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Expression and Phosphorylation of a MARCKS-Like Protein in Gastric Chief Cells: Further Evidence for Modulation of Pepsinogen Secretion by Interaction of Ca^{2+}/Calmodulin With Protein Kinase C

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Abstract In gastric chief cells, agents that activate protein kinase C (PKC) stimulate pepsinogen secretion and phosphorylation of an acidic 72-kDa protein. The isoelectric point and molecular mass of this protein are similar to those for a common PKC substrate; the MARCKS (for Myristoylated Alanine-Rich C Kinase Substrate) protein. We examined expression and phosphorylation of the MARCKS-like protein in a nearly homogeneous suspension of chief cells from guinea pig stomach. Western blotting of fractions from chief cell lysates with a specific MARCKS antibody resulted in staining of a myristoylated 72-kDa protein (pp72), associated predominantly with the membrane fraction. Using permeabilized chief cells, we examined the effect of PKC activation (with the phorbol ester PMA), in the presence of basal (100 nM) or elevated cellular calcium (1 μM), on pepsinogen secretion and phosphorylation of the 72-kDa MARCKS-like protein. Secretion was increased 2.3-, 2.6-, and 4.5-fold by incubation with 100 nM PMA, 1 μM calcium, and PMA plus calcium, respectively. A PKC inhibitor (1 μM CGP 41 251) abolished PMA-induced secretion, but did not alter calcium-induced secretion. This indicates that calcium-induced secretion is independent of PKC activation. Chief cell proteins were labeled with ^{32}P-orthophosphate and phosphorylation of pp72 was detected by autoradiography of 2-dimensional polyacrylamide gels. In the presence of basal calcium, PMA (100 nM) caused a $>$ two-fold increase in phosphorylation of pp72. Without PMA, calcium did not alter phosphorylation of pp72. However, 1 μM calcium caused an approx. 50% attenuation of PMA-induced phosphorylation of pp72. Experiments with a MARCKS "phosphorylation/calmodulin binding domain peptide" indicated that calcium/calmodulin inhibits phosphorylation of pp72 by binding to the phosphorylation/calmodulin binding domain and not by inhibiting PKC activity. These observations support the hypothesis that, in gastric chief cells, interplay between calcium/calmodulin binding and phosphorylation of a common domain on the 72-kDa MARCKS-like protein plays a role in modulating pepsinogen secretion. J. Cell. Biochem. 64:514–523.

Key words: signal transduction; stomach; hormones; phospholipase C

Pepsinogen secretion from gastric chief cells is mediated by two major signal transduction pathways [see Raufman, 1992a for review]. In one pathway, agents like secretin and vasoactive intestinal peptide (VIP) interact with specific cell membrane receptors, thereby resulting in activation of adenylyl cyclase, an increase in cellular cAMP and activation of cAMP-dependent protein kinase A (PKA). In the other pathway, agents like carbamylcholine (carbachol) and cholecystokinin (CCK) interact with specific cell membrane receptors, thereby causing activation of phospholipase C. Hydrolysis of phosphoinositol bisphosphate by phospholipase C results in the formation of inositol trisphosphate (IP_3) and diacylglycerol (DAG). Increases in cellular IP_3 stimulate the release of calcium from intracellular stores, thereby activating calcium/calmodulin kinase-II (Ca^{2+}/CM kinase-II). The increase in DAG causes activation of protein kinase C (PKC), a calcium- and phospholipid-dependent enzyme.

The importance of activation of PKA and Ca^{2+}/CM kinase-II in mediating secretagogue-

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induced pepsinogen secretion is reasonably well substantiated [Raufman, 1992a]. In contrast, the role of PKC in this process is less clear. In fact, some investigators have questioned whether the role of PKC in cellular signalling has been over-emphasized [Wilkinson and Hallam, 1994]. Nevertheless, it has been known for a decade that phorbol esters, like phorbol 12-myristate 13-acetate (PMA), that activate PKC, stimulate an increase in pepsinogen secretion [Sakamoto et al., 1985; Matsumoto et al., 1987]. Moreover, studies in dispersed chief cells have shown that PMA and a membrane-permeable DAG [1-oleoyl-2-acetylglycerol (OAG)] increase membrane-associated PKC activity [Raffaniello and Raufman, 1992a]; that PMA augments cAMP levels and pepsinogen secretion in cholera toxin-treated cells [Raufman, 1992b]; that PMA and OAG stimulate a calcium-independent increase in pepsinogen secretion from permeabilized chief cells [Raffaniello and Raufman, 1992b]; and, that PMA causes rapid in situ phosphorylation of a PKC-specific peptide substrate [Raffaniello and Raufman, 1994a]. More recently, we used quantitative immunoblotting to show that guinea pig chief cells express α and ζ isoforms of PKC, and that PMA causes an increase in membrane-associated α PKC [Raffaniello and Raufman, 1994b].

Whereas these findings indicate that activation of PKC by diacylglycerol or phorbol esters stimulates pepsinogen secretion, there is a paucity of evidence that PKC plays a role in mediating secretion caused by physiological secretagogues like cholinergic agonists or CCK. In studies evaluating in situ phosphorylation of a PKC substrate [Raffaniello and Raufman, 1994a] and translocation of the α isoform of PKC [Raffaniello and Raufman, 1994b], a 15% increase in PKC activity and a 33% increase in membrane-associated α PKC, respectively, were observed with concentrations of carbachol that are supramaximal for pepsinogen secretion. Nevertheless, activation of PKC and translocation of the α isoform could not be detected with concentrations of carbachol that are submaximal for pepsinogen secretion [Raffaniello and Raufman, 1994a,b]. These results suggest that PKC plays a modulatory, but not obligatory, role in secretagogue-induced pepsinogen secretion.

Evidence from many studies indicates that the MARCKS (for **M**yristoylated **A**lanine-**R**ich **C K**inase **S**ubstrate) protein is a major target of phosphorylation by PKC [see Blackshear, 1993, for review]. Little is known about the function of MARCKS or the role of its phosphorylation by PKC. Nevertheless, it has been suggested that binding of calmodulin to MARCKS may regulate interaction between the Ca^{2+}/calmodulin and PKC signalling systems [MacNicol and Schulman, 1992].

In the course of examining the effects of nonhydrolyzable guanine nucleotide analogues on the regulation of pepsinogen secretion from permeabilized gastric chief cells, we observed that PMA and GTPγS stimulate phosphorylation of a 72-kDa acidic protein (pp72) [Raffaniello and Raufman, 1993]. Moreover, phosphorylation of this protein was inhibited by CGP 41 251, an inhibitor of protein kinase C [Raffaniello and Raufman, 1993; Meyer et al., 1989]. Because pp72 serves as a substrate for protein kinase C and has an appropriate molecular mass, we hypothesized that this was a MARCKS-like protein.

In the present study, we present immunological and biochemical evidence that pp72 in gastric chief cells is a MARCKS-like protein. Moreover, we demonstrate that calcium inhibits PKC-mediated phosphorylation of pp72 without affecting the activity of PKC for other substrates. Hence, as with other MARCKS proteins [Hinrichsen and Blackshear, 1993; Chakravarthy et al., 1995a], PKC-induced phosphorylation and Ca^{2+}/calmodulin binding appear to compete for similar domains on pp72. These results support the hypothesis that, in gastric chief cells, pp72 is a MARCKS-like protein that may function as a calmodulin buffer under the control of PKC.

MATERIALS AND METHODS

Male Hartley guinea pigs (150–200 g) were obtained from CAMM Research Lab Animals, Wayne, NJ; HEPES from Boehringer-Mannheim Biochemicals, Indianapolis, IN; Percoll from Pharmacia, Gaithersburg, MD; collagenase (type I), leupeptin, bovine albumin (fraction V) (BSA), phorbol 12-myristate 13-acetate (PMA), EDTA, and EGTA from Sigma, St. Louis, MO; basal medium (Eagle's) amino acids (100-times concentrated), and essential vitamin solution (100-times concentrated) from GIBCO, Burlington, Ontario; ^{125}I-albumin from ICN; [^{32}P]ATP and [^{3}H]myristic acid from Du Pont-NEN. All other chemicals were of the highest purity commercially available. CGP 41251

[Meyer et al., 1989], a protein kinase C inhibitor, was a gift from Ciba-Geigy (Basel, Switzerland). The rabbit polyclonal antibody to bovine brain MARCKS [Aderem et al., 1988] was a gift from Dr. Alan Aderem (Rockefeller University, NY). The PKC pseudo-substrate peptide was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). The MARCKS phosphorylation/calmodulin binding domain peptide was obtained from Boehringer-Mannheim.

Preparation of Dispersed Chief Cells

Unless stated otherwise, 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 pyruvate, 5 mM Na fumarate, 5 mM Na glutamate, 1.5 mM $CaCl_2$, 2 mM glutamine, 0.1% (w/v) BSA, 1% (v/v) amino acid mixture and 1% (v/v) 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. Dispersed chief cells from guinea pig stomach were prepared by mucosal digestion with collagenase and cell fractionation on a Percoll density gradient as described previously [Raufman et al., 1984] and suspended in standard incubation solution. The composition of the cell suspension was originally determined by immunocytochemical techniques [Raufman et al., 1984] and was monitored daily by light microscopy using morphological criteria [Raufman et al., 1984]. In this preparation, chief cells constitute $>$ 90% of the total cell population and trypan blue exclusion is $>$ 95% [Raufman et al., 1984].

Cell Permeabilization

Dispersed chief cells, prepared as above, were permeabilized using streptolysin O (SLO) as described previously [Raffaniello and Raufman, 1992b]. Briefly, cells were washed and resuspended three times in permeabilization solution consisting of 120 mM KCl, 30 mM NaCl, 1 mM $MgCl_2$, 1 mM K_2HPO_4, 10 mM PIPES (pH 7.0), 1 mM ATP, and 1 mg/ml BSA. SLO (30 IU/ml), calcium and other agonists were added simultaneously. Free calcium concentration was adjusted using an EGTA:$CaCl_2$ buffering system [Fabiato and Fabiato, 1979].

Pepsinogen Secretion

Peptic activity was determined as described previously [Raufman et al., 1986] using ^{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. The assay was linear over a range that was at least two-fold that of the maximal value assayed.

Preparation of Subcellular Fractions

For identification and examination of the subcellular distribution of the MARCKS protein, chief cells (10^7 cells/ml) were resuspended in 50 mM β-glycerophosphate (pH 7.0), 150 mM NaCl, 1 mM EGTA, 0.3 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The cells were sonicated (3 x 10-sec bursts) and centrifuged at 100,000*g* for 40 min. The resulting supernatant represented the cytosolic fraction. Membrane proteins were extracted by resuspending the pellet in 50 mM β-glycerophosphate, 0.3 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.3% Triton X-100. After 30 min at 4°C, the extracts were centrifuged at 100,000*g* for 40 min. The supernatant represented the membrane fraction.

Western Blot and Immunostaining

Subcellular fractions were electrophoresed on SDS/8% polyacrylamide gels and the separated proteins were electroblotted to nitrocellulose membranes (Schleicher & Schnell, Keene, NH). Nitrocellulose membranes were incubated with 1% BSA in TBST [50 mM Tris (pH 7.5), 0.15 M NaCl, 0.05% Tween-20] for $>$ 2 h to block nonspecific binding. The blots were incubated overnight with TBST and BSA that included 2–5 μg/ml MARCKS antibody. Blots were washed three times for 30 min with TBST and BSA and incubated with alkaline phosphatase-conjugated anti-rabbit IgG. Following extensive washing with TBST, bands were visualized with developing solution [0.1 mM Tris (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$, 0.4 mg/ml nitroblue tetrazolium, and 0.2 mg/ml 5-bromo-4-chloro-3-indolylphosphate].

Myristoylation of Chief Cell Proteins

Chief cells (8 x 10^6 cells/ml) were incubated for 4 h with 400 μCi [^{3}H]myristic acid (10 nmol/ml). Incubations were stopped by adding ice-cold PBS containing 1 mM EDTA. The cells were washed three times with PBS and centrifuged at 10,000*g* for 2 min. Cell pellets were

resuspended in 0.5 ml of lysis buffer [50 mM glycerophosphate (pH 7.0), 1 mM EGTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin; 1 mM PMSF], mixed with 0.5 ml 2X SDS electrophoresis-sample buffer and boiled for 3 min. Samples (75 μl) were applied to 12% SDS polyacrylamide gels. Detection was by exposure to Kodak X-Omat film at -70°C for 2 months.

Phosphorylation of Proteins in Permeabilized Chief Cells

Cells that had been washed and resuspended in phosphate-free incubation solution were incubated with [^{32}P]-ortho phosphoric acid (0.5 mCi/ml) for 60 min at 37°C. The ^{32}P-loaded cells were permeabilized with SLO in the presence of calcium (0.1 or 1 μM) and/or PMA (0.1 μM) for 2 min at 37°C. The cell suspension was placed on ice and adjusted to 2% Nonidet P-40, 9.5 M urea, 5% β-mercaptoethanol, and 2% IsoLytes (consisting of 1.6% of 3–7 and 0.4% of 3–10 IsoLytes). Aliquots were subjected to isoelectric focusing (3-mm tube diameter) followed by SDS-polyacrylamide (12%) gel electrophoresis according to O'Farrell [1975]. Gels were fixed, dried, and exposed for 3—10 days to Kodak X-Omat film. The pH gradient of the isoelectric focusing gels was determined by placing segments of the tube gels in 2 ml of water for 3–4 h with constant agitation. Phosphorylation was measured using densitometry. Phosphoproteins that were not altered by agonists were used as reference standards for proteins that clearly underwent agonist-dependent changes in phosphorylation.

Effect of Calcium/Calmodulin on the Phosphorylation of Peptide Substrates by Chief Cell Lysates

PKC activity was determined using peptide substrates corresponding to the MARCKS phosphorylation/calmodulin-binding domain or the PKC pseudosubstrate domain. The peptides were preincubated without additions, or with calcium (0.25 mM) plus calmodulin (1 μg/ml) for 15 min before adding cell lysates. Chief cell lysates were assayed for peptide kinase activity in the presence or absence of phosphatidylserine (25 μg/ml), PMA (0.1 μM), and calcium (0.25 mM). The reaction was initiated by adding 10 μl lysate to 50 μl assay solution [30 mM Pipes (pH 7.5), 1 mM $MgCl_2$, 0.2 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μM ^{32}P-ATP, and 2 μM peptide substrate] and incubating the samples for 6 min at 30°C. Aliquots were spotted to P-81 cellulose ion-exchange chromotography paper, washed twice with 1% phosphoric acid and water and counted by liquid scintillation. To account for phosphorylation of endogenous proteins by PKC, assays were performed in the absence of peptide substrates and resulting values were subtracted from those obtained in the presence of substrate.

Statistics

Statistical evaluation was performed using Student's *t*-test on paired values, or ANOVA followed by Dunnett's test when comparing multiple values to a single control. P values < 0.05 were considered significant.

RESULTS

Effects of PMA and Calcium on Pepsinogen Secretion From Permeabilized Chief Cells

As reported previously [Raffaniello and Raufman, 1992], increasing ambient calcium > 300 nM caused a progressive increase in pepsinogen secretion (Fig. 1A). This observation is consistent with studies indicating that basal chief cell calcium concentration is approximately 100 nM [Raufman et al., 1986]. Adding 100 nM PMA to increasing concentrations of calcium augmented pepsinogen secretion (Fig. 1A). To determine that the actions of calcium alone were independent of activation of PKC, we examined the actions of an inhibitor of PKC, CGP 41 251 [Meyer et al., 1989]. As shown in Figure 1B, whereas the addition of 1 μM CGP 41 251 did not alter calcium-induced pepsinogen secretion, the inhibitor abolished PMA-induced secretion. Moreover, when tested with the combination of calcium plus PMA, the PKC inhibitor appeared to abolish that component of the secretory response due to activation of PKC. These results indicate that although increases in cellular calcium and activation of PKC result in augmentation of pepsinogen secretion, the contributions of these separate pathways can be distinguished by using appropriate inhibitors.

Expression and Myristoylation of the MARCKS-Like Protein in Gastric Chief Cells

As shown in Figure 2A, immunoblotting of chief cell proteins with a specific antibody to bovine brain MARCKS revealed a 72-kDa chief cell protein. This 72-kDa protein is associated with membrane fractions (Fig. 2A). Incubation

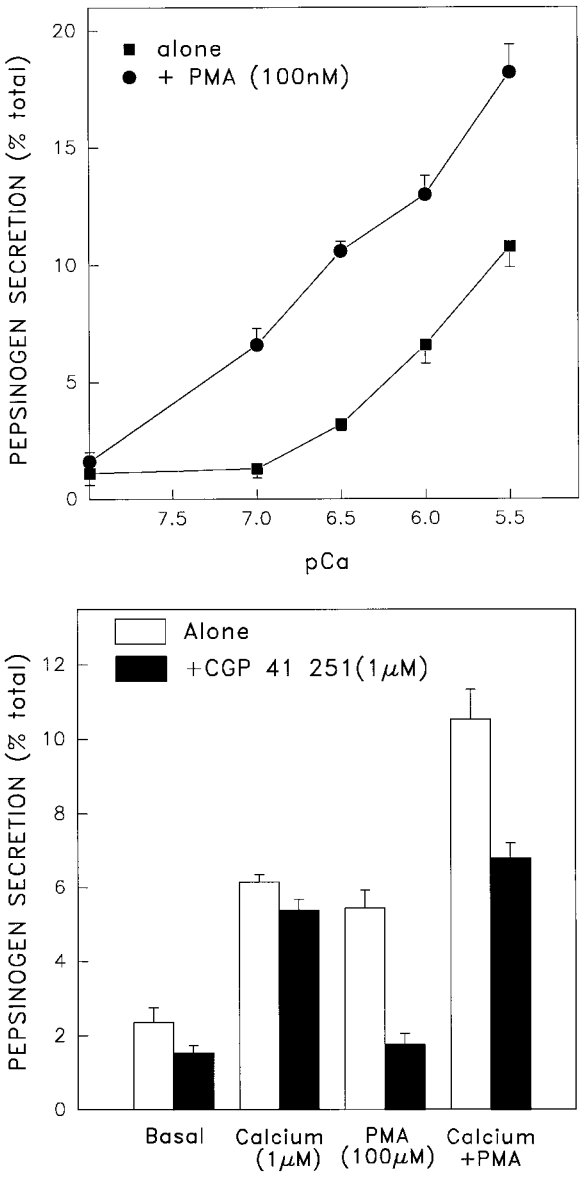

Fig. 1. Interaction of a phorbol ester and calcium on pepsinogen secretion from permeabilized chief cells. above: Effect of increasing concentrations of calcium, alone or in combination with 100 nM PMA, on secretion. below: Effect of an inhibitor of protein kinase C, CGP 41 251, on calcium- and PMA-induced secretion. Dispersed chief cells were permeabilized in the presence of calcium and the agents indicated at 37°C for 10 min. Values for pepsinogen secretion are expressed as the percent of total cellular pepsinogen present at the start of the incubation that was released into the medium after 10 min. In each experiment, values were determined in duplicate and represent mean ± S.E. from at least three separate experiments.

of chief cells with [^{3}H]myristic acid and separation on SDS-PAGE, revealed several myristoylated proteins (Fig. 2B). One of these proteins had a calculated molecular mass of 72 kDa, similar to the protein demonstrated by immunoblotting. Hence, the data shown in Figure 2 support the conclusion that the 72-kDa protein (pp72) expressed by gastric chief cells is a MARCKS-like protein.

Effects of PMA and Calcium on Phosphorylation of the Chief Cell MARCKS-Like Protein

To examine the phosphorylation of pp72 under various conditions, chief cells were incubated with [^{32}P]-orthophosphoric acid and permeabilized with SLO in the presence of agonists for 2 min at 37°C. Chief cell phosphoproteins were then examined by two-dimensional gel electrophoresis (Fig. 3). Phosphoproteins not altered by agonists were used as reference standards for proteins that clearly underwent agonist-dependent changes in phosphorylation. Increasing ambient calcium concentration from 0.1 to 1.0 μM caused a small increase in the phosphorylation of the acidic (pI approx. 4.5) pp72 (compare the protein indicated by the arrow in Fig. 3A to that in Fig. 3B). PMA alone increased phosphorylation of the MARCKS-like protein four-fold (Fig. 3C). The combination of calcium and PMA resulted in phosphorylation that was 50% of that observed with PMA alone (Fig. 3D). These results indicate that in dispersed chief cells, calcium decreases PMA-induced phosphorylation of the MARCKS-like protein.

Effect of Ca^{2+}/Calmodulin on In Vitro Phosphorylation by Chief Cell PKC of "MARCKS Phosphorylation/Calmodulin Binding Domain" and "PKC Pseudosubstrate Domain" Peptides

Tables I and II show the effects of calcium/calmodulin on phosphorylation by chief cell lysates of MARCKS "phosphorylation/calmodulin binding domain" [Graff et al., 1989] and "PKC pseudosubstrate domain" peptides. The experiment shown in Table I was designed to provide evidence for in vivo competition between Ca^{2+}/calmodulin and PKC-induced phosphorylation of the phosphorylation/calmodulin binding domain on the chief cell MARCKS-like protein. Previous studies by Verghese et al. [1993] validated use of MARCKS "phosphorylation/ calmodulin binding domain" peptides to mimic in vivo actions of MARCKS. These investigators demonstrated that, in terms of PKC substrate affinity, phosphorylation with positive cooperativity, calmodulin binding affinity, and displacement of calmodulin upon phosphorylation, this peptide behaves identically to na-

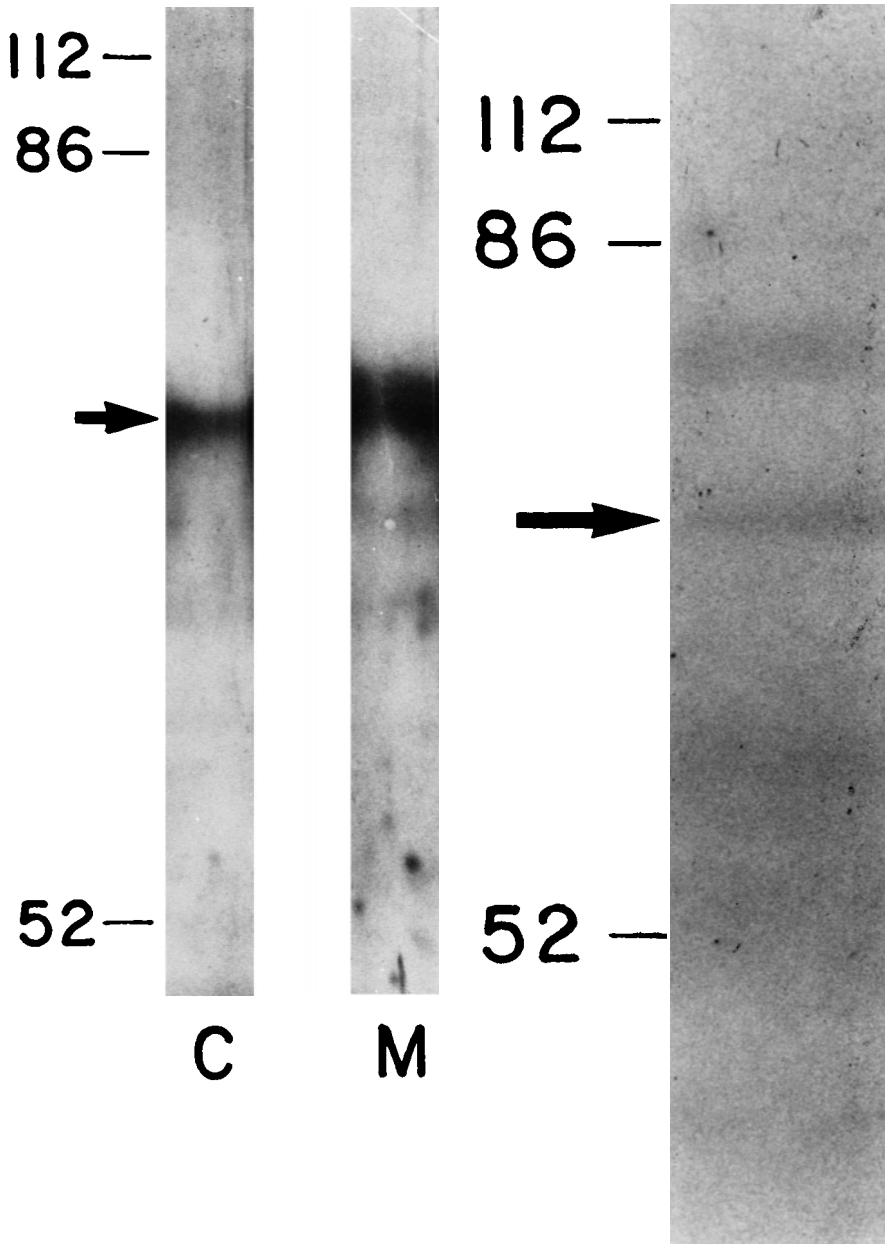

Fig 2. Expression and myristoylation of a MARCKS-like protein in chief cells. left: Immunoblotting. Subcellular fractions were electrophoresed on SDS/8% polyacrylamide gels and the separated proteins were electroblotted to nitrocellulose membranes. The blots were incubated overnight with MARCKS antibody. Blots were washed and incubated with alkaline phosphatase-conjugated anti-rabbit IgG. Bands were visualized with nitroblue tetrazolium. Arrow indicates position of the 72-dDa MARCKS-like protein. C and M indicate cytosol and membrane fractions, respectively. In the absence of antibody, the indicated bands were not observed. right: Myristoylation. Chief cells (8 x 10^6 cells/ml) were incubated for 4 h with [^{3}H]myristic acid. The cells were washed and centrifuged at 10,000*g* for 2 min. Samples of cell pellets were loaded on 12% SDS polyacrylamide gels. Detection was by exposure to Kodak X-Omat film at -70°C for 2 months. Arrow indicates position of the 72-kDa myristoylated protein. Numbers on left indicate molecular mass of protein standards. These experiment are representative of at least two others.

tive MARCKS. As shown in Table I, using chief cell lysates, phosphorylation of the "phosphorylation/calmodulin binding domain" peptide is reduced by approximately 40% when the substrate is preincubated with Ca^{2+}/calmodulin. Nevertheless, Ca^{2+}/calmodulin preincubation does not reduce PKC activity as evidenced by the lack of effect on phosphorylation of the "PKC pseudosubstrate domain" peptide (Table II). These results indicate that calcium/calmodu-

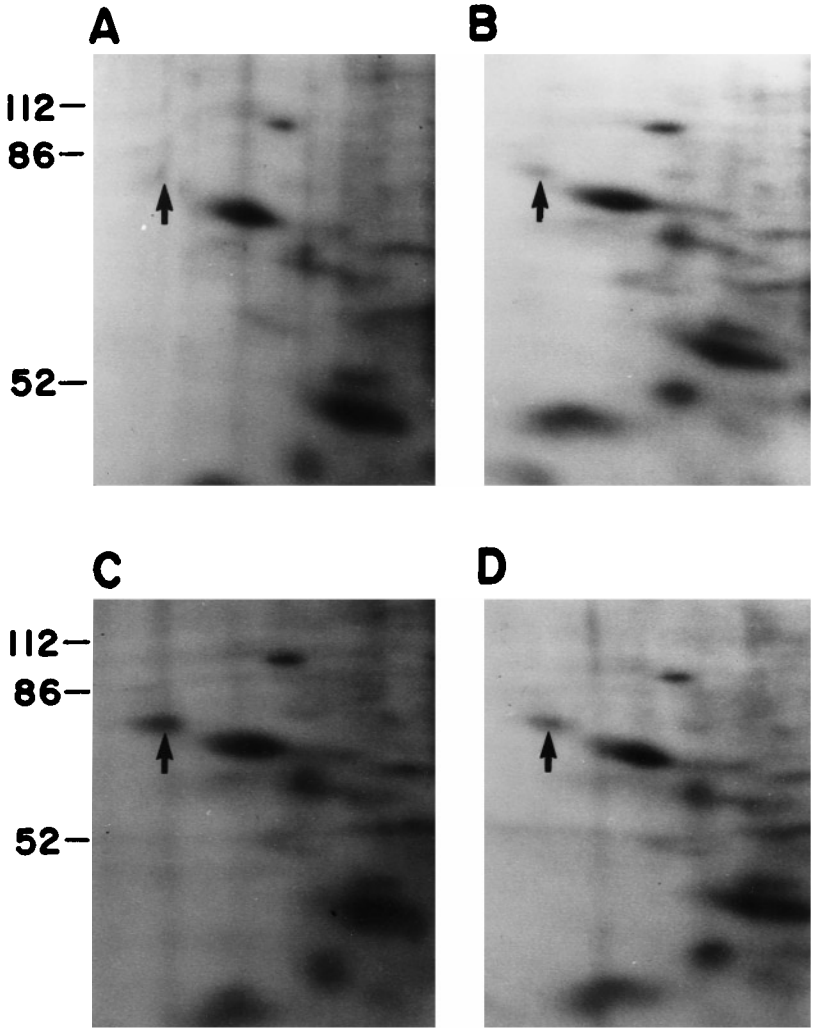

Fig. 3. Effects of calcium and activation of protein kinase C on phosphorylation of chief cell proteins. Cells were incubated with [^{32}P]-orthophosphoric acid and permeabilized with SLO in the presence of (**A**) 0.1 μM calcium; (**B**) 1.0 μM calcium; (**C**) 0.1 M PMA; and, (**D**) 1.0 μM calcium plus 0.1 μM PMA for 2 min at 37°C. Phosphoproteins were separated by two-dimensional gel electrophoresis and visualized by exposure for 3–10 days to Kodak X-Omat film. Numbers on left indicate molecular mass standards in kDa. Arrows indicate the 72-kDa MARCKS-like protein. Gels shown are representative of at least three others.

lin inhibits MARCKS phosphorylation by binding to the MARCKS phosphorylation/calmodulin binding domain and not by inhibition of PKC activity. Hence, in conjuction with the pp72 phosphorylation experiments, these data demonstrate that in gastric chief cells, as in other tissues [Graff et al., 1989; Blackshear, 1993], the newly-identified MARCKS-like protein contains a common phosphorylation-Ca^{2+}/calmodulin binding domain.

DISCUSSION

It has been suggested that a major function of PKC is to mediate cross-talk between cellular signalling pathways [see Houslay, 1991 for review]. Most investigations have focused on in

TABLE I. Effect of Calcium/Calmodulin on the Phosphorylation by Chief Cell Lysates of a MARCKS Phosphorylation/Calmodulin Binding Domain Peptide

	PKC activity (pmoles ^{32}P/10^7 cells)	
Preincubation	EGTA	PS/PMA/Ca^{2+}
No additions	0.4 ± 0.1	10.1 ± 1.9
Ca^{2+}/calmodulin	0.8 ± 0.1	6.3 ± 1.1*

PS, phosphatidyl serine; PMA, phorbol 12-myristate 13-acetate.

PKC activity was measured as described in Methods. The peptide was preincubated with no additions or Ca^{2+}/calmodulin for 15 min before adding the chief cell lysate.

*Indicates that value is significantly less than that without Ca^{2+}/calmodulin preincubation ($p < 0.05$, Student's t-test).

TABLE II. Effect of Calcium/Calmodulin on the Phosphorylation of a PKC Pseudosubstrate Domain Peptide by Chief Cell Lysates

	PKC activity (pmoles $^{32}P/10^7$ cells)	
Preincubation	EGTA	PS/PMA/Ca^{2+}
No additions	0.8 ± 0.1	6.7 ± 0.6
Ca^{2+}/calmodulin	0.9 ± 0.1	6.5 ± 0.7

PS, phosphatidyl serine; PMA, phorbol 12-myristate 13-acetate.
PKC activity was measured as described in Methods. The peptide was preincubated with no additions or Ca^{2+}/calmodulin for 15 min before adding the chief cell lysate.

teractions between the phospholipase C and adenylyl cyclase systems, but some observations, like PKC-induced stimulation of phosphatidylcholine hydrolysis [Pelech and Vance, 1989; Cook et al., 1989], suggest that the kinase may mediate interactions between limbs of the phospholipase C system. Likewise, in gastric chief cells, data presented here and elsewhere [Raffainello and Raufman, 1992] indicate that activation of PKC augments maximal calcium-induced pepsinogen secretion. Nevertheless, the mechanisms underlying this augmented response are unknown.

Earlier studies suggested that MARCKS proteins serve as Ca^{2+}/calmodulin buffers in the sense that phosphorylation of MARCKS could release calmodulin from membranes, thereby increasing cytosolic levels of this protein [Graff et al., 1989; Blackshear, 1993]. Released calmodulin would then be free to bind and activate enzymes, like Ca^{2+}/calmodulin kinase-II and calcineurin, thereby modulating the secretory response. Evidence for this includes the observations that calmodulin binds to MARCKS in a 1:1 molar ratio; PKC-mediated phosphorylation of MARCKS inhibits calmodulin binding; phosphorylation and calmodulin binding occur on the same MARCKS domain; and, a MARCKS calmodulin binding/phosphorylation domain peptide mimics the actions of MARCKS [Graff et al., 1989; McIlroy et al., 1991; Blackshear, 1993]. Both the phosphorylation site domain peptide and native MARCKS bind calmodulin with high affinity (K_d 2–5 nM) with a half-maximal calcium concentration of approximately 400 nM [Blackshear, 1993; McIlroy et al., 1991]. Phosphorylation of the phosphorylation/calmodulin-binding domain causes an approximately 200-fold decrease in its affinity for calmodulin and rapidly disrupts pre-existing peptide-calmodulin complexes [McIlroy et al., 1991]. In neuronal cell cultures, PKC activation decreases membrane-associated calmodulin and increases cytosolic calmodulin [MacNichol and Schulman, 1992]. In Paramecium, calmodulin stimulates a "forward" swimming response that is reversed by addition of the MARCKS "phosphorylation/calmodulin-binding domain" peptide which "soaks up" calmodulin [Hinrichsen and Blackshear, 1993]. PKC-induced phosphorylation of the "phosphorylation/calmodulin-binding domain" peptide causes the release of calmodulin and a reversion to "normal" swimming. In mouse keratinocytes [Chakravarthy et al., 1995a] and rat glioma cells [Chakravarthy et al., 1995b], prior treatment with calcium ionophores and calmodulin, respectively, reduce PKC-induced phosphorylation of MARCKS.

Based on the above finding in other cell types, our working hypothesis regarding PKC augmentation of calcium-induced pepsinogen secretion in gastric chief cells was that PKC-mediated phosphorylation of a MARCKS-like protein caused release of calmodulin and further activation of regulatory enzymes like Ca^{2+}/calmodulin kinase-II or calcineurin. Several observations reported in the present communication support this hypothesis:

First, we have established, using immunoblotting and a myristoylation assay, that pp72 is a membrane-associated MARCKS-like protein. Regarding the molecular mass of the identified MARCKS-like protein in chief cells, previous investigators, including Herget et al. [1992], have demonstrated that MARCKS proteins can vary in molecular mass from 60 to 90 kDa, depending on the species examined. Like pp72 (pI, 4.5), MARCKS proteins have acidic isoelectric points ranging from 4.1 to 4.5 [Blackshear, 1993; Swierzynski and Blackshear, 1995]. Myristoylation appears to play a role in anchoring MARCKS proteins to the plasma membrane by a hydrophobic interaction [Blackshear, 1993]. Nevertheless, electrostatic interaction of the phosphorylation site domain has also been implicated in promoting membrane association, which may be disrupted upon phosphorylation or calmodulin binding at this site [Thelen et al., 1991; Swierzynski and Blackshear, 1995].

Second, using two-dimensional gel electrophoresis, we showed that PKC-induced phosphorylation of pp72 is reduced by elevation of cyto-

solic calcium concentration. This observation is consistent with competition of calmodulin for the PKC phosphorylation domain.

Finally, the data concerning phosphorylation of the MARCKS "phosphorylation/calmodulin-binding domain" and "PKC pseudosubstrate domain" peptides by chief cell lysates indicate that calmodulin preincubation prevents PKC-induced phosphorylation of MARCKS without affecting PKC activity. This provides further evidence that, in chief cells, PKC-induced phosphorylation of the 72-kDa MARCKS-like protein is directed at the calmodulin binding site.

Little is known regarding the comparative phosphorylation of MARCKS by the 10 or so isoforms of PKC [Blackshear, 1993]. We have shown previously that the α and ζ isoforms of PKC are expressed in guinea pig chief cells [Raffaniello and Raufman, 1994b]. Currently it is not known if either or both of these isoforms play a role in phosphorylation of the pp72 MARCKS-like protein. Nonetheless, the observations regarding the phosphorylation-calmodulin binding domain of MARCKS proteins described previously and in the present communication indicate a complicated set of interactions that permit fine-tuning of cellular responses. Specifically, in gastric chief cells, the interplay between PKC-mediated phosphorylation and reduced binding of calmodulin to the newly identified MARCKS-like protein has the potential for activation of additional molecules of Ca^{2+}/calmodulin-dependent enzymes, like Ca^{2+}/calmodulin kinase-II and calcineurin. These events may contribute to the secretory response observed with maximal concentrations of agents, like carbachol, that activate the phospholipase C signalling pathway.

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