National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

THE ROLE OF PROTEIN KINASE C IN THE STIMULUS–SECRETION COUPLING IN RAT PAROTID GLAND

by

WEI LIN, M.D., M.Sc.

Thesis presented the Faculty of
Graduate Studies and Research
in partial fulfillment of the requirements
for the degree of MSc

Department of Anatomy
Faculty of Medicine
University of Ottawa
Ottawa, Ontario

© Wei Lin, Ottawa, Canada, 1992

National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-93596-0

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ACKNOWLEDGEMENTS

I am deeply indebted to Dr. Max Hincke, for providing the most patient guidance, helpful advice, generous support and timeless discussion throughout the course of this work.

For the friendship and competent assistance with laboratory techniques, I am grateful to Dr. Douglas. J. Franks, Department of Pathology.

The technical assiatance of Ms. Nicky Soor is sincerely appreciated.

Finally, I would like to extend thanks to the Department of Anatomy for providing me the oppotunity to study and work here.

ABSTRACT

The role of protein kinase C (PKC) in stimulus-secretion coupling in a number of endocrine and exocrine tissues remains poorly understood. In this study, the activation of a specific PKC isozyme during cAMP–mediated secretion from rat parotid acini has been examined. Hydroxylapatite chromatography and immunoblotting studies, with a specific antibody against PKC–β utilizing a sensitive enhanced chemiluminescence detection system, indicated that the β form was the major PKC isoenzyme in rat parotid gland. Isoproterenol (ISO, 0.1 μm), a β–adrenergic agonist, stimulated the translocation of PKC–β from the cytosolic to the particulate fraction. A time–course study with this agonist showed that total particulate PKC increased from 50% (resting level) to about 80% during 30 minutes of stimulation, accompanied by a decrease from 50% to 20% in the cytosolic distribution. At low concentrations (up to 0.1 μM), ISO caused significant redistribution of PKC–β during 30 min of stimulation in a dose–dependent manner (Kd=10 nM). The rate of amylase release evoked by ISO (0.1μM) was linear during 30 minutes, in good correlation with the translocation of PKC–β from cytosol to membrane. A permeant cyclic AMP derivative (dibutyryl cAMP) also caused the translocation of PKC–β in a dose–dependent manner (Kd≤50 μM). Stimulation with the phorbol ester PMA (10 nM, 100 nM) resulted in both PKC translocation and amylase secretion. Total PKC activity was assayed using a specific peptide substrate and yielded a similar pattern of stimulation of PKC translocation in response to these secretagogues. The accumulated evidence suggests that PKC–β becomes activated and translocates during β–adrenergic–stimulated amylase secretion from rat parotid acini. Therefore PKC–β activation may be the common pathway for

stimulus–secretion coupling during stimulation by agonists that cause cAMP synthesis or PIP_2 hydrolysis.

LIST OF ABREVIATIONS USED

Abreviation	Full name
PKA	Cyclic AMP–dependent protein kinase
BCA	Bicinchoninic acid
Blotto	Bovine lacto–transfer technique optimizer
BME	Basal medium Eagle
BSA	Bovine serum albumin
cAMP	Adenosine cyclic 3', 5'–monophosphate
DAG	Diacylglycerol
DiBcAMP	Dibutyryl adenosine cyclic 3', 5'–monophosphate
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
FPLC	Fast protein liquid chromatography
G protein	Guanine–nucleotide–binding protein
HTP	Hydroxylapatite
InsPt	Inositol trisphosphate
ISO	Isoproterenol
PC	Phosphotidylcholine
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol myristate acetate

TABLE OF CONTENTS

LIST OF FIGURES

Table

1. INTRODUCTION

1.1 THE RAT PAROTID GLAND

1.1.1 Model system for the study of stimulus–secretion coupling

The parotid gland is a branched acinar gland. It consists of the duct system and the secretory endpiece. The secretory portion of rat parotid gland is composed almost exclusively of serous cells. The secretory granules are rich in proteins and have a high amylase activity. Amylase is synthesized by classical route common to most secreted proteins. A lot of effort has been focused on understanding the composition and properties of the storage granules of the rat parotid gland. Through the early efforts of Schramm and collaborators (Schramm, 1961), this tissue was shown to be especially favorable for investigating stimulus–dependent exocrine secretion and for studying isolated cells that are nominally free of major proteolytic and lipolytic activities (Mangos, 1975; Putney et al, 1976). Parotid acinar cells normally devote most of their protein synthetic capability to manufacturing proteins for export, and recent studies suggest that ~85% of this production is destined for intracellular storage in granules as a reserve for stimulus–dependent release. The 1 μm diameter granules occupy nearly 30% of cytoplasmic volume (Bloom et al, 1979), and in response to β–adrenergic agonists undergo massive discharge by exocytosis (Amsterdam et al, 1969) at the apical lumen (~12% of the cell surface).

Parotid gland is known to receive both sympathetic innervation from the principal neurons of the superior cervical ganglion (SCG), and parasympathetic innervation originates in postganglionic fibers of the otic

ganglion (Lundberg et al, 1988; Shepherd, 1988) on the blood vessels and exocrine elements. Earlier ultrastructural study (Hand, 1970) revealed that unmyelinated axons, with or without their Schwann cell sheath, penetrated the basal membrane surounding the acini and came into close apposition to the acinar cells. The close apposition was made with only one cell, or in the intercellular space between adjacent acinar cells, where two or more cells were apposed. The terminals contained mitochondria, large dense–cored vesicles, and smaller vesicles resembling synaptic vesicles. The nerve terminals were often embraced by cytoplasmic folds of the acinar cell. Al-Hadithi demonstrated that the presence of nerve endings in the two sites in rat parotid gland. Those located beneath the basement membrane of acinar cells have been called hypolemmal terminals and those outside the basement membrane, epilemmal (Al-Hadithi, 1988). Both cholinergic and adrenergic terminals have been reported to contact the same cell (Hand, 1970) but Young reported that most hypolemmal terminals in the parotid gland were cholinergic (Young, 1978). It has now been shown that the co–localization of the peptides substance P (SP) and vasoactive intestinal polypeptide (VIP) occurs in the large dense–cored vesicles in the same nerve terminal. Their ultrastructure is similar to the cholinergic nerve terminals which are characterized by an abundance of small agranular vesicles and some large dense–cored vesicles in a ratio of about 9:1 (Al-Hadithi, 1988; Lundberg, 1981).

Stimulation of either sympathetic or parasympathetic nerves to the parotid gland stimulates salivary secretion, but the effects of the parasympathetic nerves are stronger and more long lasting (Kutchai, 1990). Parasympathetic stimulation with the co–release of acetylcholine (ACh), SP and VIP results in vasodilation and greatly increased blood flow to the glands, in addition to prodigious waterly saliva secretion from acinar cells through the

muscarinic cholinergic and substance P receptor–mediated Ca^{2+} pathway. On the other hand, sympathetic stimulation and circulating catecholamines stimulate transient secretion of saliva that is rich in amylase, K^+, and HCO_3^-. The amylase release will be discussed in the following section (1.1.2). In addition, sympathetic stimulation (Norepinephrine / Neuropeptide Y) constricts blood vessels, with consequent reductions in parotid gland blood flow (Lundberg, 1983).

1.1.2 Stimulus–secretion coupling

Many cell types are specialized to synthesize and secrete specific proteins. In certain cells, proteins are synthesized and secreted in a continuous fashion without prior exposure to a stimulant. Examples include albumin secretion from the liver and collagen secretion from fibroblasts. Other cell types have devised an organized machinery for regulated secretion that allows the secretory products to be stored in discrete membrane–bound compartments (granules), awaiting a specific trigger for exocytosis. Examples include secretion of neurotransmitters and peptide hormones from neurons and endocrine cells and release of digestive enzymes from salivary glands and the exocrine pancreas. These cells are programmed to direct more than 90% of their biosynthetic effort toward the production and storage of a mixture of many different enzyme precursors. The ability of parotid acinar cells to respond to a given stimulus with the coordinated release of the secretory granule content implies the existence of intracellular messengers capable of transducing the external signal into an increased rate of vesicle membrane fusion. The term *stimulus–secretion coupling* , coined by Douglas and Rubin in 1961, defines the various steps that, following the interaction of a stimulus

with its specific plasma membrane receptor, will eventually bring about the accelerated discharge of secretory products (Douglas & Rubin, 1961).

Protein and fluid secretion in rat parotid acinar cells are regulated by two separate pathways: (1) β–adrenergic receptor stimulation leads to the formation of cyclic AMP (cAMP) via activation of adenylyl cyclase by G protein (Butcher et al, 1980); amylase exocytosis is mostly induced by this pathway; (2) muscarinic–cholinergic, α–adrenergic and substance P receptor stimulation cause phosphatidylinositol turnover via G protein leading to the formation of inositol 1,4,5–trisphosphate and a rise in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) (Putney et al, 1986; Baum et al, 1987; Sugiya et al, 1989); these receptors are primarily linked to ion secretion.

Amylase release from the parotid gland is stimulated by cAMP– and Ca^{2+}–modulating agonists (Butcher et al, 1980). In the parotid gland, the cAMP–modulation system stimulates amylase release more strongly than the Ca^{2+}–system. Several studies have indicated a rise in intracellular cAMP–dependent protein kinase (PKA) activity when the parotid gland is incubated with a β–adrenergic agonist, and phosphorylation by PKA is suggested to be an essential step for amylase release (Baum et al, 1981; Kanamori et al, 1980; Baum et al, 1981; Jahn et al, 1981; Spearman et al, 1984; Quissell et al, 1985). On the other hand, there is little evidence to support a role for Ca^{2+}–dependent kinases in secretion. Ca^{2+}–dependent kinases have been detected in exocrine tissue, however a definite link between secretion and Ca^{2+}–dependent phosphorylation has not been established. Because of the generally accepted importance of Ca^{2+} in secretory processes (Rubin, 1982), there has been a continuing interest in the possible role of Ca^{2+} in the action of β–adrenoceptor agonists in the parotid gland (Butcher et al, 1980). Evidence that Ca^{2+} was involved in this pathway was several–fold: (1) prolonged depletion

of cellular Ca^{2+} stores with chelating agents partially inhibited the secretory response to β–adrenoceptor agonists or to cAMP analogues (Selinger et al, 1970; Putney et al, 1977); (2) β-adrenoceptor agonists and cAMP stimulated efflux of $^{45}Ca^{2+}$ from acinar cells (Putney et al, 1978) and (3) β–adrenoceptor agonists, albeit at high concentrations, caused a modest rise in cytosolic [Ca^{2+}] (Horn et al, 1988; Takemura et al, 1985).

It has recently been reported that the treatment with Ca^{2+}–chelators is inappropriate to evaluate the role of Ca^{2+}, because it also suppresses cellular ATP levels (Hincke, 1988; Tojyo et al, 1990). In addition, the depletion of cytosolic Ca^{2+} did not completely suppress amylase release even in saponin–permeabilized parotid cells. Results from most recent studies demonstrate that the concentration of β–adrenoceptor agonist, isoproterenol, required for Ca^{2+}–mobilization are much higher than those required for stimulating amylase secretion (Mangos et al, 1975; Takuma et al, 1988); high concentrations of isoproterenol (from 0.02 mM to 1 mM) activate α–adrenoceptors as well as β–adrenoceptors and therefore cause a rapid increase in cytosolic free Ca^{2+} in rat parotid acinar cells; while low concentration of isoproterenol (0.1 μM) evokes the maximum amylase secretion without any effect on cytosolic Ca^{2+} (Hughes et al, 1989; Tanimura et al, 1990). This suggests that large increases in cytosolic Ca^{2+} are not necessary for β–adrenoceptor agonists–induced amylase release.

Adenosine–5'–triphosphate (ATP) is stored and co–secreted with many neurotransmitters and, similarly to its metabolite adenosine, may act as a neuromodulator (Richardson et al, 1987; Burnstock et al, 1981). The effects of extracellular ATP on cells appear to be mediated through specific P_2–type purinergic receptors or ectokinases, and are pharmacologically distinct from effects mediated by adenosine (via P_1–type receptors) (Gordon et al, 1986).

Although a large number of cell types respond to extracellular ATP, the potency and effectiveness of ATP and its analogues vary widely among the systems studied (Burnstock et al, 1985). Different subtypes of P_2 purinergic receptors undoubtedly contribute to the differences in observed responses to ATP. Elevation of $[Ca^{2+}]_i$ appears to be a common effect of ATP in many tissues. Gallacher originally reported that the electrophysiological response of parotid glands from mice (but not rats) to ATP was indistinguishable from that to other Ca^{2+}–mobilizing agonists (Gallacher et al, 1982). When the effect was re–examined in dispersed rat parotid acinar cells, it was found that ATP elevated $[Ca^{2+}]_i$ in these cells in a highly specific and reversible manner consistent with the activation of a P_2–type purinergic receptor (McMillian et al, 1988). This effect on $[Ca^{2+}]_i$ was similar to that observed with carbachol, which acts via muscarinic receptors in parotid cells. Despite a larger effect on $[Ca^{2+}]_i$, ATP was much weaker than known phospholipase C–linked receptor agonists in stimulating accumulation of [^{3}H]inositol phosphates, and barely stimulated amylase exocytosis, although it activated Ca^{2+}–dependent ion conductances. This suggested that diacylglycerol is crucial for effects of phospholipase C–linked receptor agonists on exocytosis. In contrast to carbachol, the increase in $[Ca^{2+}]_i$ evoked by ATP appeared to be independent of a G protein, and there was partial additivity between maximal ATP and carbachol effects on $[Ca^{2+}]_i$. Although the mechanism by which extracellular ATP elevates $[Ca^{2+}]_i$ in parotid cells is unclear, those results suggest that it acts through a pathway distinct from that shared by parotid muscarinic, α–adrenergic and substance P receptors.

1.2 PROTEIN KINASE C

1.2.1. Members of the family

In 1977, protein kinase C (PKC) was first identified in Nishizuka's laboratory as a novel and widespread serine– and threonine–directed phosphokinase that can be proteolytically activated: many tissue extracts contained far more protein kinase C than cyclic AMP–dependent protein kinase if assayed with histone H–I as substrate (Takai et al, 1981; Nishizuka, 1983; 1984). Soon it became apparent that the intact enzyme requires calcium and phospholipid, particularly phosphatidylserine, in order to express its activity. Moreover, it is active at physiological intracellular Ca^{2+} concentrations (approx. 0.1 μM) only if the activating lipid includes a small proportion of 1,2–diacylglycerol. On the basis of this 1979 observation , Nishizuka and his colleagues proposed that the 1,2-DAG liberated during inositol lipid breakdown acts as a second messenger, with protein kinase C as its target. The mechanism by which 1,2–DAG acts is to increase the sensitivity of protein kinase C to Ca^{2+} by as much as one thousand–fold, so that the normally inactive "holoenzyme" can become fully active even at cytosolic Ca^{2+} concentrations present in resting cells: thus Ca^{2+} is an essential requirement of the enzyme but is not the key regulator of its activity. Although the 1,2–DAG that is liberated during hydrolysis of inositol lipid(s) will be mainly the 1–stearoyl, 2–arachidonoyl species, protein kinase C can be activated by other pools of diacylglycerol (Billah & Anthes, 1990) .

PKC is ubiquitous in rat tissues and is normally recovered from the soluble fraction as an inactive form and is translocated to the membrane in a Ca^{2+}–dependent fashion when cells are stimulated. The results of

chromatographic studies on rat brain and rabbit brain (Jaken & Kiley, 1987; Kiley et al, 1990) revealed that the three activities, which are eluted from hydroxylapatite (HTP) and are called type I, II and III PKC, correspond to gamma, beta I + beta II and alpha subspecies respectively. The beta I and beta II forms show nearly identical kinetic properties and cannot be separated by HTP chromatography. Epsilon PKC co–chromatographs with PKC beta (Ohno et al, 1988; Schaap et al, 1989). At last count, 8 subspecies of PKC have been found. Four conventional PKCs (alpha, beta I, beta II, gamma) emerged from the initial screening of a variety of complementary DNA libraries. More recently, at least three further novel PKCs (nPKC delta, –epsilon, –zeta) have been isolated from a rat brain library by using a mixture of alpha, beta II and gamma complementary DNAs as probes under low stringency conditions (Ono et al, 1987; 1988). These subspecies have a common structure closely related to but clearly distinct from the four subspecies initially described. Protein kinase C consists of a single (ie., β–PKC 77 kDa) polypeptide chain with two functional domains that can be separated by protease action. The hydrophilic catalytic fragment is fully active in the absence of the normal activators, so it appears that Ca^{2+}, phospholipid and 1,2–DAG must activate the intact enzyme through interactions with the smaller, hydrophobic domain. The main difference between the PKC subspecies is that the delta, epsilon and zeta subspecies lack the second conserved region C2. The βI and βII subspecies seem to be derived from a single mRNA by alternative splicing; they differ from each other only in ~50 amino acid residues at their carboxy–terminal end regions, V5, and even in this area they possess a high degree of sequence homology (Ono et al, 1987, Knopf et al, 1986; Kubo et al, 1987). The conserved region C1 contains a tandem repeat of a cysteine–rich sequence. This sequence is similar to the

consensus sequence of a cysteine–zinc–DNA binding finger that is found in many DNA–binding proteins that are related to transcriptional regulation. The C–terminal half contain regions C3 and C4 that are part of the catalytic domain. The C3 has an ATP–binding sequence. Although the conserved region C4 contains a similar sequence with C3, its significance is unknown. The sites involved in binding to calcium, diacylglycerol and phospholipid are presumably contained in C1 and C2, but they have not been identified with any certainty. The region V3 is a important area because PKC can be activated by limited proteolysis with the Ca^{2+}–dependent neutral protease–calpain. This cleavage occurs at one or two specific sites in the region V3. It may be related to down–regulation of PKC molecular itself. Osada reported that a new member of the protein kinase C family nPKC eta, predominantly expressed in lung and skin, contains a characteristic cysteine–rich repeat sequence (C1 region) and a protein kinase domain sequence (C3 region), both of which are conserved among PKC family members. However, nPKC eta lacks a putative Ca^{2+} binding region (C2 region) that is seen in conventional PKCs, but not in novel PKCs. nPKC–eta shows the highest sequence similarity to nPKC epsilon (59.4% identity). The similarity extends to the NH2–terminal sequence (E region) which corresponds to one of the divergent regions (D1 region) (Osada et al, 1990).

Proteolytic activation of PKC As described, protein kinase C is composed of two functionally different domains, one is a Ca^{2+}, phospholipid–binding regulatory domain (M_r 32,000–38,000) found in the amino–terminal side and the catalytic protein kinase domain (M_r 45,000–50,000) (Hoshijina et al, 1986; Lee & Bell, 1986; Huang & Huang, 1986; Nishizuka, 1988). Separation of these two domains is usually achieved by limited proteolysis using trypsin or calpain and generates a Ca^{2+},

phospholipid–independent catalytic fragment (Huang & Huang, 1986; Inoue et al, 1977; Kishimoto et al, 1983; Melloni, 1985; Hashimoto, et al, 1988). This was originally reported as protein kinase M (Takai et al, 1977). During the study of protein kinase C isolated from rat liver plasma membrane, two protease–activated forms of this enzyme with M_r 80,000 and M_r 50,000, were found respectively (Hashimoto et al, 1977). Although the smaller form was identified as protein kinase M, the exact mechanism of generating the larger form remains obscure. Protein kinase C contains a pseudosubstrate region near the amino–terminal end of the regulatory domain (House & Kemp, 1987), suggesting that this sequence (–R–K–G–A–L–R–) inhibits protein kinase C by interaction with its active site in the absence of Ca^{2+} and phospholipid. Recently, It was shown (Hashimoto et al, 1990) that the protease–activated form of M_r 80,000 was generated by limited proteolysis of protein kinase C at or around the pseudosubstrate region near the amino–terminal end of this enzyme.

Limited proteolysis of the three protein kinase C (α, β, and γ) isozymes generates distinctive fragments for each isozyme, indicating that each isozyme has different trypsin–sensitive sites. In addition, the various protein kinase C isozymes have different susceptibilities to proteolysis *in vitro*, when tested with trypsin, as well as with endogenous proteases in intact cells. Kishimoto also reported that several subspecies of PKC co–expressed in a single cell type disappear at different rates upon treatment with phorbol ester PMA, which may reflect the substrate specificity of the calpain action (Kishimoto et al, 1988; Ase et al, 1988).

1.2.2. Distinct expression and localization

Early studies of tissue distribution using tritiated phorbol–12, 13–dibutyrate as a probe for PKC revealed an uneven distribution of PKC in the brain (Worley et al, 1986). Northern blot analysis with specific oligonucleotide probes has suggested that some PKC subspecies are expressed specifically in certain tissues (Knopf et al, 1986; Coussens et al, 1986; Ohno et al, 1987; Ohno et al, 1988), as has *in situ* messenger RNA hybridization (Brandt et al, 1987). By using biochemical, immunological and cytochemical procedures with subspecies–specific antibodies, the relative activity and individual pattern of expression of multiple PKC subspecies in several tissues have recently been extensively examined (Wood et al, 1986; Shearman et al, 1988; Hidaka et al, 1988). Much is known about the distributions of alpha, beta and gamma, rather less about the delta, epsilon and zeta subspecies. The most striking result is that in the rat, the gamma–subspecies appears to be expressed mostly in the central nervous system after birth (Hashimoto et al, 1988; Yoshida et al, 1988), and is particularly concentrated in the hippocampus, cerebral cortex and amygdaloid complex (Roth et al, 1989). In addition, ultrastructure studies showed that it is associated with membranous structures of the cell in general except for the nucleus and mitochondria, which contain little PKC. Although it seems premature to discuss the function of PKC gamma, it may be important in specialized neuronal processes, such as long–term potentiation in the hippocampus (Roth et al, 1989). The beta subspecies is found in both the brain and peripheral tissues and accounts for the highest percentage of specific activity in the brain (Kikkawa et al, 1987; Shearman et al, 1987). The tissue distribution of the beta I and beta II subspecies shows that there is much more

beta II than beta I in the brain and many other tissues, including endocrine tissues such as pancreatic islets and the pituitary gland. Cytochemical analysis with polyclonal antibodies that distinguish the two subspecies reveals a distinct pattern of cellular expression in certain tissues (Ase et al, 1988; Huang et al, 1987). For example, in the rat cerebellar cortex the beta I subspecies is present mainly in the granule cell body and the beta II subspecies primarily in the molecular layer where it is localized in the presynaptic nerve endings terminating on the dendrites and cell body of the purkinje cells. Most tissues including liver, kidney, spleen, lung, heart and testis contain beta subspecies in variable ratios, but the alpha subspecies of PKC is the most widely distributed (Shearman et al, 1987; 1988). The alpha–, beta– and gamma–PKC are diffusely localized in the brain in part overlapping, but clearly distinct regions. There are also differences in localization to different subcellular compartments (Ase et al, 1988; Hosoda et al, 1989; Saito et al, 1989; Ito et al, 1990). This may suggest that different isoforms of PKC might be important in specialized neuronal function. The expression of PKC isotypes in different endocrine, steroidogenic cell types has been examined by using high–performance liquid chromatography and specific antipeptide antibodies against each enzyme (Pelosin et al, 1991). Their results showed that bovine and rat adrenocortical cells, as well as porcine Sertoli cells expressed only the type III PKC (alpha PKC); by contrast, Leydig cell expressed each of the isotypes I, II and III (gamma, beta and alpha) at similar levels. Taking into account the biological effect observed in these various cell types upon PKC activation, it was suggested that the PKC alpha is involved in the steroidogenic activation pathways, whereas the expression of the beta–, gamma– and alpha–PKC, in Leydig cells, may contribute to a different array of cross–talk regulation pathways in these cells.

1.2.3. Enzymatic properties of different subspecies

It is now known that *sn*–1,2–diacylglycerol, generated by receptor mediated hydrolysis of membrane phospholipids, particularly phosphatidylinositol 4,5–bisphosphate, activates PKC by increasing its affinity for calcium ions. This achieves full activation at physiological calcium concentration. Thus, the activation of PKC is thought to be biochemically dependent on Ca^{2+}, but under some conditions physiologically independent of Ca^{2+}.

Protein kinase C can also be activated by "tumor promoters'' such as phorbol myristate acetate (usually abbreviated as PMA) (Catagna et al, 1982). The most active tumor promoters are the most effective activators of protein kinase C, in accord with the idea that protein kinase C activation might be essential to the actions of these compounds. The diacyl portion of PMA is structurally analogous to 1,2–DAG and kinetic studies showed that PMA, like 1,2–DAG, activates protein kinase C by greatly increasing its Ca^{2+} sensitivity. Thus there is good evidence (Nishizuka, 1984) that PKC is the physiological receptor for tumor promoters. Tumor promoters had long been known to mimic, partially or fully, the cellular effects of receptors linked to inositol lipid breakdown in various tissues (e.g. platelet aggregation and secretion, neutrophil secretion and oxygen radical generation, lymphocyte proliferation), but the mechanism was not previously understood.

PKC can also be activated by Ca^{2+}, phospholipid (especially phosphatidylserine), and limited proteolysis with calpain. But, the activities of gamma, beta and alpha PKC are subtly different. There are kinetic variations among the subspecies: the gamma and alpha–subspecies are much less

activated by DAG in the presence of phosphatidylserine than is the mixture of beta I and beta II, which showed substantial activity in the absence of Ca^{2+} (Nishizuka, 1988). By contrast the beta form of PKC is poorly activated by free arachidonic acid, whereas the gamma form is activated by micromolar concentrations of arachidonic acid. The alpha subspecies responds to high concentrations of arachidonic acid but only at elevated concentrations of Ca^{2+}. The kinetic properties of alpha, beta, and gamma subspecies of PKC are different in the presence of several fatty acids (Shinomura et al, 1991). Although responses of the purified alpha, beta, and gamma subspecies to the lipids slightly differed from one another, the reaction velocity of these subspecies was significantly enhanced by synergistic action of diacylglycerol and a cis–unsaturated fatty acid. Arachidonic, oleic, linoleic, linolenic, and decosahexaenoic acids were active in this role, whereas saturated fatty acids such as palmitic and stearic acids were inactive. Elaidic acid was also inactive. In the presence of both phosphatidylserine and diacylglycerol, the cis–unsaturated fatty acids increase further an apparent affinity of PKC for Ca^{2+} and allow the enzyme to exhibit almost full activation at nearly basal levels of Ca^{2+} concentration. The concentration of fatty acid giving rise to the maximum activation of enzyme was approximately 20–50 μM. This implies that the receptor–mediated release of unsaturated fatty acids from phospholipids may result, in synergy with diacylglycerol, in the activation of PKC even when the Ca^{2+} concentration is low. Therefore, the activation of PKC might be an integral part of the signal–induced degradation cascade of various membrane phospholipids, which is initiated by the actions of phospholipase C and phospholipase A2.

Therefore, protein kinase C can be divided into two categories: 1) Ca^{2+}– and DAG–dependent, like alpha and gamma isoforms; 2) Ca^{2+}–independent (requires DAG alone), like delta, epsilon, and zeta isoforms which lack the Ca^{2+} binding region C_2; The beta form may also be placed in this category because of its substantial activity in the absence of Ca^{2+} (Nishizuka, 1988).

1.3 SIGNAL TRANSDUCTION PATHWAYS THAT REGULATE PROTEIN KINASE C

1.3.1 The role of G protein

In some transmembrane signaling systems, detection of the extracellular stimuli, such as neurotransmitters, hormones, epidermal and nerve growth factors, and generation of an intracellular response are properties of the same protein or protein complex. Rodbell and his collaborators were the first to provide evidence for a more complex class of signaling pathway where the sensor and intracellular effector are separate proteins that communicate through a guanine nucleotide–dependent regulatory protein or G protein (Rodbell et al, 1971). The G proteins that transmit information from receptors to their intracellular effector systems belong to a large homologous family of trimeric proteins each with an α subunit that binds guanine nucleotides, and β and γ subunits that are always tightly associated (Casperson et al, 1987; Gilman et al, 1987; Holbrook et al, 1989). Different G proteins are most readily distinguished by their α subunits, though there are also more subtle structural and functional differences in some β and γ subunits (Cerione et al, 1987). Figure 1 shows the G protein cycles between inactive GDP–bound and

active GTP–bound forms. The activation of G protein involves dissociation of the α from the $\beta\gamma$ subunits. Stimulation of a receptor by its agonist

Figure 1. Scheme for G protein signal transduction. From the unliganded state (Ⅱ), receptor binds agonist A (for example, isoproterenol, carbachol) which produces a change (Ⅲ) in receptor–G protein interaction, allowing GTP, in the presence of Mg^{2+}, to replace GDP on the α–subunit. The activated α–GTP subunit and the βγ subunits dissociate and one or both interacts with effectors (for example, adenylyl cyclase, phospholipase C) (Ⅲ). Alternatively, free βγ may bind other α subunit. The intrinsic GTPase activity of the α subunits hydrolyses GTP to GDP, releasing inorganic phosphate (Pi), and α–GDP recombines with βγ, ending the activation cycle (Ⅱ) (adapted from Neer & Clapham, 1988).

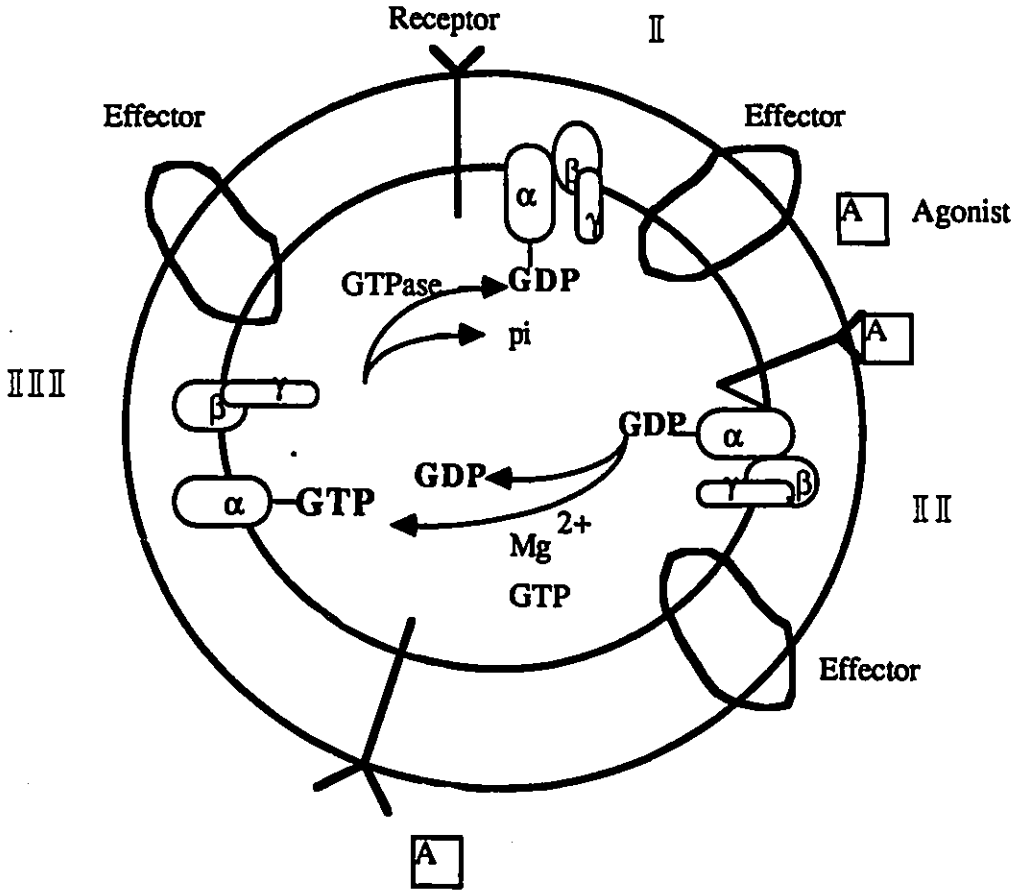
Receptor
Effector
Effector
A
Agonist
GTPase
GDP
pi
GDP
GDP
GTP
Mg 2+
GTP
A
A
Effector

Figure 1

initiates a cascade of biochemical processes that produce an intracellular signal, which ultimately causes a change in the behavior of the cell. One class of receptors, including the phototransducing molecule rhodopsin, the muscarinic acetylcholine receptor and the β–adrenergic receptors, operates by activating the G protein. The developments that followed Rodbell's pioneering studies have established that many different receptors regulate many intracellular effectors through a family of closely related G proteins (Citri et al, 1980; Rodbell et al, 1980; Schramm et al, 1984; Levitzki et al, 1988). There are several G proteins, regulating different intracellular pathways that result in changes in the activity of specific effectors, such as ion channels in plasma membrane, enzymes of phosphatidylinositol and phosphatidylcholine metabolism (phospholipase C/D) (Besterman et al, 1986; VanBlitterswijk et al, 1991), or adenylyl cyclase stimulated by β–adrenoceptor agonists to induce the formation of cAMP, therefore activates protein kinase A to initiate a series of cellular responses.

1.3.2 Diacylglycerol activates protein kinase C

Now it is believed that receptor–mediated hydrolysis of inositol phospholipids is a common mechanism for transducing various extracellular signals into the cell, such as those from certain hormones, neurotransmitters, antigens, some growth factors and many other biologically active substances. As early as 1953, Hokin and coworkers reported the response of inositol phospholipids to the stimulation of cell surface receptors (Hokin et al, 1953). They first showed that acetylcholine induced a rapid incorporation of ^{32}P into phosphatidylinositol (PI) and phosphatidic acid. This incorporation resulted from the enhanced breakdown and resynthesis of inositol phospholipid (Fig.

2). Berridge and his co–workers demonstrated that inositol–1,4,5–trisphosphate (InP_3), one of the earliest products of phosphatidylinositol 4,5–bisphosphate (PIP_2) hydrolysis is a mediator of Ca^{2+} mobilization from an internal store (Berridge et al, 1984). And later, Nishizuka suggested that the other product of PIP_2 hydrolysis: 1,2–diacylglycerol (DAG), remains in the plasma membrane, and initiates the activation of protein kinase C (PKC) (Nishizuka, 1986). It is generally accepted that both events, activation of protein kinase C and the increase in intracellular free Ca^{2+}, are produced in a fashion by any agonist that activates inositol phospholipid metabolism. However, recent evidence indicates that diacylglycerol formation and protein kinase C activation can occur independently from inositol phospholipid turnover in a variety of cell types (Rosoff et al, 1988; Augert et al, 1989). In fact, it has been suggested that the breakdown of phosphatidylcholine (PC) might be more important than that of inositol phospholipids in some signal–transducing pathways that activate protein kinase C. The list of agonists that induce PC hydrolysis is extensive, as is the number of cells and tissues showing the response (Exton et al, 1988). In 1981, it was first reported by Mufson that tumor promoter phorbol esters also stimulate PC hydrolysis (Mufson et al, 1981). They observed that phorbol esters stimulated the release of labeled choline and P–choline from mouse embryo fibroblasts previously incubated with [^{3}H]choline and concluded from measurements of labeled phospholipids that the source was PC. Similar results were later obtained by other groups utilizing other cells (Besterman et al, 1986; Daniel et al, 1986; Liscovitch et al, 1987; Schrey et al, 1987; Cabot et al, 1985; Muir et al, 1987; Takuwa et al, 1987). In 1985, the first evidence that hormones promoted PC hydrolysis was presented (Bocckino et al, 1985). It was observed that the time–course of accumulation of DAG in hepatocytes stimulated with Ca^{2+}–

Figure 2. **Turnover of inositol phospholipids and signal transduction.** Inositol phospholipids appear to be in equilibrium in membranes, although polyphosphoinositides are normally very minor components. PI, phosphatidylinositol; PIP_2 phosphatidylinositol 4,5–bisphosphate; DAG, 1,2–diacylglycerol; IP_3, inositol 1,4,5–trisphosphate; PA, phosphatidic acid; CDP–DAG, cytosine diphosphate–diacylglycerol; I, inositol; P, phosphoryl group; and R_1 and R_2, fatty acyl groups.

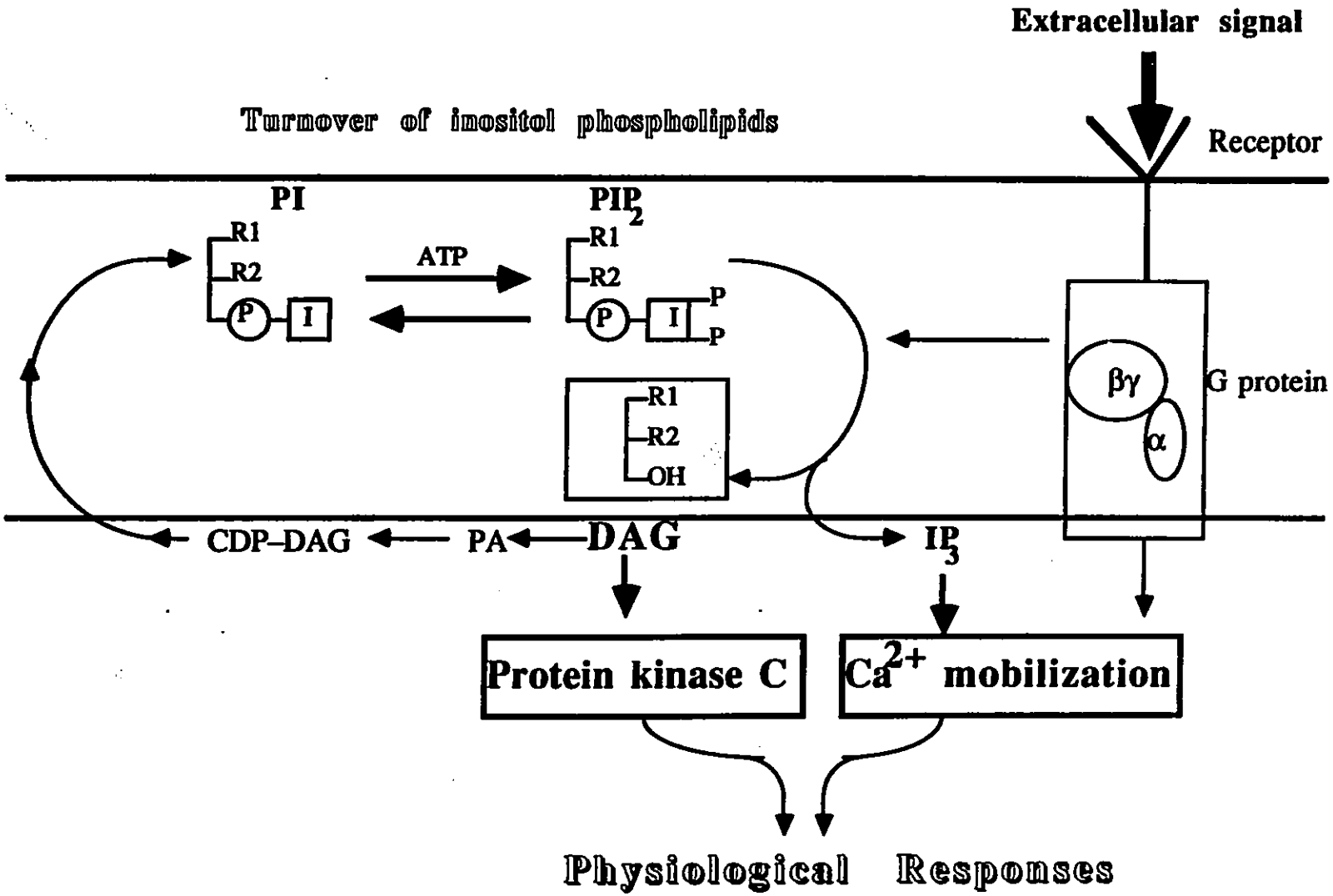
Extracellular signal
Receptor
Turnover of inositol phospholipids
PI
PIP2
R1
R2
P
I
ATP
P
P
R1
R2
OH
βγ
α
G protein
CDP–DAG
PA
DAG
IP3
Protein kinase C
Ca2+ mobilization
Physiological Responses

Figure 2

mobilizing agonists was very different from that of IP_3 (Bocckino et al, 1985; Charest et al, 1985). In addition, high pressure liquid chromatographic analysis of DAG in control and stimulated cells showed two peaks, both of which had higher contents of palmitic, oleic, and linoleic acids expected from the breakdown of PIP_2, which is enriched in stearic and arachidonic acids (Bocckino et al, 1985). The fatty acid composition of the later eluting DAG peak was particularly different from PIP_2, and it was suggested that PC was the source (Bocckino et al, 1985). The potential of alternative pathways of phospholipid turnover in hormonal signal transmission has only recently been appreciated. As shown in Figure 3 DAG can be generated directly via phospholipase C (PLC) or by the action of phospholipase D (PLD) to yield phosphatidic acid (PA), which is then cleaved to DAG by PA phosphohydrolase (PAP). In addition, hydrolysis of PC by phospholipase A_2 (PLA_2) produces lyso–PC and arachidonic acid, an immediate precursor of eicosanoids. Lyso–PC may be re–esterified to PC or catabolized to glycerophosphocholine (GPC), which can be further degraded to glycerol 3–phosphate (G3P) and choline. G3P can be converted back to DAG via PA synthesis. DAG then can react with CDP–choline to complete the PC turnover cycle.

A novel G protein, G_p, has been implicated in the coupling of hormone receptor activation to the breakdown of inositol phospholipids by PLC (Cockroft et al, 1987). Similarly, a G protein appears to mediate the P_2–purinergic–induced stimulation of PLC activity towards PC (Irving & Exton, 1987). GTP and related guanine–containing analogues were shown to stimulate PC catabolism whereas other nucleotide triphosphates had little or no effect (Irving & Exton, 1987). It seems likely from the widespread occurence of agonist–stimulated PC breakdown that it serves some physiological

function(s). One probable function relates to its ability to generate DAG for a prolonged period of time (Cabot et al, 1985; Bocckino et al, 1985; Uhing et al, 1989; Pessin et al, 1989; Agwn et al, 1989; Augert et al, 1989) and thus cause sustained activation of protein kinase C. This implies that the mechanisms involved are not subject to negative feedback and it is reasonable to propose that PC breakdown is involved in cellular control mechanisms that require prolonged activation of protein kinase C. The fact that the cellular content of PC is several hundred–fold higher than that of PIP_2 agrees with the observation that PIP_2 hydrolysis yields a relatively small amount of DAG for a short period of time compared with PC hydrolysis (Augert et al, 1989; Pessin et al, 1989). Based on analysis of the molecular species of PC (Patton et al, 1982) and of DAG sampled at late times of agonist stimulation (Pessin et al, 1989; Augert et al, 1989), PC hydrolysis produced species of DAG that are very effective activators of protein kinase C*in vitro* (Go et al, 1987). With the discovery of multiple forms of protein kinase C that vary in their tissue and subcellular distribution, substrate specificity, translocation and regulation (see section 1.2), it is possible that different molecular species of DAG may exert differential effects on various forms of protein kinase C.

Figure 3. **Phosphatidylcholine cycles for generation of second messenger diacylglycerol.** The diacylglycerols (DAG) can be generated directly via phospholipase C (PLC) or by the action of phospholipase D (PLD) to yield phosphatidic acid (PA), which is cleaved to DAG by PA phosphohydrolase (PAP). Alternatively, the phospholipase A_2 (PLA_2)–catalysed hydrolysis of PC provides for the production of lyso–PC and arachidonic acid. The lyso–PC may be re–esterified to PC or catabolized to glycerophosphocholine (GPC), which can be further degraded to glycerol 3–phosphate (G3P) and choline. G3P can be converted back to DAG via PA synthesis. DAG can react with CDP–choline to complete another PC turnover cycle (adapted from Pelech & Vance, 1989).

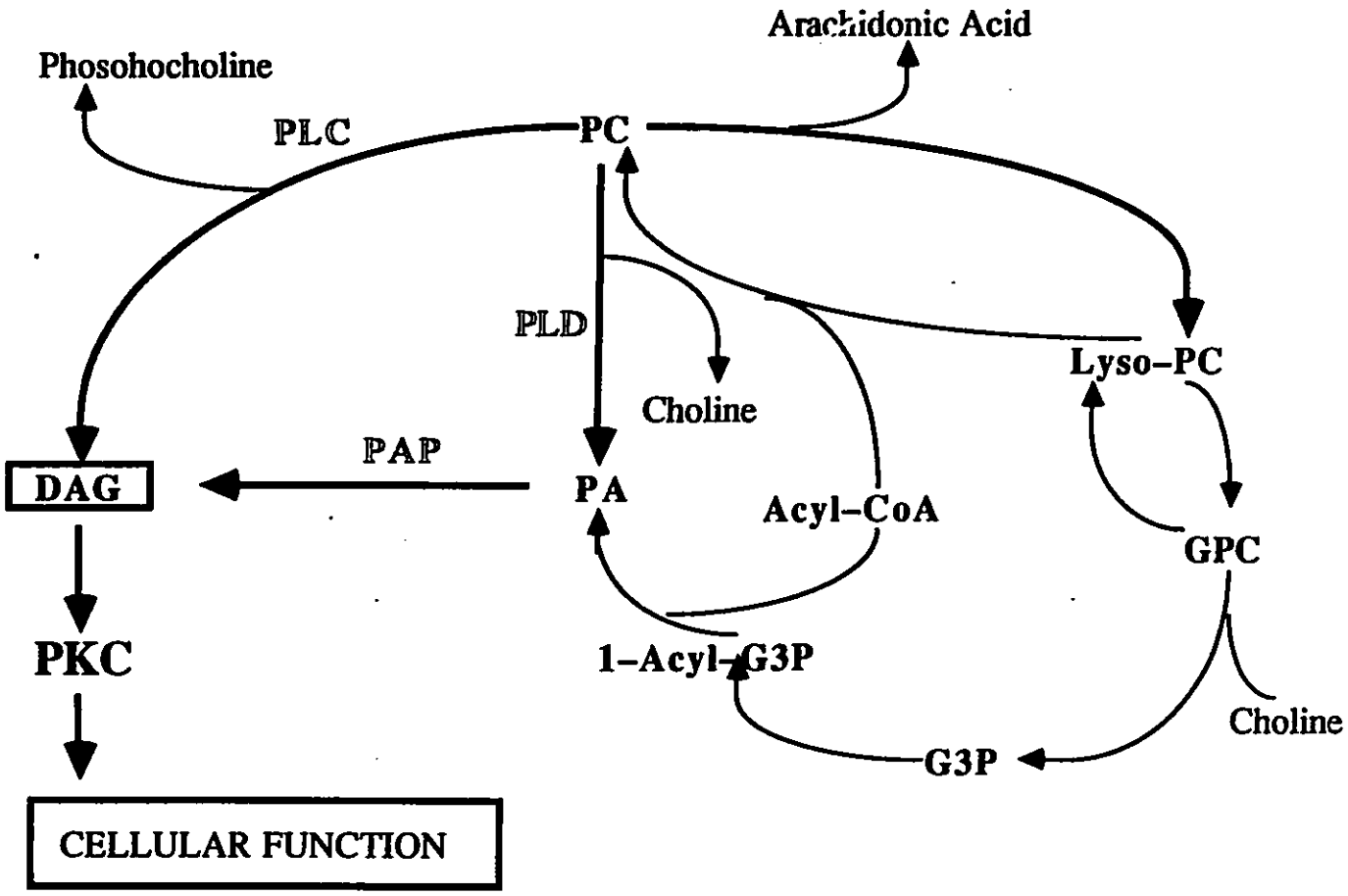
Arachidonic Acid
Phosohocholine
PLC
PC
PLD
Choline
Lyso-PC
PAP
DAG
PA
Acyl-CoA
GPC
PKC
1-Acyl-G3P
Choline
G3P
CELLULAR FUNCTION

Figure 3

1.3.3 Dual action and down regulation of protein kinase C

There have been several suggestions of possible functions for PKC, including involvement in secretion and exocytosis, modulation of ion conductance, regulation of receptor interaction with components of the signal transduction apparatus, smooth muscle contraction, gene expression and cell proliferation (Nishizuka, 1986). In addition to these positive stimulatory actions, a large body of evidence has now accumulated to indicate that PKC provides negative feedback control over various steps of the cell–signaling processes operating in both short–term and long–term responses of the cell. In the short term, for example, PKC appears to be important in decreasing the inositol–1,4,5–trisphosphate–induced elevation of intracellular Ca^{2+} levels, an effect that may occur at different points in the signaling pathway (Nishizuka, 1988). PKC may inhibit Ca^{2+} mobilization by blocking the receptor–mediated hydrolysis of inositol phospholipids and hence production of IP_3 and diacylglycerol, or by stimulating the hydrolysis of IP_3 by activating an IP_3 phosphatase (Connolly et al, 1986). Alternatively, PKC may stimulate the removal of intracellular Ca^{2+} by activation of the Ca^{2+}–transport ATPase and Na^{+}/Ca^{2+} exchange protein (Nishizuka, 1986). The negative feedback role of PKC may be extended to long–term responses, such as cell proliferation. The epidermal growth factor (EGF) receptor has been shown to be phosphorylated by PKC, both *in vivo* and *in vitro* (Schlessinger et al, 1986). This dual action of PKC provides a versatile regulatory system that is finely tuned by the transient generation of second messengers such as diacylglycerol. It is clear, however, that agents that persistently activate the enzyme, such as phorbol ester, can promote more complex and fundamental changes in its activity.

It is now well known that tumour–promoting phorbol esters (PMA) elicit pleiotropic cellular responses through selective binding to protein kinase C as a receptor (Castagna et al, 1982; Niedel et al, 1983; Leach et al, 1983). The phorbol ester promotes the translocation of PKC to the membrane and its tight binding there. This translocation is associated with activation of protein kinase C (Kraft et al, 1982; 1983) and may result in eventual proteolytic degradation or "down–regulation" of this enzyme (Rodriguez–Pena et al, 1984; Ballester & Rosen, 1985), a process thought to be involved in the secretory exocytosis of human platelets and neutrophils (Tapley et al, 1985 Melloni et al, 1986). This phorbol ester–induced down–regulation of protein kinase C is caused by an accelerated rate of degradation of the protein kinase C molecule. In 1989 Huang investigated the differential down–regulation of highly purified rat brain protein kinase C isozymes with trypsin. Their results showed that under identical conditions trypsin caused preferential degradation of types I and II enzymes, whereas the type III enzyme was relatively resistant to tryptic proteolysis (Huang et al, 1989). Degradation of the type III enzyme by trypsin could be facilitated with the addition of Ca^{2+}, phosphatidylserine, and dioleoylglycerol.

1.4 THE ROLE OF PROTEIN KINASE C IN SECRETION

Protein kinase C has been implicated in the regulation of receptor–mediated cellular events in a variety of tissues (Nishizuka, 1986). Agonist–directed redistribution of PKC activity from cytosol to membrane is considered the initial step in PKC activation (Jaken et al, 1975; Drust et al, 1985; Fearon et al, 1987; Jaken et al, 1985; Kraft et al, 1983). Agonist–stimulated phosphatidylinositol turnover in pituitary cells (Fearon et al, 1985)

has been correlated with a time– and dose–dependent decrease in soluble PKC activity, while the particulate form of the activity increases. Hirota studied the effect of a stimulus on protein kinase C distribution in a nonpermanent cell culture system (rat pituitary gonadotrophs) and found a shift of protein kinase into the membrane fraction within 10–20 min following stimulation with gonadotropin–releasing hormone (Hirota et al, 1985). Naor demonstrated the direct initiation of an exocytotic response by insertion of purified brain PKC–α and PKC–β into permeabilized rat pituitary cells that specialize in releasing luteinizing hormone (Naor et al, 1989), while PKC–γ was not effective in stimulation of the hormone secretion. This suggests that different PKC isozymes may have different effect on different cellular functions. In electropermeabilized bovine chromaffin cells, phorbol esters increase exocytosis, implying an involvement of protein kinase C. And also, in intact or digitonin–permeabilized bovine adrenal chromaffin cells, phorbol esters which activated PKC increased membrane–bound PKC activity from less than 10 to 20–50% within 30 min and stimulated secretion and protein phosphorylation (Terbush & Holz, 1986). In experiments with permanent cultured cell lines various groups have shown that receptor–mediated stimulation involving the polyphosphoinositide cycle indeed leads to a shift of protein kinase C from the cytosol to the membrane fraction (McCaffrey et al, 1984; Kraft et al, 1982; Kraft & Anderson, 1983). In the parotid gland, PKC may play a role in excitation–secretion coupling because phorbol myristate acetate (PMA) stimulates exocytosis and the release of amylase (Putney et al, 1984; Takuma et al, 1986). In addition, one of the PKC inhibitors, H-7, inhibited amylase secretion from rat parotid gland stimulated by PMA or the combination of phosphotidylserine and 1,2–diolein (Shimomure et al, 1988). Isoproterenol (20 μM) stimulates the release of amylase, but did not

significantly affect intracellular distribution of protein kinase C during the observation time of 30 min in guinea pig parotid gland (Domenech & Soling, 1987). There are three possible reasons for their inability to detect an effect of isoproterenol on the distribution of protein kinase C: 1) The total PKC activity was measured instead of examination of the change of a specific isoform of PKC 2) This high concentration of isoproterenol activates α–adrenoceptors as well as β–adrenoceptors, 20 μM ISO is not an appropriate stimulus for finding the real role of PKC; 3) And also, species difference should be taken into consideration for this inability to measure the change of PKC activity. Soling also demonstrated in guinea pig parotid that the transmission of the signal process within the cell following stimulation with either β–adrenergic or muscarinic agonists was associated with an increased *de novo* synthesis of DAG which started within the first 10 s after stimulation and returned almost to the basal level within 2 min (Soling et al, 1987; 1989). Pandol measured the effect of various pancreatic secretagogues on the PKC translocation and amylase release in dispersed pancreatic acini from the guinea pig and concluded that 1,2–DAG and protein kinase C do not have a stimulatory role in pancreatic exocytosis (Pandol & Schoeffield, 1986). Some other groups failed to observe a decrease in cytosolic protein kinase C enzyme activity during insulin treatment in several tissues (Cooper et al, 1987; Walaas et al, 1987; Egan et al, 1989; Draznin et al, 1988; Glynn et al, 1986). Although a great deal of evidence have shown that in a wide variety of cell types, i.e. rat pancreatic acini (Matozaka & Williams, 1989); cultured fibroblasts (Wright et al, 1988); BC3H–1 myocytes (Farese et al, 1988); hamster fibroblasts (Magnaldo et al, 1986); smooth muscle cells (Griendling et al, 1986); Swiss 3T3 cells (Habenicht et al, 1981), DAG formation increases in response to various stimuli, data concerning the distribution behavior of protein kinase C

and the regulatory role in rat exocrine glands in the presence of different stimuli is little and is not good enough to interprete the mechanism involved.

Although the activation of protein kinase C has been shown (Takuma, 1986; Terbush & Holz, 1986; Shinomura et al, 1988; Soling et al, 1989; VanBlitterswijk et al, 1991) during secretion by measuring the change of total activity of PKC, data concerning specific isozyme(s) of protein kinase C in stimulus–secretion coupling in rat exocrine gland is missing. In addition, there is methodological discrepency in the determination of PKC activation patterns. Cooper demonstrated that the choice of chromatography medium used to purify protein kinase C determined whether increases or decreases in cytosolic enzyme activity were observed following insulin treatment (Cooper et al, 1990). The traditional ways of measuring PKC activity are: 1) the enzyme(s) is extracted from membranes with detergents, partially purified, reconstituted with phosphatidylserine, and its activity measured with a histone substrate, histone IIIS (Kikkawa et al, 1982; Takai et al, 1979); 2) the enzyme(s) is also extracted with detergents, but the extracted enzyme activity is measured directly without partial purification using a modified histone H–I (Halsey et al, 1987); 3) the cells are permeabilized with digitonin to release soluble PKC, both the soluble and residual membrane–associated enzyme are partially trypsinized, and their activities assayed using histone H–I substrate in the presence of a cyclic AMP–dependent protein kinase inhibitor (Pelech et al, 1986); 4) PKC activity is directly measured *in vitro* while it is still associated with its native membrane, using a murine lymphoma cell extract containing a PKC–specific 85 kDa protein substrate (Chakravarthy et al, 1989). It is important to point out that the disadvantage of all of these assays is that they lack sensitivity and specificity to measure changes in a specific isoform of protein kinase C.

2. OBJECTIVES OF THE PROJECT

In order to explore the role of one specific isoform of protein kinase C, PKC–β, in the stimulus–secretion coupling in rat parotid gland, the present study includes:

1. Comparative studies of hydroxylappatite chromatographical characteristics of protein kinase C in rat parotid gland, submandibular gland and brain.

2. Development of specific procedures to measure PKC–β translocation during stimulation with different secretagogues in rat parotid acinar cells.

3. Time–course and dose–response relationship of PKC–β stimulated with different secretagogues in rat parotid acinar cells.

4. Determination of the relationship of PKC–β activation and amylase exocytosis during stimulation with different secretagogues in rat parotid acinar cells.

3. MATERIALS AND METHODS

3.1. MATERIALS

90 male Wistar strain rats (125–150 g) were used in this study. The α–amylase reagent was from Biotrol (Laboratory Imports Co., Downsview, Ont., Canada). 100% pure corn starch (Canada) was bought from a local grocery store. Procion yellow, Basal medium Eagle (BME) (with Eagle's Salts, L–glutamine and 25 mM hepes), isoproterenol, dibutyryl cAMP, tissue culture grade sodium bicarbonate, egg white protein, leupeptin, d–biotin, phorbol 12–myristate 13–acetate (PMA) and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, USA). Fatty acid free bovine serum albumin (BSA, fraction V) was from ICN (Mississauga, Ont., Canada). Collagenase (CLS2) was from Worthington (St. Laurent, Que., Canada). AquaSil and BCA protein assay reagents were purchased from Pierce (Rockford, IL, USA). Polyoxyethulene sorbitan monolaurate (tween–20), gelatin, nitrocellulose membrane and blotting filter paper were from Bio–Rad Laboratories (Mississauga, Ont., Canada). Dithiothreitol (DTT) , sodium azide, sodium choloride and sodium dodecyl sulphate (SDS) were from BDH (Toronto, Ont., Canada). Sodium phosphate dibasic and sodium phosphate monobasic was from Fisher Scientific Co. (Nepean, Ont., Canada). Anti–rabbit biotinylated species–specific IgG, streptavidin–horseradish peroxidase conjugate antibody, enhanced chemiluminescence (ECL) reagents and PKC assay kit were obtained from Amersham (Oakville, Ont., Canada). Anti-rabbit PKC–β antiserum (raised to the synthetic peptide sequence: RAKIGQGTKAPEEKTANTISK which is equal to the amino acid residues 307–327 in V_3 region) was given generously

by Dr. Bryan L. Roth (Roth & Mehegan, 1989) Adenosine–5'–[^{32}P]triphoshate was from Dupont (Mississauga, Ont., Canada). A Bio–Tek EL311 SL microplate reader was utilized in conjunction with software designed to analyze kinetic absorbance data (KinetiCalc, Bio–Tek Instruments, Inc.). The instrument was connected to the serial port of an IBM clone XT microcomputer via its RS–232 communications port. The spreadsheet software used to calculate the percentage of amylase release was from Borland International (Quattro).

3.2. METHODS

3.2.1. Tissue preparation and digestion

For each experiment, two fasted rats were used. Rats were anesthetized with 0.2 ml euthynal and then exsanguinated following cardiac puncture. The parotid glands were placed into ice–cold BME. The cleaned tissue was finely minced and incubated for 75 min in 5 ml/gland of BME containing collagenase (2.36 units per mg tissue). The suspensions were continuously gassed (O_2/CO_2; 95 : 5) and shaken in a shaking incubator (Versa–Bath S, model 224; Fisher Scientific Co.) at 37° C. At 15 min intervals the mixture was drawn up and down four times through a pasteur pipette. Following digestion, the suspension was washed four times with BME containing 0.2% BSA. The tissue was incubated for a further 45 min in the same medium and then filtered through a nylon sieve (mesh opening 420 microns). After washing once with 0.2% BSA/BME, the tissue was incubated for another 45 min. These parotid cell aggregates were utilized for the experimental protocols described below. Cell preparations were assessed for viability by examining

their ability to exclude trypan blue, and functionally, by measuring their ability to secrete amylase in reponse to a stimulus of 0.1 μM isoproterenol in each experiment.

3.2.2. Measurement of amylase release

After digestion with collagenase and final washing, cell aggregates were resuspended in BME containing 0.2% BSA and 2.0–ml aliquots were placed in siliconized glass vials for gassing (95% O2 : 5% CO2) at 37° C. Secretagogues (100X concentration) were prepared and diluted in 10 mM ascorbic acid. At timed intervals during the 30–min stimulation period (every 10 min), 50 μl supernatant was removed from the control vial and diluted into 450 μl of medium while 100 μl supernatant was taken from all the other vials and diluted in 400 μl of medium. This suspension was rapidly centrifuged (Eppendorf centrifuge model 5415) (10 s, 5000 rpm) and 10–μl duplicate samples of supernatant were removed for assay of secreted amylase. At the end of the experiment, the glass vials, containing most of the cells and medium, were frozen (–20° C) and then thawed. Distilled water (4.0 ml) was added to each vial, and the contents were sonicated (Biosonik sonicater, Bronwill Scientific.) (probe intensity was 12). After diluting a sample 5 times with 0.2% BSA/BME, duplicate aliquots of 10 μl were then removed from each vial for measurement of total activity with two methods.

Amylase activity was measured with one of two methods, which gave equivalent result:

1. **Kinetic microplate assay.** This is microplate reader–based kinetic determination of α–Amylase activity. Activity was quantified by monitoring the cleavage of *p* –nitrophenol from a chemically defined substrate at 405 nm.

The rate of increase in absorbance at 405 nm is directly proportional to the α–Amylase activity. The assay was started by rapidly pipetting 200 µl of the reconstituted reagent into each well of a microplate which contained 10 µl of sample. The microplate was then placed in the reader. The microplate reader was operated in the dual wavelength mode and the absorbance (405 nM–650 nm) measured at 1.5–min intervals for a total period of 10.5 min.

2. Procion Yellow (PY) starch assay (Jung, 1980). This method is based on the use of chemically modified starch substrate to determine amylase activity. The procion yellow chromogen, liberated from the substrate by enzymatic hydrolysis for a fixed period of time, gives a very stable color, and it has a maximum absorbance at 420 nm. Samples (10 µl) were collected in eppendorf tubes instead of microplate well. When ready to start, 0.4 ml of starch suspension was added at 15 sec intervals to the tubes, which were then capped and vortexed. Throughout the assay, the tubes were inverted. After 15 min reaction, 0.4 ml of 0.1 N HCl was added to the tubes at 15 sec intervals. The tubes were vortexed and then centrifuged (1 min, 14,000 rpm). 300 µl of each supernatant was then transfered to the wells of a microplate and the absorbance at 405 nm was measured. The absorbance is proportional to the amylase activity, and has a linear range from 0.05 to 1.5 Abs..

The results for either assay were used in calculations to convert amylase activity (ΔA405 nm/min) to release as a percentage of total cellular amylase performed using spreadsheet software (Soor and Hincke, 1990)).

3.2.3. Preparation of particulate and soluble protein kinase C

Cell aggregates were resuspended in BME containing 0.2% BSA, and then preincubated in the same solution for 45 min. The cell aggregates were

washed 3 times with BME in order to remove BSA, and then diluted in BME to an appropriate volume before they were aliquoted out into reaction vials. The reaction was started by adding 1 ml of cells to a vial containing 1 ml BME and different secretagogues (20 µl from 100 x stock). At the designated times, 1 ml of cells was sampled from the reaction vials and pipetted into labelled Eppendorf tubes. Each tube was centrifuged for 10 sec, 5,000 rpm (Eppendorf centrifuge model 5415). The supernatant was aspirated and the pellets was immediately frozen in liquid nitrogen. At the end of the experiment, frozen cells were thawed on ice and sonicated (1 sec each, 3 sec total, instrument output on lowest setting) after adding ice–cold STOP buffer (20 mM Tris–HCl, pH 7.4, 2.0 mM Na–EDTA, 1mM DTT, 10 µg/ml leupeptin, 0.1 mM PMSF). The eppendorf tubes were centrifuged at 4°C, 14,000 rpm for 30 min (eppendorf centrifuge model 5415). Higher speed centrifugation was tryed and no additional pellets was found. It was assumed that the particulate PKC fraction contained nuclei, mitochondria, membranes and secretory granules although it was not analyzed. 500 µl of the supernatant was transfered to labelled eppendorf tubes and 250 µl SDS-buffer (sample buffer : 0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol) was added to each supernatant. The rest of the supernatant was completely removed and stored in freezer (–80°C) for measurement of PKC activity. To the pellets, 250 µl STOP–buffer was added and sonicated (Biosonik sonicater) (1 sec each, 5 times, output on lowest set). The suspension was vortexed again and then quantitatively transfered (50 µl) to eppendorf tubes which were stored in freezer (–80°C) for measurement of PKC activity. Finally, 200 µl SDS–STOP was added to the rest of the pellets suspension. Supernatant and pellet samples in SDS–Stop were vortexed and heated exactly 5 min (95°C–100°C) and stored in the refrigerator.

In some preliminary experiments, samples were precipitated with 10% trichloroacetic acid for 10 min on ice. Precipitates were collected by centrifugation at 14,000 rpm for 30 min, 4°C, and the supernatant was removed completely. The precipitates were washed 4 times in diethyl ether and dryed under nitrogen. In some cases, it was necessary to adjust the pH with 1 M Tris.

3.2.4. Bicinchoninic acid protein assay

Bicinchoninic acid (BCA) protein assay was performed according to the manufacturer's instruction using bovine serum albumin as standard. Different concentrations of BSA from 1 mg/ml to 10 mg/ml were prepared, based on E_{280nm} (1%) = 6.0, and used as standard. Standards (plus a blank) and samples (10 µl) were assayed in duplicate. After adding 200 µl of working reagent, the microplate was incubated for 1 hour at room temperature and the absorbances were then read in the microplate reader at 562 nm.

3.2.5. Immunoblot analysis

Electrophoresis. SDS–PAGE (10% running gel; 4.5% stacking gel) was performed as described (Laemmli, 1970) on a protean dual vertical slab cell following the manufacturer's recommendations. DTT (to 50 mM) and bromopherol blue (to 0.0125%) were added to samples prepared in SDS–buffer. Samples (50 µg protein) were applied to the gel and run at constant voltage 60 V, overnight. Prestained standards were used for molecular weight determination and to permit visualization of transfer.

Western Blotting with Specific ECL System. The next day, the gel was equilibrated in cold, degased Towbin buffer (20 % methanol, 25 mM Tris base, 192 mM glycine, pH 8.3) for 20 min. and apposed to nitrocellulose membrane (NC) for transblotting (Towbin et al, 1979). Proteins were transfered at 100 V for 1 hour at 4°C. The NC was then equilibrated in phosphate buffered saline (PBS; 20 mM Na phosphate, pH 7.2, 0.9% NaCl) containing 0.1% Tween–20 (TPBS) for 10 min at RT and subjected to a double blocking procedure. The membrane was first blocked with solution A (TPBS containing 3% gelatin and 1.5% egg white protein) for 3 hours, washed in TPBS for 15 min (5 min x 3 times), and then incubated in solution B (TPBS containing 5% nonfat dry milk and 0.01% biotin) for 2 hours. The NC was then extensively washed with TPBS for 30 min (10 min x 3 times). The membrane was incubated with 1:800 anti PKC–β (2% BSA, PBS) 4 hours at RT or overnight at 4°C. After 30 min–wash with TPBS, the NC was incubated with 1:200 anti–rabbit biotinylated Ig–G (1% BSA, PBS) for 45 min at RT and washed (10 min x 3 times) afterwards, followed by 1:500 streptavidin–HRP (1% BSA, PBS) for 30 min at RT. Following thorough washes, the NC was incubated with the chemiluminescent substrate (ECL reagent, A:B = 1:1) for exactly 1 min and then exposed to Kodak x–ray slow film for 5 min. The immunoblot control was performed by incubating NC with the diluted antibody after it had been preabsorbed by overnight incubation with 1 μM synthetic peptide to which the anti–PKC–β antibody had been raised. The 78 Kda immunoreactive band was completely absent in samples from rat brain, parotid and submandibular gland under these conditions.

3.2.6. Quantitative densitometry

Standard curve. Parotid tissue sample was diluted in a range from full strength to 1/16, and analyzed by SDS–PAGE and Western Blotting (Fig. 4–5). The 78 Kda immunoreactive bands were scanned with the densitometer (LKB 2222–020 ultrascan XL Enhanced Laser Densitometer, LKB Bromma.). A standard curve was made using the integrated intensity of image (weight of peak) and dilution of parotid sample (Fig. 6). The relative quantity of PKC–β derived from the standard curve under the carefully controlled experimental conditions was defined as a PKC unit.

Preliminary results suggested that the film became saturated within 10 min exposure to ECL signal. As seen in Figure 7, ECL signal was long–lasting, but was greatest during the first 5 min. For these reasons, the exposure time was carefully standardized in order to yield reproducible results that could be quantitated using the same standard curve.

Quantitative analysis of the result from immunoblotting. Following film development, the bands were measured with the LKB Scanning Laser Densitometer. The 78 kDa peaks from the plotted densitometry were cut out and weighted. The data was converted to PKC unit using the standard curve, and the percentage of PKC unit in the particulate and soluble samples and the total PKC units in 100 μg protein in the original samples were calculated.

Figure 4. **Immunoblot analysis of the translocation of PKC-β with specific anti-PKC-β serum during 30 min stimulation with secretagogues.** A and B represent soluble and particulate PKC-β respectively. Dispersed rat parotid acinar cells were incubated with different concentrations of isoproterenol: control (lane 1, 9), 3 nM ISO (lane 2, 10), 10 nM (lane 3, 11), 30 nM ISO (lane 4, 12), 0.1 μM ISO (lane 5, 13), 1μM ISO (lane 6, 14), and 10 μM ISO (lane 7, 15). Lane 8 and 16 represent samples stimulated with 0.4 mM dibutyryl cAMP.

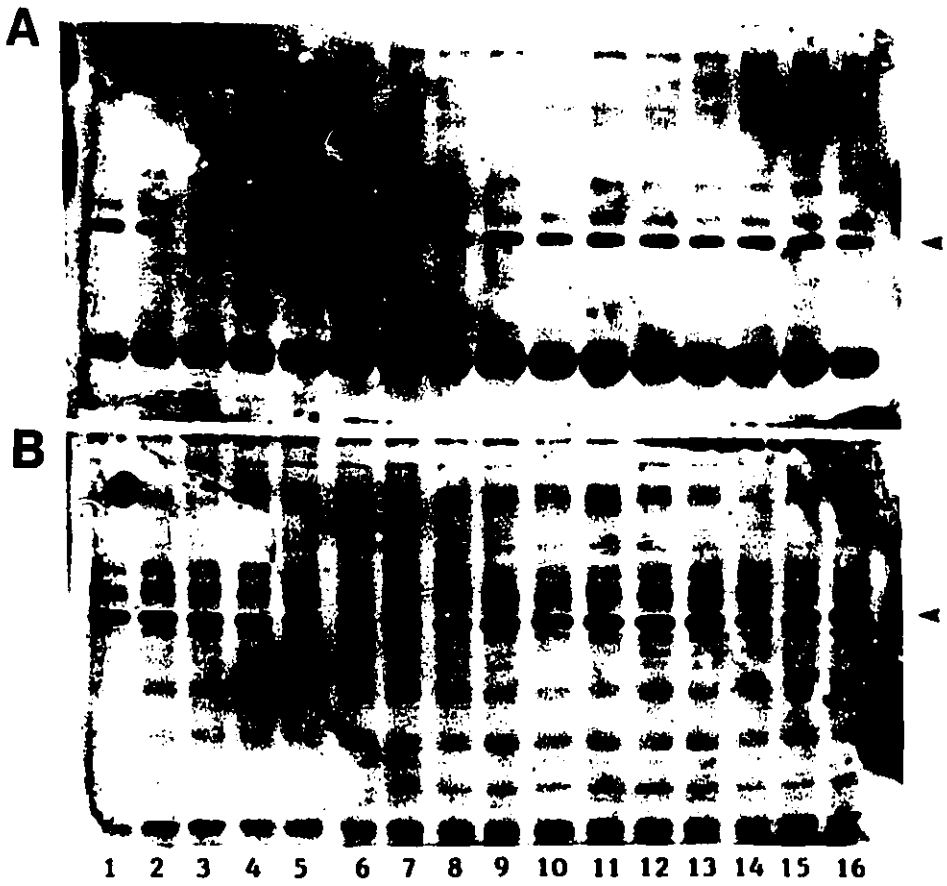

Figure 4

Figure 5. Immunoblot analysis different dilutions of protein kinase C in rat parotid gland. Parotid tissue sample was diluted from full strength (lane 6) to 1/2 (lane 5), 1/4 (lane 4), 1/8 (lane 3), 1/12 (lane 2) and 1/16 (lane 1).

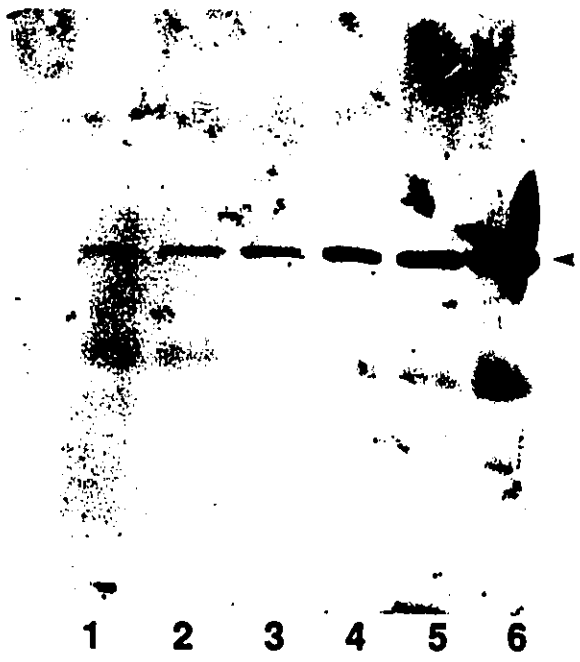

Figure 5

Figure 6. **Standard curve for quantitative analysis of the translocation of rat parotid PKC–β during stimulation with different agonists.** Dilutions of rat parotid samples were analyzed with SDS–PAGE and Western–Blotting as described under "Methods". Integrated intensity of 78 Kda immunoband scanned by densitometer reflected the amount of PKC–β in each sample.

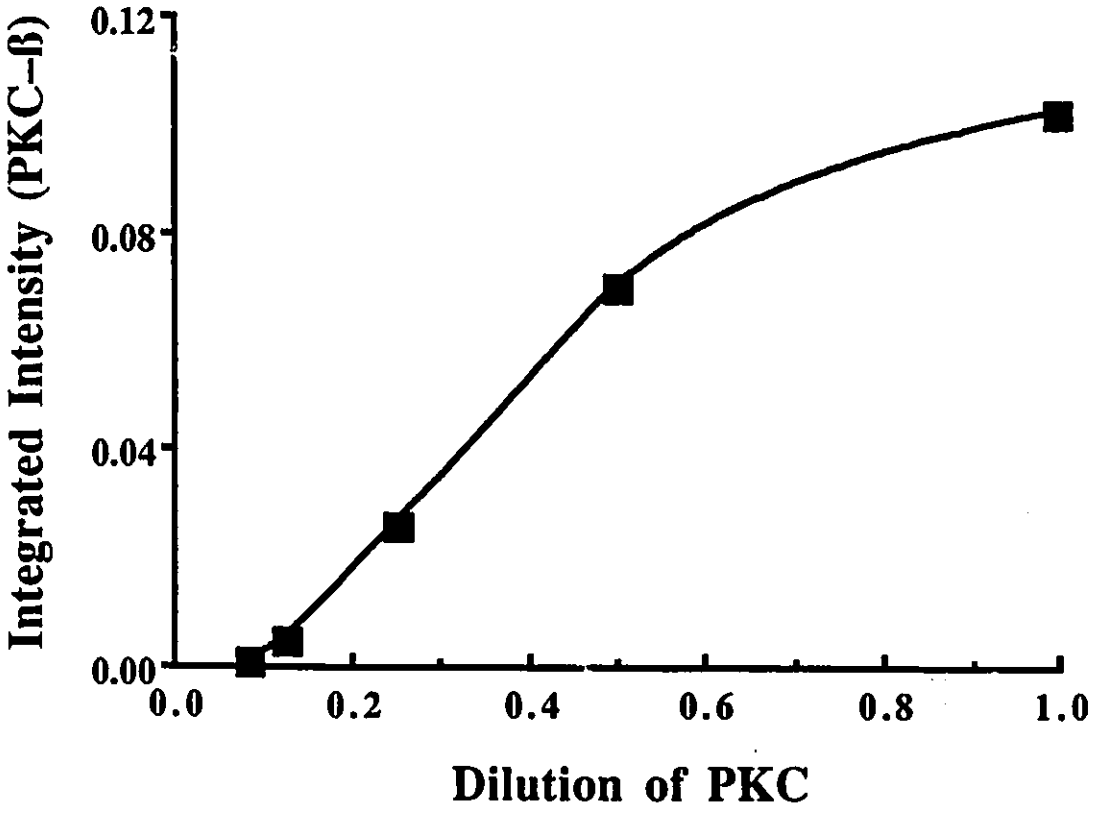
Integrated Intensity (PKC–ß)
0.12
0.08
0.04
0.00
0.0
0.2
0.4
0.6
0.8
1.0
Dilution of PKC

Figure 6

Figure 7. **Relationship between time of film exposure to nitrocellulose membrane and the intensity of 78 Kda immunoband from densitometry.** Two dilutions of parotid samples were examined: (■) undiluted and (▲) 1/4 dilution. The nitrocellulose membrane was exposed to films for 8 successive 5 min periods, and the image was quantitated by densitometry.

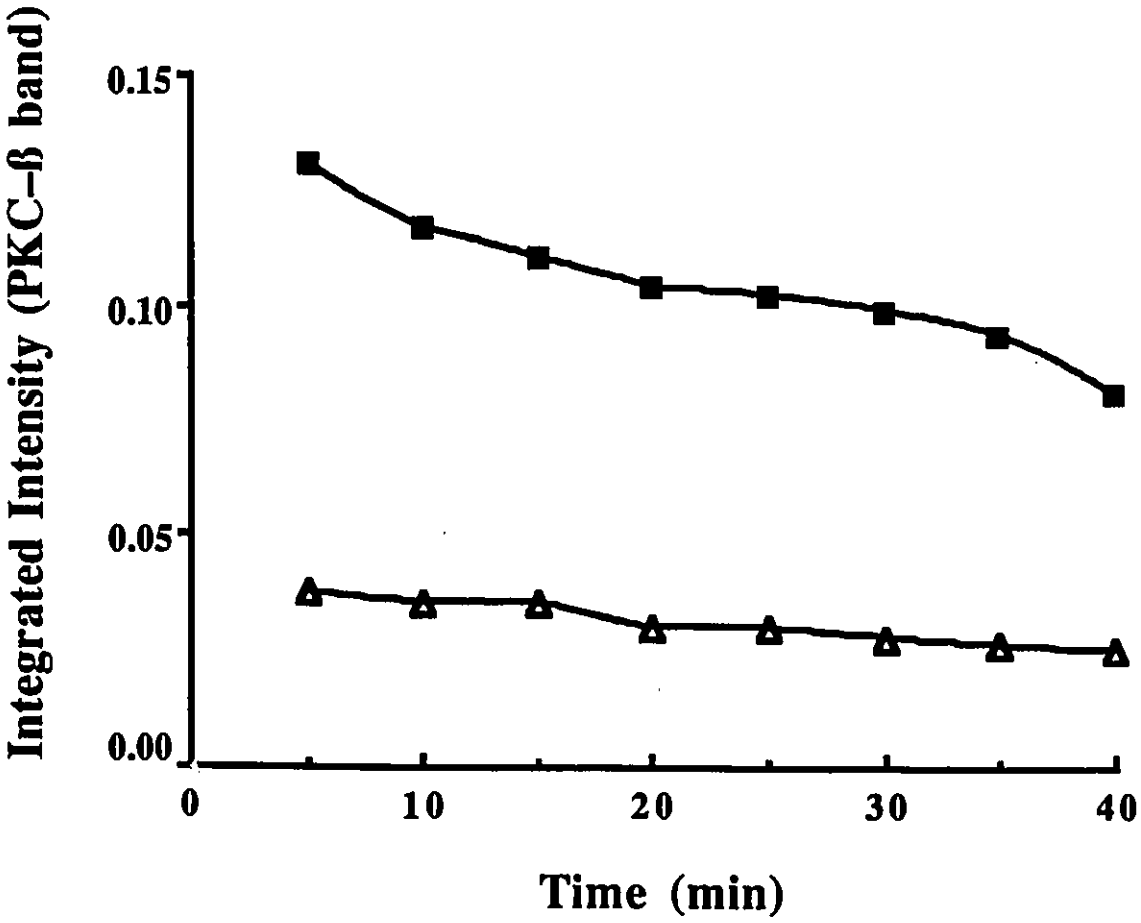
Integrated Intensity (PKC–ß band)
0.15
0.10
0.05
0.00
0
10
20
30
40
Time (min)

Figure 7

3.2.7. Separation of protein kinase C isozymes by hydroxylapatite chromatography

Protein kinase C was purified from fresh rat parotid gland, submandibular gland and brain by a modification of published procedure (Hincke, 1988). Eight rats were killed as described above. All procedures were quickly performed at 4°C. Tissues were dissected free of fat and connective tissue in a dissection solution containing 0.3 M sucrose, 20 mM Hepes, pH 7.4, 10 mM EGTA, 2 mM EDTA, 1 mM DTT, 10 µg/ml soybean trypsin inhibitor. Tissues were homogenized in 8 volumes of homogenization solution (dissection solution containing 10 µg/ml leupeptin), and the homogenate was centrifuged at 40,000 rpm for 40 min (Beckman L8–70 ultracentrifuge, Beckman Instruments Inc.). The supernatant was applied to DEAE–Sephacel column (5 x 1.5 cm), which was equilibrated with 100 ml of DEAE buffer (20 mM Hepes, pH 7.4, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM DTT). The column was then washed with DEAE buffer until the absorbance (280 nm) was back to below 0.02. The PKC activity was batch eluted with 25 ml DEAE buffer containing 10 µg/ml leupeptin and 0.3 M NaCl.

The semi–purified sample from the DEAE column was applied to HTP column (15 cm x 1.5 i.d.) equilibrated with buffer A and connected to a Pharmacia FPLC (Fast Protein Liquid Chromatography) system as previously described (Ono et al, 1987;). The whole FPLC system was equilibrated with HTP buffers: A and B (A, 10 mM K phosphate, pH 7.5, 10% glycerol, 0.5 mM K EGTA, 0.5 mM K EDTA, 1 mM DTT; B, 10% glycerol, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 300 mM K phosphate, 10 µg/ml leupeptin). After washing the HTP column with 20 ml buffer A, PKC activity was eluted with a 270 ml gradient from 10 to 300 mM K_2HPO_4 and collected

as ~2–ml fractions. HTP fractions were assayed for kinase activity. The conductivity of fractions was measured (YSI conductivity meter) in order to determine the phosphate concentration at which the PKC peaks eluted from the column.

3.2.8. Protein kinase C assay

During the separation of PKC isozymes by HTP chromatography, protein kinase C was assayed by measuring the incorporation of ^{32}Pi into histone IIIs from [γ–^{32}P] ATP in the presence of phosphotidylserine, diolein and Ca^{2+} (Hincke, 1988). The Ca^{2+}– and phospholipid–independent activity was measured under the same condition without Ca^{2+} and phospholipid but containing 1 mM EGTA. For the kinase assay, 20 µl of column fraction was mixed with 30 µl of 2 times kinase assay mixture on ice. The phosphorylation reaction was initiated by adding 10 µl [^{32}P] ATP and placing the tubes in a 30°C water bath for 10 min. 20 µl aliquot from each tube was then pipetted onto Whatman 3MM paper squares and dropped into ice–cold 10% trichloroacetic acid, 2% sodium pyrophosphate. The bath was changed every 10 min (4 times) and the wet squares were counted by cerenkov emission (Beckman LS9000 liquid scintillation counter, Beckman Instruments Inc.). The final concentrations of reagents in the standard assay mixture were 50 mM Na Hepes, pH 7.4, 5 mM DTT, 1 mg/ml histone IIIs, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.0625 mg/ml leupeptin, 50 µM/ml phosphatidylserine and 2.5 µl/ml 1,2–diolein. [^{32}P]–ATP was added to a final concentration of 0.6 mM ATP to obtain a specific activity of about 400 cpm/pmol. One unit of protein kinase activity is defined as the amount of enzyme catalyzing the

incorporation of 1 pmol of phosphate from ATP into histone IIIs per min under the standard assay condition.

3.2.9. Amersham protein kinase C assay

The assay was performed according to the manufacturer's instructions to assay soluble and particulate PKC activity in parotid samples. The assay is designed to detect PKC that will catalyze the transfer of the γ–phosphate of ATP to the threonine group on a peptide which is a specific substrate for protein kinase C. The dose–response and time–course were tested to determine the optimal conditions under which the assay gave a linear dose–dependence with the parotid samples.

Soluble and particulate PKC activities were expressed as a percentage of the total PKC activity.

3.2.10. Statistical analyses

Values are expressed as means ± SD and the significance of the difference between mean and control was determined using Student's paired *t* -test. $P<0.05$ was considered significant; n is the number of experiments.

4. RESULTS

4.1. HYDROXYLAPATITE CHROMATOGRAPHY RESOLVES TWO PEAKS FROM PAROTID HOMOGENATES

Hydroxylapatite chromatography is a biochemical method by which the major PKC isozymes (α, β and γ) found in rat brain can be separated (Jaken & Kiley, 1987; Huang et al, 1986; Sekiguchi et al, 1987; Kiley et al, 1990). Previous studies have demonstrated that rat brain PKC isolated by polylysine–agarose column chromatography was separated into three isozymic forms by hydroxylapatite chromatography (Huang et al, 1986). The three kinase activity peaks that eluted at phosphate concentrations of 0.07 M, 0.08 M and 0.15 M were designated as type I (γ), II (β I + β II), and III (α) PKC, respectively. Results from preliminary experiments in our laboratory suggested that the β–form of PKC was the major PKC isozyme in rat parotid gland. In the present studies, fresh homogenates of rat brain, parotid and submandibular glands were subjected to unexchange and HTP chromatography in order to separate protein kinase C isozymes. Two peaks of PKC activity were resolved by HTP chromatography of parotid gland (Fig. 8). HTP peak 1 was eluted with 60 mM phosphate and peak 2 was eluted with 180 mM phosphate. However, Immunoblots with specific anti–PKC–β antibody indicated that β–PKC was present in both fractions (not shown). Figure 9 shows the corresponding HTP chromatography of sample from brain homogenate. Peak 1 was eluted with 60 mM phosphate and peak 2 was eluted with 240 mM phosphate. Immunoblots indicate that only peak 1 contains PKC–β. Data from HTP chromatography of submandibular gland (Fig. 10) showed that one single peak was eluted with ~220 mM phosphate, which also had high levels of phospholipid and Ca^{2+}–

independent Histone IIIs kinase activity. This suggested that the isozyme(s) of PKC in rat submandibular gland is/are different from that those of parotid gland, although immunoblotting showed this peak did contain the β form of protein kinase C (not shown).

Figure 8. **FPLC hydroxylapatite chromatography of partially purified PKC from rat parotid gland.** PKC activity was assayed as described (section 2.2.7.) with 0.1 mM Ca^{2+} in the absence (O) or presence (●) of PS and diolein. The two peaks of kinase activities have been labeled *1* and *2* according to order of elution.

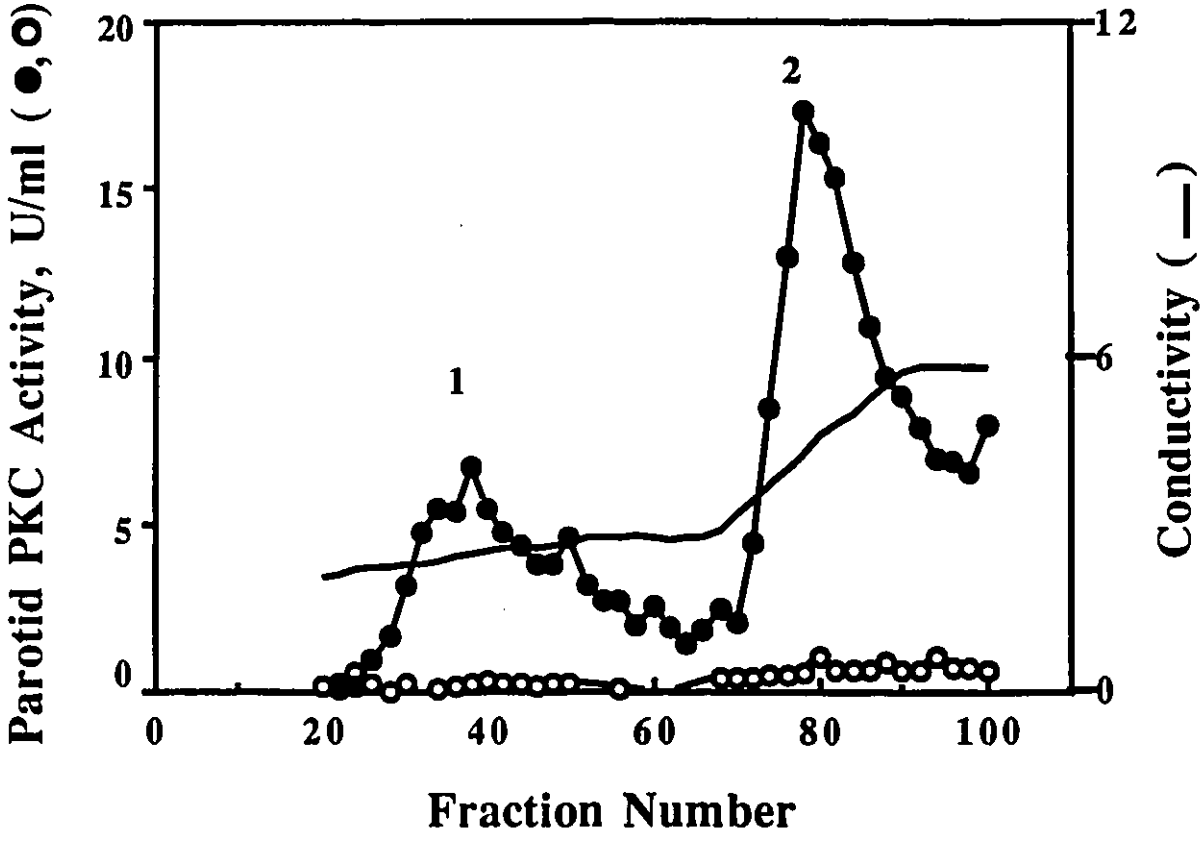
Parotid PKC Activity, U/ml (●,○)
Conductivity (—)
Fraction Number
1
2
0
5
10
15
20
0
6
12
0
20
40
60
80
100

Figure 8

Figure 9. **FPLC hydroxylapatite chromatography of partially purified PKC from rat brain.** Hydroxylapatite chromatography was performed as described under "Methods". Kinase activity was assayed in the absence (O) or presence (●) of PS and diolein. The two peaks of kinase activities separated from brain homogenates have been labeled *1* and *2* according to order of elution. Data shown are from single experiment. Four independent experiments yielded similar results.

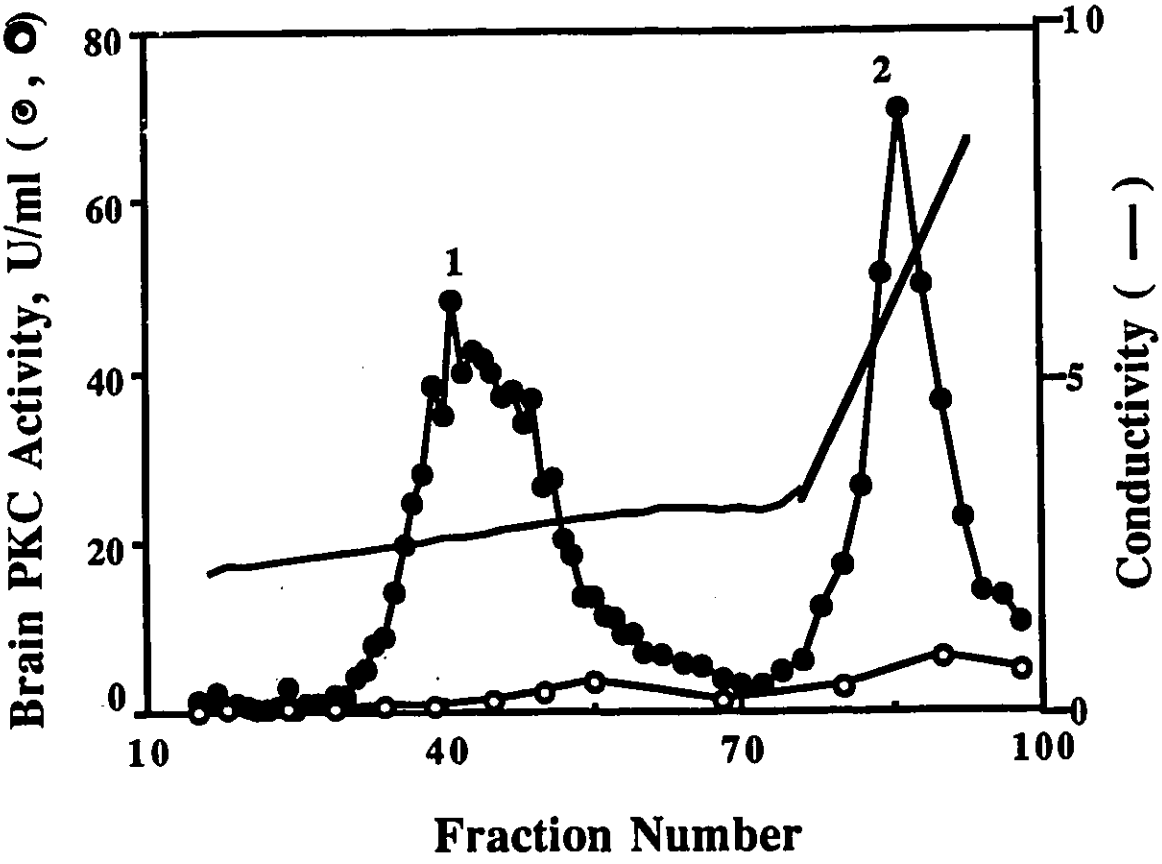
Brain PKC Activity, U/ml (◎, ●)
80
60
40
20
0
10
40
70
100
Fraction Number
1
2
10
5
0
Conductivity (—)

Figure 9

Figure 10. **Hydroxylapatite chromatography of partially purified PKC from rat submandibular gland.** Protein kinase C from rat submandibular gland was prepared as described before (section 2.2.7.) and chromatographed on hydroxylapatite. Fractions were assayed in the absence (O) or presence (●) of PS and diolein.

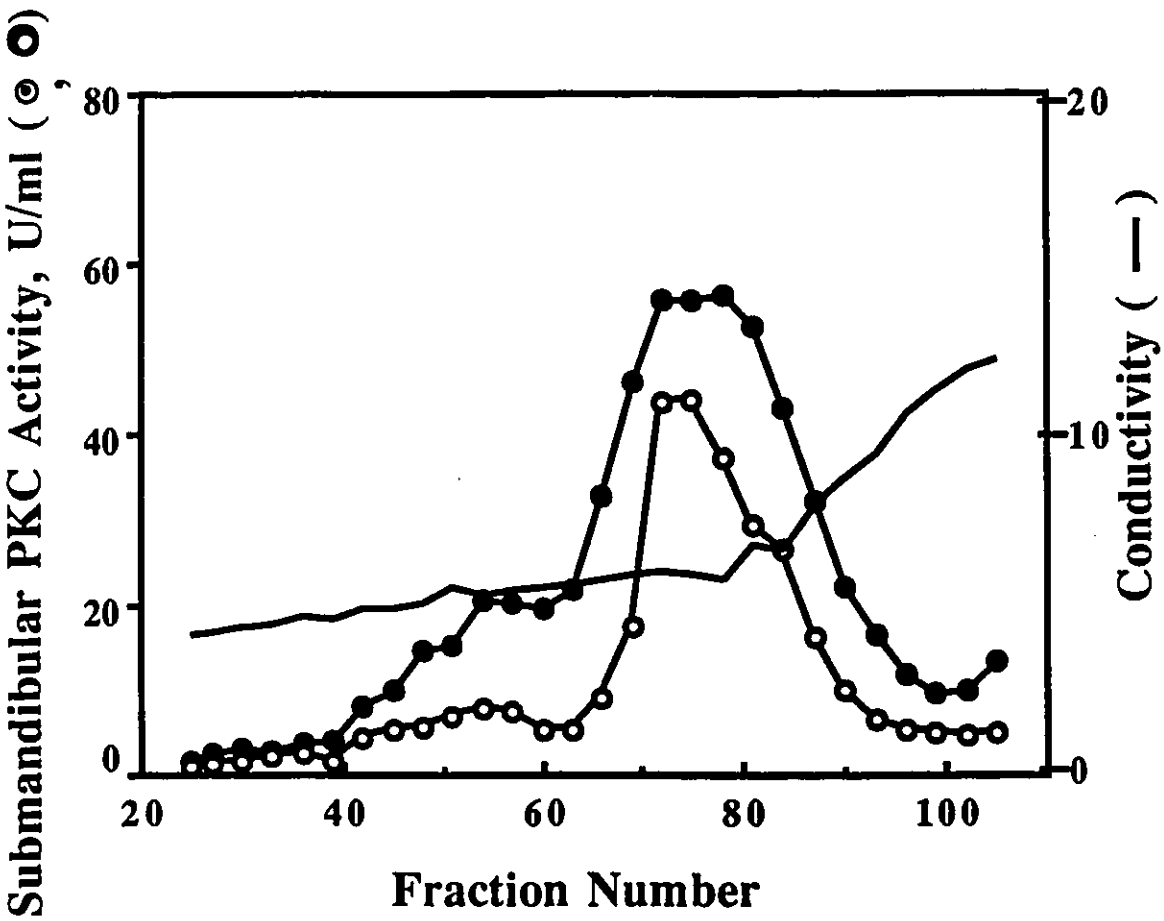
Submandibular PKC Activity, U/ml (● ○)
Conductivity (—)
Fraction Number
0
20
40
60
80
100
0
10
20

Figure 10

4.2. ACTIVATION & TRANSLOCATION OF β PROTEIN KINASE C IN PAROTID CELLS STIMULATED WITH ISOPROTERENOL, DIBUTYRYL CAMP AND PHORBOL ESTER.

PKC is a phospholipid and Ca^{2+}-dependent enzyme activated by 1,2–diacylglycerol (DAG) in response to many agonists, neurotransmitters, hormones, growth factors and electrical stimulation (Nishizuka, 1984; Nishizuka, 1986). The DAG can be generated directly via phospholipase C catalysed the hydrolysis of phosphatidylinositol 4,5–bisphosphate or by the action of phospholipase D to yield phosphatidic acid (PA) from phosphatidylcholine cycles, which is cleaved to DAG by PA phosphohydrolase without an effect on cytoplasmic Ca^{2+} level (Ase et al, 1988). Some recent studies showed that both sources of DAG activated PKC in different tissues (Hosoda et al, 1989; Saito et al, 1989; Ito et al, 1990). In vitro studies suggest that the majority of nonactivated protein kinase C is located in the cytosolic fraction of the resting cell and tends to relocate to the membrane upon activation (Drust & Martin, 1985). Thus, agonist-directed redistribution of PKC activity from cytosol to membrane has been used as an index of PKC activation (Jaken, 1985; Drust &Martin, 1985; Fearon & Tashijian, 1985; Fearon & Tashijian; Kraft & Anderson, 1983).

4.2.1. Dose–response of the translocation of β protein kinase C stimulated with ISO

In order to investigate whether PKC–β in rat parotid acinar cell is activated during the amylase exocytosis stimulated with isoproterenol (ISO), a β–adrenergic receptor agonist, different concentrations of ISO from 3 nM to

10 μM were applied to the incubation media after the cells were dispersed and washed as described under "Material and Methods". Data from immunoblots with specific anti–PKC–β antibody and densitometry showed that in the range of concentrations from 3 nM up to 0.1 μM, ISO caused significant redistribution of PKC–β from cytosolic to membrane fraction during 30 minutes of stimulation in a dose–dependent manner (Kd=10 nM) (n=4, $P \leq 0.05$); the total units of particulate PKC–β increased from 0.264 ± 0.022, the resting state, to 0.348 ± 0.013. The percentage of PKC–β increased from 41.5% to 55.48% (Fig. 11A); while the total unit of soluble PKC-ß decreased from the resting state of 0.374 ± 0.042 to 0.279 ± 0.02. The percentage of PKC–β decreased from 58.75% to 44.53% (Fig. 11B). These figures reveal that 0.1 μM ISO caused maximal translocation of PKC–β.

Figure 11 (A). **Dose–response of translocation of particulate PKC–β during 30 min of stimulation with isoproterenol in rat parotid acinar cells.** Dispersed acini were incubated with different concentrations from 3 nM to 10 μM isoproterenol as described in section 2.2.3.. Data is expressed as percent PKC–β (± SD, n=4). (*), P≤ 0.01; (**), P≤ 0.05.

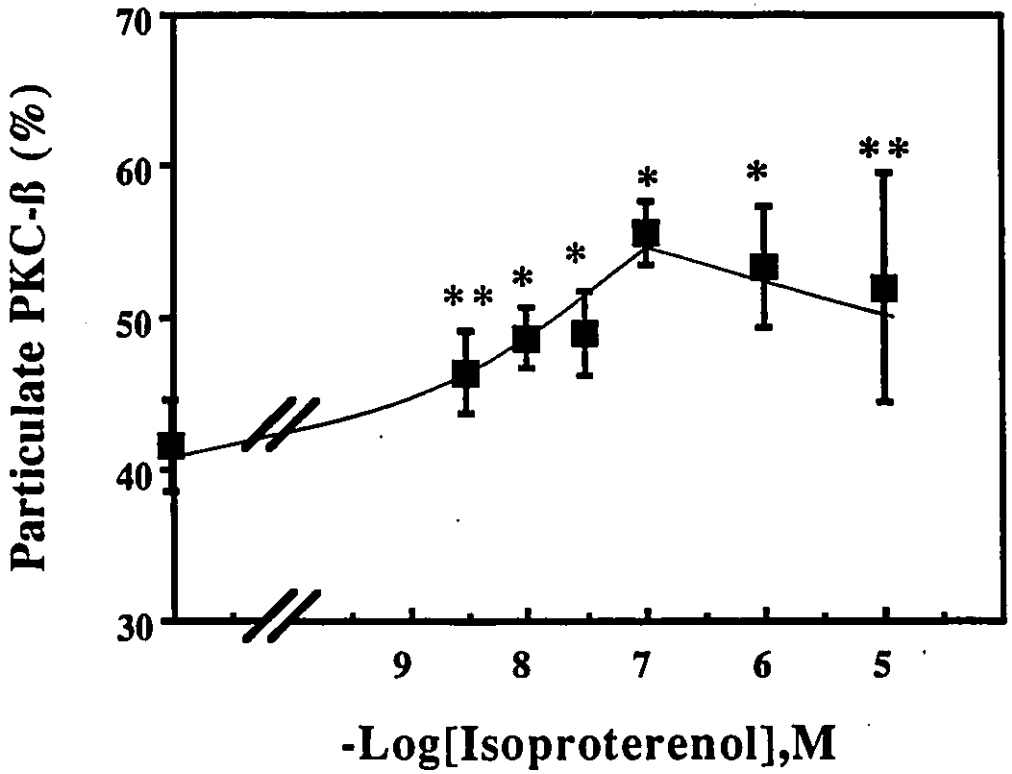

Figure 11 (A)

Figure 11 (B). **Dose–response of translocation of soluble PKC–β during 30 min of stimulation with isoproterenol in rat parotid acinar cells.** Dispersed acini were incubated with different concentrations from 3 nM to 10 μM isoproterenol as described in section 2.2.3.. Data is expressed as percent PKC–β (± SD, n=4). (*), P≤ 0.01; (**), P≤ 0.05.

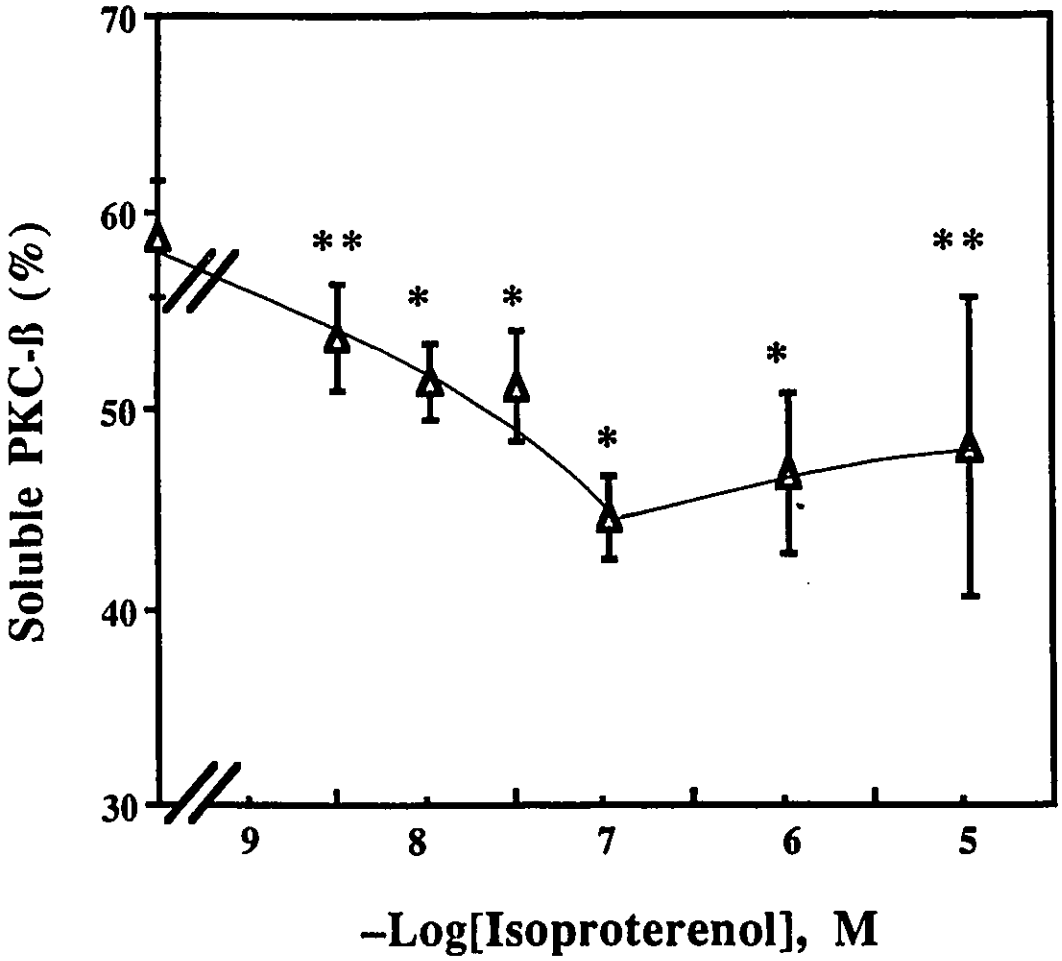

Figure 11 (B)

4.2.2. Time–course relationship of the translocation of β protein kinase C stimulated with isoproterenol

In order to examine more closely whether PKC–β is involved during amylase release stimulated with ISO in rat parotid cells, the time–course of translocation of PKC–β was determined. In addition, the activity of total soluble and particulate protein kinase C was assayed with a specific peptide substrate over the same time interval. Data from immunoblots and densitometry showed that 0.1 μM ISO caused rapid translocation of PKC–β from cytosol to membrane. The total particulate PKC–β increased from 0.372 ± 0.024 to 0.76 ± 0.085 units, translocation was progressive and continued for up to 30 min (49.9% ± 2.99 to 78.5% ± 4.95); while the total units of soluble PKC–β decreased from 0.373 ± 0.032 to 0.205 ± 0.035 (50.12% ± 2.99 to 21.5% ± 4.95) (Fig. 12).

The PKC activity was measured with a specific peptide substrate as described in the methods. Within 10 minutes, the total activity of particulate PKC increased from 3.65 ± 0.3 to 6.09 ± 0.52 pmol/min/mg (27.9% ± 2.2 to 53.7% ± 3.6); while the total activity of soluble PKC decreased from 9.44 ± 0.32 to 5.26 ± 0.58 pmol/min/mg (72.1% ± 2.2 to 46.3% ± 3.6) (n=4, $P \leq 0.05$) (Fig. 13). Between 10 and 30 minutes of stimulation, the total activity of particulate PKC and soluble PKC recovered to resting levels.

Figure 12. **Time–course of translocation of PKC–β stimulated with isoproterenol (0.1 μM) in rat parotid acinar cells.** (□), resting level of PKC–β; (■), particulate PKC–β; (▲), soluble PKC–β (± SD, n=4).

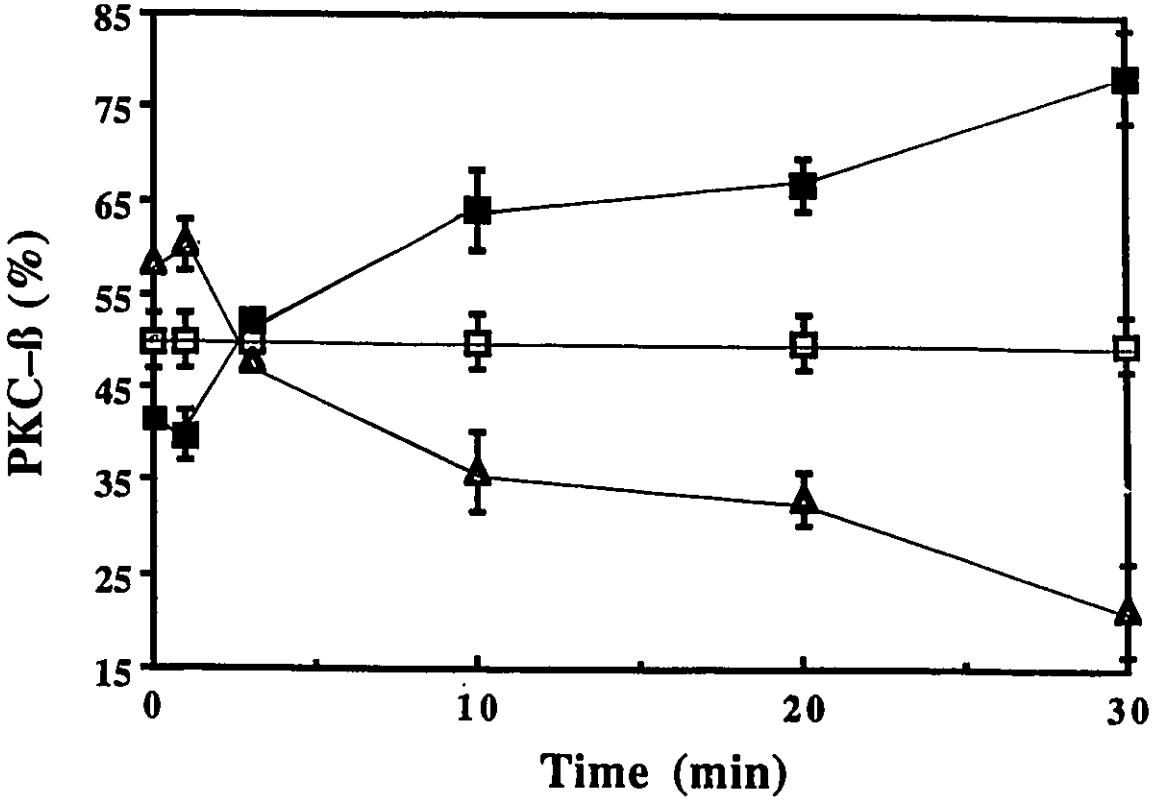

Figure 12

Figure 13. **Effect of isoproterenol on total PKC activity in rat parotid acini.** Particulate PKC activity (solid bars) and soluble PKC activity (hatched bars) stimulated with 0.1 μM isoproterenol for different designated times were measured with a specific peptide substrate (Amersham). Data is expressed as the percent PKC activity (± SD, n=4). (*), P≤ 0.01; (**), P≤ 0.05.

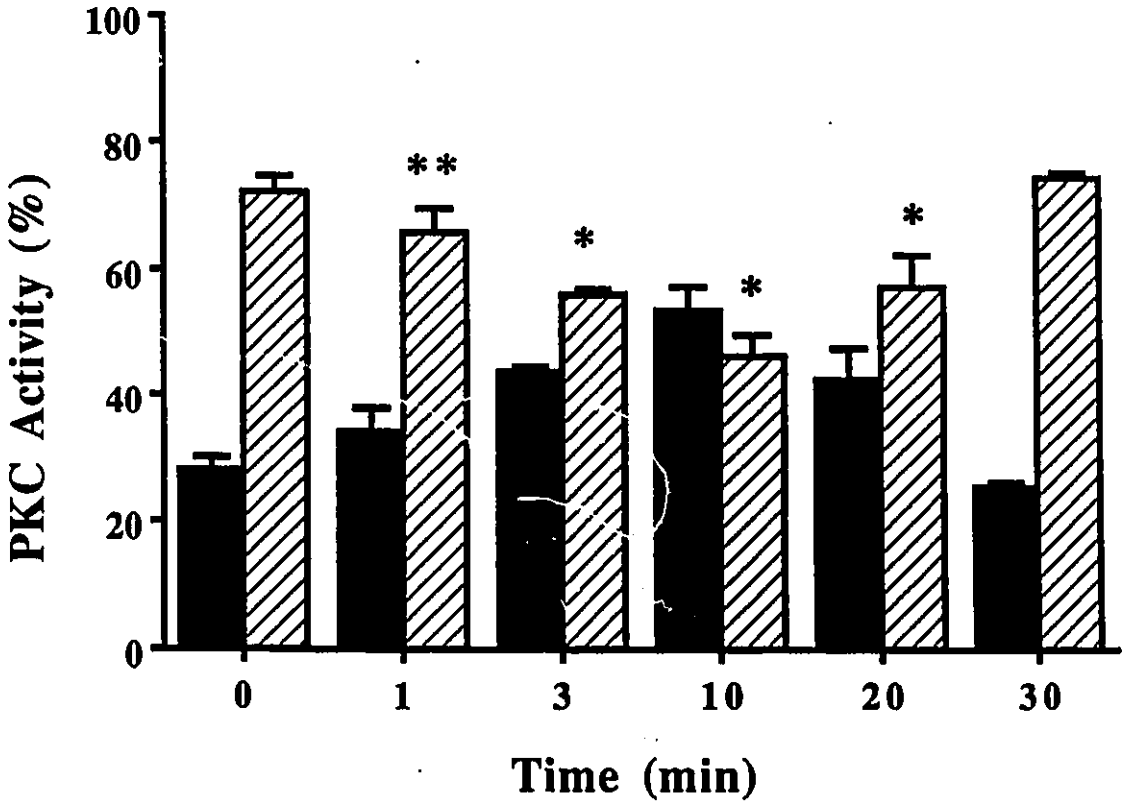

Figure 13

4.2.3. Dibutyryl cAMP activated β protein kinase C translocation

These time–course and dose–response studies indicate cyclic AMP generated through beta–adrenoceptor action is a critical modulator for both PKC–β activation and amylase secretion by the rat parotid gland. I wished to further explore 1) whether the increase in cAMP levels brings about the activation of PKC–β, or 2) it is brought about by another intracellular effector when rat parotid is stimulated with isoproterenol. Therefore, the effect of dibutyryl cAMP, a permeant cAMP derivative, on PKC–β and amylase release was examined. Dibutyryl cAMP evoked the redistribution of PKC–β in a dose–dependent manner (Fig. 14); data from immunoblots and densitometry showed that the total units of particulate PKC–β increased from 0.463 ± 0.01 to 0.68 ± 0.06, while the total unit of soluble PKC–β decreased from 0.59 ± 0.02 to 0.408 ± 0.02.

The PKC activity was also measured after cells were stimulated with dibutyryl cAMP, as above. The total activity of particulate PKC increased significantly from 11.67 ± 1.9 to 28.45 ± 6.4 pmol/min/mg (9.9% ± 1.9 to 30.2% ± 7.2) (Fig. 15); while the total activity of soluble PKC decreased from 105.82 ± 6.4 to 66.08 ± 7.9 pmol/min/mg (90.3% to 69.8%).

Figure 14. **Effect of dibutyryl cAMP on the translocation of PKC–β.** Translocation of PKC–β during 30 min of stimulation with dibutyryl cAMP was examined with SDS–PAGE and Western–Blotting. Data from densitometry was analyzed and converted to percent PKC–β (± SD, n=4). (●), particulate PKC–β; (▲), soluble PKC–ß.

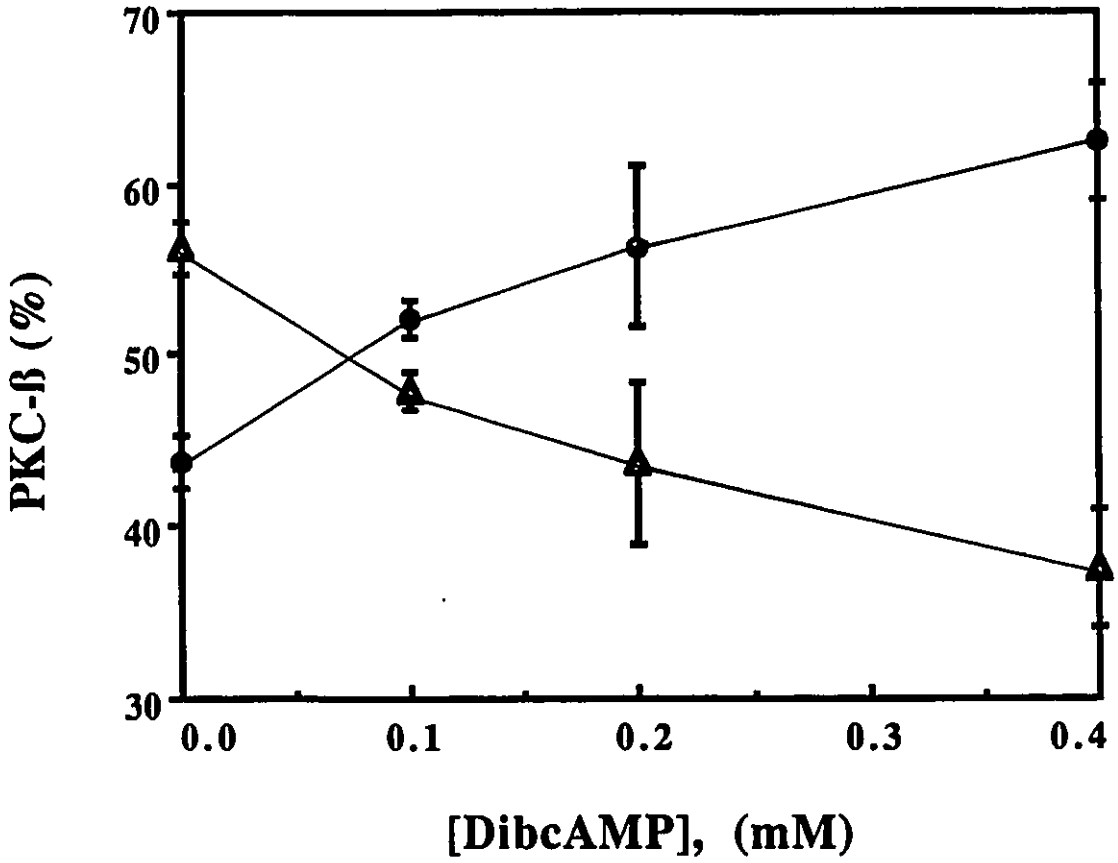

Figure 14

Figure 15. **Effect of dibutyryl cAMP on total PKC activity in rat parotid acini.** PKC activity stimulated with dibutyryl cAMP was assayed with a specific peptide substrate (Amersham). Both particulate PKC (solid bars) and soluble PKC (hatched bars) were measured after 30 min of stimulation. Data is expressed as percent PKC activity (± SD, n=4).

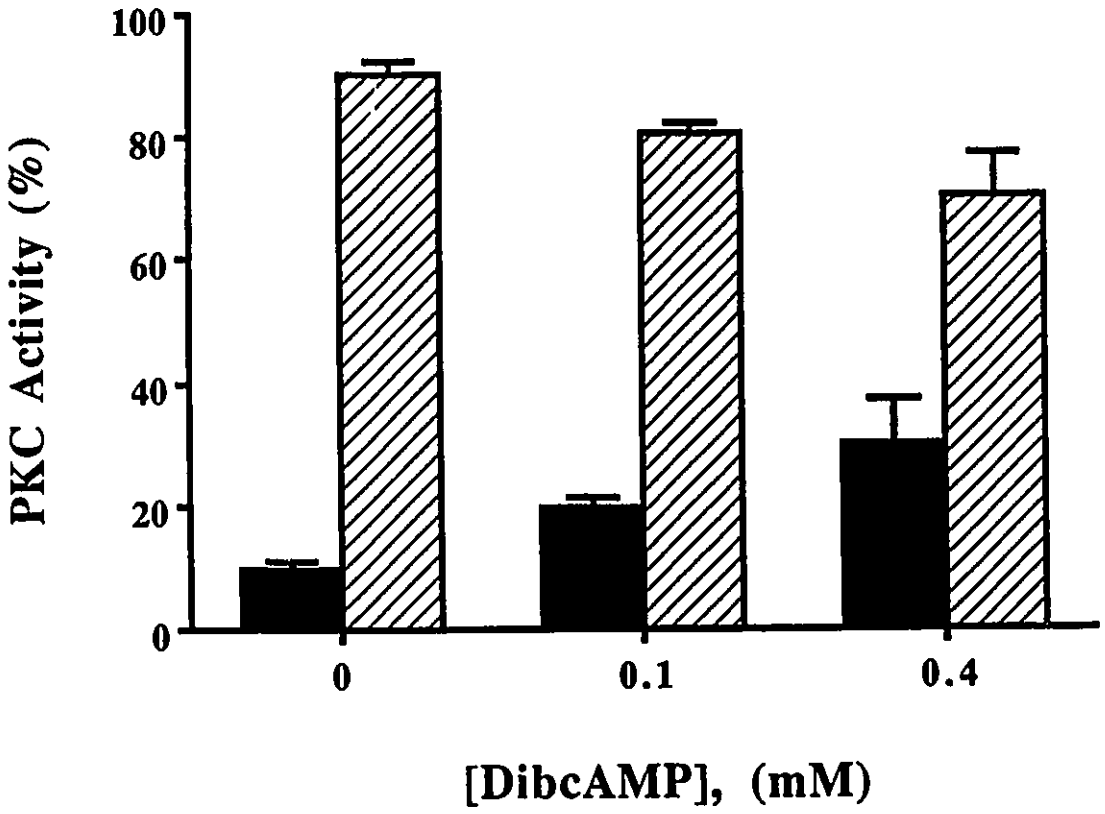

Figure 15

4.2.4. PMA stimulated β protein kinase C translocation

It is known that the tumour promoter phorbol ester (PMA) can activate and cause the translocation of protein kinase C. Data from immunoblots and densitometry showed that PMA (10 nM and 100 nM) caused significant translocation of PKC–β during the observation time of 30 min (Fig. 16). At the same time, the PKC assay indicated that the total particulate PKC increased from 11.89 ± 0.25 to 28.45 ± 6.4 pmol/min/mg (± SD, n=4, $P \leq 0.01$) (9.9% ± 1.9 to 30.2% ± 7.2); the soluble PKC decreased from 108.15 ± 4.8 to 60.08 ± 10.2 ($P < 0.01$) (90.1% ± 0.46 to 72.4% ± 2.9) (Fig. 17).

Figure 16. **Effect of phorbol ester on the translocation of PKC–β in rat parotid acini.** Translocation of PKC–β from cytosolic (hatched bars) to membrane fraction (solid bars) during 30 min of stimulation with PMA was tested as described. Data from densitometry was analyzed and converted to percent PKC–β (± SD, n=4). (*), P≤ 0.01.

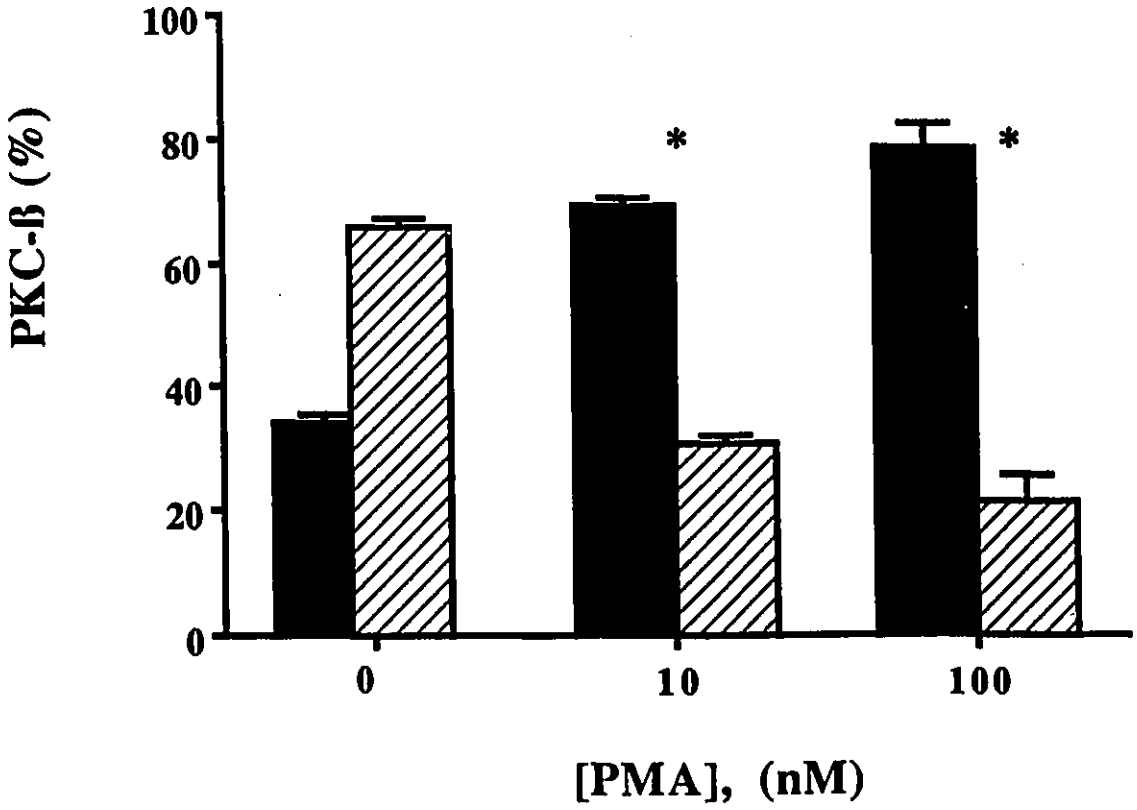

Figure 16

Figure 17. **Effect of phorbol ester on total PKC activity.** Rat parotid particulate (solid bars) and soluble (hatched bars) PKC activity stimulated with PMA for 30 min was measured with a specific peptide substrate (± SD, n=4). (*), $P \leq 0.01$.

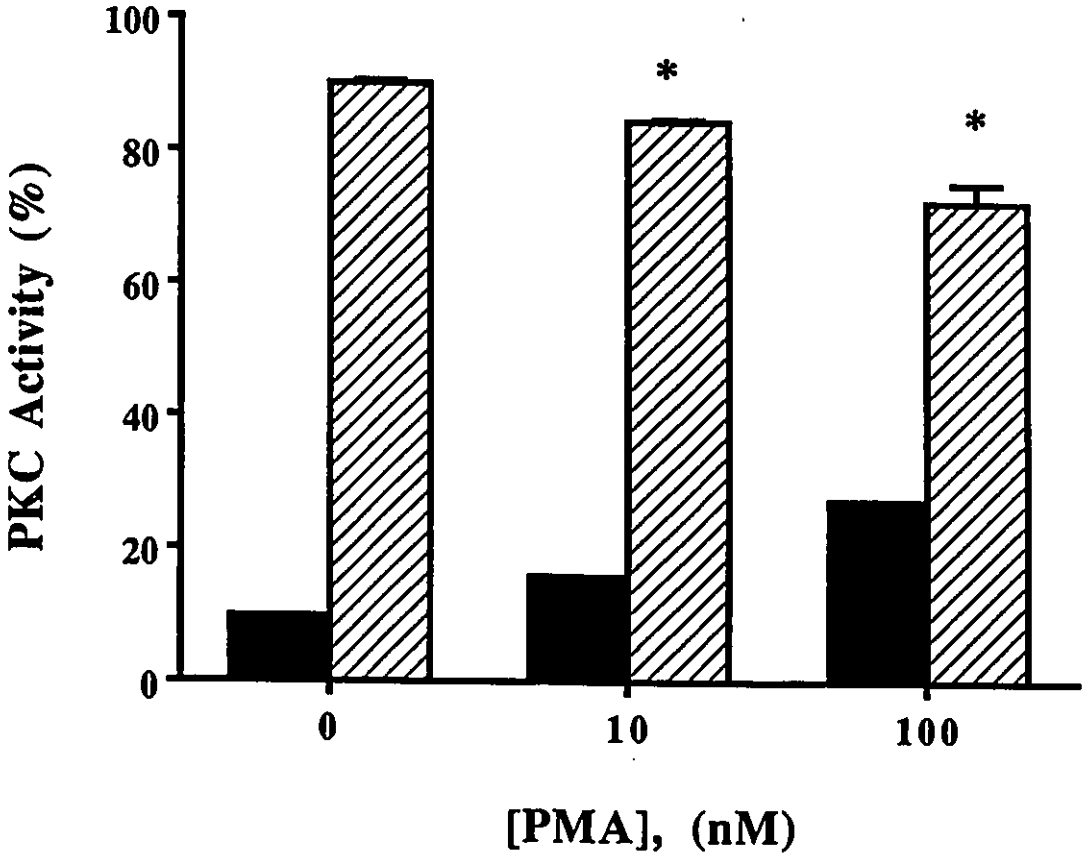

Figure 17

4.3. AMYLASE RELEASE STIMULATED WITH ISOPROTERENOL, DIBUTYRYL CAMP AND PHORBOL ESTER

4.3.1. Isoproterenol evoked linear amylase release within 30 minutes

The time–course of amylase release caused by 0.1 μM ISO was studied. During 30 minutes of stimulation, the amylase was released at a constant rate (Table 1) and correlated with the translocation of PKC–β from cytosol to membrane.

4.3.2. Dibutyryl cAMP increased amylase release in a dose–dependent manner

Amylase release was stimulated by dibutyryl cAMP in a dose–dependent manner (Kd<50 μM) (Fig. 19); 0.4 mM dibutyryl cAMP caused the maximum amylase release from rat parotid acini (n=4, ± SD).

4.3.3. Phorbol ester stimulated amylase release from rat parotid acini

PMA caused potent amylase release from parotid acini (Fig. 20).

	Amylase Release (%)			
	Time (min)			
Stimulant	10	20	30	Rate (%/min)
Control	1.89 ± 0.01	2.1 ± 0.005	3.33 ± 0.016	0.072± 0.01
ISO (0.1 μM)	10.46 ±0.73	24.99 ±1.15	33.95 ± 2.64	1.17 ± 0.06

Table 1 Time–course of amylase release stimulated with 0.1 μM isoproterenol from rat parotid acinar cells (n=8, ± SD)

Figure 18. **Dose–response of amylase release evoked by dibutyryl cAMP from rat parotid acinar cells.**

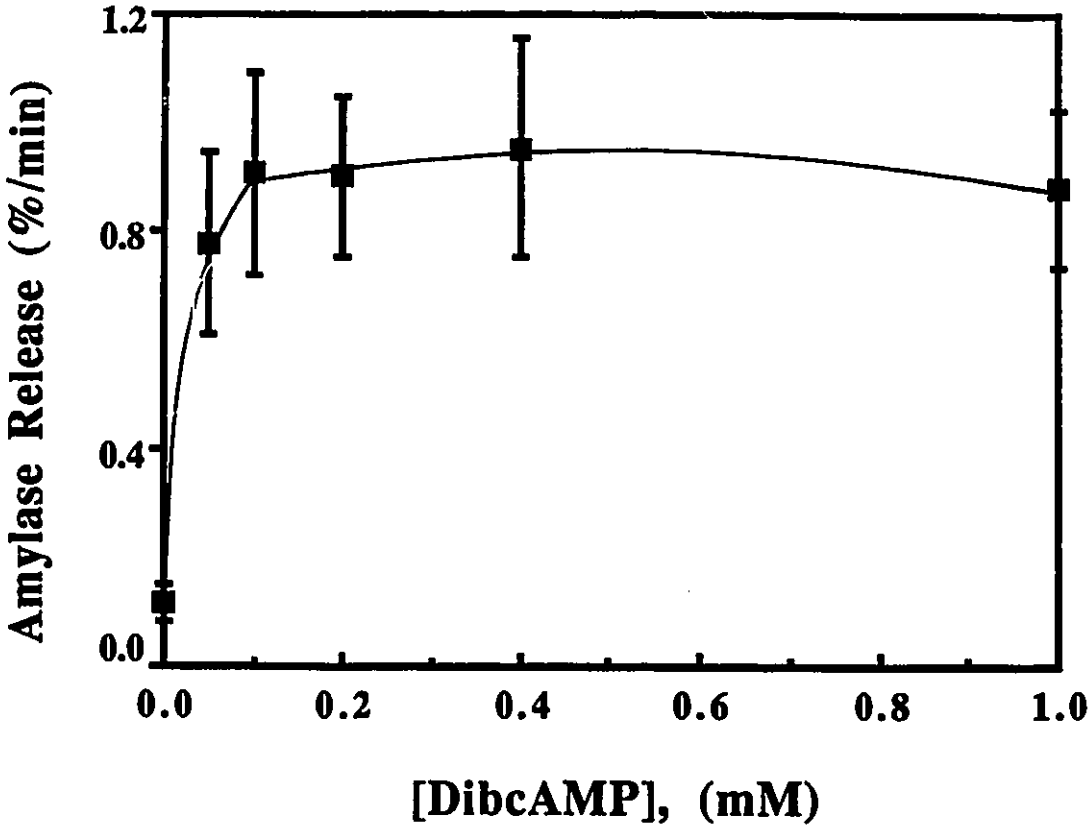

Figure 18

Figure 19. **Amylase release from rat parotid acinar cells in response to phorbol ester (PMA)** The percent of amylase release per min was measured as described (section 2.2.2.) (± SD, n=4).

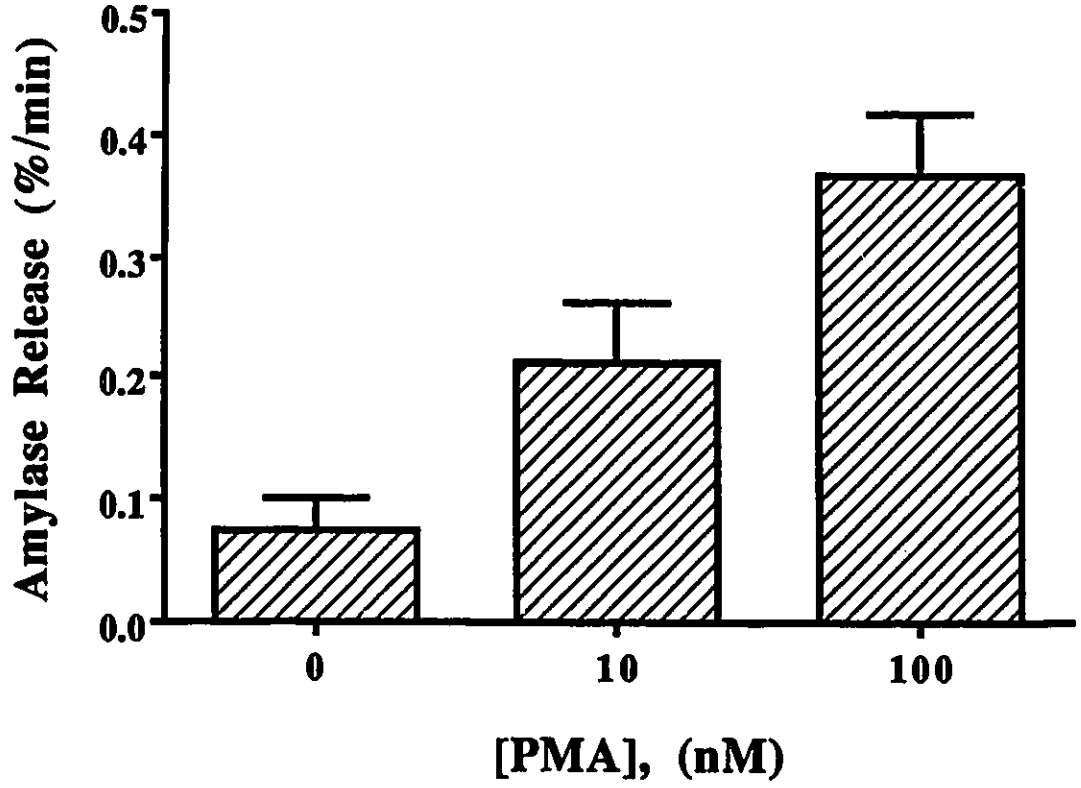

Figure 19

4.4. THE EFFECT OF BOVINE SERUM ALBUMIN ON AMYLASE RELEASE

Since Cornell reported BSA increased translocation of phosphocholine cytidylyltransferase from cytosol to membrane in Hela cells due to its ability to bind DAG (Cornell & Vance, 1987), and my preliminary result also indicated that BSA had unproducible effect on the translocation of PKC–β in dispersed rat parotid cells, BSA was washed away from incubation media just before stimulation. To determine whether medium BSA affected the amylase release evoked by secretagogue, different concentrations of BSA was added to the incubation media. Data from four independent experiments showed that BSA did not alter the rate of amylase release stimulated by ISO or dibutyryl cAMP (Fig. 21–22) .

Figure 20. **Effect of different concentrations of bovine serum albumin (BSA) on amylase release stimulated by isoproterenol from rat parotid acinar cells.** 2 mg/ml BSA (○, ●); 0.5 mg/ml BSA (□, ■); 0 mg/ml (△, ▲). The open symbols represent control and the closed symbols represent 0.1 μM isoproterenol.

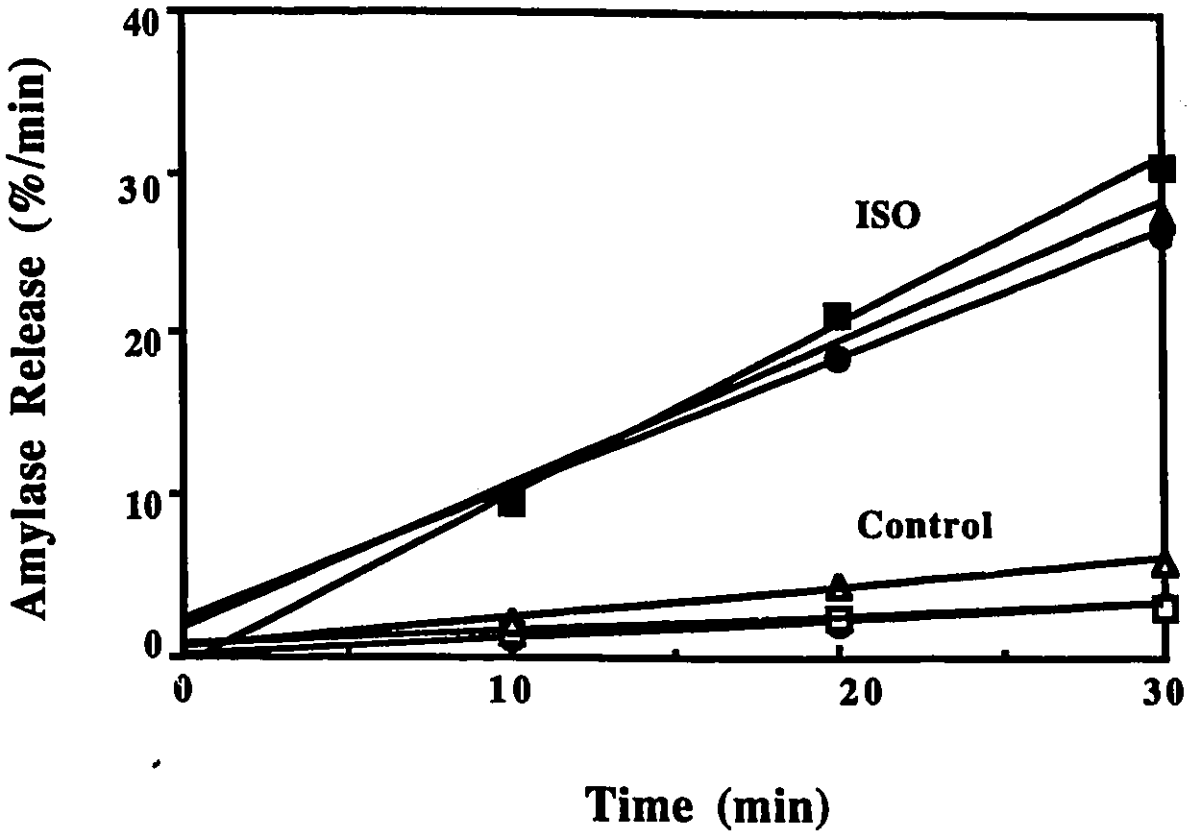
Amylase Release (%/min)
40
30
20
10
0
0
10
20
30
ISO
Control
Time (min)

Figure 20

Figure 21. **Effect of different concentrations of bovine serum albumin (BSA) on amylase release stimulated by dibutyryl cAMP (DibcAMP) from rat parotid acinar cells.** 2 mg/ml BSA (○, ●); 0.5 mg/ml BSA (□, ■); 0 mg/ml BSA (△, ▲). The open symbols represent control and the closed symbols represent 0.4 mM dibutyryl cAMP.

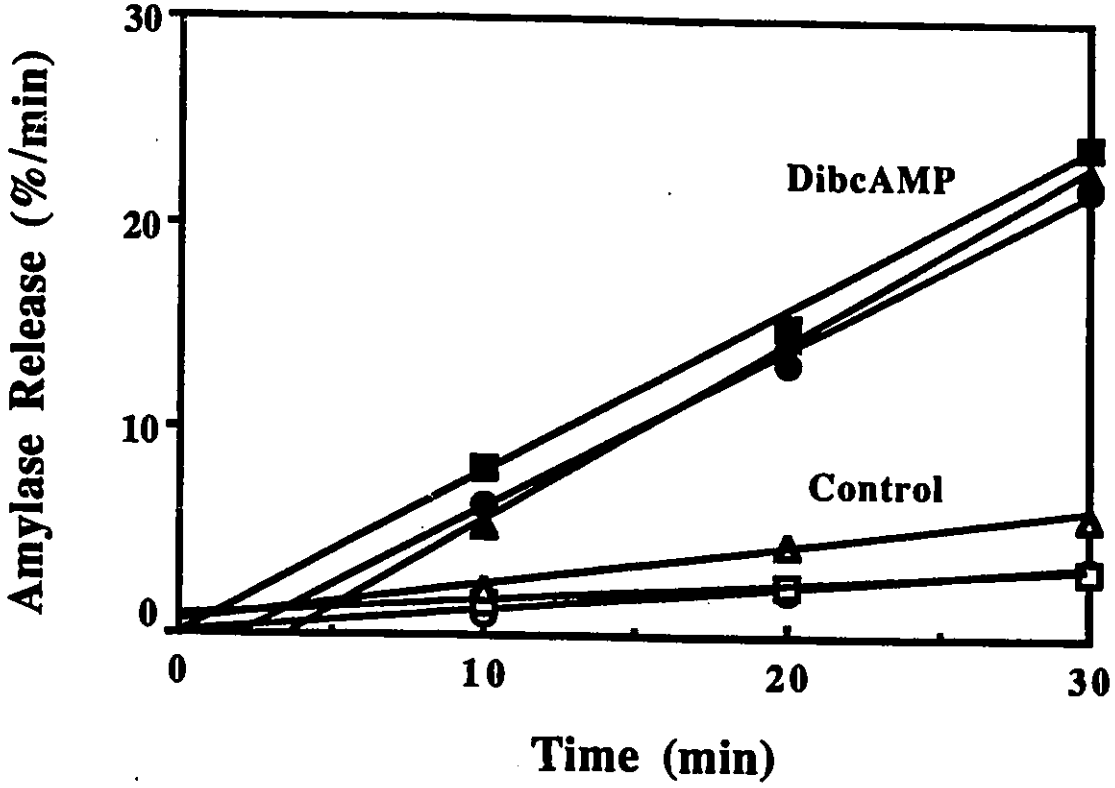
30
20
10
0
Amylase Release (%/min)
DibcAMP
Control
0
10
20
30
Time (min)

Figure 21

5. DISCUSSION

The protein kinase C enzyme family is involved in the transduction of external signals. Molecular cloning and biochemical studies indicate that the members of the PKC family exhibit differential patterns of expression in a variety of cell types and suggest that different isotypes play different roles in the cellular responses to external stimuli (Kikkawa et al, 1989). The results reported here are a exploring study of protein kinase C in rat parotid acini. Hydroxylapatite chromatography suggested that β–isoform was the major form of PKC in rat parotid gland. The presence of PKC–β was verified by Western blotting with an antibody raised to a specific synthetic peptide sequence of PKC–β (Roth et al, 1989). It had high specificity and sensitivity and recognized only the 78–kilodalton form of PKC.

There has been some evidence that some isoforms of PKC have very similar enzymatic properties (Kiley et al, 1990). For example, in clonal GH4C1 rat pituitary cells, it was found that the PKC–β and PKC–ε cochromatographed in the same peak. Therefore, the ability of HTP chromatography to resolve partially purified PKC into its component isozymes should be further re-evaluated. My results indicate that immunoblotting is a specific and sensitive method to determine the presence of PKC isoforms in rat parotid tissue.

Although some chromatographic studies on rat brain and rabbit brain (Jaken & Kiley, 1987; Kiley et al, 1990) show that there are three activities eluted from HTP column, my results from rat brain PKC only showed two peaks instead of three. The possible alternative explanations are that 1) lack of enough prepurification chromatography–DEAE–cellulose, phenyl–Sepharose, Sephacryl S–200, and polylysine–agarose (Huang et al, 1986; Kiley et al,

1990); 2) because PKC–γ is more sensitive to proteolysis, this enzyme might be degraded during tissue processing, since the brain tissue was removed from the animals following the dissection of parotid glands. Moreover, the HTP column used by some other groups (Jaken & Kiley, 1987; Huang et al, 1986; Kiley et al, 1990; Nishizuka, 1988) was from a specific manufacturer that gave the best resolution of kinase activity.

During preliminary experiments, a lot of effort was put into developing feasible protocols to measure translocation of PKC–β after stimulations. It was found eventually that bovine serum albumin (BSA) had an unpredictable effect on the translocation of PKC–β probably due to its ability to bind DAG (Cornell & Vance, 1987). In order to get the exact picture of the real distribution of PKC–β in parotid acinar cells, BSA was washed away from the incubation media just before stimulation. My data showed that omitting BSA at this stage did not affect amylase release stimulated by ISO or dibutyryl cAMP (Fig. 21–22). It is likely that homogenizing the cells in a medium containing BSA lead to post– movement of PKC due to DAG binding to BSA. Based on these preliminary results, a practical protocol was developed to pursue the project, i.e. specifically monitor the translocation of PKC–β in response to stimulation with different secretagogues. As described under "Materials and Methods", a large number of steps were involved in performing Western blotting analysis of all the samples in addition to the non–linear relationship between the film and the signal strength. It was therefore necessary to prepare a standard curve for a standardized analysis of all samples during different experiments. However, this analysis is relatively quantitative and it gave reproducible determination of the change in cytosolic and particulate PKC–β.

Protein phosphorylation stimulated by various protein kinases has been generally recognized to play crucial roles in the regulatory mechanism of diverse cellular functions. Effects of cyclic AMP are also believed to be mediated by the activation of cyclic AMP–dependent protein kinase (PKA) and the subsequent phosphorylation of specific proteins (Greengard, 1978; Krebs & Beavo, 1979). Several studies have reported that PKA was activated when the parotid gland is incubated with a β–adrenergic agonist, and phosphorylation by PKA is suggested to be an essential step for amylase release (Baum et al, 1981; Kanamori & Hayakawa, 1980; Baum et al, 1981; Jahn & Soling, 1981; Spearman et al, 1984; Quissell et al, 1985). It is well known that cyclic AMP generated through beta–adrenoceptor action appears to be a critical modulator of amylase secretion by the rat parotid gland. Although the activation of protein kinase A and the phosphorylation of some proteins are observed concurrently with amylase release evoked by β–adrenergic agonists or cyclic AMP analogues, there is conflicting evidence against direct involvement of cyclic AMP–dependent protein phosphorylation in the exocytosis of amylase. Takuma and coworkers examined the effect of H–8, a membrane–permeable inhibitor of cyclic nucleotide–dependent protein kinases, and PKI–(5–24)–peptide and PKI–(14–24)–amide, peptide fragments of the heat–stable protein kinase inhibitor (PKI), on amylase release and protein phosphorylation (Takuma, 1988). Their results demonstrated that H–8 and PKI–(5–24)–peptide strongly inhibit cyclic AMP–dependent protein phosphorylation in rat parotid cells without affecting cyclic AMP–evoked amylase release. This implies that cyclic AMP–dependent protein phosphorylation is not directly involved in the exocytosis of amylase. On the other hand, Ca^{2+}–mobilizing agonists that exert their actions through muscarinic–, alpha–adrenergic and substance P receptors cause a

predominance of water and electrolyte release (Butcher & Putney, 1980). Activation of this latter pathway takes place via stimulation of phospholipase C and results in the production of inositol 1,4,5–trisphosphate (IP_3) and diacylglycerol. IP_3 mobilizes cellular Ca^{2+} which together with diacylglycerol activates phospholipid–dependent protein kinase C to modulate physiological responses, including secretion (Berridge, 1984; Nishizuka, 1984). Experimentally, phorbol esters or synthetic diacylglycerol derivatives activate PKC by translocating it from cytosol to membrane fraction (Nishizuka, 1984). A previous study conducted on rat parotid slices has shown that phorbol 12,13–dibutyrate (PDBu) modestly stimulates amylase release without activating adenylyl cyclase or Ca^{2+} mobilization (Putney et al, 1984); Recently, Soling's group reported that in rat (Shimomura et al, 1988) and guinea pig (Soling et al, 1987; 1989) parotid glands, phorbol esters and isoproterenol initiated the activation of PKC and stimulation of amylase release. This suggests a role for protein kinase C in salivary amylase secretion. Neither the activity nor the translocation of the specific isozyme(s) of PKC have been determined. Mechanism and relationship between protein kinase C activation and amylase secretion stimulated by β–adrenergic secretagogues has not been established. In the present study, the change of distribution and the activity of PKC–β in response to a range of ISO are depicted in Figure 6–7. Analysis of this dose–response indicated that at relevant concentrations (3 nM to 0.1 μM), there was significant activation of PKC–β during 30 minutes of stimulation, especially 0.1 μM ISO caused rapid translocation of PKC–β from cytosol to membrane which was still not complete at 30 minutes. These values coincide with the time period over which ISO evoked amylase exocytosis constantly. Taken together, these

results reveal that optimal secretory activity of isoproterenol in rat parotid acinar cells is accompanied by protein kinase C activation.

Further insight into the mechanism involved was provided by the finding that 1) the stimulatory action of dibutyryl cAMP was also demonstratable as it (0.4 mM) brought about maximal redistribution of PKC–β and amylase release; 2) PMA stimulated amylase secretion and PKC–β activation to a very similar extent.

This confirmed and supported the concept that cAMP–dependent protein kinase A is not the scle factor in the β–adrenergic mechanism of salivary amylase release. Also, this results agrees with other reports (Takuma & Ichida, 1986; McKinney & Rubin, 1988) that PMA is a potent secretagogue as well as isoproterenol and dibutyryl cAMP. McKinney and Rubin (1988) demonstrated that the amount of amylase released by a combination of phorbol ester and isoproterenol was substantially greater than the sum of the release by the agents given alone. They showed that the enhancement of ISO–evoked amylase release was a function of the phorbol ester concentration. The ISO–induced amylase secretion was abolished in the presence of 1 μM propranoloi, indicating that the effect of ISO was mediated by beta adrenoceptors. They also showed that ISO produced a time–dependent increase in cAMP accumulation over a 15–minutes interval. But phorbol ester failed to elevate cAMP levels above basal values. The conclusion was that phorbol ester caused a potentiation of ISO–stimulated amylase secretion from isolated parotid cells. The effect of phorbol ester on ISO–induced amylase release is attributed to its ability to activate protein kinase C, since the degree of enhancement was related to the phorbol ester concentration and inactive phorbol ester 4α–PDD did not potentiate ISO–induced amylase secretion. Unfortunately, McKinney did not measure the change of diacylglycerol. Some

other previous studies (Dreux et al, 1986; Oron et al, 1978; Spearman et al, 1982; Takemura, 1984; Buatcher & Putney, 1980) found that carbachol potentiated isoproterenol–evoked amylase release, but not the accumulation of cAMP. Recently, Soling demonstrated the early effects (0–120 s) of the β–adrenergic secretagogue, isoproterenol (2×10^{-5} M), on various parameters of lipid and phospholipid metabolism in isolated guinea pig parotid acinar cells (Soling et al, 1987). They found that ISO stimulated DAG increased 2~3 fold over control level and interpreted the finding that ISO did not lead to a significant translocation of protein kinase C from the soluble to the particulate fraction because it stimulated preferentially the formation of 2,3–*sn* –DAG which reportedly does not activate protein kinase C (Nomura et al, 1986; Boni & Rando, 1985; Kando & Young, 1984). It is important to point out that comparing to ours, Soling's method for measuring total activity of protein kinase C is not specific or sensitive enough to show the real change in distribution of isozymes of PKC in guinea pig parotid acinar cells.

Cooper et al (1990) reported that there was a discrepancy among different methods of measuring and purifying PKC from tissues. They found that endogenous DAG remained in equilibrium with cytosolic protein kinase C following fractionation and partial purification by DEAE–Sephacel column chromatography. The traditional method for PKC assay which used Histone IIIs as substrate only measured the activity of total PKC, not any specific form of PKC, and it cannot detect PKC–ε (Schaap & Parker,1990). However, my results with a specific anti–β PKC antibody showed that in rat parotid acinar cells, 0.1 μM ISO caused significant translocation of PKC–β and also stimulated maximum amylase exocytosis. Cook's study suggested that isoproterenol could activate phospholipase C (Cook & Day, 1988). But other studies in rat parotid (Aub & Putney, 1985) and submandibular glands

(Doughney et al, 1987) demonstrated that there was no production of IP_3 observed following ISO stimulation, i.e., ISO did not activate the breakdown of phosphatidylinositol. The species difference and the difference of concentrations of isoproterenol used for stimulation should be taken for consideration when results are compared, because high concentrations of isoproterenol (0.02 mM to 1 mM) activate α–adrenoceptors as well as β–adrenoceptors while low concentration of isoproterenol (0.1 μM) evokes the maximum amylase secretion without any effect on cytosolic Ca^{2+} (Hughes et al, 1989; Tanimura et al, 1990).

Although the source of diacylglycerol was not investigated in the present study, my preliminary results showed that stimulation with low concentration of isoproterenol (0.1 μM) for 10 min caused 2~3 fold increase of 1,2–*sn*– diacylglycerol from basal level of 95.3 pmol/mg (not shown). This increase in DAG coincides with the time–course of PKC–β activation, suggesting that PKC–β could be activated by this increase in 1,2–DAG. Although the source of DAG could not be confirmed in the present study, the magnitude of the change in total DAG is in line with Soling's report in 1989. Therefore, another source of diacylglycerol than PIP_2 breakdown should be considered. For example, Soling showed in guinea pig parotid gland homogenates that cyclic AMP stimulated *de novo* synthesis of DAG. On the other hand, evidence is now accumulating to indicate that other membrane phospholipids are also degraded following cell activation. The evidence is particularly strong for phosphatidylcholine, which, unlike PIP_2, is probably hydrolysed by both a phospholipase C and a phospholipase D (Billah & Anthes, 1990) (Fig. 3). Evidence is now beginning to emerge which suggests that phospholipase D which is present in a number of tissues may have a functional role in signal transduction. Although the demonstration that

phospholipase D can be activated by both a protein kinase C–dependent route and an increase in Ca^{2+} has prompted many investigators to suggest that phospholipase D activation is dependent upon PIP_2 hydrolysis, Ca^{2+} mobilization and protein kinase C activation (Komabayashi et al, 1991; Thompson et al, 1990), there are two reports in which phospholipase D activation can be clearly dissociated from PIP_2 hydrolysis. One is in collagen–stimulated platelets treated with aspirin to inhibit thromboxane formation. In these cells phospholipase D activation occurs in the absence of significant PIP_2 hydrolysis (Narita et al, 1985; Randall et al, 1990). The other is in epidermal growth factor (EGF)–stimulated Swiss 3T3 fibroblasts, in which phospholipase D activation occurs in the absence of IP_3 formation. Inhibiting the tyrosine kinase activity of the EGF receptor blocks the activation of phospholipase D, suggesting that phospholipase D activation is mediated by tyrosine phosphorylation in these cells (Thompson et al, 1991). Although data from other cell lines might not be appropriate for interpreting the experimental phenomenon in an exocrine system, it is becoming clear that phospholipase D may bind to receptors by several different mechanisms and that these may vary among different cell types or between different receptors in the same cell. Hydrolysis of phosphatidylcholine can clearly provide an alternative source of diacylglycerol, and this pool may not activate protein kinase C in the same way as that derived from PIP_2 hydrolysis. Leach (1991) have demonstrated that in intact, thrombin–stimulated IIC–9 fibroblasts, diacylglycerol derived from phosphatidylcholine does not translocate the α–isoform of protein kinase C. Also, it does not stimulate phosphorylation of the 80 kDa endogenous protein kinase C substrate in the same way as diacylglycerol derived from PIP_2. However, a PKC activity from these cells can be activated *in vitro* by phosphatidylcholine–derived diacylglycerol extracted from the same cells. One

possible explanation for this is that the predominant diacylglycerol species derived from phosphatidylcholine may preferentially activate a Ca^{2+}–independent isoform of protein kinase C (beta, zeta, epsilon or deta) for which the 80 kDa protein may not be a physiological substrate in intact cells. There is now clear evidence that phorbol ester PMA can stimulate the breakdown of phosphatidylcholine (Thompson et al, 1991). We do not yet know whether isoproterenol or cAMP can activate phospholipase D and stimulate the formation of diacylglycerol in rat parotid gland. Further experiments are needed to investigate the sources of diacylglycerol in this bifurcating signal transduction pathway.

Figure 23 summarizes what my findings suggest:

1) protein kinase C–β and cAMP are sequential cellular signals for exocytosis;

2) the β–adrenergic secretagogue activates PKC, leading to amylase secretion in rat parotid cells, through increases in cAMP levels and activation of protein kinase A.

The conclusion is that in rat parotid gland, activation of PKC–β could be a common pathway for stimulus–secretion coupling in response to both cAMP–generating and Ca^{2+}–mobilizing secretagogues.

Figure 22. **Schematic diagram of apparent roles played by β protein kinase C and protein kinase A mediating the biological effects of secretagogues which act through cyclic AMP and diacylglycerol in stimulus–secretion coupling in rat parotid gland.** Several pathways exist in rat parotid gland: β–adrenergic agonists (ISO) activate adenylyl cyclase via G protein to produce cAMP, which activates protein kinase A to cause protein phosphorylation and bring about its physiological responses. One of these is an increase in DAG, which activates protein kinase C. Muscarinic–cholinergic, α–adrenergic and substance P receptor stimulation cause phosphatidylinositol turnover leading to the formation of DAG and activation of protein kinase C. The tumour promoter PMA activates and binds to protein kinase C directly. All events that activate PKC bring about protein secretion, whereas elevation of cellular Ca^{2+} levels leads to fluid and ion secretion through opening of ion channels.

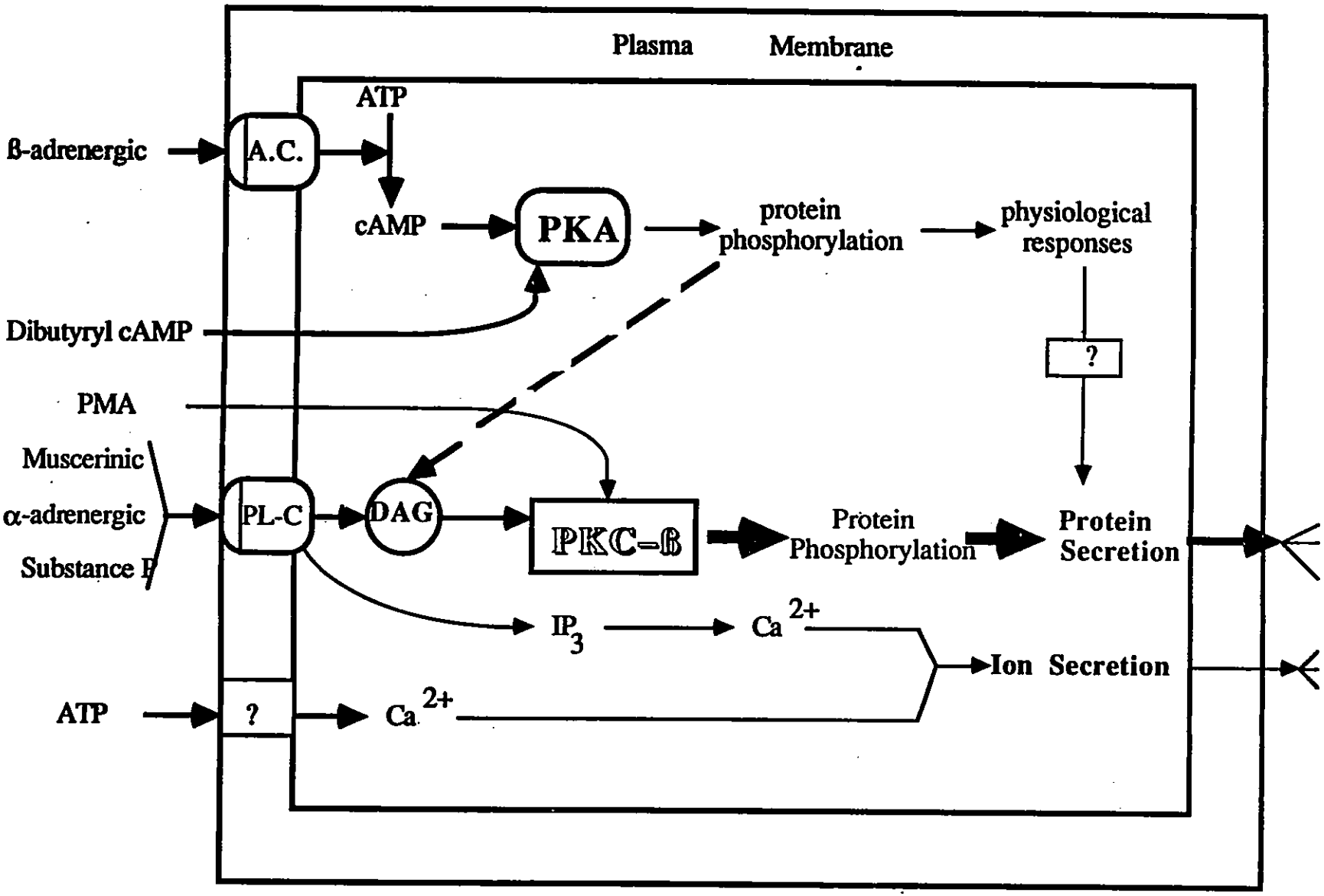
Plasma Membrane
ATP
ß-adrenergic
A.C.
cAMP
PKA
protein phosphorylation
physiological responses
Dibutyryl cAMP
?
PMA
Muscerinic
α-adrenergic
Substance P
PL-C
DAG
PKC-ß
Protein Phosphorylation
Protein Secretion
IP3
Ca 2+
Ion Secretion
ATP
?
Ca 2+

Figure 22

6. REFERENCES

Agwn, D., Mcphail, L. C., Chabot, M. C., Daniel, L. W., Wykle, R. L., McCall, C. E. (1989) Choline–linked phosphoglycerides: A sources of phosphatidic acid and diaglycerides in stimulated neutrophils. J. Biol. Chem. 264: 1405–1413.

AL-Hadithi, B. A.K., Stauber, V., and Mitchell, J. (1988) The co-localisation of substance P and VIP in cholinergic-type terminals of the rat parotid gland. 159: 83-92.

Amsterdam, A., Ohad, I., Schramm, M. (1969) Dynamic changes of the ultrastructure of the acinar cell of the rat parotid gland during the secretory cycle. J. Cell. Biol. 41: 753–773.

Ase, K., Saito, N., Shearman, M.S., Kikkawa, U., Oho, Y., Igarashi, K., Tanaka, C., and Nishizuka, Y. (1988) Distinct cellular expression of beta–1–subspecies and beta–2–subspecies of protein kinase C in rat cerebellum. J. Neurosci. 8: 3580–3856.

Aub, D. L., Putney, J. W. (1985) Properties of receptor controlled inositol trisphosphate formation in parotid acinar cells. Biochem. J. 225: 263–266.

Augert, G., Blackmore, P. F., Exton, J. H. (1989) Changes in the concentration and fatty acid composition of phosphoinositides induces by hormones in hepatocytes. J. Biol. Chem. 264: 2574–2580.

Ballester, R., Rosen, O. M. (1985) Fate of immunoprecipitable protein kinase C in GH_3 cells treated with 12–myristate 13–acetate. J. Biol. Chem. 260: 15194–15199.

Baum, B. J. (1987) Neurotransmitter control of secretion. J. Dent Res. 66: 628–632.

Baum, B. J., Colpo, F.T., and Filburn, C. R. (1981) Characterization and relationship to exocrine secretion of rat parotid gland cyclic AMP–dependent protein kinase. Arch. Oral Biol. 26: 333–337.

Baum, B. J., Freiberg, J.M. Ito, H., Roth, G. S., Filburn, C. R. (1981) Beta–adrenergic regulation of protein–phosphorylation and its relationship to exocrine secretion in dispersed rat parotid gland acinar cells. J. Biol. Chem. 256: 9731–9736.

Berridge, M. J. (1984) Inositol trisphosphate and diacylglycerol as second messengers. Biochem. J. 220: 345–360.

Besterman, J. M., Duronio, V., and Cuatrecasas, P. (1986) Rapid formation of diacylglycerol from phosphatidylcholine: A pathway for generation for a second messenger. Proc. Natl. Acad. Sci. USA. 83: 6785–6789.

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

Bloom, G. D., Carlsoo, B., Danielss, A., Gustafss, H., Henrikss, R. (1979) Quantitative structural–analysis and the secretory behavior of the rat parotid gland after long and short–term isoproterenol treatment. Med. Biol. 57: 224–233.

Bocckino, S. B., Blackmore, P. F., and Exton, J. H. (1985) Stimulation of 1,2–diacylglycerol accumulation in hepatocytes by vasopressin, epinephrine and angiotensin–II. J. Biol. Chem. 260: 4201–4207.

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

Brandt, S. J., Niedel, J. E., Bell, R. M., and Scott Young, W. (1987) Distinct patterns of expression of different protein kinase C mRNA in rat tissues. Cell. 49: 57–63.

Burnstock, G., Kennedy, C.(1985) Is there a basis for distinguishing 2 types of P_2–purinoceptor. Gen. Pharmacol. 16: 433–440.

Burnstock, G. (1981) Neurotransmitters and trophic factors in the automatic nervous system. J. Physiol. 313: 1–35.

Butcher, F. R., and Putney, J. W. (1980) Regulation of parotid gland function by cyclic–nucleotides and calcium. Adv. Cyclic Nucleotide Res. 13: 215–249.

Cabot, M. C., Welsh, C. J., Zhang, Z. C., Cao, H. M., Chabbott, H., Lebowitz, M. (1985) Vasopressin, phorbol diesters and serum elicit choline glycerophospholipid hydrolysis and diacylglycerol formation in nontransformed cells: transformed derivatives do not respond. Biochim. Biophys. Acta. 959: 46–57.

Casperson, G. F., and Bourne, H. R. (1987) Biochemical and molecular genetic analysis of hormone–sensitive adenylyl cyclase. Ann. Rev. Pharmacol. 27: 371–384.

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

Cerione, R. A., Gierschik, P., Slanizewski, C., Benoric, J. L., Codina, J., Somers, R., Birnbaumer, L., Spiegel, A. M., Lefkowitz, R. J., and Caron, M. G. (1987) Functional differences in the βγ complexes of transducin and the inhibitory guanine nucleotide regulatory protein. Biochem. 26: 1485–1491.

Chakravarthy, B. R. (1989) A novel method for measuring protein kinase C activity in a native membrane–associated state. Biochem. Biophys. Res. Commun. 160: 340–345.

Charest, R., Prpic, V., Exton, J. H., and Blackmore, P. F. (1985) Stimulation of inositol trisphosphate formation in hepatocytes by vasopressin, epinephrine and angiotensin–II and its relationship to changes in cytosolic free Ca^{2+}. Biochem. J. 227: 79–90.

Citri, Y., and Schramm, M. (1980) Resolution, reconstitution and kinetics of the primary action of a hormone receptor. Nature. 287: 297–300.

Cockroft, S. (1987) Polyphosphoinositide phosphodiesterase–regulation by a novel guanine–nucleotide binding protein–G_p. Trends Biochem. 12: 75–78.

Connolly, T. M., Lawing, W. J., and Majerus, P. W. (1986) Protein kinase C phosphorylates human–platelet inositol trisphosphate 5'–phosphomonoesterase, increasing the phosphatase activity. Cell. 46: 951–958.

Cornell, R. B., and Vance D. E. (1987) Translocation of CTP–phosphocholine cytidyltransferase from cytosol to membranes in Hela cells–stimulation by fatty acid, fatty alcohol, monoacyglycerol and diacylglycerol. Biochem. Biophys. Acta. 919: 26–36.

Cook, D. I., and Day, M. L. (1988) Ca^{2+} not cyclic AMP mediates the fluid secretory response to isoproterenol in rat mandibular salivary gland: whole cell patch–clamp studies. Eurp. J. Physiol. 413: 67–76.

Cooper, D. R., Ishizuka, T., Watlon, J. E., Standaernal, Mair, G., Farese, R. V. (1990) Protein–kinase C activation patterns are determined by methodological variations, studies of insulin action in BC3H–1 myocytes and rat adipose–tissue. Biochim. Biophys. Acta. 1054: 95–102.

Cooper, D. R., Konda, T. S., Standaert, M. L., Davis, J. S., Pollet, R. J., and Forese, R. V. (1987) Insulin increases membranes and cytosolic protein kinase C activity in BC3H–1 myocytes. J. Biol. Chem. 262: 3633–3639.

Coussens, L., Parker, P. J., Rhee, L., Yang–Feng, T. L., Chen, E., Waterfield, M. D., Francke, U., and Ullrich, A. (1986) Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. Science. 233: 859–866.

Daniel, L. W., Waite, M., Wykle, R. (1986) A novel mechanism of diacylglyceride formation: 12–O–tetradecanoglphorbol–13–acetate stimulates the cycle breakdown and resynthesis of phosphotidylcholine. J. Biol. Chem. 261: 9128–9132.

Domenech, M–D. & Soling, H. D. (1987) Effects of stimulation of muscarinic and of β–catecholamine receptors on the intracellular distribution of protein kinase C in guinea pig exocrine glands. Biochem. J. 242: 749–754.

Dougheny, C., Dormer, R. L., and Mcpherson, M. A. (1987) Adrenergic regulation of formation of inositol phosphates in rat submandibular acini. Biochem. J. 241: 705–709.

Draznin, B., Leitner, J. W., Sussman, K. E., and sherman, N. A. (1988) Insulin and glucose modulate protein kinase C activity in rat adipocytes. Biochem. Biophys. Res. Commun. 156: 570–575.

Dreux, C., Imhoff, V., Huleux, C., Busson, S., and Rossignol B, Forskolin. (1986) A tool for rat parotid secretion studies: ^{45}Ca efflux is not related to cAMP. AM. J. Physiol. 251: 754–762.

Drust, D. S., Martin, T. F. J. (1985) Protein kinase C translocates from cytosol to membrane upon hormone activation, effects of thyrotropin–

releasing hormone in GH3 cells. Biochem. Biophys. Res. Commun. 128, 531–537.

Egan, J. J., Saltis, J., Wek, S. A., Simpson, I. A., Londons, C. (1990) Insulin, oxytocin, and vasopressin stimulate protein kinase C activity in rat adipocytes plasma membranes. Proc. Natl. Acad. Sci. USA. 87: 1052–1056.

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

Fearon, C. W.m and Tashijian, A. H. (1985) Thyrotropin–releasing hormone induces redistribution of protein kinase C in GH_4C_1 rat pituitary cells. J. Biol. Chem. 260: 8366–8371.

Fearon, C. W., and Tashijian, A. H. (1987) Ionomycin inhibits thyrotropin–releasing hormone–induced translocation of protein kinase C in GH_4C_1 pituitary cells. J. Biol. Chem. 262: 9515–9520.

Gallacher, D. V. (1982) Are there purinergic receptors on parotid acinar cells. Nature. 296: 83–86.

Gilman, A. G. (1987) G–proteins–transducers of receptor–generated signals. Annu. Rev. Biochem. 56: 615–649.

Glynn, B. P., Colliton, J. W., Mcdermot, J. M., Witlers, L. A. (1986) Phorbol esters, but not insulin, promote depletion of cytosolic protein kinase C in rat adipocytes. Biochem. Biophys. Res. Commun. 135: 1119–1125.

Go, M., Sekiguch, K., Nomura, H., Kikkawa, U., and Nishizuka, Y. (1987) Further studies on the specificity of diacylglycerol for protein kinase C activation. Biochem. Biophys. Res. Commun. 144: 598–605.

Gordon, J. L. (1986) Extracellular ATP: effects, sources and fate. Biochem. J. 233: 309–319.

Greengard, P. (1978) Phosphorylated proteins as physiological effectors. Science. 199: 146–152.

Griendling, K. K., Rittenho, S. E., Brock, T. A., Gimbrone, M. A (1986) Sustained diacylglycerol formation from inositol phospholipids in angiotensin–II–stimulated rascular smooth–muscle cells. J. Biol. Chem. 261: 5901–5906.

Habenicht, A. J. R., Glomset, J. A., King, W. C., Nist, C., and Michell, C. D. (1981) Early changes in phosphatidylinositol and arachidonic acid metabolism in Guiescent Swiss 3T3 cells stimulated to divide by platelet–derived growth factor. J. Biol. Chem. 256: 2329–2335.

Halsey, D. L., Girard, P. R., Kuo, J. F., Blackshear, P. J. (1987) Protein kinase C in fibroblasts. J. Biol. Chem. 262: 2234–2243.

Hashimoto, T., Ase, K., Sawamura, S., Kikkawa, U., Saito, N., Tanaka, C., and Nishizuka, Y. (1988) Postnatal development of a brain–specific subspecies of protein kinase C in rat. J. Neurosci. 8: 1678–1683.

Hashimoto, E. (1988) Ionic strength–dependent proteolytic activation of protein kinase C by trypsin–like protease. J. Biochem. 104: 934–938.

Hashimoto, E. (1990) Mechanism of proteolytic activation of rat liver protein kinase C generating Ca^{2+}– phospholipid–independent form with apparent molecular mass of 80,000. Int. J. Biochem. 22: 865–871.

Hidaka, H., Tanaka, T., Onoda, K., Hagiwara, M., Watenabe, M., Ohta, H., Ito, Y., Tsurudome, M., and Yoshida, T. (1988) Cell type–specific expression of protein kinase C isozymes in rabbit cerebellum. J. Biol. Chem. 263: 4523–4526.

Hincke, M. T. (1988) The inhibition of cyclic AMP–mediated exocytosis from rat parotid cells by calcium chelation is due to depression of cellular ATP. Biochim. Biophys. Acta. 967: 204–210.

Hincke, M. T. (1988) Characterization of rat parotid protein kinase C. Int. J. Biol. 20: 303–307.

Hirota, K., Hirota, T., Aquilera, G., and Catt, K. J. (1985) Hormone–induced redistribution of calcium–activated phospholipid–dependent protein kinase in pituitary gonadotrophys. J. Biol. Chem. 260: 3243–3246.

Hokin, M. R., and Hokin, L. E. (1953) Enzyme secretion and the incorporation of p^{32} into phospholipids of pancreas slices. J. Biol. Chem. 203: 967–977.

Holbrook, S. R., and Kim, S–H. (1989) Molecular model of the G protein α subunit based on the crystal structure of the HRAS protein. Proc. Natl. Acad. Sci. USA. 86: 1751–1755.

Horn, V., Baum, B. J., and Ambudkar, I. S. (1988) β–adrenergic stimulation induces inositol trisphosphate production and Ca^{2+} mobilization in rat parotid acinar cells. J. Biol. Chem. 263: 12454–12460.

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

Hosoda, K., Saito, N.m Kose, A., Tsujino, T., Ogita, K., Kikkawa, U., and Ono, Y. (1989) Immunocytochemical localization of the βI subspecies of protein kinase in rat brain. Proc. Natl. Acad. Sci. USA. 86: 1393–1397.

House, C., and Kemp, B. E. (1987) Protein kinase C contains a pseudosubstrate prototype in its regulatory domain. Science. 238: 1726–1728.

Huang, K. P., Nakabayashi, H., and Huang, F. L. (1986) Isozymic forms of rat brain Ca^{2+}–activated and phospholipid–dependent protein kinase. Proc. Natl. Acad. Sci. USA. 83: 8535–8539.

Huang, K. P., Chan, K. F. J., Singh, T. J., Nakabaya, H., and Huang, F. L. (1986) Autophosphorylation of rat brain Ca^{2+}–activated and phospholipid–dependent protein kinase. J. Biol. Chem. 261: 2134–2140.

Huang, K. P., and Huang, F. L. (1986) Conversion of protein kinase C from a Ca^{2+}–dependent to an independent form of phorbol ester–binding protein by digestion with trypsin. Biochem. Biophys. Res. Commun. 139: 320–326.

Huang, F. L. (1989) Differential down–regulation of protein kinase C isozymes. J. Biol. Chem. 264: 4238–4243.

Huang, F. L., Yoshida, Y., Nakabaya, 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.

Hughes, A. R., Takemura, H., and Putney, J. W. (1989) Does β–adrenoceptor activation stimulate Ca^{2+} mobilization and inositol trisphosphate formation in parotid acinar cells ? Cell Calcium. 10: 519–525.

Inoue, M. (1977) Studies on a cyclic nucleotide–independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium–dependent protease from rat brain. J. Biol Chem. 252: 7610–7616.

Irving, H. R., and Exton, J. H. (1987) Phosphatidylcholine breakdown in rat liver plasma membrane–roles of guanine nucleotides and P_2–purinergic agonists. J. Biol. Chem. 262: 3440–3443.

Ito, K., Saito, N., Hirata, M., Kose, A., Tsujino, T., and Yoshihara, C. (1990) Immunocytochemical localization of the α subspecies of protein kinase C in rat brain. Proc. Natl. Acad. Sci. USA. 87: 3195–3199.

Jaken, S. (1975) Initial events in phorbol ester binding to GH_4C_1 rat pituitary cells. Endocrinol. 117: 2293–2300.

Jaken, S. (1985) Increased diacylglycerol content with phospholipase C or hormone treatment: inhibition of phorbol ester binding and induction of phorbol ester–like biological responses. Endocrinol. 117: 2301–2306.

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.

Jahn, R., and Soling, H. D. (1981) Phosphorylation of the same specific protein during amylase release evoked by beta–adrenergic or cholinergic agonists in rat and mouse parotid glands. Proc. Natl. Acad. Sci. USA. 78: 6903–6906.

Jung, D. H. (1980) Preparation and application of procion yellow starch for amylase assay. Clinica Chimica Acta. 100: 7–11.

Kanamori, T., and Hayakawa, T. (1980) Cyclic AMP–dependent P^{32} incorporation into a protein in rat parotid slides. Biochem. Int. 1: 395–402.

Kando, R. R., and Young, N. (1984) The stereospecific activation of protein kinase C. Biochem. Biophys. Res. Commun. 122: 818–823.

Kasaka, Y., Ogita, K., Ase, K., Nomura, H., Kikkawa, U., and Nishizuka, Y. (1988) The heterogeneity of protein kinase C in various rat tissues. Biochem. Biophys. Res. Commun. 151: 973–981.

Kikkawa, O., Ogita, K., Ono, Y., and Asaoka, Y. (1987) The common structure and activities of 4 subspecies of rat brain protein kinase C family. FEBS Letter. 223: 212–216.

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

Kikkawa, U. (1986) The role of protein kinase C in transmembrane signaling. Annu. Rev. Cell. Biol. 2: 149–178.

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

Kiley, S., Schaap, D., Parker, P., Hsieh, L. L., and Jaken, S. (1990) Protein kinase C heterogeneity in GH4C1 rat pituitary cells. J. Biol. Chem. 265: 15704–15712.

Kishimoto, A. (1983) Proteolytic activation of calcium–activated phospholipid–dependent protein kinase by calcium–dependent neutral protenase. J. Biol.Chem. 258: 1156–1164.

Knopf, J. L., Lee, M–H., Sultzman, L. A., Kriz, 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.

Komabayashi, T., Yakata, A., Izawa, T., Suda, K., Rubin, R. P., and Tsuboi, M. (1991) Protein kinase C–dependent diacylglycerol formation is mediated via Ca^{2+}/calmodulin in parotid cells. Mol. Pharmacol. 207: 175–181.

Kraft, A. S., and Anderson, W. B. (1983) Phorbol esters increase the amount of Ca^{2+}, phospholipid–dependent kinase associated with plasma membrane. Nature. 301: 621–623.

Kraft, A., Anderson, W. B., Cooper, H. L., and Sando, J. J. (1982) Decrease in cytosolic calcium/phospholipid–dependent protein kinase activity following phorbol ester treatment of EL4 thynoma cells. J. Biol. Chem. 257: 13193–13196.

Kraft, A. S., Anderson, W. B. (1983) Kinase activity following phorbol ester treatment of EL4 thynoma cells. Nature. 301: 621–623.

Krebs, E. G. and Beavo, J. A. (1979) Phosphorylation–dephosphorylation of enzymes. Annu. Rev. Biochem. 48: 923–959.

Kubo, K., Ohno, S., and Suzuki, K. (1987) Primary structure of human protein kinase C beta–I and beta–II differ only in their C–terminal sequences. FEBS Lett. 223: 138–142.

Kutchai, H. C. (1990) Gastrointestinal system in *Principles of physiology* (Berne, R. M., Levy, M. N., eds), The C. V. Mosby Com. press, 374–379.

Leach, K. L., James, M. L., Blumbery, P. M. (1983) Characterization of a specific phorbol ester aporeceptor in mouse brain cytosol. Proc. Natl. Acad. Sci. USA. 80: 4208–4212.

Leach, K. L., Ruff, V. A., Wright, T. M., Pessin, M. S. and Raben, D. M. (1991) Dissociation of protein kinase C activation and *sn* –1,2–diacylglycerol formation. J. Biol. Chem. 266: 3215–3221.

Lee, M. H., and Bell R. M.(1986) The lipid binding, regulatory domain of protein kinase C. A 32–kDa fragment contains the calcium– and phosphatidylserine–dependent phorbol ester binding activity. J. Biol. Chem. 261: 14867–14870.

Levitzki, A. (1988) From epinephrine to cyclic–AMP. Science. 241: 800–806.

Liscovitch, M., Blusztaj, J. K., Frese, A., and Wurtman, B. J. (1987) Stimulation of choline release from NG–108–15 cells by 12–O–tetradecancylphorbol 13–acetate. Biochem. J. 241: 81–86.

Lundberg, J. M., and Hokfelt, T. (1983) Coexistence of peptides and classical neurotransmitters. Trends in Neurosci. 6: 325–333.

Lundberg, J. M., Lacroix, S., Martling, C. R., Matran, R. (1988) Peptides and classical transmitters in autonomic control of airway function. Regul. Pept. 22: 417–420.

Mangos, J. A., Mucsherry, N. R., Butcher, F., Irwin, K., and barber, T. (1975) Dispersed rat parotid acinar cells. 1. Morphological and functional characterization. Am. J. Physol. 229: 553–559.

Matozaki, J., Sakamoto, C., Nagao, M., Baba, S (1986) Phorbol ester or diacylglycerol modulates somatostatin binding to its receptor on rat pancreatic acinar cell membranes. J. Biol. Chem. 261: 1414–1420.

Matozaki, J., and Williams, J. A. (1989) Multiple sources of 1,2–diacylglycerol in isolated rat pancreatic acini stimulated by cholecystokinin–involvement of phosphatidylinositol biphosphate and phosphotidylcholine hydrolysis. J. Biol. Chem. 264: 4729–4734.

McCaffrey, P. G., Friedman, B. and Rich Rosner, M. (1984) Diacylglycerol modulates binding and phosphorylation of the epidermal growth factor receptor. J. Biol. Chem. 259: 12502–12507.

McKinney, J. S., and Rubin, R. P. (1988) Enhancement of cyclic AMP modulated salivary amylase secretion by protein kinase C activations. Biochem. Pharmacol. 37: 4433–4438.

McMillian, M. K., Soltoff, S. P., Lechlecter, J. D., Cantley, L. C., and Talamd, B. R. (1988) Extracellular ATP increases free cytosolic calcium in rat parotid acinar cells. Biochem. J. 255: 291–300.

Melloni, E., Pontremo, S., Michetti, M., Sacco, O., Sparator, B., Horecher, B. L. (1986) The involvement of calpain in the activation of protein kinase C in neutrophils stimulated by phorbol myristic acid. J. Biol. Chem. 261: 4101–4105.

Melloni, E. (1985) Binding of protein kinase C to neutrophil membrane in the presence of Ca^{2+} and its activation by a Ca^{2+} requiring protenase. Proc. Natl. Acad. Sci. USA. 82: 6435–6439.

Mufson, R. A., Okin, E., Weinstei, I. B. (1981) Phorbol esters stimulates the rapid release of choline from free labeled cells. Carcenogenesis. 2: 1095–1102.

Muir, J. G., and Murray, A. W. (1987) Bombesin and phorbol ester stimulate phosphotidylcholine hydrolysis by phospholipase C–evidence for a role of protein kinase C. J. Cell. Physiol. 130: 382–391.

Naor, Z. (1989) Induction of exocytosis in permeabilized pituitary cells by α– and β– type protein kinase C. Proc. Natl. Acad. Sci. USA. 86: 4501–4504.

Narita, H., Park, H. J., Tanaka, K., Matsuura, T., and Kito, M. (1985) Insufficient mobilizing of calcium by early breakdown of phosphatidylinositol–4,5–bisphosphate for aggregation of human platelets by collagen. J. Biochem. 98: 1063–1068.

Neer, E. J. & Clapham, D. E. (1988) Roles of G protein subunits in transmembrane signalling. Nature. 333: 129–134.

Niedel, J. E., James, M. L., Blumberg, P. M. (1983) Phorbol diester receptor copurifies with protein kinase C. Proc. Natl. Acad. Sci. USA. 80: 36–40.

Nishizuka, Y. (1983) Calcium, phospholipid turnover and transmembrane signaling. Phil. Trans. Roy. Soc. Lond. 302: 101–112.

Nishizuka, Y. (1984) Turnover of inositol phospholipids and signal transduction. Science. 225: 1365–1370.

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 properties of protein kinase C. Science. 233: 305–312.

Nishizuka, Y. (1988) The molecular heterogeneity of protein kinase C and its implication of cellular regulation. Nature. 334: 661–665.

Nomura, H., Ase, K., Sekiguchi, K., Kikkawa, U., and Nishizuka, Y. (1986) Stereospecificity of diacylglycerol for stimulus–response coupling in platelets. Biochem. Biophys. Res. Commun. 140: 1143–1151.

Ohno, S., Kawasaki, H., Imajoh, S., and Suzuki, K. (1987) Tissue–specific expression of three distinct types of rabbit protein kinase C. Nature. 325: 161–166.

Ohno, S., Kawasaki, H., Konno, Y., Inagaki, M., Hidaka, H., and Suzuki, K. (1988) A fourth type of rabbit protein kinase C. Biochem. 27: 2083–2087.

Ono, Y., Kikkawa, U., Ogita, K., Fuji, T., Kurokawa, T., Asaoka, Y., Sekiguek, K., Ase, K., Igarashi, K., and Nishizuka, Y. (1987) Expression and properties of 2 types of protein kinase C alternative splicing from a single gene. Science. 236: 1116–1120.

Ono, Y., Fuji, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. (1987) Identification of three additional members of rat protein kinase C family: zeta, epsilon and sigma 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 member of the protein kinase C family. J. Biol. Chem. 263: 6927–2932.

Oron, Y., Kellogy, J., and Larner, J. (1978) Alpha adrenergic and cholinergic–muscarinic regulation of adenosine cyclic 3', 5'–monophosphate levels in the rat parotid. Mol. Pharmacol. 14: 1018–1030.

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

Pandol, S. J. and Schoeffield, M. S. (1986) 1,2–diacylglycerol, protein kinase C, and pancreatic enzyme secretion. J. Biol. Chem. 261: 4438–4444.

Patton, G. M., Fasulo, J. M., and Robins, S. J. (1982) Separation of phospholipids and individual molecular species of phospholipids by high–performance liquid chromatography. J. Lipid Res. 23: 190–196.

Pelech, S. L., Meier, K. M., Krebs, E. G. (1986) Rapid microassay for protein kinase C translocation in Swiss 3T3 cells. Biochem. 25: 8348–8353.

Pelech, S. L. & Vance, D. E. (1989) Signal transduction via phosphatidylcholine cycles. Trends Biochem. 14: 28–30.

Pelosin, J. M., Ricouart, A., Sergheraert, C., Benahmed, M., and Chambaz, E. M. (1991) Expression of protein kinase C isoforms in various steroidogenic cell types. Mole. Cell. Endocrinol. 75: 149–155.

Pessin, M. S., Raben, D. M. (1989) Molecular species analysis of 1,2–diaglycerides stimulated by α–thrombin in cultured fibroblasts. J. Biol. Chem. 264: 8727–8738.

Putney, J. W. (1986) Identification of cellular activation mechanisms associated with salivary secretion. Ann. Rev. Physiol. 48: 75–88.

Putney, J. W., Weiss, S. J., Leslie, B. A., and Marier, S. H. (1977) Is calcium final mediator of exocytosis in rat parotid gland. J. Pharm. Exp. 203: 144–155.

Putney, J. W. (1978) Role of cellular calcium in response of parotid to dibutyryl and monobutyryl cyclic–AMP. Life Sci. 22: 631–638.

Putney, J. W. (1976) Stimulation of calcium influx in rat parotid gland by carbachol. J. Pharmacol. Exp. 199: 526–537.

Putney, J. W., McKinney, J. S., Aub, D. L., Leslie, B. A. (1984) Phorbol ester–induced protein secretion in rat parotid gland. Mol. Pharmacol. 26: 261–266.

Quisell, D. O., Deisher, L. M., and Brazen, K. A. (1985) The rate–determining step in CAMP–mediated exocytosis in the rat parotid and submandibular glands appears to involve analogous 26–kDa integral membrane phosphoproteins. Proc. Natl. Acad. Sci. USA. 82: 3237–3241.

Randall, R. W., Bonser, R. W., Thompson, N. T., and Garland, L. G. (1990) A novel and sensitive assay for phospholipase D in intact cells. FEBS Lett. 264: 87–90.

Richardson, P. J. and Brown, S. J. (1987) ATP release from affinity–purified rat cholinergic nerve terminals. J. Neurochem. 48: 622–630.

Rodbell, M. (1980) The role of hormone receptors and GTP–regulatory proteins in membrane transduction. Nature. 284: 17–22.

Rodbell, M., Birnbaumer, L., Pohl, S. L., and Krans, H. M. (1971) The glucagen–sensitive adenylyl cyclase system in plasma membrane of rat liver. J. Biol. Chem. 246: 1877–1882.

Rodriguez–Pena, A., and Rozengurt, E. (1984) Disappearance of Ca^{2+}–sensitive, phospholipid–dependent protein kinase acativity in phorbol ester treated 3T3 cells. Biochem. Biophys. Res. Commun. 120: 1053–1059.

Rosoff, P. M., Savage, N., Dinarello, C. (1988) Interleukin–1 stimulates diacylglycerol production in T lymphocytes by a novel mechanism. Cell. 54: 73–81.

Roth, B. L., Mehegan, J. P., Jacobowitz, D. M., Robey, F., and Iadarola, M. J. (1989) Rat brain protein kinase C: purification, antibody production,

and quantification in discrete regions of hippocampus. J. Neurochem. 52: 215–221.

Rubin, R. P. (1982) Calcium–phospholipid interactions in secretory cells–A new perspective on stimulus–secretion coupling. Fed. Proc. 41: 2181–2187.

Saito, N., Kose, A., Ito, A., Hosoda, K., Mori, M., Hirata, M., Ogita, K., Kikkawa, U., Ono, Y., and Igarashi, K. (1989) Immunocytochemical localization of bata II subspecies of protein kinase C in rat brain. Proc. Natl. Acad. Sci. USA. 86: 3409–3413.

Schaap, D., Parker, P. J., Bristol, A., Kriz, R., and Knopf, J. (1989) Unique substrate specificity and regulatory properties of PKC–epsilon: 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.

Schramm, M., and Selinger, Z. (1984) Message transmission: receptor controlled adenylyl cyclase system. Science. 225: 1350–1356.

Schrey, M. P., Read, A. M., Steer, P. J. (1987) Stimulation of phospholipid hydrolysis and arachidonic acid mobilization in human uterine deciduea cells by phorbol ester. Biochem. J. 246: 705–713.

Sekiguchi, K., Tsukuda, M., Ogita, K., Kikkawa, U., and Nishizuka, Y. (1987) Three distinct forms of rat brain protein kinase C: differential response to unsaturated fatty acids. Biochem. Biophys. Res. Commun. 145: 797–802.

Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U., and Nishizuka, Y. (1988) Mode of activation of kinetic properties of three distinct forms of protein kinase C from rat brain. J. Biochem. 103: 759–765.

Selinger, Z., and Naim, E. (1970) Effect of calcium on amylase secretion by rat parotid slices. Biochem. Biophys. Acta. 203: 335–337.

Shearman, M. S., Naor, Z., Kikkawa, U., and Nishizuka, Y. (1987) Expression of multiple protein kinase C subspecies in rat central nervous tissue. Biochem. Biophys. Res. Commun. 147: 911–919.

Shepherd, G. M (1988) Autonomic functions in *Neurobiology*. New Youk, Oxford, Oxford university press, 376–394.

Shinomura, H., Terada, A., Hashimoto, Y., Soderling, T. R. (1988) The role of protein kinase C on amylase secretion from rat parotid gland. Biochem. Biophys. Res. Commun. 188: 1309–1314.

Shinomura, T., Asaoka, Y., Oka, M., Yoshida, K., and Nishizuka, Y. (1991) Synergistic action of diacylglycerol and unsaturated fatty acid for protein kinase C activation: its possible implications. Proc. Natl. Acad. Sci. USA. 88: 5149–5153.

Shramm, M., and Danon, D. (1961) The mechanism of enzyme secretion by the cell. I. Storage of amylase in the zymogen granules of the rat parotid gland. Biochim. Biophys. Acta. 50: 102–112.

Soling, H. D., Fest, W., Schmidt, T., Esselmann, H., and Bachmann, V. (1989) Signal transmission in exocrine cells is associated with rapid activity changes of acyltransferase and diacylglycerol kinase due to reversible protein phosphorylation. J. Biol. Chem. 264: 10643–10648.

Soling, H. D., Domenech, E. M. D., Kleineka, J., and Fest, W. (1987) Early effects of β–adrenergic and muscarinic secretagogues on lipid and phospholipid metabolism in guinea pig parotid acinar cells. J. Biol. Chem. 262: 16786–16792.

Soor, S. K. & Hincke, M. T. (1990) Microplate-reader based kinetic determination of alpha–amylase activity application to quantitation of secretion from rat parotid acini. Analyt. Biochem. 188: 187–191.

Spearman, T. N., Hurley, K. P., Olivas, R., Ulrich, R. G., and Butcher, F. R. (1984) Subcellular localization of stimulus–affected endogenuous phosphoproteins in the rat parotid gland. J. Cell. Biol. 99: 1354–1363.

Spearman, T. N., and Butcher, F. R. (1982) Rat parotid gland protein kinase activation. Relationship to enzyme secretion. Mol. Pharmacol. 21: 121–127.

Sugiya, H. (1989) The activation of Ca^{2+}–mobilizing receptors in salivary gland. Biomed. Res. 10: 111–121.

Takai, Y. Kishimoto, A., Iwasa, Y., Kawagara, Y., Mori, T., and Nishizuka, Y. (1977) Studies on a cyclic neucleotide–dependent 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. (1979) Calcium–dependent activation of a multifunctional protein kinase by membrane phospholipids. J. Biol. Chem. 254: 3692–3695.

Takai, Y., Kishimot, A., Kawahara, Y., Minkuch, R., Sano, K., Kikkawa, U., Mori, T., Yu, B., Kaibuchi, K., and Nishizuka, Y. (1981) Calcium and phosphatidylinositol turnover as signaling for transmembrane control of protein phosphorylation. Adv. Cyclic. Nucleotide. Res. 14: 301–303.

Takemura, H. (1985) Changes in cytosolic free calcium concentration in isolated rat parotid cells by cholinergic and beta–adrenergic agonists. Biochem. Biophys. Res. Commun. 131: 1048–1055.

Takemura, H. (1984) Potentiation of amylase release from isolated rat parotid cells–studies on the combination of isoproterenol and a low dose of carbachol. Jpn. J. Pharmacol. 36: 107–109.

Takuma, T., and Ichida, T. (1986) Phorbol ester stimulates amylase secretion from rat parotid acinar cells. FEBS Lett 199: 53–56.

Takuma, T. (1988) Evidence against direct involvement of cyclic AMP dependent protein phosphorylation in the exocytosis of amylase. Biochem. J. 256: 867–871.

Takuma, T. and Ichida, T. (1986) Phorbol ester stimulates amylase secretion from rat parotid cells. FEBS Lett. 199: 53–56.

Takuwa, N., Takuwa, Y., Rasmussen, H. (1987) A tumour promoter, 12–O–tetradecanoylphorbol 13–acetate, increases cellular 1,2–diacylglycerol content through a mechanism other than phosphoinositol hyddrolysis in Swiss–mouse 3T3 fibroblasts. Biochem. J. 243: 647–653.

Tanimura, A., Matsumoto, Y., Tojyo, Y. (1990) Evidence that isoproterenol–induced Ca^{2+}–mobilization in rat parotid acinar cells is not mediated by activation of β–adrenoceptors. Biochim. Biophys. Acta. 1055: 273–277.

Tapley, P. M., Murry, A. W. (1985) Evidence that treatment of platelets with phorbol ester causes proteolytic activation of Ca^{2+}–activated, phospholipid–dependent protein kinase. Eur. J. Biochem. 151: 419–423.

Tartakoff, A., Vassalli, P. (1978) Comparative studies of intracellular–transport of secretory proteins. J. Cell. Biol. 79: 694–707.

Terbush, D. R., and Holz, R. W. (1986) Effects of phorbol esters, diaglyceride, and cholinergic agonists on the subcellular distribution of protein kinase C in intact and digitonin–permeabilized adrenal chromaffin cells. J. Biol. Chem. 261: 17099–17106.

Thompson, N. T., Tateson, J. E., Randall, R. W., Spacey, G. D., Bonser, R. W., and Garland, L. G. (1990) The temporal relationship between phospholipase activation, diacylglycerol formation and superoxide production in the human neutrophil. Biochem. J. 271: 209–213.

Thompson, N. T., Bonser, R. W., and Garland, L. G. (1991) Receptor–coupled phospholipase D and its inhibition. Tips. 12: 404–408.

Tojyo, Y., Matsumot, Y. (1990) Inhibitory effects of loading with the cacium chelator 1,2–Bis (ortho aminophenoxy) ethane tetraacetic acid on amylase release and cellular ATP level in rat parotid cells. Biochem. Pharmacol. 39: 1775–1779.

Towbin, H., and Gordon, S. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets–procedure and some applications. Proc. Natl. Acad. Sci. USA. 76: 4350–4354.

Uhing, R. J., Prpic, V., Holhenbach, P. W., Adams, D. O. (1989) Involvement of protein kinase C in platelet–activating factor–stimulated diacylglycerol accumulation in murine preitoneal macrophages. J. Biol. Chem. 264: 9224–9230.

VanBlitterswijk, W. J., Hilkmann, H., Widt, J., and Van der Bend, R. L. (1991) Phospholipid metabolism in bradykinin–stimulated human fibroblasts. II. phosphatidylcholine breakdown by phospholipases C and D, involvement of protein kinase C. J. Biol. Chem. 266: 344–350.

Walaas, S. I., Horn, R. S., Adler, A., Adler, K., and Walaas, O. (1987) Insulin increases membrane protein kinase C activity in rat diaphragm. FEBS Lett. 220: 311–318.

Wood, J. G., Girard, P. R., Mazzei, G. J., Kuo, J. F. (1986) Immunocytochemical localization of protein kinase C in identified neuronal compartments of rat brain. J. Neurosci. 6: 2571–2577.

Worley, P. F., Baraban, J. M., Snyder, S. H. (1986) Heterogeneous localizations of protein kinase C in rat brain autoradiographic analysis of phorbol ester receptor–binding. J. Neurosci. 6: 199–207.

Wright, T. M., Rangan, L. A., Shin, H. S., and Raben, D. M. (1988) Kinetic analysis of 1,2–diacylglycerol mass levels in cultured fibroblasts. J. Biol. Chem. 263: 9374–9380.

Yoshida, Y., Huang, F. L., Nakabayushi, H., and Huang, K. P. (1988) Tissue distribution and developmental expression of protein kinase C isozymes. J. Biol. Chem. 263: 9868–9873.