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Characterisation of the effects of SR146131, a novel non-peptide CCK_1 receptor agonist, on IMR-32 human neuroblastoma cells

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Abstract

The effect of {2-[4-(4-chloro-2,5-dimethoxy-phenyl)-5-[2-cyclohexyl-ethyl)-thiazol-2-ylcarbamoyl]-5,7-dimethyl-indol-1-yl}-acetic acid (SR146131), a novel non-peptide agonist of cholecystokinin (CCK) CCK_1 receptors, was compared to the effect of sulphated cholecystokinin octapeptide (CCK-8-S) on CCK_1 receptors of the human neuroblastoma cell line IMR-32. SR146131 inhibited [^{125}I]CCK-8-S binding to IMR-32 cells at nanomolar concentrations. SR146131 and CCK-8-S increased intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) in the same concentration range ($EC_{50} = 6 \pm 2.3$ and 1.3 ± 0.14 nM, respectively). Although the shape of the $[Ca^{2+}]_i$ increase induced by CCK-8-S and SR146131 was slightly different, extracellular Ca^{2+} removal affected the response of both compounds to a similar degree, and the response of both compounds was essentially due to Ca^{2+} release from intracellular stores. This was also confirmed by measuring the $[Ca^{2+}]_i$ response of single cells: both compounds induced $[Ca^{2+}]_i$ oscillations at subnanomolar concentrations and elicited a large peak increase in $[Ca^{2+}]_i$ at higher concentrations ($EC_{50} = 0.5 \pm 0.04$ and 5.7 ± 1.9 nM for CCK-8-S and SR146131, respectively). Both CCK-8-S and SR146131 induced a sustained increase of phosphoinositide turnover in these cells, and acted at similar concentrations ($EC_{50} = 2.7 \pm 0.7$ and 6 ± 3.1 nM, respectively), although the maximal effect of SR146131 was somewhat lower than the effect of CCK-8-S. These data show that SR146131 activates human CCK_1 receptors on IMR-32 cells in a manner and with a potency similar to that of CCK-8-S.

Keywords: Cholecystokinin; SR146131; $[Ca^{2+}]_i$; Phosphoinositide turnover; Binding; (Human)

1. Introduction

Cholecystokinin (CCK), released into the circulation after a meal, exerts numerous effects at different levels of the digestive apparatus, acting through either CCK_1 (gall bladder, pancreas) or CCK_2 (stomach, brain) receptors (for review see Crawley and Corwin, 1994). Selective antagonists of CCK_1 (devazepide, Chang and Lotti, 1986; SR27897B, Gully et al., 1993) and CCK_2 receptors (L365260, Lotti and Chang, 1989; PD134308, Hughes et al., 1990) have been described (for review see Mahovec, 1993; D'Amato et al., 1994) and because of their possible use as regulators of food intake (Gibbs et al., 1973), CCK_1 receptor agonists have generated a considerable interest. Selective non-peptide agonists of these receptors have appeared recently in the literature but, due to their pharmacokinetics, these compounds showed poor pharmacological efficacy (Aquino et al., 1996; Henke et al., 1997). In this respect, {2-[4-(4-Chloro-2,5-dimethoxy-phenyl)-5-[2-cyclohexyl-ethyl)-thiazol-2-ylcarbamoyl]-5,7-dimethyl-indol-1-yl}-acetic acid (SR146131) has been recently described to be as potent as CCK-8-S in several models of gall bladder and gastric emptying as well as on food intake in rats and marmosets (Bignon et al., 1999b). In vitro, SR146131 inhibits sulphated cholecystokinin octapeptide (CCK-8-S) binding to human CCK_1 receptors at nanomolar concentrations, and induces $[Ca^{2+}]_i$ increase and phosphoinositide turnover both in human CCK_1 receptor-transfected cells, and in human cells naturally expressing CCK_1 receptors (Bignon et al., 1999a). However, a detailed comparison of SR146131 with CCK-8-S on human CCK_1

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receptors in a human cell environment has not yet been reported, athough it is well known that at least some aspects of CCK_1 receptor activation differ between human CCK_1 receptors transfected into animal cells and those naturally expressed in neuroblastoma cells (Rao et al., 1997).

Here, we have therefore compared the effect of SR146131 and CCK-8-S on IMR-32 cells, a human neuroblastoma cell line in which we have already characterised the presence of CCK_1 receptors (Schaeffer et al., 1994).

2. Materials and methods

2.1. Materials

Minimum essential medium (MEM) was from Sigma (Saint Quentin Fallavier, France). [Tyr(SO_3H)^{27}]cholecystokinin fragment 26–33 amide (CCK-8-S) was either from Sigma or from Neosystem (Strasbourg, France). Fetal calf serum and human fibronectin were from Boehringer (Meylan, France). *myo*-[^{3}H]inositol (100 Ci/mmol) and Bolton-Hunter-labeled CCK-8-S ([^{125}I]CCK-8-S) (2000 Ci/mmol) were from Amersham (Les Ulis, France). Devazepide, L365260, PD134308, SR27897B, and SR146131 were synthesised at Sanofi Recherche (Toulouse and Montpellier, France). The acetoxymethyl ester of fura-2 (fura-2/AM) was from Molecular Probes (Interchim, Montluçon, France).

2.2. Cell culture

IMR-32 cells were obtained from the American Type Culture Collection (Rockville, USA) (ATCC, CCL 127). IMR-32 cells were routinely cultured in 75 cm^2 flasks in MEM (Eagle) with Earle's salts, non-essential amino acids, and 5% or 10% (optimised for each batch) foetal calf serum and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml).

2.3. Binding experiments

Confluent cell monolayers were preincubated for 30 min with competitors and then incubated for 1 h at 37°C with 1.2 nM of [^{125}I]CCK-8-S (specific activity reduced to 267 Ci/mmol by unlabeled CCK-8-S) in modified physiological salt solution (PSS) (composition: NaCl 145 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1 mM, glucose 5.6 mM, HEPES/NaOH 5 mM; pH 7.4) containing bovine serum albumin 1 g/l and bacitracin 100 mg/l. The reaction was stopped by three rapid washes with ice-cold PSS, cells were digested with NaOH and radioactivity in the resulting solution determined in a γ counter. Non-specific binding was assessed by addition of 1 μM of unlabeled CCK-8-S. Experimental results were analysed by an in-house binding analysis program similar to LIGAND (Munson and Rodbard, 1980).

2.4. Cytosolic free Ca^{2+} measurements on cell populations

Neuroblastoma cells cultured in 75 cm^2 flasks were detached with a non-enzymatic cell dissociation solution (Sigma), scraped from the flasks, centrifuged and resuspended in PSS containing fura-2/AM (1 μM) and incubated at 37°C for 30 min. The cell suspension was then diluted five times with PSS, and incubated for a further 60 min at 37°C. After two washes with PSS to remove extracellular fura-2, cells were resuspended in PSS and kept in the dark at room temperature. Experiments were carried out under constant stirring in a PTI spectrofluorometer using about 300,000 cells in 3 ml fluorescence cuvettes at 37°C. Results were corrected for extracellular fura-2 by the manganese quench method and $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. (1985). CCK-8-S (100 nM) was always tested in parallel with SR146131 and results expressed relative to the $[Ca^{2+}]_i$ increase induced by CCK-8-S.

2.5. Single cell $[Ca^{2+}]_i$ measurements

Cells were plated into 35-mm diameter fibronectin-coated Petri culture dishes in which a 16-mm diameter hole had been cut in the base and replaced by a thin glass coverslip stuck with a silicon glue. After 4–6 days of culture, 3 μl fura-2/AM taken from a 3 mM stock solution in DMSO was loaded into the cells for 20 min at 20°C in the dark, at a final concentration of 3 μM in 2 ml of a HEPES–Ringer buffer (HR) pH 7.4, containing 145 mM NaCl, 5.6 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 5 mM glucose, 10 mM HEPES, and 0.1% bovine serum albumin. Cells were washed twice with the same buffer before addition of 0.4 ml HR containing 1 mM $CaCl_2$, 0.1% bovine serum albumin and 0.1% DMSO, and the dish was placed on the plate of the microscope.

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 2–4 min with the buffer. After a stable basal period (1 min 30 s), cells were exposed for at least 3 min to the concentration of the drug to be tested.

Digital imaging was performed using an IMSTAR (Paris, France) imaging system as previously described (Bignon et al, 1999a). The $[Ca^{2+}]_i$ values were calculated from the 350/380-nm fluorescence ratios as described by Grynkiewicz et al. (1985).

Except when their intracellular free calcium levels were extraordinarily high or unstable, every cell in the field of the digitised image (30–75 cells/experiment) was quantified. The $[Ca^{2+}]_i$ values generated by the IMSTAR STAR-

WISE FLUO software were transferred into an Excel software. This software first expressed free $[Ca^{2+}]_i$ as a percentage of basal levels (calculated as the mean of the 10 first values) and then allowed displaying and printing of $[Ca^{2+}]_i$ in each cell for individual analysis. Depending on the pattern of their responses, the cells were classified as non-responding, oscillating or peaking cells. EC_{50} values (concentration of the drugs inducing peaks in $[Ca^{2+}]_i$ in 50% of the cells) were calculated using the Fig. P/P. Fit software (BIOSOFT, Cambridge, UK).

2.6. Phosphoinositide turnover measurements

Confluent cell monolayers in 35 mm dishes were incubated for 24 h in normal culture medium containing 5 μCi/ml of *myo*-[^{3}H]inositol. Medium was then aspirated, the cell monolayers were washed twice with phosphate-buffered saline (PBS) and incubated for 30 min with normal medium containing antagonists or vehicle and 20 mM LiCl. Cells were then stimulated in the same medium with different concentrations of SR146131 or CCK-8-S for an additional 120 min 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 for 30 min. Extracts were then neutralised with 1 M Na_2CO_3, and [^{3}H]inositol monophosphate separated as described by Berridge et al. (1983) using columns containing 1 ml of AG1-X8 (Biorad, Ivry sur Seine, France) resin.

3. Results

As shown previously, [^{125}I]CCK-8-S binding to IMR-32 cell monolayers was inhibited by the specific CCK_1 receptor antagonists SR27897B and devazepide at low concentrations, confirming that [^{125}I]CCK-8-S binds to CCK_1 receptors in this cell line (Fig. 1; Schaeffer et al., 1994). SR146131 inhibited [^{125}I]CCK-8-S binding at low concentrations (K_i = 10 and 24 nM in two different experiments) showing that it interacts with human CCK_1 receptors on IMR-32 cells (Fig. 1).

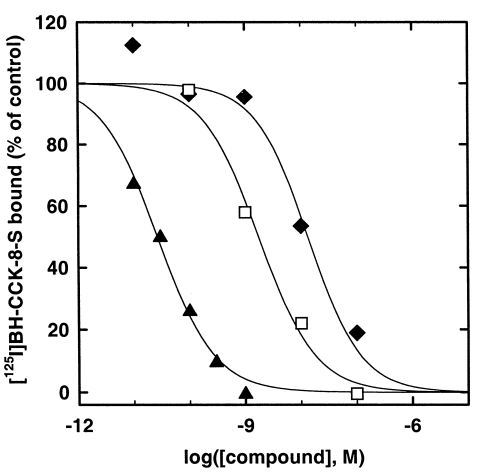

Fig. 1. Effect of devazepide (▲), SR27897 (□), and SR146131 (♦) on [^{125}I]CCK-8-S binding to IMR-32 cell monolayers. IMR-32 cells were incubated with compounds for 30 min before addition of [^{125}I]CCK-8-S, and a further incubation of 60 min at 37°C. Results are expressed as a percentage of specific binding observed under control conditions and are from one experiment representative of two experiments performed in duplicate. Non-specific binding represented from 20% to 25% of total binding under these conditions.

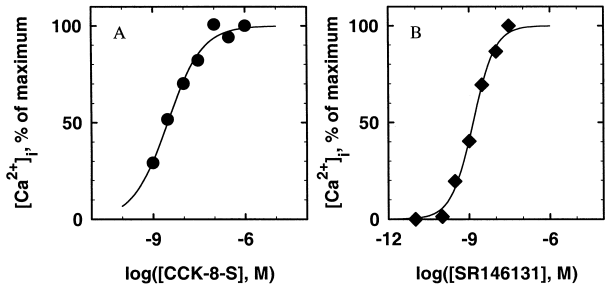

Fig. 2. Effect of CCK-8-S (A) and SR146131 (B) on $[Ca^{2+}]_i$ in IMR-32 cell suspensions. Fura-2-loaded IMR-32 cells were exposed to agonist at 37°C and $[Ca^{2+}]_i$ was measured as the increase of fluorescence ratio F_{340}/F_{380} as described under Section 2. Results are expressed as a percentage of the maximal response determined at the peak of the $[Ca^{2+}]_i$ increase. The results are from one experiment representative of a total of three experiments. The solid line represents a fit of the logistic equation to the data.

We have previously shown that activation of CCK_1 receptors by CCK-8-S or SR146131 results in an increase of $[Ca^{2+}]_i$ in IMR-32 cells (Bignon et al., 1999a). In order to further characterise the effect of SR146131 in comparison to CCK-8-S, its effect on $[Ca^{2+}]_i$ was determined in parallel with the effect of this peptide. As shown in Fig. 2, CCK-8-S, and SR146131 acted at similar concentrations to increase $[Ca^{2+}]_i$ in populations of IMR-32 cells (EC_{50} = 6 ± 2.3 and 1.3 ± 0.14 nM, (n = 3), respectively), but the maximal response of SR146131 represented only 39 ± 3 (n = 3) of the maximal response of CCK-8-S. In order to determine whether SR146131 and CCK-8-S acted through the same receptors, the effect of specific CCK_1 and CCK_2 receptor antagonists on the $[Ca^{2+}]_i$ response was assessed. Both the CCK_1 receptor antagonists devazepide and SR27897, and the CCK_2 receptor antagonists L365260 and PD134308 were able to inhibit in a concentration-dependent manner the $[Ca^{2+}]_i$ increase induced by CCK-8-S (100 nM, not shown), with the following rank order of potency (IC_{50} ± S.E.M., nM, n = 2–4): devazepide (0.10 ± 0.012) = SR27897 (0.12 ± 0.058) ≫ L365260 (54 ± 23) > PD134308 (1000 ± 540). Both CCK_1 receptor antagonists were 500 times more potent than the CCK_2 receptor antagonist L365260, and around 10,000 times more potent than PD134308. Similar results were obtained concerning the inhibitory effect of the antagonists on the $[Ca^{2+}]_i$ increase induced by SR146131 (10 nM, not shown). The rank order of potency was (IC_{50} ± S.E.M., nM, n = 3–4): SR27897 (0.23 ± 0.066) = devazepide (0.26 ± 0.13) ≫ PD134308 (770 ± 160) = L365260 (2000 ± 1600). Again, both CCK_1 receptor antagonists were equiactive

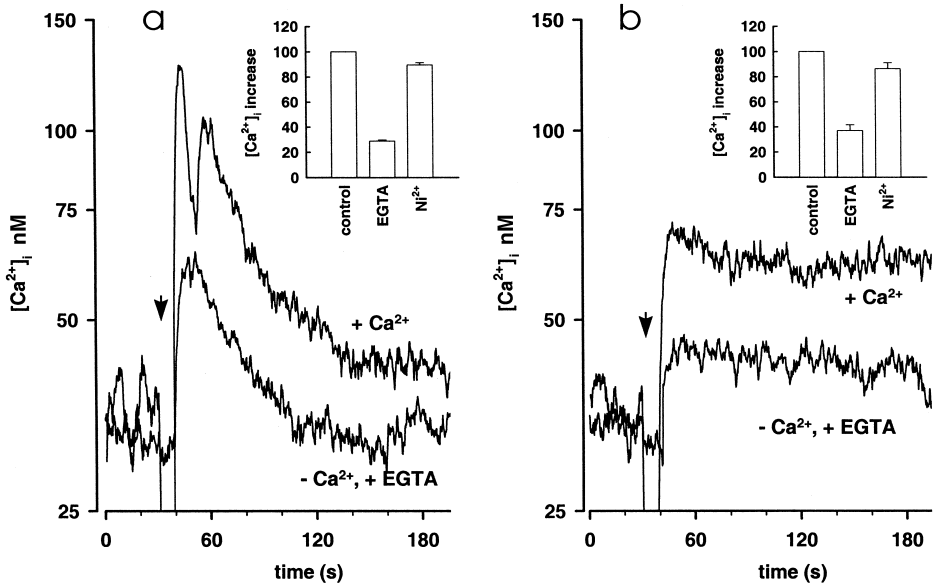

Fig. 3. Effect of CCK-8-S (100 nM, A) and SR146131 (30 nM, B) on $[Ca^{2+}]_i$ in IMR-32 cells in the presence and absence of extracellular Ca^{2+}. Fura-2-loaded IMR-32 cells were preincubated either in normal medium or in Ca^{2+}-free EGTA (200 μM)-containing solution for 1 min before exposure to the agonist (arrow) at 37°C. $[Ca^{2+}]_i$ was measured as the increase of fluorescence ratio F_{340}/F_{380} as described under Section 2. The results are from one experiment representative of two to three experiments. Inset: effect of Ca^{2+}-free (EGTA) medium and Ni^{2+} (1 mM) pretreatment on $[Ca^{2+}]_i$ increase induced by CCK-8-S (a) or SR146131 (b). Results are expressed as a percentage of the effect of each agonist under control conditions and are the mean ± S.E.M. of two to three experiments.

and were around 10,000 times more potent than the two CCK_2 receptor antagonists. These data therefore provide conclusive evidence that both CCK-8-S and SR146131 induce an increase in $[Ca^{2+}]_i$ in IMR-32 cells by acting specifically on CCK_1 receptors.

However, the profile of the response to CCK-8-S differed from the response to SR146131: CCK-8-S induced a response which was transient, whereas SR146131 induced a longer-lasting increase of $[Ca^{2+}]_i$ (Fig. 3). Interestingly, it has been shown for different agonist-induced $[Ca^{2+}]_i$ responses that transient increases are often mediated by intracellular Ca^{2+}, whereas sustained responses are due to Ca^{2+} influx (Colden-Stanfield et al., 1987; Lambert and Nahorski, 1990). In order to determine whether this difference in response kinetics was actually due to a difference in intracellular Ca^{2+} mobilisation or in extracellular Ca^{2+} influx, the effect of maximally active concentrations of CCK-8-S and SR146131 was determined after 1 min of incubation in Ca^{2+}-free medium containing the Ca^{2+} chelator EGTA (200 μM). Under these conditions, Ca^{2+} influx was abolished and the resulting $[Ca^{2+}]_i$ responses reflected the contribution of intracellular Ca^{2+} release only. As shown in Fig. 3, although the responses of both agonists were strongly decreased in the presence of EGTA, the decrease in $[Ca^{2+}]_i$ response was of similar magnitude for CCK-8-S and SR146131 (29 ± 1% and 37 ± 5%, (n = 2–3), respectively). This suggested that part of the $[Ca^{2+}]_i$ response of both agonists was due to Ca^{2+} influx. This point was further addressed by studying the effect of Ni^{2+}, which acts as an effective blocker of Ca^{2+} channels, and can thus also be used to determine the component of $[Ca^{2+}]_i$ increase ascribed to Ca^{2+} influx (Merritt et al.,

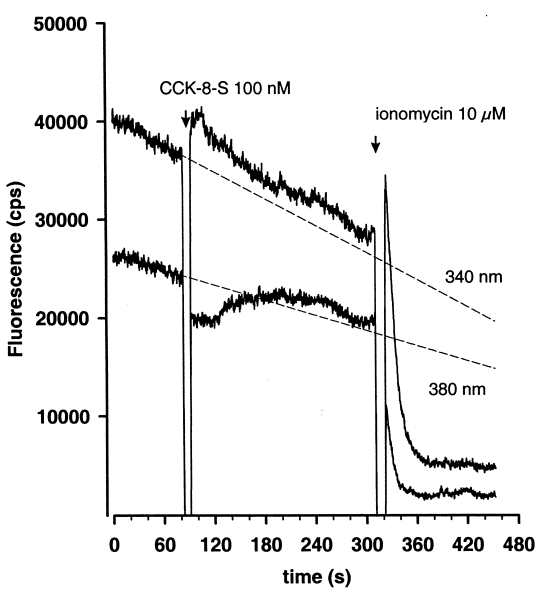

Fig. 4. Effect of the addition of CCK-8-S (100 nM) and the Ca^{2+} ionophore ionomycin (10 μM) on fura-2 fluorescence levels at 340 and 380 nm excitation wavelength in the presence of extracellular Mn^{2+} (1 mM). Fura-2-loaded IMR-32 cells were exposed to the agonists at 37°C in the presence of Mn^{2+} (1 mM). Results show fura-2 fluorescence intensity at 510 nm after excitation at 340 and 380 nm. The dotted lines represent a linear fit to the basal fluorescence decrease before the addition of agonist. The arrow represents the addition of CCK-8-S (100 nM) or ionomycin (10 μM). The results are from one experiment representative of two experiments.

1989). Interestingly, preincubation with Ni^{2+} (1 mM) had no significant effect on the response of either CCK-8-S or SR146131 (Fig. 3), suggesting that stimulated Ca^{2+} influx through Ni^{2+}-sensitive channels did not play a role in the $[Ca^{2+}]_i$ response to CCK_1 receptor activation. Another means of studying the implication of Ca^{2+} influx is the use of Mn^{2+}, which interacts with fura-2 in a manner similar to Ca^{2+}, but quenches fura-2 fluorescence once associated to fura-2. Since Mn^{2+} permeates through Ca^{2+} channels, opening of Ca^{2+} channels results in Mn^{2+} influx which quenches intracellular fura-2 fluorescence and can thus be used to indirectly determine Ca^{2+} influx (Merritt et al., 1989). As shown in Fig. 4, in the presence of Mn^{2+} (1 mM), there was a slight but steady decrease of fura-2 fluorescence. CCK-8-S induced an increase in fura-2 fluorescence at the excitation wavelength of 340 nm and a concomitant decrease at the excitation wavelength of 380 nm, as would be expected by a compound increasing $[Ca^{2+}]_i$, but neither CCK-8-S nor SR146131 (not shown) increased the rate of decrease of fura-2 fluorescence, showing that they did not induce Mn^{2+} influx into the cells. The Ca^{2+} ionophore ionomycin (10 μM) however was very effective and abolished fura-2 fluorescence at both excitation wavelengths in less than 1 min.

$[Ca^{2+}]_i$ measurements in single pancreatic cells (Matozaki et al., 1990) have shown that low concentrations of CCK-8-S induce $[Ca^{2+}]_i$ oscillations in these cells, whereas higher concentrations elicit large isolated peak responses. Compounds like CCK-JMV-180 however only induce $[Ca^{2+}]_i$ oscillations and are unable to elicit peaks, even at high concentrations (Matozaki et al., 1990). SR146131 was therefore also compared to CCK-8-S on the $[Ca^{2+}]_i$ response of single IMR-32 cells. Superfusion of IMR-32 cells with CCK-8-S resulted in an increase in $[Ca^{2+}]_i$. At low CCK-8-S concentrations, the signal consisted of $[Ca^{2+}]_i$ oscillations (repetitive transient increases in $[Ca^{2+}]_i$ levels) (Fig. 5A) but at higher concentrations, CCK-8-S induced a typical biphasic response consisting of a large peak followed by a slow return to baseline (Fig. 5B). SR146131 also induced $[Ca^{2+}]_i$ oscillations at low

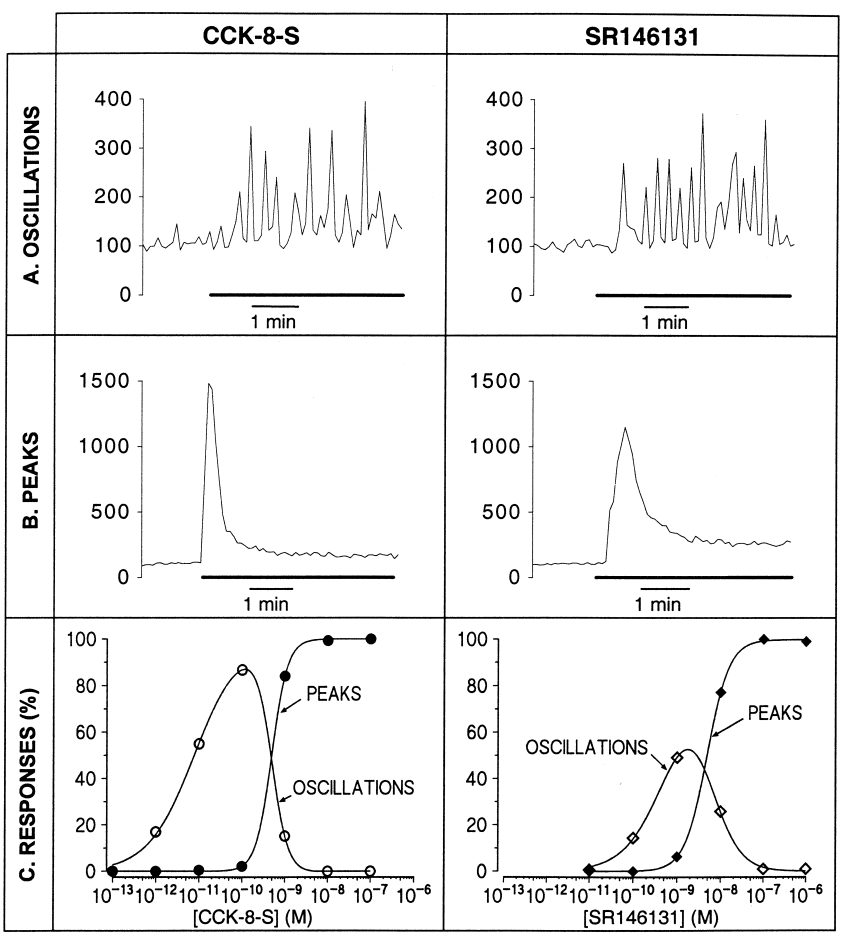

Fig. 5. Effect of CCK-8-S and SR146131 on $[Ca^{2+}]_i$ in individual IMR-32 cells. (A) Repetitive Ca^{2+} transients or oscillations induced by continuous perfusion with 100 pM CCK-8-S and 1 nM SR146131. Each plot is from one representative individual cell. The horizontal line indicates the period of perfusion of the cells with the tested compound. $[Ca^{2+}]_i$ values are expressed in percentage of basal values. (B) Peaks induced by continuous perfusion with 100 nM CCK-8S and 1 μM SR146131. (C) Concentration–response relationships. Results are expressed as a percentage of the responding cells and represent the mean of three independent experiments. Oscillations obtained with CCK-8-S (○) and SR146131 (◇); peaks obtained with CCK-8-S (●) and SR146131 (◆).

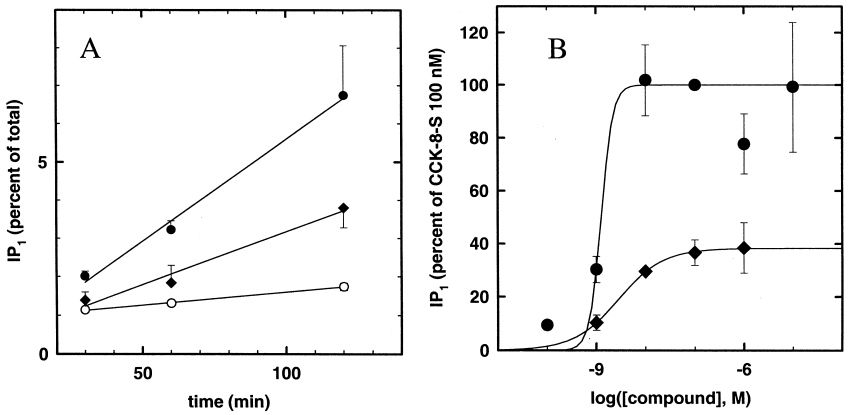

Fig. 6. Effect of CCK-8-S and SR146131 on phosphoinositide turnover in IMR-32 cells. IMR-32 cell monolayers were incubated for 30 min in Li^+ (20 mM)-containing medium before addition of the agonists. Results are the mean ± S.E.M. of two to five experiments performed in triplicate. (A) Time course of the accumulation of IP_1 under control conditions (○), or in the presence of CCK-8-S (100 nM, ●) or SR146131 (30 nM, ♦). (B) Concentration–effect relationship for the effect of CCK-8-S (●) and SR1461431 (♦) on phosphoinositide turnover. Results are expressed as a percentage of the effect induced by CCK-8-S (100 nM) which was determined as a control value in all experiments.

concentrations and provoked a large peak at higher concentrations. The effects of SR146131 and CCK-8-S on single IMR-32 cells were also compared by the analysis of the percentage of the responding cells in terms of oscillation or peaks (Fig. 5C). Low concentrations of CCK-8-S and SR146131 induced oscillations in these cells, and higher concentrations of the two agonists induced peaks with a concomitant decrease of oscillations. CCK-8-S induced peaks in 100% of the cells with an EC_{50} value of 0.50 ± 0.04 nM ($n = 3$). SR146131 also induced peaks in 100% of the cells (Fig. 5C) but was 10 times less potent than CCK-8-S with an EC_{50} value of 5.7 ± 1.9 nM ($n = 3$). Moreover, the maximum amplitudes of the peaks induced by 1 μM SR146131 were lower than those induced by 100 nM CCK-8-S (% of basal values of 979 ± 138 ($n = 2$) and 1510 ± 30 ($n = 2$), respectively). The selective CCK_1 receptor antagonist SR27897B, at a concentration of 100 nM antagonised the oscillations induced by 1 nM SR146131 or 10 pM CCK-8S, and also prevented the peaks induced by 100 nM SR146131 or 10 nM CCK-8-S (data not shown). Furthermore, in the presence of EGTA, the peak response induced by SR146131 (100 nM) and CCK-8-S (10 nM) was reduced to a similar degree (16% and 23%, respectively; mean of 50–70 cells). Thus, when measured in single IMR-32 cells, SR146131 presents a CCK_1 receptor agonist profile very close to that observed for CCK-8-S itself.

CCK-8-S has been shown to induce an increase of phosphoinositide turnover in neuroblastoma cells (Schaeffer et al., 1994). It was therefore interesting to determine whether CCK-8-S and SR146131 would act in a similar manner on this second messenger. As shown in Fig. 6, the basal level of inositol-1-phosphate (IP_1) slowly increased with time, this increase being linear for up to 2 h. In the presence of CCK-8-S and SR146131, the rate of IP_1 formation was strongly increased, but again remained linear for 2 h, with no evidence of desensitisation at this time point. The effect of both agonists on phosphoinositide turnover was concentration-dependent, and the EC_{50} values of CCK-8-S and SR146131 were similar ($EC_{50} = 2.7$ ± 0.7 and 6 ± 3.1 nM ($n = 3$-5), respectively). However, the maximal effect of SR146131 was only a fraction (38 ± 10% ($n = 5$)) of the maximal effect of CCK-8-S. The specificity of the stimulatory effect of both agonists was then determined: the effects of both CCK-8-S and SR146131 were abolished by the CCK_1 receptor antagonists devazepide (100 nM, 95 ± 3% and 86 ± 5% inhibition, respectively) and SR27897B (1 μM, 82 ± 5% and 47 ± 23% inhibition, respectively), but were not affected by the CCK_2 receptor antagonists PD134308 (10 μM) and L365260 (1 μM) (not shown).

4. Discussion

Although some synthetic agonists of CCK_1 receptors have already been described, SR146131 is the first non-peptide compound selectively activating CCK_1 receptors with a potency similar to that of the natural agonist (Bignon et al., 1999a). Here, we have found that on IMR-32 cells, a human neuroblastoma cell line which naturally expresses CCK_1 receptors (Schaeffer et al., 1994), SR146131 inhibited [^{125}I]CCK-8-S binding at nanomolar concentrations. SR146131 increased $[Ca^{2+}]_i$ in population studies of IMR-32 cells at concentrations slightly lower than those inhibiting the binding of [^{125}I]CCK-8-S, whereas CCK-8-S interacted with CCK_1 receptors in binding experiments and activated these receptors in the same concentration range (binding $IC_{50} = 4$ nM (Schaeffer et al., 1994), $[Ca^{2+}]_i$ increase in cell populations $EC_{50} = 6$ nM (this work)). This could point to a difference between SR146131 and CCK-8-S at the level of the CCK_1 receptors, but it should be noted that in single cell $[Ca^{2+}]_i$ measurements, $[Ca^{2+}]_i$ oscillations were induced by both CCK-8-S and

SR146131 at concentrations much lower than those inducing $[Ca^{2+}]_i$ peaks, showing that a direct comparison of binding data with selected functional responses is of moderate significance. Moreover, bovine serum albumin, which was present in the binding experiments but not in functional experiments (except single cell $[Ca^{2+}]_i$ determinations), has been shown to decrease the binding of [^{125}I]CCK-8-S to pancreatic CCK_1 receptors, but to enhance the effect of CCK-8-S on amylase release (Huang et al., 1995). This clearly suggests that a direct comparison between binding data and results from functional experiments may not be meaningful in the case of CCK_1 receptor binding studies.

The inhibition experiments reported here using selective CCK_1 and CCK_2 receptor antagonists unequivocally show that the $[Ca^{2+}]_i$ increase induced by both agonists was selectively mediated by the CCK_1 receptor. The fact that CCK_2 antagonists were able to inhibit the effect of both agonists at very high concentrations only suggests that these effects were due to residual CCK_1 receptor antagonist activity of these compounds, as already reported (Mahovec, 1993).

Interestingly, although both CCK-8-S and SR146131 acted at similar concentrations, the profile of the effects of both agonists and their maximal effects were slightly different. Thus, in studies on populations of IMR-32 cells, CCK-8-S induced a biphasic increase in $[Ca^{2+}]_i$ consisting of an initial peak followed by a plateau phase of a smaller amplitude, which is normally observed for compounds inducing both intracellular Ca^{2+} release and Ca^{2+} influx, like bradykinin (Colden-Stanfield et al., 1987). SR146131 only produced a long-lasting, stable increase which was reminiscent of the $[Ca^{2+}]_i$ increase induced by compounds exclusively inducing Ca^{2+} influx. However, further analysis did not corroborate this conclusion. Thus, in Ca^{2+}-free medium, the $[Ca^{2+}]_i$ increase induced by both CCK-8-S and SR146131 was reduced, but this reduction was identical for both compounds. Also, the divalent cation Ni^{2+}, which blocks Ca^{2+} influx through Ca^{2+} channels, had no effect on the $[Ca^{2+}]_i$ increase induced by both compounds, and Mn^{2+} quenching experiments showed no evidence of a significant induction of Ca^{2+} influx by either CCK-8-S or SR146131. These data suggest that CCK-8-S and SR146131 are both essentially acting by inducing Ca^{2+} release from intracellular stores and that a differential activation of Ca^{2+} release and Ca^{2+} influx cannot explain the difference in $[Ca^{2+}]_i$ increase between CCK-8-S and SR146131. The fact that the $[Ca^{2+}]_i$ response was reduced in the presence of EGTA may be due to the existence of an intracellular Ca^{2+} pool in rapid exchange with the extracellular medium. This observation shows a high analogy with the results observed for the activation of $[Ca^{2+}]_i$ increase by bradykinin in N1E-115 cells (Iredale et al., 1992).

The similarity between SR146131 and CCK-8-S was also substantiated through $[Ca^{2+}]_i$ measurement on single cells. Again, both compounds were comparable in that they induced $[Ca^{2+}]_i$ oscillations at low concentrations and a single peak at higher concentrations and that these responses were similarly resistant to extracellular Ca^{2+} removal. Again however, subtle differences between SR146131 and CCK-8-S were apparent: high concentrations of both compounds thus induced a peak increase in $[Ca^{2+}]_i$ in 100% of the cells, but the magnitude of the peak responses was slightly higher in CCK-8-S-exposed cells than in SR146131-treated cells. These slight differences between both compounds at the single cell level are totally consistent with the fact that SR146131 induced slightly lower responses than CCK-8-S in cell populations.

The fact that the maximal effect of SR146131 was lower than the maximal effect of CCK-8-S underlines the importance of the cellular environment of CCK_1 receptors on which the agonists are acting in the magnitude of the relative response of CCK-8-S and SR146131. We have already reported that SR146131 was as active as CCK-8-S in 3T3 cells expressing human CCK_1 receptors, but was less potent than CCK-8-S in CHP212 human neuroblastoma cells, suggesting that it may act as a partial agonist in cell systems where CCK_1 receptor levels are low (Bignon et al., 1999a).

The difference in peak shape between the $[Ca^{2+}]_i$ increase induced by CCK-8-S and SR146131 prompted the question as to whether similar differences might be detected on other second messenger systems. For at least one other second messenger, phosphoinositide turnover, this was not the case: both CCK-8-S and SR146131 induced a long-lasting increase in phosphoinositide turnover, the rate of which was stable for at least 2 h in both cases, although it was lower for SR146131 than for CCK-8-S. As for $[Ca^{2+}]_i$ increase, both CCK-8-S and SR146131 acted at nanomolar concentrations, and their effects were specifically inhibited by CCK_1 receptor antagonists, confirming that they activated phosphoinositide turnover through CCK_1 receptors.

In conclusion, this work shows that SR146131 acts as a specific agonist of human CCK_1 receptors with a potency similar to that of CCK-8-S. The characteristics of the $[Ca^{2+}]_i$ increase in terms of sensitivity to receptor antagonists, extracellular Ca^{2+} removal, blockade by the divalent cation Ni^{2+}, Mn^{2+} influx and induction of $[Ca^{2+}]_i$ oscillations and peaks in single cells, were identical for both agonists. Furthermore, both compounds induced a long-lasting increase in phosphoinositide turnover, which was selectively inhibited by CCK_1 receptor antagonists. Overall, this work shows that SR146131 is a potent agonist of human CCK_1 receptors closely mimicking CCK-8-S.

References

Aquino, C.J., Armour, D.R., Berman, J.M., Birkemo, L.S., Carr, R.A.E., Croom, D.K., Dezube, M., Dougherty, R.W., Ervin, G.N., Grizzle, M.K., Head, J.E., Hirst, G.C., James, M.K., Johnson, M.F., Miller,

L.J., Queen, K.L., Rimele, T.J., Smith, D.N., Sugg, E.E., 1996. Discovery of 1,5-Benzodiazepines with peripheral cholecystokinin (CCK-A) receptor agonist activity: I. Optimization of the agonist ''trigger''. J. Med. Chem. 39, 562–569.

Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P., Irvine, R.F., 1983. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem. J. 212, 473–482.

Bignon, E., Bachy, A., Boigegrain, R., Brodin, R., Cottineau, M., Gully, D., Herbert, J.M., Keane, P., Labie, C., Molimard, J.C., Olliero, D., Oury-Donat, F., Petereau, C., Prabonnaud, V., Rockstroh, M.P., Schaeffer, P., Servant, O., Thurneyssen, O., Soubrié, P., Pascal, M., Maffrand, J.P., Le Fur, G., 1999a. SR146131: a new, potent, orally active and selective non-peptide CCK_1 receptor agonist: I. In vitro studies. J. Pharmacol. Exp. Ther. 289, 742–751.

Bignon, E., Alonso, R., Arnone, M., Boigegrain, R., Brodin, R., Gueudet, C., Héaulme, M., Keane, P., Landi, M., Molimard, J.C., Olliero, D., Poncelet, M., Seban, E., Simiand, J., Soubrié, P., Pascal, M., Maffrand, J.P., Le Fur, G., 1999b. SR146131: a new, potent, orally active and selective non-peptide CCK_1 receptor agonist: II. In vivo pharmacological characterization. J. Pharmacol. Exp. Ther. 289, 752–761.

Chang, R.S.L., Lotti, V.J., 1986. Biochemical and pharmacological characterization of an extremely potent and selective nonpeptide cholecystokinin antagonist. Proc. Natl. Acad. Sci. U. S. A. 83, 4923–4926.

Colden-Stanfield, M., Schilling, W.P., Ritchie, A.K., Eskin, S.G., Navarro, L.T., Kunze, D.L., 1987. Bradykinin-induced increases in cytosolic calcium and ionic currents in cultured bovine aortic endothelial cells. Circ. Res. 61, 632–640.

Crawley, J.N., Corwin, R.L., 1994. Biological actions of cholecystokinin. Peptides 15, 731–755.

D'Amato, M., Mahovec, F., Rovati, L.C., 1994. Potential clinical applications of CCK_A receptor antagonists in gastroenterology. Drug News & Perspect. 7, 87–95.

Gibbs, J., Young, R.C., Smith, G.P., 1973. Cholecystokinin decreases food intake in rats. J. Comp. Physiol. Psychol. 84, 488–495.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Gully, D., Fréhel, D., Marcy, C., Spinazzé, A., Lespy, L., Neliat, G., Maffrand, J.P., Le Fur, G., 1993. Peripheral biological activity of SR27897: a new potent non-peptide antagonist of CCK_A receptors. Eur. J. Pharmacol. 232, 13–19.

Henke, B.R., Aquino, C.J., Birkemo, L.S., Croom, D.K., Dougherty, R.W., Ervin, G.N., Grizzle, M.K., Hirst, G.C., James, M.K., Johnson, M.F., Queen, K.L., Sherrill, R.G., Sugg, E.E., Suh, E.M., Szewczyk, J.W., Unwalla, R.J., Yingling, J., Willson, T.M., 1997. Optimization of 3-(1*H*-indazol-3-ylmethyl)-1,5-benzodiazepines as potent, orally active CCK-8-S-A agonists. J. Med. Chem. 40, 2706–2725.

Huang, S.C., Talkad, V.D., Fortune, K.P., Jonnalagadda, S., Severi, C., Delle Fave, G., Gardner, J.D., 1995. Modulation of cholecystokinin activity by albumin. Proc. Natl. Acad. Sci. U. S. A. 92, 10312–10316.

Hughes, J., Boden, P., Costall, B., Domeney, A., Kelly, E., Horwell, D.C., Hunter, J.C., Pinnock, R.D., Woodruff, G.N., 1990. Development of a class of selective cholecystokinin type B receptor antagonists having potent anxiolytic activity. Proc. Natl. Acad. Sci. U. S. A. 87, 6728–6732.

Iredale, P.A., Martin, K.F., Hill, S.J., Kendall, D.A., 1992. Agonist-induced changes in $[Ca^{2+}]_i$ in N1E-115 cells: differential effects of bradykinin and carbachol. Eur. J. Pharmacol. 226, 163–168.

Lambert, D.G., Nahorski, S.R., 1990. Muscarinic-receptor-mediated changes in intracellular Ca^{2+} and inositol 1,4,5-trisphosphate mass in a human neuroblastoma cell line, SH-SY5Y. Biochem. J. 265, 555–562.

Lotti, V.J., Chang, R.S.L., 1989. A new potent and selective non-peptide gastrin antagonist and brain cholecystokinin receptor (CCK_B) ligand: L-365,260. Eur. J. Pharmacol. 162, 273–280.

Mahovec, F., 1993. CCK-B/gastrin receptor antagonists. Drugs of the Future 18, 919–931.

Matozaki, T., Goke, B., Tsunoda, Y., Rodriguez, M., Martinez, J., Williams, J.A., 1990. Two functionally distinct cholecystokinin receptors show different modes of actions on Ca^{2+} mobilization and phospholipid hydrolysis in isolated rat pancreatic acini. J. Biol. Chem. 265, 6247–6254.

Merritt, J.E., Jacob, R., Hallam, T.J., 1989. Use of manganese to discriminate between calcium influx and mobilization from internal stores in stimulated human neutrophils. J. Biol. Chem. 264, 1522–1527.

Munson, P.J., Rodbard, D., 1980. Ligand: a versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. 107, 220–239.

Rao, R.V., Hadac, E.M., Roettger, B.F., Miller, L.J., 1997. Cholecystokinin-induced desensitization, receptor phosphorylation, and internalization in the CHP212 neuroblastoma cell line. J. Neurochem. 68, 2356–2362.

Schaeffer, P., Prabonnaud, V., Roux, M., Gully, D., Herbert, J.M., 1994. CCK-JMV-180 acts as an antagonist of the CCK_A receptor in the human IMR-32 neuroblastoma cell line. FEBS Lett. 354, 203–206.