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

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Satoh K, Matsuki-Fukushima M, Qi B, Guo M, Narita T, Fujita-Yoshigaki J, Sugiya H. Phosphorylation of myristoylated alanine-rich C kinase substrate is 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 cell proliferation and motility, phagocytosis, endocytosis, and exocytosis. The involvement of MARCKS phosphorylation in secretory function has been reported in Ca²⁺-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 PKCε inhibitor rottlerin inhibited the IPR-induced MARCKS phosphorylation and amylase release. IPR activated PKCε, 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 PKCε, which is downstream of PKA activation, is involved in the cAMP-dependent amylase release in parotid acinar cells.

PKCε; rottlerin; PKA; salivary gland; exocytosis

Phosphorylation of 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 neuropeptide secretion and MARCKS phosphorylation (16). A small interfering RNA (siRNA) to MARCKS significantly inhibited PMA-induced neuropeptide secretion, whereas overexpression of wild-type MARCKS clearly increased the neuropeptide secretion. Furthermore, siRNAs to PKC inhibited the PMA-induced MARCKS phosphorylation (16). In bovine luteal cells, prostaglandin E₂ (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 β 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 PMA-induced 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...

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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, PKCδ, a PKC isotype, was reported to be involved in exocytosis in several different cell types: mucin secretion in NHBE cells (22), neurotensin 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 PKCδ 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 isotroperonol (IPR) were obtained from Sigma (St. Louis, MO). Anti-MARCKS and anti-p-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 phalloidin, 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 -α1 luciferase were purchased from Wako (Osaka, Japan). Block Ace and IGEPAL were purchased from Yukiirui-Nyugyo (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. Dextran-tetraacetic acid (DTA) 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-GAQQSKTAAKGEAAERPGEEAVVAA (where M 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-GTAPAAEGAGAEVKRASAEKQAF. 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 Nihon University School 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 pentobarbitall (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): 116 NaCl, 5.4 KC1, 0.8 MgSO4, 1.8 CaCl2, 0.96 NaH2PO4, 25 NaHCO3, 5 HEPES (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 0.5% BSA and 0.02% trypsin inhibitor.

**Preparation of cell lysates and membrane and cytosol fractions.** Parotid acinar cell lysates were obtained by homogenization 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), CalC (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 CalC (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 a 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.

**PKC6 kinase assay.** Parotid acinar cells prepared as described above were stimulated (60 g) 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. PKC6 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 Na₃VO₄, 10 mM Na₂P₂O₇, 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 at 4°C for 60 min. After centrifugation, the supernatants were recovered and incubated with protein A-Sepharose beads with anti-PKC6 (1:100) at 4°C for overnight. Then proteins 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 MgCl₂, 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 PKC6-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 carboxylycyanide-4-(trifluoromethoxy)phenylhydrazide (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 β-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 adenylylate 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. These observations indicate that IPR treatment enhances MARCKS phosphorylation at the apical site and induces translocation of a part of MARCKS from the membrane to the cytosol. F-actin fluorescence around the exocytosed secretory granules appeared as previously reported (30), but p-MARCKS did not colocalize with them.

**Effect of H89 on IPR-induced MARCKS phosphorylation.** In parotid acinar cells, stimulation of β-adrenergic receptors enhances the activity of PKA, and PKA activation is essential for exocytotic amylase release (24). We examined the effect of the PKA inhibitor H89 (10 μM) on the IPR-induced MARCKS phosphorylation. As Fig. 3 shows, H89 inhibited the IPR-induced MARCKS phosphorylation but had no effect on the total amount of MARCKS, suggesting that IPR-induced MARCKS phosphorylation is evoked via the activation of PKA in parotid acinar cells.

**Effect of PKC inhibitors on IPR-induced MARCKS phosphorylation.** We next investigated the effects of Gö6983 (5 μM), Gö6976 (5 μM), CalC (5 μM), and rottlerin (50 μM), PKC inhibitors, on IPR-induced MARCKS phosphorylation. PKC has many isotypes (33), and PKCo, δ, ε, and ζ are expressed in rat parotid acinar cells (45). As shown in Fig. 4, Gö6983, an inhibitor of several types of PKC, inhibited IPR-
induced MARCKS phosphorylation but not Go6976, an inhibitor of Ca2+/H11001-dependent PKC. CalC, an inhibitor of diacylglycerol (DAG)-sensitive PKC (15), inhibited IPR-induced MARCKS phosphorylation, suggesting that Ca2+/H11001-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-induced MARCKS phosphorylation.
parotid acinar cells were treated with 50 nM carbachol by ATP depletion (44). In our experiments, when amylase release stimulated by secretagogues such as CCK and PMA was determined, rottlerin was reported to show the same effect as the mitochondrial uncoupler FCCP and inhibit amylase release in pancreatic acinar cells, whereas these inhibitors had no effect on the nonstimulated amylase release. These results suggest that PKCζ phosphorylation partially contributes to the IPR-induced amylase release in parotid acinar cells.

**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 PKCζ phosphorylation partially contributes to the IPR-induced amylase release in rat parotid acinar cells.

**IPR-induced PKCζ activation.** We examined novel PKCζ activation in rat parotid acinar cells stimulated with IPR. It is known that activated PKCζ, including PKCζε, 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 PKCζ was analyzed by Western blotting using an anti-PKCζ antibody. As shown in Fig. 6, A and B, IPR treatment resulted in an increase in amount of p-PKCζ in the membrane fraction in a time-dependent manner, suggesting PKCζ activation by IPR treatment. Next, we directly examined PKCζ kinase activity using PKCζ-specific immunoprecipitates. As Fig. 6C shows, PKCζ kinase activity increased in the acinar cells treated with IPR for 15 min (Fig. 6C). When the activation of PKCζ was examined by PKCζ translocation to the membrane, IPR treatment had less effect on PKCζ activation (Fig. 6D). These results suggested that PKCζ phosphorylation partially contributes to the IPR-induced amylase release in rat parotid acinar cells.

Fig. 3. Inhibition of IPR-induced MARCKS phosphorylation by the PKA inhibitor H89 in rat parotid acinar cells. A: after pretreatment without (Cont) or with H89 (10 μM) for 10 min, cells were stimulated by IPR (1 μM) for 10 min. MARCKS and p-MARCKS in the lysates (25 μg protein) were detected by Western blotting 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 the absence of IPR. Values are means ± SE from 3 independent experiments; *P < 0.05 vs. the absence of IPR.

Fig. 4. Inhibition of IPR-induced MARCKS phosphorylation by the PKC inhibitors in rat parotid acinar cells. A: after pretreatment with G66983 (5 μM), G66976 (5 μM), CalC (5 μM), and rottlerin (50 μM) for 10 min, cells were stimulated by IPR (1 μM) for 10 min. MARCKS and p-MARCKS in the lysates (25 μg protein) were detected by Western blotting 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 the absence of IPR. Values are means ± SE from 3 independent experiments; *P < 0.05 vs. the absence of IPR.
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 PKC8 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 PKC8 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

Fig. 5. Partial inhibition of IPR-induced amylase release by CalC and rottlerin in rat parotid acinar cells. After pretreatment with CalC (5 μM, triangles) (A), rottlerin (50 μM, squares) (B), or vehicle (circles) for 10 min, cells were incubated without (open symbols) or with IPR (1 μM, solid symbols) for the indicated times. IPR was added to the medium at the time indicated by the arrow. Amylase release is expressed as the percentage of total amylase activity. Values are means ± SE from 3 independent experiments.

Fig. 6. IPR-induced PKC activation in rat parotid acinar cells. Cells were stimulated by IPR (1 μM) for the indicated times. p-PKC (A) and PKC (D) in the membrane fraction (25 μg protein) were detected by Western blotting using an anti-p-PKC and anti-PKC antibodies, respectively. PKC activation is calculated as the ratio and is normalized against the stimulation at 0 min (B). PKC kinase activity is expressed as the percentage of control (C). Values are means ± SE from 3 independent experiments; *P < 0.05 vs. stimulation at 0 min.
Fig. 7. Inhibition of IPR-induced PKCα activation by PKA inhibitors in rat parotid acinar cells. A: after pretreatment with H89 (10 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-PKCα in membrane fractions (25 μg protein) was detected by Western blotting using an anti-p-PKCα antibody. B: PKCα phosphorylation 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.

Fig. 8. Partial inhibition of IPR-induced amylase release by myristoylated NH2-terminal sequence peptide of MARCKS (MANS) peptide in rat parotid acinar cells. After pretreatment with MANS (50 μM) or random RNS NH2-terminal sequence (RNS; 50 μM) peptides or without peptide for 15 min, cells were stimulated by IPR (1 μM) for 20 min. Amylase release is expressed as the percentage of total amylase activity. Values are means ± SE from 3 independent experiments; *P < 0.05 vs. presence of RNS peptide with 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>
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<tr>
<td>PKA</td>
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</tr>
<tr>
<td>Total PKC</td>
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<tr>
<td>PKCα</td>
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</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, δA-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 PKCζ-specific inhibitor rottlerin inhibited IPR-induced MARCKS phosphorylation (Fig. 4). In addition, PKCζ was activated by IPR but not PKCε (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 PKCζ 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 PKCζ activation is involved in the cAMP-dependent amylase release in parotid acinar cells.

Interestingly, we found that the PKA inhibitors H89 and PKI-(5-24)-peptide inhibit IPR-induced PKCζ activation, which suggests that PKCζ activation is downstream of PKA activation. There are two possible mechanisms for PKCζ activation by PKA. One possible mechanism is the phosphorylation and activation of PKCζ by PKA, although such a direct mechanism has not been previously reported. As another mechanism, it is known that PKCζ 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, and PLD activity in rat parotid acini has been reported to be stimulated 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, PKCζ appears to be activated by DAG which is induced by PLD via PKA activation, although further studies to characterize that mechanism are needed.

MARCKS has been proposed to function in the remodeling of the actin cytoskeleton through direct binding of F-actin (12). Moreover, a MARCKS effector domain peptide has also been shown to induce rapid actin polymerization in vitro (49). Phosphorylation at the effecter domain dissociates MARCKS from F-actin and results in the loss of actin cross-linking and polymerization activity (12). Since the actin network undergoes reorganization during regulated exocytosis, regulation of...
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REFERENCES
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