Involvement of myristoylated alanine-rich C kinase substrate phosphorylation and translocation in cholecystokinin-induced amylase release in rat pancreatic acini

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Satoh K, Narita T, Katsumata-Kato O, Sugiya H, Seo Y. Involvement of myristoylated alanine-rich C kinase substrate phosphorylation and translocation in cholecystokinin-induced amylase release in rat pancreatic acini. Am J Physiol Gastrointest Liver Physiol 310: G399–G409, 2016. First published January 7, 2016; doi:10.1152/ajpgi.00198.2015.—Cholecystokinin (CCK) is a gastrointestinal hormone that induces exocytotic amylase release in pancreatic acinar cells. The activation of protein kinase C (PKC) is involved in the CCK-induced pancreatic amylase release. Myristoylated alanine-rich C kinase substrate (MARCKS) is a ubiquitously expressed substrate of PKC. MARCKS has been implicated in membrane trafficking in several cell types. The phosphorylation of MARCKS by PKC results in the translocation of MARCKS from the membrane to the cytosol. Here, we studied the involvement of MARCKS in the CCK-induced amylase release in rat pancreatic acini. Employing Western blotting, we detected MARCKS protein in the rat pancreatic acini. CCK induced MARCKS phosphorylation. A PKC-δ inhibitor, rottlerin, inhibited the CCK-induced MARCKS phosphorylation and amylase release. In the translocation assay, we also observed CCK-induced PKC-δ activation. An immunohistochemistry study showed that CCK induced MARCKS translocation from the membrane to the cytosol. When acini were lysed by a detergent, Triton X-100, CCK partially induced displacement of the MARCKS from the GM1a-rich detergent-resistant membrane fractions (DRMs) in which Syntaxin2 is distributed. A MARCKS-related peptide inhibited the CCK-induced amylase release. These findings suggest that MARCKS phosphorylation by PKC-δ and then MARCKS translocation from the GM1a-rich DRMs to the cytosol are involved in the CCK-induced amylase release in pancreatic acinar cells.

exocytosis; PKC-δ; Syntaxin2; phosphorylation; lipid raft

It is known that cholecystokinin (CCK) activates a variety of intracellular signaling processes through G protein-coupled receptors and thereby regulates a complex array of cellular functions in pancreatic acinar cells (58). The mechanism most often studied is the coupling with the heterotrimeric G proteins of the Gq family to activate phospholipase C, leading to an increase in inositol triphosphate (IP3) and the release of intracellular Ca2+ (57). Along with protein kinase C (PKC) activation in response to the increase in diacylglycerol (DAG), this pathway stimulates the secretion of a digestive enzyme, amylase, by the process of exocytosis (29, 62). A specific PKC activator phorbol ester, which has a DAG-like structure, can also stimulate amylase secretion (56). It is thought that PKC plays an essential role in the CCK-induced pancreatic amylase release process.

PKC has a number of isotypes that fall into three groups: conventional PKC (cPKC: α-, βl-, βII-, γ-isotypes), novel PKC (nPKC: δ-, ε-, η-, θ-isotypes), and atypical PKC (aPKC: ζ-, η/θ-isotypes) (46). cPKC responds to DAG and Ca2+ signaling, nPKC responds to DAG, but not to Ca2+ signaling, and aPKC does not respond to either DAG or Ca2+ (34). PKC is a serine/threonine kinase involved in various exocytotic events in different cell types, including the secretion of mucin (37), insulin (61), neurotransmitters (47), and platelet-dense granules (31). In pancreatic acinar cells, PKC-α, PKC-δ, PKC-ε, and PKC-ζ isofoms have been identified (4, 26, 39). It has been shown that various PKC inhibitors, including staurosporine, H-7, and GF109203X, inhibit the secretion of amylase when stimulated by phorbol ester, CCK, and carbachol (CCh) (15, 26, 37, 56). Furthermore, it has been shown that the PKC-δ inhibitor rottlerin inhibits CCK-induced amylase release (26, 55). It has also been shown that the overexpression of PKC-δ increases CCK-induced amylase release, whereas the overexpression of the dominant-negative form of PKC-δ decreases the secretion (26). In contrast, one study showed no significant difference in the CCK-induced amylase release between PKC-δ/−/− and PKC-δ+/+ mice (55). Thus the specificity for a PKC isotype in the amylase release process has not been clarified. Although the molecular targets of the PKC action that regulate secretion are unknown, it has been shown that PKC activity is related to protein phosphorylation (10, 59, 60).

The myristoylated alanine-rich C kinase substrate (MARCKS) is a major cellular substrate of PKC (51). 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 (53), which is a phosphorylation site (20). It is well known that phosphorylated MARCKS (p-MARCKS) is translocated from the membrane to the cytosol because phosphorylation of the effector domain significantly decreases the binding force of MARCKS to the membrane (1). MARCKS translocation has been implicated in membrane trafficking, such as exocytosis. The NH2-terminal sequence peptide of MARCKS (MANS) has been shown to be a useful inhibitor of MARCKS function (28). In parotid acinar cells, stimulation of β-adrenergic receptors induces exocytotic amylase release. We previously reported the effect of the MANS peptide on amylase release in parotid acinar cells (44). This peptide partially inhibited the β-agonist-induced amylase re-
lease. Additionally, the β-agonist increased PKC-δ kinase activity and induced MARCKS phosphorylation. Rottler inhibited the β-agonist-induced MARCKS phosphorylation and amylase release. These observations suggest that MARCKS phosphorylation, via the activation of PKC-δ, is involved in the amylase release in parotid acinar cells. In asthma model mice, the MANS peptide inhibited methacholine-induced mucin secretion (28, 49). In normal human bronchial epithelial (NHBE) cells, phorbol ester-induced mucin secretion is regulated by the MARCKS phosphorylation via PKC-δ activation (37). Furthermore, it has been reported that PKC-δ has a strong affinity for MARCKS and can phosphorylate MARCKS both in vitro and in vivo (11, 16, 21). Taken together, these findings suggest that PKC-δ-MARCKS signaling mediates secretory function. In the present study, we demonstrated the involvement of MARCKS via activation of PKC-δ in the CCK-induced exocytotic amylase release in pancreatic acini.

MATERIALS AND METHODS

Ethical approval. In accordance with the established related guidelines, the experimental design of the study was approved by the Animal Offices at the Dokkyo Medical University (approval number: 0876), the Nihon University College of Bioresource Sciences (approval number: AP12B011), and the Nihon University School of Dentistry at Matsudo (approval number: AP14MD015).

Reagents. BSA, collagenase A from Clostridium histolyticum, protease inhibitor cocktail, and phosphatase inhibitor cocktail were purchased from Roche (Basel, Switzerland). Rabbit anti-MARCKS and rabbit anti-p-MARCKS antibodies, rottlerin, and GF109203X were purchased from Millipore (Temecula, CA). Rabbit anti-PKC-δ antibody was purchased from Oxford Biomedical (Oxford, MI). Anti-ribozyme IgG horseradish peroxidase (HRP)-linked antibody was purchased from Beckman Coulter (Fullerton, CA). Alexa Fluor 568-labeled goat anti-IgG antibody, Alexa Fluor 488-labeled phal-loidin, and Pro Long Gold antifade reagent with DAPI were purchased from Molecular Probes (Eugene, OR). HRP-linked cholera toxin B subunit (HRP-CTB), carbonyl cyanide m-chlorophenylhydrazone (CCCP), and trypsin inhibitor were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-Syntaxin2 antibody was purchased from Alomone Laboratories (Jerusalem, Israel). Enhanced chemiluminescence (ECL) Western blotting detection reagents was purchased from GE (Piscataway, NJ). CCK-octapeptide (sulfated) and Gö6976 were purchased from Tocris (Bristol, UK). Formalin and CCh were purchased from Wako (Osaka, Japan). Block Ace was purchased from Yukijirushi-Nyugyo (Sapporo, Japan). Trypan blue was purchased from Chroma (Stuttgart, Germany). The Bio-Rad protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). The ATP Assay kit (Colorimetric/Fluorometric) was purchased from Abcam (Cambridge, UK). The glass base dishes were purchased from Iwaki (Chiba, Japan). Cell-Tak was purchased from Becton Dickinson Labware (Bedford, MA). Phenylmethyl-sulfonyl fluoride (PMSF) was purchased from Nakalai Tesque (Kyoto, Japan).

MARCKS-related peptide. The MANS and the random NH₂-terminal sequence (RNS) peptides were synthesized as previously described (28) at Scrum (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 the insertion of MARCKS into the membranes: MA-GAQFSKTAAKGEAAEERPGEAA (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-GTAPAEGAGAEEKRASAEAKQAF. 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 (28).

Preparation of the pancreatic acini. The pancreatic acini were prepared as described previously (17, 35, 62) with some modifications. Sprague-Dawley rats (males, 200–250 g) were anesthetized intraperitoneally with pentobarbital (50 mg/kg), and the pancreas was removed and placed in a small volume of Krebs-Ringer bicarbonate (KRb) solution with the following composition: 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 1.8 mM CaCl₂, 0.96 mM Na₂HPO₄, 25 mM NaHCO₃, 5 mM HEPES (pH 7.4), and 11.1 mM glucose. The KRb solution was equilibrated with an atmosphere of 95% O₂-5% CO₂. The pancreas was minced with a razor and treated with digestive enzymes in KRb solution containing BSA (0.5%) and trypsin inhibitor (0.5 ml/ml); i.e., the tissues were incubated with collagenase A (1.5 mg/ml) at 37°C for 20 min. The suspension was passed through four layers of nylon mesh to separate the dispersed acini from undigested connective tissue, and the filtrate obtained was mixed with a double volume of KRb solution containing 4% BSA. After the suspension was centrifuged at 50 g for 5 min, the pelleted acini were finally suspended in appropriate amounts of KRb solution containing 0.5% BSA and 0.02% trypsin inhibitor.

Acini treatment. Pancreatic acini were prepared as described above and then stimulated by CCK (100 pM) at 37°C for the indicated times. When effects of inhibitors were examined, cells were pretreated with rottlerin (10 μM, 10 min), GF109203X (10 μM, 10 min), Gö6976 (10 μM, 10 min), CCCP (10 μM, 10 min), MANS peptide (1, 5, 10, 50, and 100 μM, 15 min), and RNS peptide (1, 5, 10, 50, and 100 μM, 15 min), and then CCK was added.

Preparation of lysate, membrane, and cytosol samples. The pancreatic acini lysates were obtained by homogenization using polypropylene homogenizer in lysis buffer (LB) with the following composition: 25 mM Tris-HCl buffer (pH 7.5), 10 mM MgCl₂, 0.3 M NaCl, phosphatase inhibitor cocktail, and protease inhibitor cocktail. To isolate the membrane and cytosol samples, acini were homogenized with 10 mM HEPES buffer (pH 7.2) containing 0.3 M sucrose, 2 mM EDTA, 0.2 mM EGTA, 1 mM PMSF, phosphatase inhibitor cocktail, and protease inhibitor cocktail and 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 samples, respectively. These samples were used for Western blotting. The protein concentrations of the samples were determined by a Bio-Rad protein assay kit using BSA as a standard (7).

Western blotting. The protein samples were resolved by SDS-PAGE under reducing conditions using a Mini-Protean II Electrophoresis Apparatus (Bio-Rad). After electrophoresis, the separated proteins were transferred onto a nitrocellulose filter by means of a Mini Transfer apparatus (Nihon Eido, Tokyo, Japan) following the standard procedure. The blots 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-PKC-δ (diluted 1:1,000), and anti-Syntaxin2 (diluted 1:600). The blots were washed three times with 10% Block Ace containing 0.05% Tween 20 and were then probed for 90 min with the anti-rabbit IgG (diluted 1:10,000) or HRP-CTB (diluted 1:5,000). Immunoreactivity was determined by the use of ECL Prime Western blotting detection reagents. Images were acquired using chemiluminescence film (GE) or EZ-Capture MG (ATTO, Tokyo, Japan). The intensities of the p-MARCKS and PKC-δ bands were measured with CS Analyzer 3.0 (ATTO).

Amylase release. The acini suspensions were passed through filter paper to separate the medium and the cells. The acini were then homogenized with phosphate buffer (pH 6.9) containing 0.01% Triton X-100 for the measurements of the total amylase activity. Amylase activity in the medium and the homogenates was assayed following the method described by Bernfeld (5). The amylase release was expressed as a percentage of the total amylase in the cells, as shown
Amylase release (%) = (amylase activity in the medium)/(amylase activities in homogenates + the medium) × 100.

**Immunohistochemistry.** Before and after stimulation, the 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 at room temperature for 15 min followed by blocking with 1% BSA in PBS. The acini were immunoreacted with 100-fold diluted rabbit anti-MARCKS and anti-p-MARCKS antibodies at 4°C overnight. After they were washed three times with PBS, the acini were reacted with 100-fold diluted Alexa Fluor 568-labeled goat anti-rabbit IgG antibody and a 100-fold diluted Alexa Fluor 488-labeled phalloidin in PBS at room temperature for 30 min to stain the MARCKS protein and F-actin. After the reaction, the antibody and phalloidin were removed by centrifugation (200 g for 1 min). The acini were then placed on a glass-base dish precoated with neutralized Cell-Tak to allow them to attach firmly to the dish. To stain the nucleus and to encapsulate the specimens, the acini were mounted in Pro Long Gold antifade reagent with DAPI. The stained specimens were observed under a Fluoview FV10i (Olympus, Tokyo, Japan) confocal microscope.

**Separation of DRMs of pancreatic acini.** The acini were stimulated by CCK (100 pM) at 37°C for 15 min. Before and after the stimulation, the acini were lysed with the LB (2×). The lysate was adjusted to 40% sucrose by the addition of the same volume of 80% sucrose in the LB (2×) and placed at the bottom of an ultracentrifuge tube. A step gradient of 5–30% sucrose (5% steps, 300 µl each) in LB was formed above the 40% cell lysate. The mixture was centrifuged at 250,000 g for 18 h at 4°C in a SW60Ti rotor (Beckman Coulter). Gradient columns were fractionated from the top of the column. In the immunoblotting analysis, the mixture was diluted by LB, and then detergent-resistant membrane fractions (DRMs) were recovered by centrifugation at 100,000 g for 1 h at 4°C.

**Cellular ATP.** The acini suspensions were incubated with rottlerin (10 µM) or CCCP (10 µM) for 25 min at 37°C. After incubation, a cell suspension was obtained, mixed with 4 M perchloric acid, and put on ice for 5 min. After the addition of 2 M KOH for neutralization, the mixture was centrifuged at 13,000 g for 15 min, and the supernatant was isolated. The ATP concentration in the supernatant was measured using an ATP assay kit.

**Cell viability.** The mixtures containing equal parts of acini suspensions and 0.5% Trypan blue solutions were collected, and then the Trypan blue negative-stained cells were counted using an optic microscope to estimate the number of live cells. Cell viability was expressed as a percentage of live cells to all of the cells in three fields of view (300–500 cells were counted in each view).

**RESULTS**

**CCK-induced MARCKS phosphorylation.** To assess whether CCK induces MARCKS phosphorylation in pancreatic acini, the acini were stimulated with CCK (100 pM), which had the maximal effect on amylase release in physiological conditions, for 2, 5, 10, and 15 min. The results of Western blotting using anti-MARCKS and anti-p-MARCKS antibodies clearly showed that CCK induced MARCKS phosphorylation in a time-dependent manner but did not affect the total amount of MARCKS (Fig. 1, A and B). PKC-δ has a strong affinity for MARCKS and can phosphorylate MARCKS (11, 16, 21). We next investigated the effects of a PKC-δ inhibitor (rottlerin: 10 µM), a potent pan-PKC inhibitor (GF109203X: 10 µM), and a cPKC inhibitor (Go6976: 10 µM) on the CCK-induced MARCKS phosphorylation. The pancreatic acini were pretreated with these inhibitors for 10 min and then stimulated with CCK (100 pM) for 15 min. As shown in Fig. 1, C and D, the rottlerin and GF109203X inhibited CCK-induced MARCKS phosphorylation, but the Go6976 did not inhibit this phosphorylation. These inhibitors did not affect the nonstimulated MARCKS phosphorylation or the total amount of MARCKS. These results suggest that CCK-induced MARCKS phosphorylation is evoked by nPKC activation. Additionally, it is likely that one of the PKC isotypes that evoke MARCKS phosphorylation is PKC-δ in rat pancreatic acini.

**The effect of rottlerin on CCK-induced amylase release.** We next examined the effect of rottlerin on CCK-induced amylase release. The pancreatic acini were pretreated with the rottlerin (10 µM) for 10 min and then were stimulated with CCK (100 pM) for 5, 10, and 15 min. As summarized in Fig. 2A, rottlerin partially inhibited the CCK-induced amylase release, whereas that inhibitor had no effect on the nonstimulated amylase release. It was well established that the time course of CCK-induced amylase release has two phases, with an early phase of −5 min and a late phase that can last 60 min (30). Thus it is likely that rottlerin had no effect on the early phase but inhibited the late phase (from 5 to 15 min). These results
This study was distinct from the ATP depletion. As shown in Fig. 2B, therefore, the rottlerin effect shown in an uncoupler of mitochondrial ATP synthesis (CCCP: 10 μM), whereas it was reduced by 54.30% in the absence of rottlerin (10 μM), kept for 25 min even in the presence of rottlerin (10 μM).

PKC-δ/H9254 is known that activated PKC, including PKC-δ, is phosphorylated and translocated from the cytosol to the membrane (44, 46). In the membrane fraction and the cell lysates stimulated with CCK (100 pM) for 5, 10, and 15 min, the translocation of PKC-δ was analyzed by Western blotting using an anti-PKC-δ antibody. As shown in Fig. 3, CCK stimulation resulted in an increase in the amount of PKC-δ in the membrane fraction in a time-dependent manner. In contrast, CCK stimulation did not affect the total amount of PKC-δ in the cell lysates. These results suggest that PKC-δ translocation from the cytosol to the membrane is induced by CCK treatment in rat pancreatic acini. Thus it is highly likely that PKC-δ is activated in CCK-treated pancreatic acini.

The effects of CCK on MARCKS distribution. We further examined the MARCKS distribution in rat pancreatic acini by immunohistochemistry using a confocal microscopy (Fig. 4A). In the nonstimulated acini, the fluorescence signal of the MARCKS was colocalized with the signal of F-actin, a marker protein of the apical membrane, visualized as yellow. The fluorescence of the MARCKS in the nonstimulated acini had less signal in the cytosol. When the acini were stimulated with CCK (100 pM) for 15 min, the fluorescence signals of the MARCKS were enhanced in the cytosol but showed less colocalization with the F-actin signals. These observations suggest that MARCKS is translocated from the apical membrane to the cytosol after CCK treatment. We next examined the localization of p-MARCKS by confocal microscopy (Fig. 4B). In the nonstimulated acini, the fluorescence signal of the p-MARCKS was lower throughout the cell. When the acini were stimulated with CCK (100 pM) for 15 min, the fluorescence signals of the p-MARCKS were dramatically enhanced in the cytosol. To confirm these observations, we conducted Western blotting using anti-p-MARCKS antibody. As shown in Fig. 4, C and D, CCK (100 pM) treatment for 15 min resulted in an increase in the amount of p-MARCKS in the cytosol fraction. Considering these results together, it is highly possible that MARCKS phosphorylation is induced by CCK treatment and that subsequently p-MARCKS is translocated from the membrane to the cytosol.

The distribution of MARCKS in DRM. It has been considered that the lipid raft of plasma membrane is involved in exocytosis through the function of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (12, 19, 22). SNARE proteins are enriched in lipid rafts, cholesterol, and sphingolipid-rich microdomain (42). In rat pancreatic acini, it has been demonstrated that Syntaxin2, one of the target plasma membrane SNAREs (t-SNAREs), is involved in regulated exocytosis (19). It has been demonstrated...
Fig. 4. Localization of MARCKS and p-MARCKS in rat pancreatic acini. Acini were stimulated by CCK (100 pM) for 15 min. A and B: acini were stained with anti-MARCKS (A) or p-MARCKS (B) antibodies, after which fluorescence was observed by use of a confocal microscope. Red, MARCKS visualized by Alexa 568-conjugated anti-rabbit IgG; green, F-actin visualized by Alexa phalloidin; blue, nucleus visualized by DAPI. Arrowheads indicate the colocalization of MARCKS and F-actin. Scale bars = 10 μm. C: p-MARCKS in the cytosol fraction (50 μg protein) was detected by Western blotting using an anti-p-MARCKS antibody. D: quantitation of the Western blotting data of the p-MARCKS was normalized against the absence of CCK. Values are shown as means ± SE from 3 independent experiments. Asterisks represent a significant difference compared with the absence of CCK (*P < 0.05, Student’s t-test).
that MARCKS is partially distributed in lipid rafts in HeLa cells (6) and mouse hippocampal neurons (25). We examined the distribution of MARCKS and Syntaxin2 in DRMs (Fig. 5). In DRMs stimulated with CCK (100 pM) for 15 min, the distribution of MARCKS, Syntaxin2, and ganglioside GM1a, a marker of the membrane microdomains, was analyzed by Western blotting using an anti-MARCKS antibody, an anti-Syntaxin2 antibody, and HRP-CTB, respectively. In the non-stimulated acini, the GM1a was detected in fractions 2–6. MARCKS was mainly detected in fractions 2–5. In addition, the Syntaxin2 was detected in fractions 3–5. These results indicate that a part of MARCKS is distributed in GM1a-rich DRMs in which Syntaxin2 is also distributed. In the CCK-stimulated acini, the distribution of GM1a was mainly detected in fractions 2–6. The Syntaxin2 was weakly detected in fractions 3–5. MARCKS was slightly detected in fractions 2–5. In the bottom-side fractions, MARCKS was weakly detected in fractions 6–9 (in 2 of 4 experiments) or in fractions 6–7 (in other 2 experiments, data not shown). These results suggested that a part of the MARCKS is released from the GM1a-rich DRMs, in which Syntaxin2 is distributed, to the cytosol by CCK treatment.

**The effects of MARCKS-related peptide on CCK-induced amylase release.** Several approaches can be used to determine whether MARCKS is directly involved in secretory function, such as using siRNA (27) or transgenic mice (23). Presently, it is known that MANS peptide is a useful inhibitor of the function of MARCKS because that peptide suppresses secretory function in the in vivo state (49), intact cells (44), and cultured cells (28). In the present study, the most striking feature was that pancreatic acini maintained the physiological condition nearly intact. Therefore, we employed the MANS peptide to examine whether or not the inhibition of the function of the MARCKS attributable to the MANS peptide would suppress the CCK-induced pancreatic amylase release. Pancreatic acini were pretreated with the MANS and RNS peptides for 15 min and then were stimulated with CCK (100 pM) for 5, 10, and 15 min. As shown in Fig. 6A, the MANS peptide inhibited CCK-induced amylase release at concentrations from 50 to 100 μM. In contrast, the RNS peptide showed no inhibitory effect at any concentration. In addition, the MANS (50 μM) and RNS (50 μM) peptide treatment maintained cell viability at a level higher than 95% (Fig. 6B). As shown in Fig. 6C, the MANS peptide (50 μM) resulted in a partial suppression of the CCK-induced amylase release, but that peptide had no effect on the nonstimulated amylase release. In contrast, the control peptide RNS (50 μM) had no effect on either CCK-induced or nonstimulated amylase release. These results suggest that MARCKS is involved in the late-phase amylase release (from 5 to 15 min) but not in the early-phase amylase release (<5 min).

The effects of MARCKS-related peptide on CCK-induced MARCKS translocation and phosphorylation and PKC-δ translocation. We checked the effect of MANS peptide on the CCK-induced MARCKS translocation and phosphorylation.

*Fig. 5. Separation of GM1a-rich detergent-resistant membrane fractions (DRMs) in rat pancreatic acini. Acini were stimulated by CCK (100 pM) for 15 min. The lysed acini were fractionated into 9 fractions using sucrose density ultracentrifugation. After fractionation, DRMs were recovered as precipitates of ultracentrifugation at 100,000 g for 1 h. MARCKS, GM1a, and Syntaxin2 in DRMs (5-μl sample) were detected by Western blotting analysis using anti-MARCKS antibody, horseradish peroxidase-linked cholera toxin B subunit, and anti-Syntaxin2 antibody, respectively.

Fig. 6. Inhibition of CCK-induced amylase release by NH2-terminal sequence peptide of MARCKS (MANS) in rat pancreatic acini. After pretreatment without (circles) or with MANS (triangles) or random NH2-terminal sequence (RNS) (squares) for 15 min, acini were incubated without (open symbols) or with CCK (100 pM, solid symbols) for 15 min (A) or the indicated times (B and C). A: effect of MANS and RNS on CCK-induced amylase release in the concentration-response curve; x-axis is indicated as logarithmic scale. B: effect of the MANS (50 μM) and RNS (50 μM) on cell viability. C: effect of MANS (50 μM) and RNS (50 μM) on CCK-induced amylase release in the time-response curve. Amylase release was expressed as the percentage of the total amylase activity. Cell viability was expressed as the percentage of Trypan blue-negative cells. CCK was added at 0 min, indicated by the arrow. Values are shown as means ± SE from 3 independent experiments. Asterisks represent a significant difference compared with the corresponding concentration of RNS (A) or period of the CCK stimulation with RNS (C) (*P < 0.05, Student’s t-test).
and PKC-δ translocation. Pancreatic acini were pretreated with the MANS (50 μM) or RNS peptide (50 μM) for 15 min. MARCKS distribution in acini was observed by immunohistochemistry using a confocal microscopy (Fig. 7, A and B). In the nonstimulated acini with MANS peptide, MARCKS was colocalized with F-actin visualized as yellow (Fig. 7A). The fluorescence of the MARCKS in the nonstimulated acini showed a lower signal in the cytosol. When the acini were stimulated with CCK (100 pm) for 15 min, the fluorescence signals of the MARCKS were enhanced in the cytosol but showed less colocalization with the F-actin signals. The MARCKS distribution in the acini pretreated with MANS peptide showed no difference from the acini pretreated with RNS peptide (Fig. 7B), with or without CCK treatment. These observations suggested that the MANS peptide had no effect on MARCKS translocation. As shown in Fig. 7, C and D, neither of the peptides, MANS or RNS, showed any effect on the amount of MARCKS and p-MARCKS, with or without CCK treatment. These results suggest that the MANS peptide had no effect on MARCKS phosphorylation. As shown in Fig. 7, E and F, neither of these peptides showed any effect on the amount of PKC-δ with CCK treatment in either the membrane fraction or the cell lysates. In addition, when both the membrane fraction and cell lysates were pretreated with these peptides, the amount of PKC-δ showed no change without CCK treatment (data not shown), suggesting that the MANS peptide had no effect on the PKC-δ activation. Therefore, these results indicate that MANS peptide did not affect the CCK-induced MARCKS translocation and phosphorylation or PKC-δ translocation.

**DISCUSSION**

In the present study, we demonstrated that 1) CCK induced MARCKS phosphorylation via PKC-δ activation, 2) CCK induced MARCKS translocation from the membrane lipid rafts to the cytosol, and 3) MANS peptide inhibited CCK-induced amylase release in rat pancreatic acini. Consequently, it is likely that MARCKS regulates pancreatic amylase release (as illustrated schematically in Fig. 8).

**PKC-δ-MARCKS signaling activated by CCK.** Secretagogue-induced MARCKS phosphorylation has been reported in several cell types. In SH-SY5Y human neuroblastoma cells, phorbol ester induces norepinephrine release and MARCKS phosphorylation (18). In chromaffin cells, PMA induces norepinephrine release and MARCKS phosphorylation (41). In bovine luteal cells, prostaglandin F₂α stimulates MARCKS phosphorylation and oxytocin secretion (43). In insulin-producing INS-1 cells, glucagon-like peptide-1 induces insulin secretion and MARCKS phosphorylation (52). Moreover, it has been reported that the secretagogue-induced MARCKS phosphorylation occurs via PKC-δ activation in BON human pancreatic carcinoid tumor cells (27), NHBE cells (37), and rat parotid acinar cells (44). We demonstrated that MARCKS phosphorylation was induced by CCK treatment in rat pancreatic acini (Figs. 1 and 4, B–D). Wishart et al. (59) previously
reported that one of the phosphorylated proteins is coincident with MARCKS in rat pancreatic acini stimulated with CCK. This observation strongly supports our results. We also demonstrated that the CCK-induced MARCKS phosphorylation is inhibited by the PKC-δ inhibitor rottlerin, as well as the potent pan-PKC inhibitor GF109203X (Fig. 1C). In contrast, the PKC-α and -βI inhibitor Go6976 did not inhibit the CCK-induced MARCKS phosphorylation (Fig. 1C). Furthermore, CCK treatment induced PKC-δ translocation from the cytosol to the membrane (Fig. 3). It is well accepted that translocation to a membrane is tightly associated with the activation of PKC (44, 46), indicating that the PKC-δ activation occurs with CCK treatment. It has been reported that the PKC-δ has a strong affinity for MARCKS and can phosphorylate MARCKS (11, 16, 21). Therefore, it is likely that MARCKS phosphorylation is induced by CCK through PKC-δ activation in rat pancreatic acinar cells.

The involvement of PKC-δ in CCK-induced amylase release. Although rottlerin has been widely used as a specific PKC-δ inhibitor, it has also been reported that this polyphenolic compound causes a general inhibition of the mitochondrial metabolism, which is correlated with a depletion of the cellular ATP concentration (50, 54). In pancreatic acinar cells incubated with rottlerin (8 μM) for 120 min, it was reported that the CCK-induced amylase release was attenuated through a decrease in the cellular ATP level (54). In contrast, we used acini incubated with 10 μM rottlerin for 25 min in the present experiments (Figs. 1C and 2). Therefore, we checked the ATP level but found that rottlerin did not reduce the ATP level in the cells under our experimental conditions (Fig. 2B). The cell viability determined by Trypan blue extrusion was maintained at >90% (data not shown). Because it has been considered that rottlerin has different effects on cell survival depending on the incubation time and the cell number (50, 54), our experimental conditions probably allow for maintenance of the cell viability. Therefore, it is unlikely that the inhibition of CCK-induced MARCKS phosphorylation by rottlerin was caused by a toxic effect, suggesting that the CCK-induced MARCKS phosphorylation was evoked by PKC-δ activation. The expression of other PKC isoforms, PKC-α, PKC-ε, and PKC-ζ, was also confirmed in pancreatic acinar cells (4, 26, 39). The translocation of the PKC-ζ from the cytosol to the membrane was not detected in CCK-treated rat pancreatic acini (26). The cPKC inhibitor did not affect the CCK-induced amylase release (26). The dominant-negative form of PKC-ε did not decrease amylase secretion, whereas PKC-δ showed a decrease using an overexpression approach (26). Therefore, it is likely that PKC-δ contributes significantly to CCK-induced amylase secretion. However, there was no significant difference in the amylase release when comparing PKC-δ−/− and wild-type (PKC-δ+/+) mice (55). Moreover, the overexpression of PKC-ε increased CCK-induced amylase secretion (26). Thus the main factor of amylase release is, not only PKC-δ, but also other factors such as PKC-ε, and they are complementary to each other through the signal transductions. We demonstrated that rottlerin had no effect on the early phase, <5 min, but it inhibited the late phase of the amylase release from 10–15 min (Fig. 2A). This time course was coincident in both the MARCKS phosphorylation (Fig. 1A) and the PKC-δ translocation (Fig. 3) experiments. It has been reported that the high-rate/low-dose secretion in the early phase is mediated by Ca2+, whereas the low-rate/high-dose secretion in the late phase is mediated by PKC (9, 26, 30). Therefore, the involvement of PKC-δ in CCK-induced amylase release may be considered only in the late phase but not in the early phase.

MARCKS translocation induced by CCK. MARCKS phosphorylation displaces the CCK protein from the membrane. When the p-MARCKS are dephosphorylated, the CCK protein is reassociated with the membrane. MARCKS binds actin and has been implicated in the arrangement of the actin cytoskeleton (1). The exocytosis process is regulated by the actin dynamics. Thus it is considered that MARCKS is involved in exocytosis through the actin skeleton. It has been proposed that MARCKS functions in the remodeling of the actin cytoskele-
ton through the direct binding of F-actin (20). Phosphorylation in the effector domain dissociates MARCKS from F-actin, resulting in the loss of actin cross linking and polymerization activity (20). In NHBE cells, it has been suggested that MARCKS functions as a molecular linker by interacting with granule membranes in its NH2-terminal domain and binding to actin filaments in its effector domain, thus tethering granules to the actin cytoskeleton for movement and exocytosis (2, 28). In SH-SY5Y cells (18), chromaffin cells (41), and luteal cells (43), the MARCKS translocation from the membrane to the cytosol interferes with MARCKS binding to F-actin. Recently, it has been reported that MARCKS plays a role as an actin-anchoring protein in exocytosis (40). Moreover, it has been considered that F-actin coating of granules may be involved in the stabilization of exocytosis in pancreatic acinar cells (33). In this study, we clearly demonstrated that MARCKS translocation from the apical membrane to the cytosol was induced in the pancreatic acini stimulated with CCK (Fig. 4A). These observations suggested that MARCKS translocation from the membrane to the cytosol is involved in CCK-induced amylase release.

The function of MARCKS in lipid rafts. Sphingolipids and cholesterol promote liquid-ordered phase formation within model membranes (3). These membrane microdomains are called lipid rafts, and they might function as a scaffold for signal transduction and vesicular transport (48). Presently, the biochemical studies conducted to characterize membrane microdomains largely depend on the analysis of detergent-resistant membranes DRMs (8). We demonstrated that MARCKS was displaced from the GM1a-rich DRMs in the CCK-treated pancreatic acini (Fig. 5). In nonstimulated acini, Syntaxin2 was detected in the GM1a-rich DRMs in which MARCKS was also distributed (Fig. 5). Syntaxin2 is known as one of the t-SNAREs. It is often assumed that secretory granules need to be docked at the plasma membrane, and SNAREs must be preassembled before exocytosis is triggered (22). It has been reported that SNAREs, such as Syntaxin1A, SNAP-25, and VAMP2, were distributed in DRMs in PC12 cells and that the depletion of cholesterol reduced the release of neurotransmitters from neuroendocrine cells (12, 24). The relationship between exocytosis and membrane microdomains was also indicated in exocrine pancreatic cells (45). Syntaxin2, 3, 4, 7, and 8 have been identified in pancreatic acinar cells (38). Butylinum neurotoxin C, which cleaved Syntaxin2 and (to a lesser extent) Syntaxin3, inhibited exocytotic amylase release in rat pancreatic acini (38). Syntaxin2 is the candidate factor in sequential exocytosis in the pancreatic exocrine gland (32), suggesting that Syntaxin2 is involved in the pancreatic amylase release. However, the regulation of SNAREs has not been clarified. In the SNARE hypothesis, a complex formation between t-SNAREs and vesicle SNAREs is required for fusion pore opening in apical membranes (32). Considered together, these findings suggest that MARCKS displacement from the membrane lipid rafts might play a role in exocytosis via the regulation of SNAREs such as Syntaxin2.

The contribution of MARCKS to exocytosis. The MANS peptide is a myristoylated cell permeable peptide corresponding to the first 24 amino acids of MARCKS that inhibit MARCKS function (28). We demonstrated that CCK-induced amylase release was inhibited in rat pancreatic acini pretreated with the MANS peptide (Fig. 6, A and C). Briefly, that peptide had no effect on the secretion in the early phase, <5 min, but it inhibited the function of MARCKS in the late phase, from 10–15 min (Fig. 6C). This time course is coincident with the MARCKS phosphorylation (Fig. 1A) experiment, suggesting that MARCKS is involved in CCK-induced amylase release in the late phase. The amylase release is induced by a muscarinic agonist CCh as well as CCK in rat pancreatic acini (29, 62). The MANS peptide (50 μM) inhibited amylase release induced by CCh (10 μM) stimulation (data not shown), implying that the MARCKS is generally involved in secretagogue-induced pancreatic amylase release. However, receptors and Ca2+ mobilization are different between CCK and CCh stimulation (62). Therefore, signal transduction of MARCKS phosphorylation and translocation should be considered separately. Further studies are needed to check the involvement of MARCKS in CCh-induced amylase release. Moreover, the effect of MANS peptide on MARCKS function has not been clarified. Chen et al. reported that MANS peptide suppresses MARCKS phosphorylation in NHBE cells (13). In contrast, it has been reported that MANS peptide had no effect on MARCKS phosphorylation in neutrophils (14) and fibroblasts (36). In addition, treatment with MANS peptide resulted in MARCKS translocation from membrane to cytosol in neutrophils under resting conditions (14). It is likely that the effect of MANS peptide on MARCKS function depends on the cell type. We checked the effect of MANS peptide on MARCKS translocation and phosphorylation and PKC-δ translocation in pancreatic acini (Fig. 7). The MANS peptide did not affect these cellular kinetics, with or without CCK treatment. We consider that MANS peptide occupies specific MARCKS binding sites on the membrane. The membrane then becomes stable in a resting condition, compared with the CCK-induced secreting condition. As shown in Fig. 5, a relationship between MARCKS and lipid raft was implied in pancreatic acini. Thus MARCKS translocation might perhaps be a switch between the resting and secreting conditions of the membrane through lipid rafts. Further studies are needed to fully characterize the function of MANS peptide on cellular kinetics.

In the present study, we demonstrated that MARCKS phosphorylation is involved in the CCK-induced amylase release in rat pancreatic acini. When pancreatic acini are stimulated by CCK, MARCKS phosphorylation occurs via PKC-δ activation, and subsequently MARCKS translocation occurs from the membrane lipid rafts to the cytosol. The lipid rafts that release the MARCKS might contribute to the exocytotic amylase release.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
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