ELSEVIER

Biochimica et Biophysica Acta 1358 (1997) 189–199

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

CCK causes rapid tyrosine phosphorylation of p125^{FAK} focal adhesion kinase and paxillin in rat pancreatic acini

Luis J. Garcia [a,1], Juan A. Rosado [a], Takaharu Tsuda [b], Robert T. Jensen [b,*]

[a] *Department of Physiology, University of Extremadura, Cáceres 10080, Spain*

[b] *Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bldg. 10, Rm. 9C-103, 10 Center Drive MSC 1804, Bethesda, MD 20892, USA*

Received 24 February 1997; accepted 2 April 1997

Abstract

Recent studies show CCK stimulates tyrosine phosphorylation (TYR PHOSP) of a number of proteins and evidence from the pancreas and other cellular systems suggest this could be important in mediating some of CCK's growth and secretory effects. In other tissues various neuropeptides such as bombesin can cause tyrosine phosphorylation of p125 focal adhesion kinase (p125^{FAK}) and paxillin which are important in mediating their growth effects. The purpose of the present study was to determine the effects of CCK in rat pancreatic acini on the TYR PHOSP of these latter proteins. In dispersed rat pancreatic acini, cell lysates were incubated with an anti-phosphotyrosine mAb (PY20) which was immunoprecipitated and then analyzed by Western blotting with anti-phosphotyrosine mAb (4G10), anti-p125^{FAK} mAb or anti-paxillin mAb. CCK-8 at 5 min increased TYR PHOSP of five proteins of molecular weight $>60\,000$ including a broad M_r 110–130 000 and M_r 70–80 000. An increase in TYR PHOSP of both p125^{FAK} and paxillin was detected within 1 min of adding CCK and reached a maximum at 2.5 min with a 9.1 ± 1.9-fold increase for p125^{FAK} and 3.6 ± 0.6-fold for paxillin. CCK-8 caused a half-maximal increase in TYR PHOSP of p125^{FAK} at 0.1 nM and paxillin at 0.03 nM. CCK-JMV also stimulated an increase in TYR PHOSP of both proteins, but was only 50% as efficacious as CCK-8. CCK-JMV caused a half-maximal increase at 10 nM and maximal at 1 μM for both proteins. To investigate whether the low affinity CCK receptor state also caused TYR PHOSP of both proteins, increasing concentrations of CCK-JMV were added to a maximally effective CCK-8 concentration (1 nM). Detectable inhibition of CCK-8-stimulated TYR PHOSP occurred with 1 μM CCK-JMV and with 3 μM CCK-JMV the CCK-8-stimulated response was inhibited 50% and was the same as that seen with CCK-JMV alone. These studies demonstrate that in rat pancreatic acini, CCK causes rapid TYR PHOSP of both p125^{FAK} and paxillin. This stimulation is mediated by both the high affinity and low affinity CCK receptor states. This phosphorylation of these proteins could be important in mediating CCK's effect on the cytoskeleton or growth effects as shown for a number of other agents (oncogenes, neuropeptides, integrins). © 1997 Elsevier Science B.V.

Keywords: Tyrosine phosphorylation; Pancreatic acini; Cholecystokinin

1. Introduction

Cholecystokinin (CCK) is a gastrointestinal peptide hormone that causes gallbladder contraction [1], pancreatic secretion [1,2], stimulates digestive en-

* Corresponding author. Fax: +1 (301) 402 0600.

[1] Visiting Scientist at the Digestive Diseases Branch, NIDDK/NIH, supported by a Grant from the Dirección Científica Y Técnica (DGICYT, PB94-1416-CO2-02).

PII S0167-4889(97)00056-6

zyme synthesis [3], causes enhancement of c-*fos*, c-*jun* and c-*myc* oncogene expression [4], and has trophic effects on the pancreas [5]. Extensive studies show that CCK causes activation of phospholipase C resulting in hydrolysis of phosphoinositides, generation of diacylglycerol, and increased inositol 1,4,5-trisphosphate with subsequent activation of protein kinase C and release of intracellular calcium and that these pathways are probably important in mediating many of the cellular effects of CCK [2]. However, recent studies suggest that the activation of tyrosine kinases is probably also an important mediator for some of the cellular effects of CCK. Recent studies showed that CCK stimulates tyrosine phosphorylation of a number of proteins and kinases including JUN kinase and MAP kinase [6–10]. Tyrosine phosphorylation has recently been implicated in the action of a number of neuropeptides that act as potent cellular growth factors through G protein-coupled receptors in both mediating their growth effects as well as cytoskeletal changes [11–15]. Bombesin, vasopressin and endothelin all stimulate tyrosine phosphorylation of multiple substrates in Swiss 3T3 cells and other cells, including the novel cytosolic tyrosine kinase p125 focal adhesion kinase ($p125^{FAK}$) [13,16] and the cytoskeleton-associated protein paxillin [17], which are associated with focal adhesion plaques [18,19]. Several investigators have recently reported that $p125^{FAK}$ and paxillin are also tyrosine phosphorylated following activation of integrins [20,21] and oncogenes [21] and addition of growth factors like PDGF [22]. Furthermore, recent studies with bombesin demonstrate activation of this pathway is necessary to mediate bombesin's growth effects in Swiss 3T3 cells [23]. Thus tyrosine phosphorylation of $p125^{FAK}$ and paxillin may represent a point of convergence in the action of mitogenic neuropeptides, integrins, oncogenes and growth factors [21].

The actions of CCK-related peptides are mediated by two different subtypes of CCK receptors [24,25]. CCK_B/gastrin receptors, which are found mainly in the central nervous system and the gastrointestinal tract, bind CCK and gastrin with approximately equal affinity. In contrast, CCK_A receptors, which are also present in the gastrointestinal tract and highly localized areas of the central nervous system, exhibit a 500-fold higher affinity for CCK than for gastrin [24,25]. A number of recent studies demonstrate that rat pancreatic acinar cells in contrast to human and guinea pig possess only CCK_A receptor [26,27]. Recent studies show this receptor can exist in multiple states [28–30], which activate different intracellular mediators, cause different biological responses and can be distinguished in rat pancreatic acini by JMV-180, a synthetic analogue of CCK-8 [31–33]. Although recent studies show activation of CCK_B receptors stably transfected into fibroblasts can increase the tyrosine phosphorylation of $p125^{FAK}$ and paxillin [34,35], at present it is unknown whether CCK_A receptor activation in the pancreas or any tissue can cause tyrosine phosphorylation of $p125^{FAK}$ and paxillin. Furthermore, if it causes such changes it will be important to determine what state of the CCK_A receptor mediates these changes.

The purpose of the present study was to determine whether activation of the CCK_A receptor in rat pancreatic acini results in increased tyrosine phosphorylation of $p125^{FAK}$ and paxillin; and, to determine which CCK_A receptor state is involved in causing CCK-induced tyrosine phosphorylation of $p125^{FAK}$ and paxillin.

2. Materials and methods

2.1. Materials

Male Sprague–Dawley rats (150–200 g) were obtained from the Small Animals Section, Veterinary Resources Branch, NIH, Bethesda, MD; purified collagenase (type CLSPA) from Worthington Biochemicals, Freehold, NJ; COOH-terminal octapeptide of cholecystokinin (CCK-8) was obtained Bayonne, NJ; phosphate buffer saline (PBS), pH 7.4, from Biofluids, Rockville, MD; anti-focal adhesion kinase ($p125^{FAK}$) monoclonal antibody, anti-paxillin monoclonal antibody and anti-phosphotyrosine monoclonal antibody (PY20) from Transduction Laboratories, Lexington, KY; anti-phosphotyrosine monoclonal antibody (clone 4G10) and recombinant protein A-agarose from Upstate Biotechnology Inc., Lake Placid, NY; 12-O-tetradecanoylphorbol-13-acetate (TPA), deoxycholic acid from Calbiochem, La Jolla, CA; soybean trypsin inhibitor (SBTI), dimethyl sulfoxide (DMSO) and Triton X-100 from Sigma, St.

Louis, MO; phenylmethanesulfonyl fluoride (PMSF) from Fluka, Ronkonkoma, MY; BME amino acids and BME vitamin solution from Gibco Laboratories, Grand Island, NY; bovine serum albumin (BSA) fraction V from Miles Inc., Kankakee, IL; aprotinin, leupeptin and HEPES from Boehringer Mannheim Biochemicals, Indianapolis, IN; rabbit anti-mouse IgG and anti-mouse IgG–horseradish peroxidase conjugate, from Pierce, Rockford, IL; sodium dodecyl sulfate (SDS), 2-mercaptoethanol, protein assay dye reagent, Tris/glycine/SDS buffer (10 times concentrated) and Tris/glycine buffer (10 times concentrated) from BIO-RAD, Richmond, CA; Hyperfilm ECL, enhanced chemiluminescence detection reagents from Amersham, Arlington Heights, IL; and nitrocellulose membrane from Schleicher and Schuell, Keene, NH.

Unless otherwise stated, the standard incubation solution contained (mM): HEPES, 25.5 (pH 7.4); NaCl, 98; KCl, 6; NaH_2PO_4, 2.5; sodium pyruvate, 5; sodium fumarate, 5; sodium glutamate, 5; glucose, 11.5; $CaCl_2$, 0.5; $MgCl_2$, 1; glutamine, 2; albumin, 1% (w/v); trypsin inhibitor, 1% (w/v); vitamin mixture, 1% (v/v) and amino acid mixture, 1% (w/v). The incubation solution was equilibrated with 100% O_2 and all incubations were performed with 100% O_2 as the gas phase.

2.2. Methods

2.2.1. Tissue preparation

Dispersed rat pancreatic acini were prepared according to the modifications [36] of the procedure published previously [37].

2.2.2. Immunoprecipitation

Dispersed acini from one rat were preincubated with standard incubation solution for 2 h at 37°C. Then dispersed acini were suspended in 15 ml of standard incubation solution and samples (1 ml) of cell suspension were treated with CCK-8 and JMV-180 at the concentrations and times indicated and sonicated for 5 s at 4°C in 1 ml of a solution containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% (w/v) NaN_3, 1 mM EGTA, 0.4 mM EDTA, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 1 mM PMSF and 0.2 mM Na_3VO_4. Lysates were centrifuged at 15 000 rpm for 15 min and the amount of protein was measured in the supernatant. The amount of protein was standardized to 500 μg/ml and 1 ml of the supernatants were incubated with 4 μg of anti-phosphotyrosine monoclonal antibody (PY20), 4 μg of goat anti-mouse IgG and 30 μl of protein A–agarose overnight at 4°C. The immunoprecipitates were washed three times

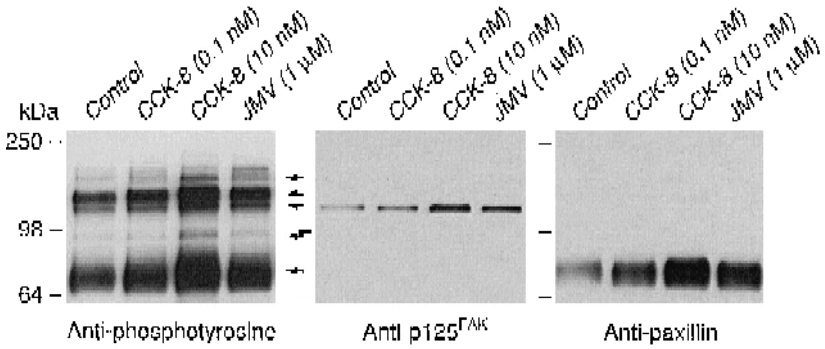

Fig. 1. Protein tyrosine phosphorylation in rat pancreatic acinar cells in response to CCK and JMV-180. Acinar cells were incubated in the absence or presence of the indicated concentrations of CCK-8 (lanes 2 and 3) or JMV-180 (lane 4) for 5 min and then lysed. Whole cell lysates were immunoprecipitates with anti-phosphotyrosine monoclonal Ab (PY20). Immunoprecipitates of proteins $>60\,000$ molecular weight were analyzed by SDS-PAGE followed by transfer of proteins to nitrocellulose membrane as described in Section 2.2. These results are representative of at least three more. *Left panel*: representative immunoblot with anti-phosphotyrosine monoclonal Ab (4G10). Bands were visualized using ECL. Arrowheads indicate the positions of the main tyrosine phosphorylated proteins >60 kDa in molecular weight. *Middle panel*: anti-phosphotyrosine immunoprecipitates were analyzed with a specific anti-$p125^{FAK}$ monoclonal Ab. *Right panel:* anti-phosphotyrosine immunoprecipitates were analyzed with an anti-paxillin monoclonal Ab immunoblot.

with PBS and further analyzed by SDS-PAGE and Western blotting.

2.2.3. Western blotting

Immunoprecipitates were fractionated by SDS-PAGE, and the proteins were transferred to nitrocellulose membranes. Membranes were blocked overnight at 4°C using blotto (5% non-fat dried milk in a solution containing 50 mM Tris–HCl, pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 0.05% (v/v) Tween 20, 0.02% NaN_3). The nitrocellulose membranes containing proteins >60 kDa were incubated for 2 h at 25°C with 1 μg/ml anti-phosphotyrosine monoclonal antibody (4G10) or with 1 μg/ml anti-p125^{FAK} monoclonal antibody or with 0.1 μg/ml anti-paxillin monoclonal antibody. The membranes were washed twice for 10 min with blotto and incubated for 40 min at 25°C with anti-mouse IgG-horseradish peroxidase conjugate. The membrane were finally washed 2 times for 10 min (80 mM NaCl, 0.05% (v/v) Tween 20, 0.02% NaN_3), incubated with enhanced chemiluminescence detection reagents (ECL) for 60 s and exposed to Hyperfilm ECL for less than 5 min. The density of bands on the film were measured using a scanning densitometer (Molecular Dynamics, Sunnyvale, CA).

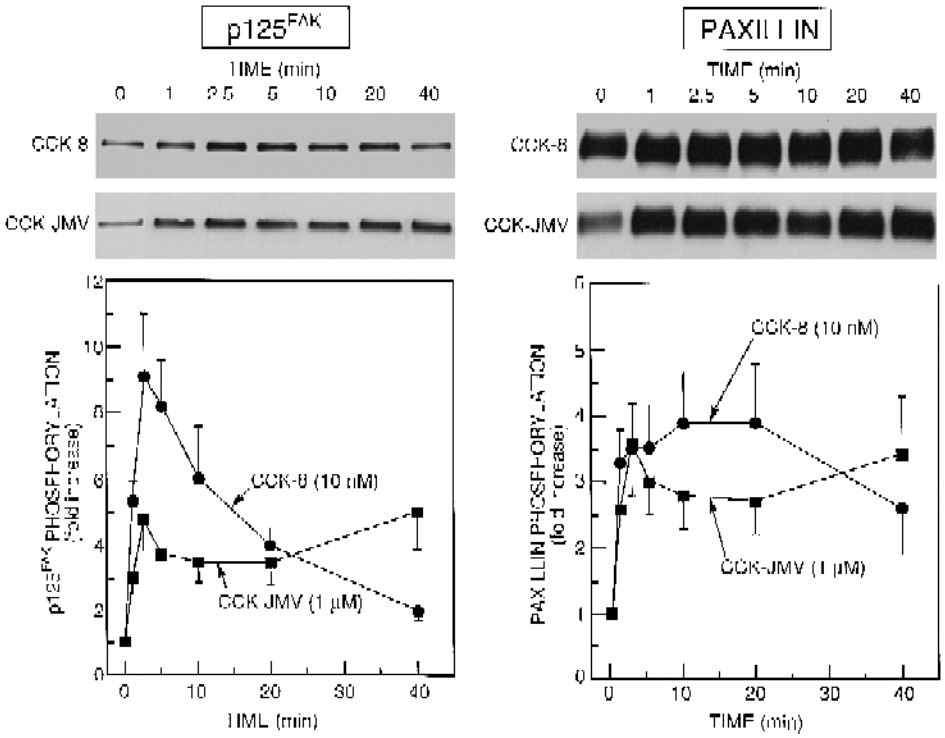

Fig. 2. Time-course of CCK-8 and JMV-180 stimulation of p125^{FAK} and paxillin tyrosine phosphorylation in rat pancreatic acinar cells. Rat pancreatic acinar cells were treated for various times as indicated with 10 nM CCK-8 or 1 μM JMV-180 and then lysed. Whole cell lysates were immunoprecipitates with anti-phosphotyrosine monoclonal Ab (PY20). Immunoprecipitates were analyzed by SDS-PAGE followed by transfer of proteins to nitrocellulose membrane. Proteins greater than 60 kDa were assessed by anti-p125^{FAK} or anti-paxillin immunoblotting as described in Section 2.2. Bands were visualized using ECL and quantification of phosphorylation was performed by scanning densitometry. *Left panel*: time-course of CCK-8 and JMV-180 stimulation of p125^{FAK} tyrosine phosphorylation. The top two panels show results from a representative experiment with both CCK-8 and JMV-180. These results are representative of at least three others. The values shown in the lower panel are the mean $\pm$ S.E. of four independent experiments and are expressed as fold increase (experimental/control). *Right panel*: time-course of CCK-8 and JMV-180 stimulation of paxillin tyrosine phosphorylation. The top two panels show results from a representative experiment with both CCK-8 and JMV-180. These results are representative of at least three others. The values shown in the lower panel are the mean $\pm$ S.E. of four independent experiments and are expressed as fold increase (experimental/control).

3. Results

Treatment of acinar cells with 0.1 nM and 10 nM CCK causes a marked increase in the tyrosine phosphorylation of at least five proteins with molecular weights > 60 kDa, which was the range examined in the present study (Fig. 1). The apparent molecular masses of these proteins was 170, 145, 126, 95 and 79 kDa (these will be referred to as p170, p145, p126, p95 and p79) as revealed by immunoprecipitation of pancreatic acinar cells lysates with anti-phosphotyrosine monoclonal antibody (PY20) followed by Western blotting of the immunoprecipitates using a second anti-phosphotyrosine monoclonal antibody (4G10) (Fig. 1, left panel, lanes 2 and 3).

To investigate whether the recently identified tyrosine kinase p125^{FAK} can serve as a substrate for CCK-stimulated tyrosine phosphorylation, pancreatic acinar cells were incubated with 0.1 nM and 10 nM CCK for 5 min, and lysates of the CCK-treated cells were immunoprecipitated with anti-phosphotyrosine monoclonal antibody (PY20) and the immunoprecipitates were analyzed by Western blotting with specific anti-p125^{FAK} monoclonal antibody. Fig. 1 (middle panel) shows that CCK (lanes 2 and 3) caused a striking increase in the tyrosine phosphorylation of a single anti-p125^{FAK} immunoreactive band. This tyrosine phosphorylated band exactly co-migrated with the protein band of 126 kDa obtained by immunoprecipitation with anti-phosphotyrosine from parallel cell lysates followed by immunoblot analysis using anti-phosphotyrosine (Fig. 1, left panel, lanes 2 and 3).

To determine whether the cytoskeleton-associated protein paxillin was a constituent of the p79 phosphotyrosine-containing band, pancreatic acinar cells lysates were immunoprecipitated with anti-phosphotyrosine monoclonal antibody (PY20) followed by immunoblotting with anti-paxillin monoclonal antibody (Fig. 1, right panel). CCK (0.1 nM and 10 nM)

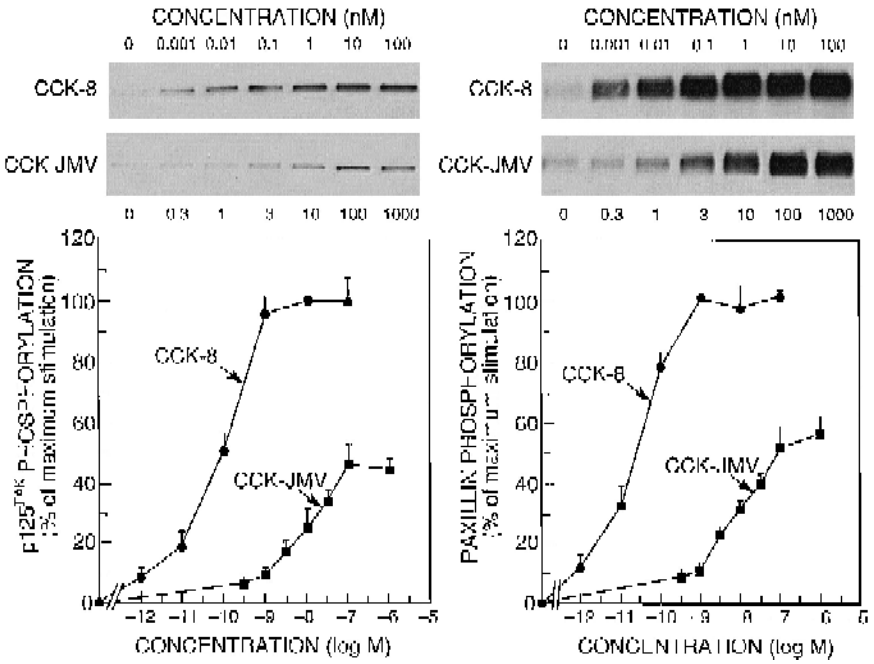

Fig. 3. Concentration-dependence of CCK-8 and JMV-180 to stimulate p125^{FAK} and paxillin tyrosine phosphorylation in rat pancreatic acinar cells. Rat pancreatic acinar cells were treated with various concentration of CCK-8 and JMV-180 as indicated for 5 min and then lysed. p125^{FAK} and paxillin tyrosine phosphorylation were determined by immunoprecipitation and Western blotting as described in Fig. 2 legend. The top two panels show p125^{FAK} tyrosine phosphorylation (*left panel*) and paxillin tyrosine phosphorylation (*right panel*). The results are from a single experiment with no additions or with various concentrations of CCK-8 or JMV-180. These results are representative of at least three others. The *bottom panel* shows the quantification of p125^{FAK} tyrosine phosphorylation (*left panel*) and paxillin tyrosine phosphorylation (*right panel*). Values are the means ± S.E. ($n = 4$) expressed as the percentage of maximal increase caused by 10 nM CCK-8 above control unstimulated values.

caused a striking increase in the amount of paxillin immunoreactivity in anti-phosphotyrosine immunoprecipitates, which migrated as a broad, diffuse band of M_r 72 000–87 000 (Fig. 1, right panel, lanes 2 and 3). This band exactly co-migrated with the p79 component revealed by anti-phosphotyrosine immunoblotting of anti-phosphotyrosine immunoprecipitates (Fig. 1, left panel, lanes 2 and 3).

Tyrosine phosphorylation of p125^{FAK} was a rapid consequence of addition of 10 nM CCK-8 to pancreatic acinar cells. An increase in tyrosine phosphorylation was detected 1 min after addition of the peptide, reached a maximum within 2.5 min with a 9.1 ± 1.9-fold increase, and decreased after 10 min (Fig. 2, left panel). Moreover, CCK-8 stimulated a rapid increase in paxillin tyrosine phosphorylation which reached maximum at 2.5 min with a 3.6 ± 0.6-fold increase and was maintained for at least 40 min (Fig. 2, right panel).

The effect of CCK on p125^{FAK} and paxillin tyrosine phosphorylation was concentration-dependent. At a 10 pM concentration, CCK-8 caused a 10% increase in tyrosine phosphorylation, a half-maximal effect occurred at 0.03 ± 0.015 nM for paxillin tyrosine phosphorylation and 0.1 nM for p125^{FAK} and a maximal effect occurred at 1 nM for both p125^{FAK} and paxillin tyrosine phosphorylation (Fig. 3).

JMV-180 is reported to be an agonist at the CCK_A high-affinity receptor state and an antagonist at the low affinity CCK_A-receptor state in rat pancreatic acinar cells [31–33]. JMV-180 (1 μM) induced tyrosine phosphorylation of several cellular proteins as

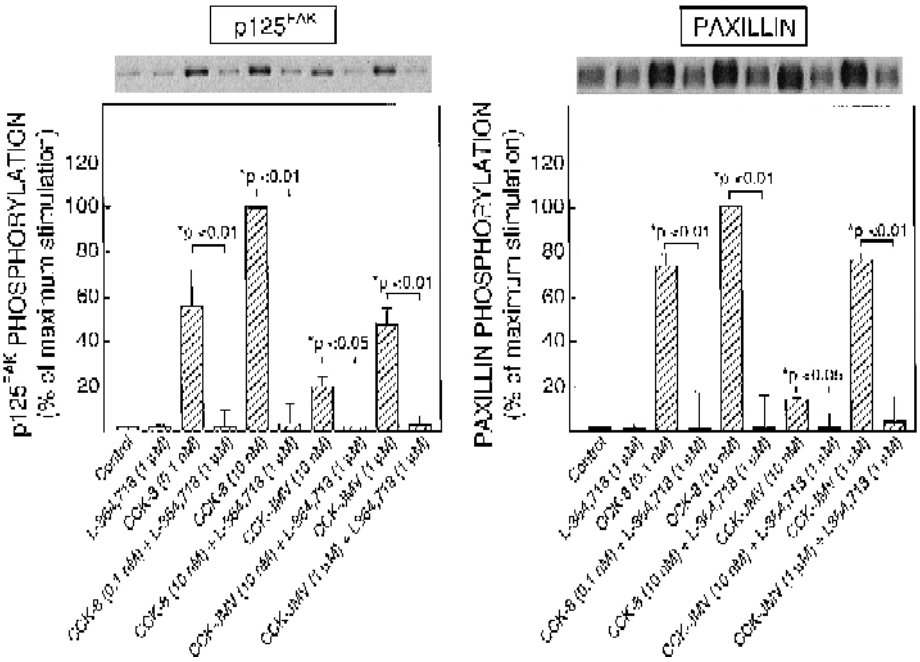

Fig. 4. Ability of the CCK_A specific receptor antagonist, L-364,718, to alter CCK-8- and JMV-180-stimulated p125^{FAK} and paxillin tyrosine phosphorylation in rat pancreatic acinar cells. Rat pancreatic acinar cells were treated with various concentrations of CCK-8 and JMV-180 either in the absence or presence of the specific CCK_A receptor antagonist L-364,718 as indicated for 5 min and then lysed. p125^{FAK} and paxillin tyrosine phosphorylation were determined as described in the legend to Fig. 2. *Left panel*: effect of the CCK_A receptor antagonist, L-364,718 on CCK-8- and JMV-180-stimulated p125^{FAK} tyrosine phosphorylation. The *top panel* shows results from a representative experiment of at least three others. The *bottom panel* shows the phosphorylation of p125^{FAK} expressed as the percentage of maximal increase caused by 10 nM CCK-8 above the control unstimulated values. Values are means $\pm$ S.E. ($n = 4$). *Right panel*: effect of the CCK_A receptor antagonist, L-364,718 on CCK-8- and JMV-180-stimulated paxillin tyrosine phosphorylation. The *top panel* shows results from a representative experiment of at least three others. The *bottom panel* shows the phosphorylation of paxillin expressed as the percentage of maximal increase caused by 10 nM CCK-8 above the control unstimulated values. Values are means $\pm$ S.E. ($n = 4$).

detected by immunoblotting with anti-phosphotyrosine monoclonal antibody (Fig. 1, left panel, lane 4). The major JMV-180-stimulated tyrosine phosphorylated proteins exactly co-migrated with the bands obtained with CCK-8 stimulation (Fig. 1, left panel, lane 3). Moreover, JMV-180 caused a striking increase in the tyrosine phosphorylation of $p125^{FAK}$ (Fig. 1, middle panel, lane 4) and paxillin (Fig. 1, right panel, lane 4).

An increase in tyrosine phosphorylation of both $p125^{FAK}$ and paxillin was detected within 1 min of adding JMV-180 and reached a maximum at 2.5 min with a 4.7 ± 0.9-fold increase in $p125^{FAK}$ tyrosine phosphorylation and 3.6 ± 0.8-fold increase for paxillin (Fig. 2). The magnitude of tyrosine phosphorylation of $p125^{FAK}$ caused by CCK-8 but not JMV-180 (Fig. 1) decreased after 2.5 min, whereas for both peptides the magnitude of tyrosine phosphorylation of paxillin remained relatively stable (Fig. 2). JMV-180 caused a detectable increase at 0.3 nM, half maximal effect at 10 nM and maximal effect at 0.1 μM in both $p125^{FAK}$ and paxillin tyrosine phosphorylation (Fig. 3).

The specific CCK_A receptor antagonist, L-364,718 (1 μM) had no effect on tyrosine phosphorylation of $p125^{FAK}$ or paxillin when added alone (Fig. 4, lane 2, left and right panels), but completely inhibited $p125^{FAK}$ (Fig. 4, lanes 4, 6, 8 and 10, left panel) and paxillin tyrosine phosphorylation (Fig. 4, lanes 4, 6, 8 and 10, right panel) in response to CCK-8 (0.1 nM and 10 nM) and to JMV-180 (10 nM and 1 μM).

Because JMV-180-stimulated $p125^{FAK}$ and paxillin tyrosine phosphorylation did not attain the full response that was obtained by CCK-8, we tested both

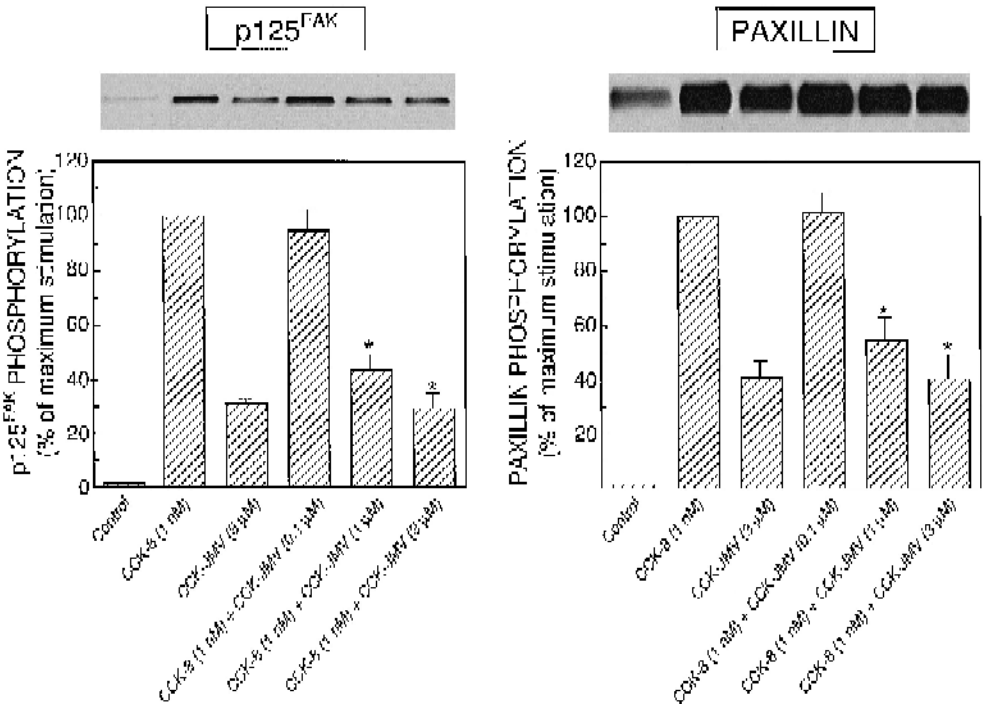

Fig. 5. Effect of CCK-8 and JMV-180 alone or in combination on $p125^{FAK}$ and paxillin tyrosine phosphorylation. Acini were stimulated with 3 μM JMV-180, with 1 nM CCK-8 alone or with 1 nM CCK-8 with various concentrations of JMV-180 for 5 min and then lysed. $p125^{FAK}$ and paxillin tyrosine phosphorylation were determined as described in the legend to Fig. 2. The *top panels* show results from a representative experiment. The *bottom panels* shows the results of $p125^{FAK}$ (*left panel*) and paxillin (*right panel*) tyrosine phosphorylation and the results are expressed as the percentage of the maximal increase caused by 1 nM CCK-8. Values are means $\pm$ S.E. for four experiments. Asterisk indicates significant differences as compared to 1 nM CCK-8 in combination with 0.1 μM JMV-180 ($P < 0.05$ with Student's *t*-test for unpaired samples).

analogues in combination. Acini were stimulated with 3 μM JMV-180 alone (Fig. 5, lane 3, left and right panel) and with 1 nM CCK-8 (Fig. 5, lane 2, left and right panel) alone or in combination with different concentrations of JMV-180 (0.1 μM, 1 μM and 3 μM) (Fig. 5, lanes 4, 5 and 6, left and right panel). Inhibition of CCK-8-stimulated p125^{FAK} and paxillin tyrosine phosphorylation was noted with 1 μM JMV-180 ($n = 4$, $P < 0.01$) (Fig. 5, lane 5, left and right panel). With 3 μM JMV-180, CCK-8-stimulated tyrosine phosphorylation decreased to the same level as that of 3 μM JMV-180 alone ($n = 4$, $P < 0.01$) (Fig. 5, lane 6, left and right panel).

4. Discussion

In the present study, we demonstrate that activation of the CCK_A receptor in rat pancreatic acinar cells causes tyrosine phosphorylation of at least five proteins of molecular weight $> 60\,000$, and demonstrate for the first time that two of these proteins are the novel tyrosine kinase p125^{FAK} and the cytoskeletal-associated protein, paxillin. Furthermore, we demonstrate that tyrosine phosphorylation of both proteins is mediated by activation of both the high affinity and the low affinity states of the CCK_A receptor.

Recent studies show a number of neuropeptides, whose receptors have no intrinsic tyrosine kinase activity, can stimulate tyrosine phosphorylation [11,12,14,15]. In previous studies, CCK-8 has been shown to cause tyrosine phosphorylation of a number of proteins [6–10]; however, except for the MAP kinases [6] (p42^{mapk} and p44^{mapk}), MEK kinases (p43 and p44) [10], and c-Jun amino-terminal kinases (p46 and p55) [9], none of the higher molecular weight proteins have been identified [6]. Furthermore, activation of the CCK_B receptor transfected into mouse 3T3 cells [35] and into rat 1 fibroblasts [34] causes tyrosine phosphorylation of p125^{FAK}, paxillin and MAP kinase. Similarly, the neuropeptides, bombesin, vasopressin, endothelin and bradykinin cause tyrosine phosphorylation of p125^{FAK} and paxillin in a number of tissues [11,12,14,15].

Recent studies [15,16,38] suggest tyrosine phosphorylation of p125^{FAK} and paxillin may be particularly important in mediating the effects of a number of neuropeptides, oncogenes and integrins particularly on growth [21]. A number of results in our study support the conclusion that CCK_A receptor activation in rat pancreatic acinar cells can cause tyrosine phosphorylation of both of these proteins. First of all, whether a mouse anti-phosphotyrosine monoclonal antibody is used initially followed by protein identification using anti-p125^{FAK} or anti-paxillin monoclonal antibodies, or when the antibodies are used in reverse order, each combination demonstrated tyrosine phosphorylation of both of these proteins. Secondly, this phosphorylation was mediated by the CCK_A receptor because it was inhibited by the specific CCK_A receptor antagonist, L-364,718 [36]. Thirdly, the dose-response curve for tyrosine phosphorylation of both proteins occurred over the same CCK-8 concentrations that cause CCK_A receptor activation and over which CCK-8 stimulates changes in cellular calcium [32,39,40], [^{3}H]thymidine incorporation [41] and MAP kinase activation [6].

Our results show similarities and differences of the ability of CCK_A receptor activation to cause tyrosine phosphorylation of these two proteins compared to the ability of CCK_B receptor activation [34,35] or the ability of other neuropeptides to cause tyrosine phosphorylation of both these proteins [13,16,17]. Similar to CCK_B, increase in p125^{FAK} and paxillin tyrosine phosphorylation was rapid, being detected as early as 1 min after addition of the peptide and reaching a maximum within 2–5 min. The CCK_A and the CCK_B receptors differ in the potency of CCK-8 at each receptor for causing tyrosine phosphorylation of both proteins. Specifically, the dose-response curve of p125^{FAK} and paxillin tyrosine phosphorylation in response to CCK_A receptor activation was one log unit to the left of that for activation of the CCK_B receptor, with the result that lower concentrations of CCK-8 caused significantly more stimulation at the CCK_A than at the CCK_B receptors. However, CCK_A receptor activation stimulates p125^{FAK} and paxillin tyrosine phosphorylation at concentrations that closely parallel those required to enhance the tyrosine phosphorylation of both proteins by bombesin in Swiss 3T3 cells [16]. The CCK_A receptor resembles the CCK_B receptor, but not the GRP receptor, with regard to their ability to cause changes in $[Ca^{2+}]_i$ and stimulate tyrosine phosphorylation of p125^{FAK} and

paxillin. Similar to the CCK_B receptor, the CCK-8 dose-response curve for $[Ca^{2+}]_i$ mobilization [32,40,42] was similar to that seen for $p125^{FAK}$ and paxillin tyrosine phosphorylation; however, with the GRP receptor activation the bombesin dose-response curve for $[Ca^{2+}]_i$ mobilization was 0.5 log unit to the right of that for tyrosine phosphorylation of both proteins in Swiss 3T3 cells [16,43].

Numerous studies demonstrate that the pancreatic CCK_A receptor exists in at least two binding states [24,28–30,33]. In one study [40] in which both states, a high affinity and a low affinity state, were described, each state had a different set of second messengers and different biological effects. In a number of studies [31–33,39], JMV-180 is reported to distinguish between these two states in rat pancreatic acini. Because of this ability, this peptide has been used to establish that CCK causes pancreatitis by the low affinity state [44], stimulates pancreatic growth by high affinity state or by both states [44,45], causes enhancement of expression of transcriptional factors such as c-*myc*, c-*jun* and c-*fos* by the low affinity state [4] and causes desensitization of stimulated pancreatic enzyme secretion by low affinity state [46]. A number of results in our study support the conclusion that CCK-8 can cause tyrosine phosphorylation of $p125^{FAK}$ and paxillin through both the high and low CCK affinity states. First of all, JMV-180 at 1 nM to 1 μM stimulates tyrosine phosphorylation of both $p125^{FAK}$ and paxillin, and causes 50% of the maximal stimulation caused by CCK-8 indicating that the high-affinity state of the CCK-receptor can cause tyrosine phosphorylation. Secondly, JMV-180 inhibited the $p125^{FAK}$ and paxillin tyrosine phosphorylation caused by a maximally effective concentration of CCK-8. This shows that JMV-180 can antagonize 60% of the ability of CCK-8 to cause tyrosine phosphorylation of both these proteins. The ability of JMV-180 to inhibit the additional increase caused by CCK-8 over JMV-180 and the fact that this only occurs at high CCK-8 concentrations, indicate that occupation of the low-affinity CCK-receptor state by CCK-8 also produces an additional stimulatory effect on tyrosine phosphorylation of $p125^{FAK}$ and paxillin. Thirdly, CCK-8-stimulated tyrosine phosphorylation of these two proteins occurred over a wide CCK-8-concentration range with a maximal effect at 1 nM. This wide range occurs over the concentration range for CCK-8 occupation of both CCK_A receptor states, the high affinity state and the low affinity state [24,28,32,33].

The cellular function caused by the tyrosine phosphorylation of $p125^{FAK}$ and paxillin by activation of the CCK_A receptor has not been addressed in this study. With other neuropeptides, such as bombesin, numerous studies demonstrate the tyrosine phosphorylation of both these proteins is particularly important in mediating both the effects on cellular morphology, such as actin stress fibre formation and focal adhesion assembly and on growth [21,23]. In preliminary studies we found that carbachol also caused tyrosine phosphorylation of $p125^{FAK}$ and paxillin in rat pancreatic acini (data not shown). Carbachol, in contrast to CCK, has minimal trophic effects on rat pancreas or pancreatic acini [47–50]. This observation raises the possibility that in contrast to the essential role of these proteins in mediating the growth effect of bombesin in Swiss 3T3 cells, with CCK and carbachol they may have a cellular function independent of growth. Stimulation with supramaximally effective concentrations of the CCK-analogue, caerulein, caused marked changes in the apical cytoskeleton of pancreatic acini [51], and in vivo causes a progressive disassembly and degradation of pancreatic acinar cell microtubules and microfilaments [52]. At present, whether tyrosine phosphorylation of $p125^{FAK}$ and paxillin is involved in these CCK_A receptor activation effects on cellular morphology or whether tyrosine phosphorylation of these proteins could be involved in CCK-8 mediated secretion is unknown. Recently, a possible role of tyrosine phosphorylation in the regulation of pancreatic secretion stimulated by CCK has been proposed. These studies [7,8,53] found that CCK-8 enhanced tyrosine phosphorylation in pancreatic acini and that genistein or various tyrphostins, two widely used tyrosine kinase inhibitors, inhibited both CCK-8-stimulated tyrosine phosphorylation and amylase release. Moreover, these studies attempted to localize the steps in the stimulus-secretion pathway that are affected by tyrosine kinase inhibition, showing that CCK-8-, NaF- or GTPγS-stimulated polyphosphoinositides hydrolysis and calcium ionophore-stimulated amylase release were significantly inhibited by tyrosine kinase inhibitors [7,8,53]. In contrast, genistein had no influence on the secretory response seen with the PKC

activator, TPA [7,8]. These results indicate that tyrosine kinase is involved in at least two separate stages in the CCK-induced amylase release, one upstream of PI hydrolysis and one downstream of increased $[Ca^{2+}]_i$. Whether tyrosine phosphorylation of $p125^{FAK}$ and paxillin is involved in mediating some of these effects is at present unknown.

References

[1] J.E. Jorpes, V. Mutt, Handbook of Experimental Pharmacology. Secretin, Cholecystokinin, Pancreozymin, Springer-Verlag, Berlin, Heidelberg, 1973, pp. 1–376.
[2] J.A. Williams, D.I. Yule, in: V.L.W. Go, E.P. DiMagno, J.D. Gardner, E. Lebenthal, H.A. Reber, G.A. Scheele (Eds.), PANCREAS: Biology, Pathobiology and Disease, Raven Press, New York, 1993, pp. 167–189.
[3] M. Korc, Y. Iwamoto, H. Sankaran, J.A. Williams, I.D. Goldfine, Am. J. Physiol. 240 (1981) G56–G62.
[4] L. Lu, C.D. Logsdon, Am. J. Physiol. 263 (1992) G327–G332.
[5] C.B. Lamers, J.I. Rotter, J.B. Jansen, Gut 29 (1988) 1358–1363.
[6] R.D. Duan, J.A. Williams, Am. J. Physiol. 267 (1994) G401–G408.
[7] R.D. Duan, A.C.C. Wagner, D.I. Yule, J.A. Williams, Am. J. Physiol. 266 (1994) G303–G310.
[8] M.P. Lutz, S.L. Sutor, R.T. Abraham, L.J. Miller, J. Biol. Chem. 268 (1993) 11119–11124.
[9] A. Dabrowski, T. Grady, C.D. Logsdon, J.A. Williams, J. Biol. Chem. 271 (1996) 5686–5690.
[10] R.D. Duan, C.F. Zheng, K.L. Guan, J.A. Williams, Am. J. Physiol. 268 (1995) G1060–G1065.
[11] W.R. Huckle, R.C. Dy, H.S. Earp, Proc. Natl. Acad. Sci. USA 89 (1992) 8837–8841.
[12] L.M.F. Leeb-Lundberg, X.H. Song, J. Biol. Chem. 266 (1991) 7746–7749.
[13] I. Zachary, J. Sinnett-Smith, E. Rozengurt, J. Biol. Chem. 267 (1992) 19031–19034.
[14] I. Zachary, J. Sinnett-Smith, E. Rozengurt, J. Biol. Chem. 266 (1991) 24126–24133.
[15] L.M.F. Leeb-Lundberg, X.H. Song, S.A. Mathis, J. Biol. Chem. 269 (1994) 24328–24334.
[16] J. Sinnett-Smith, I. Zachary, A.M. Valverde, E. Rozengurt, J. Biol. Chem. 268 (1993) 14261–14268.
[17] I. Zachary, J. Sinnett-Smith, C.E. Turner, E. Rozengurt, J. Biol. Chem. 268 (1993) 27060–27065.
[18] M.D. Schaller, C.A. Borgman, B.S. Cobb, R.R. Vines, A.B. Reynolds, J.T. Parsons, Proc. Natl. Acad. Sci. USA 89 (1992) 5192–5196.
[19] M.D. Schaller, J.T. Parsons, Curr. Opin. Cell Biol. 6 (1994) 705–710.
[20] L. Lipfert, B. Haimovich, M.D. Schaller, B.S. Cobb, J.T. Parsons, J.S. Brugge, J. Cell Biol. 119 (1992) 905–912.
[21] E. Rozengurt, Cancer Surveys 24 (1995) 81–96.
[22] S. Rankin, E. Rozengurt, J. Biol. Chem. 269 (1994) 704–710.
[23] M. Seckl, E. Rozengurt, J. Biol. Chem. 268 (1993) 9548–9554.
[24] R.T. Jensen, S.A. Wank, W.H. Rowley, S. Sato, J.D. Gardner, Trends Pharmacol Sci 10 (1989) 418–423.
[25] S.A. Wank, Am. J. Physiol. 269 (1995) G628–G646.
[26] W. Zhou, S.P. Povoski, N.A. Rosen, D.S. Longnecker, R.H. Bell Jr., Ann. N.Y. Acad. Sci. 713 (1994) 331–333.
[27] W. Zhou, S.P. Povoski, R.H. Bell Jr., J. Surg. Res. 58 (1995) 281–289.
[28] D. Menozzi, R. Vinayek, R.T. Jensen, J.D. Gardner, J. Biol. Chem. 266 (#6) (1991) 10385–10391.
[29] D.H. Yu, S.C. Huang, S.A. Wank, S. Mantey, J.D. Gardner, R.T. Jensen, Am. J. Physiol. 258 (1990) G86–G95.
[30] S.C. Huang, K.P. Fortune, S.A. Wank, A.S. Kopin, J.D. Gardner, J. Biol. Chem. 269 (1994) 26121–26126.
[31] M.C. Galas, M.F. Lignon, M. Rodriquez, C. Mendre, P. Fulcrand, J. Laur, J. Martinez, Am. J. Physiol. 254 (1988) G176–G182.
[32] S. Sato, H.A. Stark, J. Martinez, M.A. Beaven, R.T. Jensen, J.D. Gardner, Am. J. Physiol. 257 (1989) G202–G209.
[33] T. Matozaki, J. Martinez, J.A. Williams, Am. J. Physiol. 257 (1989) G594–G600.
[34] T. Seufferlein, D.J. Withers, S. Broad, T. Herget, J.H. Walsh, E. Rozengurt, Cell Growth and Differ. 6 (1995) 383–393.
[35] T. Taniguchi, T. Matsui, M. Ito, T. Murayama, T. Tsukamoto, Y. Katakami, T. Chiba, K. Chihara, Oncogene 9 (1994) 861–867.
[36] R.T. Jensen, G.F. Lemp, J.D. Gardner, J. Biol. Chem. 257 (1982) 5554–5559.
[37] S.R. Peikin, A.J. Rottman, S. Batzri, J.D. Gardner, Am. J. Physiol. 235 (1978) E743–E749.
[38] S.B. Kanner, A.B. Reynolds, R.R. Vines, J.T. Parsons, Proc. Natl. Acad. Sci. USA 87 (1990) 3328–3332.
[39] H.A. Stark, C.M. Sharp, V.E. Sutliff, J. Martinez, R.T. Jensen, J.D. Gardner, Biochim. Biophys. Acta 1010 (1989) 145–150.
[40] T. Matozaki, B. Goke, Y. Tsunoda, M. Rodriguez, J. Martinez, J.A. Williams, J. Biol. Chem. 265 (1990) 6247–6254.
[41] H. Hoshi, C. Logsdon, Am. J. Physiol. 265 (1993) G1177–G1181.
[42] C.B. Lamers, J.H.M. Van Tongeren, Gut 18 (1977) 128–134.
[43] E. Rozengurt, Ann. N. Y. Acad. Sci. 547 (1988) 277–292.
[44] A.K. Saluja, M. Saluja, H. Printz, A. Zavertnik, A. Sengupta, M.L. Steer, PNAS (USA) 86 (1989) 8968–8971.
[45] R. Dawra, A. Saluja, M.M. Lerch, M. Saluja, C. Logsdon, M. Steer, Biochem. Biophys. Res. Commun. 193 (1993) 814–820.
[46] D. Menozzi, H.A. Stark, J. Martinez, R.T. Jensen, J.D. Gardner, Peptides 10 (2) (1989) 337–341.

[47] T.E. Solomon, in: L.R. Johnson (Ed.), Physiology of the Gastrointestinal Tract, Raven Press, New York, 1981, pp. 873–892.
[48] C.D. Logsdon, Am. J. Physiol. 251 (1986) G487–G494.
[49] L.R. Johnson, Cancer 47 (1981) 1640–1645.
[50] J. Morisset, L. Jolicoeur, Y. Caussignac, T.E. Solomon, Can. J. Physiol. Pharmacol. 60 (1982) 871–876.
[51] M.S. O'Konski, S.J. Pandol, Pancreas 8 (1993) 638–646.
[52] J. Jungermann, M.M. Lerch, H. Weidenbach, M.P. Lutz, B. Kruger, G. Adler, Am. J. Physiol. 268 (1995) G328–G338.
[53] A. Piiper, D. Stryjek-Kaminska, J. Stein, W.F. Caspary, S. Zeuzem, Am. J. Physiol. 266 (1994) G363–G371.