Phosphospecific Site Tyrosine Phosphorylation of p125FAK and Proline-rich Kinase 2 Is Differentially Regulated by Cholecystokinin Receptor Type A Activation in Pancreatic Acini*

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The focal adhesion kinases, p125FAK and proline-rich kinase 2 (PYK2), are involved in numerous processes as adhesion, cytoskeletal changes, and growth. These kinases have 45% homology and share three tyrosine phosphorylation (TyrP) sites. Little information exists on the ability of stimulants to cause TyrP of each kinase site and the cellular mechanism involved. We explored the ability of the neurotransmitter/hormone, CCK, to stimulate TyrP at each site. In rat pancreatic acini, CCK stimulated TyrP at each site in both kinases. TyrP was rapid except for pY397FAK. The magnitude of TyrP differed with the different FAK and PYK2 sites. The CCK dose-response curve for TyrP for sites in each kinase was similar. CCK-JMV, an agonist of the high affinity receptor state and antagonist of the low affinity receptor state, was less efficacious than CCK at each FAK/PYK2 site and inhibited CCK maximal stimulation. Thapsigargin decreased CCK-stimulated TyrP of pY402PYK2 and pY925FAK but not the other sites. GF109203X reduced TyrP of only the PYK2 sites, pY580, GF109203X with thapsigargin decreased TyrP of pY402PYK2 and the three FAK sites more than either inhibitor alone. Basal TyrP of pY397FAK was greater than other sites. These results demonstrate that CCK stimulates tyrosine phosphorylation of each of the three homologous phosphorylation sites in FAK and PYK2. However, CCK-stimulated TyrP at these sites differs in kinetics, magnitude, and participation of the high/low affinity receptor states and by protein kinase C and [Ca2+]i. These results show that phosphorylation of these different sites is differentially regulated and involves different intracellular mechanisms in the same cell.

The closely related nonreceptor focal adhesion tyrosine kinases (FAK)1 p125FAK and PYK2 transduce key extracellular signals that are involved in mediating growth, adhesion, cytoskeletal changes, and cellular motility (1–3). These kinases are activated by such diverse stimuli as integrins, some G protein-coupled receptors, growth factors, bioactive lipids, oncogenes (2, 4–7), and mechanical factors (pressure, stretch, and shock) (2, 8–10). PYK2 and FAK share 45% overall sequence homology and undergo tyrosine phosphorylation at related sites (1–3, 11). During activation FAK tyrosine phosphorylation occurs at six or more sites in vivo (11). Two sites are located within the N-terminal domain (pY397 and pY407), two sites are within the kinase activation loop (pY576 and pY577), and two sites are within the C-terminal domain (pY861 and pY925). The pY397FAK site serves as an autophosphorylation site (11) and once phosphorylated as a binding site for c-Src family protein tyrosine kinases (12, 13) and other proteins such as phosphatidylinositol 3-kinase (14) and phospholipase Cγ (15). The phosphorylation of pY576 and pY577 in the kinase activation loop is essential for full catalytic activity (11). Phosphorylation of pY925 FAK in the C-terminal domain is followed by binding of SH-2 domain containing proteins such as Grb2 (16). The role of the phosphorylation sites pY407 and pY861 is to date unknown. Four of the six FAK tyrosine phosphorylation sites are conserved at homologous positions in PYK2 (2). The autophosphorylation sites pY402 PYK2 corresponds to pY397 FAK, pY579 PYK2 and pY580 correspond to pY576 FAK and pY577 FAK, and pY881 PYK2 correspond to pY925 FAK. It has been shown that the roles of the correspondent phosphorylation sites in both kinases are equivalent (2, 3). With both kinases, upon stimulation after the initial phosphorylation of the autophosphorylation sites, there is a recruitment of c-Src tyrosine kinases, which in turn phosphorylates the other phosphorylation sites (11). Even though these related kinases have many structural similarities (1, 2, 4), both are present in many tissues (1, 2, 17, 18), and both can be activated by similar stimuli (1, 2, 17, 18); at present little is known about the simultaneous activation events of these two kinases in the same cell by a common stimulant. Little information exists on the comparative ability (kinetics and magnitude) of a given stimulant to increase tyrosine phosphorylation of the three homologous sites in these two kinases or the participation of other cellular cascades such as the two limbs of the phospholipase C cascade in this stimulation at a given site.

It has been shown in previous studies that in rat pancreatic acini the activation of the CCK(1) receptor leads to a rapid tyrosine phosphorylation of PYK2 and FAK (9, 19). Extensive studies show that CCK(1) receptor activation causes stimulation of phospholipases (A, C, and D), activation of PKCs and mobilization of cellular calcium (20, 21). Because both kinases are expressed in rat pancreatic acini (19, 22–24) and both are tyrosine-phosphorylated upon stimulation with CCK (19, 22),
one of its main physiological stimulants (25, 26), this cell system is an ideal model to investigate the comparative ability of a given stimulant to cause site-specific differences in tyrosine phosphorylation of FAK and PYK2 in the same cell and study the intracellular mechanisms involved in the stimulation of tyrosine phosphorylation of these phosphospecific sites of these two kinases.

EXPERIMENTAL PROCEDURES

Materials

Male Harlan Sprague-Dawley rats (150–200 g) were obtained from the Small Animals Section, Veterinary Resources Branch, National Institutes of Health (Bethesda, MD). Purified collagenase (type CLSFA) was from Worthington; a C-terminal octapeptide of cholecystokinin (CCK-8) was obtained from Peninsula Laboratories (Belmont, CA); CCK-JMV was obtained from Research Plus Inc. (Bayonne, NJ); phosphate-buffered saline, pH 7.4, was from Biofluids (Rockville, MD); phosphorhodamine gels and Western blotting loading equal protein assay. The BCA protein assay is not affected by these detergents. The above methods reliably measured the protein concentration of our samples. All of the standards were prepared by adding 10% polyacrylamide gels and Western blotting loading equal amounts of cellular proteins (30 µg).

EXPERIMENTAL PROCEDURES

Materials

Preparation—Dispersion rat pancreatic acini were prepared according to the modifications of the procedure published previously (27, 28). Unless otherwise stated, the standard incubation solution contained 25.5 mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH2PO4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 11.5 mM glucose, 0.5 mM CaCl2, 1 mM MgCl2, 2 mM glutamine, 1% (w/v) albumin, 1% (w/v) tryptic inhibitor, 1% (w/v) vitamin mixture, and 1% (w/v) amino acid mixture. The incubation solution was equilibrated with 100% O2 and all of the incubations were performed with 100% O2 as the gas phase. Dispersion acini from one or two rats were preincubated with standard incubation solution without or with different inhibitors for 3 h at 37 °C. For the experiments in a calcium-free medium, acini were washed two times in calcium-free incubation buffer after 150 min of incubation prior to adding thapsigargin for 30 min. The aliquots (1 ml) were then incubated at 37 °C with different agonists at the concentrations and times indicated. Acinar lysates were obtained as described previously (19, 29). Briefly, acini were sonicated for 5 s at 4 °C in 1 ml of a solution containing 50 mM Tris/HC1 (pH 7.5), 150 mM NaCl, 1% (w/v) Triton X-100, 1% (w/v) deoxycholate, 0.1% (w/v) Na2SO4, 0.1% (w/v) EDTA, 0.4% (w/v) leupeptin, 1.5% (w/v) phenylmethylsulfonyl fluoride, and 0.2% (w/v) Na3VO4. The lysates were centrifuged at 10,000 × g for 15 min at 4 °C. Protein concentration was measured by the Bio-Rad protein assay reagent as follows. For the protein assay all of the samples were diluted 10 times. The same proportion of bysion buffer was added to all standards. All of the samples were incubated in triplicate. We confirmed that the Bio-Rad assay using the above methods reliably measured the protein concentration of our lysates, confirming that the same results were obtained using the BCA protein assay. The BCA protein assay is not affected by these deterrents even at 50 times greater concentration than was used in our protein assay. Total acinar cell lysates were analyzed by SDS-PAGE using 15% polyacrylamide gels and Western blotting loading equal amounts of cellular proteins (30 µg).

Western Blotting—Western blotting was performed as described previously (9, 19, 29). The proteins were transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature and incubated for 2 h at room temperature or overnight at 4 °C with 1 µg/ml anti-pY402 PYK2 pAb, 0.064 µg/ml anti-pY580 PYK2 pAb, 1.58 µg/ml anti-pY881 PYK2 pAb, 0.07 µg/ml anti-pY397 FAK pAb, 0.45 µg/ml anti-pY577 FAK pAb, or 0.15 µg/ml anti-pY925 FAK pAb. The membranes were washed twice and incubated 1 h at 25 °C with horseradish peroxidase-conjugated goat anti-rabbit IgG. The membranes were washed, and the proteins were visualized using ECL reagents and Hyperfilm ECL. The density of bands was measured using a Kodak Imaging System (Kodak Imaging System 440 CF, Kodak, Rochester, NY). When reprobing was necessary, the membranes were stripped of bound antibodies by incubating in stripping buffer (0.1 mM glycine, pH 2.2, 1% Tween 20, 0.1% SDS) at room temperature with agitation as described previously (29). The membranes were then incubated twice for 1 h with washing solution and blocked for 1 h at room temperature. Equal protein loading was verified using PYK2 mAb for membranes that were used for the PYK2 phosphospecific antibodies and with FAK mAb for the membranes used for FAK phosphospecific antibodies. The membranes were incubated for 1.5 h with PYK2 mAb or FAK mAb at room temperature, washed as described above, and incubated for 45 min with anti-mouse IgG horseradish peroxidase-conjugated antibody. The membranes were then washed, and the proteins were visualized as described above.

RESULTS

CCK-8 (1 nM) caused an increase in tyrosine phosphorylation of each of the three phosphorylation sites of PYK2 and FAK (Fig. 1). With each of the three tyrosine phosphorylation sites of PYK2 and FAK the increase in tyrosine phosphorylation was rapid, and the time course of tyrosine phosphorylation was biphasic with a decrease after the initial peak (Fig. 1). However, there were differences in both the magnitude and kinetics of tyrosine phosphorylation of the different sites. Maximal phosphorylation for all three PYK2 phosphorylation sites, i.e. pY402, pY580, and pY881 was reached after 0.5–1 min as it was for tyrosine phosphorylation of the FAK phosphorylation sites pY577 and pY925 (Fig. 1 and Table I). In contrast, maximal tyrosine phosphorylation of the FAK phosphorylation site pY397 was delayed to 2.5 min (Fig. 1 and Table I). Maximal fold increase in the three PYK2 tyrosine phosphorylation sites was reached at the phosphorylation site pY580 with a 5.5 ± 1.2-fold, followed by a lesser degree of stimulation of pY402 and pY81 (Fig. 1 and Table I). At the FAK phosphorylation sites, pY925 demonstrated the greatest maximal fold increase of 3.9 ± 0.5-fold, followed by pY577 and pY397 (Fig. 1 and Table I).

The magnitude of phosphorylation of the three PYK2 and the three FAK tyrosine phosphorylation sites was dependent on the concentration of CCK-8 (Fig. 2). With each of the six phosphorylation sites, CCK-8 caused a detectable increase with concentrations greater then 0.01 nM CCK-8, a half-maximal increase with 0.05–0.13 nM CCK-8, and a maximal increase with 1 nM CCK-8 (Fig. 2). Concentrations greater than 1 nM CCK-8 caused either a maximal or a slight decrease in maximal stimulation of each of the six phosphospecific sites (Fig. 2).

Numerous studies show that CCKA can activate both a high and low affinity state and can mediate different cellular responses (20, 30, 31). To establish the degree of participation of each of its main physiological stimulants (25, 26), this cell system was an ideal model to investigate the comparative ability of a given stimulant to cause site-specific differences in tyrosine phosphorylation of FAK and PYK2 in the same cell and study the intracellular mechanisms involved in the stimulation of tyrosine phosphorylation of these phosphospecific sites of these two kinases.
CCK-stimulated FAK/PYK2 Phosphospecific Phosphorylation

Fig. 1. Time course of site-specific tyrosine phosphorylation of PYK2 (left panel) and FAK (right panel) stimulated by CCK-8 in rat pancreatic acinar cells. Rat pancreatic acinar cells were treated with CCK-8 (1 nM) for the indicated times and then lysed. Whole cell lysates were analyzed by SDS-PAGE followed by transfer of proteins of molecular mass >60 kDa to nitrocellulose membranes and immunoblotted as described under “Methods” with anti-pY402 PYK2 pAb, anti-pY580 PYK2 pAb, anti-pY881 PYK2 pAb, anti-pY397 FAK pAb, anti-pY577 FAK pAb, or anti-pY925 FAK pAb. The bands were visualized using ECL, and quantification of phosphorylation was performed by scanning densitometry. The upper part of both panels shows results from a representative experiment. The left panel shows the results for the three PYK2 phosphorylation sites (pY402, pY580, and pY881) and the protein loading control assessed with PYK2 mAb after stripping the membranes. The right panel shows results for the three FAK phosphorylation sites (pY397, pY577, and pY925) with their protein loading control assessed with FAK mAb after stripping the membranes. The values shown in the bottom part are the means ± S.E. of six independent experiments and are expressed as fold increases over the pretreatment level (experiments/control).

Three FAK phosphorylation sites (i.e. from 9 to 19% of maximum) (Fig. 3 and Table I). Because at all six phosphorylation sites, CCK-JMV-stimulated tyrosine phosphorylation alone did not reach the maximal response that was obtained with CCK-8, it can be concluded that stimulation of the high affinity state by CCK-8 mediates less than 46% of tyrosine phosphorylation of the three specific sites of PYK2 and less than 20% for the FAK sites. To provide evidence that most of the stimulation of tyrosine phosphorylation at each site was mediated by CCK activating the low affinity CCK\(\alpha\) receptor state, CCK-JMV was tested in combination with CCK-8 because it is an inhibitor of the low affinity state (31–33) (Fig. 3). CCK-JMV antagonized the ability of CCK-8 to stimulate maximal tyrosine phosphorylation of the three PYK2 and the three FAK phosphorylation sites (Fig. 3), confirming that CCK-8 was mediating tyrosine phosphorylation of each of these sites by also activating the low affinity CCK\(\alpha\) receptor state.

CCK\(\alpha\) receptor stimulation activates phospholipase C, which promotes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, leading to the production of diacylglycerol (20, 21, 26), which in turn activates PKC and causes the generation of inositol phosphates that stimulate cellular calcium mobilization (20, 21, 26). Recent studies demonstrate that the phosphorylation of PYK2 and FAK in pancreatic acinar cells can be influenced by increases in cytosolic calcium and activation of PKC (9, 10, 19, 34). Therefore, we next studied whether CCK-8-stimulated phosphorylation of the three PYK2 and the three FAK phosphorylation sites is dependent on changes in cellular calcium (Fig. 4), activation of PKC (Fig. 5), or activation of both PLC cascades (Fig. 5). To determine the role of intracellular calcium changes, pancreatic acinar cells were pretreated for 30 min in a calcium-free medium (with 5 mM EGTA) with thapsigargin (10 \(\mu\)M), an agent that specifically inhibits the endoplasmatic reticulum Ca\(^{2+}\)-ATPase and depletes calcium from intracellular compartments (9, 35). These conditions have been shown to inhibit completely the [Ca\(^{2+}\)]\(_i\) increase stimulated by CCK-8 (1 nM) (9). Pretreatment of pancreatic acini with thapsigargin in a calcium-free medium showed differences in the calcium dependence of tyrosine phosphorylation of specific sites of both kinases (Fig. 4). Specifically, inhibition of changes in [Ca\(^{2+}\)]\(_i\), decreased CCK-8-stimulated phosphorylation of the PYK2 phosphorylation site pY402 to 39 ± 6% of control (Fig. 4 and Table I), whereas the phosphorylation of the other two PYK2 phosphorylation sites (pY580 and pY881) was not decreased (Fig. 4 and Table I). The CCK-8-stimulated phosphorylation of the FAK phosphorylation site pY925 decreased to 38 ± 7% of control (Fig. 4 and Table I), but the phosphorylation of the other two FAK phosphorylation sites, pY397 and pY577 was not affected (Fig. 4 and Table I).

To determine whether PKC activation was involved in mediating CCK-8-stimulated increases at the PYK2 and FAK phosphorylation sites, we used the PKC inhibitor GF109203X (5 \(\mu\)M) (36). We showed previously (19) that pretreatment of pancreatic acinar cells with 5 \(\mu\)M GF109203X for 2 h completely inhibits FAK tyrosine phosphorylation induced by 12-O-tetradecanoylphorbol 13-acetate. Pretreatment of pancreatic acinar cells with 5 \(\mu\)M GF109203X for 2 h reduced the CCK-8-stimulated tyrosine phosphorylation of the PYK2 phosphorylation site pY402 and pY580 significantly (Fig. 5 and Table I), whereas no effect was seen on the tyrosine phosphorylation of the PYK2 phosphorylation site pY881 (Fig. 5 and Table I). GF109203X pretreatment alone had no inhibitory effect on the tyrosine phosphorylation of the three FAK phosphorylation sites (Fig. 5 and Table I).

Previous studies of a number of cellular effects of CCK\(\alpha\) receptor activation demonstrate that mobilization of cellular calcium and PKC activation can have synergetic effects (9, 20, 37). To examine this possibility we determined the effect of a combination of inhibition of CCK-8-induced increases in intracellular calcium and PKC activation (Fig. 5 and Table I). Pretreatment of pancreatic acini with thapsigargin in a calcium-free medium to inhibit mobilization of intracellular calcium and with GF109203X to inhibit PKC activation reduced pY402 PYK2 tyrosine phosphorylation significantly more than seen with GF109203X alone (Fig. 5 and Table I) or with inhibition of increases in [Ca\(^{2+}\)]\(_i\) alone (Fig. 4 and Table I). In contrast, it had no effect on pY881 PYK2 tyrosine phosphorylation and no greater effect than GF109203X pretreatment on pY580 PYK2 tyrosine phosphorylation (Fig. 5 and Table I). Pretreatment of pancreatic acini with thapsigargin and with GF109203X in a...
The results are calculated from the data shown in Figs. 1–5. For CCK-JMV and the basal phosphorylation, the results are expressed as the percentages of the stimulation seen with CCK-8 (1 nm) for FAK phosphorylation site pY577 was also numerically reduced calcium-free medium had a greater inhibitory effect on the phosphorylation of the FAK phosphorylation site pY397 and pY925 FAK then either inhibition of PKC or increases in [Ca\(^{2+}\)]\(_i\), alone (Fig. 5 and Table I). The phosphorylation of the FAK phosphorylation site pY577 was also numerically reduced to a greater extent than with inhibition of [Ca\(^{2+}\)]\(_i\) or PKC increase alone, but because of the small increase this did not reach statistical significance (Fig. 5 and Table I).

The unstimulated basal state of tyrosine phosphorylation of the three PYK2 and the three FAK phosphorylation sites differed (Fig. 6). Specifically, although there was no significant difference in the magnitude of basal phosphorylation of the three PYK2 phosphorylation sites as well as in the two FAK tyrosine phosphorylation sites pY577 and pY925, the basal tyrosine phosphorylation of site pY397 FAK showed a significant higher degree of basal, unstimulated phosphorylation (Fig. 6).

**DISCUSSION**

Recent studies show that CCK\(_A\) receptor activation, similar to a number of growth factors, oncogenes, integrins, and stimulation of some other G protein-coupled receptors, causes activation and tyrosine phosphorylation of the two related kinases p125FAK and PYK2 (2, 9, 11, 19, 34, 38, 39). Activation of these kinases is important in cellular growth, motility, and cytoskeletal changes (1, 2, 39–41). FAK and PYK2 share 45\% overall sequence similarity (3). To date, six tyrosine phosphorylation sites (pY397, pY407, pY576, pY577, pY861, and pY925) are known in FAK (11, 42). Four of the six FAK phosphorylation sites are conserved at homologous positions in PYK2 (2), with pY402 PYK2 corresponding to pY397 FAK, pY579/580 PYK2 corresponding to pY576/577 FAK, and pY881 PYK2 corresponding to pY925 FAK. Some studies demonstrate that the tyrosine phosphorylation of the different sites may differ in various cells with different stimuli (43, 44). Furthermore, in some cells various stimuli that activate PYK2 or FAK cause tyrosine phosphorylation of all three PYK2 (39, 43, 45–47) or all three FAK phosphorylation sites (6, 42, 48), whereas in other cells only some of the three phosphorylation sites are phosphorylated by these stimuli (39, 49–51). At present little is known about the cellular mechanisms involved in the regulation of phosphorylation at each of the tyrosine phosphorylation sites within either of these two tyrosine kinases or whether it is similar for homologous sites within the two kinases. Furthermore, how activation of other intracellular mediators such as phospholipase C changes in [Ca\(^{2+}\)]\(_i\), or activation of PKC participate in regulation of the tyrosine phosphorylation at the different sites in the two kinases in the same cell is at present largely unclear. The present study was designed to address these issues, by investigating the effects of the hormone/neurotransmitter cholecystokinin (CCK) on one of its physiological target tissues, pancreatic acinar cells (25). In pancreatic acini, activation of CCK\(_A\) receptors stimulates rapid tyrosine phosphorylation of both FAK (34) and PYK2 (9). Furthermore, its intracellular signaling cascades have been extensively studied and shown to involve stimulation of phospholipase C with mobilization of cellular calcium and PKC activation, activation of phospholipase D and phospholipase A\(_2\), and stimulation of multiple tyrosine kinase cascades (10, 25, 52). The effect of CCK in pancreatic acini is therefore an excellent potential model to use to assess the ability of an agonist to stimulate tyrosine phosphorylation of both FAK- and PYK2-specific sites and to investigate the role of changes in [Ca\(^{2+}\)]\(_i\) and PKC activation in mediating these effects in the same cell.

In this study we demonstrate in pancreatic acini that CCK-8 stimulated tyrosine phosphorylation of each of the three homologous tyrosine phosphorylation sites in both FAK and PYK2. The ability of CCK to stimulate tyrosine phosphorylation of each of the three FAK sites differs from the stimulation of the tyrosine phosphorylation at only two of the three FAK sites reported with various growth factors (heregulin in breast cancer cells (pY577 and pY925 only) (49), transforming growth factor-\(\beta\) in mammary epithelial cells (pY397 and pY577 only) (39) and at only one site of FAK (pY397) with integrin or fibronectin stimulation in prostate cancer cells (51). Our results also differ with PYK2 from the effect of transforming growth factor-\(\beta\) in mammary cells where only two sites were tyrosine-phosphorylated (pY402 and pY881) (39). However, our results showing tyrosine phosphorylation in all three sites in FAK by CCK\(_A\) receptor activation are similar to the effects of cannabinoids in hippocampal cells (48), bone mesin in 3T3 cells (53), thrombin in platelets (6), and fibronectin in fibroblasts (42). Similarly, stimulation of tyrosine phosphorylation of all three sites of PYK2 is seen with endothelin-1 in mesangial cells (45), thrombin or histamine in HUVEC cells (43, 46), as well as the effect of transforming growth factor-\(\beta\) in mammary epithelial cells (39). These results demonstrate that different stimuli in different cells may stimulate tyrosine phosphorylation of all or only some of the potential tyrosine phosphorylation sites in FAK or PYK2.

A number of results support the conclusion that CCK stimulation of tyrosine phosphorylation of each of the different sites in FAK and PYK2 differed in kinetics and the magnitude of stimulation. The kinetics of phosphorylation of the pY397 autophosphorylation site of FAK was slower with the maximal

| Variable | PYK2 | FAK |
|----------|------|-----|
| Peak time of TyrP with CCK (min) | 0.5 ± 0.1 | 1 ± 0.4 |
| Maximum fold increase with CCK | 2.8 ± 0.3 | 1.8 ± 0.3 |
| Fold increase after 15 min with CCK | 1.7 ± 0.2 | 1.2 ± 0.4 |
| EC\(_{50}\) of CCK-8 (nm) | 0.052 ± 0.001 | 0.13 ± 0.02 |
| CCK-JMV (1 \(\mu\)M) (% of maximal) | 33 ± 8 | 19 ± 5 |
| TG with CCK (% control) | 39 ± 6 | 101 ± 4 |
| CCK stimulation with GF109203X (5 \(\mu\)M) (% control) | 59 ± 6 | 91 ± 25 |
| CCK stimulation with GF109203X (5 \(\mu\)M) and no increase in [Ca\(^{2+}\)]\(_i\) | 10 ± 7 | 0 ± 5 |
| Basal phosphorylation (% maximal) | 33 ± 2 | 63 ± 4 |
increase not seen until 2.5 min, whereas it occurred in 0.5 min with each of the other five sites of FAK and PYK2. This result would not have been anticipated, given the fact that the phosphorylation of pY397 is reported to be the first step in FAK phosphorylation (2, 11, 12, 42) and has been shown by mutational studies (11, 13, 54, 55) to be essential for the subsequent transphosphorylation of FAK. One possible explanation for the delayed maximum in pY397 FAK tyrosine phosphorylation is that for maximum phosphorylation of this site to occur, the phosphorylation of the two sites in the kinase activation loop pY376/577 is needed (54, 55). Mutational studies of pY376/577 support this possibility because without phosphorylation of these sites maximal pY397 phosphorylation does not occur (54, 55). Only limited data from other studies exist to determine whether similar kinetics exists in other cells with other stimuli. Two studies with PYK2 (47, 56) and one with FAK (44) report the kinetics of phosphorylation of all sites. Similar to our kinetic results with CCK at stimulation of PYK2 sites, thrombin or histamine in HUVEC cells (43) and electroshock in hippocampal cells (47) stimulated a rapid increase reaching a maximum at each PYK2 site within 1 min. Similar to our result with FAK, low density lipoprotein in human platelets (44) stimulated a more rapid increase in pY577 and pY925 than in pY397. However, in the same cell thrombin stimulated a similar rapid increase in all sites, which was maximal after 2.5 min (44). These results demonstrate that although the rate of tyrosine phosphorylation of the three sites in FAK and PYK2 are generally rapid, they can differ significantly in different cells with different stimuli. In pancreatic acini with CCKA receptor activation each of three sites of FAK and PYK2 demonstrated a biphasic time course with an initial peak increase and then a decrease to baseline with time. These results differ from low density lipoprotein stimulation of phosphorylation at the three sites of FAK in platelets (44), in which the stimulation remained elevated with time and with cannabinoid stimulation of pY397 FAK phosphorylation in hippocampal cells (48), where it did not decrease with time. Similar results to our finding in pancreatic acini of CCK causing a biphasic pattern of stimulation of all sites in FAK and PYK2 are results reported with thrombin stimulation of FAK tyrosine phosphorylation of all three sites in human platelets (6): endothelin stimulation in mesangial cells of pY402 and pY580 of PYK2 (45), angiotensin or lysophosphatidic acid stimulation in intestinal epithelial
cells of pY402 and pY580 of PYK2 (57), and histamine or thrombin stimulation of all three PYK2 sites in HUVEC cells (43). Because in most cells with various stimulants this biphasic pattern of stimulation of tyrosine phosphorylation at each of the PYK2 and FAK sites occurs, these results suggest coincident with their activation of FAK and PYK2 that these stimuli must also activate tyrosine phosphatases, which rapidly reverse the tyrosine phosphorylation.

We found for the first time that the magnitude of stimulation differed between the different phosphorylation sites within FAK and PYK2, as well as between the homologous sites of the two kinases. Specifically, we found that the relative magnitude of increase with PYK2 was pY580 > pY402, pY881, whereas with FAK it was pY577 > pY925 > pY397. Furthermore, whereas pY402 and pY580 of PYK2 demonstrated greater increases in tyrosine phosphorylation than the homologous pY397 and pY577 of FAK, the reverse was true with pY881 of PYK2 and pY925 of FAK. No comparable data exist with other stimulants in other cells to compare the results of the magnitude of site-specific phosphorylation of FAK to the three homologous sites in PYK2 in the same cell. However, our results have similarities and differences from studies investigating the magnitude of stimulation of the different sites in one of the kinases in a given cell. Our results with PYK2 differ from those reported with endothelin-1 in mesangial cells (45), in which all three sites had equal magnitude of phosphorylation, and from a number of cells in which the relative magnitude of stimulation of the different sites differed from ours (angiotensin or lysophosphatidic acid in mesangial cells (57), histamine in platelets (43), thrombin in HUVEC cells (46), and hepatocyte growth factor in lung cancer cells (44)). Our results with the PYK2 phosphospecific sites were similar to the magnitude of stimulation at each site in HUVEC cells with thrombin (43). However, our FAK results differ from those with cannabinoids in hippocampal cells (48), in which no differences were seen in the magnitude of stimulation at each site and differ in the relative magnitude of stimulation of each site from that reported with heregulin stimulation of breast cancer cells.
Numerous studies demonstrate that the pancreatic CCKA receptor exists in at least two binding states and that activation of the two different states causes activation of different cellular cascades (20, 21, 26, 30, 66–68). Our results support the conclusion that CCK is stimulating tyrosine phosphorylation at each of the three FAK- and PYK2-specific sites by activation of both receptor states; however, the extent of participation by each state varies with the different specific phosphorylation site. This conclusion is supported by the finding that CCK-JMV, which functions as an agonist at the high affinity receptor state and as an antagonist on the low affinity receptor state in rat pancreatic acini (31–33), stimulated tyrosine phosphorylation at each of the three sites in each kinase, demonstrating that stimulation of the CCKA receptor high affinity state participates in phosphorylation at each of three sites in both kinases. However, CCK-JMV did not stimulate to the same maximal extent as CCK, suggesting that activation of the high affinity state of the CCKA receptor only accounted for some of the stimulation caused by CCK. Furthermore, the magnitude of phosphorylation with CCK-JMV was higher in the three PYK2 than in the three FAK tyrosine phosphorylation sites, demonstrating that activation of the high affinity state of the CCKA receptor played a more important role in stimulation of the PYK2 sites than the FAK sites. CCK-JMV antagonized the ability of CCK-8 to stimulate tyrosine phosphorylation of all six phosphospecific sites. This result confirmed that activation of the low affinity CCKA receptor state, for which CCK-JMV is an antagonist (31, 33), is also involved in the ability of CCK to cause maximal stimulation of tyrosine phosphorylation at each site in each kinase. With the use of CCK-JMV, we could demonstrate that in the case of the PYK2 phosphospecific sites, ~45% of the phosphorylation was mediated by the high affinity state, whereas in the case of the three FAK phosphospecific sites, less than 20% of the phosphorylation was mediated by the high affinity state. The degree of participation of the two CCKA receptor affinity states in the site-specific phosphorylation in FAK and PYK2 has both similarities and differences from that reported with CCKA receptor-stimulated tyrosine phosphorylation of other proteins in pancreatic acini. CCKA receptor activation in rat pancreatic acini has been shown to cause tyrosine phosphorylation of paxillin (19), p130Cas (29), PKC-δ (22), Jun kinase (69), and mitogen-activated protein kinase (52). Similar to our results in the present study for FAK and PYK2 phosphospecific site phosphorylation, stimulation of tyrosine phosphorylation of paxillin in rat pancreatic acini by CCK was stimulated by activation of both the high and the low CCKA receptor states. In contrast, stimulation of tyrosine phosphorylation of PKC-δ (22) is mediated only by activation of the low affinity CCKA receptor state. These results demonstrate that not only are the phospholipase A2 and PLC cascades coupled differently to high and low affinity CCKA receptor states as described previously (68), but also the tyrosine phosphorylation cascades activated by CCKA receptor are differentially coupled to these two different receptor states.

Activation of the pancreatic CCKA receptor is known to stimulate PLC activity, resulting in the generation of inositol phosphates and diacylglycerol, which in turn results in mobilization of cellular calcium and activation of PKC, respectively (20, 33). Recent studies show that the activation of the two different limbs of the PLC cascade with changes in intracellular calcium and the activation of PKC in various cells by different stimuli can have different effects on the phosphorylation of PYK2 and FAK (9, 10, 19, 70–74). Our previous studies demonstrate that the CCKA receptor-mediated phosphorylation of PYK2 is completely controlled by PLC-dependent mechanisms (9), whereas

(49), from thrombin stimulation of platelets (6), and from transforming growth factor-β stimulation of mammary cells (39). These results demonstrate that the relative magnitude of tyrosine phosphorylation of the different sites in FAK and PYK2 stimulated by different agents in different cells varies considerably. Previous studies in other cells with other stimuli demonstrate that activation of PYK2 or FAK can result in activation of different cellular cascades. Specifically, activation of various G protein-coupled receptors results in mitogen-activated protein kinase activation that is PYK2-dependent but FAK-independent (5, 58, 59). Activation of both kinases has different effects on the regulation of the cell cycle (40, 60), the regulation of apoptosis (61), the activation of Jun kinase (40, 62), or the differential phosphorylation of substrates like p130Cas (63), PAP (64), or Nrrs (65). Our results demonstrating that CCK-stimulated phosphorylation of the homologous sites of FAK and PYK2 differs in magnitude and kinetics, supporting the conclusion that the differential activation of these two closely related kinases in pancreatic acini by CCK, may have an important role in mediating the ability of CCK to activate different cellular signaling cascades and have different cellular effects.
the CCKα receptor-mediated FAK phosphorylation is controlled by both PLC-dependent and -independent mechanisms (9, 19). A number of results in the present study support the conclusion that additional differences between the two closely related kinases exist in the role of activation of the limbs of the PLC cascade either alone or together in stimulating tyrosine phosphorylation of the three homologous sites in the two kinases. After incubation with the Ca2+/ATPase inhibitor thapsigargin in a calcium-free medium, which has been shown to completely inhibit CCK-mediated increases in [Ca2+], in pancreatic acini (9), the tyrosine phosphorylation of pY402PYK2 was inhibited by 60%, whereas the homologous pY397 site in FAK was unaffected. Conversely, the phosphorylation of pY925FAK was inhibited by 62%, whereas the homologous pY881 site in PYK2 was unaffected. In neither kinase was the kinase activation loop site (pY580PYK2 or pY577FAK) affected. These results demonstrate that the importance of increases in cytosolic calcium can vary markedly in mediating tyrosine phosphorylation at different sites in the same kinase and at similar sites in these two related kinases. Whereas no similar data exist for the role of calcium in phosphospecific site phosphorylation of FAK in other cells, our results differ from the results of the two studies (45, 47) that examined the role of calcium in tyrosine phosphorylation of specific sites in PYK2 in other cells. In human glomerular mesangial cells, endothelin-1 stimulation of tyrosine phosphorylation of pY402 PYK2, pY580 PYK2, and pY881 PYK2 is abolished by the intracellular calcium chelator BAPTA (1,2-bis(O-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl ester) (45). Similarly, in HUVEC cells, thrombin and histamine-stimulated tyrosine phosphorylation of each of the three PYK2 sites (pY402, pY580, and pY881) is decreased by treatment with BAPTA (43). These results suggest that increases in [Ca2+]i can play a varying role in mediating tyrosine phosphorylation at these different sites of PYK2 in different cells.

Our results support the conclusion that CCKα receptor activation of PKC can also differentially participate in the tyrosine phosphorylation of the three phosphorylation sites of FAK and PYK2, and the extent of participation can vary at homologous sites in the two kinases. The PKC inhibitor GF109203X at a concentration that inhibits 12-O-tetradecanoylphorbol 13-ace-tate-stimulated tyrosine phosphorylation of FAK and PYK2 (9, 19) caused a 100% decrease in CCK-stimulated tyrosine phosphorylation at the pY580 site of PYK2 while causing no decrease at the homologous pY577 site of FAK. Furthermore, GF109203X inhibited by 40% CCK-stimulated tyrosine phosphorylation at the pY402 autophosphorylation site of PYK2 while having no effect on the homologous pY397 FAK site. GF109203X caused no effect at the pY881PYK2 site or the homologous pY925 site of FAK. Finally, the inhibition of both limbs of the PLC cascade by simultaneous addition of GF109203X and thapsigargin caused greater inhibition at a number of phosphorylation sites in either kinase than inhibition of either PLC limb alone, demonstrating synergistic interaction of these two cascades. These results show that activation of the two limbs of the PLC cascade participate to different degrees in both the tyrosine phosphorylation of the different kinases within one kinase as well as at the homologous sites in the two different kinases. These results support the conclusion that the cellular mechanisms regulating tyrosine phosphorylation of the two closely related kinases are markedly different in pancreatic acinar cells. Our results with the inhibition of PKC activation in tyrosine phosphorylation of the different sites of PYK2 have similarities and differences from studies in other cells with other stimuli. In the only other study that examined the participation of PKC in tyrosine phosphorylation of the PYK2-specific sites, HUVEC cells stimulated with thrombin or histamine, the tyrosine phosphorylation of all three PKC phosphospecific sites was unchanged with addition of the PKC inhibitor Ro31-8220 (43). Similar to our result with pancreatic acini, PKC down-regulation in rat fibroblasts resulted in no change in fibronectin-stimulated pY397 phosphorylation of FAK; however, it caused an almost complete inhibition of pY925FAK phosphorylation, whereas in our study we saw no effect of PKC inhibition at this site. These results demonstrate that the participation of the two limbs of the PLC cascade mediating tyrosine phosphorylation in homologous sites of these two closely related kinases appears to vary in different cells with different stimuli.

Our results reveal that another important difference between the tyrosine phosphorylation of PYK2 and FAK is in the degree of basal phosphorylation present. The basal tyrosine phosphorylation of the autophosphorylation site pY397FAK was significantly higher than the homologous phosphorylation site pY402PYK2, as well as of the other two PYK2 and other two FAK tyrosine phosphorylation sites. A similar result was found in murine mammary epithelial cells, where pY397 was phosphorylated in the unstimulated cell to a greater extent than the other two sites in FAK or the three sites in PYK2 (39). Also, in HUVEC cells (75) and human platelets (6), pY397 basal phosphorylation was greater than that seen with pY577 or pY925 FAK; however, no comparison with PYK2 site-specific phosphorylation was performed (75). These results show that in each of the cells investigated, the basal tyrosine phosphorylation state of the pY397 FAK site is higher than in the other sites. At present the significance of this is unclear.

In conclusion, in the present study we show that each of the three homologous tyrosine phosphorylation sites in the two related kinases FAK and PYK2 undergo rapid tyrosine phosphorylation after CCK receptor activation in rat pancreatic acini. Furthermore our results demonstrate that the different phosphorylation sites differ in their regulation by the different limbs of the PLC cascade, the magnitude and degree of participation of the high and low affinity CCKα receptor states, the kinetics of stimulation, and the level of basal tyrosine phosphorylation. Although both kinases share a high degree of structure similarity, they have been shown to have different functions, such as the activation of different downstream signaling cascades and different roles in the regulation of cell cycle and apoptosis (40, 60, 61). Our results suggest that the differences shown in this study in the phosphorylation and regulation of these different sites in each of these two closely related kinases likely contribute to these different cellular effects after their activation.

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Phosphospecific Site Tyrosine Phosphorylation of $p125^{\text{FAK}}$ and Proline-rich Kinase 2 Is Differentially Regulated by Cholecystokinin Receptor Type A Activation in Pancreatic Acini

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