Co-requirement of Cyclic AMP- and Calcium-dependent Protein Kinases for Transcriptional Activation of Cholecystokinin Gene by Protein Hydrolysates*

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Little is known about the mechanisms by which protein-derived nutrients regulate hormone gene expression in the intestine. We have previously reported that protein hydrolysates (i.e. peptones), which are representative of the protein fraction in the lumen, increased cholecystokinin (CCK) gene transcription in the STC-1 enteroendocrine cell line. In the present work, we examined the intracellular events evoked by peptones to stimulate CCK gene transcription. In STC-1 cells, peptones stimulated cyclic AMP production and protein kinase A (PKA) activity. This was associated with a nuclear translocation of the PKA catalytic subunit and with a PKA-dependent phosphorylation of the CRE-binding protein (CREB) at Ser133. Using transient transfection experiments and reporter luciferase assays, we show that peptone-stimulated transcriptional activity of the CCK gene promoter was significantly decreased when the PKA pathway was inhibited. Furthermore, the intracellular calcium chelator 1,2-bis-(O-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-tetra(ace-toxymethyl)ester completely inhibited peptone-induced stimulation of the CCK gene promoter activity, phosphorylation of CREB, and PKA activity. Peptones increased, in a calcium-dependent manner, the phosphorylation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and the MEK inhibitor PD98059 decreased the peptone-induced stimulation of the CCK gene promoter activity. This stimulation was also reduced by 30% in the presence of the calcium/calmodulin-dependent protein kinase (CaMK) inhibitor KN-93. Total inhibition was obtained when the PKA, ERK, and CaMK pathways were simultaneously blocked with appropriate inhibitors to these pathways. These results demonstrate the simultaneous involvement of cAMP- and calcium-dependent protein kinases in the stimulation of intestinal CCK gene transcription by protein-derived nutrients.

Among the numerous extracellular factors that regulate gene expression, dietary compounds have received limited concern. Intestinal genes, expressed by epithelial cells of which the apical pole is in direct contact with the luminal contents, are potential targets for a nutritional control of transcription. Thus, expression of hormone genes such as those of glucose-dependent insulinotropic peptide, proglucagon, and cholecystokinin (CCK)1 has been shown to be up-regulated in the intestine of rodents by glucose, dietary fibers, or protein hydrolysates (1–3). CCK, produced by intestinal endocrine I-cells regulates several key digestive functions such as gallbladder contraction or pancreatic enzyme secretion (4). Food intake is the most potent stimulant of CCK release and there is a correlation between diet-induced CCK gene expression and peptide secretion in rat (5) and man (6). Nevertheless, these studies were based on in vivo dietary manipulations and, for that reason, did not permit identification of the molecular and cellular mechanisms involved.

Few data are available on the signaling pathways connecting dietary changes to alterations in gene expression. Most of the current knowledge concerns the effects of fatty acids and glucose in the transcriptional control of genes encoding proteins that play significant roles in lipid and glucose transport or metabolism in hepatocytes, adipocytes, or pancreatic beta cells (7, 8). The mechanisms involved were elucidated in some cases, and DNA response elements, binding transcription factors, and transduction cascades were identified. For instance, a large part of the transcriptional effects of fatty acid can be assigned to the direct activation of peroxisome proliferator-activated receptors (9). Alternatively, protein kinase C (PKC) and calcium are involved in the transcriptional induction of early response genes by palmitate and oleate in pancreatic INS-1 cells (10). The transcriptional effect of glucose has been shown to be mediated by an AMP-activated protein kinase pathway to modulate the L-type pyruvate kinase gene (11) or by a phosphatidylinositol 3-kinase (PI3K)/p38 MAPK pathway to stimulate the insulin gene (12). Much less is known about the effect of protein metabolites, and one of the few detailed studies in this field demonstrated that leucine starvation induced the stimulation of the C/EBP homologous protein promoter activity in cells of different origins. This occurred through an amino acid response element that bound the activating transcription factor 2 (13).

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1 The abbreviations used are: CCK, cholecystokinin; PepRE, peptone-response element; CREB, cAMP response element-binding protein; PKA, cAMP-dependent protein kinase; PKAα, PKA catalytic subunit; PKAβ, PKA regulatory subunit; ERK, extracellular signal-regulated protein kinase; MAPK, mitogen-activated protein kinase; MEK, MAP/ERK kinase; CaMK, calcium/calmodulin-dependent protein kinase; IBMX, isobutylmethylxanthine; BAPTA-AM, 1,2-bis-(O-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-tetra(ace-toxymethyl)ester.
the intestinal luminal dietary protein content (14), stimulated CCK gene transcription in the STC-1 enteroendocrine cell line, whereas intact proteins or free amino acids had no effect (15). The in vitro model allowed us to identify, in the CCK gene promoter, a DNA cis-element that was required for the transcriptional response to protein hydrolysates (16). This PepRE sequence binds transcription factors of the CREB family which are phosphorylated after cell treatment with protein hydrolysates. Nevertheless, the intracellular signaling involved was unknown and remained to be elucidated.

In the present work, we investigated the transcription mechanisms leading to the peptone-induced increase of CCK gene transcription in the intestinal endocrine STC-1 cells. We demonstrate that the transcriptional effect of protein hydrolysates is mediated by the concomitant participation of cAMP-dependent protein kinase (PKA), extracellular signal-regulated protein kinase (ERK), and calcium/calmodulin-dependent protein kinase (CaMK) pathways, and that intracellular calcium plays an essential role in this regulation.

EXPERIMENTAL PROCEDURES

Materials—Forskolin, 3-isobutyl-1-methylxanthine (IBMX), peptides (enzymatic hydrolysat from meat, type I), and the Escort lipofection reagent were purchased from Sigma (Saint Quentin Fallavier, France). The peptide molecular weight distribution of peptides was previously determined and showed a majority (75%) of peptides with molecular size comprised between 120 and 1,200 Da and 5% of peptides with molecular size over 5,000 Da; the rest (20%) were free amino acids (14). Cell culture reagents were from Invitrogen (Cergy Pontoise, France). PKA inhibitor H-89 (N-(2-[(2-hydroxyethyl)-N,N,N,N-tetraacetic acid (acetoxyethyl)ester], MAP/ERK kinase (MEK) inhibitor PD98059 (2-amino-3-methylxavfnone), and calcium/calmodulin-dependent protein kinase (CaMK) inhibitor KN-93 (2-N-[2-hydroxyethyl]-N-[4-methylbenzenesulfonyl]amino-N-(4-chloromethyl)-N-(methylbenzylammonium)) were from Calbiochem (Meudon, France). The mouse monoclonal anti-human PKA, antibody was obtained from Transduction Laboratories (BD PharMingen, Le Pont de Claix, France). Phosphorylation-specific and phosphorylation state-independent rabbit polyclonal p42/p44 MAPK (ERK1/2) antibodies were from Cell Signaling (Ozyme, Saint Quentin-en-Yvelines, France). Phosphorylation-specific and phosphorylation state-independent rabbit polyclonal CREB antibodies were from Cell Signaling and Santa Cruz Biotechnology (Tebu, Le Perray-en-Yvelines, France), respectively.

Plasmid Constructions—CCK gene promoter constructs have been previously described (16). Briefly, a CCK gene promoter fragment containing 700 bp of CCK gene was amplified by PCR from STC-1 cells genomic DNA using the following primers: 765 bp of the CCK gene promoter fragment was cloned into pGL3basic vector (Promega, Charbonnieres, France) upstream of the firefly luciferase reporter gene. The pGL3b/CCK-765 was deleted in 5′ of the CCK gene promoter fragment to generate pGL3b/CCK-93 and -70 constructs. Six bases of the PepRE site were mutated in pGL3b/CCK-93 to generate the -93M4 construct. The expression plasmid MT-REV(AB) was encoding a dominant negative form of the PKA regulatory subunit mutated in the cAMP-binding domains (18), was a kind gift of Dr. G. S. McKnight (Seattle, WA).

Cell Line, Culture Conditions, and Transient Transfections Experiments—STC-1 cells (gift from Dr. A. Leiter, Boston, MA) were derived from an intestinal endocrine tumor developed in a double transgenic mouse expressing the simian virus 40 large T antigen and the polyoma virus small t antigen under the control of the rat insulin promoter (19). Cells were grown in RPMI 1640 medium supplemented with 5% (v/v) fetal calf serum, 2 mM glutamine and antibiotics (100 IU penicillin/ml and 50 μg streptomycin) in a humidified CO2:air (5:95%) incubator at 37 °C. Transfection experiments were performed using the Escort lipofection reagent, according to the manufacturer’s instructions. Briefly, 1 h before transfection, STC-1 cells were seeded into 24-well plates at a density of 50,000 cells/well. For each well, 250 ng of CCK reporter plasmid was mixed with 0.5 μl of Escort reagent in 100 μl of serum-free RPMI medium. In all transfection experiments, a plasmid with a renilla reporter gene under the control of a thymidine kinase promoter (pRL-TK, 12.5 ng/well, Promega) was used as an internal control. In cotransfection experiments with MT-REV(AB) plasmid, the empty vector pUC13 (DSMZ, Braunschweig, Germany) was added to each set of transfections to ensure that each well received the same amount of DNA, and the DNA:Escort/plasmid DNA mixture for 6 h at 37 °C, then replaced in fresh complete medium for an additional 24-h period before treatment. RPMI complete medium was then replaced with RPMI without fetal calf serum but containing 0.2% bovine serum albumin in the presence or absence of the tested agents. After a 16-h incubation at 37 °C, cells were harvested in lysis buffer, and luciferase and renilla activities were measured using the Dual Luciferase Reporter assay system (Promega) in accordance with the manufacturer’s instructions.

Whole Cell and Nuclear Extract Preparations—Subconfluent (80%) STC-1 cells in 6-well plates were serum-deprived for 24 h, and washed once with serum-free RPMI medium before stimulation with various agents for the indicated times. Cells were lysed in cold solubilization buffer A containing 1% Triton X-100, 50 mM Hepes, 150 mM NaCl, 2 mM Na2VO4, 100 mM NaF, 100 IU aprotinin/ml, 20 μg leupeptin, and 0.2 mg/ml phenylmethanesulfonyl fluoride, pH 7.5. Cell extracts were then clarified (14,000 × g, 15 min, 4 °C). For nuclear extract preparation, serum-deprived STC-1 cells in 100-mm dishes were incubated at 37 °C with the tested agents for various times. Cells were washed with cold phosphate-buffered saline, and incubated in buffer B (10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2.5 mM dithiothreitol, 0.2 mg/ml phenylmethanesulfonyl fluoride, 0.5 mM leupeptin, 0.5 mM aprotinin, 20 μg/ml sodium deoxycholate, 0.5% Triton X-100, pH 7.9) for 15 min on ice before lysis with 0.6% Nonidet P-40. After centrifugation (700 × g, 5 min, 4 °C), supernatants were removed and nuclear proteins were extracted from the pellets by continuous shaking for 30 min in buffer C (20 mM Hepes, 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM dithiothreitol, 25% (v/v) glycerol, 0.2 mg/ml phenylmethanesulfonyl fluoride, 20 μg leupeptin, 100 IU aprotinin/ml, pH 7.9). Soluble nuclear extracts were obtained by centrifugation (14,000 × g, 15 min, 4 °C).

Western Blot Experiments—Cellular and nuclear extracts were diluted in 4 × SDS-PAGE sample buffer (62 mM Tris-HCl, 8% SDS, 40% glycerol, 20% 2-mercaptoethanol, and 0.16% bromophenol blue), boiled for 5 min, and resolved on 10% SDS-polyacrylamide gels. After electrophoresis, proteins were blotted onto nitrocellulose membranes. Membranes were blocked using 5% (v/v) nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20, and exposed to antibodies overnight at 4 °C in the same buffer. After incubation with appropriate secondary antibodies conjugated to horseradish peroxydase, immunoreactivity was detected using the ECL method (Pierce, Rockford, IL).

Measurement of cAMP Concentration and PKA Activity of cAMP preparations, various agents were added to 101–102 cells/well, and serum-deprived 24 h prior to stimulation. Incubations were performed for 30 min at 37 °C in phosphate-buffered saline containing 1% (v/v) bovine serum albumin, and 10 IU aprotinin/ml in the presence or absence of agonists. The reaction was stopped by addition of chilled 1 N perchloric acid. Cyclic AMP cell content was determined using a cAMP radiomunnoassay kit (New England Nuclear, Zaventem, Belgium) according to the manufacturer’s instructions.

To determine PKA activity, STC-1 cells were seeded into 60-mm dishes, and serum-starved for 24 h before stimulation. Cells were incubated at 37 °C for various times in RPMI medium containing 0.2% bovine serum albumin with or without agonists in the presence of 0.5 mM IBMX. The reaction was stopped on ice, and cells were washed twice with phosphate-buffered saline before scraping in 0.2 ml of extraction buffer (25 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM 2-mercaptoethanol, 20 μg leupeptin, 100 IU aprotinin, pH 7.4). After homogenization with a Potter homogenizer, the lysates were clarified by centrifugation at 14,000 × g for 10 min at 4 °C. PKA activity was measured with the SignaTECT cAMP-dependent protein kinase assay system (Promega). Briefly, 5 μl of cell extract were incubated in a total volume of 25 μl containing 40 mM Tris-HCl, pH 7.4, 20 mM MgCl2, 0.1 μg/ml bovine serum albumin, 0.1 μg/ml biotinylated kemptide as a specific PKA substrate, 0.1 μg/ml ATP and 20 μg/ml [γ-32P]ATP for 5 min at 30 °C. The reaction was stopped by adding 12.5 μl of 7.5 M guanidine hydrochloride, and samples were spotted onto streptavidin-coated membranes. The membranes were washed 4 times in 2 M NaCl, 4 times in 2 M NaCl in 1% H3PO4, and twice in water, and radioactivity was measured by scintillation counting. Total PKA activity was determined in the presence of 5 μM cAMP.

Data Analysis—All results were calculated as the mean ± S.E. Data were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc comparison of Fisher or Student’s t test as appropriate. Differences between two means with a p value < 0.05 were regarded as significant.
RESULTS

The cAMP/PKA Pathway Is Involved in Peptone-induced Phosphorylation of CREB in STC-1 Cells—The ability of protein hydrolysates to phosphorylate CREB has been recently demonstrated (16). Since this transcription factor can be activated by several protein kinases including PKA, we examined whether cell treatment with peptones could directly activate the cAMP/PKA pathway components and whether this pathway was effectively responsible for peptone-induced CREB stimulation.

First, we determined the effect of peptones on intracellular cAMP production and PKA enzyme activity. Fig. 1A shows that peptones induced a significant increase of the cAMP concentration in STC-1 cells (2.81 ± 0.29 vs. 1.19 ± 0.08 pmol/well, p < 0.05). Similarly, PKA activity (picomole of ATP/min/μg of protein) was significantly increased when cells were incubated with peptones from 5 min (0.395 ± 0.037 vs. 0.243 ± 0.029, p < 0.05) to 15 min (0.613 ± 0.044 vs. 0.273 ± 0.041, p < 0.05). Stimulation of PKA activity remained sustained at 30 min (0.548 ± 0.107 vs. 0.229 ± 0.037, p < 0.05). In an inactive state, PKA catalytic subunits (PKAC) are complexed with PKA regulatory subunits (PKAR) to form a holoenzyme found free in the cytosol or associated with subcellular structures through anchoring proteins (20). Under stimulation, PKAC dissociate from PKAR and then can translocate to the nucleus, while PKAR remain in the cytoplasm (21). Therefore, we analyzed whether peptone treatment was able to induce a nuclear translocation of PKAC in STC-1 cells, by determining the level of PKAC protein in nuclear extracts by Western blotting. A representative result is shown in Fig. 1C.

Treatment of cells with 2% peptones for 30 min increased the amount of PKAC in the nuclear fraction, as compared with control conditions. As a positive control, treatment of cells with forskolin/IBMX (10 μM/0.5 mM) induced a comparable increase of PKAC immunoreactivity in the nucleus. Finally, we evaluated the contribution of the peptone-stimulated PKA pathway in CREB activation. For this purpose, STC-1 cells were incubated for 45 min with peptones in the presence or absence of the selective PKA inhibitor H-89 (22), and the phosphorylation level of CREB was measured by Western blotting using an antibody directed against the Ser133-phosphorylated form of CREB. Results shown in Fig. 1D demonstrated that H-89 treatment (10 μM) led to a significant decrease in peptone-induced phosphorylation of CREB, indicating that PKA plays a role in mediating the peptone effect on this transcription factor.

Peptone-induced Stimulation of CCK Gene Transcription Is Dependent on the cAMP/PKA Signaling Pathway—To directly establish the functional link between the peptone-induced cAMP/PKA signaling pathway activation and the peptone-induced stimulation of CCK gene promoter activity, pharmacological and molecular approaches were both used. On the one hand, STC-1 cells were transiently transfected with the −765 bp CCK gene promoter construct, and treated for 16 h with peptones or forskolin/IBMX in the presence of increasing concentrations of the selective PKA inhibitor H-89 (Fig. 2A). H-89 (1–10 μM) produced a dose-dependent inhibition of the peptone-stimulated CCK gene promoter activity that reached 51.1 ± 2.9% at the highest tested concentration. A similar inhibitory effect of H-89 was registered for the stimulation of CCK gene promoter activity induced by forskolin/IBMX (Fig. 2A). Furthermore, extended exposure to agents...
that increase cAMP cell content has been shown to desensitize the cAMP-dependent pathway in cells of different origin, by down-regulating the PKAc (23–25). In STC-1 cells transfected with the −765-bp CCK gene promoter construct and with increasing amounts of the plasmid MT-REV(AB) expressing a dominant-negative mutant protein of PKA. Cells were then incubated for 16 h in the absence (control) or presence of 2% (w/v) peptones or forskolin/IBMX (10 μM/0.5 mM). B, cells were co-transfected with 500 ng of MT-REV(AB) or 500 ng of the empty vector, and with 250 ng of the different CCK gene promoter constructs. Fragments −765 bp and −93 bp contain the PepRE, whereas this sequence is mutated or deleted in fragments −93M4 and −70 bp, respectively. Cells were incubated for 16 h in the absence or presence of 2% (w/v) peptones. Relative luciferase activity was measured and results represent the mean ± S.E. of at least three independent experiments, each performed in triplicate. *p < 0.05 versus corresponding stimulated conditions without MT-REV(AB).

We next investigated the implication of intracellular Ca^{2+} in peptone-induced phosphorylation of CREB. Incubation of STC-1 cells with peptones for 45 min in the presence of 20 μM BAPTA/AM completely abolished the peptone-stimulated phosphorylation of CREB (Fig. 4B), the quantity of CREB proteins being constant in all conditions. Similarly, PKA activity stimulated by a 15-min peptone treatment was significantly reduced in the presence of BAPTA/AM (Fig. 4C). BAPTA/AM at the concentration of 50 μM led to an almost complete inhibition in these experimental conditions. Together, these findings demonstrate the requirement of intracellular Ca^{2+} in peptone-induced CCK gene transcription, as well as in cellular events leading to CREB activation.

**Activation of a Calcium-dependent ERK1/2 Pathway Participates in the Effect of Peptones on CCK Gene Promoter Activity**—Since PKA inhibition resulted in a significant but only partial decrease of the peptone-induced stimulation of CCK gene promoter activity, we searched for other calcium-dependent protein kinases responsible for the remaining peptone stimulation. In PC12 cells, the MAP kinase cascade is known to be induced by a rise of intracellular calcium (27) and therefore was a candidate to mediate the peptone signal in STC-1 cells. Incubation of cells for increasing periods of time with 2% peptones induced a marked increase of ERK1/2 phosphorylation on Thr202/Tyr204 from 5 to 30 min, as demonstrated by Western blotting (Fig. 5A). The activation of ERKs progressively decreased at longer times of exposure. ERK1/2 phosphorylation was totally impaired when cells were pretreated with 50 μM BAPTA/AM, indicating the requirement of calcium in the peptone-induced activation of ERKs in STC-1 cells (Fig. 5A).

To determine the relevance of this MAPK pathway in the stimulation of CCK gene promoter activity, the MEK inhibitor
PD98059 was used with cells transfected with the −765-bp CCK gene promoter construct. Treatment with 30 μM PD98059 led to a 60% decrease of peptone-induced stimulation of the CCK gene promoter activity (Fig. 5B). This inhibition was comparable to that obtained with the expression of the dominant-negative mutant of PKA. Simultaneous treatment with PD98059 and transfection with MT-REV(AB) inhibited 80% of the peptone-induced stimulation of promoter activity (Fig. 5B).

**Fig. 4.** Peptone-induced stimulation of CCK gene promoter activity, phosphorylation of CREB and PKA activation require intracellular calcium. A, cells were transfected with the −765-bp CCK gene promoter construct, pretreated or not with the intracellular calcium chelator BAPTA/AM (20 μM) for 30 min and then incubated for 16 h in the absence (control) or presence of 2% (w/v) peptones with or without 20 μM BAPTA/AM. Luciferase activity was then measured and normalized to the protein content. Results represent the mean ± S.E. of four independent experiments, each performed in triplicate. B, cells were pretreated or not with BAPTA/AM (20 μM) for 30 min and then incubated for 45 min in the absence or presence of 2% (w/v) peptones with or without BAPTA/AM. Total cell extracts were prepared and Ser133-phosphorylated CREB (P-CREB) protein (upper panel) or total CREB protein (lower panel) were detected by Western blotting, using appropriate antibodies. Results are representative of three independent experiments. C, cells were pretreated or not with BAPTA/AM (50 μM) for 30 min, then incubated for 15 min in the absence (control) or the presence of 2% (w/v) peptones with or without BAPTA/AM, and PKA activity was then measured as described under “Experimental Procedures.” Results represent the mean ± S.E. of three independent experiments, each performed in duplicate. *, p < 0.05 versus stimulated conditions without BAPTA/AM.

**DISCUSSION**

Nutrients are major physiological stimuli of intestinal CCK release and increase CCK gene transcription both in vivo (5, 6) and in the enteroendocrine CCK-producing STC-1 cell line (15). Mutational analysis of the 5′-flanking region of the CCK gene allowed us to identify a PepRE that binds the CREB transcription factor; the activity of this transcription factor is required for the peptone-induced activation of CCK gene promoter in STC-1 cells (16). Except for their effect on the exocytosis process, the transduction mechanisms activated by protein hydrolyases in enteroendocrine cells are unknown. In STC-1 cells, the peptone-stimulated CCK secretion involves a pertussis toxin-sensitive protein, the Rab3A small G protein and intracellular calcium (26, 28). In the rat duodenojejunum, this release and increase CCK gene transcription both in vivo (5, 6) and in the enteroendocrine CCK-producing STC-1 cell line (15).
were transfected with the \( \beta \)-H11002 gene promoter construct, pre-treated or not with the \( \beta \)-H9262 inhibitor SB203580 (10 \( \mu \)M), the PKC inhibitor GF109203X (1 \( \mu \)M), or the PKI inhibitors wortmannin (200 nM) and LY294002 (10 \( \mu \)M) and then incubated for 16 h in the absence or presence of 2\% (w/v) peptones with or without inhibitors. B, cells were co-transfected with 500 ng of MT-REVAB or with 500 ng of the empty vector, pre-treated or not with the CaMK inhibitor KN-93 (5 \( \mu \)M) in the absence or presence of PD98059 (30 \( \mu \)M) and then incubated for 16 h in the absence or presence of 2\% (w/v) peptones with or without inhibitors. Relative luciferase activity was measured and results represent the mean ± S.E. of three independent experiments, each performed in triplicate. *, \( p < 0.05 \) versus corresponding stimulated conditions without KN-93.

Expression of several gastrointestinal hormone genes, including somatostatin (30), proglucagon (31, 32), and gastrin (33) genes has been shown to be regulated by cAMP-dependent pathways. Regarding CCK gene expression, previous studies showed that the cAMP pathway was involved in its regulation. Indeed, forskolin was reported to be a potent activator of CCK gene transcription, increasing the activity of \(-200-\) and \(-100\)-bp constructs of the CCK gene promoter transiently transfected in the SK-N-MC neuroblastoma cell line (34) or inducing the stimulation of a \(-800\)-bp CCK gene promoter fragment activity in STC-1 enteroendocrine cells (15, 35). Here, we unambiguously demonstrate the functional recruitment of the cAMP/PKA pathway by protein hydrolysates. Cell exposure to peptones stimulated the activity of PKA that is able to translocate to the nucleus to control transcriptional events, such as the phosphorylation of transcription factors. A similar PKA, shift was reported in NIH-3T3 cells treated by basic fibroblast growth factor (36). Nuclear PKA, is, at least in part, responsible for the stimulation of the phosphorylation of CREB observed under peptone treatment since this stimulation was partially prevented by the H-89 treatment. However, we cannot rule out the possibility that peptones modulate the targeting of PKA to other subcellular non-nuclear compartments where it could indirectly act on transcription. Indeed PKA was described to control the activity of phospholipase \( \beta \), Rap-1, or Rap1, both possibly converging to the MAPK pathway and therefore to the nucleus (37–39). The direct relation between the stimulation of PKA activity by peptones and the concomitant stimulation of CCK gene promoter activity is further substantiated by the fact that several strategies (pharmacological inhibition, desensitization, or expression of a dominant negative mutant of PKA) resulted in similar reductions (about 50\%) of the peptone-induced stimulation of CCK gene promoter activity.

The peptidomimetic cephalosporins increase c-fos mRNA abundance in STC-1 cells through the activation of ERKs (40). In SK-N-MC cells, stimulation of the CCK gene promoter activity by basic fibroblast growth factor occurs through a CRE consensus DNA element that overlaps the PepRE, and the mechanisms mediating this effect involve p38MAPK and ERK pathways (34). These observations prompted us to evaluate the role of the ERK pathway in the peptone-induced intracellular cascade. ERK1/2 were indeed activated under peptone treatment, and the MEK inhibitor PD98059 significantly decreased the peptone-induced transcriptional effect. These results are consistent with a significant role of the ERK pathway, which accounted together with the PKA pathway for the greater part (80\%) of the peptone-induced transcriptional effect in STC-1 cells. At last, a panel of inhibitors was tested to determine the component responsible for the residual stimulation of CCK gene promoter activity. Numerous kinases capable of phosphor-yllating CREB were therefore candidates, including PKC, CaMKI, -II, and -IV, ribosomal protein S6 kinases, MAPK-activated protein kinases (MAPKAP-K2/3), mitogen- and stress-activated protein kinase 1 (41), and a novel 120-kDa CREB kinase (42). Cell treatment with KN-93 pointed to CaMK as a minor kinase mediating the effect of peptones. However, the precise nature of the type(s) and/or isoform(s) of CaMK involved remains to be identified. Furthermore, various examples of complex cross-talks between PKA pathway and ERK or CaMK pathways have been reported (43–45), and studies are needed to determine whether such connections actually occur in the enteroendocrine cell responding to protein hydrolysates. The way these different protein kinases are activated under peptone treatment remains uncertain. Activation of PKA likely occurs through the stimulation of cAMP production, thus suggesting the involvement of a membrane structure (receptor, transporter, or channel) coupled, directly or not, to adenyl cyclase. Another hypothesis is a peptone-induced entry of calcium that could activate a calcium-sensitive adenyl cyclase isoform as well as CaMK. Further studies are needed to elucidate these points.

Peptones have been shown to require Ca\(^{2+}\) for stimulation of CCK release in STC-1 cells (26). In addition, in other cell models, CREB is activated after membrane depolarization and subsequent calcium influx (46). In neurons, calcium influx through N-methyl-d-aspartate receptors also causes phosphorylation of CREB at Ser\(^{333}\) (47). Using the Ca\(^{2+}\) chelator BAPTA/AM, we here demonstrate a crucial involvement of intracellular calcium in the peptone-stimulated CCK gene transcription. The dramatic inhibitory effect obtained on CCK gene promoter activity in the presence of BAPTA/AM is likely due to the total inhibition of calcium-dependent phosphorylation of CREB, thus suppressing the final event of the different calcium-dependent kinase pathways.

Our study depicts different intracellular cascades initiated by protein-derived nutrients in intestinal cells and leading to the activation of CCK gene transcription. The multiplicity of signaling molecules recruited by the endocrine cell in response to protein hydrolysates is a feature shared by cells responding to fatty acids, that could use at least five distinct mechanisms to regulate gene expression (7). However, the connections between luminal stimuli and changes in gene expression are still incompletely understood. Theoretically, endocrine cells in the
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mucoza could respond to luminal nutrients directly through membrane structures or indirectly through nervous or paracrine intermediates (48). The effect of protein hydrolysates on STC-1 cells is in accordance with the hypothesis of a direct crine intermediates (48). The effect of protein hydrolysates on mucosa could respond to luminal nutrients directly through defined mechanism in STC-1 cells, raising the hypothesis of a presently unknown specific oligopeptide transporter. To address this point, further studies are needed to define the molecular species and/or the conformational motif responsible for the effect of protein hydrolysates. Therefore, this issue remains an exciting challenge to better understand the dietary regulation of gut hormone gene expression.

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