Protein tyrosine phosphorylation in pancreatic acini: differential effects of VIP and CCK

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Protein tyrosine phosphorylation in pancreatic acini: differential effects of VIP and CCK. Am. J. Physiol. 273 (Gastrointest. Liver Physiol. 36): G1226–G1232, 1997.—Cholecystokinin (CCK) and vasoactive intestinal peptide (VIP) stimulate enzyme secretion from pancreatic acini by binding to heptahelical receptors without intrinsic tyrosine kinase activity. Signal transduction by the CCK receptor involves activation of phospholipase C by Gq proteins and activation of tyrosine kinases, whereas occupation of VIP receptors stimulates adenylyl cyclase through binding to Gs proteins. Here, we use electrophoretic separation of cellular proteins and antiphosphotyrosine immunoblotting to demonstrate a VIP-stimulated rapid and dose-dependent increase in tyrosine phosphorylation of proteins migrating at 130, 115, and 93 kDa in freshly isolated rat pancreatic acini. Phosphorylation of these proteins was increased after direct stimulation of adenylyl cyclase or the adenine 3',5'-cyclic monophosphate (cAMP)-, and forskolin-induced protein tyrosine phosphorylation of proteins migrating at 60, 66, and 72/78 kDa. Using two-dimensional electrophoretic separation or immunoprecipitation, the 72/78-kDa phosphoprotein was identified as paxillin. We propose that paxillin might be involved in CCK- but not in VIP-induced exocytosis.

cholecystokinin; vasoactive intestinal peptide; paxillin; G protein-coupled receptors; adenylyl cyclase

CHOLECYSTOKININ (CCK) and vasoactive intestinal peptide (VIP) are gastrointestinal peptide hormones that stimulate enzyme secretion from pancreatic acinar cells by binding to heptahelical non-tyrosine kinase receptors. Whereas the intracellular signaling system activated by the CCK receptor involves activation of phospholipase C (PLC) through the Gq subtypes of heterotrimeric G proteins, VIP is known to increase adenylyl cyclase activity through G proteins of the Gs subtype. Subsequent activation of phospholipid-, Ca2+--, and adenosine 3',5'-cyclic monophosphate (cAMP)-dependent protein kinases and phosphorylation of proteins on serine and threonine residues by these kinases mediates at least part of the cellular response (32). In addition, CCK has recently been demonstrated to increase tyrosine phosphorylation of several proteins and to increase protein tyrosine kinase activity in pancreatic acini, apparently via protein kinase C- as well as Ca2+-dependent pathways (2, 8, 17, 19, 20). Whereas several reports demonstrate the ability of other phospholipase C (PLC)-activating G protein-coupled receptors to stimulate protein tyrosine phosphorylation (23), receptor-mediated or direct activation of adenylyl cyclase or cAMP-dependent protein kinase has not been related to increased tyrosine kinase activity. Similarly, protein tyrosine phosphorylation in response to stimulation of cells with VIP has not been reported.

Tyrosine phosphorylation of proteins participates in the regulation of various cellular functions, including cell proliferation, differentiation, and the formation of focal adhesions (23, 26). Increased tyrosine kinase activity is observed in response to stimulation of cells with growth factors, with several neuropeptides, or with a variety of other stimuli. Most growth factors, such as platelet-derived growth factor or epidermal growth factor, stimulate tyrosine phosphorylation through activation of receptors with intrinsic tyrosine kinase activity (23), whereas neuropeptides such as angiotensin II, vasopressin, bradykinin, or bombesin bind to heptahelical receptors that lack intrinsic tyrosine kinase activity, thus increasing tyrosine phosphorylation, probably by recruiting intracellular non-receptor tyrosine kinases (1, 30). In fibroblasts (23, 31, 35), vascular smooth muscle cells (27), and renal glomerular mesangial cells (5), these agents cause prominent tyrosine phosphorylation of proteins migrating between 110 and 130 kDa and around 70 kDa, some of which have been identified as focal adhesion kinases p125FAK and paxillin (9, 14, 23). After phosphorylation, both proteins localize to the plasma membrane of cultured cells, where they seem to participate in the regulated assembly of focal adhesion complexes (23). In pancreatic acinar cells, protein tyrosine kinase inhibitors such as genistein or tyrphostin 25 inhibit CCK-induced tyrosine phosphorylation as well as amylase secretion, and incubation of permeabilized cells with recombinant protein tyrosine phosphatase stimulates Ca2+-mediated secretion (8, 17, 19, 20), indicating that tyrosine phosphorylation of one or more proteins might be involved in the regulation of CCK-induced enzyme secretion. However, the identity of the regulated phosphoproteins has not been determined.

In this study we demonstrate VIP-, dibutyryl cAMP (DBcAMP)-, and forskolin-induced protein tyrosine phosphorylation in rat pancreatic acini. Whereas VIP and CCK both stimulate phosphorylation of proteins migrating at 115 and 130 kDa, CCK induced tyrosine phosphorylation of additional proteins migrating at 60, 66, and 72/78 kDa. One of these proteins was identified as paxillin. Because protein tyrosine kinase inhibitors reduce CCK- but not VIP-induced amylase release (3,
MATERIALS AND METHODS

Materials and animals. VIP, forskolin, DBcAMP, and the monoclonal antiphosphotyrosine antibody (PT-66) were obtained from Sigma Chemical (St. Louis, MO). CCK-8 (sulfated) was from Bachem (Bubendorf, Switzerland), peroxidase-conjugated affinity-purified rabbit anti-mouse immunoglobulin G was purchased from Dianova (Hamburg, Germany), soybean trypsin inhibitor and reagents for the amylase assay were from Boehringer (Mannheim, Germany), and collagenase was from Worthington Cell Systems (Hamburg, Germany). Tyrophostin 23 and genistein were from Calbiochem (La Jolla, CA), and essential and nonessential amino acids were purchased from Gibco (Gaithersburg, MD). Enhanced chemiluminescence reagents and films were obtained from Amersham (Braunschweig, Germany). The monoclonal antipaxillin antibody (clone 349) was from ICN (Costa Mesa, CA). All other reagents were analytical grade. Male Wistar rats (150–200 g) were bred at the Animal Care and Treatment Facility of the University of Ulm.

Preparation of isolated rat pancreatic acini. The preparation of isolated rat pancreatic acini was performed essentially as described (17). Acini were washed two times in oxygenated Krebs-Ringer-N2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer consisting of 104 mM NaCl, 5 mM KCl, 1 mM KH2PO4, 1.2 mM MgCl2, 2 mM CaCl2, 0.2% (wt/vol) bovine serum albumin, 0.01% (wt/vol) soybean trypsin inhibitor, 10 mM glucose, and 25 mM HEPES-NaOH, pH 7.4, and supplemented with minimal essential amino acid solution and glutamine. Cell viability, as assessed by trypan blue exclusion, exceeded 95%. All preincubation and incubation steps were carried out at 37°C.

Examination of protein tyrosine phosphorylation. To examine secretagogue-induced tyrosine phosphorylation, isolated pancreatic acini were equilibrated for 10 min at 37°C. In some experiments the acini were preincubated for an additional 10 min with protein kinase inhibitor or appropriate vehicle. Secretagogue or vehicle was then added for the indicated time periods, and the incubation was terminated by suspending the acini in an excess volume of ice-cold Krebs-Ringer-HEPES buffer. The acini were then pelleted by centrifugation at 300 g (4°C). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), cellular protein was extracted by trituration in 400 µl lysis buffer consisting of 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4, 30 mM Na2HPO4, 95 mM NaCl, 1% (wt/vol) Triton X-100, 0.5 mM sodium orthovanadate, 0.1% (wt/vol) soybean trypsin inhibitor, 10 µg/ml leupeptin, 10 µg/ml apronin, 5 µg/ml pepstatin A, 1 mM benzamidine, and 5 µM ZnCl2. After removal of insoluble material by centrifugation, 50 µg of soluble protein was separated according to the method of Laemmli (13). For two-dimensional separation, cells were extracted in sample buffer (12.25 M urea, 0.125 mM dithiothreitol, 2.5% Triton X-100, 2.5% Servalyte 7–9). In the first dimension, 40 µg of protein was separated in polyacrylamide tube gels containing 2% Servalyte 3–10, 3% Servalyte 4–6.5, and 4.5% Servalyte 5–8, using a Mini-Protean II system (Bio-Rad, Hercules, CA) according to the manufacturer’s guidelines. SDS-PAGE was then performed as described above. Gel-resolved proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore). Membranes were incubated overnight in blocking buffer [50 mM Tris-HCl, pH 7.8, 100 mM NaCl, 0.5% (wt/vol) Tween 20, 2% (wt/vol) bovine serum albumin], followed by two washes with Tris-buffered saline supplemented with 0.1% (wt/vol) Tween 20. The membranes were then incubated for 1 h with primary antibodies (3:1,000) in blocking buffer. After an additional three washes with Tris-buffered saline supplemented with 0.1% (wt/vol) Tween 20, antigen-antibody complexes were visualized using secondary peroxidase-conjugated antibody and the enhanced chemiluminescence system by exposure to Kodak X-OMAT AR films for 1–2 min. Quantitation was performed by densitometry using Phoretix 1D gel analysis software (Phoretix, Newcastle upon Tyne, UK).

Data presentation. All experiments were repeated at least three times with acini from different preparations. Data shown are means ± SE. Statistical analysis was performed using Student’s t-test for paired values.

RESULTS

Tyrosine phosphorylation in response to VIP. Phosphotyrosine-containing proteins were examined in freshly isolated rat pancreatic acini under basal conditions and after stimulation with VIP. Probing Western blots with antiphosphotyrosine antibodies revealed that several protein bands were already phosphorylated under basal conditions. Incubation of the acini with VIP resulted in an increase in tyrosine phosphorylation of several protein bands, with the most prominent phosphoproteins migrating at 130, 115, and 93 kDa on SDS-polyacrylamide gels. These proteins are therefore referred to as p130, p115, and p93 (Fig. 1A). The phosphorylation signal of the VIP-responsive proteins was maximal after 2–5 min of stimulation (Fig. 1B). Densitometric quantitation of the bands revealed that the maximum intensity of tyrosine phosphorylation of these phosphoproteins was 1.6- to 2-fold greater than signal intensity under basal conditions and was observed at VIP concentrations of 10 nM or higher (Fig. 1C). As shown in Fig. 2, preincubation with the protein kinase inhibitors genistein (0.1 mM) or tyrphostin 23 (0.1 mM) decreased the VIP-induced change in protein tyrosine phosphorylation of p130 completely and reduced the signal intensity of p115 to less than basal levels. In comparison to other studies (3, 17), the current experiments do not show prominent phosphoproteins migrating below 60 kDa, which may be explained by molecular weight-dependent changes in blotting efficiency. In the present setting, conditions of electrophoretic transfer of proteins from the gel to the membrane were adjusted to allow maximum transfer of high molecular weight proteins, whereas some of the low molecular weight proteins migrated through the blotting membrane.

Tyrosine phosphorylation in response to forskolin or DBcAMP. The VIP-induced signaling cascade involves activation of adenyllyl cyclase via Gs proteins, which leads to an increase in the intracellular cAMP level (32) and to stimulation of the cAMP-dependent protein kinase. To examine whether this signaling pathway mediates VIP-induced protein tyrosine phosphorylation, pancreatic acini were stimulated with forskolin or DBcAMP, which activate adenyllyl cyclase or the CAMP-
dependent protein kinase directly, thus bypassing receptor activation of G proteins. As shown in Fig. 3, forskolin (0.1 mM) or DBCAMP (2 mM) increased tyrosine phosphorylation of the same protein bands as VIP, i.e., p130, p115, and to a lesser extent p93.
Moreover, the magnitude and the time course of the effect of forkolin or DBcAMP on tyrosine phosphorylation of these phosphoproteins were similar to those observed in response to VIP.

Comparison of VIP- and CCK-induced protein tyrosine phosphorylation patterns. Both VIP and CCK are able to induce tyrosine phosphorylation of several cellular proteins in a dose- and time-dependent manner. However, only CCK-stimulated acinar cell secretion is sensitive to inhibition of tyrosine kinases (3, 19). We have therefore examined whether this differential sensitivity toward tyrosine kinase inhibition is accompanied by a differential pattern of protein tyrosine phosphorylation. Acini were incubated with maximal stimulatory concentrations of VIP (10 nM) or CCK (10 nM) with respect to tyrosine phosphorylation to enable direct comparison between VIP and CCK-induced protein tyrosine phosphorylation. As shown in Fig. 4, the VIP-sensitive phosphoproteins p130 and p115 were phosphorylated on tyrosine residues in response to both VIP and CCK. Most important, CCK caused a strong increase in tyrosine phosphorylation of a broad band migrating at 72/78 kDa and to a lesser extent of two proteins migrating at 66 and 60 kDa, whereas VIP had no effect on the phosphorylation state of these proteins.

Identification of p72/78. To clearly distinguish individual tyrosine-phosphorylated proteins, total cellular extracts were separated by two-dimensional gel electrophoresis and were again analyzed by antiphosphotyrosine immunoblotting. As shown in Fig. 5A, several proteins were phosphorylated on tyrosine residues under basal conditions, with subsets increasing in signal intensity after stimulation with 10 nM CCK (Fig. 5B). As observed after one-dimensional separation in SDS-polyacrylamide gels, the most prominent protein migrated as a broad band between 70 and 80 kDa in the pH range of ~6–7. Again, stimulation of cells with VIP did not induce signal intensity of this phosphoprotein (Fig. 5C). Two-dimensional separation and antiphosphotyrosine Western blotting further revealed tyrosine phosphorylation of several additional proteins that were not recognized after one-dimensional SDS-PAGE with the same antibody, whereas the signals of higher molecular weight proteins became less intense. This effect is probably due to better isoelectric focusing of lower molecular weight proteins, whereas higher molecular weight proteins might not be focused and run off or not enter the gel. Proteins that were stimulated by CCK as well as by VIP migrated at 50 kDa at an estimated pH of 5.5 and 6 and at 40 kDa at an estimated pH of 7. All signals could be suppressed by competition with soluble phosphotyrosine. Because activation of several Gq protein-coupled heptahelical re-

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Fig. 4. Comparison of effects of VIP and CCK on protein tyrosine phosphorylation. Representative immunoblot analysis of isolated rat pancreatic acini after stimulation with 10 nM VIP for 3 min (left) or after stimulation with 10 nM CCK for 2 min (right).

Fig. 5. Two-dimensional analysis of tyrosine-phosphorylated proteins. Rat pancreatic acini were stimulated with VIP (10 nM) or CCK (10 nM). Total cellular extracts of control cells (A) and of cells after stimulation with CCK (B and D) or VIP (C) were separated by two-dimensional gel electrophoresis and analyzed by antiphosphotyrosine immunoblotting (A-C) or by staining with antipaxillin antibodies (D). Immunoblots shown are representative of at least 3 independent experiments.
receptors in cultured cell systems is known to cause phosphorylation of paxillin (23), membranes were incubated with paxillin-specific antibodies, and the signal was compared with the migration pattern of tyrosine-phosphorylated proteins. As shown in Fig. 5D, the 72/78-kDa band did indeed comigrate with paxillin. Similar results were obtained after the one- and two-dimensional blots were stripped and reprobed with antipaxillin antibody. Furthermore, the CCK-induced increase in tyrosine phosphorylation of paxillin could be confirmed by immunoprecipitation of paxillin from CCK-stimulated and control extracts (data not shown).

**DISCUSSION**

CCK stimulates secretion of digestive enzymes as well as cell growth of pancreatic acinar cells through binding to the CCK-A receptor (21). On activation, this heptahelical transmembrane receptor then couples to G_q proteins, which in turn activate theinositol phospholipid-specific PLC-β (33). Furthermore, CCK has been shown to increase protein tyrosine phosphorylation of cellular proteins as well as protein tyrosine kinase activity, and a role for tyrosine phosphorylation events in mediating the physiological cellular response to CCK has been suggested (8, 17, 19, 20). In contrast, VIP stimulates pancreatic acinar cell enzyme secretion by occupying receptors that couple to G_s proteins and thus activate adenyl cyclase and the cAMP-dependent protein kinase (33).

In this study, we demonstrate that VIP stimulates protein tyrosine phosphorylation in freshly isolated pancreatic acinar cells. Direct activation of adenyl cyclase with forskolin or DBCAMP elicited a similar phosphorylation response, indicating that activation of adenyl cyclase in pancreatic acini and the subsequent stimulation of CAMP-dependent protein kinase is sufficient to mediate at least part of the VIP-induced protein tyrosine phosphorylation and that this can occur independently from direct activation of tyrosine kinases by G proteins. Even though tyrosine phosphorylation is a well-known signaling mechanism of various G_s protein-coupled receptors (15, 23, 30), stimulation of tyrosine phosphorylation by heptahelical receptors that couple to G_s proteins has not been reported. Therefore, our finding may be interpreted in at least two ways. One explanation is that the freshly isolated acinar cells used in this study represent a more sensitive experimental system than cultured or receptor-transfected cells, which are commonly employed to examine signal transduction mechanisms. In addition, acinar cells might employ a signaling system that couples activation of adenyl cyclase to the stimulation of protein tyrosine kinases. Another possibility would be cross-activation of G_s proteins or phospholipases by the VIP receptor or other downstream signaling molecules similar to dual coupling of the luteinizing hormone receptor (6). However, VIP is not known to stimulate second messenger pathways other than the G_s protein-adenyl cyclase cascade, and we were able to stimulate phosphorylation by direct activation of adenyl cyclase or the cAMP-dependent protein kinase (33). Hence VIP-stimulated tyrosine phosphorylation seems to represent a new signal transduction mechanism of the G_s protein-coupled VIP receptor, which is mediated by activation of the CAMP-dependent protein kinase through adenyl cyclase.

CCK and VIP both are able to stimulate pancreatic enzyme secretion. To establish a role for tyrosine phosphorylation events in regulating pancreatic acinar cell secretion, we and others have examined amylase secretion from pancreatic acini in the presence of tyrosine kinase inhibitors. In these experiments, genistein, tyrphostin 25, and herbimycin were able to decrease CCK- or Ca^{2+}-induced amylase secretion (17) but had no effect on VIP-stimulated secretion (3, 19). Similar results were observed with tyrphostin 23 or with the peptide pp60v-src-(137—157) in permeabilized acini, which was used as a more specific approach for tyrosine kinase inhibition (A. Piiper and M. P. Lutz, unpublished observations). On the basis of these data it was suggested that protein tyrosine kinases are involved in the CCK-stimulated signal transduction cascade leading to amylase release in response to activation of the PLC-Ca^{2+} pathway.

To date, the tyrosine-phosphorylated proteins and protein tyrosine kinases involved in the regulation of CCK-induced amylase secretion from pancreatic acini have not been identified, even though several of the phosphorylated proteins have been described (2, 4, 9, 28). As shown, CCK and VIP both stimulate pancreatic acinar cell tyrosine phosphorylation, yet only the CCK-induced tyrosine phosphorylation events seem to be involved in enzyme secretion. Therefore, identification of a subset of proteins that are tyrosine phosphorylated in response to CCK but not in response to VIP might help to identify proteins involved in the regulation of CCK-induced acinar cell secretion. Comparison of VIP- and CCK-induced protein tyrosine phosphorylation patterns revealed that several tyrosine-phosphorylated proteins, namely p130, p115, and p93, are stimulated by both secretagogues. Thus tyrosine phosphorylation of these substrates can be achieved either by activation of the PLC or the adenyl cyclase pathways, and the observation that protein tyrosine kinase inhibitors did not affect VIP-induced amylase secretion suggests that p130, p115, or p93 are unlikely to be involved in regulating this cellular function. In contrast, only CCK caused an increase in tyrosine phosphorylation of p66 and p72/78. Whereas phosphorylation of these proteins was not observed in response to stimulation of acini with VIP, forskolin, or DBCAMP, increased tyrosine phosphorylation of p72/78 has been reported on incubation with a Ca^{2+} ionophore or a COOH-terminal phenyll ethyl ester analog of CCK (CCK-OE), which elicits little or no PLC activation but a clear intracellular Ca^{2+} response, or by direct activation of protein kinase C with 12-O-tetradecanoylphorbol 13-acetate (17), indicating that tyrosine phosphorylation of p72/78 is mediated by Ca^{2+}-protein kinase C pathway and not by activation of adenyl cyclase. In addition, tyrosine kinase...
inhibitors were able to inhibit the secretory response to bombesin, carbachol, CCK-OPE, and to Ca\(^{2+}\) ionophores (3, 17, 19), and we therefore propose that p72/78 might play a role in the Ca\(^{2+}\)-mediated secretory response to CCK.

To identify p72/78 we did compare protein tyrosine phosphorylation events in CCK-stimulated acinar cells with patterns reported after stimulation of other heptahelical G\(_3\) protein-coupled receptor systems. Interestingly, the pattern observed in acinar cells in response to CCK was strikingly similar to that reported in cultured cells in response to stimulation with vasopressin, bradykinin, angiotensin II, bombesin, or endothelins (15, 27, 35). In most of these systems, paxillin was identified as one of the most prominent phosphoproteins migrating at or around 70 kDa (23), and paxillin phosphorylation in acinar cells has been reported in response to CCK (9).

Using two-dimensional electrophoretic protein separation as well as immunoprecipitation of total cellular extracts with antipaxillin antibody, we were able to confirm that p72/78 comigrates with paxillin. Thus paxillin is phosphorylated on tyrosine residues in response to stimulation of pancreatic acinar cells with CCK but not in response to stimulation with VIP. Paxillin is a 68-kDa cytoskeletal protein concentrated in focal adhesions, i.e., multimeric protein complexes that occur at sites where cultured cells adhere to the extracellular matrix (25). To date, the functional role of paxillin has not been firmly established. Phosphorylation of paxillin correlates with the formation of focal adhesions in a human colon cancer cell line (24) and in cultured rat aortic smooth muscle cells (29) and with the formation of actin stress fibers as well as the assembly of focal contacts in Swiss 3T3 fibroblasts (12), where it colocalizes with the focal adhesion kinase and integrin receptors (18). Activation of these proteins, e.g., by hormonal stimulation or adhesion to the extracellular matrix, leads to changes in cell adhesion properties, cell motility, and cell shape (22, 23). Paxillin has been implicated in cell signaling because it directly binds to the paxillin binding site on the focal adhesion kinase and associates with pp60src or the COOH-terminal Src kinase (7). Even though paxillin is thought to be involved in the formation of focal adhesions, these complexes are typically observed in cultured cells only (10), and the presence of focal adhesion complexes has not been demonstrated in pancreatic acinar cells. However, acinar cells do need an intact actin filament system for regulated enzyme secretion (11), and it is likely that actin filaments in pancreatic acinar cells are attached to the plasma membrane by anchoring systems that resemble the focal adhesion complexes in adhering cultured cells. Therefore, we propose that phosphorylated paxillin binds to and may even participate in the regulation of protein complexes that mediate the turnover and composition of the actin filament system in acinar cells. Because the actin filament system is thought to be essential for regulated fusion of zymogen granules with the plasma membrane (11), paxillin might participate in the regulation of CCK-induced enzyme secretion at this final step. The role of VIP-induced protein tyrosine phosphorylation as well as the tyrosine kinases activated by VIP remain to be identified.

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