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Cholecystokinin$_B$ Receptor from Human Jurkat Lymphoblastic T Cells Is Involved in Activator Protein-1-Responsive Gene Activation

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SUMMARY

The aim of this study was to analyze the role of cholecystokinin (CCK_B) receptor in human lymphoblastic Jurkat T cells. We investigated the trophic effect resulting from activation of such a receptor by using the reporter gene strategy. For this purpose, we transiently transfected Jurkat T cells with the reporter plasmid p[(TRE)3-tk-Luc] and found that CCK-8 was able to dose-dependently induce luciferase expression related to activator protein-1 (AP-1) activation with a maximal response identical to that obtained with compounds known to activate AP-1 complex (quantitatively, the same level of induction was obtained with 1 nM 12-*O*-tetradecanoylphorbol-13-acetate, 100 μM diacylglycerol, or 4 nM epidermal growth factor). The involvement of the CCK_B receptor in such a stimulation was demonstrated by the inhibiting effect of the selective CCK_B receptor antagonist PD-135,158. This effect was confirmed in COS-7 cells transfected with the cDNA of CCK_B receptor cloned from Jurkat T cells. To better understand the AP-1-dependent luciferase expression in Jurkat T cells, we tested two specific inhibitors of serine/threonine phosphatases-1 and -2A: okadaic acid and calyculin A. These compounds strongly increased the phorbol-12-myristate-13-acetate response, whereas we have not observed a contribution of phosphatase inhibitors on a CCK-8-induced luciferase activity. To confirm that CCK_B receptors are involved in AP-1 response, we investigated the CCK-8 effect on interleukin-2 expression, a natural endogenous gene regulated by several factors, including AP-1. In Jurkat T cells activated by phorbol-12-myristate-13-acetate and phytohemagglutinin, CCK-8 induced IL-2 expression. This induction was abolished by PD-135,158. Our results indicate that CCK-8 exerts a trophic effect in Jurkat T cells through stimulation of CCK_B receptors by modulation of expression of AP-1-regulated genes.

Several studies have shown that various gastrointestinal peptides may be involved in the control of proliferation of various tissues and neoplastic cells (1). For example, CCK was shown to increase growth of tumors in nude mice bearing transplanted pancreatic cancer tissues (2). CCK is also known to increase the number of animals developing nitrosamine-induced pancreatic cancers (3), and CCK was shown to increase the rate of growth of cultured pancreatic cancer cells (2). Similar observations were described for bombesin/gastrin-releasing peptide in human glioblastoma *in vitro* and *in vivo* in small-cell lung carcinoma, prostatic, mammary, and pancreatic cancer cell lines (1). In addition, gastrointestinal peptides can function as autocrine growth factors in neoplastic tissues as shown for bombesin/gastrin-releasing peptide in small-cell lung carcinoma cells, for gastrin and

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ABBREVIATIONS: CCK, cholecystokinin; CCK-8, H-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2; ^{125}I-BH-CCK-8, Bolton-Hunter cholecystokinin(26–33); gastrin(5–17), H-Leu-(Glu)$_5$-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2; BOC-CCK-4, *N-tert*-butoxycarbonyl-Trp-Met-Asp-Phe-NH_2; YM-022, (*R*)-1-[2,3-dihydro-1-(2′-methyl-phenacyl)-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea; PD-135,158 [4([2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[[1.7.7-trimethyl-bicyclo[2.2.1]hept-2-yl)oxy]carbonyl]amino]propyl]amino]1-phenylethyl]amino-4-oxo-[1*S*-1α.,2β[*S**(*S**)]4α]]butanoate *N*-methyl-D-glucamine (bicyclo system 1*S*-endo); L-365,260, (3*R*)-(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-*N*-(3-methyl-phenyl)urea; L-364,718, (3*S*)-(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-1*H*-indole-2-carboximide; AP-1, activator protein-1; TRE, 12-*O*-tetradecanoylphorbol-13-acetate-responsive element; Luc, luciferase; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; PHA, phytohemagglutinin; FCS, fetal calf serum; PCR, polymerase chain reaction; IL-2, interleukin-2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PP-1, protein phosphatase-1; PP-2A, protein phosphatase-2A.

CCK in colon and pancreatic carcinoma, and for vasoactive intestinal peptide in neuroblastoma (1). Cell growth is a biological complex response involving several regulatory pathways; however, in gastrointestinal tissues and neoplastic cells in which gastrointestinal peptides control cell proliferation, one can suggest, at least in part, the involvement of their specific receptors because specific antagonists inhibit their effect. Moreover, specific receptors for gastrointestinal peptides have been detected in gastrointestinal tissues and neoplastic cells (1). CCK receptors are currently classified into two main subtypes: CCK_A and CCK_B. This classification is based on their relative affinities for sulfated and unsulfated CCK agonists and for selective synthetic antagonists. CCK_A receptors, which occur mainly in the periphery (4), have high affinity for sulfated forms of CCK and for the antagonist L-364,718. CCK_B receptors, which are widely distributed in the brain, have high affinity for both sulfated and unsulfated CCK, for the carboxyl-terminal tetrapeptide of CCK, and for the antagonist L-365,260. In the past few years, the CCK_B receptor was cloned from tissues of different species (5–10). Recently, a preferential splice donor site was found in exon 4 of the human CCK_B receptor gene (11, 12), leading to two receptor isoforms (short and long forms) that differed by five amino acids in their putative third cytoplasmic domain.

We previously reported on the presence of CCK_B receptors in Jurkat cells, a human T lymphoblastic cell line (13, 14). Because no biological activity was connected to activation of this receptor, this study was performed to characterize its putative role. Taking into account the role of CCK receptors on the growth and differentiation of cells (1, 15–17), we investigated the putative action of this receptor on AP-1-responsive gene expression in Jurkat T cells. Such a study was performed by transient transfection with a reporter plasmid [p(TRE)3-tk-Luc], leading to Luc expression after AP-1 activation. The role of CCK_B receptor was confirmed by analyzing the AP-1 activation induced by CCK-8 in COS-7 cells cotransfected with the cDNA encoding the major isoform of the CCK_B receptor that we cloned from Jurkat T cells and the plasmid p(TRE)3-tk-Luc. In Jurkat T cells, we studied the effect of CCK-8 on IL-2 expression, a T cell factor involved in trophic effect whose expression also depends on AP-1 activation.

Experimental Procedures

Materials. CCK-8, its radiolabeled analogue ^{125}I-BH-CCK-8, gastrin(5–17), BOC-CCK-4, and YM-022, a specific CCK_B receptor antagonist, were synthesized in our laboratory. The ^{125}I-labeled Bolton-Hunter reagent (2000 Ci/mmol) was purchased from Amersham International (Buckinghamshire, UK). The CCK_B receptor antagonist PD-135,158 was a gift from Dr. D. Horwell (Parke-Davis Neuroscience Research Center, Cambridge, UK). L-365,260 and L-364,718 (MK-329 or Devazepide) were gifts from Dr. P. Anderson (Merck Sharp and Dohme Research Laboratories, West Point, PA). Luciferin, PMA, PHA, okadaic acid, and calyculin A were from Sigma Chemical (St. Louis, MO). Cell culture mediums were from GIBCO (France). The reporter plasmid p(TRE)3-tk-Luc previously described (18, 19) was a gift from Dr. M. Pons (Institut National de la Santé et de la Recherche Médicale U439, Montpellier, France).

Cell lines and cell culture conditions. Jurkat cells (lymphoblastic T cells) were obtained from Dr. J. Dornand (Université Montpellier II, Sciences et Techniques du Languedoc, Montpellier, France). They were grown in RPMI 1640 medium supplemented with 10% (v/v) FCS and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin). COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS (v/v), glutamine (2 mM), and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin). The growth of cells was performed in a humidified incubator at 37° under an atmosphere of 5% CO_2 in air. As indicated below, transfections were realized in medium supplemented or not supplemented with 10% (v/v) FCS, whereas incubation of cells with the various tested compounds was performed in appropriate medium without FCS.

Cloning of the CCK_B receptor from Jurkat T cells. Total RNAs were extracted from 60×10^6 Jurkat T cells using the RNA Plus reagent (Bioprobe Systems, Montreuil-sous-Bois, France). Oligo(dT)-primed cDNA was synthesized using Superscript II reverse transcriptase (GIBCO BRL, Gaithersburg, MD) from 10 μg of total RNAs. Double-stranded cDNA (5 ng) served as template for PCR with 0.5 μM oligonucleotide sequences P1 (5′-GGCCATGGAGCTGCTCAAGCTG-3′) and P2 (5′-TCAGCCAGGGCCCAGTGTGCT-3′) (Institut Pasteur, Paris, France) localized at 5′ and 3′ ends of the published coding sequence of the human CCK_B receptor (12). PCR was performed with a thermoblock Biometra apparatus in a 50-μl solution containing 5 μl of 10 × *Pyrococcus furiosus* buffer, 5 μl of dimethylsulfoxide, 0.5 mM concentration of dNTPs, 0.4 μM concentration of primers, 5 ng of cDNA sample, and 2.5 units of recombinant *P. furiosus* polymerase (Stratagene, Cambridge, UK). The following cycle temperatures and times were used under standard PCR conditions: 35 cycles of denaturation at 95° for 1 min, annealing at 60° for 1 min, and extension at 73° for 5 min with a final extension period of 10 min. The PCR products were separated on a 1% agarose gel. The band localized at 1341 bp was purified with the GeneClean II kit (Bio 101, Vista, CA). Then, the cDNA was phosphorylated and ligated to dephosphorylated blunt-ended pCI Neo Mammalian Expression Vector (Promega, Madison, WI) first linearized by the restriction enzyme *Sma*I (Eurogentec, Seraing, Belgium). This construct, p(CCK_B/pCI Neo), was used for transient expression in COS-7 cells. After transformation of competent cells (*Escherichia coli*, JM109) by electroporation, plasmids containing insert were isolated and purified by the alkaline lysis method using a Plasmid Maxi Kit (Qiagen, Studio City, CA). cDNA insert was sequenced by Genome Express (Grenoble, France). This experiment was repeated several times before deducing the precise cDNA sequence encoding the CCK_B receptor from Jurkat T cells.

Transient transfection experiments. For binding experiments, COS-7 cells (2×10^6 cells/dish) were plated for 3 hr in 10-cm tissue culture dishes (Corning Glassworks, Corning, NY) in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS, 2 mM glutamine, and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin) in a humidified incubator under an atmosphere of 5% CO_2 in air at 37°. Then, 2 μg of p(CCK_B/pCI Neo) was transfected according to the calcium phosphate coprecipitation method. As control, one dish of COS-7 cells was transfected with pCI Neo. Cells were incubated with the precipitate for 24 hr and then washed twice and incubated for 24 hr in the medium described above. For reporter gene experiments, COS-7 cells were transiently cotransfected according to the calcium phosphate coprecipitation procedure with the plasmids p(TRE)3-tk-Luc and p(CCK_B/pCI Neo). As control, COS-7 cells were cotransfected with both the plasmids p(TRE)3-tk-Luc and pCI-Neo. Typically, experiments were performed in six-well tissue culture cluster plates; cells were seeded at 200–500 × 10^3 cells/well in medium supplemented with 10% (v/v) FCS. Each well then received 1 μg of the DNA precipitate [0.5 μg of the reporter plasmid p(TRE)3-tk-Luc and 0.5 μg of the plasmid p(CCK_B/pCI Neo)]. After an overnight incubation time, the precipitate was removed, cells were then washed and cultured in the absence of FCS. Jurkat T cells were transiently transfected using electroporation (Easyject Plus; Eurogentec, Seraing, Belgium) or using a synthetic cationic lipopolyamine molecule (Transfectam Reagent; Promega). For electroporation, 10–

20×10^6 Jurkat T cells were resuspended in 800 μl of complete culture medium. The cell suspension was then added to the 4-mm electrode gap cuvette before receiving DNA (12–24 μg). After mixing, electroporation was carried out at room temperature according to the following parameters: single pulse, 250 V, 1500 μF, and infinite internal resistance (under these conditions, the pulse time was 22 msec). After electroporation, cells were immediately withdrawn and transferred into a tissue culture flask containing 15 ml of growth medium. The following day, cells were rinsed twice and refed with growth medium. Such an experiment is needed for 12–24 experimental points. For transfection of Jurkat T cells by using Transfectam reagent, the protocol followed was that recommended by the manufacturer. Jurkat T cells (10^7) were extensively washed and then resuspended in 18 ml of culture medium without FCS. The cell suspension was then mixed with 36 ml of culture medium without FCS and containing DNA (36 μg) and Transfectam Reagent (72 μl). The following day, the cells were rinsed twice and refed with growth medium. Such an experiment is needed for 36 experimental points.

Receptor binding experiments. At 48 hr after transient transfection, COS-7 cells were rinsed with ice-cold 25 mM HEPES-buffered Hanks' balanced salt solution, pH 7.4, supplemented with 0.1% (w/v) bovine serum albumin. Cells were scrapped from the dishes, harvested by centrifugation (1000 rpm, 5 min), and then resuspended in the same solution to obtain an appropriate concentration of 2×10^5 cells/ml. Kinetic experiments were performed at 37° in a final volume of 250 μl containing 10 pM ^{125}I-BH-CCK-8. Displacement experiments were performed at 37° for 60 min by adding 10 pM ^{125}I-BH-CCK-8 plus different drug concentrations in a final volume of 250 μl. The nonspecific binding was determined in the presence of 1 μM CCK-8. The incubation was terminated by adding 2 ml of ice-cold 50 mM Tris buffer, pH 7.4, containing 0.01% (w/v) bovine serum albumin. Aliquots were then centrifuged at 4° for 10 min at 3000 rpm. Supernatants were discarded, and the radioactivity bound to the pellet was measured. Nonspecific binding was always <10% of the total binding. Data are expressed as mean ± standard error.

Luciferase assays. At 48 hr after transfection of Jurkat T cells, they were rinsed and suspended in the appropriate culture medium in absence of FCS. The cell suspension was then plated onto six-well tissue culture cluster plates at 200–500 $\times 10^3$ cells/well. At 48 hr after transfection of COS-7 cells, the cells were washed in culture medium without FCS and then maintained in the culture medium in absence of FCS. The Jurkat T or COS-7 cells were then incubated with the tested compounds (CCK-8 with or without selective CCK_B receptor antagonist PD-135,158 or as indicated) for 6–8 hr at 37° before determination of the luciferase activity (19). After incubation, cells were extensively washed with PBS and then resuspended in 300 μl of lysis buffer [25 mM Tris/phosphoric acid, pH 7.8, 2 mM 2-mercaptoethanol, 2 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100]. Then, 100 μl of the cellular homogenate was introduced in luminometer fitted test-tubes for assay (1251 luminometer; LKB Wallac, Sundyberg, Sweden). Each test-tube was automatically injected in the luminometer with 100 μl of detection buffer (10 mM luciferin, 40 mM Tris·HCl, pH 7.8, 2.1 mM $MgCl_2$, 5.4 mM $MgSO_4$, 0.2 mM EDTA, 66 mM 2-mercaptoethanol, 10 mM ATP, pH 8, 10 mM Coenzyme A). The corresponding luciferase activity was then determined by integrating the luminescence signal for 15 sec. Results were expressed as percentage of the maximal response and as arbitrary units (18, 19).

IL-2 assays. Experiments were performed in Jurkat T cells previously cultured in the absence of FCS for 24 hr. Jurkat T cells were preincubated for 20–30 min at 37° with different CCK-8 concentrations, alone or in the presence of the selective CCK_B receptor antagonist PD-135,158. After preincubation, PMA (10 ng/ml) alone or in combination with PHA (10 μg/ml) was added to the cells. Incubation was then continued for 24 hr at 37° by the addition of culture medium free of FCS (each experimental point contained 1–2 $\times 10^6$ cells in a final volume of 1 ml). After incubation, aliquots of 30 and 100 μl of the corresponding supernatants were diluted 10 times before assayed for their IL-2 content using an IL-2 enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). The protocol was that recommended by the manufacturer. The IL-2 content (mean values ± standard error) was checked periodically (from 25 to 120 min) and expressed as units/ml by using a standard curve.

Results

Effect of activation of the CCK_B receptor in Jurkat T cells: induction of a reporter activity under AP-1 control. No biological role for CCK_B receptors was clearly demonstrated in Jurkat T cells. Several studies have shown that gastrointestinal peptides exert trophic effect on both gastrointestinal and neoplastic cells (1). We thus investigated the effect of CCK-8 on AP-1 regulated genes because activation of nuclear oncogenes like Fos and Jun is believed to be involved in events directly related to growth and differentiation of cells.

Our study was performed using transient transfection of Jurkat T cells [cells known to possess CCK_B receptors (13, 14)] with the reporter plasmid p(TRE)3-tk-Luc. This plasmid carries the firefly Luc expression under control of an AP-1 regulatory sequence (19). Such a sequence, called TRE, was extensively described as enhancing expression from both homologous and heterologous promoters after Fos and Jun activation (20–22). As shown in Fig. 1A, Jurkat T cells transiently transfected with p(TRE)3-tk-Luc were able to induce luciferase expression in a CCK-8 concentration-dependent manner, reaching a maximal response at ~20 nM CCK-8. As reflected by the EC_{50} value (3–5 nM), this chimeric response is in agreement with classic biological responses involving CCK_B receptor activation by CCK-8. Under our experimental conditions, the value of maximal stimulation of luciferase activity was 2–3 times higher than the basal level. This relative induction ratio is similar to that obtained with different compounds known to activate the AP-1 pathway. For example, this induction level corresponded to that obtained with 1 nM PMA (Fig. 1C). Moreover, in the MDA MB231 breast cancer cell line stably transfected with p(TRE)3-tk-Luc (19), 100 μM diacylglycerol and 4 nM epidermal growth factor induced luciferase activity to the same level than that observed with 10 nM CCK-8 in Jurkat T cells. The implication of CCK_B receptor in such a stimulation is shown in Fig. 1B: the luciferase activity induced by 10 nM CCK-8 was completely inhibited by 100 nM concentration of the potent and selective CCK_B receptor antagonist PD-135,158. As a control of specificity, in MDA MB231 cells free of CCK_B receptors and stably transfected with the plasmid p(TRE)3-tk-Luc, CCK-8 was without effect, whereas PMA was able to induce luciferase activity (Ref. 19 and data not shown).

To characterize the CCK_B receptor expressed in human Jurkat T cells and to confirm its involvement in such a phenomenon, it was cloned by reverse transcription-PCR. As indicated in Experimental Procedures, a 1341-bp fragment was amplified. Because this size corresponded well to the cDNA encoding human CCK_B receptor, this cDNA was purified and subcloned into a pCI Neo Mammalian Expression Vector [p(CCK_B/pCI Neo)] before sequencing. The amino acid sequence deduced from the cloned cDNA showed 100% identity to the CCK_B receptor expressed in human brain (12). However, the cloned cDNA carried two silent mutations (A12 and T936). To confirm that the cloned cDNA well encodes the

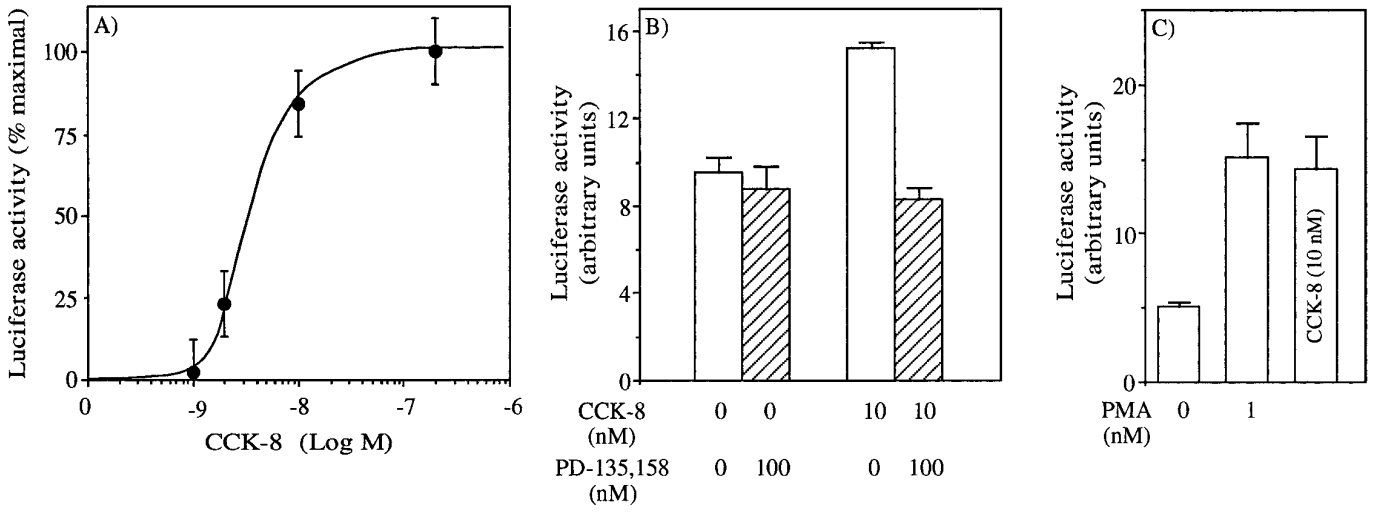

Fig. 1. Induction of Luc activity by CCK-8 in Jurkat T cells. As described in Experimental Procedures, Jurkat T cells were transiently transfected using electroporation (A) or Transfectam Reagent (B and C) with the plasmid p(TRE)3-tk-Luc. At 48 hr after transfection, CCK-8 was added alone (A and B, □) or in the presence of 100 nM antagonist PD-135,158 (B, ▨). C, As a control, the effect of 1 nM PMA is shown. Luc activity was then determined, and results (mean ± standard error values) were expressed as a percentage of the maximal induction or arbitrary units as previously described (19). The presented experiment is a typical result obtained from three separate experiments.

CCK_B receptor from Jurkat T cells, we determined the pharmacological profile of the recombinant receptor expressed in COS-7 cells. The ability of ^{125}I-BH-CCK-8 to bind to COS-7 cells transiently transfected with p(CCK_B/pCI Neo) was investigated. As control, no specific binding was found when the COS-7 cells were transfected with pCI-Neo (data not shown). As shown in Fig. 2A, the ^{125}I-BH-CCK-8 binding was time dependent and reached a steady state within 50 min at 37° with an apparent pseudo-first-order rate constant K_{+1} (app) of 2.2×10^7 M^{-1} min^{-1}. The plateau remained stable for almost 2 hr. The dissociation kinetic of ^{125}I-BH-CCK-8 was studied by the addition of 1 μM CCK-8 (Fig. 2B). The dissociation curve appeared biphasic with two values of dissociation constants: ($K_{-1} = 3.7 \times 10^{-2}$ min^{-1} and (K_{-1})′ = 9.4×10^{-3} min^{-1}. This biphasic dissociation suggested that the radioligand interacts with two affinity states of the CCK_B receptor, as previously suggested (23). This hypothesis is confirmed by the Hill coefficient value calculated by linear regression analysis from nine displacement curves clearly inferior to 1 (Hill coefficient = 0.5 ± 0.1; $n = 9$). These results are strongly similar to the biphasic dissociation observed in the Jurkat T cells (data not shown). Displacement experiments of bound ^{125}I-BH-CCK-8 by various agonists and antagonists of the CCK_B and CCK_A receptors were also investigated. Competition binding studies demonstrated inhibition of specific binding in a concentration-dependent manner by CCK agonists CCK-8, [Leu^{11}]gastrin(5–17), and BOC-CCK-4 (Fig. 3A) and antagonists L-365,260, L-364,718, PD-135,158, and YM-022 (Fig. 3B). The concentrations required to inhibit 50% of the specific binding (IC_{50}) are reported in Table 1. They are consistent with our previous report characterizing CCK_B receptors in Jurkat T cells (13, 14) and agree with a CCK_B receptor profile (5–8).

To confirm the role of CCK_B receptor on AP-1-regulated genes, we transiently cotransfected cells free of CCK_B receptors with both the plasmids p(TRE)3-tk-Luc and p(CCK_B/pCI Neo). As shown in Fig. 4A, in COS-7 cells, CCK-8 induced Luc activity in a dose-dependent manner ($EC_{50} \sim$ 3–5 nM). The induction was similar to that observed in Jurkat T cells, and

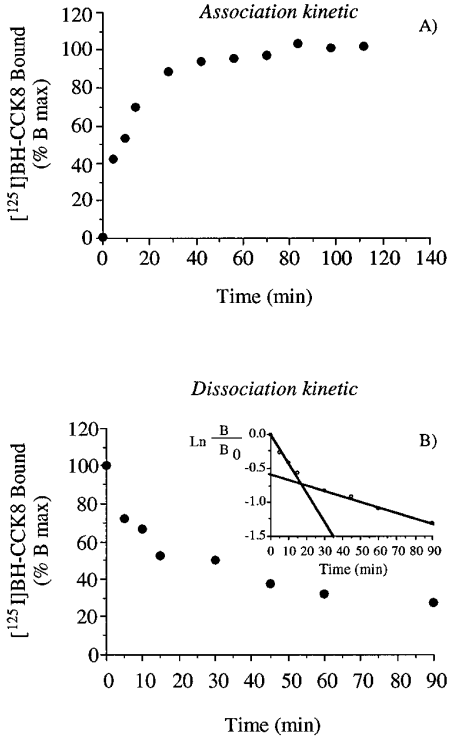

Fig. 2. Association and dissociation kinetics of ^{125}I-BH-CCK8 on COS-7 cells transiently expressing the recombinant CCK_B receptor of Jurkat T cells. COS-7 cells were transiently transfected with the plasmid p(CCK_B/pCI Neo). A, At 48 hr after transfection, cells were incubated at 37° with 10 pM ^{125}I-BHCCK-8 in the presence or absence of 1 μM CCK-8 for association kinetics. B, For dissociation kinetics, cells were incubated in the same conditions for 90 min, and CCK-8 (1 μM final concentration) was added, and the amount of specifically bound ^{125}I-BHCCK-8 was determined as a function of time. The presented experiment is a typical result obtained from three separate experiments.

the half-maximal Luc induction occurred at the same CCK-8 concentration. The direct implication of CCK_B receptor is shown in Fig. 4B: the Luc activity induced by 20 nM CCK-8 was completely inhibited by 200 nM concentration of the

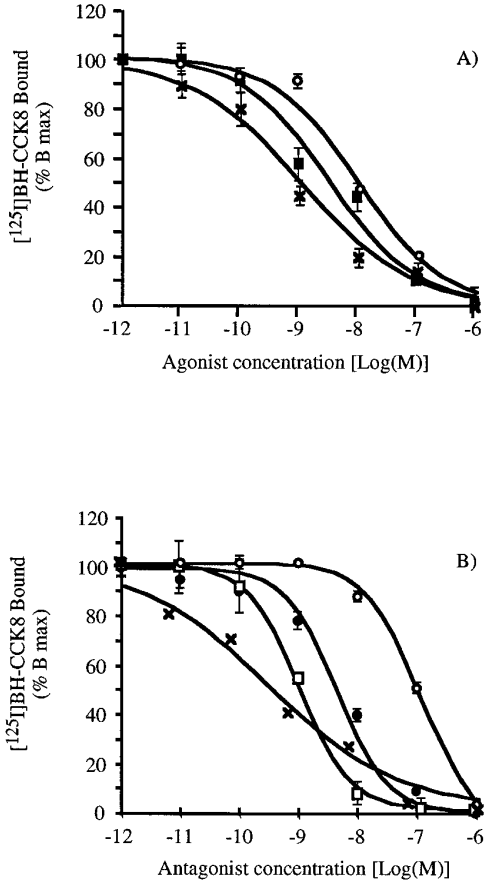

Fig. 3. Binding experiments of ^{125}I-BHCCK-8 on COS-7 cells transiently expressing the recombinant CCK_B receptor of Jurkat T cells. COS-7 cells were transiently transfected with the plasmid p(CCK_B/pCI Neo), and at 48 hr after transfection, binding experiments were performed as indicated in Experimental Procedures. The specific binding of 10 pM ^{125}I-BHCCK-8 was measured in the presence of various concentrations of CCK-8 (✕), gastrin(5–17) (○), and CCK-4 (■) (A) and L-365,260 (●), L-364,718 (○), PD-135,158 (✕), and YM-022 (□) (B).

TABLE 1

Abilities of different CCK/gastrin agonists and antagonists to inhibit binding of [^{125}I]BH-CCK-8 to COS-7 cells transiently transfected with the plasmid p(CCK-B/pCI Neo)

Ligand	IC_{50} nM	
	COS-7 cells[a]	Jurkat cells[b]
CCK-8	1 ± 0.2	0.3 ± 0.04
gastrin(5–17)	9.5 ± 1	0.4 ± 0.03
BOC-CCK-4	6 ± 2	1 ± 0.1
L-365,260	7.3 ± 1.5	3.3 ± 0.5
L-364,718	100 ± 15	100 ± 6
PD-135,158	1.6 ± 0.6	0.5 ± 0.09
YM-022	1.3 ± 0.5	0.6 ± 0.1

[a] The IC_{50} values were determined from competition binding assays with 10 pM ^{125}I-BH-CCK-8 in COS-7 cells transiently transfected with p(CCK-B/pCI Neo). Each value represents the mean ± standard deviation of at least three separate experiments performed in duplicate.

[b] IC_{50} values obtained from our previous study in Jurkat T cells (13). Each value represents the mean ± standard deviation of at least three separate experiments performed in duplicate.

CCK_B receptor antagonist PD-135,158. We realized the same kind of experiments in CV-1 cells free of CCK_B receptors. After cotransfection of both of the same plasmids, we found that CCK-8 induced Luc activity in a dose-dependent manner. This induction was similar to that obtained in COS-7 or

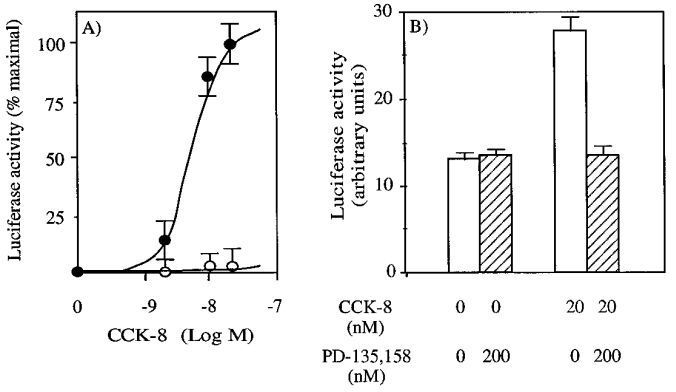

Fig. 4. CCK-8-induced Luc activity in COS-7 cells. As described in Experimental Procedures, COS-7 cells were transiently cotransfected with the plasmid p(TRE)3-tk-Luc and the plasmid p(CCK_B/pCI Neo) (●) or with the plasmid p(TRE)3-tk-Luc and the plasmid pCI Neo (○). At 48 hr after transfection, CCK-8 was added alone (A and B, □) or in the presence of 200 nM antagonist PD-135,158 (B, ▨). The presented experiment is a typical result obtained from three separate experiments. Luc activity was determined and results (mean ± standard error values) expressed as a percentage of the maximal induction or arbitrary units as previously described (19).

Jurkat T cells and was inhibited by PD-135,158 (data not shown). As a control of specificity, we found that in COS-7 cells free of CCK_B receptors cotransfected with both plasmids p(TRE)3-tk-Luc and pCI Neo, CCK-8 was unable to induce Luc activity (Fig. 4A).

To further characterize this phenomenon, we investigated the effect of CCK-8 in the absence and presence of okadaic acid or calyculin A in Jurkat T cells transiently transfected with the plasmid p(TRE)3-tk-Luc. These compounds are well known as inhibitors of serine/threonine-specific PP-1 and PP-2A. As a control and in agreement with the literature (19), in the presence of okadaic acid or calyculin A, the Luc expression induced by PMA was drastically increased (Fig. 5, *top*). Under the same experimental conditions, we did not observe any contribution of phosphatase inhibitors to the CCK-8-induced Luc expression (Fig. 5, *bottom*). There was no additional effect when the Jurkat T cells were incubated with the combination of phosphatase inhibitors and CCK-8, suggesting that the CCK_B receptor and the phosphatase inhibitors are acting by a similar mechanism.

Effect of activation of the CCK_B receptor in Jurkat T cells: induction of IL-2. To confirm that CCK-8 exerts a regulatory effect on natural and endogenous AP-1 regulated genes, we investigated its effect on IL-2 production in Jurkat T cells. AP-1 has been extensively described as one of the factors involved in IL-2 gene expression by acting via two TRE sequences (24, 25). Because determining IL-2 dose by measuring the growth of CTLL cells remained an indirect method (26), we directly evaluated IL-2 production by immunoassay. As shown in Fig. 6, 100 nM CCK-8 had no effect when added to 10 nM PMA. The combination of 10 nM PMA and 10 μM PHA led to an important secretion of IL-2 by Jurkat T cells, whereas PMA alone had no effect, and PHA alone had only a moderate effect. CCK-8 (1 or 100 nM) added to Jurkat T cells in combination with 10 nM PMA and 10 μM PHA significantly increased ($p < 0.05$ and $p < 0.001$, respectively) the effect of PMA and PHA. Quantification of IL-2 by radioimmunoassay clearly indicated that CCK-8 increased the effect of PMA and PHA by 10 units of IL-2/ml, corre-

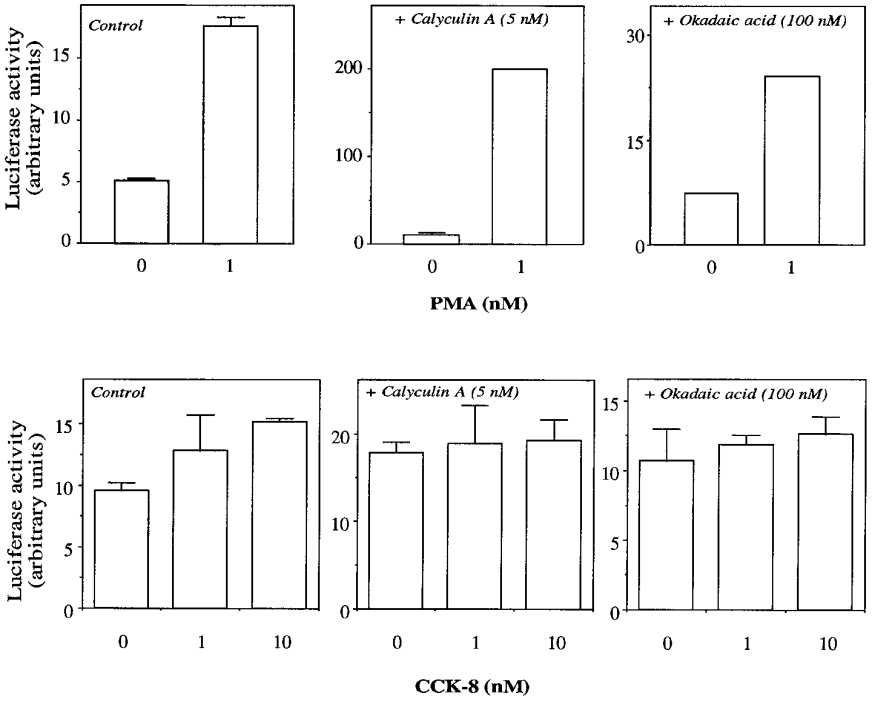

Fig. 5. Effect of serine/threonine-specific PP-1 and -2A inhibitors on the CCK-8-induced Luc activity in Jurkat T cells. Using Transfectam Reagent, Jurkat T cells were transiently transfected with the plasmid p(TRE)3-tk-Luc and treated as described in Experimental Procedures. The effect of serine/threonine-specific PP-1 and PP-2A inhibitors (100 nM okadaic acid or 5 nM calyculin A) was determined as a control in PMA-treated Jurkat T cells (*top*) and in CCK-8-treated Jurkat T cells (*bottom*). Results (mean ± standard error values) are expressed as arbitrary units (see legend to Fig. 1).

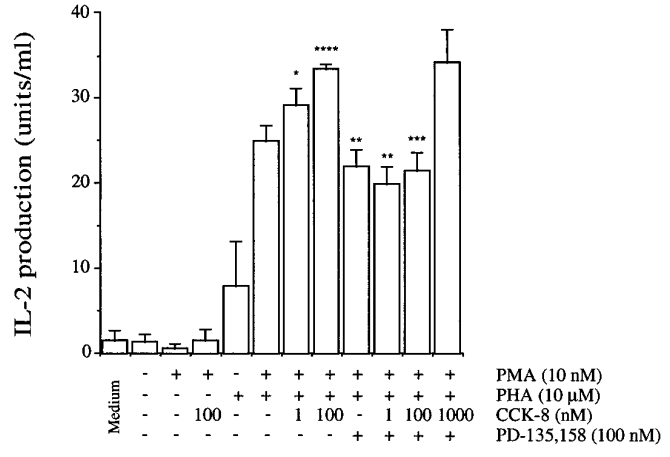

Fig. 6. IL-2 assay of CCK-8-treated Jurkat T cells. As described in Experimental Procedures, Jurkat T cells underwent various treatments, and the corresponding supernatants were tested for their IL-2 content by immunoassay. Values are mean from three independent experiments per dose in triplicate, and results are expressed as units of IL-2/ml. *Bars*, mean ± standard error values. *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$; ****, $p < 0.001$ versus 10 nM PMA/10 µM PHA (Student's *t* test).

sponding to 30% increase in the PMA/PHA effect. Although it was without any effect when tested alone, 100 nM concentration of the selective CCK_B receptor antagonist PD-135,158 completely inhibited the effect of 1 and 100 nM CCK-8 ($p < 0.02$ and $p < 0.01$, respectively), confirming the involvement of CCK_B receptors in this natural response induced by CCK. As a control, this inhibitory effect can be reversed by increasing the CCK-8 concentration to 1 µM.

Discussion

We studied the potential role of CCK-8 as a trophic agent in Jurkat T cells. Three main points justified such a study: (i) we previously reported the presence of CCK_B receptor on Jurkat T cells (13, 14) and no biological role of this receptor has been reported; (ii) under our experimental conditions, we found no specific binding of ^{125}I-BH-CCK-8 on normal lymphocytes (27)[1]; and (iii) G protein-coupled receptors (including CCK_B receptors), like several other membrane receptors (e.g., tyrosine kinase receptors, cytokine receptors), are involved in complex biological responses such as cell growth and differentiation. Although neuropeptide receptors do not generally possess intrinsic protein tyrosine kinase but signal through G proteins, it has been suggested that on activation by CCK-8, CCK_B receptors may induce cell proliferation in various cancerous cell lines through tyrosine kinase activity and stimulation of the mitogen-activated protein kinase cascade (8, 11, 28–30).

We thus investigated the trophic effect of CCK_B receptor in Jurkat T cells by studying the effect of CCK-8 on expression of genes controlled by AP-1. This was realized by using transient transfection of Jurkat T cells with the reporter plasmid p(TRE)3-tk-Luc. Such a plasmid led to expression of the firefly Luc when the *trans*-activator protein AP-1 was activated. We showed that CCK-8 was able to stimulate luciferase activity in a dose-dependent manner with a maximum induction occurring at ~20 nM CCK-8. As revealed by the EC_{50} value (~3–5 nM), this chimeric response occurred in the same CCK-8 concentration range as other classic and natural responses induced by CCK-8. The potent and selective CCK_B receptor antagonist PD-135,158 completely inhibited this CCK-8 effect, showing the direct involvement of the CCK_B receptor. This was also confirmed by testing the effect of CCK-8 in the absence of CCK_B receptors. In a breast cancer cell line (MDA MB231) stably transfected with the p(TRE)3-tk-Luc, we have not observed CCK-8 effect. This result clearly rules out any nonspecific effect of CCK-8 toward the p(TRE)3-tk-Luc construction. The maximal CCK-8 induction of Luc expression was identical to that obtained with 1 nM

[1] M.-F. Lignon and J. Martinez, unpublished observations.

PMA on Jurkat T cells and with epidermal growth factor or diacylglycerol in MDA MB 231 breast cancer cells stably transfected with p(TRE)3-tk-Luc (Ref. 19 and data not shown).

To confirm the role of CCK_B receptor in such a phenomenon, the cDNA encoding the CCK_B receptor from Jurkat T cells was cloned. It showed 100% sequence identity with the CCK_B receptor expressed in human brain (12), despite two silent mutations. When transiently expressed in COS-7 cells, this receptor had a similar behavior as that in Jurkat T cells as revealed by binding parameters of several ligands. We noted, however, some small differences in affinities (especially for the agonists) between the CCK_B receptor expressed in Jurkat T cells and the recombinant receptor transiently expressed in COS-7 cells. One possible explanation could reside in the nature of the cell type. Another possible explanation could be the density of the CCK_B receptor transiently expressed in COS-7 cells; agonists, in contrast to antagonists, tend to have lower affinities in systems in which receptors are overexpressed.

To study the CCK-8 effect on AP-1-regulated genes, we realized the same kind of experiments initially described in Jurkat T cells. COS-7 cells were transiently transfected both with the plasmid p(TRE)3-tk-Luc and the plasmid encoding the CCK_B receptor from Jurkat T cells (p(CCK_B/pCI Neo)). Like in Jurkat T cells, CCK-8 was able to stimulate luciferase activity in a dose-dependent manner ($EC_{50} \sim 5$ nM) with a maximum induction at ~20 nM. To prove the direct involvement of the CCK_B receptor in this Luc activation, we tested the effect of CCK-8 in cells free of CCK_B receptor. In COS-7 cells (or CV-1 cells) cotransfected both with the plasmids p(TRE)3-tk-Luc and pCI Neo, we showed that CCK-8 was unable to induce luciferase expression. The direct implication of the CCK_B receptor was confirmed by using the specific CCK_B receptor antagonist PD-135,158. Luc activity induced by 20 nM CCK-8 was totally inhibited by 200 nM of PD-135,158. The maximal response obtained on luciferase expression by CCK-8 activation in COS-7 cells transiently cotransfected with p(TRE)3-tk-Luc and p(CCK_B/pCI Neo) was similar to that obtained in Jurkat T cells transiently transfected with the plasmid p(TRE)3-tk-Luc (~2–3-fold). These results clearly indicate the direct implication of CCK_B receptor in AP-1-regulated gene expression.

To further investigate the mechanism of action of CCK-8 on Jurkat T cells transiently transfected with the plasmid p(TRE)3-tk-Luc, we studied the effect of CCK-8 in the absence and presence of okadaic acid and calyculin A, two serine/threonine-specific PP-1 and PP-2A inhibitors. As a control, we showed that these two compounds drastically increased the Luc expression induced by 1 nM PMA (current study and Ref. 19). Because PP-1 and PP-2A are generally considered to reverse PKC-mediated phosphorylations (31), our results are in agreement with several studies indicating that PMA activates AP-1 through a PKC-dependent pathway (17, 19, 22, 32). In contrast, under the same experimental conditions, okadaic acid and calyculin A have no effect on Luc expression induced by CCK-8, suggesting that CCK-8 could activate AP-1 through an independent PKC pathway. These results clearly differentiate the effect of CCK-8 from that of PMA to activate expression of an AP-1-regulated gene in Jurkat T cells. They are in agreement with a recent study in Rat1 fibroblasts transfected with the cDNA encoding the human CCK_B receptor showing that CCK-8 may activate mitogen-activated protein kinase in a PKC-independent manner (30). These authors pointed out that the signal transduction pathway resulting from activation of the CCK_B receptor to stimulate mitogen-activated protein kinase is different from that of another seven-transmembrane domain receptor such as bombesin receptor. In a previous study performed in our laboratory using Swiss 3T3 cells transfected with the plasmid p(TRE)3-tk-Luc, we also observed such a different behavior between CCK-8 and bombesin on Luc expression in the presence of okadaic acid or calyculin A.[2] These results suggest that in contrast to bombesin and PMA, CCK-8 could induce AP-1 activation through an independent PKC pathway, in accordance with Seufferlein *et al.* (30). Another possible explanation of the mechanism resides in a CCK-8 activation of phorbol ester-insensitive PKC isoforms (e.g., PKCζ), which may have different sensitivities to phosphatase inhibitors. Interestingly, it has been demonstrated that CCK_A receptor subtypes were phosphorylated on serine residues in response to CCK and that receptor phosphorylation was transient, even in the continued presence of CCK (33–35). In these experiments, the addition of okadaic acid increased the extent and duration of CCK-induced receptor phosphorylation. We cannot exclude that serine/threonine-specific protein phosphatase inhibitors may abolish CCK_B receptor activation by directly acting on the phosphorylation/dephosphorylation CCK_B receptor state. Another possible explanation would be that CCK-8 acts via a mechanism similar to that of phosphatase inhibitors because the effects were nonadditive. Among the multiple factors controlling differentiation and proliferative T cell processes, the T cell growth factor IL-2 plays a key role. Activation, differentiation, and growth of peripheral T lymphocytes as well as other lymphoid cells are mainly controlled by IL-2. Deregulation of IL-2 production is involved in maintaining the growth and the tumorigenicity of transformed T cells (36, 37). IL-2 also plays an important role in enhancing early thymocyte development, and it is expressed by thymocytes early during embryonic ontogeny (38, 39). It is known that Jurkat T cells are models for IL-2-secreting cells and that IL-2 is an important mediator of proliferation. However, it is known that IL-2 expression depends, at least in part, on AP-1 regulation (24, 25). No effect on IL-2 production could be seen when Jurkat T cells were treated with various doses of CCK-8. However, CCK-8 was able to increase the IL-2 production of Jurkat T cells activated by PHA plus PMA. The fact that the selective CCK_B receptor antagonist PD-135,158 completely abolished this response and that this inhibitory effect can be reversed by increasing CCK-8 concentrations determined the direct involvement of the CCK_B receptor in IL-2 production by activated Jurkat T cells. This study showed that CCK-8 was able to activate IL-2, a natural gene known to be regulated at least in part by AP-1, through the CCK_B receptor expressed on Jurkat T cells.

Taken together, these results showed that CCK-8 exerts a trophic effect on Jurkat T cells by increasing expression of AP-1-regulated genes via activation of the CCK_B receptor. Our conclusion is in agreement with Iwata *et al.* (40), who showed that CCK_B receptors mediate cell proliferation of several leukemia cells in a ligand-dependent manner.

[2] D. Gagne and J. Martinez, unpublished observations.

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