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Cellular Distribution of Gastric Chief Cell Protein Kinase C Activity: Differential Effects of Diacylglycerol, Phorbol Esters, Carbachol, and Cholecystokinin

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Abstract Stimulation of chief cells with carbachol or cholecystokinin (CCK) results in the production of inositol trisphosphate (IP_3) and diacylglycerol (DAG). Although IP_3 increases cell calcium concentration, thereby stimulating pepsinogen secretion, the role of DAG and its target, protein kinase C (PKC), is less clear. To examine the relation between the cellular distribution of PKC activity and pepsinogen secretion, we determined PKC activity in cytosolic and membrane fractions from dispersed chief cells from guinea pig stomach. To validate our assay, we studied the actions of the phorbol ester PMA. PMA caused a rapid, dose-dependent, 6-fold increase in pepsinogen secretion and membrane-associated PKC activity. Similarly, dose-response curves for pepsinogen secretion and the increase in membrane-associated PKC activity induced by a membrane-permeant DAG (1-oleoyl-2-acetylglycerol) were superimposable. In contrast, CCK (0.1 nM to 1.0 μM) and carbachol (0.1 μM to 1.0 mM) caused a 4-fold increase in pepsinogen secretion, but did not alter the distribution of PKC activity. These results indicate that in gastric chief cells, PMA- and DAG-induced pepsinogen secretion is accompanied by increased membrane-associated PKC activity. However, the cellular distribution of PKC activity is not altered by CCK or carbachol.

Key words: pepsinogen secretion, signal transduction, stomach, translocation, hormones

In most tissues, a major signal transduction pathway involves the hydrolysis of phosphatidyl inositol 4,5-bisphosphate to form inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Berridge, 1987). IP_3 causes mobilization of calcium from intracellular stores (Berridge et al., 1983), whereas DAG can activate protein kinase C (PKC), a lipid- and calcium-dependent kinase (Nishizuka, 1986). Although elevation in cytosolic calcium concentration appears to be a ubiquitous stimulant of cellular responses, the contribution of PKC activation to stimulation of cellular responses differs from cell to cell (Nishizuka, 1988).

In gastric chief cells, cholecystokinin (CCK)- and carbachol-induced pepsinogen secretion appear to be mediated by increases in cytosolic calcium levels. This is evidenced by the observations that these agents stimulate an increase in chief cell IP_3, followed by an initial transient release of calcium from internal stores (Chew and Brown, 1986). Subsequently, a more sustained increase in cytosolic calcium is dependent on influx of extracellular calcium (Tsunoda et al., 1988).

In contrast to that of calcium, the role, if any, of PKC in mediating pepsinogen secretion remains unclear. Assumptions regarding the role of PKC in signal transduction have been made from the observation that phorbol esters, agents that mimic the actions of DAG by binding and activating PKC (Castagna et al., 1982), stimulate pepsinogen secretion (Sakamoto et al., 1985; Matsumoto et al., 1987). Moreover, CCK stimulates a biphasic increase in DAG in chief cells (Nakano et al., 1990). Hence, investigators (Sakamoto et al., 1985; Matsumoto et al., 1987; Nakano et al., 1990) have concluded that CCK-induced increases in chief cell DAG result in activation of PKC and thereby contribute to the stimulation of pepsinogen secretion. Potential flaws in this approach to evaluating the role of PKC in signal transduction have been reviewed by Blackshear (1988). Although activation of PKC by phorbol esters causes secretion,

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it cannot be assumed that PKC is involved in pepsinogen secretion induced by physiological secretagogues. Nor can it be assumed that a secretagogue-induced rise in chief cell DAG results in activation of PKC. Experiments using putative inhibitors of PKC may be misleading because of non-specific actions on other kinases (Blackshear, 1988). Presently, there is no direct evidence for the involvement of PKC in pepsinogen secretion from chief cells.

Alterations in PKC activity in cytosolic and membrane fractions, an indicator of the kinase's involvement in cellular events (Kraft and Anderson, 1983), have been studied in a variety of secretory cells (Ishizuka et al., 1987; Terbush and Holz, 1986; Persaud et al., 1989). To define more precisely the role of PKC in mediating pepsinogen secretion, we examined secretagogue-induced changes in PKC activity in cytosolic and membrane fractions from dispersed chief cells from guinea pig stomach.

METHODS

Materials

Male Hartley guinea pigs (150–200 g) were obtained from CAMM Research Lab Animals (Wayne, NJ); Hepes from Boeringer Mannheim (Indianapolis, IN); collagenase (type I), bovine albumin (fraction V), cholecystokinin (26–33) (CCK-8), EGTA, phorbol 12-myristate 13-acetate (PMA), leupeptin, 1-oleoyl-2-acetyl-rac-glycerol (OAG), phenylmethylsulfonylfluoride (PMSF), phosphatidyl serine, 1,2-dioleoylglycerol, and adenosine triphosphate (ATP) from Sigma (St. Louis, MO); basal medium (Eagle) amino acids and essential vitamin solution from Grand Island Biological (Grand Island, NY); Percoll from Pharmacia (Piscataway, NJ); Ecoscint liquid scintillation fluid from National Diagnostics (Manville, NJ); 0.45 μM nitrocellulose filters from Millipore (Bedford, MA); ^{32}P-ATP from New England Nuclear (Boston, MA).

Incubation Solution

The standard incubation solution contained 24.5 mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM KH_2PO_4, 1 mM $MgCl_2$, 11.5 mM glucose, 5 mM Na fumarate, 5 mM Na pyruvate, 5 mM Na glutamate, 1.5 mM $CaCl_2$, 2 mM glutamine, 0.1% (wt/vol) albumin, 1% (vol/vol) amino acid mixture, and 1% (vol/vol) essential vitamin mixture. The standard incubation solution was equilibrated with 100% O_2 and all incubations were performed with 100% O_2 as the gas phase.

Tissue Preparation

Dispersed chief cells from guinea pig stomach were prepared as described previously (Raufman et al., 1984), except that following collagenase digestion, the cells were dispersed by incubation for 20 min in standard solution without Ca^{++}, Mg^{++}, or EGTA. EGTA was omitted from this step to avoid potential damage to membrane calcium channels (Uemura et al., 1990).

Cell Fractionation and Protein Kinase C Assay

One milliliter of cell suspension (3–6 × 10^6 cells/ml) was incubated with the appropriate agent for a specified time. Incubation was terminated by adding 3 ml of ice-cold isolation solution that consisted of 20 mM Tris (pH 7.4), 2 mM EDTA, 0.25 M sucrose, 50 mM mercaptoethanol, 1 mM PMSF, and 10 μg/ml leupeptin. The cells were immediately pelleted (3 min at 800*g*), washed, and resuspended in 1 ml isolation solution. The cells were then sonicated (3 × 5 sec bursts) and the homogenate centrifuged at 140,000*g* for 40 min. The supernatant (cytosolic fraction) was stored at 4°C and the pellet was resuspended in extraction solution (isolation solution plus 10 mM EGTA and 0.1% Triton X-100) by sonication, and placed on ice for 30 min. After a 40-min centrifugation (140,000*g*), the supernatant (membrane fraction) was removed and kept at 4°C.

The cytosolic and membrane fractions were assayed for PKC activity as described by Noguchi et al. (1985). The assay solution contained: 25 mM Pipes (pH 7.0), 20 μg/100 μl histone-III, 2.5 μg/100 μl phosphatidylserine, 0.2 μg/100 μl 1,2-dioleoylglycerol, 0.66 mM $CaCl_2$, 10 mM $MgCl_2$, 0.2 mM dithiothreitol, 1 mM PMSF, and 10 μg/ml leupeptin. $CaCl_2$ (2.1 mM) was added to the membrane extract assay solution to neutralize EGTA. $CaCl_2$, phosphatidylserine, and 1,2-dioleoylglycerol were omitted from control solutions and 0.5 mM EGTA was added to obviate potential effects of contaminating lipids (Kikkawa et al., 1983). The final assay volume was 100 μl and contained 10 μl of cytosolic or membrane fraction. The assay was initiated by adding ^{32}P-ATP (10 μl; 15 μM final) and terminated, after 2 min at 30°C, by adding 3 ml ice-cold stop solution (5% TCA; 1.5% Na pyrophosphate; 1% $NaPO_4$). The precipitates were collected on Millipore filters (Type HA, 0.45 μm pore size) and

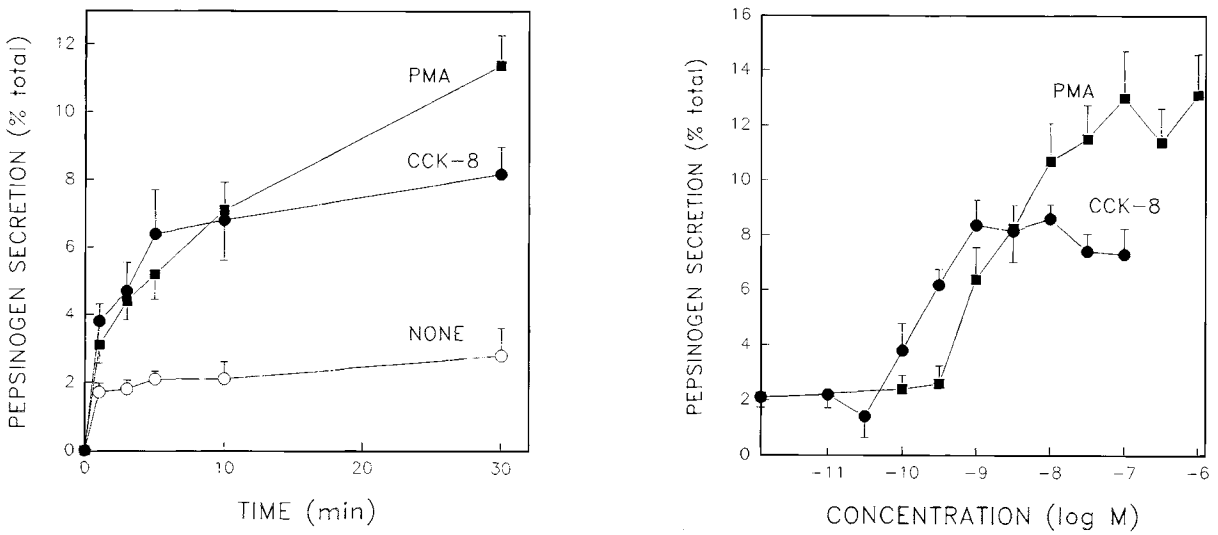

Fig. 1. Effects of CCK-8 and PMA on pepsinogen secretion from dispersed chief cells. **Left:** Time-course. Cells were incubated with 3 nM CCK-8 (closed circles), 100 nM PMA (squares), or no additions (open circles) for the times indicated. **Right:** Dose-response curve. Dispersed chief cells were incubated for 10 min with the indicated concentrations of CCK-8 (circles) or PMA (squares). In each experiment, each value was determined in duplicate and results given are means ± SE from 4 separate experiments.

the tubes and filters were washed 4 times with stop solution. Radioactivity associated with the filters was determined by adding scintillation fluid and counting in a LKB 1211 Rackbeta liquid scintillation counter. Results are expressed as picomoles phosphate transferred to the protein substrate per minute per μg protein. Protein in the cytosolic and membrane fractions was determined using the Bio-Rad (Richmond, CA) method with bovine albumin (fraction V) (Sigma) standards. Using this assay, kinase activity increased 4- to 5-fold compared with basal when calcium and lipids were added.

Pepsinogen Secretion

Pepsinogen secretion was determined as described previously (Raufman et al., 1986) using ^{125}I-albumin as substrate. Values were expressed as the percentage of total cellular pepsinogen that was released into the medium during incubation.

Statistical Analysis

Significance between two means was determined by Student's t-test. Differences between several means were determined by analysis of variance, followed by Dunnett's test or the t test for differences among several means. P values < 0.05 were considered significant.

RESULTS

Effects of CCK-8 and PMA on Pepsinogen Secretion

As seen in Figure 1 (left), CCK-8 (3 nM) and PMA (100 nM) caused a rapid increase in pepsinogen secretion during the first 5 min of incubation. After 5 min the rate of CCK-induced pepsinogen secretion was similar to that from control cells. In contrast, the rate of PMA-induced pepsinogen secretion after 5 min remained greater than control.

The dose-response curves for the effects of PMA and CCK-8 on pepsinogen secretion are also shown in Figure 1 (right). After a 10-min incubation, an increase in secretion was detected with 0.1 and was maximal with 1 nM CCK-8. With PMA, an increase in secretion was detected with 0.3 and was maximal with 100 nM. Maximal PMA-induced secretion (5.5-fold) was greater than that observed with CCK-8 (3-fold) ($P < 0.05$).

Effects of Inhibitors of PKC

Staurosporine, H7, and other purported specific inhibitors of PKC have been used to determine whether or not the actions of an agonist are mediated by this kinase (Blackshear, 1988; Verme et al., 1989). However, in dispersed chief cells, concentrations of staurosporine and H7

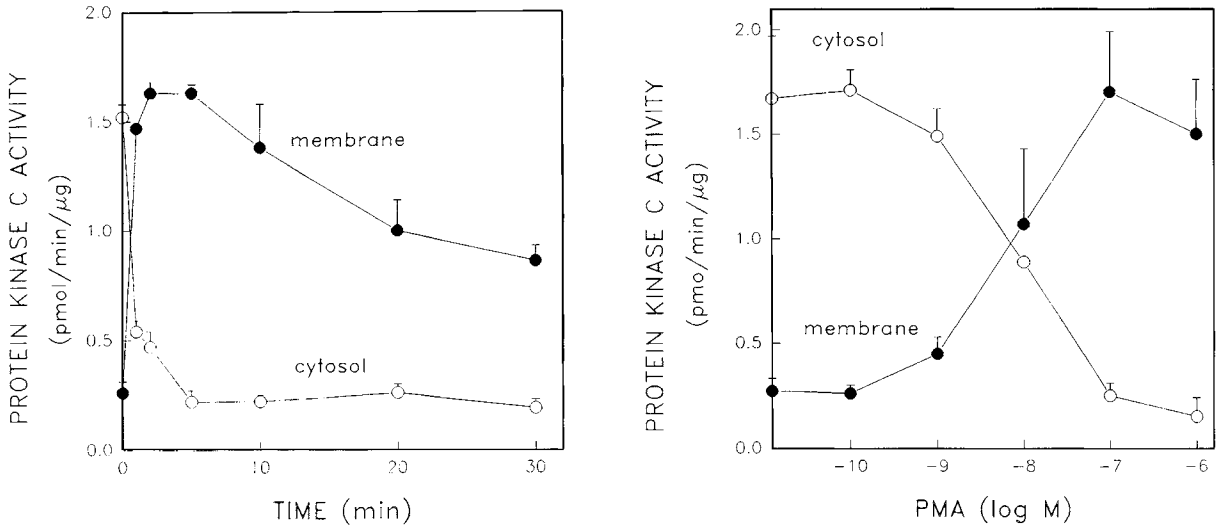

Fig. 2. Actions of PMA on the distribution of chief cell PKC activity. **Left:** Time-course. Chief cells were incubated with PMA (100 nM) for the times indicated. **Right:** Dose-response curve. Chief cells were incubated for 5 min with the indicated concentrations of PMA. Cytosolic (open circles) and membrane-associated (closed circles) PKC activity was determined as described. Results given are means ± SE from 5 separate experiments.

that have been used to inhibit PKC stimulated an increase in pepsinogen secretion. For example, 1 μM staurosporine induced a 2- to 3-fold increase in pepsinogen secretion compared with control cells (results not shown)..Cell activation by these inhibitors has been observed in other systems (Felipo et al., 1990; Ederveen et al., 1990). Also, these inhibitors may interact with cellular mediators other than PKC (Ruegg and Burgess, 1989). Hence, we believe that these agents cannot be used to determine the role of PKC in mediating pepsinogen secretion induced by physiological agonists. Down-regulation of PKC activity following prolonged incubation with phorbol esters, another method of evaluating the role of the kinase in signal transduction (Blackshear, 1988), is not feasible in dispersed chief cells because the secretory response diminishes greatly after 3–4 h (results not shown).

Effect of PMA on Chief Cell PKC Activity

Consequently, we examined PKC activity in chief cell cytosolic and membrane fractions. As shown in Figure 2 (left), within 1 min, 100 nM PMA caused a marked increase in membrane-associated PKC activity. Control values for PKC activity in cytosolic and membrane fractions were 1.52 ± 0.60 and 0.26 ± 0.05 pmol/min/μg protein, respectively. At 5 min, membrane-associated PKC activity in PMA-treated cells increased about 6-fold, whereas cytosolic activity decreased to 0.22 ± 0.05 pmol/min/μg. After 5 min, membrane-associated PKC activity steadily declined, while cytosolic activity remained low.

Maximal membrane-associated PKC activity was observed with 100 nM PMA (Fig. 2, right). The 90% reduction in cytosolic kinase activity observed with 0.1 to 1 μM PMA is further evidence that this assay measures PKC activity. Comparison of Figures 1 and 2 reveals the close relation between the time-courses and dose-response curves for PMA-induced pepsinogen secretion and alterations in the distribution of chief cell PKC activity.

Lack of Effect of CCK on Distribution of Chief Cell PKC Activity

CCK-8 did not alter chief cell distribution of PKC activity at time points from 0.5 to 10 min (Fig. 3, left). As shown in Figure 3 (right), a slight decrease in cytosolic PKC activity was observed with increasing concentrations of CCK-8, but this decrease was significant only at the highest concentration tested (1 μM), a concentration of CCK-8 that is 1,000-fold maximal for pepsinogen secretion (Fig. 1, right). Membrane PKC activity was not altered by any concentration of CCK-8 tested. Likewise, carbachol (0.1 μM to 1.0 mM) did not alter chief cell distribution of PKC activity (results not shown).

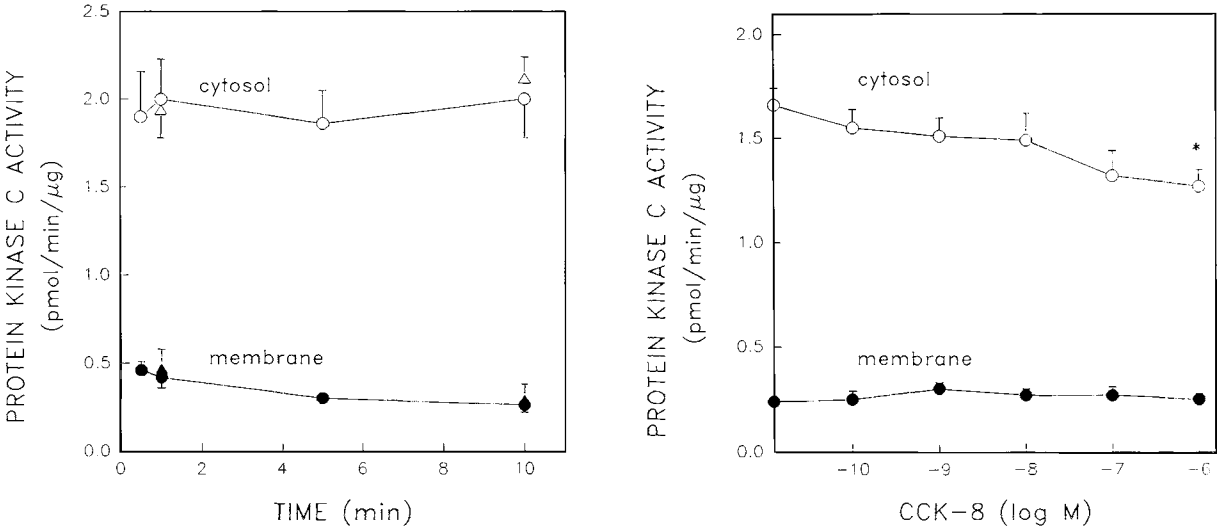

Fig. 3. Lack of effect of CCK-8 on chief cell distribution of PKC activity. **Left:** Time-course. Chief cells were incubated with CCK-8 (3.0 nM) for indicated time. **Right:** Dose-response curve. Chief cells were incubated for 1 min with the indicated concentrations of CCK-8. Cytosolic (open circles) and membrane-associated (closed circles) PKC activity was determined as described. In the time course, cytosolic (open triangles) and membrane-associated (closed triangles) PKC activity was determined in control cells at the times indicated. Results given are means ± SE from 4 separate experiments. *Indicates value that is significantly lower ($P < 0.05$) than the basal value.

Effects of OAG on Pepsinogen Secretion and PKC Activity

It has been suggested that PKC present on the cell membrane may be activated by small amounts of DAG produced under physiological conditions and that activation of this resident enzyme is sufficient to induce or enhance cell activation (Diaz-Guerra and Bosca, 1990). If this is true, then changes in the cellular distribution of PKC activity may not be required for the enzyme to mediate, totally or in part, cell function. Thus, in dispersed chief cells, CCK-induced increases in DAG may activate pre-existing membrane-bound PKC. To address this possibility, we examined the actions of OAG, a membrane-permeant DAG that activates PKC (Kaibuchi et al., 1983), on pepsinogen secretion and membrane-associated PKC activity.

Addition of 250 μM OAG to the cells caused a 3-fold increase in membrane-associated PKC activity, with a concomitant decrease in cytosolic activity, that was maximal by 2 min and began to decrease by 5 min (data not shown). Cells were incubated with 0.1–250 μM OAG for 2 min and membrane-associated PKC activity was determined. OAG-induced pepsinogen secretion was determined after 10-min incubation. As shown in Table I, significant ($P < 0.05$) increases in membrane-associated PKC activity and pepsinogen secretion were observed with OAG concentrations ≥ 1 μM. The lowest concentration of OAG tested (0.1 μM) did not alter chief cell distribution of PKC activity or pepsinogen secretion. The relation between OAG-induced changes in chief cell distribution of PKC activity and pepsinogen secretion is shown in Figure 4. These data indicate that PKC-medi-

TABLE I. Effect of OAG on Pepsinogen Secretion and Membrane-Associated PKC Activity in Dispersed Chief Cells*

OAG added (μM)	Membrane-associated PKC activity (pmol/min/μg)	Pepsinogen secretion (% total)
None	0.38 ± 0.03	2.1 ± 0.2
0.1	0.41 ± 0.05	2.3 ± 0.9
1	0.51 ± 0.03	4.0 ± 0.1
10	0.71 ± 0.04	10.5 ± 0.7
100	1.42 ± 0.17	14.3 ± 0.5
250	1.41 ± 0.18	16.9 ± 1.2

*Cells were incubated with the indicated concentrations of OAG for 2 min (PKC activity) or 10 min (pepsinogen secretion) at 37°C. In each experiment each value was determined in duplicate and results given are means ± SE for at least 3 separate experiments. Values for membrane-associated PKC activity and pepsinogen secretion were significantly greater than basal values with OAG concentrations ≥ 1 μM ($P < 0.05$).

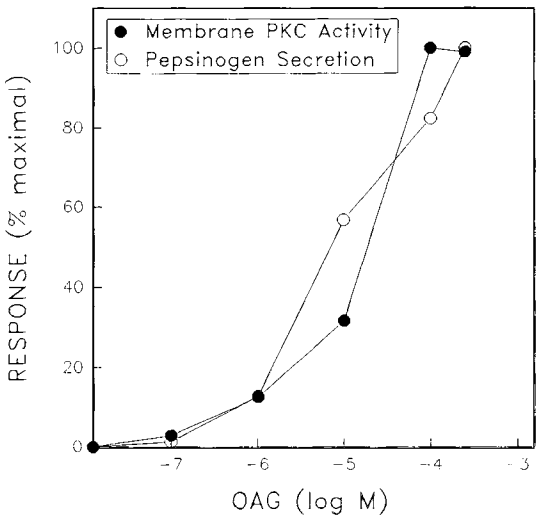

Fig. 4. Comparison of dose-response curves for OAG-induced increases in pepsinogen secretion and membrane-associated PKC activity. Data from Table I were taken and plotted as a percentage of the maximal response for pepsinogen release (open circles) and membrane PKC activity (closed circles).

ated pepsinogen secretion (that is, PMA- or DAG-induced secretion) is consistently accompanied by increases in membrane-associated PKC activity.

DISCUSSION

The results of this study indicate that in gastric chief cells there is a close relation between the abilities of phorbol esters, like PMA, and diacylglycerols, like OAG, to increase membrane-associated PKC activity and the abilities of these agents to stimulate pepsinogen secretion. In contrast, we were unable to detect an increase in membrane-associated PKC activity with a cholinergic agonist or CCK, a secretagogue that has been shown to increase chief cell DAG levels (Nakano et al., 1990).

Hydrolysis of PIP_2 without alteration of the cellular distribution of PKC activity has been observed in other cells. Examples include fibroblasts stimulated with epidermal growth factor (Thompson et al., 1989) and hepatocytes stimulated with vasopressin (Diaz-Guerra et al., 1990). From these observations, and the present study in gastric chief cells, one may conclude that secretagogue-induced phosphatidylinositol hydrolysis and pepsinogen secretion can occur in the absence of a change in the cellular distribution of PKC activity. However, alternative explanations must be considered.

Using phospholipid vesicles, investigators have suggested that secretagogue-induced increases in DAG and calcium may result in the formation of a labile PKC-membrane association (Bazzi and Nelsestuen, 1988a,b). Reversible binding and activation of bovine brain PKC in response to small increases in calcium concentration have been demonstrated, using such vesicles by Bazzi and Nelsestuen (1989). Hence, the failure to detect an increase in membrane-associated PKC activity with secretagogues may result from the disruption of such a labile association by cellular fractionation procedures. Nevertheless, our ability to detect the formation of a stable PKC-membrane association with OAG in chief cells indicates that it is unlikely that carbachol- or CCK-induced DAG production results in a labile PKC-membrane association.

Diaz-Guerra et al. (1990) suggested that activation of pre-existing membrane-bound PKC in some cells is sufficient to alter function. This hypothesis assumes that membrane-associated PKC is inactive under basal conditions and can be activated by small increases in DAG or calcium when the cells are stimulated. Our experiments with OAG in chief cells do not support this hypothesis. With OAG, an increase in pepsinogen secretion occurred only when an increase in membrane-associated PKC activity was detected.

While CCK-8 alone did not alter the distribution of PKC activity, it is possible that the increase in second messengers induced by CCK-8 is sufficient to modulate increases in membrane-associated PKC activity caused by other agents. In fact, adding CCK-8 (3 nM) enhanced the increase in membrane-associated PKC activity caused by a sub-maximal concentration of PMA (10 nM) (data not shown).

Stimulation of dispersed chief cells with calcium ionophores can elicit a secretory response of equal or greater magnitude than that observed with CCK or carbachol (Raufman et al., 1984). Therefore, CCK- or carbachol-stimulated pepsinogen secretion may be mediated largely by calcium-dependent mechanisms involving calmodulin-dependent kinases. For example, in parotid acini and gastric parietal cells, the effects of cholinergic stimulation appear to be mediated primarily by calcium/calmodulin-dependent mechanisms (Schepp et al., 1989; Manganel and Turner, 1990). Further studies are necessary to determine the role, if any, of PKC

in mediating carbachol- and CCK-induced pepsinogen secretion.

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