Bombesin stimulates cholecystokinin secretion through mitogen-activated protein-kinase-dependent and -independent mechanisms in the enteroendocrine STC-1 cell line

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Bombesin has been reported to stimulate cholecystokinin (CCK) secretion from rat duodeno-jejunal I-cells. Bombesin was shown to activate mitogen-activated protein kinases (MAPKs) in cell types such as Swiss 3T3 fibroblasts and rat pancreatic acinar cells. No information is available on whether MAPK is activated in intestinal endocrine cells upon bombesin stimulation. This was studied by using the CCK-producing enteroendocrine cell line STC-1. Bombesin stimulated markedly and transiently both $p42^{MAPK}$ and $p44^{MAPK}$, with a maximum at 2 min, and a decrease to basal levels within 10 min. As expected, bombesin stimulated MAPK kinase 1 (MEK-1) activity. Activation of protein kinase C (PKC) with PMA also stimulated $p42^{MAPK}$, $p44^{MAPK}$ and MEK-1. Treatment of cells with PD 098059 (at 10 μM or 30 μM), which selectively inhibits MEK phosphorylation, blocked bombesin-induced $p42^{MAPK}$ and $p44^{MAPK}$ activation for at least 90 min. However, PD 098059 inhibited bombesin- and PMA-stimulated CCK secretion during the first 15 min, but failed to significantly reduce CCK release at later times. Inhibition of PKC with staurosporine, or PKC down-regulation by prolonged treatment with PMA, both drastically decreased MEK-1, $p42^{MAPK}$ and $p44^{MAPK}$ activation upon bombesin stimulation. Additionally, PKC activation appeared to be required for both MAPK-dependent (early) and -independent (late) CCK responses to bombesin. It is concluded that the early CCK secretory response of STC-1 cells to bombesin involves MAPK pathway activation through a PKC-dependent mechanism, whereas the late phase of bombesin-induced CCK secretion, that also requires PKC, appears to result from a MAPK-independent process.

INTRODUCTION

Cholecystokinin (CCK), a polypeptide hormone produced by endocrine I-cells located in the upper small intestine, is involved in the regulation of gall bladder contraction, pancreatic secretion, gastric emptying, intestinal motility and satiety [1]. CCK release is modulated at the luminal side by nutrients [2] and releasing factors [3–6], while neuropeptides and hormones act at the basolateral side of I-cells [1]. The amphibian peptide bombesin, and its mammalian counterpart gastrin-releasing peptide (GRP), have been reported to stimulate potently CCK release [2,4,7–10].

Bombesin-related peptides interact with G-protein-coupled receptors, especially of the bombesin/GRP subtype, such as in Swiss 3T3 fibroblasts [11]. Receptor occupation is followed by activation of phospholipase C and generation of the two secondary messengers inositol-1,4,5-trisphosphate and diacylglycerol, which results in mobilization of intracellular Ca^{2+} and protein kinase C (PKC) activation [12]. Furthermore, bombesin receptor occupation can lead to activation of focal adhesion-associated proteins (FAK), c-Jun amino-terminal kinases (JNKs) and S6 kinases [13–16]. Recent studies indicate that bombesin activates the mitogen-activated protein kinase (MAPK) family of enzymes in various cells or tissues, e.g. in Swiss 3T3 fibroblasts [17–20], in Rat1 fibroblasts [21], in rat pancreatic acini [14,22] and in rabbit rectosigmoid smooth-muscle cells [23].

The MAPK family consists of highly conserved serine/threonine kinases, also known as extracellular signal-regulated kinases (ERKs), that are activated in response to a wide range of extracellular signals, including growth factors, hormones and neuropeptides (reviewed in [24–26]). These kinases transduce extracellular stimuli into intracellular signals that affect the expression of genes linked to cell proliferation and differentiation. The best characterized isoforms $p42^{MAPK}$ (ERK2) and $p44^{MAPK}$ (ERK1) are directly activated through phosphorylation on specific threonine and tyrosine residues by the dual specificity MAPK kinase (MEK), of which at least two isoforms have been identified in mammalian cells. MEK, in turn, is regulated by the Raf kinase proteins Raf-1 and/or B-Raf. Kinases of the Raf family can be activated through tyrosine kinase receptors and $p21^{ras}$-GTP accumulation, or through G-protein-linked receptors via PKC-dependent or -independent pathways.

Recently, isoforms of MEK and MAPK were detected in insulin-secreting cells, and their activation was shown to correlate with the secretory response to glucose, PMA and nerve growth factor [27]. Whether the MAPK pathway is activated in intestinal endocrine cells and contributes to hormone secretion has not yet been investigated. We report here that bombesin evokes a rapid and transient stimulation of MEK-1, $p42^{MAPK}$ and $p44^{MAPK}$ through PKC activation in the enteroendocrine CCK-secreting cell line STC-1. Using PD 098059, a selective inhibitor of MEK

Abbreviations used: CCK, cholecystokinin; GRP, gastrin-releasing peptide; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK or ERK kinase; PACAP, pituitary adenylate-cyclase-activating peptide 27; IBMX, 3-isobutyl-1-methylxanthine; PKC, protein kinase C; MBP, bovine brain myelin basic protein.

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activation, we show that the early phase of bombesin-induced CCK secretion requires MAPK cascade activation. Finally, we demonstrate that PKC activation is a critical event in the bombesin-induced secretion of CCK during both the early MAPK-dependent and the late MAPK-independent phases of CCK release.

MATERIALS AND METHODS

Materials

Rabbit polyclonal antisera to the 12-amino-acid C-termini of $p44^{MAPK}$ and $p42^{MAPK}$, and to the 17 N-terminal residues of MEK-1 (peptide synthesis by Neosystem, Strasbourg, France) respectively were generated as described previously [28]. Bombesin, pituitary adenylate-cyclase-activating peptide 27 (PACAP), PMA, 4-α-phorbol, staurosporine, BSA, Triton X-100, Protein A–Sepharose CL-4B, sodium orthovanadate, aprotinin, leupeptin, PMSF and bovine brain myelin basic protein (MBP) were from Sigma (Saint Quentin Fallavier, France). [γ-^{32}P]ATP was from Amersham (Les Ulis, France). Cell culture reagents were from Life Technologies (Cergy Pontoise, France). PD 098059 was a generous gift from Dr. A. R. Saltiel (Ann Arbor, MI, U.S.A.).

Cell culture

The STC-1 cell line (gift from Dr. A. Leiter, Boston, MA, U.S.A.) was derived from an intestinal endocrinal tumour that developed in a double-transgenic mouse expressing the rat insulin promoter linked to the simian virus 40 large T antigen and the polyoma virus small t antigen [29]. Cells were grown in RPMI-1640 medium supplemented with 5% (v/v) fetal calf serum, 2 mM glutamine and antibiotics (100 units/ml penicillin and 50 μM streptomycin) in a humidified CO_2:air 5%:95% incubator at 37 °C. Cells were plated at 0.3×10^6 cells/cm² (dishes of 2 cm² for CCK release, 4.5 cm² for MAPK determination and 10 cm² for MEK assay) and cultured for 24 h until 80% confluency was obtained.

Immunopurification of $p44^{MAPK}$ and MEK-1 from STC-1 cell extracts

STC-1 cells were first starved for 1.5 h in RPMI medium with 0.2% (w/v) BSA, then stimulated at 37 °C with the different effectors, as indicated. Experiments were stopped by quickly aspirating the medium and solubilizing cells for 15 min on ice in a buffer containing 1% (v/v) Triton X-100/50 mM Hepes (pH 7.5)/150 mM NaCl/10 mM $Na_4P_2O_7$/10 mM EDTA/2 mM sodium vanadate/100 mM NaF/100 units/ml aprotinin/20 μM leupeptin/0.2 mg/ml PMSF. Cell extracts were clarified by centrifugation for 15 min at 14000 ***g***, and then incubated for 2 h with either $p44^{MAPK}$ or MEK-1 antisera, both antisera being pre-adsorbed to Protein A–Sepharose beads. Following the immunoprecipitation period, the beads were washed twice with solubilization buffer. All manipulations were performed at 4 °C.

$p44^{MAPK}$ assay

Protein A–Sepharose beads with immunoprecipitated $p44^{MAPK}$ were washed twice with HNTG buffer (50 mM Hepes (pH 7.5)/150 mM NaCl/10% (v/v) glycerol/0.1% (v/v) Triton X-100/0.2 mM sodium vanadate), dehydrated using a syringe, and resuspended in 50 μl of HNTG buffer supplemented with 100 units/ml aprotinin, 20 μM leupeptin and 0.2 mg/ml PMSF. The kinase reaction was started by the addition of the following, given at their final concentrations: 150 μg/ml MBP (as a MAPK substrate), 10 mM magnesium acetate, 1 mM dithiothreitol and [γ-^{32}P]ATP (5 μM, 30 Ci/mmol). The phosphorylation reaction was allowed to proceed for 30 min at room temperature, and was stopped by spotting Whatman P-81 filter papers with an aliquot of the reaction mixture and dropping them into 0.1% (v/v) orthophosphoric acid. The papers were washed overnight in this solution with several replacements of bathing solution, washed once in ethanol and dried. Radioactivity associated with the papers was determined by Čerenkov counting. The reaction blank (a sample in which cell lysate had been omitted during immunoprecipitation, but which was otherwise treated like any other sample) was subtracted from all values.

$p42^{MAPK}$ mobility-shift assay and Western blotting

Activation of $p42^{MAPK}$ was determined by the appearance of a slower migrating form in gel electrophoresis owing to phosphorylation, which is known to occur on specific threonine and tyrosine residues within subdomain VIII. Serum-starved cultures of STC-1 cells were treated with factors as indicated, and cells were subsequently lysed in cold solubilization buffer. Cell extracts were clarified at 14000 ***g*** for 15 min at 4 °C, and aliquots of the supernatants (25 μg) were diluted in 4 × SDS/PAGE sample buffer (62 mM Tris/HCl/8% (w/v) SDS/40% (v/v) glycerol/20% (v/v) 2-mercaptoethanol/0.16% (w/v) Bromophenol Blue), boiled for 5 min and resolved by SDS/PAGE (7.5% gels). After SDS/PAGE, proteins were transferred to nitrocellulose membranes. Membranes were blocked using 5% (w/v) non-fat dried milk in Tris-buffered saline containing 0.2% Nonidet P-40 (LDB) and incubated with a rabbit polyclonal $p42^{MAPK}$ antiserum (1:1000) overnight at 4 °C. After 4 washes in LDB, membranes were incubated for 3 h at 4 °C with horseradish-peroxidase-conjugated goat anti-(rabbit IgG) (Nordic Immunological Laboratories, Tilburg, The Netherlands). Peroxidase activity was developed using the enhanced chemiluminescence method (ECL; Pierce, Rockford, IL, U.S.A.), after several rinsing cycles in Tris-buffered saline.

MEK-1 assay

The kinase activity of MEK-1 was measured in a reconstitution assay by the ability of purified MEK-1 to activate bacterially expressed recombinant rat glutathione S-transferase $p44^{MAPK}$ (a gift from Dr. J. Avruch, Boston, MA, U.S.A.), the activity of which was measured using MBP as substrate. Protein A–Sepharose beads with immunoprecipitated MEK-1 were washed twice with 50 mM Hepes, pH 7.4, dehydrated using a syringe, and resuspended in 50 μl of Hepes buffer, pH 7.4, containing recombinant $p44^{MAPK}$/0.2 mM sodium vanadate/100 units/ml aprotinin/20 μM leupeptin/0.2 mg/ml PMSF. The phosphorylation cascade *in vitro* was initiated by the addition of 50 μM [γ-^{32}P]ATP (30 Ci/mmol), 150 μg/ml MBP, 15 mM $MgCl_2$ and 1 mM EGTA, all values given at final concentrations. The phosphorylation reaction was allowed to proceed for 20 min at room temperature, and was stopped as described previously for the $p44^{MAPK}$ assay. Control experiments showed that omission of MBP or recombinant MAPK from the phosphorylation reaction mixture reduced radioactivity in the filter papers by 90%.

Measurement of cholecystokinin secretion

On the day of the experiment, culture medium was removed and dishes were incubated at 37 °C with Kreb's Ringer bicarbonate buffer [118 mM NaCl/20 mM Hepes/12 mM $NaHCO_3$/10 mM glucose/4.6 mM KCl/1 mM $MgCl_2$/0.5 mM $CaCl_2$/0.2% (w/v)

BSA, (pH 7.4)] with test agents, as indicated. The incubation was stopped by cooling the cell culture plates on ice. The medium was collected, and immediately centrifuged at 4 °C for 5 min at 100 *g* to remove any detached cells. Thereafter, the medium was frozen until the CCK assay was performed. For determination of CCK cell content, cells were homogenized in 2 ml extraction medium (2 M acetic acid/20 mM HCl). The homogenate was sonicated, boiled at 100 °C for 10 min, neutralized with ammonia, and stored at −20 °C. CCK immunoreactivity was measured with a previously described radio-immune assay [8,30] using antiserum 39A obtained from a New Zealand White rabbit after repeated injections of highly purified CCK-33 (a gift from Dr. V. Mutt, Karolinska Institute, Stockholm, Sweden) conjugated to BSA. Its reactivity was measured as 100 % for CCK-33 and sulphated CCK-8, 5 % for unsulphated CCK-8 and less than 0.1 % for unsulphated CCK-7-Gly. The detection limit and the ID_{50} (concentration required to give 50 % inhibition) were 0.5 pM and 4.0 pM respectively.

Statistical analysis

CCK concentrations, MEK activity and MAPK activity were analysed by one-way analysis of variance (ANOVA) followed by the *post hoc* comparison of Fisher. Differences between two means with a P value < 0.05 were regarded as significant. All values were expressed as means ± S.E.M.

RESULTS

Bombesin activates the MAPK pathway in STC-1 cells

To examine the effect of bombesin on the MAPK pathway, cell lysates from STC-1 cells treated with 100 nM bombesin for various times were analysed for $p44^{MAPK}$ and $p42^{MAPK}$ activation (Figure 1). $p44^{MAPK}$ was immunopurified from cell extracts, and its activity was measured *in vitro* using MBP as a substrate. Bombesin induced a rapid increase of $p44^{MAPK}$ activity in STC-1 cells, reaching a maximum at 2 min after stimulation (470 ± 67 % of basal levels, $P < 0.05$). The kinase activity swiftly declined to 27 ± 5 % over basal at 10 min, and remained near basal throughout the incubation period (Figure 1A). Stimulation of STC-1 cell $p44^{MAPK}$ activity by bombesin at 2 min was dose-dependent, the half-maximum and maximum concentrations being about 1 nM and 10 nM respectively (Figure 1B). $p42^{MAPK}$ activation was determined using the gel mobility-shift assay, by the appearance of a slower migratory form , which results from phosphorylation on specific threonine and tyrosine residues. Bombesin induced a rapid and transient stimulation of $p42^{MAPK}$, as judged by the mobility shift (Figure 1C). The time course of $p42^{MAPK}$ activation was similar to that of $p44^{MAPK}$, reaching a maximum at 2 min and declining thereafter. Phosphorylation of $p42^{MAPK}$ was first significant at 10 nM bombesin, and maximal from concentrations of bombesin of 100 nM and higher (Figure 1D). In addition, a significant increase in MEK-1 activity was observed after a 2 min incubation with 100 nM bombesin (334 ± 30 % of basal activity, $P < 0.05$). Overall, these data indicated that bombesin stimulated the MAPK cascade in the endocrine intestinal cell line STC-1.

PD 098059 inhibits the bombesin-induced MAPK activation in STC-1 cells

In order to explore further the MAPK pathway in the STC-1 cell CCK response, we tested the effects of the specific MEK inhibitor PD 098059 [31] on bombesin-dependent activation of $p44^{MAPK}$ and $p42^{MAPK}$ for a 2 min period (Figure 2). Pretreatment of cells with either 10 μM or 30 μM PD 098059 had no effect on $p44^{MAPK}$ and $p42^{MAPK}$ basal activities (Figures 2A and 2C). Pretreatment of cells with 10 μM PD 098059 for 30 min inhibited the peak $p44^{MAPK}$ activity by 71 %, following a 2 min incubation with bombesin (Figure 2A). Increasing the concentration to 30 μM did not markedly enhance the inhibitory effect of the compound on $p44^{MAPK}$ activation. Similar results were observed with the $p42^{MAPK}$ isoform, i.e. PD 098059 treatment of cells strongly reduced the $p42^{MAPK}$ phosphorylation induced by a 2 min incubation period in the presence of bombesin (Figure 2C). The duration of PD 098059 inhibitory effects on bombesin activation of $p44^{MAPK}$ and $p42^{MAPK}$ in STC-1 cells was assessed at regular intervals of PD 098059 pretreatment. The $p44^{MAPK}$ activity (Figure 2B) and the phosphorylation of $p42^{MAPK}$ (Figure 2D) in response to a 2 min stimulation by bombesin, applied after 30, 60 or 90 min incubation with 10 μM PD 098059, were both drastically lower than in the absence of inhibitor.

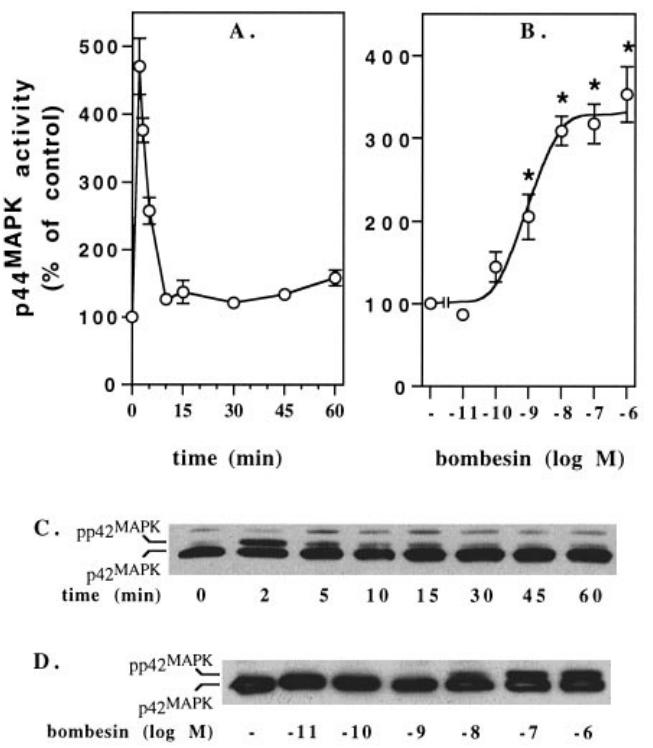

Figure 1 Effects of bombesin on $p44^{MAPK}$ activity (A,B) and on $p42^{MAPK}$ phosphorylation (C,D) in STC-1 cells

STC-1 cells were treated at 37 °C for serial times with 100 nM bombesin (**A**,**C**) or for 2 min at 37 °C with increasing concentrations of bombesin (**B**,**D**). (**A**) and (**B**) The kinase activity of $p44^{MAPK}$, immunopurified from the cell extracts, was measured *in vitro* using MBP as a substrate, as described in the Materials and Methods section. Data are expressed as percentages of control activity in untreated cells (659 ± 53 c.p.m./10^6 cells) and represent the means ± S.E.M. of three separate experiments, each performed in triplicate. *, $P < 0.05$ compared with basal activity. (**C**) and (**D**) Cell extracts were analysed by Western blotting with anti-$p42^{MAPK}$ polyclonal antibody. The positions of non-phosphorylated $p42^{MAPK}$ and the slower migrating phosphorylated form, $pp42^{MAPK}$, are indicated (representative of three independent experiments).

Bombesin stimulates CCK secretion through MAPK-dependent and -independent mechanisms in STC-1 cells

To determine whether MAPK pathway activation is required for bombesin-stimulated CCK release, cells were treated in the presence or absence of 10 μM PD 098059 during 30 min before

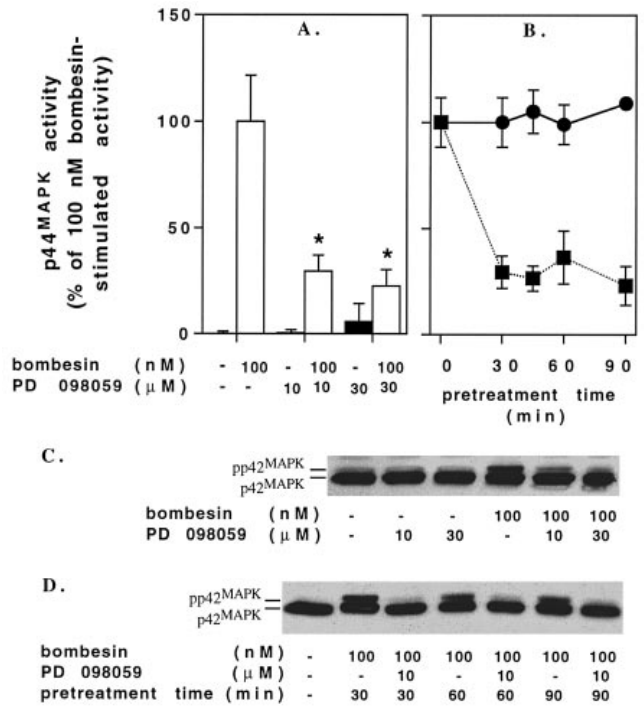

Figure 2 Effects of PD 098059 on bombesin-stimulated p44^{MAPK} activity (A,B) and p42^{MAPK} phosphorylation (C,D) in STC-1 cells

(**A**) and (**C**) STC-1 cells were preincubated for 30 min with 10 μM and 30 μM PD 098059 or vehicle (DMSO), followed by a 2 min exposure to 100 nM bombesin. (**B**) and (**D**) STC-1 cells were pretreated with (■) or without (●) 10 μM PD 098059, for the indicated periods of time. At each of these preincubation times, bombesin (100 nM) was added for 2 min before MAPK determination. (**A**) and (**B**) Cell lysates were immunoprecipitated with anti-p44^{MAPK} antiserum, and the phosphorylation of MBP was assayed. Results were expressed as percentages of bombesin-stimulated p44^{MAPK} activation without PD 098059, which represented 456 $\pm$ 77 % of the control. Data are the means $\pm$ S.E.M. of three experiments, each performed in triplicate. *, $P < 0.05$ compared with bombesin-stimulated enzyme activity without inhibitor. (**C**) and (**D**) Cell extracts were analysed by Western blotting with anti-p42^{MAPK} polyclonal antibody. The positions of non-phosphorylated p42^{MAPK}, and the slower migrating phosphorylated form, pp42^{MAPK}, are indicated (representative of three independent experiments).

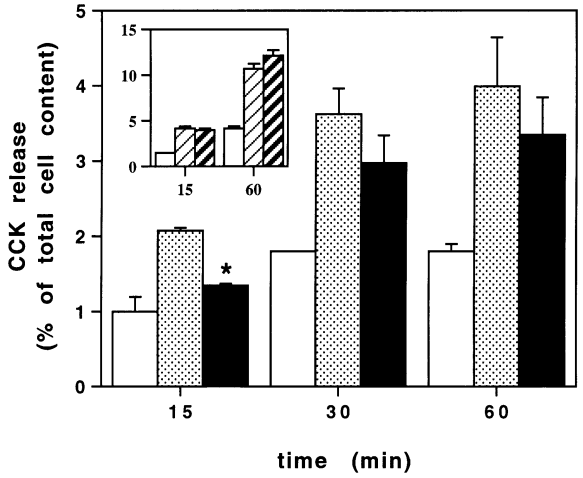

Figure 3 Effects of PD 098059 on bombesin-induced CCK release in STC-1 cells.

STC-1 cells were pretreated for 30 min with 10 μM PD 098059 (solid bars) or DMSO (open and stippled bars) before the addition of bombesin, and during the bombesin incubation period. Cells were stimulated with 100 nM bombesin (stippled and solid bars) or vehicle (open bars) for 15, 30 or 60 min. CCK concentrations were determined in both medium and cells by radioimmune assay. Total CCK cell contents after DMSO- and PD 098059-pretreatments were 2875 $\pm$ 130 and 3063 $\pm$ 392 fmol/10^6 cells respectively. Values of basal release at 15, 30 and 60 min were 30 $\pm$ 5, 51 $\pm$ 1 and 54 $\pm$ 3 fmol/10^6 cells respectively. The inset shows parallel experiments performed with a 100 nM PACAP/1 mM IBMX mixture (hatched bars) versus control vehicle (open bars), following a 30 min pretreatment of the cells with 10 μM PD 098059 (thick stripes) or DMSO (thin stripes). Results (means $\pm$ S.E.M., $n = 3$) were expressed as percentages of total CCK cell content [(peptide released in medium $\times$ 100)/(peptide released in medium + cell content at the end of the incubation)]. *, $P < 0.05$ compared with bombesin-stimulated CCK secretion without PD 098059 pretreatment.

the addition of 100 nM bombesin (Figure 3). CCK was then measured in the medium 15, 30 and 60 min later. PD 098059 alone modified significantly neither the total CCK cell content (3063 $\pm$ 392 versus 2875 $\pm$ 130 fmol/10^6 cells in untreated cells), nor the basal CCK secretion at 15 min (1.0 $\pm$ 0.3 % versus 1.0 $\pm$ 0.2 % of total CCK cell content without inhibitor), 30 min (1.7 $\pm$ 0.2 % versus 1.8 $\pm$ 0.1 % of total CCK cell content) or 60 min (1.9 $\pm$ 0.2 % versus 1.8 $\pm$ 0.1 % of total CCK cell content). Treating cells with PD 098059 significantly decreased the bombesin-stimulated CCK secretion at 15 min (1.4 $\pm$ 0.1 % versus 2.1 $\pm$ 0.1 % of the total cell content in the absence of inhibitor, $P < 0.05$), but did not significantly modify the amount of CCK released at 30 and 60 min post-bombesin addition (3.0 $\pm$ 0.4 % versus 3.6 $\pm$ 0.3 % and 3.3 $\pm$ 0.5 % versus 4.0 $\pm$ 0.7 % of the total cell content respectively). PACAP and 3-isobutyl-1-methylxanthine (IBMX), that have been shown to increase cAMP levels in STC-1 cells [32], induced CCK release (Figure 3, inset, and [32]). However, CCK secretion induced by either a 15 min or 60 min incubation in the presence of a 100 nM PACAP/1 mM IBMX mixture, which failed to activate p42^{MAPK} and p44^{MAPK} (results not shown), was not affected by PD 098059 pretreatment of the cells (Figure 3, inset). Overall, these results suggest that the MAPK pathway is involved specifically in the early phase of bombesin-induced CCK secretion.

PKC is involved during MAPK-dependent and -independent CCK secretion induced by bombesin in STC-1 cells

Because PKC activation has been shown to be linked to bombesin-induced CCK secretion [32], we examined whether the bombesin-induced, MAPK-dependent and -independent CCK secretion could be mediated by PKC activation. For these experiments, STC-1 cells were preincubated for 30 min with 1 μM staurosporine, or exposed to 100 nM PMA for 48 h. CCK release was determined after 15 min and 60 min incubation with bombesin. Preincubation for 30 min with staurosporine alone did not significantly modify either total CCK cell content (2622 $\pm$ 185 versus 2745 $\pm$ 212 fmol/10^6 cells in untreated cells) or basal CCK secretion at 15 min (2.0 $\pm$ 0.2 % versus 2.0 $\pm$ 0.3 % of total CCK cell content without inhibitor) and 60 min (5.4 $\pm$ 0.3 % versus 5.5 $\pm$ 0.6 % of total CCK cell content). Similarly, PMA pretreatment for 48 h did not alter basal CCK secretion (Figure 4, right panel) or total CCK cell content (2613 $\pm$ 237 versus 2069 $\pm$ 123 fmol/10^6 cells without PMA pretreatment). In contrast, staurosporine and PKC down-regulation both inhibited the CCK response to 100 nM PMA at 15 min (2.1 $\pm$ 0.2 % and 2.1 $\pm$ 0.1 % versus 4.5 $\pm$ 0.7 % and 3.3 $\pm$ 0.2 % of total CCK cell content in untreated cells respectively) and at 60 min (6.8 $\pm$ 0.5 % and 3.9 $\pm$ 0.1 % versus 14.7 $\pm$ 1.7 % and 12.8 $\pm$ 1.3 % of total CCK cell content in untreated cells respectively).

CCK release induced by 15 min stimulation with bombesin was significantly decreased by 30 min pretreatment with 1 μM staurosporine (Figure 4, left; 2.5 $\pm$ 0.3 % versus 3.9 $\pm$ 0.5 % of

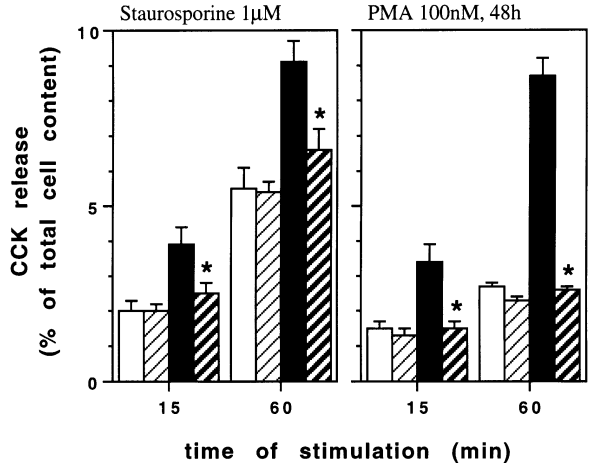

Figure 4 Effect of staurosporine, and PKC desensitization by PMA, on CCK secretion induced by bombesin in STC-1 cells

STC-1 cells were pretreated for 30 min with 1 μM staurosporine (left panel) or for 48 h with 100 nM PMA (right panel). STC-1 cells were then exposed to 100 nM bombesin for 15 or 60 min. CCK concentrations were determined in medium and cells by radioimmune assay. The left panel shows total CCK cell contents, after pretreatment with DMSO and staurosporine, were 2745 ± 212 and 2622 ± 185 fmol/10^6 cells respectively; values of basal release in untreated cells were 33 ± 7 and 96 ± 13 fmol/10^6 cells at 15 and 60 min respectively. The right panel shows total CCK cell contents with or without PMA treatment were 2609 ± 123 and 2613 ± 237 fmol/10^6 cells respectively; basal secretions represented 39 ± 5 and 70 ± 3 fmol/10^6 cells in untreated cells at 15 and 60 min respectively. Data are the means $\pm$ S.E.M. of three experiments, each performed in quadruplicate. In both panels, the symbols used correspond to the following: no stimulant (open bars); 100 nM bombesin (closed bars); and pretreated cells either without stimulant (hatched bars, thin stripes) or with 100 nM bombesin (hatched bars, thick stripes); *, $P < 0.05$ compared with the corresponding bombesin-stimulated CCK secretion without cell pretreatment.

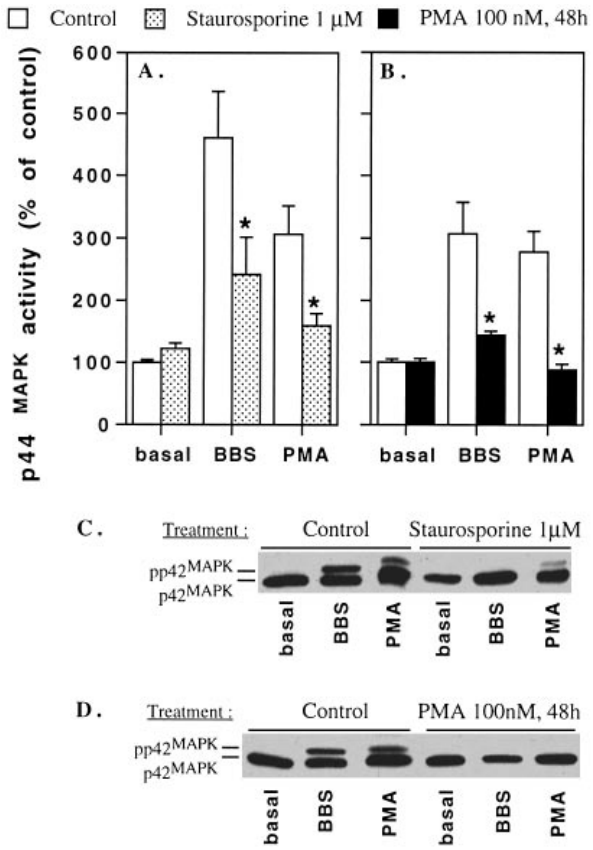

Figure 5 Effects of staurosporine, and PKC desensitization by PMA, on $p44^{MAPK}$ (A,B) and $p42^{MAPK}$ (C,D) activation by bombesin and PMA in STC-1 cells

(**A**) and (**C**) STC-1 cells were preincubated with 1 μM staurosporine (stippled bars) or with the control vehicle DMSO (open bars) for 30 min. (**B**) and (**D**) STC-1 cells were pretreated with 100 nM PMA (solid bars) or with the control vehicle DMSO (open bars) for 48 h. In all panels, cells were then stimulated with 100 nM bombesin (BBS) for 2 min or with 100 nM PMA for 15 min. (**A**) and (**B**)The kinase activity of $p44^{MAPK}$, immunopurified from the cell extracts, was measured *in vitro* using MBP as a substrate, and was expressed as percentages of basal activity in untreated cells, which represented 399 ± 15 and 270 ± 14 c.p.m./10^6 cells for staurosporine and PMA experiments, respectively. Data are the means $\pm$ S.E.M. of three separate experiments, each performed in triplicate. *, $P < 0.05$ compared with the corresponding bombesin- or PMA-stimulated $p44^{MAPK}$ activity without cell pretreatment. (**C**) and (**D**) Cell extracts were analysed by Western blotting with anti-$p42^{MAPK}$ polyclonal antibody. The positions of non-phosphorylated $p42^{MAPK}$ and the slower migrating phosphorylated form $pp42^{MAPK}$ are indicated (representative of three independent experiments).

total cell content in the absence of the inhibitor, $P < 0.05$). Similarly, prolonged exposure of cells to 100 nM PMA reduced by 90 % the bombesin-stimulated CCK release at 15 min (Figure 4, right).

In contrast to the effects of PD 098059, CCK secretion induced by a 60 min bombesin stimulation was inhibited by STC-1 cell exposure to 1 μM staurosporine (Figure 4, left; 6.6 ± 0.6 % versus 9.1 ± 0.6 % of total cell content in the absence of the inhibitor, $P < 0.05$). Prolonged exposure of cells to 100 nM PMA reduced by 95 % the CCK release induced by a 60 min bombesin-incubation period (Figure 4, right). Overall, these results indicate that both the early and late phases of the CCK response involve a phorbol-ester-sensitive PKC-dependent pathway.

PKC activation leads to MAPK cascade stimulation

Because CCK release is both MAPK- and PKC-dependent during the early phase of secretion, we examined whether PKC activation could induce MAPK pathway activation. The PKC activator PMA enhanced both $p44^{MAPK}$ activity and $p42^{MAPK}$ phosphorylation (Figure 5), whereas the inactive phorbol ester 4-α-phorbol was ineffective (results not shown). In contrast to the effect of bombesin, the response reached a maximum after 15 min (593 ± 36 % of $p44^{MAPK}$ basal activity) and was sustained for at least 1 h (318 ± 4 % of $p44^{MAPK}$ basal activity). Additionally, exposure of STC-1 cells to 100 nM PMA increased MEK-1 activity (312 ± 23 % of MEK-1 basal activity after 15 min).

To gain a further insight into the relationship between PKC activation and the MAPK pathway, we used the specific inhibitor of MEK-1, PD 098059. This inhibitor prevented $p42^{MAPK}$ phosphorylation and $p44^{MAPK}$ activation by PMA (results not shown). In addition, MAPK pathway inhibition by PD 098059 pretreatment decreased the PMA-induced CCK secretion at 15 min (1.4 ± 0.2 % versus 2.1 ± 0.3 % of the total cell content in the absence of inhibitor, $P < 0.05$), but did not significantly alter it at 60 min (5.1 ± 0.5 % versus 6.1 ± 0.4 % of the total cell content without inhibitor). Thus, MAPK pathway is involved during the early phase of CCK secretion induced by PKC activation only.

Bombesin-induced MAPK pathway stimulation requires PKC activation

Because MAPK pathway activation by bombesin has been reported in various cell systems to occur through either PKC-dependent [17,20] or -independent [21] pathways, we examined

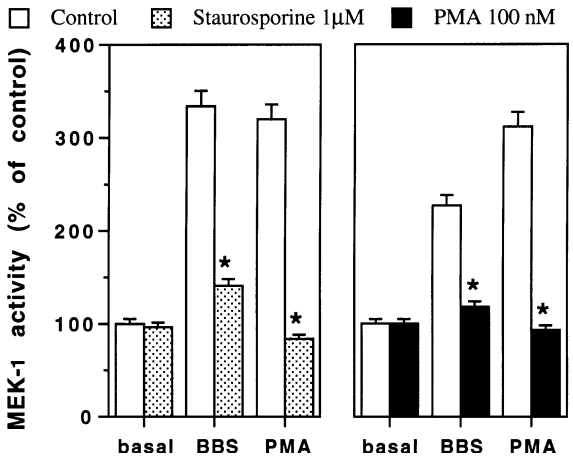

Figure 6 Effects of staurosporine, and PKC desensitization by PMA, on MEK-1 activity induced by bombesin and PMA in STC-1 cells

On the left, STC-1 cells were preincubated with 1 μM staurosporine (stippled bars) or with the control vehicle DMSO (open bars) for 30 min. On the right, STC-1 cells were pretreated with 100 nM PMA (solid bars) or with the control vehicle DMSO (open bars) for 48 h. In both panels, cells were then stimulated with 100 nM bombesin (BBS) for 2 min, or with 100 nM PMA for 15 min, and MEK-1 was immunoprecipitated from the solubilized cell extracts. The activity of the immunopurified MEK-1 was measured in a reconstitution assay by its ability to activate recombinant $p44^{MAPK}$, the activity of which was measured using MBP as a substrate. The activity of MEK-1 was expressed as a percentage of basal activity without staurosporine (274 ± 28 c.p.m./10^6 cells, left) or PMA (198 ± 20 c.p.m./10^6 cells, right) pretreatments. Data are the means $\pm$ S.E.M. of two experiments, each performed in triplicate. *, $P < 0.05$ compared with the corresponding bombesin- or PMA-stimulated MEK-1 activity without cell pretreatment.

whether PKC activation is required for MAPK pathway stimulation in response to bombesin in STC-1 cells. Pretreatment of STC-1 cells with 1 μM staurosporine for 30 min, or with 100 nM PMA for 48 h, prevented the stimulatory effect of PMA on $p44^{MAPK}$ (Figures 5A and 5B), $p42^{MAPK}$ (Figures 5C and 5D) and MEK-1 (Figure 6). With the same experimental conditions, bombesin-induced $p44^{MAPK}$, $p42^{MAPK}$ and MEK-1 activation were strongly decreased in comparison with control experiments without pretreatment (Figures 5 and 6). Together, these data suggest that bombesin stimulates $p42^{MAPK}$ and $p44^{MAPK}$ by activating MEK-1 through a PKC-dependent mechanism.

DISCUSSION

The present study was aimed at the signalling pathway that links bombesin-receptor occupation to CCK secretion in the enteroendocrine cell line STC-1. Bombesin induced MEK-1 activation, and a rapid and transient activation of $p42^{MAPK}$ and $p44^{MAPK}$, through a pathway requiring phorbol-ester-sensitive PKC isoform(s). The specific MEK inhibitor PD 098059 abolished the early phase of the CCK response to bombesin and PMA, providing the first evidence for a significant role of the MAPK pathway in the stimulated secretion of an intestinal hormone. In contrast, PD 098059 failed to modify significantly CCK release at later times, in spite of its sustained ability to suppress MAPK activation. Interestingly, stimulation of CCK secretion by bombesin was PKC-dependent during both the early and the late phases of CCK response.

The level of MAPK activation could be of importance for cell proliferation. It has been proposed that mitogenic agents induce persistent stimulation of the MAPK cascade [33]. Bombesin indeed is a potent stimulant of DNA synthesis and cell proliferation, and induces a marked and sustained increase in $p42^{MAPK}$ activity in Swiss 3T3 fibroblasts [19,20]. Using the MEK inhibitor PD 098059 [31,34] or interfering mutants of MEK-1 stably transfected into Swiss 3T3 cells, it was recently shown that abolishing the MAPK pathway prevented both bombesin-induced DNA synthesis and the transition of quiescent cells to the S-phase of the cell cycle [20]. The effect reported here with STC-1 cells was a transient increase in $p44^{MAPK}$ and $p42^{MAPK}$ activities. It is noteworthy that bombesin in our hands failed to increase DNA synthesis in STC-1 cells (Némoz-Gaillard, E., Chevrier, A. M., Bernard, C. and Abello, J., unpublished results). These results are therefore consistent with the hypothesis that the time-restricted MAPK response to bombesin in STC-1 cells would be sufficient for hormone release, but not for cell proliferation.

Previous observations indicated that PKC is involved in MAPK activation and DNA synthesis stimulation by bombesin [17,20,35]. However, a novel PKC-independent pathway for MAPK activation upon bombesin stimulation was recently observed in Rat1 fibroblasts [21]. In STC-1 cells, inhibition of PKC with staurosporine, or desensitization by prolonged exposure to PMA, decreased bombesin-stimulated MEK-1 and MAPK activities. Thus our results demonstrate that phorbol-ester-sensitive PKC activation is a critical event of the bombesin-induced signalling pathway leading to MAPK activation in STC-1 cells. In the AR4-2J pancreatic acinar cell line, PKC activation by PMA induces tyrosine phosphorylation of SHC, that subsequently binds to Grb2 and the guanine nucleotide-exchange factor Sos, leading to $p44^{MAPK}$ activation [36]. It was shown in other systems that PKC directly phosphorylates and activates Raf proteins (namely Raf-1 and B-Raf) both *in vivo* and *in vitro* [37–39]. Additional investigations are needed to define the molecular and functional links between PKC and MEK in STC-1 cells.

The downstream events which follow a transient MAPK activation and lead to CCK secretion in intestinal-type endocrine cells are currently unknown. It was shown recently that synapsin I, a protein involved in the regulation of neurotransmitter release, could be phosphorylated by MAPK in neuronal preparations [40,41]. Because endocrine cells and neurons share several basic mechanisms for hormone and neurotransmitter release, it is possible that activation of the MAPK cascade could affect some components of the secretory machinery. In contrast, our experiments showed that CCK secretion could also occur independently of MAPK activation, even though the mechanism required phorbol-ester-sensitive PKC isoform(s). Further studies are necessary to delineate the impact of MAPK and PKC activation respectively on exocytosis.

In conclusion, the results presented here indicate that the MAPK pathway is involved in the early CCK response of the enteroendocrine cell line STC-1 to bombesin via a PKC-dependent pathway. In addition, PKC also appears to be required for the MAPK-independent (late) CCK secretion. Both the cytoplasmic events that are triggered by MAPK activation and the PKC-dependent mechanisms that underlie the MAPK-independent (late) phase of bombesin-stimulated CCK release remain to be investigated.

We thank Dr. A. Saltiel (Parke Davis, Ann Arbor, MI, U.S.A.) for providing PD 098059. We also thank V. Baron, A. M. Chevrier, P. Gual and I. Mothe for helpful discussions at various stages of this work.

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Received 15 October 1997/5 January 1998; accepted 9 January 1998