JNK Kinases Are Rapidly Activated by Cholecystokinin in Rat Pancreas both in Vitro and in Vivo*

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Stimulation of pancreatic acini from male Sprague-Dawley rats by both cholecystokinin (CCK)-8 and anisomycin caused an increase in p46\textsuperscript{nk} and p55\textsuperscript{nk} activities. Both forms of c-jun amino-terminal kinase (J NK) were slightly activated at 5 min, reached a maximum at 30 min, and remained significantly increased at 60 min of CCK stimulation. By contrast, p42\textsuperscript{mapk} was activated fully by 5 min. In pancreatic acini stimulated with different concentrations of CCK for 30 min, the minimal and maximal J NK responses were observed at 30 pm and 100 nm CCK, respectively; p42\textsuperscript{mapk} activation was, as previously reported, much more sensitive, with maximal activation by 1 nm CCK. Carbachol and bombesin also stimulated J NK activity, while vasoactive intestinal peptide did not. Neither activating protein kinase C nor increasing intracellular Ca\textsuperscript{2+} significantly activated J NK. In in vivo experiments, rats were infused intravenously for 5 and 15 min with a secretory (0.1 \mu g/kg/h) or supramaximal (10 \mu g/kg/h) dose of the CCK analog caerulein (CER). Secretory doses of CER induced a 4-fold increase of both forms of J NK in pancreatic tissue at 5 and 15 min, while at the same time points, supramaximal stimulation with CER caused 4- and 27-fold increases, respectively, of these kinase activities. The secretory dose of CER slightly increased the activities of both forms of mitogen-activated protein kinase, while the supramaximal dose induced a 10-fold increase of p42\textsuperscript{mapk} at 5 min. In conclusion, J NKs and mitogen-activated protein kinases are rapidly activated in rat pancreatic acini stimulated with CCK as well as in pancreatic tissue during in vivo stimulation with CER. The large response to supramaximal CER stimulation may be of importance in the early pathogenesis of acute pancreatitis.

Mitogen-activated protein kinases (MAPKs),\textsuperscript{1} also known as extracellular signal-regulated kinases, are serine/threonine protein kinases that are rapidly activated by a variety of cell-surface receptors (1–3). They function in signal cascade pathways that control the expression of genes involved in many cellular processes, including cell growth and differentiation (3, 4). Blocking the function of MAPK prevents cell proliferation in response to a number of growth-stimulating agents (5, 6).

Recently, a novel signal cascade of mammalian enzymes closely related to that culminating in MAPK activation has been identified. The kinases related to MAPKs were identified by virtue of their ability to phosphorylate the amino terminus of the c-jun transcription factor, and they were therefore termed c-jun amino-terminal kinases (J NKs) (7–9). Two forms were identified with molecular masses of 46 and 55 kDa, both of which are activated by dual phosphorylation on threonine and tyrosine residues, similar to the activation of MAPKs. J NKs can be potently activated by inhibitors of protein synthesis such as cycloheximide and anisomycin, inflammatory cytokines such as interleukin-1 and tumor necrosis factor \(\alpha\), heat shock, changes in osmolarity, and ultraviolet irradiation and are also referred to as stress-activated protein kinases (8, 10–20). J NKs are believed to be responsible for phosphorylating the transactivating domain of c-jun protein in vivo (8–10), which can then dimerize or bind with fos as a heterodimer and can then control the expression of a number of genes, including c-jun itself (4, 21–23).

Cholecystokinin (CCK) regulates a variety of pancreatic functions, including secretion of pancreatic juice (24), stimulation of pancreatic growth (25, 26), digestive enzyme synthesis (27), and enhancement of expression of transcriptional factors such as c-myc, c-jun, and c-fos (28). Stimulation with a supramaximal dose of the CCK analog caerulein (CER) is known to induce acute pancreatitis in the rat (29). The trigger and exact mechanism of this phenomenon are not completely understood. Recently, we found that CCK activates p42\textsuperscript{mapk} and p44\textsuperscript{mapk} as well as other upstream components of the MAPK signaling cascade, including Ras and MEK, in isolated rat pancreatic acini (30, 31). The aim of this study was therefore to evaluate whether J NKs are activated in pancreatic acini as well as in pancreatic tissue during in vivo stimulation with CER and to compare their activation to that of MAPK.

EXPERIMENTAL PROCEDURES

Materials—The following reagents were purchased. Guanidine hydrochloride and minimal essential amino acids were from Life Technologies, Inc. Leupeptin and aprotinin were from Boehringer Mannheim. [γ\textsuperscript{32P}]ATP (3000 Ci/mmol) was from DuPont NEN. CCK octapeptide (CCK-8) was a gift from the Squibb Research Institute (Princeton, NJ). Vasoactive intestinal peptide and bombesin were from Bachem California (Torrance, CA). Chromatographically purified collagenase was from Worthington. Caerulein was from Research Plus (Bayonne, NJ). All other reagents were obtained from Sigma.

Preparation of Pancreatic Acini and Cell-free Extract—The preparation of pancreatic acini was carried out according to Williams and co-workers (30, 32). Briefly, pancreata from Sprague-Dawley rats were digested by collagenase, mechanically dispersed, and passed through a 150-μm mesh nylon cloth. Acini were then purified by centrifugation at 50 \(\times\) g for 3 min in a solution containing 4% bovine serum albumin and were resuspended in incubation buffer that consisted of a HEPES-buffered Ringer solution supplemented with 11.1 mM glucose, Eagle's...
minimal essential amino acids, 0.1 mg/ml soybean trypsin inhibitor, and 10 mg/ml bovine serum albumin. Acini were preincubated at 37°C with minimal shaking for 120 min, followed by stimulation with different agonists in 1-ml aliquots in 25×55-mm polystyrene vials for the indicated times. Acini were then pelleted; washed once with 1 ml of ice-cold phosphate-buffered saline containing 1 mM Na3VO4, pH 7.4; and resuspended for 5 s in 0.5 ml of ice-cold lysis buffer (50 mM Hepes, pH 7.4, 15 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 1 mM dithiothreitol, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, pH 7.4). The lysates were then centrifuged in a microcentrifuge at 4°C for 15 min, and the supernatant was assayed for MAPK and JNK activities. The amount of protein in the cell extracts was assayed by the Bio-Rad protein assay reagent.

In Vivo Stimulation with Caerulein—Under sterile conditions, rats were anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg). A polyethylene 50 cannula (Biolab Products, Lake Havasu City, AZ) was introduced through the femoral vein into the inferior vena cava. The cannula was tunneled subcutaneously and brought out behind the head in the cervical region to allow complete freedom of movement. The cannula potency was maintained by a heparin lock (1000 units/ml), and the animal was allowed to recover overnight, fasted with water ad libitum. The following morning, the cannula was flushed with 0.9% NaCl, and patency was confirmed by the entry of blood into the cannula following slight negative pressure. Rats with patent cannulas were infused with 0.9% NaCl (controls), a bolus secretion of CCK-8 or 50 μg/ml anisomycin, and 0.9% NaCl, and patency was confirmed by the entry of blood into the cannula following slight negative pressure. Rats with patent cannulas were infused with 0.9% NaCl (controls), a bolus secretion of CCK-8 or 50 μg/ml anisomycin, and 10 mg/ml bovine serum albumin. Acini were preincubated at 37°C in Krebs-Ringer bicarbonate buffer containing minimal essential amino acids, 0.1 mg/ml soybean trypsin inhibitor, 10 mM Na3VO4, 10 mM 2-mercaptoethanol, and 10 mM sodium pyrophosphate, 1.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 1 mM dithiothreitol, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, pH 7.4. The lysates were then centrifuged in a microcentrifuge at 4°C for 15 min, and the supernatant was assayed for MAPK and JNK activities.

RESULTS

Previously, we demonstrated that CCK rapidly activates MAPK in rat pancreatic acini (30, 31). Since anisomycin is known as a strong activator of another member of the MAPK family, J NKS, we decided to compare the effects of CCK and anisomycin in pancreatic acini. Using an in-gel kinase assay, we found that both agents significantly stimulated the activities of two kinases with apparent molecular masses of 46 and 55 kDa, which were able to phosphorylate GST-c-Jun-(1–79) peptides (Fig. 1, left panel). The response to 10 nm CCK was in the same range of magnitude as observed after 30 min of stimulation with anisomycin (50 μg/ml). The same samples were subjected to in-gel assay with GST copolymerized into the gel (Fig. 1, right panel). In the positions corresponding to 46 and 55 kDa, only very faint bands were occasionally visible, indicating slight phosphorylation of GST or minimal autophosphorylation of the kinases. Because of the mass, substrate specificity, and activation by anisomycin, these kinases can be identified as p46 and p55, respectively. In-gel MAPK assay of the samples using myelin basic protein as substrate showed strong activation of both forms of MAPK (p42 and p44) after CCK stimulation, while anisomycin had much less effect (data not shown).

Fig. 2 presents the time course of CCK-induced activation of J NKS and MAPKs in pancreatic acini. In the upper panel of Fig. 2A, the radioactivity of the p46 and p55 bands is shown. The integrated density of the bands was calculated and is illustrated in the lower panel of Fig. 2A. CCK-8 slowly increased the activities of both J NKS, reaching a maximum at 30 min, when 2.5-fold increases of p46 and p55 activities were noted. The activity remained elevated at 45 min. In another experiment, activity was similar at 30 and 60 min. The same samples were subjected to in-gel MAPK assay. CCK-8 rapidly increased the activities of both MAPKs, reaching a maximum within 5 min, when 7.5- and 6-fold increases of p42 and p44 activities, respectively, were noted over the activities at time 0 (Fig. 2B). The activities of both kinases remained elevated at 45 min of CCK-8 stimulation.

Pancreatic acini were then incubated for 30 min with different doses of CCK-8 (Fig. 3). The minimal response of J NKS to CCK stimulation was observed at a 30 pmM concentration of the hormone, while half-maximal and maximal responses were observed at 3 and 100 nmM, respectively (Fig. 3, A and C). The activities of both kinases remained elevated at 1 μM CCK. In the same samples, p42 and p44 activities were much more elevated at 30 pm CCK-8 compared with the effect on J NKS, reached a maximum at 1 nmM, and remained at the similar level of activation up to 1 μM CCK-8 (Fig. 3, B and C).

It is known that CCK, after binding its receptor, triggers hydrolysis of polyphosphoinositide, generating inositol 1,4,5-triphosphate and diacylglycerol, which mobilize intracellular calcium and activate protein kinase C, respectively (35). We investigated whether one of these signal transduction pathways was responsible for activation of J NKS by determining the effects of various agonists on these kinase activities in...
Tetradecanoylphorbol-13-acetate, a potent stimulator of pro-

the activities of both JNKs (Fig. 4). CCK, bombesin, and carbacho-
significantly stimulated comparison with the effect on the MAPK signaling pathway

without 10 nM CCK-8 for the indicated times and then sonicated in lysis buffer and boiled for 5 min in stop solution. Samples were run separately on gels containing either GST-c-Jun-(1–79) or myelin basic protein, and in-gel kinase assays were performed in duplicate. The intensity of phosphorylation was measured with the use of a Model GS-250 molecular imager and is expressed as a percentage of the average value at time 0. The data presented are from one experiment that is repre-
sentative of three different experiments.

comparison with the effect on the MAPK signaling pathway (Fig. 4). CCK, bombesin, and carbachol significantly stimulated the activities of both J NKs (Fig. 4A). The efficacy of CCK was −2 times higher than that of bombesin and carbachol. 12-O-Tetradecanoylphorbol-13-acetate, a potent stimulator of protein kinase C, as well as the Ca²⁺-ATPase inhibitor cyclopia-
zonic acid had only a minimal effect on JNK activity, while vasoactive intestinal peptide, which stimulates pancreatic acini via a pathway related to cyclic AMP, had no effect. As shown before (30), CCK, bombesin, carbachol, and 12-O-tetradecanoylphorbol-13-acetate significantly stimulated MAPK activity in pancreatic acini, while the effect of cyclopiazonic acid was much less. Incubation of acini with a combination of cyclo-
piazonic acid and 12-O-tetradecanoylphorbol-13-acetate resulted in additive effects on MAPK activity. Vasoactive intes-
tinal peptide had no effect on MAPK activity in pancreatic acini.

To examine the regulation of these kinases in the intact pancreas, we examined the effect of in vivo stimulation with the CCK analog CER on the activities of JNKs and MAPKs in pancreatic tissue (Fig. 5). Stimulation of rats with a secretory dose of CER (0.1 µg/kg) resulted in a significant 3-fold increase of both forms of JNK activity as early as 5 min of intravenous infusion, while at 15 min, the activities of these kinases were slightly higher. In the same animals, pancreatic p42⁰⁰ mapk activity was increased 2-fold at 5 min, while at 15 min of stimulation, the levels returned to those seen in control animals infus-
ed with 0.9% NaCl. Stimulation with a supramaximal dose of CER (10 µg/kg), which is known to induce acute pancreatitis, induced a 4-fold increase of both JNKs at 5 min, while 15 min of stimulation resulted in a 27-fold elevation of these kinase activities. In the same animals, a 9-fold increase of p42⁰⁰ mapk was observed in pancreatic tissue at 5 min, while at 15 min, it was significantly decreased, but remained at 2.5 times above control levels. It is noteworthy that basal levels of both JNKs and MAPKs appear much lower in the intact pancreas versus dis-
pered acini. Previously, we had found that basal levels of MAPK and JNK declined after a 2-h preincubation in vitro, suggesting that the preparation of acini increases the activities of both kinases from those found in the in situ pancreas.

**DISCUSSION**

We recently reported that CCK activates p42⁰⁰ mapk and p44⁰⁰ mapk as well as other upstream components of the MAPK signaling cascade, including Ras and MEK, in isolated rat pancreatic acini (30, 31). In the present study, we have demon-
strated for the first time that CCK activates two forms of JNK (p46⁰⁰ jnk and p55⁰⁰ jnk) in isolated rat pancreatic acini. Interestingly, the effectiveness of CCK was the same or even higher than that of anisomycin, which is known as one of the strongest JNK activators (2, 13). In response to CCK, both JNKs were slowly activated in pancreatic acini, with maximum activation at 30 min, compared with p42⁰⁰ mapk and p44⁰⁰ mapk, which reached a maximum at 5 min. Furthermore, the minimal CCK concentration that activated JNKs was 10 times higher than that which activated the MAPKs (p42⁰⁰ mapk and p44⁰⁰ mapk) (30). Correspondingly, a 100 times greater CCK concentration was necessary to induce the maximal response of JNKs in pancreatic acini when compared with MAPKs. Thus, CCK activation of JNKs occurs slower and requires higher concentrations of CCK compared with the activation of MAPKs.

Since JNKs are known to be activated by a signal cascade distinct from that of MAPKs, it is not surprising that there were some differences in the activation of JNKs and MAPKs in acini in response to intracellular messengers. In acini, CCK, carbachol, and bombesin are all known to interact with hetero-
trimeric G proteins and thereby activate a phospholipase C
that hydrolyzes phosphatidylinositol bisphosphate, generating inositol 1,4,5-triphosphate and diacylglycerol, which mobilize intracellular Ca$^{2+}$ and activate protein kinase C, respectively. Increasing intracellular Ca$^{2+}$ with cyclodiacylionic acid failed to activate either JNKs or MAPKs. Activation of protein kinase C with active phorbol ester, which is known to stimulate MAPK activity in acini (30), had its expected effect, but showed a minimal effect on JNKs. Although CCK stimulates cAMP formation in acini at high concentrations, this does not appear to be important in activating JNKs as vasoactive intestinal peptide, CCh, or carbachol; BBS, bombesin; CPA, cyclopiazonic acid.

The data were presented as the mean $\pm$ S.D. of eight experiments for CCK and three experiments for the other agonists, with each experiment performed in duplicate. TPA, 12-O-tetradecanoylphorbol-13-acetate; VIP, vasoactive intestinal peptide; CCh, carbachol; BBS, bombesin; CPA, cyclopiazonic acid.

In the in vivo part of our study, rats were stimulated intravenously with different doses of the CCK analog CER for 5 or 15 min. Stimulation with the lower dose of CER, known to stimulate pancreatic secretion, produced a mild increase of JNK activity in pancreatic tissue at 5 and 15 min. Under the same conditions, p42$^{\text{mapk}}$ and p44$^{\text{mapk}}$ were only transiently activated at 5 min. These data suggest that both MAPKs and JNKs may be activated physiologically by CCK. Stimulation with a supramaximal dose of CER, known to induce acute pancreatitis, produced significant increases of p42$^{\text{mapk}}$ and p44$^{\text{mapk}}$ at 5 min of stimulation. This increased activity of both MAPKs was transient in that they were markedly decreased at 15 min. In the same group of animals, JNK activity was moderately elevated at 5 min and dramatically increased (27-fold) following 15 min of hyperstimulation. In our study, hyperstimulation both in vitro and in vivo was accompanied by significant activation of JNKs that may reflect the response to cellular stress. It is noteworthy that after CCK or CER stimulation in vitro as well as in vivo, the activation of p42$^{\text{mapk}}$ and p44$^{\text{mapk}}$ preceded the activation of JNKs. A similar phenomenon, in response to cellular stress, was recently observed by others in a rat model of kidney ischemia-reperfusion (44). These authors suggested that strong activation of the stress-activated protein kinase family of serine/threonine kinases leading to JNK activation (37, 38). The authors of these studies suggested that G protein- and tyrosine kinase-linked receptors may activate Rac and Cdc42 directly or through activation of Ras. This then results in activation of a kinase cascade with MEK kinase-phosphorylating and activating JNK, which dually phosphorylates JNK (39–43). Since MEK kinase and JNK kinase are expressed in most tissues, it seems likely that such a pathway occurs in acinar cells. An uncertainty in all types of cells is how MEK kinase is activated. Thus, how JNKs are activated in pancreatic acini remains to be determined, but our data clearly support the existence of a pathway distinct from that activating the MAPKs.

In the present study, we have found that increased cellular Ca$^{2+}$ leads to JNK activation (37, 38). The authors of these studies suggested that G protein- and tyrosine kinase-linked receptors may activate Rac and Cdc42 directly or through activation of Ras. This then results in activation of a kinase cascade with MEK kinase-phosphorylating and activating JNK, which dually phosphorylates JNK (39–43). Since MEK kinase and JNK kinase are expressed in most tissues, it seems likely that such a pathway occurs in acinar cells. An uncertainty in all types of cells is how MEK kinase is activated. Thus, how JNKs are activated in pancreatic acini remains to be determined, but our data clearly support the existence of a pathway distinct from that activating the MAPKs.

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very early after reperfusion of ischemic kidney may transduce an important signal to the nucleus and trigger the complex genetic response to ischemia. An important role of oxygen radicals is suggested in the early pathogenesis of both experimental models: CER-induced acute pancreatitis and organ ischemia-reperfusion (45). Since it is also known that reactive oxygen species are capable of different protein kinase activation (46, 47), it is possible that they may be partially responsible for the activation of MAPKs and JNKs in these experimental models.

Taken together, CCK stimulation of dispersed acini or the intact pancreas stimulates two separate signaling cascades leading to activation of JNKs and MAPKs. The extensive activation of both MAPKs and JNKs during supramaximal stimulation with CER may be of importance in the early pathogenesis of acute pancreatitis and/or may reflect a universal reaction of the organism responding to cellular stress.

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