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COMPLEX ROLE OF PROTEIN KINASE C IN MEDIATING THE SUPRAMAXIMAL INHIBITION OF PANCREATIC SECRETION OBSERVED WITH CHOLECYSTOKININ

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Summary: Protein kinase C appears to play an important, yet complex role in the supramaximal inhibition of pancreatic acinar cell secretion observed in response to cholecystokinin (CCK). The addition of protein kinase C activation to the concentration-response curve of a partial agonist acting at the CCK receptor (a phenethyl ester analogue of CCK), transforms a curve without supramaximal inhibition to a full agonist curve typical of CCK. This effect can be elicited by low concentrations of phorbol ester (50pM to 1nM 12-O-tetradecanoyl-phorbol-13-acetate) or by hormonal agonists (0.1μM carbamylcholine, 10pM bombesin, 1pM CCK-8) which activate protein kinase C, but not by agonists acting via alternate second messengers (VIP). Of interest, this effect is dependent on preincubation of the acinar cells with the protein kinase C activator at 37°C, with the effect rapidly reversed by transient exposure of the cells to lower temperature. This is consistent with mediation by a phosphorylation event. However, the requirement for an extended (>15 min) preincubation period when using minimal kinase activation suggests that this phenomenon is more complicated than a simple bimolecular phosphorylation event and likely includes a series of events such as translocation of substrates and/or enzymes involved.

Introduction: It is well recognized that certain agonists have bimodal effects on their targets, in which increasing concentrations stimulate a response to a maximum, while further increases in agonist concentration actually inhibit the response. The molecular basis of the supramaximal inhibition of action, however, is unclear. The major physiologic agonist for stimulating pancreatic exocrine secretion, the peptide hormone cholecystokinin (CCK), has this type of concentration-response curve. Of interest is the description of analogues of this hormone, with the carboxyl-terminal phenylalanine amide of CCK replaced by a phenethyl ester group (OPE), which are equally-efficacious pancreatic agonists, but which do not express the supramaximal inhibition of secretion typical of native CCK (1,2). Thus, they appear to be only partial agonists. Further, evaluation of radioligand

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binding and affinity labeling using probes derived from CCK and OPE directly demonstrate that they both initiate their different responses by interacting with the same receptor molecule (2). Thus, the actions of these agonists at the CCK receptor offers a useful model to provide insight into the molecular basis of this type of concentration-response curve.

Our experimental approach involved the addition of a well-defined cellular activity to the partial agonist (OPE) in an attempt to elicit a full agonist response typical of native CCK, in which supramaximal inhibition of secretion was observed. In this work, we focused on protein kinase C for the added activity for the following reasons: (i) CCK is known to be a strong stimulant of the cascade initiated by phospholipase C activation which results in phosphatidylinositol hydrolysis and activation of protein kinase C, while phenethyl ester analogues elicit no such response (1,3); (ii) strong direct activation of protein kinase C has been reported to inhibit CCK-stimulated secretory responses (4); and (iii) protein kinase C activity can be easily stimulated in the intact pancreatic acinar cell using the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (5).

METHODS

Tissue preparations. Dispersed pancreatic acini from 125-150g male Sprague-Dawley rats were prepared by sequential enzymatic and mechanical dissociation according to the method of Schultz et al. (6). All incubations were performed in Krebs-Ringers-Hepes medium, containing 25mM Hepes, pH 7.4, 104mM NaCl, 5mM KCl, 1.2mM $MgSO_4$, 2mM $CaCl_2$, 1mM KH_2PO_4, 2.5mM D-glucose, 0.2% bovine serum albumin, and 0.01% soybean trypsin inhibitor, unless otherwise specified.

Secretion studies. Pancreatic acini (1.5 to 2 million cells) were incubated with agonists for 40 min at 37°C, followed by the separation of supernatant from cells by centrifugation (2). In mixing experiments, the acini were typically preincubated at 37°C for 15 min with selected agents in a total volume of 0.9 ml, after which 0.1ml of OPE was added to the tubes to yield the stated concentrations, and incubation was carried out for another 40 min at 37°C. In selected experiments, the conditions of the preincubation period were varied as noted. Amylase was assayed by the generation of maltose from starch according to the method of Bernfeld (7).

Radioligand binding studies. ^{125}I-OPE was prepared as reported (2), and purified by reversed phase HPLC to yield a radioligand with specific radioactivity of approximately 2000Ci/mmol. Eight to 10pM radioligand was added to the assay tubes, and incubations were performed as described above, except extending the incubations to 60 min to permit the attainment of steady state conditions (2). Bound was separated from free radioligand by rapid filtration with a Skatron cell harvester (Sterling VA), using receptor-binding filtermats (2). Non-specific binding, determined in the presence of 0.1μM CCK-8 or 1μM OPE, was less than 15% of total binding. Data were analyzed using the LIGAND program of Munson and Rodbard (8).

Statistical analysis. Results are expressed as means±SEM, and were analyzed for significance using Student's T-test for unpaired data.

RESULTS

Secretagogue effects on pancreatic secretion. Figure 1 shows concentration-response curves for CCK, OPE, and TPA to stimulate dispersed rat pancreatic acini to secrete amylase. Confirming observations from different laboratories at different times (1-3,9), CCK-8 and OPE were equally-efficacious secretagogues, with CCK more potent than OPE, and with CCK eliciting supramaximal inhibition of secretion not observed with OPE. TPA also stimulated acinar cell secretion in a concentration-dependent manner, reaching a plateau in response to 1μM or higher concentrations of TPA which represented 50.6±1.7% of the maximum observed with CCK or OPE.

Of note, this high concentration of TPA (1μM) has been reported to stimulate a protein kinase C response in excess of five times that observed with any concentration of CCK (10), while low concentrations of TPA (50pM to 1nM) are reported to stimulate more physiologic protein kinase C responses while having minimal effects on secretion (5).

Effects of TPA on OPE-stimulated secretion. If protein kinase C-stimulated events are responsible for the supramaximal inhibition observed in response to CCK, we would expect the addition of TPA to supramaximal concentrations of OPE to inhibit the secretory response to OPE alone. However, Figure 2 shows that the simultaneous addition of 1nM TPA to 3μM OPE did not change the secretory response to OPE alone.

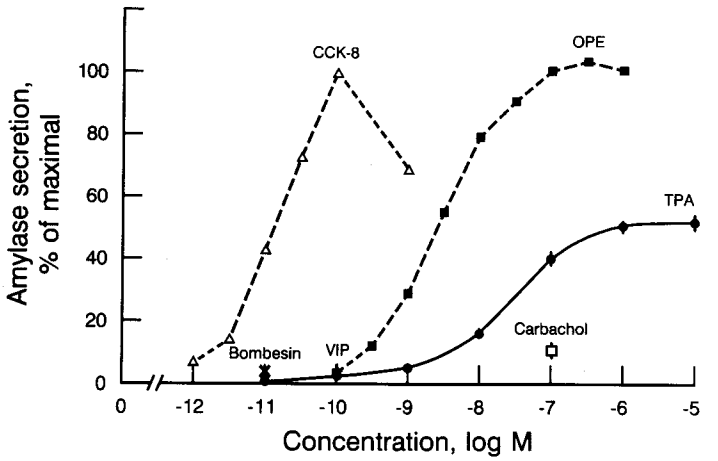

Figure 1. Secretagogue Effects on pancreatic secretion. Shown are full concentration-response curves of CCK-8, OPE and TPA performed on the same dispersed rat pancreatic acinar cell preparation (n≥4, mean±SEM). Also shown are secretagogue effects of the concentrations of each of the agents used in mixing experiments shown in Figs 3 and 4.

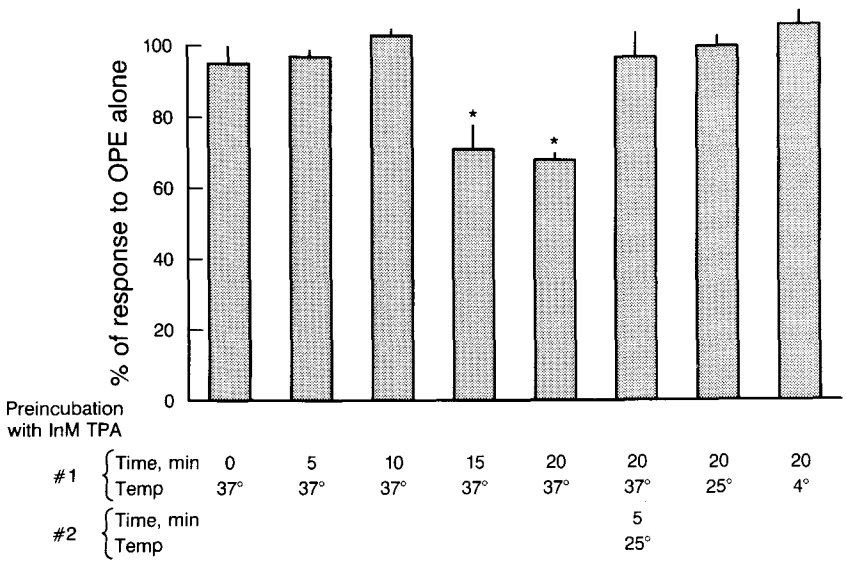

Figure 2. Effects of different time and temperature conditions of TPA preincubation on OPE-stimulated amylase-secretion. Bars represent means±SEM of secretory responses to 3μM OPE (a supramaximal concentration) after the noted preincubation conditions, with 100% representing the peak response to OPE in the absence of an additional secretagogue in the preincubation.

Because the direct phosphorylation effects of protein kinase C are known to be transient, rapidly reversible, and temperature-dependent (11), and since the events which occur in the cell are affected by signals traversing space over time, this experiment was repeated varying the time and temperature conditions of a preincubation period with TPA (Fig 2). Preincubations with the same concentration of TPA (1nM) at 37°C for 15 min or longer did elicit supramaximal inhibition of the OPE secretory response. Shorter preincubation times with this same concentration of TPA at this same temperature had no effect. Similar duration preincubations with the same concentration of TPA at lower temperature (25°C or 4°C) were also not effective. Additionally, the effect of a 20 min preincubation with 1nM TPA at 37°C to lead to supramaximal inhibition was reversed by a 5 min interval at 25°C.

Indeed, using this experimental protocol (a 15 min 37°C preincubation period in which cells were exposed to the TPA prior to OPE stimulation), we observed the predicted effect of low concentrations of TPA (ranging between 50pM and 1nM TPA) to change the OPE concentration-response curve into a CCK-like curve which included supramaximal inhibition (Fig 3). The maximal secretory response was unchanged by this treatment and no inhibition was observed on the stimulatory portion of the OPE concentration-response curve. Concentrations of TPA lower than 10pM, previously shown to have no effect on

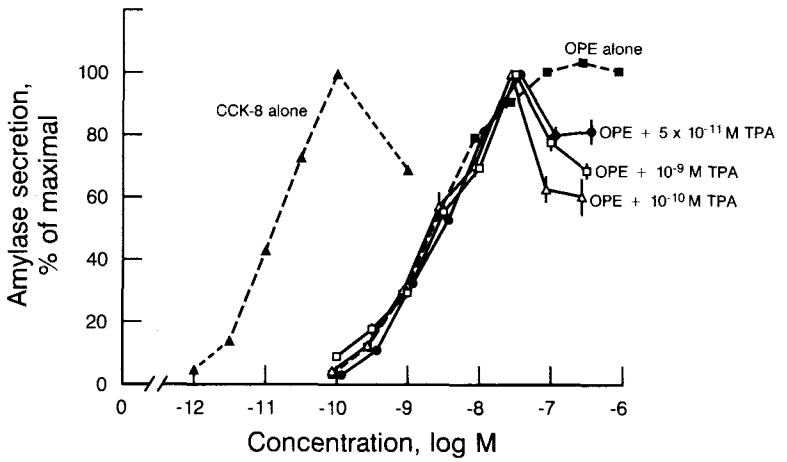

Figure 3. Effect of preincubation with different concentrations of TPA on OPE-stimulated amylase secretion. Shown are concentration-response curves for each control agent, CCK-8 and OPE, as well as the OPE concentration-response curves performed after a 15 min preincubation with the noted concentrations of TPA at 37°C ($n \geq 3$, mean±SEM).

protein kinase C in these cells (5), had no effect on the OPE concentration-response curve (data not shown).

Effect of hormonal agonists on OPE-stimulated secretion. Like TPA, hormonal agonists which are known to stimulate activation of protein kinase C after binding to their own distinct receptors on pancreatic acinar cells transformed the OPE concentration-response curve into a CCK-like curve (Fig 4). The agonists studied included 10pM bombesin, 0.1μM carbamylcholine, and even 1pM CCK-8 itself. These represent the concentrations of these agents which alone are minimally-stimulatory of pancreatic secretion (less than 5% of maximal) (Fig 1). Once again, the 15 min preincubation period at 37°C was critical for this observation, with coincubation experiments not having any significant effect on the OPE concentration-response curve (data not shown). Preincubations with analogous minimally-secretory concentrations of vasoactive intestinal polypeptide (0.1nM VIP), a cyclic AMP stimulating hormone, (Figs 1,4) and of OPE (0.1nM) (data not shown) had no effect on the OPE concentration-response curve.

Effects of TPA and agonists on OPE binding. Under the conditions utilized in the mixing experiments to transform the OPE concentration-response curve into a CCK-like curve, none of the agents had a significant effect on OPE competition for the binding of ^{125}I-OPE to intact pancreatic acini (K_d=14.2±5.7nM). This supports the interpretation that the mechanism of the change in biological responses to OPE effected by these agents is mediated by a post-receptor event.

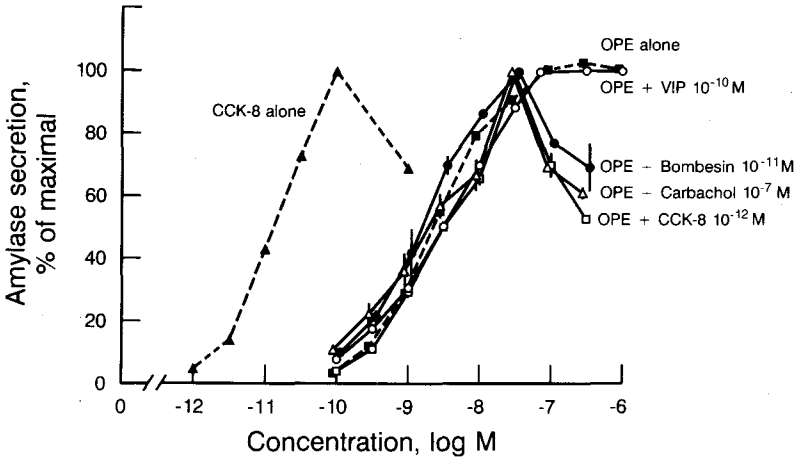

Figure 4. Effects of preincubations with hormonal agonists on OPE-stimulated amylase secretion. Shown are concentration-response curves for each control agent, CCK-8 and OPE, as well as the OPE concentration-response curves performed after a 15 min preincubation with the noted concentrations of hormones which are known to stimulate protein kinase C (bombesin, carbachol, and CCK-8) and VIP ($n \geq 3$, mean±SEM).

DISCUSSION

In this work, we have studied the molecular basis of supramaximal inhibition of pancreatic acinar cell secretion observed in response to CCK. We have focused on the role of protein kinase C in this biological response, since this enzyme has been implicated in inhibition of pancreatic secretion (4,12), it is activated by CCK agonists which elicit supramaximal inhibition of secretion (10), and it can be readily manipulated using TPA and hormonal agonists (5,12). Key for our approach, was the use of intact cells as bioassay for relevant cellular processes, maintaining the integrity of mechanisms of signal amplification and interacting regulatory cascades. This assay system was more sensitive than using existing direct biochemical assays of enzymatic activity. Using this approach, we have demonstrated a critical role for protein kinase C in supramaximal inhibition of secretion, by activating that enzyme, and thereby transforming a partial agonist concentration-response curve into a typical full agonist curve including this response.

In the presence of direct activation of protein kinase C by the phorbol ester, TPA (5), we were able to transform the OPE concentration-response curve into a CCK-like curve. This occurred using TPA concentrations in the range between 50pM and 1nM. The protein kinase C activation elicited by these low concentrations of TPA is believed to be in the range stimulated by physiologic agonists (10). Lower concentrations of TPA which have not been observed to

affect protein kinase C (5) had no effect. Of note, the upstroke of the OPE concentration-response curve was not inhibited by the concentrations of TPA used, suggesting that this was not a simple inhibition of secretion. Higher concentrations of TPA were not used, since these are recognized as being capable of stimulating pancreatic secretion (4,5) and causing down-regulation of the high affinity CCK receptor (13), and they have been shown to elicit greater than five times the protein kinase C activity that is stimulated by maximally-secretory concentrations of CCK (10). As further control, the concentrations of TPA used here were demonstrated to have no effect on the binding of OPE to pancreatic acinar cells.

Of particular interest, this effect of TPA could not be elicited by the simple addition of this agent to OPE, but rather required an extended (15 min) preincubation period at 37°C with the phorbol ester. Additionally, this effect was reversed by a transient drop in temperature prior to addition of OPE. This is consistent with the transient, temperature-dependent, and reversible nature of a phosphorylation event. The long duration of the preincubation, however, suggests that it is likely more complicated than a simple bimolecular interaction, and may well require translocation of enzyme(s) and/or substrates involved.

The transformation of the OPE concentration-response curve into a CCK-like curve could also be accomplished using similar preincubations with acinar cell secretagogues which are known to act via G protein association leading to phospholipase C activation, phosphatidylinositol hydrolysis, and protein kinase C translocation. This was not observed with secretagogues acting via a different mechanism. Included among the effective agents were the heterologous agonists acting at other receptors, carbamylcholine and bombesin, as well as native CCK itself. Of note, all of these agonists were used in very low concentrations (1pM CCK, 0.1μM carbamylcholine, 10pM bombesin), representing the minimal concentrations which alone stimulate pancreatic secretion. These concentrations have been shown to have an effect on protein kinase C activation (14), and we show here that each has no detectable effect on OPE binding to the acinar cell preparation. Like TPA, concentrations of these agonists higher than those used in this study can be demonstrated to produce high affinity CCK receptor down-regulation (13), inhibition of intracellular calcium (15), and acinar cell secretion (13).

Perhaps the most interesting mixing experiment utilized native CCK itself in 1pM concentration. If CCK and OPE interacted with the same receptors to elicit the same cellular responses, their mixing should have been additive throughout their concentration-response curves (taking the differences of potency into consideration). Clearly, this was not the case, with the upstroke of the OPE concentration-response curve essentially unaffected by this very low concentration of native hormone, and the

supramaximal portion of the OPE curve transformed into a CCK-like curve. This offers further evidence that OPE is a partial agonist, with at least some of the difference in biological responses to these two agents explained by post-protein kinase C events.

These observations also have important implications for our understanding of events which occur at the level of the receptor. The bimodal shape of the CCK concentration-response curve has been previously attributed to the presence of high affinity receptors thought to mediate stimulation of secretion, and low affinity receptors thought to mediate inhibition of secretion (9,16). Since the CCK effect is observed with such a low concentration of this agent, it is unlikely that its effect to elicit supramaximal inhibition was mediated by its low affinity receptor.

We would propose an alternate mechanism for the differences observed between CCK and OPE. There are recent data which suggest that the high and low affinity receptors for CCK represent inter-convertible states of the same molecule, rather than distinct molecules (17,18). These affinity states are regulated by association of a G protein with the CCK receptor (18), an event which seems to be differentially stabilized by CCK (19) and OPE (2). We postulate that OPE is a partial agonist as a result of being less effective than CCK to stabilize the ternary complex with G_q, thereby stimulating less phospholipase C activation, phosphatidylinositol hydrolysis, and protein kinase C activation.

The most interesting aspect of this hypothesis is the mechanism whereby both CCK and OPE do stimulate pancreatic acinar cell secretion. If we eliminate the traditional cascade initiated by G_q association (as described), we must postulate the existence of a novel alternate cascade. Perhaps this is initiated by receptor association with a different G protein, or even possibly by a G protein-independent cascade. Proof for such a mechanism will require additional experimentation.

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