Carbachol activates protein kinase C in dispersed gastric chief cells

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We used an '*in situ*' kinase assay to examine agonist-induced protein kinase C (PKC) activation in dispersed chief cells from guinea-pig stomach. Phorbol 12-myristate 13-acetate (PMA), a phorbol ester, and carbamoylcholine, a cholinergic agent, caused a 4- and 3-fold increase in pepsinogen secretion from dispersed chief cells respectively. Whereas PMA caused a rapid 3-fold increase in peptide kinase activity, carbachol caused a 15% increase in activity that was inhibited by the PKC inhibitor, CGP 41 251. Concentrations of carbamoylcholine and a Ca^{2+} ionophore that were sub-maximal for stimulation of pepsinogen secretion did not cause PKC activation. These results indicate that, in the absence of PKC activation, other mechanisms, most likely involving changes in cellular Ca^{2+}, are sufficient to stimulate pepsinogen secretion. Nevertheless, carbamoylcholine stimulated maximal secretion of pepsinogen only at concentrations that also resulted in activation of PKC. Moreover, these data indicate that relatively small increases in PKC activity (5–10%) can stimulate pepsinogen secretion from dispersed chief cells.

INTRODUCTION

In gastric chief cells, at least two major cellular pathways mediate agonist-induced pepsinogen secretion (see [1] for review). Interaction of agents such as secretin, vasoactive intestinal peptide, prostaglandins or cholera toxin with cell membrane receptors causes activation of adenylate cyclase, an increase in cellular cyclic AMP, and activation of cyclic-AMP-dependent protein kinases [1]. Interaction of carbamoylcholine (carbachol), cholecystokinin or gastrin with cell membrane receptors causes activation of phospholipase C, production of inositol trisphosphate and a subsequent rise in cytosolic Ca^{2+} [2,3]. Agonist-receptor-mediated activation of phospholipase C also results in the formation of diacylglycerol, an activator of protein kinase C (PKC) [4]. Hence it has been suggested that activation of PKC may contribute to agonist-induced pepsinogen secretion.

Several lines of evidence suggest a role for PKC in agonist-induced secretion. Activators of PKC, such as phorbol esters or membrane-permeant diacylglycerol analogues, cause redistribution of PKC activity to the membrane and stimulate pepsinogen secretion from dispersed chief cells [5]. Moreover, phorbol 12-myristate 13-acetate (PMA) potentiates Ca^{2+}-dependent secretion from permeabilized chief cells [6] and causes an increase in phosphorylation of a 72 kDa chief-cell phosphoprotein [7]. Recently, using immunoblotting, we detected a conventional (α) [8] and a novel (ζ) (R. D. Raffaniello and J.-P. Raufman, unpublished work) PKC isoform in chief-cell lysates. Nevertheless, activation of PKC by carbachol has not been demonstrated in chief cells, and the possibility exists that the secretory response elicited by this agent is mediated exclusively by Ca^{2+}-dependent mechanisms.

In the present study, agonist-induced PKC activation was examined by an '*in situ*' kinase assay. This assay employs a PKC-specific peptide substrate, and does not require harsh extraction procedures that may alter kinase activity or distribution [9,10]. Our results indicate that, although both carbachol and PMA activate PKC in dispersed chief cells, the effects of these agents on kinase activity are distinct.

MATERIALS AND METHODS

Male Hartley guinea pigs (150–200 g) were obtained from CAMM Research Lab Animals (Wayne, NJ, U.S.A.); collagenase (type I), BSA (fraction V), carbachol, EGTA, PMA, leupeptin and ATP were from Sigma; basal medium (Eagle) amino acids, PKC-(19–31)Ser^{25} and essential vitamin solution were from Grand Island Biological, Grand Island, NY, U.S.A.; Percoll was from Pharmacia; ^{125}I-albumin was from ICN; [^{32}P]ATP was from New England Nuclear.

Tissue preparation

Dispersed chief cells from guinea-pig stomach were prepared as described previously [11]. The standard incubation solution was equilibrated with 100% O_2, and all incubations were performed with 100% O_2 as the gas phase.

Pepsinogen secretion

Peptic activity was determined as described previously [12], with ^{125}I-albumin as substrate. Pepsinogen secretion was expressed as the percentage of total cellular pepsinogen at the start of the incubation that was released into the medium during the incubation.

'*In situ*' kinase assay

To measure agonist-induced kinase activity, dispersed chief cells (100000 cells/0.5 ml) were incubated with or without a specific agent, and the incubation was terminated by centrifugation at 10000 ***g*** for 15 s and immediately placing the tubes in an ice/water bath. The incubation medium was rapidly aspirated and the pellets were kept on ice until assayed for PKC activity (usually within 10 min).

The pellets were assayed for PKC activity with 40 μl of assay buffer, which consisted of the following: 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM K_2HPO_4, 50 μg/ml digitonin, 10 mM $MgCl_2$, 20 mM Hepes (pH 7.0), 25 mM β-glycerophosphate, 100 μM [^{32}P]ATP, 5 mM EGTA, 2.5 mM $CaCl_2$

Abbreviations used: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.
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and 100 μM PKC-(19–31)Ser^{25} as substrate. Pilot studies were performed to determine the optimal assay conditions with respect to chief-cell, digitonin and peptide substrate concentrations. Kinase reactions were allowed to proceed at 30 °C for 6 min, after which samples were spotted on 2 cm × 2 cm phosphocellulose strips. The strips were washed in two changes of 1% H_3PO_4 and distilled water for 5 min each, then placed in minivials with 5 ml of scintillation cocktail and counted for radioactivity. Endogenous phosphorylation in the absence of the peptide substrate was unaltered by agonists and was subtracted from all measurements. In a representative experiment, endogenous phosphorylation resulted in the retention of 6227 c.p.m. on the phosphocellulose strips, whereas in the presence of peptide substrate (no agonist added) 47511 c.p.m. was retained on the strips.

Statistics

Significance between two means was determined by Student's t test or the Mann and Whitney test (U-test); $P < 0.05$ was considered significant.

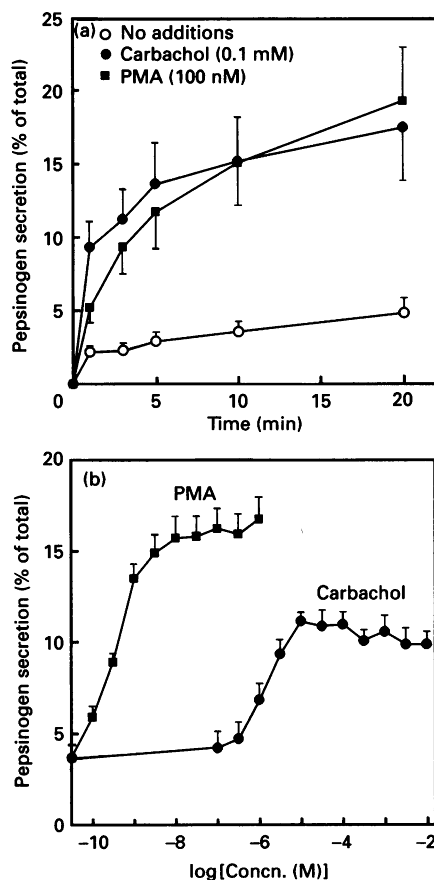

Figure 1 (a) Time course for carbachol- and PMA-induced pepsinogen secretion from dispersed chief cells, and (b) dose–response for carbachol- and PMA-induced pepsinogen secretion

(a) Chief cells were incubated with 0.1 mM carbachol (●), 100 nM PMA (■) or no additions (○) for the times indicated, and pepsinogen release was determined. (b) Chief cells were incubated with the indicated concentration of carbachol (●) or PMA (■) for 20 min, and pepsinogen release was determined. In each experiment, each value was determined in duplicate, and results given are means ± S.E.M. for at least three experiments.

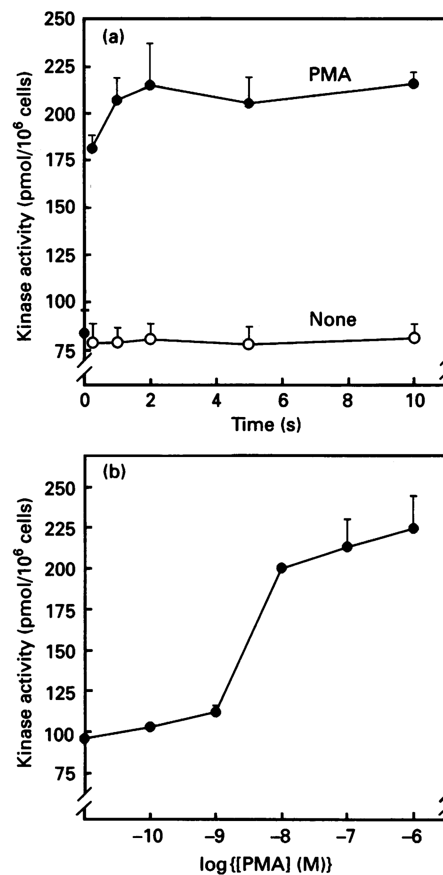

Figure 2 Effect of PMA on peptide kinase activity in dispersed chief cells

(a) Time course. Chief cells were incubated with PMA (100 nM) (●) or no additions (○) for the times indicated. (b) Dose–response curve. Chief cells were incubated for 2 min with the indicated concentrations of PMA. Peptide kinase activity was determined as described in the Materials and methods section. In each experiment, each value was determined in duplicate, and results given are means ± S.E.M. for at least three experiments.

RESULTS AND DISCUSSION

Carbachol- and PMA-induced pepsinogen secretion from dispersed chief cells

As shown in Figure 1(a), carbachol (0.1 mM) and PMA (100 nM) caused a rapid increase in pepsinogen secretion. After 5 min, the rate of carbachol-induced secretion was not different from basal. In contrast, the rate of PMA-induced secretion declined after 5 min, but remained slightly greater than basal. After a 20 min incubation, carbachol-induced secretion was detected with 1 μM and was maximal with 10 μM of the cholinergic agonist (Figure 1b). PMA-induced secretion was observed with 0.1 nM and was maximal with 100 nM phorbol ester (Figure 1b).

Effect of PMA on peptide kinase activity in dispersed chief cells

Agonist-induced activation of PKC was examined with an '*in situ*' kinase assay that employs a PKC-specific peptide substrate. As shown in Figure 2(a), PMA (100 nM) caused a rapid increase in peptide phosphorylation. Maximal peptide phosphorylation with PMA (3-fold) was observed by 1 min, and remained elevated throughout the incubation. A modest increase in peptide phosphorylation was observed with 1 nM PMA, and higher concentrations ($\geqslant$ 10 nM) caused a 2–3-fold increase (Figure 2b).

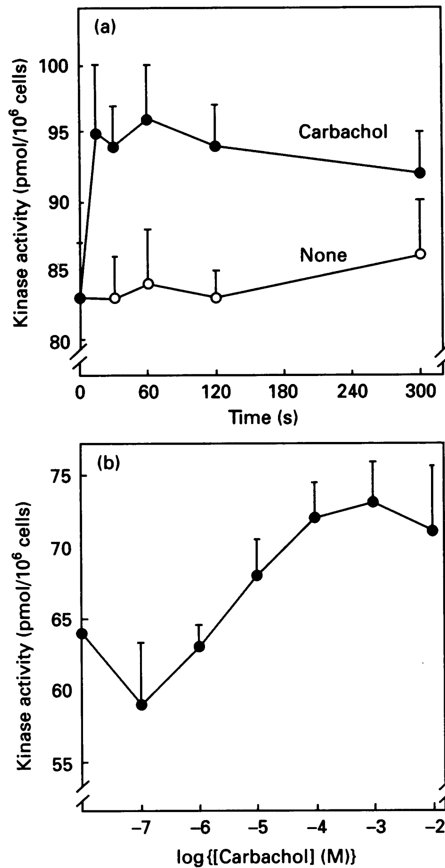

Figure 3 Effect of carbachol on peptide kinase activity in dispersed chief cells

(**a**) Time course. Chief cells were incubated with carbachol (0.1 mM) (●) or no additions (○) for the times indicated. (**b**) Dose–response curve. Chief cells were incubated for 30 s with the indicated concentrations of carbachol. Peptide kinase activity was determined as described in the Materials and methods section. In each experiment, each value was determined in duplicate, and results given are means ± S.E.M. for three to five experiments.

Table 1 Effect of kinase inhibitors on carbachol-induced peptide kinase activity

Chief cells were incubated without or with 0.1 mM carbachol for 1 min, then assayed for peptide kinase activity in the presence of 1 μM CGP 41 251, 3 μM KN-62 or no additions. Values represent carbachol-induced kinase activity minus basal activity. Results given are means ± S.E.M. for at least three experiments: *indicates that the value in the presence of CGP 41 251 was significantly ($P < 0.05$) less than that observed in the absence of the inhibitor.

Inhibitor	Peptide kinase activity (pmol/10^6 cells)
None	15.2 ± 2.1
CGP 41 251 (1 μM)	1.3 ± 0.9*
KN-62 (3 μM)	14.8 ± 3.9

Effect of carbachol on peptide kinase activity in dispersed chief cells

As shown in Figure 3(a), carbachol (0.1 mM) caused a small increase in peptide phosphorylation that was maximal by 15 s

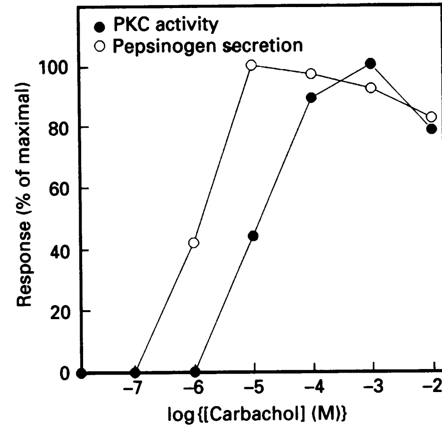

Figure 4 Comparison of dose–response curves for carbachol-induced increases in pepsinogen secretion and kinase activation

Data from Figures 1(b) and 3(b) were plotted as a percentage of the maximal response for pepsinogen secretion (○) and kinase activation (●).

and remained elevated above basal throughout the incubation. Carbachol-induced peptide phosphorylation was dose-dependent and was significantly ($P < 0.05$) greater than basal with concentrations $\geqslant$ 0.1 mM (Figure 3b). Maximal peptide phoshorylation was observed with 1.0 mM carbachol (15% greater than basal) and was a fraction of that observed with PMA. This is not particularly surprising, since carbachol-induced activation of PKC is dependent on small, transient, changes in diacylglycerol and Ca^{2+} [2,4], whereas PMA is a pharmacological activator of PKC that is not degraded in the cell [13,14]. Moreover, a modest increase in PKC activation appears sufficient to stimulate pepsinogen secretion. PKC activation with 1 nM PMA was only 17% greater than basal (Figure 2b) (comparable with that observed with maximal concentrations of carbachol). Nonetheless, 1 nM PMA stimulated a 3-fold increase in pepsinogen secretion (Figure 1b).

Effect of protein kinase inhibitors on carbachol-induced peptide phosphorylation

To ensure that the peptide kinase activity being measured was attributable to PKC, cell pellets were assayed for peptide kinase activity after treatment with or without carbachol (0.1 mM) for 1 min, in the absence or presence of CGP 41 251 (1 μM), a specific PKC inhibitor [15], or KN-62 (3 μM), an inhibitor of Ca^{2+}/calmodulin kinase II [16]. In the absence of kinase inhibitors, carbachol treatment resulted in an increase in peptide phosphorylation (Table 1). This increase was not altered by the Ca^{2+}/calmodulin kinase II inhibitor. However, carbachol-induced increases in peptide kinase activity were almost totally abolished by the PKC inhibitor CGP 41 251 (Table 1). Moreover, CGP 41 251 inhibited basal peptide kinase activity by nearly 80%, whereas KN-62 was without effect (results not shown). These data indicate that the carbachol-induced peptide phosphorylation observed in these experiments can be attributed to PKC activation.

Relationship between carbachol-induced PKC activation and pepsinogen secretion

To examine the relationship between carbachol-induced kinase activation and pepsinogen secretion, the dose–response curves

Table 2 Effect of sub-maximal concentrations of PMA, carbachol and A23187 on peptide kinase activity and pepsinogen secretion

Chief cells were incubated with the indicated concentrations of the agonists for 1 min for peptide kinase activity or for 10 min to determine pepsinogen secretion. Peptide kinase activity and pepsinogen secretion were determined as described in the Materials and methods section. Values for peptide kinase activity are representative of three experiments. Values for pepsinogen secretion represent means ± S.E.M. for four separate experiments: * indicates values that are significantly greater ($P < 0.05$) than basal.

Additions	Peptide kinase activity (pmol/10^6 cells)	Pepsinogen secretion (% of total)
None	83	3.7 ± 0.4
PMA (0.01 nM)	83	3.8 ± 0.4
PMA (0.3 nM)	87	10.3 ± 0.8*
Carbachol (1 μM)	79	8.4 ± 1.4*
Carbachol (10 μM)	89	14.0 ± 1.8*
A23187 (1 nM)	80	3.4 ± 0.3
A23187 (10 nM)	82	12.1 ± 1.9*

for these events were expressed as a function of maximal response. As shown in Figure 4, a sub-maximal concentration of carbachol (1 μm) stimulated pepsinogen secretion independently of kinase activation, suggesting that PKC activation is not obligatory for carbachol-induced secretion.

To test the hypothesis that sub-maximal pepsinogen secretion with carbachol is independent of PKC activation and that other mechanisms mediate secretion, cells were incubated with sub-maximal concentrations of PMA, carbachol and the Ca^{2+} ionophore A23187. Previous studies from this laboratory indicate that 10 nM A23187 causes a 2-fold increase in chief-cell Ca^{2+} concentration [12]. As shown in Table 2, concentrations of PMA that do not activate PKC do not induce secretion. That is, 0.01 nM PMA did not alter peptide kinase activity or pepsinogen secretion, whereas 0.3 nM PMA, which causes a small increase in kinase activity ($P < 0.05$, Mann and Whitney test, $n = 3$ experiments), caused a 3-fold increase in secretion, indicating that PMA-induced pepsinogen secretion is dependent on activation of PKC. In contrast, 1 μM carbachol did not alter peptide kinase activity, but caused a 2-fold increase in secretion. These data indicate that other mechanisms, most likely involving Ca^{2+}, mediate secretion at sub-maximal concentrations. However, as shown by the results with 10 μM carbachol, the maximal secretory response elicited by carbachol is observed only at concentrations that also activate PKC ($P < 0.05$, Mann and Whitney test, $n = 3$ experiments). Moreover, the results with the Ca^{2+} ionophore indicate that changes in chief-cell Ca^{2+} concentration are sufficient to stimulate pepsinogen secretion in the absence of PKC activation (Table 2).

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Previous studies have suggested a modulatory role for PKC in regulating Ca^{2+}-induced secretion. For example, Ca^{2+}-induced catecholamine release from adrenal chromaffin cells is enhanced by phorbol-ester-induced activation of PKC [17]. However, PKC activation alone does not induce secretion from chromaffin cells [17], whereas agents that activate PKC, such as phorbol esters and diacylglycerol analogues, are among the most efficacious effectors of pepsinogen secretion [5]. In pancreatic acini, down-regulation of PKC [18] or preincubation with an inhibitor of PKC [19] resulted in a nearly 50 % decrease in carbachol-induced amylase secretion. These data suggest that, although PKC activation is not required for agonist-induced secretion, the kinase plays an important modulatory role and may be necessary for maximal secretion.

In a previous study using permeabilized gastric chief cells, we observed that PKC activators potentiated Ca^{2+}-induced secretion [6]. Likewise, in intact chief cells, carbachol-induced PKC activation may potentiate the effects of increased cytosolic Ca^{2+}, resulting in the observed maximal secretory response. Studies are underway to determine the relative importance of the two different PKC isoforms with respect to agonist-induced activation and secretion.

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