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Cholecystokinin stimulates the recruitment of the Src–RhoA–phosphoinositide 3-kinase pathway by Vav-2 downstream of $G_{\alpha 13}$ in pancreatic acini ☆

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Abstract

In isolated rat pancreatic acini, Src, RhoA, PI3-K, Vav-2, $G_{\alpha 12}$, and $G_{\alpha 13}$ were detected by immunoblotting. CCK enhanced the levels of these proteins, and the levels of Src and RhoA were reduced by the Src inhibitor herbimycin A and the Rho inhibitor pravastatin. The PI3-K inhibitor wortmannin reduced the level of PI3-K. These inhibitors also decreased amylase secretion in CCK-treated pancreatic acini without altering basal secretion. Immunoprecipitation studies indicated that CCK caused Src to associate with Vav-2, RhoA, and PI3-K and RhoA and Src to associate with Vav-2. Ras, RasGAP, and SOS did not coimmunoprecipitate with Vav-2, and RasGAP and SOS did not coimmunoprecipitate with RhoA. CCK also enhanced Vav-2 and RhoA to coimmunoprecipitate with $G_{\alpha 13}$. We conclude that CCK stimulates the recruitment of the Src–RhoA–PI3-K signaling pathway by Vav-2 downstream of $G_{\alpha 13}$ in pancreatic acini.

Keywords: Vav-2; $G_{\alpha 12/13}$; Src; Phosphoinositide 3-kinase RhoA; CCK; Pancreas; Rat

Cholecystokinin (CCK)-A receptors on pancreatic acini are members of the seven-pass transmembrane molecule G-protein-coupled receptor superfamily, which are widely distributed in mammals. The stimulation of these receptors leads to the activation of heterotrimeric G-proteins [1,2], which consist of α, β, and γ subunits. These subunits transduce extracellular stimuli to intracellular signaling networks [3]. G_q, a member of the G_α subunit superfamily (includes G_s, G_i, G_q, and G_{12}), is thought to be associated with Ca^{2+} signaling events that lead to enzyme secretion in pancreatic acini [4,5].

We previously demonstrated that the CCK-A receptor is coupled to signaling pathways via the protein tyrosine kinase $pp60^{c\text{-}src}$, which plays an important role in mediating extracellular Ca^{2+}-dependent amylase secretion in rat pancreatic acini. We also showed that this pathway involves activation of phosphoinositide 3-kinase (PI3-K) and the small G-protein RhoA downstream of $G_{q/11}$ protein [6]. Rho family GTPases were identified based on their structural homology to the Ras proto-oncogene [7]. Rho regulates the assembly and organization of the actin cytoskeleton. The Rho proteins cycle between an inactive GDP-bound state and an active GTP-bound state. In addition, the activity of Rho proteins is regulated by three molecules: Rho guanine nucleotide exchange factors (RhoGEFs), Rho GTPase activating proteins, and Rho guanine nucleotide dissociation inhibitors. RhoGEFs are thought to be the main regulators of Rho activity because they control the exchange of GDP for GTP. When bound to GTP, Rho proteins interact with and regulate the activity and/or localization of their downstream effectors.

☆ *Abbreviations:* CCK, cholecystokinin; PTK, protein tyrosine kinase; PI3-K, phosphoinositide 3-kinase; PSS, physiological salt solution; RhoGEF, Rho guanine nucleotide exchange factor; PH, pleckstrin homology; DH, Dbl2 homology; SH2, Src homology 2; SH3, Src homology 3.

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The Vav proteins, which include three families, Vav-1, Vav-2, and Vav-3, belong to the more than 40-member-Dbl family of RhoGEFs [8]. Although recent reports suggest that newly identified G-protein families, G_{12} and G_{13}, regulate the activity of Rho through Rho GEF in several cell types [9–11], it is still unknown whether Vav and $G_{\alpha 12/13}$ are involved in signaling pathways in pancreatic acinar cells. The aim of this study was to investigate the role of Vav and $G_{\alpha 12/13}$ in CCK stimulation of pancreatic acini via the Src–RhoA–PI3-K pathway.

Materials and methods

Materials. Male Sprague–Dawley rats (200–250 g) were obtained from Saitama Experimental Animal Supply (Saitama, Japan). Pravastatin was kindly provided from Sankyo (Tokyo, Japan). CCK-8 was from Sigma Chemical (St. Louis, MO). Antibodies to RhoA, Vav-2, $G_{\alpha 12}$, $G_{\alpha 13}$, and RasGAP were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to Src, PI3-K N-SH2, Ras, and Sos1 were from Upstate Biotechnology (Lake Placid, NY).

Isolation of pancreatic acinar cells and amylase measurement. Isolated rat pancreatic acini were prepared by collagenase digestion of pancreatic tissue samples obtained from male Sprague–Dawley rats [12]. Acini were suspended in physiological saline solution (PSS; 0.1% bovine serum albumin, 0.1 mg/ml soybean trypsin inhibitor, 137 mM NaCl, 4.7 mM KCl, 0.56 mM $MgCl_2$, 1.28 mM $CaCl_2$, 1 mM NaH_2PO_4, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 2 mM L-glutamine, 5.5 mM D-glucose, and Eagle's minimum essential amino acids adjusted to pH 7.35 with NaOH and equilibrated with 100% O_2). Isolated acini were preincubated for 40 min at 37 °C in 40 ml PSS, washed twice, and resuspended in 40 ml of fresh PSS. Aliquots distributed into flasks were preincubated with herbimycin A, pravastatin, or wortmannin for 10 min and further coincubated with CCK-8 for 60 min at 37 °C. The incubation was terminated by centrifugation at 10,000*g* for 50 s at 4 °C in a microfuge. The amylase released into the supernatant and remaining in the pellet was assayed using procion yellow starch as a substrate. Amylase secretion was expressed as a percentage of the total amylase content in each sample.

Immunoprecipitation. Isolated acinar cells were immunoprecipitated as follows. Acini (2×10^6 cells) in 1 ml PSS were incubated with reagents for the indicated periods. The incubation was terminated by adding 1 ml chilled 8 mM Hepes buffer (buffer A, pH 7.4) containing 1 mM $NaVO_4$, 0.5 mM Na_2HPO_4, 109.5 mM NaCl, 4.7 mM KCl, and 1.13 mM $MgCl_2$. The suspension was immediately centrifuged at 10,000*g* for 15 s at 4 °C. The supernatant was discarded and the pellet was resuspended in chilled lysis buffer (buffer B, pH 7.4) containing 25 mM β-glycerophosphate, 0.2 mM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 25 mM sodium fluoride, 0.2% Triton X-100, 10 μg/ml leupeptin, and 0.05 trypsin inhibitor units/ml aprotinin). Each suspension was sonicated, vortexed for 30 s, and centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was diluted with buffer B to 1 mg protein/ml. Samples (0.5 ml) were incubated overnight at 4 °C with the indicated antibody followed by 2 h at 4 °C with 50 μl of protein G–Sepharose (Santa Cruz, CA). The immunoprecipitates were washed three times with 100 mM Tris(hydroxymethyl)aminoethane, pH 7.5, containing 150 mM NaCl and 0.015% (v/v) Tween 20 and then analyzed by SDS–PAGE and immunoblotting.

Immunoblotting. Acinar cells (2×10^6 cells) in 1 ml PSS were incubated with reagents for the indicated periods. The incubation was terminated by adding 1 ml buffer A followed by centrifugation at 10,000*g* for 15 s at 4 °C. The suspension was discarded, and the pellet was resuspended in 150 μl chilled lysis solution (buffer C, pH 7.4) containing 66.7 mM β-glycerophosphate, 1 mM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride, 1.5 mM EGTA, 1 mM dithiothreitol, 150 mM NaCl, 1% Triton X-100, 10 μg/ml leupeptin, and 0.05 trypsin inhibitor units/ml aprotinin. Each suspension (25 μl; 80 μg protein) was mixed with 2.5 μl of 100% β-mercaptoethanol and 12.5 μl Laemmli buffer. The samples were heated at 95 °C for 5 min, separated by SDS–PAGE, and electrophoretically transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Src, RhoA, and PI3-K were detected using anti-Src, anti-RhoA, and anti-PI3-K as primary antibodies and using peroxidase-conjugated goat anti-mouse IgG as a secondary antibody. Vav-2, $G_{\alpha 12}$, and $G_{\alpha 13}$ were detected using anti-Vav-2, anti-$G_{\alpha 12}$, and anti-$G_{\alpha 13}$ as primary antibodies and using peroxidase-conjugated mouse anti-goat IgG or anti-rabbit IgG as secondary antibodies. After incubation with primary and secondary antibodies, immunoreactive proteins were detected using enhanced chemiluminescent reagents (Amersham, Arlington Heights, IL).

Results

Expression of Src, RhoA, and PI3-K proteins and effects of inhibitors in CCK-treated isolated rat pancreatic acini

The anti-Src, anti-RhoA, and anti-PI3-K (N-SH2) antibodies detected protein bands at 60, 21, and 85 kDa, respectively, in unstimulated rat pancreatic acinar cells. Treatment of pancreatic acini with CCK-8 (10 pM and 10 nM) for 20 min enhanced the expression of Src, RhoA, and PI3-K proteins (Fig. 1). Furthermore, the Src inhibitor herbimycin A (3 μM) (Fig. 2A) and the Rho inhibitor pravastatin (100 μM) (Fig. 2B) reduced the ability of CCK to increase the level of RhoA and PI3-K proteins. The PI3-K inhibitor wortmannin (3 μM) also reduced the ability of CCK to increase the level of PI3-K protein but had no effect on the level of RhoA protein (Fig. 2C). These results and our previous data [4,6] suggest that RhoA and PI3-K are involved in Src signaling pathways.

Effects of the Src, RhoA, and PI3-K inhibitors on CCK-stimulated pancreatic amylase secretion

We previously reported that Src may interact with PI3-K in mediating CCK-stimulated pancreatic amylase secretion [6] and that RhoA plays an important role in mediating stimulus-secretion coupling and interacts with Src in acinar cells [4]. Pretreatment with herbimycin A (3 μM), pravastatin (100 μM), or wortmannin (3 μM) inhibited amylase secretion without altering basal secretion in CCK-treated pancreatic acini (Fig. 3). These results sup-

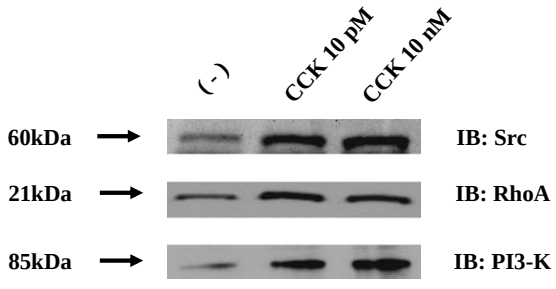

Fig. 1. Western immunoblotting (IB) of Src, RhoA, and PI3-K. Pancreatic acini were treated with or without CCK (10 pM or 10 nM) for 20 min at 37 °C. (−) Unstimulated cells. Similar results were observed in four independent experiments.

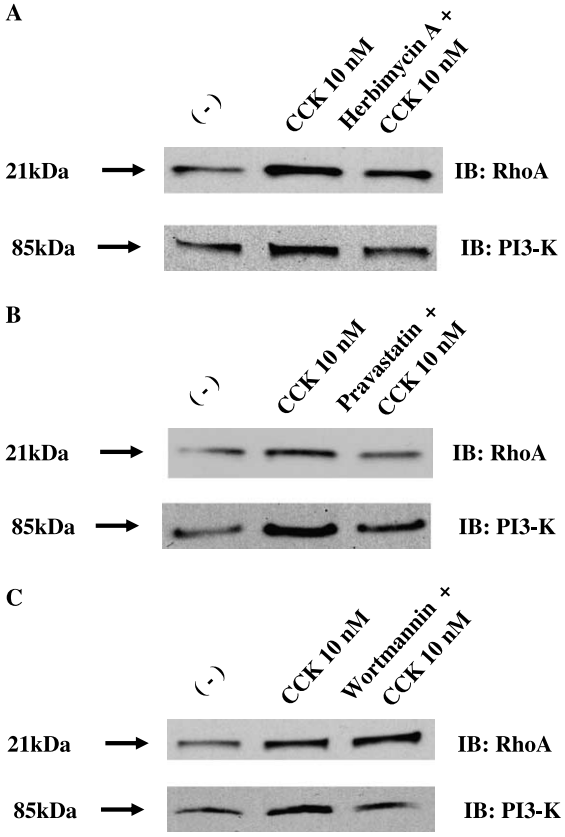

Fig. 2. Effects on herbimycin A, pravastatin, and wortmannin on the levels of Src, RhoA, and PI3-K proteins following CCK stimulation. Acini were pretreated with either (A) herbimycin A (3 μM), (B) pravastatin (100 μM), or (C) wortmannin (3 μM) for 5 min and then coincubated with CCK (10 nM) for 20 min at 37 °C. The levels of Src, RhoA, and PI3-K were determined by immunoblotting (IB). Similar results were found in three independent experiments.

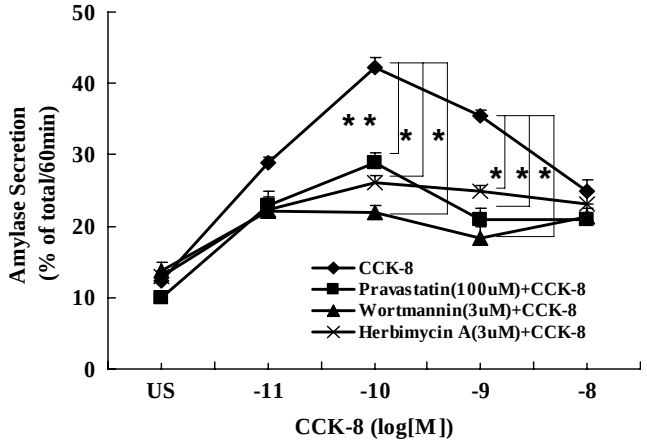

Fig. 3. Effect on herbimycin A, pravastatin, and wortmannin on CCK-stimulated amylase secretion. Acini were pretreated with or without herbimycin A, pravastatin, or wortmannin for 10 min at 37 °C and then further coincubated with various concentrations of CCK for 60 min. Results represent the mean amylase secretion ± SEM from five independent experiments (4–12 determinations per experiment). $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with CCK alone according to a two-tailed unpaired t test.

port the idea that Src, RhoA, and PI3-K are involved in pancreatic amylase secretion.

Existence of Vav-2, $G_{\alpha 12}$, and $G_{\alpha 13}$ in pancreatic acini

We next examined the expression of Vav-2, $G_{\alpha 12}$, and $G_{\alpha 13}$ protein in pancreatic acini by immunoblotting. We detected Vav-2 as 95-kDa band, and $G_{\alpha 12}$ and $G_{\alpha 13}$ as 45-kDa bands, respectively. In addition, the levels of these proteins were enhanced by treatment with CCK (Fig. 4).

Immunoprecipitation of Src, Vav-2, RhoA, and PI3-K

We found that Src coimmunoprecipitated with Vav-2, RhoA, and PI3-K (N-SH2) following stimulation with CCK, indicating that Src forms a complex with Vav-2, RhoA, and PI3-K (Fig. 5). We also found that RhoA coimmunoprecipitated with Src and Vav-2 following CCK stimulation (Fig. 6), indicating that RhoA forms complexes with Vav-2 and Src.

Association of Vav-2 and RhoA with Ras, RasGAP, and Sos1

We next examined the association of Vav-2 with Ras, RasGAP, and Sos1. As shown in Fig. 7A, the anti-Vav-2

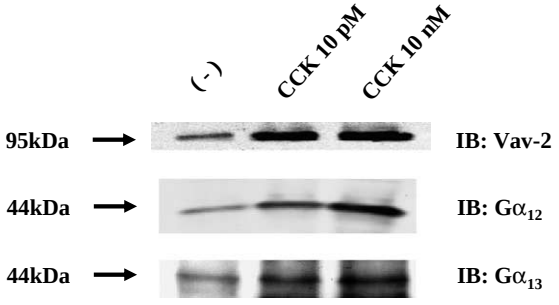

Fig. 4. Immunoblotting (IB) for Vav-2, $G_{\alpha 12}$, and $G_{\alpha 13}$. Pancreatic acini were treated with or without CCK (10 pM and 10 nM) for 20 min at 37 °C. (−) Unstimulated cells. Similar results were observed in four independent experiments.

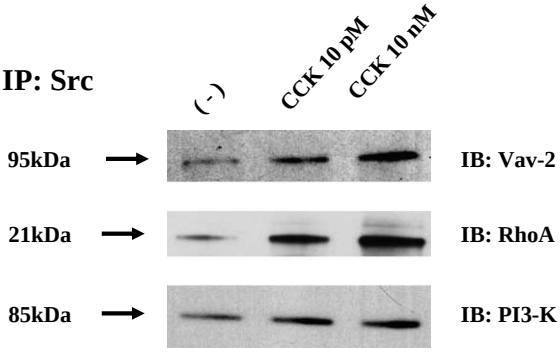

Fig. 5. Association of Src with Vav-2, RhoA, and PI3-K in isolated rat pancreatic acini. Proteins were immunoprecipitated (IP) with an anti-Src antibody and then analyzed by immunoblotting (IB) for Vav-2, RhoA, and PI3-K. Results are representative of three independent experiments.

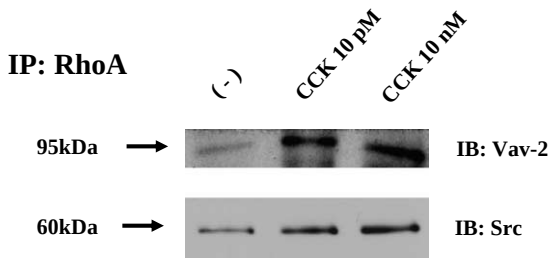

Fig. 6. Association of RhoA with Vav-2 and Src in isolated rat pancreatic acini. Proteins were immunoprecipitated (IP) with an anti-RhoA antibody and then analyzed by immunoblotting (IB) for Vav-2 and Src. Results are representative of four independent experiments.

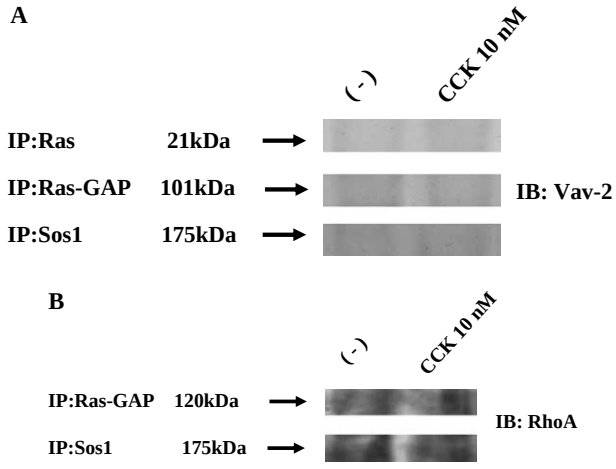

Fig. 7. Association of Ras, RasGAP, and Sos1 with Vav-2 and RhoA in isolated rat pancreatic acini. Proteins were immunoprecipitated (IP) with antibodies to Ras, RasGAP, and Sos1, and then analyzed by immunoblotting (IB) for Vav-2 (A) and RhoA (B). Results are representative of three independent experiments.

antibody failed to immunoprecipitate Ras, RasGAP, or Sos1 in the presence or absence of CCK. Similarly, as shown in Fig. 7B, RasGAP and Sos1 did not coimmunoprecipitate with RhoA in the presence or absence of CCK. These data suggest that Vav-2 and RhoA are not involved in Shc-Sos-Grb-Ras signaling in CCK-stimulated pancreatic acini.

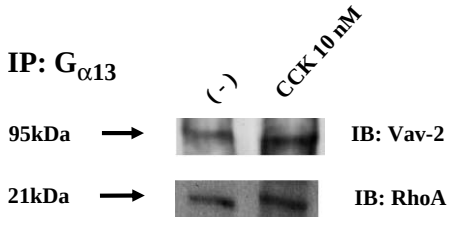

Fig. 8. Association of $G_{\alpha 13}$ with Vav-2 and RhoA in isolated rat pancreatic acini. Proteins were immunoprecipitated (IP) with an antibody to $G_{\alpha 13}$ and then analyzed by Immunoblotting for Vav-2 and RhoA. Results are representative of three independent experiments.

Association of $G_{\alpha 13}$ with Vav-2 and RhoA

We further examined the association of $G_{\alpha 13}$ with Vav-2 and RhoA. As shown in Fig. 8, $G_{\alpha 13}$ coimmunoprecipitated with Vav-2 and RhoA. These results suggest that $G_{\alpha 13}$ forms a complex with Vav-2 and RhoA. The association of $G_{\alpha 12}$ with Vav-2 and RhoA was not detected (data not shown).

Discussion

Although it is well known that Rho regulates the assembly and organization of the actin cytoskeleton in several cell types [7], the role of Rho in pancreatic acini is still not clear. We previously demonstrated that RhoA cascades are regulated by the sequential stimulation of protein kinase C and Src downstream of $G_{q/11}$ and phospholipase C [4,5], and another group reported that Rho participates in the tyrosine phosphorylation of $p125^{FAK}$ and paxillin [13]. The actin cytoskeleton and Rho are thought to be involved in pathophysiological events in pancreatitis as well as in normal pancreatic enzyme secretion. Supramaximal doses of the CCK analogue caerulein induce pancreatitis and disruption of the acinar cytoskeleton and tight junctions [14]. The disassembly and degradation of microtubular–microfilamentous systems induce basolateral secretion and subsequent access of active enzymes to interstitial or perivascular spaces [15–17] and may lead to missorting of proteins and vacuole formation [7]. Our previous study also suggested that RhoA and the Rho-effector ROCK-II play important roles in mediating normal pancreatic enzyme secretion and preventing the pathogenesis of caerulein-induced acute pancreatitis [18].

The relationship between Rho and Src or PI3-K may vary in different cells. Our study showed that RhoA is regulated by Src. Specifically, we found that the Src inhibitor herbimycin A inhibits CCK-induced expression of RhoA. Although RhoA interacts 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, several signaling molecules, including RhoGEFs, Rho GTPase activating proteins, and Rho guanine nucleotide dissociation inhibitors, may mediate this interaction. For example, protein kinase C stimulates Src activity, which in turn leads to an increased activity of Rho in A7r5 vascular smooth muscle cells [19]. On the other hand, in Swiss 3T3 cells, translocation of Src kinase to the cell periphery is mediated by the actin cytoskeleton, which is under the control of the Rho family GTPases [20]. Also, activation of PI3-K in Swiss 3T3 cells is dependent on Rho [21]. These reports and our present study suggest that Src, Rho, and PI3-K closely interact in cell systems.

Vav proteins are well-known Rho GEFs [8]. These proteins are composed of seven different structural domains: a calponin homology region, which functions as an actin-binding domain in other proteins; an acidic domain; a Dbl homology (DH) region, which exhibits GEF activity

towards Rho; a pleckstrin homology (PH) domain, which interacts with polyphosphoinositides; a zinc finger butterfly domain; and a single Src homology 2 (SH2) domain and two Src homology 3 (SH3) domains, which mediate protein–protein interactions [22]. Vav proteins are the only Rho GEFs that are known to possess DH, PH, SH2, and SH3 motifs in the same protein. The different Vav family proteins show significant regulatory differences; however, they possess similar structures and biochemical activities. Vav-1 acts preferentially on Rac-1, Rac-2, and RhoG small GTPases, whereas Vav-2 and Vav-3 act mainly on RhoG and RhoA [23]. Since the intensity of the Vav-1 and Vav-3 bands was weak and variable in immunoblotting and immunoprecipitation (data not shown), we focused on Vav-2 in the present study. We detected Vav-2 as a 95-kDa protein in the pancreatic acinar cells, and its expression was enhanced by a 20-min stimulation with CCK. Furthermore, Vav-2 was coimmunoprecipitated with Src and RhoA. These results suggest that Vav-2 forms a complex with Src and RhoA and is recruited into the Src–RhoA–PI3-K pathway. This possibility is supported by several reports. First, the tyrosine phosphorylation of Vav-1 by a Src-related tyrosine kinase or other kinases plays an important role in the macrophage response. Second, Vav-2-mediated activation of Rho family GTPases plays a significant role in transducing receptor signaling in maturing T cells [24,25]. Third, Vav acts as an adaptor protein responsible for targeting the p85 regulatory subunit of PI3-K rather than as a GEF [26].

Activation of CCK-A receptors results in pancreatic enzyme secretion *via* $G_{q/11}$, which activates phospholipase C and leads to an increase in intracellular Ca^{2+} and activation of protein kinase C. Previous reports suggested that protein kinase C-dependent activation of Shc–Grb2–Sos complex formation and mitogen-activated protein kinase signaling cascades result in Ras activation in pancreatic acinar cells [27,28]. However, we showed that Vav-2 does not coimmunoprecipitate with Ras, RasGAP, or Sos and that RhoA does not coimmunoprecipitate with RasGAP and Sos. These results suggest that Vav-2 and the Src–RhoA–PI3-K pathways are independent of Shc–Grb2–Sos complex formation.

Recent reports have suggested that $G_{\alpha 12/13}$-mediated signaling cascades promote several cellular responses, for example stress fiber formation and focal adhesion assembly [29], via Rho-dependent pathways and RhoGEFs, such as p115RhoGEF [9], PDZ-RhoGEF [30], and leukemia-associated RhoGEF [31]. These RhoGEFs possess the similar structural domain, regulators of G-protein signaling, and directly link $G_{\alpha 12/13}$ to the activation Rho. Furthermore, CCK-A receptors activate Rho through $G_{\alpha 12/13}$, most specifically, $G_{\alpha 13}$, and remodels the cytoskeleton in NIH3T3 cells [32]. Our present study showed that $G_{\alpha 13}$ not but $G_{\alpha 12}$ coprecipitates with Vav-2 and RhoA. Although Vav-2 does not have the regulators of G-protein signaling and the precise mechanism of these interactions remains unclear, the DH, PH, SH2, and SH3 in Vav-2 may be involved. The PH domain of the DH–PH pair may block access to the binding site for the Rac/Rho small G-protein on the DH domain. Phosphoinositide binding to the PH domain could allow access to the DH domain [33]. Further studies are required to identify functional differences between $G_{\alpha 12}$ and $G_{\alpha 13}$ in pancreatic acini. Our previous studies demonstrated that, in rat pancreatic acini, RhoA pathways are involved in the activation of protein kinase C and $pp60^{c\text{-}src}$ cascades via G_q and phospholipase C [4]. Galanin binding to GALR2 simultaneously activates multiple classes of G-proteins and signals through the G_q-PKC-PLC-Ca^{2+} sequence and a G_{q12}/Rho pathway in small cell lung cancer [34]. These studies suggest that $G_{\alpha 12/13}$ as well as G_q can regulate Rho activity.

In conclusion, our findings indicate that Vav-2 recruits the Src–RhoA–PI3-K signaling pathway downstream of $G_{\alpha 13}$ in rat pancreatic acini.

Acknowledgment

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