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Effect of Basic Fibroblast Growth Factor on Cholecystokinin-Induced Amylase Release and Intracellular Calcium Increase in Male Rat Pancreatic Acinar Cells

Ana I. Lajas, María J. Pozo,† Ginés M. Salido* and Jose A. Pariente*‡*

Department of Physiology, Faculty of Veterinary Science and Department of Nursing, University of Extremadura, 10071 Caceres, Spain

ABSTRACT. Isolated rat pancreatic acinar cells were used to investigate the effect of basic fibroblast growth factor (bFGF) on both amylase secretion and intracellular free calcium concentration ($[Ca^{2+}]_i$) in response to the calcium-mobilizing secretagogue cholecystokinin-octapeptide (CCK-8). Our data show that bFGF inhibited CCK-8-induced amylase release in a concentration-dependent manner and decreased the CCK-8-induced rise in $[Ca^{2+}]_i$. This inhibitory effect of bFGF on both amylase secretion and $[Ca^{2+}]_i$ increase in response to CCK-8 was reverted when acinar cells were pretreated with 100 μM tyrphostin A25, a tyrosine kinase inhibitor. Tyrphostin A25 also inhibited Ca^{2+} influx induced by CCK-8. These results show that bFGF inhibits CCK-8-induced pancreatic response by a tyrosine kinase-dependent mechanism. A role for tyrosine phosphorylation in capacitative Ca^{2+} entry is suggested. BIOCHEM PHARMACOL **55**;6:903–908, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. basic fibroblast growth factor; cholecystokinin; tyrosine kinase; amylase secretion; calcium; pancreatic acinar cells

The main function of pancreatic acinar cells is to synthesize, store and then undergo regulated secretion of digestive enzymes. An important early signal in the stimulation of secretion by phosphoinositide-linked agonists, such as cholecystokinin (CCK)§, is an increase in intracellular free calcium concentration ($[Ca^{2+}]_i$). It is well established that the increase in $[Ca^{2+}]_i$ is an aggregate of two general processes: initially inositol-1,4,5 trisphosphate (IP3) induces the release of Ca^{2+} from intracellular stores, an event which is followed by the capacitative Ca^{2+} entry across the plasma membrane [1, 2]. Changes in $[Ca^{2+}]_i$ are observed in response to a large number of hormones, neurotransmitters, and growth factors, and these changes in $[Ca^{2+}]_i$ are involved in the regulation of a number of different cellular functions [2].

Basic fibroblast growth factor (bFGF) is a mitogenic polypeptide that belongs to a family of heparin-binding proteins that modulate numerous cellular processes [3]. Initiation of cellular responses by bFGF begins with binding to a specific cell-surface tyrosine kinase receptor [4, 5]. However, only a few controversial studies have attempted to examine the regulation and intracellular events of pancreatic secretion by growth factors. In pancreatic acinar cells, bFGF induces IP3 accumulation, an increase in $[Ca^{2+}]_i$, and amylase release [6]. Epidermal growth factor (EGF), on the other hand, produces a small calcium transient and exerts statistically insignificant stimulatory effects on both IP3 levels and amylase release [6]. In the presence of CCK, however, EGF inhibits both CCK-8-induced IP3 production, intracellular calcium increase, and activation of the Cl^- conductance in zymogen granules [7–9], whereas it has dual effects on CCK-8-induced amylase secretion: inhibition at lower EGF concentrations and stimulation at higher EGF concentrations [10]. In addition, it has been reported that EGF reduces both amylase release by submaximal bFGF concentrations [11] and secretagogue-induced cyclic AMP production [12], but increases amylase output in anaesthetized rat [13]. However, the effect of bFGF on CCK-induced-amylase release and calcium mobilization remains unclear.

Therefore, in this study we investigated the effect of bFGF on CCK-8-induced amylase release as well as on CCK-8-stimulated increase in $[Ca^{2+}]_i$ in rat pancreatic acinar cells. Additionally, a tyrosine kinase inhibitor tyrphostin A25 was used to study whether these effects involve tyrosine kinase-dependent mechanisms.

‡ Corresponding author: Dr. José A. Pariente, Department of Physiology, Faculty of Veterinary Science, University of Extremadura, P.O. Box 643, 10071 Cáceres, Spain. Tel./FAX: +34-27-257154; E-mail: pariente @cc.unex.es

§ *Abbreviations:* bFGF, basic fibroblast growth factor; CCK-8, cholecystokinin-octapeptide; IP3, inositol-1,4,5 trisphosphate; EGF, epidermal growth factor; PSS, physiological salt solution; $[Ca^{2+}]_i$, intracellular free calcium concentration.

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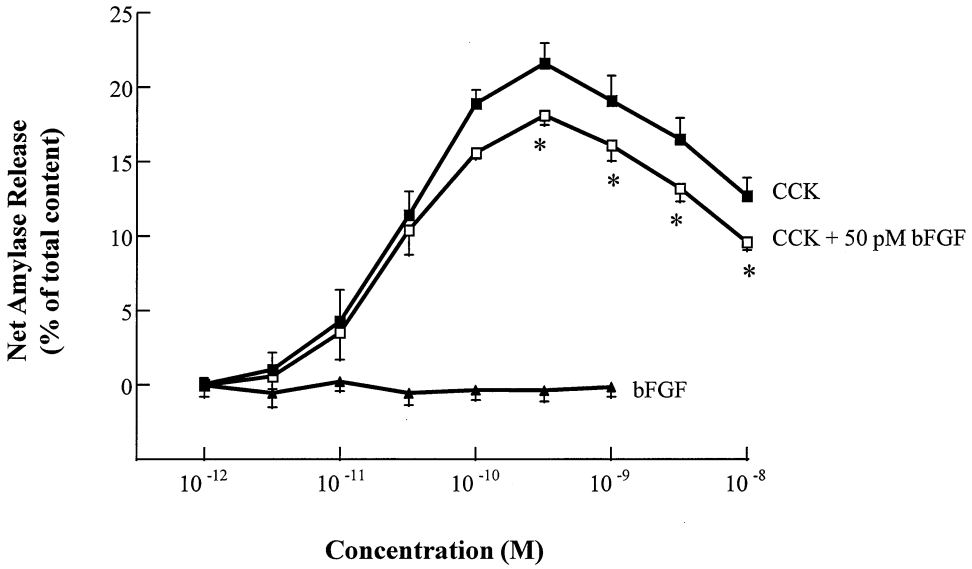

FIG. 1. Net amylase release induced by bFGF and CCK-8. Acini were incubated with various concentrations of bFGF (solid triangles) or CCK-8 (squares) either in the absence (solid squares) or presence (open squares) of 50 pM bFGF. Basal amylase release was 5.96 ± 0.27 of total amylase content of the acini at the beginning of the experiment. Results are means ± SEM of 11–13 different experiments. * Indicates a value significantly different from CCK-8 alone.

MATERIALS AND METHODS

Chemicals

All reagents were obtained from the Sigma Chemical Co. except collagenase CLSPA, which was obtained from Worthington Biochemical Corporation.

Preparation of Isolated Rat Pancreatic Acini

Acinar cells were prepared by methods as previously described [14, 15]. In brief, pancreata were excised from overnight-fasted male Wistar rats (150–200 g) and acinar cells prepared by enzymatic digestion with collagenase. Acinar cells were then suspended in a physiological salt solution (PSS) containing 1.0 mL/L essential amino acid mixture, 0.1 mg/mL soybean trypsin inhibitor and (in mM): 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 10 HEPES, 10 glucose, 1 $CaCl_2$. The pH was adjusted to 7.4 and equilibrated with 100% oxygen. In experiments where the protein tyrosine kinase inhibitor was used, tyrphostin A25 or the same volume of its vehicle dimethyl sulfoxide (DMSO) as control was added, and a preincubation was performed at 37° for 30 min. After preincubation, acinar cells were pelleted and resuspended in PSS containing the same concentrations of tyrphostin A25 or DMSO. Cell viability, monitored with trypan blue, was always greater than 95% and was not significantly reduced by either the secretagogues or the protein tyrosine kinase inhibitor concentrations used in this work.

Measurement of Amylase Release

To measure amylase secretion, aliquots (500 μL) of fresh or tyrphostin-treated acinar cells were incubated with appropriate secretagogue at 37° for 30 min followed by centrifugation at 1000 *g* for 30 sec. Amylase release was measured as described previously [16]. Amylase activities in the supernatant were determined using the Phadebas blue starch method [17] and expressed as percent of the total content of amylase at the beginning of the incubation.

Measurement of $[Ca^{2+}]_i$

Cell suspensions were loaded with 4 μM of fura-2 acetoxymethyl ester (fura-2/AM) in presence of 0.025% pluronic acid at room temperature for 40 min and then washed and resuspended in fresh PSS. For quantification of fluorescence, aliquots of cell suspension were placed in a perifusion chamber placed in an inverted epifluorescence microscope and continuously superfused at a flow rate of 1.5 mL/min. To monitor changes in $[Ca^{2+}]_i$, samples were excited at 350/380 nm and the resultant emission at 505 nm was recorded by a cooled CCD camera using a dedicated software (Newcastle Photometric Systems). $[Ca^{2+}]_i$ values were calculated after calibration using standard methods [18, 19].

Statistical Analysis

Values given are means ± SEM. Statistical significance was calculated by one-way analysis of variance. $p < 0.05$ was considered as statistically significant.

RESULTS

Basal amylase secretion was approximately 5% of total amylase content present at the beginning of the incubation.

TABLE 1. Effect of different agonists on amylase release

Treatment	Amylase Release (% of Maximum)	*n*
None	20.66 ± 1.10	11
CCK 320 pM	100	11
bFGF 50 pM	19.89 ± 1.42	13
EGF 3.2 nM	20.45 ± 1.10	7
EGF 1 nM	21.10 ± 1.20	7
EGF 320 pM	22.77 ± 1.77	7
Tyrphostin A25 100 μM (Tyrphos)	22.29 ± 1.72	20
CCK 320 pM + EGF 1 nM	97.80 ± 2.46	10
CCK 320 pM + bFGF 50 pM	87.41 ± 1.43*	13
CCK 320 pM + bFGF 50 pM + Tyrphos 100 μM	101.02 ± 4.45†	13
CCK 320 pM + Tyrphos 100 μM	96.87 ± 5.2	13

Values are means ± SEM of n experiments.
*, Significantly different from CCK alone.
†, Significantly different from CCK plus bFGF.

Fig. 1 shows the effect of different concentrations of either CCK-8 or bFGF on amylase secretion from pancreatic acinar cells. The results show that CCK-8 can evoke a biphasic increase in amylase release with a maximal effect of 21.57 ± 1.35% of total above basal occurring at 320 pM. Higher concentrations resulted in a decrease in amylase release. In contrast, when pancreatic acinar cells were incubated in the presence of bFGF at concentrations ranging from 1 pM to 1 nM, no significant increase in amylase secretion could be observed.

Figure 1 also shows the effect of bFGF on the CCK-8-evoked secretory response. Acinar cells were incubated with a range of concentrations of CCK-8 alone or in the presence of bFGF, and amylase release was measured. The results show that 50 pM bFGF attenuates the secretory effect of CCK-8 compared to the response of CCK-8 in the absence of bFGF. This concentration of bFGF was employed to study its interaction with CCK-8, because our initial experiments demonstrated maximal inhibition of the CCK-8-evoked response. In addition, the same concentration was also employed in other studies [6, 20]. This attenuation proved to be significantly different at higher concentrations of CCK-8. The sensitivity of acini to CCK-8 was not changed by bFGF, inasmuch as the dose-response curve did not shift. However, increasing concentrations of EGF had no significant influence on either basal or CCK-8-evoked amylase secretion (Table 1).

Figure 2 shows the effect of different doses of bFGF (0.5–500 pM) on the 320 pM concentration of CCK-8 that induced maximal secretory response. The results show that bFGF can attenuate CCK-8-evoked amylase secretion in a concentration-dependent manner. The IC_{50} for bFGF-induced inhibition of the CCK-8-stimulated amylase release was 7.5 pM. In an attempt to find out whether the attenuating effect of bFGF on CCK-8-induced amylase secretion was associated with the tyrosine kinase pathway, we used the tyrosine kinase inhibitor tyrphostin A25. The results show that the inhibitory effect of bFGF on CCK-8-induced amylase was completely abolished in the entire range of bFGF concentrations tested when acinar cells were

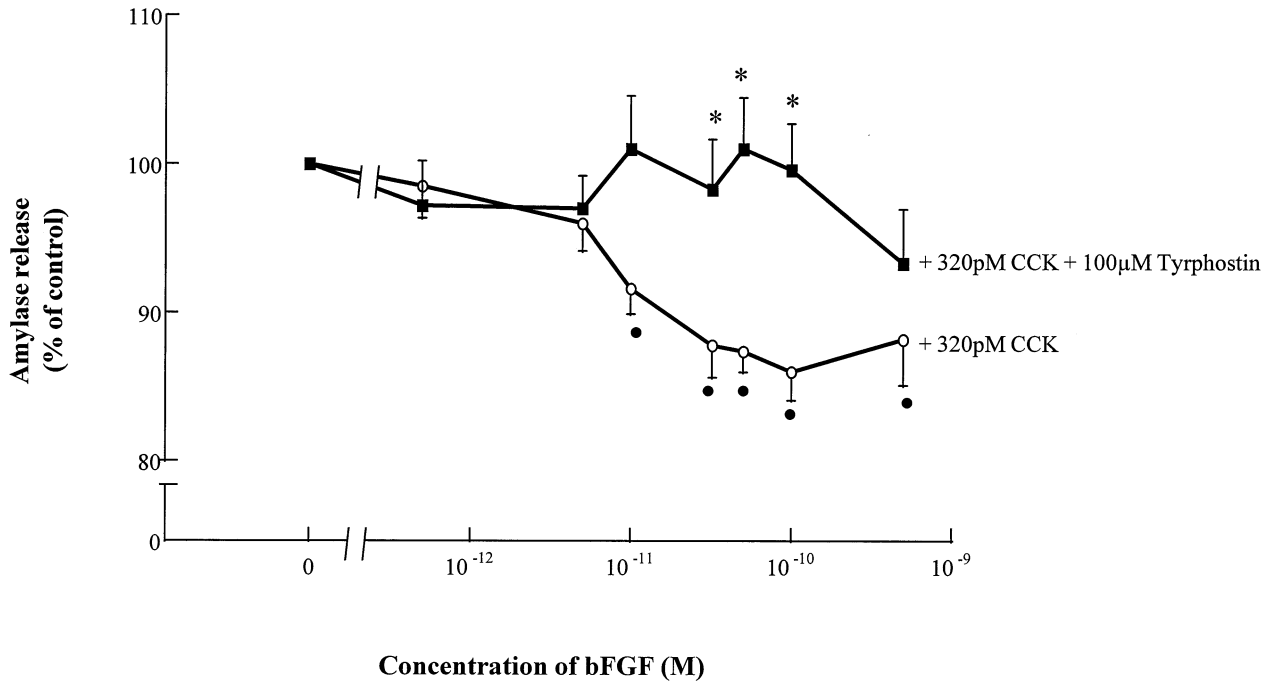

FIG. 2. Dose-response curve for bFGF-induced inhibition of CCK-8-induced amylase secretion. Acini were incubated with various concentrations of bFGF plus 320 pM CCK-8 either in the absence (open circles) or presence (solid squares) of 100 μM tyrphostin A25. Results are means ± SEM of 13 different experiments and are expressed as percentage of maximal response in the presence of CCK-8 alone. ●, Significantly different from CCK-8. *, Significantly different from CCK-8 plus bFGF.

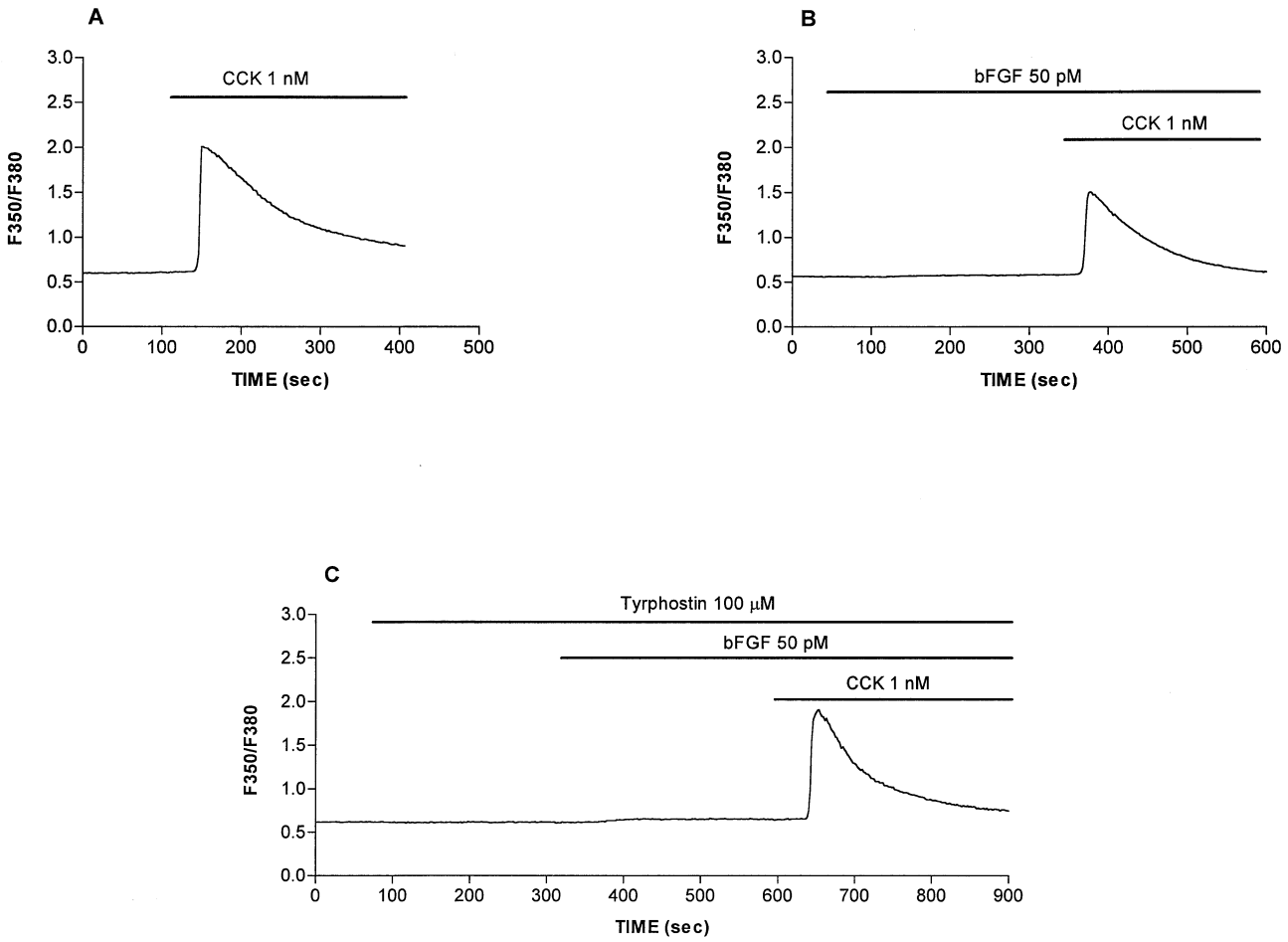

FIG. 3. Representative $[Ca^{2+}]_i$ tracings in pancreatic acinar cells. A, Application of CCK-8 (1 nM). B, Cells were perfused with bFGF (50 pM) followed by CCK-8 (1 nM). C, Cells were perfused with tyrphostin A25 (100 μM) followed by bFGF (50 pM) and CCK-8 (1 nM). All traces are representative of seven to eight independent experiments.

preincubated for 30 min with 100 μM tyrphostin (Figure 2). In addition, tyrphostin had no effect on basal amylase release but slightly inhibited the secretory response to CCK-8 (Table 1).

To investigate whether the inhibitory effect of bFGF on CCK-8-stimulated amylase release correlates with inhibition of Ca^{2+} transient, we examined the effect of bFGF on CCK-8-induced $[Ca^{2+}]_i$ increase. As shown in Fig. 3A, stimulation of pancreatic acinar cells with 1 nM CCK-8 induced a large transient increase in $[Ca^{2+}]_i$ followed by a gradual and slow recovery to prestimulated levels. When cells were pretreated with 50 pM bFGF (Fig. 3B), the CCK-8-evoked increase in $[Ca^{2+}]_i$ was reduced compared to the response of CCK-8 alone (384 ± 12 nM versus 502 ± 18 nM; Table 2). This reduction was observed in 80 of 102 studied cells and was estimated to be 24%. The inhibitory effect of bFGF on CCK-8-induced $[Ca^{2+}]_i$ increase was abolished in 43 of 52 acinar cells when they were

TABLE 2. Ca^{2+} responses in pancreatic cells stimulated by various agonists

Treatment	Peak $[Ca^{2+}]_i$ (nM)	Net $[Ca^{2+}]_i$ (nM) (above basal value)	*n*
None	97 ± 4		232
CCK 1 nM	502 ± 18	404 ± 16	78
bFGF 50 pM	101 ± 4	3 ± 0.5	102
Tyrphostin A25 100 μM	104 ± 4	7 ± 0.7	52
CCK 1 nM + bFGF 50 pM	384 ± 12*	287 ± 9*	102
CCK 1 nM + bFGF 50 pM + Tyrphos 100 μM	483 ± 20†	386 ± 20†	52

Values are means ± SEM from n acinar cells.
*, Significantly different from CCK alone.
†, Significantly different from CCK plus bFGF.

pretreated with 100 μM tyrphostin (Fig. 3C). Treatment with either bFGF or tyrphostin alone had no effect on the intracellular free Ca^{2+} (Table 2).

In Fig. 3C, it can be also observed that the application of tyrphostin before CCK-8 stimulation resulted in a rapid recovery of $[Ca^{2+}]_i$ to the prestimulation level together with no effect on the initial release of Ca^{2+}. The rate of tyrphostin-induced recovery was much faster than the rate of recovery during the stimulation with a maximal dose of CCK-8 (Fig. 3A). The rate of recovery was calculated by fitting single exponential to the decay of Ca^{2+} response for 200 sec after spike, and the time constant was obtained for each cell. In the presence of tyrphostin before CCK-8-stimulation the time constant was significantly smaller to that obtained during the stimulation with CCK-8 alone (46.6 ± 1.2 sec vs. 55.0 ± 1.3 sec, $p < 0.001$). The application of bFGF before CCK-8-stimulation had no effect on the time constant of Ca^{2+} recovery compared to that obtained during stimulation of CCK-8 alone (54.36 ± 0.79 sec vs. 55.0 ± 1.3 sec).

DISCUSSION

In the present study, we have tested the effect of bFGF on both CCK-8-induced amylase release and $[Ca^{2+}]_i$ increase in rat pancreatic acinar cells. In previous studies, it has been shown that EGF inhibits CCK-8-induced cellular responses such as IP3 production [7, 8, 10, 11], release of calcium from intracellular stores [8], activation of Cl^- conductance in isolated zymogen granules [9], and amylase secretion [7, 8, 10, 11]. In our work, we have demonstrated for the first time that like EGF, bFGF can also exert an inhibitory effect on both amylase secretion and $[Ca^{2+}]_i$ in response to the calcium-mobilizing secretagogue, CCK-8. However, we could not detect any effect on amylase release or $[Ca^{2+}]_i$ following stimulation of acinar cells with bFGF alone, whereas it was reported previously that bFGF induce IP3 accumulation, release of intracellular calcium, and amylase release [6]. The reasons for these differences in the results are yet unknown. It is possible that the discrepancies may be related to the strain and age of rats or differences both in isolation procedures and experimental conditions. It is worthwhile to note that we have employed Wistar rats whereas Chandrasekar and Korc [6] used Sprague-Dawley rats.

As bFGF and EGF are thought to bind a similar kind of cell-surface tyrosine kinase receptor, it is likely that they have similar actions. Nevertheless, our findings did not support this observation, because in agreement with previous reports [6, 7, 11], EGF had no significant effect on either basal or CCK-8-induced amylase secretion under our experimental conditions. These unexpected results could be explained by the finding that bFGF receptor exhibits a tyrosine kinase domain separated into two contiguous regions and an extracellular domain that has immunoglobulin-like regions, whereas the EGF receptor is monomeric [21]. Therefore, it is possible that bFGF receptor stimulation could induce different effects from those activated by the EGF receptor.

Our EGF results disagree with those obtained by Stryjek-Kaminska *et al.* [10], who found contradictory results when they studied the effect of EGF on pancreatic acini stimulated by CCK-8. Low concentrations of EGF caused a reduction in CCK-8-stimulated amylase release, whereas the amylase release increased in response to high EGF concentrations plus CCK-8 as compared with CCK-8 alone [10]. Our lack of effect cannot be due to the doses used, because we used several doses that were both lower and higher than those used by these authors. Therefore, differences in the functional state of the pancreatic acini or in the method of preparation of isolated rat pancreatic acinar cells could be responsible for these controversial findings.

We used the tyrosine kinase inhibitor tyrphostin A25 to determine whether tyrosine kinases are involved in the inhibitory effects of bFGF on both amylase secretion and $[Ca^{2+}]_i$ in response to CCK-8. In previous studies, tyrphostin A25 has been demonstrated to be a potent, specific and effective inhibitor of tyrosine kinase [22]. We found that tyrphostin abolished the inhibitory effects of bFGF on CCK-8-induced pancreatic response. The bFGF receptor possesses a ligand-regulated cytoplasmic tyrosine kinase domain [23]. Thus, although bFGF failed to elicit a stimulation on both amylase release and $[Ca^{2+}]_i$ in pancreatic acini, it is not surprising that tyrphostin reverted the attenuating effect of bFGF on CCK-8-induced amylase release as well as on CCK-8-stimulated increase in $[Ca^{2+}]_i$.

Recently, a possible importance of tyrosine phosphorylation in the secretory mechanism stimulated by CCK-8 has been suggested [24–26]. Furthermore, tyrosine kinase inhibitors have been reported to attenuate agonist-stimulated enzyme secretion from rat pancreatic acinar cells [25–27]. A potential site of action of these inhibitors is at the level of Ca^{2+} homeostasis; indeed, these inhibitors have been shown to attenuate Ca^{2+} influx in fibroblast and platelets but to be without effect on Ca^{2+} release from intracellular stores [27, 28]. Our findings demonstrate that the application of tyrphostin before CCK-8 stimulation resulted in a much faster recovery of $[Ca^{2+}]_i$ than that detected during the stimulation of CCK-8 alone, and had no effect on the initial release of Ca^{2+}. This much faster rate of tyrphostin-induced recovery could be due to an inhibition of capacitative Ca^{2+} entry into the cell. Similar findings have been obtained by Yule *et al.* [29], who found that other tyrosine kinase inhibitors such as genistein inhibited Ca^{2+} influx stimulated by thapsigargin- and carbachol-induced intracellular store depletion in a concentration-dependent manner without affecting the initial release of Ca^{2+}. These data indicate that inhibition of Ca^{2+} influx could in part underlie our own and previous results [25–27] which have shown an inhibition of CCK-evoked amylase secretion by tyrosine kinase inhibitors.

In summary, our results have demonstrated the inhibitory effects of bFGF on both amylase secretion and $[Ca^{2+}]_i$ in response to the calcium-mobilizing secretagogue CCK-8,

indicating that bFGF decreases CCK-8-induced pancreatic response via tyrosine kinase-dependent mechanisms. Our data indicate a physiological role for tyrosine phosphorylation in Ca^{2+} homeostasis during secretagogue (i.e., CCK)-evoked secretory responses in the exocrine pancreas.

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