Protein kinase C modulates mitogenic signalling by Epidermal Growth Factor in T51B rat liver cells.

by

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DEDICATION

To my parents for their love, and...

To my brother Rajesh Sharma, who is always in our memories.

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ACKNOWLEDGEMENTS

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ABSTRACT

The regulation of the proliferation of non-neoplastic T51B rat liver epitheloid cells involves a complex interplay between the epidermal growth factor (EGF) and the protein kinase C (PKC) signal-transduction pathways. T51B cells can be made proliferatively quiescent upon prolonged serum deprivation, which results in their growth-arrest at the G1 phase of the cell cycle. Addition of EGF (1.5ηM) or, re-addition of serum (10% BCS) to the conditioned medium initiates DNA synthesis, within 8 hours, which reaches a maximum by 18-24 hours, when a significant number of cells are in the S phase. The DNA synthetic responses of EGF and serum are additive. Growth factor or, serum-stimulated DNA synthesis is followed by an increase in cell number. The mitogenic response of EGF can be blocked by 15-20μM genistein, a tyrosine kinase inhibitor.

EGF activates particulate PKC within 15 minutes, and the stimulation reaches a maximum by 35-45 minutes. PKC activity then remains elevated for up to 24 hours. The tumour promoting phorbol ester, TPA, which is known to activate PKC, is not mitogenic to T51B cells. TPA modulates the responses of EGF or, serum depending upon the culture conditions. When TPA is added to serum-deprived cells in the presence of EGF or serum, it potentiates DNA synthesis within 18 hours, during which time it down-regulates PKC. On the other hand, if TPA is present in the medium during serum-deprivation, the response to EGF in these PKC-inactivated cells is as mitogenic as that caused by serum. However, TPA

pretreatment makes the cells less responsive to EGF or serum in that, the net DNA synthesis is significantly reduced (>30%) in control, and in treated cells. The inhibitory response of genistein is slightly attenuated in the presence of EGF and TPA.

The data presented in this thesis suggest that the mitogenic response of EGF is mediated by its receptor-associated tyrosine kinase activity. PKC modulates this response; down-regulation of PKC results in enhanced growth of T51B cells.

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LIST OF ABBREVIATIONS

A431	an epidermoid carcinoma cell line
ATP	Adenosine triphosphate
BCS	Bovine Calf Serum
BME	Eagle's Basal Medium
c-fos	cellular fos (a nuclear proto-oncogene)
c-myc	cellular myc (a nuclear proto-oncogene)
cAMP	Adenosine 3, 5'-cyclic monophosphate
cDNA	Complimentary deoxyribonucleic acid
CSF-R	Colony Stimulating Factor-Receptor
DAG	Diacylglycerol
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGF-R	Epidermal Growth Factor-Receptor
EGTA	Ethylene bis(oxyethylene-nitrilo)-tetra-acetic acid
G	Guanine nucleotide binding
Gαs	G-stimulatory protein α-subunit
GDP	Guanosine diphosphate
GME	Glucose EDTA solution
GTP	Guanosine tri-phosphate
I-R	Insulin-Receptor
IGF	Insulin-like Growth Factor
IGF1-R	Insulin-like Growth Factor 1-Receptor
IP_3	Inositol-1,4,5-trisphosphate
KCl	potassium chloride
$MgCl_2$	magnesium chloride
mRNA	messenger Ribonucleic acid
Na_2HPO_4	sodium phosphate dibasic
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NGF	Nerve Growth Factor
PAGE	Polyacrylamide Gel Electrophoresis
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCA	perchloric acid
4αPDD	4-α-phorbol-12,13-didecanoate
PDGF	Platelet Derived Growth Factor
PI	phosphatidylinositol
PIP_2	phosphatidylinositol-4,5-bisphosphate

PKC	protein kinase C
PKM	a catalytic fragment of PKC
PLC	phospholipase C
PLD	Phospholipase D
PMSF	Phenylmethylsulphonylfluoride
PNF	post nuclear fraction
PS	phosphatidylserine
RNase	Ribonuclease
S49	a murine lymphoma cell line
SDS	sodium dodecyl sulphate
SH	Src Homology
T51B	a rat liver epithelial cell line
Tdr	deoxythymidine
TGFα	Transforming Growth Factor α
TPA	12-O-tetradecanoyl-phorbol-13-acetate
V-erbB	a virus nuclear oncoprotein
VVGF	Vaccinia Virus Growth Factor
WB	a nontransformed rat liver epithelial cell line

Chapter 1.

INTRODUCTION

Transmembrane signal-transduction is a complex process by which extracellular information is translated into an intracellular physiological response. Various growth factors stimulate quiescent cells to re-enter the cell cycle by binding to specific cell surface receptors which initiate a sequence of events leading to DNA synthesis and mitosis. The links between occupation of the cell-surface receptors and nuclear events remains poorly understood.

Two major signal-transduction pathways have been shown to be involved in cell growth stimulation:

1. Protein tyrosine phosphorylation: this pathway plays an important role in the mitogenic action of growth factors such as epidermal growth factor (EGF), Insulin like growth factor-I (IGF-I), and platelet derived growth factor (PDGF) (Letterio, Coughlin and Williams, 1986; Chambard, Paris, L'Allemain and Oouyssegur, 1987).

2. Breakdown of phosphoinositides to give inositol trisphosphates and diacylglycerol: mitogenic signal-transduction of bombesin, thrombin and, bradykinin seems to depend mainly on this pathway; these growth factors activate phospholipase C (PLC) to hydrolyze phosphatidyl-inositol bisphosphate and the resultant inositol trisphosphate mobilizes calcium from the endoplasmic reticulum whereas diacylglycerol stimulates protein kinase C (PKC) (Nishizuka, 1984; Berridge and Irvine, 1989). PDGF and EGF, in addition to their tyrosine kinase

mediated processes, can induce rapid activation of PLC (Berridge, Heslop, Irvine and Brown, 1984; Pike and Eakes, 1987) and PKC (Rozengurt, Rodriguez-Pena and Smith, 1983; Kazlauskas and Cooper, 1988; Matuoka, Fukami, Nakamishi, Kawai and Takenawa, 1988) in some cell systems. The role of calcium in cell proliferation is clearly seen in the development of lymphocytes or, in fertilized eggs of fishes and sea urchins (Whitfield, Durkin, Franks, Kleine, Reptis, Rixon, Sikorska and Walker, 1987). Penetration of just one small sperm cell into an egg releases cortical Ca^{++} ions and cause a pH_i surge, which together sets off an explosion of events, such as the production of Ca^{++}-calmodulin complex, a burst of cAMP production, the prompt inactivation of the cytostatic factors that held the unfertilized egg in the metaphase II,etc., that ultimately results in chromosomal replication and mitosis (Woodland, 1982). In many cultured cell lines, progression through the cell cycle is Ca^{++}-dependent such that, lowering Ca^{++} concentrations in the medium brings them to growth arrest in the late G1 phase (Boynton, Kleine, Whitfield and Bossi, 1985). Increasing the Ca^{++} in the medium allows them to resume DNA synthesis (Whitfield, MacManus and Gillan, 1973).

1.1 PROTEIN KINASE C

Protein kinase C (PKC) was initially identified in rat brain by Nishizuka in 1977, as a novel type of serine- and threonine-specific protein kinase which was proteolytically activated from a proenzyme (Inuoue, Kishimoto, Yakai and Nishizuka, 1977; Takai, Kishimoto, Inuoe and Nishizuka, 1977). Soon after, it was realized that apart from proteolysis the enzyme could also be reversibly activated

in vitro by unsaturated diacylglycerol (DAG) in the presence of acidic phospholipids such as phosphatidyl-serine (PS) (Takai, Kishimoto, Iwasa, Kawahara, Mori and Nishizuka, 1979a). This observation provided the link between receptor-induced phosphatidyl inositol (PI) breakdown and protein phosphorylation (Kishimoto, Takai, Mori, Kikkawa, Mori and Nishizuka, 1979). In most tissues other than brain, this enzyme is recovered from the soluble fraction as an inactive form, and is translocated to membranes in a Ca^{++}-dependent manner when cells are stimulated (Kikkawa, Takai, Minakachi, Inohara and Nishizuka, 1982; Kraft and Anderson, 1981). It could be activated by limited proteolysis with the neutral protease calpain (Inuoe et al., 1977, Takai et al., 1977), which is active at the micromolar range (0.1μM-1μM) of calcium (Kikkawa, Kishimoto and Nishizuka, 1989; Millgren, 1980; Murachi, Tanaka, Hatanaka and Murakami, 1981). Calpain cleaves PKC in the presence of PS and DAG or the tumour promoting phorbol ester TPA implying that the activated form of PKC is a target of the calpain action (Kishimoto, Kazikawa, Shiota and Nishizuka, 1983). This limited proteolysis of PKC generates a catalytically fully active fragment, called PKM, and a regulatory fragment (Inuoe et al., 1977; Takai et al., 1977; Kishimoto et al., 1983; Hoshijama, Kikuda, Tanimoto, Kaibuchi and Takai, 1986; Lee and Bell, 1986; Huang and Huang, 1986). PKM is not always recovered from the cell, and is probably degraded further by the action of other proteases.

1.1.1 Molecular heterogeneity and Structural features of Protein kinase C family.

Nine members of the PKC family have been identified to date by molecular cloning (Bell and Burns, 1991; Parker, Cousseus, Totty, Rhee, Young, Chen, Stabel, Waterfield and Ullrich, 1986). Many cell-types express more than one sub-species of PKC in variable ratios, and their intracellular distribution may depend on the state of activation of the cells (Kikkawa et al., 1989). All the sub-species are widely distributed but enriched in the nervous tissues of animals (Huang, 1989). The isolation of PKC cDNA clones (Huang, 1989; Nishizuka, 1988) and the discovery of multiple PKC isozymes (Nishizuka, 1988; Schaap, Parker, Bristol, Kriz and Knopf, 1989) have established the molecular diversity of this enzyme family into two groups, (group A designated as PKC α, β and γ, and group B designated δ, ε, ζ, and η), based on the sequence similarity at several coding regions. The PKC βI and βII mRNAs are generated by alternative splicing of the most C-terminal exon of the β-gene producing two polypeptides (approx. 77 kDa.) which only differ in the sequence of their 50 amino acids at the C-terminal (Cousseus, Rhee, Parker and Ullrich, 1987; Kubo, Ohno and Suzuki, 1987; Ono, Fujii, Ogita, Kikkawa, Igarashi and Nishizuka, 1988). An analogous situation may exist for PKC ε, where PKC ε_2 differs from PKC ε_1 by the absence of the 240 N-terminal amino acids of PKC ε_1 and a substitution by a sequence carrying a stop codon, which results in a smaller PKC ε_2 by approximately 27 kDa. (Housey, O'Brian, Johnson, Kirschmeier and Weinstein, 1987; Ohno, Fujii, Ogita, Kakkawa, Igarashi and Nishizuka, 1988).

Four species (α, βI, βII and γ) were isolated from the initial screening of a variety of cDNA libraries (Parker et al., 1986; Knopf, Lee, Sultzman, Kriz, Loomis, Hewich and Bell, 1986; Kakkawa and Nishizuka, 1987). Three further sub-species (δ, ε and ζ) were isolated from a rat brain library by using a mixture of α, βII and γ cDNA as probes under low stringency conditions (Ono et al., 1988; Ono, Fujii, Ogita, Kikkawa, Igarashi and Nishizuka, 1987). The screening of the two epidermis cDNA libraries under reduced stringency with α, β, γ, ε and ζ probes identified PKC η, and PKC L, which was strongly expressed in skin and lung, and only weakly in brain (Bacher, Zisman, Berent and Livneh, 1991; Osada, Mizuno, Saida, Akita, Suzuki, Kuroki and Ohno, 1990). These findings indicate that newer subspecies of PKC might exist which are preferentially or exclusively expressed in other tissues than the ones used for screening so far. Whether each of these different subspecies play a specific role in cell type-specific processes like endocytosis, secretion, transmission of electric potentials, growth or differentiation, is unknown. All the members of the family are composed of a single polypeptide chain (Mr= 80,000 kDa. for the most common α and β isozymes, fig. 1) with each member of the group α-, βI-, βII- and γ-subspecies having four conserved regions C1 to C4, between which are the variable regions of lower homology (V1-V5) (Cousseus et al., 1986; Kikkawa et al., 1987; Nishizuka, 1989; Bell and Burns, 1991). The region C2 is conspicously absent from the δ, ε, ζ, η and L subspecies, whose activities are Ca^{++}-independent, suggesting that the C2 region confers Ca^{++}-dependence for the activity of Ca^{++}-dependent PKC isozymes (α, βI, βII and γ) (Bell and

FIGURE 1. STRUCTURAL FEATURES OF PROTEIN KINASE C SPECIES.

Group A (α, βI, βII, γ) species consist of two constant regions (C1, C2) in their regulatory domain, while there is only one such region C1 at the amino terminus (NH_2) of group B species (δ, ε, ζ). The C1 region contains a pseudosubstrate sequence (PS) and two cysteine-rich (Cys) repeats. The C3 region contains the ATP-binding site. Constant regions are flanked by the variable regions (V1-V5), V5 at the carboxyl terminus (COOH) (reproduced from Huang, 1989).

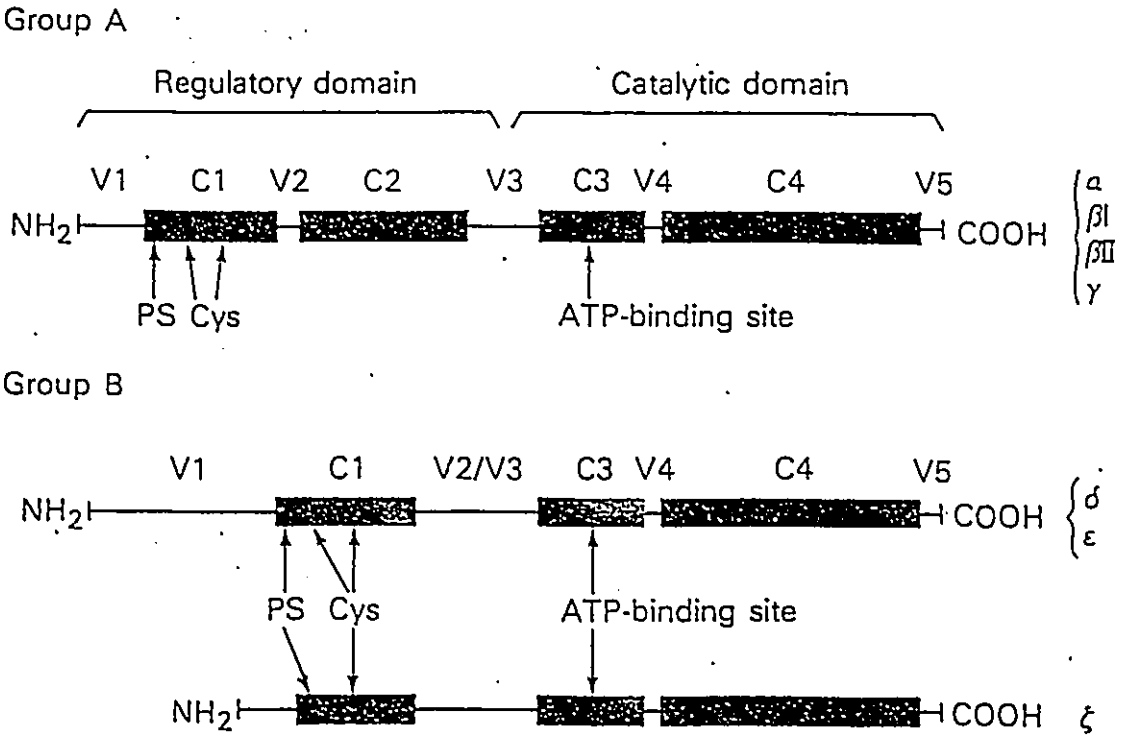

Group A
Regulatory domain
Catalytic domain
V1
C1
V2
C2
V3
C3
V4
C4
V5
NH2
COOH
α
βI
βII
γ
PS
Cys
ATP-binding site
Group B
V1
C1
V2/V3
C3
V4
C4
V5
NH2
COOH
δ
ε
PS
Cys
ATP-binding site
NH2
COOH
ζ

FIGURE 1

Burns, 1991). The N-terminal half of the polypeptide containing V1-C2 represents the regulatory domain (Mr= 32,000-38,000). It carries binding sites for the active phorbol esters (like TPA) (Huang et al., 1986; Kaibuchi, Fukomoto, Oku, Takai, Arai and Muramatsu, 1989) and a pseudosubstrate region. C1 contains a tandem repeat of a cysteine-rich sequences. This sequence is similar to the consensus sequence of a cysteine-zinc-DNA-binding finger that is found in many metalloproteins and DNA-binding proteins (Berg, 1986). Not all subspecies have two zinc-fingers (PKC ζ has one such structure), and the C1 domain is preceded by an N-terminal extension of varying length with the type ε and η having the longest extensions among the mammalian PKCs identified so far (Stabel and Parker, 1991). The regulatory domain also contains the DAG binding site, and segments which interact with phospholipid by either Ca^{++}-dependent or -independent mechanisms (Bell and Burns, 1991). There is no evidence of Zn-finger associated PKC binding to DNA (Ono, Fujii, Igarashi, Kuno, Tanaka, Kikkawa and Nishizuka, 1989).

The removal of the regulatory domain by mild proteolysis leads to constitutive and activator-independent kinase activity (Mr=45,000-50,000) (Inoue et al., 1977; Mochley-Rosen, Henrich, Cheever, Khaner and Simpson, 1990). The carboxyl terminal half of each enzyme containing the regions C3 and C4 constitute the catalytic domain and shows large clusters of sequences that resemble many protein kinases (Nishizuka, 1988). The conserved region C3 has an ATP-binding site Gly-x-Gly-xx-Gly----Lys (Hunter and Cooper, 1985). The variable V3 region of the α species contains a potential Ca^{++}-binding site. However, this sequence is not

conserved in either β or γ-subspecies (Huang, 1989). The V3 region also contains the calpain and trypsin-sensitive sites of these enzymes (Huang, Yoshida, Melo-Cunha, Beaver and Huang, 1989). Proteolysis cleaves at V3 to separate the catalytic and regulatory domains, and makes the catalytic fragment constitutively active in the absence of Ca^{++}.

1.1.2 Differential expression and distribution of PKC isozymes.

Early studies using autoradiographic analysis after tritiated phorbol-12,13-dibutyrate-binding have shown an uneven distribution of PKC in the brain (Worley, Bareban, Desouza and Snyder, 1986). Northern blot analysis with specific oligonucleotide probes and in situ mRNA hybridization has also suggested a tissue-specific expression of some PKC subspecies. Using a combination of biochemical, immunological, and cytochemical analyses with subspecies-specific antibodies indicate that the relative activity and differential pattern of expression of multiple PKC subspecies vary from cell to cell (Noar, Shearman, Kishimoto and Nishizuka, 1988). PKC α is widely distributed in many tissues and cell-types. Most tissues, including liver, kidney, spleen, and testis, also contain β-subspecies in variable amounts (Kikkawa et al., 1989). Some tissues such as, heart, lung, and platelets possess several undefined subspecies (Kikkawa et al., 1989). T-lymphocytes, and various cloned cell lines express α-, βI-, and βII- subspecies in different ratios, whereas cultured fibroblast express α- subspecies only (Kikkawa et al., 1989). PKC βI and PKC βII also display differential expression in the brain and many other tissues, including endocrine tissues such as the pituitary gland

(Noar et al., 1989) and pancreatic islets. In the rat cerebellar cortex, PKC βI- and βII- subspecies are localized differentially. PKC-γ is expressed solely in the brain and spinal cord and is not found in other tissues and cell types (Huang, Yoshida, Nakayabashi and Huang, 1987). Mitochondria generally lack or poorly express PKC (Huang et al., 1987). The distribution and biochemical properties of δ-, ε-, ζ-, η-, and L- subspecies have not been elucidated, but their expression in several tissues and organs have been demonstrated (Housey et al., 1987; Ohno et al., 1988).

1.1.3 Activation of Protein kinase C.

Activation of PKC requires the 'unmasking' of the regulatory domain from the catalytic site. The conformational change is brought about by the interaction of PKC with cofactors. The monomeric PKC, four molecules of PS, one molecule of DAG or phorbol ester, and at least one Ca^{++}-ion combine to form an active enzyme (Hannun, Loomis and Bell, 1986). The PKC, Ca^{++}, PS, and DAG or phorbol ester interaction could also lead to a delayed formation of a membrane-inserted or irreversibly bound form of PKC, whose activity is independent of the exogenously added Ca^{++} and phorbol ester (Bazzi and Nelsestuen, 1988). Like many kinases PKC can undergo autophosphorylation (Nishizuka, 1988).

Diacylglycerol is the most potent physiological activator of PKC. Naturally occurring 1,2-sn-DAG, but not its 2,3-sn-enantiomer nor the 1,3-sn-diastereomer, is capable of activating PKC (Boni and Rando, 1985). DAG may be derived from the hydrolysis of phosphoinositides or phosphatidylcholine by each of their specific

phospholipases (Exton, 1988). Berridge, in 1983, demonstrated that the first phosphoinositide to be cleaved following cell stimulation was phosphatidyl Inositol-4,5-bisphosphate (PIP_2), forming DAG and inositol-1,4,5-trisphosphate (Berridge, 1983). Binding of extracellular agent such as hormone, neurotransmitter, or growth factor to a specific cell-surface receptor activates a heterotrimeric GTP-binding protein (G-protein) which is functionally analogous to that of the cAMP pathway and is presumed to consist of an α, β and γ subunits (Rana and Hokin, 1990). When activated, GTP exchanges for bound guanosine diphosphate (GDP), the GTP.α complex dissociates from the $\beta.\gamma$ dimer. The GTP.α complex stimulates a second-messenger-forming enzyme, in this case a phosphatidyl-inositol-specific-phospholipase C that cleaves PIP_2, releasing two second messengers DAG, and Inositol-trisphosphate (IP_3) (fig. 2). IP_3 is a small water soluble molecule, which binds to its specific receptor on the endoplasmic reticulum (or related organelle), causing release of stored Ca^{++} (Rana and Hokin, 1990). DAG remains membrane-bound and activates PKC, while the increase in free Ca^{++} concentration may enhance PKC activation as well as stimulate a number of other Ca^{++}-dependent enzymes and Ca^{++}-binding regulatory proteins. DAG can also be derived from lipids other than phosphoinositides (Ganong, 1991; Exton, 1990). Phosphatidylcholine, upon activation of PLC or, PLD followed by phosphatase action also produces DAG (Rana and Hokin, 1990). Another source of DAG is generated by the action of insulin and Nerve Growth Factor (NGF) (Chan, Chao and Saltiel, 1989). Insulin and NGF evoke the hydrolysis of a novel inositol-containing glycolipid, glycosyl-phosphatidyl-inositol to generate a structurally distinct DAG and an inositol phosphate glycan. Phospholipase A_2 catalyzes the

FIGURE 2. PHOSPHATIDYL-INOSITOL, PHOSPHATIDYL-CHOLINE, AND ARACHIDONATE PATHWAYS TO ACTIVATE PROTEIN KINASE-C.

Inositol phospholipids (PI) are hydrolyzed by phospholipase C (PI-PLC) to give inositol phosphates (IP_n), which mobilize intracellular calcium from endoplasmic reticulum (ER); the other product diacylglycerol (DG) activates PKC. When phosphatidylcholine is the substrate, either PLC and/or phospholipase D (PLD) coupled to phosphatidate phosphatase (PA-P), which carries out the same reaction as PLC, but on phosphatidic acid (PA), produces DG. Phospholipase A_2 (PLA_2) catalyzes the conversion of PC into arachidonate (AA) and a lysophospholipid, Lyso-PC. Choline phosphate (Choline-P) is one of the by-products of PC pathway. Phosphatidylinositol-4-phosphate PI(4)P, phosphatidylinositol -4,5-bisphosphate ($PI(4,5)P_2$), inositol-1,4,5-trisphosphate ($I(1,4,5)P_3$) (adapted from Dennis, Rhee, Billah and Hannun, 1991; Rana and Hokin, 1990).

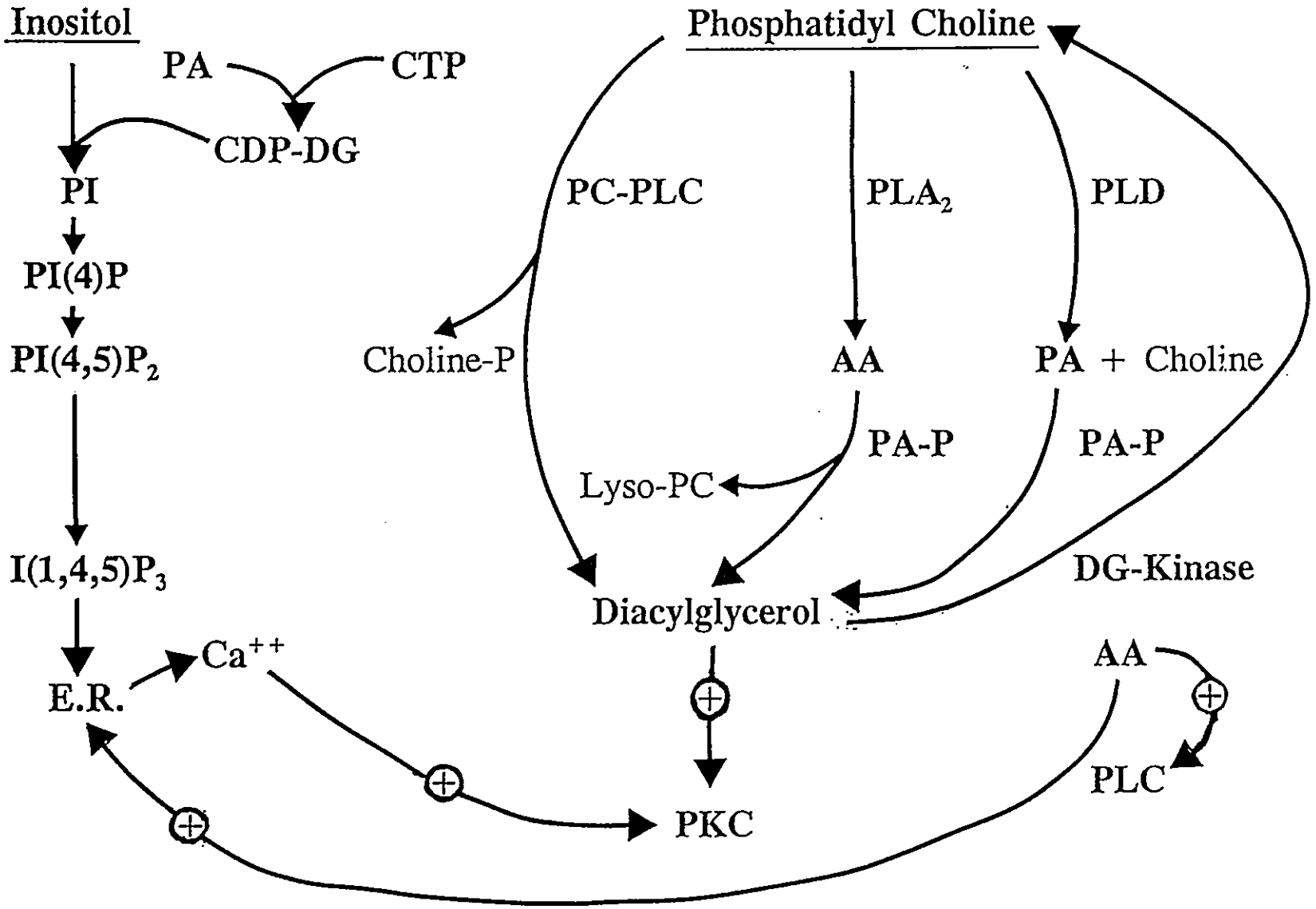

Inositol
PA
CTP
CDP-DG
PI
PI(4)P
PI(4,5)P2
I(1,4,5)P3
E.R.
Ca++
Phosphatidyl Choline
PC-PLC
PLA2
PLD
Choline-P
AA
PA + Choline
PA-P
PA-P
Lyso-PC
DG-Kinase
Diacylglycerol
AA
PLC
PKC

FIGURE 2

hydrolysis of phospholipids to release lysophospholipid and free fatty acids, usually arachidonic acid. Free arachidonate and lipoxin A (Ganong, 1991), an oxygenated metabolite of arachidonic acid, as well as lysophosphatidylcholine (Oishi, Raynor, Charp and Kuo, 1988) are activators of PKC. DAG dramatically increases the affinity of PKC for Ca^{++} and thereby renders it fully active without a net increase in the Ca^{++} concentration (Kaibuchi, Takai and Nishizuka, 1981). Tumour-promoting phorbol esters, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA), have a structure very similar to diacylglycerol and activate PKC directly both in vitro and in vivo (Nishizuka, 1986). Like DAG, phorbol ester dramatically increase the affinity of this enzyme for Ca^{++}, resulting in its full activation at physiological Ca^{++} concentrations. Several lines of evidence support the notion that PKC is the receptor for tumour-promoting phorbol esters (Ashandel, Staller and Boutwa, 1983; Ashendal, 1985). The effect of phorbol esters was initially described as "translocation" of PKC to the membrane (Kraft and Anderson, 1983) since phorbol esters caused a shift in the PKC population from the cytosolic to the membrane-bound form.

1.1.4 Short-term and Long-term dual actions of Protein kinase C.

Several physiological functions have been assigned to PKC, including its involvement in secretion and exocytosis, modulation of ion-conductance, interaction and down-regulation of receptors, smooth muscle contraction, gene expression, and cell-proliferation (Schlessinger, 1988; Moolener et al., 1988). However, the biological roles of individual PKC subspecies is little known at present. The

synergistic interaction between PKC and Ca^{++} pathways underlies a variety of cellular responses to external stimuli (Nishizuka, 1984). A short-term positive action of PKC is followed by a negative feedback control over various steps of the cell-signalling processes, operating to produce short-term and long-term cellular responses. In short-term responses, for instance, a major role of PKC appears to be to decrease the Ca^{++} concentration. In various cell-types, PKC activates the Ca^{++}-transport ATPase and the Na^{+}/Ca^{++} exchange protein, both of which remove Ca^{++} from the cytosol (Nishizuka, 1986). PKC may inhibit Ca^{++} mobilization by blocking the receptor-mediated hydrolysis of inositol phospholipids, thereby blocking the action of the Ca^{++} signalling pathway. The recent observations that TPA inhibits the frequency of repetitive Ca^{++} transients recorded from single cells (Woods, Cuthberston and Cobbold, 1987) could be explained for such an inhibitory action of PKC. The positive action of PKC seems to be in the control of gene expression, such as induction of the interleukin-2 receptor and the activation of some proto-oncogenes, such as c-fos and c-myc (Nishizuka, 1986). The appearance of second messengers such as DAG, is normally very transient (within few seconds to a minute). The negative feedback role of PKC is extended to long-term responses such as cell proliferation. The receptor for epidermal growth factor has repeatedly been shown to be phosphorylated by PKC, resulting in a rapid decrease in high affinity binding of EGF as well as inhibition of the ligand-induced tyrosine phosphorylation (Schlessinger, 1986). It is plausible that the treatment of cells with TPA frequently causes depletion of the PKC molecule and thereby relieves the cell from down-regulation of the growth factor receptor,

so that uncontrolled cell-proliferation might occur in the presence of the mitogenic stimulus. Such a dual effect of PKC is also seen in T-lymphocyte proliferation, where the synergistic action of TPA and Ca^{++}-ionophore has been demonstrated (Kaibuchi, Takai and Nishizuka, 1983). The TPA and Ca^{++}-ionophore activated PKC has a negative feedback action in these cells, as it phosphorylates and thereby induces the down-regulation of the T-cell receptor, preventing any further stimulation by antigen to proliferation (Cantrell, Davies and Crompton, 1985). A consequence of chronic activation of PKC in vivo is down-regulation. This has been shown to be due to increased proteolysis (Woodgett and Hunter, 1987; Young et al., 1987). PKC is cleaved by the Ca^{++}-dependent protease, calpain, in the presence of PS and DAG or TPA, implying that the activated PKC is a target of calpain action (Kishimoto et al., 1983). Several subspecies of PKC co-expressed in a single cell type have been found to disappear at different rates upon treatment with TPA (Nishizuka, 1988), which may reflect the substrate specificity of the calpain action. The mechanism involved in the increased proteolysis has not been elucidated, however the increased susceptibility of the active conformation of PKC to proteases in vitro (Kishimoto, Mikawa, Hasimoto, Yasuda, Tanaka, Tommaga, Kuroda and Nishizuka, 1989) suggests that in this passive manner, increased proteolysis occurs in vivo. Perhaps distinct pathways are involved for different PKC isozyme down-regulation.

1.2 EPIDERMAL GROWTH FACTOR.

One of the best studied growth regulators is epidermal growth factor (EGF),

initially identified and characterized by Cohen and his coworkers (Carpenter and Cohen, 1979). EGF is a single polypeptide chain of 53 amino acid residues, devoid of alanine, phenylalanine, and lysine (Carpenter and Cohen, 1979). The three disulphide bonds in mouse EGF are required for biological activity (Taylor, Mitchell and Cohen, 1972) and exhibits identical biological activities and some common antigenic sites with the human EGF (Carpenter and Cohen, 1979). EGF is constitutively expressed in kidney and salivary gland and detectable in plasma, platelets, and urine (Carpenter and Wahl, 1990). EGF is a potent mitogen for a variety of cells both in vitro and in vivo (Hanguel-Demouzon, Csermely, Zoppini and Kahn, 1992; Schlessinger, 1988). It has also been found to be an inhibitor of growth and proliferation (Macleod, Luk, Castagnola, Cronin and Mendelson, 1986). A number of EGF-like molecules, which belong to the family of peptide mitogens are encoded by distinct genes (Carpenter and Cohen, 1990). These include the Transforming growth factor alpha (TGF-α), Vaccinia virus growth factor (VVGF) and amphiregulin (Carpenter and Wahl, 1990). They resemble EGF, in that, they have cysteine-rich sequences, and they bind to the EGF-receptor with high affinity, inducing mitogenic responses in EGF-sensitive cells.

The biological activity of EGF can be divided into early and delayed responses. The short-term biological effects of EGF can be measured within seconds to minutes and include effects on calcium flux (Macara, 1986), activation of phospholipid metabolism and stimulation of the expression of a set of cellular genes called "immediate-early" genes (Hanguel-Demonzon et al., 1992). Two of these genes are the nuclear proto-oncogenes c-fos and c-myc which are activated

by EGF and a variety of other polypeptide growth factors (Muller, Bravo, Burckhardt and Curran, 1984), and have been associated with stimulation of cell proliferation by hormones and serum (Muller et al., 1984). Stimulation of DNA synthesis in cultured cells is regarded as a delayed effect of EGF, since it starts several hours (6-8 hours) after the treatment with EGF (Hanguel-Demouzon et al., 1992).

1.3 THE EGF RECEPTOR (EGF-R).

EGF-R belongs to a family of receptors, including the avian erythroblastosis virus protein (v-erbB), c-erbB2 (the neu-oncoprotein) and c-erbB3 (Merlino, 1990). All these proteins possess tyrosine kinase that are highly conserved. The EGF-R is also related to other cell-surface tyrosine kinase families including the family of platelet-derived growth factor receptor (PDGF-R), colony stimulating factor receptor (CSF-R), and the family containing the insulin receptor (I-R) and the insulin-like growth factor 1 receptor (IGF1-R) (Carpenter, 1987).

1.3.1 Structure of EGF-R.

The mature EGF receptor is composed of a single polypeptide chain of 1186 amino acids residues (Mr= 170 kDa.) and an N-linked oligosaccharide (Carpenter and Cohen, 1990). It consists of three domains: a 621 amino acids extracellular amino portion that is heavily glycosylated, contains a high percentage of cysteine residues, and is capable of binding EGF, TGFα, and VVGF at high affinity. This region is anchored to the plasma membrane by a single 23 amino

acid hydrophobic transmembrane region, and a 542 amino acid intracellular carboxy domain that contains an ATP-binding site and a potent tyrosine kinase (Carpenter, 1987). The integrity of the transmembrane region seems less important for signal-transduction (Carpenter and Cohen, 1990) as it was shown that EGF was able to enhance the kinase activity and stimulate various responses in cells expressing mutant EGF-R bearing altered transmembrane regions (Kashles, Szapary, Bellot, Ullrich, Schlessinger and Schmidt, 1988). Thus, the transmembrane region plays a passive role in signal-transduction. The cytoplasmic domain has high homology to the avian erbB oncogene product (Ullrich and Schlessinger, 1990), which are derived from the avian gene for the EGF-R (Maihle and Kung, 1988). The cytoplasmic domain contains a 300 amino acids region that is homologous to the catalytic domains of the protein tyrosine kinase encoded by the src gene family of oncogenes (Schlessinger, 1986). It contains a consensus lysine (Lys721) residue and a consensus sequence Gly-x-Gly-xx-Gly-x(15-20)-Lys, which together with the Lys721 form part of the ATP-binding site (Schlessinger, 1988; Yarden and Ullrich, 1988). Replacement of the consensus Lys residue of the ATP-binding site in the EGF-R completely abolishes its kinase activity in vitro and in vivo (Kashles et al., 1988). There are four autophosphorylation sites located in the C-terminal portion that include Tyr 1068, Tyr 1148, Tyr 1173, and Tyr 1086 (White, 1991). Removal of the C-terminus may increase the biological sensitivity of the EGF-R, suggesting that the C-terminal phosphorylation sites may play a regulatory role through competitive inhibition rather than through activation (White, 1991) of the EGF-R.

1.3.2 EGF-Receptor trafficking.

The EGF-R is initially monodisperse on the surface of target cells. When EGF binds to the EGF-R, it rapidly becomes associated with and concentrated in clathrin-coated pits. After one to two minutes, EGF-R and bound EGF are internalized in multivesicular bodies. These bodies are degraded within the lysosomal compartment (Felder, Miller, Moehren, Ullrich, Schlessinger and Hopkins, 1990). Intrinsic kinase activity also appears necessary for the normal trafficking of internalized receptor. Studies with point mutant EGF-Rs where Lys721 or Lys726 and Thr654 are replaced (Felder et al., 1990), indicate that kinase negative EGF-Rs are neither down-regulated nor degraded. Kinase-negative EGF-Rs recycle to cell surface unlike the wild-type EGF-Rs, in response to EGF (Felder et al., 1990).

1.3.3 EGF-Receptor function.

The binding of EGF to the receptor induces the activation of the protein tyrosine kinase, which phosphorylates various cellular proteins as well as the EGF-R itself. It has been suggested that the autophosphorylation of EGF-R regulates its capacity to phosphorylate exogenous substrates (Schlessinger, 1986). Tyrosine kinase activity is essential for, and is the first catalytic step in the EGF signal-transduction pathway. There are various tyrosine kinase substrates that are phosphorylated by the EGF-R. The best characterized substrate for the EGF-R is PLC-γ1. PLC-γ1 is a unique PLC isozyme in that it contains sequence shared with several cytoplasmic tyrosine kinases (Pawson, 1988). These regions are referred

to as the src homology region SH2 and SH3, and they may have regulatory functions (Pawson, 1988). Phosphorylation of PLC is also associated with the activation of PKC. Not all cell types which have a moderate number of EGF-Rs, respond to EGF with PLC-γ1 phosphorylation (Nishibe and Carpenter, 1990). The effect of the phosphorylation of PLC-γ on its activity has not been demonstrated. The magnitude of phosphorylation of PLC-γ on tyrosine is variable. Kim et al. (Kim, Sim, Kim, Nishibe, Wahl, Carpenter and Rhee, 1990) have demonstrated that the EGF-R kinase phosphorylates PLC-γ stoichiometrically in vitro on tyrosines 472, 771, 783 and 1254. However, the phosphorylation PLC had no effect on PLC activity as measured using PIP_2 as a substrate (Kim et al., 1990). In contrast, PLC-γ isolated from A431 epidermoid carcinoma cells, phosphorylated in vitro with EGF-R kinase was shown to increase its activity by three-fold (Nishibe, Wahl, Harnandez-Sotomayer, Tonks, Rhee and Carpenter, 1990).

1.3.4 EGF and the Phosphatidyl Inositol Pathway.

One of the earliest measurable responses of certain cells to EGF is the stimulation of the PI pathway to generate the second messengers IP_3 and DAG (Pike and Eakes, 1987, Wahl, Sweatt and Carpenter, 1987). This phenomenon is dependent on the tyrosine kinase activity of the EGF-R. Activation of PLC via EGF-R tyrosine kinase activity results in IP_3 production in A431 cells (Hepler, Nakahata, Lovenberg, Diguiseppi, Herman, Earp and Harden, 1987). This effect of EGF on PI breakdown is also observed in several non-transformed cell-lines (Moscat, Molloy, Flemming and Aaronson, 1988). However, EGF can also exert its

mitogenic effect without detectable PI hydrolysis (Taylor, Uhing, Blackmore, Prpie and Exton, 1985; L'Allemain and Ponyssegur, 1986).

1.3.5 Protein kinase C and the EGF receptor.

Activation of protein kinase C via the PLC pathway, or by the phorbol ester TPA, leads to many cellular responses. One important function of PKC is the phosphorylation on serine and threonine residues of the EGF-R (Downward, Waterfield and Parker, 1985). EGF-R is also phosphorylated by cAMP-dependent protein kinase (Ghosh-Daster and Fox, 1984). Phosphorylation of EGF at the Thr654 by PKC has been studied extensively (White, 1991; Carpenter and Cohen, 1990). Thr654 is one of the major PKC phosphorylation sites located in the cytoplasmic domain, which is surrounded by the basic residues. In cells treated with the PKC-activator TPA, Thr654 phosphorylation has been found to reduce the autophosphorylation in response to EGF (King and Cooper, 1986). Phosphorylation of the EGF-R by PKC was shown to cause a 3-fold decrease in the affinity of purified EGF-R for EGF and to reduce the receptor kinase activity (Carpenter and Cohen, 1990). TPA caused a decrease in high affinity binding of EGF to wild type (Thr 654) as well as cells expressing mutant receptors containing either Ala654, or Tyr654 instead of Thr654 (Davis, 1988; Livneh, Reiss, Berent, Ullrich and Schlessinger, 1987). Therefore although the regulation of apparent affinity of the EGF-R is independent of Thr654 or other major sites of serine- and threonine-phosphorylation of EGF-R (Countaway, McQuilkin, Girones and Davis, 1990), the

phosphorylation of Thr654 by Protein kinase C appears to provide a control mechanism for EGF-induced mitogenesis (Livneh, Dull, Berent, Prywes, Ullrich and Schlessinger, 1988; Ullrich and Schlessinger, 1990; Davis, 1988).

PKC activators have shown to inhibit EGF-stimulated PI hydrolysis in WB cells (a nontransformed rat liver epithelial cell line) (Huckle, Hepler, Rhee, Harden and Earp, 1990). This hydrolysis was potentiated in cells depleted of PKC. Thus, there is a strong association between PKC activation and EGF-R transmodulation by providing negative feedback for the receptor activity control.

1.3.6 EGF and DNA synthesis.

It has been suggested that EGF-R tyrosine kinase activity is essential for stimulation of DNA synthesis. This is examplified by the inability of an in vitro mutant of the EGF-R, having a non functional ATP-binding site to induce this deiayed response of EGF (Honegger, Dull, Bellot, Van Obberghen, Szapary, Schmidt, Ullrich and Schlessinger, 1988). However, EGF must remain in the extracellular environment for nearly eight hours before cells become committed to DNA synthesis (Carpenter and Cohen, 1990). Using CHO cells that express EGF-Rs that were constitutively phosphorylated at residue 654 in the absence of PKC activation, it was demonstrated that this phosphorylation blocks the mitogenic response to EGF (Bowen, Stanley, Selva and Davis, 1991). PKC mediated phosphorylation of the EGF-R decreases the receptor kinase and affinity for EGF.

1.4 THE T51B CELL LINE AS A MODEL SYSTEM.

The T51B rat liver cell is a non-neoplastic epitheloid derivative of a cell line

obtained from the liver of a normal adult Fisher rat (Swierenga, 1984). Their proliferative response is under stringent control by growth factor, calcium, cAMP, and phospholipid signalling pathways. DNA replication in the proliferatively quiescent T51B cells is tightly controlled by the Ca^{++} concentration in the extracellular media. Lowering the Ca^{++} concentration from 1.8mM to 0.02-0.10mM arrests the growth of approximately 85-90% of the cells at the G0/G1 and the G1/S boundaries (Boynton, Kleine, Whitfield and Bossi, 1985). These non-tumourigenic cells, unlike their pre-neoplastic or neoplastic clones, remain viable and quiescent for prolonged periods of time. In contrast the tumourigenic T51B clones continue to proliferate even under Ca^{++}-deprived conditions (Hill et al., 1989). Adding back Ca^{++} to the medium to the normal level removes the blocks and allows the cells to re-enter the cell cycle. Ca^{++}-deprived T51B cells also undergo DNA synthesis in the presence of the PKC activator TPA and inositol (1,3,4,5) tetrakis-phosphate (Hill, Zwitter and Boynton, 1989). Cyclic AMP elevating agents and DAG are also able to release the low Ca^{++}-mediated G1/S block and initiate T51B cells to undergo DNA synthesis within one hour (Boynton, Kleine and Whitfield, 1985). Mitogenic effects of EGF on T51B cells are dependent on the extracellular Ca^{++} in the medium, but are independent of freshly added BCS to conditioned low-serum medium (Hill, Kindmark and Boynton, 1988). Thus, all these three membrane signalling systems seem to play their roles in the growth regulation of T51B cells (Whitfield et al., 1987). Therefore, the T51B cell is a useful model system along with the two transformed clones, that show less calcium dependence, for the study of the regulation of mitogenesis.

Chapter 2.

METHODS

2.1 Cell culture.

T51B rat liver epithelial cells were cultured in a medium consisting of 90% (v/v) Eagle's Basal Medium (BME) (GIBCO. Montreal, Canada), and 10% (v/v) Bovine Calf Serum (BCS) (Colorado Serum Co. Denver, USA) containing 0.1 g/L gentamycin sulfate (Sigma Chemical Co. Ltd. St.Louis, USA). The conditions were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Prior to experiments, cells were detached by brief exposure to 0.25% (w/v) trypsin and 1mM EDTA in phosphate buffered saline (PBS) (all the reagents were purchased from Sigma Chem. Co. Ltd.). The cells were plated in 100 mm dishes in 10 ml BME (+ 10% BCS) or, 60 mm dishes in 5 ml BME (+ 10% BCS) and incubated at 37°C for 3-4 days after which they reached confluence. Cells were sub-cultured in 6-well multi-dishes (culture dishes purchased from Nunc, USA; Sarstedt, Germany) for cell counting and DNA synthesis experiments. Cell passages from 8 to 16 only, were used in the experiments. The vehicles for all the agents to be added were BME, PBS or, PBS/DMSO (1:1 v/v). When DMSO was used the total DMSO concentration in the medium never exceeded 0.14M. No significant changes in the morphology or, the metabolic response was observed due to the vehicles alone.

2.2 Cell proliferation.

2.2.1 Flow cytometry.

Flow cytometric determinations were performed as described in Boynton and Whitfield (1979). Confluent monolayers of cells were treated with various reagents as described in the figure legends. The cells were trypsinized with 0.25% trypsin and 1mM EDTA by incubating at 37°C for 5 minutes. The rounded, and detached cells were washed with 2 ml of GME (6mM glucose, 0.14M NaCl, 5.4mM KCl, 1.1mM $Na_2HPO_4.12H_2O$, 1.1mM KH_2PO_4, 0.3mM EDTA in H_2O; pH 7.0). The dispersed cells were counted and then centrifuged for 5 min. at 500 x g. The cell pellet was re-dispersed in 400 µl GME. The cell clumps were dispersed using a 21 gauge needle, and 1.2 ml ethanol (95%, stored at -20°C) was added slowly while gently mixing on a vortex. This procedure gave approximately 5-10 million cells per ml. An aliquot of the cell suspension (100 µl) was mixed with 2 ml propidium iodide (0.1%) and RNase (0.1%) solution ("Coulter stain"), and vortexed for 1 minute at room temperature. The stained cells were left for at least 15 minutes before flow cytometric analysis of DNA content.

Flow cytometric analysis was performed on the Epics-Profile II cytofluorometer (Coulter Electronics Co., Florida, USA.) and peaks were analyzed using the "Elite" software (Coulter Co., USA.).

2.2.2 Cell number.

Cells were subcultured in 6-well multidishes until they were approximately 50% confluent (2 days). Cells were fluid changed into low serum (0.2% BCS) media for 48 hours prior to various treatments. Epidermal Growth Factor (UBI.

Lake Placid, NY, USA), BCS or, TPA (Sigma Chem. Co. Ltd.) were added in various combinations for 24 hours, after which the cells were trypsinized with 2 ml trypsin (0.25%) / EDTA (1mM). "Isoton" (20 ml) (Coulter Co.) was added to each cell suspension and two aliquots of 100 µl were counted in a Coulter counter (Model C-1000, Coulter Co., USA.).

2.2.3 DNA synthesis.

[^{3}H]-thymidine incorporation into DNA was performed as described by Franks, Plamondon, and Hamet (1984). T51B cells were subcultured in 3 ml BME (+ 10% BCS) in 6-well multidishes. After the cells reached confluence, they were serum-deprived by fluid changing to BME containing 0.2% BCS for 48 hours prior to addition of the agents to be studied. DNA synthesis was assessed by the addition of 0.5 µCi/ml [^{3}H-methyl thymidine (NEN Research Products. DuPont, Montreal, Canada) for 24 hours (except stated otherwise). At the end of this time period, the medium was removed, cells were washed with 3 ml ice-cold PBS and fixed with PBS/formalin (80% PBS: 10% formalin: 10% acetic acid) (all other reagents were purchased from Fisher Scientific Co. or, BDH Chemicals, Ottawa, Canada). Acid soluble material was removed by treating the fixed cells with 0.5N Perchloric acid (PCA) at 4°C for 15 minutes, and washing with cold PCA (0.5N). DNA was solubilized by heating the acid insoluble material at 95°C for 20 minutes. Radioactivity was determined in the solubilized fraction, with 10 ml scintillation cocktail (NEN Research Products, DuPont, Canada) in a liquid scintillation spectrometer (LKB, model 1214 Rack Beta).

2.2.4 Autoradiography.

Cells were grown on 100 mm coverslips in 6-well multidishes. Confluent cells were serum deprived for 48 hours prior to various treatments and labelling. [^{3}H]-thymidine labelling of cell nuclei was performed as described (Boynton and Whitfield, 1976), with minor modifications. Cells were treated with various factors, and [^{3}H]-thymidine (0.5 µCi/ml) was added for 24 hours. The coverslips were washed twice with cold PBS, and fixed with 4% paraformaldehyde for at least 30 minutes at 4°C. The coverslips were dried and mounted on glass microscope slides with permount. The dried coverslips were dipped in photographic emulsion (Kodak, Rochester, NY) and exposed in the dark at 4°C for 5 days. The slides were developed and stained with haematoxylin / eosin. Photographs were taken with a camera mounted on a Ziess (Model D7082, Carl Zeiss Co., Germany) microscope.

2.3 Protein kinase C activity.

2.3.1 S49 cyc$^-$ cell extract phosphorylation.

Murine S49 cyc$^-$ lymphoma cells are a variant of S49 wild type lymphoma cells lacking the Gαs subunit of adenylate cyclase (Johnson, Kaslow and Bourne, 1978). A 85 kDa. protein isolated from S49 cyc$^-$ cells has been used as a PKC-specific substrate by Chakravarthy (1989) (Chakravarthy, Franks, Whitfield, and Durkin, 1989). PKC activity was measured as described in Chakravarthy et al., (1989). Briefly, dishes of confluent T51B cells were washed twice with cold PBS and cells were scraped into a small volume of hypotonic lysing buffer consisting of 1mM $NaHCO_3$, 5mM $MgCl_2$ and 100µM PMSF. The scraped cells were fully

lysed by vortexing for 2 minutes. Tris-HCl (pH7.5, 500mM) was added to the cell lysate to a final concentration of 50mM. Nuclei and cell debris were removed by centrifugation at 500 x g for 5 minutes at 4°C. The post-nuclear fraction (PNF) was further centrifuged at 14,000 x g for 15 minutes to obtain a cytosolic and crude membrane fraction, respectively. Separated fractions of proteins were quantified with the Bio-Rad protein assay kit (Bio-Rad Richmond, CA) and BSA as a standard. Approximately 15µg of protein preparation was used in each assay, since dose experiment indicated that the phosphorylation assay was linear up to 50µg. At this concentration, there was little interference due to the autophosphorylation of 'conventional PKCs' (since PKC's α, β, and γ have molecular weights ~80 kDa.). PKC activity was measured by phosphorylation of the 85 kDa. substrate. The assay mixture consisted of 1mM $NaHCO_3$, 50mM Tris-HCL (pH 7.5), 5mM $MgCl_2$, 100µM $CaCl_2$, 100µM PMSF, 100µM sodium vanadate and 100µM sodium pyrophosphate. The reaction was initiated by adding the 85 kDa. protein substrate and γ[^{32}P]-ATP (20µM, 4000 cpm/pmol) (NEN Research Products, DuPont, Canada) for 10 minutes at 37°C. The reaction was stopped by adding EGTA to a final concentration of 1mM. Particulate material was removed by centrifugation, and the supernatant was used for SDS-PAGE and autoradiographic analysis.

2.3.2 SDS-Polyacrylamide gel electrophoresis of phosphorylated substrate.

The phosphorylated proteins in the supernatant were solubilized in SDS sample buffer (10 % SDS, 0.025% bromophenol blue, 5% β-mercapto-ethanol, and 10% glycerol in 0.5M Tris-HCl, pH 6.8) and boiled for 5 minutes. SDS-PAGE (10%

running gel; 4.0% stacking gel) was performed as described (Laemmli, 1970) on a mini-protean II dual vertical slab cell following the manufacturer's recommendations (Bio Rad, Richmond, CA). 50-60 µl of samples from the solubilized supernatant were applied to the gel, which was run at constant voltage (65 volts/cell) for approximately 1 hour at room temperature. A constant volume of sample was loaded in each lane in any given experiment. Low molecular weight standards (10,000-100,000 Da., Bio-Rad, CA), were used as molecular weight markers. The separated proteins were identified after 1/2 hour staining with Coomassie blue solution (0.1% Coomassie blue R-250 in 40% methanol, 10% acetic acid) and destaining (40% methanol and 10% acetic acid) for 1-2 hours.

2.3.3 Autoradiography and densitometry of gels.

The destained gels were soaked in 5% (v/v) glycerol solution for 1.5 hours. Gels were dried overnight in Bio-gel wrap (Bio Design Inc., New York, NY) at room temperature, and placed with an X-ray film (Kodak XAR-5, Rochester, NY) in the dark for 24-48 hours at -70°C. The 85 kDa. phosphorylated substrate was identified after developing the autoradiogram. The autoradiographs were analyzed on a Molecular Dynamics Computing Densitometer (Model 300A) and integrations were performed by the "Image Quant" software.

2.3.4 Amersham PKC assay.

Protein kinase C activity was measured in particulate fractions, using an Amersham PKC assay kit (Amersham, Oakville,ONT., Canada). The suppliers instructions were followed essentially. Cells were lysed, and the particulate fraction was isolated and quantified as described before. Particulate PKC activity was

measured in aliquots containing 5μg of protein. Triplicate samples were taken for each point. Since endogenous phosphorylation in the absence of peptide or, Ca^{++}/phospholipid was less than 20% of the activity in the presence of PBS/DMSO vehicle control, the blank was determined as the radioactivity in the absence of sample fraction. Calculations were performed as stated in the suppliers instructions.

2.4 Statistical analysis.

The statistical analysis was performed on the data by computer aided two-tailed paired or independent student's t-test as indicated. The p-levels below 0.05 were accepted as significant for two data points. P-levels below 0.0001 were rounded off.

Chapter 3.

RESULTS

3.1 Effects of EGF on Mitogenesis of Growth-arrested T51B Cells.

Epidermal growth factor is a potent mitogen for serum-deprived T51B cells. A series of experiments were performed to examine the mitogenic response to EGF under different culture conditions.

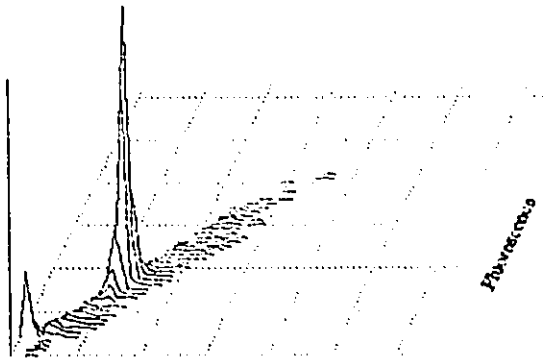
3.1.1 Effects of Serum-deprivation on the Cell-cycle.

When confluent cultures of T51B cells were fluid changed to low serum (0.2% BCS) containing medium (BME), approximately 85% of the cells were growth-arrested in G1, within 48 hours of serum-deprived condition (fig. 3A), less than 10% were still in the replicative (S) phase. Upon addition of EGF to cells in the serum-deprived conditioned medium, the proliferatively quiescent cells entered S phase and resumed cycling (fig. 3B, table 1). A significant number of cells underwent replication in 24 hours (approx. 33% in S phase after 24 hours) (fig. 3B). Long-term serum-deprived cells (serum-deprived for more than five days including the incubation period with EGF or serum in the serum deprived conditioned medium) responded weakly to EGF and 10% serum replenishment probably due to old / dead cells in the culture. Therefore, 48 hours of serum deprivation was performed in the subsequent experiments of this study.

FIGURE 3. FLOW CYTOMETRIC ANALYSIS OF T51B CELLS.

Flow cytometric profiles of the serum-deprived cells (48 hours)(A) (%G1 = 82.6, %G2 = 7.7, %S = 9.6), and the same cells after 24 hours in the presence of 1.5ηM EGF (B) (%G1 = 63.9, %G2 = 2.9, %S = 33.2). Flow cytometric data analysis was performed as described in the methods.

A



B

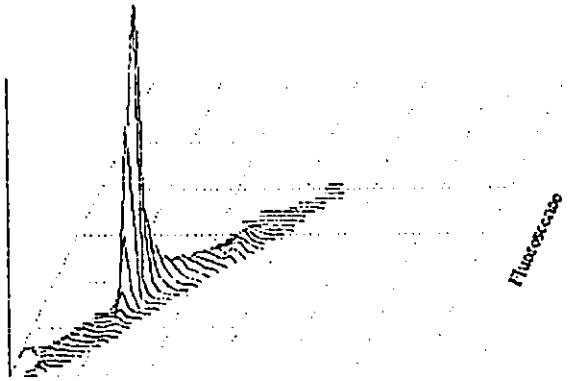


TABLE 1: MITOGENIC EFFECTS OF EPIDERMAL GROWTH FACTOR AND SERUM ON CELL NUMBER OF SUBCONFLUENT CULTURES.

T51B cells were seeded at a density of 1.8×10^4 cells in 6-well multidishes. Sparse cultures were serum changed (0.2% BCS in BME) after two days for 48 hours. Cells were counted after 24 hours of the various treatments: EGF (1.5ηM), and BCS (10%). "No Add" is the cell count in serum deprived conditioned medium (for 48 + 24 hours). Data is expressed as the average of three dishes each counted in duplicate (mean ± SEM).

TABLE 1.

Treatment	Cell number ($\times 10^{-6}$)(± SEM)
No Add	2.30 ± 0.19
EGF	3.40 ± 0.08
10%BCS	3.45 ± 0.08

3.1.2 [^{3}H]-Thymidine Incorporation into Cell Nuclei and Cell Proliferation.

Autoradiographic analysis of the cells serum deprived for 48 hours, confirmed the data obtained from the flow cytometry experiment. A small proportion of cells appeared to be replicating their DNA in the serum deprived condition, and therefore, few nuclei were labelled when cells were incubated in the presence of [^{3}H]-thymidine for 24 hours (fig. 4A). When the cells were labelled either in the presence of EGF (fig. 4B) or, 10% serum (fig. 4C), a dramatic increase in the number of labelled nuclei was observed. A significant number of cells underwent mitosis as can be seen by the increase in cell numbers in response to EGF and serum (table 1).

3.1.3 Time course and Dose response of EGF on DNA synthesis in T51B cells.

EGF stimulated the incorporation of [^{3}H]-thymidine into DNA in serum deprived cells, in a dose-dependent manner. There was a 4-5 fold increase in DNA synthesis with 1ηM EGF within 24 hours, after which a plateau in the rate of DNA synthesis was observed (fig. 5). However, there was no significant cell-death (floating cells) observed with EGF concentrations up to 10ηM. The DNA synthesis response due to EGF was time-dependent (fig. 6). EGF had to be present for a minimum of eight hours before increased DNA synthesis was observed (fig. 6). The rate of DNA replication was maximal between 18-30 hours after which the rate declined. Exposure to EGF for longer periods (up to 56 hours) resulted in no further increase in the rate of DNA synthesis, and in fact led to cell death. Serum

FIGURE 4. [3H]-THYMIDINE INCORPORATION IN T51B CELL NUCLEI.

Eosin/hematoxylin stained autoradiograph of serum-deprived T51B cells untreated (4A), treated with EGF (1.5ηM) (4B), or 10% BCS (4C), in the presence of [^{3}H]-thymidine (0.5μCi/ml) for 24 hours is presented. Silver grains representing the incorporation of [^{3}H]-thymidine are shown as dark patches. Magnification is 400x.

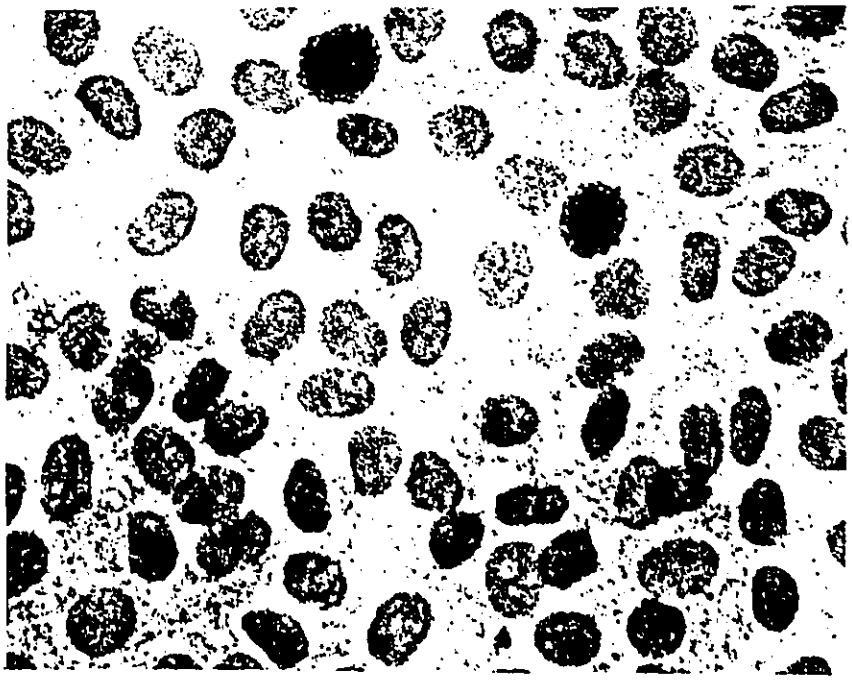


FIGURE 4(A)

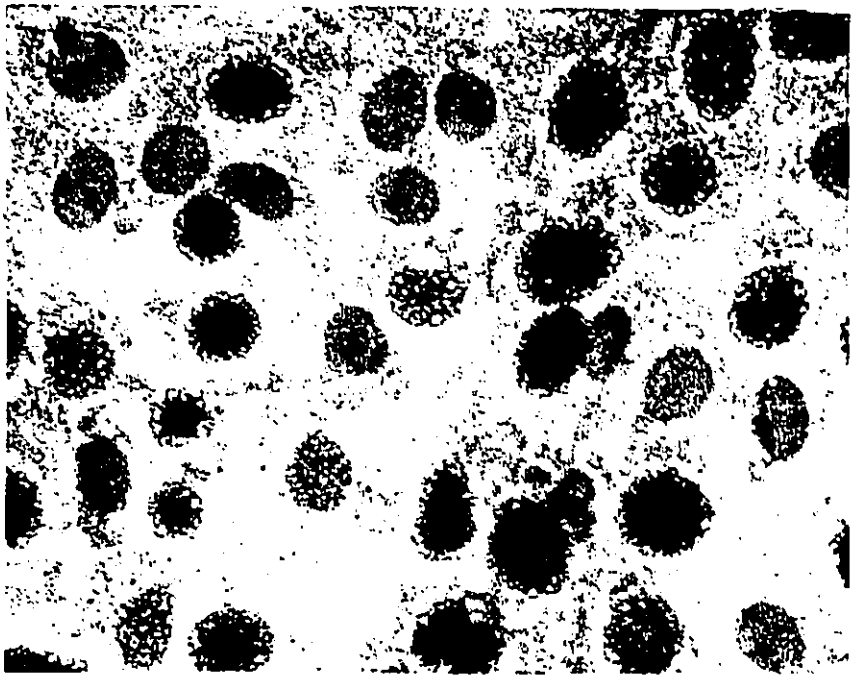


FIGURE 4(B)

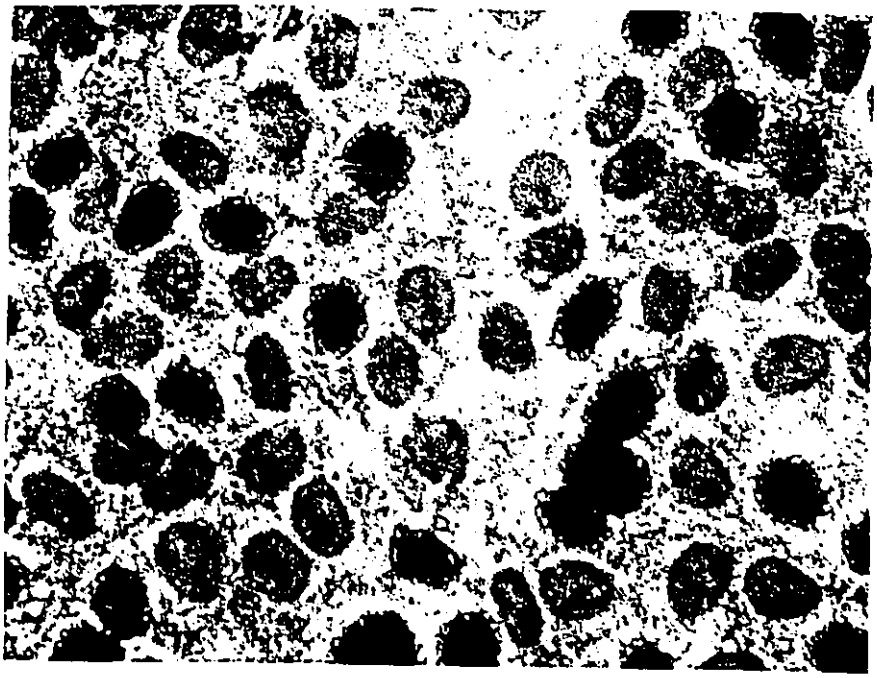
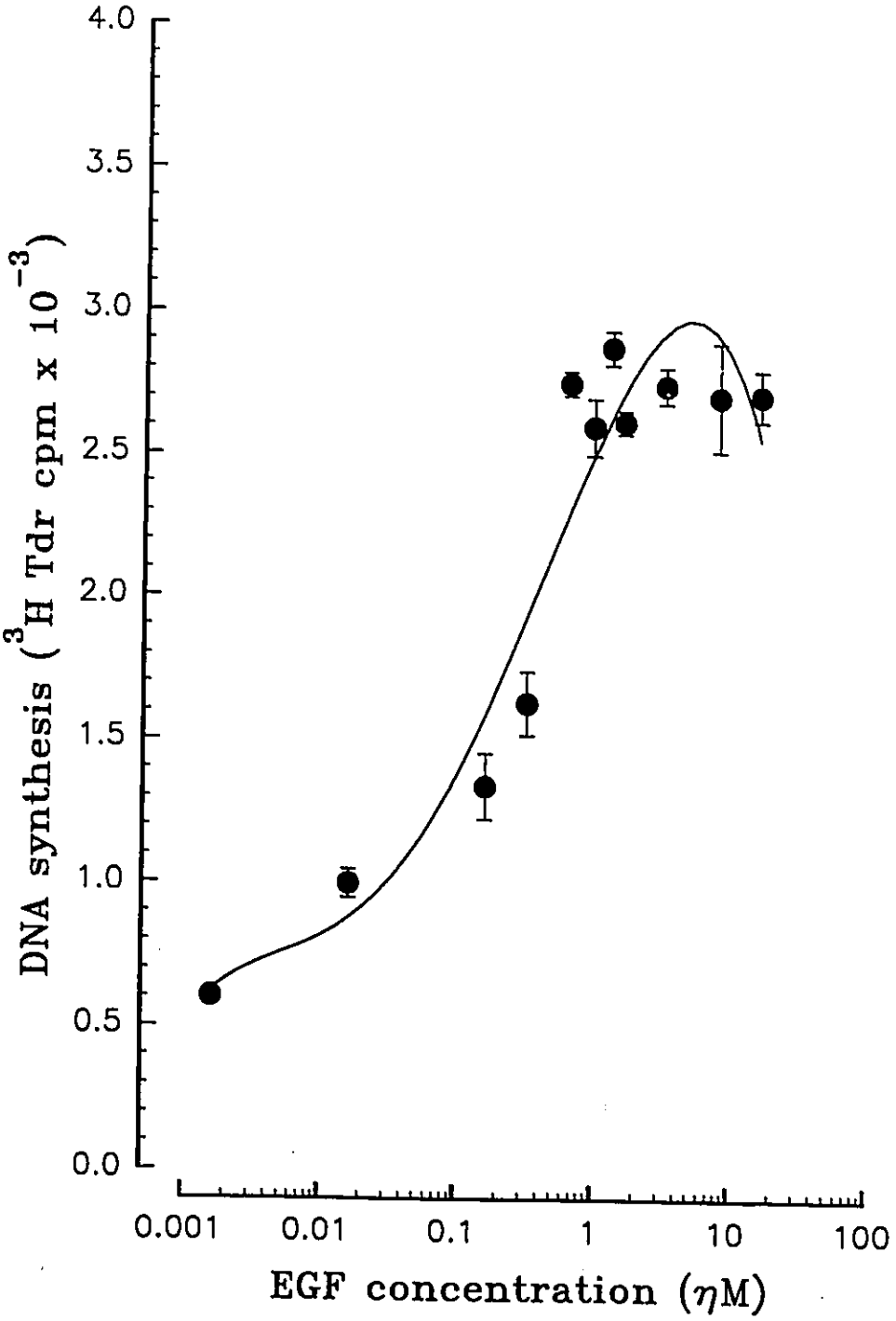


FIGURE 4(C)

FIGURE 5. DOSE RESPONSE OF EGF ON DNA SYNTHESIS IN T51B CELLS.

Confluent and serum-deprived (0.2% serum for 48 hours) cells were treated with 0.5μCi/ml [^{3}H]-thymidine and increasing concentrations of EGF, for 24 hours. DNA synthesis was measured as described in methods. Values are mean ± SEM of four determinations, each performed in triplicate.


4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
DNA synthesis (^{3}H Tdr cpm x 10^{-3})
0.001
0.01
0.1
1
10
100
EGF concentration (ηM)

replenishment showed higher incorporation of radioactivity in 56 hours (fig. 6).

3.1.4 Effects of Protein Kinase Inhibitors on EGF Induced DNA Synthesis.

Mitogenic signalling mediated by EGF acts through its receptor on the cell surface. The EGF receptor associated tyrosine kinase activity is a key regulator of various short and long-term responses in a variety of cells. Therefore, the effects of the tyrosine kinase inhibitors, tyrphostin and genistein, and a PKC inhibitor (Tamaoki, Nomoto, Takahashi, Kato, Morimoto, and Tomita, 1986) and p60 v-src tyrosine kinase inhibitor (Nakana, Koboyashi, Takahashi, Tamaoki, Kuzuu, and Iba, 1987) staurosporine, were studied on the EGF-stimulated DNA synthesis in the quiescent and serum-deprived cells. [^{3}H]-thymidine incorporation, in response to EGF (1.5ηM), was inhibited in the presence of tyrphostin, genistein and staurosporine in a dose-responsive manner (figs. 7,8). Genistein was the most effective of the two specific tyrosine kinase inhibitors tested (fig. 7). Approximately 50% inhibition of DNA synthesis occurred in the presence of 10μM genistein, and 25μM tyrphostin, whereas concentrations of genistein 25μM and higher were cytotoxic to cells. Staurosporine, also a PKC inhibitor, was most potent of all. Concentrations of staurosporine as low as 1μM inhibited EGF-stimulated DNA synthesis by more than 60% (fig. 8). Concentrations above 5μM staurosporine were cytotoxic to cells. Inhibition of DNA synthesis was also observed in control cells treated with the three agents used. This could be explained by the fact that cells in the serum deprived conditioned state maintain a basal activity of DNA

FIGURE 6. TIME COURSE INCORPORATION OF [^{3}H]-THYMIDINE IN THE PRESENCE OF EGF.

EGF (1.5ηM) was added to confluent and serum-deprived culture, for the times indicated. [^{3}H]-thymidine was present for at least 24 hours. DNA synthesis was measured as described in methods. Values are the mean ± SEM of 4 separate determinations, each performed in triplicate. The serum-deprived control values were not significantly different for the three time points (24, 30, and 54 hours) measured. (cpms were 88,471 ± 15,356, 87,448 ± 15,841, 103,436 ± 13,787, respectively). Data is plotted against the percent of 54 hours serum-deprived control. BCS (10%) was more potent mitogen (769.9 ± 7.3 % of control, in 46 hours) than EGF present for the same time period.

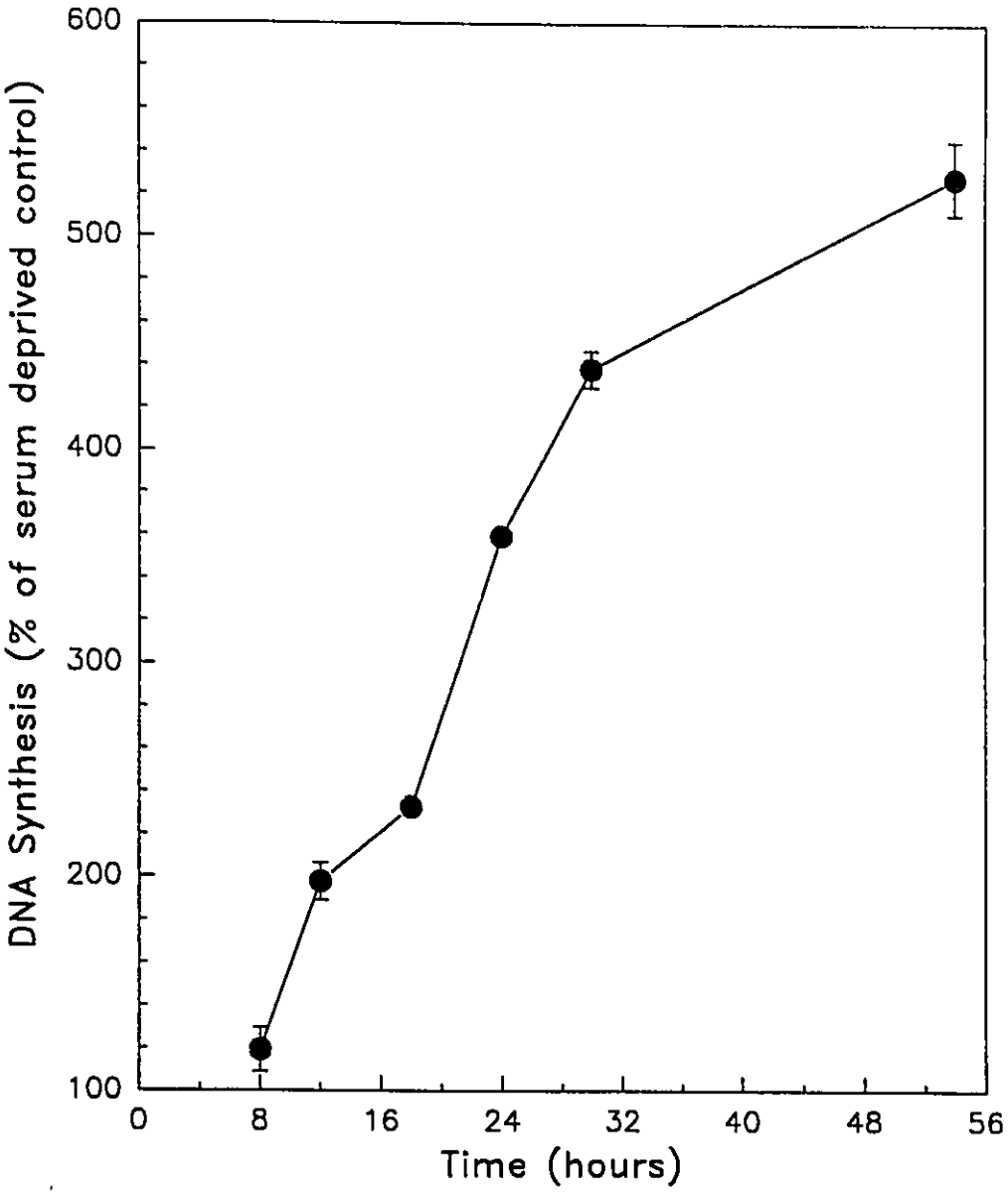

600
500
400
300
200
100
DNA Synthesis (% of serum deprived control)
0
8
16
24
32
40
48
56
Time (hours)

FIGURE 7. EFFECTS OF TYROSINE KINASE INHIBITORS GENISTEIN AND TYRPHOSTIN ON EGF STIMULATED DNA SYNTHESIS.

Confluent and serum-deprived T51B cells were incubated with the indicated concentrations of genistein, or tyrphostin in the presence of EGF (1.5ηM) and [^{3}H]-thymidine. DNA synthesis was measured after 24 hours, as described in methods. Values are expressed as the percent of serum-deprived conditioned medium control (mean ± SEM) from three dishes. The p-values for each agent were calculated by the two-tailed paired t-test analysis of the means with respect to the corresponding untreated (EGF 24 hours, only) control. The p-values for all the data points (except for the data with 10μM tyrphostin and EGF) are significant (<0.002).

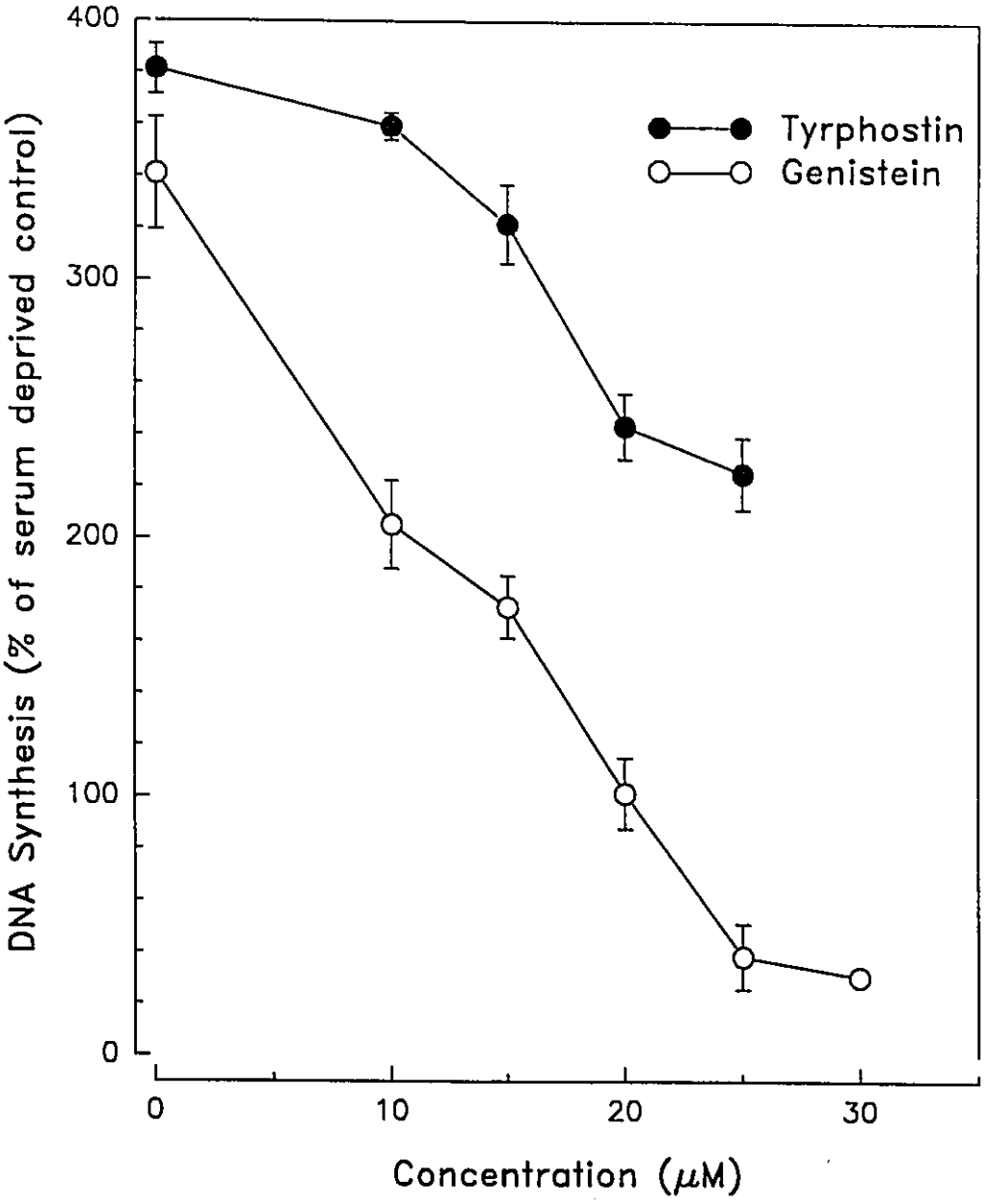
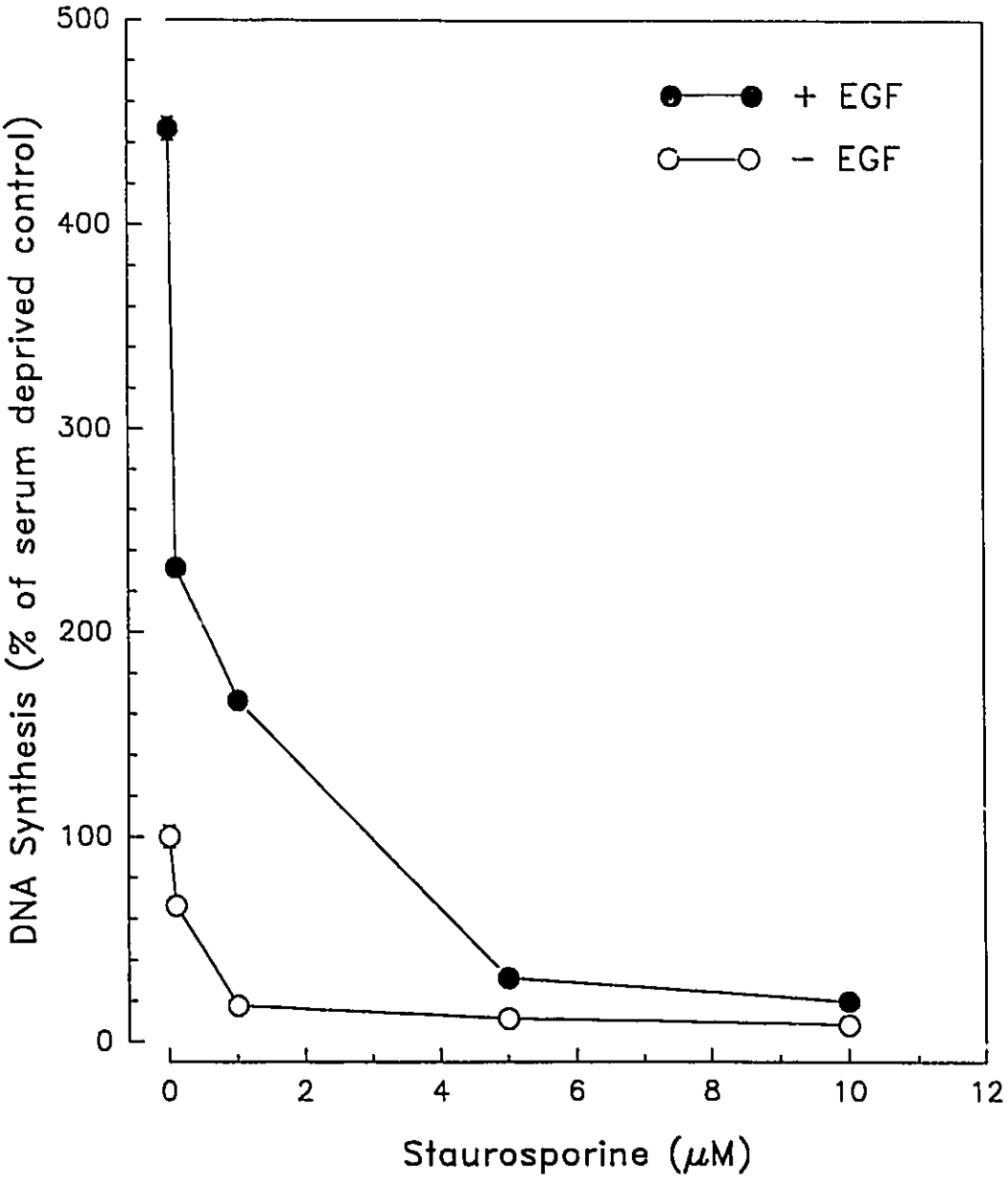

400
300
200
100
0
0
10
20
30
DNA Synthesis (% of serum deprived control)
Concentration (μM)
Tyrphostin
Genistein

FIGURE 8. EFFECT OF STAUROSPORINE ON EGF-STIMULATED DNA SYNTHESIS.

Confluent and serum-deprived cells were incubated with [^{3}H]-thymidine and the indicated concentrations of staurosporine in the absence or presence of EGF (1.5ηM). DNA synthesis was measured as described in the methods. Values are expressed as the percent of serum-deprived conditioned medium control (mean ± SEM) from three dishes.


500
400
300
200
100
0
DNA Synthesis (% of serum deprived control)
+ EGF
– EGF
0
2
4
6
8
10
12
Staurosporine (μM)

synthesis / repair, which is regulated by kinase activity although active at lower levels. The inhibitory response of genistein (10μM) on EGF or, serum-stimulated DNA synthesis was significant (table 2). The effective inhibition of serum-stimulated DNA synthesis by genistein could also account for the presence of other kinases activated by serum factors that are also inhibitable by genistein. Although tyrphostin being a EGF-R tyrosine kinase specific inhibitor, genistein was more effective in inhibiting DNA synthesis at a lower concentrations used in our experimental conditions. Therefore, genistein was chosen as the tyrosine kinase inhibitor used in further studies. Genistein inhibited EGF-mediated DNA synthesis dose-dependently (fig. 9. EGF response was completely blocked by 20μM genistein (fig. 10). However, genistein did not inhibit [^{3}H]-thymidine incorporation as effectively (only 20% inhibition), when it was added eighteen hours after EGF addition to serum-deprived cells (fig. 11), during which time DNA synthesis was maximal as shown earlier (fig. 6).

3.1.5 Effects of PKC Down-Regulation on the Inhibitory Responses of Genistein.

Since prolonged exposure of TPA causes down-regulation of PKC, the effect of TPA on EGF-mediated DNA synthesis was studied. TPA was added to serum-deprived and quiescent T51B cells in the presence or absence of EGF and genistein. DNA synthesis in the presence of TPA (100μM) alone, remained the same (fig. 12). There was also no detectable effect of TPA on cell number (table 3). However, TPA enhanced the mitogenic response to EGF and serum in 24

TABLE 2: INHIBITORY EFFECTS OF GENISTEIN ON EGF AND SERUM STIMULATED DNA SYNTHESIS.

Confluent and quiescent (serum-deprived for 48 hours) T51B cells were treated with various agents (EGF, 1.5ηM; Genistein, 10μM; BCS, 10% v/v) in the presence of [^{3}H]-thymidine. DNA synthesis was measured in the untreated and serum-deprived ('No add'), and treated dishes after 24 hours, as described in the methods. Data are expressed as the mean ± SEM from 3 separate experiments, n = 3 for each point. P-values are obtained from the two-tailed paired t-test compared with the means of the corresponding genistein untreated samples.

TABLE 2.

TREATMENT	CPM (MEAN ± SEM) ($x10^{-3}$)	P-VALUE
No Add	74.5 ± 7.8	0.0003
Genistein	25.4 ± 2.5	
EGF	323.3 ± 18.3	<0.0001
EGF + Genistein	163.6 ± 14.6	
BCS	704.7 ± 25.4	<0.0001
BCS + Genistein	250.3 ± 16.1	

FIGURE 9. DOSE RESPONSE OF GENISTEIN ON EGF-STIMULATED DNA SYNTHESIS.

Increasing concentrations of genistein was added to confluent and serum-deprived T51B cells in the absence (open circles), or, the presence of 1.5ηM EGF (closed circles). DNA synthesis was measured by the [^{3}H]-thymidine incorporation in 24 hours. Values are expressed as the percent of serum-deprived control (mean ± SEM) from three separate experiments, each performed in triplicate. P-values calculated by two-tailed paired t-test for all the EGF and genistein treated data points (closed circles) are significant (<0.006) when compared to the cpm counts from EGF treated cells.

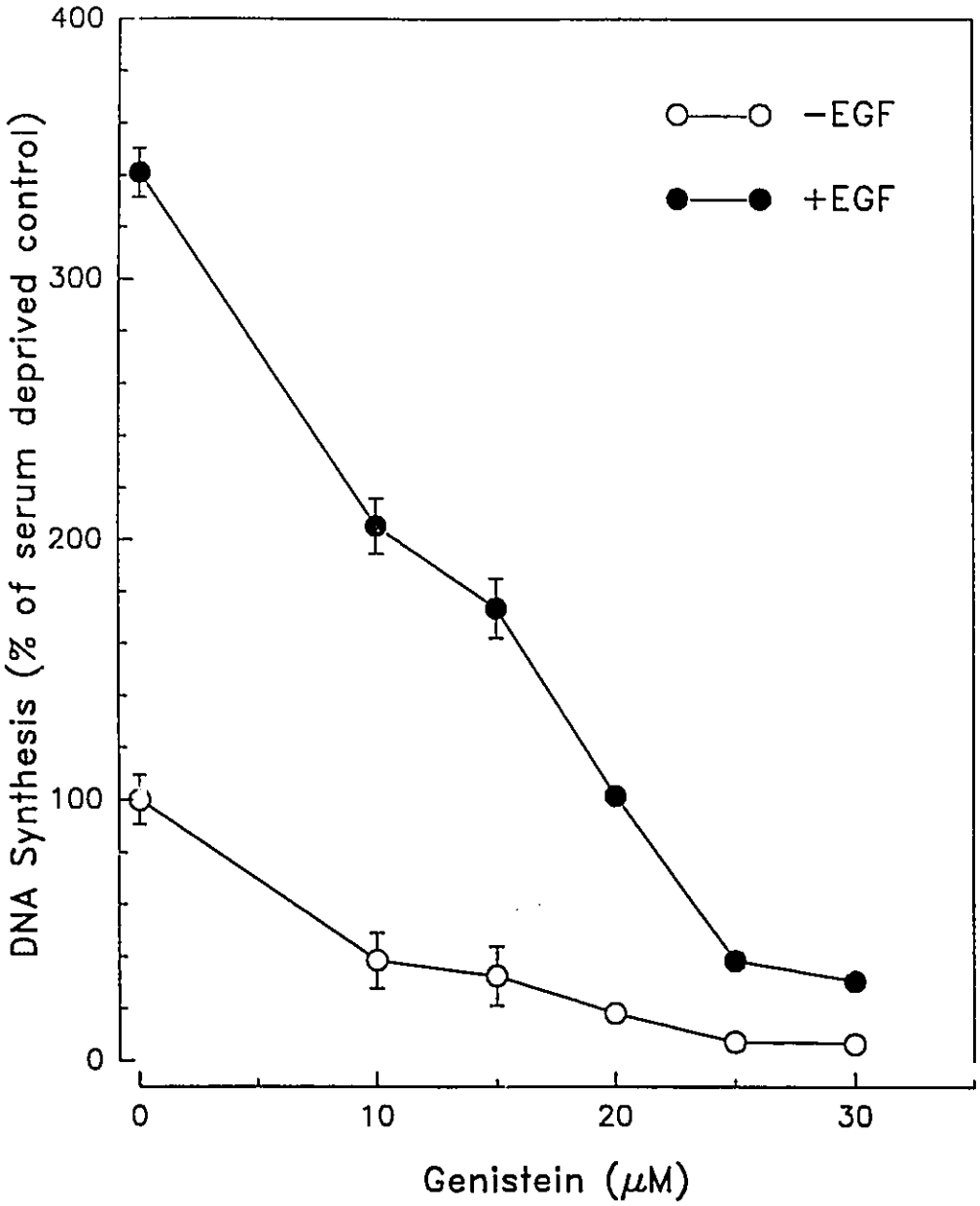

400
300
200
100
0
DNA Synthesis (% of serum deprived control)
−EGF
+EGF
0
10
20
30
Genistein (μM)

FIGURE 10. INHIBITION OF EGF-STIMULATED DNA SYNTHESIS IN THE PRESENCE OF GENISTEIN.

The experimental data presented in figure 9 are expressed as the percent inhibition in the response due to EGF alone in 24 hours (mean ± SEM) by the increasing concentrations of genistein present for the same 24 hour period. Data are from three separate experiments, each performed in triplicate.
* indicates that the p-value obtained by two-tailed paired t-test was 0.025 when compared with the values obtained in the presence of EGF and 10μM genistein.

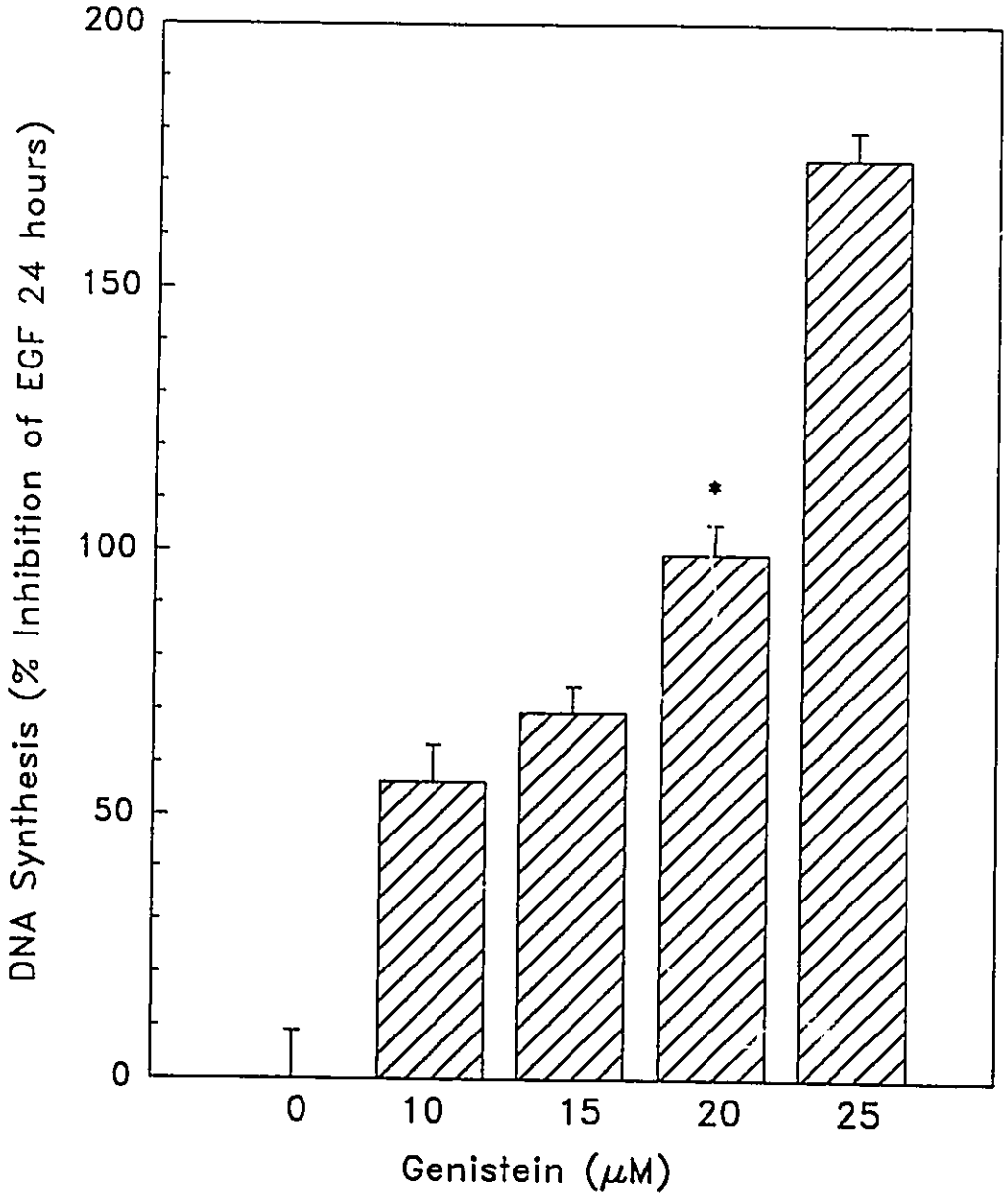
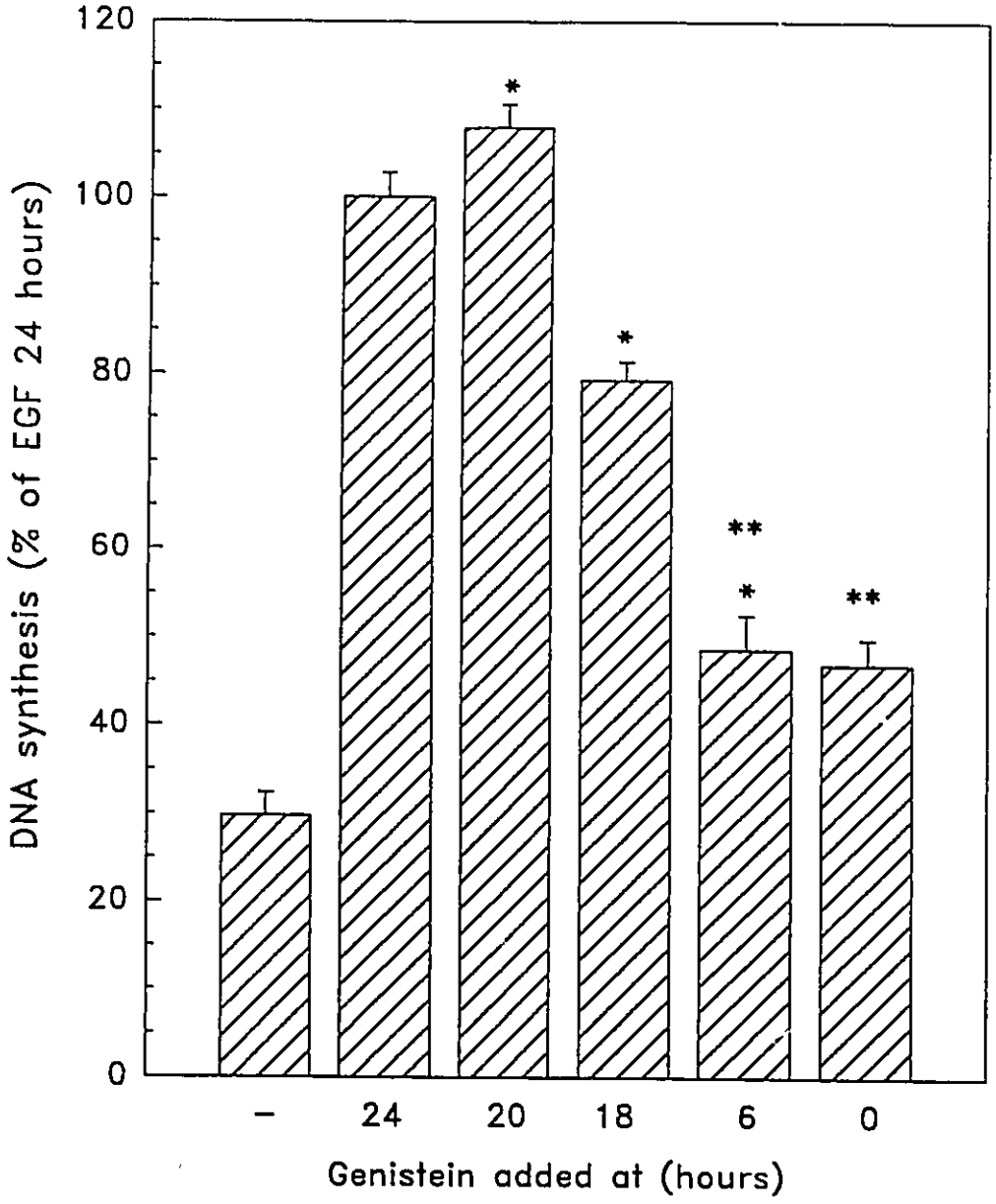

200
150
100
50
0
DNA Synthesis (% Inhibition of EGF 24 hours)
*
0
10
15
20
25
Genistein (μM)

FIGURE 11. TIME COURSE EFFECT OF GENISTEIN ON EGF-STIMULATED DNA SYNTHESIS.

Confluent cells were serum-deprived as described in the methods. [^{3}H]-thymidine and EGF (1.5ηM) were added at zero hours (i.e., after 48 hours of serum deprivation). Genistein (15μM) was added at the times indicated. Serum-deprived control is plotted as '-'. Reaction was stopped at 24 hours and DNA synthesis was measured as described in the methods. Data are obtained from three separate experiments, each performed in triplicate, and is expressed as percent of EGF stimulated DNA synthesis (mean ± SEM). $p < 0.0001$ (*) when data point was compared with 24 hours control (-); $p < 0.0001$ (**) when data point was compared with the values for genistein (20 hours) in the presence of EGF (24 hours). Statistical analysis performed was the two-tailed paired t-test.


120
100
80
60
40
20
0
DNA synthesis (% of EGF 24 hours)
*
*
**
*
**
–
24
20
18
6
0
Genistein added at (hours)

hours (fig. 12, table 3). Genistein (10μM), whether added to control or TPA treated cells, inhibited [^{3}H]-thymidine incorporation in the absence or the presence of EGF. Although genistein inhibited the responses due to EGF or EGF and TPA by almost half (40% inhibition of the combined effects of TPA and EGF verses 55% inhibition of the EGF response), it did not bring the DNA synthesis down to the same level as it did in the presence of EGF alone. Therefore, in the presence of TPA, the inhibitory effect of genistein was slightly attenuated.

3.2 Effects of EGF on Protein kinase C.

EGF has been shown to activate phospholipase C and increase DAG levels in many cell lines including T51B cells (Hill et al., 1988). The effects of EGF on PKC activity was determined by the phosphorylation of the 85 kDa. specific substrate obtained from S49 cyc$^-$ lymphoma cells in serum-deprived T51B cells. EGF (1.5ηM) increased the membrane-associated PKC activity within 15 minutes and the increase was maximal within 35-45 minutes (figs. 13,14, table 4). The cytosolic activity measured in repeated experiments showed an inconsistent response over short-term incubation with EGF. However, overall there was no significant change detected in the cytosolic PKC activity as measured by the phosphorylation of the 85 kDa. substrate. Therefore, only particulate PKC activity was measured in the subsequent experiments that follow. Even in the particulate fractions the fold stimulation varied from one experiment to the other probably due to the different age (passage number) as evident from the data summarized from the densitometric scans of the autoradiographed SDS-polyacrylamide gels.

FIGURE 12. EFFECT OF PKC DOWNREGULATION BY PROLONGED EXPOSURE TO TPA ON THE DNA SYNTHESIS INHIBITORY RESPONSE OF GENISTEIN.

TPA (100ηM), EGF (1.5ηM), or genistein (10μM), were added alone or in various combinations to the confluent and serum-deprived cells, as indicated. [^{3}H]-thymidine incorporation was measured after 24 hours, as described in the methods. Values are the mean ± SEM of serum-deprived control from 4 separate experiments, n = 3 for each experiment.

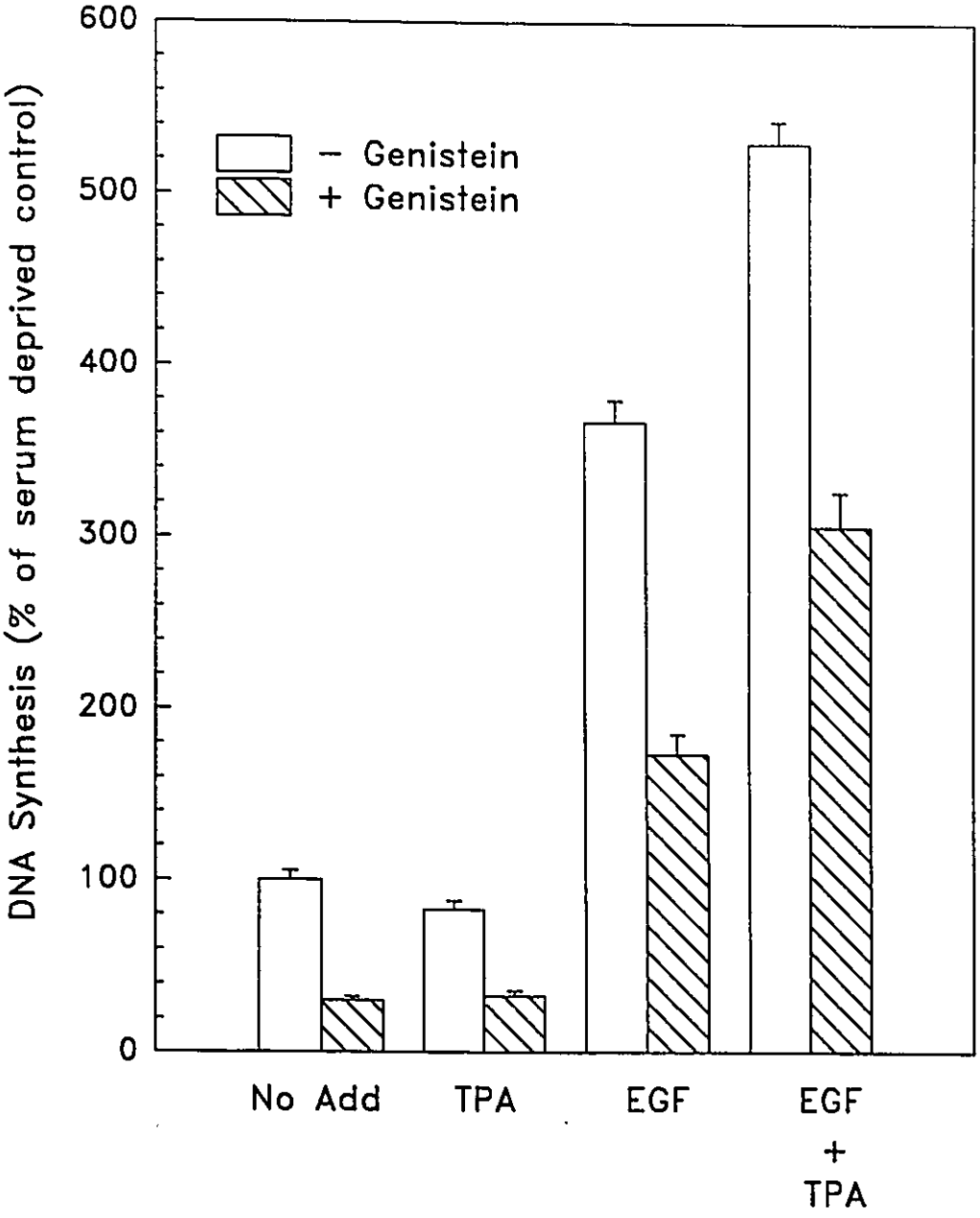

600
500
400
300
200
100
0
DNA Synthesis (% of serum deprived control)
− Genistein
+ Genistein
No Add
TPA
EGF
EGF
+
TPA

TABLE 3: MITOGENIC EFFECTS OF EPIDERMAL GROWTH FACTOR, TPA, AND SERUM ON CELL NUMBER OF SUBCONFLUENT CULTURES.

T51B cells were seeded at a density of 1.8 x 10^4 cells in 6-well multidishes. Sparse cultures were serum changed (0.2% BCS in BME) after two days for 48 hours. Cells were counted after 24 hours of the various treatments: EGF (1.5ηM), TPA (100ηM), and BCS (10%). "No Add" is the count of cells in serum-deprived conditioned medium (for 48 + 24 hours). Data is expressed as the average of three dishes each counted in duplicate (mean ± SEM).

TABLE 3.

Treatment	Cell number ($x10^{-6}$)(± SEM)	Fold Increase (± SEM)
No Add	2.30 ± 0.19	1.00 ± 0.08
EGF	3.40 ± 0.08	1.47 ± 0.04
10%BCS	3.45 ± 0.08	1.50 ± 0.04
TPA	2.18 ± 0.02	0.95 ± 0.01
EGF + TPA	3.98 ± 0.21	1.73 ± 0.01
BCS + TPA	4.42 ± 0.15	1.92 ± 0.07

FIGURE 13. EFFECT OF EGF ON PARTICULATE PROTEIN KINASE C ACTIVITY.

Confluent and serum-deprived (48 hours) cells were incubated with EGF (1.5ηM) for the indicated times. Particulate PKC activity was measured by its ability to phosphorylate an 85 kDa. substrate isolated from S49 cyc⁻ lymphoma cells as described in the methods. The autoradiograph of 10% SDS-PAGE gel is shown. Arrow indicates the phosphorylated 85 kDa. PKC substrate.

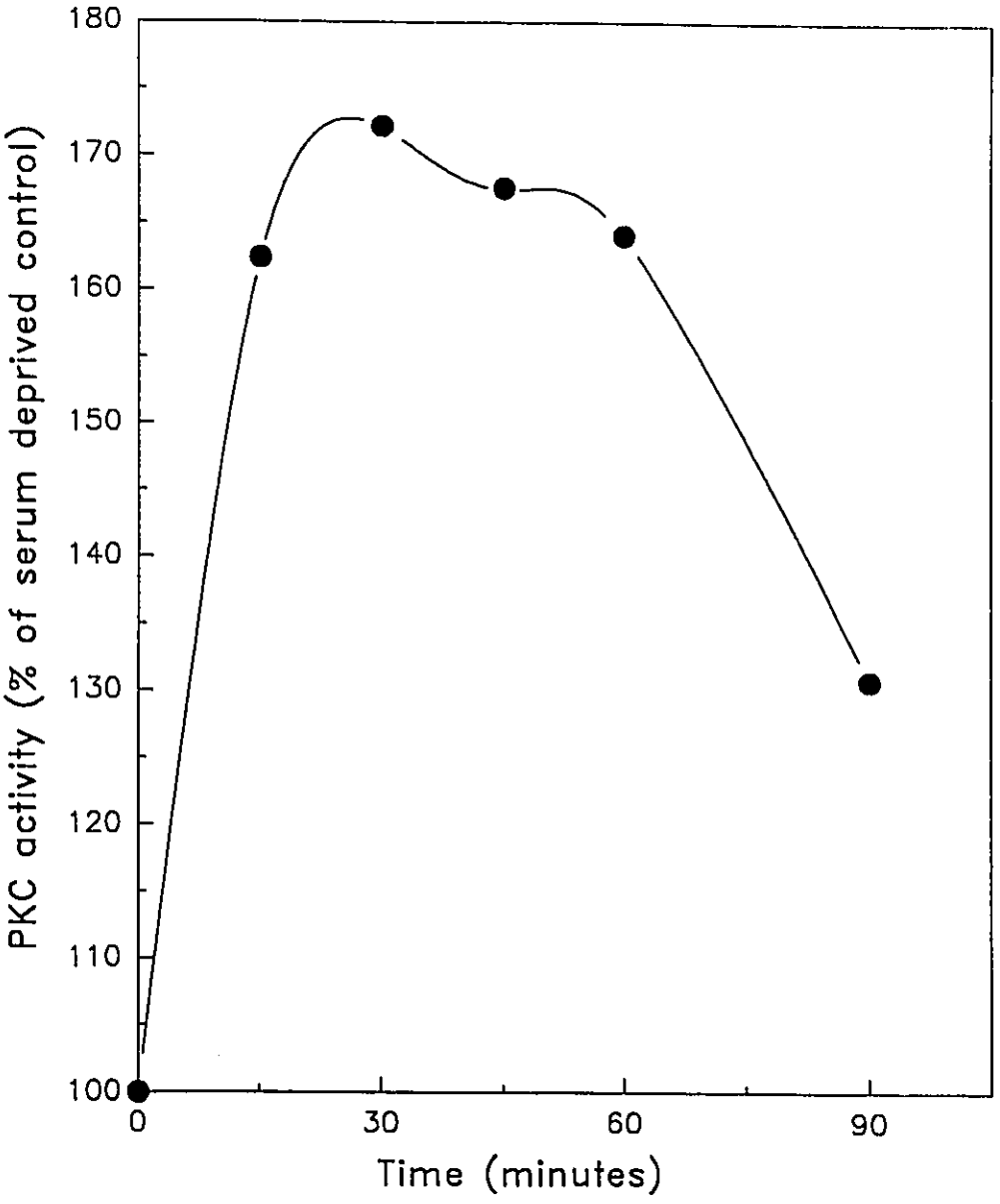
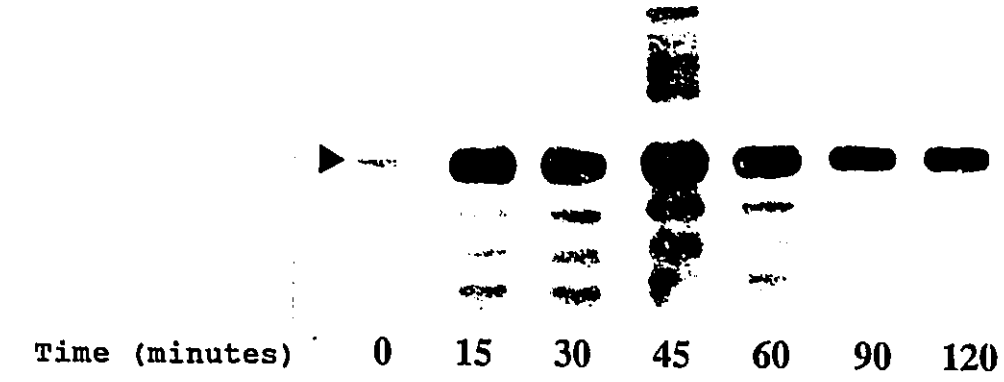

180
170
160
150
140
130
120
110
100
PKC activity (% of serum deprived control)
0
30
60
90
Time (minutes)

FIGURE 14. EFFECT OF EGF ON PARTICULATE PROTEIN KINASE C ACTIVITY.

Particulate PKC activity was measured by the phosphorylation of an 85 kDa. substrate as shown in the figure 13. Data are expressed as the percent of zero minute serum-deprived conditioned medium control, obtained from the densitometric scan (pixels units) of the 85 kDa. band from the autoradiogram. Similar profiles were obtained in identical experiments, the data for which is summarized in table 4.


Time (minutes)
0
15
30
45
60
90
120

PKC activity in the particulate fraction remained elevated for up to 24 hours in the presence of EGF (figs. 15, 16), thereafter it continued to decline in the serum-deprived conditioned cells incubated alone for 24 hours (table 4, legend; fig. 17).

3.3 Effects of TPA on Down-Regulation of Protein kinase C.

Long-term responses of cells to TPA often involve down-regulation of PKC. Attempts were made to quantitate PKC levels in T51B cells pretreated with TPA, by Western immunoblot analysis. However, I was unsuccessful in measuring the Protein kinase C levels under my experimental conditions, when the anti-PKC antibodies against the alpha and beta isoforms of PKC were used.

Therefore, PKC activity had to be examined instead of the levels of PKC. As shown in the figure 17, and in table 4, serum deprivation for an additional 24 hours, following the initial 48 hour period reduced PKC activity by approximately 50%. PKC activity, on the other hand, remained elevated in the presence of EGF for 24 hours. TPA (100ηM), when added six hours after the EGF addition , reduced the level of PKC activity elicited by EGF, close to the untreated control level. TPA also decreased PKC activity in the untreated cells. However, the inhibitory effect of TPA was more pronounced on EGF pretreated cells (>50% inhibition), than on the untreated cells (table 5).

TABLE 4: EFFECT OF EPIDERMAL GROWTH FACTOR ON PARTICULATE PROTEIN KINASE C ACTIVITY IN SERUM-DEPRIVED CELLS.

Confluent T51B cells were serum-deprived (0.2% BCS containing BME) for 48 hours. Short-term and long-term incubations with EGF (1.5ηM) were performed in the various experiments. Particulate PKC activity was measured by the 85 kDa. protein phosphorylation (see methods). Densitometric scan of the various autoradiographs was calculated in pixels, and expressed as the percent of zero minute untreated control (24 hours control from an experiment was 96.8% of the 0 minute control).

TABLE 4.

Time (hours)	Particulate PKC activity (% of control)					
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6
0.00	100	100	100	100	100	100
0.25	108	360	99	112	136	162
0.50	136	432	128	154	134	172
0.75	152	468	-	-	141	168
1.00	130	374	103	114	155	164
1.50	-	336	114	131	165	131
2.00	-	282	116	134	157	-
4.00	-	-	143	178	-	-
8.00	-	-	139	171	-	-
12.0	-	-	120	141	-	-
16.0	-	-	134	164	-	-
24.0	-	-	118	138	-	-

FIGURE 15. LONG TERM EFFECTS OF EGF ON PARTICULATE PROTEIN KINASE C ACTIVITY.

Particulate PKC activity was measured by the 85 kDa. protein phosphorylation as detailed in the methods. The autoradiogram of the 10% SDS-PAGE gel is shown. Arrow indicates the 85 kDa. phosphorylated protein. Lanes 1 & 13, 48 hours serum-deprived control; lanes 2,3,5,6,7,8,9 & 10 are the PKC activity in serum-deprived (48 hours) cells treated with EGF (1.5ηM) for 24,16,12,8,4,2,1.5, and 1 hours, respectively. Lanes 11 & 12 are the 30, and 15 minutes treatments, respectively with EGF.

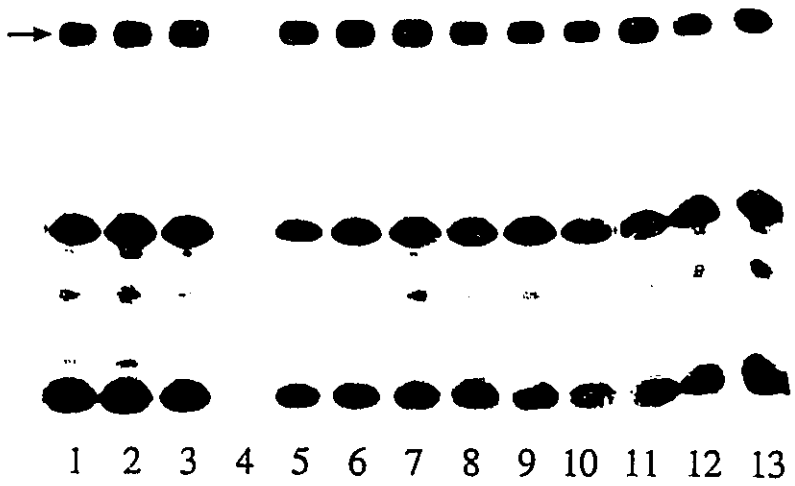

1 2 3 4 5 6 7 8 9 10 11 12 13

FIGURE 16. LONG TERM EFFECTS OF EGF ON PARTICULATE PROTEIN KINASE C ACTIVITY.

Data are from a densitometric scan (pixels) of the autoradiogram developed after SDS-PAGE of the phosphorylated substrate (figure 15), and is expressed as the percent of zero minute untreated serum-deprived control. Similar profiles were obtained from identical experiments repeated several times, and the data from each densitometric scan is summarized in table 4.

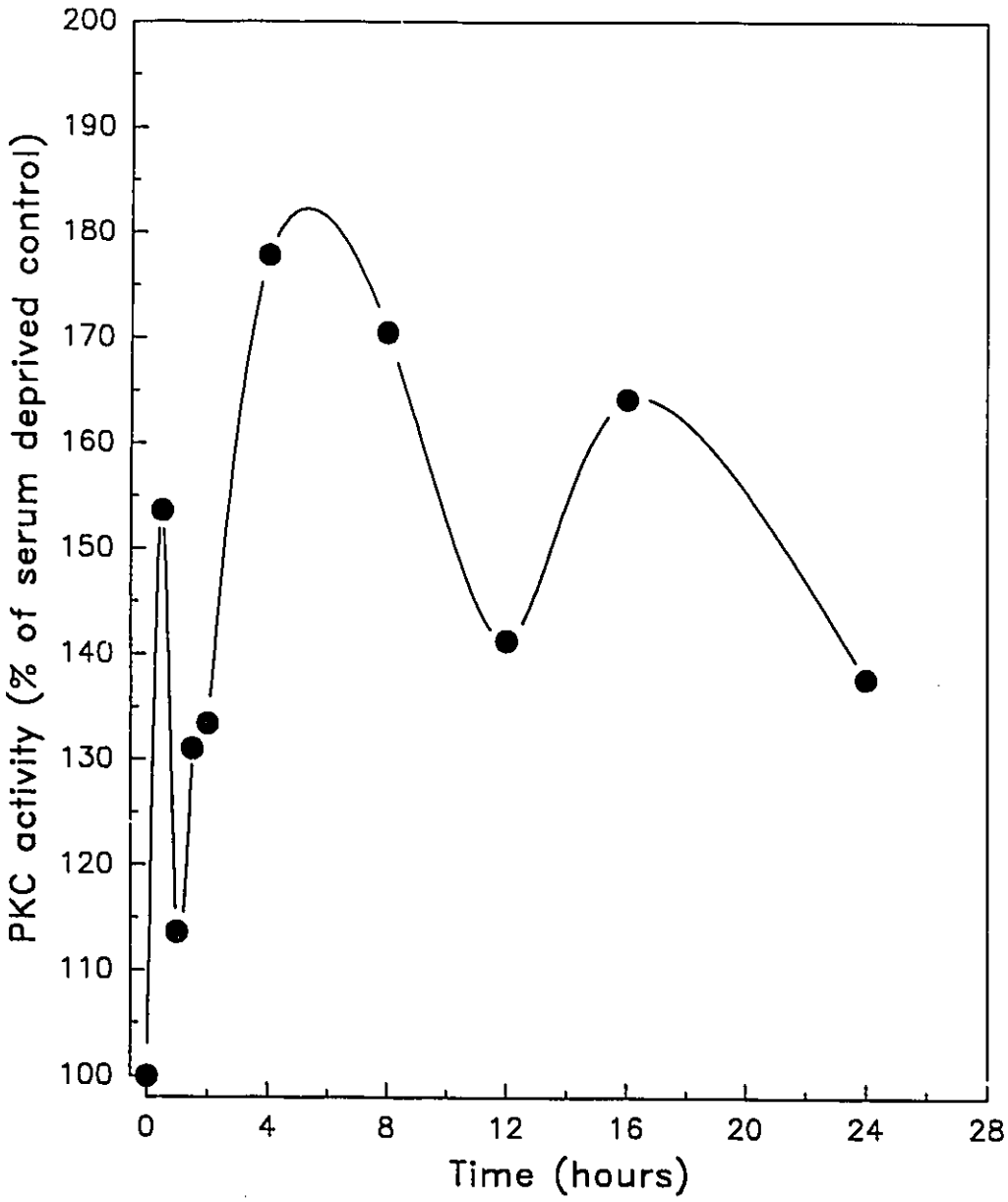
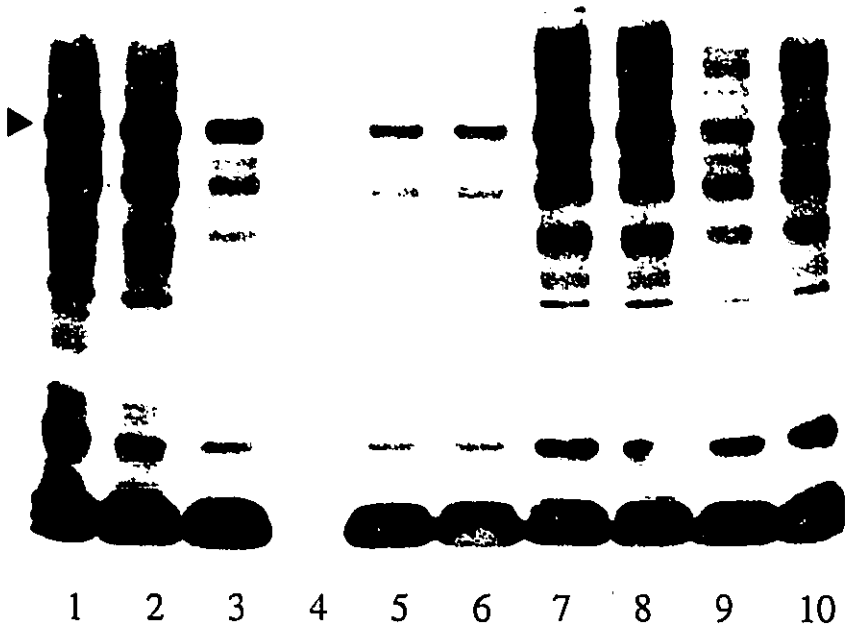

200
190
180
170
160
150
140
130
120
110
100
PKC activity (% of serum deprived control)
0
4
8
12
16
20
24
28
Time (hours)

FIGURE 17. LONG TERM EFFECTS OF EGF AND TPA ON PROTEIN KINASE C ACTIVITY.

Particulate PKC activity was measured as described in the methods. The autoradiogram developed after 10% SDS-PAGE is shown. Serum-deprived for 48 hours (lanes 1&2), followed by additional incubation in the same conditioned medium for 24 hours (No add, lane 3), molecular weight standards (lane 4), No add (as in lane 3) including final 18 hours with 100nM TPA (lanes 5&6), EGF (1.5ηM) for 24 hours after the 48 hours serum-deprivation of cells (lanes 7&8), and EGF (as in lanes 7&8) with the final 18 hours of TPA (lanes 9&10). Arrow indicates the 85 kDa. phosphorylated protein.


1 2 3 4 5 6 7 8 9 10

3.4 Specificity of EGF Stimulated DNA synthesis Regulation by TPA.

The effects of TPA and its inactive analog 4α phorbol di-decanoate (4αPDD) on EGF-mediated DNA synthesis was studied in the serum-deprived T51B cells. TPA addition to EGF or serum-treated cells enhanced their mitogenic responses, as discussed in section 3.1.5, whereas 4αPDD was ineffective (fig. 18). However, both TPA and 4αPDD were without effect on DNA synthesis in the presence of serum-deprived conditioned medium alone. When the time course of the TPA effect was examined, it was observed that TPA was only effective in potentiating the EGF-stimulated DNA synthesis, when it was present for at least 18 hours (fig. 19), during which time, TPA down-regulated PKC in these cells (fig. 17, table 5). Thus TPA mediated PKC inhibition / inactivation was further studied when cells were differently treated during the 48 hours 0.2% serum treatment. When cells were serum-deprived as in the previous experiments (0.2% BCS in BME), EGF (1.5ηM) was an incomplete mitogen under these conditions, since 10% BCS produced a much higher stimulation of DNA synthesis (fig. 20). This serum response was additive when EGF was also present (table 6). The combined effects of EGF, serum and TPA were not significantly higher than that of EGF and serum (table 6). But the potentiating effect of TPA was most evident with EGF or BCS (table 6) as before. When the cells were treated with TPA (100ηM) in the final 18 hours of the 48 hours serum deprivation pretreatment period, EGF or serum were

FIGURE 18. EFFECTS OF PHORBOL ESTERS ON EGF STIMULATED DNA SYNTHESIS.

Confluent and serum-deprived cells were incubated in the absence or, presence of EGF (1.5ηM), or serum (10%). TPA (100ηM), or 4αPDD (100ηM), when added, were present for the final 18 hours of the 24 hour incubation with [^{3}H]-thymidine and the agents mentioned above. DNA synthesis was measured as described in the methods. Data are expressed as the percent of serum-deprived control (mean ± SEM) from 3-6 separate experiments, each performed in triplicate. A significant increase in the DNA synthesis was observed (* indicates p=0.0004, and ** indicates p=0.006). The two-tailed paired t-test compared the means of DNA synthesis in the presence of EGF with and without TPA (*) or, DNA synthesis in the presence of 10% serum with and without TPA (**).

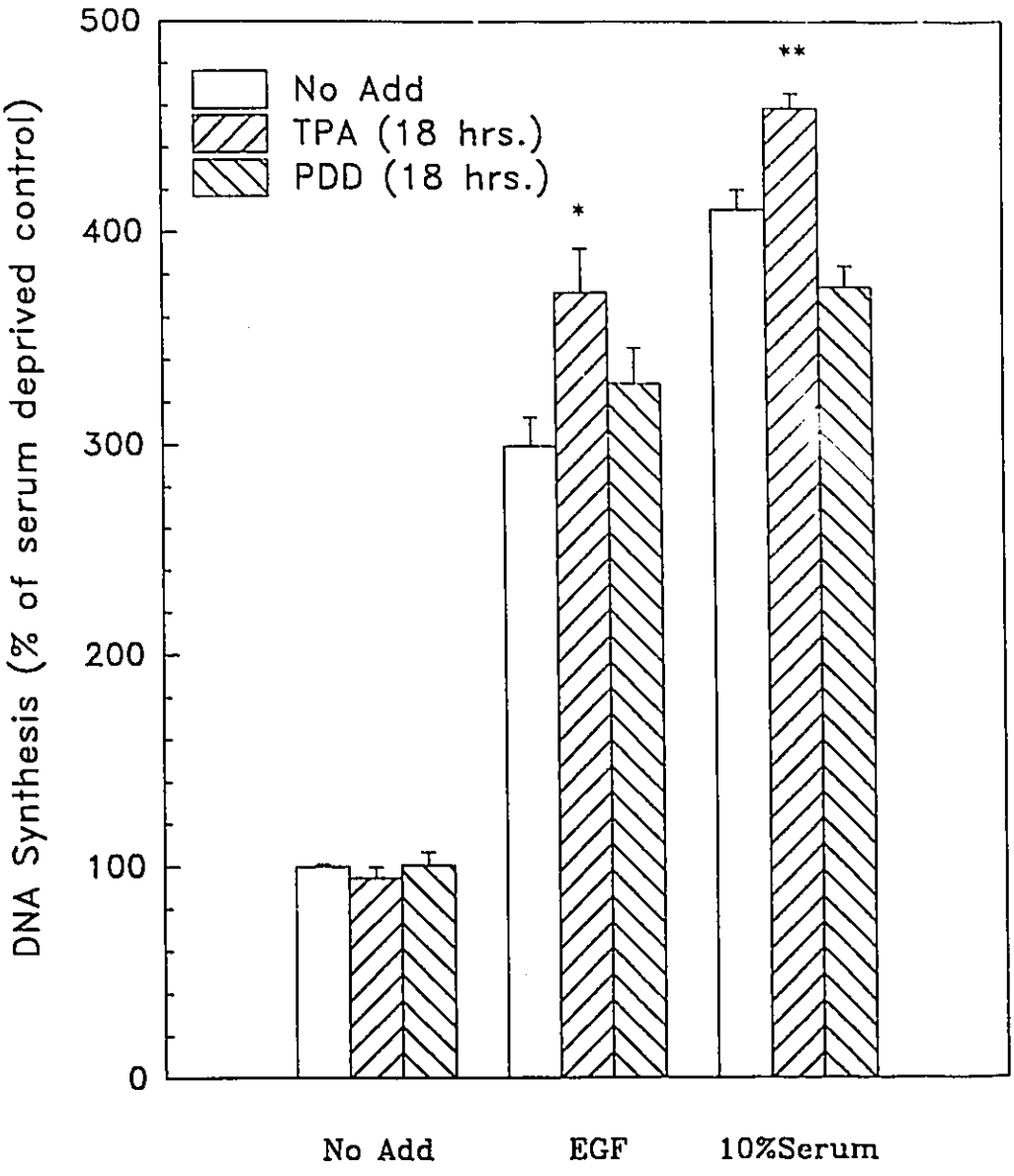

500
400
300
200
100
0
DNA Synthesis (% of serum deprived control)
No Add
TPA (18 hrs.)
PDD (18 hrs.)
*
**
No Add
EGF
10%Serum

FIGURE 19. TIME COURSE EFFECT OF TPA ON EGF-STIMULATED DNA SYNTHESIS.

Confluent and serum-deprived T51B cells were incubated with TPA (100ηM) for the indicated time intervals in the absence, or presence of EGF (1.5ηM)), and [3H]-thymidine for 24 hours. DNA synthesis was measured as described in methods. Data are expressed as the percent of serum-deprived control (mean ± SEM) from three separate experiments, each performed in triplicate.

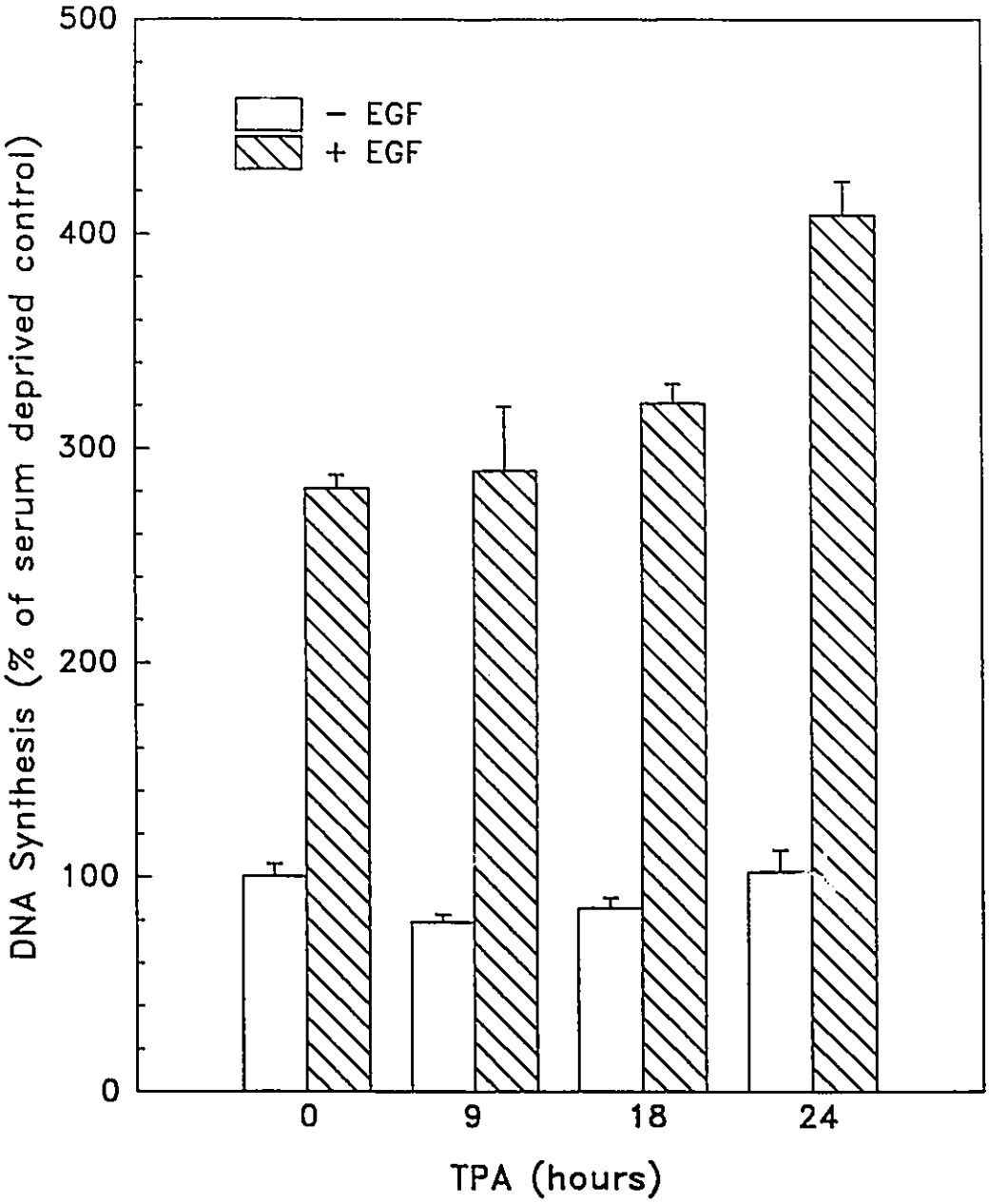

500
400
300
200
100
0
DNA Synthesis (% of serum deprived control)
− EGF
+ EGF
0
9
18
24
TPA (hours)

TABLE 5: EFFECTS OF TPA AND EGF ON PARTICULATE PROTEIN KINASE C ACTIVITY IN SERUM-DEPRIVED CELLS.

Confluent T51B cells were medium changed to 0.2% BCS containing BME (serum-deprived) for 48 hours. Cells were collected immediately (No Add), or taken after leaving for an additional 24 hours in the serum-deprived conditioned medium (A). EGF (1.5ηM) was added to the 48 hours serum-deprived cells, for 24 hours (B). TPA (100μM), where present, was added for the final 18 hours of treatment. Particulate PKC activity was measured by the phosphorylation of 85 kDa. protein as described in the methods. The data are expressed as pixel counts (arbitary units) obtained from the densitometric scan of the autoradiogram from the 10% SDS-PAGE gels.

TABLE 5.

	Treatment after 48 hours of low-serum incubation (0.2% BCS containing BME) (Particulate Protein kinase C activity in Net Pixels)				
	No Add	+24 hrs serum deprived medium (A)	(A) including 18 hrs. TPA	+ EGF 24 hours(B)	(B) including 18 hrs. TPA
Expt.1	466 469	251	145 149	433 470	188 219
Expt.2	-	426	241	763	262
Expt.3	-	1671 1591	145	2086	245

equally mitogenic (fig. 20). However, there was an overall decrease in total cpm in the cells pretreated with TPA during serum-deprivation (table 6). There was 60% less incorporation of [^{3}H]-thymidine in the control cells (table 6), but when these cells were examined by flow cytometry, there were no significant changes in the G1, G2, or S-phase DNA content. The percent increase in EGF response is due to the decreased basal level of DNA synthesis (table 6). The extent of [^{3}H]-thymidine incorporation by cells treated with EGF and serum, was still higher in cells pretreated with TPA, and an additional dose of TPA (100ηM) further elevated the increase in DNA synthesis due to either EGF or, serum alone. Hence, neither EGF, nor BCS were maximally mitogenic under these conditions when cells were pretreated with TPA.

3.5 Effects of TPA pretreatment on EGF-stimulated PKC activity.

Long-term serum-deprivation (48 hours) of confluent T51B cells showed high levels of particulate PKC activity (fig. 21, lane 1), which was down-regulated by TPA (fig. 21, lane 2). The reduced PKC activity in response to TPA remained low for at least 24 hours, under the serum-deprived conditions (fig. 21, lane 3). EGF activated membrane PKC, although this EGF-stimulated PKC activity was less in TPA pretreated cells (fig.21, lanes 5&6) in comparison to the untreated cells (fig. 21, lane 9). EGF-stimulated membrane-associated PKC activity, in TPA pretreated cells, was further down-regulated by an additional dose of TPA (fig 21, lanes 7&8). Similar results were obtained by measuring the PKC activity using a peptide substrate (fig. 22). The effect of TPA on untreated cells was not significant

FIGURE 20. EFFECT OF PKC DOWNREGULATION ON DNA SYNTHESIS IN T51B CELLS.

Confluent cells were serum deprived for 48 hours as before or, TPA (100ηM) was added (TPA pretreated) after 30 hours during the 48 hours serum-deprivation period. EGF (1.5ηM), or serum (10% v/v) were then added, where indicated, in the presence of [^{3}H]-thymidine for 24 hours. DNA synthesis was measured as described in methods. Data are expressed as the percent of corresponding serum-deprived control (mean ± SEM) from three separate experiments, n = 3 for each treatment.

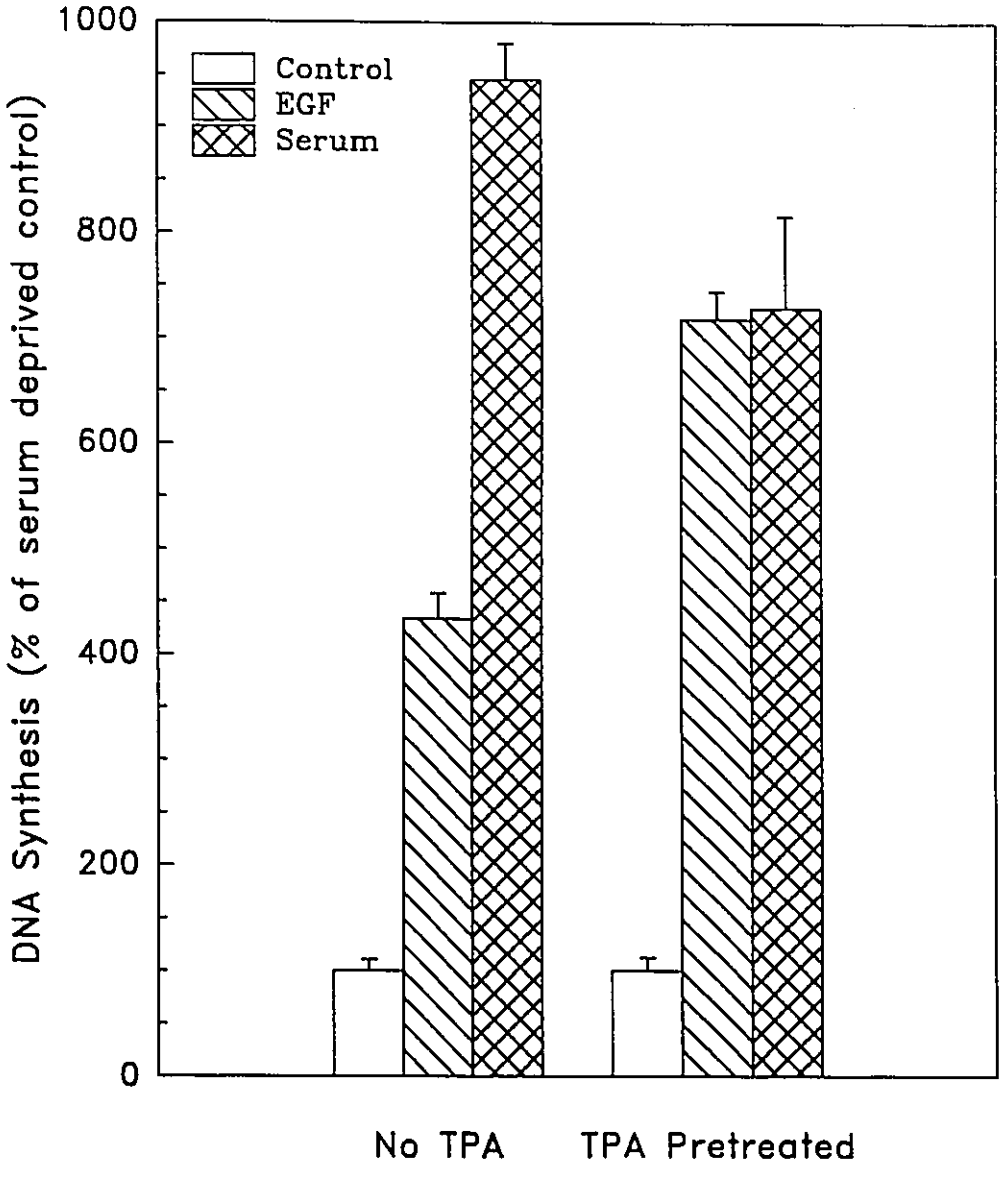

1000
800
600
400
200
0
DNA Synthesis (% of serum deprived control)
Control
EGF
Serum
No TPA
TPA Pretreated

TABLE 6: DNA SYNTHESIS IN RESPONSE TO EPIDERMAL GROWTH FACTOR IN T51B CELLS TREATED DIFFERENTLY DURING SERUM DEPRIVATION.

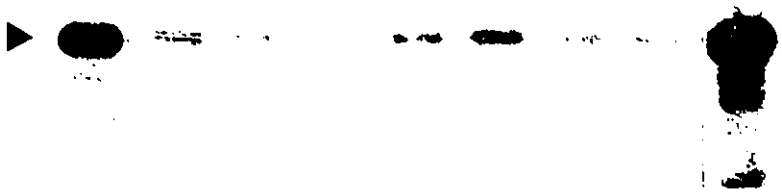
Confluent cells made quiescent by 0.2% serum change, for 48 hours (No TPA) or, TPA (100ηM) was added for the final 18 hours of the 48 hours serum-deprivation period (TPA pretreated). EGF (1.5ηM), BCS (10% v/v) or, TPA (100ηM) were then added alone or, in various combinations, with [^{3}H]-thymidine (0.5μCi/ml) for 24 hours. DNA synthesis was measured as described in the methods. Values are expressed in cpm (mean ± SEM) from three separate experiments, n = 3 for each point in an experiment. P values obtained from the two-tailed paired t-test are highly significant ($P<0.0001$), when compared with their corresponding 'No Add' (serum-deprived and untreated) controls (except the cpm value for TPA (68.0 ± 4.1 ($x10^3$)), which is not significantly different from its corresponding 'No Add' control).

TABLE 6.

Treatment	0.2% Serum for 48 hours.	0.2% Serum for 48 hours (including 18 hr.TPA).
	cpm (x 10^{-3}) (mean ± SEM)	
No Add	74.5 ± 7.8	31.2 ± 3.9
EGF	323.3 ± 18.3	224.2 ± 8.1
10% BCS	704.7 ± 25.4	227.5 ± 27.3
EGF + BCS	991.5 ± 12.3	739.1 ± 13.8
TPA	68.0 ± 4.1	16.4 ± 1.7
EGF + TPA	505.0 ± 15.5	330.6 ± 11.4
BCS + TPA	750.8 ± 40.8	263.2 ± 1.2
BCS+EGF +TPA	1,017.2 ± 40.2	746.4 ± 30.4

FIGURE 21. EFFECTS OF TPA PRETREATMENT DURING SERUM DEPRIVATION ON THE REGULATION OF PKC ACTIVITY BY EGF.

Protein kinase C activity was measured in the particulate fractions of the cells differently pretreated during the 48-hours serum-deprivation. Autoradiogram of a 10% SDS-PAGE gel shows the 85 kDa. phosphorylated protein by cells, serum-deprived (48 hours) (lane 1), serum-deprived for 48 hours, including TPA (100ηM) for 18 hours (lane 2), cells treated as in lane 2, followed by additional incubation for 24 hours (lane 3), or EGF (1.5ηM) added for 24 hours to the cells in lane 2 (lanes 5&6) or, EGF added (as in lanes 5&6) with an additional dose of TPA (100ηM) in the final 18 hours (lanes 7&8). Lane 9 shows the particulate-PKC activity due to EGF treated (1.5ηM, for 24 hours) cells which were serum-deprived for 48 hours (as in lane 1).


1 2 3 4 5 6 7 8 9

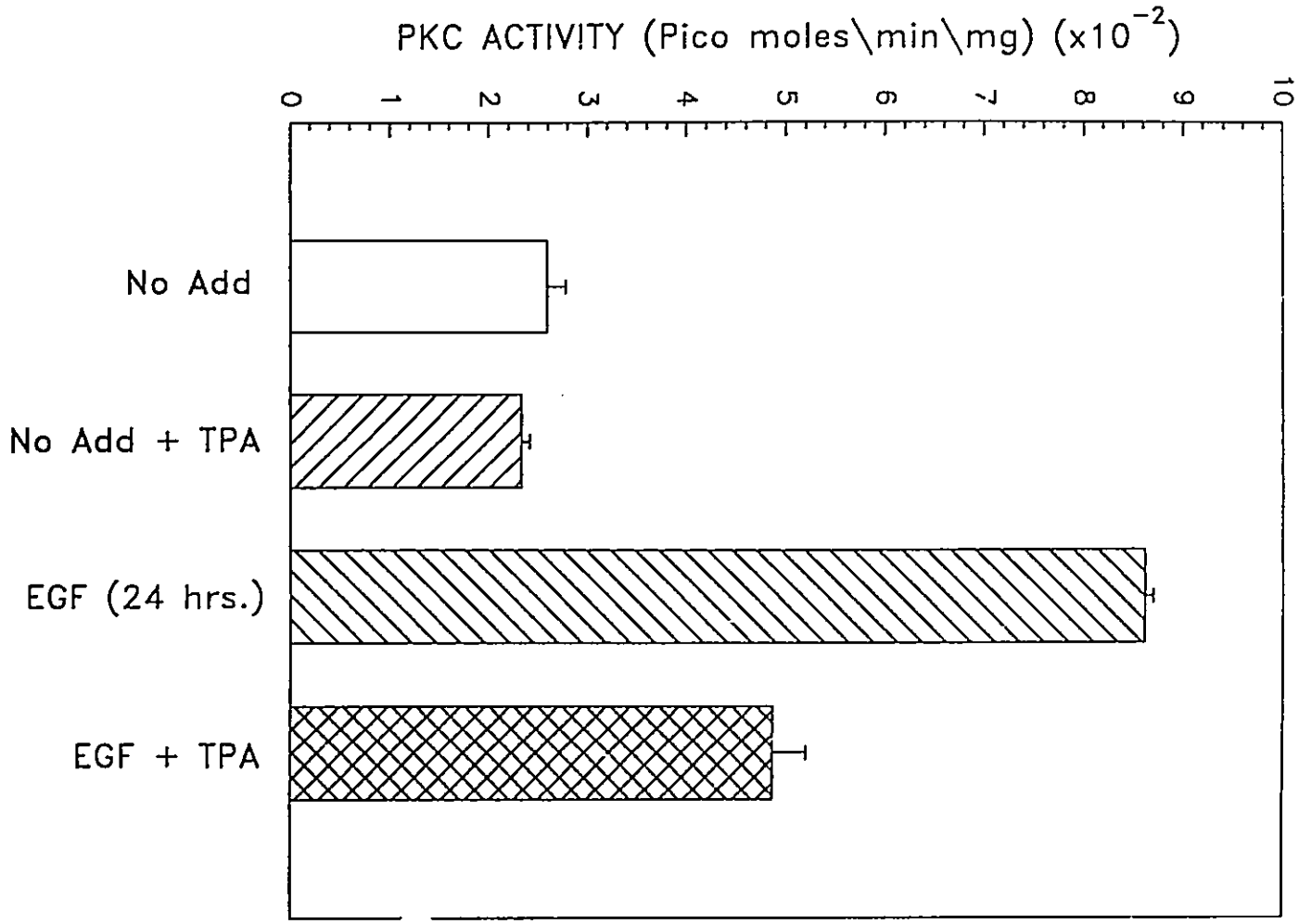
in this experiment. However, the EGF response to PKC activation in 24 hours was significantly reduced in the presence of TPA (fig. 22). These results, combined with the DNA synthesis data presented in table 6, show that PKC down-regulation, either by prolonged TPA-pretreatment, or TPA treatment of serum-deprived cells in the presence of EGF, leads to an increased mitogenic response in T51B cells.

3.6 Summary.

To summarize the results presented here, EGF is mitogenic to T51B cells in a dose and time-dependent manner. This effect is blocked by the tyrosine kinase inhibitors tyrphostin and genistein, as well as staurosporine, which is also an effective inhibitor of PKC. EGF stimulates PKC activity in the cell membrane in both the short and long-term, which is down-regulated by TPA. At the same time, EGF-mediated DNA synthesis is potentiated by TPA. TPA pretreatment of cells during serum-deprivation decreases the basal cell-proliferation, but EGF's mitogenic response in these serum deprived and TPA pretreated cells is doubled within 24 hours. An additional down-regulation of PKC by the second TPA treatment, further potentiates the mitogenic response of EGF under these conditions.

FIGURE 22. EFFECT OF TPA AND EGF ON PARTICULATE PKC ACTIVITY.

Particulate PKC activity was measured by the phosphorylation of synthetic peptide substrate, using the Amersham PKC assay kit. Serum-deprived for 72 hours (No Add), EGF added for 24 hours after the 48 hours serum-deprivation (EGF 24 hours). TPA, when present, was added for the final 18 hours. Data are represented as mean ± SEM, n = 3 for each data point.


PKC ACTIVITY (Pico moles\min\mg) (x10^{-2})
0
1
2
3
4
5
6
7
8
9
10
No Add
No Add + TPA
EGF (24 hrs.)
EGF + TPA

Chapter 4.

Discussion

The biochemical basis for signal-transduction across the cell membrane is a subject that has attracted a great deal of interest over the past several years. Considerable efforts have been made to understand the elaborate network of intracellular signalling systems that eventually lead to the regulation of physiological processes such as cell-cell communication, cell proliferation, and differentiation. Many growth factors and growth-modulating agents activate protein kinases that mediate signal transduction and regulation of cell growth. The binding of epidermal growth factor to its cell surface receptor induces its intrinsic tyrosine kinase activity. Many studies have shown the presence of two types of EGF-Rs, a high affinity class comprising generally 1-10% of the EGF-Rs and a larger proportion of a low affinity class of the EGF-Rs (Bowen et al., 1991). EGF binding presumably to the high affinity class, stimulates pleiotropic effects ranging from early events, such as activation of the receptor tyrosine kinase and changes in ion flux, to later events including the induction of proto-oncogenes and stimulation of DNA synthesis (Friedman, Fujiki and Rosner, 1990).

The present study examined the "cross-talk" among two major cell-signalling systems, at the level of the plasma membrane-particulate fraction. The effects of epidermal growth factor and protein kinase C activation on the growth-regulation of T51B rat liver epitheloid cells were studied using several experimental approaches.

4.1 Effect of serum on growth arrest of T51B cells.

When T51B cells were serum-changed to 0.2% serum containing medium, most of the cells became quiescent within 48 hours as evidenced by the flow cytometric and radiolabeling analyses. Earlier experiments in our laboratory, as well as by Boynton et al.(1977) and Boynton and Whitfield (1979), have shown that either serum-deprivation or, lowering the calcium concentration in normal serum-containing medium stopped the proliferation of T51B cells early in the G1 phase of their growth division cycle. The data presented (figs.3 & 4) demonstrate that confluent T51B cells can be made quiescent with prolonged serum-deprivation, without loss of viability, or loss of responsiveness to various growth factors and inhibitors. Serum-deprivation has been used successfully to induce growth-arrest of many cell types (Issandou and Darbon, 1991). In the case of T51B cells, autoradiographic analysis (fig. 4) showed that the cells under serum-deprived condition, still maintain a general "house-keeping" basal DNA synthesis and repair activity despite being in a synchronized and quiescent state.

4.2 EGF-stimulated mitogenesis of serum-deprived cells.

Epidermal growth factor is mitogenic to serum-deprived T51B cells in a progressive time-dependent and dose-responsive manner. However, doses exceeding 10ηM did not significantly change the total radioactivity incorporated, relative to the peak response at 24 hour. Similar response to high concentrations of EGF (greater than 10ηM) have been observed in other cell lines (Bagby, O'Reilly, Kirk, Mitchell, Stenberg, Makler and Bakke, 1992). This mitogenic response is maximal within 18-20 hours. The prolonged presence of EGF required

in the culture medium to elicit its maximal mitogenic response on serum-deprived T51B cells supports similar observations made in earlier studies in other cell lines (Carpenter and Cohen, 1990). However, the mitogenic response to EGF was not maximal when compared to the effect of 10% serum (fig. 20, table 6). There was further increase in DNA synthesis when cells were treated with EGF and serum, which suggested that even though serum is considered to be a 'complete' mitogen, its proliferative response could be further enhanced by EGF in these cells. The combined effect observed, was more additive than synergistic under these conditions. This is evident from the incorporation of [^{3}H]-thymidine in EGF, or 10% serum-treated cells alone, verses that due to serum and EGF together, which, in repeated experiments, appeared to be approximately additive (table 6). There was a substantial variation in the response of T51B cells to EGF. This could be explained by the different passages of the cells. Older passages of T51B cells, in our laboratory, have been found to become less responsive to various agents which is why passages up to seventeen only were studied. Identical conditions and passage numbers were used for experiments wherever possible for the experiments in this study. However, due to the limited availability of a single passage of cells at a given time, and time constraints for certain experiments, it was not possible to maintain a certain passage for long periods of time. Even in the similar set of culture conditions for a given experiment, the response variability could be seen because of the different stock cultures. However, these variations were minimized, where possible.

4.3 Effects of tyrosine kinase inhibitors.

Several lines of evidence indicate that activation of the EGF receptor tyrosine kinase is required for the induction of mitogenesis (Friedman et al., 1990). No stimulation of cell division was observed when tyrosine kinase activity was abolished by site-directed mutagenesis (Schlessinger, 1986; Chen, Lazar, Poeinic, Tsein, Gill and Rosenfield, 1987). Genistein has been shown to block a number of EGF dependent phenomenon, including both receptor autophosphorylation and histone phosphorylation. Genistein inhibited EGF-stimulated DNA synthesis dose-dependently with an IC_{50} of 10-15μM, when present for 24 hours. However, once the cells were committed to DNA synthesis (i.e., after 18 hours of EGF treatment) and had entered S phase, genistein had no inhibitory effect on DNA synthesis. Therefore, the inhibitory action of genistein on the EGF-stimulated mitogenic response is an early phenomenon that is associated with its receptor tyrosine kinase activity. Once the cells become committed to DNA synthesis, inhibition of the EGF-receptor tyrosine kinase activity by genistein is upstream of some of the signals needed to undergo replication of cells. The inhibitory response of genistein in the presence of serum was also significant as it reduced the DNA synthesis to approximately 50% of that due to serum alone. This suggests that the mitogenic response to other components (growth factors and hormones) present in the serum could also be inhibited by genistein, either via inhibition of receptor tyrosine kinase or by an as yet unidentified effect of genistein not related to tyrosine kinase inhibition. Such a nonspecific response could also explain the inhibition of labelled thymidine incorporation in quiescent cells, in which cells were incubated in serum-

deprived medium for two days, in the absence of any growth-stimulating agents. The inhibitory effect of genistein has also been observed in a fibroblast cell line (Linassier, Pierre, LePeco and Pierre, 1990), in which genistein inhibited the mitogenic effects of thrombin, although the thrombin receptor does not involve any protein tyrosine kinase activity. In addition the inhibition of topoisomerase II by genistein (Linassier et al., 1990) which also contributes to the inhibition of DNA synthesis can not be excluded. The lower inhibitory response of tyrphostin on EGF-stimulated DNA synthesis in T51B cells could be due to its relatively slower rate of entry into the cells, as well as the rapid loss of its inhibitory effect on autophosphorylation of EGF-R (Lyall, Zilberstein, Gazit, Gilon, Levitzki and Schlessinger, 1988) as well as due to its specificity for EGF-R tyrosine kinase. Staurosporine is also a potent tyrosine kinase inhibitor. Initially characterized as a protein kinase C inhibitor (Tamaoki, Nomoto, Takahashi, Morimoto and Tomita, 1986), staurosporine also inhibits c-AMP dependent protein kinase in vitro (Tamaoki et al., 1986) and p60v-src tyrosine kinase (Nakano, Kobayashi, Takahashi, Tamaoki, Kuzuu and Iba, 1987). The IC_{50} for inhibition of PKC, c-AMP-dependent PKA, and p60v-src by staurosporine are within the same order of magnitude ranging from 3-30ηM (Tamaoki et al., 1986; Nakamo et al., 1987). Staurosporine has also been found to antagonize the inhibition of high affinity binding of the EGF receptor induced by phorbol esters and growth factors (Friedman et al., 1990). The same authors have also shown that staurosporine enhances EGF binding to the receptor, which is accompanied by a reduction in the phosphorylation state of the EGF receptor, raising the possibility that the high

affinity state of EGF receptor is normally suppressed as a consequence of receptor phosphorylation. Taken together, these observations justify the highly potent inhibitory role of staurosporine on EGF stimulated as well as basal DNA synthesis in T51B cells.

4.4 EGF and Protein kinase C.

EGF activated particulate PKC activity within 10-15 minutes (fig. 14). Activation of EGF receptor-tyrosine-kinase leads to the stimulation of phosphoinositide hydrolysis and increases in the cytosolic Ca^{++} (Pike and Eakes, 1987; White, 1991). However, EGF does not activate PI hydrolysis in T51B cells, as well as in a number of other cell types, even though it does cause an increase in DAG via phosphatidylcholine breakdown, which eventually leads to an intracellular increase in Ca^{++} (Hill et al., 1988) and PKC activation. Whether the early tyrosine kinase activation also regulates PKC in T51B cells remains unclear. Repeated attempts were made to study the short-term response of genistein on EGF-stimulated PKC activity, however no significant changes in the EGF-stimulated PKC activity were observed (data not shown). It has been demonstrated by Dean et al, (1987) that EGF induces an increase in DAG which is inhibited by genistein. The observation by the same authors, that EGF-mediated accumulation of DAG is independent of PIP_2 hydrolysis and increased Ca^{++}, suggests that other pathways lead to DAG accumulation and PKC activation (fig. 2). Since low concentrations of genistein, which were inhibitory to the long-term EGF-stimulated DNA synthesis, may not be sufficient to have significant effect on shorter-term PKC activation by EGF, additional experiments using higher genistein

concentrations are needed to closely follow the interaction between EGF-mediated PKC activation and its interaction with the EGF-R tyrosine kinase. Unlike TPA, which is structurally related to DAG and acts at the cell-membrane, the large benzene-ring structure of genistein may not easily traverse the cell-membrane. In addition, it is possible that the transient PKC activation by the generation of "second messengers", in response to EGF-R tyrosine kinase may occur long before genistein could block this effect. Therefore, longer times of incubation in the presence of genistein may be required to improve its intracellular entry and effectiveness, before the EGF treatment of T51B cells. The EGF-stimulated PKC activity remained high for up to 24 hours. Although the extent of activation varied from one experiment to the other due to the differences in PKC activity levels in untreated serum-deprived cells, the activation followed the same consistent pattern. The poor reproducibility and inconsistent levels of cytosolic PKC activity in these cells as measured by the S49 cyc⁻ PKC substrate phosphorylation, could result from the presence of constitutively active catalytic fragments of PKC (also called PKM) as well as the presence of other PKC isoforms which may not necessarily require Ca^{++} for activation. It is generally accepted that TPA 'activates' PKC by promoting its translocation from cytosol to the plasma membrane, when the enzyme is activated (Anderson, Estival, Tapiovaera and Gopalakrishna, 1985). TPA has been shown to decrease cytosolic PKC activity temporally in T51B cells (Aasheim, Kleine and Franks, 1989). Earlier experiments in Dr. Kleine's laboratory have shown that, as early as 3-5 minutes TPA treatment increases particulate PKC activity in T51B cells. Long-term TPA treatment of serum-deprived T51B cells

down-regulated particulate PKC activity (figs.18, 21 & 22). Further study is required to determine the regulation of various PKC isozymes by TPA, and EGF using Western immunoblotting and immuno-cytochemistry. This would allow to further investigate the roles of specific PKC isoforms in T51B cell-signalling.

4.5 **Effect of TPA on EGF-stimulated DNA synthesis.**

In serum-deprived T51B cells, neither TPA nor its inactive analog 4αPDD alone, were able to stimulate DNA synthesis (fig. 18). Similar observations were made by Hill et al. (1988), who showed that TPA was not mitogenic to T51B cells. However, TPA added in combination with EGF enhanced the EGF-induced DNA synthesis to a higher rate by 24 hours (fig. 19), as well as significantly increasing the rate of cell-proliferation (table 3). The combined effects of serum and TPA on T51B cell-proliferation (table 3) was most pronounced under these conditions. This effect could be accounted for by the presence of additional agents in the serum which could act synergistically with TPA. The attenuation of the inhibitory effect of genistein on EGF-stimulated DNA synthesis, in the presence of TPA suggests additional EGF-dependent pathways that may not necessarily involve EGF-R tyrosine kinase activation. Similar observations were made with insulin, another growth factor, which acts through its receptor-tyrosine-kinase in rat adipocytes (Alber, Smith, Randazzo, Rottenberg and Jarett, 1992). It is possible that the combined stimulatory effects of TPA on EGF-mediated mitogenic signalling is inhibited to the same extent (50%) by genistein, even when the combined effects of EGF and TPA are higher than that due to EGF alone (fig. 12). A more direct approach to verify the interaction between TPA and genistein effects on the EGF-

mediated mitogenic signalling is needed. Detection and characterization of the EGF-R and its associated tyrosine kinase in T51B cells would be a major step in this direction.

4.5.1 Regulation of EGF-mediated mitogenesis by PKC.

The data presented in this report, as well as preliminary data from Western immunoblot analysis with an anti-EGF-R antibody in Dr. Kleine's laboratory, strongly suggests the presence of EGF-R in T51B cells. Experiments with hepatocytes in primary culture have shown a time-dependent increase in the sensitivity of the growth stimulatory effect of EGF (Sand and Christoffersen, 1987) associated with a progressive loss in high affinity EGF-R binding (Gladhaug, Refnes, Sand and Christoffersen, 1988). TPA has been shown to down-regulate the EGF-R (Thompson and Gill, 1985). However, it is not clear whether TPA inhibits EGF binding only, or converts the high affinity receptor into a low affinity state. It has been suggested that the high affinity EGF-Rs are more sensitive to negative modulation by TPA, presumably as a result of PKC activation (Gladhaug, Refsnes and Christoffersen, 1992). If a similar situation exists for the putative EGF-R in T51B cells, then phorbol-ester-induced down-regulation of the EGF-R could account for the decreased net [^{3}H]-thymidine incorporation in T51B cells when they were pretreated with TPA during serum-deprivation (table 6).

The strong association between TPA-induced PKC activation and the various EGF-mediated short-term and long-term responses have been studied by many investigators. Huckle et al., (1990) have shown that acute PKC activation by TPA inhibited EGF-stimulated tyrosine phosphorylation of PLCγ and [^{3}H]-inositol

phosphate accumulation in WB rat liver epithelial cells. Prolonged treatment of WB cells with TPA resulted in depletion of PKC activity and loss of the capacity of TPA to inhibit EGF stimulated IP accumulation (Huckle et al., 1990). In T51B cells, EGF caused a higher fold stimulation of DNA synthesis either in the presence of TPA or when cells were pretreated with TPA. By then, the PKC activity was already downregulated (figs. 21 & 22) prior to EGF addition to the incubation medium. The regulation of EGF-R function by PKC has been studied in detail. PKC activation results in the phosphorylation of the EGF-R at Thr654 (Countaway, Nairn and Davis,1992). However, phosphorylation of Thr654 is not necessarily required for the EGF-R affinity decrease, since other serine / threonine sites on the EGF-R are also phosphorylated (Davis, 1988). Therefore, it is possibly the extent of receptor phosphorylation which regulates the receptor-mediated phenomena. Bowen et al. (1991) have shown that a single point-mutant EGF-R where Thr654 is replaced by Ser654, is constitutively phosphorylated in response to EGF. [Ala654] EGF-R is not phosphorylated by PKC activation (Bowen et al,1991). TPA caused an inhibition of the high affinity binding of ^{125}I-EGF to cells expressing [Thr654], [Ser654], and [Ala654] EGF-Rs. Nevertheless, EGF-stimulated mitogenesis was higher in [Ala654] EGF-R only. Therefore it was suggested that the site of PKC action, Thr654 phosphorylation in wild type EGF-R inhibits EGF-stimulated mitogenic signal-transduction. As mentioned earlier, the high affinity EGF-Rs are selectively lost early on in the signal-transduction process and the high affinity receptors are also associated with the early responses due to EGF, such as tyrosine kinase activation and second messenger generation. If such is the case

in T51B cells, then serum-deprivation would result in the loss of the EGF receptors (the high affinity EGF-Rs prefentially) with increasing time in culture. At the same time, the sensitivity to growth in respose to EGF would increase. EGF addition would result in DNA synthesis that would be mediated by the remaining EGF receptors. During this process, PKC activated in the presence of EGF would modulate the EGF receptor affinity / tyrosine kinase activity by a negative feedback mechanism. Addition of TPA to the EGF treated cells would cause the downregulation of PKC activity and therefore, would potentiate the mitogenic response to EGF. Long term TPA pretrearment of serum-deprived cells, before the addition of EGF would result in the maximal loss of high affinity receptors as well as the overall receptor losses due to the combined effects of TPA and serum-deprivation. Protein kinase C would also be significantly depleted in the presence of TPA, before EGF is added. EGF treatment under these conditions, would minimize the inhibition of EGF receptors and DNA synthesis would be the least inhibited. However, there will be an overall decrease in DNA synthesis due to the receptor losses, as well as due to minimal "newly recruited" active PKC in the presence of EGF. The same results were obtained when EGF was as mitogenic as 10% serum when cells were pretreated with TPA (fig. 20, table 6). EGF-R transmodulation via its phosphorylation at multiple sites by PKC, suggests its potential importance in the regulation of EGF receptor function. It however, remains unproven whether TPA or PKC modification of integral membrane proteins (or lipids) indirectly results in the altered EGF receptor numbers and affinity, or whether their loss is a consequence of a direct effect of PKC catalyzed EGF

receptor phosphorylation. Numerous studies including a recent study by Gladhaug et al., (1992) have suggested that increased mitogenic sensitivity to EGF is inversely associated with the receptor level. However a causal relationship in which a subpopulation of low affinity receptors remain after down-regulation and transduces a stronger mitogenic signal than that transmitted by the entire surface receptor pool has not been proven. In this thesis, I hypothesize that it is the initial activation of PKC via the EGF-dependent pathway, which transmodulates the ligand-induced EGF-R and causes the selective loss of high affinity binding. This sets the stage for quiescent T51B cells in serum-deprived medium and TPA-mediated PKC down-regulation, to become maximally responsive to EGF's mitogenic response. If it is the inhibition/downregulation of PKC, which results in an un-inhibited EGF-mediated mitogenic response, then additional studies with PKC activators like diacylglycerols, or vasopressin would permit us to determine if constitutive PKC activation could cause, (a) elevated EGF-R phosphorylation at selected serine / threonine residues including residue 654 and its subsequent inhibition of tyrosine kinase activity, (b) inhibition of DNA synthesis and cell proliferation, and (c) the cell surface EGF-R loss with time. This, combined with assays to measure PKC levels by immunoblot analysis could answer whether high PKC activity and PKC levels are a pre-requisite for the proliferatively quiescent state, inhibiting cell-proliferation. An additional approach to study the initial inhibition of PKC vs. enhanced EGF responses, would be to use specific PKC inhibitors, for e.g. peptide inhibitors, which cell permeable and selectively inhibit PKC. Last, but not least, the possible involvement of G-proteins would address another link between EGF-R and protein kinase C "cross-talk".

Chapter 5.

CONCLUSIONS

T51B rat liver epithelial cells serve as a good working model for studying the two cell-signalling pathways: EGF-mediated mitogenic signalling and, protein kinase C "cross-talk" with the EGF receptor. The following conclusions can be drawn from the results in this study.

1. Serum-deprived, proliferatively quiescent T51B cells respond to the growth factor EGF and, serum by increased DNA synthesis and cell-proliferation.
2. EGF is an incomplete mitogen; addition of serum to EGF treated cells further enhanced the mitogenic effect.
3 The mitogenic response of EGF can be inhibited by a tyrosine kinase inhibitor, genistein, when it is added early enough (in the first 8 hours of EGF addition), before the cells have committed themselves for DNA replication.
4. The phorbol-ester TPA, or its inactive analog 4αPDD alone, are not mitogenic to serum-deprived cells. However, when TPA is added in the presence of EGF or serum, it potentiates their mitogenicity.
5. EGF activates protein kinase C. The prolonged treatment of cells with TPA (18 hours) results in the inactivation of untreated as well as EGF-stimulated PKC.
6. Long-term TPA pretreatment (18 hours) of cells during serum-deprivation

makes them equally sensitive to EGF or serum, but the overall DNA synthesis is reduced.

7. There is a correlation between TPA induced PKC-inactivation (down-regulation) and, its potentiating effects on EGF-stimulated mitogenicity in T51B cells.

REFERENCES

Aasheim, L.H., Kleine, L.P. and Franks, D.J. (1989) Activation of protein kinase C sensitizes the cyclic AMP signalling system of T51B rat liver cells. Cell. Signalling 1, 617-625.

Alber, A., Smith, J.A., Randazzo, P.A., Rothenberg, P.L. and Leonard, J. (1992) Genistein differentially inhibits post receptor effects of insulin in rat adipocytes without inhibiting the insulin receptor kinase. J. Biol. Chem. 267, 3496-3951.

Anderson, W.B., Estival, A., Tapiovaera, H and Gopalakrishna, R. (1985) Altered subcellular distribution of protein kinase C (a phorbol ester receptor). Possible role in tumor promotion and the regulation of cell growth relationship to changes in adenylate cyclase activity. Adv. Cyclic Nucleotide Prot. Phosphorylation Res. 19, 287-306.

Argiles, A., Kraft, N., Ootaka, T., Hutchinson, P and Atkins R.C. (1992) EGF and TGF Stimulate or inhibit Proliferation of a human renal adrenocarcinoma cell line depending on cell status: Differentiation of the two pathways by G Protein involvement. Cancer Res., 52, 4356-4360.

Ashendel, C. L. (1985) The phorbol ester receptor: a phospholipid-regulated kinase Biochim. Biophys. Acta 822, 219-242.

Ashendel, C. L., Staller, J. M. and Boutwell, R. K. (1983) Protein kinase C activity associated with a phorbol ester receptor purified from mouse brain. Cancer Res. 43, 4333-4337.

Bacher, N., Zisman, Y., Berent, E. and Livneh, E. (1991) Isolation and characterization of PKC-L, a new member of the protein kinase C-related gene family specifically expressed in lung, skin and heart. Molec. Cell Biol. 11, 126-133.

Bagby, S.P., O'Reilly, M.M., Kirk, E.A., Mitchell, L.H. Stenberg, P.E., Makler, T. and Bakke, A.C. (1992) EGF is incomplete mitogen in porcine aortic smooth muscle cells: DNA synthesis without cell division. Am. J. Physiol., 262, C578-C588.

Bazzi, M. D., and Nelsestuen, G. L. (1988) Association of protein kinase C with phospholipid monolayers: two stage irreversible binding. Biochemistry 27, 6776-6783.

Bell, R.M. and Burns, D.J. (1991) Lipid activation of protein kinase C. J. Biol. Chem. 266, 4661-4664.

Berg, J.(1986) Potential metal-binding domains in nucleic acid binding proteins. Science 232, 485-487.

Berridge, M. J. (1983) Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyze polyphosphoinositides instead of phosphatidylinositol. Biochem. J. 212, 849-858.

Berridge, M.J., Heslop, J.P., Irvine, R.F. and Brown, K.D. (1984) Inositol trisphosphate formation and calcium mobilization in Swiss 3T3 cells in response to platelet-derived growth factor. Biochem. J. 222, 195-201.

Berridge, M.J. and Irvine,R.F.(1989) Inositol phosphates and cell signalling. Nature 341, 197-205.

Besterman, J.M., Watson, S.P. and Cuatrecasas, P. (1986) Lack of association of epidermal growth factor-, insulin-, and serum-induced mitogenesis with stimulation of polyphosphoinositide degradation in BALB/c 3T3 fibroblast. J.Biol.Chem. 261, 723-727.

Billah, M. M. and Canthes, J. C. (1990) The regulation and cellular functions of phosphatidylcholine hydrolysis. Biochem. J. 269, 281-291.

Boni, L.T. and Rando, R.R. (1985) The nature of protein kinase C activation by physically defined phospholipid vesicles and diacylglycerols. J Biol. Chem. 260, 10819-10825.

Bowen, S., Stanley, K., Selva, E. and Davis, R.J. (1991) Constitutive phosphorylation of the epidermal growth factor receptor blocks mitogenic signal transduction. J. Biol. Chem. 266, 1162-1169.

Boynton, A.L., Whitfield, J.F., Issacs, R.J., Trembley, R.G. (1977) Cancer Res. 37, 2657-2661.

Boynton, A.L.and Whitfield, J.F. (1976) The different actions of normal and supranormal calcium concentrations on the proliferation of BALB/c 3T3 mouse cells. In Vitro 12, 479-484.

Boynton, A.L. and Whitfield, J.F. (1979) The cyclic AMP-dependent initiation of DNA synthesis in T51B rat liver epitheloid cells. J.Cell Physiol. 101, 139-148.

Boynton, A., Whitfield, J., Bossi, D., Durkin, J., Franks, D., Kleine, L. and Raptis, L. (1985a) In: Cell Membranes and Cancer, Eds: Galleotti et al., (Elsevier) 417-430.

Boynton, A.L., Kleine, L.P., Whitfield, J.F. and Bossi, D. (1985b) Involvement of the Ca^{++}/phospholipid-dependent protein kinase in the G1 transit of T51B rat liver epithelial cells. Expt. Cell Res. 160, 197-205.

Boynton, A.L., Kleine, L.P. and Whitfield, J.F. (1985c) In: Control of Animal cell Proliferation, Eds: A.L. Boynton and H.L. Leffert (Academic Press, Orlando, FL.) Vol 1, 121-149.

Cantrell, D. A., Davies, A. A., Crumpton, M. J. (1985) Activators of protein kinase C down-regulate and phosphorylate the T3/T-cell antigen receptor complex of human T lymphocytes. Proc. Natl.Acad. Sci. USA 82, 8158-8162.

Carpenter, G. and Cohen, S. (1979) Epidermal growth factor. Ann. Rev. Biochem. 48, 193-216.

Carpenter, G. and Cohen,S. (1990) Epidermal growth factor, J. Biol. Chem. , 265, 7709-7712.

Carpenter, G. (1987) Receptors for epidermal growth factor receptor and other polypeptide mitogens. Ann. Rev. Biochem. 56, 881-914.

Carpenter, G. and Wahl, M.I. (1990) The epidermal growth factor family. Handb. Exp. Pharmacol. 951,69-171.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257, 7847-7851.

Chakravarthy, B.R., Franks, D.J., Whitfield, J.F. and Durkin, J.P. (1989) A novel method for measuring protein kinase C activity in a native membrane-associated state. Biochem. biophys. Res. Commun. 160, 340-345.

Chambard, J.C., Paris, S., L'Allemain, G. and Pouyssegur, J.(1987) Two growth factor signalling pathways in fibroblasts distinguished by pertussis toxin. Nature 326, 800-803.

Chan, B. L., Chao, M. V. and Saltiel, A. R. (1989) Nerve growth factor stimulates the hydrolysis of glycosylphosphatidylinositol in PC-12 cells: a mechanism of protein kinase C regulation. Proc. Natl.Acad. Sci. USA 86, 1756-1760.

Chen, W.S., Lazar, C.S., Poenie, M., Tsien, R.Y., Gill, G.N. and Rosenfield, M.G. (1987) Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. Nature , 328, 820-823.

Countaway, J.L., Nairn, A.C. and Davis, R.J. (1992) Mechanism of desensitization of the epidermal growth receptor protein- tyrosine kinase. J. Biol. Chem. 267, 1129-1140.

Coussens, L., Parker, P.J., Rhee, L., Yang - Fang, T.L., Chem, E., Waterfield, M.D. Franke, U. and Ullrich A. (1986) Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signalling pathways. Science 233, 859-866.

Coussens, L., Rhee, L., Parker, P. J. and Ullrich, A., (1987) Alternative splicing increases the diversity of the human protein kinase C family. DNA 6, 389-394.

Davis, R.J. (1988) Independent mechanisms account for the regulation by protein kinase-C of the EGF receptor affinity and tyrosine-protein kinase activity. J.Biol.Chem. 263, 9462-69.

Downward, J., Waterfield, M.D. and Parker, P.J. (1985) Autophosphorylation and protein kinase C phosphorylation of the epidermal growth factor receptor. Effect on tyrosine kinase activity and ligand binding affinity. J.Biol.Chem. 260, 14538-14546.

Earp, H.S., Hepler, J.R., Petch, L.A., Miller, A., Berry, A.R., Harris, J., Raymond, V.W., McCune, B.K., Lee, L.W., Grisham, J.W. and Harden, T.K. (1988) Epidermal growth factor (EGF) and hormones stimulate phosphoinositide hydrolysis and increase EGF receptor protein synthesis and mRNA levels in rat liver epithelial cells. J.Biol.Chem. 263, 13868-13874.

Exton, J. H. (1988) Mechanisms of action of calcium-mobilizing agonists: some variations on a young theme.FASEB J. 2, 2670-2676.

Exton, J. H. (1990) Signalling through phosphatidylcholine breakdown. J. Biol. Chem. 265,1-4.

Farago, A. and Nishizuka, Y. (1990) Protein kinase C in transmembrane signalling. FEBS Lett. 268, 350-354.

Felder, S., Miller, K., Moehren, G., Ullrich, A., Schlssinger, J., and Hopkins, C.R. (1990) Kinase activity controls the sorting of EGF receptor within the multivesicular body. Cell 61, 623-634.

Franks, D.J., Plamondon, J. and Hamet, P. (1984) An increase in adenylate cyclase activity precedes DNA synthesis in cultured vascular smooth muscle cells. J.Cell. Physiol. 119, 41-45.

Friedman, B.A., Fujiki, H. and Rosner, M.R. (1990) Regulation of the epidermal growth factor receptor by growth-modulating agents: effects of staurosporine, a protein kinase inhibitor. Cancer Res. 50, 533-538.

Ganong, B.R., Loomis, C.R., Hannun, Y.A. and Bell, R.M. (1986) Specificity and mechanism of protein kinase C activation by sn-1,2-diacylglycerols. Proc. Natl. Acad. Sci. USA 83, 1184-1188.

Ganong, B.R. (1991) Roles of lipid turnover in transmembrane signal transduction. Amer J. Med. Sc. 302(5), 304-312.

Ghosh-Dastidar, P. and Fox, C.F. (1984) c-AMP dependent protein kinase stimulates epidermal growth-factor-dependent phosphorylation of epidermal growth factor receptors.
J.Biol.Chem. 259, 3864-3869.

Gladhaug, I.P., Refsnes, M. Sand, T.E. and Christoffersen, T. (1988) Effects of butyrate on epidermal growth factor receptor binding, morphology, and DNA synthesis is cultured rat hepatocytes. Cancer Res. 48, 6560-6564.

Gladhaug, I.P., Refsnes, M. and Christoffersen, T. (1992) Regulation of surface expression of high affinity receptors for epidermal growth factor (EGF) in hepatocytes by hormones, differentiating agents, and phorbol ester. Digestive Dis. Sci. 37, 233-239.

Hannun, Y. A., Loomis, C. R., and Bell, R. M. (1986) Protein kinase C activation in mixed micelles. Mechanistic implications of phospholipid, diacylglycerol, and calcium interdependies. J Biol. Chem. 261, 7184-7190.

Hansson, A., Serhan, C. N., Haeggstrom, J., Ingieman-Sunderg, M. and Samuelsson, B. (1986) Activation of protein kinase C by lipoxin A and eicosinoids. Intracellular action of oxygenation products of arachidonic acid. Biochem. biophys. Res. Commun. 134,1215-1222.

Hauguel-Demouzon, S., Csermely, P., Zoppini, G. and Kahn, C. R. (1992) Quantitative dissociation between EGF effects on c-myc and c-fos gene expression, DNA synthesis, and epidermal growth factor receptor tyrosine kinase C activity. J. Cell Physiol. 150. 180-187.

Hepler, J.R., Nakahata, N., Lovenberg, T.W., DiGuiseppi, J., Herman, B., Earp, H.S., and Harden, T.K. (1987) Epidermal growth factor stimulates the rapid accumulation of inositol (1,4,5)-trisphosphate and a rise in cytosolic calcium mobilized from intracellular stores in A431 cells. J.Biol.Chem. 262, 2951-2956.

Hill, T.D., Kindmark, H. and Boynton, A.L. (1988) Epidermal growth factor-stimulated DNA synthesis requires an influx of extracellular calcium. J. Cell. Physiol. 38, 137-144.

Hill, T.D., Zwiller, J. and Boynton, A.L. (1989) Inositol 1,3,4,5-tetrakisphosphate stimulates the initiation of DNA synthesis in Ca^{++}-deprived rat liver cells. J. Cell. Physiol. 140, 403-407.

Honegger, A., Dull, Bellot, T.J., Van Obberghen, F., Szapary, E., Schimdt, D., Ullrich, A. and Schlessinger, J. (1988) Biological activity of EGF-receptor mutants with individually altered autophosphorylation sites. EMBO J. 7, 3045-3052.

Hoshijina, M., Kikuchi, A., Tanimoto, T., Kaibuchi, K. and Takai, Y., (1986) Formation of a phorbol ester-binding fragment from protein kinase C by proteolytic digestion. Cancer Res. 46, 3000-3004.

Housey, G. M., O'Brian, C.A., Johnson, M. D., Kirschmeier, P., and Weinstein, B.(1987) Isolation of cDNA clones encoding protein kinase C: evidence for a protein kinase C-related gene family. Proc. Natl. Acad. Sci. USA 84, 1065-1069.

Huang, K.-P., Nakabayashi, H. and Huang, F. L. (1986) Isozymic forms of rat brain Ca^{++}-activated and phospholipid-dependent protein kinase. Proc. Natl. Acad. Sci. USA 83, 8535- 8539.

Huang, K.-P. and Huang, F. L. (1986) Conversion of protein kinase C from a Ca^{++}-dependent to an independent form of phorbol ester-binding protein by digestion with trypsin. Biochem. biophys. Res. Commun. 139, 320-326.

Huang, F. L., Yoshida, Y., Nakabayashi, H. and Huang, K.-P. (1987) Differential distribution of protein kinase C isozymes in the various regions of brain. J.Biol.Chem. 262, 15714-15720.

Huang, F. L., Yoshida, Y., Melo-Cunha, J.R., Beaven, M. A., and Huang, K-P. (1989) Differential down-regulation of protein kinase C isozymes. J. Biol. Chem. 264, 4238-4243.

Huang, K.P. (1989) The mechanism of protein kinase C activation. TINS 12, 425-432.

Huckle, W.R., Hepler, J.R., Rhee, S.G., Harden, T.K. and Earp, H.S. (1990) Protein kinase C Inhibits epidermal growth factor-dependent tyrosine phosphorylation of phospholipase Cγ and activation of phosphoinositide hydrolysis. Endorcinol. 127, 1697-1705.

Hunter, T. and Cooper, J.A. (1985) Protein tyrosine kinases. Ann. Rev. Biochem. 54, 897,-930.

Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues.II. J. Biol. Chem. 252, 7610-7616.

Issandou, M. and Darbon, J.M. (1991) Activation of protein kinase C by phorbol esters induce DNA synthesis and protein phosphorylations in glomerular mesangial cells. FEBS Lett. 281, 196-200.

Jaken, S. and Kiley, S.C. (1987) Purification and characterization of three types of protein kinase C from rabbit brain cytosol. Proc. Natl. Acad. Sci. USA 84, 4418-4422.

Johnson, G.L., Kaslow, H.R. and Bourne, H.R. (1978) Genetic evidence that cholera toxin substrates are regulatory components of adenylate cyclase. J. Biol. Chem. 253, 6401-6412.

Johnson, R.M. and Garrison, J.C. (1987) Epidermal growth factor and angiotesin II stimulate formation of 1,4,5- and inositol 1,3,4-trisphosphate in hepatocytes. J.Biol.Chem. 262,17285-17293.

Kaibuchi, K., Takai, Y. and Nishizuka, Y. (1981) Cooperative roles of various membrane phospholipids in the activation of calcium-activated, phospholipid-dependent protein kinase. J. Biol. Chem. 256, 7146-7149.

Kaibuchi, K., Takai, Y. and Nishizuka, Y. (1983) Protein kinase C and calcium ion in mitogenic response of macrophage-depleted human peripheral lymphocytes. J. Biol. Chem. 260, 1366-1369.

Kaibuchi, K., Fukomoto, Y., Oku, N., Takai, Y., Arai, K.-I. and Muramatsu, M. (1989) Molecular genetic analysis of the regulatory and catalytic domain of protein kinase C. J. Biol. Chem. 264, 13489-13496.

Kashles, O., Szapary, D., Bellot, F., Ullrich, A., Schlssinger, J., and Schmidt, A. (1988) Ligand-induced stimulation of EGF- receptor mutants with altered transmembrane regions. Proc. Natl. Acad. Sci. USA 85, 9567-9571.

Kazlauskas, A. and Cooper, J.A.(1988) Protein kinase C mediates platelet-derived growth factor-induced tyrosine phosphorylation of p42. J. Cell Biol. 106, 1395-1402.

Kikkawa, U., Takai, Y., Minaguchi, R., Inohara, S. and Nishizuka, Y., (1982) Calcium-activated, phospholipid-dependent protein kinase from rat brain. J. Biol. Chem. 257, 13341-13348.

Kikkawa U., Ono, Y., Ogita, K., Fujii, T., Asaoka, Y., Sekiguichi, K., Kosaka, Y., Igarishi, K. and Nishizuka, Y. (1987) identification of the structures of multiple subspecies of protein kinase C expressed in rat brain. FEBS Lett. 217, 227-231.

Kikkawa, U., Kishimoto, A. and Nishizuka, Y., (1989) The protein kinase C family: heterogeneity and its implications. Ann. Rev. Biochem. 58, 31-44.

Kim, J.W., Sim, S.S., Kim, U., Nishibe, S., Wahl, M.I., Carpenter, G. and Rhee, S.G. (1990) Tyrosine residues in bovine phospholipase C-γ phosphorylated by the epidermal growth factor receptor in vitro. J. Biol. Chem. 265, 3940-3943.

King, C.S. and Cooper, J.A. (1986) Effects of protein kinase C activation after epidermal growth factor binding on epidermal growth factor receptor phosphorylation. J.Biol.Chem. 262, 10073-10078.

Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y., (1980) Activation of calcium- and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. J. Biol. Chem. 255, 2273-2276.

Kishimoto, A., Kajikawa, N., Shiota, N. and Nishizuka, Y. (1983) Proteolytic activation of calcium-activated phospholipid-dependent protein kinase C by calcium-dependent neutral protease. J. Biol. Chem. 258, 1156-1164

Kishimoto, A., Mikawa, K., Hasimoto, K., Yasuda, I. Tanaka, S.-I., Tommaga, M., Kuroda, T. and Nishizuka, Y. (1989) Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). J. Biol. Chem. 264, 4088-4092.

Knopf, J. L., Lee, M.-H., Sultzman, L.A., Kniz, R. W., Loomis, C. R., Hewick, R. M. and Bell, R. M. (1986) Cloning and expression of multiple protein kinase C cDNAs. Cell 46, 491-502.

Kraft, A.S., Anderson, W.B., Cooper, H.L. and Sando, J.J. (1982) Decrease in cytosolic calcium/phospholipid-dependent kinase activity following phorbol ester treatment of EL_4 thynoma cells. J. Biol. Chem. 257, 13193-13196.

Kraft, A. S. and Anderson, W. B. (1983) Phorbol esters increase the amount of Ca^{++}, phospholipid-dependent protein kinase associated with plasma membrane. Nature 301, 621-623.

Kubo, K., Ohno, S. and Suzuki, K., (1987) Primary structures of human protein kinase C β_1 and β_2 differ only in their C-terminal sequences. FEBS Lett. 223, 138-142.

L'Allemain, G. and Ponyssegar, J. (1986) EGF and insulin action in fibroblasts. Evidence that phosphoinositide hydrolysis is not an essential mitogenic signalling pathway. FEBS Lett. 197, 344-348.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227, 680-685.

Letterio, J.J., Coughlin, S.R. and Williams, L.T.(1986) Pertussis toxin-sensitive pathway in the stimulation of c-myc expression and DNA synthesis by bombesin. Science 234, 1117-1119.

Lin, R.C. Chen, S. W., Lazar, C. S., Carpenter, C. D., Gill, G. N., Evans, R. M. and Rosenfeld M. G. (1986) Protein kinase C phosphorylation at Thr 654 of the unoccupied EGF receptor and EGF binding regulate functional receptor loss by independent mechanisms. Cell 44, 839-848.

Linassier, C., Pierre, M., Le Peco, J.-B and Pierre, J. (1990) Mechanism of action in NIH 3T3 cells of genistein, an inhibitor of EGF receptor tyrosine kinase activity. Biochem. Pharmacol. 39, 187-193.

Litosch, I. and Fain, J.N. (1986) Minireview. Regulation of phosphoinositide breakdown by guanine nucleotides. Life Science. 39,187-194.

Livneh, E., Reiss,N., Berent, E., Ullrich, A. and Schilessinger, J. (1987) An insertion mutant of epidermal growth factor receptor allows dissection of diverse receptor function. EMBO J. 6, 2669-2676.

Lyall, R.M., Zilberstein, A., Gazit, A., Gilon, C., Levitzki, A. and Schlessinger, J. (1988) Tyrphostins inhibit epidermal growth factor (EGF) receptor tyrosine kinase activity in living cells and EGF-stimulated cell proliferation. J. Biol. Chem. 264, 14503-14509.

Macara, I.G. (1986) Activation of ^{45}Ca influx and $^{22}Na^+/H^+$ exchange by epidermal growth factor and vanadate in A431 cells is independent of phosphatidylinositol turnover and is inhibited by phorbol ester and diacylglycerol. J. Biol. Chem. 261, 9321-9327.

Macleod, C. L., Luk, A., Castagnola, J., Cronin, M. and Mendelsohn, F. (1986) EGF induces cell cycle arrest of A431 human epidermoid carcinoma cells. J.Cell Physiol., 127, 175-182.

Maihle, N.J. and Kung, H.S. (1988) C-erbB and the epidermal growth factor receptor: a molecule with dual identity. Biochim. Biophys. Acta 948, 287-304.

Matuoka, K., Fukami, K., Nakanishi, O., Kawai, S. and Takenawa, T.,(1988) Mitogenesis in response to PDGF and bombesin abolished by microinjection of antibody to PIP_2. Science 239, 640-643.

McPhail, L. C., Clayton, C. C. and Snyderman, R. (1984) A potential second messenger role for unsaturated fatty acids: Activation of Ca^{++}-dependent protein kinase. Science 224,622-625.

Mellgren, R.I. (1980) Canine cardiac calcium-dependent proteases: resolution of two forms with different requirements for calcium. FEBS Lett. 109, 129-133.

Merlino,G.T. (1990) Epidermal growth factor receptor regulation and function. Seminars in Cancer Biology 1, 277-284.

Mochley-Rosen, D., Henrich, C.J., Cheever, L., Khaner, H. and Simpson, P.C. (1990) A protein kinase C isozyme is translocated to cytoskeletal elements on activation. Cell Regul. 1, 693-706.

Moolenar, W. H., Bierman, A. J., Tilly, B. C., Verlaan, I., Defize, L.H.K., Honegger, A.M., Ullrich, A. and Schlessinger, J. (1988) A point mutation at the ATP-binding site of the EGF-receptor abolishes signal transduction. EMBO J. 7, 707-710.

Moscat, J., Molloy, C.J., Hemming, T.P. and Aaronson, S.A. (1988) Epidermal growth factor activates phosphoinositide turnover and protein kinase C in BALB/MK keratinocytes. Mol. Endocrinol. 2, 799-805.

Muller, R., Bravo, R., Burckhad, S. and Curran, T. (1984) Induction of c-fos gene and protein by growth factors precedes activation of c-myc. Nature 312, 716-720.

Murachi, T., Tanaka, K., Hatanaka, M. and Murakami, T., (1981) Intracellular Ca^{++}-dependent protease (calpain) and its high-molecular-weight endogenous inhibitor (calpastatin). Adv. Enzyme Regul. 19, 407-424.

Nair, B.G., Parikh, B., Milligan, G. and Patel, T.B. (1990) Gsα mediates epidermal growth factor-elicited stimulation of rat cardiac adenylate cyclase. J. Biol. Chem. 265, 21317-21322.

Nakano, H., Kobayashi, E., Takahashi, I., Tamaoki, T., Kuzuu, Y. and Iba, H. (1987) Staurosporine inhibits tyrosine-specific protein kinase activity of Rous sarcoma virus transforming protein p60. J. Antibiot. (Tokyo) 40, 706-708.

Naor, Z., Shearman. M. S., Kishimoto, A., Nishizuka, Y. (1988) Calcium-independent activation of hypothalamic type I protein kinase C by unsaturated fatty acids. Molec. Endocrinol. 2, 1043-1048.

Nelsestuen, G.L. and Bazzi, M.D. (1991) Activation and regulation of Protein kinase C enzymes. J. Bioenergetics. Biomembranes. 23(1), 43-61.

Nishibe, S. and Carpenter, G. (1990) Tyrosine phosphorylation and the cell growth: growth factor stimulated tyrosine phosphorylation of phospholipase C. Seminars in Cancer Biology 1, 285-292.

Nishibe, S. Wahl, M.I., Harnandez-Sotomayor, S.M.T., Tonks, N.K., Rhee, S.G., and Carpenter, G. (1990) Increase of the catalytic activity of phospholipase C-γ1 by tyrosine phosphorylation. Science 250, 1253-1256.

Nishizawa, N., Okana, Y., Chatani, Y., Amano, F., Tanaka, E., Nomoto, H., Nozawa, Y. and Kohno, M.,(1990) Mitogenic signalling pathways of growth factors can be distinguished by the involvement of pertussis toxin-sensitive guanosine triphosphate-binding protein and of protein kinase C.Cell Regulation 1, 747-761.

Nishizuka, Y.(1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308, 693-698.

Nishizuka, Y., (1986) Studies and perspectives of protein kinase C. Science 233, 305-312.

Nishizuka, Y.(1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334,661-665.

Ohno, S., Kawasaki, H., Imajho, S., Sizuki, K., Inagaki, M., Yokokura,H., Sakohi, T. and Hidaka, H.(1987) Tissue-specific expression of three distinct types rabbit protein kinase C. Nature 325, 161-166.

Ohno, S., Akita, Y., Komuo, Y., Imajoh, S. and Suzuki, K. (1988) A novel phorbol ester receptor/protein kinase, ηPKC, distantly related to the protein kinase C family. Cell 53, 731-741.

Oishi, K. Raynor, R. L., Charp, P. A. and Kuo, J. F. (1988) Regulation of protein kinase C by lysophospholipids. J. Biol. Chem. 263, 6865-6871.

Ono, Y., Kurokawa, T., Fujii, T., Kawahara, K., Igarashi, K., Kikkawa, U., Oqita, K.and Nishizuka, Y. (1986) Two types of complementary DNAs of rat brain protein kinase C. FEBS Lett. 206, 347-352.

Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarshi, K., and Nishizuka, Y. (1987) Identification of three additional members of rat protein kinase C family; δ-, ε- and ζ-subspecies. FEBS Lett. 226: 125-128.

Ono, Y., Fujii, T., Ogita, K., Kikkawa, u., Igarashi, K., and Nishizuka, Y., (1988) The structure, expression and properties of additional members of the protein kinase C family. J.Biol. Chem. 263, 6927-6932.

Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U. and Nishizuka, Y. (1989) Phorbo; ester binding of protein kinase C requires a cysteine-rich zinc-finger-like sequence. Proc.Natl. Acad. Sci. USA. 86, 4868-4871.

Osada, S., Mizuno, K., Saido, T.C., Akita, Y., Suzuki, K., Kuroki, T. and Ohno, S. (1990) A phorbol ester receptor/protein kinase C PKCη, a new member of the protein kinase C family predominantly expressed in lung and skin. J. Biol. Chem. 265, 22434-22440.

Parker, P., Coussens,L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. and Ullrich, A., (1986) The complete primary structure of protein kinase C- the major phorbol ester receptor. Science 233, 853-859.

Parker, P.J., Kour, G., Marais, R.M., Mitchell, F., Pears, C.J., Schaap, D., Stabel, S. and Webster, C., (1989) Protein kinase C- a family affair. Mol. cell. Endocrinol. 65,1-11.

Pawson, T. (1988) Noncatalytic domains of cytoplasmic protein-tyrosine kinases: regulatory elements in signal transduction. Oncogene 3, 491-495.

Pike, L.J. and Eakes, A.T. (1987) Epidermal growth factor stimulates the production of phosphatidylinositol monophosphate and the breakdown of polyphosphoinositides in A431 cells. J. Biol. Chem. 262, 1644-1651.

Qiu, F.H., Rav, P., Brown, K., Barker, P.E., Jhanwar, S., Ruddle, F.H. and Besmer, P. (1988) Primary structure of c-kit: relationship with the CSF-1/PDGF receptor kinase family-oncogenic activation of v-kit involves deletion of extracellular domain and C terminus. EMBO J. 7,1003-1011.

Rozengurt, E., Rodriguez-Pena, M. and Smith, K.A.(1983) Phorbol esters, phospholipase C, and growth factors rapidly stimulate the phosphorylation of a Mr 80,000 protein in intact quiescent cells. Proc. Natl. Acad. Sci. USA 80, 7244-7248.

Sand, T.E. and Christoffersen. T. (1987) Temporal requirement for epidermal growth factor and insulin in the stimulation of hepatocyte DNA synthesis. J. Cell. Physiol. 131, 141-148.

Savage, C.R., Inagami, T. and Cohen, S. (1972) The primary struture of epidermal growth factor. J. Biol. Chem. 247, 7612-7621.

Schaap, D., Parker, P.J., Bristol, A., Kriz, R., and Knopf,J. (1989) Unique substrate specificity and regulatory properties of PKC-ε: a rationale for diversity. FEBS Lett. 243, 351-357.

Schlessinger, J. (1986) Allosteric Regulation of the epidermal growth factor receptor kinase. J. Cell. Biol. 103, 2067-2072.

Schlessinger, J. (1988a) Signal transduction by allosteric receptor oligomerization. Trends Biochem. Sci. 13, 443-497.

Schlessinger, J. (1988b) The epidermal growth factor receptor as a multifunctional allosteric protein. Biochem. 27, 3119-3123.

Shearman, M. S., Berry, N., Oda, T., Ase., K., Kikkawa, U. and Nishizuka, Y.(1988) Isolation of protein kinase C subspecies from a preparation of human T lymphocytes. FEBS Lett. 234, 387-391.

Stabel, S. and Parker, P.J. (1991) Protein kinase C. Pharmac. Ther. 51, 71-95.

Swierenga, S.H.H. (1984) In vitro Models for Cancer Research (Webber, M.M. and Sekely,L.I., Eds.) CRC Press, Bocaraton, FL. Vol.2, 61-89.

Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues.I. Purification and characterization of an active enzyme from bovine cerebellum. J. Biol. Chem. 252, 7603-7609.

Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y., (1979a) Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. J. Biol Chem. 254, 3692-3695.

Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. and Nishizuka, Y., (1979b) Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase by membrane phospholipids. Biochem. biophys. Res. Commun. 91, 1218-1224.

Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Staurosporine, a protein inhibitor of phospholipid /Ca^{++}-dependent protein kinase. Biochem. Biophys. Res. Commun. 135, 397-402.

Taylor, D., Uhing, R.G., Blackmore, P.F., Prpie, V. and Exton, J.H. (1985) Insulin and epidermal growth factor do not effect PI metabolism in rat liver plasma membranes and hepatocytes. J.Biol.Chem. 260,2011-2014.

Teitelbaum, I., Stersheim, A. and Berl, T. (1990) Epidermal growth factor stimulated phosphoinositide hydrolysis in cultured rat inner medullary collecting tubule cells. Regulation by G protein, calcium, and protein kinase C. J Clin Invest. 85,1044-1050.

Thompson, D.M. and Gill, G.N. (1985) The EGF receptor: structure regulation and potential role in malignancy. Cancer Surv. 4, 767-768.

Uhing, R.J.,Prpic, V., Jiang, H. and Exton, J.H. (1986) Hormone-stimulated polyphosphoinositide breakdown in rat liver plasma membranes. J.Biol.Chem. 261, 2140-2146.

Ullrich, A., Cousseus, L., Hayflick, J.S., Dull, T. J., Gray A., Tam, A.N., Lee, J., Mayes, E.L.V., Whittle, N., Waterfield, M. D., and Seeburg, P.H. (1984) Nature 309, 418-424.

Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzzelli, L.M., Dull, T.J., Gray, A., Caussens, L., Liao, Y.C., Tsubokawa, M., Mason, A., Seeburg, P.H., Grunfeld, C., Rosen, O.M., Ramachandran, J. (1985) Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature 313, 756-761.

Ullrich, A., and Schlessinger, J. (1990) Signal transduction by receptor tyrosine kinase activity. Cell 61,203-212.

Wahl, M.I., Sweatt, J.D. and Carpenter, G. (1987) Epidermal growth factor (EGF) stimulates inositol trisphosphate formation in cells which overexpress the EGF receptor. Biochem. Biophys. Res. Commun. 142, 688-695.

White, M. F. (1991) Structure and function of tyrosine kinase receptors. J. Bioenergetics and Biomembranes 23(1), 63-82.

Woodgett, J.R. and Hunter, T. (1987) Immunological evidence for two physiological forms of protein kinase C. Mol. cell. Biol. 7, 85-96.

Woods, N.M., Cuthberston, K.S.R. and Cobbold, R.H. (1987) Phorbol ester-induced alterations of free calcium ion transients in single rat hepatocytes. Biochem. J. 246, 619-623.

Worley, P. F., Baraban, J. M., DeSouza, E. B. and Snyder, S. H. (1986) Mapping second messenger systems in the brain: Differential localization of adenylate cyclase and protein kinase C. Proc. Natl. Acad. Sci. USA 83, 4053-4057.

Yarden, Y., Escobedo, J.A., Kuang, W. J., Yang-Feng, T. L., Daniel, T.O., Tremble, P. M., Chen, E. Y., Ando, M. E., Harkins, R. N., Franks, U., Fried, U. A., Ullrich A. and William, L. T. (1986) Nature 323, 226-232.

Yarden, Y. and Ullrich A. (1988) Growth factor receptor tyrosine kinases. Ann. Rev. Biochem. 57, 443-478.

Young,S., Parker, P.J., Ullrich, A. and Stable, S. (1987) Down-regulation of protein kinase C is due to an increased rate of degradation. Biochem. J. 244, 775-779.