Phosphorylation of myristoylated alanine-rich C kinase substrate is involved in the cAMP-dependent amylase release in parotid acinar cells

Keitaro Satoh,1 Miwako Matsuki-Fukushima,1 Bing Qi,1 Ming-Yu Guo,1 Takanori Narita,1,2 Junko Fujita-Yoshigaki,1,2 and Hiroshi Sugiy1,2

1Department of Physiology and 2Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba, Japan

Submitted 10 September 2008; accepted in final form 10 April 2009

Satoh K, Matsuki-Fukushima M, Qi B, Guo M, Narita T, Fujita-Yoshigaki J, Sugiy H. Phosphorylation of myristoylated alanine-rich C kinase substrate in involved in the cAMP-dependent amylase release in parotid acinar cells. Am J Physiol Gastrointest Liver Physiol 296: G1382–G1390, 2009. First published April 16, 2009; doi:10.1152/ajpgi.90536.2008.–Myristoylated alanine-rich C kinase substrate (MARCKS) is known as a major cellular substrate for protein kinase C (PKC). MARCKS has been implicated in the regulation of brain development and postnatal survival, cellular migration and adhesion, as well as phagocytosis, endocytosis, and exocytosis. The involvement of MARCKS phosphorylation in secretory function has been reported in C2+/-mediated exocytosis. In rat parotid acinar cells, the activation of β-adrenergic receptors provokes exocytotic amylase release via accumulation of intracellular cAMP levels. Here, we studied the involvement of MARCKS phosphorylation in the cAMP-dependent amylase release in rat parotid acinar cells. MARCKS protein was detected in rat parotid acinar cells by Western blotting. The β-adrenergic agonist isoproterenol (IPR) induced MARCKS phosphorylation in a time-dependent manner. Translocation of a part of phosphorylated MARCKS from the membrane to the cytosol and enhancement of MARCKS phosphorylation at the apical membrane site induced by IPR were observed by immunohistochemistry. H89, a cAMP-dependent protein kinase (PKA) inhibitor, inhibited the IPR-induced MARCKS phosphorylation. The PKC6 inhibitor rottlerin inhibited the IPR-induced MARCKS phosphorylation and amylase release. IPR activated PKC8, and the effects of IPR were inhibited by the PKA inhibitors. A MARCKS-related peptide partially inhibited the IPR-induced amylase release. These findings suggest that MARCKS phosphorylation via the activation of PKC8, which is downstream of PKA activation, is involved in the cAMP-dependent amylase release in parotid acinar cells.

PKC8; rottlerin; PKA; salivary gland; exocytosis

Myristoylated alanine-rich C kinase substrate (MARCKS) is a major cellular substrate for protein kinase C (PKC) (37). MARCKS has been implicated in various cellular functions such as motility, phagocytosis, membrane trafficking, and mitogenesis (3, 16, 26). MARCKS binds to the plasma membrane via the dual actions of a hydrophobically myristoylated NH2 terminus and a polybasic stretch within the so-called effector domain (39) which is a phosphorylation site (12). It has been demonstrated that phosphorylated MARCKS (p-MARCKS) translocates from the membrane to the cytosol, because phosphorylation of the effector domain significantly decreases the binding force of MARCKS to the membrane (1).

MARCKS phosphorylation has been reported to be involved in secretory functions. In SH-SY5Y human neuroblastoma cells, phorbol-12-myristate-13-acetate (PMA), a PKC activator, enhanced norepinephrine release and MARCKS phosphorylation (10), and those effects were inhibited by a PKC inhibitor (10). In chromaffin cells, PMA-induced norepinephrine release and MARCKS phosphorylation were demonstrated (27, 47). A peptide of the MARCKS phosphorylation site domain sequence inhibited the PMA-induced norepinephrine release in permeabilized cells (27, 47). In BON human pancreatic carcinoma cells, PMA induced neurotensin secretion and MARCKS phosphorylation (16). A small interfering RNA (siRNA) to MARCKS significantly inhibited PMA-induced neurotensin secretion, whereas overexpression of wild-type MARCKS clearly increased the neurotensin secretion. Furthermore, siRNAs to PKC inhibited the PMA-induced MARCKS phosphorylation (16). In bovine luteal cells, prostaglandin F2α (PGF2α) stimulated MARCKS phosphorylation and oxytocin secretion (28). However, PGF2α failed to stimulate oxytocin exocytosis in cells expressing a mutant MARCKS that cannot be phosphorylated by PKC (28). In insulin-producing INS-1 cells, glucagon-like peptide-1 (GLP-1) induced insulin secretion, PKCo and ε activation, and MARCKS phosphorylation (38). A PKC inhibitor inhibited MARCKS phosphorylation and insulin secretion coupled to GLP-1-induced signaling (38). These observations indicate that MARCKS phosphorylation is involved in Ca2+-dependent secretion. In normal human bronchial epithelial (NHBE) cells, a cholinergic agonist induced mucin secretion via cGMP signaling (17). A myristoylated NH2-terminal sequence peptide of MARCKS (MANS) inhibited the mucin secretion induced by 8-bromo-cGMP with PMA (17) or a cholinergic agonist (2, 34). Therefore, MARCKS phosphorylation has also been suggested to be involved in cGMP-dependent secretion.

In parotid acinar cells, stimulation of β-adrenergic receptors induces exocytotic amylase release (24). The amylase release is regulated by intracellular cAMP levels without elevation of Ca2+, because a β-agonist will provoke increases in intracellular cAMP levels but has no effect on intracellular Ca2+ levels (13, 43). In the cAMP-dependent amylase release, activation of cAMP-dependent protein kinase (PKA) is thought to be an essential step in the process. During amylase secretion from parotid acinar cells, the activity of PKA was enhanced (24). PKA inhibitors such as H89 and PKI-(5-24)-peptide inhibited the cAMP-dependent amylase release (31, 40, 41). Insertion of PKA induced amylase release in permeabilized parotid acinar cells (40). Therefore, it is most likely that PKA phosphorylates proteins involved in amylase exocytosis. However, it is not clear which protein phosphorylation is crucial for triggering
cAMP-dependent exocytosis, although several proteins have been reported to be phosphorylated upon β-adrenergic stimulation in parotid glands (5, 14, 36).

On the other hand, it has been thought that PKC activation is involved in amylase release in parotid acinar cells, since the PKC activator 4β-phorbol-dibutyrate induced protein secretion in parotid gland (23) and amylase release in parotid acinar cells (52). Amylase release induced by the stimulation of β-adrenergic receptors was inhibited by the PKC inhibitor H7 (32). Recently, PKCh, a PKC isotype, was reported to be involved in exocytosis in several different cell types: mucin secretion in NHBE cells (22), neurotransin secretion in BON cells (16), insulin secretion in cultured clonal β-cells (HIT-T15) (51), and dense granule secretion in human platelets (18). In the present study, we demonstrate the involvement of MARCKS phosphorylation via PKC activation in the CAMP-dependent amylase release in parotid acinar cells.

EXPERIMENTAL PROCEDURES

Materials. Collagenase A, hyaluronidase, and bovine serum albumin (BSA) were purchased from Roche (Basel, Switzerland). H89, forskolin, trypsin (type III), trypsin inhibitor (type I-S), luciferase, and isoproterenol (iPR) were obtained from Sigma (St. Louis, MO). Anti-MARCKS and anti-phosphorylated MARCKS antibodies were obtained from Chemicon (Temecula, CA). Anti-PKCδ, anti-phosphorylated PKCδ (p-PKCδ), and anti-PKCε antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Cell Signaling (Beverly, MA), and Abcam (Cambridge, MA), respectively. Alexa Fluor 568 goat anti-rabbit IgG, Alexa Fluor 488 phallolidin, and ProLong Gold antifade reagent were purchased from Molecular Probes (Eugene, OR). Anti-rabbit and anti-mouse IgG alkaline phosphatase-linked antibodies and ECL Western blotting detection reagents were purchased from GE Healthcare (Piscataway, NJ). Calphostin C (CalC), Go6983, Go6976, rottlerin, and PKI(5-24)-peptide were purchased from Calbiochem (San Diego, CA). Nonradioactive PKC and PKA kinase activity kits were purchased from Assay Designs (Ann Arbor, MI). PKA and β-luciferin were purchased from Wako (Osaka, Japan). Block Ace and IGEPAL were purchased from Yukiurushi-Nyugo (Sapporo, Japan) and MP (Solon, OH), respectively. Dibutyryl-cAMP was purchased from Biomol (Plymouth Meeting, PA). Radioactive PKC assay kit and [γ-32P]ATP were purchased from Upstate (Charlottesville, VA) and PerkinElmer (Waltham, MA), respectively. Protease inhibitor cocktail was purchased from Cytoskeleton (Denver, CO).

MARCKS-related peptide. The MANS and the random NH2-terminal sequence (RNS) peptides were synthesized as previously described (17) at Bex (Tokyo, Japan). The MANS peptide consisted of a sequence identical to the first 24 amino acids of MARCKS, the myristoylated NH2-terminal region that mediates MARCKS insertion into membranes: MA-GAQFSKTAAKGEAAEPRGEAAVA (where MA is the NH2-terminal myristate chain). The corresponding control peptide (RNS) contained the same amino acid composition as the MANS but arranged in random order: MA-GTAPAAEGTAAEKSAEAKQAFF. The presence of the hydrophobic myristate moiety in these synthetic peptides enhances their permeability to plasma membranes, enabling the peptides to be taken up readily by cells (17).

Preparation of parotid acinar cells. All animal protocols were approved by the Laboratory Animal Committee of the All University announces of Dentistry at Matsudo. Parotid acinar cells were prepared as previously described (9). Sprague-Dawley (SD) rats (male, 200–250 g) were intraperitoneally anesthetized with pentobarbital (50 mg/kg), and the parotid glands were removed and placed in a small volume of Krebs-Ringer-bicarbonate (KRB) solution with the following composition (in mM): NaCl, 116.5 KCl, 0.8 MgSO4, 1.8 CaCl2, 0.96 NaH2PO4, 25 NaHCO3, 5 HEPS (pH 7.4), and 11.1 glucose.

The KRB solution was equilibrated with an atmosphere of 95% O2-5% CO2. After being minced with a razor, the parotid glands were treated with KRB solution containing 0.5% BSA in the presence or absence of enzyme. First, the glands were incubated with trypsin (0.5 mg/ml) at 37°C for 5 min, after which the trypsin-treated glands were removed by centrifugation at 200 g for 1 min. The glands were subsequently incubated in Ca2+-Mg2+-free KRB solution containing 1 mM EGTA and trypsin inhibitor (0.5 mg/ml) at 37°C for 5 min. After the solution was removed by centrifugation (200 g for 1 min), the glands were incubated in Ca2+-Mg2+-free KRB solution without trypsin inhibitor at 37°C for 5 min. After the solution was removed by centrifugation (200 g for 1 min), the glands were incubated in KRB solution with collagenase A (1.5 mg/ml) at 37°C for 20 min. The suspension was passed through eight layers of nylon mesh to separate the dispersed cells from undigested connective tissue and then was placed on KRB solution containing 4% BSA. After centrifugation (50 g for 5 min), the cells were suspended in appropriate amounts of KRB solution containing 5% BSA and 0.02% trypsin inhibitor.

Preparation of cell lysates and membrane and cytosol fractions. Parotid acinar cell lysates were obtained by homogenizing using polypropylene homogenizer in 25 mM Tris-HCl buffer (pH 7.5) containing 10 mM MgCl2, 0.3 M NaCl, 2% IGEPAL, and protease inhibitor cocktail. To isolate membrane and cytosolic fractions, cells were homogenized with 10 mM HEPES buffer (pH 7.2) containing 0.3 M sucrose, 2 mM EDTA, 0.2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail and were then centrifuged at 750 g at 10 min to remove the nuclear fraction. The postnuclear supernatant fraction was centrifuged at 100,000 g for 60 min. The pellet and supernatant were collected as crude membrane and cytosol fractions, respectively.

Protein phosphorylation. Parotid acinar cells prepared as described above were stimulated by iPR (1 μM), forskolin (100 μM), dibutyryl-cAMP (1 mM), and PMA (100 nM) at 37°C for the indicated times. When the effects of Go6983 (5 μM), Go6976 (5 μM), CaCl2 (5 μM), rottlerin (50 μM), H89 (10 μM), and PKI-(5-24)-peptide (10 μM) were examined, cells were pretreated with those drugs for 10 min and then iPR was added. After treatment, cell suspensions were centrifuged and the media were removed. Protein samples of lysates and membrane and cytosol fractions were obtained as described above. MARCKS, p-MARCKS, p-PKCδ, and PKCε were detected by Western blotting analysis.

Western blotting. Protein concentrations were determined by the method of Bradford (7). Proteins were resolved by 7.5% SDS-PAGE and were then transferred to nitrocellulose membranes (12.5 V, overnight). The membranes were blocked at room temperature for 50 min in Block Ace, and then were probed for 120 min with the primary antibodies anti-MARCKS (diluted 1:1,000), anti-p-MARCKS (diluted 1:2,000), anti-p-PKCδ (diluted 1:1,000), and anti-PKCε (diluted 1:100). The blots were washed three times with 10% Block Ace containing 0.05% Tween 20 and were then probed for 90 min with the secondary antibody, anti-rabbit or anti-mouse IgG (diluted 1:10,000). Immunoreactivity was determined by use of ECL Western blotting detection reagents. Intensities of specific proteins’ bands were measured by Image J (NIH) from the image translated using a computer.

Amylase release. Parotid acinar cells prepared as described above were stimulated by iPR (1 μM) at 37°C for 20 min. The effects of the CaCl2 (5 μM), rottlerin (50 μM), and MANS peptide (50 μM) were examined by pretreating cells with those drugs for 10 min or 15 min, and then adding iPR. The cell suspensions were diluted with phosphate-buffered saline (PBS) and passed through filter paper. Released and total amylase activities, respectively, in the medium and in acinar cells homogenized with phosphate buffer (pH 6.9) containing 0.01 % Triton X-100 were measured according to the method of Bernfeld (6).

Immunohistochemistry. SD rats (male, 200–250 g) were anesthetized, and their parotid glands were removed and placed in a small volume of KRB solution. Parotid acini were isolated by use of hyaluronidase (0.5 mg/ml) and collagenase A (2 mg/ml) in KRB.
solution at 37°C for 30 min. The suspensions were passed through eight layers of nylon mesh to separate the dispersed acini from undigested connective tissue and were then placed in KRB solution containing 4% BSA. After centrifugation (50 g for 5 min), the acini were suspended in the appropriate amounts of KRB solution containing 0.5% BSA and 0.02% trypsin inhibitor. The suspensions were stimulated by IPR (1 μM) at 37°C for 10 min. After the treatment, acini were fixed with 4% paraformaldehyde for 20 min at room temperature. After three washes with PBS, the acini were permeabilized with 0.3% Triton X-100 for 15 min. After blocking step with 1% BSA in PBS, the acini were incubated with anti-p-MARCKS antibody (diluted 1:100) with 1% BSA in PBS for overnight at 4°C. Acini were washed three times with PBS and then were incubated with Alexa Fluor 568 goat anti-rabbit IgG antibody (diluted 1:100), Alexa Fluor 488 phalloidin (diluted 1:100), and To-pro-3 iodide (diluted 1:500) in 1% BSA in PBS. Acini were encapsulated by Pro Long Gold antifade reagent on glass-based dishes. Fluorescence images were observed using LSM-510 META (Carl Zeiss) confocal microscope.

**PKCα kinase assay.** Parotid acinar cells prepared as described above were stimulated by IPR (1 μM) at 37°C for 15 min. When the effects of MANs peptide (50 μM) was examined, cells were pre-treated with this drug for 15 min and then IPR was added. After treatment, cell suspensions were centrifuged and the media were removed. PKCα was purified from cell lysate by immunoprecipitation and the kinase activity was measured according to the method of Satoh et al. (29). Parotid acinar cell lysates were obtained by homogenization by using a polypropylene homogenizer in 50 mM Tris-HCl buffer (pH 7.5) containing 130 mM NaCl, 5 mM EGTA, 5 mM EDTA, 1.5 mM MgCl2, 10 mM NaF, 1 mM Na4P2O7, 10 mM Na3PO4, 1 mM PMSF, 10% (vol/vol) glycerol, and protease inhibitor cocktail and then incubated for 45 min at 4°C. After the centrifugation for 15 min at 15,000 g, protein A-Sepharose beads conjugated with normal rabbit IgG were added to the lysate, which was rotated 4°C for 60 min. After centrifugation, the supernatants were recovered and incubated with protein A-Sepharose beads with anti-PKCα (1:100) at 4°C for overnight. Then protein A-Sepharose beads were collected by centrifugation, and the beads were resuspended in final 50 μl of 20 mM MOPS buffer (pH 7.2) containing 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na3VO4, and 1 mM dithiothreitol. The kinase assay was performed by using the radioactive PKC assay kit. The assay was started with the addition of a magnesium-ATP mixture (75 mM MgCl2 and 0.5 mM ATP) containing 10 μCi of [γ-32P]ATP to the sample containing 10 μl of the PKCα-specific immunoprecipitate, 30 μl of buffer, and 40 μM of substrate, and the reaction was incubated for 10 min at 30°C. Reactions were stopped by the addition of 50 μl of 0.75% phosphoric acid, and the samples were applied onto p81 phosphocellulose paper. The p81 papers were washed three times with 0.75% phosphoric acid and once with acetone. The amount of 32P was determined by liquid scintillation counting. Background measurements of 32P were determined from incubations conducted in the absence of substrate and were subtracted from the 32P values in experimental samples.

**Total PKC and PKA kinase assay.** Parotid acinar cell lysates prepared as described above were incubated with or without the MANs peptide (50 μM) for 15 min at 30°C. The kinase activities of total PKC and PKA were determined by using the nonradioactive PKC and PKA kinase assay kits, respectively, according to the manufacturer’s instruction.

**ATP assay.** Parotid acinar cell suspensions prepared as described above were incubated with rottlerin (50 μM) or carboxybenzoyl-carnitoyl-(trifluromethoxy)phenylhydrazone (FCCP; 10 μM) for 30 min at 37°C. After incubation, cell suspension was taken, mixed with 35% perchloric acid, and put on ice for 30 min. After addition of 17.5% potassium hydroxide was added to the mixture for neutralization, the mixture was centrifuged at 10,000 g for 5 min, and supernatant was isolated. The ATP concentration in the supernatant was measured by luminometric assay using d-luciferin and luciferase (50).

**RESULTS**

**IPR-induced MARCKS phosphorylation.** To assess whether a β-adrenergic agonist induces MARCKS phosphorylation in parotid acinar cells, the cells were stimulated with IPR (1 μM) for 2, 5, 10, and 15 min. In Western blotting using anti-MARCKS and anti-p-MARCKS antibodies, IPR clearly induced MARCKS phosphorylation in a time-dependent manner but did not affect the total amount of MARCKS (Fig. 1, A and B). When the cells were treated with the PKC activator PMA (100 nM) as the positive control, MARCKS phosphorylation also occurred (Fig. 1E). The adenylyl cyclase activator forskolin (100 μM) and the cAMP analog dibutyryl-cAMP (1 mM) induced MARCKS phosphorylation (Fig. 1, C and D). These results suggest that MARCKS phosphorylation is induced by IPR via the increase in intracellular cAMP levels.

Because it has been demonstrated that unphosphorylated MARCKS binds to the cell membrane (1) and p-MARCKS changes its localization from the membrane to the cytosol (38), we next examined localization of p-MARCKS by Western blotting (Fig. 2A). In rat parotid acinar cells, p-MARCKS was mainly localized in the membrane fraction. When cells were stimulated with IPR (1 μM) for 2, 5, 10, and 15 min, the amount of p-MARCKS in the cytosol fraction increased in a time-dependent manner (Fig. 2A), although most of p-MARCKS remained in the membrane fraction.

We further examined p-MARCKS localization in rat parotid acini by immunohistochemistry using confocal microscopy (Fig. 2B). In the control, p-MARCKS localized on or near the membrane but had less signal of immunofluorescence in cytosol. p-MARCKS less colocalized with F-actin, a marker protein of the apical membrane, visualized as yellow. When the cells were stimulated with IPR for 10 min, signal of immunofluorescence was enhanced. In the cells, p-MARCKS appeared in the cytosol, and p-MARCKS colocalized with F-actin became clear on June 28, 2017 http://ajpgi.physiology.org/ Downloaded from
induced MARCKS phosphorylation but not Gö6976, an inhibitor of Ca²⁺-dependent PKC. CalC, an inhibitor of diacylglycerol (DAG)-sensitive PKC (15), inhibited IPR-induced MARCKS phosphorylation, suggesting that Ca²⁺-independent and DAG-sensitive PKC, i.e., novel PKC such as PKCδ and ε, is involved in IPR-induced MARCKS phosphorylation. Of those, it has been reported that PKCδ is involved in MARCKS phosphorylation in secretory cells (16, 22). Therefore, we examined the effect of rottlerin, a PKCδ-specific inhibitor, on IPR-induced MARCKS phosphorylation. The rottlerin inhibited IPR-in-

**Fig. 1.** Isoproterenol (IPR)-induced myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation in rat parotid acinar cells. Cells were stimulated by IPR (1 μM) for the indicated times. A: MARCKS and phosphorylated (p)-MARCKS in lysates (25 μg protein) were detected by Western blotting analysis using anti-MARCKS and anti-p-MARCKS antibodies, respectively. B: MARCKS phosphorylation is calculated as the ratio of p-MARCKS to MARCKS and is normalized against stimulation at 0 min. C: cells were stimulated by IPR (1 μM), forskolin (FSK, 100 μM) and dibutyryl-cAMP (DcAMP, 1 mM) for 15 min. MARCKS and p-MARCKS in lysates (25 μg protein) were detected by Western blotting analysis using anti-MARCKS and anti-p-MARCKS antibodies, respectively. D: MARCKS phosphorylation is calculated as the ratio of p-MARCKS to MARCKS and is normalized against control (Cont). E: PMA (100 nM) stimulation was used as the positive control. Values are means ± SE from 3 independent experiments; *P < 0.05 vs. stimulation at 0 min (B) or control (D).

**Fig. 2.** Localization of p-MARCKS in rat parotid acinar cells. Cells were stimulated by IPR (1 μM) for the indicated times. A: p-MARCKS in cytosol and membrane fraction (50 μg protein) was detected by Western blotting analysis using an anti-p-MARCKS antibody. B: acini were stimulated by IPR for 10 min, after which fluorescence was observed by use of a confocal microscope. Red, p-MARCKS visualized by Alexa568 conjugated anti-rabbit IgG; green, actin visualized by Alexa phalloidin. Scale bars equal 10 μm.
parotid acinar cells were treated with 50 μM carbachol by ATP depletion (44). In our experiments, when carbachol release stimulated by secretagogues such as CCK and effect as the mitochondrial uncoupler FCCP and inhibit amy-

parotid acinar cells, rottlerin was reported to show the same with a depletion of the cellular ATP concentration (35, 44). In our experimental conditions probably allow maintenance of the cell viability. Therefore, it is unlikely that the inhibition of IPR-induced MARCKS phosphorylation by rottlerin is caused by a toxic effect and suggests that the IPR-induced MARCKS phosphorylation is evoked by PKCε activation.

Effect of CalC and rottlerin on IPR-induced amylase release. Next we examined the effect of CalC and rottlerin on IPR-induced amylase release in parotid acinar cells. As summarized in Fig. 5, CalC (5 μM) and rottlerin (50 μM) partially inhibited the IPR-induced amylase release in parotid acinar cells, whereas these inhibitors had no effect on the nonstimulated amylase release. These results suggest that PKCε activation partially contributes to the IPR-induced amylase release in rat parotid acinar cells.

IPR-induced PKCε activation. We examined novel PKC activation in rat parotid acinar cells stimulated with IPR. It is known that activated PKC, including PKCε, is phosphorylated and translocated from the cytosol to the membrane fraction (33). In the membrane fraction of cells stimulated with IPR (1 μM) for 2, 5, 10, and 15 min, and the activation of PKCε was analyzed by Western blotting using an anti-p-PKCε antibody. As shown in Fig. 6, A and B, IPR treatment resulted in an increase in amount of p-PKCε in the membrane fraction in a time-dependent manner, suggesting PKCε activation by IPR treatment. Next, we directly examined PKCε kinase activity using PKCε-specific immunoprecipitates. As Fig. 6C shows, PKCε kinase activity increased in the acinar cells treated with IPR for 15 min (Fig. 6C). When the activation of PKCε was examined by PKCε translocation to the membrane, IPR treatment had less effect on PKCε activation (Fig. 6D). These
observations suggest that IPR induces PKC activation in rat parotid acinar cells.

Effect of PKA inhibitors on IPR-induced PKC activation. It has not been previously reported whether PKA activates PKC in rat parotid acinar cells, so we investigated the effect of the PKA inhibitors H89 and PKI-(5-24)-peptide on IPR-induced PKC activation in those cells. When the cells were pretreated with H89 (10 μM) or PKI-(5-24)-peptide (10 μM) for 10 min, IPR (1 μM) failed to stimulate PKC activation (Fig. 7). These results suggest that PKC is activated via PKA activation in the IPR-stimulated rat parotid acinar cells.

Effect of MARCKS-related peptide on IPR-induced amylase release. Several approaches can be used to determine whether MARCKS is directly involved in secretory function, such as using siRNA (16) or transfection of a dominant-negative construct (22). Currently, MANS has been demonstrated to be an useful inhibitor of MARCKS function, because that peptide suppresses secretory function in cultured cells (17) and in vivo (2, 34). Thus we examined the effect of the MANS peptide on IPR-induced amylase release. Parotid acinar cells were preincubated with the MANS peptide (50 μM) for 15 min and then were stimulated with IPR (1 μM) for 20 min. As shown in Fig. 8, the MANS peptide resulted in a partial suppression of the IPR-induced amylase release, but the control peptide RNS (50 μM) had no effect. We further checked the effect of MANS peptide on kinase activities of PKA and PKC. However, kinase activities of PKA, total PKC, and PKC in the parotid acinar cells pretreated with MANS peptide (50 μM) for 15 min were not significantly different from that in the control cells (Table 1). In addition, the MANS peptide had no effect on IPR-induced increase in PKC kinase activity in parotid acinar cells, 109.21 ± 4.61% (n = 3) of nontreated MANS peptide. Therefore, these results suggest that MARCKS is involved in the IPR-induced amylase release in rat parotid acinar cells.

DISCUSSION

In parotid acinar cells, IPR provokes an increase in intracellular cAMP levels, activates PKA, and induces the exocytotic
After pretreatment with MANS (50 μM), PKI-(5-24)-peptide (PKI, 10 μM) or vehicle (Cont) for 10 min, cells were stimulated by IPR (1 μM) for 15 min. p-PKC activity is calculated as the ratio and is normalized against the absence of IPR. Values are means ± SE from 4 independent experiments; *P < 0.05 vs. the absence of IPR.

**Table 1. Effects of MANS peptide on kinase activities of PKA and PKC in rat parotid acinar cells**

<table>
<thead>
<tr>
<th>Protein Kinase</th>
<th>MANS (+) Activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKA</td>
<td>95.52 ± 1.07</td>
</tr>
<tr>
<td>Total PKC</td>
<td>105.48 ± 5.45</td>
</tr>
<tr>
<td>PKC8</td>
<td>108.94 ± 6.44</td>
</tr>
</tbody>
</table>

Values are means ± SE from 3 independent experiments. Samples were preincubated with or without myristoylated NH2-terminal sequence peptide of myristoylated alanine-rich C kinase substrate (MANS; 50 μM) peptide for 15 min. Protein kinase activity is expressed as the percentage of absence of MANS peptide.

α, βI, βII, γ-isotypes), novel PKC (nPKC: δ, ε, η, θ-isotypes), and atypical PKC (aPKC: ζ, θ/A-isotypes) (33). cPKC responds to DAG and Ca2+ signaling, nPKC responds to DAG but not to Ca2+ signaling, and aPKC does not respond to DAG or Ca2+ (21). In rat parotid acinar cells, IPR-induced MARCKS phosphorylation is inhibited by CalC (Fig. 4), which has been reported to be a DAG-sensitive PKC inhibitor (15). IPR (1 μM) has no effect on Ca2+ mobilization in parotid acinar cells (13, 43). Therefore, IPR-activated PKC is probably a Ca2+-independent and DAG-sensitive kinase, i.e., an nPKC. PKCα, δ, ε, and ζ have been reported to be expressed in parotid acinar cells (45). We demonstrated that the PKCδ-specific inhibitor rottlerin partially inhibited IPR-induced MARCKS phosphorylation (Fig. 4). In addition, PKCδ was activated by IPR but not PKCe (Fig. 6) in rat parotid acinar cells. Furthermore, we demonstrated that rottlerin partially inhibits IPR-induced amylase release in rat parotid acinar cells (Fig. 5). The involvement of MARCKS phosphorylation by PKCδ in secretory function has been reported in BON cells (16) and in NHBE cells (22). Taken together, it is likely that MARCKS phosphorylation induced by PKCδ activation is involved in the Ca2+-dependent amylase release in parotid acinar cells.

Interestingly, we found that the PKA inhibitors H89 and PKI-(5-24)-peptide inhibit IPR-induced PKCδ activation, which suggests that PKCδ activation is downstream of PKA activation. There are two possible mechanisms for PKCδ activation by PKA. One possible mechanism is the phosphorylation and activation of PKCδ by PKA, although such a direct mechanism has not been previously reported. As another mechanism, it is known that PKCδ is activated by the increase in DAG. The adenylate cyclase activator forskolin was reported to activate nPKC via the increase in DAG content in INS-1 cells (38). Phospholipase D (PLD) is a regulator for DAG production, and PLD activity in rat parotid acini has been reported to be stimulated by Ca2+ independently (11). It has also been reported that PLD activation partially contributes to the IPR-induced amylase release in rat parotid acinar cells (9). Taken together, PKCδ appears to be activated by DAG which is induced by PLD via PKA activation, although further studies to characterize that mechanism are needed.
the actin cytoskeleton structure is considered to be involved in exocytosis (4). In NIHBE cells, MARCKS has been suggested to function as a molecular linker by interacting with granule membranes at its NH2-terminal domain and binding to actin filaments at its effector domain, thus tethering granules to the actin cytoskeleton for movement and exocytosis (16). In rat parotid acinar cells, the IPR-induced amylase release is inhibited by the actin filament-disrupting reagent cytochalasin D (19, 42, 46) and the actin stabilizing reagent jasplakinolide (19). Furthermore, secretory granules at the apical membrane site are rapidly coated with F-actin after the fusion with the apical membrane in the IPR-stimulated parotid acinar cells, whereas F-actin is localized mainly underneath the apical membrane in the absence of stimulation (30). Such F-actin coating of granules has been considered to be involved in stabilization of exocytosis in pancreatic acinar cells (20). These observations suggest that regulation of the actin cytoskeleton is involved in the IPR-induced amylase release in parotid acinar cells. We demonstrated that MARCKS phosphorylation was enhanced at the apical site in the parotid acinar cells stimulated with IPR (Fig. 2B). Therefore, MARCKS phosphorylation occurred in on or near the apical membrane induced by IPR appears to contribute to the F-actin mobilization. It has been shown that MARCKS is localized at the cell membrane in resting cells (1) and that p-MARCKS changes its localization from the membrane to the cytosol (27, 38). In SH-SY5Y cells (10), chromaffin cells (27, 47), and luteal cells (28), MARCKS phosphorylation by PKC causes MARCKS translocation from the membrane to the cytosol and also interferes with MARCKS binding to F-actin. However, we demonstrated that p-MARCKS mainly localized on or near the membrane in rat parotid acinar cells without stimulation and a part of p-MARCKS translocated to the cytosol after IPR stimulation (Fig. 2, A and B). It has reported that MARCKS phosphorylation by PMA was confined to the membrane fraction in N1E-115 neuroblastoma cells and the amount of p-MARCKS increased in both membrane and cytosolic fractions after activation of phosphorylation in C6 glioma cells (8). Therefore, our observation indicates that MARCKS phosphorylation is not always paralleled by a translocation from membrane to cytosolic fractions in the rat parotid acinar cells. We need further study with the interaction of F-actin and MARCKS phosphorylation as previously reported in N1E-115 neuroblastoma cells. We need further study with the interaction of F-actin and MARCKS phosphorylation in cAMP-regulated exocytosis of rat parotid acinar cells. 

**REFERENCES**


