A Role for Cholecystokinin-stimulated Protein Tyrosine Phosphorylation in Regulated Secretion by the Pancreatic Acinar Cell*

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Cholecystokinin (CCK) is a gastrointestinal hormone that acts through a G protein-coupled receptor to stimulate pancreatic enzyme secretion. In this work, we demonstrate that CCK stimulation of dispersed pancreatic acini results in increased tyrosine phosphorylation. This is mediated by a calcium-dependent pathway, also activated by a phenethyl ester analogue of CCK and calcium ionophores, and by a protein kinase C-dependent cascade, also activated by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate. All demonstrable stimulated tyrosine phosphorylation events were inhibited by genistein, with different subsets of proteins affected by staurosporine and H-7. The importance of tyrosine phosphorylation events in agonist-stimulated amylase secretion was studied using genistein and staurosporine as protein kinase inhibitors. Genistein inhibited the secretory response to CCK, its phenethyl ester analogue, and calcium ionophores, all known to stimulate secretion in a calcium-dependent fashion. In contrast, genistein had no effect on the secretory response to 12-O-tetradecanoylphorbol-13-acetate, suggesting that the protein kinase C-dependent tyrosine phosphorylation events were not involved in the secretory mechanism. Furthermore, CCK-induced secretion was not affected by relatively specific protein kinase C inhibitors. Staurosporine, an inhibitor of both protein kinase C and tyrosine kinase activities in these cells, provides evidence that acinar cell tyrosine phosphorylation is stimulated by agonists acting via calcium-dependent and protein kinase C-dependent pathways, with only the calcium-dependent tyrosine phosphorylation cascade involved in triggering hormone-induced amylase release.

Cholecystokinin (CCK) is a gastrointestinal peptide hormone that functions as the major physiological pancreatic secretagogue as well as a pancreatic acinar cell mitogen. Its plasmalemmal receptor is a member of the superfamily of G protein-coupled receptors and contains no consensus sequence to suggest endogenous tyrosine kinase activity. The intracellular effects of cholecystokinin are classically thought to be mediated by a cascade consisting of G protein activation of phospholipase C, leading to the production of inositol phosphates and diacylglycerol. The downstream mechanisms affected by this hormone-stimulated cascade are believed to be dependent on a rise in intracellular calcium levels and the activation of protein kinase C. Consistent with the activation of this and other kinases in this well-described signaling cascade, multiple serine/threonine phosphorylation events have been observed in acinar cells in response to agonist stimulation (3–5). Missing from this paradigm has been a role for protein tyrosine phosphorylation in the stimulus-secretion coupling pathway in acinar cells.

Precedent for the abilities of G protein-coupled receptor agonists to stimulate tyrosine phosphorylation comes from the recent reports of such effects by angiotensin, vasopressin, and bombesin in tissue culture cell lines (6–9). All of these agents are reported to be trophic in the studied cell lines, and it was suggested that the stimulated tyrosine phosphorylation may play a role in that process. Indeed, protein tyrosine phosphorylation has become a major theme in cellular regulation, particularly as it relates to the induction of cell growth and mitogenesis. The transmembrane receptors for several polypeptide growth factors express a ligand-regulated tyrosine kinase domain. Of interest, the patterns of protein tyrosine phosphorylation stimulated by the receptor tyrosine kinase and the G protein-coupled receptor families appear to be different (6). There is now evidence, however, for interrelationships and cross-activation between tyrosine phosphorylation and serine/threonine phosphorylation events (10). For example, certain receptor-linked protein-tyrosine kinases are indirectly linked to the serine/threonine kinase protein kinase C via the tyrosine kinase substrate phospholipase C, whose activation leads to production of diacylglycerol (11, 12). Conversely, direct activation of protein kinase C by phorbol esters has been shown to activate tyrosine kinases (13).

It would be particularly interesting if agonist-stimulated protein tyrosine phosphorylation could be clearly linked to a cell process other than mitogenesis. A potential role for tyrosine phosphorylation in regulating pancreatic acinar cell secretion was suggested in a recent report by Jena et al. (14), in which the introduction of a recombinant tyrosine-specific phosphatase from brain into permeabilized pancreatic acini resulted in increased amylase secretion in response to calcium. It is not clear, however, whether that manipulation resulted in a decrease or increase in net intracellular tyrosine phosphorylation. For example, the introduction of a tyrosine phosphatase might paradoxically activate src-like protein-tyrosine kinases by dephosphorylating a negative regulatory phosphotyrosyl residue (15).

This study was therefore undertaken to determine if hor-
monal stimulation of the CCK receptor in freshly isolated, nontransformed, intact pancreatic acinar cells could lead to a change in the state of tyrosine phosphorylation of proteins. These observations were then expanded using a variety of well-characterized pancreatic secretagogues and enzyme inhibitors to gain insight into the intracellular mediation of protein tyrosine phosphorylation. Additionally, tyrosine kinase inhibitors were used to study the role of tyrosine phosphorylation in mediating cellular responses other than cell growth, namely the regulated secretion of amylase from these healthy, intact pancreatic acinar cells.

**MATERIALS AND METHODS**

Reagents and Animals—Bovine serum albumin (Cohn fraction V) was from Armour; soybean trypsin inhibitor and collagenase were from Worthington. A23187, ionomycin, 12-O-tetradecanoylphorbol-13-acetate (TPA), staurosporine, and genistein were from Calbiochem; H-7 was from Saikagaku (Rockville, MD). Monoclonal anti-phosphotyrosine antibodies (4G10) were from Upstate Biotechnology Corp.; H-7 was from Saikagaku (Rockville, CA); and the chemiluminescent ECL reagent was from Amersham Corp. The Phadebas reagent was purchased from Pharmacia LKB Biotechnology Inc. Other reagents were analytical grade.

Synthetic CCK-8 (CCK-(26-33)) was purchased from Peninsula Laboratories, Inc. (Belmont, CA); and the COOH-terminal phenethyl ester analogue of cholecystokinin, D-Tyr-Gly-[Nle<sup>3</sup>]-CCK-26-32 phosphaapeptide was synthesized as described (16).

Male Sprague-Dawley rats weighing 125-150 g were used as the source of pancreatic acini. The protocols were reviewed and approved by the Mayo Clinic Animal Care and Use Committee.

Pancreatic Acinar Cell Preparation—Dispersed rat pancreatic acini were prepared by sequential enzymatic and mechanical dissociation as described by Schultz et al. (17) or by Jena et al. (14). After three washes, the acini were resuspended in oxygenated Krebs-Ringer/Hepes buffer containing 25 mM Hepes, pH 7.4, 104 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 1.2 mM MgSO₄, 2 mM CaCl₂, 2.5 mM d-glucose, essential and nonessential amino acids, 0.2% bovine serum albumin, and 0.01% soybean trypsin inhibitor at 37 °C. The viability of cells, as assessed by trypsin blue exclusion, remained in excess of 95% after 1 h of incubation and was not significantly reduced by either the secretagogues or the protein kinase inhibitors used in this work.

Tyrosine Phosphorylation Studies—For the analysis of phosphorylated bands, acini were preincubated in the presence of well-characterized pancreatic secretagogues and enzyme inhibitors to gain insight into the intracellular mediation of protein tyrosine phosphorylation. An example of this is shown in the right, top three panels. The major stimulated bands were observed in the range below 60 kDa and at 93 kDa; however, these did not significantly change in intensity upon hormonal stimulation. The phosphorylation of all of the responsive proteins increased in a change in intensity upon hormonal stimulation. The phosphorylation of all of the responsive proteins increased in a

**RESULTS**

Tyrosine Phosphorylation Studies—The presence of phosphotyrosine-containing proteins in acinar cell suspensions was studied in resting acini and in response to stimulation of these cells with various well-characterized pancreatic secretagogues. Nonstimulated cells contained several bands that reacted with the 4G10 anti-phosphotyrosine antibodies (Fig. 1, left). The intensity of the labeling of these bands did not change during preincubation of acini at 37 °C for up to 30 min, suggesting that these proteins exhibited a basal level of phosphotyrosine in nonstimulated acinar cells in vivo. After exposure of cells to CCK, the major hormonal stimulant of pancreatic secretion, increases in the tyrosine phosphorylation of several proteins could be observed (Fig. 1, left and right, top three panels). The major stimulated tyrosine-phosphorylated proteins migrated on sodium dodecyl sulfate-polyacrylamide gels at apparent molecular masses of 66, 72, 78, 93 kDa, and 120 kDa (these will be referred to as p66, p72, p78, p93, and p120, respectively). Several additional tyrosine-phosphorylated bands were observed in the range below 60 kDa and at 93 kDa; however, these did not significantly change in intensity upon hormonal stimulation. The phosphorylation of all of the responsive proteins increased in a

To elucidate the role of individual second messenger cascades in the observed events, phosphorylation was stimulated with the carboxyl-terminal phenethyl ester analogue of CCK, OPE, a partial CCK receptor agonist that elicits little or no phospholipase C activation, but a clear intracellular calcium response (20). This analog increased phosphorylation of the same bands as CCK (Fig. 1, right, top three panels). However, there were quantitative differences in the phosphorylation responses to this secretagogue compared with those to the native hormone.

Acinar cells were also stimulated with the phorbol ester TPA to activate protein kinase C directly and with the calcium ionophores A23187 and ionomycin to increase intracellular calcium levels. TPA stimulated only a portion of the CCK or
Tyrosine Phosphorylation in Amylase Secretion

FIG. 1. Protein tyrosine phosphorylation in rat pancreatic acinar cells in response to various stimuli. Left, representative anti-phosphotyrosine immunoblot of total cellular proteins from nonstimulated and CCK-stimulated acinar cells; right, top three panels, densitometric quantitation of representative bands from gels in which acinar cells were stimulated with the indicated concentrations of CCK and OPE for 2 min and with TPA and A23187 for 5 min. Phosphoproteins p72 and p78 behaved similarly to p120, so quantitative analyses of those bands are not illustrated. Values represent means ± S.E. of at least three independent experiments. Asterisks indicate significant differences as compared to nonstimulated controls (p ≤ 0.05 with Student’s t test for unpaired samples). Bottom panel, densitometric quantitation of time courses of stimulation of representative bands.

OPE response, namely phosphorylation of p72, p78, and p120. Interestingly, exposure of the cells to ionophores stimulated responses similar to and approximately of the same magnitude as OPE (Fig. 1, right, top three panels).

To distinguish further these pathways and to evaluate the involvement of tyrosine kinases in the observed phenomena, we examined the effects of several protein kinase inhibitors on CCK-dependent tyrosine phosphorylation in acinar cells (Fig. 2). Genistein was used as a relatively specific tyrosine kinase inhibitor (21); staurosporine was used at a concentration known to inhibit both tyrosine kinases and protein kinase C (22); and H-7 was employed to inhibit protein kinase C activity without affecting tyrosine kinases directly (23). All three modified the tyrosine phosphorylation responses to CCK in these cells. Genistein eliminated all of the tyrosine phosphorylation stimulated by CCK, strongly suggesting that tyrosine kinases were activated by stimulation of pancreatic acinar cells with CCK. In contrast, the responses to staurosporine and H-7 could be separated into two different patterns. Staurosporine inhibited the CCK-stimulated phosphorylation
of p105 and p120, whereas the phosphorylation of p66 on tyrosine residues was actually enhanced by this inhibitor. H-7 significantly inhibited the tyrosine phosphorylation of only p120 and, like staurosporine, enhanced the phosphorylation of p66. The CCK-stimulated tyrosine phosphorylation of p72 and p78 was not affected by H-7, but was inhibited by staurosporine.

In control experiments, we observed that treatment of pancreatic acinar cells with genistein or staurosporine alone, in the absence of secretagogues, reduced the basal phosphotyrosine content of the p120 and p105 substrates. This result is likely explained by a drug-induced inhibition of basal protein-tyrosine kinase activity in these cells, which, in the presence of constitutive intracellular phosphatase activity, leads to a decreased steady-state level of phosphotyrosine. This effect on basal levels of tyrosine phosphorylation is reflected in some of the secretagogue-stimulated data. For example, staurosporine or genistein alone reduced the basal activity of p120 to 48 ± 10 and 60 ± 12% of that observed in untreated cells, respectively. After stimulation with CCK, the phosphorylation intensity of this band was 41 ± 12 and 64 ± 14% of controls in the presence of staurosporine and genistein, respectively (Fig. 2). Thus, the apparent inhibition only reflects a decrease in basal phosphorylation and does not suggest secretagogue-induced inhibition of kinase activity. Furthermore, in the presence of these tyrosine kinase inhibitors, CCK does not stimulate tyrosine phosphorylation above relevant basal levels.

Like the CCK-stimulated protein tyrosine phosphorylation events, the tyrosine phosphorylation events stimulated by OPE, calcium ionophore, and TPA were all completely eliminated by genistein (data not shown).

**Amylase Secretion Studies**—To study the potential role of tyrosine phosphorylation in regulated amylase secretion, we tested the effects of kinase inhibitors on stimulated secretion in dispersed intact rat pancreatic acinar cells. Cholecystokinin stimulated acinar cell secretion in a concentration-dependent manner, with maximal secretion of 28.5 ± 1.1% of total cellular amylase content stimulated by 10^{-9} M CCK and with reduced secretion observed in response to higher hormone concentrations. Genistein partially inhibited CCK-stimulated amylase secretion (Fig. 3). This inhibitory effect varied depending on the concentration of CCK used and was most marked at maximally stimulatory CCK concentrations, at which inhibition was 41%. The inhibition of acinar cell secretion stimulated by a maximal concentration of CCK was also dependent on the concentration of genistein used, reaching its maximum between 50 and 100 μg/ml concentrations of this reagent and with concentrations as high as 200 μg/ml not increasing this effect further.

The partial agonist analogue of CCK (OPE) was tested in a similar fashion (Fig. 4). As previously reported (16), OPE stimulated amylase secretion with efficacy similar to that of CCK (31.0 ± 1.0% maximal release); however, in contrast to CCK, OPE elicited no supramaximal inhibition of secretion. Genistein again was able to significantly decrease amylase secretion induced by maximally stimulatory concentrations of this agonist by 46%.

Both CCK and OPE are known to increase intracellular calcium. To determine if the genistein action to inhibit amylase secretion might be distal to the calcium step, the effect on ionophore-stimulated secretion was studied (Fig. 4). Consistent with previous reports (24), the calcium ionophores A23187 and ionomycin increased the level of secreted amylase by 16.5 ± 2.6%, representing about half of the stimulated secretion observed with CCK and its OPE analogue. Inhibition of A23187-stimulated amylase secretion by genistein was detectable at 1 μM A23187. Similar results were obtained using ionomycin; genistein reduced secretion by 7.5% with 0.3 μM ionomycin and by >35% with 1 μM ionomycin.

Cholecystokinin is known to activate phospholipase C, leading to phosphatidylinositol hydrolysis, diacylglycerol release, and protein kinase C activation. This is the cascade of events that OPE appears not to stimulate (16, 20). The ability of genistein to inhibit OPE-stimulated secretion might suggest...
that this effect is not distal to protein kinase C. This was tested more directly using the phorbol ester TPA to directly activate protein kinase C (Fig. 4). TPA stimulated acinar cell secretion in a concentration-dependent manner, with maximal release at 16.0 ± 1.0% of total cellular amylase content. In contrast to the results obtained with the hormonal secretagogues or with the calcium-stimulating agents, secretion stimulated by the phorbol ester was not affected by tyrosine kinase inhibition with genistein.

These results support the existence of two CCK receptor-coupled signaling cascades with differing involvement of tyrosine phosphorylation. One cascade is calcium-dependent and is sensitive to tyrosine kinase inhibition. The other is protein kinase C-dependent and does not involve genistein-sensitive tyrosine phosphorylation events. We more closely investigated this second cascade by examining the effects of other kinase inhibitors on stimulated secretion. Staurosporine was used as combined inhibitor of protein kinase C and tyrosine kinases, and H-7 was employed to inhibit protein kinase C more selectively (Fig. 5). Staurosporine significantly inhibited CCK-stimulated acinar cell secretion, whereas H-7 had no significant effect on CCK-stimulated acinar cell secretion and had a tendency to actually increase CCK-stimulated secretion. In control experiments, the same concentration of H-7 was sensitive and significant in inhibiting TPA-stimulated acinar cell secretion with a secretagogue concentration as high as 1 μM TPA.

**DISCUSSION**

Cholecystokinin is the major physiological pancreatic secretagogue, known to act via a G protein-coupled receptor. In this work, we have demonstrated for the first time that this hormone stimulates protein tyrosine phosphorylation in freshly isolated, healthy pancreatic acinar cells. Furthermore, the inhibition of tyrosine kinases reduced CCK-stimulated secretion by these cells, suggesting a role for CCK-stimulated tyrosine phosphorylation in augmenting and regulating this cellular function. By using a variety of different pancreatic secretagogues and kinase inhibitors, it has been possible to gain insight into the mechanisms of cellular mediation of tyrosine phosphorylation as well as insights into the cascade of events responsible for the observed effect of tyrosine phosphorylation on cellular secretion.

It has recently been appreciated that protein tyrosine phosphorylation can be stimulated by occupancy of G protein-coupled receptors that have no intrinsic tyrosine kinase activity. Examples of this include stimulation of specific phosphorylation events in tissue culture cells by angiotensin and bombesin (6-9). The pattern of tyrosine phosphorylation in these cells is quite characteristic and is different from that stimulated by ligation of members of the receptor tyrosine kinase family, which express an intrinsic tyrosine kinase domain. Indeed, we observed an increase in signal intensity of several phosphoproteins on anti-phosphotyrosine immunoblots of pancreatic acinar cells stimulated by CCK. These included substantial increases in phosphosubstrates designated p120, p105, p66, p72, and p78 on the basis of their apparent molecular masses when separated on SDS-polyacrylamide gels. Phosphorylation occurred in an agonist concentration-dependent manner and could be inhibited by protein-tyrosine kinase inhibitors. The pattern of stimulated tyrosine phosphorylation was reminiscent of that seen after angiotensin or bombesin stimulation of cultured WB cells (6) or 3T3 fibroblasts (7, 9) and was clearly distinct from the pattern typically observed after polypeptide growth factor stimulation (6).

There are several potential mechanisms for stimulating protein-tyrosine kinases. By studying a number of pancreatic secretagogues with differing mechanisms of action, including the OPE analogue of cholecystokinin, phorbol esters, and calcium ionophores, we were able to gain insight into the signaling pathway responsible for stimulated protein tyrosine phosphorylation in these cells. Like the recent reports of Huckle et al. (6) and Leeb-Lundberg and Song (8), all of the calcium-mobilizing agonists stimulated qualitatively similar increases in cellular tyrosine phosphoproteins. Of interest, TPA, which stimulates secretion via a calcium-independent cascade, also stimulated an increase in cellular tyrosine phosphoproteins, although these represented only a subset of those stimulated by the calcium-mobilizing agonists.

Thus, in contrast to the studies performed with angiotensin or bombesin, the tyrosine phosphorylation events observed after hormonal stimulation of acinar cells seem to be the result of the activation of two different, potentially interactive pathways, one depending on an increase in the intracellular calcium concentration and the other activated by protein kinase C. One possible explanation for this difference could be that the studies cited above used cultured cells, in which a stimulated response may be different from that in normal tissue. Another difference could relate to intrinsic differences in the receptor systems studied, with additional novel intracellular events activated by the CCK receptor only.

An increase in phosphorylation may reflect an increase in the activity of a relevant kinase and/or a decrease in the activity of a phosphatase. In this work, we have focused on the kinase side of this equation. By demonstrating that the increase in protein tyrosine phosphorylation stimulated by these agonists was globally inhibited by genistein, a role for tyrosine kinase was strongly suggested. The possible existence of two complementary cascades of stimulation of tyrosine kinase was further supported by studying the differential effects of protein kinase inhibitors. The mixed inhibitor (stau-rosorine) or the more selective protein kinase C inhibitor (H-7) showed two different types of responses, either inhibiting or stimulating distinct tyrosine phosphoprotein bands, whereas genistein decreased all bands stimulated by all agonists. These observations further support the suggestion from the studies with differing agonists that two different pathways for agonist-stimulated activation of tyrosine kinases exist in these cells, one calcium-dependent and the other protein kinase C-dependent.
Tyrosine Phosphorylation in Amylase Secretion

The role of protein tyrosine phosphorylation in cell growth and differentiation has been extensively described. Although CCK, like bombesin or angiotensin, is known to be mitogenic for its target cell (1), this hormone is the major physiological stimulant of pancreatic secretion. We attempted to determine if tyrosine phosphorylation might also affect secretion stimulated by CCK. To do this, we measured the release of amylase from acinar cells in the presence or absence of kinase inhibitors.

Genistein has been widely used as a selective inhibitor of protein-tyrosine kinases relative to serine/threonine kinases (21). As expected, we have shown that this inhibitor decreases tyrosine phosphorylation in acinar cells normally seen in response to hormonal stimulation. In addition, incubation of acini with genistein significantly decreased amylase secretion in response to CCK, OPE, and calcium ionophores. In contrast, genistein had no influence on the secretory response seen with TPA, confirming that the effect of this reagent on secretion was not due to a generalized inhibitory effect on the cell’s secretory machinery. All members of the first group of secretagogues stimulate increased intracellular calcium levels, whereas TPA activates protein kinase C directly and does not change intracellular calcium levels.

A possible interpretation of these observations would therefore be that the tyrosine phosphorylation pathway functions to increase the amplitude of the secretory response rather than to provide an obligate signal for this response to occur. The relevant events occur in a calcium-dependent cascade, rather than being dependent on the tyrosine phosphorylation events known to be stimulated by protein kinase C. This was further demonstrated by comparing the effects of H-7 and staurosporine. These agents both inhibit protein kinase C, but differ in their abilities to inhibit tyrosine kinases (25–27).

Staurosporine, which affects both types of kinase activities at the concentration used in these studies, significantly decreased CCK-stimulated amylase secretion, whereas the more selective protein kinase C inhibitor (H-7) did not inhibit CCK-induced secretion. Of note, the concentration of H-7 was clearly capable of inhibiting secretion stimulated by TPA, which demonstrates that H-7 effectively inhibits protein kinase C under these treatment conditions. Similar effects of these drugs on pancreatic secretion have been reported by several groups (25–27). We believe that the differential abilities of these agents to inhibit tyrosine kinases may well explain this difference in their biological responses.

This potential involvement of changes in tyrosine phosphorylation in regulating secretion was first suggested by Jena et al. (14), who showed that the introduction of a tyrosine phosphatase into a preparation of permeabilized acinar cells increased calcium-mediated secretion. One might expect the same net effect on the phosphorylation state of substrate proteins from either the increase in tyrosine phosphorylation activity or the inhibition of a phosphorylating tyrosine kinase. However, the results of Jena et al. (14) are apparently opposite to those that might be extrapolated from this work with either of the tyrosine kinase inhibitors, genistein or staurosporine. Several plausible explanations might account for this. The most obvious are the differences between permeabilized and intact cells. Permeabilized cells do not allow the study of complex intracellular signaling cascades since some of the key cytosolic components leak out and cannot be replaced. Another possible difference relates to the specificity of the phosphatase preparation used in the study of Jena et al., which could not be expected to match the phosphatases present in the intact acinar cell. It is even possible that the tyrosine phosphatase introduced in that study increased the catalytic activities of src-like protein-tyrosine kinases by dephosphorylating a regulatory carboxyl-terminal phosphotyrosyl residue (Tyrr27 in p68src) (15). Although this possibility was not evaluated in that study, activation of src-like kinases has been linked to secretory events in other cell types (28).

In conclusion, we provide direct evidence for the activation of tyrosine phosphorylation events in intact acinar cells in response to CCK by at least two different signaling pathways. These phosphorylation events seem to play a role in the regulation of secretion in response to the hormonal agonist, with the relevant mechanisms activated in a calcium-dependent manner. The specific enzymes and substrates involved in this process remain to be determined.

Acknowledgments—We thank Delia Pinon and Eileen Holicky for excellent technical assistance.

REFERENCES