Cholecystokinin-stimulated tyrosine phosphorylation of p125^{FAK} and paxillin is mediated by phospholipase C-dependent and -independent mechanisms and requires the integrity of the actin cytoskeleton and participation of p21^{rho}

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Recent studies show that the effects of some oncogenes, integrins, growth factors and neuropeptides are mediated by tyrosine phosphorylation of the cytosolic kinase p125 focal adhesion kinase (p125^{FAK}) and the cytoskeletal protein paxillin. Recently we demonstrated that cholecystokinin (CCK) C-terminal octapeptide (CCK-8) causes tyrosine phosphorylation of p125^{FAK} and paxillin in rat pancreatic acini. The present study was aimed at examining whether protein kinase C (PKC) activation, calcium mobilization, cytoskeletal organization and small G-protein p21^{rho} activation play a role in mediating the stimulation of tyrosine phosphorylation by CCK-8 in acini. CCK-8-stimulated phosphorylation of p125^{FAK} and paxillin reached a maximum within 2.5 min. The CCK-8 dose response for causing changes in the cytosolic calcium concentration ($[Ca^{2+}]_i$) was similar to that for p125^{FAK} and paxillin phosphorylation, and both were to the left of that for receptor occupation and inositol phosphate production. PMA increased tyrosine phosphorylation of both proteins. The calcium ionophore A23187 caused only 25 % of the maximal stimulation caused by CCK-8. GF109203X, a PKC inhibitor, completely inhibited phosphorylation with PMA but had no effect on the response to CCK-8. Depletion of $[Ca^{2+}]_i$ by thapsigargin had no effect on CCK-8-stimulated phosphorylation. Pretreatment with both GF109203X and thapsigargin decreased CCK-8-stimulated phosphorylation of both proteins by 50 %. Cytochalasin D, but not colchicine, completely inhibited CCK-8- and PMA-induced p125^{FAK} and paxillin phosphorylation. Treatment with *Clostridium botulinum* C3 transferase, which inactivates p21^{rho}, caused significant inhibition of CCK-8-stimulated p125^{FAK} and paxillin phosphorylation. These results demonstrate that, in pancreatic acini, CCK-8 causes rapid p125^{FAK} and paxillin phosphorylation that is mediated by both phospholipase C-dependent and -independent mechanisms. For this tyrosine phosphorylation to occur, the integrity of the actin, but not the microtubule, cytoskeleton is essential as well as the activation of p21^{rho}.

INTRODUCTION

Cholecystokinin (CCK)-related peptides are widely distributed in both the central and the peripheral nervous system, where they function as neurotransmitters and neuromodulators, and in the gastrointestinal tract, where they function as a neurotransmitter or hormone [1]. In the gastrointestinal tract CCK has numerous diverse effects, including stimulation of gall bladder contraction, pancreatic secretion, trophic effects and altering the motility of both the colon and stomach [1–3]. The actions of CCK-related peptides are mediated by two different subtypes of CCK receptors: a CCK_A subtype receptor with high affinity only for sulphated CCK peptides, and a CCK_B receptor with high affinity for both CCK and gastrin [4,5].

The action of CCK in pancreatic acinar cells, which are one of the main physiological sites of action of CCK, has been extensively used to investigate its cellular basis of action [3,4,6]. CCK interacts with CCK_A receptors on these cells, and numerous studies demonstrate that it activates phospholipase C (PLC), resulting in the generation of inositol phosphates and diacylglycerol, which release intracellular calcium and activate protein kinase C (PKC) [3,6]. The ability of CCK to activate these pathways has been extensively studied [3,4,6]. Recent studies suggest that, similar to a number of other neuropeptides [7–9], the activation of tyrosine kinases is probably also an important mediator of some of the cellular effects of CCK [10,11]. Recent studies showed that CCK causes tyrosine phosphorylation of numerous pancreatic proteins, including mitogen-activated protein kinases, mitogen-activated protein kinase kinases and c-Jun N-terminal kinases [12–14]. However, little is known about the mechanisms of the ability of CCK to activate this pathway.

Tyrosine phosphorylation by a number of other neuropeptides has been shown to be particularly important in mediating some of the growth effects caused by activation of their G-protein-coupled receptors, as well as in causing various cytoskeletal changes [9,15,16]. Particularly important in this respect is tyrosine phosphorylation of the cytosolic tyrosine kinase p125 focal adhesion kinase (p125^{FAK}) and the cytoskeleton-associated protein paxillin [7,8,17,18]. Several investigations have recently reported that p125^{FAK} and paxillin tyrosine phosphorylation are stimulated by oncogenes, integrins and growth factors [9]; thus they represent a convergence of the actions of numerous mitogenic stimuli. Recent studies show that the integrity of the actin cytoskeleton [8,18–22] and the participation of the Ras-related

Abbreviations used: CCK, cholecystokinin; $[Ca^{2+}]_i$, cytosolic calcium concentration; fura 2/AM, fura 2 acetoxymethyl ester; GRP, gastrin-releasing peptide; IP, inositol phosphates; IP_3, inositol 1,4,5-trisphosphate; FAK, focal adhesion kinase; NMB, neuromedin B; NMB-R, NMB receptor; PKC, protein kinase C; PLC, phospholipase C.

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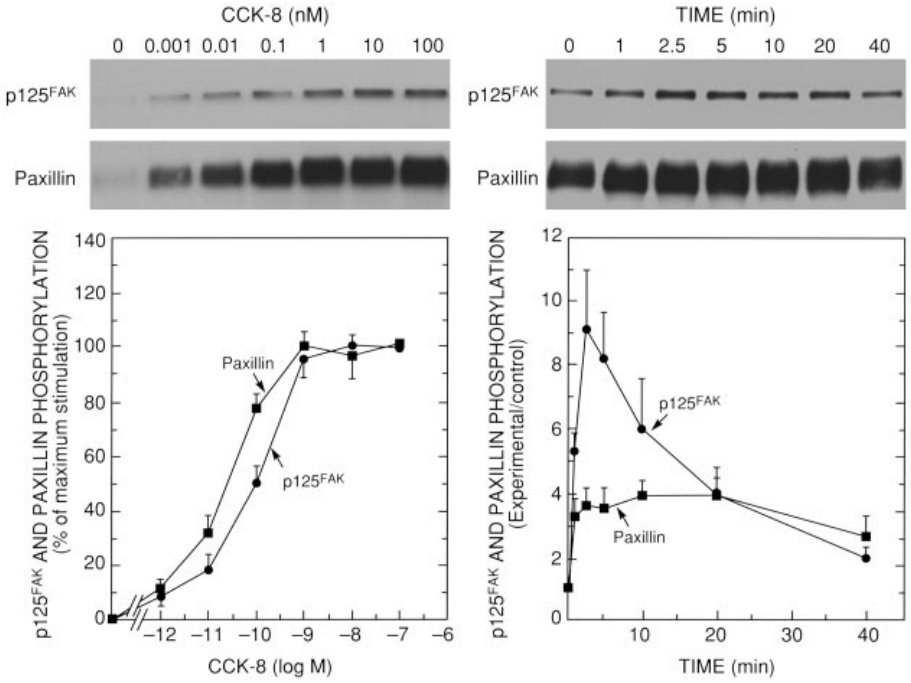

Figure 1 Concentration dependence (left panel) and time-course (right panel) of CCK-8 stimulation of p125^{FAK} and paxillin tyrosine phosphorylation in rat pancreatic acinar cells

Rat pancreatic acinar cells were treated with the indicated concentrations of CCK-8 at the indicated times and then lysed. Whole cell lysates were immunoprecipitated with anti-phosphotyrosine monoclonal antibody (PY20). Immunoprecipitates were analysed by SDS/PAGE followed by transfer of proteins of molecular mass $>$ 60 kDa to nitrocellulose membrane and anti-p125^{FAK} or anti-paxillin immunoblotting as described in the Materials and methods section. Bands were revealed using enhanced chemiluminescence, and quantification of phosphorylation was performed by scanning densitometry. Left panel: Rat pancreatic acini were incubated for 5 min with the indicated concentrations of CCK-8. The upper part shows p125^{FAK} and paxillin tyrosine phosphorylation results from a representative experiment with no additions or with various concentrations of CCK-8. These results are representative of at least three others. The bottom part shows the quantification of p125^{FAK} and paxillin tyrosine phosphorylation. Values are the means $\pm$ S.E.M. ($n = 4$) expressed as the percentage of maximal increase caused by 10 nM CCK-8 above control unstimulated values. Right panel: The upper part shows results from a representative experiment with CCK-8 (10 nM) for both proteins. These results are representative of at least three others. The values shown in the bottom part are the mean $\pm$ S.E.M. of four independent experiments and are expressed as fold increase over the pretreatment level (experimental/control).

small G-protein p21^{rho} [23,24] may be important for this stimulation. We have recently demonstrated in rat pancreatic acini that CCK rapidly increases the tyrosine phosphorylation of both p125^{FAK} and paxillin [25]. However, what remains unclear are the mechanisms of the stimulation of p125^{FAK} and paxillin tyrosine phosphorylation by the CCK_A receptor activation. It is unclear whether CCK-induced PKC activation or changes in cytosolic calcium are involved in the stimulation of tyrosine phosphorylation of these two proteins, and whether cytoskeletal components in the acini or small G-proteins are also important in mediating these changes.

The purpose of the present study was to determine whether PKC activation, calcium mobilization, cytoskeletal organization and p21^{rho} activation play a role in mediating p125^{FAK} and paxillin tyrosine phosphorylation stimulated by CCK_A receptor activation in rat pancreatic acinar cells.

MATERIALS AND METHODS

Materials

Male Sprague–Dawley rats (150–200 g) were obtained from the Small Animals Section, Veterinary Resources Branch, NIH, Bethesda, MD, U.S.A.; purified collagenase (type CLSPA) was from Worthington Biochemicals (Freehold, NJ, U.S.A.); anti-p125^{FAK} monoclonal antibody, anti-paxillin monoclonal antibody and anti-phosphotyrosine monoclonal antibody (PY20) were from Transduction Laboratories (Lexington, KY, U.S.A.); recombinant-Protein A–agarose was from Upstate Biotechnology (Lake Placid, NY, U.S.A.); anti-RhoA monoclonal antibody (sc-418) was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); *Clostridium botulinum* C3 transferase was from List Biological Laboratories (Campbell, CA, U.S.A.); thapsigargin and GF109203X were from Biomol (Plymouth Meeting, PA, U.S.A.); rabbit anti-mouse IgG and anti-mouse IgG–horseradish peroxidase conjugate were from Pierce (Rockford, IL, U.S.A.); ^{125}I-labelled Bolton–Hunter reagent–CCK-8 (^{125}I-CCK-8; 2200 Ci/mmol) and *myo*-[2-^{3}H]inositol (16–20 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.); nitrocellulose membrane was from Schleicher & Schuell (Keene, NH, U.S.A.); and fura 2 acetoxymethyl ester (fura 2/AM) was from Molecular Probes (Eugene, OR, U.S.A.).

Tissue preparation

Dispersed rat pancreatic acini were prepared according to the modifications [26] of the procedure published previously [27]. 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), 1% (w/v) albumin, 1% (w/v) trypsin inhibitor, 1% (v/v) vitamin mixture and 1% (w/v) amino acid mixture. The incubation solution was equilibrated with 100% O_2, and all incubations were performed with 100% O_2 as the gas phase.

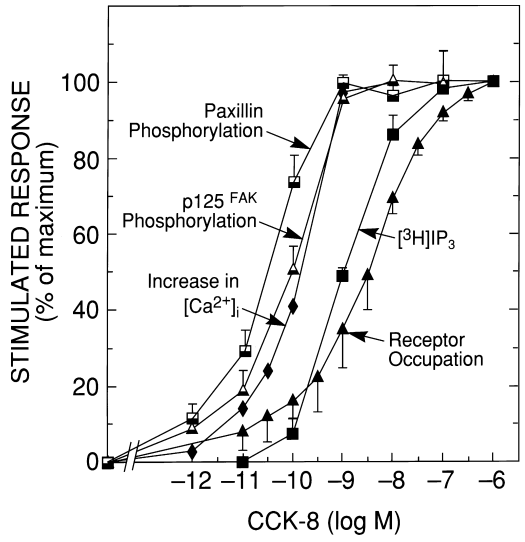

Figure 2 Relationship between the ability of CCK-8 to occupy CCK receptors, stimulate p125^{FAK} and paxillin tyrosine phosphorylation, alter cytosolic calcium and stimulate [^{3}H]IP$_3$ accumulation

p125^{FAK} and paxillin tyrosine phosphorylation was determined as described in the Figure 1 legend after an incubation time of 5 min. Results are expressed as the percentages of the maximal increase caused by 10 nM CCK-8 above the unstimulated control values. The ability of CCK-8 to inhibit binding of 50 pM ^{125}I-CCK-8 to rat pancreatic acini was determined at 37 °C after a 60 min incubation. Results were calculated as the percentage of receptors occupied with the indicated CCK-8 concentration, where maximal occupation occurred with 1 μM CCK-8. The peak increase in $[Ca^{2+}]_i$ at each CCK-8 concentration was determined, and results are expressed as the percentages of the maximal increase caused by 10 nM CCK-8. Control and 10 nM CCK-8-stimulated $[Ca^{2+}]_i$ were 140 ± 16 (S.E.M.) nM and 827 ± 65 nM respectively ($n = 20$). Dispersed acini were incubated with *myo*-[2-^{3}H]inositol for 2 h, washed and incubated either alone or with various concentrations of CCK-8 for 15 min at 37 °C. [^{3}H]IP were determined as described in the Materials and methods section. Results are expressed as the percentages of the maximal increase in [^{3}H]IP$_3$ caused by 10 nM CCK-8. Control and 10 nM CCK-8 values ($n = 4$) were 271 ± 13 and $13\,998 \pm 779$ d.p.m. respectively.

Binding of ^{125}I-CCK-8

Dispersed acini from one rat were suspended in 10 ml of standard incubation solution. Samples (500 μl) of cell suspension were incubated with the appropriate peptides plus 50 pM ^{125}I-CCK-8 for 60 min at 37 °C. Binding of ^{125}I-CCK-8 was determined as described previously [26]. Non-saturable binding of ^{125}I-CCK-8 was the amount of radioactivity associated with the acini when the medium contained 1 μM CCK-8. All values for binding of ^{125}I-CCK-8 are for saturable binding, i.e. total binding minus non-saturable binding. Non-saturable binding was always less than 14 % of total binding.

Intracellular Ca^{2+} imaging

To determine changes in cytosolic calcium ($[Ca^{2+}]_i$) in individual pancreatic acini, cells were resuspended in NaHepes medium contained (in mM) NaCl (130), KCl (5), Hepes (20), KH_2PO_4 (1.2), D-glucose (10), $CaCl_2$ (1) and 0.1 mg/ml trypsin inhibitor, and were incubated with 2 μM fura 2/AM for 30–40 min at 25 °C. The cells were then washed and used within the next 3 h. For determination of the concentration-dependent effect of CCK-8 on $[Ca^{2+}]_i$, the cells were placed on a glass coverslip attached to an open Perspex perifusion chamber and were continuously perifused with NaHepes solution without or with various concentrations of CCK-8 ranging from 1 pM to 0.1 μM. All experiments were carried out at 37 °C. The hardware used for image capture consisted of a Nikon Diaphot inverted microscope, Digital computer hardware with a dedicated image analysis system and an intensified charge-couple-device camera (Newcastle Photometric System, Newcastle, U.K.). Excitation wavelengths were selected by two interference filters centred at 340 and 380 nm placed in the light path of a 100 W Xenon lamp. The $[Ca^{2+}]_i$ imaging data were recorded at an effective sample rate of 1.6 s for each ratio image. The images were ratioed (340/380) pixel by pixel, and the values were proportional to $[Ca^{2+}]_i$. Fura-2 fluorescence was calibrated by using cells loaded with the dye and exposed to either 10 mM EGTA or 10 mM Ca^{2+} in the presence of 1.5 μM ionomycin, in a manner similar to that described by Grynkiewicz et al. [28] and using a dissociation constant for the Ca^{2+}–fura 2 complex of 224 nM.

To examine the effect of preincubation in a calcium-free medium with thapsigargin (10 μM), after loading with 2 μM fura-2/AM, pancreatic acini were washed three time in NaHepes medium. For measurements of $[Ca^{2+}]_i$, 2 ml of acinar cells were placed in quartz cuvettes in a Delta PTI Scan-1 spectrometer (PTI Instruments, Gaithersburg, MD, U.S.A.), and values for $[Ca^{2+}]_i$ were calculated as described previously [29,30].

Measurement of inositol phosphates

Changes in total inositol phosphates were determined in pancreatic acini as described previously [31]. Briefly, dispersed acini were incubated with 100 μCi/ml of *myo*-[2-^{3}H]inositol for 120 min. Acinar cells were washed and incubated in phosphoinositide buffer (standard incubation buffer containing 10 mM LiCl) for 15 min and then for 30 min at 37 °C with CCK-8 at various concentrations. Reactions were ended by adding 750 μl of a mixture consisting of chloroform/methanol/4 M HCl (100:200:2, by vol.), and total [^{3}H]inositol phosphates (IP) were isolated by anion-exchange chromatography as described previously [31].

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 different agonists and inhibitors 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 % (w/v) Triton X-100, 1 % (w/v) 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 10000 ***g*** for 15 min at 4 °C. Protein concentration was measured by the Bio-Rad protein assay reagent, and the volume was adjusted such that 1 ml aliquots of acinar lysates containing the same amount of protein (500 μg/ml) 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 with PBS and were analysed further by SDS/PAGE and Western blotting.

Western blotting

Immunoprecipitates were fractionated by SDS/PAGE using 10 % polyacrylamide gels, and proteins with molecular masses higher than 60 kDa 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 and 0.02% (w/v) NaN_3] and incubated for 2 h at 25 °C with 1 μg/ml anti-p125^{FAK} monoclonal antibody or with 0.1 μg/ml anti-paxillin monoclonal antibody. For RhoA studies, equal amounts of cellular proteins (30 μg) from acinar lysates were fractionated by SDS/PAGE using 14% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes. Membranes were blocked for 1 h at 25 °C using blotto and incubated for 2 h at 25 °C with 0.5 μg/ml anti-RhoA monoclonal antibody (sc-418). The membranes were washed twice for 10 min each with blotto and incubated for 40 min at 25 °C with anti-mouse IgG–horseradish peroxidase conjugate. The membranes were finally washed twice for 10 min each with blotto and twice for 10 min each with washing solution [50 mM Tris/HCl, pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 0.05% (v/v) Tween 20 and 0.02% (w/v) NaN_3], incubated with enhanced chemiluminescence detection reagents for 60 seconds and exposed to Hyperfilm ECL. The density of bands on the film was measured using a scanning densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RESULTS

CCK-8 (10 nM) caused rapid tyrosine phosphorylation of p125^{FAK} (Figure 1, right panel). An increase in tyrosine phosphorylation was detected 1 min after addition of the peptide, reached a maximum within 2.5 min with a 9±2 (S.E.M.) -fold increase, and decreased after 10 min. Moreover, CCK-8 stimulated a rapid increase in paxillin tyrosine phosphorylation, which reached maximum at 2.5 min with a 3.5±0.5-fold increase and was maintained for at least 20 min (Figure 1, right panel).

The effect of CCK-8 on p125^{FAK} and paxillin tyrosine phosphorylation was concentration dependent (Figure 1, left panel). CCK-8 caused a detectable increase at 1 pM, half-maximal effect at 0.1 nM for p125^{FAK} tyrosine phosphorylation and at 0.03 nM for paxillin tyrosine phosphorylation, and maximal effect at 1 nM for both p125^{FAK} and paxillin tyrosine phosphorylation. After CCK-8 binds to the CCK_A receptor it causes activation of PLC, which results in production of inositol 1,4,5-trisphosphate (IP_3), which in turn mediates the rapid mobilization of Ca^{2+} from intracellular stores and enzyme secretion [3,4]. We compared the ability of various concentrations of CCK-8 to alter these different cellular responses with its ability to cause tyrosine phosphorylation of p125^{FAK} and paxillin and with its ability to occupy the CCK_A receptor (Figure 2). In pancreatic acinar cells CCK-8 caused detectable receptor occupation at 0.03 nM, half maximal at 10 nM and maximal occupation at 1 μM (Figure 2). CCK-8 caused a detectable increase in [^{3}H]IP_3 accumulation at 0.1 nM, a half-maximal increase at 1 nM and a maximal increase at 100 nM (Figure 2). The CCK-8 dose response for causing changes in $[Ca^{2+}]_i$ was similar to that for stimulation of p125^{FAK} and paxillin tyrosine phosphorylation, and each were to the left of that for receptor occupation with a half-maximal effect at 0.03–0.1 nM (Figure 2).

In addition to increasing the production of IP, which causes mobilization of cellular calcium, activation of PLC by CCK-8 promotes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, leading to production of diacylglycerol, which in

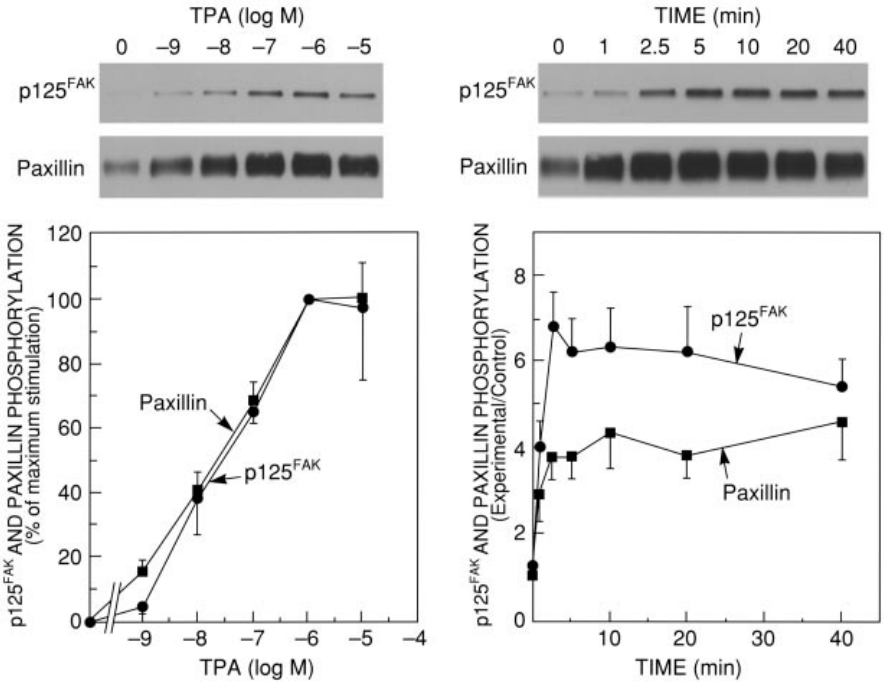

Figure 3 Concentration dependence (left panel) and time course (right panel) of the ability of the phorbol ester PMA (TPA) to stimulate p125^{FAK} and paxillin tyrosine phosphorylation in rat pancreatic acinar cells

Rat pancreatic acinar cells were treated with the indicated concentrations of PMA and for the indicated times and then lysed. p125^{FAK} and paxillin tyrosine phosphorylation were determined by immunoprecipitation and Western blotting as described in the Figure 1 legend. The organization of the Figure is the same as that of Figure 1, with results from a representative experiment at the top and average data at the bottom. Left panel: Rat pancreatic acini were treated with the indicated concentrations of PMA for 5 min. The top results are representative of at least three others. In the bottom part are shown the means±S.E.M. of four experiments expressed as the percentage of maximal increase caused by 1 μM PMA above the control unstimulated values. Right panel: The results from one experiment with PMA (1 μM) at the top are representative of at least three others. The values shown in the bottom part are the means±S.E.M. of four independent experiments and are expressed as the fold increase over the pretreatment value (experimental/control).

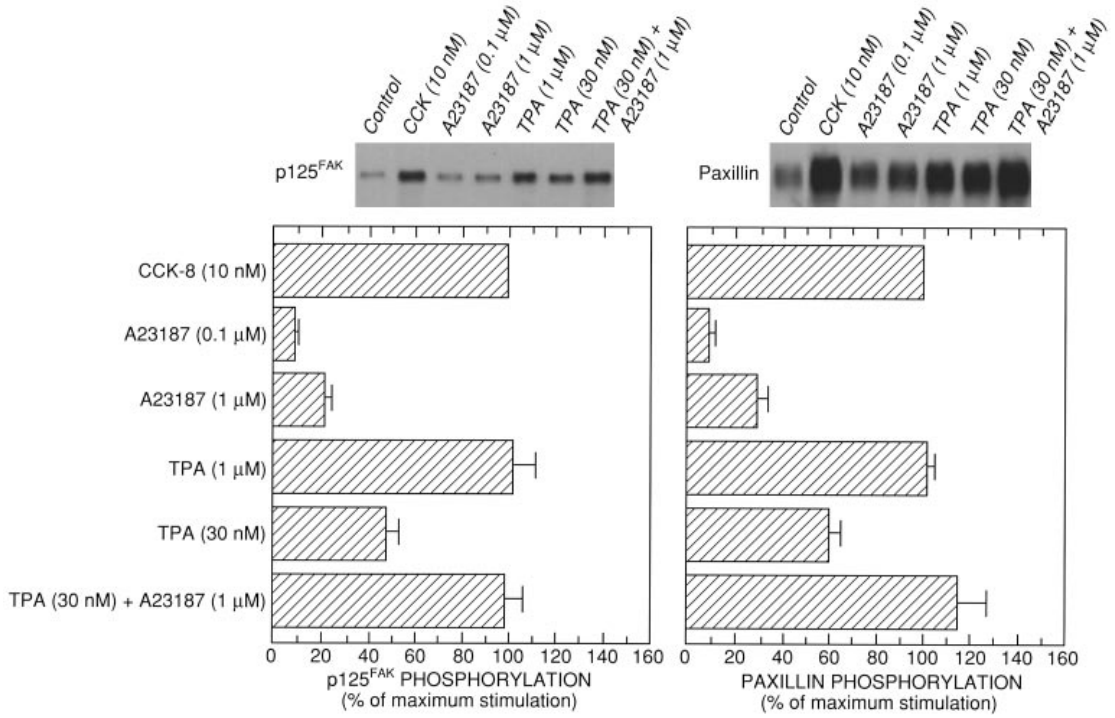

Figure 4 Effect of the calcium ionophore A23187, alone or in combination with the phorbol ester PMA (TPA), on stimulation of p125FAK and paxillin tyrosine phosphorylation in rat pancreatic acinar cells

Pancreatic acinar cells were treated with the indicated agents for 5 min at 37 °C, and p125FAK and paxillin tyrosine phosphorylation was determined as described in the Figure 1 legend. Results shown in the top panel are from a typical experiment representative of three others, and those in the bottom panel are the means ± S.E.M. of four experiments. In both panels the values are the means ± S.E.M. ($n = 4$) expressed as the percentage of the maximal increase in p125FAK (left) and paxillin (right) tyrosine phosphorylation caused by 10 nM CCK-8 above control.

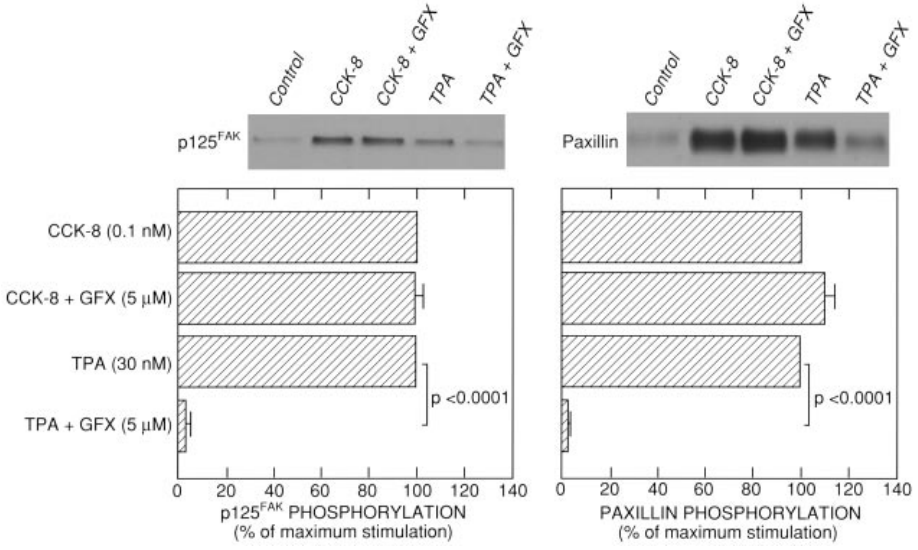

Figure 5 Effect of the selective PKC inhibitor GF109203X (GFX) on stimulation of p125FAK and paxillin tyrosine phosphorylation by PMA (TPA) and CCK-8

Pancreatic acinar cells were pretreated for 2 h in either the absence or the presence of 5 μM GF109203X. Control cells received an equivalent volume of solvent. Acini were then incubated for a further 5 min with no additions (control), with 0.1 nM CCK-8 or with 30 nM PMA. p125FAK and paxillin tyrosine phosphorylation was determined as described in the Figure 1 legend. The top panels show a single experiment representative of three others. In the lower panels are shown the means ± S.E.M. from four experiments for p125FAK (left) and paxillin (right) tyrosine phosphorylation. The values with GF109203X present are expressed as the percentages of the maximal increase in phosphorylation caused by PMA or CCK-8 without GF109203X present.

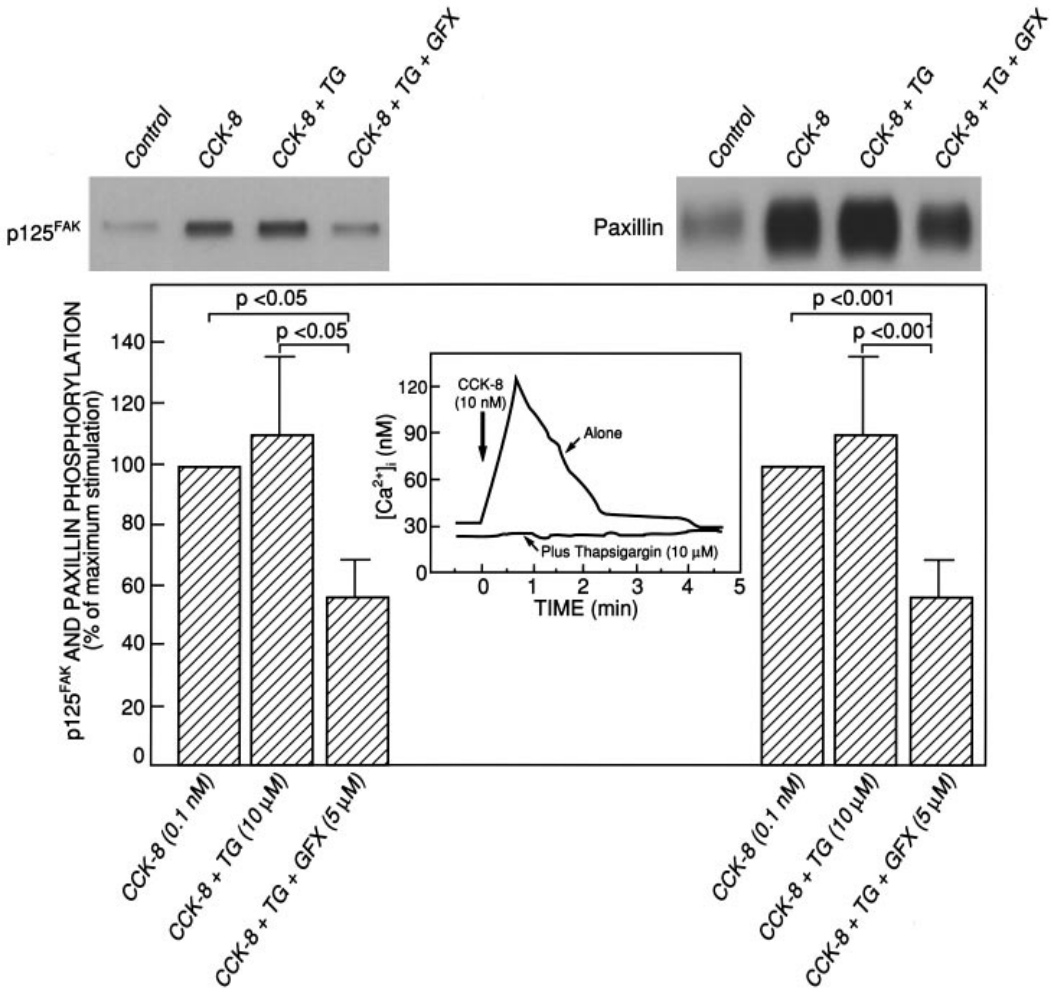

Figure 6 Effect of thapsigargin (TG), alone or in combination with GF109203X (GFX), on CCK-8 stimulation of p125^{FAK} and paxillin tyrosine phosphorylation or CCK-8-stimulated changes in $[Ca^{2+}]_i$ (inset)

Pancreatic acinar cells were pretreated with 10 μM thapsigargin for 1 h in calcium-free medium (with EGTA 5 mM) either in the absence or presence of 5 μM GF109203X. Acini were then incubated for a further 5 min with no additions (control) or with 0.1 nM CCK-8. p125^{FAK} and paxillin tyrosine phosphorylation was determined as described in the Figure 1 legend. The top panels show a single experiment representative of three others. In the lower panel are shown the means $\pm$ S.E.M. from four experiments; the data are expressed as the percentages of the maximal increase in phosphorylation caused by 0.1 nM CCK-8 above the control level. Brackets indicate significant differences as compared with acinar cells pretreated with 10 μM thapsigargin in combination with 5 μM GF109203X ($P < 0.05$ with Student's t test for unpaired samples). Inset: Effect of 10 nM CCK-8 on $[Ca^{2+}]_i$ in pancreatic acinar cells pretreated with or without 10 μM thapsigargin for 1 h in calcium-free medium with EGTA (5 mM).

turn activates PKC [3,31]. We next attempted to determine whether CCK-8's activation of one or both of these second messengers was needed for its ability to cause tyrosine phosphorylation of p125^{FAK} and paxillin. To determine whether activation of PKC could increase the tyrosine phosphorylation of p125^{FAK} and paxillin, pancreatic acinar cells were treated with the phorbol ester PMA, which directly activates PKC. PMA (1 μM) stimulated a rapid increase in p125^{FAK} and paxillin tyrosine phosphorylation, which reached maximum at 2.5 min and was maintained for at least 40 min (Figure 3, right panel). The effect of PMA on p125^{FAK} and paxillin tyrosine phosphorylation was concentration dependent (Figure 3, left panel). PMA caused a half-maximal effect at 30 nM and maximum effect at 1 μM on tyrosine phosphorylation of both proteins.

To determine whether increased $[Ca^{2+}]_i$ either alone or in combination with activation of PKC could alter tyrosine kinase pathway, we compared the ability of the calcium ionophore A23187 to cause p125^{FAK} and paxillin tyrosine phosphorylation in pancreatic acinar cells when present alone or with PMA (Figure 4). A23187 (0.1 μM) caused only 9 ± 1.5 (S.E.M.) % and 6 ± 3 % of the maximal stimulation caused by CCK-8 (10 nM) in p125^{FAK} and paxillin tyrosine phosphorylation respectively. A 10-fold higher concentration of A23187 (1 μM) caused 22 ± 4 % and 31 ± 4 % of the maximal stimulation caused by CCK-8 (10 nM) in both proteins respectively. In contrast, PMA (1 μM) caused a 102 ± 9 % and 95 ± 7 % of the maximal stimulation caused by CCK-8 (10 nM) in p125^{FAK} and paxillin tyrosine phosphorylation respectively (Figure 4). A lower concentration of PMA (30 nM) caused 50 ± 5 % and 66 ± 1 % of the maximal stimulation caused by CCK-8 (10 nM) in p125^{FAK} and paxillin tyrosine phosphorylation respectively (Figure 4). The simultaneous stimulation with both PMA (30 nM) and A23187 (1 μM) increased p125^{FAK} and paxillin tyrosine phosphorylation to 99 ± 9 % and 125 ± 10 % of the stimulation caused by CCK-8

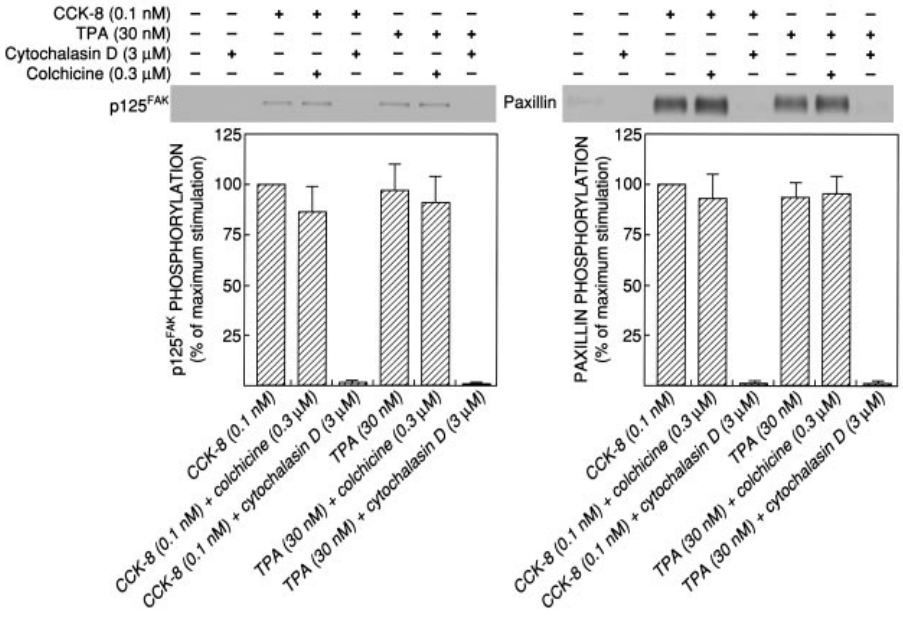

Figure 7 Effect of cytochalasin D or colchicine on CCK-8 or PMA (TPA) stimulation of p125FAK and paxillin tyrosine phosphorylation in pancreatic acinar cells

Pancreatic acinar cells were pretreated for 2 h in either the absence or the presence of 3 μM cytochalasin D or 0.3 μM colchicine. Acini were then incubated for a further 5 min with no additions (control), with 0.1 nM CCK-8 or with 30 nM PMA. p125FAK and paxillin tyrosine phosphorylation was determined as described in the Figure 1 legend. The top panels show the results from one experiment representative of three others. In the lower panels are shown the means $\pm$ S.E.M. from four experiments; the data are expressed as the percentages of the maximal increase in phosphorylation caused by 0.1 nM CCK-8 in rat pancreatic acini not treated with cytochalasin D or colchicine.

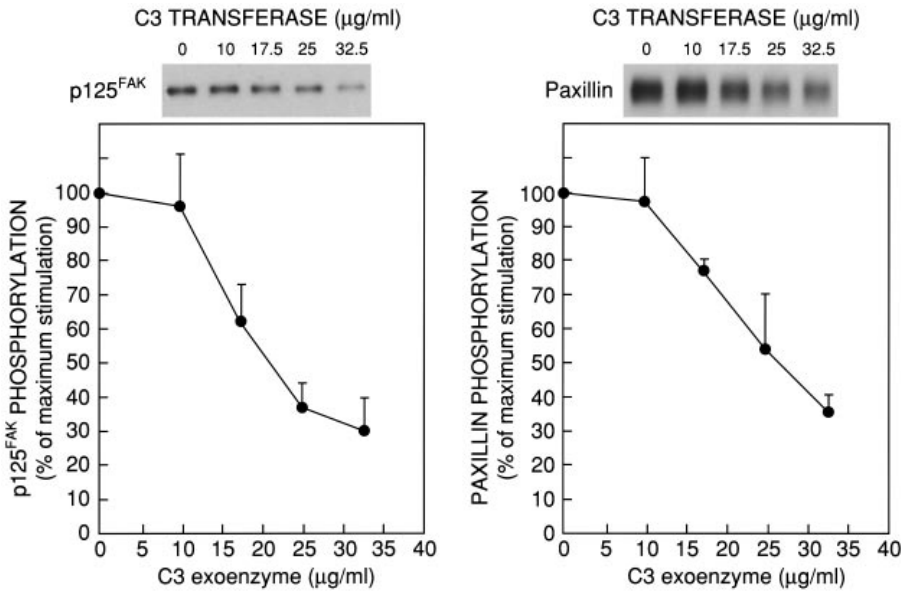

Figure 8 Effect of different concentrations of *C. botulinum* C3 transferase on CCK-8 stimulation of p125FAK and paxillin tyrosine phosphorylation in pancreatic acinar cells

Pancreatic acinar cells were pretreated for 3 h in either the absence or the presence of various concentrations of C3 transferase as indicated. Acini were then incubated for a further 5 min with 0.1 nM CCK-8. p125FAK and paxillin tyrosine phosphorylation was determined as described in the Figure 1 legend. The top panels show the results from one experiment representative of three others. In the lower panels are shown the means $\pm$ S.E.M. from four experiments for p125FAK (left panel) and paxillin (right panel); the data are expressed as percentages of the maximal increase in phosphorylation caused by 0.1 nM CCK-8 in acinar cells not treated with C3 transferase.

(10 nM). This increase with both agents together was greater than the sum of the values obtained with each alone (Figure 4) and equal to the stimulation caused by a maximally effective concentration of PMA alone (i.e. 1 μM) (Figure 4).

To determine whether PKC activation might be involved in mediating the CCK-8-stimulated changes in p125^{FAK} and paxillin tyrosine phosphorylation, we examined the effect of a PKC inhibitor, GF109203X [32], on both PMA and CCK-8 stimulation of p125^{FAK} and paxillin tyrosine phosphorylation (Figure 5). Pretreatment of pancreatic acinar cells with 5 μM GF109203X for 2 h did not modify significantly basal tyrosine phosphorylation (results not shown), but caused complete inhibition of tyrosine phosphorylation of p125^{FAK} (Figure 5, left panel) and paxillin (Figure 5, right panel) tyrosine phosphorylation induced by PMA (30 nM). However, GF109203X did not alter stimulation of tyrosine phosphorylation of p125^{FAK} or paxillin by 0.1 nM CCK-8 (Figure 5), a concentration that caused a similar degree of stimulation as 30 nM PMA did. To examine the effect of changes in $[Ca^{2+}]_i$ alone or in combination with PKC activation by CCK-8, the effect of thapsigargin, an agent that specifically inhibits the endoplasmic reticulum Ca^{2+}-ATPase and thereby depletes Ca^{2+} from intracellular compartments [29,30], was examined alone or with PKC inhibitor GF109203X present (Figure 6). Treatment with 10 μM thapsigargin for 1 h in a calcium-free medium (with 5 mM EGTA), alone, inhibited completely the increase in $[Ca^{2+}]_i$ induced by subsequently added CCK-8 (10 nM) (Figure 6, inset), but had no effect on the increase in p125^{FAK} (Figure 6, upper left) and paxillin (Figure 6, upper right) tyrosine phosphorylation caused by CCK-8 (0.1 nM) and did not modify significantly basal tyrosine phosphorylation in control cells (results not shown). However, the combination of GF109203X and thapsigargin decreased CCK-8-stimulated tyrosine phosphorylation of p125^{FAK} and paxillin by 44 ± 12 % and 52 ± 4 % respectively (Figure 6) without having any significant effect on basal tyrosine phosphorylation (results not shown).

Given the cellular localization of FAK and paxillin to focal adhesions that are in proximity with actin stress fibers [16], we considered whether the integrity of the cytoskeleton and its interaction with the actin-filament network might be necessary for the p125^{FAK} and paxillin tyrosine phosphorylation. To test this possibility, pancreatic acinar cells were pretreated for 2 h with cytochalasin D (3 μM), a selective disrupter of the actin-filament network [33], and then incubated with CCK-8 (0.1 nM) or PMA (30 nM) for another 5 min (Figure 7). Treatment with cytochalasin D completely inhibited CCK-8- and PMA-stimulated p125^{FAK} and paxillin tyrosine phosphorylation. In contrast, pretreatment with colchicine (0.3 μM), which inhibits microtubule synthesis [34,35], had no effect in p125^{FAK} and paxillin tyrosine phosphorylation stimulated by CCK-8 (0.1 nM) or PMA (30 nM) (Figure 7).

The small G-protein p21^{rho} has been implicated in mitogen-stimulated formation of focal adhesions and stress fibres [36–38]. Moreover, previous studies in Swiss 3T3 cells showed that the activation of p21^{rho} is required for the tyrosine phosphorylation of p125^{FAK} and paxillin caused by such neuropeptides as bombesin [23,24]. To determine whether this small G-protein could be involved in the ability of CCK-8 to stimulate tyrosine phosphorylation of p125^{FAK} and paxillin in pancreatic acinar cells, we examined the effect of pretreatment for 3 h with different concentrations of the exoenzyme *C. botulinum* C3 transferase on CCK-8-stimulated changes in p125^{FAK} and paxillin tyrosine phosphorylation (Figure 8), because the C3 transferase has been shown to cause ADP ribosylation of p21^{rho}, which inactivates p21^{rho} [39–41]. Pretreatment with C3 transferase inhibited CCK-8-stimulated p125^{FAK} and paxillin tyrosine phosphorylation in a concentration-dependent manner (Figure 8). Pretreatment with 32.5 μg/ml of C3 transferase for 3 h decreased CCK-8-stimulated p125^{FAK} and paxillin tyrosine phosphorylation by 70 ± 23 (S.E.M.) % and 64 ± 9 % respectively (Figure 8). C3 transferase pretreatment of control cells in the absence of CCK-8 did not modify significantly basal tyrosine phosphorylation below 25 μg/ml (results not shown). Previous studies showed that treatment with C3 transferase and ADP ribosylation of RhoA resulted in a marked diminution in the amount of immunodetectable RhoA [42,43]. Pretreatment of pancreatic acinar cells for 3 h with different concentrations of C3 transferase results in a concentration-dependent loss of immunodetectable RhoA (Figure 9, top). Moreover, our results show a close relationship between inhibition of CCK-8-stimulated p125^{FAK} tyrosine phosphorylation and loss of immunodetectable RhoA after C3 transferase pretreatment (Figure 9, bottom). In addition, the activation of PLC by CCK-8 (0.1 nM) was not altered after C3 transferase treatment at any concentration used (Figure 9, bottom). To investigate the possible site of action of p21^{rho} in CCK-8 stimulation of tyrosine phosphorylation of p125^{FAK} and paxillin, we determined the ability of C3 transferase to alter the effects of the calcium ionophore A23187 alone, the effects of the PKC activator PMA alone or the effects of both together (Figure

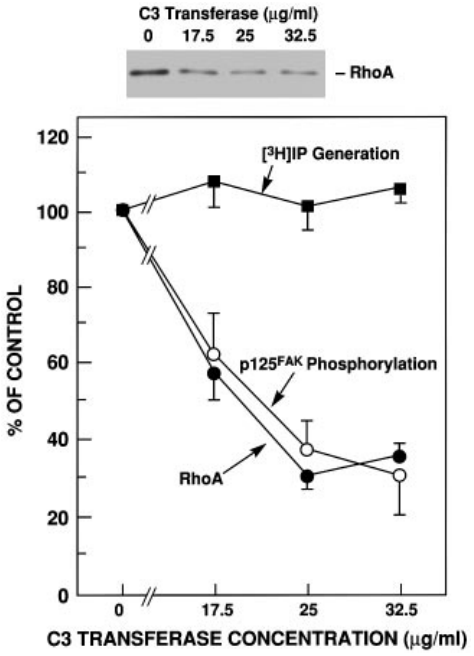

Figure 9 Effects of C3 transferase on CCK-8 stimulation of [^{3}H]IP generation and p125^{FAK} tyrosine phosphorylation and on the total immunodetectable RhoA in rat pancreatic acinar cells

Top panel: Pancreatic acinar cells were pretreated for 3 h at 37 °C in either the presence or the absence of different concentrations of C3 transferase and then lysed. Acinar lysate proteins (30 μg) were subjected to SDS/PAGE on a 14% gel. RhoA was identified by Western blotting using anti-RhoA mAb (sc-418) as described in the Materials and methods section. These results are representative of at least three others. Bottom panel: Immunodetectable RhoA present in acinar cells treated with C3 transferase was determined as described above and in the Materials and methods section and is expressed as the percentage of the total immunodetectable RhoA present in control cells without C3 transferase treatment. The results of total [^{3}H]IP generation and p125^{FAK} tyrosine phosphorylation in acinar cells treated with C3 transferase are expressed as percentages of the maximal increase caused by 0.1 nM CCK-8 in cells not treated with C3 transferase (control). The [^{3}H]IP values for the control and with CCK-8 stimulation were 2550 ± 200 and 10460 ± 640. Results are means $\pm$ S.E.M. from four separate experiments.

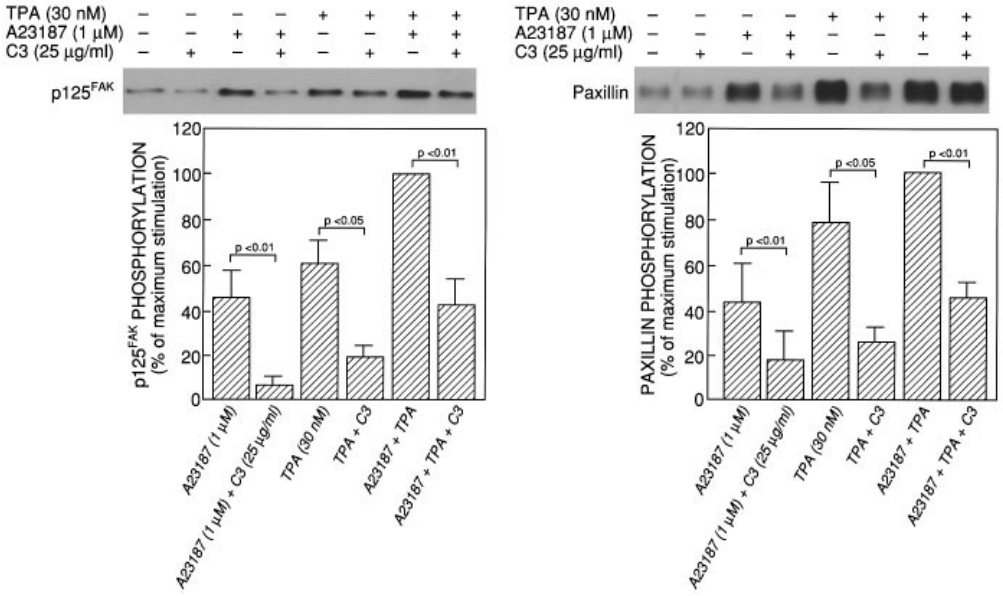

Figure 10 Effect of C3 transferase (C3) on stimulation by A23187 or PMA (TPA) of p125^{FAK} and paxillin tyrosine phosphorylation in pancreatic acinar cells

Pancreatic acinar cells were pretreated for 3 h either in the absence or presence of 25 μg/ml of C3 transferase. Acini were then incubated for a further 5 min with no additions (control), with 1 μM A23187, with 30 nM PMA or with both. p125^{FAK} and paxillin tyrosine phosphorylation was determined as described in the Figure 1 legend. The top panels show the results from one experiment representative of three others. The lower panels show the means $\pm$ S.E.M. from four experiments; the data are expressed as percentages of the maximal increase in phosphorylation caused by 1 μM A23187 together with 30 nM PMA in acinar cells not pretreated with the C3 transferase.

10). Pretreatment with 25 μg/ml C3 transferase for 3 h caused a significant ($P < 0.05$) decrease in p125^{FAK} and paxillin tyrosine phosphorylation stimulated by A23187 [71 $\pm$ 14 (S.E.M.) and 59 $\pm$ 22 % decrease], PMA (61 $\pm$ 16 and 74 $\pm$ 6 % decrease) or both together (58 $\pm$ 11 and 55 $\pm$ 6 % decrease) (Figure 10).

DISCUSSION

Numerous studies have demonstrated that with CCK receptor activation [10–14,44,45], similar to the activation of a number of other G-protein-coupled receptors for neuropeptides that have no intrinsic tyrosine kinase activity, stimulation of tyrosine phosphorylation of a number of proteins occurs and is probably an important intracellular signalling cascade [18,46–48]. Recent studies [8,18,49] suggest that with these neuropeptides, similar to the effect seen with oncogenes and integrins, the tyrosine phosphorylation of p125^{FAK} and the cytoskeletal protein paxillin may be particularly important in mediating numerous cellular effects, especially on growth. A previous study by us [25] demonstrated CCK-8 can cause tyrosine phosphorylation of both of these proteins in rat pancreatic acinar cells by activating both low- and high-affinity sites of the CCK_A receptors. Furthermore, in other studies [44,45] CCK-8 has been shown also to cause tyrosine phosphorylation of these proteins by activation of CCK_B receptors in other cells. In addition to causing tyrosine phosphorylation of these two proteins, by interacting with CCK_A receptors on rat pancreatic acinar cells, CCK-8 and other agonists cause activation of PLC, stimulating the generation of IP_3 and diacylglycerol, causing mobilization of cellular calcium and activation of PKC [3,4]. The ability of CCK-8 at the CCK_A receptor to activate the PLC cascade and the interaction of both arms of the PLC cascade have been extensively studied [3,4]. However, little is known about the mechanism of the ability of CCK-8 to stimulate tyrosine phosphorylation of p125^{FAK} and paxillin or the possible relationships between the ability of CCK_A-receptor stimulation to activate the PLC cascade and its ability to cause phosphorylation of p125^{FAK} and paxillin.

To provide insights into the relationship of the ability of CCK to activate these different intracellular cascades, we first investigated the stoichiometric relationship between the ability of CCK-8 to cause tyrosine phosphorylation of p125^{FAK} and paxillin and its ability to stimulate the PLC cascade compared with its ability to cause CCK_A-receptor occupation. CCK-8 stimulated phosphorylation of both proteins over the same concentration range that it caused changes in $[Ca^{2+}]_i$, and the dose–response curves for p125^{FAK} and paxillin phosphorylation and calcium mobilization were similar. However, both of these dose–response curves are one log to the left of the ability of CCK-8 to cause receptor occupation or increase $[^3H]IP_3$ accumulation. These results demonstrate that for both CCK-8-stimulated changes in tyrosine phosphorylation and changes in cytosolic calcium, in contrast to stimulation of phosphoinositide breakdown, maximal changes in tyrosine phosphorylation and cytosolic calcium occur with submaximal receptor activation. Therefore, there is receptor spareness for tyrosine phosphorylation and changes in intracellular calcium but not for stimulation of the generation of IP. These results have similarities and differences from the coupling of other neuropeptide receptors to these various intracellular pathways. The results are similar to activation of gastrin-releasing peptide (GRP) receptors on Swiss 3T3 cells [8,50], neuromedin B (NMB) receptor (NMB-R) activation on C-6 glioblastoma cells and NMB-R-transfected cells [51,52] and to activation of CCK_B receptors transfected into rat1 fibroblasts [45], in that in each case submaximal receptor activation results in maximal changes in p125^{FAK} and paxillin phosphorylation and calcium mobilization, whereas the generation of IP is closely coupled to receptor occupation. In contrast, coupling of CCK_A receptors to these transduction pathways in rat pancreatic acini

differs from GRP or NMB-Rs in the extent of receptor spareness for the different pathways. Whereas the stoichiometric relationship between receptor occupation and stimulation of tyrosine phosphorylation of these proteins and agonist-induced changes in cellular calcium are similar in the CCK_A receptor, with both GRP receptors and NMB-Rs the extent of receptor spareness for these two different intracellular cascades differs [8,50–52].

A number of recent results suggest that with some receptors activation of the PLC cascade may be important for the agonist to stimulate tyrosine phosphorylation of $p125^{FAK}$ and paxillin. Angiotensin II in liver cells increases tyrosine phosphorylation of a 125 kDa protein through a Ca^{2+}-dependent pathway [46], and epinephrine stimulation of $p125^{FAK}$ phosphorylation in platelets is blocked by inhibition of increases in $[Ca^{2+}]_i$ [22]. $p125^{FAK}$ possesses a PKC phosphorylation sequence [53], and activation of PKC by phorbol ester causes tyrosine phosphorylation of $p125^{FAK}$ in a number of cells [8]. Recent studies demonstrate that epinephrine stimulation of $p125^{FAK}$ phosphorylation in platelets is inhibited when PKC activation is suppressed [22]. In contrast, neither $p125^{FAK}$ tyrosine phosphorylation by GRP receptors in Swiss 3T3 cells [8] nor NMB-Rs in NMB-R-transfected cells or C-6 glioblastoma cells [51] is affected by changes in $[Ca^{2+}]_i$ or PKC inhibition. Moreover, paxillin phosphorylation in response to GRP receptor activation is not affected by complete inhibition of bombesin-induced mobilization of intracellular calcium or PKC activation [7]. These results demonstrated that with different receptors activation of the PLC pathways has a different importance in the ability of the agonist to cause tyrosine phosphorylation of $p125^{FAK}$ and paxillin. In the present study the calcium ionophore A23187 caused only a minimal stimulation of phosphorylation of $p125^{FAK}$ and paxillin, demonstrating that increases in $[Ca^{2+}]_i$ alone had a minimal effect in pancreatic acini. The fact that increases in $[Ca^{2+}]_i$ alone are not essential for the ability of CCK-8 to stimulate tyrosine phosphorylation of $p125^{FAK}$ and paxillin was shown directly by pretreatment with thapsigargin to deplete the intracellular calcium stores. Under these conditions CCK-8 caused no changes in $[Ca^{2+}]_i$; however, tyrosine phosphorylation of $p125^{FAK}$ and paxillin was not altered. In contrast to the minimal effect of increases in $[Ca^{2+}]_i$, PKC activation by phorbol esters caused a rapid increase in tyrosine phosphorylation of both of these proteins, and the magnitude of the increase was equal to that seen with CCK-8. CCK_A receptor activation has been recently shown to cause activation of PKC isoforms δ and ϵ in pancreatic acinar cells [54]; therefore these results raise the possibility that PKC could be important in mediating CCK-8's tyrosine phosphorylation of these two proteins. However, our results suggest that it is unlikely that PKC activation by CCK-8 alone is involved in stimulating tyrosine phosphorylation of these two proteins. This conclusion is strongly supported by the fact that pretreatment with the PKC inhibitor GF109203X, at a concentration that completely inhibited PMA stimulation of phosphorylation of these two proteins, had no effect on stimulation of tyrosine phosphorylation caused by a similarly efficacious concentration of CCK-8. These results suggest that, similar to changes in $[Ca^{2+}]_i$, PKC activation by CCK-8 alone is not important in mediating tyrosine phosphorylation of these two proteins by CCK-8.

It has been shown that simultaneous activation of the PKC pathway and increases in $[Ca^{2+}]_i$ can have synergistic effects on a number of cellular responses, such as phosphorylation of various proteins [3,55,56], secretion of aldosterone in adrenal cortex [57], amylase release from pancreatic acini [3,6] or pepsinogen release from chief cells [58]. In our study simultaneous stimulation of both the PKC pathway with PMA and increased $[Ca^{2+}]_i$ by the calcium ionophore A23187 caused a greater increase in $p125^{FAK}$ and paxillin phosphorylation than seen with either alone, and the combined increase resembled the maximal increase obtained after CCK-8 stimulation. Thus simultaneous PKC activation and intracellular calcium mobilization could be important for stimulation of $p125^{FAK}$ and paxillin phosphorylation by CCK-8 in rat pancreatic acinar cells. This conclusion was supported by the fact that the combination of GF109203X and thapsigargin, at a concentration that completely inhibited PMA-stimulated increases in tyrosine phosphorylation and CCK-8-stimulated increases in intracellular calcium respectively, inhibited CCK-8-stimulated phosphorylation of $p125^{FAK}$ and paxillin by 50%. These results demonstrate that CCK-8 stimulates tyrosine phosphorylation of $p125^{FAK}$ and paxillin by both calcium–PKC-dependent and -independent pathways (Figure 11). This result is

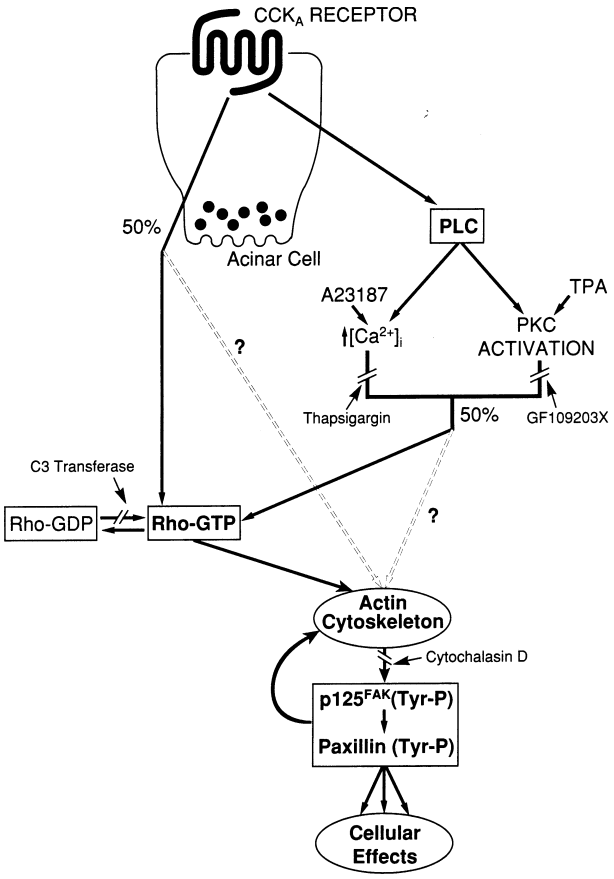

Figure 11 Putative signal-transduction pathways involved in the stimulation of $p125^{FAK}$ and paxillin tyrosine phosphorylation (Tyr-P) by CCK_A receptors activation

After binding of the ligand (CCK-8) to CCK_A receptors, activation of PLC occurs, stimulating the activation of PKC and increasing $[Ca^{2+}]_i$ in pancreatic acinar cells. Activation of both arms of the PLC cascade accounts for 50% of the increase in the tyrosine phosphorylation of $p125^{FAK}$ and paxillin by CCK-8. The remaining 50% of CCK stimulation of $p125^{FAK}$ and paxillin phosphorylation is independent of PKC activation and calcium mobilization. The site of action of C3 transferase by inactivating $p21^{rho}$ is shown. In the conditions used in our study C3 transferase treatment did not completely inhibit $p21^{rho}$; therefore, we cannot exclude other PLC-dependent and -independent pathways that do not involve $p21^{rho}$, and these possible pathways are indicated by question marks. The sites of action of the PKC inhibitor GF109203X the PKC activator PMA (TPA), the calcium ionophore A23187, cytochalasin D, which disrupts the actin cytoskeleton, and thapsigargin, which inactivates the endoplasmic reticulum Ca^{2+}-ATPase and depletes calcium from the intracellular stores, are shown. The evidence for the different pathways is summarized in the last paragraph of the Discussion section. Rho-GDP and Rho-GTP refer to the small G-protein $p21^{rho}$ in the inactive and active states respectively.

consistent with a recent study [22] examining thrombin-induced p125^{FAK} tyrosine phosphorylation in platelets. In that study the p125^{FAK} phosphorylation in response to thrombin was not impaired by BAPTA-AM, a chelator of intracellular calcium, and only partially inhibited by GF109203X, whereas the inhibition of both pathways completely inhibited thrombin-induced p125^{FAK} phosphorylation [22]. One could propose at least two possible explanations for the ability of inhibition of both limbs of the PLC cascade to cause partial inhibition of tyrosine phosphorylation, whereas inhibition of each alone has no effect. One possibility is that potentiation between PKC activation and changes in $[Ca^{2+}]_i$ could be so large that even small changes in either pathway, which are undetectable by the methods used, can cause a potentiated maximal response in either pathway. Therefore, even though each of the PKC and calcium pathways appears completely inhibited when a single inhibitor is present, small changes could possibly be occurring that potentiate the other cascade and result in a maximal response. We cannot exclude this possibility; however, this seems unlikely, particularly in the case of changes in $[Ca^{2+}]_i$, because the instrumentation used is sufficiently sensitive that even very small changes in $[Ca^{2+}]_i$ could be detected and yet were not seen when thapsigargin was used to deplete completely the intracellular pool. Another possibility is that the stimulation of either changes in $[Ca^{2+}]_i$ or of PKC is sufficient alone to give the 50% maximal stimulation that occurs due to activation of the PLC-dependent pathway by CCK.

Focal adhesions form a link between the cell cytoskeleton and the extracellular matrix, and from these structures emanate the actin stress fibers [16]. Recent studies show that tyrosine phosphorylation of p125^{FAK} and paxillin by growth factors, neuropeptides and bioactive lipids requires the integrity of the actin cytoskeleton [8,18–22]. Previous studies have demonstrated that activation of human CCK_B receptors expressed in mouse Swiss 3T3 and NIH 3T3 fibroblasts can cause rapid formation of actin stress fibers [59]. Moreover, in rat pancreatic acinar cells activation of CCK_A receptors with supramaximal concentrations of caerulein, a CCK-8 analogue, causes marked morphologic effect on cytoskeletal organization [60,61]. In the present study, in rat pancreatic acini we found that cytochalasin D, which selectively disrupts actin microfilaments [33], completely inhibited CCK-8- or PMA-stimulated p125^{FAK} and paxillin tyrosine phosphorylation. This effect appeared specific for inhibition of actin microfilaments, because colchicine, which disrupts microtubules [34,35], had no effect on CCK-8- or PMA-stimulated p125^{FAK} or paxillin tyrosine phosphorylation. These results demonstrate that, similar to a number of other neuropeptides and growth factors in different tissues, phosphorylation of p125^{FAK} and paxillin in rat pancreatic acini stimulated by CCK-8 or after PKC activation depends on the integrity of the actin cytoskeleton.

Recent studies suggest that tyrosine phosphorylation and some of the cytoskeletal changes induced by various neuropeptides and growth factors require the involvement of p21^{rho} [23,24,36–38], one of the Ras-related small G-proteins [62]. This conclusion is supported by the fact that the microinjection of p21^{rho} into fibroblasts induces tyrosine phosphorylation of p125^{FAK} and paxillin [63] and rapidly induces the formation of actin stress fibres and focal adhesions [37,64]. Furthermore, inhibition of p21^{rho} either by *C. botulinum* C3 transferase, dominant negative mutants or inhibitory fragments of p21^{rho} results in decreased tyrosine phosphorylation of p125^{FAK} and paxillin in some cells [23,24,65,66]. Consequently, we determined whether p21^{rho} activation was involved in CCK-8-stimulated tyrosine phosphorylation of p125^{FAK} and paxillin. To inactivate p21^{rho} we incubated the pancreatic acini with C3 transferase from *C. botulinum*, which has been shown to ADP ribosylate p21^{rho} on asparagine residue 41, which prevents the interaction of p21^{rho} with its downstream targets [39]. In our study, the C3 transferase markedly inhibited CCK-8-stimulated p125^{FAK} and paxillin tyrosine phosphorylation, providing evidence that p21^{rho} is involved in the ability of CCK_A receptor activation to stimulate tyrosine phosphorylation of p125^{FAK} and paxillin. In contrast, treatment with C3 transferase had no effect on the ability of CCK-8 to stimulate changes in phosphoinositides, demonstrating it was not having a general cell-inhibitory effect. Furthermore, previous studies have demonstrated that when C3 transferase causes ADP ribosylation of RhoA there is a diminution of the immmunodetectable amount of RhoA [42,43]. In our study the dose–response curve for the ability of C3 transferase to cause a decrease in immunoreactive RhoA was superimposable on that for its ability to cause a decrease in the ability of CCK-8 to cause tyrosine phosphorylation of p125^{FAK} and paxillin. Both of these latter results are consistent with the C3 transferase having a specific effect on RhoA in pancreatic acini. We found that C3 transferase inhibited the tyrosine phosphorylation of p125^{FAK} and paxillin by the calcium ionophore, A23187, by the PKC activator, PMA or by the combination of both. These results demonstrated that the participation of p21^{rho} is necessary both for CCK_A receptor-mediated p125^{FAK} and paxillin tyrosine phosphorylation and for stimulation of tyrosine phosphorylation of both of these proteins by a post-receptor mechanism. This result suggests p21^{rho} probably participates at a distal post-receptor site of action (Figure 11). These results demonstrate that p21^{rho} is necessary for tyrosine phosphorylation to occur by activation of the PLC-dependent pathway. At present it remains unclear whether p21^{rho} is important in the PLC-independent stimulation caused by CCK-8, because the C3 transferase under our conditions did not cause complete inhibition of CCK-8-stimulated p125^{FAK} and paxillin tyrosine phosphorylation.

In summary, in our study we found that CCK-8 causes a rapid, concentration-dependent tyrosine phosphorylation of p125^{FAK} and paxillin in pancreatic acinar cells. In Figure 11 we propose a model summarizing the likely signal-transduction pathways involved in the tyrosine phosphorylation of both proteins after CCK_A receptor activation, based on our results. The binding of CCK-8 to the CCK_A receptor causes activation of PLC, stimulates the activation of PKC, and causes mobilization of cellular calcium. The interaction between both arms of the PLC cascade mediates 50% of the increase in the phosphorylation of p125^{FAK} and paxillin after CCK_A receptor activation. This conclusion is based on the observation that thapsigargin and GF109203X together inhibit only 50% of the CCK-stimulated tyrosine phosphorylation of these two proteins. The remaining 50% of tyrosine phosphorylation of p125^{FAK} and paxillin stimulated by CCK_A receptor activation is dependent on PLC-independent mechanisms (Figure 11). Activation of the small G-protein p21^{rho} is involved in CCK-8-mediated phosphorylation, because pretreatment with C3 transferase, which ADP ribosylates and inactivates p21^{rho}, inhibits CCK-8-mediated tyrosine phosphorylation of these two proteins by 70%. The site of action of p21^{rho} is localized downstream of PLC, because pretreatment with C3 transferase had no effect on CCK-stimulated PLC activation. Activated p21^{rho} is probably involved in the PLC-dependent increase in the tyrosine phosphorylation of p125^{FAK} and paxillin, because the C3 transferase has an inhibitory effect on the tyrosine phosphorylation of p125^{FAK} and paxillin stimulated by the calcium ionophore A23187, by the PKC activator PMA, or by the combination of both. Activated p21^{rho} is also probably involved in the PLC-independent pathway, because we obtained greater than 50% inhibition of CCK's ability to cause

tyrosine phosphorylation after C3 transferase pretreatment. We can not exclude the possibility that other pathways may exist that do not involve activated $p21^{rho}$ (Figure 11, question marks), because in our experimental conditions C3 transferase treatment did not cause complete inhibition of $p21^{rho}$. We suggest that $p21^{rho}$'s action is upstream of the actin cytoskeleton, because studies in other cell systems [37,64] demonstrated that the microinjection of activated $p21^{rho}$ into fibroblasts rapidly induces the formation of actin stress fibres and focal adhesion. Our results suggest a central role for the actin cytoskeleton organization in the ability of CCK to cause $p125^{FAK}$ and paxillin tyrosine phosphorylation, because pretreatment with cytochalasin D, which disrupts actin microfilaments, causes a full inhibition of $p125^{FAK}$ tyrosine phosphorylation. Other studies [67] using tyrosine kinase inhibitors suggest that $p125^{FAK}$ tyrosine phosphorylation may also affect the actin cytoskeleton in addition to causing numerous cellular effects, such as on growth and cellular motility.

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