Involvement of RhoA and its interaction with protein kinase C and Src in CCK-stimulated pancreatic acini

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Involvement of RhoA and its interaction with protein kinase C and Src in CCK-stimulated pancreatic acini. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G915–G923, 1999.—We evaluated intracellular pathways responsible for the activation of the small GTP-binding protein Rho p21 in rat pancreatic acini. Intact acini were incubated with or without CCK and carbachol, and Triton X-100-soluble and crude microsomes were used for Western immunoblotting. When a RhoA-specific antibody was used, a single band at the location of 21 kDa was detected. CCK (10 pM–10 nM) and carbachol (0.1–100 µM) dose dependently increased the amount of immunodetectable RhoA with a peak increase occurring at 3 min. High-affinity CCK-A receptor agonists JMV-180 and CCK-OPE (1–1,000 nM) did not increase the intensities of the RhoA band, suggesting that stimulation of RhoA is mediated by the low-affinity CCK-A receptor. Although an increase in RhoA did not require the presence of extracellular Ca2+, the intracellular Ca2+ chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-AM abolished the appearance of the RhoA band in response to CCK and carbachol. The Gq protein inhibitor G protein antagonist-2A (10 µM) and the phospholipase C (PLC) inhibitor U-73122 (10 µM) markedly reduced RhoA bands in response to CCK. The protein kinase C (PKC) activator phorbol ester (10–1,000 nM) dose dependently increased the intensities of the RhoA band, which were inhibited by the PKC inhibitor K-252a (1 µM). The pp60-src inhibitor herbimycin A (6 µM) inhibited the RhoA band in response to CCK, whereas the calmodulin inhibitor W-7 (100 µM) and the phosphoinositide 3-kinase inhibitor wortmannin (6 µM) had no effect. RhoA was immunoprecipitated with Src, suggesting association of RhoA with Src. Increases in mass of this complex were observed with CCK stimulation. In permeabilized acini, the Rho inhibitor Clostridium botulinum C3 exoenzyme dose dependently inhibited amylase secretion evoked by a Ca2+ concentration with an IC50 of C3 exoenzyme at 1 ng/ml. We concluded that the small GTP-binding protein RhoA p21 exists and appears to mediate pancreatic enzyme secretion evoked by cholecystokinin (CCK) and carbachol. The activation of RhoA involves the sequential stimulation of protein kinase C (PKC) and Src kinase cascades via the Gq protein and phospholipase C (PLC) pathway.

METHODS

Materials. Chemicals were purchased from the following sources. CCK-8, carbachol, creatinine phosphohatase, creatine phosphokinase, ATP, 12-O-tetradecanoylphorbol 13-acetate (TPA), and wortmannin were from Sigma Chemical (St. Louis, MO). K-252a, W-7, and 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-AM (BAPTA-AM) were from Calbiochem (La Jolla, CA). Clostridium botulinum C3 exoenzyme, U-73122, G protein antagonist-2A (GP antagonist-2A), and herbimycin A were from Biomol (Plymouth Meeting, PA). Anti-RhoA mouse monoclonal antibody was from Research Plus (Bayonne, NJ). Protein G-Sepharose was from Pharmacia Biotech (Uppsala, Sweden). Streptolysin O (STO) was from Gibco BRL (Grand Island, NY). JMV-180 and CCK-OPE were from Neotrace (Uppsala, Sweden).

Isolation of pancreatic acinar cells. Isolated rat pancreatic acini were prepared by collagenase digestion with pancreas obtained from male Sprague-Dawley rats (33). Acini obtained were suspended in a physiological salt solution (PSS). The PSS contained 0.1% bovine serum albumin, 0.1 mg/ml soy-
bean trypsin inhibitor, and (in mM) 137 NaCl, 4.7 KCl, 0.56 MgCl₂, 1.28 CaCl₂, 1 NaH₂PO₄, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 5.5 d-glucose, and 2 L-glutamine with Eagle’s minimum essential amino acid neutralized with NaOH. The PSS was adjusted to pH 7.4 and equilibrated with 100% O₂. Isolated acini were preincubated for 40 min at 37°C in 40 ml of PSS, washed twice, and resuspended in 40 ml of fresh PSS.

Preparation of SLO-permeabilized pancreatic acinar cells and measurement of amylase secretion. Permeabilized pancreatic acinar cells were prepared as described previously (31). Dispersed pancreatic acini suspended with the fresh PSS were washed twice and resuspended with 40 ml of cytosol buffer. The cytosol buffer (pH 7.2 at 37°C) contained 0.2% bovine serum albumin, 1 mM ATP, 1 mM creatine phosphate, 25 µg/ml creatine phosphokinase, and (in mM) 20 NaCl, 0.5 MgSO₄, 100 KCl, 0.2 NaH₂PO₄, 0.8 Na₂HPO₄, 10 HEPES, and 10 glucose. One hundred nanomolar free-Ca²⁺ solution was prepared with Ca²⁺-Mg²⁺-ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) buffer (in mM: 2.55 CaSO₄, 1.64 MgSO₄, and 5 EGTA). Solutions with different Ca²⁺ concentrations ([Ca²⁺⁺]; 100 pM to 2 µM) were prepared by altering concentrations of both CaSO₄ and MgSO₄ in the cytosol buffer, which contained 5 mM EGTA. Aliquots of SLO (1 U/ml) were added to a 40-ml cell suspension that was suspended in 100 mM Ca²⁺ cytosol buffer, incubated for 20 min, and gassed with 100% O₂. After centrifugation (50 g), cells were washed twice, resuspended with a 20-ml fresh and SLO-free cytosol buffer (100 nM Ca²⁺), and incubated in the presence or absence of various concentrations of C3 exoenzyme for 30 min at 37°C. After centrifugation and removal of C3 exoenzyme, aliquots of the acinar cell suspension (2 ml each < 10 samples) were then incubated with buffers containing various [Ca²⁺⁺] gassed with 100% O₂ for 30 min at 37°C. The incubation was terminated by centrifugation (10,000 rpm) for 50 s at 4°C in a microcentrifuge. The amylase that released into the supernatant (1 ml of PSS × 2 in 10 groups) and that remained in the pellet (1 ml of Triton X-100 × 2 in 10 groups) in each microcentrifuge was assayed by using procion yellow starch as a substrate. Amylase secretion was expressed as the percentage of the total content in each sample.

Immunoprecipitation. Acinar cells (2 × 10⁶) in 1 ml of PSS were incubated with reagents for the indicated periods. The incubation was stopped with 1 ml of chilled 8 mM HEPES buffer (pH 7.4) containing (in mM) 1 sodium orthovanadate, 0.5 Na₂HPO₄, 109.5 NaCl, 4.7 KCl, and 1.13 MgCl₂. The suspension was immediately centrifuged (10,000 rpm) for 15 s at 4°C. The supernatant was discarded, and the resultant pellet was resuspended in 150 µl of lysis solution (pH 7.4) containing (in mM) 66.7 β-glycerophosphate, 1 sodium orthovanadate, 1 phenylmethylsulfonyl fluoride, 1.5 EGTA, 1 dithiothreitol, 1% Triton X-100, 10 mg/ml leupeptin, and 0.05 TIU/ml aprotinin. Each suspension was sonicated, vortexed for 30 s at 4°C, and centrifuged at 10,000 rpm for 10 min. The supernatant (25 µl, ~80 µg protein), which contained crude microsomes, was mixed with 2.5 ml of 100% mercaptoethanol and 12.5 µl of Laemmli buffer (pH 7.8), containing 62.5 mM Tris base, 2.3% SDS, 7.5% glycerol, and 0.1% bromphenol blue. Protein was determined by the method of Bradford (3). The solution was heated at 95°C for 5 min and separated by SDS-PAGE on 15% polyacrylamide gels and electrophoretically transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA) for 1 h at 100 V at 4°C. Immunoblotting was performed with a mouse monoclonal RhoA antibody or a rabbit polyclonal RhoB antibody (1 µg/ml). The membrane was then incubated with peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (1:3,000 dilution). The blots were developed and visualized with a chemiluminescent horseradish peroxidase substrate (enhanced chemiluminescence, Amersham, Arlington Heights, IL).

RESULTS

CCK-8 and carbachol but not high-affinity CCK-A-receptor agonists increased the density of the immunodetectable RhoA band. When we applied the RhoA (26C4) mouse monoclonal IgG, a single band at the location of 21 kDa was detected in unstimulated rat pancreatic acinar cells (Fig. 1). Treatment of pancreatic acini with CCK-8 (10 pM to 10 nM) for 20 min resulted in a quantitative increase in the intensities of the RhoA p21 (1–1,000 nM) did not increase the intensities of the RhoA band. Similarly, carbachol (0.1–100 µM) also increased the immunodetectable RhoA bands in a dose-dependent manner (Fig. 1B). On the other hand, the high-affinity CCK-A agonists JMV-180 and CCK-OPC (1–1,000 nM) did not increase the intensities of the RhoA band over basal (Fig. 1, C and D). Densitometry data from the scanned gels are summarized in Fig. 2. These results indicate that stimulation of the M₃ muscarinic receptor as well as the low- (but not high-) affinity CCK-A receptor resulted in a quantitative increase in RhoA in crude microsomes. CCK-8 at 1, 10, and 100 pM increased the density of the RhoA band with a peak increase occurring at 3 min, which was sustained up to 20–30 min (Fig. 3). Note that all immunoblotting or immunoprecipitation data presented in Figs. 1–9 were performed with the same experimental conditions (e.g., exposure time and amounts of protein). Each figure was prepared from data conducted on the same series of experiments.

RhoB could not be detected in pancreatic acini. To determine whether other members of the subfamily of Rho proteins are present in rat pancreatic acini, we...
performed Western immunoblotting using RhoB and RhoA antibodies. It should be noted that the RhoA monoclonal antibody does not cross-react with RhoC, RhoG, Rac1, Rac2, or Cdc42Hs proteins, whereas the RhoB polyclonal antibody has no cross-reactivity with RhoA, RhoC, or other members of the Ras gene superfamily (tested by Santa Cruz Biotechnology). In contrast to RhoA, no RhoB bands were detected in unstimulated and CCK-stimulated pancreatic crude microsomes (Fig. 4).

Stimulation of RhoA was independent of extracellular \([\text{Ca}^{2+}]\) but required intracellular \([\text{Ca}^{2+}]\). We next examined the requirement of \([\text{Ca}^{2+}]\) on CCK-stimulated RhoA activation. Elimination of extracellular \([\text{Ca}^{2+}]\) (by the addition of 1 mM EGTA plus 0 CaCl\(_2\) in the PSS) did not inhibit mass increases in RhoA in response to various concentrations of CCK-8 and carbachol (Fig. 5, A–D). On the other hand, preloading of pancreatic acini with the intracellular \([\text{Ca}^{2+}]\) chelator BAPTA-AM at 100.
µM for 10 min abolished the immunodetectable RhoA band in CCK- and carbachol-stimulated pancreatic crude microsomes (Fig. 5, E and F, respectively). These results suggest that stimulation of RhoA is independent of extracellular \([\text{Ca}^{2+}]_o\) but requires intracellular \([\text{Ca}^{2+}]_i\).

Increases in immunodetectable RhoA were mediated by the Gq protein and PLC pathway. We have recently demonstrated that, depending on the CCK agonists used, the CCK-A receptor in rat pancreatic acini is coupled to three different effectors and/or signal transduction pathways (30–32, 34, 35). The low-affinity CCK-A receptor is coupled to the conventional Gq/PLC-β pathway, resulting in the production of d-myo-inositol 1,4,5-trisphosphate (to release Ca\(^{2+}\) and activate calmodulin) and diacylglycerol (to activate PKC). In addition, the low-affinity CCK-A receptor also appears to be coupled to the nonreceptor protein tyrosine kinase pathway, in which pp60-src-phosphoinositide 3 kinase (PI 3-kinase) are the key enzymes in mediating \([\text{Ca}^{2+}]_i\)-dependent pancreatic exocytosis. On the other hand, the high-affinity CCK-A receptor is coupled to the Gq\(_b\)-cytosolic phospholipase A\(_2\) (cPLA\(_2\)) pathway to produce arachidonic acid, which enhances intracellular Ca\(^{2+}\) oscillations. To determine which pathways are involved in functional roles of the RhoA protein, we examined the effects of specific inhibitors of these three signal transduction pathways. The specificity of these inhibitors has been previously determined (30–32, 34, 35). The G protein antagonist GP antagonist-2A (10 µM), which selectively inhibits the activation of Gq, and the PLC inhibitor U-73122 (10 µM) inhibited the intensities of the RhoA band evoked by CCK-8 (Fig. 6, A and B). In contrast, the calmodulin kinase inhibitor W-7 (100 µM) and the PI 3-kinase inhibitor wortmannin (6 µM) had no effect (Fig. 6, C and E). The Src inhibitor herbimycin A (6 µM) decreased the RhoA band in response to CCK-8 (Fig. 6D). In separate experiments, we showed that the PKC activator TPA (10–1,000 nM) increased the intensities of the RhoA band, whereas the PKC inhibitor K-252a, a substance closely related to staurosporine, inhibited the action of CCK-8 (Fig. 7, A and B). Densitometry data from these results are shown in Fig. 8. These results suggest that increases in immunodetectable RhoA are mediated by the stimulation of PKC and Src cascades via the Gq protein and PLC pathway.

RhoA was immunoprecipitated with Src. To investigate possible interactions between RhoA and Src, we performed immunoprecipitation studies. Previously, we...
demonstrated that CCK stimulated phosphotransferase activities of Src in rat pancreatic acini (34). This study showed that immunoblotting with Src antibody revealed the presence of a single band corresponding to p60 in crude microsomes (Fig. 9A). This band was quantitatively increased in response to CCK-8 stimulation in a dose-dependent manner. Association of Src with RhoA was subsequently examined by the probing of Src immunoprecipitates with RhoA antibody or vice versa (i.e., probing of RhoA immunoprecipitates with Src antibody). Each immunoprecipitation revealed the presence of the band corresponding to p60 in crude microsomes (Fig. 9A) and pp60<br>
c-src (Fig. 9B). CCK-8 (10 pM - 10 nM) caused a quantitative increase in these bands in a dose-dependent manner (Fig. 9D). These observations suggest that Src and RhoA formed an immunocomplex and CCK-8 enhanced a significant increase in this complex.

C3 exoenzyme inhibited amylase secretion in SLO-permeabilized pancreatic acini. We next examined the functional role of RhoA in the mediation of pancreatic enzyme secretion. The Clostridium botulinum C3 exoenzyme has been shown to selectively ADP-ribosylate RhoA, -B, and -C, but not Rac and Cdc42, at the asparagine-41 residue, blocking their action (1, 20, 21, 26). In rat pancreatic acini, it has been shown that C3 exoenzyme inhibited the increase of immunodetectable RhoA in response to CCK-8 without affecting the production of inositol polyphosphates (15). Because C3 exoenzyme is not cell permeable (tested by Biomol), we evaluated the effect of C3 exoenzyme in SLO-permeabilized pancreatic acini. Increasing [Ca2+]1 from 100 pM to 2 µM resulted in an increase in amylase secretion (in %total/30 min) from 6.3 ± 0.6 (n = 13 determinations) to 9.7 ± 0.7 (P < 0.005; n = 17 determinations). Pretreatment of permeabilized acini with an inhibitor of Rho, C3 exoenzyme, for 30 min followed by incubation with 2 µM Ca2+ for 30 min at 37°C resulted in a concentration-dependent inhibition of amylase secretion with an IC50 of C3 exoenzyme at 1 ng/ml and the maximal inhibition occurring at 10 ng/ml (Fig. 10A). This IC50 value is one log unit lower than that required to inhibit activities of the Rho subfamily in other cell types (1, 21, 26). On the other hand, C3 exoenzyme did not affect the minimum amylase secretion stimulated by 100 pM Ca2+, which was lower than the basal cytosolic [Ca2+] (Fig. 10A). Increasing [Ca2+] from 0.2 to 2 µM in the absence of C3 exoenzyme resulted in a significant increase in amylase secretion. This [Ca2+] dependent amylase secretion was significantly inhibited by 10 ng/ml of C3 exoenzyme (Fig. 10B). These observations suggest that RhoA is involved in [Ca2+] dependent pancreatic exocytosis.

**DISCUSSION**

Several lines of evidence suggest that the monomeric small GTP-binding protein Rho plays an important role in mediating signal transduction in various cell types (16). In this study, we demonstrated the existence of Rho protein in rat pancreatic acini and its functional role in mediating pancreatic enzyme secretion. We showed that, with the use of an anti-RhoA mouse monoclonal antibody (26C4), a single band (p21) could
be detected by Western immunoblotting during the basal state in rat pancreatic acini. On stimulation with CCK-8 or carbachol, an increase in RhoA band intensities was observed. Peak increases in the p21 RhoA band were observed when intact acini were stimulated with submaximal concentrations of CCK-8 (≤1 nM). Reduction of the intensities of the RhoA band occurred with supramaximal concentrations of CCK-8 (>1 nM). This phenomenon is similar to the biphasic pancreatic amylase secretion stimulated by CCK-8, suggesting a functional relationship between RhoA and amylase secretion. If RhoA mediates cytoskeletal assembly, it is conceivable that high concentrations of CCK-8 may disrupt the cytoskeletal system, resulting in the reduction of enzyme secretion (19, 22).

In several cell types, it has been shown that the Rho protein may translocate from the cytosol to the plasma membrane during receptor activation (13, 14, 27, 36). Because we used Triton X-100-soluble and crude microsomal fractions (10,000 rpm, supernatant of the cell sonicates) for immunoblotting, it is unclear whether quantitative increases in the RhoA band during cell stimulation are due to translocation of RhoA from the cytosol to the plasma membrane or actual activation of membrane RhoA proteins. One possibility is that RhoA may translocate from Triton X-100-insoluble to-soluble fractions after cell stimulation. Because RhoA but not RhoB proteins were increased during CCK and carbachol stimulation, it appears that activation of the Rho family by these agonists is protein specific. Our studies also demonstrated that [Ca^{2+}]_o is not necessary for increases in RhoA in response to either CCK or carbachol. On the other hand, application of the intracellular Ca^{2+} chelator BAPTA-AM significantly inhibited the appearance of the RhoA band in response to CCK or carbachol stimulation, suggesting that [Ca^{2+}]_o, at the basal level, is required for stimulation of RhoA.

The interrelationship between Rho and other serine/threonine kinases or tyrosine kinases may vary in different cell systems. For example, the tyrosine kinase pp60^{src} regulates rearrangement of actin cytoskeleton and p190Rho-GTPase activating protein (GAP) after stimulation by epidermal growth factor in fibroblast cell lines (6). On the other hand, it has been shown that, in Swiss 3T3 cells, translocation of Src kinase to the cell periphery is mediated by the actin cytoskeleton under the control of the Rho family (12). Furthermore, activation of PI 3-kinase in Swiss 3T3 cells and platelets is dependent on Rho, suggesting that PI 3-kinase lies downstream of Rho (20, 36). These observations suggest that there is a close interaction between Rho and protein tyrosine kinases. On the other hand, in several cell types, the serine/threonine kinase PKC has been shown to activate Rho (28, 29). Our studies demonstrated that, in rat pancreatic acini, increases in immunodetectable RhoA are mediated by Gq and PLC pathways via the PKC cascade but not calmodulin pathways. Furthermore, we showed that, in contrast to CCK-8 and carbachol, high-affinity CCK-A-receptor agonists, which fail to stimulate PKC (35), do not increase Rho. Thus it appears that RhoA activation may depend on PKC stimulation at the basal level of [Ca^{2+}]_o rather
than on the Ca\textsuperscript{2+} signal frequency and amplitude (Ref. 4 and this study). In addition, in response to CCK-8 stimulation, PKC translocation (30 s) precedes Rho activation (3 min), suggesting that PKC lies upstream of Rho (Refs. 4 and this study). It is still unknown, however, whether PKC directly or indirectly mediates increases in immunodetectable Rho. In rat pancreatic acini, it has been shown that CCK stimulates the formation of Shc-Grb2-SOS complex through a PKC-dependent mechanism (7). It is conceivable that this complex may activate Rho to form "GTP-bound Rho" in a manner similar to Ras (7, 10). Because PKC is a serine/threonine kinase and Shc is a tyrosine-phosphorylated protein, the precise mechanism by which PKC activates Shc remains to be determined.

Alternatively, our study showed that RhoA is also regulated by pp60\textsuperscript{c-src}. This is because herbinycin A, which abolishes Src kinase activities in response to CCK in rat pancreatic acini (34), inhibits RhoA intensities stimulated by CCK. Because RhoA was coimmunoprecipitated with Src, RhoA may interact with Src. The precise mechanism by which Src forms the immunocomplex with Rho remains to be determined. Because Rho does not possess Src homology domains, this interaction may be mediated by Rho-GAP, which possesses Src homology domains. Indeed, p190Rho-GAP is considered to be a preferred substrate of c-Src in fibroblast cell lines (6). This possibility is supported by the fact that Ras-GAP is known to be associated with pp60\textsuperscript{c-src}, and the observation that tyrosine phosphorylation of Ras-GAP (by Src) decreases its ability to enhance the

Fig. 9. Effects of CCK-8 on Src and association of Src with RhoA. Acini were incubated with CCK-8 for 20 min at 37°C. Triton X-100-soluble and crude microsomes (10,000 rpm supernatant of sonicates) were immunoprecipitated (IP) with anti-Src antibody (AB), analyzed by SDS-PAGE, and immunoblotted (IB) with either anti-Src AB (Src-AB; A) or anti-RhoA AB (Rho-AB; B). C: RhoA-AB was immunoprecipitated and then Src-AB was used for blotting. D: quantification of intensities of Src and RhoA bands was performed by scanning densitometry. Data are means ± SE from 4 separate experiments.

Fig. 10. Effects of Rho inhibitor Clostridium botulinum C3 exoenzyme on amylase secretion with streptolysin O (SLO)-permeabilized pancreatic acini. A: intact acini were permeabilized by 1 U/ml SLO for 20 min at 37°C in 10\textsuperscript{-7} M Ca\textsuperscript{2+} cytosol buffer. After washout of SLO, acini were incubated with various concentrations of C3 (20 min; 37°C) at 10\textsuperscript{-7} M Ca\textsuperscript{2+} and permeabilized cells were resuspended in either 10\textsuperscript{-10} M or 2 × 10\textsuperscript{-6} M Ca\textsuperscript{2+} cytosol buffer. Amylase secretion was measured at 30 min. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control (C3 at 0 ng/ml).

B: intact acini were permeabilized by 1 U/ml SLO for 20 min at 37°C at 10\textsuperscript{-7} M Ca\textsuperscript{2+} cytosol buffer. After washout of SLO, acini were incubated with various concentrations of Ca\textsuperscript{2+} (2 × 10\textsuperscript{-7} to 2 × 10\textsuperscript{-6} M) with (10 ng/ml) or without C3. Data are means ± SE from 4–8 separate experiments (8–17 determinations). *P < 0.05 compared with C3 control by unpaired t-tests.
GTPase activity, maintaining Ras in its activated GTP-bound state (11). Because Rho possesses the formin homology (FH) 1 domain, which is enriched in proline, this domain may directly interact with the Src homology (SH) 3 domain of Src. Alternatively, p34\superscript{Src}-c2, a substrate for pp60\superscript{Src}, may be an intermediate between Src and Rho (34). Our laboratory (34) previously identified pp60\superscript{Src} in rat pancreatic acini, characterized its functional role in mediating pancreatic enzyme secretion, and suggested its association with PI 3-kinase in response to CCK. Several lines of evidence reveal that PI 3-kinase is a direct substrate of pp60\superscript{Src} (5). In other cell types, PI 3-kinase binds directly to Rho guanosine 5’-O-(3-thiotriphosphate) (GTP\superscript{S}) and is thereby activated, probably via the Rho-GAP-like domain located on the p85 regulatory subunit of PI 3-kinase (36). These observations suggest possible interactions among Src, Rho, and PI 3-kinase. Furthermore, it has recently been reported (15) that, in rat pancreatic acini, Rho plays a role in the ability of CCK to stimulate tyrosine phosphorylation of focal adhesion kinase (p125\superscript{FAK}) and the cytoskeletal protein paxillin utilizing PLC-dependent and -independent pathways. Because pancreatic acini possess actin and myosin and actin-associated proteins (e.g., vinculin and villin) that are preferred substrates of Src (9, 18), it is conceivable that Src, RhoA, PI 3-kinase, and focal adhesion kinase may reciprocally act on the actin cytoskeleton in mediating pancreatic exocytosis. Finally, we determined the functional role of RhoA in the mediation of pancreatic enzyme secretion. We demonstrated that the Rho inhibitor C3 exoenzyme inhibited [Ca\superscript{2+}]\superscript{S} dependent amylase secretion in permeabilized acini, suggesting that RhoA is likely to play a role in pancreatic exocytosis. Although C3 exoenzyme inhibited [Ca\superscript{2+}] dependent enzyme secretion in permeabilized acini, the high-affinity CCK-A-receptor agonist MV-180 and CCK-OPE, which elicited intracellular Ca\superscript{2+} oscillations (30–32, 35), did not increase the amount of Rho. This suggests that [Ca\superscript{2+}]\superscript{S} in permeable cells may directly act on the Rho-associated actin cytoskeleton in mediating enzyme secretion, in which small soluble proteins are extruded from the cells. It has recently been reported that pretreatment of intact rat pancreatic acini with the high concentration of C3 exoenzyme (25 μg/ml) for 120 min resulted in a significant inhibition of amylase secretion elicited by either CCK or TPA. This result further supports the interaction between PKC and Rho on pancreatic enzyme secretion (25). In conclusion, the small GTP-binding protein RhoA p21 appears to be involved in the mediation of pancreatic enzyme secretion. RhoA pathways are involved in the activation of PKC and pp60\superscript{Src} cascades via G\subscript{q} protein and PLC.

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