MECHANISMS OF SIGNAL TRANSDUCTION IN THE EXOCRINE PANCREAS

P.H.G.M. WILLEMS

MECHANISMS OF SIGNAL TRANSDUCTION IN THE EXOCRINE PANCREAS

MECHANISMS OF SIGNAL TRANSDUCTION IN THE EXOCRINE PANCREAS

een wetenschappelijke proeve op het gebied van de
WISKUNDE EN NATUURWETENSCHAPPEN

Proefschrift
ter verkrijging van de graad van doctor
aan de Katholieke Universiteit te Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
vrijdag 11 maart 1988
des namiddags te 1.30 uur precies

door

Petrus Henricus Gerardus Maria Willems

geboren op 20 oktober 1952
te Grave

Druk: Reprografie Geneeskunde Faculteit Nijmegen, 1988

Promotor: Prof. Dr. J.J.H.H.M. De Pont

Voor Jeannette,
Neeltje, Fieke
en Sanne.

CONTENTS

Chapter 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Communication between cells is required to coordinate their diverse activities and must have been a prerequisite for the evolution of multicellular organisms. Cells which are not in direct physical contact in that they do not form gap junctions allowing the cytoplasms of the interacting cells to join directly, communicate by means of signaling molecules. These signaling molecules are either released by the cell or displayed on the outer surface of the cell and they are recognized by other cells and sometimes also by the signaling cell itself. In multicellular organisms, the extracellular medium contains a wide variety of signaling molecules. Some of these signaling molecules are released in the immediate vicinity of the target cell(s) where they are rapidly taken up or degraded so that they act only on a limited group of cells (local chemical mediators) or even only on one single cell (neurotransmitters). Others are released into the bloodstream and transported to essentially all parts of the body (hormones).

Intercellular communication by means of signaling molecules implies the presence of a cell-specific set of receptors enabling a particular cell to select those signaling molecules which are released to control its activity. The fact that most, if not all, cells possess more than one type of receptor indicates already that various signaling molecules apparently act in concert to regulate the activity of a particular cell. As a consequence, the cell has an important integrative function in that it translates all the information it receives into the most appropriate response.

Basically, by virtue of localization of the ligand-binding domain, two different kind of receptors are recognized: (1) cytosolic receptors and (2) cell surface receptors.

Cytosolic receptors interact with ligands such as steroid hormones, which, because of their lipophilic nature, can easily pass the plasma membrane. On the other hand, cell surface receptors expose their ligand-binding site into the extracellular space. Agonist-binding leads to receptor activation and depending on the pathway employed to convey information across the plasma membrane, cell surface receptors which do not need intervention of other membrane-associated molecules are differentiated from those who do.

Recently, molecular cloning of some of the receptors, which require other membrane-associated molecules to pass information to the cell interior, has allowed to predict the topographical orientation of such receptor proteins with respect to the plasma membrane (Nathans and Hogness, 1984; Zuker et al., 1985; Yarden et al., 1986; Hall, 1987). The receptors in question are the β-adrenergic receptor, the muscarinic cholinergic receptor and the light receptor. Several regions are present, which because of their hydrophobicity, are potentially membrane spanning. These presumptive membrane spanning regions, or transmembrane segments, are linked by hydrophilic regions, forming extracellular and intracellular loops. Although it is obvious that ligands which can not pass the plasma membrane bind to the hydrophilic extracellular domains, preliminary evidence suggests that agonist-binding may also occur in the hydrophobic domains (Yarden et al., 1986; Ratnam et al., 1986). Receptor activation is thought to promote the binding between hydrophilic intracellular domains and a membrane-associated molecule functioning as a transducer. As a consequence, the transducer becomes activated and, in turn, acts to regulate second messenger generating enzymes (Rodbell, 1985; Gilman, 1987; Litosch, 1987; Levitzki, 1987) or, in some cases, acts to regulate ion channels directly (Pfaffinger et al., 1985; Logothetis et al., 1987; Bourne, 1987).

Cell surface receptors which do not need intervention of a transducer and a second messenger generating enzyme, are receptors whose intracellular hydrophilic domains possess tyrosine kinase activity. Profoundly studied are the insulin receptor and the epidermal growth factor receptor. Upon receptor activation, tyrosine kinase activity increases and a specific set of substrate proteins will be phosphorylated.

In addition, an autophosphorylation site is present as well as sites which can be phosphorylated by protein kinases present in the cytosol (Takayama et al., 1984; Hunter et al., 1984; Moon et al., 1984; Bollag et al., 1986; Nishizuka, 1986). Cell surface receptors which act through transducer molecules, can also be phosphorylated (Kelleher et al., 1984; Nishizuka, 1986; Lefkowitz et al., 1986).

The presence of these phosphorylation sites is indicative for the existence of devices enabling the cell to adjust the activity of a particular receptor to the overall information it receives.

This thesis deals with cell surface receptors which are thought to interact with transducer molecules. There is good evidence now for the existence of such functional transducing molecules which, after being activated by the receptor, pass the signal to a second membrane- associated molecule functioning as an amplifier. The latter molecule is an enzyme which generates intracellular signaling molecules or second messengers. These second messengers either remain associated with the plasma membrane or are released into the cytosol. A wide variety of intracellular events often influencing each other and leading thereby to the most appropriate response, is then initiated by these messenger molecules.

With regard to the amplifier molecule which is switched on, stimuli which bind to cell surface receptors can be devided into two groups: (1) stimuli which activate the phosphatidylinositol 4,5-specific phosphodiesterase (PIP_2 phosphodiesterase) and, (2) stimuli which either activate or inhibit adenylate cyclase. Activation of the PIP_2 phosphodiesterase results in the formation of the intracellular messenger molecules 1,2-diacylglycerol and inositol 1,4,5-trisphosphate ((1,4,5-)IP_3). Activation of adenylate cyclase leads to the formation of adenosine (cyclic) 3':5'-monophosphate (cAMP). Since stimuli which activate the PIP_2 phosphodiesterase increase also the free cytosolic calcium concentration, they are said to activate the calcium messenger system, whereas stimuli which increase the intracellular cAMP concentration activate the cAMP messenger system. Both messenger systems will be discussed in more detail and with particular interest in the pancreatic acinar cell, in the following sections.

THE cAMP MESSENGER SYSTEM

As outlined in the above section, the cAMP messenger system is build up of cell surface receptors, a transducing system and an effector. The effector is the enzyme adenylate cyclase and stimulation of its activity results in the conversion of adenosine triphosphate into adenosine (cyclic) 3':5'-monophosphate (cAMP). In 1958 Sutherland and Rall were the first to discover cyclic AMP and in 1968 the role of cAMP as an intracellular signaling molecule had been established in a number of hormonally-regulated physiological functions (Robison et al., 1968).

The involvement of a transducing molecule in transmembrane signaling was first suggested by Rodbell et al. (1971) to explain the requirement for guanosine triphosphate (GTP) for hormonal activation of adenylate cyclase and because of its affinity for GTP the membrane-bound transducer was called G protein (guanine nucleotide-binding regulatory protein). In subsequent years, it was found that the requirement for GTP for hormonal regulation of adenylate cyclase activity was paralleled by GTP-induced decreases in affinity of the hormones for their receptors (Maguire et al., 1976). In 1976-1977 Cassel and Selinger demonstrated for the first time that hormonal stimulation increased GTPase activity in isolated membranes. Based on these and similar observations, the following model for hormonal regulation of adenylate cyclase activity involving G proteins has evolved. Binding of a ligand to its receptor induces a conformational change in the receptor. This change is transmitted through the cell membrane to the G protein, increasing its susceptibility to GTP. The binding of GTP induces another conformational change of the G protein enabling it to activate the catalytic subunit of adenylate cyclase. Activation of the cyclase is terminated by the hydrolysis of GTP to GDP which is catalyzed by the enzyme GTPase. Dissociation of GDP occurs as a result of the rate-limiting process which is accelerated by interaction between

G protein and receptor (Gilman, 1987). Dissociation of GTP is followed by binding of GTP (GDP-to-GTP exchange) (Levitzki, 1987).

The activity of the catalytic subunit of adenylate cyclase is controlled by two different G proteins, a stimulatory one (G_s) and an inhibitory one (G_i). An extracellular signaling molecule which activates adenylate cyclase binds to a receptor whose action is mediated by G_s. The stimulatory effect of such a stimulus can be inhibited by an extracellular signaling molecule which binds to a receptor whose action is mediated by G_i (Gilman, 1984).

G proteins are heterotrimers, with subunits designated α, β and γ in order of decreasing mass. The subunit defining the G protein appears to be the α subunit. The α subunits of some G proteins have been shown to be substrate for certain bacterial toxins. The α subunit of G_s is ADP-ribosylated by cholera toxin (Moss and Vaughan, 1977; Gill and Meren, 1978), whereas the α subunit of G_i is ADP-ribosylated by pertussis toxin (Katada and Ui, 1982a,b). Toxin-induced ADP-ribosylation of the α subunit of a G protein has been shown to block the flow of information from receptor to amplifier. In case of cholera toxin, in one way or another, the off-reaction which terminates adenylate cyclase activation is blocked, leading to continuous activation of the cyclase. On the other hand, pertussis toxin-induced modification of the α subunit of G_i blocks the on-reaction, in one way or another, thus preventing inhibitory extracellular signaling molecules from exerting their inhibitory action on the activity of adenylate cyclase.

Hormonal stimulation of adenylate cyclase activity leads to enhanced formation of cAMP. Depending on the activity of the enzyme catalyzing the hydrolysis of cAMP to AMP, the cellular cAMP level will be increased and cAMP-dependent protein kinases will be activated. Each cAMP-dependent kinase or A-kinase has two parts, one acting as a regulatory subunit and one acting as a kinase. Binding of cAMP to the regulatory subunit results in the dissociation of both subunits and it is the liberated catalytic subunit which is enzymatically active and which will catalyze the phosphorylation of a specific set of substrate proteins. Phosphorylation of these proteins will induce conformational changes of these proteins and their biological activities will change. However, in most biological systems, including the pancreatic acinar cell, the steps leading from protein phosphorylation to the final cellular response are still poorly understood.

The cAMP messenger system in the pancreatic acinar cell

Originally, the presence of a membrane-bound adenylate cyclase in the pancreas was shown in particulate fractions of homogenates of the whole organ (Rutten et al., 1972; Marois et al., 1972; Kempen et al., 1974; Deschodt-Lanckman et al., 1974; Schulz et al., 1974) and it was not before 1977 that a hormone-sensitive adenylate cyclase was shown to be present in particulate fractions of pancreatic acinar cells (Kempen et al., 1977a; Long and Gardner, 1977).

Adenylate cyclase activity in particulate fractions of pancreatic tissue is stimulated by secretin, vasoactive intestinal polypeptide (VIP) and the C-terminal octapeptide of cholecystokinin (CCK-8).

In all species tested, CCK-8 has been shown to stimulate adenylate cyclase activity in a particulate fraction of homogenates of the whole pancreas (Rutten et al., 1972; Marois et al., 1972; Kempen et al., 1974; Deschodt-Lanckman et al., 1974; Schulz et al., 1974; Bonting et al., 1977; Sjodin and Gardner, 1977; De Pont et al., 1979). This suggests the presence of cholecystokinin receptors able to activate adenylate cyclase via G_s and indeed Lambert et al. (1985) were able to solubilize cholecystokinin receptors associated with both G_s and G_i from rat pancreatic plasma membranes as a reversible complex with ^{125}I-labelled CCK-9. Moreover, they showed that the GTP-induced increase in dissociation rate was preserved in the soluble state, indicating that the solubilized CCK receptors remained functionally coupled with the guanine nucleotide binding site modulating the affinity for CCK.

In order to account for the action of cholecystokinin on enzyme secretion from pancreatic acini, the hypothesis has been developed that pancreatic acinar cells possess one single class of cholecystokinin receptors and each receptor has two distinct, interacting binding sites, one with a high affinity for cholecystokinin and one with a low affinity for cholecystokinin (Collins et al., 1981a). The high affinity binding site has been associated with enzyme secretion, whereas the low affinity binding site has been associated with activation of adenylate cyclase.

In the early studies mentioned above, it was generally found that in particulate fractions of pancreatic tissue CCK-8 increased adenylate cyclase activity equally well and sometimes even better than secretin or VIP. For instance, in a comparative study it was found that CCK-8 stimulated adenylate cyclase activity in all species tested, whereas secretin was active in guinea pig, cat and rat, but not in mouse and rabbit (De Pont et al., 1979).

Studies in which the binding of ^{125}I-VIP and ^{125}I-secretin was measured, revealed that the guinea pig pancreatic acinar cell has two functionally distinct classes of receptors each of which interact with VIP and secretin. One class has a high affinity for VIP and a low affinity for secretin (VIP-preferring receptors); the other class has a high affinity for secretin and a low affinity for VIP (secretin-preferring receptors) (Gardner and Jensen, 1981; Jensen and Gardner, 1981). Using isolated pancreatic acinar cells of the guinea pig, it has been shown that occupation of VIP-preferring receptors by secretin or VIP leads to the formation of cAMP and increased enzyme secretion, whereas occupation of secretin-preferring receptors by secretin or VIP, despite significant increases in cAMP does not lead to enhanced enzyme secretion (Jensen et al., 1981; Raufman et al., 1982). These observations have been explained by compartimentalization of cAMP. Only the cAMP that is produced upon activation of VIP-preferring receptors is in the appropriate compartment to cause stimulation of enzyme secretion (Gardner et al., 1982). In addition, it has been suggested that the cAMP formed in response to occupation of secretin-preferring receptors is involved in stimulation of fluid and electrolyte secretion from pancreatic acinar cells (Hootman et al., 1983).

In the pancreatic acinar cell of the rat, the picture is somewhat different and four distinct classes of receptors have been recognized. One class has a high affinity for VIP and does not interact with secretin. Occupation leads to the formation of cAMP and to stimulation of enzyme secretion. A second class has low affinities for secretin and VIP and occupation does not lead to cAMP formation or enzyme secretion. A third class has a high affinity for secretin and does not interact with VIP, and occupation leads to cAMP formation and enzyme secretion. Finally, a fourth class has low affinity for secretin and does not interact with VIP. Occupation of these secretin receptors leads to enhanced enzyme secretion by a mechanism not mediated by cAMP (Bisonnette et al., 1984). The latter observation may be explained by the recent observation of Trimble et al. (1987a,b) that in rat pancreatic acinar cells secretin, but not VIP, in addition to its stimulatory effect on acinar cell adenylate cyclase, stimulates the production of (1,4,5)IP_3 and increases the free cytosolic calcium concentration.

In general secretin, VIP and cholecystokinin readily stimulate adenylate cyclase activity in particulate fractions of pancreatic tissue. However, increases in pancreatic acinar cell cAMP concentration, evoked by the above secretagogues, are much more difficult to measure and whereas slight increases are measured in response to secretin or VIP (Gardner et al., 1982), no increases are measured in response to CCK-8. Only when a suitable inhibitor of cyclic nucleotide phosphodiesterase activity is present, small CCK-8-induced increases in cAMP concentration can be observed (Kempen et al., 1977a,b; Renckens et al, 1980; Gardner et al., 1983).

As mentioned already, both in guinea pig and in rat, secretin and VIP stimulate enzyme secretion from pancreatic acinar cells. However, in the guinea pig, VIP already maximally stimulated pancreatic enzyme secretion at concentrations which did not lead to measurable increases in cellular cAMP

concentration (Gardner et al., 1983). Yet, adenylate cyclase activity appeared to be increased since in the presence of an inhibitor of cyclic nucleotide phosphodiesterase activity, marked increases in cAMP were measured as well as marked increases in enzyme secretion. On the other hand, secretin stimulated enzyme secretion only at concentrations at which measurable increases in acinar cell cAMP concentration occurred.

Also in the rat, secretin and VIP stimulate pancreatic enzyme secretion, but whereas both secretagogues increase the acinar cell cAMP concentration to similar levels, secretin is much more efficacious than VIP as a secretory agent (Heisler et al., 1974; Vandermeers et al., 1984; Bisonnette et al., 1984; Dehaye et al., 1985).

In other species, like dog, cat and mouse, secretin has been shown to increase cAMP, without having a stimulatory effect on pancreatic enzyme secretion (Robberecht et al., 1977), whereas in another study, secretin did not stimulate adenylate cyclase activity in a particulate fraction of mouse and rabbit, but did stimulate enzyme secretion from cat pancreatic tissue (De Pont et al., 1979).

The presence of a hormone-sensitive adenylate cyclase in pancreatic acinar cells suggests the involvement of cAMP in the mechanism of action of the respective hormones. In order to establish a second messenger function for cAMP it has to be shown, that an increase in cellular cAMP concentration alone is sufficient to lead to the response.

There are several ways to increase the acinar cell cAMP concentration without receptor activation. For instance inhibitors of cyclic nucleotide phosphodiesterase activity such as theophylline, 3-isobutyl-1-methylxanthine (IBMX) or Ro 20-1724, can be used to prevent enzymatic breakdown of cAMP formed under unstimulated conditions. Another way to increase the intracellular cAMP concentration, is the use of lipophilic derivatives of cAMP such as dibutyryl cAMP or 8-bromo cAMP, which easily pass the plasma membrane and bypass receptor activation. In mouse and rabbit, enzyme secretion increased in response to dibutyryl cAMP and inhibitors of cyclic nucleotide phosphodiesterase activity (Kulka and Sternlicht, 1968; Ridderstap and Bonting, 1969; Malamud, 1972). In guinea pig, IBMX increased the acinar cell cAMP concentration and stimulated enzyme secretion (Gardner et al., 1982). And in the latter species, enzyme secretion was also enhanced by dibutyryl cAMP and 8-bromo cAMP, which had to be used, however, in relatively high concentrations (Gardner and Jackson, 1977). Also in the rat, exogenous derivatives of cAMP stimulated enzyme secretion (Stock et al., 1977; Bauduin et al., 1977).

Increases in acinar cell cAMP concentration can also be obtained by activating post-receptor elements of the cellular adenylate cyclase system. As has been discussed already, cholera toxin modifies the stimulatory G protein of the adenylate cyclase system in such a way that it will permanently activate the cyclase. In the rat, pretreatment of pancreatic slices with cholera toxin resulted in an increase in adenylate cyclase activity when assayed in a particulate fraction prepared from toxin-treated slices (Kempen et al., 1975). However, cholera toxin topically applied to the cannulated rat pancreas, neither affected basal enzyme secretion nor cholecystokinin-stimulated enzyme secretion (Kempen et al., 1975; Smith and Case, 1975). Similar observations were reached for the perfused cat pancreas (Smith and Case, 1975). However, using acini isolated from the rat pancreas, it was found that pretreatment with cholera toxin besides increasing the acinar cell cAMP level, significantly potentiated CCK-8-induced enzyme secretion (Pan et al., 1982).

In the guinea pig, pretreatment of pancreatic acini with cholera toxin increased both the cellular cAMP concentration and enzyme secretion, whereas the cAMP response to secretin or VIP was markedly augmented in toxin-treated acini (Gardner and Rottman, 1979).

Forskolin, a diterpene extracted from the roots of *Coleus forskohlii* interacts directly and/or indirectly (through a G protein) with the catalytic subunit of adenylate cyclase, thereby increasing its activity and thus providing another powerful tool to activate the cyclase at a level beyond receptor activation (Seamon and Daly, 1981,1983; Ross, 1982; Wong and Martin, 1983). In the rat, forskolin

slightly increased enzyme secretion, whereas it markedly potentiated the secretory response to CCK-8 (Heisler, 1983).

Summary

(1) The pancreatic acinar cell contains a membrane-bound adenylate cyclase which, in a particulate fraction, is stimulated by cholecystokinin, by VIP and apparently with some exceptions (mouse and rabbit) by secretin.
(2) In guinea pig and rat, cholera toxin increased the acinar cell cAMP level, indicating that G_s is present.
(3) In intact acinar cells, secretin and VIP, but not cholecystokinin, increase cAMP when measured in the absence of suitable inhibitors of cyclic nucleotide phosphodiesterase activity.
(4) Similar increases in cAMP concentration induced by secretin or VIP do not lead to similar secretory responses and species differences are observed.

Aims of this part of the investigation

Using rabbit pancreatic acini we have studied whether forskolin-induced increases in acinar cell cAMP concentration lead to enhanced enzyme secretion (**Chapter 2**).

Despite large increases in cellular cAMP concentration, enzyme secretion was not stimulated. However, the stimulatory effect of the C-terminal octapeptide of cholecystokinin was markedly potentiated and we concluded that the role of cAMP in pancreatic enzyme secretion is rather a modulatory one.

In a previous study it was found that secretin did not stimulate adenylate cyclase activity in a particulate fraction of the rabbit pancreas. This has led us to investigate whether secretin is able to increase cAMP in intact rabbit pancreatic acinar cells (**chapter 9**).

It was found that secretin, both in the absence and in the presence of an inhibitor of cyclic nucleotide phosphodiesterase activity, increased the the acinar cell cAMP level. However, enzyme secretion, even in the presence of the phosphodiesterase inhibitor, when the cAMP level in response to secretin was markedly increased, was not enhanced. We showed also that VIP increased the acinar cell cAMP level and again no stimulatory effect on enzyme secretion was observed. It is not clear yet why in the earlier study secretin did not stimulate adenylate cyclase activity in a particulate fraction of the rabbit pancreas.

Evidence for the presence of G_i was sought by treating pancreatic acini with pertussis toxin and measuring cAMP production both under unstimulated and stimulated conditions (**chapter 3**).

Pretreatment of rabbit pancreatic acinar cells with pertussis toxin facilitated CCK-8-induced activation of adenylate cyclase. This observation led to the hypothesis that CCK-8, by activating the calcium messenger system, activates G_i. Activation of G_i then leads to inhibition of CCK-8-induced activation of the cyclase via G_s. Experiments were developed to test this hypothesis (**chapter 9**).

THE CALCIUM MESSENGER SYSTEM

Back in 1953, Hokin and Hokin were the first to demonstrate stimulus-induced changes in phospholipid metabolism *(phospholipid effect)*. However, it was not before methods became available to separate individual phospholipids, that the phospholipids showing the phospholipid effect were determined. It was found that in pigeon pancreas slices, cholinergic stimulation enhanced incorporation of $^{32}P_i$ in phosphatidic acid and phosphatidylinositol *(phosphatidylinositol effect)* (Hokin and Hokin, 1955,1958). Subsequently, it was found that cholinergic stimulation of avian salt gland tissue, prelabelled with [^{14}C]glycerol, did not result in increased incorporation of label into phosphatidic acid and it was concluded that the increased incorporation of $^{32}P_i$ into phosphatidic acid was not due to *de novo* synthesis of this phospholipid but to increased phosphorylation of diacylglycerol (Hokin and Hokin, 1965). The hypothe-

sis was developed that upon cholinergic stimulation phosphatidylinositol was hydrolysed in a phospholipase C-like manner, yielding diacylglycerol and inositol monophosphate and that the diacylglycerol produced was rapidly phosphorylated by the enzyme diacylglycerol kinase. Finally, phosphatidic acid was converted back to phosphatidylinositol by the sequential actions of CTP-phosphatidic acid cytidyl transferase and phosphatidylinositol synthase. The scheme was called the *phosphatidylinositol-phosphatidic acid cycle.*

As to the physiological relevance of the phosphatidylinositol- phosphatidic acid cycle, it was not before 1975 that Michell presented an exhaustive list of tissues which showed the phosphatidylinositol effect in response to a wide variety of stimuli, showing that it were the very same receptors which mediated changes in free cytosolic calcium concentration and phosphatidylinositol metabolism. Michell then postulated (1975) that agonist-induced breakdown of phosphatidylinositol might play a key role in the reactions that couple receptor activation to changes in free cytosolic calcium concentration.

Next, it was found that in most tissues, including the exocrine pancreas (Hokin-Neaverson, 1974; Farese et al., 1980; 1981a,b; 1982a,b), stimulation resulted in a substantial decrease in mass of phosphatidylinositol. This decrease in mass of phosphatidylinositol was shown to be calcium-dependent and to be mimicked by calcium ionophores, which finding was apparently in conflict with the hypothesis put forward by Michell (1975). On the other hand, the increased labelling of phosphatidic acid and phosphatidylinositol were found to occur also in the absence of extracellular calcium.

Finally, in a wide variety of tissues, including iris smooth muscle (Abdel-Latif et al., 1977; Akhtar and Abdel-Latif, 1980), liver (Kirk, 1982, Creba et al., 1983), parotid gland (Putney, 1982; Weiss et al., 1982; Downes and Wusteman, 1983), platelets (Billah and Lapetina, 1982), exocrine pancreas (Putney et al., 1983; Orchard et al., 1984) and insect salivary gland (Berridge, 1983; Litosch et al., 1984), agonist-induced increases in ^{32}P-labelling of phosphatidic acid and phosphatidylinositol were shown to be preceded by stimulated breakdown of ^{32}P-prelabelled phosphatidylinositol 4,5-bisphosphate (PIP_2) rather than of phosphatidylinositiol. Receptor-mediated hydrolysis of PIP_2 was independent of calcium and a second messenger function for the products formed, diacylglycerol (Nishizuka, 1984) and inositol trisphosphate (Berridge et al., 1983; Downes and Wusteman, 1983), is generally accepted now (Berridge, 1984,1985,1987a,b; Berridge and Irvine, 1984; Nishizuka, 1986).

For a long, time it was poorly understood how receptors were coupled to the PIP_2 specific phosphodiesterase. In 1982 (Goodhardt et al.), preliminary evidence was provided that a G protein may also function in signal transduction for calcium-mobilizing receptors. Analogous to observations with the adenylate cyclase system, it was found that GTP reduced the affinity of noradrenaline for the α_1-adrenergic receptor. Somewhat later, it was found that in several cell types pretreatment with pertussis toxin, known to modify the G proteins G_i, G_o and transducin (Gilman, 1984; Ui, 1984), inhibited subsequent stimulation with calcium-mobilizing stimuli, suggesting the involvement of a G protein sensitive to pertussis toxin (Molski et al., 1984; Nakamura and Ui, 1985; Brandt et al., 1985; Bradford and Rubin, 1985). However, in other tissues no such effect of pertussis toxin was found (Masters et al., 1985; Lynch et al., 1986; Schimmel and Elliott, 1986). Stimulatory effects of GTP (or non-hydrolysable analogues of GTP) on secretion in permeabilized mast cells (Gomperts, 1983) and diacylglycerol formation in permeabilized platelets (Haslam and Davidson, 1984) are also in line with the hypothesis that a G protein mediates between receptor and PIP_2 phosphodiesterase. Finally, it has been shown that in isolated membranes of several cell types (summarized by Cockcroft, 1987), guanine analogues alone and in combination with agonists stimulate the phospholipase C-like hydrolysis of PIP_2. However, the G protein of the phosphatidylinositol/calcium system, termed G_p, has not been purified and sequenced yet.

The (1,4,5-)IP_3/calcium pathway

In 1983, Streb et al., using permeabilized pancreatic acinar cells, were the first to show that one of the breakdown products of receptor-mediated cleavage of PIP_2, inositol 1,4,5-trisphosphate ((1,4,5-)IP_3), released calcium from the intracellular store they had characterized before as vesicular, ATP-dependent and non-mitochondrial. Similar observations have now been reported for many other cell types (Berridge, 1987a: Table 1) and it is generally accepted now that this intracellular agonist-sensitive calcium pool is the endoplasmic reticulum, or at least part of these internal membrane systems that actively sequester calcium (Streb et al., 1984; Taylor and Putney, 1985; Biden et al., 1986).

Only shortly thereafter, it was found that much of the inositol trisphosphate that accumulated upon cholinergic stimulation of parotid glands, prelabelled with [^{3}H]inositol, was inositol 1,3,4-trisphosphate ((1,3,4-)IP_3) rather than (1,4,5-)IP_3 (Irvine et al., 1984). However, whereas the 1,4,5-isomer increased within seconds after stimulation, a time lag was observed in the formation of the 1,3,4-isomer (Irvine et al., 1985; Burgess et al., 1985). In addition to both inositol trisphosphates, inositol 1,3,4,5- tetrakisphosphate ((1,3,4,5-)IP_4) was found to be formed nearly as rapidly as (1,4,5-)IP_3 (Batty et al., 1985). Finally, in GH4 cells prelabelled with [^{3}H]inositol, also the presence of inositol pentaphosphate and inositol hexaphosphate was shown (Heslop et al., 1985). However, the concentrations of the latter inositol polyphosphates did not markedly change upon stimulation.

In addition to the inositol phosphates mentioned above, cyclic inositol phosphates have also been found to be increased after stimulation with calcium-mobilizing stimuli (Wilson et al., 1985). However, whether these compounds are involved in signal transduction remains an open question (Michell, 1986a).

Recently, it has been suggested that inositol tetrakisphosphate, which does not release calcium from intracellular stores (Irvine et al., 1986a), may function to control calcium entry across the plasma membrane (Hansen et al., 1986; Irvine and Moor, 1986,1987; Michell, 1986b). Moreover, Petersen recently provided evidence that in the lacrimal gland acinar cell only in combination, (1,4,5-IP)$_3$ and (1,3,4,5-)IP_4 mimicked the acetylcholine-induced increase in plasma membrane permeability for calcium (Morris et al., 1987). However, it has also been suggested that the (1,4,5-)IP_3-induced discharge of the intracellular calcium pool is the trigger for the entry of external calcium (Putney, 1986). In addion, it has been suggested that the receptor-mediated decrease in PIP_2 leads to inhibition of the plasma membrane calcium pump, which seems to require phosphoinositides for optimal functioning (Charest et al., 1985). As a consequence, passive influx of calcium exceeds active extrusion.

With respect to the (1,4,5-)IP_3/calcium pathway it is the current working hypothesis that receptor-mediated phospholipase C-like hydrolysis of PIP_2 yields (1,4,5-)IP_3. The 1,4,5-isomer of IP_3 releases calcium from the endoplasmic reticulum and is rapidly converted to (1,3,4,5-)IP_4 (Irvine et al., 1986b), a process which is enhanced by calcium (Rossier et al., 1986,1987). Inositol 1,3,4,5-tetrakisphosphate, in turn, may act as the intracellular messenger coupling receptor activation to increased plasma membrane permeability for calcium. Inositol tetrakisphosphate is either phosphorylated to inositol pentaphosphate, or dephosphorylated to (1,3,4-)IP_3. Next, the 1,3,4-isomer is dephosphorylated, leading to the formation of inositol 1,4-bisphosphosphate ((1,4-)IP_2). However, it has to be taken into account that part of the 1,4,5-isomer appears to be dephosphorylated directly. Further breakdown of inositol 1,4-bisphosphate occurs both via inositol 1- and inositol 4- monophosphate. Finally, the monophosphates are dephosphorylated to yield inositol, which is then used for the resynthesis of phosphatidylinositol, phosphatidylinositolmonophosphate and phosphatidylinositolbisphosphate.

The diacylglycerol/protein kinase C pathway

As mentioned already, the receptor-mediated breakdown of PIP_2 yields both the hydrophilic (1,4,5-)IP_3 molecule, which is rapidly released into the cytosol, and the lipophilic diacylglycerol molecule, which remains within the membrane where it is biologically active and where it is metabolized. Diacylglycerol can be metabolized either by a kinase, yielding phosphatidic acid (Hokin, 1985), or by a lipase, yielding arachidonic acid and monoacylglycerol (Hasegawa-Sasaki, 1985). Phosphatidic acid is converted via CMP-phosphatidate to phosphatidylinositol. Arachidonic acid is precursor in the formation of leukotrienes and prostaglandins, which may also have second messenger functions (Macphee et al., 1984).

In 1977 Inoue et al. isolated a protein kinase, which could only be activated by the calcium-dependent thiol protease. In most,if not all, mammalian tissues the enzyme is present, where it is recovered mainly from the soluble fraction in its inactive form. To prevent proteolytic activation by calpain (a calcium-dependent protease), the enzyme is usually extracted in the presence of high concentrations of calcium-chelating agents. As a consequence, the exact intracellular localization of the enzyme is unknown. However, recent immunocytochemical studies, using monoclonal antibodies, have revealed that the localization of the enzyme varies with cell types, but that the enzyme is poorly represented in the cell nucleus (Nishizuka, 1986). In 1979 it was found by Takai et al. that the enzyme, isolated in the presence of calcium chelators, could be activated, other than proteolytically, by phospholipids, especially phosphatidylserine (Nishizuka, 1983,1986; Takai et al., 1984), in the presence of calcium (Takai et al., 1979a). Of major importance was the observation (Takai et al., 1979b), that diacylglycerol dramatically increased the affinity of the *calcium/phospholipid-dependent protein kinase (C)* for calcium. A second major breakthrough was the observation that the above action of diacylglycerol was completely mimicked by the tumor promoting phorbol esters, present in oil expressed from the seeds of the tree *Croton tiglium* (Castagna et al., 1982). In subsequent years, these phorbol esters proved to be powerful tools in investigating the role of protein kinase C in signal transduction, since they enabled activation of the kinase in intact cells without receptor activation. Recently, Nishizuka (1986) presented an exhaustive list of proposed substrate proteins of protein kinase C and of possible roles of protein kinase C in cellular responses.

By inducing the hydrolysis of PIP_2, calcium-mobilizing agonists stimulate the generation of two different intracellular signaling molecules, each activating a specific sequence of intracellular events. Recently, evidence is accumulating that activation of protein kinase C both positively and negatively influences cellular processes induced by calcium-mobilizing agonists as well as by agonists which stimulate the formation of cAMP (Berridge, 1987a). Some of these effects will be discussed in more detail in the next section, which deals with the calcium messenger system in pancreatic acinar cells.

The calcium messenger system in the pancreatic acinar cell

The most studied stimulants for pancreatic enzyme secretion are the neurotransmitter acetylcholine and the peptide hormone cholecystokinin (formerly: pancreozymin). Both secretagogues are of the calcium- mobilizing type. In addition, pancreatic enzyme secretion can be stimulated by other calcium-mobilizing stimulants. The calcium- mobilizing secretagogues gastrin and caerulein (a decapeptide isolated from the skin of the amphibian *Hyla caerulea*), have structural analogies with cholecystokinin. The calcium- mobilizing stimulants bombesin (amphibian skin), litorin (amphibian skin), physalaemin (amphibian skin) and eledoisin (isolated from the posterior salivary gland of the octopod *Eledone moschata*), are dissimilar to cholecystokinin (Schulz and Stolze, 1980; Jensen and Gardner, 1981).

Normally, when pancreatic exocrine tissue is incubated and enzyme secretion is measured, there is a certain rate of basal enzyme secretion (Beaudoin and Grondin, 1987; Arvan and Castle, 1987). When one of the above stimulants is added, and medium amylase activity is measured, a rapid increase is observed within minutes. During the first 5 to 10 min, the rate of enzyme secretion is high (initial rapid phase of enzyme secretion), thereafter, it rapidly declines but remains elevated for the time the secretagogue remains present (sustained phase of enzyme secretion) (Gardner and Jensen, 1981; Pandol et al, 1985a,b).

A role for intracellular calcium ions in the mechanism of action of the above-mentioned calcium-mobilizing pancreatic secretagogues is deduced from several observations.

First, upon preincubation of pancreatic acini in calcium-free medium, the rate of enzyme secretion in response to these secretagogues is increased for some minutes (initial phase) but then rapidly declines to prestimulatory levels, indicating that long-term secretion (sustained phase) is lost (Scheele and Haymovits, 1979; Williams, 1980; Pandol et al., 1985b). Similar observations were reached with the isolated rabbit pancreas (Schreurs et al., 1976). Moreover, the latter study showed that upon readdition of calcium to the incubation medium, enzyme secretion of the isolated pancreas, stimulated in the absence of extracellular calcium, markedly increased, indicating that the sustained response was restored.

Second, secretion can be stimulated by calcium ionophores, like A23187 and ionomycin. These calcium ionophores not only cause a large influx of extracellular calcium (Gardner et al., 1980) but also cause mobilization of cellular calcium (Gardner and Jensen, 1980). The latter ability of these ionophores explains their stimulatory effect during the first 5-10 min in the absence of extracellular calcium (Gardner et al., 1979, 1980; Pandol et al., 1985b).

Third, the secretagogues mentioned above cause a rapid efflux of $^{45}Ca^{2+}$ from prelabelled pancreatic acinar cells, even in the absence of extracellular calcium (Case and Clausen, 1973; Matthews et al., 1973; Kondo and Schulz, 1976a; Schreurs et al., 1976), suggesting that intracellularly accumulated calcium is released (Williams, 1980; Schulz, 1980). Stimulation in the presence of radioactive calcium showed an increased influx, indicating that in addition to the release of intracellularly stored calcium, the permeability of the plasma membrane for calcium is increased (Kondo and Schulz, 1976a,b; Renckens et al., 1978; Dormer et al., 1981).

Fourth, using calcium-selective microelectrodes (Stark and O'Doherty, 1982), the calcium-activated photoprotein, aequorin (Dormer, 1983) and the calcium-selective fluorescent indicator, quin2 (Ochs et al., 1983, 1985; Pandol et al, 1985a; Bruzzone et al., 1986; Ansah et al., 1986), rapid increases in the free cytosolic calcium concentration in response to the above secretagogues could be directly visualized. Measurements with quin2 showed a rapid, but transient (2-3 min), increase in free cytosolic calcium concentration. During this initial period, calcium is thought to mediate the rapid and extracellular calcium-independent phase of enzyme secretion (Pandol et al., 1985a).

Fifth, using permeabilized pancreatic acinar cells, Knight and Koh (1984) showed that enzyme secretion increased dose-dependently with the medium calcium concentration.

The above experiments strongly suggest the involvement of calcium in the mechanism of action of calcium-mobilizing pancreatic secretagogues.

Calcium-mobilizing secretagogues dose-dependently stimulate enzyme secretion. However, once the maximally stimulatory concentration is reached the magnitude of the secretory response decreases again as the concentration of secretagogue increases further. This phenomenon is termed *restricted stimulation*. In case of cholecystokinin as a stimulant, the hypothesis has been developed that this submaximal stimulation by supramaximal concentrations of secretagogue is mediated by a receptor with a low affinity for the secretagogue (Collins et al., 1981a). On the other hand, (sub)maximal stimulation by (sub)maximal concentrations of cholecystokinin is thought to be mediated by a receptor with a high affinity for the secretagogue. According to this hypothesis, occu-

pation of the high affinity site is a prerequisite for occupation of the low affinity site.

When pancreatic acini are first incubated in the presence of a supramaximal concentration of a calcium-mobilizing secretagogue, washed to remove free secretagogue, and then reincubated, two phenomena occur. First, enzyme secretion is still stimulated and this phenomenon has been termed *residual stimulation* (Collins et al., 1981a,b). In case of cholecystokinin, residual stimulation is thought to be mediated by the low affinity site. Second, subsequent stimulation by the same or any other calcium-mobilizing secretagogue, but not by secretagogues which increase cAMP, is inhibited (Abdelmoumene and Gardner, 1980). This phenomenon has been termed *desensitization*.

Recently, Pandol et al. (1985a) showed that when the cholinergic receptor antagonist, atropine, and the secretagogue cholecystokinin were added simultaneously to acini preincubated in the presence of a supramaximal concentration of the cholinergic agonist, carbachol, both the initial rapid phase of cholecystokinin-induced enzyme secretion and the cholecystokinin-induced increase in free cytosolic calcium concentration were abolished. They concluded that calcium did not increase as a result of carbachol-induced depletion of the intracellular calcium store and they suggested that this might be the cause for agonist-induced desensitization.

As discussed already, the ability of pancreatic secretagogues of the calcium-mobilizing type to stimulate the breakdown of PIP_2 led to the discovery of the phospholipid effect by Hokin and Hokin (1953). More recently, these decreases in PIP_2 have been shown to take place within seconds after the onset of stimulation (Putney et al., 1983; Orchard et al., 1984; Pandol et al., 1985b) and are generally accepted now to preceed the secretagogue-induced increase in free cytosolic calcium concentration. In addition, secretagogue-induced increases in (1,4,5-)IP_3 and (1,3,4,5-)IP_4 have also been shown to be fast enough to preceed the increase in cytosolic calcium concentration (Streb et al., 1985; Merritt et al., 1986a; Trimble et al., 1987a; Doughney et al., 1987). And finally, using permeabilized pancreatic acinar cells, Streb et al. (1983) were the first to show that (1,4,5-)IP_3 releases actively stored calcium.

Concerning the involvement of a G protein in secretagogue-induced PIP_2 hydrolysis, Merritt et al. recently described that a non-hydrolysable GTP analogon stimulated the formation of inositol trisphosphate in electrically permeabilized pancreatic acinar cells prelabelled with [^{3}H]inositol (Merritt et al., 1986b). In addition, Williams and McChesney reported that cholecystokinin induced the interaction of its receptor with a G protein (Williams and McChesney, 1987).

Phorbol esters, thought to activate protein kinase C in a diacylglycerol-like manner, have been used in a wide variety of cell types to investigate the role of protein kinase C in stimulus response coupling (Nishizuka, 1986). The presence of the calcium-activated, phospholipid-dependent protein kinase (C) in the pancreatic acinar cell has recently been shown by Noguchi et al. (1985). Using an enriched preparation of protein kinase C, which contained no cyclic nucleotide- dependent or calcium-dependent kinase activity, they showed an absolute requirement for calcium and phospholipid for its activation, and the phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA), markedly increased its affinity for calcium. In the presence of TPA, the enzyme was active at submicromolar calcium concentrations. When applied to intact pancreatic acini the phorbol esters TPA and phorbol dibutyrate significantly stimulate pancreatic enzyme secretion, without affecting the free cytosolic calcium concentration (Merritt and Rubin, 1985; Pandol et al., 1985a), suggesting an important role for protein kinase C in the mechanism of action of calcium-mobilizing pancreatic secretagogues (De Pont and Fleuren-Jakobs, 1984; Merritt and Rubin, 1985; Nogochi et al., 1985; Pandol et al., 1985a).

Receptor-mediated breakdown of PIP_2 leads to the formation of two second messenger molecules, diacylglycerol and (1,4,5-)IP_3, which has led to the dual signal hypothesis of cell activation (Nishizuka, 1984;1986; Kikkawa and Nishizuka, 1986;

Berridge, 1985,1987a,b). In the pancreatic acinar cell, activation of either of both limbs of the pathway, bifurcating at the level of receptor-mediated PIP_2 breakdown, leads to an increase in enzyme secretion. This is concluded from experimental data obtained with calcium ionophores and phorbol esters, which activate the (1,4,5-)IP_3/calcium limb and the diacylglycerol/protein kinase C limb, respectively, without receptor activation.

In the guinea pig, maximal phorbol ester-induced enzyme secretion is comparable to maximal secretagogue-induced secretion (Pandol et al., 1985a). On the other hand, in rat (Merritt and Rubin, 1985) and rabbit (De Pont and Fleuren-Jakobs, 1984), maximal phorbol ester-induced secretion is significantly less. Moreover, in all three species, although less obvious in rat, ionophore-induced secretion is less than maximal secretagogue-induced secretion. In combination, the stimulatory effects of calcium ionophores and phorbol esters are additive, indicating that both limbs of the bifurcating calcium messenger system act synergistically in generating the final response. This synergistic role of the two routes has been shown for many biological systems (Nishizuka, 1986; Kikkawa and Nishizuka (1986) Berridge, 1987a).

Recently, activation of the C kinase route has been shown to lead to inhibition of agonist-induced PIP_2 breakdown, calcium mobilization and cellular responses (Zavoico et al., 1985; Drummond, 1985; Brock et al., 1985; Watson and Lapetina, 1985; Kojima et al., 1986; Tohmatsu et al., 1986). In hepatocytes, this effect of phorbol esters was found to be paralleled by inhibition of agonist-binding, which suggests an effect at the level of the receptor (Lynch et al., 1985). Cholinergic stimulation of rat pancreatic acini pretreated with phorbol ester resulted also in an impaired calcium response (Ansah et al., 1986), whereas secretagogue-induced inositol trisphosphate production was unimpaired by phorbol ester treatment (Merritt et al., 1986a).

Similar observations as those described above, summarized by Nishizuka (1986), Kikkawa and Nishizuka (1986) and Berridge (1987a,b), lay emphasis on the important role for protein kinase C in regulation of cellular activities.

Summary

(1) It is well established now that the pancreatic secretagogues acetylcholine and cholecystokinin are of the calcium-mobilizing type.

Preliminary evidence suggests the presence of a G protein (G_p), coupling calcium-mobilizing receptors to the effector enzyme PIP_2 phosphodiesterase.

(3) Both secretagogue-induced decreases in radiolabelled PIP_2 and increases in radiolabelled (1,4,5-)IP_3 occur fast enough to preceed secretagogue-induced calcium mobilization. Inositol 1,4,5-trisphosphate releases calcium accumulated actively in permeabilized acinar cells. In addition, calcium-mobilizing secretagogues increase the permeability of the plasma membrane for calcium, leading to influx of calcium.

(4) Protein kinase C is present in the pancreatic acinar cell and the partially purified enzyme is activated in the presence of phosphatidylserine and synthetic diacylglycerols or phorbol esters by submicromolar concentrations of calcium.

(5) Calcium ionophores and phorbol esters bypass receptor activation and stimulate enzyme secretion, indicating that activation of either of the two routes is sufficient to induce the response. The effects of ionophores and phorbol esters are additive which shows that both routes act synergistically in generating the final response.

(6) In addition to its stimulatory effect on enzyme secretion, protein kinase C inhibits secretagogue-induced calcium mobilization. Therefore, the diacylglycerol/protein kinase C pathway also functions as an internal feedback system.

Aims of this part of the investigation

Using rabbit pancreatic acini we have investigated whether the G protein, thought to mediate between calcium-mobilizing receptors and PIP_2 phosphodiesterase, is sensitive to pertussis toxin **(chapter 3).**

Pretreatment of pancreatic acini with the toxin did not alter rapid increases in free cytosolic calcium concentration ($[Ca^{2+}]_i$) induced by the C-terminal octapeptide of cholecystokinin (CCK-8), as judged from changes in fluorescence obtained from quin2-loaded acini. On the other hand, toxin treatment markedly potentiated CCK-8-induced enzyme secretion and cAMP formation, especially when measured in the presence of an inhibitor of cyclic nucleotide phosphodiesterase activity. These observations suggest that in the pancreatic acinar cell G_i, but not G_p, is modified by pertussis toxin. Again, increases in cAMP concentration alone did not lead to enhanced enzyme secretion and the potentiating effect on CCK-8-induced secretion is in line with a modulatory role for the cyclic nucleotide in pancreatic enzyme secretion.

The involvement of extracellular calcium in secretagogue-induced changes in $[Ca^{2+}]_i$ was investigated by means of the fluorescent calcium indicator, quin2 (**chapter 4**).

It was shown that the initial and rapid secretagogue-induced increase in $[Ca^{2+}]_i$ was independent of extracellular calcium. In the presence of extracellular calcium, $[Ca^{2+}]_i$ did not decline to prestimulatory levels, but remained elevated in the presence of the secretagogue. In the absence of extracellular calcium, the $[Ca^{2+}]_i$ rapidly declined to prestimulatory levels, but increased again upon readdition of calcium to the medium. The putative intracellular calcium antagonist 3,4,5-trimethoxybenzoate 8-(diethylamino)-octyl ester (TMB-8), did not affect the secretagogue-induced extracellular calcium-independent increase in $[Ca^{2+}]_i$. However, the secretagogue-induced extracellular calcium-dependent increase in $[Ca^{2+}]_i$ was inhibited by the drug. Our results indicate that TMB-8 does not inhibit the stimulated release of intracellularly stored calcium, but rather prevents the occurrence of long-term elevated calcium levels in the stimulated acinar cell.

The putative intracellular calcium antagonist 3,4,5-trimethoxybenzoate 8-(diethylamino)-octyl ester (TMB-8) was used in an effort to ascertain the role of the secretagogue-induced increase in free cytosolic calcium concentration in pancreatic enzyme secretion (**chapter 5**).

At concentrations between 10 and 100 μM, the drug inhibited carbachol-, but not CCK-8-, induced increases in $[Ca^{2+}]_i$ and enzyme secretion. Inhibition is apparently due to its action as a cholinergic receptor antagonist. Our results show that the drug does not impair secretagogue- induced calcium mobilization. However, at concentrations between 50 and 100 μM, TMB-8 potentiated the stimulatory effect of submaximal concentrations of secretagogue as well as the stimulatory effects of the calcium ionophore, A23187, and the phorbol ester, TPA. At concentrations beyond 100 μM, the potentiating effect of the drug declined and at a concentration of 500 μM the stimulatory effect of CCK-8 was abolished completely. However, severe cytotoxic effects were observed at this relatively high concentration.

The phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA), thought to activate protein kinase C in a diacylglycerol-like manner, was used to investigate whether activation of the diacylglycerol/protein kinase C pathway may underly secretagogue- induced desensitization (**chapter 6**).

Rabbit pancreatic acini were incubated in the presence of TPA, washed and reincubated. Enzyme secretion was still enhanced in phorbol ester- treated acini, indicating that the C kinase is still active. However, the dose-response curves for CCK-8-stimulated enzyme secretion and calcium mobilization were shifted to the right, suggesting an inhibitory effect of protein kinase C on secretagogue- induced calcium mobilization. This inhibitory effect was not paralleled by inhibition of CCK-8-induced PIP_2 hydrolysis. From these observations the conclusion was drawn that protein kinase C acts at a level beyond receptor-mediated PIP_2 breakdown.

In a subsequent study it was investigated whether phorbol ester pretreatment impaired (1,4,5-)IP_3-induced calcium release in permeabilized pancreatic acinar cells (**chapter 7**).

It was found, that at a free calcium concentration of 0.1 μM and in the presence of an inhibitor of mitochondrial calcium uptake, pretreatment with TPA significantly inhibited (1,4,5-)IP_3- induced calcium release in permeabilized pancreatic acinar cells. No effect was observed on ATP-dependent calcium accumulation. This observation indicates that protein kinase C is able to modulate the activity of the (1,4,5-)IP_3/calcium route, without impairing the formation of its own stimulant diacylglycerol.

In addition to its effect on secretagogue-induced calcium mobilization, the effect of phorbol ester pretreatment on secretagogue-induced cAMP formation was investigated (**chapter 9**).

In a previous study, it was found that pretreatment with pertussis toxin facilitated CCK-8-induced activation of adenylate cyclase in intact acinar cells. Since, the toxin is thought to act by inactivating G_i, it was proposed that the relative inability of CCK-8 to stimulate cyclase activity in the intact cell might be due to activation of G_i by some component of the calcium messenger system, thus preventing the secretagogue from activating the cyclase via G_s. The observation that in the rabbit pancreatic acinar cell, neither secretin nor VIP stimulated the breakdown of ^{32}P-labelled PIP_2, is in line with the above proposal. Pretreatment with phorbol ester did not significantly affect basal adenylate cyclase activity, but inhibited both CCK-8- and VIP- induced increases in acinar cell cAMP concentration. These results indicate that protein kinase C modulates also secretagogue-induced cAMP formation. However, only the cAMP response to relatively low concentrations of secretagogue was inhibited. Therefore, CCK-8-induced activation of protein kinase C does not explain the relative inability of this secretagogue to stimulate adenylate cyclase in the intact acinar cell.

Phospholipids, phosphoinositides in particular, play a crucial role in signal transduction. In this study (**chapter 8**) , we investigated both the effect of the secretagogue CCK-8 and of the phorbol ester TPA on the incorporation of ortho[^{32}P]phosphate in acinar cell phospholipids.

Only in the presence of CCK-8, labelling of PIP_2 reached equilibrium within 60 min. Compared to control acini or acini labelled in the presence of TPA, the amounts of labelled PIP_2, extracted from cells labelled in the presence of CCK-8, were significantly reduced and subsequent stimulation with carbachol did not lead to any further decrease in the amount of ^{32}P-labelled PIP_2. These observations suggest that the amount of PIP_2 is markedly decreased in stimulated cells and that this may be the reason for the lack of effect of other stimuli of the calcium-mobilizing type. In the presence of TPA, labelling of PIP_2 appeared to be increased. Compared to acini labelled in the presence of CCK-8, no increased labelling of phosphatidic acid and phosphatidylinositol was observed. However, labelling of phosphatidylcholine was significantly enhanced. The latter observations indicate that phorbol esters seriously affect phospholipid metabolism in a manner different from calcium-mobilizing secretagogues.

REFERENCES

Abdel-Latif, A.A., Akhtar, R.A. and Hawthorne, J.N. (1977) Biochem. J. 162, 61-73
Abdelmoumene, S. and Gardner, J.D. (1980) Am. J. Physiol. 239, G272-G279
Akhtar, R.A. and Abdel-Latif, A.A. (1980) Biochem. J. 192, 783-791
Ansah, T.-A., Dho, S. and Case, R.M. (1986) Biochim. Biophys. Acta 889, 326-333
Arvan, P. and Castle, J.D. (1987) J. Cell Biol. 104, 243-252
Batty, I.R., Nahorsky, S.R. and Irvine, R.F. (1985) Biochem. J. 232, 211-215
Bauduin, H., Stock, C., Launay, J.F., Vincent, D., Potvliege, P. Grenier, J.F. (1977) Pflügers Arch. 372, 69-76
Beaudoin, A.R. and Grondin, G. (1987) Life Sci. 40, 2453-2460
Berridge, M.J. (1983) Biochem. J. 212, 849-858
Berridge, M.J. (1984) Biochem. J. 220, 345-360
Berridge, M.J. (1985) Sci. Am. 253, 124-134
Berridge, M.J. (1987a) Ann. Rev. Biochem. 56, 159-193
Berridge, M.J. (1987b) Biochim. Biophys. Acta 907, 33-45
Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315-321
Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P. and Irvine, R.F. (1983) Biochem. J. 212, 473-482
Biden, T.J., Wollheim, C.B. and Schlegel, W. (1986) J. Biol. Chem. 261, 7223-7229
Billah, M.M. and Lapetina, E.G. (1982) J. Biol. Chem. 257, 12705-12708
Bisonnette, B.M., Collen, M.J., Adachi, H., Jensen, R.T. and Gardner, J.D. (1984) Am. J. Physiol. 246, G710-G717
Bollag, G.E., Roth, R.A., Beaudoin, J., Mochly-Rosen, D. and Koshland, D.E., Jr. (1986) Proc. Natl. Acad. Sci. USA 83, 5822-5824
Bonting, S.L., De Pont, J.J.H.H.M., Kempen, H.J.M., Case, R.M., Smith, P.A. and Scratcherd, T. (1977) Br. J. Pharmacol. 61, 243-250
Bourne, H.R. (1987) Nature 325, 296-297
Bradford, P.G. and Rubin, R.P. (1985) FEBS Lett. 183, 317-320
Brandt, S.J., Dougherty, R.W., Lapetina, E.G. and Niedel, J.E. (1985) Proc. Natl. Acad. Sci. USA 82, 3277-3280
Brock, T.A., Rittenhouse, S.E., Powers, C.W., Ekstein, L.S., Gimbrone, M.A. and Alexander, R.W. (1985) J. Biol. Chem. 260, 14158-14162
Bruzzone, R., Pozzan, T. and Wollheim, C.B. (1986) Biochem. J. 235, 139-143
Burgess, G.M., McKinney, R.F., Irvine, R.F. and Putney, J.W. Jr. (1985) Biochem. J. 232, 237-243
Case, R.M. and Clausen, T. (1973) J. Physiol. London 235, 75-102
Cassel, D. and Selinger, Z. (1976) Biochim. Biophys. Acta 452, 538-551
Cassel, D. and Selinger, Z. (1977a) Proc. Natl. Acad. Sci. USA 74, 3307-3311
Cassel, D. and Selinger, Z. (1977b) J. Cycl. Nucl. Res. 3, 11-22
Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y (1982) J. Biol. Chem. 257, 7847-7851
Charest, R., Prpic, V., Exton, J.H. and Blackmore, P.F. (1985) Biochem. J. 227, 79-90
Cockcroft, S. (1987) TIBS 12, 75-78
Collins, S.M., Abdelmoumene, S., Jensen, R.T. and Gardner, J.D. (1981a) Am. J. Physiol. 240, G459-G465
Collins, S.M., Abdelmoumene, S., Jensen, R.T. and Gardner, J.D. (1981b) Am. J. Physiol. G466-G471
Creba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H. and Kirk, C.J. (1983) Biochem. J. 212, 733-747
Dehaye, J.-P., Gillard, M., Poloczek, P., stievenart, M., Winand, J. and Christophe, J. (1985) J. Cycl. Nucl. Prot. Phosph. Res. 10, 269-280
De Pont, J.J.H.H.M. and Fleuren-Jakobs, A.M.M. (1984) FEBS Lett. 170, 64-68
De Pont, J.J.H.H.M., Luyben, D. and Bonting, S.L. (1979) Biochim. Biophys. Acta 584, 33-42
Deschodt-Lanckman, M., Svoboda, M. and Christophe, J. (1974) Arch. Intern. Physiol. Biochem. 82, 981-982
Dormer, R.L. (1983) Biosci. Rep. 3, 233-240
Dormer, R.L., Poulsen, J.H., Licko, V. and Williams, J.A. (1981) Am. J. Physiol. 240, G38-G49
Doughney, C., Brown, G.R., McPherson, M.A. and Dormer, R.L. (1987) Biochim. Biophys. Acta 928, 341-348
Downes, C.P. and Wusteman, M.M. (1983) Biochem. J. 216, 633-640
Drummond, A.H. (1985) Nature 315, 752-755
Farese, R.V., Larson, R.E. and Sabir, M.A. (1980) Biochim. Biophys. Acta 633, 479-484
Farese, R.V., Larson, R.E. and Sabir, M.A. (1981a) Diabetes 30, 396-401
Farese, R.V., Sabir, M.A. and Larson, R.E. (1981b) Biochim. Biophys. Acta 665, 463-470
Farese, R.V., Larson, R.E. and Sabir, M.A. (1982a) Biochim. Biophys. Acta 710, 391-399
Farese, R.V., Larson, R.E. and Sabir, M.A. (1982b) Arch. Biochem. Biophys. 219, 204-208
Gardner, J.D. and Jackson, M.J. (1977) J. Physiol. London 270, 439-454
Gardner, J.D. and Jensen, R.T. (1980) Am. J. Physiol. 238, G63-G66

Gardner, J D and Jensen, R T (1981) In Physiology of the Gastrointestinal Tract, edited by Johnson, L R , pp 831-871 Raven Press, New York
Gardner, J D and Rottman, A J (1979) Biochim Biophys Acta 585, 250-265
Gardner, J D , Costenbader, C L and Uhlemann, E R (1979) Am J Physiol. 236, E754-E762
Gardner, J D , Walker, M D and Rottman, A J (1980) Am J Physiol 238, G458-G466
Gardner, J D , Korman, L., Walker, M D and Sutliff, V E (1982) Am J Physiol 242, G547-G551
Gardner, J D , Sutliff, V E , Walker, M D and Jensen, R T (1983) Am J Physiol 245, G676-G680
Gill, D M and Meren, R (1978) Proc Natl Acad Sci USA 75, 3050-3054
Gilman, A G (1984) Cell 36, 577-579
Gilman, A G (1987) Ann. Rev Physiol 48, 103-117
Gomperts, B D (1983) Nature 306, 64-66
Goodhardt, M , Ferry, N , Geynet, P and Hanoune, J (1982) J Biol Chem 257, 11577-11583
Hall, Z W (1987) TINS 10, 99-101
Hansen, C A , Mah, S and Williamson, J R (1986) J Biol Chem 261, 8100-8103
Hasegawa-Sasaki, H (1985) Biochem J 232, 99-109
Haslam, R and Davidson, M (1984) J Recept Res 4, 605-629
Heisler, S (1983) Can J Physiol Pharmacol 61, 1168-1176
Heisler, S , Grondin, G and Forget, G (1974) Life Sci 14, 631-639
Heslop, J P , Irvine, R F , Tashjian, A H and Berridge, M J (1985) J Exp Biol 119, 395-401
Hokin, L E (1985) Ann Rev Biochem 54, 205-235
Hokin, L E and Hokin, M R (1953) J Biol Chem 203, 967-977
Hokin, L E and Hokin, M R (1955) Biochim Biophys Acta 18, 102-110
Hokin, L E and Hokin, M R (1958) J Biol Chem 233, 805-810
Hokin, M R and Hokin, L E (1965) In Metabolism and Physiological Significance of Lipids, edited by Dawson, R M C and Rhodes, D N , pp 423-434, John Wiley and Sons, Ltd London
Hokin-Neaverson, M R (1974) Biochem Biophys Res Commun 58, 763-768
Hootman, S R , Ernst, S A and Williams, J A (1983) Am J Physiol 245, G339-G346
Hunter, T , Ling, N and Cooper, J A (1984) Nature 311, 480-483
Inoue, M , Kishimoto, A , Takai, Y and Nishizuka, Y (1977) J Biol Chem 252, 7610-7616
Irvine, R F and Moor, R M (1986) Biochem J 240, 917-920
Irvine, R F and Moor, R M (1987) Biochem Biophys Res Commun 146, 284-290
Irvine, R F , Letcher, A J , Lander, A J and Downes, C P (1984) Biochem J 223, 237-243
Irvine, R F , Anggard, E E , Letcher, A J and Downes, C P (1985) Biochem J 229, 505-511
Irvine, R F , Letcher, A J , Lander, D J and Berridge, M J (1986a) Biochem J 240, 301-304
Irvine, R F , Letcher, A J , Heslop, J P and Berridge, M J (1986b) Nature 320, 631-634
Jensen, R T and Gardner, J D (1981) Fed Proc 40, 2486-2496
Jensen, R T , Tatemoto, K , Mutt, V , Lemp, G F and Gardner, J.D (1981) Am J Physiol. 241, G498-G502
Katada, T and Ui, M (1982a) J Biol Chem 257, 7210-7216
Katada, T and Ui, M (1982b) Proc Natl Acad Sci USA 79, 3129-3133
Kelleher, D J , Pessin, J E , Ruoho, A E and Johnson, G L (1984) Proc Natl Acad Sci USA 81, 4316-4320
Kempen, H J M , De Pont, J J H H M and Bonting, S L (1974) Biochim Biophys Acta 370, 573-584
Kempen, H J M , De Pont, J J H H M and Bonting, S L (1975) Biochim Biophys Acta 392, 276-287
Kempen H J M , De Pont, J J H H M and Bonting, S L (1977a) Biochim Biophys Acta 496, 521-531
Kempen, H J M , De Pont, J J H H M and Bonting, S L (1977b) Biochim Biophys Acta 496, 65-76
Kikkawa, U and Nishizuka, Y (1986) Ann Rev Cell Biol. 2, 149-178
Kirk, C J (1982) Cell Calcium 3, 399-411
Knight, D E and Koh, E. (1984) Cell Calcium 5, 401-418
Kojima, I , Shibata, H , Ogata, E (1986) Biochem J 237, 253-258
Kondo, S and Schulz, I (1976a) J Membr Biol 29, 185-203
Kondo, S and Schulz, I (1976b) Biochim Biophys Acta 419, 76-92
Kulka, R G and Sternlicht, E (1968) Proc Natl Acad Sci USA 61, 1123-1128
Lambert, M , Svoboda, M , Furnelle, J and Christophe, J (1985) Eur J Biochem 147, 611-617
Lefkowitz, R J , Benovic, J L , Kobilka, B and Caron, M G (1986) TIPS 7, 444-448
Levitzki, A (1987) TIPS 8, 299-303
Litosch, I , Lee, H S and Fain, J N (1984) Am J Physiol 246, C141-C147
Logothetis, D E , Kurachi, Y , Galper, J , Neer, E J and Clapham, D E (1987) Nature 325, 321-326
Long, B W and Gardner, J D (1977) Gastroenterology 73, 1008-1014
Lynch, C J , Charest, R , Bocckino, S B , Exton, J H and Blackmore, P F (1985) J Biol Chem 260, 2844-2851
Lynch, C J , Prpic, V , Blackmore, P F and Exton, J H (1986) Mol Pharmacol 29, 196-203
Macphee, C H , Drummond, A H., Otto, A M and Jiminez de Asua, L (1984) J Cell Physiol 119, 35-40
Maguire, M E , Van Arsdale, P M and Gilman, A G (1976) Mol Pharmacol 12, 335-339

Malamud, D. (1972) Biochim. Biophys. Acta 279, 373-376
Marois, C., Moriset, J. and Dunnigan, J. (1972) Rev. Can. Biol. 31, 253-257
Masters, S.B., Martin, M.W., Harden, T.K. and Brown, J.H. (1985) Biochem. J. 227, 933-937
Matthews, E.K., Peterson, O.H. and Williams, J.A. (1973) J. Physiol. London 234, 689-701
Merritt, J.E. and Rubin, R.P. (1985) Biochem. J. 230, 151-159
Merritt, J.E., Taylor, C.W., Rubin, R.P. and Putney, J.W., Jr. (1986a) Biochem. J. 238, 825-829
Merritt, J.E., Taylor, C.W., Rubin, R.P. and Putney, J.W., Jr. (1986b) Biochem. J. 236, 337-343
Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81-147
Michell, R.H. (1986a) Nature 319, 176-177
Michell, R.H. (1986b) Nature 324, 613
Molski, T.F.P., Naccache, P.H., Marsh, M.L., Kermode, J., Becker, E.L. and Sha'afi, R.L. (1984) Biochem. Biophys. Res. Commun. 124, 644-650
Moon, S.O., Palfrey, H.C. and King, A.C. (1984) Proc. Natl. Acad. Sci. USA 81, 2298-2302
Morris, A.P., Gallacher, D.V., Irvine, R.F. and Petersen, O.H. (1987) Nature 330, 653-655
Moss, J. and Vaughan, M. (1977) J. Biol. Chem. 252, 2455-2457
Nakamura, T. and Ui, M. (1985) J. Biol. Chem. 260, 3584-3593
Nathans, J. and Hogness, D.S. (1984) Proc. Natl. Acad. Sci. USA 81, 4851-4855
Nishizuka, Y. (1983) TIBS 8, 13-16
Nishizuka, Y. (1984) Nature 308, 693-694
Nishizuka, Y. (1986) Science 233, 305-311
Noguchi, M., Adachi, H., Gardner, J.D. and Jensen, R.T. (1985) Am. J. Physiol. 248, G692-G701
Ochs, D.L., Korenbrot, J.I. and Williams, J.A. (1983) Biochem. Biophys. Res. Commun. 117, 122-128
Ochs, D.L., Korenbrot, J.I. and Williams, J.A. (1985) Am. J. Physiol. 249, G389-G398
Orchard, J.L., Davis, J.S., Larson, R.E. and Farese, R.V. (1984) Biochem. J. 217, 281-287
Pan, G.-Z., Collen, M.J. and Gardner. J.D. (1982) Biochim. Biophys. Acta 720, 338-345
Pandol, S.J., Schoeffield, M.S., Sachs, G. and Muallem, S (1985a) J. Biol. Chem. 260, 10081-10086
Pandol, S.J., Thomas, M.W., Schoeffield, M.S., Sachs, G. and Muallem, S. (1985b) Am. J. Physiol. 248, G551-G560
Pfaffinger, P.J., Martin, J.M., Hunter, D.D., Nathanson, N.M. and Hille, B. (1985) Nature 317, 536-538
Putney, J.W. Jr. (1982) Cell Calcium 3, 369-383
Putney, J.W. Jr. (1986) Cell Calcium 7, 10-12
Putney, J.W. Jr., Burgess, G.M., Halenda, S.P., McKinney, J.S. and Rubin, R.P. (1983) Biochem. J. 212, 483-488
Ratnam, M., Nguyen, D.L., Rivier, J., Sargent, P.B. and Lindstrom, J. (1986) Biochemistry 25, 2633-2643
Raufman, J.-P., Jensen, R.T., Sutliff, V.E., Pisano, J.J. and Gardner, J.D. (1982) Am. J. Physiol. 242, G470-474
Renckens, B.A.M., Schrijen, J.J., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1978) Biochim. Biophys. Acta 544, 338-350
Renckens, B.A.M., van Emst-de Vries, S.E., De Pont, J.J.H.H.M. and Bonting, S.L. (1980) Biochim. Biophys. Acta 630, 511-518
Ridderstap, A.S. and Bonting, S.L. (1969) Pflüger's Arch. 313, 62-70
Robberecht, P., Deschodt-Lanckman, M., Lammens, M., DeNeef, P. and Christophe, J. (1977) Gastroenterol. Clin. Biol. 1, 519-525
Robison, G.A., Butcher, R.W. and Sutherland, E.W. (1968) Ann. Rev. Biochem. 37, 149-174
Rodbell, M. (1985) TIBS 10, 461-464
Rodbell, M., Birnbaumer, L., Pohl, S.L. and Krans, H.M.J. (1971) J. Biol. Chem. 246, 1877-1882
Ross, E.M. (1982) J. Biol. Chem. 257, 10751-10758
Rossier, M.F., Dentand, I.A., Lew, P.D., Capponi, A.M. and Vallotton, M.B. (1986) Biochim. Biophys. Res. Commun. 139, 259-265
Rossier, M.F., Capponi, A.M. and Vallotton, M.B. (1987) Biochem. J. 245, 305-307
Rutten, W.J., De Pont, J.J.H.H.M. and Bonting, S.L. (1972) Biochim. Biophys. Acta 274, 201-213
Scheele, G. and Haymovits, H. (1979) J. Biol. Chem. 254, 10346-10353
Schimmel, R.J. and Elliott, M.E. (1986) Biochem. Biophys. Res. Commun. 135, 823-829
Schreurs, V.V.A.M., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1976) Biochim. Biophys. Acta 419, 320-330
Schulz, I. (1980) Am. J. Physiol. 239, G335-G347
Schulz, I. and Stolze, H.H. (1980) Ann. Rev. Physiol. 42, 127-156
Schulz, I., Pedersen, R., Wizeman, V. and Kondo, S. (1974) In: Secretory Mechanisms of Exocrine Glands, edited by Thorn, N.A. and Petersen, O.H., pp. 88-95. Munksgaard, Copenhagen
Seamon, K.B. and Daly, J.W. (1981) J. Cycl. Nucl. Res. 7, 201-224
Seamon, K.B. and Daly, J.W. (1983) TIPS 4, 120-123
Sjodin, L. and Gardner, J.D. (1977) Gastroenterology 73, 1015-1018
Smith, P.A. and Case, R.M. (1975) Biochim. Biophys. Acta 399, 277-290

Stark, R.J. and O'Doherty, J. (1982) Am. J. Physiol. 242, G513-G521
Stock, C., Launay, J.F. and Grenier, J.F. (1977) Biochem. Biophys. Res. Commun. 76, 217-223
Streb, H., Irvine, R.F., Berridge, M.J. and Schulz, I. (1983) Nature 306, 67-69
Streb, H., Bayerdorffer, E., Haase, W., Irvine, R.F. and Schulz, I. (1984) J. Membr. Biol. 81, 241-253
Streb, H., Heslop, J.P., Irvine, R.F., Schulz, I. and Berridge, M.J. (1985) J. Biol. CHem. 260, 7309-7315
Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y (1979a) J. Biol. Chem. 254, 3692-3695
Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. and Nishizuka, Y. (1979b) Biochem. Biophys. Res. Commun. 91, 1218-1224
Takai, Y., Kikkawa, U., Kaibuchi, K. and Nishizuka, Y. (1984) Adv. Cycl. Nucl. Prot. Phosph. Res. 18, 119-158
Takayama, S., White, M.F., Lauris, V. and Kahn, C.R. (1984) Proc. Natl. Acad. Sci. USA 81, 7797-7801
Taylor, C.W. and Putney, J.W. Jr. (1985) Biochem. J. 232, 435-438
Tohmatsu, T., Hattori, H., Nagao, S., Ohki, K. and Nozawa, Y. (1986) Biochem. Biophys. Res. Commun. 134, 868-875
Trimble, E.R., Bruzzone, R., Meehan, C.J. and Biden, T.J. (1987a) Biochem. J. 242, 289-292
Trimble, E.R., Bruzzone, R., Biden, T.J., Meehan, C.J., Andreu, D. and Merrifield, R.B. (1987b) Proc. Natl. Acad. Sci. USA 84, 3146-3150
Ui, M. (1984) TIPS 5, 277-279
Vandermeers, A., Vandermeers-Piret, M.C., Rathe, J., Dehaye, J.P., Winand, J. and Christophe, J. (1984) Peptides 5, 359-365
Watson, S.P. and Lapetina, E.G. (1985) Proc. Natl. Acad. Sci. USA 82, 2623-2626
Weiss, S.J., McKinney, J.S. and Putney, J.W. Jr. (1982) Biochem. J. 206, 555-560
Williams, J.A. (1980) Am. J. Physiol. 238, G269-G279
Williams, J.A. and McChesney, D.J. (1987) Regul. Peptides 18, 109
Wilson, D.B., Bross, T.E., Sherman, W.R., Berger, R.A. and Majerus, P.W. (1985) Proc. Natl. Acad. Sci. USA 82, 4013-4017
Wong, S.K.-F. and Martin, B.R. (1983) Biochem. J. 216, 753-759
Yarden, Y., Rodriguez, H., Wong, S.K.-F., Brandt, D.R., May, D.C., Burnier, J., Harkins, R.N., Chen, E.Y., Ramachandran, J., Ullrich, A. and Ross, E.M. (1986) Proc. Natl. Acad. Sci. USA 83, 6795-6799
Zavoico, G,B., Halenda, S.P., Sha'afi, R.I. and Feinstein, M.B. (1985) Proc. Natl. Acad. Sci. USA 82, 3859-3862
Zuker, C.S., Cowman, A.F. and Rubin, G.M. (1985) Cell 40, 851-858

Chapter 2

Potentiating role of cyclic AMP in pancreatic enzyme secretion demonstrated by means of forskolin.

WILLEMS, P.H.G.M.,

FLEUREN-JAKOBS, A.M.M.,

DE PONT, J.J.H.H.M. and

BONTING, S.L.

Department of Biochemistry, University of Nijmegen,
P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

Biochimica et Biophysica Acta, 802 (1984) 209–214
Elsevier

BBA 21903

POTENTIATING ROLE OF CYCLIC AMP IN PANCREATIC ENZYME SECRETION, DEMONSTRATED BY MEANS OF FORSKOLIN

P H G M. WILLEMS, A M M. FLEUREN-JAKOBS, J.J.H H.M. DE PONT and S.L BONTING

Department of Biochemistry, University of Nijmegen, 6500 HB Nijmegen (The Netherlands)

(Received May 17th, 1984)

Key words: cyclic AMP, Forskolin, Amylase, Cholecystokinin; Enzyme secretion; (Pancreas)

The role of cyclic AMP in the regulation of enzyme secretion by the rabbit pancreas has been investigated by means of forskolin, an activator of the catalytic subunit of adenylate cyclase. Forskolin increases the cyclic AMP level in isolated pancreatic acini in a dose-dependent way. Basal amylase release, however, remains unchanged. Forskolin potentiates the increase in amylase release induced by the C-terminal octapeptide of cholecystokinin (CCK-8). Potentiation is already apparent at hormone concentrations which are only marginally effective in stimulating amylase secretion. CCK-8 alone does not raise the cellular cAMP level, but it potentiates the forskolin-induced increase. In relative terms, potentiation is higher with decreasing concentration of forskolin. These results indicate that cAMP alone does not play a direct role in CCK-stimulated pancreatic enzyme secretion in the rabbit, but it potentiates enzyme secretion already stimulated through a cAMP-independent process.

Introduction

Considerable evidence has accumulated suggesting that an increase in cytosolic Ca^{2+} may mediate the action of CCK on pancreatic enzyme secretion [1–3]. However, it is still uncertain whether and, if so, how cyclic AMP is involved in the mechanism of action of this hormone.

In all species studied, the acinar cell contains an adenylate cyclase, which is stimulated by CCK [4–6]. However, a detectable increase in the acinar cAMP level is only found in the presence of a suitable inhibitor of cAMP phosphodiesterase activity [7–9]. Species differences in the effects of cholera toxin and the hormone secretin further complicate the picture. Cholera toxin increases the cAMP level in guinea pig and rat acini, but only in the guinea pig does it stimulate the enzyme secretion [10–12]. In both species, cholera toxin potentiates the CCK-stimulated enzyme secretion [10,13]. Secretin increases the acinar cAMP level as well as the enzyme secretion in guinea pig and rat [14,15], while the hormone also stimulates the adenylate cyclase [14]. However, in the rabbit secretin neither stimulates the adenylate cyclase nor enhances enzyme secretion [14]. Hence, we have chosen the rabbit pancreas to investigate the CCK-stimulated pathway of enzyme secretion.

Forskolin, a diterpene extracted from the roots of *Coleus forskohlii*, is known to activate the catalytic subunit of nearly all eukaryotic adenylate cyclases [16]. This has led us to use this substance in a study of the involvement of cAMP in the regulation of pancreatic enzyme secretion by CCK. We have investigated the effects of forskolin, with and without CCK, on amylase release from rabbit pancreatic acini, and correlated these with the cAMP levels in the acini.

Abbreviations CCK, cholecystokinin-pancreozymin, CCK-8, C-terminal octapeptide of cholecystokinin-pancreozymin; VIP, vasoactive intestinal peptide.

The results presented here show that an increase in cellular cAMP, caused by forskolin, does not stimulate enzyme secretion by itself, but that it potentiates the effect of CCK on enzyme secretion.

Materials and Methods

Pancreatic acini

Rabbit pancreatic acini are prepared essentially as described for acinar cells by Amsterdam and Jamieson [17], except that the divalent cation chelating step is omitted. The method is based on two successive incubations with collagenase and hyaluronidase in a medium containing 0.1 mM Ca^{2+}. After this digestion, the tissue is incubated in a medium containing 1.2 mM Ca^{2+} and is dispersed by pipetting. The suspension is filtered through nylon gauze and purified by centrifugation through a 4% albumin layer. The intactness of the isolated acini has been demonstrated by Trypan blue exclusion.

For secretion studies, a Krebs-Ringer bicarbonate medium (pH 7.4) containing 119 mM NaCl, 3.5 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.8 mM glucose, 1% bovine serum albumin and an amino acid mixture according to Eagle [18] is used at 37°C. The stimulants are added after 20 min, and samples of the incubation mixture are removed at appropriate times for determination of the amylase activity. At the end of the experiment the acini are homogenized in the incubation medium. Amylase activity is measured in the samples after rapid sedimentation of the acini and in the acinar homogenate by means of the Phadebas test.

Determination of cAMP

cAMP is determined by the method described by Kempen et al. [19]. Acini prepared from two pancreases are suspended in 20 ml incubation medium and incubated in a volume of 1.5 ml. At appropriate times, 300-μl samples are removed and mixed with 300 μl 10% trichloroacetic acid. After centrifugation at 10000 × *g* for 1 min, 500 μl supernatant is extracted three times with 5 ml of water-saturated diethyl ether. After addition of 2 pmol [^{3}H]cAMP, the supernatant is further purified on a Dowex-50W column, according to the method of Krishna et al. [20]. Standard solutions of cAMP in incubation medium are treated in the same way.

The cAMP content of the extract is determined by saturation assay with cAMP binding protein, as described by Brown et al. [21]. By this method, between 0.5 and 100 pmol cAMP per sample can be measured. The cAMP content of the acini is expressed in pmol/mg protein. Protein concentrations are determined by the Lowry method, using bovine serum albumin as a standard.

Materials

The cAMP binding protein is isolated from bovine adrenal cortex as described by Brown et al. [21]. The C-terminal octapeptide of cholecystokinin has been synthesized by Dr. H.M. Rajh in the Department of Organic Chemistry, University of Nijmegen. [^{3}H]cAMP (15 Ci/mmol) is purchased from ICN, Irvine, CA, U.S.A. Soybean trypsin inhibitor and bovine serum albumin are obtained from Sigma, St. Louis, MO, USA; cAMP from Boehringer, Mannheim, F.R.G.; Dowex 50W-X8 from Fluka, Buchs, Switzerland; activated charcoal (Norit, SX-1) from Norit, Amersfoort, The Netherlands. Forskolin is purchased from Calbiochem, La Jolla, CA, U.S.A., and the Phadebas test kit is from Pharmacia, Uppsala, Sweden. All other chemicals are of reagent grade.

Results

Effects on amylase release

Incubation of rabbit pancreatic acini in the presence of $1 \cdot 10^{-8}$ M CCK-8 produces a nearly linear increase in amylase release with time during the first 20 min (Fig. 1). In the presence of $3 \cdot 10^{-5}$ M forskolin (without CCK-8), the basal amylase release remains virtually unchanged. CCK-8 with forskolin, however, gives a more than additive effect on amylase secretion. This potentiation of the CCK-8 response by forskolin occurs mainly during the first 10 min.

The dose-response curve for the effect of CCK-8 alone on amylase release shows a maximal effect at a concentration of $1 \cdot 10^{-8}$ M, beyond which it decreases (Fig. 2). In the presence of $3 \cdot 10^{-5}$ M forskolin the curve has a similar shape, but at all concentrations of CCK-8 the response is signifi-

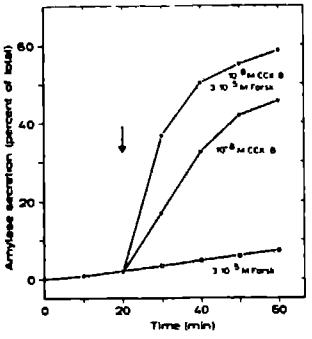


Fig. 1 Effects of CCK-8 and forskolin (Forsk), alone and in combination, on amylase release from rabbit pancreatic acini Acini are preincubated for 20 min at 37°C and are then incubated for 40 min with 1 10^{-8} M CCK-8, (○) 3 10^{-5} M forskolin (□) or both (●) Amylase release is measured at the indicated times, and is expressed as percentage of the total amylase content of the acini at the beginning of incubation Basal release from 20 to 60 min is the same as that in the presence of forskolin (without CCK-8) This figure is typical for seven experiments.

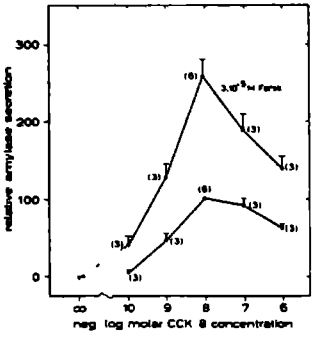


Fig. 2 Effect of forskolin (Forsk) on the dose-response curve for CCK-8 stimulation of amylase secretion by rabbit pancreatic acini Acini are incubated for 35 min at 37°C The indicated concentrations of CCK-8, with (●) or without (○) forskolin (3 10^{-5} M), are added at 20 min Amylase release is measured at 0, 10, 15 and 30 min and is expressed as percentage of total amylase originally present in the acini The increase in amylase release at 30 min is corrected for the basal release at that time, determined by extrapolation from the release values at 0, 10 and 15 min in each experiment The value obtained with 1 10^{-8} M CCK-8 alone (12 9%, S E. 1 5, $n = 6$ of total amylase originally present in the acini) is set at 100%, to which all other values are related The number of experiments is given in parentheses.

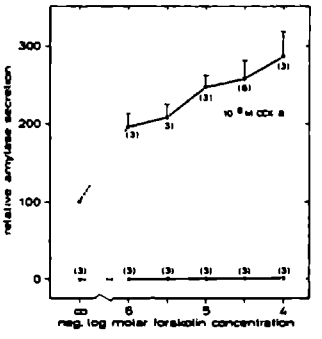


Fig. 3 Effect of CCK-8 on the dose-response curve for forskolin stimulation of amylase secretion by rabbit pancreatic acini Acini are incubated for 35 min at 37°C The indicated concentrations of forskolin, with (●) or without (○) CCK-8 (1 10^{-8} M), are added at 20 min Details on sampling and calculations are given in the legend of Fig. 2 The number of experiments is given in parentheses

cantly higher. The potentiation in absolute terms is maximal at a CCK-8 concentration of $1 \cdot 10^{-8}$ M, but at lower hormone concentrations the relative potentiation is even higher (700% at $1 \cdot 10^{-10}$ M, 170% at $1 \cdot 10^{-9}$ M, 150% at $1 \cdot 10^{-8}$ M).

Fig. 3 shows a dose-response curve for forskolin. With forskolin alone the basal amylase release remains unchanged at all concentrations tested In combination with $1 \cdot 10^{-8}$ M CCK-8, potentiation is alreay 100% at a forskolin concentration of only $1 \cdot 10^{-6}$ M. Potentiation of the hormone response increases with increasing concentration of forskolin, and at a concentration of $1 \cdot 10^{-4}$ M it is almost 200%.

Effects on cAMP content

Incubation of rabbit pancreatic acini in the presence of $3 \cdot 10^{-5}$ M forskolin produces a steep increase in the cellular cAMP level during the first 4 min after addition of the agent (Fig. 4). The response is maximal after approx. 15 min, when a new steady state level of cAMP is established. Addition of CCK-8 alone does virtually not change the basal cellular cAMP concentration (see also Fig. 5, lower line) However, a combination of the two agents potentiates the forskolin effect. Potentiation is greatest during the first 8 min after

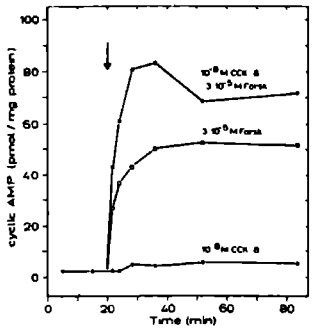


Fig 4 Effects of forskolin (Forsk) and CCK-8, alone and in combination, on the cAMP content of rabbit pancreatic acini Acini are preincubated for 20 min at 37°C and are then incubated for 40 min with 1 10^{-8} M CCK-8 (○), 3 10^{-5} M forskolin (■) or both (●) cAMP is determined at the indicated times This figure represents a single experiment

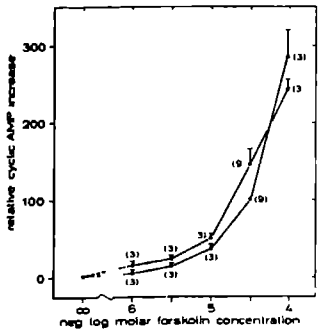


Fig. 6 Effect of CCK-8 on the dose-response curve of the forskolin-induced increase of the cAMP content of rabbit pancreatic acini Acini are incubated for 35 min at 37°C The indicated concentrations of forskolin, with (●) and without (○) CCK-8 (1 10^{-8} M), are added at 20 min Details on sampling and calculations are given in the legend of Fig 5 The number of experiments is given in parentheses

addition, reaches a maximum at about 15 min, and then decreases slightly to a new steady cAMP level in about 30 min

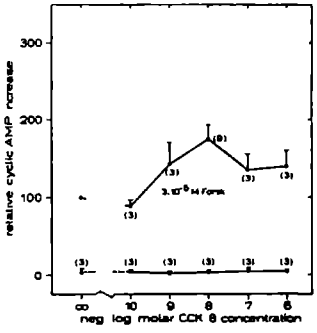


Fig 5 Effect of forskolin (Forsk) on the dose-response curve for the CCK-8-induced increase of the cAMP content of rabbit pancreatic acini Acini are incubated for 35 min at 37°C The indicated concentrations of CCK-8, with (●) and without (○) forskolin (3 10^{-5} M), are added at 20 min Samples for cAMP determination are taken at 5 min and 35 min The increase in cAMP is expressed as percentage of that obtained with 3 10^{-5} M forskolin measured in the same experiment In unstimulated acini the cAMP level is 3 1 (S E 0 8, $n = 9$) pmol/mg protein With 3 10^{-5} M forskolin, the increase in cAMP is 41 (S E 5 6, $n = 9$) pmol/mg protein The number of experiments is given in parentheses.

In further experiments, the difference in the cAMP level, measured 15 min before and after addition of CCK-8 and/or forskolin, is used for expressing the effects of the added compounds Fig 5 shows the dose-response curve for CCK-8 At none of the concentrations tested, the hormone increases the cAMP level significantly in the absence of forskolin In the presence of 3 10^{-5} M forskolin, potentiation of the response begins at 1 10^{-9} M CCK-8, reaches a maximum at 1 10^{-8} M, and then decreases slightly The maximum effect coincides with that on the amylase release, which also occurs at 1 10^{-8} M CCK-8 (Fig 2)

The dose-response curve for forskolin (Fig 6) shows that its effect on the cAMP level is clearly dose-dependent At a concentration of 1 10^{-4} M, the cAMP level is increased about 40-fold CCK-8 (1 10^{-8} M) has a small, but significantly positive effect on the cAMP level in the presence of forskolin, as compared to forskolin alone Only at the highest forskolin concentration (1 10^{-4} M) tested, the effect of CCK-8 is not significant In absolute terms, potentiation is maximal at a concentration of forskolin of $3 \cdot 10^{-5}$ M, whereas in relative terms, it is higher at lower concentrations of forskolin

Discussion

Our finding that CCK-8 does not increase the cellular cAMP level in rabbit pancreatic acini confirms the earlier findings of many other investigators [22]. Only in the presence of a cAMP-phosphodiesterase inhibitor in high concentration and at high ($> 1 \cdot 10^{-8}$ M) CCK-8 concentrations is there an increase in the cAMP concentration [8,9]. This virtual failure of the hormone to increase the cellular cAMP level suggests that stimulation of pancreatic adenylate cyclase, which has been detected in isolated pancreas membranes [4–6] and which originates from the acinar cell [6,7] is not involved in the mechanism of action of CCK in intact cells.

Forskolin has been used with succes to elucidate the role of cAMP in the intracellular control mechanisms of several secretory systems [16], and most recently also in enzyme secretion by the rat pancreas [23]. In contrast to cholera toxin, which acts by ADP-ribosylation [24] of the noncatalytic G-subunit, forskolin activates the catalytic subunit of adenylate cyclase in a direct way [25]. We have now demonstrated that forskolin increases the cellular cAMP level in rabbit pancreatic acini. Whereas the effect of cholera toxin only becomes visible after relatively lengthy incubation periods [10–12], the effect of forskolin is already apparent within a few minutes after addition. Therefore, forskolin appears to be a useful tool in investigating the role of cAMP in pancreatic enzyme secretion.

In the rabbit pancreatic acini the increase in cellular cAMP concentration, caused by forskolin, is not paralleled by an increase in the secretion of amylase. In the guinea pig, however, an increase in cAMP, caused by either cholera toxin [10], or secretin or VIP [10,14,15], may lead to enzyme secretion. In the rat the situation is complicated, since secretin and VIP increase cAMP and give a minor increase in amylase secretion [7,15], while cholera toxin alone increases cAMP without enhancing enzyme secretion [11–13]. In the rabbit pancreas secretin has neither an effect on the acinar cell adenylate cyclase activity, nor on the pancreatic enzyme secretion [14]. The use of rabbit pancreatic acini thus has the advantage that a secretin-stimulated cAMP pathway seems to be absent.

In contrast to the findings of Heisler [23] for rat acinar cells, we do not find forskolin alone to stimulate enzyme secretion by the rabbit pancreatic acini. In agreement with his findings, we find that forskolin potentiates the stimulatory effect of CCK-8 on enzyme secretion. The latter observation parallels the finding of Pan et al. [13] that cholera toxin potentiates the effect of CCK-8 on enzyme secretion in the rat. It is also in agreement with the report of Gardner et al. [9] that the increase in the cellular cAMP concentration, caused by CCK-8 in the presence of an inhibitor of cAMP phosphodiesterase activity, potentiates the effect of this hormone on amylase secretion from guinea pig pancreatic acini. For the rabbit pancreatic acini we find that marginally effective concentrations of CCK-8 greatly stimulate enzyme secretion, when the cellular cAMP level is increased by forskolin. These observations suggest that whereas in guinea pig a mere increase in cellular cAMP concentration already results in an enhanced enzyme secretion, in rabbit another intracellular factor seems to be additionally required. This intracellular factor may well be an increase in cytosolic Ca^{2+}, since Heisler [23] has found that forskolin potentiates the ionophore A23187-induced enzyme secretion from rat pancreatic acinar cells. For the rabbit pancreatic acini we find that forskolin ($3 \cdot 10^{-5}$ M) increases enzyme secretion induced by ionophore A23187 ($1 \cdot 10^{-6}$ M) by 180% (S.E. 66; $n = 3$).

The mechanism by which the forskolin-induced increase in cAMP leads to stimulation of amylase secretion is not yet understood. The molecular details of the stimulus-secretion coupling process are still insufficiently known. Hence, an explanation for the effect of cAMP in this process can only be speculative. One possibility could be that cAMP has an effect on the cytosolic calcium concentration, and thus may indirectly stimulate enzyme secretion. Another possibility could be that cAMP would affect the phosphatidylinositol metabolism, which is stimulated by CCK. It could also be that the phosphorylation of a protein involved in one of the final steps of the stimulus-secretion coupling process could be induced by cAMP and Ca^{2+}, acting on different sites. In the rabbit pancreas, phosphorylation on a cAMP-dependent site alone would not be sufficient to in-

duce secretion. However, it would potentiate secretion induced by phosphorylation on a Ca^{2+}-dependent site. Further studies are needed to distinguish between these various possibilities.

Another observation made in our study is that the forskolin-induced increase of the cellular cAMP concentration is potentiated by CCK-8. It is not clear whether this increase is due to a direct activation of the adenylate cyclase by the hormone or to an indirect effect of the hormone through the increased cytosolic Ca^{2+} level, e.g., by inhibition of cAMP phosphodiesterase activity.

In conclusion, we can say that although cAMP is not directly involved in the mechanism of action of CCK, it may potentiate the stimulatory effect of the hormone on the secretion of enzymes by the rabbit pancreatic acinar cell. However, it cannot be stated at this moment whether it would do this directly or indirectly.

References

1 O'Doherty, J and Stark, R.J. (1982) Am. J. Physiol. 242, G513–521
2 Ochs, D L., Korenbrot, J.I. and Williams, J.A. (1983) Biochem. Biophys. Res. Commun. 117, 122–128
3 Schreurs, V.V.A.M., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1976) Biochim. Biophys. Acta 419, 320–330
4 Rutten, W.J., De Pont, J.J.H.H.M. and Bonting, S.L. (1972) Biochim. Biophys Acta 274, 201–213
5 Marois, C., Moriset, J and Dunnigan, J. (1972) Rev. Can. Biol. 31, 253–257
6 Long, B.W. and Gardner, J.D. (1977) Gastroenterology 73, 1008–1014
7 Kempen, H.J.M., De Pont, J.J H H M. and Bonting, S.L. (1977) Biochim Biophys Acta 496, 521–531
8 Renckens, B A M., Van Emst-De Vries, S.E, De Pont, J J H.H M. and Bonting, S L (1980) Biochim Biophys. Acta 630, 511–518
9 Gardner, J.D., Sutliff, V.E, Walker, M.D. and Jensen, R.T. (1983) Am J. Physiol 245, G676–G680
10 Gardner, J.D. and Rottman, A.J (1979) Biochim Biophys. Acta 585, 250–265
11 Kempen, H.J.M., De Pont, J J H H M and Bonting, S L (1975) Biochim. Biophys. Acta 392, 276–287
12 Smith, P.A and Case, R M. (1975) Biochim. Biophys. Acta 399, 277–290
13 Pan, G-Z., Collen, M.J. and Gardner, J.D (1982) Biochim. Biophys. Acta 720, 338–345
14 De Pont, J.J H.H M., Luyben, D. and Bonting, S.L (1979) Biochim Biophys. Acta 584, 33–42
15 Robberecht, P., Deschodt-Lanckman, M., Lammens, M, De Neef, P. and Christophe, J. (1977) Gastroenterol. Clin. Biol. 1, 519–525
16 Seamon, K.B. and Daly, J.W. (1983) Trends Pharmacol. Sci. 4, 120–123
17 Amsterdam, A. and Jamieson, J.D. (1974) J Cell Biol. 63, 1037–1056
18 Eagle, H. (1959) Science 130, 432–437
19 Kempen, H.J M, De Pont, J.J H H M. and Bonting, S.L. (1977) Biochim. Biophys Act 496, 65–76
20 Krishna, G., Weiss, B and Brodie, B.B. (1968) J. Pharmacol Exp. Ther. 163, 379–385
21 Brown, B.L., Albano, J.D M., Ekins, R P and Sgherzi, A.M (1971) Biochem J 121, 561–563
22 Gardner, J.D. and Jensen, T (1981) in Physiology of the Gastrointestinal Tract (Johnson, L R., ed.), pp 831–871, Raven Press, New York
23 Heisler, S. (1983) Can. J. Physiol Pharmacol. 61, 1168–1176
24 Cassel, D. and Pfeuffer, T (1978) Proc. Natl. Acad. Sci. USA 75, 2669–2673
25 Seamon, K.B., Padgett, W. and Daly, J W. (1981) Proc. Natl. Acad. Sci. USA 78, 3363–3367

Chapter 3

Pertussis toxin stimulates cholecystokinin-induced cyclic AMP formation but is without effect on secretagogue-induced calcium mobilization in exocrine pancreas.

WILLEMS, P.H.G.M.,

TILLY, R.H.J. and

DE PONT, J.J.H.H.M.

Department of Biochemistry, University of Nijmegen,
P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

Biochimica et Biophysica Acta 928 (1987) 179–185
Elsevier

BBA 12008

Pertussis toxin stimulates cholecystokinin-induced cyclic AMP formation but is without effect on secretagogue-induced calcium mobilization in exocrine pancreas

P.H.G.M. Willems, R.H.J. Tilly and J.J.H.H.M. de Pont

Department of Biochemistry, University of Nijmegen, Nijmegen (The Netherlands)

(Received 13 November 1986)

Key words Pertussis toxin, Cholecystokinin, cyclic AMP, Calcium ion mobilization, (Pancreas)

The role of a pertussis toxin sensitive GTP-binding protein in mediating between cholecystokinin receptors and phosphatidylinositol 4,5-bisphosphate phosphodiesterase as well as in preventing cholecystokinin from increasing cellular cyclic AMP has been investigated using dispersed acini from rabbit pancreas. Pertussis toxin pretreatment (500 ng/ml, 2 h) did not affect cholecystokinin(octapeptide) (CCK-8) -induced increases in cytosolic free Ca^{2+} as judged from changes in fluorescence obtained from quin2-loaded acini. Although pretreatment with pertussis toxin was also without effect on resting acinar cell cyclic AMP levels, adenylate cyclase activity was increased, since inhibition of cyclic AMP phosphodiesterase activity by isobutylmethylxanthine (IBMX) resulted in an additional increase in cyclic AMP levels in toxin-treated acini, indicating that acinar cell adenylate cyclase activity is under some tonic inhibitory control by the pertussis toxin-sensitive inhibitory GTP-binding protein (G_i) of the adenylate cyclase system. CCK-8 gave an increase in cyclic AMP levels in both control (1.6-fold) and toxin-treated (2.3-fold) acini, leading to cyclic AMP levels in the toxin-treated acini 2-times as high as those in control acini. In the presence of IBMX, the cyclic AMP response to CCK-8 was again markedly enhanced in acini pretreated with the toxin (3.2- vs. 1.8-fold), resulting in cAMP levels in the toxin-treated acini 3.7-times those in the absence of IBMX, 2.5-times those in control acini in the presence of IBMX and 7.0-times those in control acini in the absence of IBMX. Neither the pretreatment with pertussis toxin, nor the presence of IBMX alone, nor the combination had an effect on basal amylase secretion. However, all three treatments potentiated the stimulatory effect of CCK-8 on amylase secretion and the amount of potentiation was proportional to the cyclic AMP levels reached. Our findings suggest that in the intact pancreatic acinar cell G_i inhibition of the catalytic subunit of the adenylate cyclase may largely be responsible for preventing cholecystokinin from increasing cellular cyclic AMP. They moreover show that cyclic AMP is a modulatory agent in rabbit pancreatic enzyme secretion, not able to stimulate secretion itself, but potentiating effects mediated by the phosphatidylinositol-calcium pathway.

Abbreviations CCK-8, cholecystokinin (octapeptide) GTP, guanosinetriphosphate, G_s, stimulatory GTP-binding protein of the adenylate cyclase system, G_i, inhibitory GTP-binding protein of the adenylate cyclase system, PIP_2, phosphatidylinositol 4,5-bisphosphate, IP_3, inositol 1,4,5-trisphosphate, TPA, 12-*O*-tetradecanoylphorbol 13-acetate, IBMX, 3-isobutyl-1-methylxanthine, Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Correspondence J.J.H.H.M. de Pont, Department of Biochemistry, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

Introduction

It is the current working hypothesis that calcium-mobilizing hormones exert their actions on target cells by stimulating phosphodiesteric cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2), yielding the intracellular messengers inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol, mediating intracellular Ca^{2+} mobilization and protein kinase C activation, respectively [1,2]

Recent studies have revealed that in the exocrine pancreas receptors for the secretagogue cholecystokinin are coupled to PIP_2-phosphodiesterase, since within seconds the hormone decreases membrane-bound PIP_2 and increases the cytosolic concentrations of IP_3 and Ca^{2+} [3–8]

In broken cell preparations, however, cholecystokinin has also been shown to be able to stimulate acinar cell adenylate cyclase activity [9–11] Yet, in intact acinar cells no cholecystokinin-induced increases in cellular cyclic AMP have been observed [12–14] This suggests that in acinar cells one or more of the following possibilities occur (i) cholecystokinin-induced coupling of its receptors to adenylate cyclase may be blocked, (ii) the enzyme may be under inhibitory control or (iii) cyclic AMP formed in response to the hormone may rapidly be eliminated

It is well established that hormonal stimulation of adenylate cyclase activity is mediated by a stimulatory GTP-binding protein (G_s), whereas an inhibitory one (G_i) mediates hormonal inhibition [15–17] Pertussis toxin, an exotoxin of *Bordetella pertussis*, has been shown to inactivate G_i [16,18,19] This has led us to use this substance alone and in combination with isobutylmethylxanthine (IBMX), a well-known inhibitor of cyclic AMP phosphodiesterase activity, to investigate whether G_i and cyclic AMP phosphodiesterase are involved in preventing cholecystokinin from increasing acinar cell cyclic AMP

In addition we investigated whether the GTP-binding protein postulated to mediate between calcium-mobilizing receptors and PIP_2-phosphodiesterase [20–24], as already described in some tissues [25–32], is also sensitive to pertussis toxin in the exocrine pancreas

Materials and Methods

Dispersed acini from rabbit pancreas were prepared using the method reported previously [8,14] and were suspended in a Krebs-Ringer bicarbonate medium (pH 7 4), containing 119 mM NaCl, 3 5 mM KCl, 1 2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1 2 mM $CaCl_2$, 1 2 mM $MgCl_2$, 5 8 mM glucose, 1% bovine serum albumin, 0 2 mg/ml soybean trypsin inhibitor and an amino acid mixture according to Eagle [33] Acini were incubated in the absence or presence of 500 ng/ml pertussis toxin for 2 h at 37°C Pertussis toxin was removed by centrifugation and the acini were resuspended in Krebs-Ringer bicarbonate medium to which 0 or 10^{-4} M isobutylmethylxanthine (IBMX) was added for enzyme secretion and cAMP formation studies and to which quin2/AM was added for measurement of cytosolic free calcium as described below Acini were then incubated for another 65 min and samples were removed at 0, 15, 30, 45 and 60 min for measuring amylase activity and at 35 and 65 min for determination of the cyclic AMP concentration At the beginning of the experiment an aliquot of the acini suspension was removed and the acini were homogenized in the medium Amylase activity was measured in the samples after rapid sedimentation of the acini and in the acinar cell homogenate by means of the Phadebas test [34] Amylase activity was expressed as a percentage of total amylase present in the acini at the beginning of the experiment Cyclic AMP was measured as described previously [14] and the cyclic AMP content in the acini was expressed as pmol cyclic AMP per mg protein

Quin2 loading of control and pertussis toxin-treated acini and measurement of quin2 fluorescence was essentially the same as reported before [8], with the exception that fluorescence measurement was performed in a Hepes/Tris medium medium (pH 7 4) containing 133 mM NaCl, 4 2 mM KCl, 1 0 mM $CaCl_2$, 1 0 mM $MgCl_2$, 5 8 mM glucose, 0 2 mg/ml soybean trypsin inhibitor and 10 mM Hepes, adjusted with Tris to pH 7 4 and thoroughly gassed with 100% O_2 Maximal fluorescence was obtained using the calcium ionophore ionomycin at a concentration of 10^{-5} M and $[Ca^{2+}]_i$ was calculated according to Tsien et al [35]

Materials

Purified pertussis toxin (batch 91) was purchased from PHLS Centre for Applied Microbiology and Research, Salisbury, U K The C-terminal octapeptide of cholecystokinin has been synthesized by Dr H M Rajh in the Department of Organic Chemistry, University of Nijmegen The cAMP binding protein was isolated from bovine adrenal cortex as described by Brown et al [36] Ionomycin was obtained from Behring Diagnostics, La Jolla, CA, U S A, 3-isobutyl-1-methylxanthine (IBMX) from Aldrich Chemical Company, Inc, Milwaukee, WI, U S A, [^{3}H]cAMP (15 Ci/mmol) from ICN, Irvine, CA, U S A, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), soybean trypsin inhibitor, bovine serum albumin and quin2/AM from Sigma, St Louis, MO, U S A, cAMP and hyaluronidase from Boehringer, Mannheim, F R G, collagenase from Cooper Biomedical Inc, Malvern, PA, U S A, Dowex 50W-X8 from Fluka, Buchs, Switzerland and activated charcoal (Norit, SX-1) from Norit, Amersfoort, The Netherlands The Phadebas test kit was purchased from Pharmacia, Uppsala, Sweden All other chemicals were of reagent grade

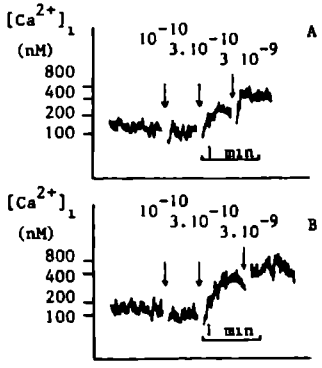


Fig. 1 The effect of CCK-8 on cytosolic free Ca^{2+} levels in quin2 loaded rabbit pancreatic acini pretreated with pertussis toxin Rabbit pancreatic acini were incubated for 2 h either in the absence or in the presence of pertussis toxin (500 ng/ml) During the last 20 min of this preincubation quin2/AM was added at a concentration of 50 μM Excess quin2/AM was removed by centrifugation and the acini were resuspended in fresh Krebs-Ringer bicarbonate medium. Samples removed for fluorescence measurements were centrifuged and the acini were resuspended in a Hepes/Tris medium (pH 7 4) thoroughly gassed with 100% O_2 CCK-8 (10^{-10} $3\,10^{-10}$ and $3\,10^{-9}$ M) was added at the indicated times This figure is typical for five experiments

Results

Effects on cytosolic free calcium

In acini pretreated with pertussis toxin (500 ng/ml, 2 h), the effect of CCK-8 on cytosolic free Ca^{2+} had been measured with the quin2 technique

Basal cytosolic free calcium concentrations, calculated to be 137 nM (S E 21, $n = 5$) in pertussis toxin-treated acini, were not significantly different from those in control acini (150 nM, S E 9, $n = 5$) Fig 1 shows that in toxin-treated acini (B) and in control acini (A) CCK-8 at a concentration of 10^{-10} M was without effect on cytosolic-free Ca^{2+} At a concentration of $3\,10^{-10}$ M, however, CCK-8 increased $[Ca^{2+}]_i$ in both toxin-treated and control acini to the same extent (338 nM, S E 43; $n = 5$ and 431 nM, S E 55, $n = 5$, respectively) as did $3\,10^{-9}$ M CCK-8 (745 nM, S E. 118, $n = 5$ and 635 nM, S E 99, $n = 5$, respectively) The fact that the dose-response curve for the CCK-8-induced initial increase in $[Ca^{2+}]_i$ is not shifted to the right excludes the possibility that in the exocrine pancreas a pertussis toxin-sensitive GTP-binding protein mediates between the cholecystokinin receptor and $PI P_2$-phosphodiesterase

Effects on basal amylase release and resting cellular cyclic AMP levels

From Table I it is clear that both basal amylase release (first line) and resting cyclic AMP levels (fourth line) remained virtually unchanged upon pretreatment with pertussis toxin However, acinar cell cyclic AMP levels, measured after 25 min, were substantially increased in control as well as in toxin-treated acini, when 10^{-4} M IBMX was included in the medium The levels reached in the presence of IBMX were significantly higher (1 4-times) in the toxin-treated acini than those in control acini Neither IBMX alone nor the combination pertussis toxin/IBMX had a clear effect on basal amylase secretion

TABLE I

EFFECTS OF PERTUSSIS TOXIN ON AMYLASE RELEASE AND cAMP LEVELS IN CONTROL ACINI AND IN ACINI STIMULATED WITH CCK 8 OR TPA

Rabbit pancreatic acini incubated in the absence or presence of pertussis toxin (500 ng/ml) for 2 h at 37°C were resuspended in fresh incubation solution containing 0 or 10^{-4} M IBMX. After 30 min either CCK-8 (10^{-8} M), TPA (10^{-6} M) or no stimulant was added. Samples for measurement of amylase activity in the medium were removed at 0, 15, 30, 45 and 60 min and amylase activity is expressed as percentage of total amylase originally present in the acini. The increase in amylase activity in the incubation medium at 60 min was calculated from the amylase activity values at 30, 45 and 60 min and was corrected for the basal release at that time, determined by extrapolation from the amylase activity values at 0, 15 and 30 min in each experiment. Samples for cAMP measurement were removed at 25 and 65 min and cAMP levels are expressed in pmol/mg protein. Results shown are the means ± S.E. of the amount of experiments given in parentheses.

	Acini not treated with pertussis toxin				Acini treated with pertussis toxin (500 ng/ml)			
	−IBMX		+IBMX		−IBMX		+IBMX	
Basal amylase secretion (% of total)	1.6±0.1	(14)	1.8±0.1	(28)	1.5±0.2	(14)	2.3±0.1[d]	(28)
Stimulated amylase secretion (% of total)								
CCK 8	11.9±1.7[b]	(8)	23.2±2.8[b,d]	(9)	18.3±3.1[b]	(8)	40.0±4.4[b,d]	(9)
TPA	3.7±0.8[a]	(6)	6.9±0.7[c]	(6)	5.2±1.1	(6)	9.1±1.2[e]	(6)
cAMP before stimulation (pmol/mg protein)	2.9±0.3	(14)	6.3±0.4[d]	(28)	3.8±0.6	(14)	9.1±0.7[d,f]	(28)
cAMP after stimulation (pmol/mg protein)								
–	–		7.4±0.9	(13)	–		10.9±0.9[d,e]	(13)
CCK 8	4.7±0.7[b]	(8)	13.1±3.0[a]	(9)	8.9±2.3	(8)	32.8±4.7[b,d,f]	(9)
TPA	3.5±0.7	(6)	5.9±1.2[c]	(6)	3.4±0.5	(6)	8.6±1.0[d]	(6)

[a] Compared with control without stimulus ($P < 0.05$)
[b] The same as [a] ($P < 0.01$)
[c] Compared with same conditions in the absence of IBMX ($P < 0.05$)
[d] The same as [c] ($P < 0.01$)
[e] Compared with same conditions but not treated with pertussis toxin ($P < 0.05$)
[f] The same as [e] ($P < 0.01$)

After 65 min of incubation in the presence of IBMX, the intracellular cyclic AMP concentration in toxin-treated acini was still significantly higher than that in control acini (Table I, fifth line). However, compared to the levels after 25 min, in both control and toxin-treated acini, the 65 min levels were not statistically different ($P > 0.10$).

Effects on CCK-8- and TPA-induced amylase secretion

30 min after the incubation was started, either CCK-8 (10^{-8} M), TPA (10^{-6} M) or no stimulant was added. The increase in amylase secretion above basal was calculated for the 30 min period after stimulation. CCK-8 stimulated amylase secretion in control and in pertussis toxin-treated acini with and without IBMX present (Table I, second line). Although the stimulatory effect of CCK-8 appeared to be potentiated in toxin-treated acini incubated in the absence of IBMX, this effect was not statistically significant ($0.05 < P < 0.10$). IBMX gave a 2-fold increase of the CCK-8 effect in both control and toxin-treated acini. In the presence of IBMX, the potentiating effect of pertussis toxin on CCK-8-stimulated amylase release was significant ($P < 0.01$).

The stimulatory effect of TPA (10^{-6} M) on amylase secretion, which was much smaller than that of CCK-8 (10^{-8} M), was also potentiated in the presence of IBMX ($P < 0.01$ and $P < 0.05$ in control and toxin-treated acini, respectively). Pertussis toxin itself, however, did not significantly affect TPA-induced amylase secretion ($P > 0.10$) and also the potentiating effect of IBMX on the TPA response in toxin-treated acini was not significantly different from that in control acini ($P > 0.10$).

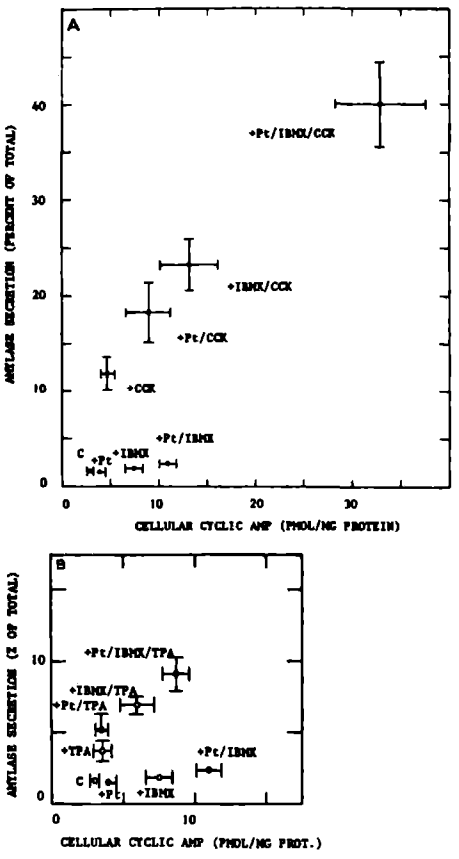


Fig. 2. The potentiating effect of increased cellular cyclic AMP levels on amylase secretion induced by either CCK-8 or TPA. Rabbit pancreatic acini incubated in the absence or presence of pertussis toxin (500 ng/ml) for 2 h at 37°C were resuspended in fresh incubation solution containing 0 or 10^{-4} M IBMX. After 30 min either 10^{-8} M CCK-8 (A), 10^{-6} M TPA (B) or no stimulant was added. Details on sampling and calculations are given in the legend of Table I.

Effects on cellular cyclic AMP levels in CCK-8 and TPA-stimulated acini

Acinar cell cyclic AMP levels were measured 35 min after stimulation. Upon stimulation with CCK-8, cyclic AMP levels were slightly but significantly increased above control levels in both control and toxin-treated acini (Table I, sixth line). The acinar cell cyclic AMP concentration in toxin-treated acini was about 2-times as high as in control acini.

In the presence of IBMX, 10^{-8} M cholecystokinin increased the cellular cAMP concentration 1.8-times that in control acini and 3.2-times that in toxin-treated acini, resulting in final cyclic AMP concentrations in toxin-treated acini 2.5-times those in control acini.

The effects of IBMX and/or pertussis toxin pretreatment on cyclic AMP levels remained virtually unchanged in the presence of 10^{-6} M TPA, indicating that TPA does not directly or indirectly influence acinar cell cyclic AMP levels.

In Fig. 2 acinar cell cyclic AMP levels are plotted against amylase secretion. This figure shows that the increase in cyclic AMP concentration alone did not lead to enhanced amylase secretion, but dose-dependently potentiated the secretory response to CCK-8 (A) as well as to TPA (B).

Discussion

The main observation in this paper is that cholecystokinin, one of the calcium-mobilizing pancreatic secretagogues, is able to significantly activate rabbit pancreatic acinar cell adenylate cyclase provided that the inhibitory guanine nucleotide-binding regulatory protein of the adenylate cyclase system (G_i) is inactivated.

Inactivation of G_i was achieved by incubating pancreatic acini with pertussis toxin under the conditions (500 ng/ml, 2 h) described in the literature as being appropriate for the toxin to exhibit its modifying effect on the α-subunit of G_i [29–30,37]. The results obtained indicate that the acinar cell G_i was indeed inactivated by pretreat-

ment with pertussis toxin since, as in other toxin-treated tissues (for a review, see Ref 38), the cyclic AMP response to an agent which stimulates adenylate cyclase activity was found to be enhanced

Our data show that the basal adenylate cyclase activity is normally slightly inhibited by G_i since, provided that IBMX is present, the increase in cellular cyclic AMP concentration in acini pretreated with pertussis toxin is about 1 6-times as high as in control acini This observation is in agreement with the finding by Cerione et al [39], i e , that reconstitution of G_i purified from human erythrocytes together with the adenylate cyclase catalytic subunit purified from bovine caudate nucleus markedly reduced basal adenylate cyclase activity In the absence of IBMX pertussis toxin pretreatment does not significantly affect acinar cell cyclic AMP levels This indicates that the relatively slightly increased cyclic AMP production is fully counteracted by the activity of cyclic AMP phosphodiesterase

Both in the absence and in the presence of IBMX, in control as well as in pertussis toxin-pretreated acini, basal amylase secretion is hardly or not affected Therefore, there is no indication that pertussis toxin interferes with pancreatic enzyme secretion other than by altering adenylate cyclase activity

The fact that pertussis toxin was found to increase the ability of hormones to stimulate adenylate cyclase activity and cyclic AMP accumulation has also been described for a variety of tissues [38] It suggests that in the intact pancreatic acinar cell the ability of cholecystokinin to activate adenylate cyclase greatly depends upon the activity of G_i and in fact is largely suppressed by G_i This is further supported by our observation that CCK-8, even in the absence of IBMX, substantially increases cellular cyclic AMP in acinar cells pretreated with pertussis toxin Apparently cyclic AMP phosphodiesterase activity is too low to fully eliminate the amount of cyclic AMP then produced

Our observation that cholecystokinin is able to increase cAMP levels in intact acini is in line with earlier reports that the hormone stimulates adenylate cyclase in broken cell preparations [9–11] It suggests the presence of cholecystokinin receptors able to couple with the stimulatory guanine nucleotide-binding regulatory protein (G_s) of the acinar cell adenylate cyclase system The presence of both a cholera toxin-sensitive α_s-subunit of G_s and a pertussis toxin-sensitive α_i-subunit of G_i coupled to the adenylate cyclase system in rat pancreatic membranes has already been reported by Lambert et al [40]

Now that we have shown that inactivation of G_i enables CCK-8 to substantially stimulate acinar cell adenylate cyclase activity, it remains to be elucidated whether the inhibitory action of G_i on CCK-8-induced activation of the adenylate cyclase is due to (i) the inability of the secretagogue, when used at concentrations which optimally stimulate enzyme secretion, to overcome some tonic inhibitory control exerted by G_i over the activity of the adenylate cyclase (ii) direct activation of G_i by CCK-8 binding to an inhibitory receptor, or (iii) indirected activation of G_i by CCK-8 activating the calcium messenger system In the latter case it may be speculated that one of the crucial enzymes of this messenger system, protein kinase C, is involved The fact that pancreatic secretagogues such as vasoactive intestinal peptide or secretin, which do not activate the calcium messenger system, substantially activate the acinar cell adenylate cyclase and increase the acinar cell cAMP concentration in the absence of pertussis toxin and/or IBMX may be in favour of the third explanation Coupling of G_i to the catalytic subunit of the adenylate cyclase as a consequence of the activation of the calcium messenger system may also explain the fact that in broken cell preparations the adenylate cyclase system is much better activated by cholecystokinin than in intact cells It also may explain the inhibitory effect of CCK-8 on cAMP levels increased by secretin and vasoactive intestinal polypeptide in guinea pig acinar cells [10]

The present results demonstrate that a positive correlation exists between cellular cyclic AMP levels and the extent of potentiation of the secretory response to an agent (cholecystokinin) activating the calcium messenger system and an agent (TPA) activating the C-kinase part of this system This is in line with our earlier observation that forskolin-induced increases in acinar cell cyclic AMP potentiate the secretory response to

CCK-8 as well as to the calcium ionophore A23187, without affecting basal amylase secretion [14] Therefore, the role for cyclic AMP in pancreatic enzyme secretion stimulated by cholecystokinin seems rather to be a modulatory one

Finally, the fact that pertussis toxin pretreatment of pancreatic acinar cells does not result in inhibition of CCK-8-induced increases in $[Ca^{2+}]_i$ or amylase secretion clearly demonstrates that, as in some [41–45], but not all [25–32] tissues, the GTP-binding protein postulated to mediate between calcium-mobilizing receptors and $PI\,P_2$-phosphodiesterase in the pancreatic acinar cell is not sensitive to pertussis toxin. The same conclusions were drawn by Merritt et al. [46] who recently showed that in permeabilized rat pancreatic acinar cells $GTP_\gamma S$-induced inositol trisphosphate formation was not inhibited by pertussis toxin.

References

1 Williamson, J R , Cooper, R H , Joseph, S K and Thomas, A P (1985) Am J Physiol 248 C203–C216
2 Berridge, M J (1985) Sci Am 253, 124 134
3 Orchard, J L , Davis, J S , Larson, R E and Farese, R V (1984) Biochem J 217, 281–287
4 Putney, J W , Burgess, G M , Halenda, S P , McKinney, J S and Rubin, R P (1983) Biochem J 212, 483–488
5 Pandol, S J , Thomas, M W , Schoeffield, M S , Sachs, G and Muallem, S (1985) Am J Physiol 248, G551–G560
6 Rubin, R P , Godfrey, P P , Chapman, D A and Putney, J W (1984) Biochem J 219, 655–659
7 Ochs, D L , Korenbrot, J I and Williams, J A (1985) Am J Physiol 249, G389–G398
8 Willems, P H G M , Van Nooij, I G P and De Pont, J J H H M (1986) Biochim Biophys Acta 888, 255–262
9 Rutten, W J , De Pont, J J H H M and Bonting, S L (1972) Biochim Biophys Acta 274, 201–213
10 Long, B W and Garner, J D (1977) Gastroenterology 73, 1008–1014
11 Svoboda, M , Lambert, M and Christophe, J (1981) Biochim Biophys Acta 675, 46–61
12 Renckens, B A M , Van Emst-De Vries, S E , De Pont, J J H H M and Bonting, S L (1980) Biochim Biophys. Acta 630, 511–518
13 Gardner, J D , Suthiff, V E , Walker, M D and Jensen, R T (1983) Am J Physiol 245, G676–G680
14 Willems, P H G M , Fleuren-Jakobs, A M M , De Pont, J J H H M and Bonting, S L (1984) Biochim Biophys Acta 802, 209–214
15 Rodbell, M (1980) Nature 284, 17–22
16 Gilman, A G (1984) Cell 36, 577–579
17 Houslay, M D (1984) Trends Biochem Sci 9, 39–40
18 Ui, M (1984) Trends Pharmacol Sci. 5, 277–279
19 Bokoch, G M , Katada, T , Northup, J K , Ui, M and Gilman, A G (1984) J Biol Chem 259, 3560–3567
20 Haslam, R J and Davidson, M M L (1984) J Receptor Res 4, 605–629
21 Cockcroft, S and Gomperts, B D (1985) Nature 314, 534–536
22 Joseph, S K (1985) Trends Biochem Sci 10, 297–298
23 Litosch, I , Wallis, C and Fain, J N (1985) J Biol Chem 260, 5464–5471
24 Smith, C D , Cox, C C and Snyderman, R (1986) Science 232, 97–100
25 Brandt, S J , Dougherty, R W , Lapetina, E G and Niedel, J E (1985) Proc Natl Acad Sci USA 82, 3277–3280
26 Krause, K H , Schlegel, W , Wollheim, C B , Andersson, T , Waldvogel, F F A and Lew, P D (1985) J Clin Invest. 76, 1348–1354
27 Smith, C D , Lane, B C , Kusaka, I , Verghese, M W and Snyderman, R (1985) J Biol Chem 260, 5875–5878
28 Nakamura, T and Ui, M (1985) J Biol Chem 260, 3584–3593
29 Volpi, M , Naccache, P H , Molski, T F P , Shefcyk, J , Huang, C-K , Marsh, M L , Munoz, J , Becker, E L and Sha'afi, R (1985) Proc Natl Acad Sci USA 82, 2708–2712
30 Molski, T F P , Naccache, P H , Marsh, M L , Kermode, J , Becker, E L and Sha'afi, R L (1984) Biochem Biophys Res Commun 124, 644–650
31 Bradford, P G and Rubin, R P (1985) FEBS Lett 183, 317–320
32 Backlund, P S , Jr , Meade, B D , Manclark, C R , Cantoni, G L and Aksamit, R R (1985) Proc Natl Acad Sci USA 82, 2637–2641
33 Eagle, H (1959) Science 130, 432–437
34 Ceska, M , Hultman, E and Ingelman, B (1969) Experientia 25, 555–556
35 Tsien, R Y , Pozzan, T and Rink, T J (1982) Nature 295, 68–71
36 Brown, B L , Albano, J D M , Ekins, R P and Sgherzi, A M (1971) Biochem. J 121, 561–563
37 Bokoch, G M and Gilman, A G (1984) Cell 39, 301–308
38 Hewlett, E L , Cronin, M J , Moss, J , Anderson, H , Myers, G A and Pearson, R D (1984) Adv Cyclic Nucl Protein Phosphoryl Res 17, 173–182
39 Cerione, R A , Staniszewski, C , Caron, M G , Lefkowitz, R J , Codina, J and Birnbaumerr, L (1985) Nature 318, 293–295
40 Lambert, M , Svoboda, M , Furnelle, J and Christophe, J (1985) Eur J Biochem 147, 611–617
41 Masters, S B , Martin, M W , Harden, T K and Brown, J H (1985) Biochem J 227, 933–937
42 Lynch, C J , Prpic, V , Blackmore, P F and Exton, J H (1986) Mol Pharmacol 29, 196–203
43 Grandt, R , Greiner, C , Zubin, P and Jakobs, K H (1986) FEBS Lett 196, 279–283
44 Martin, T F J , Lucas, D O , Bajjaheh, S M and Kowalchyk, J A (1986) J Biol Chem. 261, 2918–2927
45 Schimmel, R J and Elliott, M E (1986) Biochem Biophys Res Commun. 135, 823–829
46 Merritt, J E , Taylor, C W , Rubin, R P and Putney, J W (1986) Biochem. J 236, 337–343

Chapter 4

Inhibitory effect of TMB-8 on cholecystokinin-induced long-term elevated free cytosolic calcium levels in rabbit pancreatic acinar cells.

WILLEMS, P.H.G.M. and

DE PONT, J.J.H.H.M.

Department of Biochemistry, University of Nijmegen,
P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

The abbreviations are: CCK-8, cholecystokinin octapeptide; CCh, carbachol; $[Ca^{2+}]_i$, free cytosolic calcium concentration; quin2, 2-((2-(bis((carbonyl)methyl)amino)-5-methylphenoxy)-methyl-6-methoxy-8-(bis((carbonyl)methyl)amino)quinoline; quin2/AM, quin2-tetra(acetoxymethyl)ester; HEPES,4-(2-hydroxyethyl)- 1-piperazin-ethanesulfonic acid; EGTA, ethyleneglycolbis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

SUMMARY

The effects of the potent muscle relaxant 8 (N,N-diethylamino)-octyl 3,4,5-trimethoxybenzoate (TMB-8) on secretagogue-induced changes in free cytosolic calcium levels ($[Ca^{2+}]_i$) in rabbit pancreatic acinar cells were investigated using the calcium selective indicator, quin2.

Both the C-terminal octapeptide of cholecystokinin (CCK-8) and the cholinergic receptor agonist, carbachol, increased $[Ca^{2+}]_i$ from a resting level of 150 nM to a peak value of about 700 nM, independent of the presence of extracellular calcium. In the absence of extracellular calcium, however, $[Ca^{2+}]_i$ then rapidly decreased to the resting level, whereas in the presence of extracellular calcium, this decrease was more slowly and a post-stimulatory $[Ca^{2+}]_i$ of approximately 200 nM, which was significantly higher than before stimulation, was maintained for longer periods of time, The higher steady-state level, reached within 2 min after the onset of stimulation, is probably due to a stimulus-induced increase in plasma membrane permeability for calcium, since both the presence of a receptor antagonist and the absence of extracellular calcium prevented stimuli from maintaining $[Ca^{2+}]_i$ elevated for longer periods of time.

TMB-8 only slightly and transiently decreased $[Ca^{2+}]_i$ in unstimulated acinar cells. The drug did not affect the CCK-8-induced increase in $[Ca^{2+}]_i$. The CCK-8-induced, extracellular calcium dependent long-term elevated $[Ca^{2+}]_i$, however, was blocked by TMB-8. Furthermore, in acinar cells stimulated in the absence of extracellular calcium, TMB-8 decreased $[Ca^{2+}]_i$ to below the resting level. However, resting levels were restored upon the readdition of calcium to the medium.

Our results suggest that in rabbit pancreatic acinar cells, TMB-8 lowers the free cytosolic calcium concentration by stimulating the calcium extrusion mechanism rather than by blocking the secretagogue-induced increase in plasma membrane permeability for calcium.

INTRODUCTION

Enzyme secretion studies have shown that in pancreatic exocrine tissue stimulated with either cholecystokinin (CCK) or acetylcholine the rate of enzyme secretion is initially relatively high but then rapidly declines to a much lower level, which is maintained, however, for prolonged periods of time [1-3]. It has also been shown that the initial phase of the secretory response is independent of the presence of extracellular calcium [1,2], whereas the sustained response is blocked completely in its absence [4,5]. In the latter case, stimulated enzyme secretion was shown to be restored upon readdition of calcium to the medium.

Experiments in which $^{45}Ca^{2+}$ has been used to measure calcium fluxes also show a biphasic pattern in that a stimulated calcium efflux is followed by a stimulated influx [6-8]. In these studies the secretagogue-induced calcium influx was interpreted to be due to a secretagogue-induced increase in the plasma membrane permeability for calcium [9,10], resulting in a net accumulation of calcium [7].

Finally, experiments in which changes in free cytosolic calcium concentration ($[Ca^{2+}]_i$) were measured by means of the fluorescent calcium indicator, quin2, have shown that the secretagogues CCK and acetylcholine induce an almost instantaneous increase in $[Ca^{2+}]_i$ followed by a more gradual decrease to near resting levels [11-14]. The secretagogue-induced initial increase in $[Ca^{2+}]_i$ is independent of the presence of extracellular calci-

um, suggesting that it represents the inositol 1,4,5-trisphosphate (IP_3) stimulated release of calcium from an intracellular store [11,12,15]. On the other hand, after stimulation with CCK or a cholinergic receptor agonist, $[Ca^{2+}]_i$ does not return to prestimulatory levels but remains elevated for prolonged periods of time [11,13]. This sustained calcium response, however, is not observed in the absence of extracellular calcium [11]. Therefore, there are good indications that intracellularly stored calcium plays a role in the initial secretory response, whereas extracellular calcium is involved in sustained secretion.

The potent muscle relaxant 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB-8) has been postulated to act as an intracellular calcium antagonist [16-18]. Inhibitory effects of this drug on stimulus-evoked processes have therefore frequently been used to support other evidence that release of calcium from an intracellular pool is involved in the mechanism of action of calcium mobilizing stimuli (for a review, see Ref.19). Recently, however, we [20] and others [21] have shown that TMB-8 does not affect stimulus-evoked initial increases in $[Ca^{2+}]_i$, but has several other effects among which a potentiating effect [20,22-24] and an antimuscarinic effect [20,25].

In this study we investigated whether (part of) the effects of TMB-8 could be due to inhibition of the sustained calcium influx which is essential for prolonged enzyme secretion. We therefore applied the quin2 technique on rabbit pancreatic acinar cells and demonstrated indeed the presence of a secretagogue-induced permeability increase for calcium. TMB-8 was shown to be able to block the increase in cytosolic calcium, but the results indicate that TMB-8 activates the calcium extrusion mechanism rather than that it inhibits the permeability increase.

MATERIALS AND METHODS

Preparation of rabbit pancreatic acinar cells, loading of the acinar cells with the calcium selective fluorescent indicator, quin2 and fluorescence measurements were carried out as previously described [15,20]. Cytosolic free calcium concentrations were calculated according to Tsien et al.[26].

The C-terminal octapeptide of cholecystokinin has been synthesized by Dr. H.M. Rajh in the Department of Organic Chemistry, University of Nijmegen. Carbachol was purchased from ACF Chemiefarma, Maarsen, The Netherlands. Atropine was obtained from Merck, Darmstadt, F.R.G.; TMB-8 from Aldrich Chem. Co., Milwaukee, WI, U.S.A.; soybean trypsin inhibitor and quin2/AM from Sigma, St. Louis, MO, U.S.A.; collagenase from Cooper Biomedical Inc., Malvern, PA, U.S.A. and hyaluronidase from Boehringer, Mannheim, F.R.G. All other chemicals were of reagent grade.

RESULTS

Rabbit pancreatic acinar cells loaded with the fluorescent calcium indicator, quin2, were used to calculate the free cytosolic calcium concentration both under stimulated and unstimulated conditions.

In unstimulated acinar cells $[Ca^{2+}]_i$ was calculated to be 150 nM (S.E. 9; 5 different cell preparations). When acinar cells were resuspended in medium to which no calcium was added and the extracellular calcium concentration was reduced further by adding 0.2 mM of the calcium chelating agent, EGTA, no decrease in $[Ca^{2+}]_i$ was observed. $[Ca^{2+}]_i$ did also not increase in those experiments in which, after a 1 min incubation in the presence of EGTA, the medium calcium concentration was increased to 1.0 mM.

Both CCK-8 (3.10^{-8} M) and carbachol (10^{-5} M) increased $[Ca^{2+}]_i$ within seconds to about 800 nM. Figure 1 shows that this initial increase was independent of the presence of extracellular calcium. In the absence of extracellular calcium, however, $[Ca^{2+}]_i$ more rapidly declined and returned to prestimulatory levels, whereas in its presence, $[Ca^{2+}]_i$ remained elevated at approx. 200 nM for prolonged periods of time. Readdition of calcium to the medium, after stimulation in the absence of

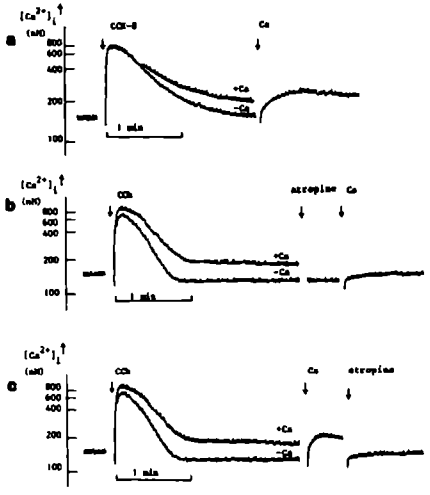


Figure 1. Effects of extracellular calcium on CCK-8- and carbachol-induced changes in $[Ca^{2+}]_i$ in quin2-loaded rabbit pancreatic acinar cells. Immediately before use, acinar cells loaded with quin2 were resuspended in a HEPES/TRIS medium (pH 7.4) to which no calcium was added and which was thoroughly gassed with O_2. The cells were transferred to a cuvet placed in a spectrofluorometer and preincubated for 3 min at 37°C. Stirring of the cell suspension was started and 0.2 mM EGTA was added (-Ca). In those experiments in which extracellular calcium was present (+Ca), after 1 min the medium calcium concentration was increased to 1.0 mM. CCK-8 (3.10^{-8} M), Carbachol (CCh; 10^{-5} M), atropine (3.10^{-5} M) and calcium (final concentration 1.0 mM) were added at the times indicated. In the recordings shown, corrections were made for the effects of EGTA and calcium, due to the presence of extracellular quin2. This figure is typical for three different acinar cell preparations.

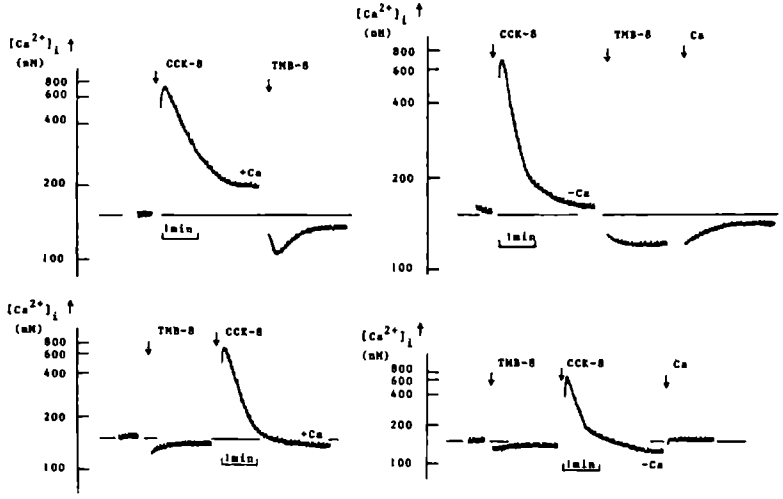


Figure 2. Effects of TMB-8 on CCK-8-induced changes in $[Ca^{2+}]_i$ in quin2-loaded rabbit pancreatic acinar cells. After 3 min of preincubation in the spectrofluorometer (see legends to figure 1), stirring of the cell suspension was started and 0.2 mM EGTA was added (-Ca). In those experiments in which extracellular calcium was present (+Ca), after 1 min the medium calcium concentration was increased to 1.0 mM. CCK-8 (3.10^{-9} M), TMB-8 (250 µM) and calcium (final concentration 1.0 mM) were added at the times indicated. In the recordings shown, corrections were made for the effects of EGTA and calcium, due to the presence of extracellular quin2. This figure is typical for five different acinar cell preparations.

extracellular calcium, resulted in a rapid increase in $[Ca^{2+}]_i$ to the same elevated levels as observed in cells stimulated in the presence of extracellular calcium. The latter increase in $[Ca^{2+}]_i$ was blocked when the cholinergic receptor antagonist atropine was added prior to calcium (Fig. 1b). In the presence of extracellular calcium, the carbachol-induced long-term elevated $[Ca^{2+}]_i$ decreased immediately to prestimulatory levels when atropine was added (Fig. 1 b,c).

Figure 2 shows that addition of 250 μM TMB-8 to unstimulated acinar cells resulted in a small but transient decrease in $[Ca^{2+}]_i$ (Fig. 2 c,d). As shown before [20], the CCK-8-induced initial increase in $[Ca^{2+}]_i$ was not affected by the drug. However, after stimulation with CCK-8 in the presence of extracellular calcium and TMB-8, $[Ca^{2+}]_i$ did not return to the sustained elevated levels but decreased to the prestimulatory level (Fig. 2c), whereas in the absence of extracellular calcium, $[Ca^{2+}]_i$ decreased even to below the resting level (Fig. 2d). In the latter case prestimulatory free cytosolic calcium levels were restored upon readdition of calcium to the medium. Figure 2a shows that, in the presence of extracellular calcium, TMB-8 almost immediately decreased the CCK-8-induced long-term elevated $[Ca^{2+}]_i$ to below the resting level followed by a more gradually increase to prestimulatory levels. Addition of TMB-8 to acinar cells, stimulated in the absence of extracellular calcium, at the time $[Ca^{2+}]_i$ had reached resting levels again, resulted in an almost immediate, but not a transient, decrease in $[Ca^{2+}]_i$ (Fig. 2b). In the latter case, $[Ca^{2+}]_i$ did not return to prestimulatory levels unless calcium was readded to the medium.

TMB-8, at a concentration of 250 μM did not affect the intactness of the acinar cells as was demonstrated by Trypan blue exclusion immediately after measurement of fluorescence.

DISCUSSION

At least three structures are involved in regulating the free cytosolic calcium concentration: plasma membrane [27], mitochondria [28] and a non-mitochondrial pool probably the endoplasmic reticulum [6,29,30]. In the resting cell, calcium pumps localized in the above structures maintain the $[Ca^{2+}]_i$ at about 150 nM. The fact that in the absence of extracellular calcium no decrease in $[Ca^{2+}]_i$ is observed, indicates that the passive leakage of calcium is very low and/or that a very efficient compensatory mechanism exists. Addition of 250 μM TMB-8 to unstimulated acinar cells slightly but transiently decreased $[Ca^{2+}]_i$, suggesting that the calcium balance is temporary disturbed. In adrenal glomerulosa cells, TMB-8 was also found to decrease the resting $[Ca^{2+}]_i$ [31]. In these cells, however, the TMB-8-induced decrease was permanent and it was suggested that the drug affected basal calcium influx.

Upon stimulation with CCK-8 or carbachol an almost instantaneous increase in $[Ca^{2+}]_i$ is observed which is independent of the presence of extracellular calcium and which is therefore thought to represent the inositol 1,4,5-trisphosphate-mediated release of calcium from an intracellular store, most probably the endoplasmic reticulum [32]. As we have shown before, this initial phase of the calcium response was not affected by TMB-8 [20].

After having reached its peak value, $[Ca^{2+}]_i$ decreased within 2 min to a plateau level of about 200 nM, which is maintained for a prolonged period of time. This sustained phase of the calcium response clearly depends on the presence of extracellular calcium and is therefore most likely due to an increased influx of calcium. The fact that that the sustained phase of the calcium response is blocked completely by a receptor antagonist, indicates that receptor occupation is required. Although it is not clear yet which mechanism underlies this stimulus-induced increase in plasma membrane permeability for calcium, there are some indications that inositol 1,3,4,5-tetrakisphosphate, might be involved [33]. Most interestingly, TMB-8 blocks this CCK-8-induced long-term elevated $[Ca^{2+}]_i$. Inhibition was complete at a concentration of 250 μM, whereas at lower concentrations (50 - 100 μM), the drug markedly but not completely decreased this long-term elevated $[Ca^{2+}]_i$

(data not shown). Observations like these strongly suggest that TMB-8 inhibits the CCK-8-induced increase in plasma membrane permeability for calcium. In fact, in adrenal glomerulosa cells, TMB-8 is thought to inhibit angiotensin-2 stimulated aldosterone secretion by inhibiting the influx of extracellular calcium [34], whereas in cultured bovine adrenal chromaffin cells it is speculated that the drug inhibits calcium uptake by blocking a cholinergic receptor-coupled calcium channel [35]. Our observation, however, that in acinar cells stimulated in the absence of extracellular calcium, TMB-8 decreases $[Ca^{2+}]_i$ to below the resting level and that the resting level is restored upon readding calcium to the medium, is not in line with the above hypothesis. This observation rather suggests that the drug activates some kind of calcium extrusion mechanism. If this is the case then the transient decrease in $[Ca^{2+}]_i$ observed upon addition of TMB-8 to unstimulated cells can be explained by a stimulated calcium extrusion process, followed by a compensatory release of calcium from an intracellular store. In stimulated cells this intracellular store is emptied but in the presence of extracellular calcium, the influx of calcium compensates for the increased extrusion. In the absence of extracellular calcium no compensatory calcium pool is present and $[Ca^{2+}]_i$ decreases to below the resting level, which is restored upon readdion of calcium to the medium.

From the results presented in this study it is clear that TMB-8 inhibits secretagogue-induced long-term elevated free cytosolic calcium levels in rabbit pancreatic acini and that this inhibitory action of TMB-8 is rather due to a stimulatory effect on the process of calcium extrusion than to an inhibitory effect on the receptor-mediated increase in plasma membrane permeability for calcium. More detailed investigations are needed, however, to clarify the exact mechanism of action of this drug.

REFERENCES

1. Schulz, I. (1980) Am. J. Physiol. 239, G335-G347
2. Pandol, S.J., Thomas, M.W., Schoeffield, G.S., Sachs, G. and Muallem, S. (1985) Am. J. Physiol. 248, G551-G560
3. Campbell, A.K. (1983) Intracellular Calcium: Its universal role as regulator, John Wiley and Sons Limited, Chichester
4. Schreurs, V.V.A.M., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1976) Biochim. Biophys. Acta 419, 320-330
5. Petersen, O.H. and Ueda, N. (1976) J. Physiol. 254, 583-606
6. Stolze, H. and Schulz, I. (1980) Am. J. Physiol. 238, G338-G348
7. Renckens, B.A.M., Schrijen, J.J., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1978) Biochim. Biophys. Acta 544, 338-350
8. Gardner, J.D., Conlon, T.P., Klaeveman, H.L., Adams, T.D. and Ondetti, M.A. (1975) J. Clin. Invest. 56, 366-375
9. Kondo, S. and Schulz, I. (1976) Biochim. Biophys. Acta 419, 76-92
10. Kondo, S. and Schulz, I. (1976) J. Membr. Physiol. 29, 185-203
11. Ochs, D.L., Korenbrot, J.I. and Williams, J.A. (1985) Am. J. Physiol. 249, G389-G398
12. Pandol, S.J., Schoeffield, M.S., Sachs, G. and Muallem, S. (1985) J. Biol. Chem. 260, 10081-10086
13. Merritt, J.E. and Rubin, R.P. (1985) Biochem. J. 230, 151-159
14. Bruzzone, R., Pozzan, T. and Wollheim, C.B. (1986) Biochem. J. 235, 138-143
15. Willems, P.H.G.M., van Nooij, I.G.P., Haenen, H.E.M.G. and De Pont, J.J.H.H.M. (1987) Biochim. Biophys. Acta 930, 230-236
16. Malagodi, M.H. and Chiou, C.Y. (1974) Eur. J. Pharmacol. 27, 25-33
17. Malagodi, M.H. and Chiou, C.Y. (1974) Pharmacol. 12, 20-31
18. Chiou, C.Y. and Malagodi, M.H. (1975) Br. J. Pharmacol. 53, 279-285
19. Brand, M.D. and Felber, S.M. (1984) Biochem. J. 224, 1027-1030
20. Willems, P.H.G.M., van Nooij, I.G.P. and De Pont, J.J.H.H.M. (1986) Biochim. Biophys. Acta 888, 255-262

21. Simpson, A.W.M., Hallam, T.J. and Rink, T.J. (1984) FEBS Lett. 176, 139-143
22. Shaw, J.O. and Lyons, R.M. (1982) Biochim. Biophys. Acta 714, 492-499
23. Gomez-Cambronero, J., Inarrea, P., Alonso, F. and Sanchez Crespo, M (1984) Biochem. J. 219, 419-424
24. Wiedenkeller, D.E. and Sharp, G.W.G. (1984) Endocrinol. 114, 116-119
25. Tennes, K.A., Kennedy, J.A. and Roberts, M.L. (1983) Biochem. Pharmacol. 32, 2118-2120
26. Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) Nature 295, 68-71
27. Petersen, O.H. and Iwatsuki, N. (1978) Ann. N.Y. Acad. Sci. 307, 599-617
28. Dormer, R.L. and Williams, J.A. (1981) Am. J. Physiol. 240, G130-G140
29. Streb, H.,Irvine, R.F., Berridge, M.J. and Schulz, I.(1983) Nature 306, 67-69
30. Bayerdorffer, E., Eckhardt, W.,Haase, W. and Schulz, I. (1984) J. Membr. Biol. 81, 69-82
31. Kojima, I., Shibata, H. and Ogata, E. (1986) Biochim. Biophys. Acta 888, 25-29
32. Berridge, M.J. (1985) Sci. Am. 253, 124-134
33. Irvine, R.F. and Moor, R.M. (1986) Biochem. J. 240, 917-920
34. Kojima, I., Kojima, K and Rasmussen, H (1985) Biochem. J. 232, 87-92
35. Sasakawa, N., Yamamoto, S., Ishii, K. and Kato, R. (1984) Biochem. Pharmacol. 33, 4063-4067

Chapter 5

Stimulatory and inhibitory effects of TMB-8 on pancreatic enzyme secretion.

WILLEMS, P.H.G.M.,

VAN NOOIJ, L.G.P. and

DE PONT, J.J.H.H.M.

Department of Biochemistry, University of Nijmegen,
P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

Biochimica et Biophysica Acta 888 (1986) 255–262
Elsevier

BBA 11853

Stimulatory and inhibitory effects of TMB-8 on pancreatic enzyme secretion

P.H.G.M. Willems, I.G.P. Van Nooij and J.J.H.H.M. De Pont

Department of Biochemistry, University of Nijmegen, P O Box 9101, 6500 HB Nijmegen (The Netherlands)

(Received 1 May 1986)

Key words TMB-8, Enzyme secretion, Phorbol ester, (Pancreas)

The putative intracellular calcium antagonist 3,4,5-trimethoxybenzoate 8-(diethylamino)-octyl ester (TMB-8) affects carbachol-induced enzyme secretion from rabbit pancreatic acini in a different way than it does that induced by either the C-terminal octapeptide of cholecystokinin (CCK-8), the phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or the calcium ionophore, A23187. In the presence of TMB-8 the dose-response curve for carbachol-induced amylase release shifts to the right, suggesting competitive antagonism at the muscarinic receptor. The hypothesis that TMB-8 acts as a muscarinic receptor antagonist is supported by the observation that TMB-8 dose-dependently inhibits the carbachol-, but not CCK-8-induced increases in cytosolic free calcium, measured in acinar cells by means of the fluorescent calcium indicator quin2. At a concentration of 100 μM, TMB-8 maximally potentiates the secretory response to suboptimal, but not (supra)optimal, concentrations of CCK-8. At the same concentration the drug also potentiates TPA- and A23187-induced enzyme secretion. Cytosolic free calcium levels and CCK-8-induced increases in cytosolic free calcium remain unaffected by 100 μM TMB-8. The above results strongly suggest that potentiation occurs at or beyond the site of interaction between the diacylglycerol- and the Ca^{2+}-activated pathways. At concentrations beyond 100 μM the potentiating effect of TMB-8 declines and, finally, at a concentration of 500 μM the drug completely abolishes the secretory response to CCK-8 and TPA. Basal enzyme secretion, however, remains unaffected. At 500 μM severe side effects are observed as is shown by Trypan blue uptake, lactic dehydrogenase release and release of trapped quin2. It is concluded that at lower concentrations TMB-8 does not act as a specific intracellular calcium antagonist in pancreatic enzyme secretion and that inhibitory effects obtained with rather high concentrations of this drug should be treated with caution.

Introduction

In the exocrine pancreas both the receptor for cholecystokinin (CCK) and for acetylcholine are coupled to the calcium messenger system [1–4]. In secretion studies, the initial response to the above secretagogues has been shown to be independent of the presence of extracellular calcium, whereas in its absence the sustained response is abolished [1,5,6] Moreover, upon preincubation with calcium chelating agents the secretory response in the absence of extracellular calcium has been shown to diminish with time [1,5,7,8] Observations of the kind described above have led to the hypothesis that during the initial phase of the response, calcium is mobilized from intracellular stores, whereas the influx of extracellular calcium is needed to maintain the response [1,3,9,10]

The drug TMB-8 has been postulated to act as

Abbreviations TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)-octyl ester, CCK(-8), cholecystokinin(octapeptide), TPA, 12-*O*-tetradecanoylphorbol 13-acetate, quin2, 2-[(2-bis-(carboxymethyl)amino-5-methylphenoxy)methyl]-6-methoxyquinoline, quin2/AM, quin2-tetra-(acetoxymethyl)ester

Correspondence address Dr J.J.H.H.M. de Pont, Department of Biochemistry, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

an intracellular calcium antagonist [11–13] It could, therefore, be a useful tool to assess the relative requirement for calcium of intracellular and extracellular origin in pancreatic enzyme secretion

In preliminary experiments, however, a slight stimulatory effect of TMB-8 on the secretory response to the octapeptide of cholecystokinin (CCK-8) was observed This has led us to study the mechanism of action of this drug in pancreatic enzyme secretion in more detail

We conclude that TMB-8 has several effects in exocrine pancreatic secretion but is not a specific intracellular calcium antagonist in this system

Materials and Methods

Rabbit pancreatic acini were prepared essentially as described for acinar cells by Amsterdam and Jamieson [14], except that the divalent cation chelating step was omitted The method is based on two successive incubations with collagenase and hyaluronidase in a medium containing 0 1 mM Ca^{2+} After this digestion the tissue was dispersed by pipetting The suspension was filtered through nylon gauze and purified by centrifugation through a 4% albumin layer For the preparation of acinar cells the procedure of Amsterdam and Jamieson [14] including the calcium chelating step has been applied The intactness of the isolated acini and acinar cells has been demonstrated by Trypan blue exclusion

For secretion studies, acini were incubated in a Krebs-Ringer bicarbonate medium (pH 7 4) containing 119 mM NaCl, 3 5 mM KCl, 1 2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1 2 mM $CaCl_2$, 1 2 mM $MgCl_2$, 5 8 mM glucose, 1% bovine serum albumin, 0 2 mg/ml soybean trypsin inhibitor and an amino acid mixture according to Eagle [15] and 37°C The stimulants were added as indicated in the legends to the figures, and samples of the incubation mixture were removed at appropriate times for determination of both the amylase and lactic dehydrogenase activities At the end of the experiment, the acini were homogenized in the incubation medium Amylase activity was measured in the samples after rapid sedimentation of the acini and in the acinar homogenate by means of the Phadebas test [16] Lactic dehydrogenase activity was assayed with the technique of Kubowitz and Ott [17]

Intracellular calcium measurements have been carried out in acinar cells loaded with the fluorescent calcium indicator, quin2, as described by Ochs et al [18] After a 20 min incubation in the presence of 50 μM quin2/AM at 37°C, the medium was diluted 8-times and the cells were incubated for another 45 min Excess quin2/AM was then removed by two centrifugation steps for 5 min at $100 \times g$ Quin2-loaded acinar cells were resuspended in the above medium and kept at room temperature Samples removed for fluorescence measurements were centrifuged and the cells were resuspended in the above medium containing 1 0 mM $CaCl_2$, 1 0 mM $MgCl_2$ and 0 1% bovine serum albumin Fluorescence measurements were carried out at 37°C, using a Shimadzu RF-510 spectrofluorometer equipped with a magnetic stirrer and a thermostated cuvet holder For calibration of the quin2 signals digitonin (15 μg/ml, maximal fluorescence), Tris base (10 mM) and EGTA (2 mM, minimum fluorescence) were added sequentially In each experiment the same amount of unloaded acinar cells was treated in exactly the same way and corrections were made for the effects of the compounds added Calcium concentrations were calculated according to Tsien et al [19]

Materials

The C-terminal octapeptide of cholecystokinin was synthesized by Dr H M Rajh in the Department of Organic Chemistry, University of Nijmegen Carbachol was purchased from ACF Chemiefarma, Maarsen, The Netherlands A23187 was obtained from Calbiochem, La Jolla, CA, U S A , TMB-8 was from Aldrich Chem Co , Milwaukee WI, U S A , TPA, soybean trypsin inhibitor, bovine serum albumin and quin2/AM were from Sigma, St Louis, MO, U S A , collagenase was from Cooper Biomedical Inc , Malvern, PA, U S A and hyaluronidase was from Boehringer, Mannheim, F R G The Phadebas test kit for determination of amylase activity was purchased from Pharmacia, Uppsala, Sweden All other chemicals were commercial preparations of the highest obtainable purity

Results

Effects on CCK-8 induced amylase release

The increase in amylase release from rabbit pancreatic acini stimulated by the secretagogue CCK-8 is nearly linear with time during the first 30 min (Fig 1) But, whereas in the presence of 100 μM TMB-8 basal amylase release remains virtually unchanged, the secretory response to 10^{-8} M CCK-8 is potentiated. On the other hand, enzyme secretion induced by CCK-8 at a concentration of 10^{-6} M is not potentiated but slightly inhibited To investigate the effects of TMB-8 on CCK-8-induced amylase release in more detail, dose-response curves for TMB-8 in the presence of suboptimal (10^{-9} M and 10^{-8} M), optimal (10^{-7} M) and supraoptimal (10^{-6} M) concenntrations of CCK-8 were produced.

Fig 2 shows that the effect of TMB-8 on CCK-8 stimulated amylase release depends both on its own concentration and that of the secretagogue At TMB-8 concentrations above 100 μM the secretory response to CCK-8 is inhibited dose dependently leading to complete abolishment at 500 μM. Basal amylase secretion, however, remains unaffected, even with TMB-8 concentrations as high as 500 μM. At concentrations between 50 and 100 μM, TMB-8 potentiates the secretory response to suboptimal (10^{-9} M and 10^{-8} M) concentrations of CCK-8, whereas the stimulatory effect of optimal (10^{-7} M) and supraoptimal (10^{-6} M) concentrations of the hormone is slightly inhibited.

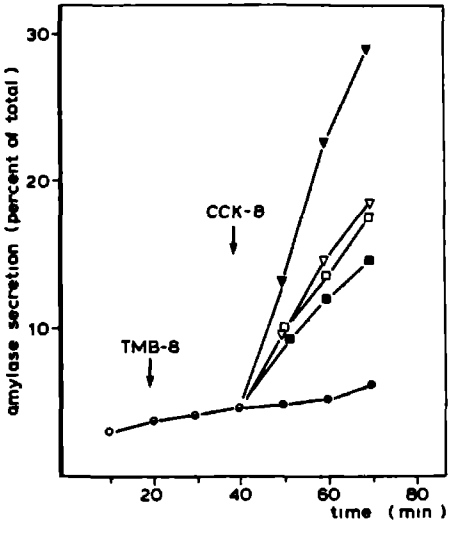


Fig. 1 Effects of CCK-8 and TMB-8, alone and in combination, on amylase release from rabbit pancreatic acini After preincubation for 20 min at 37°C TMB-8 (100 μM closed symbols) is added CCK-8 (10^{-8} M, ▽,▼, and 10^{-6} M, □,■) was added 20 min later Amylase release was measured at the indicated times, and is expressed as a percentage of the total amylase content of the acini at the beginning of incubation Basal release from 20 to 70 min is the same as that in the presence of TMB-8 (without CCK-8) This figure is typical of four experiments

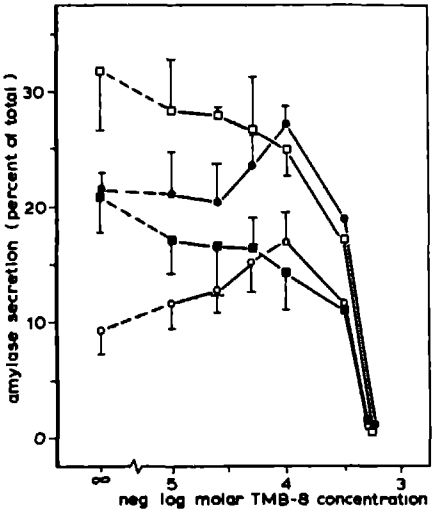


Fig. 2 Dose-inhibition curve for TMB-8 on CCK-8 stimulated amylase secretion from rabbit pancreatic acini Acini were incubated for 70 min at 37°C TMB-8, at the indicated concentrations, and CCK-8 (10^{-9} M, ○, 10^{-8} M, ●, 10^{-7} M, □; and 10^{-6} M, ■) were added after 20 min and 40 min, respectively Amylase release was measured at 0, 20, 40 and 70 min and is expressed as percentage of total amylase originally present in the acini The increase in amylase release at 70 min was corrected for the basal release at that time, determined by extrapolation from the release values at 0, 20 and 40 min in each experiment The values presented are the means ± S E. of 3–4 experiments

Effects on carbachol-induced amylase release

The dose-response curve for the effect of carbachol alone on amylase release from rabbit pancreatic acini shows a maximal effect at a concentration of 3 10^{-5} M (Fig 3). As with CCK-8 the stimulatory effect of carbachol is completely abolished at 500 μM TMB-8 At concentrations between 10 and 100 μM, however, TMB-8 affects carbachol-induced amylase release differently from the response to CCK-8. The stimulatory effect of suboptimal ($3 \cdot 10^{-6}$ M and 10^{-5} M) concentrations of carbachol is effectively inhibited by TMB-8 at concentrations ranging from 10 to about 100 μM The secretory response to optimal ($3 \cdot 10^{-5}$ M) and supraoptimal (10^{-4} M) concentrations of carbachol, however, is slightly inhibited at low (10–25 μM) and potentiated at intermediate (25–50 μM) concentrations of the drug Therefore, the potentiating effect of TMB-8 seems to overrule an inhibitory effect which is not seen upon stimulation with CCK-8. This observation would be in line with a competitive action of TMB-8 on the cholinergic receptor The latter is more apparent by the right-hand shift of the dose-response curve for carbachol in the presence of TMB-8 (Fig 4)

One interesting feature of potentiation by TMB-8 is that in the presence of the drug, amylase release does not exceed that induced by the maximally stimulating concentration of secretagogue alone (Figs 2 and 3)

Effects on TPA- and A23187-induced amylase release

The mechanism of action of TMB-8 on pancreatic enzyme secretion was examined further by investigating the effect of 100 μM TMB-8, shown to maximally potentiate the response to CCK-8, on amylase release induced by the phorbol ester,

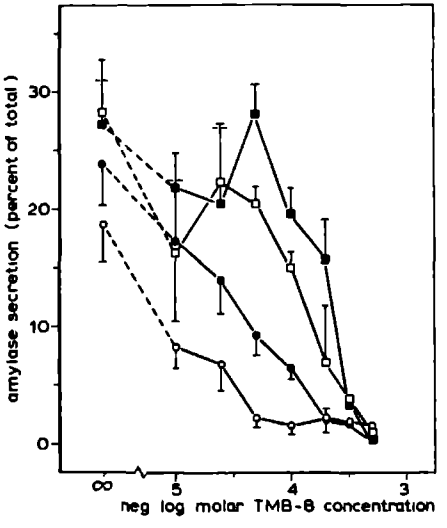


Fig. 3 Dose-inhibition curve for TMB-8 on carbachol stimulated amylase release from rabbit pancreatic acini Acini were incubated for 70 min TMB-8, at the indicated concentrations, and carbachol (3 10^{-6} M, ○, 10^{-5} M, ●, 3 10^{-5} M, □, and 10^{-4} M, ■) were added after 20 min and 40 min, respectively Details on sampling and calculations are given in the legend of Fig. 2 The values presented are the means ± S E. of 3–4 experiments.

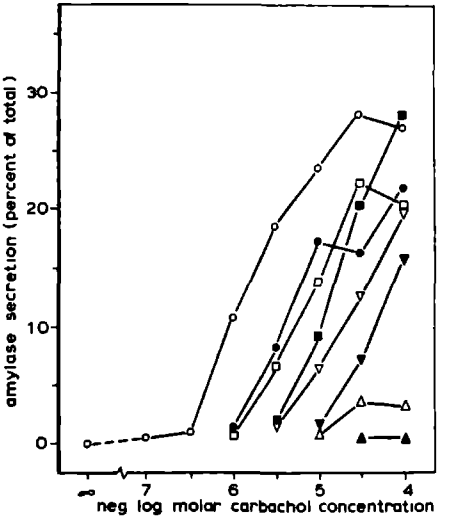


Fig 4 Effect of TMB-8 on the dose-response curve for carbachol stimulation of amylase secretion from rabbit pancreatic acini Acini were incubated for 70 min TMB-8 (0 μM, ○, 10 μM, ●, 25 μM, □, 50 μM, ■, 100 μM, ▽, 200 μM, ▼, 300 μM, △, and 500 μM, ▲) and carbachol, at the indicated concentrations, were added after 20 min and 40 min, respectively Details on sampling and calculations are given in the legend of Fig. 2 The values presented are the means of 3–4 experiments.

TPA, known to activate protein kinase C [20], and the calcium ionophore A23187.

Fig. 5 shows that 100 μM TMB-8 potentiates the slight stimulatory effect of TPA at all concentrations (1 nM to 10 μM) tested. At the same concentration TMB-8 also potentiates the secretory response to 10^{-6} M A23187 (Fig. 6). As with CCK-8 and carbachol, TPA-induced amylase secretion is completely inhibited at 500 μM TMB-8. However, at a concentration of 500 μM, TMB-8 neither potentiates nor inhibits the response to 10^{-6} M A23187 (data not shown).

Effects on cytosolic free calcium

In order to investigate more directly the possibility that intracellular calcium levels are affected by TMB-8, pancreatic acinar cells were loaded with the fluorescent calcium indicator quin2. In unstimulated acinar cells cytosolic free calcium, $[Ca^{2+}]_i$, is calculated to be 149 nM (S.E. 10 nM; 5 different cell preparations). Fig. 7 shows that the addition of TPA (10^{-5} M) does not lead to any change in $[Ca^{2+}]_i$. Upon stimulation with 10^{-9} M CCK-8, however, an almost immediate increase in $[Ca^{2+}]_i$ up to about 800 nM is observed, followed by a relatively slow decay (Fig. 8, top). When pancreatic acinar cells are preincubated with 100 μM TMB-8, shown before to maximally potentiate thesecretory response to either CCK-8, TPA or A23187, for 20 min, neither the basal $[Ca^{2+}]_i$ nor the CCK-8 (10^{-9} M)-induced initial increase in $[Ca^{2+}]_i$ is altered (Fig. 8, top).

Fig. 8 (bottom) shows that the addition of 10^{-5} M carbachol results in an almost immediate increase in $[Ca^{2+}]_i$ up to about 800 nM. This figure also shows that upon subsequent stimulation with 10^{-8} M CCK-8, there is no further increase in $[Ca^{2+}]_i$. However, when 50 μM TMB-8 is added just before carbachol, the effect of carbachol on $[Ca^{2+}]_i$ is almost abolished, whereas the effect of CCK-8 remains unchanged (Fig. 8, bottom). A

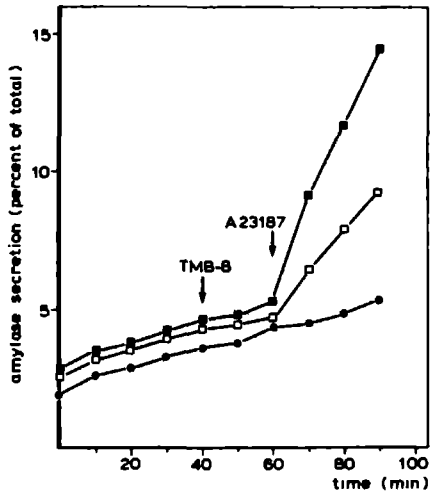


Fig. 6. Effects of A23187 and TMB-8, alone and in combination, on amylase release from rabbit pancreatic acini. After preincubation for 40 min at 37°C, TMB-8 (100 μM, closed symbols) was added. A23187 (10^{-6} M, □, ■) was added 20 min later. Details on sampling and calculations are given in the legend of Fig. 1. Basal release from 40 to 90 min was the same as that in the presence of TMB-8 alone. This figure is typical of three experiments.

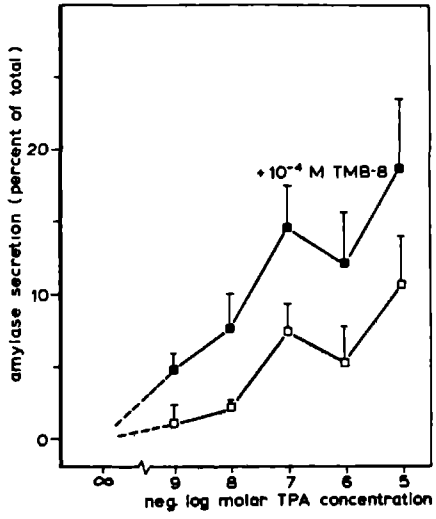


Fig. 5. Effect of TMB-8 on the dose-response curve for TPA stimulation of amylase release from rabbit pancreatic acini. Acini were incubated for 70 min at 37°C. TMB-8 (100 μM) and TPA, at the indicated concentrations, were added after 20 min and 40 min, respectively. Details on sampling and calculations are given in the legend of Fig. 2. The values presented are the means ± S.E. of 3–4 experiments.

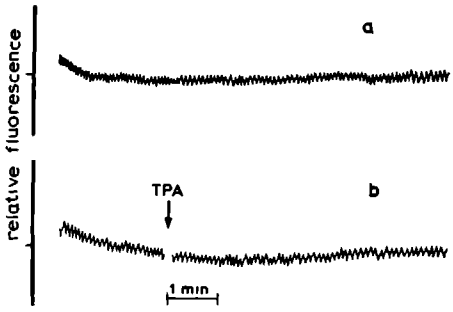


Fig. 7. The effect of TPA on $[Ca^{2+}]_i$ in quin2-loaded rabbit pancreatic acinar cells. Quin2-loaded cells were incubated either in the absence (a) or in the presence (b) of TPA (10^{-5} M). This figure is typical of five experiments.

dose-response curve for the inhibitory effect of TMB-8 on the initial increase in $[Ca^{2+}]_i$ induced by 10^{-5} M carbachol (Fig. 9) indicates again that

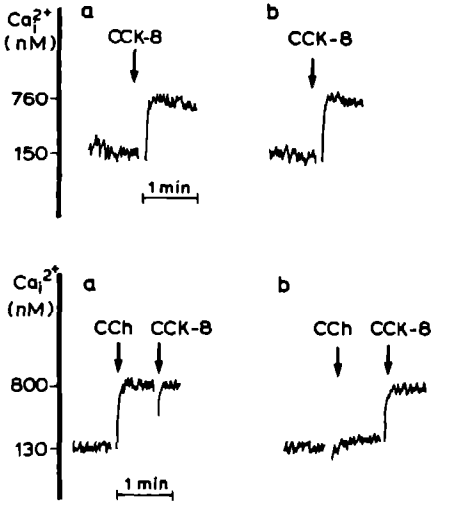


Fig 8 The effect of TMB-8 on the carbachol- and CCK-8-induced increase in $[Ca^{2+}]_i$ in quin 2-loaded rabbit pancreatic acinar cells. Top: quin2-loaded cells were preincubated for 20 min either in the absence (a) or presence (b) of TMB-8 (100 μM) Fluorescent measurement was started and CCK-8 (10^{-9} M) was added Bottom: quin2-loaded cells were incubated either in the absence (a) or presence (b) of TMB-8 (50 μM). Carbachol (CCh; 10^{-5} M) and CCK-8 (10^{-8} M) were added at the indicated times. This figure is typical of four experiments.

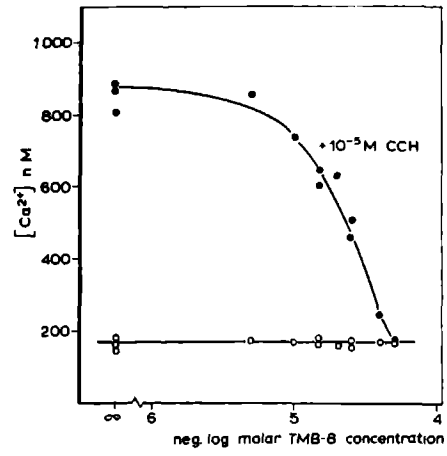


Fig. 9. Dose-inhibition curve for TMB-8 on the carbachol-induced initial increase in $[Ca^{2+}]_i$ in quin2-loaded rabbit pancreatic acinar cells. Quin2-loaded cells were incubated for 1 min in the presence of TMB-8, at the indicated concentrations. After 1 min carbachol (CCh, 10^{-5} m) is added

TMB-8 is a cholinergic receptor antagonist. The inhibitory action of 50 μM TMB-8 is relieved when the drug is removed. TMB-8, at concentrations up to 250 μM, has no effect on $[Ca^{2+}]_i$ (data not shown). At the relatively high concentration of 500 μM, however, TMB-8 increases the fluorescence obtained from unloaded as well as quin2-loaded cells. Similar observations with relatively high concentrations of TMB-8 are reported by Simpson et al. [21].

Effects on lactic dehydrogenase retention and Trypan blue exclusion

The viability of the acinar cells is routinely checked during the incubation period using the vital stain Trypan blue. The addition of carbachol, CCK-8, TPA (dissolved in dimethylsulfoxide) or A23187 (dissolved in ethanol) does not effect the viability of the acinar cells. Also TMB-8 alone, at concentrations as high as 500 μM, has no effect on Trypan blue exclusion over a period of at least 50 min. However, pancreatic acinar cells preincubated with 500 μM TMB-8 lose their capability to exclude Trypan blue within 20 min after the

addition of either 10^{-6} M A23187 or CCK-8 at concentrations at or above 10^{-9} M

The suggestion that TMB-8 in combination with a stimulant has a cytotoxic effect on pancreatic acinar cells is supported by the fact that, whereas no lactic dehydrogenase is released in the presence of TMB-8 (500 μM) alone, addition of either CCK-8 or A23187 leads to a nearly complete release of lactic dehydrogenase within 20–30 min In the presence of 100 μM TMB-8, however, none of the stimulants used induced the release of lactic dehydrogenase

Discussion

The mechanism of action of the potent muscle relaxant TMB-8 has been investigated by Malagodi and Chiou [11–13] Since TMB-8 blocked muscle contraction in response to agents which increase cytosolic calcium and the more since the drug inhibited caffeine-induced calcium release from isolated sarcoplasmic reticulum TMB-8 was concluded to act by preventing the release of calcium from intracellular stores This finding has led many investigators to use TMB-8 in studies on the role of intracellular calcium pools in stimulus-response coupling (for a review, see Ref 22)

Pancreatic enzyme secretion is stimulated by the neurotransmitter acetylcholine and the peptide hormone cholecystokinin Since both secretagogues are thought to activate the same intracellular pathways [1–4], it was quite surprising to us that in rabbit pancreatic acini, TMB-8 affected carbachol-induced amylase release in a significantly different way than the secretory response to CCK-8, TMB-8 inhibition of enzyme secretion stimulated by carbachol was more effective, whereas amylase release induced by CCK-8 was even potentiated under certain circumstances

The experimental data obtained strongly suggest that in the exocrine pancreas TMB-8 acts as a cholinergic receptor antagonist Our results support those of Tennes et al [23], who reported that in mouse, [^{3}H]quinuclidinol benzoate binding was inhibited by TMB-8 both in pancreatic acini and submandibular glands, indicating that in these tissues TMB-8 blocks the muscarinic receptor Inhibitory effects of TMB-8 on carbachol-induced pancreatic enzyme secretion were also reported by Ikeda et al [24] An anticholinergic effect of TMB-8 was excluded by these authors because the drug also inhibited CCK-8 stimulated enzyme secretion The type of inhibition, however, was not investigated in more detail

An interesting finding made in this study is that under certain conditions TMB-8 potentiated the stimulatory effect of the secretagogue used Potentiating effects of TMB-8 have been described in the literature [25–27] In none of these studies, however, is the mechanism of action of TMB-8 examined in more detail The observation that secretagogue-induced enzyme secretion potentiated by TMB-8 did not exceed that induced by the optimal concentration of secretagogue, suggests that the drug accelerates some important step in stimulus-secretion coupling up to the same level as is reached with optimal concentrations of secretagogue The fact that TMB-8 alone had no effect on pancreatic enzyme secretion seems to rule out a direct activation by the drug itself of crucial steps leading to secretion

Our results do not support the hypothesis that the potentiating effect of TMB-8 is mediated by calcium [26,27] This is because the drug neither increased $[Ca^{2+}]_i$, nor induced an additional increase in $[Ca^{2+}]_i$ upon stimulation with secretagogue Moreover, TMB-8 also potentiated the secretory response to calcium ionophore A23187

Since TMB-8 potentiated both the response to A23187 and TPA, the drug seems to act at or distal from the site of interaction between Ca^{2+} and TPA It remains to be elucidated, whether potentiation occurs at the level of protein kinase C itself, or at some step beyond the activation of this enzyme

At TMB-8 concentrations above 100 μM, the drug becomes inhibitory to the stimulatory effects of CCK-8, carbachol and TPA Although an explanation for the inhibitory effect of 100–500 μM TMB-8 can hardly be given, it is clear that at 500 μM cytotoxic effects are observed The acinar cells rapidly lost their integrity in the presence of secretagogue, as has been observed by Di Virgilio and Gomperts [28] for TMB-8, in combination with monensin, at concentrations at or above 300 μM in rabbit neutrophils At relatively high concentrations, TMB-8 has been described to decrease significantly intracellular ATP levels [22,29]

and to act as a general membrane disrupting reagent [30]. Therefore, inhibitory effects of relatively high concentrations of TMB-8 found on stimulus-evoked processes should be treated with caution

From the results presented in this paper, it is concluded that in the exocrine pancreas TMB-8 is not a selective inhibitor of secretagogue-induced calcium mobilization Inhibitory effects of TMB-8 are either due to its anticholinergic activity or to the cytotoxic activity it has at relatively high concentrations. At 100 μM, TMB-8 maximally potentiates enzyme secretion induced by suboptimal concentrations of CCK-8 It is argued that this potentiating effect of TMB-8 is not likely to be due to an (additional) increase in $[Ca^{2+}]_i$. Since 100 μM TMB-8 also potentiates the response to A23187 and TPA, it is suggested that potentiation occurs at or beyond the site of interaction between Ca^{2+} and TPA Detailed investigations are needed, however, the clarify to exact mechanism of the stimulatory action of TMB-8 in pancreatic enzyme secretion

References

1 Schulz, I (1980) Am J Physiol 239, G335–G347
2 Jensen, R T and Gardner, J D (1981) Fed Proc. 40, 2486–2496
3 Williams, J A (1984) Ann Rev Physiol 46, 361–375
4 Petersen, O H (1985) Gastroenterology 89, 109–117
5 Pandol, S J, Thomas, M W, Schoeffield, G S, Sachs, G and Muallem, S (1985) Am J Physiol 248, G551–G560
6 Campbell, A K (1983) Intracellular Calcium Its Universal Role as Regulator, John Wiley and Sons Limited, Chichester
7 Gardner, J D, Costenbader, C L and Uhleman, E R (1979) Am J Physiol 236, E754–E762
8 De Pont, J J H H M and Fleuren-Jakobs, A M M (1984) FEBS Lett 170, 64–68
9 Laugier, R and Petersen, O H (1980) J Physiol London 303, 61–72
10 Schreurs, V V A M, Swarts, H G P De Pont J J H H M and Bonting, S L (1976) Biochim Biophys Acta 419, 320–330
11 Malagodi, M H and Chiou, C Y (1974) Eur J Pharmacol 27, 25–33
12 Malagodi, M H and Chiou, C Y (1974) Pharmacology 12 20–31
13 Chiou, C Y and Malagodi, M H (1975) Br J Pharmacol 53, 279–285
14 Amsterdam, A and Jamieson, J D (1974) J Cell Biol 63, 1037–1056
15 Eagle, H (1959) Science 130, 432–437
16 Ceska, M, Hultman, E and Ingelman B (1969) Experientia 25, 555–556
17 Kubowitz, F and Ott, P (1943) Biochem Z 314 94
18 Ochs, D L, Korenbrot, J I and Williams, J A (1983) Biochem Biophys Res Commun 117, 122–128
19 Tsien, R Y, Pozzan, T and Rink, T J (1982) Nature 295 68–71
20 Takai, Y, Kikkawa, U, Kaibuchi, K and Nishizuka, Y (1984) Adv Cyclic Nucl Prot Phosphorylation Res 18, 119–158
21 Simpson, A W M, Hallam, T J and Rink, T J (1984) FEBS Lett 176, 139–143
22 Brand, M D and Felber, S M (1984) Biochem J 224, 1027–1030
23 Tennes, K A, Kennedy, J A and Roberts, M L (1983) Biochem Pharmacol 32, 2118–2120
24 Ikeda, M, Nagai, M, Suzuki, S, Niwa, H, Oka, H, Fujino, M and Suzuki, H (1984) Life Sci 34, 529–533
25 Shaw, J O and Lyons, R M (1982) Biochim Biophys Acta 714, 492–499
26 Gómez-Cambronero, J, Inarrea, P, Alfonso, F and Sánchez Crespo, M (1984) Biochem J 219 419–424
27 Wiedenkeller, D E. and Sharp, G W G (1984) Endocrinol 114, 116–119
28 Di Virgilio, F and Gomperts, B D (1983) Biochim Biophys Acta 763, 292–298
29 Spearman, T N and Butcher, F R (1983) Pflugers Arch 397, 220–224
30 Mürer, E H, Steward, G J, Davenport, K and Siojo, E. (1981) Biochem Pharmacol 30, 523–530

Chapter 6

Phorbol ester inhibits cholecystokinin octapeptide-induced amylase secretion, but is without effect on secretagogue-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate in rabbit pancreatic acini.

WILLEMS, P.H.G.M.,

VAN NOOIJ, L.G.P.,

HAENEN, H.E.M.G. and

DE PONT, J.J.H.H.M.

Department of Biochemistry, University of Nijmegen,
P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

230

Biochimica et Biophysica Acta 930 (1987) 230–236
Elsevier

BBA 12095

Phorbol ester inhibits cholecystokinin octapeptide-induced amylase secretion and calcium mobilization, but is without effect on secretagogue-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate in rabbit pancreatic acini

P.H.G.M. Willems, I.G.P. van Nooij, H.E.M.G. Haenen and J.J.H.H.M. de Pont

Department of Biochemistry, University of Nijmegen, Nijmegen (The Netherlands)

(Received 1 April 1987)

Key words: Phorbol ester; Cholecystokinin octapeptide; Amylase secretion; Calcium mobilization; Inositol phosphate; (Rabbit acini)

The effect of prolonged protein kinase C activation on cholecystokinin octapeptide (CCK-8)-induced amylase secretion from rabbit pancreatic acini was studied by means of the phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA). The phorbol ester itself increased basal amylase secretion but inhibited completely the secretory response to relatively low concentrations of CCK-8. The inhibitory action of TPA on CCK-8-induced amylase secretion was paralleled by inhibition of CCK-8-induced calcium mobilization but not by inhibition of CCK-8-induced breakdown of ^{32}P-labelled phosphatidylinositol 4,5-bisphosphate. The results presented suggest that protein kinase C, or one of its phosphorylated products, inhibits the CCK-8-stimulated pathway leading to secretion at a level beyond the secretagogue-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate. Inhibition of the initial, inositol 1,4,5-trisphosphate-mediated and extracellular calcium-independent, increase in free cytosolic calcium concentration, together with the findings of others, suggests that the efficacy of this inositolphosphate to release calcium is reduced.

Introduction

Activation of the calcium messenger system implies the receptor-mediated breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2), yielding 1,2-diacylglycerol and inositol 1,4,5-trisphosphate ($(1,4,5)IP_3$). Both of these products possess important second messenger functions. Diacylglycerol activates the calcium/phospholipid-dependent protein kinase (C) and $(1,4,5)IP_3$ releases calcium from an intracellular store [1,2].

In the exocrine pancreas, the increase in free cytosolic calcium concentration in response to the calcium-mobilizing secretagogues cholecystokinin and acetylcholine has been shown to take place within seconds after stimulation, followed by a somewhat slower decay to nearly resting levels reached within 2 min [3,4]. So far, however, increases in diacylglycerol have only been measured upon supramaximal stimulation and were found

Abbreviations: CCK-8, cholecystokinin octapeptide, TPA, 12-*O*-tetradecanoylphorbol 13-acetate, quin2, 2-((2-(bis((carbonyl)methyl)amino)-5-methylphenoxy)methyl-6-methoxy-8-(bis-((carbonyl)methyl)amino)quinoline, quin2/AM, quin2-tetra(acetoxymethyl)ester, PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate, $[Ca^{2+}]_i$, free cytosolic calcium concentration; DMSO, dimethyl sulfoxide, Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Correspondence: P H G M Willems, Department of Biochemistry, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

to take place more gradually with time, reaching plateau values which were maintained as long as the calcium-mobilizing receptor was occupied by its agonist [5]

It is well documented that prolonged stimulation of exocrine pancreatic tissue with a secretagogue of the calcium-mobilizing type reduces the secretory response to subsequent stimulation with a calcium-mobilizing secretagogue (for a review, see Ref 6) More recently, this phenomenon of secretagogue-induced desensitization has been explained by assuming that the intracellular calcium pool is emptied by the first stimulus and is not refilled before the stimulus is removed [4] In various biological systems, however, protein kinase C activation has also been implicated in the process of desensitization (for a review, see Ref 28)

The experiments described in this paper were undertaken to investigate whether activation of the protein kinase C pathway of the calcium messenger system can also attribute to the reduced efficacy of the second stimulus To achieve protein kinase C activation without receptor activation we used the phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), known to activate protein kinase C in a diacylglycerol-like manner [7]

The results presented here show that TPA reduces the secretory response to cholecystokinin-octapeptide (CCK-8) by inhibiting CCK-8-induced calcium mobilization The CCK-8-induced breakdown of ^{32}P-labelled PIP_2, however, is not affected by TPA This suggests that protein kinase C or one of its phosphorylated products exerts a negative feed-back control over some step beyond the level of PIP_2 hydrolysis leading to calcium mobilization

Materials and Methods

Methods

Preparation of rabbit pancreatic acini Rabbit pancreatic acini were prepared as previously described [8,9]

Amylase secretion experiments For amylase secretion experiments rabbit pancreatic acini resuspended in a Krebs-Ringer bicarbonate medium (pH 7 4) containing 119 mM NaCl, 3 5 mM KCl, 1 2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1 2 mM $CaCl_2$, 1 2 mM $MgCl_2$, 5 8 mM glucose, 1% bovine serum albumin, an amino acid mixture according to Eagle [10] and 0 2 mg/ml soybean trypsin inhibitor, were incubated for 30 min either in the absence or in the presence of 10^{-6} M TPA The suspension was then centrifuged and the acini were resuspended in fresh incubation solution to which no additional TPA was added Amylase secretion was measured over a period of 50 min Except for the controls, CCK-8 at the indicated concentrations was added 20 min after the incubation was started At appropriate times, samples were removed for determination of medium amylase activity At the beginning of the experiment, an aliquot of the suspension was removed and the acini were homogenized for determination of total amylase activity Amylase activity was measured by means of the Phadebas test and medium amylase activity was expressed as percent of total amylase present in the acini at the beginning of incubation

Free cytosolic calcium concentration measurements Rabbit pancreatic acini resuspended in the above Krebs-Ringer bicarbonate medium were loaded with quin2 by a 20 min incubation in the presence of 50 μM quin2/AM The suspension was then diluted six times and half of the suspension was incubated further in the presence of 10^{-6} M TPA To the remaining part of the suspension dimethyl sulfoxide, the solvent for TPA, was added After 30 min the suspensions were centrifuged and the acini were resuspended in fresh incubation solution Immediately before fluorescence measurement, samples were removed, rapidly centrifuged (Eppendorf minifuge) and resuspended in a Hepes/Tris medium (pH 7 4) containing 133 mM NaCl, 4 2 mM KCl, 5 8 mM glucose, 1 0 mM $CaCl_2$, 1 0 mM $MgCl_2$, 0 2 mg/ml soybean trypsin inhibitor and 10 mM Hepes Before use, the medium was thoroughly gassed with 100% O_2 Details on fluorescence measurement and calibration of intracellular calcium concentrations are described elsewhere [9,11]

Phosphatidylinositol 4,5-bisphosphate breakdown measurements Rabbit pancreatic acini were resuspended in the above Krebs-Ringer bicarbonate medium containing 10 μM KH_2PO_4, 0 5% bovine serum albumin and 25 μCi/ml ortho[^{32}P]phosphate and were incubated for 60 min at 37°C TPA (10^{-6} M) and DMSO (control acini) were

present during the last 30 min of the labelling period After 60 min of labelling, CCK-8 at the indicated concentrations was added and 400-μl samples were removed and vigorously mixed with 1 8 ml ice-cold extraction medium (dichloromethane/methanol/concentrated HCl, 20 40 . 1) immediately before and 8 and 30 s after stimulation ^{3}H-labelled PIP_2 was added to the extraction medium for recovery measurements. Phase separation was obtained by adding 400 μl dichloromethane and 400 μl water followed by centrifugation (5 min, 700 × *g*). After collection of the organic phase, the aqueous phase was extracted again and the combined organic phases were blown to dryness with nitrogen. The extracts were then dissolved in dichloromethane/ methanol/ water (75 : 25 : 2) and the phospholipids were separated by thin-layer chromatography using potassium oxalate-treated high-performance plates (Merck). The plates were developed with dichloromethane/ methanol/ methylamine (20%) (60 : 36 . 10) PIP_2 spots were identified by both the tritiated PIP_2 marker and by unlabelled PIP_2 and were scraped off. Scintillation fluid (Aqualuma) and water (10%, v/v) were added and ^{3}H and ^{32}P radioactivity were measured using a Philips PW 4510 liquid scintillation analyzer After correction for the ^{3}H-labelled PIP_2 recovery the amount of ^{32}P-labelled PIP_2 present in the CCK-8 stimulated acini was expressed as percent of the amount present immediately before stimulation.

Materials

The C-terminal octapeptide of cholecystokinin has been synthesized by Dr. H.M. Rajh in the Department of Organic Chemistry, University of Nijmegen, Bovine serum albumin, soybean trypsin inhibitor, TPA, phosphatidylinositol 4,5-bisphosphate and quin2/AM were obtained from Sigma, St. Louis, MO, U S A.; HPTLC plates silica gel 60 were from Merck, Darmstadt, F.R G., phosphatidyl[2-^{3}H]inositol 4,5-bisphosphate was from Amersham Laboratories, U K., ortho[^{32}P]phosphate was from New England Nuclear, Boston, MA, U.S.A.; ionomycin was from Behring Diagnostics, La Jolla, CA, U S A ; collagenase was from Cooper Biomedical Inc, Malvern, PA, U S A and hyaluronidase was from Boehringer, Mannheim, F.R G. The Phadebas test kit was purchased from Pharmacia, Uppsala, Sweden. All other chemicals were of reagent grade

Results

Rabbit pancreatic acini were preincubated with the phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate, at a concentration of 10^{-6} M for 30 min and then resuspended in fresh incubation solution without additional TPA. Amylase secretion was measured over the next 50 min of incubation. Upon stimulation of DMSO-pretreated acini with 10^{-6} M TPA at 20 min, the amount of amylase released during the subsequent 30 min increased from 1 8% (S E , 0.2; $n = 13$) to 5.7% (S E., 0 6; $n = 6$), which was not significantly different from the rate of amylase secretion observed for the TPA-pretreated acini over the same period of time (5.5%, S.E., 0 6; $n = 12$)

Fig 1 shows that CCK-8 at concentrations of $3 \cdot 10^{-10}$–10^{-9} M did not significantly alter amylase secretion from TPA-treated acini, whereas in control acini the secretory response dose-dependently increased. At $3 \cdot 10^{-9}$–10^{-8} M, however, the amount of amylase released during the subsequent 30 min of incubation was not different from that in control acini.

In order to examine the inability of CCK-8 at concentrations of $3 \cdot 10^{-10}$–10^{-9} M to stimulate amylase secretion from TPA-treated acini in more detail, we investigated the effects of TPA pretreatment on CCK-8-induced increases in $[Ca^{2+}]_i$ measured by means of quin2 At a concentration of 3 10^{-10} M, CCK-8 increased the free cytosolic calcium concentration within seconds, to about 200 nM. Fig. 2 (A, B) shows that this initial increase in $[Ca^{2+}]_i$ in response to the secretagogue was independent of the presence of extracellular calcium. In the absence of extracellular calcium, however, the fluorescence intensity declined more rapidly and returned to basal levels within 2 min When the acini were pretreated with 10^{-6} M TPA and then stimulated with $3 \cdot 10^{-10}$ M CCK-8, the increase in $[Ca^{2+}]_i$ was completely abolished, even in the presence of extracellular calcium (Fig. 2 (C))

Fig 3 shows that TPA pretreatment inhibited completely the CCK-8-induced initial increase in $[Ca^{2+}]_i$ at the lower concentrations (10^{-10}–10^{-9}

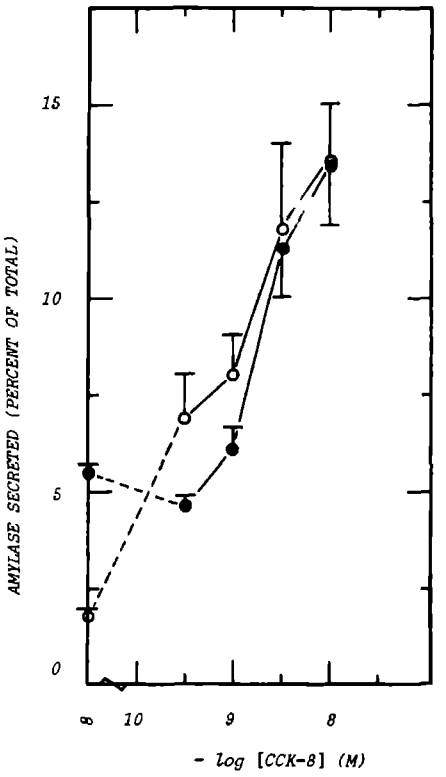


Fig 1 Effect of TPA on the dose-response curve for CCK-8 stimulation of amylase secretion from rabbit pancreatic acini Rabbit pancreatic acini were incubated for 30 min either in the absence (○) or in the presence (●) of 10^{-6} M TPA The suspensions were then centrifuged and the acini were resuspended in fresh incubation solution to which no additional TPA was added Amylase secretion was measured over a period of 50 min Except for the controls, CCK-8 at the indicated concentrations was added at 20 min Medium amylase activity was measured at 0, 20, 30, 50 and 60 min and the increase in amylase release over the last 30 min of incubation is expressed as percentage of total amylase originally present in the acini The values presented are the mean ± S E of 6–9 experiments.

M) of the secretagogue At or beyond $3 \cdot 10^{-9}$ M, CCK-8 also increased $[Ca^{2+}]_i$ in TPA-treated acini. The dose-response curve, however, was shifted markedly to the right.

Since the CCK-8-induced initial increase in free

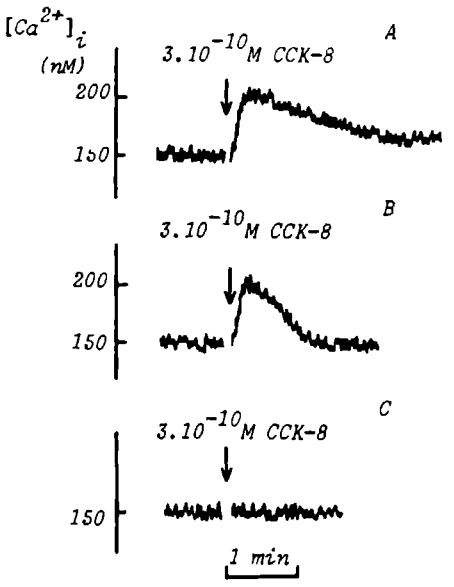


Fig 2 Effects of extracellular calcium and TPA on CCK-8-induced increases in $[Ca^{2+}]_i$ in quin2-loaded rabbit pancreatic acini Rabbit pancreatic acini were incubated with quin2/AM and TPA (10^{-6} M) Immediately before use, pancreatic acini loaded with quin2 were resuspended in a Hepes/Tris medium (pH 7 4) to which no calcium was added and which was thoroughly gassed with O_2 The acini were transferred to a cuvette placed in a spectrofluorometer and preincubated for 3 min at 37 °C before measurement was started Stirring of the acini suspension was then started and 0 2 mM EGTA was added In those experiments in which extracellular calcium was present, after 1 min the medium calcium concentration was increased to 1 0 mM DMSO-treated acini were stimulated with 3 10^{-10} M CCK-8 in the presence (A) and in the absence (B) of extracellular calcium TPA-treated acini were stimulated with CCK-8 in the presence of extracellular calcium (C)

cytosolic calcium concentration is thought to represent the (1,4,5)IP_3-mediated release of Ca^{2+} from an intracellular store [3,4], we investigated the effects of the phorbol ester on CCK-8-induced PIP_2 breakdown Rabbit pancreatic acini were labelled with ortho[^{32}P]phosphate for 1 h During the last 30 min of that period either TPA or DMSO was present. Fig 4 shows that subsequent stimulation with CCK-8 resulted in a dose- and time-dependent decrease in the amounts of radiolabelled PIP_2 both in control and in TPA-treated

234

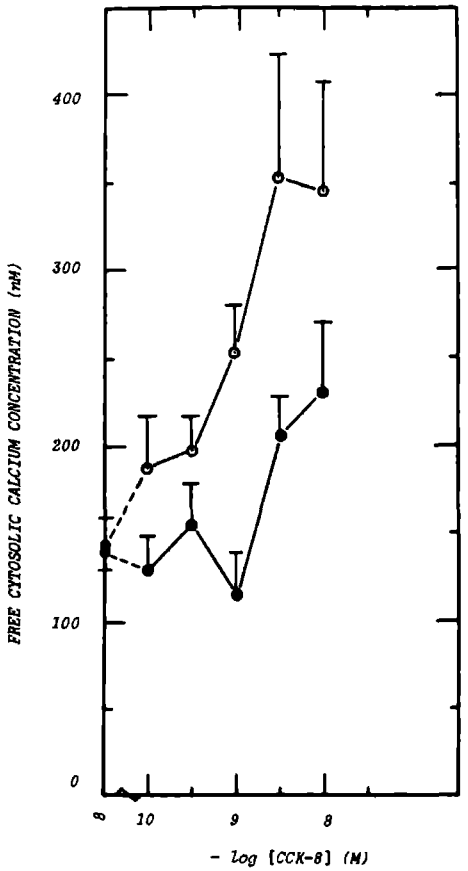


Fig. 3 Effect of TPA on the dose-response curve for CCK-8-induced initial increases in free cytosolic calcium concentration in rabbit pancreatic acini. A suspension of rabbit pancreatic acini, incubated in the presence of 50 μM quin2/AM for 20 min, was diluted 6 times. Half of the suspension was then incubated in the presence of 10^{-6} M TPA (●), whereas DMSO was added to the remaining half (○). At 30 min, the suspensions were centrifuged and the acini were resuspended in fresh incubation solution to which no additional TPA was added. Immediately before fluorescence measurement samples were removed, rapidly centrifuged (Eppendorf minifuge) and resuspended in a Hepes/Tris medium (pH 7.4). Fluorescence measurements were carried out at 37 °C using a spectrofluorometer equipped with a thermostatically controlled cuvette holder and a magnetic stirrer. After 5 min of equilibration, CCK-8 was added at the indicated concentrations. The initial increase in $[Ca^{2+}]_i$, observed within seconds, was calculated according to the method of Tsien et al. [27] using 10^{-5} M ionomycin to determine maximal fluorescence. The values presented are the mean ± S.E. of 3–5 experiments.

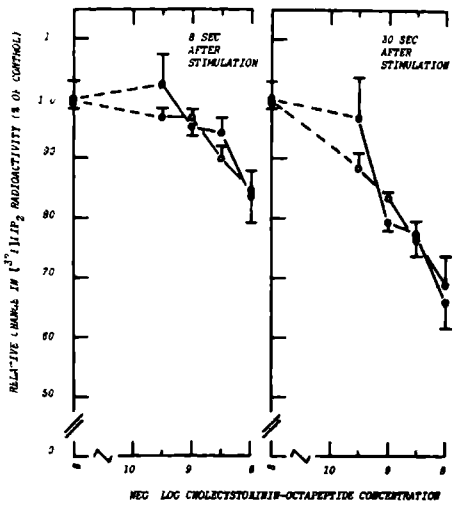


Fig. 4 Effect of TPA on the dose-response curve for CCK-8-induced breakdown of ^{32}P-labelled PIP_2. Rabbit pancreatic acini were incubated in the presence of ortho[^{32}P]phosphate for 60 min at 37 °C. At 30 min 10^{-6} M TPA (○) was added to one half of the suspension while DMSO (●) was added to the other half. After 60 min of labelling, the acini were stimulated with CCK-8 at the indicated concentrations and samples for measurement of radiolabelled PIP_2 were removed immediately before and 8 (A) and 30 (B) s after stimulation. ^{32}P-labelled PIP_2 at 8 and 30 s after stimulation was expressed as percentage of radiolabelled PIP_2 immediately before stimulation. The values presented are the mean ± S.E. of 3–7 experiments.

acini. No inhibitory effect of TPA (10^{-6} M) was observed on PIP_2 breakdown induced by 10^{-9} M CCK-8, at which concentration the stimulatory effect on calcium mobilization was completely inhibited by pretreatment with TPA (10^{-6} M). Even at a concentration of 10^{-5} M TPA, the stimulatory effect of CCK-8 (10^{-9} M) on PIP_2 hydrolysis was not attenuated, since the residual ^{32}P-labelled PIP_2 content, 83.0% (S.E., 4.4, $n = 3$), was not significantly different from that in control acini (79.7%, S.E., 1.7, $n = 7$) when measured 30 s after stimulation.

Discussion

In the intact cell, phorbol esters are generally believed to act by mimicking the stimulatory ef-

fect of endogenous diacylglycerol on the calcium- and phospholipid dependent-protein kinase C [7] In recent years many studies have revealed the ability of phorbol esters to stimulate pancreatic enzyme secretion [4,9,12–15], thus establishing the important role protein kinase C plays in the mechanism of action of pancreatic secretagogues that initiate phosphatidylinositol turnover

One of the main observations presented in this paper is that the phorbol ester, TPA, in addition to its stimulatory effect on pancreatic enzyme secretion also inhibits the secretory response to submaximal concentrations of the secretagogue CCK-8 This suggests that protein kinase C is also involved in negative feed-back regulation of pancreatic enzyme secretion

Cholecystokinin stimulates the enzymatic breakdown of PIP_2 ([16–18] and this study), yielding the intracellular messengers diacylglycerol [5] and (1,4,5)IP_3 [19], involved in protein kinase C activation [7] and intracellular calcium mobilization [20], respectively A rapid but transient increase in the free cytosolic calcium concentration ($[Ca^{2+}]_i$) is observed when pancreatic acinar cells, loaded with the fluorescent calcium indicator, quin2, are stimulated with CCK-8 ([3–5] and this study) This initial increase in $[Ca^{2+}]_i$ is independent of the presence of extracellular calcium (Refs 3 and 4 and this study), and is therefore thought to be mediated by (1,4,5)IP_3

In order to study the intracellular mechanism underlying the inhibitory action of TPA on CCK-8-induced pancreatic enzyme secretion in more detail, we investigated whether CCK-8-induced increases in $[Ca^{2+}]_i$ were affected by phorbol ester treatment From the results obtained it is evident that inhibition of CCK-8-stimulated amylase secretion is paralleled by the failure of CCK-8 to increase $[Ca^{2+}]_i$ in these cells The observation that TPA-stimulated amylase secretion is only then increased when the concentration of CCK-8 is high enough to overcome the inhibitory action of TPA on secretagogue-induced calcium mobilization supports the important role calcium is thought to play in pancreatic enzyme secretion and makes it unlikely that the intracellular pathways leading to CCK-8-induced enzyme secretion are inhibited at or beyond the level of protein kinase C activation Inhibitory effects of TPA on carbachol-induced increases in $[Ca^{2+}]_i$ have recently been described for guinea pig pancreatic acini by Ansah et al [13] However, they did not investigate whether carbachol-induced amylase secretion was also affected by the phorbol ester

The possibility that in TPA-treated acini CCK-8-induced increases in $[Ca^{2+}]_i$ are not observed because of protein kinase C-mediated stimulation of calcium extrusion and/or sequestration is not supported by the observation that when stimulated with 10^{-10}–10^{-9} M CCK-8, no increase in $[Ca^{2+}]_i$ is observed at all Stimulated calcium extrusion and/or sequestration has been suggested by Drummond [29] to explain the fact that in GH_3 cells TPA transiently lowers $[Ca^{2+}]_i$ elevated by high potassium

The fact that the initial increase in $[Ca^{2+}]_i$, which is independent of the presence of extracellular calcium and which is therefore thought to be mediated by (1,4,5)IP_3, is inhibited in phorbol ester-treated pancreatic acini led us to investigate the effect of TPA on the CCK-8-induced hydrolysis of PIP_2 The results surprisingly demonstrate that TPA is without effect on CCK-8-induced PIP_2 breakdown, even then when the secretagogue is used at concentrations which fail to increase $[Ca^{2+}]_i$ in phorbol ester-treated acini This observation strongly suggests that protein kinase C or one of its phosphorylated products does not act at the level of secretagogue-induced PIP_2 breakdown Our results support those of Merritt et al [19], who reported that in rat pancreatic acini the phorbol ester, phorbol dibutyrate, is without effect on the formation of (1,4,5)IP_3 and (1,3,4)IP_3 induced by maximal as well as submaximal concentrations of caerulein It was concluded by these authors that in the exocrine pancreas phorbol esters are not involved in desensitization of calcium-mobilizing receptors They did not investigate, however, whether phorbol dibutyrate affected caerulein-induced calcium mobilization and/or enzyme secretion

Comparison of the effect of the higher concentrations of CCK-8 ($3\ 10^{-9}$–10^{-8} M) on amylase secretion and calcium mobilization shows that pretreatment with TPA has no effect on enzyme secretion (Fig 1), whereas the stimulatory effect on cytosolic calcium is reduced by at least 50% (Fig 3) This strongly suggests that in the

presence of TPA only a partial increase of the free cytosolic calcium concentration is sufficient for maximal stimulation of enzyme secretion

The possibility that in TPA-treated acini the synthesis of PIP_2 is reduced, thus leading to less $(1,4,5)IP_3$ formed upon stimulation with CCK-8, seems to be ruled out, since in TPA-treated acini [^{32}P]PIP_2 amounted to 84 3% (S E , 14 7, $n = 7$) of the control value On the other hand, the amount of [^{32}P]phosphatidylcholine was 164 6% (S E , 24 3, $n = 11$) of the control, indicating an enhanced phosphorylation of this phospholipid Effects of phorbol esters on the metabolism of phospholipids have been described for a variety of tissues. In human platelets TPA was found to increase, not to decrease, the labelling of phosphatidylinositol and phosphatidylinositolmonophosphate in the presence of [^{32}P]P_i [21], whereas an enhanced biosynthesis of phosphatidylcholine has been observed in phorbol ester-treated neuroblastoma-glioma (NG 108-15) cells [22], human leukemic cells [23], HeLa cells [24], myoblasts [25] and Krebs 11 ascites cells [26]

Together, our results and those of Ansah et al [13] and Merritt et al [19] suggest that protein kinase C exerts its negative feed-back control over secretagogue-induced pancreatic enzyme secretion, not at the level of the secretagogue-induced hydrolysis of PIP_2 ([19] and this study), nor at the level of the (de)phosphorylation of $(1,4,5)IP_3$ [19], but maybe at the level of the $(1,4,5)IP_3$-activated release mechanism for calcium from the intracellular store More detailed investigations are needed, however, to test this possibility

References

1 Williamson, J R., Cooper, R H , Joseph, S K and Thomas, A P (1985) Am J Physiol 248, C203–C216
2 Berridge, M J (1985) Sci Am 253, 124–134
3 Ochs, D L , Korenbrot, J I and Williams, J A (1985) Am J Physiol 249, G389–G398
4 Pandol, S J , Schoeffield, M S , Sachs, G and Muallem, S (1985) J Biol Chem 260, 10081–10086
5 Pandol S J and Schoeffield, M S (1986) J Biol Chem. 261, 4438–4444
6 Gardner, J D and Jensen, R T (1981) in Physiology of the Gastrointestinal Tract (Johnson, L R , ed), pp 831–871, Raven Press, New York
7 Takai, Y , Kikkawa, U , Kaibuchi, K and Nishizuka, Y (1984) Adv Cycl Nucl Prot Phosph Res. 18, 119–158
8 Willems, P H G M , Fleuren-Jakobs, A M M , De Pont, J J H H M and Bonting, S L (1984) Biochim Biophys Acta 802, 209–214
9 Willems, P H G M., Van Nooij, I G P and De Pont, J J H H M (1986) Biochim Biophys Acta 888, 255–262
10 Eagle, H (1959) Science 130, 432–437
11 Willems, P H G M , Tilly, R H J and De Pont, J J H H M (1987) Biochim Biophys Acta 928, 179–185
12 Burnham, D B , Munowitz, P , Hootman, S R and Williams, J A (1986) Biochem J 235, 125–131
13 Ansah, T-A , Dho, S and Case, R M (1986) Biochim. Biophys Acta 889, 326–333
14 De Pont, J J H H M and Fleuren-Jakobs, A M M (1984) FEBS Lett 170, 64–68
15 Merritt, J E. and Rubin, R P (1985) Biochem. J 230, 151–159
16 Putney, Jr , J W , Burgess, G M , Halenda, S P , McKinney, J S and Rubin, R P (1983) Biochem J 212, 483–488
17 Orchard, J L., Davis, J S., Larson, R E. and Farese, R V (1984) Biochem. J 217, 281–287
18 Pandol, S J , Thomas, M W , Schoeffield, M S , Sachs, G and Muallem, S (1985) Am J Physiol 248, G551–G560
19 Merritt, J E., Taylor, C W , Rubin, R P and Putney, J W., Jr (1986) Biochem J 238, 825–829
20 Streb, H , Irvine, R F , Berridge, M J and Schulz, I (1983) Nature 306, 67–69
21 Halenda, S P and Feinstein, M B (1984) Biochem. Biophys Res. Commun 124, 507–513
22 Luscovitch, M , Freese, A., Bluestajn, J K and Wurtman, R J (1986) J Neurochem. 47, 1936–1941
23 Lockney, M W , Golomb, H M and Dawson, G 91984) Biochim Biophys. Acta 796, 384–392
24 Cook, H W and Vance, D E. (1985) Can. J Biochem Cell. Biol 68, 145–151
25 Hill, S A , McMurray, W C and Sanwal, B.D (1984) Can. J Biochem Cell Biol 62, 369–374
26 Fjose, A , Pryme, I F and Lillehaug, J R. (1983) Mol. Cell Biochem. 56, 137–144
27 Tsien, R Y , Pozzan, T and Rink, T J (1982) Nature 295, 68–71
28 Nishizuka, Y (1986) Science 233, 305–312
29 Drummond, A H (1985) Nature 315, 752–755

Chapter 7

Desensitization of inositol 1,4,5-trisphosphate-induced Ca^{2+} release in permeabilized pancreatic acinar cells by phorbol ester pretreatment.

WILLEMS, P.H.G.M.,*

VAN DEN BROEK, L.A.M.,**

DE HAAN, A.F.J.,***

VAN OS, C.H.,** AND

DE PONT, J.J.H.H.M.*

Departments of Biochemistry,* Physiology** and
Statistical Consultation***
P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

Abbreviations: (1,4,5-)IP_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; TPA, 12-O-tetradecanoylphorbol 13- acetate; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N'-tetra acetic acid; HEEDTA, N-(2-hydroxy ethyl) ethylene diamine-N,N',N'-triacetic acid; HEPES, 4-(2-hydroxy ethyl)-1-piperazine ethane sulphonic acid; NTA, nitrilotriacetic acid.

SUMMARY

Stimulus-induced increases in free cytosolic Ca^{2+} concentration reflect changes in the Ca^{2+} permeability of plasma membrane and/or intracellular Ca^{2+} stores. In the pancreatic acinar cell the initial secretagogue-induced increase in cytosolic Ca^{2+} is independent of extracellular Ca^{2+} and represents release from intracellular Ca^{2+} stores[1-3]. Receptor-mediated Ca^{2+} mobilization is preceded by phosphatidylinositol 4,5-bisphosphate (PIP_2) breakdown[4], yielding inositol 1,4,5-trisphosphate ((1,4,5-)IP_3), which has originally been shown to be a potent promotor of the release of actively stored Ca^{2+} in permeabilized pancreatic acinar cells[5]. The kinetics of stimulus-induced increases in (1,4,5-)IP_3 concentration are compatible with the idea that this compound is involved in the receptor-mediated increase in Ca^{2+} permeability of the intracellular Ca^{2+} store[6-9]. Receptor-mediated hydrolysis of PIP_2 also results in formation of diacylglycerol and thus in activation of protein kinase C[4]. Using a phorbol ester, which substance mimicks the stimulatory effect of diacylglycerol on protein kinase C[10], we[3] and others[11] have recently shown that in pancreatic acinar cells, as in other cell types[4], agonist-induced Ca^{2+} mobilization can be inhibited. However, unlike in other cell types this inhibitory action of the phorbol ester was not paralleled by inhibition of receptor-mediated breakdown of PIP_2 (Ref. 3), suggesting an effect at the level of the (1,4,5-)IP_3-induced release of Ca^{2+} from the intracellular store, or at the level of the metabolism of (1,4,5-)IP_3. We have now examined the effects of phorbol ester pretreatment on ATP-dependent Ca^{2+} uptake and (1,4,5-)IP_3-induced Ca^{2+} release from rabbit pancreatic acinar cells, permeabilized by saponin treatment. The (1,4,5-)IP_3-induced release of actively stored Ca^{2+} was significantly inhibited in phorbol ester-treated acinar cells. This is in support of our earlier hypothesis[3] that activation of protein kinase C leads to negative feed-back control on agonist-induced Ca^{2+} mobilization at a level beyond receptor-mediated formation of (1,4,5-)IP_3.

RESULTS AND DISCUSSION

Dispersed rabbit pancreatic acinar cells were incubated in the presence of 12-O-tetradecanoylphorbol 13-acetate (TPA) (10^{-6}M) or dimethylsulfoxide (0.1 %, v/v) for 30 min at 37°C. Permeabilization and $^{45}Ca^{2+}$ uptake experiments were performed essentially as described for enterocytes by van Corven et al.[12], with the exception that $^{45}Ca^{2+}$ uptake was measured at pH 7.1 and at 37°C.

At a free Ca^{2+} concentration of 0.1 µM and in the presence of ruthenium red, an inhibitor of mitochondrial Ca^{2+} uptake, Ca^{2+} uptake in permeabilized pancreatic acinar cells was ATP-dependent and linear with time during the first 60 sec (0.60 nanomole Ca^{2+}/mg protein, S.D. 0.08, n=7) (Fig. 1A). At 10 min, a plateau value of 1.34 nanomole Ca^{2+}/mg protein (S.D. 0.47, n=7) was reached which was maintained for the next 5 min. Phorbol ester pretreatment neither significantly affected the initial rate of Ca^{2+} uptake, determined at 60 sec (0.58 nanomole Ca^{2+}/mg protein, S.D. 0.06, n=7), nor the maximum amount of Ca^{2+} accumulated, determined at 10 min (1.36 nanomole Ca^{2+}/mg protein, S.D. 0.44, n=7). Therefore, the non-mitochondrial Ca^{2+} uptake system, recently characterized as a Ca^{2+} + K^+-stimulated Mg^{2+}-dependent transport ATPase[13] does not seem to be influenced by phorbol ester-pretreatment.

When (1,4,5-)IP_3 (2 µM) was added at 10 min, the residual Ca^{2+} content decreased linear with

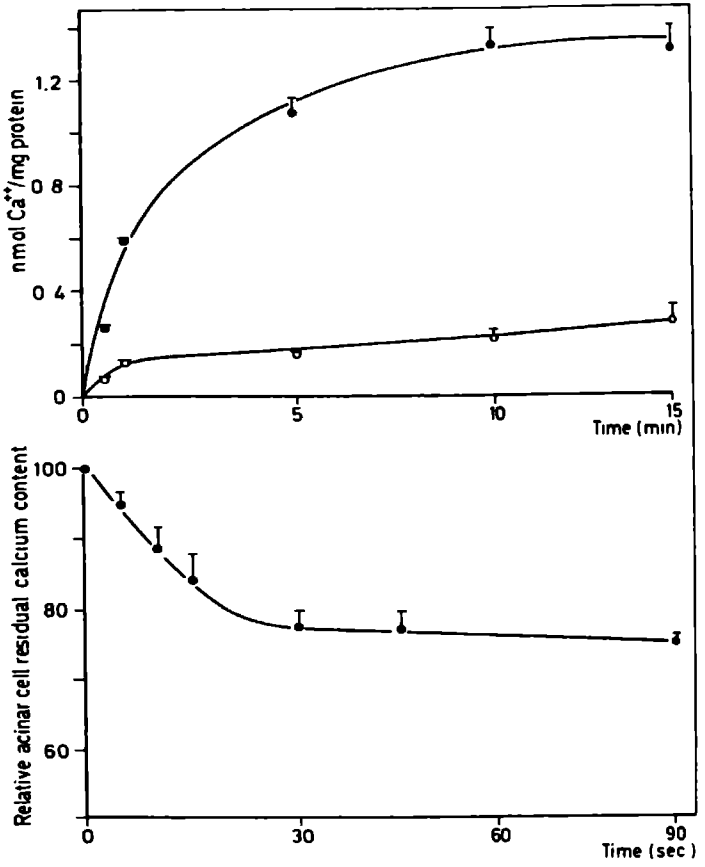


Figure 1. Time-dependence of Ca^{2+} uptake (A) and (1,4,5-)IP_3-induced Ca^{2+} release (B) in permeabilized pancreatic acinar cells. Isolated rabbit pancreatic acinar cells[3] were pelleted twice, resuspended in a high potassium medium (1 mg protein/ml) containing (mM): 135 KCl; 1.0 $MgCl_2$; 1.2 KH_2PO_4; 0.1 phenylmethylsulphonylfluoride and 10 Hepes (pH 7.4) and permeabilized with saponin (30 µg/ml, 10 min, 25°C). After saponin-treatment less than 25 % of the cells excluded trypan blue. The permeabilized cells were washed twice and resuspended (5-8 mg protein/ml) in a "Ca^{2+} uptake" medium containing (mM): 120 KCl; 1.0 $MgCl_2$; 1.2 KH_2PO_4; 5 pyruvate; 5 succinate; 0.5 EGTA; 0.5 NTA and 0.5 HEEDTA and adjusted to pH 7.1 with KOH. The mitochondrial Ca^{2+} uptake inhibitor ruthenium red (10 µM) was added and the permeabilized cells were kept at 0°C for 45 min. Calcium uptake at 37°C was started by adding 100 µl of permeabilized cells to 900 µl Ca^{2+} uptake medium which contained in addition: 10 mM creatine phosphate; 10 Units creatine kinase; 5 µCi/ml $^{45}Ca^{2+}$ and 0 (o) or 10 mM (•) MgATP. The free Mg^{2+} and Ca^{2+} concentrations were adjusted to 1.45 mM and 0.1 µM, respectively, according to Van Heeswijk et al[14]. After 10 min, maximal ATP-dependent Ca^{2+} accumulation was reached (A) and (1,4,5-)IP_3 was added (B). At the times indicated, 100 µl aliquots were quenched in 1.0 ml ice-cold "stop solution" containing (mM): 150 KCl; 5.0 $MgCl_2$; 1.0 EGTA and 20 Hepes-KOH (pH 7.1) and the suspension was rapidly filtered (Schleicher and Schüll, ME 25, 0.45 µm). The filters were washed twice with 2.0 ml ice-cold stop solution, dissolved in scintillation fluid and counted. Total Ca^{2+} was calculated and expressed as nanomole per mg protein (A). When (1,4,5-)IP_3-induced Ca^{2+} release was measured, in each experiment the Ca^{2+} content at 10 min was set at 100%, to which all other values were related (B). Protein was determined with a commercial Coomassie blue kit (Biorad, Richmond, USA) after treatment of the cells with 0.1 % Triton X-100. The values presented are the means ± S.E. of 5-7 (A) and 3-4 (B) cell preparations.

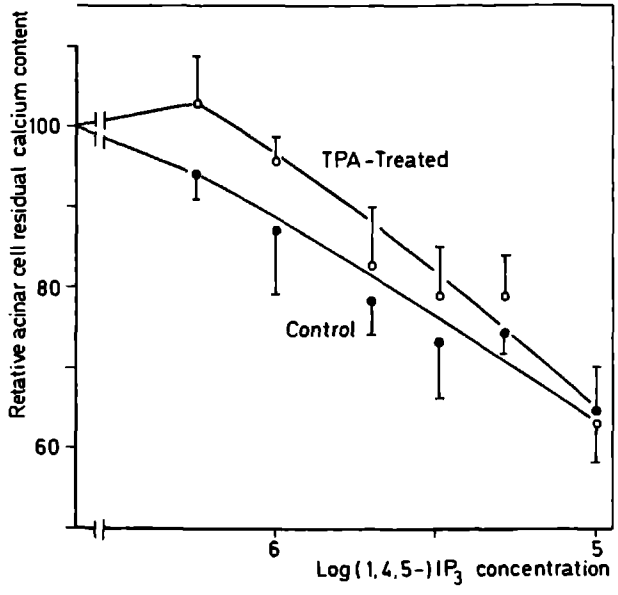


Figure 2. Effect of TPA-pretreatment on (1,4,5-)IP_3-induced Ca^{2+} release in permeabilized pancreatic acinar cells. Isolated rabbit pancreatic acinar cells were incubated in the presence of 10^{-6}M TPA (o) or 0.1% (v,v) dimethylsulfoxide (•) for 30 min at 37°C. After treatment the cells were permeabilized and resuspended in calcium uptake medium as described in the legend to figure 1. ATP-dependent calcium uptake was started by adding 10 µl of permeabilized cells to 90 µl Ca^{2+} uptake medium. At 10 min maximal ATP-dependent Ca^{2+} accumulation was reached and (1,4,5-)IP_3 was added in the concentrations indicated. After 30 sec 1.0 ml ice-cold stop solution was added and the suspension was rapidly filtered. The filters were washed, dissolved in scintillation fluid and radioactivity was counted. Total Ca^{2+} was calculated and expressed as nanomole per mg protein. In every experiment, for dimethylsulfoxide- as well as phorbol ester- treated cells, each concentration of (1,4,5-)IP_3 was tested in triplicate. In between two concentrations of (1,4,5-)IP_3 its solvent was added and the mean of the latter triplicate was set at 100%, to which the means of the other two triplicates were related. The values presented are the means ± S.E. of three different cell preparations. A rather high variation was observed among the three cell preparations used in this investigation with respect to the effectiveness of (1,4,5-)IP_3 to release actively accumulated Ca^{2+}. However, multivariate analysis of variance revealed that over the whole range of (1,4,5-)IP_3 concentrations tested, the (1,4,5-)IP_3-induced decrease in residual Ca^{2+} content was significantly less in phorbol ester-treated cells ($p = 0.0027$). It was calculated that in phorbol ester-treated cells the residual Ca^{2+} content was 5.5% (S.E. 1.6%) above the value obtained in control cells, indicating that in phorbol ester-treated cells the dose-response curve for (1,4,5-)IP_3-induced Ca^{2+} release is significantly shifted to the right.

time during the first 15 sec and a maximal effect was observed within 30 sec (Fig. 1B). No reuptake of Ca^{2+} occurred during the next 60 sec, which suggests that the (1,4,5-)IP_3 receptor remains occupied and (1,4,5-)IP_3 breakdown is still not completed. In subsequent experiments, in which the residual Ca^{2+} content was measured 30 sec after the onset of stimulation, (1,4,5-)IP_3 decreased the residual Ca^{2+} content dose-dependently in both dimethylsulfoxide- and phorbol ester-treated acinar cells (Fig. 2). However, in TPA-treated cells the dose-response curve was significantly shifted to the right. Since the (1,4,5-)IP_3-induced release of Ca^{2+} at a maximally effective concentration of 10 μM (Ref. 5) was not markedly inhibited in phorbol ester-treated cells, the above observation suggests that (1,4,5-)IP_3-induced Ca^{2+} release from intracellular Ca^{2+} stores in phorbol ester-treated cells has a lower affinity for (1,4,5-)IP_3 than in untreated cells.

Since phorbol esters act by mimicking the stimulatory effect of diacylglycerol on protein kinase C[10], the data obtained in the present study provide evidence for protein kinase C-induced desensitization at the level of the (1,4,5-)IP_3 receptor, which is in support of the hypothesis that activation of protein kinase C leads to negative feedback control over the stimulus-induced release of intracellularly stored Ca^{2+} at a level beyond the receptor-mediated hydrolysis of PIP_2[3].

REFERENCES

1. Ochs, D.L., Korenbrot, J.I., Williams, J.A. (1985) Am. J. Physiol. 249, G389-G398
2. Pandol, S.J., Schoeffield, M.S., Sachs, G. and Muallem, S. (1985) J. Biol. Chem. 260, 10081-10086
3. Willems, P.H.G.M., van Nooij, I.G.P., Haenen, H.E.M.G. and De Pont, J.J.H.H.M. (1987) Biochim. Biophys. Acta 930, 230-236
4. Berridge, M.J. (1987) Ann. Rev. Biochem. 56, 159-193
5. Streb, H., Irvine, R.F., Berridge, M.J. and Schulz, I. (1983) Nature 306, 67-69
6. Streb, H., Irvine, R.F., Schulz, I. and Berridge, M.J. (1985) J. Biol. Chem. 260, 7309-7315
7. Merritt, J.E., Taylor, C.W., Rubin, R.P. and Putney, J.W., Jr. (1986) Biochem. J. 238, 825-829
8. Trimble, E.R., Bruzzone, R., Meehan, C.J. and Biden, T.J. (1987) Biochem. J. 242, 289-292
9. Doughney, C., Brown, G.R., McPherson, M.A. and Domner, R.L. (1987) Biochim. Biophys. Acta 928, 341-348
10. Nishizuka, Y. (1986) Science 233, 305-312
11. Ansah, T.-A., Dho, S. and Case, R.M. (1986) Biochim. Biophys. Acta 889, 326-333
12. van Corven, E.J.J.M., Verbost, P.M., de Jong, M.D. and van Os, C.H. (1987) Cell Calcium 8, 197-206
13. Imamura, K. and Schulz, I. (1985) J. Biol. Chem. 260, 11339-11347
14. Van Heeswijk, M.P.E., Geertsen, J.A.M. and van Os, C.H. (1984) J. Membr. Biol. 79, 19-31

Chapter 8

Differential effects of cholecystokinin octapeptide and 12-O-tetradecanoylphorbol 13-acetate on ^{32}P-labelling of phospholipids in rabbit pancreatic acini.

WILLEMS, P.H.G.M.

VAN NOOIJ, L.G.P. and

DE PONT, J.J.H.H.M.

Department of Biochemistry, University of Nijmegen,
P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

The abbreviations are: CCK-8, C-terminal octapeptide of cholecystokinin; PI, phosphatidylinositol; PC, phosphatidylcholine; PA, phosphatidic acid; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; (1,4,5-)IP_3, inositol 1,4,5-trisphosphate; (1,3,4-)IP_3, inositol 1,3,4-trisphosphate; (1,3,4,5-)IP_4, inositol 1,3,4,5-tetrakisphosphate.

SUMMARY

We have studied the effects of both the C-terminal octapeptide of cholecystokinin (CCK-8) and the phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA), on the labelling of pancreatic acinar cell phospholipids by ortho[^{32}P]phosphate.

CCK-8, but not TPA, markedly enhanced ^{32}P-labelling of phosphatidic acid and phosphatidylinositol. Only in the presence of CCK-8, labelling of phosphatidylinositol 4,5-bisphosphate (PIP_2) reached equilibrium within 60 min. Equilibrium was reached at 40 min and the amount of labelled PIP_2 was reduced by some 50% compared to control acini or acini labelled in the presence of TPA. This suggests that in the presence of CCK-8, but not of TPA, PIP_2 levels are significantly decreased. Additional stimulation through the cholinergic receptor of acini, labelled in the presence of CCK-8, did not lead to a further decrease in the amount of ^{32}P-labelled PIP_2.

In the presence of TPA, labelling of PIP_2 appeared to be increased, suggesting a stimulatory effect on de- and/or rephosphorylation rates. TPA did not inhibit the CCK-8 induced decrease in ^{32}P-labelled PIP_2.

Finally, labelling of phosphatidylcholine was markedly enhanced in the presence of TPA, but not in the presence of CCK-8. It remains to be elucidated whether the lack of effect of the secretagogue on the labelling of phosphatidylcholine reflects inhibitory pathways not activated by the phorbol ester alone.

INTRODUCTION

Stimulation of pancreatic enzyme secretion with acetylcholine or cholecystokinin is accompanied by rapid changes in phospholipid metabolism. Within seconds after stimulation marked decreases in [^{32}P]orthophosphate- or [^{3}H]inositol-prelabelled phosphatidylinositol 4,5-bisphosphate (PIP_2) are observed, followed by rapid incorporation of label in phosphatidic acid and phosphatidylinositol in the presence of these radioactive tracers [1-3]. The breakdown products of PIP_2, diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) have important second messenger functions in that they activate the calcium- and phospholipid- dependent protein kinase C and release calcium from an intracellular store, respectively [4-6]. Phorbol esters, like 12-O-tetradecanoylphorbol 13-acetate (TPA), are able to activate protein kinase C, probably because of their structural analogy with diacylglycerol [7].

Recently we [8] and others [9] showed that activation of protein kinase C also leads to inhibition of secretagogue-induced calcium mobilization and thus may attribute to secretagogue-induced desensitization. However, in our experiments the CCK-8-induced PIP_2 hydrolysis was not impaired by pretreatment with TPA [8].

In the latter study [8], the effect of TPA on the CCK-8-induced breakdown of PIP_2 was investigated in acinar cells prelabelled with ortho[^{32}P]phosphate for 60 min, during the last 30 min of which the phorbol ester was present. We found that TPA significantly stimulated the incorporation of label into phosphatidylcholine (PC), whereas the labelling of PIP_2 appeared to be slightly reduced. This has led us to compare the effects of TPA on the labelling of phospholipids in rabbit pancreatic acinar cells with that of the calcium mobilizing secretagogue cholecystokinin.

MATERIALS AND METHODS

Preparation of rabbit pancreatic acini. Rabbit pancreatic acini were prepared as previously described [8,10].

^{32}P-labelling, extraction and analysis of phospholipids. For labelling studies acini were resuspended in a Krebs-Ringer bicarbonate medium (pH 7.4) containing 119 mM NaCl, 3.5 mM KCl, 10 μM KH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.8 mM glucose, 0.5% bovine serum albumin, an amino acid mixture according to Eagle [11.], 0.2 mg/ml soybean trypsin inhibitor and 25 μCi/ml ortho[^{32}P]phosphate and were incubated for 70 min at 37°C. CCK-8 (10^{-8} M), TPA (10^{-5} M) or the solvent dimethylsulfoxide, were present during the entire incubation period. At 60 min, 10^{-5} M carbachol was added to the CCK-8-treated acini, whereas 10^{-8} M CCK-8 was added to the TPA- and dimethylsulfoxide-treated acini.

At appropriate times 200 μl samples were removed in duplo and vigorously mixed with 0.9 ml ice-cold extraction medium (dichloromethane: methanol: concentrated HCl, 20:40:1). Phase separation was obtained by adding 200 μl dichloromethane and 200 μl water, followed by centrifugation (5 min, 700xg). After collection of the organic phase, the aqueous phase was re-extracted and the combined organic phases were blown to dryness with nitrogen. The extracts were then dissolved in dichloromethane/ methanol/ water (75:25:2) and divided over two potassium oxalate-treated high performance plates (Merck). One was developed with dichloromethane/ methanol/ 20% methylamine (60:36:10) and the other with dichloromethane/ acetone/ methanol/ acetic acid/ water (40:15:13:12:8). The first system separates PIP_2, PIP, PC and PE, but not PI from PA and the second system separates PIP_2, PIP, PA and PE, but not PI from PC. Radioactive spots were visualized by means of autoradiography, scraped off and counted.

Under the above conditions of labelling with ortho[^{32}P]phosphate, PS was not labelled, whereas PE-labelling was very weak. In each experiment, for each individual phospholipid, the amount of labelling reached in the dimethylsulfoxide-treated control acini at 60 min was set at 100%, to which all other values were related.

Materials. The C-terminal octapeptide of cholecystokinin was synthesized by Dr. H.M. Rajh in the Department of Organic Chemistry, University of Nijmegen. Carbachol was purchased from ACF Chemiefarma, Maarsen, The Netherlands. Atropine and silica gel 60 HPTLC plates were obtained from Merck, Darmstadt, F.R.G.; bovine serum albumin, soybean trypsin inhibitor and TPA were from Sigma, St. Louis, MO, U.S.A.; collagenase was from Cooper Biomedical Inc., Malvern, PA, U.S.A.; hyaluronidase was from Boehringer, Mannheim, F.R.G. and ortho[^{32}P]phosphate was from New England Nuclear, Boston, MA, U.S.A. All other chemicals were of reagent grade.

RESULTS

The incorporation of ortho[^{32}P]phosphate in pancreatic acinar cell phospholipids was followed both under unstimulated (dimethylsulfoxide-treated control acini) and stimulated conditions. In the latter case either CCK-8, at a concentration (10^{-8} M) which nearly maximally stimulates enzyme secretion [10], or the phorbol ester, TPA (10^{-5} M), known to activate the calcium- and phospholipid-dependent protein kinase C [7], was present during the entire period of labelling.

As expected, the most dramatic effects of CCK-8 were observed on the labelling of PA and PI (Fig. 1). At 60 min, the ^{32}P-labelled PA content was 2.6-fold the control value and the ^{32}P-labelled PI content 3.6-fold. Of more interest is the observation that in the presence of CCK-8, ^{32}P-labelling of PIP_2 reached equilibrium within 40 min, whereas in control acini labelling of PIP_2 appeared to increase nearly linear with time during at least 60 min. At 60 min, the ^{32}P-labelled PIP_2 content in acini labelled in the presence of CCK-8 was about 50% the control value. Labelling of PIP appeared to be slightly reduced in the presence of CCK-8. However, both in control acini and in CCK-8 stimulated acini the ^{32}P-labelled PIP content increased virtually linear with time. Finally, both in control acini and in acini labelled in the presence of CCK-8, the rate of incorporation of label in PC was relatively low during the first 40 min and increased thereafter.

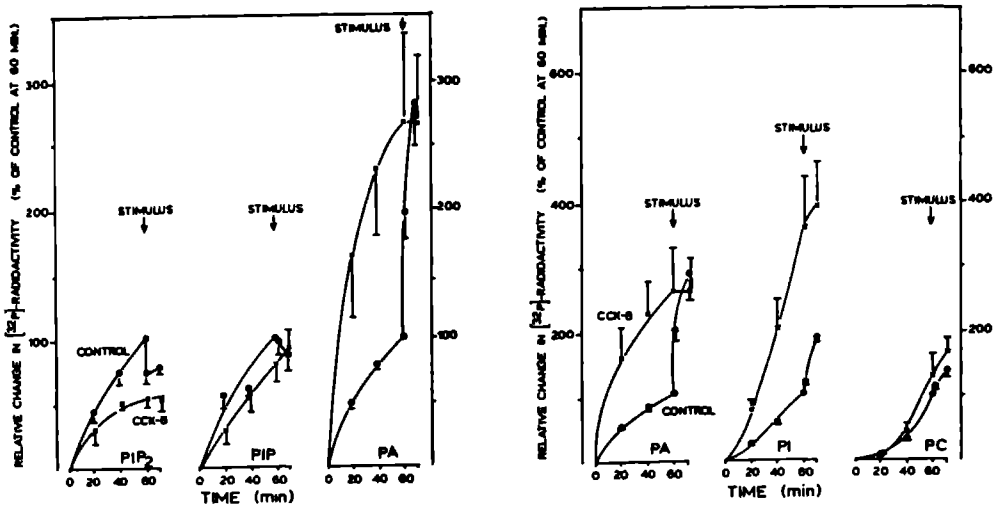


Figure 1. Effect of CCK-8 on ^{32}P-labelling of pancreatic acinar cell phospholipids. Rabbit pancreatic acini were incubated in the presence of ortho[^{32}P]phosphate and either CCK-8 (10^{-8}M) (x) or dimethylsulfoxide (•) for 70 min at 37°C. Samples for extraction and analysis of ^{32}P-labelled phospholipids were removed at the indicated times. After 60 min of labelling, the dimethylsulfoxide-treated acini were stimulated with CCK-8 (10^{-8}M) and the CCK-8-treated acini with carbamylcholine (10^{-5}M). In each experiment, for each individual phospholipid, the amount of label incorporated in the dimethylsulfoxide-treated acini at 60 min is set at 100%, to which all other values are related. The values presented are the mean ± S.E. of three experiments.

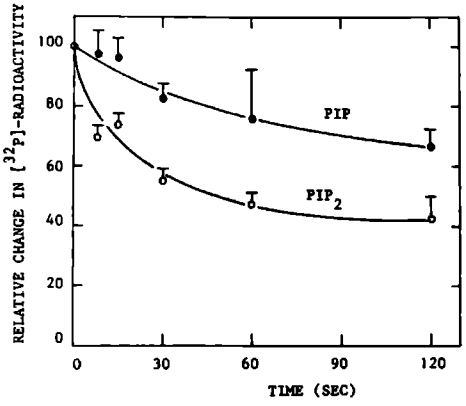


Figure 2. Time-dependence of CCK-8-induced decreases in ^{32}P-labelled PIP_2 and ^{32}P-labelled PIP in rabbit pancreatic acini prelabelled with ortho[^{32}P]phosphate. Rabbit pancreatic acini were incubated in the presence of ortho[^{32}P]phosphate for 60 min at 37°C. At 60 min the acini were stimulated with 10^{-8}M CCK-8. Samples for extraction and analysis of ^{32}P-labelled phospholipids were removed at the times indicated. For each phospholipid, the amount of label incorporated at 60 min is set at 100%, to which all other values are related. The values presented are the means of two separate experiments.

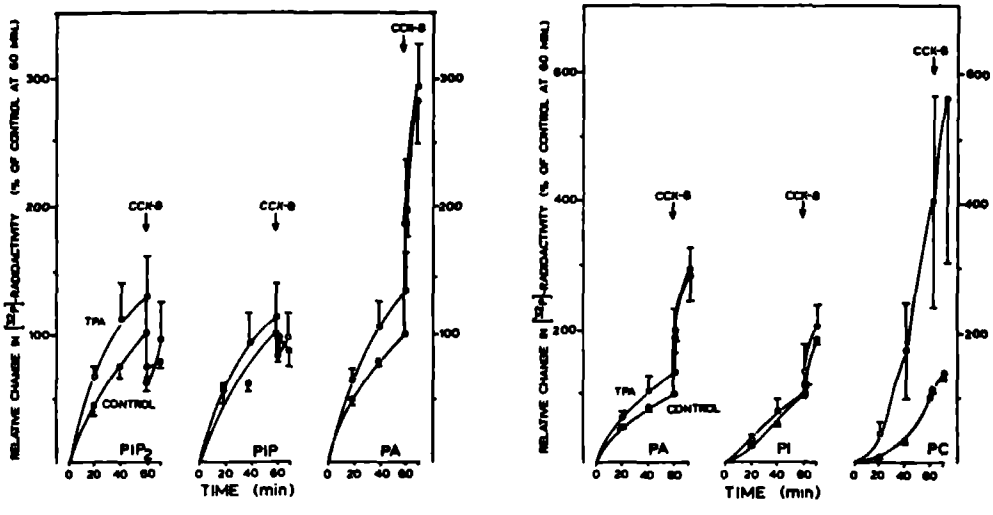


Figure 3. Effects of TPA on ^{32}P-labelling of pancreatic acinar cell phospholipids. Rabbit pancreatic acini were incubated in the presence of ortho[^{32}P]phosphate and either TPA (10^{-5}M) (o) or dimethylsulfoxide (•) for 70 min at 37°C. At 60 min the acini were stimulated with CCK-8 (10^{-8}M). Details on sampling and calculations are given in the legend of Figure 1. The values presented are the means ± S.E. of three experiments.

At 60 min, CCK-8-treated acini were stimulated with 10^{-5} M carbachol and control acini with 10^{-8} M CCK-8. In control acini, already 8 sec after the onset of stimulation, a substantial decrease in the amount of ^{32}P-labelled PIP_2 was observed (Fig. 2). A maximal effect was apparently reached within 2 min of stimulation (see also, Fig. 1). At 30 sec, the CCK-8-induced decrease in the amount of labelled PIP_2 was nearly 80% of maximal. No CCK-8-induced decrease in the amount of ^{32}P-labelled PIP was observed within 15 sec of stimulation. At 30 sec, the decrease was about 50% the decrease observed after 2 min. However, the latter decrease was apparently not maximal, since 10 min after the onset of stimulation, the amount of labelled PIP was still further decreased (Fig. 1).

Additional stimulation of CCK-8 stimulated acini with carbachol did not change the ^{32}P-labelled PIP_2 content when measured 10 min after the onset of stimulation (Fig. 1). Neither were marked effects observed on ^{32}P-labelling of PIP, PA or PI. On the other hand, a rapid decrease in the amount of ^{32}P-labelled PIP_2 was observed in control acini as well as rapid increases in labelled PA and PI. At 30 sec, the ^{32}P-labelled PIP content was hardly affected. Ten minutes after the onset of stimulation, the amount of ^{32}P-labelled PIP_2 was still decreased, whereas a decrease in the ^{32}P-labelled PIP content was more obvious now.

In the presence of TPA, labelling of PIP_2 and to a lesser extent of PIP and PA appeared to be enhanced, whereas labelling of PI was virtually not affected (Fig. 3). The most dramatic effect of TPA was on the labelling of PC. At 60 min, the ^{32}P-labelled PC content was about 5.4-fold the control value. Preincubation of the acini in the presence of the phorbol ester neither affected the CCK-8-induced decrease in ^{32}P-labelled PIP_2, nor the CCK-8-induced increases in ^{32}P-labelled PA and PI. However, ^{32}P-labelling of PIP_2 and PIP appeared to be increased in phorbol ester treated acini when measured 10 min after the onset of stimulation.

DISCUSSION

In unstimulated pancreatic acinar cells synthesis and breakdown of PIP and PIP_2 are in balance. The main enzymatic reactions involved are de- and rephosphorylation reactions. Under unstimulated conditions the incorporation of ortho-[^{32}P]phosphate in PIP and PIP_2 occurs by (re)phosphorylation and, as was found in the present study, did not reach equilibrium during a 60 min period of labelling.

Upon cholinergic stimulation or upon stimulation with cholecystokinin, the balance of de- and rephosphorylation of the phosphoinositides is disturbed, primarily because of the rapid breakdown of PIP_2. In acinar cells, prelabelled with ortho[^{32}P]phosphate, this rapid secretagogue-induced breakdown of PIP_2 is reflected by a rapid decrease in the amount of ^{32}P-labelled PIP_2, which in this study was nearly maximal within 30 sec after the onset of stimulation. After this initial decrease, the amount of ^{32}P-labelled PIP_2 hardly increased over the next 10 min, indicating that a lower steady-state level of PIP_2 was established. However, it must be taken into account that at the time of stimulation, ^{32}P-labelling of PIP_2 had not reached equilibrium, so that the sustained secretagogue-induced decrease in ^{32}P-labelled PIP_2 is somewhat underestimated because of a concommitant increase in specific labelling to reach equilibrium.

When acinar cells were labelled with ortho[^{32}P]phosphate under stimulatory conditions, the amounts of ^{32}P-labelled PIP_2 extracted at 60 min were significantly less than in unstimulated cells. Moreover, ^{32}P-labelling of this polyphosphoinositide reached equilibrium within 40 min of labelling. These observations are in support of a lower steady-state level of PIP_2 and disagree with the hypothesis that in stimulated cells the amount of PIP_2 increases again to prestimulatory levels [5].

Stimulation of pancreatic acini, prelabelled with [^{3}H]inositol, has been shown to result in accumulation of labelled (1,3,4-)IP_3 with time [12-14]. Since (1,3,4-)IP_3 is formed upon dephosphorylation of (1,3,4,5-)IP_4, the phosphorylation product

of (1,4,5-)IP_3, this observation strongly suggests that PIP_2 breakdown is increased during the entire period of stimulation and we provide evidence now that this occurs at a lower steady-state level of PIP_2.

In studies dealing with secretagogue-induced desensitization [15,16], inhibition of subsequent stimulation is most obvious when pancreatic acinar cells are treated first with supramaximally stimulatory concentrations of secretagogue. When adding cholecystokinin together with a cholinergic receptor antagonist to pancreatic acini incubated in the presence of a supramaximally stimulatory concentration of carbachol, no increase in free cytosolic calcium concentration was observed by Pandol et al. [17]. They concluded that this was due to carbachol-induced depletion of the intracellular calcium store. However, we find now that the rate of PIP_2 hydrolysis reached with a nearly maximally stimulatory concentration of 10^{-8}M CCK-8 [10] appears to be maximal, since additional stimulation with a nearly maximally stimulatory concentration of 10^{-5}M carbachol [10] did not result in any further decrease in the amount of ^{32}P-labelled PIP_2. This suggests that the findings of Pandol et al.[17] may also be explained by an already maximally carbachol-induced decrease in the amount of PIP_2.

Stimulation of rabbit pancreatic acini, prelabelled with [^{32}P]phosphate, did not result in an initial fast, but in a sustained slow decrease in the amount of ^{32}P-labelled PIP. This observation is in line with the hypothesis that stimulation results primarily in the enhanced breakdown of PIP_2, leading to the formation of (1,4,5-)IP_3, generally accepted to be the intracellular messenger mediating the release of intracellularly stored calcium [4,5]. However, an almost instantaneous and substantial decrease in the amount of PIP_2, must result in a rapid decrease in the rate of dephosphorylation of PIP_2. Assuming that the rate of phosphorylation of PIP remains unaltered, the fact that no rapid decrease in ^{32}P-labelled PIP was observed, suggests that the rate of dephosphorylation of PIP_2 is relatively low compared to the stimulated rate of PIP_2 hydrolysis to yield diacylglycerol and (1,4,5-)IP_3. As a consequence, the rate of phosphorylation of PIP has also to be relatively low, which means that after an initial large production of (1,4,5-)IP_3, the rate of formation of this inositolphosphate rapidly declines.

However, the fact that the rapid and substantial decrease in the amount of ^{32}P-labelled PIP_2 was not followed by a rapid decrease in the amount of labelled PIP may also be explained by the fact that labelling of PIP had not reached equilibrium at the time of stimulation. In that case, lower specific labelling of PIP compared to PIP_2 would result in less pronounced secretagogue-induced decreases in labelled PIP.

Interestingly enough, the decrease in the amount of labelled PIP, underestimated because of the fact that labelling of PIP had not reached equilibrium at the time of stimulation, appeared to be more sustained. This may very well reflect stimulated breakdown of PIP through mechanisms other than phosphorylation to yield PIP_2. For instance, it may be due to an increased phospholipase C-like hydrolysis of PIP to yield diacylglycerol and inositol 1,4-bisphosphate.

Labelling of PIP under stimulatory conditions did not reach equilibrium during a 60 min period of incubation in the presence of ortho[^{32}P]phosphate. This observation is not in line with the observation that 10 min after the onset of stimulation of prelabelled cells, the amount of ^{32}P-labelled PIP is decreased and also seems to disagree with the observation that labelling of PIP_2 does reach equilibrium. However, one explanation may be that only part of the ^{32}P-labelled PIP, formed under stimulatory conditions, is used for the formation of PIP_2. If this is the case, compartimentalization may be involved. There is some evidence for compartimentalization since the major site of resynthesis of PI, the precursor for PIP, is in the endoplasmic reticulum and not in the plasma membrane [18].

The receptor-mediated increases in ^{32}P-labelling of PA and PI are of course well known phenomena, demonstrated first by Hokin and Hokin back in 1953 [19] and representing the stimulated breakdown and resynthesis of PI [4,5]. Additional cholinergic stimulation of CCK-8 stimulated acinar cells did not lead to any further enhancement of the rates at which both phospholipids were phosphorylated.

In the presence of TPA, the accumulation of label into PIP_2 appeared to be increased. Similar observations with phorbol esters have been reached in other cell types [20-23]. Increased labelling of PIP_2 can be explained by inhibition of unstimulated breakdown of PIP_2, by an increase in the rate of phosphorylation of PIP, or by increased rates of de- and re- phosphorylation of PIP_2. Whether or not the amount of PIP_2 is increased in acinar cells, incubated in the presence of TPA, cannot be concluded from the present labelling experiments, since no equilibrium was reached. Effects of TPA on the labelling of PIP and PA were less obvious, whereas the labelling of PI was virtually not affected by the phorbol ester.

Conform the results presented before [8], the CCK-8-induced decrease in the amount of ^{32}P-labelled PIP_2 was not inhibited in TPA-treated acini, neither were the CCK-8-induced increases in labelled PA and PI. In TPA-treated acini, the CCK-8-induced decrease in the amount of ^{32}P-labelled PIP, when measured 30 sec after the onset of stimulation, was much more pronounced than in control acini, which may be in line with a TPA-induced increase in the rate of phosphorylation of PIP.

It is also noteworthy that 10 min after stimulation of TPA-treated acini, the amounts of ^{32}P-labelled PIP_2 and ^{32}P-labelled PIP appeared to be increased again. This observation may suggest that in TPA-treated cells the sustained, and not the initial, secretagogue-induced breakdown of PIP_2 is inhibited.

The most remarkable effect of TPA was the enhanced labelling of PC. Stimulatory effects of phorbol esters on the metabolism of PC have been described for a number of cell types [24-29]. It has been shown that phorbol esters as well as synthetic diacylglycerols induce the translocation of the rate limiting enzyme in the synthesis of PC, CTP:phosphocholine cytidylyltransferase, from the cytosol where it is inactive to the membrane where it is active [30,31]. On the other hand, phorbol esters also activate the PC specific phospholipase C, yielding diacylglycerol and phosphocholine [32,33], and the diacylglycerol produced is used for the resynthesis of PC [33]. In the latter study it was argued that the diacylglycerol produced was not used for the synthesis of phosphatidic acid because of an inhibitory effect of TPA on diacylglycerol kinase activity. In our experiments, however, in which acini, labelled in the presence of TPA, were stimulated with CCK-8, no inhibition of ^{32}P-labelling of phosphatidic acid was observed.

In 3T3 L1 cells it has been shown that stimulation with platelet derived growth factor results also in the activation of the PC specific phospholipase C [32]. In contrast, no stimulatory effect of CCK-8 on the labelling of PC was observed in the present investigation. It is not clear whether the lack of effect of CCK-8 on the labelling of PC reflects inhibitory pathways not activated by the phorbol ester alone.

The data provided in this study show that in pancreatic acinar cells, secretagogues of the calcium mobilizing type evoke a sustained decrease in PIP_2 content. Under maximally stimulatory conditions, additional stimulation through a different receptor does not lead to any further decrease in the amount of ^{32}P-labelled PIP_2, which may explain some aspects of desensitization. The main effect of the phorbol ester was the enhanced labelling of PC. However, also some interesting but less obvious effects were observed on the labelling of the polyphosphoinositides, before as well as after stimulation, which certainly need closer examination.

REFERENCES

1. Putney, J.W.Jr., Burgess, G.M., Halenda, S.P., McKinney, J.S. and Rubin, R.P. (1983) Biochem. J. 212, 483-488
2. Orchard, J.L., Davis, J.S., Larson, R.E. and Farese, R.V. (1984) Biochem. J. 217, 281-287
3. Pandol, S.J., Thomas, M.W., Schoeffield, M.S., Sachs, G. and Muallem, S. (1985) Am. J. Physiol. 248, G551-G560

4. Berridge, M.J. (1985) Sci. Am. 253, 124-134
5. Berridge, M.J. (1987) Ann. Rev. Biochem. 56, 159-193
6. Nishizuka, Y. (1986) Science 233, 305-312
7. Castagna, M., Takai, Y., Kaikuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851
8. Willems, P.H.G.M., Van Nooij, I.G.P., Haenen, H.E.M.G. and De Pont, J.J.H.H.M. (1987) Biochim. Biophys. Acta 930, 230-236
9. Ansah, T.-A., Dho, S. and Case, R.M. (1986) Biochim. Biophys. Acta 889, 326-333
10. Willems, P.H.G.M., Van Nooij, I.G.P. and De Pont, J.J.H.H.M. (1986) Biochim. Biophys. Acta 888, 255-262
11. Eagle, H. (1959) Science 130, 432-437
12. Merritt., J.E., Taylor, C.W., Rubin, R.P. and Putney, J.W., Jr. (1986) Biochem. J. 238, 825-829
13. Trimble, E.R., Bruzzone, R., Meehan, C.J. and Biden, T.J. (1987) Biochem. J. 242, 289-292
14. Doughney, C., Brown, G.R., McPherson, M.A. and Dorner, R.L. (1987) Biochim. Biophys. Acta 928, 341-348
15. Abdelmoumene, S. and Gardner, J.D. (1980) Am. J. Physiol. 239, G272-G279
16. Barlas, N., Jensen, R.T. and Gardner, J.D. (1982) Am. J. Physiol. 242, G464-G469
17. Pandol, S.J., Schoeffield, M.S., Sachs, G and Muallem, S. (1985) J. Biol. Chem. 260, 10081-10086
18. Hokin, L.E. (1985) Ann. Rev. Biochem. 54, 205-235
19. Hokin, L.E. and Hokin, M.R. (1953) J. Biol. Chem. 203, 967-977
20. De Chaffoy de Courcelles, D., Roevens, P. and van Belle, H. (1984) FEBS Lett. 173, 389-393
21. Halenda, S.P. and Feinstein, M.B. (1984) Biochem. Biophys. Res. Commun. 124, 507-513
22. Taylor, M.V., Metcalfe, J.C., Hesketh, T.R., Smith, G.A. and Moore, J.P. (1984) Nature 312, 462-465
23. Boon, A.M., Beresford, B.J. and Mellors, A. (1985) Biochem. Biophys. Res. Commun. 129, 431-438
24. Weinstein, L.B. (1981) J. Supramol. Struct. 17, 99-120
25. Pelech, S.L. and Vance, D.E. (1984) Biochim. Biophys. Acta 779, 217-251
26. Lockney, M.W., Golomb, H.M. and Dawson, G. (1984) Biochim. Biophys. Acta 796, 384-392
27. Cook, H.W. and Vance, D.E. (1985) Can. J. Biochem. Cell. Biol. 68, 145-151
28. Hill, S.A., McMurray, W.C. and Sanwal, B.D. (1984) Can. J. Biochem. Cell. Biol. 62, 369-374
29. Wright, P.S., Morand, J.N. and Kent, C. (1985) J. Biol. Chem. 260, 7919-7926
30. Pelech, S.L., Paddon, H.B. and Vance, D.E. (1984) Biochim. Biophys. Acta 795, 447-451
31. Daniel, L.W., Waite, M. and Wykle, R.L. (1986) J. Biol. Chem. 261, 9128-9132
32. Besterman, J.M., Duronio, V. and Cuatrecasas, P. (1986) Proc. Natl. Acad. Sci. Usa. 83, 6785-6789
33. Takuwa, N., Takuwa, Y. and Rasmussen, H. (1987) Biochem. J. 243, 647-653

Chapter 9

Secretagogue-induced cyclic AMP formation in rabbit pancreatic acini: inhibitory effects of 12-O-tetradecanoylphorbol-13-acetate.

WILLEMS, P.H.G.M.

VAN NOOIJ, I.G.P. and

DE PONT, J.J.H.H.M.

Department of Biochemistry, University of Nijmegen,
P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

The abbreviations are: CCK-8, cholecystokinin octapeptide; VIP, vasoactive intestinal polypeptide; TPA, 12-O-tetradecanoylphorbol 13-acetate; IBMX, isobutylmethylxanthine; $[Ca^{2+}]_i$, free cytosolic calcium concentration; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; quin2, 2-((2-(bis((carbonyl)methyl) amino)-5-methylphenoxy)-methyl)-6-methoxy-8-((bis(carbonyl)methyl)amino)quinoline; quin2/AM, quin2-tetra(acetoxymethyl)ester.

SUMMARY

It has been demonstrated that the C-terminal octapeptide of cholecystokinin (CCK-8) is a potent stimulator of adenylate cyclase activity in broken cell preparations of pancreatic exocrine tissue. In the presence of isobutylmethylxanthine (IBMX), an inhibitor of cyclic nucleotide phosphodiesterase activity, CCK-8 only slightly increased the acinar cell cyclic AMP level. On the other hand, secretin and vasoactive intestinal polypeptide (VIP) markedly enhanced cyclic AMP accumulation.

In contrast to CCK-8, neither VIP nor secretin activated the acinar cell calcium messenger system, since both secretagogues did not induce the breakdown of ^{32}P-labelled phosphatidylinositol 4,5- bisphosphate, the ^{32}P-labelling of phosphatidic acid nor the increase in free cytosolic calcium concentration. This leaves the possibility that the simultaneous activation of the phosphatidylinositol-calcium pathway by CCK-8 underlies its inability to significantly activate the adenylate cyclase in the intact acinar cell.

To test the possibility that activation of the protein kinase C pathway of the calcium messenger system is involved in this process, the effects of TPA on secretagogue-induced cyclic AMP formation were investigated. The phorbol ester itself did not significantly affect the cellular cyclic AMP concentration in acini incubated in the presence of IBMX. The response to relatively low concentrations of CCK-8 and VIP, however, was markedly inhibited in TPA-treated acini, whereas cyclic AMP formation in response to relatively high concentrations of CCK-8 remained virtually unaffected.

The observation that the cyclic AMP response to the higher concentrations of VIP and CCK-8 was not inhibited in TPA-treated acini indicates that protein kinase C activation alone cannot account for the relative inability of CCK-8 to activate the adenylate cyclase in the intact acinar cell.

INTRODUCTION

An intriguing property of the peptide hormone cholecystokinin is its ability to activate both the phosphatidylinositol 4,5-bisphosphate (PIP_2) specific phosphodiesterase and the adenylate cyclase in the intact pancreatic acinar cell. But whereas in the intact acinar cell cholecystokinin readily increases diacylglycerol [1], inositol 1,4,5-trisphosphate (IP_3) [2,3] and free cytosolic calcium [4-7], a suitable inhibitor of cyclic nucleotide phosphodiesterase activity has to be present to enable measurement of cholecystokinin-induced increases in cyclic AMP [8,9].

Recently we have shown that pretreatment of rabbit pancreatic acini with pertussis toxin facilitated cholecystokinin octapeptide (CCK-8)-induced activation of the adenylate cyclase in intact acinar cells [10]. Since pertussis toxin is thought to inactivate the inhibitory guanine nucleotide-binding regulatory protein of the adenylate cyclase system (G_i), we postulated that activation of this regulatory protein by some component of the calcium messenger system might underlie the inability of cholecystokinin to significantly activate the adenylate cyclase in the intact pancreatic acinar cell.

It was the aim of the present investigation to test the above hypothesis by examining the effects of the phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA), known to activate the protein kinase C pathway of the calcium messenger system [11], on secretagogue-induced cyclic AMP formation in rabbit pancreatic acini. In addition we investigated whether vasoactive intestinal polypeptide (VIP) and/or secretin, two potent activators of the acinar cell adenylate cyclase, activate the calcium messenger system in rabbit pancreatic acini.

This because secretin has recently been shown to stimulate inositol phosphate production and intracellular calcium mobilization in rat exocrine pancreas [12,13]. If this stimulation would also occur in the rabbit pancreas, the hypothesis that activation of this messenger system negatively influences activation of the adenylate cyclase could be rejected beforehand.

The results presented in this paper do not provide any evidence for effects of secretin and VIP on the calcium-phosphatidylinositol system. They moreover, indicate that although phorbol ester treatment attenuates secretagogue-induced activation of the rabbit pancreatic acinar cell adenylate cyclase, activation of protein kinase C alone cannot account for the inability of cholecystokinin to significantly activate the adenylate cyclase in the intact cell.

MATERIALS AND METHODS

Preparation of rabbit pancreatic acini. Rabbit pancreatic acini were prepared as previously described [14].

Amylase secretion experiments. For amylase secretion experiments, rabbit pancreatic acini, resuspended in a Krebs-Ringer bicarbonate medium (pH 7.4) containing 119 mM NaCl, 3.5 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $CaCl_2$, 1.2 mM glucose, 1 % bovine serum albumin, an amino acid mixture according to Eagle [15] and 0.2 mg/ml soybean trypsin inhibitor, were preincubated in the absence or presence of 10^{-4} M isobutylmethylxanthine (IBMX) for 30 min at 37°C. Secretin and CCK-8 were then added and samples for measurement of medium amylase activity were removed immediately before and 15 min after stimulation. At the beginning of the experiment, an aliquot of the suspension was removed and the acini were homogenized for determination of total amylase activity. Amylase activity was measured by means of the Phadebas test and medium amylase activity was expressed as percent of total amylase present in the acini at the beginning of incubation.

Cyclic AMP measurements. Acini, resuspended in the above Krebs-Ringer bicarbonate medium (pH 7.4), were preincubated in the absence or presence of 10^{-4} M IBMX and either TPA (10^{-6} M) or dimethylsulfoxide for 30 min at 37°C. Stimulants were then added and samples for determination of the cyclic AMP concentration were removed at appropriate times and immediately mixed with ice-cold trichloroacetic acid (final concentration, 5 % (w/v)). Cyclic AMP was measured as previously described [16] and the acinar cell cyclic AMP content was expressed as pmol cyclic AMP per mg protein. Protein concentrations were determined by the Lowry method, using bovine serum albumin as a standard.

Quin2 fluorescence measurements. Measurement of quin2 fluorescence was performed as previously described [6].

Analysis of ^{32}P-labelled phospholipids. Labelling of the acini with ortho[^{32}P]phosphate, extraction of the various phospholipids and thin layer chromatography was essentially the same as reported before [6] with the exception that the extracts were devided over two potassium oxalate treated high performance thin layer chromatography plates (Merck). One plate was developed with dichloromethane, methanol and 20% methylamine (60 : 36 : 10), separating PIP_2, phosphatidylinositol 4-monophosphate (PIP), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) but not phosphatidylinositol (PI) from phosphatidic acid (PA). The second plate was developed with dichloromethane, aceton, methanol, acetic acid and water (40 : 15 : 13 : 12 : 8), separating PIP_2, PIP, PA and PE but not PI from PC. Radioactive spots localized by means of autoradiography were scraped off and counted as described before [6]. The amount of ^{32}P-labelled phospholipid was expressed as percent of the amount extracted from unstimulated control acini.

Materials The C-terminal octapeptide of cholecystokinin has been synthesized by Dr. H.M. Rajh in the Department of Organic Chemistry, University of Nijmegen. The cyclic AMP binding protein was

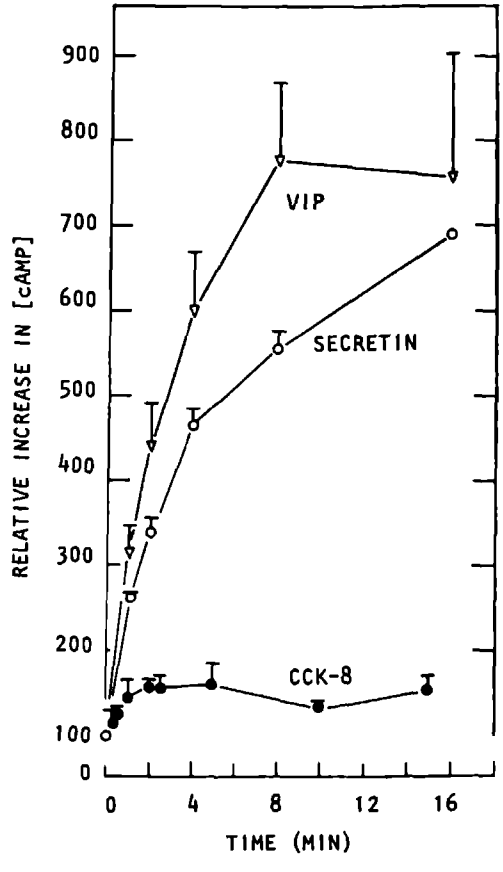


Figure 1. Time-courses for secretagogue-induced cyclic AMP formation in rabbit pancreatic acini. Rabbit pancreatic acini were preincubated in the presence of 10^{-4} M IBMX for 30 min at 37°C. CCK-8 (10^{-8} M, •), secretin (1.5×10^{-6} M, o) or VIP (10^{-6} M, v) were then added and samples for cyclic AMP measurement were removed at the indicated times. The acinar cell cyclic AMP concentration measured immediately before stimulation (6.3 pmol/mg protein, S.E. 0.3, n=4) is set at 100%, to which all other values are related. The values presented are the means ± S.E. of 3 experiments.

isolated from bovine adrenal cortex as described by Brown et al. [17]. Synthetic secretin was a gift from Dr. H. Beyerman (Department of Organic Chemistry, Technical University, Delft, The Netherlands). Carbachol was purchased from ACF Chemiefarma, Maarsen, The Netherlands. Forskolin was obtained from Calbiochem, La Jolla, CA, U.S.A.; IBMX from Aldrich Chemical Company, Inc., Milwaukee, WI, U.S.A.; VIP (porcine), TPA, bovine serum albumin, quin2/AM and soybean trypsin inhibitor, from Sigma, St. Louis, MO, U.S.A.; hyaluronidase and cyclic AMP from Boehringer, Mannheim, F.R.G.; collagenase from Cooper Biomedical Inc., Malvern, PA, U.S.A.; [^{3}H]cAMP (15 Ci/mmol) from ICN, Irvine, CA, U.S.A.; ortho[^{32}P]phosphate from New England Nuclear, Boston, MA, U.S.A.; Dowex 50W-X8 from Fluka, Buchs, Switzerland; activated charcoal (Norit, SX-1) from Norit, Amersfoort, The Netherlands and HPTLC plates silica gel 60, from Merck, Darmstadt, F.R.G. The Phadebas test kit was purchased from Pharmacia, Uppsala, Sweden. All other chemicals were of reagent grade.

RESULTS

Effects of CCK-8 and secretin on cyclic AMP formation and amylase secretion. When the acini were stimulated with secretin ($1.5x10^{-6}$ M) and the increase in medium amylase activity was measured 15 min after the onset of stimulation, no stimulatory effect was observed (Table I). Acinar cell cyclic AMP levels, however, were significantly increased by secretin (5-fold). On the other hand, CCK-8 (10^{-8} M) clearly stimulated amylase secretion (2.4-fold) without altering the acinar cell cyclic AMP concentration.

In the presence of IBMX, the resting cyclic AMP concentration was increased 3.1-fold and stimulation with secretin resulted in a 14-fold increase in acinar cell cyclic AMP concentration as compared to unstimulated acini incubated in the absence of IBMX. Despite the substantial increase in cyclic AMP level, amylase secretion remained unchanged. With CCK-8, the increase in acinar cell cyclic AMP concentration, measured in the presence of IBMX, was less pronounced (6-fold), whereas the secretory response was considerably potentiated (2.8-fold) as compared to the CCK-8-induced response in the absence of IBMX.

Time-course for secretagogue-induced cyclic AMP formation. Rabbit pancreatic acini were preincubated in the presence of 10^{-4} M IBMX for 30 min and then stimulated with either CCK-8 (10^{-8} M), VIP (10^{-8} M) or secretin ($1.5x10^{-6}$ M). Figure 1 shows the respective time-courses for cyclic AMP formation. After 30 min of incubation in the presence of 10^{-4} M IBMX, the acinar cell cyclic AMP concentration was 6.3 pmol/mg protein (S.E. 0.3; n=4). Both secretin and VIP markedly enhanced the acinar cell adenylate cyclase activity and the increase in cellular cyclic AMP concentration was nearly linear with time during the first 8 min after the onset of stimulation.

We have previously shown, that, in the absence of IBMX, CCK-8 at a concentration of 10^{-8} M not only maximally stimulates rabbit pancreatic enzyme secretion, but also maximally potentiates forskolin-induced cyclic AMP formation [16]. Hence, a concentration of 10^{-8} M was used in this investigation to determine the time-dependency for the cyclic AMP response to CCK-8. With CCK-8, only a small increase in acinar cell cyclic AMP concentration was observed and maximal cyclic AMP levels were already reached within 2 min of stimulation.

Effects of CCK-8, VIP and secretin on the free cytosolic calcium concentration. The effects of CCK-8, VIP and secretin on the acinar cell free cytosolic calcium concentration ($[Ca^{2+}]_i$) were measured with the quin2 technique. Neither VIP, at concentrations of 5.10^{-10} -10^{-6} M (Fig. 2), nor secretin, at a concentration of $1.5x10^{-6}$ M (data not shown), changed the resting $[Ca^{2+}]_i$, whereas CCK-8, at a concentration of 5.10^{-10} M, increased the free cytosolic calcium concentration within seconds (Fig. 2).

Effects of CCK-8, VIP and secretin on [^{32}P]-PIP_2 hydrolysis. In agreement with previous findings

	amylase secretion		acinar cell cAMP concentration	
	- IBMX	+ IBMX	- IBMX	+ IBMX
unstimulated	2.7 ± 0.3 (4)	3.2 ± 0.4 (4)	2.7 ± 0.4 (6)	8.5 ± 0.3 (6)
secretin (1.5×10^{-6} M)	2.8 ± 0.1 (3)	3.4 ± 0.3 (3)	13.9 ± 4.0 (6)	37.8 ± 7.6 (6)
CCK-8 (10^{-8} M)	6.5 ± 1.1 (4)	18.3 ± 3.1 (4)	3.7 ± 0.9 (3)	16.2 ± 1.1 (3)

Table I. Effects of CCK-8 and secretin on amylase secretion and cyclic AMP formation in rabbit pancreatic acini. Rabbit pancreatic acini were incubated in the absence or presence of 10^{-4} M IBMX for 30 min at 37°C. The indicated concentrations of CCK-8 and secretin were then added. The increase in medium amylase activity and acinar cell cyclic AMP content was measured 15 min after the onset of stimulation and expressed as percentage of amylase total and as pmol/mg protein, respectively. The values presented are the means ± S.E. of the number of experiments given in parentheses.

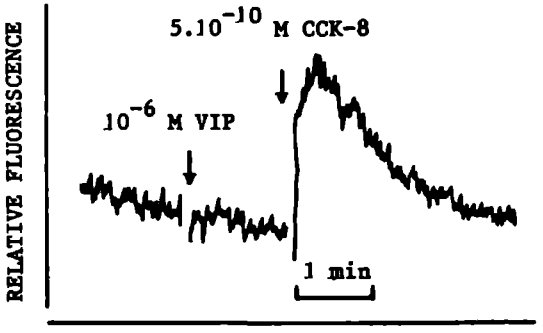


Figure 2. Effects of VIP and CCK-8 on the free cytosolic calcium concentration in rabbit pancreatic acini. Rabbit pancreatic acini resuspended in a Krebs-Ringer bicarbonate medium (pH 7.4), were incubated in the presence of quin2/AM (50 µM) for 20 min. At 20 min, the suspension was diluted 6 times and incubated for another 30 min. The suspension was then centrifuged and the acini were resuspended in fresh incubation solution. Immediately before fluorescence measurement samples were removed, rapidly centrifuged (Eppendorf minifuge) and resuspended in a HEPES/TRIS medium (pH 7.4). Fluorescence measurements were carried out at 37°C using a spectrofluorometer equipped with a thermostated cuvet holder and a magnetic stirrer. After 5 min of equilibration, VIP (5.10^{-10} - 10^{-6} M) or secretin (1.5×10^{-6} M, data not shown) and CCK-8 (5.10^{-10} M) were added successively. This figure is typical for all concentrations of VIP tested.

	[^{32}P]-PIP$_2$	[^{32}P]-PIP	[^{32}P]-PI	[^{32}P]-PA	[^{32}P]-PC	[^{32}P]- PE
unstimulated	100	100	100	100	100	100
CCK-8 (10^{-8} M)	66.5 ± 8.5	71.3 ± 5.1	101.7 ± 7.9	247.8 ± 20.1	95.5 ± 14.7	98.7 ± 2.5
secretin (1.5×10^{-6} M)	103.1 ± 1.5	121.8 ± 5.7	112.7 ± 2.7	101.2 ± 9.1	119.1 ± 2.2	116.4 ± 6.8
VIP (10^{-6} M)	108.3 ± 9.2	116.3 ± 1.1	108.3 ± 9.6	103.2 ± 11.6	105.5 ± 12.6	98.8 ± 7.3

Table II Effects of CCK-8, secretin and VIP on ^{32}P-labelling of phospholipids in rabbit pancreatic acini Rabbit pancreatic acini were incubated in the presence of ortho[^{32}P]phosphate for 60 min at 37°C After 60 min of labelling, the stimulants were added as indicated and samples for determination of radiolabelled phospholipids were removed 2 min after the onset of stimulation The amounts of ^{32}P-labelled phospholipids were expressed as percent of the amounts extracted from unstimulated control acini The values presented are the means of two separate experiments.

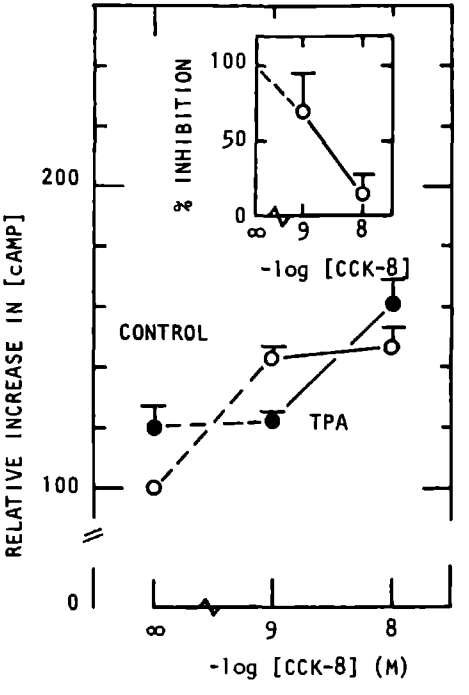


Figure 3 Effects of TPA on CCK-8-induced cyclic AMP formation in rabbit pancreatic acini Rabbit pancreatic acini were preincubated in the presence of 10^{-4} M IBMX and either dimethylsulfoxide (o) or 10^{-6} M TPA (•) for 30 min at 37°C The indicated concentrations of CCK-8 were added at 30 min and samples for cyclic AMP measurement were removed 2 min after the onset of stimulation The acinar cell cyclic AMP concentration measured in the dimethylsulfoxide-treated acini immediately before stimulation (5 9 pmol/mg protein, S E 0 3, n=4) is set at 100%, to which all other values are related. To calculate the percentage of inhibition (insertion), in each single experiment the relative increase in cyclic AMP concentration in control acini is set at 100%, to which the relative increase in TPA-treated acini is related The values presented are the means ± S E of 4 experiments

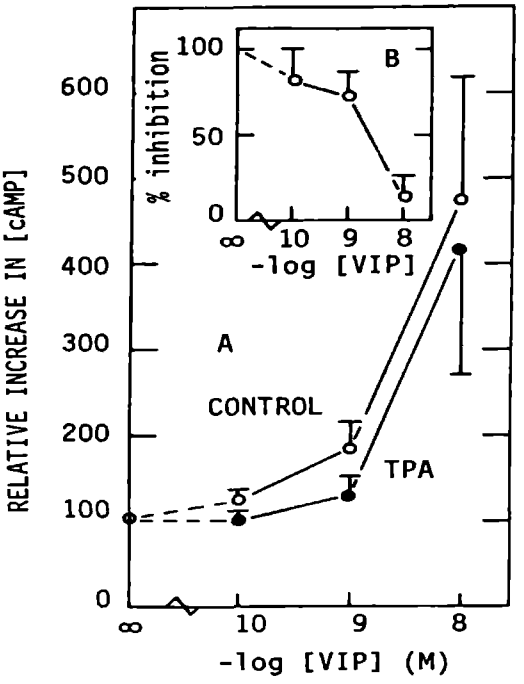


Figure 4. Effect of TPA on the dose-response curve for VIP-induced cyclic AMP formation in rabbit pancreatic acini. Rabbit pancreatic acini were preincubated in the presence of 10^{-4} M IBMX and either dimethylsulfoxide (o) or 10^{-6} M TPA (•) for 30 min at 37°C. The indicated concentrations of VIP were added at 30 min and samples for cyclic AMP measurement were removed 2 min after the onset of stimulation. The acinar cell cyclic AMP concentration measured immediately before stimulation and given in the legend to figure 3 is set at 100%, to which all other values are related (A). The percentage of inhibition (B) is calculated as described in the legend to figure 3. The values presented are the means ± S.E. of 3 experiments.

	relative acinar cell cAMP concentration
unstimulated	6.5 ± 2.3 (5)
forskolin (10^{-5} M)	56.0 ± 5.6 (5)
forskolin (10^{-5} M) + CCK-8 (10^{-8} M)	101.7 ± 9.2 (4)
forskolin (10^{-5} M) + carbachol (10^{-5} M)	100 (5)
forskolin (10^{-5} M) + CCK-8 (10^{-8} M) + carbachol (10^{-5} M)	113.5 ± 15.7 (3)
forskolin (10^{-5} M) + TPA (10^{-6} M)	61.1 ± 9.3 (5)

Table III. Effects of carbachol, CCK-8 and TPA on forskolin-induced cyclic AMP formation in rabbit pancreatic acini. Rabbit pancreatic acini were incubated in the presence of 10^{-4} M IBMX for 30 min at 37°C. At 30 min, the stimulants were added as indicated. Samples for cyclic AMP measurement were removed 15 min after the onset of stimulation. The acinar cell cyclic AMP concentration in acini stimulated with the combination of carbachol and forskolin is set at 100%, to which all other values are related. In unstimulated acini the cyclic AMP concentration was 7.5 pmol/mg protein (S.E. 2.7; n=5). The values presented are the means ± S.E. of the number of experiments given in parentheses.

[6], CCK-8 (10^{-8} M) significantly decreased the acinar cell [^{32}P]-PIP_2 content within 2 min. Table II shows that over the same period of time the acinar cell [^{32}P]-PIP content also decreased, whereas the labelling of PA markedly increased. Labelling of the other phospholipids, including PI, PC and PE remained unchanged upon stimulation with CCK-8. Secretin, however, when added at a concentration of 1.5×10^{-6} M, at which concentration cyclic AMP formation was considerably stimulated (Fig. 1), did not change the ^{32}P-labelling of the phospholipids extracted 2 min after the onset of stimulation. Also when ^{32}P-labelled acini were stimulated with 10^{-6} M VIP, no changes in [^{32}P]-PIP_2, [^{32}P]-PIP or [^{32}P]-PA were observed. At this concentration VIP, in the presence of IBMX, increased the acinar cell cyclic AMP level within 2 min to 640% (S.E. 200; n=3) of the control.

Effects of TPA on secretagogue-induced cyclic AMP formation. When acini were preincubated in the presence of IBMX and TPA (10^{-6} M) for 30 min, the acinar cell cyclic AMP concentration was 7.1 pmol/mg protein (S.E. 0.4; n=4), whereas in the presence of IBMX and dimethylsulfoxide, the cyclic AMP level was 5.9 pmol/mg protein (S.E. 0.3; n=4). Figure 3 shows that in control acini CCK-8 at both 10^{-9} and 10^{-8} M increased the acinar cell cyclic AMP level to the same extent. In TPA-treated acini, however, cyclic AMP formation induced by 10^{-9} and 10^{-8} M CCK-8 was inhibited by 70 and 15 %, respectively (Fig. 3, insertion).

VIP dose-dependently increased the acinar cell cyclic AMP concentration in acini preincubated in the presence of IBMX and either dimethylsulfoxide or TPA (10^{-6} M) (Fig. 4A). Figure 4B demonstrates that in TPA-treated acini only the response to the lower concentrations of VIP was significantly inhibited, whereas the cyclic AMP response to the higher concentration of VIP remained virtually unaffected.

Effects of TPA, carbachol and CCK-8 on forskolin-induced cyclic AMP formation. Table III summarizes the effects of TPA (10^{-6} M), carbachol (10^{-5} M) and CCK-8 (10^{-8} M) on the forskolin-induced increase in cellular cyclic AMP concentration measured 15 min after the onset of stimulation. In the presence of IBMX, the phorbol ester alone did not affect the cyclic AMP response to forskolin. The response to forskolin was almost doubled, however, when the drug was added in combination with either carbachol or CCK-8 or in combination with both secretagogues.

DISCUSSION

Stimulation of pancreatic acinar cells with cholecystokinin or with the cholinergic receptor agonist, carbachol, leads within seconds, to the enzymatic breakdown of PIP_2 [6,18,19], yielding diacylglycerol [1], and inositol 1,4,5-trisphosphate (IP_3) [2,3] and to the mobilization of intracellular calcium [4-7]. In addition, cholecystokinin induces an increase in the acinar cell cyclic AMP concentration within 30-60 sec after the onset of stimulation (Fig. 1), indicating that not only the PIP_2 specific phosphodiesterase but also the adenylate cyclase is rapidly activated by cholecystokinin. Cholecystokinin-induced increases in acinar cell cyclic AMP concentration, however, can only be measured when cyclic AMP phosphodiesterase activity is inhibited [8,9 and this publication]. In the presence of the cyclic nucleotide phosphodiesterase inhibitor, IBMX, CCK-8 stimulated cyclic AMP accumulation is only observed during the first 2 min, whereas cyclic AMP accumulation in response to VIP or secretin continues for at least 8 min with final cyclic AMP concentrations reached far above those for CCK-8 stimulated acini. This observation and the fact that in broken cell preparations of the rabbit pancreas CCK-8 proved to be even a better activator of the adenylate cyclase than secretin [20], might suggest that after the initial activation of the adenylate cyclase by cholecystokinin, a negative feed-back mechanism becomes rapidly activated preventing the secretagogue from further activation of the adenylate cyclase.

In addition, our recent observation that pretreatment of the acini with pertussis toxin considerably facilitates CCK-8-induced activation of the adenylate cyclase [10], may suggest the involvement of the inhibitory guanine nucleotide-binding protein (G_i) of the adenylate cyclase system in such

a negative feed-back mechanism. Our findings with pertussis toxin led us to speculate that CCK-8-induced activation of the calcium messenger system might lead to activation of G_i thus inhibiting CCK-8-induced activation of the acinar cell adenylate cyclase via the stimulatory G-protein (G_s). In fact, it has been shown by Katada et al. [21] that protein kinase C can directly phosphorylate the α-subunit of G_i. Phorbol esters are generally believed to exert their biological effects by activating protein kinase C in a diacylglycerol-like manner [11] and have extensively been used to investigate the role of protein kinase C in a wide variety of biological systems [22]. We now find that upon pretreatment of pancreatic acini with the phorbol ester TPA, the cyclic AMP response to the lower concentrations of both CCK-8 and VIP is inhibited. The response to the higher concentrations of secretagogue, however, is hardly affected by pretreatment with the phorbol ester and especially the finding that VIP-induced cyclic AMP formation is not lowered to the levels of CCK-8-induced cyclic AMP formation indicates that protein kinase C activation alone cannot account for the difference observed between cholecystokinin- and VIP- or secretin- induced activation of the adenylate cyclase in intact acinar cells. The fact that TPA pretreatment inhibits the cyclic AMP response to the lower concentrations of both CCK-8 and VIP indicates that in the pancreatic acinar cell protein kinase C can modulate secretagogue-induced cyclic AMP formation. One possibility is that modulation occurs at the level of G_i [21]. Another possibility is that protein kinase C acts at the level of the catalytic subunit of the adenylate cyclase [25]. TPA-induced desensitization of receptor-mediated stimulation of the adenylate cyclase has also been found for duck [23] and turkey [24] erythrocytes and was shown to be accompanied by phosphorylation of the β-adrenergic receptor thus providing a third possibility.

We have previously shown that under the same experimental conditions, employed in the present study, TPA also inhibits CCK-8-induced calcium mobilization and amylase secretion [6]. As in the present study, only the response to the relatively low concentrations of CCK-8 (10^{-10} - 10^{-9} M) was inhibited. This may suggest that the same mechanism underlies both the inhibitory action of TPA on CCK-8-induced calcium mobilization and cyclic AMP formation. This in turn would mean that the inhibitory action of TPA has to occur at the level of the CCK receptor and that this receptor is coupled both to the PIP_2 specific phosphodiesterase and the adenylate cyclase. However, the phorbol ester-induced inhibition of calcium mobilization was not paralleled by inhibition of receptor-mediated PIP_2-breakdown, suggesting a more distal site of action of TPA. We now find that the cyclic AMP response to both CCK-8 and VIP is inhibited in TPA-treated acini, suggesting that either both receptors are desensitized or that inhibition occurs more distally as discussed above.

We have shown before that the dose-response curve for CCK-8-induced potentiation of the cyclic AMP response to forskolin has the same shape as the dose-response curve for CCK-8-induced amylase secretion, with a maximum at 10^{-8} M CCK-8 and submaximal potentiation at the higher (supramaximal) concentrations of secretagogue [16]. This observation together with the present finding that the cholinergic receptor agonist carbachol, like CCK-8, when given at a concentration which maximally stimulates amylase secretion [14], potentiates the cyclic AMP response to forskolin and that the potentiating effects of CCK-8 and carbachol are not superimposable, suggests that activation of the adenylate cyclase by secretagogues which primarily act by stimulating the phosphatidylinositol-calcium pathway might be a side-effect of receptor-mediated activation of the latter pathway.

Recently it has been reported that secretin, but not VIP, stimulated IP_3 formation and calcium mobilization in rat pancreatic acini, indicating that secretin receptors are coupled to the PIP_2-specific phosphodiesterase [12,13]. In order to test whether activation of the calcium messenger system underlies the inability of CCK-8 to significantly activate the adenylate cyclase in the intact acinar cell, we investigated whether secretin and/or VIP stimulated the phosphatidylinositol-calcium pathway in rabbit pancreatic acinar cells. None of the cellular responses typical for CCK-8-induced activation of

the calcium messenger system: stimulated breakdown of [^{32}P]-PIP_2, enhanced ^{32}P-labelling of PA and rapid increases in free cytosolic calcium concentration were observed for secretin and/or VIP, indicating that at least in rabbit pancreatic acini secretin and VIP do not activate the calcium messenger system. Moreover, both secretin and VIP, despite significant increases in cellular cyclic AMP concentration, do not stimulate amylase secretion in the rabbit pancreas, whereas we have shown before that the secretory response to calcium mobilizing secretagogues is markedly potentiated once the acinar cell cyclic AMP concentration is increased [14].

From the results presented, it is concluded that protein kinase C, or one of its phosphorylated products, can modulate secretagogue-induced activation of the acinar cell adenylate cyclase in a negative sense, but that protein kinase C activation alone cannot account for the inability of CCK-8 to significantly activate the adenylate cyclase in the intact cell.

REFERENCES

1. Pandol, S.J. and Schoeffield, M.S. (1986) J. Biol. Chem. 261, 4438-4444
2. Merritt, J.E., Taylor, C.W., Rubin, R.P. and Putney, Jr., J.W. (1986) Biochem. J. 238, 825-829
3. Doughney, C., Brown, G.R., Mc Pherson, M.A. and Dormer, R.L. (1987) Biochim. Biophys. Acta 928, 341-348
4. Ochs, D.L., Korenbrot, J.I. and Williams, J.A. (1985) Am. J. Physiol. 249, G389-G398
5. Pandol, S.J., Schoeffield, M.S., Sachs, G and Muallem, S. (1985) J. Biol. Chem. 260, 10081-10086
6. Willems, P.H.G.M., Van Nooij, I.G.P., Haenen, H.E.M.G. and De Pont, J.J.H.H.M. (1987) Biochim. Biophys. Acta 930, 230-236
7. Merritt, J.E. and Rubin, R.P. (1985) Biochem. J. 230, 151-159
8. Renckens, B.A.M., van Emst-de Vries, S.E., De Pont, J.J.H.H.M. and Bonting, S.L. (1980) Biochim. Biophys. Acta 630, 511-518
9. Gardner, J.D., Sutliff, V.E., Walker, M.D. and Jensen, R.T. (1983) Am. J. Physiol. 245, G676-G680
10. Deschodt-Lanckman, M., Robberecht, P., De Neef, P., Labrie, F. and Christophe, J. (1975) Gastroenterology 68, 318-325
11. Long, B.W. and Gardner, J.D. (1975) Gastroenterology 73, 1008-1014
12. Trimble, E.R., Bruzzone, R., Biden, T.J. and Farese, R.V. (1986) Biochem. J. 239, 257-261
13. Trimble, E.R., Bruzzone, R., Biden, T.J., Meehan, C.J., Andreu, D. and Merrifield, R.B. (1987) Proc. Natl. Acad. Sci. USA 84, 3146-3150
14. Willems, P.H.G.M., Tilly, R.H.J. and De Pont, J.J.H.H.M. (1987) Biochem. Biophys. Acta 928, 179-185
15. Takai, Y., Kikkawa, U., Kaibuchi, K. and Nishizuka, Y. (1984) Adv. Cycl. Nucl. Prot. Phosph. Res. 18, 119-158
16. Willems, P.H.G.M., Van Nooij, I.G.P. and De Pont, J.J.H.H.M. (1986) Biochim. Biophys Acta 888, 255-262
17. Eagle, H. (1959) Science 130, 432-437
18. Willens, P.H.G.M., Fleuren-Jakobs, A.M.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1984) Biochim. Biophys. Acta 802, 209-214
19. Brown, B.L., Albano, J.D.M., Ekins, R.P. and Sgherzi, A.M. (1971) Biochem. J. 121, 561-563
20. Orchard, J.L., Davis, J.S., Larson, R.E. and Farese, R.V. (1984) Biochem. J. 217, 281-287
21. Putney, J.W., Burgess, G.M., Halenda, S.P., McKinney, J.S. and Rubin, R.P. (1983) Biochem. J. 212, 483-488
22. De Pont, J.J.H.H.M., Luyben, D. and Bonting, S.L. (1979) Biochim. Biophys Acta 584, 33-42
23. Katada, T., Gilman, A.G., Watanabe, Y., Bauer, S. and Jakobs, K.H. (1985) Eur. J. Biochem. 151, 431-437
24. Nishizuka, Y. (1986) Science 233, 305-312
25. Sibley, D.R., Nambi, P., Peters, J.R. and Lefkowitz, R.J. (1984) Biochem. Biophys. Res. Commun. 121, 973-979
26. Kelleher, D.J., Pessin, J.E.,Ruoho, A. and Johnson, G.L. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 4316-4320
27. Yoshimassa, T., Sibley, D.R., Bouvier, M., Lefkowitz, R.J. and Caron, M.G. (1987) Nature, 327, 67-70

Chapter 10

GENERAL DISCUSSION AND SUMMARY / SAMENVATTING EN DISCUSSIE

GENERAL DISCUSSION AND SUMMARY

Pancreatic enzyme secretion is stimulated by activating one of two functionally distinct processes. Depending on the process which is activated, two groups of pancreatic secretagogues are generally recognized (Gardner, 1979; Jensen and Gardner, 1981; Gardner and Jensen, 1980,1981,1986; Schulz and Stolze, 1980).

Some secretagogues, such as vasoactive intestinal polypeptide (VIP) and secretin, are thought to stimulate enzyme secretion by increasing the cellular cAMP concentration. Binding of these agents to their specific receptors on the basolateral surface of the acinar cell, initiates a sequence of events including, activation of the stimulatory G protein (G_s) of the adenylate cyclase system, activation of the cyclase, increases in cellular levels of cAMP, activation of protein kinase A, phosphorylation of a specific set of cellular proteins and, after a series of presently undefined steps, fusion of zymogen granules with the apical membrane.

Other secretagogues, such as acetylcholine, cholecystokinine, physalaemin, bombesin, litorin, eledoisin and the cholecystokinin similar peptides gastrin and caerulein, cause increases in the free cytosolic calcium concentration. Binding of these agents to their specific receptors, initiates a sequence of events including, activation of a G protein (G_p), activation of the PIP_2-specific phosphodiesterase, increases in levels of diacylglycerol and (1,4,5-)IP_3, activation of protein kinase C and mobilization of intracellular calcium stores, phosphorylation of a specific set of cellular proteins by kinase C and modulation of the activity of a specific set of cellular proteins by calcium and, after a series of presently undefined steps, discharge of zymogens via exocytosis.

The cAMP messenger system

In the present study *(chapter 9)*, both secretin and VIP are found to increase the cAMP concentration in the intact rabbit pancreatic acinar cell. This indicates that also in the rabbit, secretin and VIP bind to receptors which are functionally coupled to adenylate cyclase via G_s. However, in a previous study, it was found that secretin did not increase the activity of adenylate cyclase in a particulate fraction of the rabbit pancreas, whereas it did in rat and guinea pig (De Pont et al., 1979). Moreover, it was found that CCK-8 stimulated adenylate cyclase equally well in all three species and in rat and guinea pig, the stimulatory effect of CCK-8 was comparable to the stimulatory effect of secretin. In the latter study, no GTP was added to the assay medium and it is now known that the presence of GTP is required for hormonal activation of the cyclase (Rodbell, 1980,1985; Gilman, 1984,1987; Levitzki, 1987). It has been shown in rat, that the apparent affinity of the adenylate cyclase system for GTP is 6-7 -fold higher in the presence of CCK-8 than in the presence of secretin (Svoboda et al., 1980). Therefore, the inability of secretin to stimulate adenylate cyclase in a particulate fraction of the rabbit pancreas may be explained by lack of GTP, whereas there is enough GTP present to allow activation of the cyclase by CCK-8. The species differences observed for secretin-induced activation of the cyclase may be explained either by differences in the amounts of residual GTP in the different preparations or by species-dependent differences in apparent affinity of the adenylate cyclase system for GTP in the presence of secretin. In addition, it has been shown that reducing agents such as dithiothreitol, added to protect the cyclase against oxydation of -SH groups, decrease the stimulatory effects of secretin and VIP, but not of CCK-8 (Robberecht et al., 1984).

Although secretin and VIP, both in the absence and in the presence of the inhibitor of cyclic nucleotide phosphodiesterase activity, isobutylmethylxanthine (IBMX), significantly increase the cAMP concentration in rabbit pancreatic acinar cells, enzyme secretion is not enhanced *(chapter 9)*. Again, species differences seem to be involved since both in guinea pig and in rat, secretin and VIP stimulate enzyme secretion (Kempen et al., 1975; Smith and Case, 1975; Gardner and Jackson,

1977; Singh, 1979; Gardner et al., 1979a,1982,1983). However, in both species similar increases in cellular cAMP concentration induced by either VIP or secretin do not lead to similar secretory responses (Gardner et al., 1982; Vandermeers et al., 1984; Bisonnette et al., 1984). In the rat, secretin, acting through the high affinity secretin receptor, is much more efficacious as a secretory agent than VIP, acting through the high affinity VIP receptor (Vandermeers et al., 1984; Bisonnette et al., 1984). On the other hand, in guinea pig, VIP already maximally stimulates enzyme secretion at concentrations which hardly increase the cellular cAMP concentration, whereas secretin does not become secretory active before its effect on the acinar cell cAMP level is nearly maximal (Gardner et al., 1982). Moreover, in guinea pig, secretin-induced enzyme secretion increases still dose-dependently when the cAMP concentration is already maximally elevated. The above observations for rat and guinea pig have been explained by compartimentalization of the cAMP produced (Gardner and Jensen, 1986). In case of the guinea pig, secretin is thought to stimulate cAMP formation by activating a secretin-preferring receptor at lower concentrations and a VIP-preferring receptor at higher concentrations. According to the above hypothesis, it is the cAMP produced in response to activation of the VIP-preferring receptor which is in the appropriate compartment to cause stimulation of enzyme secretion. In the presence of IBMX, the secretory response to secretin and VIP is markedly augmented in guinea pig (Gardner et al., 1982), but not in rabbit *(chapter 9)*.

Similar to secretin and VIP, forskolin-induced substantial increases in cellular cAMP concentration do not lead to enhanced enzyme secretion in rabbit *(chapter 2)*. On the other hand, in rat, forskolin does stimulate enzyme secretion (Heisler, 1983; Dehaye et al., 1985). However, the stimulatory effect of forskolin is weak and comparable to that of VIP. In our opinion, the latter observation does not support the hypothesis that the stimulatory effect of cAMP on pancreatic enzyme secretion depends on the compartment in which it is produced. There is no indication that forskolin selectively activates adenylate cyclase molecules localized in the immediate vicinity of VIP receptors.

Neither VIP nor secretin induces the breakdown of ^{32}P-labelled PIP_2 in rabbit pancreatic acini and no increases in free cytosolic calcium concentration are observed in response to these two secretagogues *(chapter 9)*. On the other hand, secretin, but not VIP, has recently been shown to stimulate the formation of (1,4,5-)IP_3 and to increase the free cytosolic calcium concentration in rat pancreatic acini (Trimble et al., 1987a,b). In our study and in the studies of Trimble et al., rather high concentrations of secretin are used, indicating that species differences may be involved.

In summary, agents such as forskolin and IBMX, but also cholera toxin and lipophilic derivatives of cAMP, which all bypass receptor activation in increasing the acinar cell cAMP concentration, stimulate enzyme secretion in guinea pig and rat but not in rabbit. However, compared to secretin in rat and to VIP in guinea pig, the secretory effect of the above compounds is weak in both species. In rabbit, neither secretin nor VIP activates the calcium messenger system, whereas in rat secretin, but not VIP, activates this messenger system and is also more efficacious than VIP as a secretory agent. Together, these observations suggest that in rat and guinea pig, but not in rabbit, cAMP is a weak activator of enzyme secretion and that the additional effect of secretin on enzyme secretion in rat may be due to its ability to activate the calcium messenger system.

- o -

CCK-8 is a potent activator of adenylate cyclase in isolated membranes of exocrine tissue in all species tested (Rutten et al., 1972; Kempen et al., 1974; Schulz et al., 1974; Bonting et al., 1977; Long and Gardner, 1977; Svoboda et al., 1978; De Pont et al., 1979). Yet, stimulation of the intact acinar cell with CCK-8 does not lead to any detectable increase in cAMP concentration *(chapter 2)*, unless a suitable inhibitor of cyclic nucleotide phosphodiesterase activity is present *(chapters 3 and 9)*. And, even in the presence of such an inhibitor, the effect of CCK-8 is weak compared to the effects exerted by secretin or VIP *(chapter 9)*. In the

intact acinar cell, CCK-8-induced activation of adenylate cyclase is improved by pretreatment with pertussis toxin *(chapter 3)*. Effects exerted by this toxin are generally ascribed to its ability to modify G proteins including the inhibitory G protein of the adenylate cyclase system (G_i) (Gilman, 1984; Ui, 1984; Bokoch et al., 1984). In case of G_i, modification by pertussis toxin results in inactivation. As a consequence, receptor-mediated inhibition of adenylate cyclase is abolished in toxin-treated cells. Our results with pertussis toxin may suggest that CCK-8 not only activates the cyclase via G_s, but also inactivates the cyclase via G_i. Inactivation of the cyclase via G_i is normally the result of receptor-mediated activation of G_i. This would imply the presence of inhibitory cholecystokinin receptors which upon activation interact with G_i.

In rabbit, CCK-8, but not secretin or VIP, activates the calcium messenger system *(chapter 9)*. This has led us to put forward the hypothesis that CCK-8 activates G_i via some component of the activated calcium messenger system. We have used the phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA), which mimicks the stimulatory effect of diacylglycerol on protein kinase C (Castagna et al., 1982), to test the hypothesis that activation of the diacylglycerol/protein kinase C pathway underlies the relative inability of CCK-8 to activate adenylate cyclase in the intact acinar cell. Pretreatment of pancreatic acinar cells with TPA does not significantly alter basal adenylate cyclase activity *(chapter 9)*. On the other hand, activation of the cyclase by CCK-8 or by VIP is inhibited in phorbol ester-treated cells. However, inhibition is only observed at the lower concentrations of secretagogue. This indicates, that activation of the diacylglycerol/protein kinase C pathway alone cannot explain the relative inability of CCK-8 to activate adenylate cyclase in the intact acinar cell.

In hepatocytes, glucagon binds to two different receptors. One receptor, the GR2 receptor, interacts with G_s and activates adenylate cyclase. The other receptor, the GR1 receptor, interacts with G_p and activates the calcium messenger system. In this tissue, phorbol esters do inhibit glucagon-induced activation of the cyclase (Heyworth et al., 1984; Murphy et al., 1987). Therefore, in hepatocytes, secretagogue-induced activation of protein kinase C may underly secretagogue-induced inhibition of adenylate cyclase. In addition, inhibitory effects of phorbol esters on hormone-sensitive adenylate cyclase have been described for human T lymphocytes (Beckner and Farrar, 1987). At present, it is generally believed that the inhibitory effect of phorbol esters on hormonal stimulation of adenylate cyclase is due to phosphorylation of the receptor by protein kinase C. Evidence for this hypothesis comes from the β-adrenergic receptor which has been shown to be phosphorylated and desensitized by phorbol ester treatment of cells bearing this receptor (Sibley et al., 1984; Kelleher et al., 1984).

In other cell types, including frog erythrocytes (Sibley et al., 1986), S49 lymphoma cells (Bell et al., 1985), rat pinealocytes (Sugden et al., 1985), Swiss 3T3 cells (Rozengurt et al., 1987) and pheochromocytoma cells (Hollingsworth et al., 1986), phorbol ester pretreatment results in increased agonist-stimulated as well as basal, guanine nucleotide- and fluoride ion- stimulated adenylate cyclase activities. In pancreatic acinar cells, TPA does not increase basal activity of the cyclase *(chapter 9)*. Even in cells pretreated with pertussis toxin, TPA has no effect on basal cyclase activity *(chapter 3)*. It has been shown that TPA produces phosphorylation of the catalytic subunit of adenylate cyclase in frog erythrocytes (Yoshimasa et al., 1987). This suggests that phosphorylation of the catalytic subunit of the cyclase by protein kinase C may be involved in phorbol ester-induced enhancement of adenylate cyclase activity. In Swiss 3T3 cells, forskolin- induced cAMP formation is enhanced by receptor-mediated as well as phorbol ester-induced activation of protein kinase C (Rozengurt et al., 1987). In cells pretreated with pertussis toxin, the enhancing effect of the phorbol ester is lost again. In the pancreatic acinar cell, forskolin- induced cAMP formation is enhanced by CCK-8 and by carbachol, but not by TPA *(chapters 2 and 9)*. In blood platelets, protein kinase C phosphorylates G_i, so reducing the pathway which inhibits adenylate cyclase (Katada et al., 1985; Jakobs et al., 1985; Olianas and Onali, 1986). Although CCK-8 and carbachol increase forskolin-induced cAMP formation in the rabbit pancreatic acinar cell, this is apparently not due to

protein kinase C-induced phosphorylation of G_i, since TPA does not enhance the cAMP response to forskolin *(chapter 9)*.

In summary, compared to secretin or VIP, CCK-8 is a weak activator of adenylate cyclase in the intact pancreatic acinar cell. The stimulatory effect of CCK-8 is apparently not due to activation of protein kinase C, since it is not mimicked by TPA. Moreover, CCK-8-induced activation of the cyclase is markedly enhanced by pretreatment of the acinar cells with pertussis toxin, which may suggest that G_i is involved in preventing CCK-8 from significantly activating the cyclase. However, CCK-8-induced activation of protein kinase C, leading to activation of G_i, cannot explain the relative inability of CCK-8 to activate the adenylate cyclase, since phorbol ester treatment only slightly inhibited secretagogue-induced activation of adenylate cyclase. Therefore, the best explanation may be the presence of three different cholecystokinin recptors on the surface of the acinar cell. One receptor which interacts with G_p, a second receptor which interacts with G_s and a third receptor which interacts with G_i.

- o -

Enzyme secretion induced by CCK-8 is substantially augmented in acinar cells in which the cAMP concentration is increased *(chapters 2, 3 and 9)*. In rabbit, increases in acinar cell cAMP concentration alone do not lead to enhanced enzyme secretion, whereas in guinea pig and rat the stimulatory effect of cAMP is relatively weak. These observations strongly suggest that cAMP rather is a modulatory agent in pancreatic enzyme secretion.

In acinar cells in which the cAMP concentration is increased, also the secretory responses to the calcium ionophore, A23187, and the phorbol ester, TPA, are potentiated *(chapters 2 and 3)*. This suggests that protein kinase A or one of its phosphorylated products, acts at or beyond the site of interaction between the (1,4,5-)IP_3/calcium limb and the diacylglycerol/protein kinase C limb of the bifurcating calcium messenger system. To clarify the molecular basis of potentiation, detailed information is needed on these later steps in the process of secretagogue-induced enzyme secretion.

In summary, pancreatic enzyme secretion induced by calcium-mobilizing secretagogues is markedly potentiated by cAMP. Normally, calcium- mobilizing secretagogues do not increase the acinar cell cAMP concentration. Physiologically, substantial increases in acinar cell cAMP concentration can be produced by secretin or VIP, which in rabbit pancreas do not stimulate enzyme secretion.

- o -

The calcium messenger system

In all species studied, pancreatic secretagogues of the calcium- mobilizing type are much more efficacious as a secretory agent than secretagogues which increase the cellular cAMP concentration. The cholinergic receptor agonist, carbachol, and the C-terminal octapeptide of cholecystokinin, are among the best studied pancreatic secretagogues of the calcium-mobilizing type.

Upon stimulation with carbachol or CCK-8, the free cytosolic calcium concentration ($[Ca^{2+}]_i$) increases within seconds, as is judged from pancreatic acinar cells loaded with the fluorescent calcium indicator, quin2 *(chapters 3-6 and 9)*. This initial increase in $[Ca^{2+}]_i$ is independent of the presence of extracellular calcium *(chapters 4 and 6)* and is thought to represent the (1,4,5-)IP_3-induced release of calcium from the intracellular calcium store. After having reached its maximum height, the $[Ca^{2+}]_i$ rapidly declines to a poststimulatory level which is maintained as long as the receptor remains occupied by the agonist *(chapter 4)*. The prestimulatory $[Ca^{2+}]_i$ is calculated to be 150 nM *(chapters 3-6)*. The poststimulatory $[Ca^{2+}]_i$, established after stimulation with nearly maximally stimulatory concentrations of CCK-8 or carbachol *(chapter 5)*, is calculated to be 200 nM *(chapter 4)*. The occurrence of poststimulatory-elevated calcium levels depends on the presence of extracellular calcium *(chapter 4)*. This suggests that hormonal stimulation increases

the plasma membrane permeability for calcium, which leads to the influx of calcium and to elevated free cytosolic calcium levels. Similar conclusions were drawn from experiments in which the influx of $^{45}Ca^{2+}$ was found to be stimulated by secretagogues of the calcium-mobilizing type (Kondo and Schulz, 1976; Dormer et al., 1981).

The potent muscle relaxant TMB-8, originally shown to inhibit caffeine- induced calcium release from isolated sarcoplasmic reticulum (Malagodi and Chiou, 1974a,b; Chiou and Malagodi, 1975), does not inhibit the CCK-8-induced, extracellular calcium-independent, initial increase in $[Ca^{2+}]_i$ in the pancreatic acinar cell *(chapters 4 and 5)*. This suggests that the drug has no effect on the secretagogue-induced and (1,4,5-)IP_3-mediated release of calcium from the intracellular store. On the other hand, TMB-8 effectively inhibits carbachol-induced increases in $[Ca^{2+}]_i$. The latter effect of the drug is most probably due to competitive antagonism at the muscarinic receptor as is judged from the rightward shift of the dose-response curve for carbachol-induced enzyme secretion in the presence of TMB-8 *(chapter 5)*. Moreover, Tennes et al. (1983) showed that TMB-8 inhibited [^{3}H]QNB binding in mouse pancreatic acini.

The presence of TMB-8 prevents CCK-8 from establishing poststimulatory- elevated calcium levels. This effect of TMB-8 may be explained by an inhibitory effect of the drug on the CCK-8-induced increase in plasma membrane permeability for calcium. In the absence of extracellular calcium, TMB-8 decreases $[Ca^{2+}]_i$ to below the prestimulatory level in CCK-8-stimulated cells only. This observation suggests a stimulatory effect on the calcium extrusion mechanism and/or an inhibitory effect on the release of calcium from an intracellular store which plays a role in maintaining $[Ca^{2+}]_i$ at the prestimulatory level in cells stimulated in the absence of extracellular calcium. TMB-8 has little or no effect on $[Ca^{2+}]_i$ in unstimulated acinar cells which suggests that the drug does not affect basal calcium influx *(chapter 4)*. The same conclusion is reached from the observation that in CCK-8- stimulated acinar cells, the $[Ca^{2+}]_i$, decreased by TMB-8 to below the prestimulatory level in the absence of extracellular calcium, returns to the prestimulatory level when calcium is readded to the medium. In the latter experiment, $[Ca^{2+}]_i$ does not increase to the poststimulatory-elevated level which supports an inhibitory effect on the CCK-8-induced increase in plasma membrane permeability for calcium.

TMB-8 has been used in a wide variety of cell types to investigate the role of intracellular calcium in stimulus-evoked processes. In most tissues, inhibitory effects are observed at millimolar concentrations of the drug. In pancreatic acini, CCK-8-induced enzyme secretion is abolished completely at 0.5 mM TMB-8 *(chapter 5)*. However, at this rather high concentration of TMB-8, severe side effects are observed. This has led us to conclude that inhibitory effects of relatively high concentrations of TMB-8 should be treated with caution.

At concentrations between 50 and 100 µM, TMB-8 potentiates the secretory response to submaximally stimulatory concentrations of CCK-8. The drug potentiates also the secretory response to A23187 and to TPA. This suggests that TMB-8, like cAMP, acts at or beyond the site of interaction between the (1,4,5-)IP_3/calcium limb and the diacylglycerol/protein kinase C limb of the bifurcating calcium messenger system. But, whereas forskolin potentiates also the secretory response to (supra)maximally stimulatory concentrations of CCK-8 *(chapter 2)*, TMB-8 inhibits enzyme secretion induced by (supra)maximal concentrations of CCK-8 *(chapter 5)*. Therefore, the mechanism of action of TMB-8 differs from the mechanism of action of cAMP.

In summary, calcium-mobilizing secretagogues, increase the permeability for calcium of both the intracellular calcium store, leading to a rapid but transient increase in $[Ca^{2+}]_i$, and the plasma membrane, leading to a sustained but small increase in $[Ca^{2+}]_i$. The putative intracellular calcium antagonist, TMB-8, has no effect on the secretagogue-induced release of calcium from the intracellular store, but prevents the onset of the secretagogue-induced poststimulatory-elevated calcium level. However, the effects of TMB-8 on the poststimulatory-elevated calcium level are not sim-

ply explained by inhibition of the secretagogue-induced increase in plasma membrane permeability for calcium.

Inhibitory effects of the drug on carbachol-induced cellular processes are apparently due to competitive antagonism at the muscarinic receptor.

Inhibitory effects by relatively high concentrations of TMB-8 most probably reflect cytotoxic activities of the drug.

At moderate concentrations TMB-8 potentiates the secretory response to CCK-8 as well as A23187 and TPA. This suggests that the drug acts at or beyond the site of interaction between the two limbs of the calcium messenger system. However, TMB-8-induced potentiation differs from cAMP-induced potentiation in that cAMP potentiates also the secretory response to (supra)maximal concentrations of secretagogue. Detailed information on cellular processes influenced by protein kinase C and/or calcium is needed to elucidate the exact site of action of TMB-8.

- o -

In a number of cell types, including neutrophils (Verghese et al., 1985; Bradford and Rubin, 1985), leukocytes (Ohta et al., 1985), mast cells (Nakamura and Ui, 1984) and adipocytes (Moreno et al., 1983), pertussis toxin inhibits cellular processes induced by calcium-mobilizing stimuli. Since the toxin is known to modify G proteins (Gilman, 1984; Ui, 1984; Bokoch et al., 1984), the above results were explained by pertussis toxin-induced inactivation of G_p. However, pretreatment of pancreatic acinar cells with the toxin has no inhibitory effect on CCK-8-induced calcium mobilization and enzyme secretion *(chapter 3)*. This observation suggests that in the pancreatic acinar cell, as in other cell types, including hepatocytes (Uhing et al., 1986) and brown fat cells (Schimmel and Elliott, 1986), G_p is insensitive to pertussis toxin. Our findings support those of Merritt et al. (1986b), who reported that in permeabilized rat pancreatic acinar cells GTPγS-induced inositol trisphosphate formation is not inhibited by pertussis toxin. According to Taylor and Merritt (1986), inhibitory effects of pertussis toxin on cellular events induced by calcium-mobilizing stimuli should be treated with caution, since toxin-induced inhibition of G_i might lead to cAMP-dependent inhibition of cellular responses induced by calcium-mobilizing stimuli. Evidence for the existence of G_p in the pancreatic acinar cell is recently provided by Williams and McChesney (1987), who reported that cholecystokinin induced the interaction of its receptor with a G protein.

In summary, preliminary evidence suggests that G_p is present in the pancreatic acinar cell. However, in the pancreatic acinar cell this G protein is also insensitive to pertussis toxin.

- o -

In addition to the stimulatory effect of TPA on pancreatic enzyme secretion (De Pont and Fleuren-Jakobs, 1984; Merritt and Rubin, 1985; Nogochi et al., 1985; Pandol et al., 1985a, *chapters 3, 5 and 6)*, the phorbol ester attenuates CCK-8-induced enzyme secretion *(chapter 6;* Lee et al., 1987). In rabbit, the inhibitory effect of TPA on CCK-8-induced enzyme secretion is paralleled by inhibition of CCK-8-induced increases in free cytosolic calcium concentration *(chapter 6)*. Inhibitory effects of phorbol esters on secretagogue-induced increases in free cytosolic calcium concentration are apparently not restricted to one type of stimulus, since in rat pancreas it has been shown that TPA also inhibits carbachol-induced increases in free cytosolic calcium concentration (Ansah et al., 1986). Attenuation of agonist-induced increases in free cytosolic calcium concentration by phorbol esters have been observed in a wide variety of cell types including, platelets (Zavoico et al., 1985; Drummond, 1985), hepatocytes (Cooper et al., 1985; Lynch et al., 1985), neutrophils (Naccache et al., 1985), smooth muscle cells (Brock et al., 1985) and glomerulosa cells (Kojima et al., 1986).

In rabbit pancreatic acini, the inhibitory effect of TPA on CCK-8- induced increases in free cytosolic calcium concentration is not paralleled by inhibition of CCK-8-induced PIP_2 breakdown *(chapter 6)*. This observation is rather unique,

since in an increasing number of tissues pretreatment with phorbol esters is found to lead to inhibition of agonist-induced hydrolysis of PIP_2 (Zavoico et al., 1985; Brock et al., 1985; Kojima et al., 1986). In hepatocytes, phorbol esters inhibit PIP_2 breakdown and calcium mobilization induced by α_1-agonists, but not by other calcium-mobilizing agents such as vasopressin and angiotensin II (Corvera and Garcia-Sainz, 1984; Cooper et al., 1985). This selective action of a phorbol ester suggests that the diacylglycerol/protein kinase C pathway exerts its negative feedback by phosphorylating particular receptors rather than G_p or the PIP_2-specific phosphodiesterase. Evidence for such a mechanism of action of protein kinase C is provided by the observation that in phorbol ester-treated smooth muscle cells, α_1-agonist-induced inhibition of PIP_2 hydrolysis is paralleled by phosphorylation of the α_1-adrenergic receptor (Leeb-Lundberg et al., 1985). Recently, Lee et al. (1987) reported that in TPA-treated rat pancreatic acinar cells, attenuation of CCK-8-induced enzyme secretion is paralleled by a reduction in specific binding of ^{125}I-labelled CCK-8. This observation suggests that protein kinase C exerts its effect at the receptor level. However, the effects of phorbol ester treatment on CCK-8-induced PIP_2 breakdown were not investigated in this study.

In rabbit, TPA inhibits both both CCK-8-induced cAMP formation and calcium mobilization at the same concentrations of secretagogue *(chapters 6 and 9)*. Assuming that protein kinase C acts at the receptor level, as proposed by Lee et al. (1987), this observation may suggest that the very same cholecystokinin receptor is coupled to both G_p and G_s. It may even be speculated that G_p itself has some stimulatory effect on adenylate cyclase.

In some tissues, including GH3 cells (Drummond, 1985) and neutrophils (Rickard and Sheterline, 1985), phorbol esters have been shown to reduce increases in free cytosolic calcium concentration induced by high potassium or calcium ionophores. Observations like these suggest that activation of protein kinase C may also lead to activation of the calcium extrusion mechanism. However, in TPA-treated pancreatic acinar cells the calcium response to the lower concentrations of CCK-8 is abolished completely, which suggests that no calcium is released into the cytosol *(chapter 6)*.

Another possibility is that TPA does not inhibit CCK-8-induced PIP_2 breakdown, but stimulates the enzyme that hydrolyses (1,4,5-)IP_3. However, it has been reported by Merritt et al. (1986b) that in rat pancreatic acini, phorbol ester treatment has no effect on caerulein-induced formation of (1,4,5-)IP_3.

In the rabbit pancreatic acinar cell, phorbol esters do not affect the CCK-8-induced breakdown of PIP_2 or the agonist-induced increase in (1,4,5-)IP_3 but inhibit the (1,4,5-)IP_3-induced release of calcium in permeabilized cells *(chapter 7)*. It is generally accepted now that the agonist-sensitive calcium pool of the cell is the endoplasmic reticulum (Streb et al., 1984; Prentki et al., 1984; O'Rourke et al., 1985). Accumulation of calcium by the endoplasmic reticulum is ATP-dependent. At a free calcium concentration of 100 nM, calcium is rapidly accumulated by this system in the permeabilized acinar cell and phorbol ester treatment has no effect on the rate at which calcium is accumulated. Therefore, the calcium pump of the endoplasmic reticulum does not seem to be controlled by protein kinase C. The rapidity and the relative insensitivity to temperature of the (1,4,5-)IP_3-induced release of calcium, suggests the presence of an (1,4,5-)IP_3-operated calcium channel in the endoplasmic reticulum (Chueh et al., 1987). Our observations suggest that activation of protein kinase C leads to desensitization of this (1,4,5-)IP_3-operated calcium channel.

In summary, phorbol ester treatment of pancreatic acinar cells results in inhibition of CCK-8-induced enzyme secretion. This inhibitory action of phorbol esters is paralleled by inhibition of CCK-8-induced increases in free cytosolic calcium concentration. Pretreatment with phorbol ester has no effect on CCK-8-stimulated breakdown of PIP_2, but reduces (1,4,5-)IP_3-induced release of calcium from the intracellular calcium store. These observations suggest that in the pancreatic acinar cell the diacylglycerol/protein kinase C pathway exerts a negative feedback on the

(1,4,5-)IP_3/calcium pathway at the level of the (1,4,5-)IP_3-operated calcium channel of the endoplasmic reticulum.

- o -

Pretreatment of pancreatic acinar cells with TPA has no effect on the CCK-8-induced breakdown of ^{32}P-labelled PIP_2 *(chapters 6 and 8)*. On the other hand, labelling of PIP_2 with ortho[^{32}P]phosphate appears to be enhanced in the presence of TPA. Similar effects of phorbol esters have been observed in other tissues (De Chaffoy de Courcelles et al., 1984; Halenda and Feinstein, 1984; Taylor et al., 1984; Boon et al., 1985). These results suggest that protein kinase C may exert a positive feedback on inositol lipid metabolism by enhancing the formation of PIP_2 (Berridge et al., 1987a). Additional evidence for this hypothesis is provided by the observation that in most cell types, prestimulatory levels of PIP_2 are restored again, soon after their substantial stimulus-induced decrease. However, in the pancreatic acinar cell, the amount of ^{32}P-labelled PIP_2 is still maximally decreased 10 min after the onset of stimulation *(chapter 8)*. Moreover, labelling of acinar cells in the presence of a nearly maximally stimulatory concentration of CCK-8, results in a substantial decrease in the amounts of ^{32}P-labelled PIP_2, whereas the rate at which labelling of PIP_2 reaches equilibrium, is markedly enhanced. This observation suggests that in the presence of secretagogue the prestimulatory level of PIP_2 is not restored. Additional stimulation of acinar cells labelled in the presence of CCK-8 through the cholinergic receptor does not lead to any further decrease in the amount of ^{32}P-labelled PIP_2. Therefore, reduced levels of PIP_2, caused by a maximally stimulatory concentration of secretagogue, may protect the cell against overstimulation and may explain some aspects of desensitization.

Labelling of pancreatic acinar cells in the presence of TPA results in increased incorporation of label in phosphatidylcholine *(chapter 8)*. This effect of the phorbol ester has been observed for several other tissues (Weinstein, 1981; Pelech and Vance, 1984; Hill et al., 1984). In the preadipocyte cell line 3T3-L1 (Besterman et al., 1986) as well as in Swiss-mouse 3T3 fibroblasts (Takuwa et al., 1987), phorbol esters induced the prompt increase in diacylglycerol. These observations suggest that phorbol esters activate the phosphatidylcholine-specific phospholipase C. No increase in ^{32}P-labelled phosphatidylcholine is observed in acinar cells labelled in the presence of CCK-8. It is not clear whether this lack of effect of the secretagogue on the labelling of phosphatidylcholine reflects inhibitory pathways not activated by the phorbol ester alone.

In summary, secretagogues of the calcium-mobilizing type apparently evoke a sustained decrease in the amount of PIP_2. Under maximally stimulatory conditions, additional stimulation through a different receptor does not lead to any further decrease in the amount of PIP_2, which may explain some aspects of desensitization. The main effect of the phorbol ester is the enhanced labelling of phosphatidylcholine. However, it remains to be elucidated whether this effect of the phorbol ester is caused by activation of protein kinase C, since no increased incorporation of label is observed in the presence of calcium-mobilizing secretagogues. Moreover, it remains to be elucidated whether the enhanced incorporation of label in phosphatidylcholine also in the pancreatic acinar cell reflects increased phospholipase C-like hydrolysis of this phospholipid.

- o -

Perspectives

Future studies will concentrate on the mechanism of action of protein kinase C in the phorbol ester-induced processes described above. Protein kinase C has been partially purified from rabbit pancreatic tissue by A.G.H. Ederveen in our department. It will be investigated whether protein kinase C inhibits (1,4,5-)IP_3-induced calcium release in a microsomal fraction, enriched in endoplasmic reticulum membranes. In addition, it will be investigated whether protein kinase C enhances the formation of the polyphosphoinositides and the

turnover of phosphatidylcholine in a microsomal fraction, enriched in plasma membranes. Finally, the protein kinase C preparation will be used in isolated membranes to study the effects of kinase C on receptor-mediated hydrolysis of PIP_2 and activation of adenylate cyclase.

Protein kinase C exerts positive forward as well as negative feedback controls over various steps of its own and other signaling pathways (Nishizuka, 1986; Kikkawa and Nishizuka, 1986; Berridge, 1987a,b). Diacylglycerol is the natural activator of protein kinase C and future investigations will concentrate on the metabolism of this important intracellular messenger.

SAMENVATTING EN DISCUSSIE

De alvleesklier of pancreas is een orgaan met een tweeledige functie. Enerzijds controleert het *endocriene* deel, dat de hormonen insuline en glucagon afgeeft aan de bloedbaan, de bloedsuikerspiegel. Anderzijds is het *exocriene* deel van de pancreas, dat zijn producten afgeeft aan het darmkanaal, betrokken bij de spijsvertering.

Anatomisch gezien bestaat de exocriene pancreas uit een zich steeds fijner vertakkend stelsel van afvoergangen, wier wanden gevormd worden door de *ductulaire* cellen, en die uiteindelijk uitmonden in blaasvormige ruimten omzoomd door de *acineuze* cellen. Dergelijke groepjes van acineuze cellen, gelegen aan het begin van de afvoergangen worden *acini* genoemd en vormen de kleinste functionele eenheden van de exocriene pancreas. De acineuze cellen staan met elkaar in contact via zogenaamde ' *gap junctions* ', welke een belangrijke rol spelen bij het op elkaar afstemmen van het secretoire gedrag van de cellen (Petersen and Iwatsuki, 1979; Meda et al., 1986,1987; Bruzzone et al., 1987).

De bestanddelen van het pancreassap kunnen naar afkomst onderscheiden worden in de spijsverteringsenzymen (amylase, trypsine, carboxypeptidasen, lipasen), welke worden aangemaakt en afgegeven door de acineuze cellen, en de bicarbonaatrijke vloeistof, wier uiteindelijke samenstelling wordt bepaald door de ductulaire cellen (Kuijpers en De Pont, 1987) en wier functie vooral is gelegen in het neutraliseren van de zure voedselbrij welke afkomstig is uit de maag. Dit laatste is nodig willen de spijsverteringsenzymen optimaal kunnen functioneren.

Zodra de zure maaginhoud wordt geleegd in de twaalfvingerige darm, geven in de darmwand gelegen endocriene cellen een hormoon af aan het bloed, het *cholecystokinine* (vroeger pancreozymine genaamd), dat de acineuze cellen aanzet tot de afgifte van de spijsverteringsenzymen. Een andere belangrijke stimulator van de enzymsecretie is de neurotransmitter, *acetylcholine*.

Om adequaat te kunnen reageren op de in het extracellulaire milieu aanwezige stimuli moet de cel functionele *receptoren* bezitten. Naast de receptoren voor bovengenoemde stimuli, de cholecystokinine receptor en de muscarine cholinerge receptor, bezit de acineuze cel nog tal van andere receptoren (Gardner, 1979; Schulz and Stolze, 1980; Jensen en Gardner, 1981; Gardner en Jensen, 1980,1981,1986). De experimenten beschreven in dit proefschrift werden uitgevoerd met de cholinerge receptor agonist, carbachol, het C-terminale octapeptide van cholecystokinine (CCK-8), secretine en het vasoactieve intestinale polypeptide (VIP). Geen van de genoemde stimuli is in staat de celwand (plasmamembraan) te passeren zodat het gedeelte van de receptor dat de stimulus herkent, buiten de plasmamembraan moet zijn gelegen. Geen van deze receptoren behoort tot het type van de transmembraanreceptoren dat zelf enzymatisch actief is. Andere met de plasmamembraan geassocieerde moleculen zijn daarom nodig om het door de receptor opgevangen signaal naar binnen toe door te geven. Dit doorgeven geschiedt door de vorming van *intracellulaire boodschappers* welke in de cel tal van biochemische processen initiëren, uiteindelijk leidend tot het beoogde gedrag van de cel.

Afhankelijk van de weg waarlangs het door de receptor opgevangen signaal wordt doorgegeven, kunnen stimuli worden onderverdeeld in twee groepen. In geval van cholecystokinine en acetylcholine leidt receptoractivering uiteindelijk tot de aanmaak van de intracellulaire boodschappers: *inositol 1,4,5-trisfosfaat (IP_3)* (Streb et al., 1985; Merritt et al., 1986a; Trimble et al., 1986,1987a,b; Dougney et al., 1987) en *diacylglycerol* (Banschbach et al., 1974; Pandol en Schoeffield, 1986). Stimulering van acineuze cellen met secretine of VIP leidt daarentegen tot de vorming van het *adenosine (cyclisch) 3',5'-monophosphate (cAMP)* (Rutten et al., 1972; Kempen et al., 1977a; Christophe et al., 1976; Robberecht et al., 1976; Gardner et al., 1976a,b; Gardner en Rottman, 1979; Gardner en Jensen, 1981).

Het cyclisch AMP boodschapper systeem

Langs welke weg receptoractivering leidt tot de aanmaak van de intracellulaire boodschapper is in meer detail bekend voor het cyclisch AMP systeem. Het membraangeassocieerde enzym dat de aanmaak van het cAMP bevordert is het *adenylaatcyclase* en reeds lang is bekend dat de activiteit van dit enzym gecontroleerd wordt door twee zogenaamde *G eiwitten* (guanine nucleotide-bindende regulatoire eiwitten) (Rodbell, 1980,1985; Gilman, 1984,1987; Houslay, 1984; Im et al., 1987; Litosch, 1987; Levitzki, 1987). Activering van het stimulerende G-eiwit (G_s) leidt tot een toename van de activiteit van het adenylaatcyclase (Smigel et al., 1985; Gilman, 1986; Pace en Lancet, 1986). Activering via het stimulerende G-eiwit wordt teniet gedaan wanneer het tweede G-eiwit, het remmende (G_i), wordt geactiveerd (Northrup et al., 1980; Codina et al., 1983; Katada et al., 1984a,b; Jakobs et al., 1984).

Zoals beschreven in hoofdstuk 9 leidt stimulering van acineuze cellen met secretine of VIP tot een stijging van de intracellulaire cAMP concentratie. De receptoren voor beide stimuli zijn daarmee naar alle waarschijnlijkheid via het stimulerende G eiwit gekoppeld aan het adenylaatcyclase. De door secretine gestimuleerde cAMP productie blijkt geremd te worden door somatostatine (Taparel et al., 1985), waarmee de somatostatine receptor gekoppeld lijkt aan het remmende G eiwit.

In hoofdstuk 9 is aangetoond dat in intacte acineuze cellen van de konijnepancreas zowel secretine als VIP het adenylaatcyclase activeren. Dit in tegenstelling tot eerdere experimenten waarin de stimuleerbaarheid van het adenylaatcyclase door secretine werd onderzocht voor geïsoleerde membranen van onder andere de konijnepancreas (De Pont et al., 1979). De aanwezigheid van GTP is een vereiste voor hormonale stimulering van het adenylaat cyclase. In laatstgenoemde experimenten werd geen GTP toegevoegd aan het assaymedium, zodat enkel GTP, achtergebleven in het membraanpreparaat, aanwezig was in het assaymedium. Bovendien is voor geïsoleerde membranen van de rattepancreas aangetoond dat de schijnbare affiniteit van het adenylaatcyclase systeem voor GTP in aanwezigheid van cholecystokinine 6 tot 7 maal hoger was dan in aanwezigheid van secretine (Svoboda et al., 1980). Het wel optreden van activering van het cyclase door cholecystokinine, maar niet door secretine, zou dan verklaard kunnen worden door de aanwezigheid van juist voldoende GTP voor activering door het eerste hormoon, maar onvoldoende voor activering door het tweede hormoon. Veelal worden membranen geïsoleerd in aanwezigheid van reducerende verbindingen als dithiothreitol, welke oxidatie van -SH groepen van het adenylaatcyclase moeten tegengaan. Aangetoond is dat in dat geval activering van het cyclase door secretine en VIP, maar niet door CCK-8, sterk geremd wordt (Robberecht et al., 1984).

Een van de criteria voor een rol als intracellulaire boodschapper is dat een verhoging van de intracellulaire concentratie van het betreffende molecuul leidt tot de respons. Voor de exocriene pancreas wordt het onderzoek naar de rol van cAMP in stimulus-secretie koppeling sterk beïnvloed door speciësverschillen. Terwijl in de cavia (Gardner en Jackson, 1977; Gardner et al., 1979a,1982,1983) en in de rat (Kempen et al., 1975; Smith en Case, 1975; Singh, 1979), secretine en VIP de enzymsecretie stimuleren, blijkt een stimulerend effect in de hond, de kat en de muis twijfelachtig (Robberecht et al., 1977; De Pont et al., 1979) en in het konijn afwezig (Hoofdstuk 9 en De Pont et al., 1979). In het laatste geval treedt wel een significante stijging van de cAMP concentratie in de acineuze cellen op (Hoofdstuk 9). Bovendien wordt in de rat, bij vergelijkbare cAMP stijgingen, de enzymsecretie door secretine aanzienlijk beter gestimuleerd dan door VIP (Vandermeers et al., 1984; Bisonnette et al., 1984).

Verhoging van de intracellulaire cAMP concentratie buiten de receptor om, bijvoorbeeld door forskoline, een verbinding die rechtstreeks het adenylaatcyclase activeert (Seamon et al., 1981), leidt in de konijnepancreas evenmin tot stimulering van de enzymsecretie (Hoofdstuk 2). In de rattepancreas wordt daarentegen met forskoline een stimulering van de enzymsecretie gevonden, welke verge-

lijkbaar is met die voor VIP, maar welke sterk achterblijft bij die voor secretine of cholecystokinine (Heisler, 1983; Dehaye et al., 1985). Het niet optreden van vergelijkbare secretoire responsen bij vergelijkbare verhogingen van de cAMP concentratie, na stimulering met secretine respectievelijk VIP, heeft geleid tot het concept van *compartimentalisatie* (Gardner en Jensen, 1986). In de cavia leidt een door VIP geïnduceerde, nauwelijks meetbare, stijging van de cAMP concentratie reeds tot de maximale secretoire respons op dit secretagoog. Daarentegen treedt de secretoire respons op secretine eerst op bij aanzienlijk sterkere stijgingen van de cAMP concentratie (Gardner et al., 1982). Het cAMP aangemaakt in geval van stimulering met VIP, en in de rat met secretine, zou zich dan in het juiste compartiment bevinden om de enzymsecretie te stimuleren. De waarneming dat in de rattepancreas na stimulering met forskoline de enzymsecretie sterk achterblijft bij die na stimulering met secretine (Dehaye et al., 1985), is echter niet in overeenstemming met bovengenoemde theorie daar geen enkele reden bestaat aan te nemen dat forskoline niet het door secretine stimuleerbare adenylaatcyclase zou activeren.

Recentelijk is aangetoond dat in de rattepancreas, secretine, en niet VIP, het calciumsysteem activeert (Trimble et al., 1987a,b). Activering van het calciumsysteem leidt in alle speciës tot enzymsecretie. Het is daarom niet ondenkbaar dat het vermogen van secretine om in de rat de enzymsecretie te stimuleren verband houdt met het vermogen van dit secretagoog om in dit speciës het calciumsysteem te activeren. In de konijnepancreas induceert noch secretine noch VIP de hydrolyse van PIP_2 (Hoofdstuk 9). Evenmin stimuleren beide secretagogen de enzymsecretie. Daarentegen neemt de cAMP productie sterk toe na stimulering met secretine of VIP. Het beeld dat ontstaat is, dat in de konijnepancreas de verhoging van cAMP onvoldoende is om de enzymsecretie te stimuleren en dat noch secretine noch VIP het calciumsysteem activeren. In de rat is de stijging van cAMP op zich al voldoende is om de enzymsecretie te stimuleren en secretine geeft een extra stimulering omdat het bovendien het calciumsysteem kan activeren.

Op grond van bovenstaande waarnemingen is geconcludeerd dat in ieder geval in de konijnepancreas cAMP niet voldoet aan de criteria voor intracellulaire boodschapper. Toch heeft cAMP wel degelijk een effect op de enzymsecretie en wel een *potentiërend* effect. Wordt namelijk het fosfatidylinositol/calciumsysteem geheel of gedeeltelijk geactiveerd, hetgeen wel leidt tot stimulering van de enzymsecretie, dan blijkt in geval van een verhoogde intracellulaire cAMP spiegel de secretoire respons aanzienlijk te worden versterkt (Deschodt-Lanckman et al., 1975; Collen et al, 1982; Dehaye et al., 1985; Hoofdstukken 2, 3 en 9). De conclusie is dat in de exocriene pancreas van het konijn de rol van cAMP een modulerende is.

Een merkwaardig verschijnsel is dat het adenylaatcyclase van de acineuze cel van de exocriene pancreas geactiveerd kan worden door een stimulus, cholecystokinine, welke primair via het fosfatidylinositol/calcium systeem lijkt te werken en ook duidelijk wel de enzymsecretie stimuleert (Rutten et al., 1972; Kempen et al., 1974; Schulz et al., 1974; Bonting et al., 1977; Long en Gardner, 1977; Svoboda et al., 1978; De Pont et al., 1979; Hoofdstukken 3 en 9). Uit het voorgaande mag blijken dat als het cAMP al een rol speelt bij de door cholecystokinine gestimuleerde enzymsecretie, er hooguit sprake kan zijn van een modulerende rol. Een modulerende rol die duidelijk tot uiting komt in de aanzienlijke potentiëring van de door cholecystokinine gestimuleerde enzymsecretie (Hoofdstukken 2, 3 en 9). Onder normale condities treedt echter geen meetbare stijging op van de intracellulaire cAMP concentratie wanneer gestimuleerd wordt met cholecystokinine. Enkel wanneer het enzym dat het cAMP afbreekt wordt geremd kan een geringe stijging van het cAMP worden waargenomen (Kempen et al., 1977a,b; Renckens et al., 1980; Gardner et al., 1983; Hoofdstukken 3 en 9). De door cholecystokinine geïnduceerde stijging van de intracellulaire cAMP spiegel staat echter in geen verhouding tot die welke geïnduceerd wordt door secretine of VIP (Hoofdstuk 9).

Zoals gezegd spelen twee G eiwitten een sleutelrol bij de overdracht van informatie van de

receptor naar het adenylaatcyclase. Beide G eiwitten blijken gemodificeerd te kunnen worden door bacteriële toxines (Gilman, 1987). Het cholera toxine modificeert het stimulerende G eiwit zodanig dat dit in een blijvend geactiveerde staat komt. Het gevolg is een aanhoudende stimulering van het adenylaatcyclase zonder dat van enige receptoractivering sprake is en de intracellulaire cAMP spiegel stijgt sterk (Smith and Case, 1975; Kempen et al., 1975; Gardner en Rottman, 1979; Gardner en Jensen, 1981).

Het remmende G eiwit wordt gemodificeerd door het pertussis toxine en wel zodanig dat het in een blijvend-geinactiveerde toestand komt (Gilman, 1984; Ui, 1984; Bokoch et al., 1984). Het gevolg is dat de activiteit van het adenylaatcyclase niet langer geremd kan worden door stimuli die via het remmende G eiwit het adenylaatcyclase controleren. Na behandeling van de acineuze cellen van de konijnepancreas met pertussis toxine, blijkt het vermogen van CCK-8 om het adenylaatcyclase te activeren sterk te zijn toegenomen (Hoofdstuk 3).

Een mogelijke verklaring zou kunnen zijn dat er twee typen cholecystokinine receptoren zijn. Een ervan zou dan, door tussenkomst van het stimulerende G eiwit, het adenylaatcyclase activeren, terwijl de andere, door tussenkomst van het remmende en door pertussis toxine inactiveerbare G eiwit, activering door de eerstgenoemde zou tegengaan. Een mogelijke ondersteuning voor deze hypothese is dat in membranen van de pancreas, cholecystokinine receptoren, geassocieerd met cholera toxine- en pertussis toxine-gevoelige eiwitten, konden worden aangetoond (Lambert et al., 1985).

Een andere mogelijke verklaring voor het feit dat het adenylaatcyclase beter geactiveerd wordt door CCK-8 in acineuze cellen welke behandeld zijn met pertussis toxine, zou kunnen zijn dat activering van het fosfatidylinositol/calciumsysteem door CCK-8 leidt tot activering van het remmende G eiwit en daarmee tot remming van de activering van het adenylaatcyclase via het stimulerende G eiwit. Om deze hypothese te kunnen testen werd de diacylglycerol/proteïne kinase C route van het fosfatidylinositol/calciumsysteem buiten de receptor om geactiveerd door middel van de forbolester, 12-O-tetradecanoylforbol 13-acetaat (TPA) (Hoofdstuk 9). De stimulering van het adenylaatcyclase door zowel CCK-8 als VIP blijkt te zijn geremd in cellen welke zijn voorbehandeld met forbolester. De mate van remming is echter gering en geeft aan dat enkel de activering van de diacylglycerol/proteïne kinase C weg onvoldoende is om het niet optreden van een significante activering van het adenylaatcyclase door CCK-8 te kunnen verklaren.

Een van de eigenschappen van stimuli die via het stimulerende G-eiwit het adenylaatcyclase activeren is dat zij de door forskoline gestimuleerde cAMP productie potentiëren. In de acineuze cellen van de exocriene pancreas blijken zowel cholecystokinine (Hoofdstukken 2 en 9) als de cholinerge receptor agonist, carbachol, (Hoofdstuk 9) de door forskoline gestimuleerde cAMP productie te verhogen. Dat dit potentiërende effect niet het gevolg is van activering van de diacylglycerol/proteïne kinase C route blijkt uit het feit dat TPA geen effect heeft op de door forskoline gestimuleerde cAMP productie (Hoofdstuk 9).

Het fosfatidylinositol/calciumsysteem.

Hormonaal geïnduceerde veranderingen in het metabolisne van fosfolipiden, in het bijzonder van fosfatidylinositol, werden voor het eerst beschreven door Hokin en Hokin (1953). Stimulering van pancreasweefsel met acetylcholine bleek in aanwezigheid van $^{32}P_i$ te leiden tot een verhoogde incorporatie van label in fosfatidylinositol en fosfatidezuur (Hokin en Hokin, 1953, 1954, 1958). Al snel bleek uit prelabelingsexperimenten met [^{3}H]inositol dat er sprake moest zijn van een gestimuleerde afbraak van fosfatidylinositol gevolgd door resynthese van dit fosfolipide via diacylglycerol en fosfatidezuur (Hokin en Hokin, 1964). De hormonaal-gestimuleerde incorporatie van $^{32}P_i$ in fosfatidylinositol bleek vervolgens onafhankelijk van de aanwezigheid van extracellulair calcium te zijn (Hokin, 1966; Farese et al, 1982a,b).

Werd gestimuleerd met acetylcholine, dan leidde dit bovendien tot afname van de totale hoeveelheid fosfatidylinositol (Hokin-Neaverson, 1974) en tot toename van de totale hoeveelheden diacylglycerol (Banschbach et al., 1974) en fosfatidezuur (Hokin-Neaverson, 1974). Merkwaardig genoeg bleken deze stimulus-geïnduceerde veranderingen van de totale hoeveelheden fosfatidylinositol en fosfatidezuur wel afhankelijk van extracellulair calcium (Farese et al., 1980; 1981a,b; 1982a,b). Bovendien werd voor een aantal celtypen, inclusief de acineuze cel van de pancreas (Farese et al., 1980), gevonden dat verhoging van de intracellulaire calciumconcentratie, door middel van de calciumionofoor A23187, eveneens leidde tot veranderingen van de totale hoeveelheden fosfatidylinositol en fosfatidezuur. Geconcludeerd werd dat stimulering van de acineuze cel van de rattepancreas met een calciummobilizerend secretagoog leidt tot een calcium-onafhankelijke toename van de turnover van fosfatidylinositol (toename van de incorporatie van ^{32}P in fosfatidylinositol) en tot een calcium-afhankelijke afname van de totale hoeveelheid fosfatidylinositol (Farese et al., 1982a,b).

Recentelijk werd echter aangetoond dat cholecystokinine in acineuze cellen van de caviapancreas ook in afwezigheid van extracellulair calcium en zelfs bij volledige depletie van de intracellulaire opslagplaats voor calcium, een daling geeft van de totale hoeveelheid fosfatidylinositol (Pandol et al., 1985b). Depletie van de intracellulaire opslagplaats voor calcium heeft tot gevolg dat geen door cholecystokinine-geïnduceerde calciumstijging kan optreden. De calciumionofoor A23187 bleek daarentegen alleen in calciumhoudend medium een afname van de totale hoeveelheid fosfatidylinositol te geven. In calciumhoudend medium veroorzaken calciumionoforen, door de inlux van calcium te bevorderen, een langdurige en sterke verhoging van de intracellulaire calciumconcentratie (Gardner et al., 1980). In afwezigheid van extracellulair calcium trad wel stimulering op van de $^{45}Ca^{2+}$ efflux, en daarmee een tijdelijke verhoging van de intracellulaire calciumconcentratie, maar geen stimulering van de afbraak van fosfatidylinositol. Een tijdelijke verhoging van de intracellulaire calciumconcentratie lijkt daarmee onvoldoende om de afbraak van fosfatidylinositol te stimuleren. Dat cholecystokinine in afwezigheid van extracellulair calcium in de caviapancreas wel en in de rattepancreas niet de totale hoeveelheid fosfatidylinositol verlaagt kan zowel liggen aan speciësverschillen als aan verschillen in de relatieve snelheden waarmee fosfatidylinositol wordt afgebroken en aangemaakt (Michell, 1975).

Het feit dat A23187 alleen in calciumhoudend medium de totale hoeveelheid fosfatidylinositol verlaagt en cholecystokinine zelfs wanneer de stijging van de intracellulaire vrije calciumconcentratie volledig wordt geremd, duidt erop dat het effect van calciummobilizerende secretagogen op het metabolisme van fosfatidylinositol ten dele onafhankelijk is van de geïnduceerde stijging van de cellulaire calciumconcentratie.

De calcium-afhankelijkheid van de stimulus-geïnduceerde afbraak van fosfatidylinositol was daarom zo belangrijk omdat inmiddels in 1975 door Michell was gepostuleerd dat deze afbraak een rol zou spelen bij de koppeling van receptoractivering aan veranderingen van de cellulaire calciumconcentratie. De wijze waarop was vooralsnog onduidelijk maar in 1977 werd door Abdel-Latif et al. voor het eerst aangetoond dat receptoractivering leidt tot de afbraak van het polyfosfoinositide, PIP_2. Deze afbraak bleek in het gebruikte gladde spierweefsel van de iris van het konijn echter afhankelijk te zijn van extracellulair calcium (Akhtar en Abdel-Latif, 1978) en geïnduceerd te kunnen worden door de calciumionofoor A23187 (Akhtar en Abdel-Latif, 1980). In de meeste andere celtypen (Kirk et al., 1981; Weiss et al., 1982; Billah en Lapetina, 1982), inclusief de acineuze cel van de pancreas (Putney et al., 1983; Orchard et al., 1984; Pandol et al., 1985b), is echter gebleken dat de stimulus-geïnduceerde PIP_2 afbraak onafhankelijk is van calcium en inderdaad de schakel vormt tussen receptoractivering en calciummobilizering (Berridge, 1985, 1987a,b).

De laatste jaren pas is meer inzicht verkregen in het werkingsmechanisme van stimuli, waarvan reeds lang bekend was dat zij én de intracellulaire vrije calciumconcentratie verhogen én het metabolisme van een van de membraanfosfolipiden, het fosfatidylinositol, stimuleren. Ook nu weer lijken G eiwitten betrokken bij de overdracht van het signaal naar het enzym dat de aanmaak van de intracellulaire boodschappers bevordert (Joseph, 1985; Cockcroft en Gomperts, 1985; Taylor en Merritt, 1986; Litosch en Fain, 1986; Litosch, 1987; Gilman, 1987; Berridge, 1987a,b). In tegenstelling tot de G eiwitten van het cAMP systeem is tot op heden nog geen G eiwit van het fosfatidylinositol/calciumsysteem geïsoleerd.

Een sterke aanwijzing voor het bestaan van een dergelijk G eiwit is de stimulering door (slecht hydrolyseerbare) GTP (analoga) van de PIP_2 afbraak en de $(1,4,5)IP_3$ vorming in gepermeabiliseerde cellen, zoals onlangs aangetoond voor acineuze cellen van de rattepancreas door Merritt et al. (1986b).

In een aantal celtypen bleek behandeling met het eerder genoemde pertussis toxine te leiden tot remming van responsen opgewekt door calciummobilizerende stimuli (Brandt et al., 1985; Krause et al., 1985; Nakamura and Ui, 1985; Bradford en Rubin, 1985). Door gebruik te maken van een verbinding welke sterker begint te fluoresceren zodra zij calcium bindt, het quin2 (Rink en Pozzan, 1985), kon worden nagegaan dat behandeling van acineuze cellen van de exocriene pancreas van het konijn met pertussis toxine niet leidt tot een remming van de door cholecystokinine geïnduceerde stijging van de intracellulaire vrije calciumconcentratie (Hoofdstuk 3). Daarmee blijkt in geval van de acineuze cel van de exocriene pancreas het G eiwit, dat verondersteld wordt receptoren als die voor het cholecystokinine en het acetylcholine te koppelen aan het PIP_2 fosfodiesterase, ongevoelig te zijn voor pertussis toxine.

Het enzym dat in dit systeem de aanmaak van de intracellulaire boodschappers bevordert is het fosfatidylinositol 4,5-bisfosfaat- specifieke fosfodiesterase (PIP_2 fosfodiesterase). Het substraat, PIP_2, wordt door tussenkomst van dit enzym op een zodanige wijze afgebroken dat als producten het diacylglycerol en het $(1,4,5)IP_3$ ontstaan (Berridge, 1985,1987a,b). De door cholecystokinine gestimuleerde afbraak van PIP_2 kan, voor de hogere concentraties van het hormoon, reeds na 8 sec gemeten worden en is duidelijk dosis-afhankelijk (Hoofdstuk 6). Noch het in deze studies gebruikte synthetische secretine noch het VIP stimuleren de afbraak van PIP_2 (Hoofdstuk 9), hetgeen in overeenstemming is met het feit dat zij geen van beiden de enzymsecretie stimuleren (Hoofdstuk 9). Recentelijk is echter gevonden dat stimulering van acineuze cellen van de rattepancreas met supramaximale concentraties secretine leidt tot een stijging van de $(1,4,5)IP_3$ concentratie en de vrije calciumconcentratie (Trimble et al., 1986, 1987a,b). Bovendien is gebleken dat stimulering van acineuze cellen van de rattepancreas met dibutyryl cAMP leidt tot een van calcium afhankelijke afbraak van fosfatidylinositol (Farese et al., 1981b).

Hoewel secretine het fosfatidylinositol/calcium systeem alleen activeert bij supramaximale concentraties, is toch interessant dat VIP dit systeem in de rattepancreas niet activeert (Trimble et al., 1986). En terwijl beide secretagogen de cAMP productie in dezelfde mate stimuleren, is het secretoire effect van VIP veel geringer en volledig vergelijkbaar met dat van forskoline (Vandermeers et al., 1984; Bisonnette et al., 1984). Een mogelijke verklaring zou kunnen zijn dat, in de rattepancreas, forskoline en VIP de enzymsecretie stimuleren door stimulering van de calcium-afhankelijke afbraak van het fosfatidylinositol, terwijl secretine daarnaast de calcium-onafhankelijke afbraak van fosfatidylinositol via PIP_2 stimuleert en daarmee in veel sterkere mate de enzymsecretie.

De inositolpolyfosfaat/calcium route

Binnen enkele seconden na stimulering kan reeds een verhoging van de $(1,4,5)IP_3$ concentratie worden gemeten (Streb et al., 1985; Merritt et al., 1986a; Trimble et al., 1987b; Doughney et al.,

1987). Iets later in de tijd treedt een verhoging op van de inositol 1,3,4,5-tetrakisfosfaat ((1,3,4,5)IP_4) concentratie, gevolgd door een stijging van de concentratie van het inositol 1,3,4-trisfosfaat ((1,3,4)IP_3) (Trimble et al., 1987b; Doughney et al., 1987). Inositol 1,3,4,5-tetrakisfosfaat wordt door fosforylering gevormd uit (1,3,4)IP_3, waarna uit (1,3,4,5)IP_4 door defosforylering (1,3,4)IP_3 wordt gevormd (Berridge, 1985,1987a,b). Inositoltrisfosfaten worden achtereenvolgens gedefosforyleerd tot inositolbisfosfaat, inositolmonofosfaat en inositol. Het gevormde inositol wordt vervolgens gebruikt voor de resynthese van fosfatidylinositol. Uit fosfatidylinositol wordt dan weer via fosfatidylinositol 4-monofosfaat (PIP), PIP_2 gevormd (Berridge, 1985,1987a,b).

De concentraties van het (1,4,5)IP_3 en het (1,3,4,5)IP_4 bereiken vrij snel na aanvang van stimulering een plateau, terwijl de concentratie van (1,3,4)IP_3 blijft stijgen in de tijd (Trimble et al., 1987b; Doughney et al., 1987). Bij aanhoudende stimulering blijft de afbraak van PIP_2 dus doorgaan. Een dergelijke verhoogde turnover verklaart waarom bij de labeling van PIP_2 met ^{32}P eerder een evenwicht wordt bereikt in aanwezigheid van cholecystokinine (Hoofdstuk 8).

Van het (1,4,5)IP_3 werd in 1983 (Streb et al., 1983) bekend dat het calcium vrij kan maken uit een gesloten, ATP-afhankelijke en niet-mitochondriële intracellulaire opslagplaats. Thans lijkt vrij aannemelijk gemaakt dat deze intracellulaire opslagplaats voor calcium het endoplasmatisch reticulum is (Streb et al., 1984). Het oorspronkelijke experiment van Streb et al. (1983) werd uitgevoerd met gepermeabilizeerde acineuze cellen van de exocriene pancreas en met gebruik making van een zeer gevoelige calcium-selectieve electrode. Hoofdstuk 7 beschrijft hetzelfde type experiment, uitgevoerd met gepermeabilizeerde cellen van de konijnepancreas. De door (1,4,5)IP_3 gestimuleerde efflux van calcium werd gemeten met behulp van $^{45}Ca^{2+}$. Bij een concentratie van 10 µM blijkt (1,4,5)IP_3 in 30 sec ongeveer 35 % van het in aanwezigheid van ATP geaccumuleerde calcium vrij te maken.

De door (1,4,5)IP_3 gemedieerde stijging van de cytoplasmatische vrije calciumconcentratie kan worden geregistreerd met behulp van quin2, een verbinding welke sterker fluoresceert naarmate meer calcium wordt gebonden (Rink en Pozzan, 1985). Wordt deze stof geïntroduceerd in het cytoplasma van de cel, dan kan de vrije calciumconcentratie aan de hand van het verkregen fluorescentiesignaal berekend worden. In de acineuze cel van de konijnepancreas blijkt de vrije calciumconcentratie te liggen rond de 150 nM (Hoofdstukken 3-6). Zodra gestimuleerd wordt met cholecystokinine of carbachol treedt binnen enkele seconden een sterke stijging op van de intracellulaire calciumconcentratie en nadat een dosis-afhankelijk maximum is bereikt, daalt de calciumconcentratie weer vrij snel (Hoofdstuk 4). De zeer snelle, initiële stijging van de vrije calciumconcentratie blijkt onafhankelijk van de aanwezigheid van extracellulair calcium (Ochs et al., 1984,1985; Pandol et al., 1985a; Hoofdstukken 4 en 6) en representeert daarmee de, door (1,4,5)IP_3 stimuleerbare, afgifte van calcium uit de intracellulaire opslagplaats.

Wordt gestimuleerd met een concentratie van cholecystokinine of carbachol welke een bijna maximale enzymsecretie geeft, dan keert de intracellulaire calciumspiegel duidelijk niet terug naar het prestimulatoire niveau maar naar een niveau van om en nabij de 200 nM (Ochs et al., 1985; Merritt en Rubin, 1985; Hoofdstuk 4). Dit poststimulatoire niveau wordt na ongeveer 1 min bereikt en blijft gehandhaafd voor de duur van de receptoractivering. Voor het instandhouden van dit poststimulatoire calciumniveau is de aanwezigheid van extracellulair calcium vereist, hetgeen duidt op een verhoogde influx van calcium. Experimenten met $^{45}Ca^{2+}$ laten inderdaad zien dat een verhoogde influx van calcium optreedt na hormonale stimulering (Kondo en Schulz, 1976; Renckens et al., 1978; Dormer et al., 1981). Recente waarnemingen aan zeeëgeleieren lijken te wijzen op een gezamenlijke rol van het (1,4,5)IP_3 en het (1,3,4,5)IP_4 bij de hormonaal-geïnduceerde stijging van de plasmamembraanpermeabiliteit voor calcium (Irvine en Moor, 1986,1987).

De veronderstelde remmer van de hormonaal-gestimuleerde afgifte van calcium uit de intracellulaire opslagplaats, TMB-8 (Chiou en Malagodi, 1975), blijkt geen effect te hebben op de door cholecystokinine gestimuleerde initiële calciumstijging (Hoofdstukken 4 en 5). Deze verbinding heeft wel duidelijke effecten op de poststimulatoire blijvend-verhoogde calciumspiegel (Hoofdstuk 4). Het niet optreden hiervan zou kunnen duiden op remming van bovenbeschreven hormonaal-geïnduceerde stijging van de plasmamembraanpermeabiliteit voor calcium. Toediening van TMB-8 aan gestimuleerde cellen, leidt, in afwezigheid van extracellulair calcium echter tot een verdere daling van de cytoplasmatische vrije calciumconcentratie. Dit duidt erop dat TMB-8 mogelijk processen beïnvloedt welke bijdragen aan handhaving van het calciumniveau in cellen gestimuleerd in afwezigheid van extracellulair calcium. Wordt de extracellulaire calciumconcentratie weer op peil gebracht, dan stijgt de intracellulaire calciumspiegel weliswaar in aanwezigheid van TMB-8, maar niet tot het poststimulatoir-verhoogde niveau. Een observatie welke weer in in overeenstemming is met een remming door TMB-8 van de hormonaal-geïnduceerde stijging van de plasmamembraanpermeabiliteit voor calcium. Het exacte werkingsmechanisme van TMB-8 is vooralsnog echter onbekend.

Aanwijzingen voor een directe rol van calcium in de stimulus-secretie koppeling komen vrijwel uitsluitend van werk met calciumionoforen. Calciumionoforen als het A23187 en het ionomycine verhogen, buiten de receptor om, de intracellulaire calciumconcentratie, hetgeen voldoende is om de enzymsecretie te stimuleren (Schulz, 1980; Gardner et al., 1980; Ponnappa en Williams, 1980; Pandol et al., 1985a; Merritt en Rubin, 1985; Hoofdstukken 4 en 6). Op een directere wijze hebben Knight en Koh (1984) aangetoond dat verhoging van de calciumconcentratie van het incubatiemedium leidt tot stimulering van de enzymsecretie door gepermeabilizeerde acineuze cellen. Calcium lijkt dus te voldoen aan het voor intracellulaire boodschappers opgestelde criterium dat enkel een verhoging van de concentratie leidt tot de respons. Een ander criterium is dat remming van de hormonaal-gestimuleerde stijging van de concentratie van de boodschapper leidt tot remming van de respons. Van de verbinding TMB-8 was beschreven dat deze de stijging van de intracellulaire calciumconcentratie zou remmen en in die zin zou het een belangrijk hulpmiddel kunnen zijn in het onderzoek naar de betekenis welke de stijging van de vrije calciumconcentratie heeft voor de secretoire respons. Uit metingen van de hormonaal-geïnduceerde veranderingen van de vrije calciumconcentratie blijkt dat de door carbachol en niet de door CCK-8 geïnduceerde calciumstijging door TMB-8 wordt geremd (Hoofdstuk 5). Deze remming treedt reeds op bij micromolair concentraties TMB-8 en geconcludeerd is, mede op grond van gegevens uit de literatuur betreffende de verdringing van [^{3}H]QNB door TMB-8 (Tennes et al., 1983), dat TMB-8 een cholinerge receptorantagonist is. De door carbachol gestimuleerde enzymsecretie wordt reeds bij lage concentraties van TMB-8 geremd. De secretoire respons op CCK-8 wordt daarentegen pas bij veel hogere concentraties van TMB-8 geremd, nadat eerst zelfs het stimulerende effect van CCK-8 wordt gepotentieerd. Interessant is dat deze potentiëring alleen optreedt bij submaximaal stimulerende concentraties secretagoog. De wijze waarop TMB-8 zijn potentiërende werking uitoefent, is onbekend. De duidelijke remming van de hormonaal-gestimuleerde enzymsecretie welke optreedt bij de hogere concentraties van TMB-8 lijkt gepaard te gaan met cytotoxische effecten. Vreemd genoeg echter worden de granula, waarin de spijsverteringsenzymen intracellulair gelocaliseerd zijn, niet beschadigd.

De secretoire respons van acineuze cellen van de pancreas is bifasisch. Gedurende de eerste 5 minuten na stimulering is de secretiesnelheid hoog. Daarna daalt de secretiesnelheid tot een niveau, hoger dan het ongestimuleerde, dat gehandhaafd blijft voor de duur van de receptoractivering (Schulz, 1980; Campbell, 1983; Pandol et al., 1985a,b).

Worden acineuze cellen van de caviapancreas gestimuleerd met carbachol en wordt vervolgens activering van de cholinerge receptor geblokkeerd door middel van een cholinerge receptorantagonist, atropine, dan daalt de snelheid waarmee de spijsverteringsenzymen afgegeven worden onmiddelijk

tot het ongestimuleerde niveau (Pandol et al., 1985a). Wordt nu gelijktijdig met atropine, cholecystokinine toegevoegd, dan wordt de enzymsecretie wel gestimuleerd. De initiële fase van de secretoire respons, welke gekenmerkt wordt door de hoge secretiesnelheid, treedt echter niet op. Onder dezelfde condities leidt stimulering met cholecystokinine niet tot verhoging van de intracellulaire calciumconcentratie en geconcludeerd wordt dat de initiële calciumstijging van belang is voor de, gedurende de eerste 5 min na stimulering optredende, hoge secretiesnelheid. Het niet optreden van een door cholecystokinine geïnduceerde calciumstijging wordt, als eerder beschreven door Stolze en Schulz (1980), toegeschreven aan depletie van de intracellulaire opslagplaats ten gevolge van de voorafgaande stimulering met carbachol.

Dat de hormonaal-geïnduceerde stijging van de intracellulaire calciumconcentratie inderdaad een rol speelt bij de secretoire respons blijkt ook uit het feit dat de respons duidelijk geremd is in cellen die opgeladen zijn met het calcium-bindende quin2 (Ochs et al., 1985), of in cellen waarin door middel van osmotische zwelling andere calciumchelatoren zijn geïntroduceerd (Dormer, 1984).

Terwijl bovengenoemde initiële fase van de secretoire respons afhankelijk is van de aanwezigheid van calcium in de intracellulaire opslagplaats, is de daaropvolgende fase van de respons, gekenmerkt door de geringere maar nog steeds verhoogde secretiesnelheid, wel afhankelijk van extracellulair calcium (Gardner et al., 1979b,1980; Williams, 1980; Schulz, 1980; Argent et al., 1982). De secretoire respons op forbolesters lijkt daarentegen, of veel minder (Gunther, 1981), of nauwelijks (Merritt en Rubin, 1985) afhankelijk van extracellulair calcium. Dit duidt erop dat calcium, maar nu van extracellulaire origine, ook een rol speelt gedurende de fase van de geringere secretiesnelheid (Schreurs et al., 1976; Petersen en Ueda, 1976). In dit verband is het interessant dat het potentiërende effect van TMB-8 bij die concentraties waarbij het remmende effect op de poststimulatoir-verhoogde calciumspiegel zichtbaar wordt, begint af te nemen en overgaat in een remmend effect (Hoofdstukken 4 en 5).

De diacylglycerol/proteïne kinase C route

Behalve tot de vorming van (1,4,5)IP_3, leidt de hormonaal gestimuleerde afbraak van PIP_2 ook tot de vorming van het diacylglycerol.

In 1977 werd door de groep van Nishizuka een proteïne kinase beschreven dat enkel proteolytisch (door een calcium-afhankelijk thiol protease) geactiveerd kon worden (Inoue et al, 1977). Het niet proteolytisch geactiveerde enzym bleek vervolgens toch geactiveerd te kunnen worden door de combinatie van calcium en fosfolipiden (Takai et al., 1979a). Vervolgens bleek dat wel de calciumafhankelijkheid, maar niet de fosfolipideafhankelijkheid, sterk afnam in aanwezigheid van diacylglycerol (Takai et al, 1979b). Tenslotte werd gevonden dat de tumor-bevorderende forbolesters, welke qua moleculaire structuur sterke gelijkenis vertonen met diacylglycerolen, eveneens de calcium-afhankelijkheid van het proteïne kinase C, zoals het enzym inmiddels genoemd werd, verlaagden (Castagna et al., 1982). Sindsdien zijn forbolesters, zoals het 12-O-tetradecanoylforbol 13-acetaat (TPA) overal waar mogelijk gebruikt om de rol van proteïne kinase C te bestuderen (Ashendel, 1985; Nishizuka, 1986; Kikkawa en Nishizuka, 1986). Onlangs nog is door Nishizuka (1986) een overzicht gepubliceerd betreffende de processen welke door proteïne kinase C beïnvloed worden.

Wanneer acineuze cellen van de exocriene pancreas gestimuleerd worden met forbolesters, dan leidt dit tot toename van de enzymsecretie (De Pont en Fleuren-Jakobs, 1984; Merritt en Rubin, 1985; Pandol et al., 1985a; Burnham et al., 1986; Ansah et al., 1986; Hoofdstukken 3, 5 en 6). Net als bij cAMP lijkt ook bij forbolesters de mate waarin de enzymsecretie gestimuleerd wordt afhankelijk van het speciës. In het konijn wordt de enzymsecretie door zowel TPA alleen, als door de calciumionofoor, A23187, alleen, relatief slecht gestimuleerd indien vergeleken met maximaal effectieve concentraties van carbachol of cholecystokinine (De Pont en Fleuren-Jakobs, 1984 en Hoofdstuk 5). De combinatie van beiden geeft echter een maximale stimulering van de enzymsecretie (De Pont en Fleuren-Jakobs, 1984). In de cavia verschilt de

maximale respons op TPA nauwelijks van die op cholecystokinine (Pandol et al., 1985a) en toevoeging van A23187 heeft in dat geval nauwelijks nog extra effect op de enzymsecretie. Het synergistisch effect van forbolesters en calciumionoforen zoals dat waargenomen wordt in de exocriene pancreas (De Pont en Fleuren-Jakobs, 1984; Merritt en Rubin, 1985) blijkt op te treden in tal van celtypen en ondersteunt de hypothese dat slechts de gezamenlijke activering van zowel de inositolfosfaat/calcium route als de diacylglycerol/proteïne kinase C route, leidt tot een volledige respons (Nishizuka, 1983).

Wanneer acineuze cellen van de pancreas worden gestimuleerd met cholecystokinine, vervolgens verschillende malen gewassen en daarna opnieuw gestimuleerd, dan blijkt de respons aanzienlijk geremd (Abdelmoumene en Gardner, 1980). De mate van desentizering blijkt toe te nemen met de concentratie van het cholecystokinine tijdens de eerste incubatie. Bovendien wordt niet alleen de respons op cholecystokinine maar ook die op andere calciummobilizerende hormonen geremd. De respons op secretagogen welke cAMP verhogen wordt daarentegen niet geremd (Gardner en Jensen, 1981).

De mogelijkheid dat activering van de diacylglycerol/proteïne kinase C route een rol speelt bij desentizering werd onderzocht door acineuze cellen van de konijnepancreas voor te behandelen met TPA, te wassen en vervolgens te stimuleren met cholecystokinine (Hoofdstuk 6). Voorbehandeling met TPA blijkt, zoals verwacht, te leiden tot stimulering van de enzymsecretie (activering van de diacylglycerol/proteïne kinase C route). Wordt vervolgens gestimuleerd met cholecystokinine dan blijkt een verdere verhoging van de TPA-gestimuleerde enzymsecretie eerst op te treden bij relatief hoge concentraties van het hormoon. Het onvermogen van de lagere hormooncentraties om de TPA-gestimuleerde secretie verder te verhogen blijkt samen te vallen met een remming van het vermogen van het hormoon om bij deze lagere concentraties de intracellulaire vrije calciumconcentratie te verhogen (zie ook: Ansah et al., 1986). Een mogelijke verklaring zou kunnen zijn dat voorbehandeling met forbolester leidt tot stimulering van het calciumextrusiemechanisme en zo een hormonaal-geïnduceerde calciumstijging teniet doet (Berridge, 1987a). Hiertegen spreekt echter dat de door cholecystokinine geïnduceerde calciumstijging volledig wordt geremd.

Remming van stimulus-geïnduceerde stijgingen van de vrije calciumconcentratie door forbolesters is ook gevonden voor andere celtypen (Zavoico et al., 1985; Sagi-Eisenberg et al., 1985; McIntyre et al., 1985; Kojima et al., 1986) en blijkt in tal van celtypen gepaard te gaan met remming van de stimulus-geïnduceerde afbraak van PIP_2 (Watson en Lapetina, 1985; Orellana et al., 1985; Vicentini et al., 1985; Drummond, 1985). Voor enkele celtypen is bovendien aangetoond dat behandeling met forbolesters leidt tot fosforylering van de α_1-adrenerge receptor en langs die route tot ontkoppeling van de receptor van de fosfatidylinositol/calcium route (Leeb-Lundberg et al., 1985). In hepatocyten is gebleken dat forbolester-geïnduceerde remming van de hormonaal-gestimuleerde afbraak van PIP_2 wel optreedt voor de α_1-receptor maar nauwelijks of niet voor de vasopressine of angiotensine-2 receptor (Cooper et al., 1985; Corvera en Garcia-Sainz, 1984), hetgeen erop zou kunnen duiden dat het remmende effect van forbolesters optreedt op receptorniveau en niet op het niveau van het G eiwit of het PIP_2 fosfodiesterase (Berridge, 1987a).

In het licht van de bovenbeschreven observaties is het tamelijk exceptioneel dat in acineuze cellen van de konijnepancreas voorbehandeld met TPA, de cholecystokinine-geïnduceerde afbraak van [^{32}P]PIP_2 niet wordt geremd (Hoofdstuk 6). Dit betekent dat de hormonaal-geïnduceerde productie van (1,4,5)IP_3 niet geremd is in acineuze cellen voorbehandeld met forbolester, hetgeen overeenstemt met de observatie van Merritt et al. (1986a). De observatie van Merritt et al. (1986a) betekent bovendien dat een verhoogd metabolisme van (1,4,5)IP_3 (Molina y Vedia en Lapetina, 1986; Connolly et al., 1986) niet de oorzaak zou zijn van remming van de door cholecystokinine geïnduceerde calciumstijging. Recentelijk is echter gevonden dat TPA-voorbehandeling van acini van

de rattepancreas wel leidt tot remming van de hormonaal- geïnduceerde (1,4,5)IP_3 stijging (Bruzzone en Wollheim, 1987).

Remming van de hormonaal-gestimuleerde calciumstijging zonder remming van de hormonaal-geïnduceerde PIP_2 afbraak kan behalve uit een verhoogd metabolisme van de, voor stimulering van de calciumafgifte in aanmerking komende polyinositolfosfaten, ook verklaard worden uit een desentizering van de intracellulaire calciumopslagplaats voor deze inositolpolyfosfaten. Wanneer acineuze cellen van de konijnepancreas worden voorbehandeld met TPA en vervolgens gepermeabilizeerd met saponine dan blijkt de ATP-afhankelijke opname van Ca^{2+} niet beïnvloed, terwijl de (1,4,5)IP_3-gestimuleerde afgifte van calcium, ondanks grote verschillen in stimuleerbaarheid van de verschillende celpreparaten, significant is geremd (Hoofdstuk 7). Gemiddeld over alle (1,4,5)IP_3 concentraties blijft ongeveer 5,5% meer calcium achter in de met forbolester behandelde cellen. De dose-respons curve is daarmee naar rechts verschoven ten opzichte van die voor de onbehandelde cellen, hetgeen betekent dat de affiniteit voor (1,4,5)IP_3 verlaagd is. In dat geval leidt activering van de diacylglycerol/proteïne kinase C route tot desentizering van de intracellulaire calciumopslagplaats, met als gevolg remming van de door cholecystokinine gestimuleerde afgifte van calcium.

Worden acineuze cellen van de pancreas gelabeld met ^{32}P in aanwezigheid van een calciummobilizerend secretagoog zoals cholecystokinine, dan treedt, als reeds uitvoerig beschreven, een sterke incorporatie van label op in fosfatidylinositol en fosfatidezuur (Hoofdstukken 8 en 9). De incorporatie van label in PIP_2 blijft echter sterk achter bij die in controle cellen terwijl evenwichtslabeling duidelijk eerder wordt bereikt, hetgeen, zoals reeds ter sprake is gekomen, verklaard zou kunnen worden uit de verhoogde turnover van dit polyfosfoinositide bij een gedaalde totale hoeveelheid. De bevinding dat de totale hoeveelheid PIP_2 verlaagd is in gestimuleerde cellen is nieuw. Berridge (1987a) concludeert namelijk uit de toename van de hoeveelheid ^{32}P-PIP_2, welke optreedt na de stimulus-geïnduceerde snelle en sterke daling, dat in gestimuleerde cellen de totale hoeveelheid PIP_2 na stimulering eerst af- en dan toeneemt en soms zelfs het prestimulatoire niveau overschrijdt. Op grond van het feit dat forbolesters de labeling van PIP_2 met ^{32}P bevorderen (De Chaffoy de Courcelles et al., 1984; Halenda en Feinstein, 1984; Taylor et al., 1984; Boon et al., 1985), zoals ook gevonden is voor de konijnepancreas (Hoofdstuk 8), wordt een positief feedback effect van het geactiveerde proteïne kinase C gesuggereerd.

Vreemd genoeg bereikt de labeling van PIP geen evenwicht, terwijl de turnover toch verhoogd moet zijn om het benodigde PIP_2 te kunnen leveren. Een mogelijke verklaring zou kunnen zijn dat het PIP dat gelabeld wordt in aanwezigheid van secretagoog niet de precursor is voor het secretagoog-gevoelige PIP_2. Dit zou ook kunnen verklaren waarom stimulering niet in alle gevallen leidt tot een duidelijke afname van de hoeveelheid ^{32}P-PIP (Putney et al., 1983; Orchard et al., 1984; Pandol et al., 1985b).

Wordt gelabeld in aanwezigheid van TPA, dan blijkt vooral de incorporatie van ^{32}P in PIP_2 gestimuleerd. Een dergelijke door forbolester geïnduceerde verhoogde incorporatie van label in PIP_2 is, als boven beschreven, eerder gevonden voor andere celtypen en is mogelijk het gevolg van een verhoogde de- en/of refosforylering van PIP_2 en PIP. Een zelfde effect op de labeling van PIP is minder duidelijk, terwijl de labeling van fosfatidylinositol nauwelijks beïnvloed lijkt. Opvallend is dat stimulering van acineuze cellen gelabeld in aanwezigheid van forbolester wel leidt tot een duidelijke en snelle daling van de hoeveelheid ^{32}P-PIP en zoals reeds eerder vermeld is de afbraak van ^{32}P-PIP_2 niet geremd. Bovendien lijkt in aanwezigheid van TPA de hoeveelheid ^{32}P-PIP_2 na de stimulus-geïnduceerde daling wel toe te nemen.

Het meest opvallend is echter dat in aanwezigheid van forbolester de labeling van fosfatidylcholine zeer sterk wordt gestimuleerd, een effect dat

niet optreedt in aanwezigheid van cholecystokinine. Het stimulerende effect van forbolesters op het metabolisme van fosfatidylcholine is beschreven voor tal van celtypen en is in verband gebracht met de tumor- bevorderende werking van deze verbindingen (Weinstein, 1981; Pelech en Vance, 1984).

Het CTP:fosfocholine cytidylyltransferase katalyseert de snelheidsbepalende stap in de synthese van fosfatidylcholine. Bevindt dit enzym zich in het cytosol dan is het inactief. Membraangebonden daarentegen is het enzym wel actief. Vetzuren (Cornell en Vance, 1987), diacylglycerol (Wright et al., 1985) en forbolesters (Pelech et al., 1984) bevorderen de translocatie van dit enzym en stimuleren daarmee mogelijk de synthese van fosfatidylcholine. Forbolesters stimuleren ook de hydrolyse van fosfatidylcholine. Het enzym dat wordt geactiveerd is het fosfatidylcholine-specifieke fosfolipase C en de producten zijn fosfocholine en diacylglycerol (Daniel et al, 1986; Besterman et al., 1986). Er bestaat echter verschil tussen het effect van forbolesters en dat van synthetische diacylglycerolen als het 1,2-dioctanoylglycerol of het 1-oleoyl-2-acetylglycerol. De laatsten stimuleren de hydrolyse van fosfatidylcholine op fosfolipase A_2-achtige wijze met als producten lysofosfatidylcholine en arachidonzuur (Kolesnick en Paley, 1987).

In 3T3 L1 cellen werd gevonden dat niet alleen forbolesters, maar ook stimuli als de platelet derived growth factor (PDGF), het fosfatidylcholine-specifieke fosfolipase C activeren, gemeten aan de toename van fosfo[^{3}H]choline. Downregulatie van proteïne kinase C door langdurige voorbehandeling met forbolester bleek vervolgens wel de respons op forbolester, maar in veel mindere mate die op PDGF, te remmen. Hieruit werd geconcludeerd dat PDGF niet alleen door activering van de diacylglycerol/proteïne kinase C route van het fosfatidylinositol/calciumsysteem, maar ook langs een van proteïne kinase C onafhankelijke route het fosfatidylcholine-specifieke fosfolipase C activeert (Besterman et al., 1986).

Werden 3T3 cellen gestimuleerd met forbolester dan bleek de totale hoeveelheid fosfatidylcholine niet te veranderen. Dit ondanks een verhoogde turnover van dit fosfolipide. Ook de totale hoeveelheid fosfatidezuur veranderde niet. Wel echter bleek de hoeveelheid diacylglycerol aanzienlijk toegenomen en geconcludeerd werd dat de forbolester het diacylglycerol-kinase remt (Takuwa et al., 1987).

In acineuze cellen van de konijnepancreas wordt de labeling van fosfatidylcholine niet of nauwelijks beïnvloed door stimulering met cholecystokinine, terwijl de labeling van fosfatidezuur en fosfatidylinositol sterk is toegenomen. Dit duidt erop dat het gevormde diacylglycerol in hormonaal-gestimuleerde cellen niet het substraat is voor het CDP-choline-diacylglycerol-transferase maar voor het diacylglycerol-kinase. Voor cellen gestimuleerd in aanwezigheid van forbolester is het beeld precies omgekeerd. De forbolester heeft geen effect op de labeling van fosfatidylinositol, terwijl die van fosfatidylcholine significant is toegenomen, hetgeen aangeeft dat het diacylglycerol nu substraat is voor het CDP-choline-diacylglycerol-transferase.

Aangezien zowel diacylglycerol als forbolesters de translocatie van het CTP:fosfocholine cytidylyltransferase stimuleren, moet het verschil in effect later optreden. Een mogelijkheid is dat forbolesters inderdaad het diacylglycerol-kinase remmen (Takuwa et al., 1987). Dit lijkt echter in geval van de acineuze cel niet waarschijnlijk daar na stimulering met cholecystokinine in aanwezigheid van forbolester de incorporatie van label in fosfatidezuur niet is geremd. Een andere verklaring zou kunnen zijn dat de vetzuursamenstelling van het uit PIP_2 gevormde diacylglycerol zodanig verschilt van het uit fosfatidylcholine gevormde diacylglycerol dat dit bepalend is voor de wijze waarop het wordt gemetabolizeerd.

Bij de behandeling van het cAMP systeem is reeds ter sprake gekomen dat activering van de diacylglycerol/proteïne kinase C route ook leidt tot een remming van het vermogen van cholecystokinine (en VIP) om het adenylaatcyclase te activeren en de intracellulaire cAMP-spiegel te doen stijgen. Ook in dit geval treedt de remming enkel op voor de relatief lage concentraties van het cholecystokinine (Hoofdstuk 9). Een dergelijke observatie doet vermoeden dat een en dezelfde, door de

forbolester- geïnduceerde, modificering ten grondslag ligt aan de remming van zowel de door cholecystokinine geïnduceerde activering van het cAMP systeem als de door cholecystokinine geïnduceerde activering van het fosfatidylinositol/ calciumsysteem. In dat geval is voor de hand liggend dat remming optreedt op het niveau van de receptor, hetgeen betekent dat dezelfde receptor gekoppeld is aan beide systemen. Hiertegen spreekt echter dat voorbehandeling met TPA niet leidt tot remming van de door cholecystokinine gestimuleerde afbraak van PIP_2 (Hoofdstuk 6). Op welk niveau de forbolester de hormonaal- gestimuleerde aanmaak van cAMP remt is onduidelijk. In geval van hepatocyten bleek TPA activering van het adenylaatcyclase door glucagon te remmen zonder de binding van glucagon te beïnvloeden (Heyworth et al., 1984), terwijl in erythrocyten van de kalkoen de door forbolester geïnduceerde desentizering van de β-adrenerge receptor gepaard gaat met fosforylering van de receptor (Kelleher et al., 1984). In weer andere celtypen, zoals pinealocyten, potentiëren forbolesters juist de hormonaal-gestimuleerde cAMP productie (Sugden et al., 1985). Aangetoond is verder dat proteïne kinase C zowel het remmende G eiwit van het adenylaatcyclase systeem als de katalytische subunit van dat enzym kan fosforyleren (Yoshimasa et al., 1987).

Conclusies

In het voorgaande is uitvoerig ingegaan op het werkingsmechanisme van die hormonen en neurotransmitters welke, omdat zij niet in staat zijn de plasmamembraan te passeren, extracellulair binden aan hun specifieke receptoren. In het onderzoek naar de wijze waarop het signaal vanaf het receptormolecuul naar het inwendige van de cel wordt doorgegeven speelde en speelt de geïsoleerde acineuze cel van de exocriene pancreas een belangrijke rol. De redenen hiervoor zijn onder andere dat functionele acineuze cellen relatief gemakkelijk en in grote aantallen te isoleren zijn.

Het hormoon cholecystokinine (CCK) en de neurotransmitter acetylcholine stimuleren de enzymsecretie primair door activering van het calciumsysteem. Het C-terminale octapeptide van cholecystokinine (CCK-8) en de cholinerge receptor agonist carbachol induceren dalingen van de hoeveelheid PIP_2 en stijgingen van de hoeveelheid (1,4,5-)IP_3 welke voldoende snel zijn om vooraf te kunnen gaan aan de stijging van de cytoplasmatische vrije calciumconcentratie. Bovendien bestaan nu sterke aanwijzingen dat (1,4,5-)IP_3 de lang gezochte schakel vormt tussen de gestimuleerde afbraak van fosfoinositiden en de gestimuleerde afgifte van calcium vanuit een intracellulaire opslagplaats. Het metabolisme van de inositolfosfaten staat in het middelpunt van de belangstelling en mogelijk speelt het (1,3,4,5-)IP_4 een rol bij de receptor-geïnduceerde verhoging van de plasmamembraanpermeabiliteit voor calcium. Wij hebben aangetoond dat na stimulering met CCK-8 of carbachol, de plasmamembraanpermeabiliteit voor calcium blijvend verhoogd is in aanwezigheid van het secretagoog. De vrije calciumconcentratie, 150 nM in de ongestimuleerde cel, wordt daarbij gehandhaafd op 200 nM, mits extracellulair calcium aanwezig is. De veronderstelde intracellulaire calciumantagonist TMB-8, remt niet de door CCK-8 geïnduceerde afgifte van calcium uit de intracellulaire calciumopslagplaats maar wel de door CCK-8 geïnduceerde langdurig- verhoogde vrije calciumconcentratie. Remming door TMB-8 van de door CCK-8 geïnduceerde stijging van de plasmamembraanpermeabiliteit voor calcium kan onze waarnemingen niet volledig verklaren en toekomstig onderzoek moet uitwijzen op welk niveau TMB-8 exact aangrijpt.

Het tweede product dat ontstaat bij de receptorgestimuleerde afbraak van PIP_2 is het diacylglycerol. Deze verbinding blijft in de plasmamembraan en activeert daar het proteïne kinase C, een naar het lijkt cruciaal enzym in de regulatie van cellulaire processen. Belangrijke hulpmiddelen bij de bestudering van dit enzym in de intacte cel zijn de forbolesters welke, zonder tussenkomst van receptoractivering, het kinase activeren. Wij hebben de forbolester 12-O-tetradecanoylphorbol 13-acetaat (TPA) gebruikt om de rol van proteïne kinase C in de acineuze cel van de pancreas nader te bestude-

ren. De forbolester stimuleert de enzymsecretie. Activering van proteïne kinase C lijkt daarmee voldoende om de acineuze cel aan te zetten tot secretoire activiteit. Het maximale effect van TPA blijft echter sterk achter bij dat van CCK-8 of carbachol. Pas in combinatie met een calciumionofoor, welke buiten de receptor om de intracellulaire calciumconcentratie verhoogt en waarvan het maximale stimulerende effect ook duidelijk achterblijft bij dat van CCK-8 of carbachol, treedt een stimulering op van de enzymsecretie welke vergelijkbaar is met die van CCK-8 of carbachol. Het effect van de forbolester en de calciumionofoor is additief. Dit additieve effect wordt algemeen beschouwd als bewijs voor een synergistische werking van de (1,4,5-)IP_3/calcium route en de diacylglycerol/ proteïne kinase C route bij het tot stand komen van de uiteindelijke respons. Dit synergistisch effect blijkt ondermeer nog uit onze waarneming dat CCK-8 in acineuze cellen, welke zijn voorbehandeld met forbolester, de door de forbolester gestimuleerde enzymsecretie pas bij die concentraties verder doet toenemen welke een stijging geven van de vrije calciumconcentratie.

Activering van de enzymsecretie bleek echter niet het enige effect van de forbolester. Voorbehandeling van acineuze cellen met TPA leidde namelijk ook tot remming van de door CCK-8 geïnduceerde stijging van de cytoplasmatische calciumconcentratie en daarmee van de door de forbolester gestimuleerde enzymsecretie. Deze remming bleek niet op te treden op het niveau van de door CCK-8 geïnduceerde afbraak van PIP_2. Wel echter kon worden aangetoond dat de voorbehandeling met TPA leidde tot remming van de door (1,4,5-)IP_3 geïnduceerde afgifte van ATP-afhankelijk opgeslagen calcium in gepermeabiliseerde acineuze cellen. De voorlopige werkhypothese zal zijn dat het in het endoplasmatisch reticulum aanwezige (1,4,5-)IP_3-gevoelige calciumkanaal door proteïne kinase C zelf wordt gefosforyleerd. Bovendien is gevonden dat voorbehandeling met carbachol een zo mogelijk nog sterkere remming geeft van de door (1,4,5-)IP_3 geïnduceerde afgifte van ATP-afhankelijk opgeslagen calcium in gepermeabilizeerde cellen.

Op ons laboratorium is inmiddels een sterk gezuiverd proteïne kinase C preparaat verkregen uit exocrien pancreasweefsel (A.G.H. Ederveen). Een volgende stap zal dan ook zijn een aan endoplasmatisch reticulum verrijkte membraanfractie te preïncuberen met gezuiverd proteïne kinase C en te onderzoeken of de door (1,4,5-)IP_3 geïnduceerde afgifte van ATP-afhankelijk opgeslagen calcium opnieuw geremd is.

Voorbehandeling met forbolester leidt ook tot veranderingen in het metabolisme van fosfolipiden. De meest in het oog springende verandering is wel de sterk verhoogde incorporatie van $^{32}P_i$ in fosfatidylcholine. Deze verhoogde incorporatie van label in fosfatidylcholine treedt niet op in aanwezigheid van CCK-8. De vraag rijst dan ook of dit effect van TPA al dan niet het gevolg is van activering van proteïne kinase C. Zo ja, welk mechanisme voorkomt dan dat activering van proteïne kinase C door CCK-8 niet leidt tot dit effect. Bovendien is tot nu toe in een celtype gevonden dat activering van het metabolisme van fosfatidylcholine door forbolesters leidt tot een aanzienlijke verhoging van de hoeveelheid diacylglycerol. Hoe verstrekkend een dergelijke werking van forbolesters is, is op het moment nog onduidelijk maar verdient zeker in verband met het tumorbevorderende karakter van deze verbindingen nader onderzoek.

De incorporatie van label in de polyfosfoinositiden, in het bijzonder in PIP_2, lijkt eveneens gestimuleerd door TPA. De forbolester heeft geen effect op de zeer snelle door CCK-8 of carbachol gestimuleerde daling van de hoeveelheid gelabeld PIP_2. Na deze hormonaal- geïnduceerde daling lijkt in aanwezigheid van forbolester de hoeveelheid gelabeld PIP_2 weer toe te nemen, terwijl dat niet het geval is in controle cellen. Een correcte interpretatie van bovengenoemde waarnemingen wordt echter bemoeilijkt door het feit dat op het moment van stimulering geen evenwicht was bereikt met betrekking tot de labeling van de beide polyfosfoinositiden. Een langere labelingsduur is daarom noodzakelijk. Dit laatste is zeker het geval wanneer gelabeld wordt met verbindingen als inositol, arachidonzuur of glycerol. Het zal daarom nodig zijn om te zoeken naar een manier om de vers

geïsoleerde acineuze cellen langer in leven te houden. Het alternatief is te gaan werken met een cellijn.

Wordt gelabeld in aanwezigheid van een hoeveelheid CCK-8 welke de enzymsecretie maximaal stimuleert, dan blijkt de labeling van PIP_2 wel tot evenwicht te komen. De hoeveelheid gelabeld PIP_2 is echter aanzienlijk verlaagd ten opzichte van cellen gelabeld in afwezigheid van secretagoog. Worden eerstgenoemde cellen vervolgens maximaal gestimuleerd met carbachol, dan treedt geen verdere daling op van de hoeveelheid PIP_2. Deze observatie geeft aan dat zolang een maximaal effectieve stimulus aanwezig is een tweede stimulus geen effect kan hebben omdat de hydrolyse van PIP_2 al maximaal geactiveerd is en kan mede ten grondslag liggen aan het verschijnsel van desentizering.

Voorbehandeling met forbolester blijkt geen effect te hebben op de basale activiteit van het adenylaatcyclase. Toch wordt het cAMP systeem beïnvloed door TPA. Zowel de door CCK-8 als de door VIP geïnduceerde stijging van de intracellulaire cAMP concentratie is geremd in cellen welke zijn voorbehandeld met TPA. Remming treedt echter alleen op wanneer gestimuleerd wordt met lage concentraties van de betreffende secretagogen. Toekomstig onderzoek zal moeten uitwijzen op welk niveau remming optreedt. Een mogelijkheid biedt de geïsoleerde membraanfractie. Deze fractie kan zowel gebruikt worden om het effect van voorbehandeling met forbolester als het effect van preïncubatie gezuiverd proteïne kinase C op de hormonale stimuleerbaarheid van het adenylaatcyclase te bestuderen.

Stimulering van het cAMP systeem leidt in de exocriene pancreas van het konijn niet tot enzymsecretie. Wel wordt bij verhoogde intracellulaire cAMP concentraties de door calciummobilizerende secretagogen gestimuleerde secretie aanzienlijk versterkt. De rol van cAMP lijkt daarmee een meer modulerende te zijn. Omdat cAMP ook de secretoire respons op calciumionoforen en forbolesters versterkt, is geconcludeerd dat het niveau waarop cAMP aangrijpt gelegen moet zijn na het punt waar de (1,4,5-)IP_3/calcium route en de diacylglycerol/ proteïne kinase C route bij elkaar komen. Een eerste aanzet om deze hypothese te onderzoeken is na te gaan of mogelijk een van de substraateiwitten voor proteïne kinase C ook gefosforyleerd wordt door een cAMP-afhankelijk kinase.

Gezien de belangrijke rol die proteïne kinase C speelt in de regulatie van de cellulaire activiteit is, behalve de isolatie en karakterisatie van dit enzym en het gebruik van gezuiverd enzym om na te gaan wat voor effecten het heeft op tal van intracellulaire processen, van belang het metabolisme van de cellulaire activator van proteïne kinase C, het diacylglycerol, te onderzoeken. Dit laatste zal dan ook het hoofddoel zijn van het vervolgonderzoek.

REFERENCES

Abdelmoumene, S. and Gardner, J.D. (1980) Am. J. Physiol. 239, G272-G279
Abdel-Latif, A.A., Akhtar, R.A. and Hawthorne, J.N. (1977) Biochem. J. 162, 61-73
Akhtar, R.A. and Abdel-Latif, A.A. (1978) J. Pharmacol. Exp. Ther. 204, 655-668
Akhtar, R.A. and Abdel-Latif, A.A. (1980) Biochem. J. 192, 783-791
Ansah, T.-A., Dho, S. and Case, R.M. (1986) Biochim. Biophys. Acta 889, 326-333
Argent, B.E., Case, R.M. and Hirst, F.C. (1982) J. Physiol. 323, 339-352
Ashendel, C.L. (1985) Biochim. Biophys. Acta 822, 219-242
Banschbach, M.W., Geison,R.L. and Hokin-Neaverson, M. (1974) Biochem. Biophys. Res. Commun. 58, 714-718
Beckner, S.K. and Farrar, W.L. (1987) Biochem. Biophys. Res. Commun. 145, 176-182
Bell, J.D., Buxton, L.O. and Brunton, L.L. (1985) J. Biol. Chem. 260, 2625-2628
Berridge, M.J. (1985) Sci. Am. 253, 124-134
Berridge, M.J. (1987a) Ann. Rev. Biochem. 56, 159-193
Berridge, M.J. (1987b) Biochim. Biophys. Acta 907, 33-45
Besterman, J.M., Duronio, V. and Cuatrecasas, P. (1986) Proc. Natl. Acad. Sci. Usa. 83, 6785-6789
Billah, M.M. and Lapetina, E.G. (1982) J. Biol. Chem. 257, 12705-12708
Bisonnette, B.M., Collen, M.J., Adachi, H., Jensen, R.T. and Gardner, J.D. (1984) Am. J. Physiol. 246, G710-G717
Bokoch, G.M., Katada, T., Northrup, J.K., Ui, M. and Gilman, A.G. (1984) J. Biol. Chem. 259, 3560-3567
Bonting, S.L., De Pont, J.J.H.H.M., Kempen, H.J.M., Case, R.M., Smith, P.A. and Scratcherd, T. (1977) Br. J. Pharmacol. 61, 243-250
Boon, A.M., Beresford, B.J. and Mellors, A. (1985) Biochem. Biophys. Res. Commun. 129, 431-438
Bradford, P.G. and Rubin, R.P. (1985) FEBS Lett. 183, 317-320
Brandt, S.J., Dougherty, R.W., Lapetina, E.G. and Niedel, J.E. (1985) Proc. Natl. Acad. Sci. USA. 82, 3277-3280
Brock, T.A., Rittenhouse, S.E., Powers, C.W., Ekstein, L.S., Gimbrone, M.A. and Alexander, R.W. (1985) J. Biol. Chem. 260, 14158-24162
Bruzzone, R. and Wollheim, C.B. (1987) Digestion 38, 8
Bruzzone, R., Trimble, E.R., Gjinovci, A., Traub, O., Willecke, K. and Meda, P. (1987) Pancreas 2, 262-271
Burnham, D.B., Munowitz, P., Hootman, S.R. and Williams, J.A. (1986) Biochem. J. 235, 125-131
Campbell, A.K. (1983) Intracellular Calcium: Its universal role as regulator, John Wiley and Sons Limited, Chichester
Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851
Chiou, C.Y., and Malagodi, M.H. (1975) Br. J. Pharmacol. 53, 279-285
Christophe, J.P., Conlon, T.P. and Gardner, J.D. (1976) Gastroenterology 251, 4629-4634
Chueh, S.-H., Mullaney, M., Ghosh, T.K., Zachary, A.L. and Gill, D.L. (1987) J. Biol. Chem. 262, 13857-13864
Cockcroft, S. and Gomperts, B.D. (1985) Nature, 314, 834-836
Codina, J., Hildebrandt, J.D., Iyengar, R., Birnbaumer, L., Sekura, R.D. and Manclark, C.R. (1983) Proc. Natl. Acad. Sci. USA. 77, 4276-4280
Collen, M.J., Sutliff, V.E., Pan, G.Z. and Gardner, J.D. (1983) Am. J. Physiol. 242, 6423-6428
Connolly, T.M., Lawing, W.J., Jr. and Majerus, P.W. (1986) Cell 46, 951-958
Cooper, R.H., Coll, K.E. and Williamson, J.R. (1985) J. Biol. Chem. 260, 3281-3288
Cornell, R. and Vance, D.E. (1987) Biochim. Biophys. Acta 919, 26-36
Corvera, S. and Garcia-Sainz, J.A. (1984) Biochem. Biophys. Res. Commun. 119, 1128-1133
Daniel, L.W., Waite, M. and Wykle, R.L. (1986) J. Biol. Chem. 261, 9128-9132
De Chaffoy de Courcelles, D., Roevens, P. and van Belle, H. (1984) FEBS Lett. 173, 389-393
Dehaye, J.-P., Gillard, M., Poloczek, P., Stievenart, M., Winand, J. and Christophe, J. (1985) J. Cycl. Nucl. Prot. Phosph. Res. 10, 269-280
De Pont, J.J.H.H.M. and Fleuren-Jakobs, A.M.M. (1984) FEBS Lett. 170, 64-68
De Pont, J.J.H.H.M., Luyben, D. and Bonting S.L. (1979) Biochim. Biophys. Acta 584, 33-42
Deschodt-Lanckman, M., Robberecht, P., De Neef, P., Labrie, F. and Christophe, J. (1975) Gastroenterology 68, 318-325
Dorner, R.L. (1984) Biochem; Biophys. Res. Commun. 119, 876-883
Dorner, R.L., Poulsen, J.H., Licko, V. and Williams, J.A. (1981) Am. J. Physiol. 240, G38-G49
Doughney, C., Brown, G.R., McPherson, M.A. and Dorner, R.L. (1987) Biochim. Biophys. Acta 928, 341-348
Drummond, A.H. (1985) Nature 315, 752-755
Farese, R.V., Larson, R.E. and Sabir, M.A. (1980) Biochim. Biophys. Acta 633, 479-484
Farese, R.V., Larson, R.E. and Sabir, M.A. (1981a) Diabetes 30, 396-401
Farese, R.V., Sabir, M.A. and Larsen, R.E. (1981b) Biochem. Biophys. Acta 665, 463-470
Farese, R.V., Larson, R.E. and Sabir, M.A. (1982a) Biochim. Biophys. Acta 710, 391-399
Farese, R.V., Larson, R.E. and Sabir, M.A. (1982b) Arch. Biochem. Biophys. 219, 204-208

Gardner, J.D. (1979) Gastroenterology 76, 202-214
Gardner, J.D. and Jackson, M.J. (1977) J. Physiol. 270, 439-454
Gardner, J.D. and Jensen, R.T. (1980) Am. J. Physiol. 238, G63-G66
Gardner, J.D. and Jensen, R.T. (1981) In: Physiology of the Gastrointestinal Tract, edited by L.R Johnson, pp. 831-871, Raven Press, New York
Gardner, J.D. and Jensen, R.T. (1986) Ann. Rev. Physiol. 48, 103-117
Gardner, J.D. and Rottman, A.J. (1979) Biochim. Biophys. Acta 585, 250-265
Gardner, J.D., Conlon, T.P. and Adams, T.D. (1976a) Gastroenterology 70, 29-35
Gardner, J.D., Conlon, T.P., Fink, M.L. and Bodanszky, M. (1976b) Gastroenterology 71, 965-970
Gardner, J.D., Rottman, A.J., Natarajan, S. and Bodanszky, M. (1979a) Biochim. Biophys. Acta 583, 491-503
Gardner, J.D., Costenbader, C.L. and Uhlemann, E.R. (1979b) Am. J. Physiol. 236, E745-E762
Gardner, J.D., Walker, M.D. and Rottman, A.J. (1980) Am. J. Physiol. 238, G458-G466
Gardner, J.D., Korman, L.Y., Walker, M.D. and Sutliff, V.E. (1982) Am. J. Physiol. 242, G547-G551
Gardner, J.D., Sutliff, V.E., Walker, M.D. and Jensen, R.T. (1983) Am. J. Physiol. 245, G676-G680
Gilman, A.G. (1984) Cell 36, 577-579
Gilman, A.G. (1986) TINS 100, 460-463
Gilman, A.G. (1987) Ann. Rev. Physiol. 56, 615-649
Gunther, R. (1981) J. Biol. Chem. 256, 12040-12045
Halenda, S.P. and Feinstein, M.B. (1984) Biochem. Biophys. Res. Commun. 124, 507-513
Heisler, S. (1983) Can. J. Physiol. Pharmacol. 61, 1168-1176
Heyworth, C.M., Whetton, A.D., Kinsella, A.R. and Housley, M.D. (1984) FEBS Lett. 170, 38-42
Hill, S.A., McMurray, W.C. and Sanwal, B.D. (1984) Can. J. Biochem. Cell. Biol. 62, 369-374
Hokin, L.E. (1966) Biochim. Biophys. Acta 115, 219-221
Hokin, L.E. and Hokin, M.R. (1953) J. Biol. Chem. 203, 967-977
Hokin, L.E. and Hokin, M.R. (1958) J. Biol. Chem. 233, 805-810
Hokin, M.R. and Hokin, L.E. (1954) J. Biol. Chem. 209, 549-558
Hokin, M.R. and Hokin, L.E. (1964) In: Metabolism and Significance of Lipids, edited by Dawson, R.M.C. and Rhodes, D.N., pp. 423-434, John Wiley and Sons, Ltd. London
Hokin-Neaverson, M. (1974) Biochem. Biophys. Res. Commun. 58, 763-768
Hollingsworth, E.B., Ukena, D. and Daly, J.W. (1986) FEBS Lett. 196, 131-134
Houslay, M.D. (1984) TIBS 9, 39-40
Im, M.-J., Holzhofer, A., Keenan, A.K., Gierschik, P., Hekman, M., Helmreich, E.J.M. and Pfeuffer, T. (1987) J. Rec. Res. 7, 17-42
Inoue, M., Kishimoto,A., Takai, Y. and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7610-7616
Irvine, R.F. and Moor, R.M. (1986) Biochem. J. 240, 917-920
Irvine, R.F. and Moor, R.M. (1987) Biochem. Biophys. Res. Commun. 146, 284-290
Jakobs, K.H., Aktories, K. and Schultz, G. (1984) Adv. Cycl. Nucl. Prot. Phosph. Res. 17, 135-143
Jakobs, K.H., Bauer, S. and Watanabe, Y. (1985) Eur. J. Biochem. 151, 425-430
Jensen, R.T. and Gardner, J.D. (1981) Fed. Proc. 40, 2486-2496
Joseph, S.K. (1985) TIBS 10, 297-298
Katada, T., Bokoch, G.M., Northrup, J.K., Ui, M. and Gilman, A.G. (1984a) J. Biol. Chem. 259, 3568-3577
Katada, T., Bokoch, G.M., Smigel, M.D., Ui, M. and Gilman, A.G. (1984b) J. Biol. Chem. 259, 3586-3595
Katada, T., Gilman, A.G., Watanabe, Y., Bauer, S. and Jakobs, K.H. (1985) Eur. J. Biochem. 151, 431-437
Kelleher, D.J., Pessin, J.E., Ruoho, A.E. and Johnson, G.L. (1984) Proc. Natl. Acad. Sci. USA. 81, 4316-4320
Kempen, H.J.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1974) Biochim. Biophys. Acta 370, 573-584
Kempen, H.J.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1975) Biochim. Biophys. Acta. 392, 276-287
Kempen, H.J.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1977a) Biochim. Biophys. Acta 496, 521-531
Kempen, H.J.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1977b) Biochim. Biophys. Acta 496, 65-76
Kikkawa, U. and Nishizuka, Y. (1986) Ann. Rev. Cell Biol. 2, 149-178
Kirk, C.J., Creba, J.A., Downes, C.P. and Michell, R.H. (1981) Biochem. Soc. Trans. 9, 377-379
Knight, D.E. and Koh, E. (1984) Cell Calcium 5, 401-418
Kojima, I., Shibata, H. and Ogata,E. (1986) Biochem. J. 237, 253-258
Kolesnick, R.N. and Paley, A.E. (1987) J. Biol. Chem. 262, 9204-9210
Kondo, S. and Schulz, I. (1976) Biochim. Biophys. Acta 419, 76-92
Krause, K.H., Schlegel, W., Wollheim, C.B., Andersson, T., Waldvogel, F.P.A. and Lew, P.D. (1985) J. Clin. Invest. 76, 1348-1354
Kuijpers, G.A.J. and De Pont (1987) Ann. Rev. Physiol. 49, 87-103
Lambert, M., Svoboda, M., Furnelle, J. and Christophe, J. (1985) Eur. J. Biochem. 147, 611-617
Lee,P.C., Leung, Y.K., Srimaruta, N., Cumella, J. and Rossi, T. (1987) Biochim. Biophys. Acta 931, 101-109
Leeb-Lundberg, L.M.F., Cotecchia, S., Lomasney, J.W., DeBernardis, J.F., Lefkowitz, R.J. and Caron, M.G. (1985) Proc. Natl. Acad. Sci. 82, 5651-5655

Levitzki, A. (1987) TIPS 8, 299-303
Litosch, I. (1987) Life Sci. 41, 251-258
Litosch, I and Fain, J.N. (1986) Life Sci. 39, 187-194
Long, B.W. and Gardner, J.D. (1977) Gastroenterology 73, 1008-1014
Lynch, C.J., Charest, R., Bocckino, S.B., Exton, J.H. and Blackmore, P.F. (1985) J. Biol. Chem. 260, 2844-2851
MacIntyre, D.E., McNicol, A. and Drummond, A.H. (1985) FEBS Lett 180, 160-164
Malagodi, M.H. and Chiou, C.Y. (1974a) Eur. J. Pharmacol. 27, 25-33
Malagodi, M.H. and Chiou, C.Y. (1974b) Pharmacology 12, 20-31
Meda,P., Bruzzone, R., Knodel,S. and Orci, L. (1986) J. Cell. Biol. 103, 475-483
Meda, P., Bruzzone, R., Chanson, M., Bosco, D. and Orci, L (1987) Proc. Natl. Acad. Sci. USA. 84, 4901-4904
Merritt, J.E. and Rubin, R.P. (1985) Biochem. J. 230, 151-159
Merritt, J.E., Taylor, C.W., Rubin, R.P. and Putney, J.W., Jr. (1986a) Biochem. J. 238, 825-829
Merritt, J.E., Taylor, C.W., Rubin, R.P. and Putney, J.W., Jr. (1986b) Biochem. J. 236, 337-343
Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81-147
Molina y Vedia, L.M. and Lapetina, E.G. (1986) J. Biol. Chem. 261, 10493-10495
Morena, F.J., Mill, I., Garcia-Sainz, J.A. and Fain, J.N. (1983) J. Biol. Chem. 258, 10938-10943
Murphy, G.J., Hruby, V.J., Trivedi, D., Wakelam, M.J.O. and Houslay, M.D. (1987) Biochem. J. 243, 39-46
Naccache, P.H., Molski, T.F.P., Borgeat, P., White, J.R., Sha'afi, R.I. (1985) J. Biol. Chem. 260, 2125-2131
Nakamura, T. and Ui, M. (1984) FEBS Lett. 173, 414-418
Nakamura, T. and Ui, M. (1985) J. Biol. Chem. 260, 3584-3593
Nishizuka, Y. (1983) Phil. Trans. R. Soc. Lond. B 302, 101-112
Nishizuka, Y. (1986) Science 233, 305-312
Nogochi, M., Adachi, H., Gardner, J.D. and Jensen, R.T. (1985) Am. J. Physiol. 248, G692-G701
Northrup, J.K., Sternwise, P.C., Smigel, M.D., Schleifer, L.S., Ross, E.M. and Gilman, A.G. (1980) Proc. Natl. Acad. Sci. USA. 74, 6516-6520
Ochs, D.L., Korenbrot, J.I. and Williams, J.A. (1983) Biochem. Biophys. Res. Commun. 117, 122-128
Ochs, D.L., Korenbrot, J.I. and Williams, J.A. (1985) Am. J. Physiol. 249, G389-G398
Ohta, H., Okajima, F. and UI, M. (1985) J. Biol. Chem. 260, 15771-15780
Olianas, M.C. and Onali, P.J. (1986) J. Neurochem. 47, 890-897
Orchard, J.L., Davis, J.S., Larson, R.E. and Farese, R.V. (1984) Biochem. J. 217, 281-287
Orellana, S.A., Solski, P.A. and Brown, J.H. (1985) J. Biol. Chem. 260, 5236-5239
O'Rourke, F.A., Halenda, S.P., Zavoico, G.B. and Feinstein, M.B. (1985) J. Biol. Chem. 260, 956-962
Pace, U. and Lancet, D. (1986) Proc. Natl. Acad. Sci. USA. 83, 4947-4951
Pandol, S.J. and Schoeffield, M.S. (1986) J. Biol. Chem. 261, 4438-4444
Pandol, S.J., Schoeffield, M.S., Sachs, G. and Muallem, S. (1985a) J. Biol. Chem. 260, 10081-10086
Pandol, S.J., Thomas, M.W., Schoeffield, M.S., Sachs, G. and Muallem, S. (1985b) Am. J. Physiol. 248, G551-G560
Pelech, S.L. and Vance, D.E. (1984) Biochim. Biophys. Acta 779, 217-251
Pelech, S.L., Paddon, H.B. and Vance, D.E. (1984) Biochim. Biophys. Acta 795, 447-451
Petersen, O.H. and Iwatsuki, N. (1979) In: Hormone receptors in digestion and nutrition, edited by Rosselin, G., Fromageot, P. and Bonfils, S., pp. 191-202, Elsevier/North Holland Biomedical Press, Amsterdam
Petersen, O.H. and Ueda, N. (1976) J. Physiol. 254, 583-606
Ponnappa, B.C. and Williams, J.A. (1980) Cell Calcium 1, 267-278
Prentki, M., Biden, T.J., Janjic, D., Irvine, R.F., Berridge, M.J. and Wollheim, C.B. (1984) Nature 309, 562-564
Putney, J.W.,Jr., Burgess, G.M., Halenda, S.P., McKinney, J.S. and Rubin, R.P. (1983) Biochem. J. 212, 483-488
Renckens, B.A.M., van Emst-de Vries, S.E., De Pont, J.J.H.H.M. and Bonting, S.L. (1980) Biochim. Biophys. Acta 630, 511-518
Rickard, J.E. and Sheterline, P. (1985) Biochem. J. 231, 623-628
Rink, T.J. and Pozzan,T. (1985) Cell Calcium 6, 133-144
Robberecht,P., Conlon, T.P. and Gardner, J.D. (1976) J. Biol. Chem. 251, 4635-4639
Robberecht, P., Deschodt-Lanckman; M., Lammens, M., DeNeef, P. and Christophe, J. (1977) Gastroenterol. Clin. Biol. 1, 519-525
Robberecht, P., Waelbroeck, M., Camus, J.-C., De Neef, P. and Christophe, J. (1984) Biochim. Biophys. Acta 773, 271-278
Rodbell, M. (1980) Nature 284, 17-22
Rodbell, M. (1985) TIBS 10, 461-464
Rozengurt, E., Murray, M., Zachary, I. and Collins, M. (1987) Proc. Natl. Acad. Sci. USA 84, 2282-2286
Rutten, W.J., De Pont, J.J.H.H.M. and Bonting, S.L. (1972) Biochim. Biophys. Acta 274, 201-213
Sagi-Eisenberg, R., Lieman, H. and Pecht, I. (1985) Nature 313, 59-60
Schimmel, R.J. and Elliot, M.E. (1986) Biochem. Biophys. Res. Commun. 135, 823-829

Schreurs, V.V.A.M., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1976) Biochim. Biophys. Acta 419, 320-330
Schulz, I. (1980) Am. J. Physiol. 239, G335-G347
Schulz, I. and Stolze, H.H. (1980) Ann. Rev. Physiol. 42, 127-156
Schulz, I., Pedersen, R., Wizemann, V. and Kondo, S. (1974) In: Secretory Mechanisms of Exocrine Glands, edited by Thorn, N.A. and Petersen, O.H., pp. 88-95, Munksgaard, Copenhagen
Seamon, K.B., Padgett, W. and Daly, J.W. (1981) Proc. Natl. Acad. Sci. USA. 78, 3363-3367
Sibley, D.R., Nambi, P., Peters,J.R. and Lefkowitz, R.J. (1984) Biochem. Biophys. Res. Commun. 121, 973-979
Sibley, D.R., Jeffs, R.A., Daniel, K., Nambi, P. and Lefkowitz, R.J. (1986) Archs. Biochem. Biophys. 244, 373-381
Singh, M. (1979) J. Physiol. 296, 159-176
Smigel, M.D., Ferguson, K.M. and Gilman, A.G. (1985) Adv. Cycl. Nucl. Prot. Phosph. Res. 19, 103-112
Smith, P.A. and Case, R.M. (1975) Biochim. Biophys. Acta 399, 277-290
Stolze, H. and Schulz, I. (1980) Am. J. Physiol. 238, G338-G348
Streb, H., Irvine, R.F., Berridge, M.J. and Schulz, I. (1983) Nature 306, 67-69
Streb, H., Bayerdorffer, E., Haase, W., Irvine, R.F. and Schulz, I (1984) J. Membrane Biology 81, 241-253
Streb, H., Heslop, J.P., Irvine, R.F., Schulz, I. and Berridge, M.J. (1985) J. Biol. Chem. 260, 7309-7315
Sugden, D., Vanecek, J., Klein, D.C., Thomas, T.P. and Anderson, W.B. (1985) Nature 314, 359-361
Svoboda, M., Robberecht, P., Camus, J., Deschodt-Lanckman, M. and Christophe, J. (1978) Eur. J. Biochem. 83, 287-297
Svoboda, M., Furnelle, J., Eckstein, F. and Christophe, J. (1980) FEBS Lett. 109, 275-279
Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979a) J. Biol. Chem. 254, 3692-3695
Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. and Nishizuka, Y. (1979b) Biochem. Biophys. Res. Commun. 91, 1218-1224
Takuwa, N., Takuwa, Y. and Rasmussen, H. (1987) Biochem. J. 243, 647-653
Taparel, D., Susini, C., Esteve, J.P., Diaz, J., Cazaubon, C., Vaysse, N. and Ribet, A. (1985) Peptides 6, 109-114
Taylor, C.W. and Merritt, J.E. (1986) TIPS 7, 238-242
Taylor, M.V., Metcalfe, J.C., Hesketh, T.R., Smith, G.A. and Moore, J.P. (1984) Nature 312, 462-465
Tennes, K.A., Kennedy, J.A. and Roberts, M.L. (1983) Biochem. Pharmacol. 32, 2118-2120
Trimble, E.R., Bruzzone, R., Biden, T.J., and Farese, R.V. (1986) Biochem. J. 239, 257-261
Trimble, E.R., Bruzzone, R., Biden, T.J., Meehan, C.J., Andreu, D. and Merrifield, R.B. (1987a) Proc. Natl. Acad. Sci. USA 84, 3146-3150
Trimble, E.R., Bruzzone, R., Meehan, C.J. and Biden, T.J. (1987b) Biochem. J. 242, 289-292
Ui, M. (1984) TIPS 5, 277-279
Uhing, R.J., Prpic, V., Jiang, H. and Exton, J.H. (1986) J. Biol. Chem. 261, 2140-2146
Vandermeers, A., Vandermeers-Piret, M.C., Rathe, J., Dehaye, J.P., Winand, J. and Christophe, J (1984) Peptides 5, 359-365
Verghese, M.W., Smith, C.D. and Snyderman, R. (1985) Biochem. Biophys. Res. Commun. 127, 450-457
Vicentini, L.M., Di Virgilio, F., Ambrosini, A., Pozzan, T. and Meldolesi, J. (1985) Biochem. Biophys. Res. Commun. 127, 310-317
Watson, S.P. and Lapetina, E.G. (1985) Proc. Natl. Acad. Sci. USA 82, 2623-2626
Weinstein, L.B. (1981) J. Supramol. Struct. 17, 99-120
Weiss, S.J., McKinney, J.S. and Putney, J.W., Jr. (1982) Biochem. J. 206, 555-560
Williams, J.A. (1980) Am. J. Physiol. 238, G269-G279
Williams, J.A. and McChesney, D.J. (1987) Reg. Peptides 18, 109
Wright, P.S., Morand, J.N. and Kent, C. (1985) J. Biol. Chem. 260, 7919-7926
Yoshimasa, T., Sibley, D.R., Bouvier, M., Lefkowitz, R.J. and Caron, M.G. Nature 327, 67-70
Zavoico, G.B., Halenda, S.P., Sha'afi, R.I. and Feinstein, M.B. (1985) Proc. Natl. Acad. Sci. USA 82, 3859-3862

DANKWOORD

Aan het tot stand komen van dit proefschrift hebben velen bijgedragen en mede namens Jeannette, Neeltje, Fieke en Sanne wil ik hen op deze plaats van harte bedanken.

Zonder anderen te kort te willen doen dank ik met name *Irene van Nooij* en dan niet alleen voor haar deskundige hulp en haar bereidheid om langer te blijven als de werkzaamheden weer eens uitliepen, maar ook voor haar belangstelling in het wel en wee van ons gezin. Zij heeft dacht ik toch meestal wel begrepen wat ik eigenlijk bedoelde te zeggen.

Ben van den Broek wil ik eveneens met name danken en dan vooral ook voor de prettige en vlotte samenwerking. Zijn onnavolgbare incubatieschema's zijn hem hierbij vergeven.

Verder wil ik *Sjenet van Emst-de Vries en Annie Fleuren-Jakobs* noemen die de bioloog begeleid hebben bij zijn eerste biochemische schreden.

De bijdragen van *Roland Tilly, Bert Haenen, Silvia Pelgrom, Herman van den Berg en Twan Rutten* mogen niet onvermeld blijven. Hun resultaten zijn in een aantal gevallen verwerkt in dit proefschrift en/ of vormen de basis voor verder onderzoek. Met *Riny Crul* heb ik prettig samengewerkt in een gezamenlijk project met de afdeling neurologie.

Herman Swarts wil ik vooral bedanken voor zijn onmisbare hulp tijdens de spannende uurtjes van het 'computerizeren' van het manuscript en *Twan Ederveen*, die steeds weer kleine foutjes vond in uiteindelijke versies, voor de discussies op het werk, over het werk en op de fiets.

Albert Peters en *Leo Jansen* van het Centraal Dierenlaboratorium wil ik noemen voor hun snelle en deskundige hulp.

Tot slot wil ik al de niet met name genoemde medewerkers en mede koffiedrinkers van de afdeling biochemie danken voor hun interesse in het pancreasonderzoek en de medewerkers van de afdeling Fysiologie voor hun bereidheid tot twee maal toe een plaatsje voor mij in te ruimen.

Onze ouders en familieleden bedanken wij voor hun belangstelling en vooral ook voor hun steun gedurende de afgelopen jaren.

Jeannette, door het overnemen van steeds meer ouderlijke taken gedurende de afgelopen jaren en door je geduld en optimisme heb heb je in mijn ogen de meest wezenlijke bijdrage geleverd aan het tot stand komen van dit proefschrift.

0-0-0

PUBLICATIONS

Verburg-van Kemenade, B.M.L., Willems, P.H.G.M., Jenks, B.G. and van Overbeeke, A.P. (1984) The development of the pars intermedia and its role in the regulation of dermal melanophores in the larvae of the amphibian *Xenopus laevis*.
Gen. Comp. Endocrinol. 55, 54-65

Willems, P.H.G.M., Fleuren-Jakobs, A.M.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1984) Potentiating role of cyclic AMP in pancreatic enzyme secretion demonstrated by means of forskolin.
Biochim. Biophys. Acta 802, 209-214

Willems, P.H.G.M., van Nooij, I.G.P. and De Pont, J.J.H.H.M. (1986) Stimulatory and inhibitory effects of TMB-8 on pancreatic enzyme secretion.
Biochim. Biophys. Acta 888, 255-262

Willems, P.H.G.M., Tilly, R.H.J. and De Pont, J.J.H.H.M. (1987) Pertussis toxin stimulates cholecystokinin-induced cyclic AMP formation but is without effect on secretagogue-induced calcium mobilization in exocrine pancreas.
Biochim. Biophys. Acta 928, 179-185

Willems, P.H.G.M., van Nooij, I.G.P., Haenen, H.E.M.G. and De Pont, J.J.H.H.M. (1987) Phorbol ester inhibits cholecystokinin octapeptide-induced amylase secretion and calcium mobilization, but is without effect on secretagogue-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate in rabbit pancreatic acini.
Biochim. Biophys. Acta 930, 230-236

CURRICULUM VITAE

Peter H.G.M. Willems werd geboren op 20 oktober 1952 te Grave. In 1972 behaalde hij het diploma HBS-B aan het Dominicus College te Nijmegen. Na zijn militaire dienstplicht werkte hij van 1974 tot 1977 als laborant bij Organon International b.v. te Oss op het farmacologisch controle laboratorium. In de avonduren werd de HBO-A opleiding zoölogisch analist, fysiologisch-farmacologische richting, gevolgd. Het diploma werd behaald in 1977. In datzelfde jaar begon hij met de studie Biologie aan de Katholieke Universiteit van Nijmegen. In 1979 behaalde hij het diploma deskundigheid stralingshygiene voor een C-laboratorium en in 1980 legde hij het kandidaatsexamen af met hoofdvak Biologie en bijvak Geologie (B_1G). Het doctoraalexamen met hoofdvak Ontwikkelingsbiologie der Dieren (Prof. Dr. J.M. Denucé) en bijvakken Dierfysiologie (Prof. Dr. A.P. van Overbeeke) en Biochemie (Prof. Dr. J.J.H.H.M. De Pont en Prof. Dr. S.L. Bonting) werd afgelegd in september 1984.

Vanaf juli 1984 is hij werkzaam als wetenschappelijk assistent in de groep van prof. dr. J.J.H.H.M. De Pont bij de vakgroep Biochemie M.F. van de Katholieke Universiteit van Nijmegen. In het kader van zijn onderwijstaak begeleidde hij doctoraalstudenten Biologie en leverde hij een bijdrage aan het onderwijs aan eerste en tweede jaars studenten Geneeskunde.

Per 1 juni 1988 is hij aangesteld als post-doc medewerker in de groep van prof. dr. J.J.H.H.M. De Pont.

In 1979 trouwde hij met Jeannette Donjacour en geboren werden Neeltje (1981), Fieke (1982) en Sanne (1984).

STELLINGEN

I

De hypothese van Gardner en Jensen dat het verschil in secretoire activiteit tussen VIP en secretine berust op compartimentalisatie van het gevormde cyclisch AMP, wordt niet ondersteund door de bevinding van Dehaye et al., dat forskoline de enzymsecretie slechts in geringe mate stimuleert.

Gardner, J.D. and Jensen, R.T. (1986) Ann. Rev. Physiol. 48, 103-117

Dehaye, J.-P., Gillard, M., Poloczek, P., Stievenart, M., Winand, J. and Christophe, J. (1985) J. Cycl. Nucl. Prot. Phosph. Res. 10, 269-280

II

Dat het netto stimulerende effect van cholecystokinine geringer is in acini die voorbehandeld zijn met forbol ester, bewijst niet dat het aantal receptoren voor het betreffende hormoon is afgenomen, maar dient veeleer verklaart te worden uit het feit dat een van beide routes waarlangs het hormoon de enzymsecretie stimuleert reeds door de forbol ester is geactiveerd.

Lee, P.C., Leung, Y.K., Srimaruta, N., Cumella, J. and Rossi, T. (1987) Biochim. Biophys. Acta 931, 101-109

Willems, P.H.G.M., Van Nooij, I.G.P., Haenen, H.E.M.G. and De Pont, J.J.H.H.M. (1987) Biochim. Biophys. Acta 930, 230-236

III

Uit het feit dat in aanwezigheid van fosfatidylserine enkele laag-moleculaire eiwitten gefosforyleerd worden dienen Burnham en Williams wel degelijk te concluderen dat proteïne kinase C activiteit aanwezig is in de 'particulate' fractie van de pancreas.

Burnham, D.B. and Williams, J.A. (1984) Am. J. Physiol. 246, G500-G508

IV

De mogelijkheid dat de door forbol esters geïnduceerde remming van hormonaal gestimuleerde calciumstijgingen berust op desentizering van de voor IP_3 gevoelige intracellulaire opslagplaats voor calcium, is ten onrechte onvermeld gebleven in de recentelijk door Berridge gepubliceerde overzichtsartikelen.

Hoofdstuk 7 van dit proefschrift.

Berridge, M.J. (1987) Ann. Rev. Biochem. 56, 159-193

Berridge, M.J. (1987) Biochim. Biophys. Acta 907, 33-45

V

De door Hanai et al. gebruikte methode om de Na^+/Ca^{2+}- exchange te meten in intacte niercellen is onbevredigend, daar de efflux van Ca^{2+} in aanwezigheid van Na^+ gelijk blijkt te zijn aan die in aanwezigheid van K^+.

Hanai, H., Ishida, M., Liang, C.T. and Sacktor, B. (1986) J. Biol. Chem. 261, 5419-5425

Van den Broek, L.A.M. and Van Os, C.H., unpublished results.

VI

De specificiteit van het $(Na^+ + K^+)$-ATPase voor ATP is niet zozeer gelegen in de fosforyleringsdeelreactie, als wel in de conformatieovergang die direct aan deze reactie voorafgaat.

Schuurmans Stekhoven, F.M.A.H., Swarts, H.G.P., Zhao, R.S. and De Pont, J.J.H.H.M. (1986) Biochim. Biophys. Acta 861, 259-266

VII

De door Hooper en Taylor beschreven reactie van T cellen tegen autologe erythrocyten is geen argument voor de theorie dat clonale deletie geen rol speelt bij zelftolerantie.
Hooper, D.C. and Taylor, R.B. (1987) Eur. J. Immunol. 17, 797-802

VIII

Het meten van hormonaal gestimuleerde calciumstijgingen met behulp van intracellulaire calcium indicatoren als quin2 en fura2, biedt de mogelijkheid het aantal proefdieren voor biologische ijkingen drastisch te verminderen.

IX

De 'pakkans' van een artikel lijkt evenredig aan het aantal in de Current Contents gebezigde trefwoorden vervat in de titel.

X

De stelling dat het onderzoek naar de rol van inositollipiden overeenkomsten heeft met televisieseries als Dallas en Dynasty, bevestigt eens te meer dat promovendi geen tijd hebben om naar de TV te kijken.
Stelling 10, proefschrift M.L. de Jong, 1986,

XI

De vasthoudendheid waarmee 'aangeschoten' politici hun positie verdedigen doet vermoeden dat Tilburg voorlopig de laatste vacature is.

XII

De discussienota Liberaal Bestek '90 laat zich het best lezen als in de tekst het voorvoegsel 'kans' wordt weggelaten.

XIII

Uit het oogpunt van 'Public Relations' zou het voor chemische bedrijven aantrekkelijk moeten zijn hun verouderde producten terug te nemen.

XIV

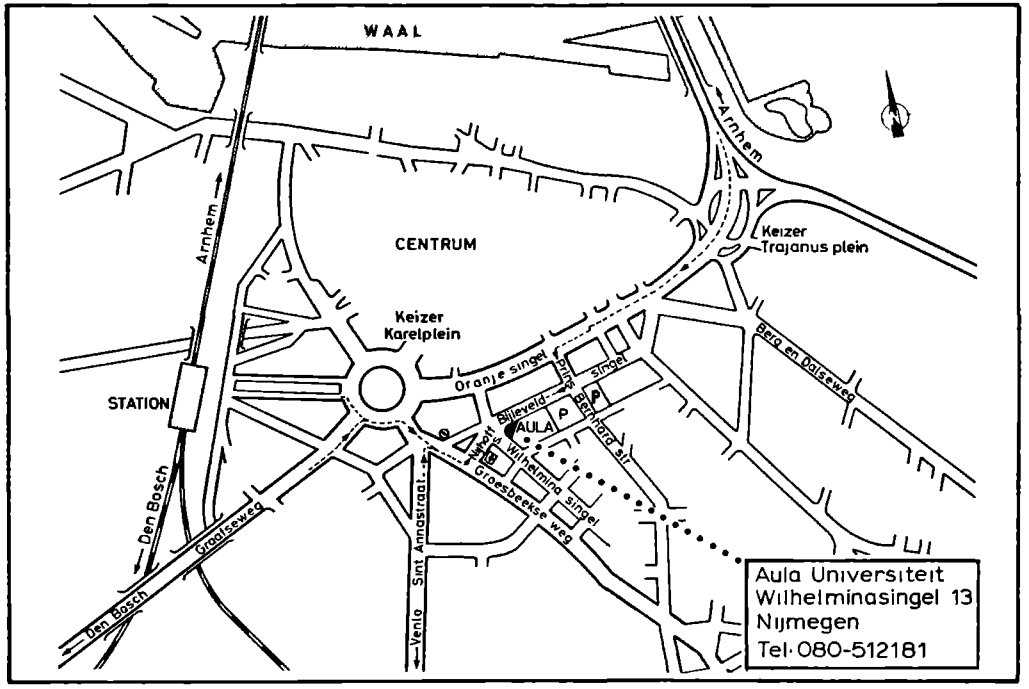
Het is onverantwoord de verwerking van radioactief afval over te laten aan het particuliere initiatief.

XV

De hoop is ijdel dat het huidige cellentekort leidt tot een toekomstig delinquententekort.

P.H.G.M. Willems,
11-maart-1988.

RECEPTIE NA AFLOOP VAN DE PROMOTIE
IN DE AULA
VAN DE KATHOLIEKE UNIVERSITEIT,
WILHELMINASINGEL 13,
NIJMEGEN


WAAL
Arnhem
CENTRUM
Keizer Karelplein
Keizer Trajanus plein
Arnhem
STATION
Oranje singel
Prins Bernhard singel
Berg en Dalseweg
Bijleveld-
AULA
Wilhelmina singel
Groesbeekse weg
Sint Annastraat
Den Bosch
Graafseweg
Den Bosch
Venlo
Aula Universiteit
Wilhelminasingel 13
Nijmegen
Tel. 080-512181