponding determination of the cAMP synthesis in the absence and in the presence of TPA for each time point with IPR.

TPA alone had little or no effect on the cyclic AMP production in T51B cells. Addition of TPA alone for up to 10 minutes had no effect on cyclic AMP synthesis. However, when $1\ \mu\text{M}$ IPR was added for the final 1 minute of the incubation with 100nM TPA cyclic AMP production was increased to a maximum within 3 minutes of incubation with TPA (figure 11A). The difference in the fold stimulation caused by TPA in the absence and presence of IPR show a pattern similar to that of cAMP synthesis stimulated by TPA in the presence of IPR (figure 11B). At three minutes incubation with TPA the largest difference of the fold stimulation can be seen between IPR alone and IPR and TPA together however this difference is not significant ($p < 0.08$). Figure 11 is a combination of four different experiments and the statistical analysis performed is the independent t-test comparing the difference of the fold stimulation by IPR alone (at

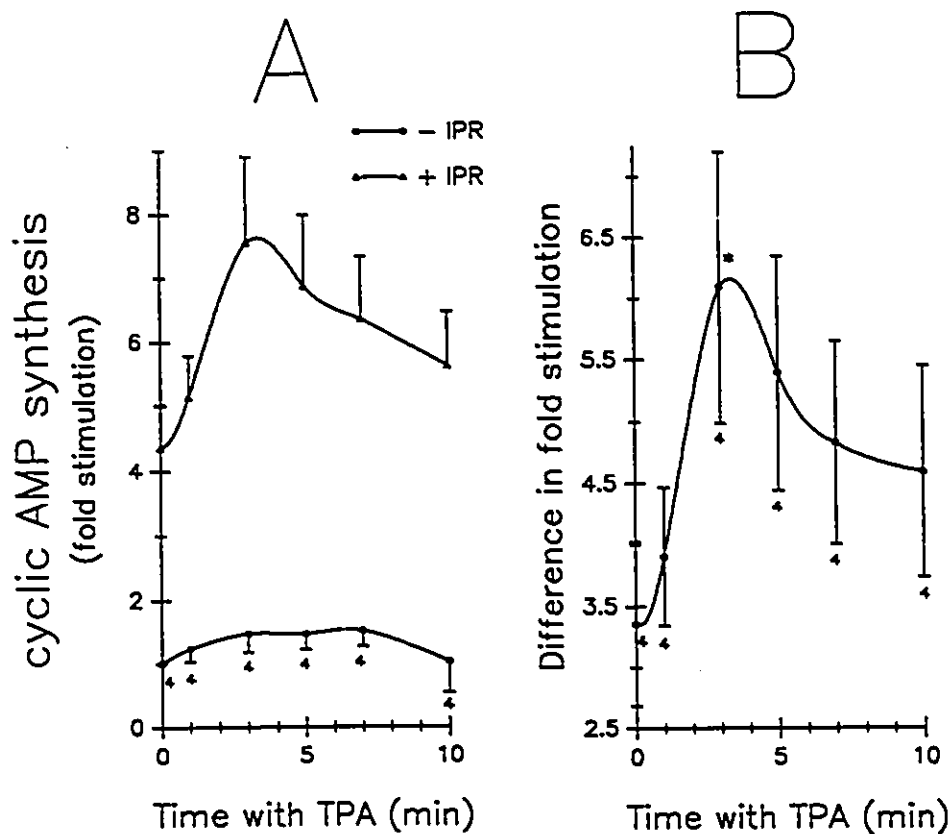


FIGURE 11: TIME COURSE FOR TPA STIMULATION OF CYCLIC AMP SYNTHESIS *IN VIVO* IN THE ABSENCE AND PRESENCE OF IPR.

A: T51B cells were treated with 100nM TPA in the absence or presence of 1 μ M IPR for 1 minute. The results are expressed as a fold stimulation above basal value of the conversion ratio (10^{-3} cpm [3 H]cAMP per cpm [3 H]ATP per mg protein). The time course experiment is represented as the mean \pm S.E. of four different experiments each with one determination at the various times. The numbers on the graph indicates the number of determinations of that value.

B: The difference of the fold stimulation of cAMP synthesis between IPR stimulation alone (at time 0 min.) and IPR stimulation in the presence of TPA was calculated. Here presented as a mean \pm S.E. A difference between the mean of cyclic AMP synthesis in the presence of IPR alone (at time 0 min.) and that of IPR in the presence of TPA is indicated with * for $p < 0.08$. The means of the values in the absence and the presence of IPR were analyzed by the two-tailed independent t-test.

time 0 min.) and the difference in fold stimulation by IPR and TPA at the times indicated.

A dose response of cyclic AMP synthesis to TPA is shown in figure 12. Again TPA alone had little or no effect at all the concentrations used. In the presence of IPR (1 μ M, 1 min.) addition of TPA for 5 minutes caused a significant increase in cAMP production at concentration 100nM ($p < 0.05$). This correlates well with the doses found to stimulate PKC. Figure 12 is a combination of seven different experiments. Part A shows the actual graphs of the fold stimulation and part B shows the means of the difference in the fold stimulation in the absence and the presence of IPR. The statistical analysis performed in this figure is the independent t-test performed on the difference of the fold stimulation by IPR alone (at time 0 min.) and the difference in fold stimulation by IPR and TPA at the concentrations indicated.

Figure 13 shows the dose response curve for IPR in the absence and presence of TPA. Concentrations of IPR that caused a stimulation of cyclic AMP synthesis were enhanced by addition of TPA (5 min., 100nM) with the maximum being obtained at approximately 1 μ M IPR. Addition of TPA enhanced the IPR stimulation of adenylate cyclase and this enhancement was greatest at 1 μ M IPR. The IPR dose response shown in fig. 13 is a combination of seven different experiments. Again the statistical analysis performed here is the independent t-test comparing the means of the difference in fold stimulation between TPA alone and the mean of difference in fold stimulation obtained with the TPA and IPR at the concentrations indicated.

The enhancement by phorbol esters on cyclic AMP synthesis in response to IPR was also seen with other adrenergic agents. Table 1 shows that adenylate cyclase was stimulated by adrenergic agents according to the β -adrenergic potency of the molecule.

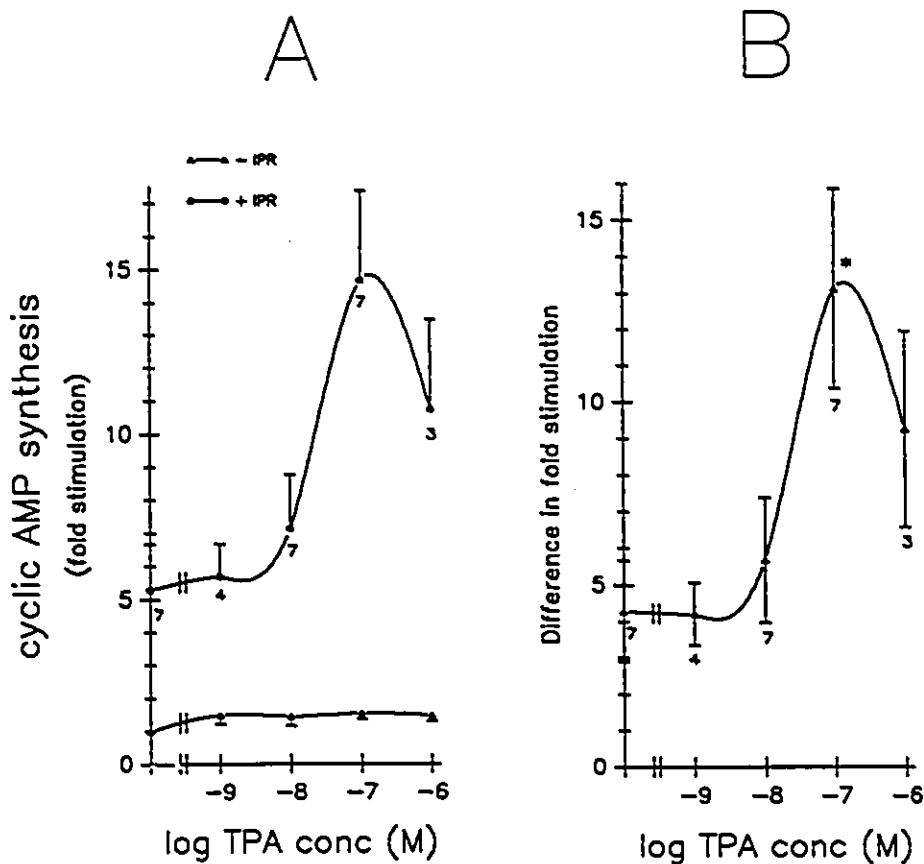


FIGURE 12: DOSE RESPONSE TO TPA IN THE ABSENCE OR PRESENCE OF ISOPROTERENOL (IPR).

A: T51B cells were treated with TPA at the indicated concentrations for 5 minutes. When present, IPR was added at $1\mu\text{M}$ concentration for the final 1 minute of the incubation. The dose response is presented as the mean \pm S.E. of seven different experiments and the numbers under the error-bars indicate the number of determinations for the dose of TPA indicated. The results are expressed as a fold stimulation above basal value of the conversion ratio (10^3 cmp [^3H]cAMP per cmp [^3H]ATP per mg protein).

B: The difference of the fold stimulation between IPR stimulation alone (at time 0 min.) and IPR stimulation in the presence of TPA at the times indicated is pictured here as a mean \pm S.E. TPA treatment caused a significant increase in cAMP production in the presence of IPR (* $p < 0.05$) when comparing the IPR stimulation alone. The statistical analysis used here is the two-tailed independent t-test of the means of the fold stimulations.

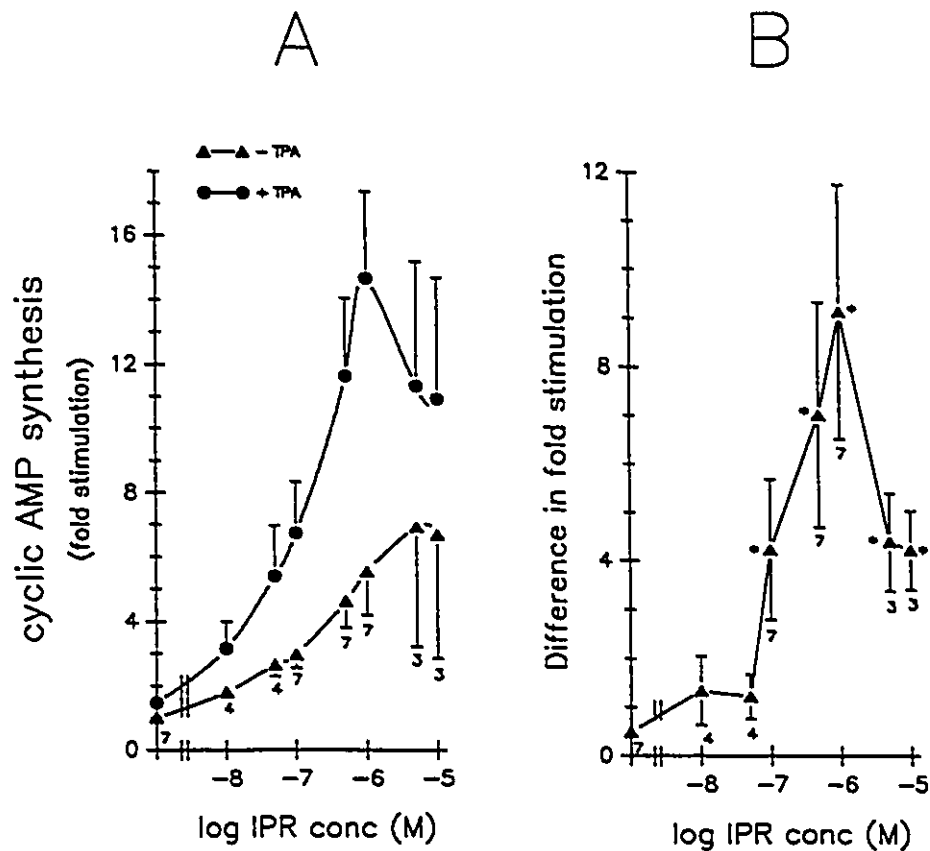


FIGURE 13: DOSE RESPONSE TO IPR IN THE ABSENCE OR PRESENCE OF TPA.

A: T51B cells were treated with IPR at the indicated concentrations for 1 minute. When present, 100 nM TPA was added 4 minutes prior to IPR. The dose response curve is presented as the mean \pm S.E. of seven different experiments and the number of determinations per point is indicated. The results are expressed as the fold stimulation above basal value of the conversion ratio (10^3 cpm [3 H]cAMP per cpm [3 H]ATP per mg protein).

B: The difference of the values between TPA stimulation alone and IPR stimulation in the presence of TPA is pictured here as a mean \pm S.E. A significant difference between the fold stimulation by TPA alone and the fold stimulation by TPA and IPR at the times indicated are marked by * for $p < 0.05$. The statistical analysis used in this part was the two-tailed independent t-test on the means of the difference in fold stimulation.

ADRENERGIC AGONISTS AND ANTAGONISTS STIMULATION OF CYCLIC AMP SYNTHESIS (conversion ratio)			
	CONTROL	+ TPA	FOLD STIMULATION
Part A:			
BASAL	0.32 ± 0.70	0.16 ± 0.14	-
IPR	10.30 ± 0.23	14.39 ± 0.23 **	1.4
EPI	7.85 ± 0.24	10.01 ± 0.77 *	1.3
NOREPI	1.07 ± 0.18	1.34 ± 0.15	1.3
PHENEPI	0.14 ± 0.16	0.27 ± 0.16	-
Part B:			
IPR	10.08 ± 1.24	14.76 ± 1.94 *	1.5
IPR + PRO	3.20 ± 1.02	2.91 ± 0.95	-

TABLE 1: EFFECT OF VARIOUS ADRENERGIC AGENTS ON CYCLIC AMP SYNTHESIS IN VIVO IN THE ABSENCE OR PRESENCE OF TPA.

T51B cells were treated with the adrenergic agents indicated at the concentration of 1 μ M for 1 minute. When present, 100 nM TPA was added for 5 minutes. Part A shows an experiment where TPA treatment caused a significant enhancement of different β -adrenergic agents (* indicates $p < 0.05$ and ** $p < 0.0005$). The agents were used as part of other experiments under the same conditions with similar results. Data are presented as mean of triplicate dishes from one experiment +/- S.E.. Part B represents an average of three different experiments combined expressed as mean +/- S.E.. The TPA treatment causes a significant enhancement in IPR stimulation (* $p < 0.05$). The results are expressed as conversion ratio (10^{-3} cmp [3 H]cAMP per cmp [3 H]ATP per mg protein). Statistical analysis used in this figure is the two-tailed independent t-test.

The enhancement by TPA in all cases where there was a stimulation of cAMP production resulted in a fold stimulation between 1.3 and 1.5 although the enhancement was only statistically significant with isoproterenol ($p < 0.0005$) and epinephrine ($p < 0.05$) which increased adenylate cyclase activity the most. This indicates that the effect of TPA is independent of the level of stimulation of adenylate cyclase via the β -adrenergic receptor. The β -adrenergic antagonist propranolol drastically reduces the stimulation of cyclic AMP production caused by IPR. There was no enhancement by TPA when IPR and propranolol were added in equimolar concentrations. The result shown in table 1 is obtained from one experiment performed with triplicate dishes and the result is shown as a mean of the conversion ratio \pm S.E.. The table also gives an idea of the low variability in this method of measuring cAMP production if factors such as ATP-pool size, protein content and actual growth state of the cells could be kept constant from experiment to experiment. The statistical analysis of the result for this figure was done with the independent t-test comparing the mean of the conversion ratio between the different treatments.

3.2.2 Phorbol ester specificity.

The specificity of the phorbol ester enhancement of adenylate cyclase was examined. Figure 14 shows that addition of TPA causes a significant enhancement of the IPR stimulated cAMP production ($p < 0.001$) and that the inactive TPA analog, 4 α -phorbol-12,13-didecanoate (4 α PDD), had no effect on the IPR stimulation in T51B cells. An analog to the natural PKC activator diacylglycerol, 1,2-dioctanoyl-glycerol (DiC8) gave a significant enhancement ($p < 0.0005$) similar to that seen with TPA on IPR

T51B cells

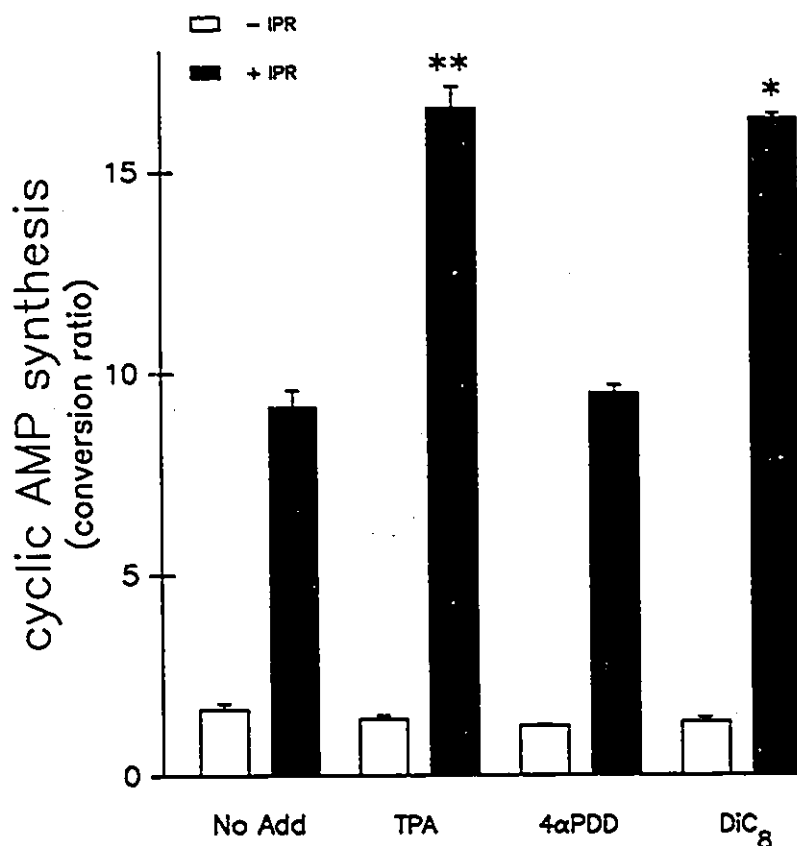


FIGURE 14: EFFECT OF DIFFERENT PHORBOL ESTERS AND DIACYLGLYCEROL ON IPR-STIMULATED CYCLIC AMP SYNTHESIS *IN VIVO*.

T51B cells were incubated with the agents indicated for 5 minutes. The concentrations used were for TPA and 4αPDD 100nM and for DiC₈ 100μM. When present, 1μM IPR was added for the final 1 minute of the incubation. The results are expressed as conversion ratio (10^{-3} cmp [³H]cAMP per cmp [³H]ATP per mg protein). The agents were used as a part in other experiments with similar results. Data are presented as mean of triplicate dishes in one experiment +/- S.E.. The enhancement of cAMP production in response to IPR after TPA and DiC₈ treatment are significant (* indicates $p < 0.0005$ and ** $p < 0.001$) when analyzed with the two-tailed independent t-test on the means of conversion ratios.

stimulated cAMP production. This strongly suggests that the effect of TPA is due to an effect of PKC on one or more components of the adenylate cyclase system. The result in figure 14 is expressed as the mean of the conversion ratio (10^{-3} cpm [^3H]cAMP per cpm [^3H]ATP per mg protein) obtained from one experiment using three separate dishes for each treatment. The statistical analysis performed for this figure is the independent t-test on the means of the different treatments.

3.2.3 Effect of TPA treatment on forskolin and cholera toxin stimulation.

It has been reported that activation of PKC by phorbol esters leads to phosphorylation of β -adrenergic receptors (55,56).

To examine if the effect seen on cAMP synthesis by TPA addition is due to receptor phosphorylation, the effect of TPA on agents that do not involve receptors was measured.

Stimulation of adenylate cyclase by C.T. is due to ADP-ribosylation of G_s which inhibits its GTPase activity thus leaving it in a permanently active state. Cyclic AMP production in response to C.T. reached a maximum after 60 minutes in the presence of 0.1 $\mu\text{g}/\text{ml}$ C.T. and thereafter declined (figure 15A). TPA caused a large increase of this stimulation ($p < 0.01-0.05$) and as with IPR stimulation this increase is largest where the cyclic AMP production is the largest (figure 15B). The time course for C.T. is a combination of five individual experiments. The statistical analysis performed is the independent t-test on the difference in fold stimulation caused by TPA in the absence of C.T. (at time 0 min.) and the difference in fold stimulation caused by TPA after C.T. treatment at the times indicated.

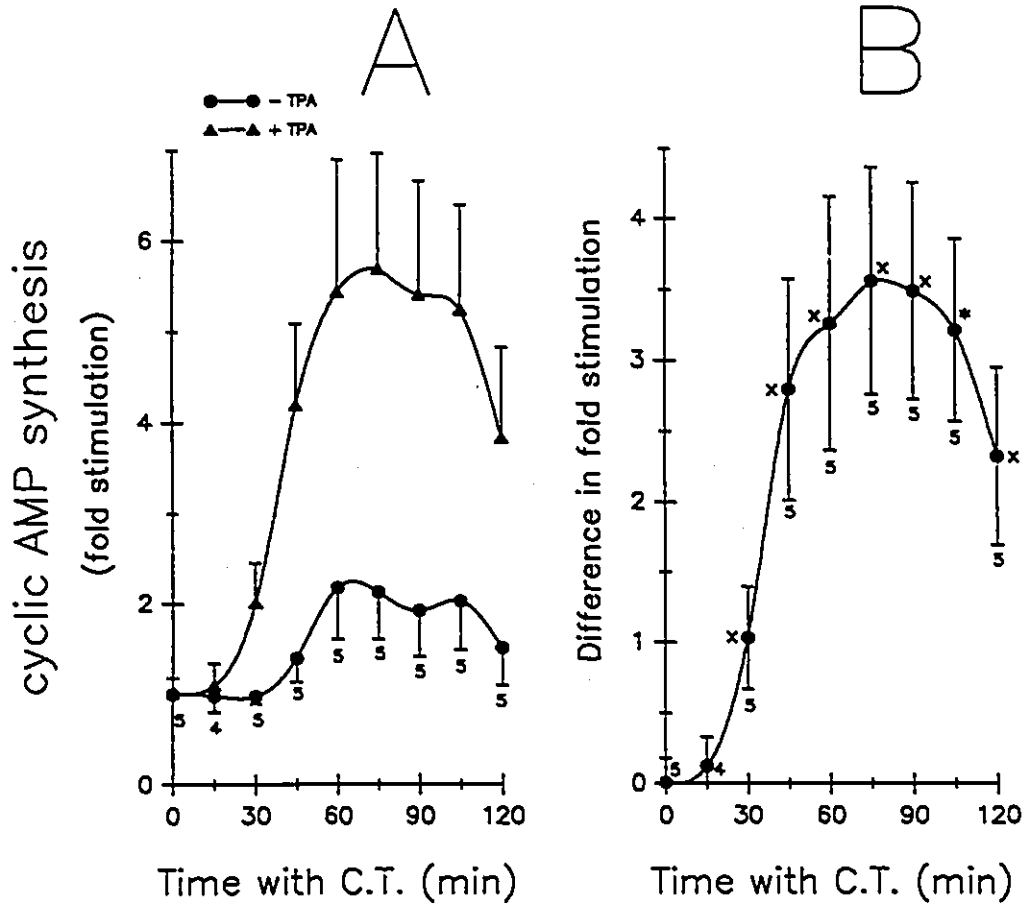


FIGURE 15: TIME COURSE OF CYCLIC AMP SYNTHESIS *IN VIVO* IN RESPONSE TO CHOLERA TOXIN.

A: T51B cells were incubated with 0.1 $\mu\text{g/ml}$ cholera toxin (C.T.) for the times indicated. When present, 100 nM TPA was added for the final 5 minutes. The results are expressed as a fold stimulation above basal value of the conversion ratio (10^3 cmp [^3H]cAMP per cmp [^3H]ATP per mg protein). The time course experiment is presented as the mean \pm S.E. of five different experiments and the numbers on the graph indicate the number of determinations at that time point.

B: The difference of the values between TPA stimulation and C.T. stimulation in the presence of TPA is pictured here as a mean \pm S.E. A significant increase in fold stimulation by TPA is indicated by * for $p < 0.01$ and x for $p < 0.05$. The statistical analysis used in this part were the two-tailed independent t-test comparing the difference in fold stimulation caused by TPA above basal and the difference in fold stimulation by TPA after C.T. treatment at the times indicated.

Forskolin (FSK), which stimulate cAMP synthesis directly by acting on the catalytic subunit, and cholera toxin (C.T.) were used in combination. Stimulation of adenylate cyclase by both FSK and C.T. together leaves only the inhibitory arm of the adenylate cyclase system available. In other words if TPA enhances cAMP production by eliminating the G_i pathway then addition of TPA should enhance the stimulation by FSK and C.T. together. In table 2 TPA causes an enhancement of the stimulation by FSK ($p < 0.01$) and by C.T. ($p < 0.001$) alone but when the two agents are added together there is no significant enhancement by TPA. Addition of C.T. and FSK together caused a 84 fold stimulation, however there was no further increase by TPA. The result in table 2 is the mean of two experiments each done in triplicate. The result is expressed as a fold stimulation above basal and the statistical analysis performed is the paired t-test comparing the means of the fold stimulation obtained from the different treatments.

3.3 Effect of pertussis toxin on adenylate cyclase activity.

Incubation of whole cells with pertussis toxin did not have any effect on the cyclic AMP synthesis *in vivo* in T51B cells. As a control experiment an *in vivo* pertussis toxin incubation was followed by an *in vitro* treatment in the presence of ^{32}P -NAD. ^{32}P labelled membrane proteins were separated by SDS-PAGE and identified by autoradiography. Figure 16 shows the result of this experiment. There is one major substrate about 40 kDa in size which would correspond to the α -subunit of G_i . There was a slight change in the intensity of the labelling over the time course of the pretreatment with P.T.. The labelling intensity from the second *in vitro* P.T. treatment increased above the non-treated sample and never decreased to a level below that level even after 22 hrs

FORSKOLIN AND CHOLERA TOXIN STIMULATION OF cAMP PRODUCTION:

EFFECT OF TPA

(fold stimulation)

	CONTROL	+ TPA	DIFFERENCE IN FOLD STIMULATION
BASAL	0.997 ± 0.117	-	-
FSK	20.75 ± 3.21	25.15 ± 2.89 **	4.41 ± 1.13
C.T.	13.23 ± 1.04	17.90 ± 1.38 *	4.58 ± 0.65
FSK + C.T.	84.05 ± 12.24	78.40 ± 6.89	-5.65 ± 6.61

TABLE 2: EFFECT OF FORSKOLIN AND CHOLERA TOXIN ON CYCLIC AMP SYNTHESIS IN VIVO IN THE ABSENCE OR PRESENCE OF TPA.

T51B cells were treated with forskolin (FSK, 10 μ M, 3 min.) and cholera toxin (C.T., 0.1 μ g/ml, 60 min.). When present, 100nM TPA was added for 5 minutes. The results are expressed as a fold stimulation above basal value of the conversion ratio (10^3 cmp [3 H]cAMP per cmp [3 H]ATP per mg protein). The result is a mean of two experiments performed with triplicate dishes \pm S.E.. There is a significant enhancement by TPA of FSK and C.T. stimulation of cAMP synthesis (* indicates $p < 0.001$ and ** $p < 0.01$). The statistical analysis used for this table is the two-tailed independent t-test on the means of the fold stimulation caused by the agents indicated.

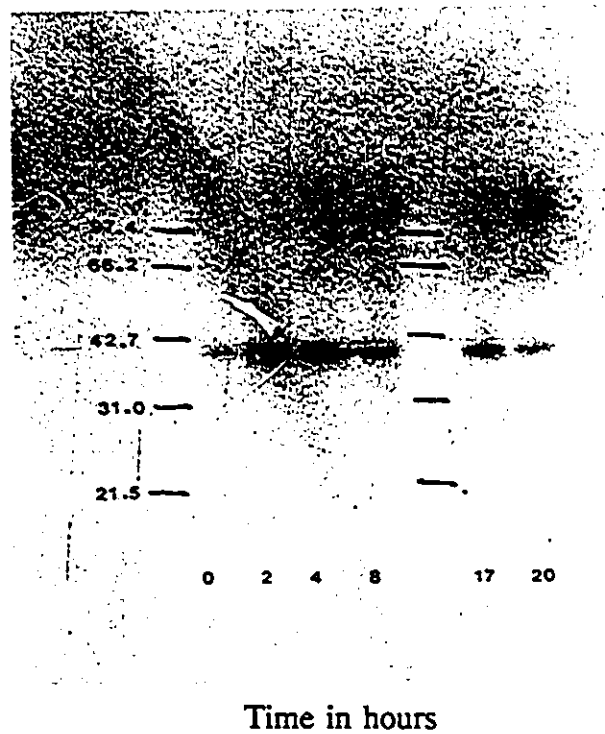


FIGURE 16: EFFECT OF PERTUSSIS TOXIN TREATMENT ON T51B CELLS.

Autoradiograph of pertussis toxin treatment of T51B cells. T51B cells were pretreated with pertussis toxin *in vivo* for the times indicated. The membranes were then treated *in vitro* with pertussis toxin in the presence of ^{32}P -NAD. SDS-PAGE and autoradiography were performed on the isolated membranes as described in Methods.

pretreatment. If the preincubation with pertussis toxin had led to an ADP-ribosylation of α_1 or any of the other α -subunits, the second P.T. treatment *in vitro* would have shown a decrease in the labelling intensity.

3.4 Effect of TPA on PKC activity.

As outlined in the introduction 1.1.10.1 and 1.1.10.2 phorbol esters can directly activate PKC and also cause a translocation of the enzyme from the soluble fraction to the membrane. The sensitization of adenylate cyclase could be caused by translocation of PKC to the membrane or by activation of PKC already present there. To verify this, T51B cells were incubated with TPA and PKC activity was measured.

3.4.1 Soluble PKC activity.

Figure 17 shows the time course for changes in PKC activity in the soluble fraction (measured as PKC activity in the EDTA/EGTA extractable fraction). TPA treatment of T51B cells shows a decrease in PKC activity with a different time course from that of adenylate cyclase enhancement. After 5 minutes treatment there was no measurable difference in the soluble PKC activity and even after 10 minutes the drop in PKC activity was not significant. At 20 minutes the PKC activity was lowered to less than half and levelled off by 60 minutes ($p < 0.05$). It is unlikely that the decrease in PKC activity is due to proteolytic degradation since the basal level of kinase activity (in the absence of PS and diolin) did not change over the time course of the experiment.

T51B cells

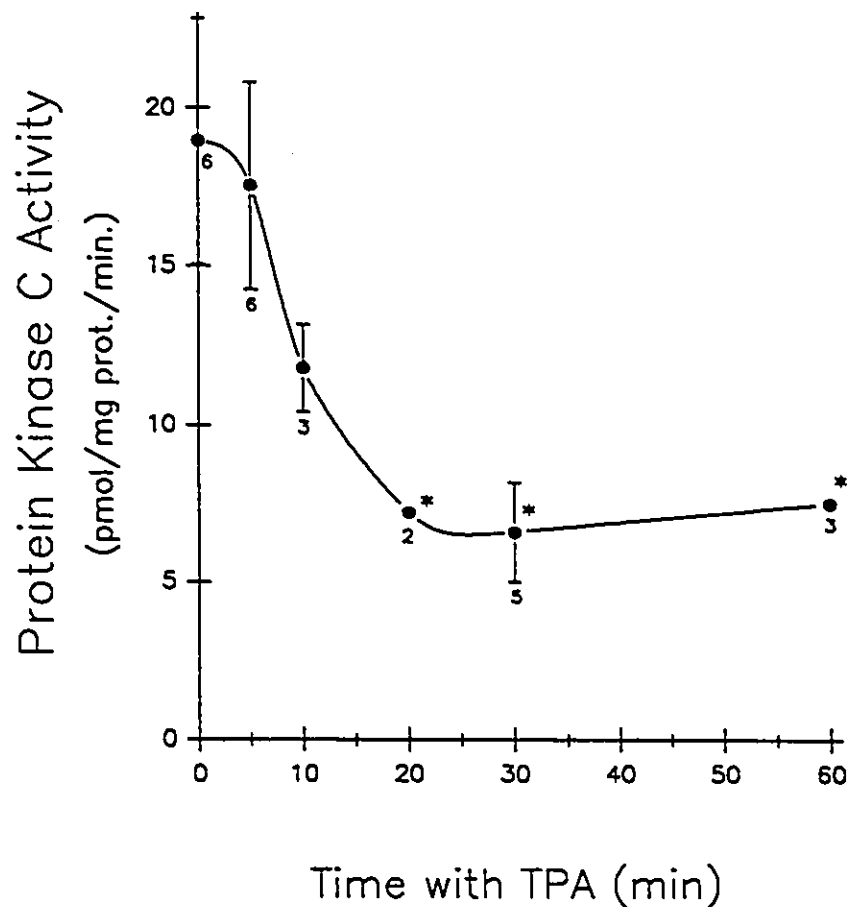


FIGURE 17: EFFECT OF TPA ON THE SOLUBLE PKC ACTIVITY.

T51B cells were treated with 100 nM TPA for the times indicated. The soluble PKC activity was extracted in a solution containing EDTA and EGTA and assayed as described in Methods section 2.5. PKC activity *in vitro* is measured as the difference in the absence and the presence of PS and TPA expressed as pmol ^{32}P incorporated into histone per mg protein per minute. After 20 minutes TPA treatment PKC activity decreased significantly (* indicates $p < 0.05$) when comparing the activity to that at time 0 min. using the two-tailed independent t-test. The values are a combination of six different experiments and expressed as means of specific PKC activity \pm S.E.. The numbers on the graph indicates the number of determinations at that time point. The statistical analysis used in this figure is the two-tailed independent t-test comparing the mean PKC activity without TPA treatment with the mean in PKC activity after addition of TPA for the times indicated.

3.4.2 Membrane PKC activity.

Measurement of membrane PKC activity normally requires extraction with non-ionic detergents. In a conventional assay this detergent has to be removed for measurement of PKC activity.

3.4.2.1 In vitro PKC assay.

Table 3 shows the results of some of the experiments done to try to accurately measure the membrane PKC activity in T51B cells after treatment with TPA for different times. The results obtained from these experiments were inconclusive. There did not seem to be any pattern to the results obtained. TPA treatments gave an increased PKC activity in one experiment and a decreased activity in another. One of the problems was the method by which the detergent was removed. The DEAE-Sephacel columns could have different elution-times. An increase in the elution time seemed to reflect a decrease in the PKC activity most likely by a proteolytic cleavage of the regulatory domain from the catalytic domain. Proteolytic breakdown of PKC results in two major fragments, the catalytic domain and the regulatory domain leaving the catalytic domain active and unregulatable. This is reflected in a higher background count in a particular sample and this was often observed in the experiments.

Membrane Protein Kinase C Activity (pmol ³² P/mg prot./min.)						
Treatment	Ex 22/3	Ex 30/3	Ex 19/4	Ex 16/5	Ex 20/5	Ex 6/6
No Add	ND	78.9 ± 42.7	34.5 ± 9.65	ND	9.76 ± 25.4	99.8 ± 13.0
2.5 min.	-	-	-	-	15.0 ± 31.8	-
5.0 min.	24.0 ± 3.30	ND	ND	11.4 ± 24.2	ND	84.7 ± 11.4
10 min.	-	-	-	-	ND	-
15 min.	-	-	-	29.0 ± 69.9	-	43.9 ± 7.9
30 min.	ND	121 ± 148	20.9 ± 7.90	35.4 ± 21.3	12.6 ± 2.9	85.0 ± 8.18
60 min.	-	-	-	-	-	70.7 ± 14.6

TABLE 3: EFFECT OF TPA TREATMENT ON MEMBRANE PKC ACTIVITY.

T51B cells were treated *in vivo* with 100 nM TPA for the times indicated. The membrane PKC activity was extracted in a buffer containing NP-40. The detergent was removed using a DEAE-Sephacel column and PKC activity was determined as described in section 2.5. Each column of the table represents an individual experiment. Data are presented as mean of triplicate +/- S.E.. ND indicates non-detectable levels of PKC activity.

3.4.2.2 In situ protein kinase gel assay.

The idea behind this assay was to separate the different kinases on a non-denaturing gel. By doing so the detergent required to extract PKC from the membrane is removed. The gel is then incubated with substrate and ^{32}P -ATP and the level of phosphorylation can be visualised by autoradiography. The separation was poor and no distinct protein bands were seen but merely some diffuse spots. Hence it was concluded that this assay was not sensitive enough to measure changes in membrane PKC activity.

3.4.3 PDBu binding assay.

As an alternative to direct measurement of PKC, phorbol ester binding can be used to identify the amount of PKC that is present in different sub-cellular fractions. Using a phorbol ester binding assay it was shown that the T51B cell line has an almost equal amount of PKC in the cytosolic fraction and in the plasma membrane fraction measured as fmol PDBu bound per mg protein (table 4). This indicates that there should be an adequate amount of PKC already present in the membrane to account for the enhancement of adenylate cyclase activity by TPA treatment.

3.5 Alternative protein kinase C assays.

A new method to measure PKC activity in membrane fractions has recently been developed (75). It utilizes a soluble physiological substrate for PKC isolated from S49 cyc- lymphoma cells. After isolation of membrane fractions containing PKC activity,

T51B CELL PDBu BINDING ASSAY

TOTAL HOMOGENATE	11.37 ± 3.01	fmol/mg prot.
CYTOSOL	7.85 ± 1.93	fmol/mg prot.
MEMBRANE	8.79 ± 1.87	fmol/mg prot.

TABLE 4: PDBu BINDING ASSAY ON DIFFERENT FRACTIONS OF THE T51B CELLS.

T51B cells were separated in the different sub-cellular fractions indicated and the PDBu binding was measured. The results are expressed as the difference between the fmol/mg protein in the absence and the presence of TPA in a 1000-fold excess. The data are the mean of triplicates determination in one cell fractionation +/- S.E.

the membranes are mixed with the S49-substrate and assayed in the presence of ^{32}P -ATP. After incubation, the membranes are removed by centrifugation and the supernatant can be analyzed by SDS-PAGE and autoradiography. Due to initial problems with isolation of the substrate and lack of time it was not possible to use this method. However this will be part of future experiments in the Laboratory of Dr. D. Franks.

4. DISCUSSION

4.1 Adenylate cyclase activity (in vitro).

Earlier studies using n-NRK and tsK-NRK cells indicated that activated viral K-*ras* proteins increase the responsiveness of adenylate cyclase to stimulation by cholera toxin, forskolin, GTP analogs and sodium fluoride (71-73). It had been shown that the K-*ras* protein could cooperate with activated PKC to sensitize adenylate cyclase responsiveness to various agents (74). One of the ways by which PKC might sensitize the adenylate cyclase system is by phosphorylation of one or more of its regulatory components (see section 1.2.2).

As a means of determining changes in the activity of either the stimulatory or the inhibitory G protein, G_s or G_i , it is possible to measure adenylate cyclase activation by guanine nucleotide at high (eg. 10mM) and at low (eg. 1mM) Mg^{2+} concentrations. Activation of G_i is observed at a concentration one order of magnitude lower than activation of G_s (24).

Initial experiments were performed to examine the effect of different Mg^{2+} concentrations on adenylate cyclase activity *in vitro*. Normal NRK cells were used for these experiments. A dose response curve to GTP was determined in the presence of IPR (0.5mM). Concentrations below 0.1 μ M GTP did not activate adenylate cyclase, however at low Mg^{2+} the activation reaches a maximum at about 10 μ M GTP and weak inhibition is seen at concentrations above that. At high Mg^{2+} concentrations (10 mM) the activation of adenylate cyclase by GTP follows the curve measured at low Mg^{2+} up to 10 μ M GTP and reaches a plateau at about 50 μ M GTP but no inhibition is seen. Similar

results are seen with nonhydrolyzable GTP analogs. From these experiments it can be seen that comparing adenylate cyclase *in vitro* assays performed at low Mg^{2+} (1 mM) with those at high Mg^{2+} (10 mM) concentrations the activity of in the inhibitory G-protein can be observed.

4.1.1 Effect of *in vitro* TPA treatment on adenylate cyclase activity.

Studies using the rat kidney cell line infected by a temperature-sensitive mutant Kirsten sarcoma virus (tsK-NRK) have indicated that reactivating the *K-ras* protein seems to interfere with the inhibitory action of G_i (72) and that PKC is involved in the process. In the next series of experiments an attempt was made to reproduce the enhancement shown with *K-ras* on adenylate cyclase by activating PKC with phorbol esters such as TPA. For these experiments the tsK-NRK cell line was used. To eliminate any interference from the *ras* p21 protein in these experiments all the cultures were incubated at the nonpermissive temperature (41°C).

Initially the TPA treatment was performed on whole cell homogenates to try to reproduce the enhancement seen when intact cells were treated with phorbol esters. The TPA treatment of homogenates indicated that activation of PKC leads to an enhancement of adenylate cyclase activity in response to various agents. TPA treatment of tsK-NRK cell homogenates showed that the $GTP\gamma S$ dose response was enhanced the most at the concentrations showing maximum activation. When adenylate cyclase activity was measured at low Mg^{2+} (1mM) the inhibition seen at high $GTP\gamma S$ concentrations did not change. This is an indication that the mechanism of TPA pretreatment on cell homogenate is probably not by inactivation of the inhibitory G-protein (G_i).

Unfortunately the *in vitro* pretreatment experiments were poorly reproducible. The effect did not seem to only be related to the phorbol esters activation of PKC. The time course and dose response experiments failed to give any conclusive results. There seemed to be an enhancement of adenylate cyclase activity after a preincubation even if no PKC activating agent was added. This could be because PKC is already partly activated in the cells and during the preincubation it is in contact with components of the adenylate cyclase system. Furthermore PKC could be degraded during the preincubation despite the fact that leupeptin was continually present as a protease inhibitor. All cells have a variety of proteases capable of hydrolysing PKC in such a way that the catalytic domain is uncoupled from the regulatory domain. The catalytic domain is active but is no longer regulated by the normal agents (37). This could lead to an activation of PKC independent of the phorbol ester added and occur merely as a result of the homogenization and incubation. Also it has been suggested that adenylate cyclase could be directly effected by proteases leading to an increased activity. Finally a less likely explanation is that the difference could be because the tsK-NRK cells were maintained at 41°C, a nonphysiological temperature. This may have had an effect on the initial enzyme activities leading to a pre-activation of adenylate cyclase and/or PKC. However since the same enhancement of adenylate cyclase activity was seen using the T51B cell line that was cultured at 37°C, it is not likely that the culture temperature has an effect. Time did not permit further analysis of these problems.

Figures 4 to 8 are combinations of different individual experiments and the mean of adenylate cyclase activity is shown. In many cases it was not possible to show a significant enhancement after TPA treatment. The only conclusion that can be made from these experiments is that activation of PKC probably does modify the cyclic AMP

system in these cells since there was either no effect or an enhancement. The TPA pretreatment did not give a decrease in adenylate cyclase stimulability.

The first experiments, although not too much emphasis can be put on them, give an indication that it is unlikely that the enhancement of adenylate cyclase activity by PKC activation is due to G_i inactivation. This is drawn from the results in figure 4, in which TPA pretreatment did not abolish the inhibition of adenylate cyclase activity by high $GTP\gamma S$ concentrations when the assay was performed at low Mg^{2+} concentrations. Further studies will have to be done to prove these speculations.

The initial hypothesis that the effect of activated PKC and the presence of p21 K-*ras* cause a deactivation of the inhibitory pathway of adenylate cyclase therefore was not confirmed. There was no evidence that activation of PKC caused an inhibition of G_i . After the first series of experiments it was decided to investigate the effect of PKC activation on adenylate cyclase activity. Because of the variability observed in the *in vitro* TPA treatments it was necessary to change the system in which the cross-talk between adenylate cyclase and PKC could be studied. A liver cell line exists that was shown to have a normal responding adenylate cyclase system and PKC activity that responds to agents such as phorbol esters. Furthermore, the growth of these cells has been shown to be regulated by growth factors, Ca^{2+} and cAMP.

4.1.2 T51B cell/adenylate cyclase activity (*in vitro*).

Due to the problems mentioned above a new approach was taken. TPA had been shown to stimulate DNA synthesis in T51B rat liver cells (60). Furthermore the proliferation of these cells had been shown to be regulated by cAMP and Ca^{2+} (59).

Therefore these cells were chosen for future experiments.

Adenylate cyclase activity *in vitro* in the T51B cells was shown to give a normal response to hormonal stimulation by agents such as the β -adrenergic agonist isoproterenol (IPR) and glucagon. The stimulation by glucagon in the presence of GTP was only 1.5 fold above that seen with GTP alone. Stimulation with IPR showed a 5 fold increase over that obtained with GTP alone. Due to this greater stimulation IPR was chosen for the next series of experiments.

4.1.3 Effect of *in vivo* TPA treatment on cyclic AMP synthesis.

To demonstrate effects of TPA a whole cell adenylate cyclase assay was used. For the next part of this project an *in vivo* prelabelling assay was used in which the intracellular ATP pool was labelled by incubating the cells with ^3H -adenine. The advantage of this assay is that it measures the *de novo* synthesis of cAMP (i.e. adenylate cyclase activity in intact cells) without any disruption of the cellular structure. This should eliminate the problems with inconsistency due to disruption of the cellular integrity. The activation of PKC will no longer be effected by homogenization and preincubation but represent a more natural system responding to agents added to the intact cells.

Earlier reports by Houslay had shown that treatment of hepatocytes with TPA led to an inhibition of glucagon stimulation of adenylate cyclase activity (76). The same group later showed that this effect was cAMP independent and likely due to a change in the interaction between the glucagon receptor and the stimulatory G protein, G_s (77).

Using the T51B rat liver cell line, it was shown that TPA caused an enhancement

of the cAMP production in response to the cAMP elevating agents tested. The time course and dose response experiments showed that cAMP production is specifically enhanced by PKC activating agents. The effect of TPA was only seen in situations where adenylate cyclase was activated. TPA treatment did not change the time or the dose that caused the maximum stimulation but merely enhanced the maximum response. The concentration of TPA that induced the enhancement of cAMP production was identical to that which is reported to give a maximum stimulation of PKC. The phorbol ester specificity of the enhancement is also as would be expected from PKC activating ability.

The prelabelling assay proved to be very consistent with respect to the TPA enhancement seen on adenylate cyclase activation in response to agents such as IPR and C.T.. The actual value for the conversion ratio varied between experiments since it is dependent on the specific activity of the ^3H -ATP pool and the amount of cellular protein. The level of the ^3H -ATP pool was always determined in experiments even though the intracellular ATP concentration was not measured.

In the experiments performed on hepatocytes by Houslay's group the activation with TPA was different. The incubation was a 15 minute pretreatment of cells prior to measurement of adenylate cyclase activity in isolated membranes. This difference in methods of measuring adenylate cyclase could be one of the reasons for the different results. Furthermore they later showed that the effect on adenylate cyclase was due to an uncoupling of the receptor from the stimulatory G-protein. The TPA effect on T51B cells is different from that of Houslay in that the time course experiments showed no inhibition at times up to 60 minutes (data not shown). Finally the cell lines used were different. Houslay's group used primary hepatocyte cultures while the experiments in this study were performed on an established rat liver cell line. It is not unlikely that

properties or quantities of adenylate cyclase and/or PKC change as a cell line such as the T51B cell line goes through an increasing number of passages.

4.1.3.1 Effect of TPA treatment on β -adrenergic stimulation (*in vivo*).

The activation of cyclic AMP synthesis by β -adrenergic agents was enhanced by TPA. This enhancement was specific to the activation mediated via the β -adrenergic receptor as seen by the fact that the TPA effect was inhibited by the β -adrenergic antagonist propranolol (table 1). The enhancement was also independent of the level of stimulation. The fold stimulation by TPA was between 1.3-1.5 with adrenergic agents that stimulated cyclic AMP production between 3-30 times above basal levels (table 1).

4.1.3.2 Various effects of TPA.

From the literature there are conflicting results with respect to the interaction of PKC and adenylate cyclase. Enhancement by TPA of adenylate cyclase has previously been shown using the S49 lymphoma cell line. Phorbol esters were shown to enhance cyclic AMP production in response to cholera toxin in wild type S49 cells (49). Their conclusion was that TPA causes a facilitation of the interaction of α_s -GTP with adenylate cyclase via phosphorylation of one or more components of the adenylate cyclase system (possibly the catalytic component or α_s). Others have shown that TPA treatment of S49 cyc- cells impairs GTP-dependent and hormone-induced inhibition of adenylate cyclase (52,78). The conclusion from these experiments was that TPA treatment caused a phosphorylation of G_i impairing its action. It is further speculated

that this inactivation of G_i involves increased hydrolysis of bound GTP to GDP and association of α and $\beta\gamma$ -subunits.

Other conflicting results have been reported on the effect of activated PKC on the adenylate cyclase system *in vivo* and *in vitro* depending on the cell type used as mentioned in the introduction. TPA treatment of intact human platelets largely impairs the GTP-dependent, hormone-sensitive inhibitory pathway of adenylate cyclase *in vitro* (78). The same group later showed that incubation of purified PKC with human platelet membranes resulted in phosphorylation of a 41 kDa protein corresponding to the inhibitory G-protein α -subunit, α_i (52).

To verify if the effect of TPA treatment on T51B cells was caused by inactivation of G_i , addition of TPA to the cells should result in a decreased inhibition of adenylate cyclase activity. If that is the case TPA treatment should abolish the effect of agents that activate the inhibitory pathway. All attempts to show any effect of agents activating the inhibitory pathway of adenylate cyclase in the T51B cell line were unsuccessful. This could be due to a lack of receptors to the agents tested such as somatostatin and angiotensin II. Treatment with pertussis toxin will lead to a deactivation of G_i by ADP-ribosylation of α_i , causing an increase in adenylate cyclase activity. It has also not been possible to show any effect of pertussis toxin on cyclic AMP synthesis *in vivo*. Treatment of whole cells with pertussis toxin which have resulted in an increased cAMP synthesis *in vivo* in other cells did not have any effect on cAMP levels in the T51B cells. Control experiments were performed in which an *in vivo* pertussis toxin treatment was first performed for times from 0-22 hours. This was followed by an *in vitro* treatment of the plasma membranes with pertussis toxin in the presence of ^{32}P -NAD. These experiments showed that there is a substrate for pertussis toxin *in vitro* with a molecular size

corresponding to α_i . However the *in vivo* pretreatment did not decrease in intensity on the autoradiograph to a level below that of untreated cells (figure 16). If pertussis toxin had caused an ADP-ribosylation in whole cells the labelling of the second P.T. treatment *in vitro* should have decreased with time since α_i only has one site for ADP-ribosylation. The reason for the absence of an effect of P.T. treatment is uncertain. One explanation could be a problem with penetration of the toxin through the cell membrane so that the toxin never gets in contact with its substrate in the cell. However the lack of effect of other agents that act through the inhibitory receptors may indicate that T51B cells have a reduced G_i activity. The P.T. substrate identified in the *in vitro* experiments could be α_o that also is a substrate for P.T. and has a molecular size about 40 kDa. α_o has been proposed as the ϵ -subunit of another G-protein involved in membrane ion channels commonly found in cells. The molecular weight of α_i and α_o are so similar that they do not separate well on a normal SDS-PAGE gel. The best way to distinguish the two proteins are a combination of 2-D gel electrophoresis and immuno-chemistry. There are antibodies that will only recognize α_i . The presence of α_o instead of α_i does not explain the lack of ADP-ribosylation *in vivo*.

TPA has been shown to induce phosphorylation of the catalytic subunit of adenylate cyclase in frog erythrocytes *in vivo*. Also, purified PKC can phosphorylate the catalytic subunit *in vitro* (50). The phosphorylation of the catalytic subunit led to an enhanced adenylate cyclase activation in response to GTP, prostaglandin E_1 (PGE_1), IPR and NaF (50).

Phosphorylation of the catalytic subunit seems to be a likely explanation for the effect of TPA on the T51B cells. This conclusion is based on the fact that TPA treatment enhances any response leading to an elevated cyclic AMP level. Activation

through receptors, through the stimulatory guanine nucleotide binding protein G_s , or directly on the catalytic subunit are all enhanced by TPA treatment. Further evidence for such a site of action comes from the experiment in which the combined activation of adenylate cyclase by FSK and C.T. was investigated (table 2). If TPA treatment inactivated G_i , then activation by the other two pathways should still be enhanced by TPA addition. However, when the stimulatory pathway was activated by cholera toxin and the catalytic component was activated directly by FSK, the activity could not be further enhanced by the addition of TPA.

No effect of any G_i stimulation or inhibition has been observed in the T51B cells. Since G_i can not be activated by any natural means, it is unlikely that the effect of PKC activation on adenylate cyclase is due to α_i phosphorylation. However, time did not permit rigorous proof of this hypothesis.

4.1.3.3 Phorbol ester specificity.

The effect of TPA on adenylate cyclase is likely due to PKC activation. The concentration of TPA used in these experiments was 100 nM. This was shown to give a maximum effect. Activation of protein kinase C by TPA has been shown to be maximal at the same concentration (40). The specificity of the phorbol ester enhancement was also identical to their ability to activate PKC (40). The inactive phorbol ester 4 α PDD used here was shown to have no effect on the IPR activation of cyclic AMP production. The diacylglycerol DiC₈ enhanced the cAMP levels to the same extent as did TPA. This strongly supports the hypothesis that the action of TPA is through PKC activation.

4.1.3.4 TPA effect on PKC.

In the cell PKC is found mainly anchored in and loosely associated with the plasma membrane. PKC can be translocated to and tightly associates with the plasma membrane after stimulation by phorbol esters. Experiments were performed to try to clarify which compartment of PKC is responsible for the enhancement of the cAMP-system.

The time course for the TPA induced translocation of PKC showed that this is a relatively slow process. There was no significant disappearance of PKC activity in the soluble fraction until after 15 minutes incubation and it appeared to level off at about half of the original level after 20 minutes. This is as expected compared to results from other laboratories using various other cell lines (39). These results indicate that the enhancement of adenylate cyclase seen after TPA treatment is not caused by translocation of PKC to the membrane but by activation of PKC already present in the membrane. The translocation experiments were combined in figure 17 and the average of the means is shown with standard errors. The statistical analysis used is the independent student t-test of the combined means. The translocation levelled off to just under half in all cases and the decrease in activity was significant after about 20 minutes. The decrease in PKC activity seen after addition of TPA is likely due to a redistribution within the cells likely a translocation to the membrane. This is further supported by the fact that the basal level of phosphorylation of histone in the assay did not change over the course of the experiment. Hence, it is likely the PKC fraction

anchored in the plasma membrane that is producing the effect on the adenylate cyclase system.

4.1.3.5 Membrane PKC activity.

Attempts were made to measure the PKC activity in the membranes. The first approach was to measure the activity by extracting PKC from the membranes with nonionic detergents. Before the PKC activity can be measured the detergent has to be removed for example by ion exchange chromatography using a DEAE-sephacel column. As can be seen from table 3, these experiments did not give any conclusive information. This is probably due to instability of PKC while the sample is eluted off the column. Indications of this can be seen by the fact that the sample eluting off from the slowest column in general had the lowest PKC activity. In addition, some of the samples from slow columns with low PKC activity also had a high background kinase activity. Due to these difficulties a new approach was taken.

The next approach was to try an *in situ* kinase assay. The samples were applied to a non-denaturing poly-acrylamide gel which separated PKC from other kinases and from the detergent used to extract it from the membranes. The whole gel was incubated with protamine and ^{32}P -ATP followed by autoradiography. However there was a lack of separation of the samples into individual protein bands and the following incubation led to even more diffuse bands. For these reasons this method was abandoned.

Finally a newly developed method to measure membrane PKC activity utilizing a natural substrate isolated from S49 cyc- cells was tried. The plasma membranes were isolated and they were incubated with the substrate and ^{32}P -ATP. The membranes and

the substrate were isolated by centrifugation. The supernatant containing the S49 cyc-substrate was identified by SDS-PAGE and autoradiography. Due to problems of stability of the substrate and other start-up problems this method did not give any usable results. However it has since been used successfully to verify that membrane associated PKC activity is activated within 5 minutes of exposing T51B cells to TPA.

An alternative to directly measure PKC activity is to measure phorbol ester binding. The PDBu binding assay performed on the different fractions of the T51B cells showed that the amount of PKC in the soluble and the membrane fraction is about equal. This indicates that the amount of PKC activity in the membrane should be measurable if the instability problem could be solved. The amount of PKC present in the membranes should be large enough to cause the effect seen on the adenylate cyclase activity through activation alone so no translocation is necessary. It is not possible to measure the changes in PKC distribution after TPA treatment of the cells using the PDBu binding assay. This is because TPA binds more strongly to the same binding site on PKC as does PDBu. In fact non-specific PDBu binding is measured by determining the binding of the ^3H labelled phorbol ester in the presence of a 100 fold excess cold phorbol ester.

4.2 Conclusion and future direction.

The following conclusions can be drawn from the results obtained from this study:

1. TPA treatment of T51B cells causes an enhancement of all the cyclic AMP stimulating agents tested.

2. The effect of TPA treatment is through activation of PKC already present in the membrane and not through translocation.

3. The component that is most likely affected by the PKC activation is the catalytic subunit of the adenylate cyclase system but the stimulatory G-protein (G_s) can not be ruled out.

The effect of the PKC activation is likely to phosphorylate one or more of the components of the adenylate cyclase system. To further study this hypothesis it would be necessary to show a direct phosphorylation of one or more components of the adenylate cyclase system *in vivo* and *in vitro*. It would be important to show that the different components of the adenylate cyclase system can be phosphorylated by partially purified PKC *in vitro*. Furthermore phosphorylation of the adenylate cyclase system components should be shown by prelabelling the cells with ^{32}P and stimulating PKC with agents such as phorbol esters. Identification of the different components of the adenylate cyclase system can be done by separating the proteins by 2-dimensional-electrophoresis followed by Western-blotting and identification using specific antibodies and/or radio labelled ligands such as forskolin.

To show how PKC is causing the enhancement of the adenylate cyclase activity it is also important to develop a method sensitive enough to measure the membrane activity in resting cells.

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