FEBS Letters 354 (1994) 203–206

FEBS 14727

CCK-JMV-180 acts as an antagonist of the CCK_A receptor in the human IMR-32 neuroblastoma cell line

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Received 29 September 1994

Abstract [^{125}I]Cholecystokinin-8-S (CCK-8-S) bound to a single class of saturable binding sites on the human neuroblastoma cell line IMR-32 (K_D = 4 ± 1.5 nM, B_{max} = 10,500 ± 3,500 sites/cell (n = 6)). These binding sites were of the CCK_A type, as demonstrated by the differential inhibition of the binding of [^{125}I]CCK-8-S and CCK-8-S-induced $^{45}Ca^{2+}$ efflux by the specific CCK_A antagonist SR 27897 and the specific CCK_B antagonist PD 134,308. CCK-JMV-180, an analogue of CCK-8-S which has been shown to activate $^{45}Ca^{2+}$ efflux in rat cells in a manner similar to CCK-8-S, acted as a potent antagonist of CCK-8-S-induced $^{45}Ca^{2+}$ efflux (IC_{50} = 50 nM) and inhibited [^{125}I]CCK-8-S binding to IMR-32 cells (IC_{50} = 1.7 nM). These results show that, unlike its CCK-like effect in various animal systems, CCK-JMC-180 acts as an antagonist of CCK_A receptors in the human neuroblastoma cell line IMR-32.

Key words: CCK-JMV-180; IMR-32; CCK_A receptor; $^{45}Ca^{2+}$ efflux; SR 27897

1. Introduction

The existence of two distinct types of cholecystokinin (CCK-8-S) receptors, CCK_A and CCK_B receptors, is now clearly established since the determination of their deduced amino acid sequence [1,2]. Whereas CCK_A receptors are prominent in pancreatic acinar cells and gallbladder smooth muscle cells, CCK_B receptors are primarily localized on gastrointestinal smooth muscle cells and in the brain [3,4]. CCK_A receptors have been studied essentially in rat and mouse pancreatic acini where cholecystokinin octapeptide (CCK-8-S) induces amylase release [5]. The concentration–effect relationship of CCK-8-S in this tissue is somewhat peculiar, because release of amylase is stimulated at low concentrations of CCK-8-S, but decreases at higher concentrations of the peptide, although only one CCK_A receptor seems to be present on these cells [5]. Interestingly, a peptide analogue of CCK-8-S, CCK-JMV-180, induces amylase release without a decrease at higher concentrations, and is able to inhibit the decrease produced by CCK-8-S [6]. Several explanations have been considered to explain these findings. Clearly, amylase release is Ca^{2+}-dependent, because it is abolished by removal of extracellular Ca^{2+}, and both CCK-8-S and CCK-JMV-180 induce an efflux of $^{45}Ca^{2+}$ in pancreatic acini [7,8]. However, the intracellular mechanism inducing this Ca^{2+} release is not yet clearly established. Considering the fact that IP_3 production is not measurable at low concentrations of CCK-8-S and at any concentration of CCK-JMV-180 [7], and that inhibition of phospholipase C [9] or of intracellular IP_3 receptors [10] inhibits the effects of CCK-8-S, but not those of CCK-JMV-180 on intracellular free Ca^{2+}, it has been proposed that the second inhibitory phase of amylase release is due to the activation of phosphoinositide hydrolysis, whereas the first phase is due to an unknown second messenger able to induce Ca^{2+} oscillations in these cells [9,10]. This may correlate with the existence of at least two [5] but perhaps three [11], different states of the CCK_A receptor in pancreatic acini, with a higher affinity state mediating the activation and a lower affinity state the inhibition of amylase release [5]. This has led to the hypothesis that responses which are activated by CCK-JMV-180 are due to the higher affinity receptor state, whereas responses inhibited by CCK-JMV-180 are mediated by the lower affinity receptor state [12,13]. However, recent studies show that in CHO cells expressing high levels of cloned rat CCK_A receptors, CCK-JMV-180 is able to activate phosphoinositide hydrolysis [14], suggesting that in different cells CCK-8-S and CCK-JMV-180 will have contrasting effects depending on the level of receptors and the second messenger systems present. Actually, whereas CCK-JMV-180 inhibits the CCK-8-S-induced inhibition of food intake in rats, it acts as an agonist of this response in mice [13].

It was therefore interesting to determine how the effects of CCK-8-S and CCK-JMV-180 compare on human receptors. We show here that in the human neuroblastoma cell line IMR-32 [15], CCK-JMV-180 binds to CCK_A receptors with an affinity close to that of CCK-8-S and acts as an antagonist of CCK_A receptor-induced $^{45}Ca^{2+}$ efflux.

2. Materials and methods

2.1. Compounds

SR 27897 and PD 134308 were synthesized by Sanofi Recherche (Toulouse, France). [Tyr(SO_3H)^{27}]Cholecystokinin fragment 26-33 amide (CCK-8-S) and CCK-8-NS were from Sigma Chemical Co. (St. Louis, MO). CCK-JMV-180 was purchased from Research Plus (Bayonne, NJ). $^{45}CaCl_2$ (10–40 mCi/mmol) and [^{125}I]CCK-8-S (2,000 Ci/mmol) were from Amersham (Les Ulis, France). Tissue culture reagents were from Boehringer-Mannheim (Mannheim, Germany).

2.2. Cell culture

IMR-32 cells were obtained from American Type Culture Collection (Rockville, MD) (ATCC, CCL 127). IMR-32 cells were routinely cultured in 75 cm^2 culture flasks in minimum essential medium (MEM, Eagle) with Earle's salts, non-essential amino acids and 10% fetal calf serum. Three days before the experiment, cells were seeded at $1.5 \cdot 10^6$ cells/well in 35 mm culture dishes which had been pre-coated with human fibronectin at 5 $\mu g/cm^2$. Cells were used between passages 53 and 68.

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Abbreviations: CCK, cholecystokinin; PSS, physiological salt solution; K_D, dissociation constant; B_{max}, maximum binding capacity.

SSDI 0014-5793(94)01114-1

2.3. Binding experiments

Confluent cell monolayers were 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 unlabelled CCK-8-S) in physiological salt solution (PSS, composition: NaCl 145 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1 mM, glucose 5.6 mM, bovine serum albumin 1 g/l, bacitracin 100 mg/l, HEPES-NaOH 5 mM, pH 7.4). The compounds to be tested were added simultaneously in solution in PSS. The reaction was stopped by three rapid washes with ice-cold PSS, cells were digested with NaOH 0.1 N and radioactivity in the resulting solution was determined in a gamma-counter. Non-specific binding was assessed by addition of 1 μM of unlabelled CCK-8-S. In the absence of cells, the specific binding of [^{125}I]CCK-8-S to fibronectin-treated cell culture dishes was negligible. All experiments were carried out in duplicate. Experimental results were analysed by an in-house binding analysis program similar to LIGAND [16].

2.4. $^{45}Ca^{2+}$ efflux experiments

Confluent cell monolayers were incubated overnight with medium containing 5 μCi/ml of $^{45}CaCl_2$. The experiment was started by removal of the medium and replacement by PSS devoid of $^{45}Ca^{2+}$. The solution was removed every 30 s and replaced by fresh PSS. Radioactivity in the solution was determined by scintillation counting. Results were expressed as fractional rate (R) of ^{45}Ca efflux:

$$R_i = \frac{Q_i}{Q_c + \sum_{n=i}^{last} Q_n}$$

where Q_i = quantity of $^{45}Ca^{2+}$ lost from the cells during time period i and Q_c = quantity of $^{45}Ca^{2+}$ in the cells at the end of the experiment. $^{45}Ca^{2+}$ efflux was stimulated by addition of CCK-8-S to the washing solution after 8 medium changes. When the effect of antagonists were studied, they were present from the beginning of the experiment, i.e. for 4 min before the addition of CCK-8-S. Inter-experiment variability of basal rates of $^{45}Ca^{2+}$ efflux and of the maximal stimulation observed with CCK-8-S being quite high (80%), data were expressed as stimulation ratio's (i.e. the ratio of the fractional rate of $^{45}Ca^{2+}$ efflux during the stimulation interval divided by the rate of efflux during the interval immediately preceding stimulation. Under these conditions, variations were lower (20%). For inhibition studies, controls were always run in parallel, inhibition percentages under these conditions being highly reproducible (S.E.M. represented less than 5% of the mean). IC_{50} values and slope factors as well as the corresponding standard error were determined by fitting the logistic equation [17] to the pooled data from several experiments using the curve fitting package supplied with the SIGMAPLOT program (Jandel Scientific, Erkrath, Germany).

3. Results and discussion

[^{125}I]CCK-8-S binding was studied on IMR-32 cell monolayers at 37°C. Since non-specific binding represented 40–50% of total binding at 1 nM of ligand, experiments were performed by isotopic dilution of a fixed amount of [^{125}I]CCK-8-S (1.2 nM). As shown in Fig. 1A, [^{125}I]CCK-8-S bound to a single class of saturable, non-interacting binding sites, with a dissociation constant (K_D) of 4 ± 1.5 nM and a maximum binding capacity (B_{max}) of 10,500 ± 3,500 sites/cell (n = 6). These results were similar to those obtained in COS cells transfected with the rat pancreatic CCK_A receptor, where [^{125}I]CCK-8-S bound to a single class of binding sites with a dissociation constant of 1.5 nM [11]. Since at least two different classes of binding sites have been reported in other cell types like pancreatic acini, we took great care to detect other binding sites but we were unable to demonstrate the presence of low affinity binding sites on the surface of IMR-32 cells. However, as already observed in transfected COS cells, this may be due to the fact that only high affinity binding sites can be reliably detected in whole cells [11]. The dissociation constant of [^{125}I]CCK-8-S for its receptors was determined by association and dissociation kinetic experiments:

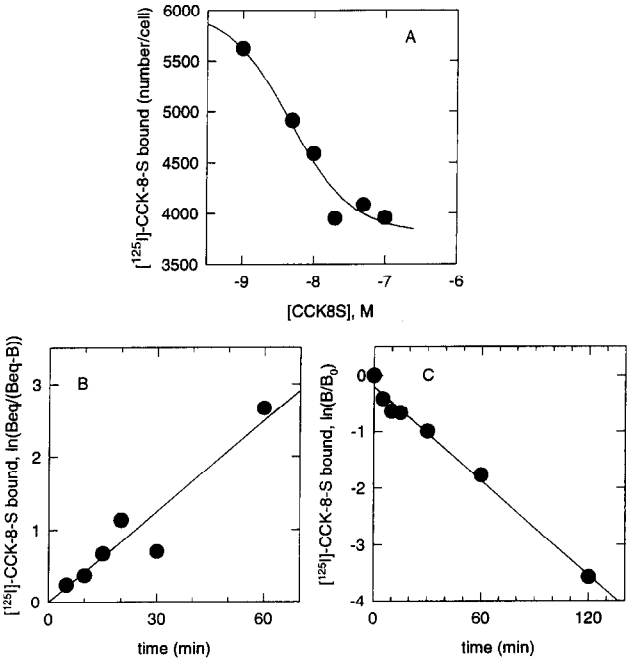

Fig. 1. [^{125}I]CCK-8-S binding to IMR-32 cells. (A) Equilibrium binding experiments. Cells were incubated for 1 h at 37°C with 1.2 nM of [^{125}I]CCK-8-S and increasing concentrations of unlabelled CCK-8-S. Data shown are the mean of two experiments and represent the total binding to the cells. The solid line represents the results of a non-linear regression analysis of the data to a one-site binding equation. (B) Association kinetics of [^{125}I]CCK-8-S (1.2 nM) binding to IMR-32 cells. (C) Dissociation kinetics. [^{125}I]CCK-8-S (1.2 nM) was incubated with cell monolayers for 1 h, then dissociation was induced by 1 μM of CCK-8-S.

as shown in Fig. 1B, association of [^{125}I]CCK-8-S (1.2 nM) proceeded with a pseudo-first order kinetics, as evidenced by the linear plot. A mean k_{obs} value of 0.034 min^{-1} could thus be determined, corresponding to a half-time of association ($t_{1/2}$) of 20 min. Dissociation of the [^{125}I]CCK-8-S/receptor complex, initiated by the addition of an excess of unlabelled CCK-8-S (1 μM) proceeded in a mono-exponential manner (Fig. 1C) with a half-life of 29 min. From these results the dissociation rate constant value was found to be $4 \cdot 10^{-4}$ s^{-1}. From the relationship $k_{obs} = k_{+1}([^{125}I]CCK\text{-}8\text{-}S) + k_{-1}$, the association rate constant k_{+1} was found to be $1.4 \cdot 10^5$ $M^{-1} \cdot s^{-1}$. Calculating the dissociation constant value (K_d) from these data gave $K_d = k_{-1}/k_{+1}$ = 2.8 nM, a value close to the K_d value determined previously in the equilibrium binding experiments.

The inhibition of [^{125}I]CCK-8-S binding to IMR-32 cells by different compounds was then studied. As shown in Fig. 2A, binding of [^{125}I]CCK-8-S was inhibited by unlabelled CCK-8-S with an IC_{50} value (concentration which inhibited 50% of the specific binding) of 4.2 nM. The non-sulphated analogue of CCK-8-S (CCK-8-NS), known to be active on the CCK_B receptor [19], displaced [^{125}I]CCK-8-S from its binding sites on IMR-32 cells only when tested at high concentrations (Fig. 2B). [^{125}I]CCK-8-S (1 nM) binding to IMR-32 cells was inhibited by low concentrations of SR 27897, a specific non-peptide inhibitor of CCK-8-S binding to CCK_A receptors [20] (IC_{50} = 1.5 nM), whereas higher concentrations of the CCK_B antagonist

PD 134,308 [4] were necessary to interfere with the binding of [^{125}I]CCK-8-S to these cells (IC_{50} = 0.36 μM). Although the inhibitory concentration of PD 134,308 was slightly lower than what could be expected from previous results under other experimental conditions, the fact that non-sulphated CCK-8 was nearly inactive on [^{125}I]CCK-8-S binding, and that PD 134,308 was two orders of magnitude less active than the CCK_A-selective compound SR 27897, confirms that, on this cell type, [^{125}I]CCK-8-S is binding to CCK_A receptors. [^{125}I]CCK-8-S binding was inhibited by the CCK-8-S analogue CCK-JMV-180, which was active at concentrations very similar to those of CCK-8-S (IC_{50} = 1.7 nM).

We then studied a functional response of CCK_A receptor activation in IMR-32 cells: $^{45}Ca^{2+}$ efflux was studied because in rat pancreatic acini this response was induced by both CCK-8-S and CCK-JMV-180 [7,8]. As shown in Fig. 3, CCK-8-S (100 nM) induced a rapid and transient increase in $^{45}Ca^{2+}$ efflux from IMR-32 cells. $^{45}Ca^{2+}$ efflux was maximal during the first 30 s of the presence of CCK-8-S and declined rapidly thereafter. This result was in close analogy to the transient increase in Ca^{2+}_i observed after CCK-8-S stimulation of rat pancreatic acinar cells [7]. The $^{45}Ca^{2+}$ efflux during the first 30 s of exposure was therefore routinely used to determine the effect of CCK-8-S and antagonists. CCK-8-NS did not induce any effect on $^{45}Ca^{2+}$ efflux (2% increase in the presence of 1 μM CCK-8-NS). Since

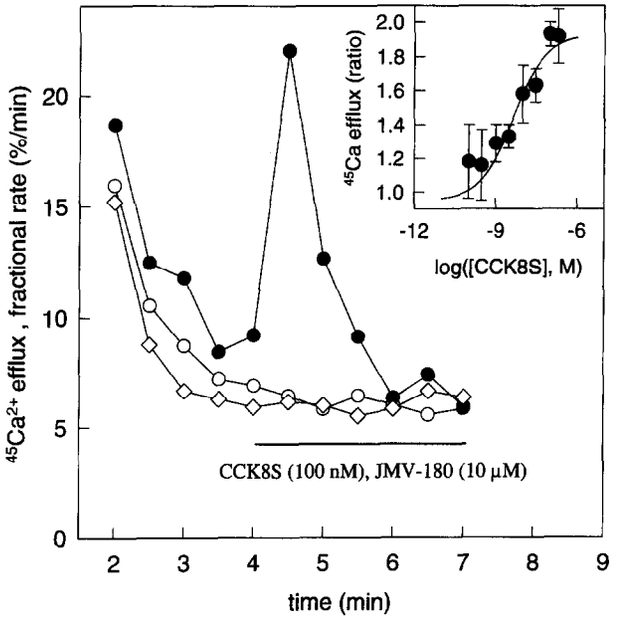

Fig. 3. Effect of CCK-8-S on $^{45}Ca^{2+}$ efflux in IMR-32 cell monolayers. Medium was replaced every 30 s and $^{45}Ca^{2+}$ determined by scintillation counting. The horizontal bar denotes the presence of CCK-8-S (100 nM) (●), CCK-JMV-180 (10 μM) (◇) or buffer (○). Data are the mean of three determinations on the same batch of cells. (Inset) Concentration-effect relationship of the stimulatory effect of CCK-8-S. Data are given as stimulation ratio vs. the pre-stimulatory period and are the mean ± S.E.M. of at least 5 determinations.

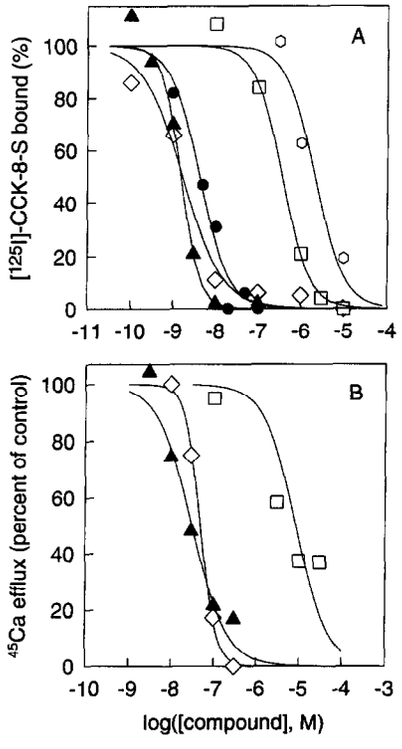

Fig. 2. Effect of SR 27897 (▲), CCK-JMV-180 (◇), CCK-8-S (●), CCK-8-NS (○) and PD 134,308 (□) on [^{125}I]CCK-8-S binding (1 nM) (A) and CCK-8-S (100 nM)-induced $^{45}Ca^{2+}$ efflux (B). Results are expressed as percent of total binding or control response ($^{45}Ca^{2+}$ efflux). Data are the mean of two determinations for binding experiments and of at least six determinations for $^{45}Ca^{2+}$ efflux experiments. The solid lines represent a fit of the logistic equation to the data.

CCK-8-NS is as active as CCK-8-S on CCK_B receptors [19], but less active on CCK_A receptors, these data further suggest that CCK-8-S acts through CCK_A receptors to induce an increase in $^{45}Ca^{2+}$ efflux in IMR-32 cells.

Despite the fact that the $^{45}Ca^{2+}$ efflux response was of a highly variable nature at low concentrations of CCK-8-S, a dose–effect relationship could be established (Fig. 3, inset). CCK-8-S increased $^{45}Ca^{2+}$ efflux with an EC_{50} value of 4 nM. This concentration is higher than that which stimulates $^{45}Ca^{2+}$ release in pancreatic acinar cells [7], but close to the K_D value obtained previously in the binding experiments and also similar to the concentration inducing an increase in phosphatidylinositol turnover, as has already been reported in CHP 212 neuroblastoma cells [21]. This discrepancy between pancreatic acinar cells and neuroblastoma cells could be due to the lower receptor density in neuroblastoma cells [21]. CCK-JMV-180 had no effect on $^{45}Ca^{2+}$ efflux up to a concentration of 10 μM, that is, a concentration 100-times higher than the concentration giving a maximal effect of CCK-8-S (Fig. 3). However, as shown in Fig. 2B, CCK-JMV-180 inhibited CCK-8-S (100 nM)-induced $^{45}Ca^{2+}$ efflux at much lower concentrations. This effect occurred in a dose-dependent manner, with an IC_{50} value of 50 nM.

In order to confirm that the effects of CCK-8-S in these cells were mediated by CCK_A receptors, the effects of SR 27897, a specific antagonist of CCK-8-S binding to the CCK_A receptors, and PD 134308, specific for the CCK_B receptors were determined. As shown in Fig. 2B, both compounds inhibited

CCK-8-S-stimulated $^{45}Ca^{2+}$ efflux, but a 1000-fold difference was observed between both compounds, SR 27897 being active at very low concentrations (IC_{50} = 32 nM). These data confirm that SR 27897, which has been shown to be a selective and high affinity antagonist of CCK_A receptors in rat acini [19], is a potent antagonist of CCK-8-S on human CCK_A receptors in neuroblastoma cells.

From Fig. 2 it clearly appears that the rank order of potency of the antagonists is identical between [^{125}I]CCK-8-S binding and CCK-8-S-induced $^{45}Ca^{2+}$ efflux. The concentrations necessary for inhibition of the Ca^{2+} response were about 30-times higher than those effective on [^{125}I]CCK-8-S binding but this may be due to the fact that near-maximal concentrations of CCK-8-S had to be used in order to obtain reliable responses with regard to $^{45}Ca^{2+}$ efflux.

The finding that CCK-JMV-180 acts as an antagonist of CCK-8-S-induced $^{45}Ca^{2+}$ efflux in IMR-32 cells raises controversy with other results showing that, like CCK-8-S, this compound acts as an agonist of the high affinity state of the CCK_A receptor [6–10]. However, it can not be excluded that a difference between human and rat receptors may result in CCK-JMV-180 acting as an agonist on rat receptors and an antagonist on human receptors. This is reminiscent of the fact that in mice, CCK-JMV-180 acts as a high affinity agonist of the second inhibitory phase of amylase release and of food intake, whereas it inhibits these responses in the rat [13]. However, the finding that CCK-JMV-180 inhibits CCK-8-S-induced $^{45}Ca^{2+}$ efflux is also consistent with the idea that this compound acts as a partial agonist of the CCK_A receptors, acting as an agonist in tissues were receptor-response coupling is strong and as an antagonist in other tissues [6]. This would explain why CCK-JMV-180 is able to activate phosphoinositide hydrolysis in CHO cells where CCK_A receptors have been over-expressed, but not in rat pancreatic acini where the CCK_A receptor density is lower [11].

Whatever the mechanism distinguishing the effect of CCK-JMV-180 from that of CCK-8-S, this compound is a very interesting tool because it might be able to functionally discriminate CCK_A-related responses in different tissues. However, our results represent another example showing that caution must be taken in extrapolating results obtained in animal tissues to that in the human situation.

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