SR146131: A New Potent, Orally Active, and Selective Nonpeptide Cholecystokinin Subtype 1 Receptor Agonist. I: In Vitro Studies

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ABSTRACT

SR146131 inhibited the binding of [125I]-Bolton Hunter (BH)-sulfated cholecystokinin octapeptide (CCK-8S) for the human recombinant cholecystokinin subtype 1 (CCK_1) receptor (IC\textsubscript{50} = 0.56 nM) with high (300-fold) selectivity to the CCK\textsubscript{2} receptor. The biological activity of SR146131 was characterized in vitro in a NIH-3T3 cell line expressing the human recombinant CCK\textsubscript{1} receptor (3T3-hCCK\textsubscript{1}). Measuring intracellular calcium release, SR146131 behaved as a full agonist with an efficacy comparable with that of CCK-8S (EC\textsubscript{50} = 1.38 ± 0.06 nM). On individual cells, SR146131 induced, like CCK-8S, Ca\textsuperscript{2+} oscillations at subnanomolar concentrations and sustained responses at higher concentrations. Like CCK-8S, SR146131 also fully stimulated inositol monophosphate formation (EC\textsubscript{50} = 18 ± 4 nM). SR146131 partially activated mitogen-activated protein kinase and enhanced the expression of the immediate early gene krox 24. In the human CHP212 and IMR32 neuroblastoma cell lines, which constitutively express the CCK\textsubscript{1} receptor, SR146131 behaved as a partial agonist on intracellular calcium release and inositol monophosphate formation. All of these effects of SR146131 were inhibited by the CCK\textsubscript{1} receptor antagonists SR27897B and devazepide, suggesting that the effects of SR146131 were entirely mediated by the CCK\textsubscript{1} receptor. In contrast, high concentrations (>1 μM) of SR146131 had only minimal effects on CCK-8S-stimulated and unstimulated Chinese hamster ovary (CHO) cells expressing the human CCK\textsubscript{2} receptor, indicating that SR146131 is functionally inactive on the CCK\textsubscript{2} receptor. In conclusion, these in vitro experiments show that SR146131 is a highly potent and selective agonist of the CCK\textsubscript{1} receptor.

Two members of the cholecystokinin (CCK) receptor family have been identified to date (Ulrich et al., 1993; de Weerth et al., 1993; Wank et al., 1994). These subtypes were termed CCK\textsubscript{1} (or CCK\textsubscript{A}) and CCK\textsubscript{2} (or CCK\textsubscript{B}) due to their predominant occurrence in the digestive tract and in the brain, respectively. However, it is now clear that CCK\textsubscript{1} receptors are also present in the central and peripheral nervous system (Crawley and Corwin, 1994), whereas CCK\textsubscript{2} receptors, which are identical to gastrin receptors (Lee et al., 1993), are also found at the periphery (e.g., stomach and kidney).

CCK receptor subtypes have been characterized by various ligands. CCK\textsubscript{1} receptors show a high affinity for the sulfated cholecystokinin octapeptide (CCK-8S) and the nonpeptide specific receptor antagonists SR27897B (Gully et al., 1993) and devazepide (Chang and Lotti, 1986). CCK\textsubscript{2} receptors have a high affinity for CCK-8S, cholecystokinin tetrapeptide, gastrin, and the nonpeptide specific receptor antagonists L365,260 (Lotti and Chang, 1989) and PD134,308 (Hughes et al., 1990).

The signal transduction pathways linked to CCK\textsubscript{1} receptor activation have been extensively studied in pancreatic acinar cells, where CCK stimulates amylase secretion. CCK activates the hydrolysis of polyphosphoinositides by phospholipase C and the subsequent formation of the second messengers inositol 1,4,5-trisphosphate (Yule et al., 1993; Dunlop et al., 1997) and 1,2-diacylglycerol, leading to the release of intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) (Yule et al., 1993; Dunlop et al., 1997) and the activation of protein kinase C, respectively. In addition, stimulation of the CCK\textsubscript{1} receptor activates other intracellular events. CCK stimulates mitogen-activated protein kinase (MAPK) (Duan and Williams, 1994; Dabrowski et al., 1996, 1997; Tateishi et al., 1998).
obesity. As a consequence, a whole range of different CCK1 receptor agonists has been reported. The peptide derivatives, A-71623 (Lin et al., 1991), A-71378 (Lin et al., 1990; Holladay and Lin, 1992), and ARL-15849 (Simmons et al., 1998) were found to act as full agonists of CCK receptors in the guinea pig in vitro. Furthermore, GW5823, a nonpeptide compound, was also described to bind to the human CCK1 receptor with moderate affinity, to contract in vitro isolated gallbladder of the guinea pig, and to be a full agonist for calcium mobilization on Chinese hamster ovary (CHO) cells expressing the human CCK1 receptor (Henke et al., 1997).

However, the characterization of CCK1 agonists is complicated by the fact that the biological responses following CCK1 receptor activation vary across different species. This can be exemplified by JMV-180, a CCK7 analog, which stimulates amylase secretion from rat pancreatic acini to the same extent as CCK-8S but, unlike CCK-8S, does not reduce amylase secretion at high concentrations (Galas et al., 1988). JMV-180 even inhibits the decrease of amylase secretion produced by CCK-8S, and thus acts as an antagonist in this case. Considering these results, it has been proposed that JMV-180 may act as an antagonist at the high-affinity state of CCK receptors and as an agonist at the low-affinity state of CCK receptors (Stark et al., 1989). However, the profile of JMV-180 also varies considerably among different species. Thus, JMV-180 exhibits a full agonist profile as activator of amylase secretion in the mouse but behaves as a partial agonist in the guinea pig and rat (Matozaki et al., 1989; Bianchi et al., 1994). Such species-dependent differences in profile of JMV-180 also seem to be related to different effects of the compound on feeding behavior in vivo (Asin and Bednarz, 1992). Similarly, the secretory profile of tetrapeptide analogs of CCK, such as A70,784 and A57,282, varies considerably among these species (Bianchi et al., 1994). The activity of JMV-180, A70,784, and A57,282 on inositol phosphate formation in pancreatic acini was also shown to be species dependent (Bianchi et al., 1994). In this context, studies on the activation of CCK1 receptor-mediated responses in animal tissues are insufficient to ensure that a compound will be an agonist of the human CCK1 receptor. Studies on human tissues or cells are clearly required in this framework.

In the present study, we report the characterization of SR146131, a new potent and selective nonpeptide agonist of the CCK1 receptor. This compound is chemically related to the selective CCK1 receptor antagonist SR27897B (Fig. 1). The aim of this study was to characterize its in vitro properties at the level of the human recombinant and constitutive CCK1 receptors and to assess its specificity using the human recombinant CCK2 receptors (the in vivo evaluation is described in the companion paper by Bignon et al.).

**Experimental Procedures**

**Materials**

SR146131 (2-[4-(4-chloro-2,5-dimethoxyphenyl)-5-(2-cyclohexylethyl)thiazol-2-ylcarbamoyl]-5,7-dimethyl-indol-1-yl-1-acetic acid), was synthesized by Sanofi Recherche (Montpellier, France). SR27897B (Lintitripert), devazepide, L365,260 and PD134,308 were synthesized by Sanofi Recherche (Toulouse, France). [125I]-Bolton Hunter (BH)-CCK-8S (2000 Ci/mmol) and myo-[3H]inositol (80–120 Ci/mmol) were obtained from Amersham International (Buckinghamshire, UK). CCK-8S was obtained from NeoSystem (Strasbourg, France).

Fura-2 acetylomethyl ester (fura-2-AM) was from Molecular Probes (Interchim, Montluçon, France). Cell culture media, antibiotics, calf serum, and fetal calf serum were obtained from Gibco (Cergy Pontoise, France).

**Cell Cultures**

NIH-3T3 cells stably transfected with the human CCK1 receptor (3T3-hCCK1) were kindly provided by Dr. S. Wank (the National Institutes of Health, Bethesda, MD). 3T3-hCCK1 cells were routinely cultured in 175-cm2 flasks in Dulbecco’s minimum essential medium (DMEM) supplemented with 10% heat inactivated calf serum and the antibiotic gentamicin (G418) at a concentration of 300 µg/ml.

CHO cells stably transfected with the human CCK2 receptor (CHO-hCCK2) were kindly provided by Dr. D. Fourny (Institut National de la Santé et de la Recherche Médicale, Unité U151, Toulouse, France). CHO-hCCK2 cells were routinely cultured in 175-cm2 flasks in αMEM supplemented with 2.2 g/liter NaHCO3, 10% heat inactivated fetal calf serum, 100 µg/ml streptomycin, 100 µg/ml penicillin, and gentamicin (G418) at a concentration of 200 µg/ml.

The human neuroblastoma IMR32 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were routinely cultured in 75-cm2 culture flasks in MEM with Earle’s salts, nonessential amino acids, and 10% fetal calf serum.

The human neuroblastoma CHP212 cells were kindly provided by Dr. J. Biedler (Memorial Sloan-Kettering Cancer Center, New York). Cells were routinely cultured in 75-cm2 culture flasks in MEM with Earle’s salts, nonessential amino acids, and 10% fetal calf serum.

**[125I]-BH-CCK-8S Binding to CCK Receptors**

Preparation of Membrane Homogenates. Cells were cultured to confluence and the flasks were washed with 10 ml of PBS and
filled with an equal volume of PBS. Cells were detached from the flasks with a cell scraper. After centrifugation at 800g for 5 min, the cell pellet from 40 flasks was homogenized at 4°C using a Polytron (setting 4, 3 times 30 s) in 40 ml of buffer A (50 mM Tris-HCl pH 7.4, 5 mM MgCl₂, 0.1 g/liter phenylmethylsulfonyl fluoride, 0.1 g/liter bacitracin) in the case of 3T3-hCCK₁ cells, or buffer B (10 mM HEPES pH 6.5, 5 mM MgCl₂, 130 mM NaCl, 1 mM EGTA, 0.25 g/liter bacitracin) for CHO-hCCK₂ cells. After centrifugation at 45,000g (3T3-hCCK₁) or 30,000g (CHO-hCCK₂) for 15 min at 4°C, the cell pellet was homogenized at 4°C using a Teflon/glass potter in 30 ml of buffer A. Aliquots of the membrane suspension obtained were stored in liquid nitrogen.

**[125I]-BH-CKK-8S Binding to CCK Receptors.** [125I]-BH-CKK-8S binding to CCK receptors was performed according to a modification of the method described by Steigerwalt and Williams (1981) with 10 μg protein/tube of 3T3-hCCK₁, cell membranes and 45 pM of radiolabeled CCK. Agonists and antagonists were added in 1% DMSO (final concentration). After incubation at 25°C for 40 min, the incubation mixture was filtered on Whatman GF/B filters presoaked as above. The filters were washed with ice-cold buffer B. After incubation at 25°C for 40 min, the incubation mixture was washed with ice-cold buffer B and the dish was placed on the plate of the microscope.

**Measurement of [Ca²⁺], Release in Individual Cells**

3T3-hCCK₁ cells were plated in culture medium at a density of 0.5 to 1 × 10⁶ cells/well, into 35-mm diameter filament-coated Petri culture dishes in which a 16-mm diameter hole had been made on the bottom and covered by a thin (0.17-mm) glass coverslip stuck with a silicon glue.

**Loading Procedure.** After 1 to 3 days of culture, cells were incubated for 60 min at 20°C in the dark with 3 μM fura-2-AM in HEPES-Ringer buffer, pH 7.4, containing 145 mM NaCl, 5.6 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, 1 mM Na₂HPO₄, 5 mM glucose, 10 mM HEPES, and 0.1% BSA. Cells were washed twice with the same buffer and the dish was placed on the plate of the microscope.

**Measurement of [Ca²⁺]**

The bath was continuously perfused at 2 ml/min using a peristaltic pump equipped with silicon-coated tubes. The buffer was warmed to 37°C by passing through a temperature-controlled heating coil placed immediately before the cells. Before beginning the recording, cells were perfused for 2 to 4 min with the buffer, which contained, as needed, no added drug or one of the test compounds. The cells were allowed to stabilize for 90 s and were then exposed to the test substance. Fluorescent [Ca²⁺] images were measured on single isolated cells. Digital imaging was performed using an IMSTAR (Paris, France) imaging system. Cells were viewed with a Nikon (Tokyo, Japan) Diaphot-TMD microscope through a Nikon UV-Fluor 40× (NA 1.3) oil-immersion objective. Fura-2 fluorescence was excited alternatively at 350 and 380 nm. Cellular fluorescence was filtered by a 490- to 530-nm bandpass filter (Nikon) and measured with a Darkstar-800 CCD camera (Photonic Sciences, Millham, UK). Images were digitized via an 8-bit Imaging Technology Inc., Video Frame Grabber 512 × 512 × 4 numerization card into a PC 486/50 MHz computer and analyzed using IMSTAR STARWISE FLUO software. Ratiometric Ca²⁺ images were generated at 5-s intervals. Background compensation was performed by subtracting the illumination from an area of the image that contained no cells. The [Ca²⁺]⁰ values were calculated from the 350/380-nm fluorescence ratios as described by Grynkiewicz et al. (1985), using Rₘᵢₙ and Rₘₐₓ values of 0.35 and 8.00, respectively; the Kₐ of Ca²⁺ for fura-2 was assumed to be 224 nM (Grynkiewicz et al., 1985). Except when their [Ca²⁺] values were extraordinarily high or unstable, every cell in the field of the digitized image was quantified. The [Ca²⁺] values generated by the IMSTAR STARWISE FLUO software were transferred into an Excel software, which allowed displaying and printing of [Ca²⁺], in each cell for individual analysis.

**Determination of Inositol Phosphate Formation**

Cells cultured on six-well plate dishes were incubated for 24 h in culture medium containing 5 μCi/ml of myo-[³H]inositol. The cell monolayers were washed twice with PBS and incubated for 15 min with culture medium supplemented with 20 mM LiCl. Cells were then stimulated in the same medium containing the test compounds for an additional 40 min (CHO-hCCK₂ cells) or 120 min (3T3-hCCK₁ and neuroblastoma cells) at 37°C. At the end of the incubation period, the buffer was aspirated and the cells were extracted with an ice-cold methanol/HCl 0.1 N (50:50) solution. Extracts were then neutralized with 1 M Na₂CO₃, and [³H]inositol monophosphate (IP₁) separated as described by Berridge (1983) using columns containing 1 ml of AG1-X8 resin. IP₁ was eluted with 0.2 M ammonium formate/0.1 M formic acid. Radioactivity was quantified by liquid scintillation counting.

**MAPK Activity Measurements**

3T3-hCCK₁ cells grown to confluence in 6-well cluster plates were washed with fresh medium. Twenty-four hours later, the cells were stimulated for 15 min with various concentrations of CCK-8S or SR146131. The reaction was stopped by aspiration of the medium and by rapid addition of a lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₂HPO₄, 1 mM NaF, 0.25% Nonidet P40, 0.5% sodium.
deoxycholate, 1 mM dithiothreitol, 10 μg/ml leupeptin, and 10 μg/ml apro Cin. After 15 min of incubation at 4°C, the lysates were collected and centrifuged for 10 min at 800 × g. The MAPK activity present in the 800g supernatant was determined with the MAPK assay kit from Amersham.

Immediate Early Gene Activation

Isolation of RNA and S1 Nuclease Analysis. Cytoplasmic RNA was extracted from confluent cell monolayers lysed in extraction buffer (50 mM Tris-HCl, pH 8, 100 mM NaCl, 5 mM MgCl2, and 0.5% Nonidet P-40). After proteinase K (0.15 mg/ml) treatment and phenol extraction, RNA was ethanol precipitated, and the pellets were resuspended in water.

Quantitative S1 analyses were performed using 32P-labeled oligonucleotide probes complementary to krox 24 mRNA. The sizes and complementary positions of mouse oligonucleotide sequence (M22326 from GenBank) was: 45mer-krox 24 (1767 to 1811).

Solution hybridization was carried out overnight on 25 to 50 μg of cytoplasmic RNA at 52°C with 5 × 106 cpm of oligonucleotide (109 cpm/μg) in 0.16 M HEPES, pH 7.5, 0.33 mM EDTA, and 1 M NaCl. The excess probe was digested with 270 μl of buffer containing an S1 nuclease digestion solution (0.28 M NaCl, 0.05 M Na-acetate, pH 5.2, 4.5 mM ZnSO4, and 1000 U/ml of S1 nuclease) for an additional 60 min at 37°C. The reaction was stopped with 5 mM EDTA, and hybrids were precipitated with ethanol using 10 μg of transfer RNA as a carrier. The pellets were resuspended in the loading buffer (80% formamide, 10 mM EDTA, 1 mg/ml of xylene cyanol, and bromophenol blue) and analyzed on denaturing polyacrylamide/8 M urea gel. Autoradiography of dry gels was performed at ~80°C with an intensifying screen for 16 h. After densitometric analysis of the autoradiograms (Spot.sam system, Unilog, Grenoble, France), the amounts of mRNA were expressed as optical densities (O.D.) of protected bands normalized by the amount of hybridized cytoplasmic RNA. The data are expressed as percentage of respective control values and are the means ± S.E.M. of four independent determinations.

Data Analysis

Specific binding was defined as the difference between total and nonspecific binding. IC50 values were determined using a nonlinear least square regression analysis (Munson and Rodbard, 1980) using RS/1 (BBN Software Product Corporation, Cambridge, MA) and an internal computerized interactive procedure. Values were expressed as the mean ± S.E.M. of at least three determinations.

[Ca2+]i concentration increase in cell population, IP1 formation, and MAPK activity upon treatment with drug was defined as the difference between the level obtained in the absence (baseline) and in the presence of the drug. Results were expressed as a percentage of the level obtained in the same experiment with CCK-8S; they are the mean ± S.E.M. of at least three independent determinations. EC50 and IC50 values were determined using weighted least square fitting of experimental data to a four-parameter logistic model (Ratkowsky and Reedy, 1986) with a nonlinear curve fitting RS/1 program (BBN Software Products Corporation). Stimulation at the highest dose of SR146131 was compared with stimulation at the highest dose of CCK-8S (100%) by calculating the 95% confidence interval of the mean ratio. Paired Student’s t test was applied in the IP1 study in experiments, intergroup comparisons were performed using ANOVA, followed as appropriate by Dunnett’s test (for comparisons with a single control group), or Duncan’s test (for all pairwise comparisons).

Results

Affinity of SR146131 for Human CCK1, and CCK2 Receptors

As shown in Fig. 2A, SR146131 inhibited in a concentration-dependent manner the binding of [125I]-BH-CCK-8S to CCK1 sites on 3T3-hCCK1 cell membranes with an IC50 value of 0.56 ± 0.10 nM (mean ± S.E.M., n = 3), which was slightly better than that of CCK-8S (IC50 of 1.17 ± 0.11 nM). At much higher concentrations, SR146131 also inhibited the binding of radiolabeled CCK to CCK2 sites in CHO-hCCK2 membranes with an IC50 of 162 ± 27 nM. Under the same experimental conditions, CCK-8S inhibited the binding of radiolabeled CCK to CCK2 sites with an IC50 value of 0.31 ± 0.07 nM (Fig. 2B). Moreover, SR146131 did not interact (IC50 > 10 μM) with over 50 receptors of nonpeptide (e.g., histamine, α- and β-adrenergics, opiates, dopamine, serotonin, adenosine, benzodiazepine, and L-type calcium channel) or peptide ligands (e.g., neuropeptide Y, vasopressin, galanin, and endothelin) (data not shown).

Because the biological activity of CCK1 receptor agonists is often species dependent (Matozaki et al., 1989; Bianchi et al., 1994), and because the in vivo pharmacological effects of the compounds were determined in rodents (Bignon et al., companion paper), we studied the effect of SR146131 on the binding of [125I]-BH-CCK-8S to rat pancreatic membranes. SR146131 inhibited the binding of [125I]-BH-CCK-8S to CCK1 receptors on these membranes, with an IC50 value of 0.84 ± 0.09 nM, which was slightly better than that of CCK-8S (IC50 = 1.65 ± 0.16 nM) (data not shown).

Effects of SR146131 on 3T3-hCCK1 Cells

[Ca2+]i Increase in Cell Population. In 3T3-hCCK1 cells, CCK-8S and SR146131 induced a strong and transient
increase of [Ca\(^{2+}\)]. As shown in Fig. 3A, CCK-8S and SR146131 stimulated in a concentration-dependent manner the increase of [Ca\(^{2+}\)]. SR146131 demonstrated an efficacy comparable with that of CCK-8S (EC\(_{50}\) of 1.38 ± 0.06 nM and 1.08 ± 0.28 nM, respectively), with a mean maximum stimulation of 81% of CCK-8S, (minimum, 70%; maximum, 99%, P > .05), thus indicating that SR146131 is a full CCK1 receptor agonist on this cell system response. The selective CCK1 receptor antagonists SR27897B and devazepide totally inhibited the increase of [Ca\(^{2+}\)] induced by 100 nM SR146131 (IC\(_{50}\) of 1.53 ± 0.32 nM and 2.07 ± 0.19 nM, respectively; Fig. 3B), suggesting that these effects of SR146131 were entirely mediated by the CCK1 receptor.

[Ca\(^{2+}\)] Increase in Individual Cells. Superfusion of 3T3-hCCK1 cells with CCK-8S resulted in an increase in [Ca\(^{2+}\)]. At low CCK-8S concentrations, the signal consisted of [Ca\(^{2+}\)] oscillations (repetitive transient increases in free [Ca\(^{2+}\)] levels) (Fig. 4A), but at higher concentrations, CCK-8S induced a typical biphasic response consisting of a large peak followed by a slow return to baseline (Fig. 4B). SR146131 also induced [Ca\(^{2+}\)] oscillations at low concentrations and provoked a large peak at higher concentrations. The effects of SR146131 and CCK-8S on single 3T3-hCCK1 cells were also compared by the analysis of the percentage of the responding cells in terms of oscillation or peaks (Fig. 4C). Low concentrations of CCK-8S and SR146131 induced oscillations in these cells, and higher concentrations of the two agonists induced peaks with a concomitant decrease of oscillations. CCK-8S induced peaks in 100% of the cells with an EC\(_{50}\) value of 0.20 ± 0.08 nM (n = 5). SR146131 was as potent as CCK-8S and also induced peaks in 100% of the cells (Fig. 4C), with an EC\(_{50}\) value of 0.75 ± 0.20 nM (n = 5). The maximum amplitudes of the peaks induced by 10 nM CCK-8S and 100 nM SR146131 were similar [% of basal values of 316 ± 40 (n = 5), and 302 ± 47 (n = 5), respectively; data not shown]. The selective CCK1 receptor antagonist SR27897B, at a concentration of 100 nM, antagonized the oscillations induced by 1 nM SR146131 or 100 pM CCK-8S, and also prevented the peaks induced by 10 nM SR146131 or 1 nM CCK-8S (inhibition of 89% and 75%, respectively; data not shown). Similar results were obtained with the CCK1 receptor antagonist devazepide. Thus, when measured in single 3T3-hCCK1 cells, SR146131 presents a CCK1 receptor agonist profile very close to that observed for CCK-8S itself.

Inositol Phosphate Formation. As shown in Fig. 5A, in 3T3-hCCK1 cells, CCK-8S as well as SR146131 stimulated IP3 formation in a concentration-dependent manner with EC\(_{50}\) values of 2.2 ± 0.4 nM and 18 ± 4 nM, respectively. For
SR146131, the maximal activation of IP₃ formation, occurring at approximately 100 nM, was comparable with that of CCK-8S (88% of CCK-8S effect: minimum, 81%; maximum, 95%, P > .05). Both specific CCK₁ receptor antagonists SR27897B and devazepide inhibited IP₃ formation induced by 10 nM SR146131 with IC₅₀ values of 2.4 ± 1.3 nM and of 3.3 ± 1.0 nM, respectively (Fig. 5B).

**MAPK Activity.** The capacity of CCK-8S and SR146131 to stimulate MAPK activity in 3T3-hCCK₁ cells is shown in Fig. 6. As found for [Ca²⁺], and IP₃ formation, CCK-8S activated MAPK activity at subnanomolar concentrations (EC₅₀ of 0.55 ± 0.15 nM), whereas SR146131 was less potent in inducing this effect (EC₅₀ of 290 ± 80 nM). The maximum MAPK activation observed at 10 µM SR146131, although not significantly different than that observed with 1 µM CCK-8S, represented only 73% (minimum, 54%; maximum, 83%, P > .05) of the CCK-8S effect. The MAPK activity induced by 1 µM SR146131 was completely and concentration dependently inhibited by both SR27897B and devazepide, with IC₅₀ values of 14 ± 2 nM and 2.5 ± 1.0 nM, respectively, demonstrating the CCK₁ receptor-dependence of this effect (Fig. 6B).

**Immediate Early Gene Activation.** Preliminary experiments indicated that the maximal effect of CCK-8S on krox 24 mRNA levels in the 3T3-hCCK₁ cells was obtained at 10 nM (data not shown). In the present study, CCK-8S (10 nM), but not SR146131 (10 nM) increased krox 24 mRNA after 15 min of incubation (Fig. 7A). The maximum effect of CCK-8S was observed after 30 min of incubation, and SR146131 also increased krox 24 mRNA levels at this time. The effect of both compounds was less after 60 min, remaining significant for CCK-8S but not for SR146131. As shown in Fig. 7B, SR146131 induced a gradual, concentration-dependent increase in krox 24 mRNA expression, which attained statistical significance at the concentration of 10 nM. However, the increase in krox 24 mRNA produced by SR146131 (100 nM) was significantly lower than that produced by CCK-8S (10 nM) (t = 8.53, P < .05). SR27897B (100 nM) antagonized the increase in krox 24 mRNA produced by both CCK-8S [10 nM, F(2,8) = 145.25, P < .01] and SR146131 [100 nM, F(2,9) = 33.87, P < .01] (data not shown).

**Effects of SR146131 in Human CHP212 and IMR32 Neuroblastoma Cells**

Two human neuroblastoma cell lines, IMR32 and CHP212, have been reported to constitutively express a small number of CCK₁ receptors (Barrett et al., 1989; Schaeffer et al., 1994). We showed previously that [¹²⁵I]-BH-CCK-8S specifically labels CCK₁ receptors on IMR32 neuroblastoma cells (Schaeffer et al., 1994). SR146131 inhibited [¹²⁵I]-BH-CCK-8S with an IC₅₀ value of 31 ± 17 nM, showing that it interacts with CCK₁ receptors on IMR32 cells. However, SR146131 not only bound to CCK₁ receptors on these cells,
but also activated these receptors: as shown in Fig. 9A, 30 nM SR146131 induced a clear but transient increase of [Ca\(^{2+}\)]\(_i\) in IMR32 neuroblastoma cells (higher concentrations of SR146131 could not be studied in this particular assay because the autofluorescence of the compound interfered with the measurement). This effect (mean maximum stimulation of 39 ± 3% (minimum, 35%; maximum, 43%) was significantly different (P < .05) from the maximal effect of CCK-8S (100 nM). The effect of SR146131 was related to CCK\(_1\) receptor activation, because it was abolished by low concentrations (10 nM) of the CCK\(_1\) receptor antagonists SR27897B (83 ± 4% inhibition, n = 4) or devazepide, (78 ± 6% inhibition, n = 4).

Further evidence for the activation of CCK\(_1\) receptors by SR146131 was obtained by studying the effect of the agonist on phosphoinositide turnover in these cells. As shown in Fig. 8B, incubation with 1 \(\mu\)M SR146131 nearly doubled the level of IP\(_1\) in these cells. This effect of SR146131, which was observed at low concentrations of the compound (EC\(_{50} = 6.0 ± 3.1\) nM), represented 38 ± 10% (minimum, 20%; maximum, 68%, P < .05) of the maximal effect of CCK-8S (100 nM) and was CCK\(_1\) receptor-mediated, because it was strongly decreased by the addition of 1 \(\mu\)M SR27897B (47 ± 23% inhibition, n = 3) or 100 nM devazepide (86 ± 5% inhibition, n = 3).

Similar effects were observed in CHP212 neuroblastoma cells (Fig. 9A) in which SR146131 (30 nM) induced an increase in [Ca\(^{2+}\)]\(_i\), which represented 54 ± 4% (minimum, 50%; maximum, 58%, P < .05) of the maximal effect of CCK-8S (100 nM). This effect of SR146131 was strongly inhibited by 100 nM SR27897B (75 ± 5% inhibition, n = 3) as well as by 10 nM devazepide (50 ± 13% inhibition, n = 3). SR146131 also stimulated phosphoinositide turnover in these cells, with an EC\(_{50}\) value of 9.3 ± 2.8 nM (Fig. 9B). This effect of SR146131, which reached 66 ± 8 (minimum, 54%; maximum, 86%, P < .05) of the maximal effect of CCK-8S (100 nM), was again due to a selective activation of the CCK\(_1\) receptor (Fig. 9B), because it was abolished by 100 nM SR27897B (99 ± 1% inhibition, n = 4) and devazepide (100 ± 1% inhibition, n = 3).

Further evidence for the agonist activity of SR146131 was obtained on the increase of [Ca\(^{2+}\)]\(_i\) in individual CHP212 cells (data not shown). SR146131 induced peaks in 61% of the cells with an EC\(_{50}\) value of 9.3 ± 3.4 nM (n = 5). CCK-8S was slightly more potent and induced peaks in 92% of the cells, with an EC\(_{50}\) value of 2.8 ± 0.9 nM (n = 4). The maximal amplitudes of the peaks induced by 1 \(\mu\)M SR146131 or CCK-8S were similar [256 ± 43 (n = 5), and 271 ± 49 (n = 4) % of basal values, respectively].

Effects of SR146131 in Human CHO-hCCK\(_2\) Cells

Functional activity at the CCK\(_2\) receptor was determined in the CHO-hCCK\(_2\) cell line. In these cell line, CCK-8S stimulated IP\(_1\) formation with an EC\(_{50}\) value of 0.94 ± 0.11 nM (Fig. 10A). This effect was completely inhibited by the selective CCK\(_2\) antagonists L365,260 and PD134,308, with IC\(_{50}\) values of 25 ± 5 nM and 12 ± 2 nM, respectively (Fig. 10B). At a 10-\(\mu\)M concentration, SR146131 did not stimulate IP\(_1\) formation (Fig. 10A) and slightly (30%) but significantly (t(2) = 9.41, P < .05) inhibited 3 nM CCK-8S induced IP\(_1\) formation (Fig. 10B).

Discussion

Although molecular cloning of CCK\(_1\) receptors has revealed a high degree of homology across species (Wank, 1998), and CCK-8S has similar pharmacological profiles in a variety of animal species including humans, the biological activity of synthetic CCK\(_1\) agonists is often species-dependent. For example, the CCK\(_1\) receptor agonists JMV-180 and cholecystokinin tetrapeptide derivatives elicited responses varying from antagonist to full agonist activities (relative to CCK) on [Ca\(^{2+}\)]\(_i\) uptake or phosphoinositide breakdown in human neuroblastoma cells or pancreas of rats, mice, and guinea-pigs (Schaeffer et al., 1994; Bianchi et al., 1994). For this reason, and with the objective to develop a therapeutic agent, we determined the biochemical characteristics of SR146131 on the human CCK\(_1\) and CCK\(_2\) receptors.

SR146131 is the first potent nonpeptide, selective CCK\(_1\) receptor agonist reported to date. Moreover, it is one of the rare examples in which we were able, starting from a full antagonist SR27897B, to design an agonist of a neuropeptide receptor. SR146131 binds with a very high affinity (IC\(_{50} = 0.56\) nM) to the human CCK\(_1\) receptor. The affinity of
SR146131 for the CCK₁ receptor was slightly better than that of CCK-8S (IC₅₀ of 1.17 ± 0.11 nM). SR146131 shows a high level of selectivity for the CCK₁ receptor versus the CCK₂ receptor (300-fold) and a variety of receptors of non-peptide or peptide ligands. In this respect, SR146131 has a much higher affinity for the human CCK₁ receptor and higher selectivity over the CCK₂ receptor compared with the recently reported CCK₁ receptor agonist: the 1,5-benzodiazepine GW5823 (Henke et al., 1997).

In vitro biological characterization of SR146131 in 3T3-hCCK₁ cells showed that SR146131 is a full agonist on [Ca²⁺]ᵢ release with an efficiency comparable with that of CCK-8S (EC₅₀ ~ 1 nM). The increase of [Ca²⁺]ᵢ is probably due to calcium release from intracellular stores, because the measurements were performed in the absence of extracellular calcium. The effects of CCK-8S were similar to those reported in CHO cells expressing the CCK₁ receptor (Dunlop et al., 1997). SR146131 is more potent than GW5823, reported to be a full agonist in CHO cells bearing the human CCK₁ receptor (Henke et al., 1997). On individual 3T3-hCCK₁ cells, SR146131 induced, like CCK-8S, oscillations and peaks of calcium: the proportion of the cells that responded with oscillations or peaks varied as a function of the concentrations tested. The maximum amplitudes of the peaks induced by CCK-8S and SR146131 were very similar, confirming that SR146131 is a potent and full CCK₁ receptor agonist on this type of response. These results suggest that SR146131 is a much more potent CCK₁ receptor agonist than JMV180, which, in contrast, appears to exhibit a partial agonist or agonist-antagonist profile at the CCK₁ receptor (Schaeffer et al., 1994). For example, JMV180 induces sustained calcium...

Fig. 8. Effect of SR146131 on [Ca²⁺]ᵢ and phosphoinositide turnover in IMR32 human neuroblastoma cells. A, effect of SR146131 (30 nM) on [Ca²⁺]ᵢ in IMR32 cells. Arrow indicates time at which vehicle or SR146131 (30 nM) were added to fura-2-loaded neuroblastoma cells. Data are representative of three to seven experiments. B, effect of SR146131 on phosphoinositide turnover in IMR32 cells. Cell monolayers were incubated with increasing concentrations of SR146131 for 120 min at 37°C and IP₃ formation was determined as described in Experimental Procedures. Data are representative of three to five experiments performed in triplicate. Bars represent S.E.M.

Fig. 9. Effect of SR146131 on [Ca²⁺]ᵢ and phosphoinositide turnover in CHP212 human neuroblastoma cells. A, effect of SR146131 (30 nM) on [Ca²⁺]ᵢ in CHP212 cells. Arrow indicates time at which vehicle or SR146131 (30 nM) were added to fura-2-loaded CHP212 cells. Data are representative of two to three experiments. B, effect of SR146131 on phosphoinositide turnover in CHP212 cells. Cell monolayers were incubated with increasing concentrations of SR146131 for 120 min at 37°C and IP₃ formation was determined as described in Experimental Procedures. Data are representative of four experiments performed in triplicate. Bars represent S.E.M.
responses in only 65% of CHO cells stably transfected with the cloned rat CCK₁ receptor (Yule et al., 1993), and in rat pancreatic acini, the peptide elicits only calcium oscillations and acts as an antagonist to block the effect of high concentrations of CCK-8S to induce large transient increases of [Ca²⁺]. (Matozaki et al., 1990).

The present study also clearly demonstrates that SR146131 stimulates with a high efficacy (EC₅₀ of 18 ± 4 nM) IP₁ formation in 3T3-hCCK₁ cells. The amplitude of the effect induced by SR146131 was similar to that elicited by CCK-8S, demonstrating that SR146131 is a full agonist on this response. These results again indicate a stronger CCK₁ receptor agonist activity of SR146131 versus that observed for JMV180, because the latter could only slightly stimulate IP₁ formation in isolated pancreatic acini from rodents (Bianchi et al., 1994) and in CHO cells stably transfected with the cloned rat CCK₁ receptor (Yule et al., 1993). In 3T3-hCCK₁ cells, the agonist activity of SR146131 on calcium release and IP₁ formation (EC₅₀: 1.38 and 18 nM, respectively) was consistent with its affinity for the CCK₁ receptor. Moreover, the selective CCK₁ receptor antagonists SR27897B or devazepide inhibited in a concentration-dependent manner these agonist effects of SR146131, therefore suggesting that the observed effects of SR146131 are entirely mediated by the CCK₁ receptor.

It has been shown previously that CCKₐ receptors are capable of interacting with multiple G proteins and that the signal observed upon stimulation depends on the nature of the host cell and its diversity of intracellular signaling components (Yule et al., 1993). In the present study, we investigated the effect of SR146131 on the activity of MAPK, another coupling mechanism previously described to be linked to CCK₁ receptor activation (Duan and Williams, 1994). SR146131, like CCK-8S, stimulated MAPK activity in 3T3-hCCK₁ cells. However, the maximal stimulation produced by SR146131 was lower than that of CCK-8S, and higher concentrations of SR146131 were required to activate MAPK than to stimulate [Ca²⁺], or IP₁ formation. Nevertheless, we found that this response was antagonized in a concentration-dependent manner by SR27897B and devazepide, demonstrating a selective CCK₁ receptor-dependent activation by SR146131.

Recently, Day et al. (1994) showed that the selective activation of CCK₁ receptors increased the levels of a number of immediate early gene mRNAs, including krox 24 (nerve growth factor-induced gene A) and c-fos in several brain regions in vivo. We have found that the incubation of 3T3-hCCK₁ cells with SR146131 and CCK-8S increased mRNA of krox 24 (and also of c-fos, our unpublished observation), supporting the observation of Day et al. (1994), and indicating that SR146131 behaved as a CCK₁ receptor agonist on immediate early gene expression. However, SR146131 was unable to activate this early gene to the same maximum extent as CCK-8S. Furthermore, the effect of SR146131 differs from that of JMV-180, which has been shown not to stimulate the mRNA levels of transcription factors but rather to inhibit the increase in c-fos mRNA induced by CCK-8S (Lu and Logsdon, 1992).

Collectively, these results suggest that SR146131 elicits a more efficient coupling of the CCK₁ receptor with phospholipase C than with the krox 24 and MAPK pathways, MAPK activation and krox 24 expression probably being linked, as already suggested in another receptor-coupled system (Bouaboula et al., 1995).

SR146131 activated CCK₁ receptors not only in transfected cells, but also in cells expressing this receptor in a constitutive manner: IMR32 and CHP212 human neuroblastoma cell lines. In IMR32 cells, SR146131 inhibited the binding of radiolabeled CCK-8S and increased [Ca²⁺], and IP₁ formation. Although maximal activation of [Ca²⁺] increase and IP₁ formation was lower than that observed for CCK-8S, SR146131 appeared as a much more stronger agonist than the CCK analog JMV180, which was devoid of agonistic activity but acted as an antagonist in this cell line (Schaeffer et al., 1994). Similarly in CHP212 cells, SR146131 stimulated phosphoinositide turnover and [Ca²⁺] increase to 54% and 66%, respectively, of the maximal effect of CCK-8S. The partial agonist effect of SR146131 on [Ca²⁺] in this cell population might be related to the fact that only 61% of the cells induced peaks (when measured in individual cells) during SR146131 perfusion (versus 92% with CCK-8S). On the other hand, the maximal amplitudes of the peaks induced in the responding cells by SR146131 and CCK-8S were similar.

Because the in vivo pharmacological effects of the compounds were determined in rodents (Bignon et al., companion...
SR146131 is a very potent and selective nonpeptide agonist of CCK receptor in CHO cells expressing the human recombinant CCK-1 receptor. Our in vitro study demonstrated that SR146131 is a potent CCK-A agonist on several intracellular events linked to CCK receptor activation in various cell types: on Ca" release and IP3 formation, SR146131 appears as a full CCK-1 receptor agonist in the 3T3-hCCK1 cells, but a partial CCK-1 receptor agonist on MAPK activation and early gene expression in this cell line. SR146131 also acts as a partial agonist in the two neuroblastoma cell lines that we have studied. SR146131 appears to be much more potent than the previously described nonpeptide CCK-1 receptor agonists. In summary, SR146131 is a very potent and selective nonpeptide agonist of the CCK-1 receptor, which might be useful for the treatment of eating disorders and obesity.

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