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YM022 [(*R*)-1-[2,3-dihydro-1-(2′-methylphenacyl)-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea]: An Irreversible Cholecystokinin Type-B Receptor Antagonist

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ABSTRACT. A functional evaluation of the recently developed cholecystokinin type-B (CCK-B) receptor antagonist YM022 [(*R*)-1-[2,3-dihydro-1-(2′-methylphenacyl)-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea] was undertaken in Chinese hamster ovary cells stably expressing the human CCK-B receptor gene (hCCK-B.CHO). YM022 exhibited high affinity and selectivity for the CCK-B receptor subtype as estimated from [^{125}I]CCK8S displacement studies using membranes derived from hCCK-B.CHO and hCCK-A.CHO cells. Functional antagonist activity of YM022 was demonstrated employing CCK-4-stimulated Ca^{2+} mobilization in hCCK-B.CHO cells. In the presence of 30 nM YM022, the maximum effect of CCK-4 was suppressed to 48 ± 11% of control, an effect that was accompanied by a modest rightward shift in the CCK-4 concentration–response curve. In contrast, the structurally similar CCK-B receptor antagonist L-365,260 [3*R*(+)-*N*-[2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-*N*′-(methylphenyl)urea; 30 nM–10 μM] produced progressive rightward shifts in the CCK-4 concentration–response curve, with no effect observed on the CCK-4 maximum response. Further characterization using the technique of microphysiometry revealed that the agonist activity of CCK-4 was not restored following washout after exposure to YM022. The antagonist activity of L-365,260, however, was found to be fully reversible in this system. Thus, YM022 behaves as an irreversible antagonist, whilst its structural analogue L-365,260 exhibits properties consistent with a competitive antagonist. BIOCHEM PHARMACOL 54;1:81–85, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. cholecystokinin; CCK-B receptor; YM022; irreversible antagonist

The brain/gut peptide CCK‡ is distributed widely throughout both the CNS and the gastrointestinal tract. Peripheral actions of CCK, including stimulation of pancreatic enzyme secretion, gallbladder contraction, and gut motility, are mediated via activation of CCK-A receptors, which represent the predominant peripheral CCK receptor subtype [1]. In contrast, the CCK-B receptor subtype represents the predominant central receptor population [2].

To investigate the physiological role of CCK in the CNS, numerous selective CCK-B receptor antagonists have been developed [3, 4]. In addition, recent ideas concerning the therapeutic potential of CCK-B receptor antagonists have resulted in considerable investment in the development of potent and selective antagonists of this receptor subtype. A number of studies have implicated the anxiolytic, anti-panic, and anti-nociceptive potential of CCK-B receptor antagonists [4–6].

Recently, YM022 has been reported as a new potent and selective CCK-B receptor antagonist. Thus, YM022 displaced [^{125}I]CCK-8 binding to the human CCK-B receptor with an IC_{50} value of 0.34 nM [7]. Further studies comparing the binding affinity of YM022 for rat brain CCK-B receptors with rat pancreatic CCK-A receptors revealed a 1000-fold selectivity for the rat CCK-B receptor subtype [8]. Functional studies *in vivo* employing pentagastrin-stimulated gastric acid secretion in anaesthetized rats [8], and *in vitro* employing CCK-8 stimulated Ca^{2+} mobilization in GH3 cells [9] have demonstrated the antagonist activity of YM022.

In this study, the CCK-B receptor antagonist activity of YM022 was evaluated in a Chinese hamster ovary cell line transfected with the human CCK-B receptor (hCCK-B.CHO cells; [10]). Since a number of studies have demonstrated the coupling of CCK-B receptor activation to mobilization of intracellular Ca^{2+} [11–13], this signal transduction pathway has been employed as a functional assay. Additionally, the technique of microphysiometry, which monitors the acidification rate of the extracellular medium in response to agonist stimulation [14], was used to further

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‡ *Abbreviations:* CCK, cholecystokinin; YM022, (*R*)-1-[2,3-dihydro-1-(2′-methylphenacyl)-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea; and L-365,260, 3*R*(+)-*N*-[2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-*N*′-(methylphenyl)urea.

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characterize the antagonist activity of YM022. These studies revealed that YM022 has properties consistent with those of an irreversible antagonist, whilst the structurally similar CCK-B receptor antagonist L-365,260 behaves in a manner consistent with competitive antagonism.

MATERIALS AND METHODS

Cell Lines

The hCCK-B.CHO and hCCK-A.CHO cell lines were obtained from Dr. Alan Kopin (Tufts University, Boston, MA, U.S.A.). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum and penicillin/streptomycin. YM022 and L-365,260 were synthesized in the Department of Chemistry, Wyeth–Ayerst Research (Princeton, NJ, U.S.A.).

Radioligand Binding Studies

The CCK receptor binding affinity of YM022 was estimated using the displacement of [^{125}I]CCK-8S from membranes derived from hCCK-B.CHO or hCCK-A.CHO cells. Membranes were resuspended in assay buffer (20 mM HEPES, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2% bacitracin, pH 6.5) at room temperature at a final protein concentration of 20–40 μg/mL, as determined by the commercially available Bio–Rad assay kit. Binding assays comprised 20 pM [^{125}I]CCK-8S, YM022, or L-365,260 in the concentration range 10^{-12} to 10^{-5} M and membrane preparation. Non-specific binding was determined in the presence of 0.1 μM CCK-8S. Incubation at room temperature was terminated after 90 min by rapid filtration through Whatman GF/B filters followed by two washes with 5 mL of ice-cold Tris–HCl buffer (pH 7.4). Radioactivity retained on the filters was measured by liquid scintillation counting. The IC_{50} values were estimated from displacement curves by non-linear regression analysis using Kaleidagraph™ software for the Apple Macintosh.

Ca^{2+}-Mobilization Measurements

Functional antagonism was evaluated in hCCK-B.CHO cells employing CCK-4-stimulated Ca^{2+} mobilization, measured with the Ca^{2+}-sensitive fluorescent probe FURA-2 [10]. Briefly, hCCK-B.CHO cells were loaded in the presence of 5 μM FURA-2-AM for 30 min at 37°, followed by two washes with a HEPES-buffered physiological saline (HBS). Cells were resuspended at 0.5×10^6 cells/mL and kept at room temperature until used for Ca^{2+} measurements.

Cytoplasmic free Ca^{2+} was measured in a Shimadzu RF-5001PC spectrofluorimeter employing dual wavelength excitation at 340/380 nm, and emission was monitored at 505 nm. Cells (1.5 mL) were equilibrated to 37° in a stirred thermostatically controlled cuvette before data collection was undertaken. Antagonists, at concentrations indicated in the figures, were added at the start of data collection followed by the addition of CCK-4 at 30 sec. The ratios of fluorescent intensities 340:380, collected at 2-sec intervals, were used to calculate cytoplasmic free Ca^{2+} [15]. Maximum and minimum fluorescence intensities were determined at the end of each experimental run by the addition of 50 μM digitonin and 5 mM EGTA, respectively.

TABLE 1. CCK receptor binding affinities estimated from [^{125}I]CCK-8S displacement curves

	IC_{50} (nM)	
	CCK-B	CCK-A
YM022	0.05 ± 0.02 (3)	316 ± 86 (3)
L-365,260	12.6 ± 2.8 (6)	589 ± 107 (4)
CCK-4	23.4 ± 3.8 (6)	15,848 ± 4,164 (4)

Data represent means ± SEM from the number of experiments indicated in parentheses. The CCK-B and CCK-A cell lines expressed 0.65 and 0.49 pmol/mg receptor, respectively.

Microphysiometer

The Cytosensor™ microphysiometer was used to further analyze the functional antagonist activity of YM022 and L-365,260 against the CCK-4-mediated response in hCCK-B.CHO cells. The microphysiometer measures cellular responses by monitoring changes in extracellular acidification rate following exposure to receptor agonists. Essentially the instrument functions as an extremely sensitive pH electrode monitoring changes in cellular metabolic activity which occur as a consequence of agonist stimulation [14]. Briefly, cells were seeded at a density of 3×10^5 cells/mL in Cytosensor capsule inserts 18 hr prior to experimental work. The capsule inserts, mounted in sensor chambers [14], were placed on the instrument, and the cells were washed for a period of 2 hr to stabilize extracellular acidification rates. The medium used to maintain the cells throughout the experiments was DMEM minus bicarbonate to reduce the buffering capacity of the medium. Cells were stimulated three times by the introduction of a 30-sec pulse of the CCK-B selective agonist CCK-4 (30 nM) with a 1-hr recovery period between pulses. The antagonists (30 nM) were introduced 15 min prior to the introduction of the second agonist stimulation and were present throughout the agonist pulse. Results are expressed as a percentage of the basal acidification rate measured immediately prior to the introduction of the agonist stimulation.

RESULTS

Binding Parameters

The IC_{50} values estimated for the displacement of [^{125}I]CCK-8S from CCK-A and CCK-B receptor sites in the presence of YM022, L-365,260, and CCK-4 are presented in Table 1. From these values it can be calculated that YM022 exhibits 6000-fold selectivity for the human CCK-B receptor subtype, whilst L-365,260 is 50-fold selective for this site relative to the CCK-A receptor subtype.

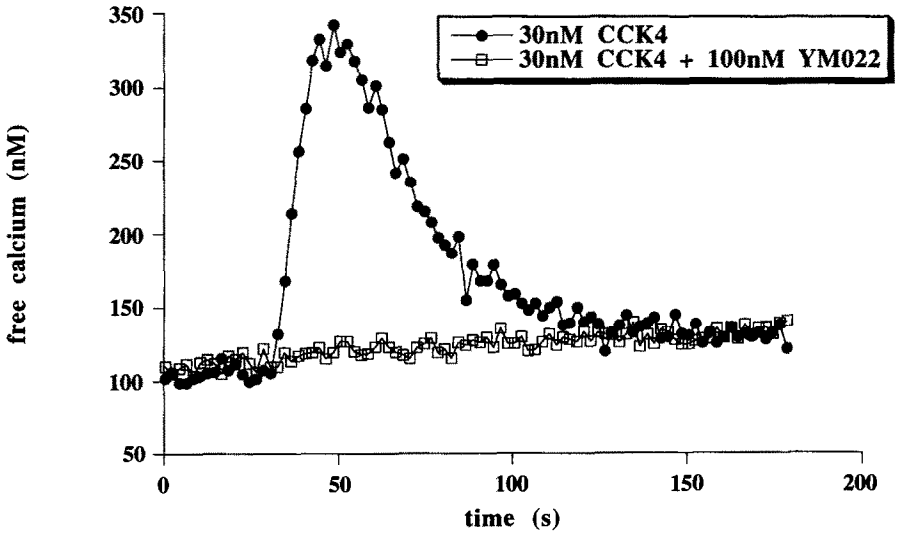

FIG. 1. Effect of YM022 on CCK-4-stimulated Ca^{2+} mobilization in hCCK-B.CHO cells. YM022 was introduced at the beginning of data collection, and CCK-4 was added at 30 sec. A representative profile is presented.

Ca^{2+}-Mobilization Experiments

The addition of 30 nM CCK-4 to FURA-2-loaded hCCK-B.CHO cells resulted in an elevation of free intracellular Ca^{2+} concentration from 105 ± 1 nM (basal) to 329 ± 5 nM at the top of the Ca^{2+} peak (Fig. 1). As is evident from Fig. 1, the response to CCK-4 was abolished completely by the prior addition of 100 nM YM022. YM022 alone had no effect on basal Ca^{2+} levels.

The concentration–response profile to CCK-4 in hCCK-B.CHO cells (Fig. 2) yielded a half-maximal effective concentration of 36 nM. In the presence of 3 nM YM022, the estimated EC_{50} for CCK-4 was 141 nM and the maximum response to CCK-4 was reduced to 70 ± 4% of control. In the presence of 30 nM YM022, there was no further shift in the estimated EC_{50} for CCK-4 (151 nM); however the maximum response to CCK-4 was further reduced to 48 ± 11% of control (Fig. 2). Figure 3 illustrates the effect of increasing concentrations of L-365,260 on CCK-4-stimulated Ca^{2+} mobilization in hCCK-B.CHO cells. In the presence of L-365,260, the following apparent EC_{50} values for CCK-4 were estimated; 295 nM (30 nM L-365,260), 603 nM (100 nM L-365,260), 741 nM (300 nM L-365,260), and 115 μM (10 μM L-365,260). Figure 3 further illustrates that L-365,260 did not suppress the maximum response to CCK-4 when compared with the data presented in Fig. 2 for YM022.

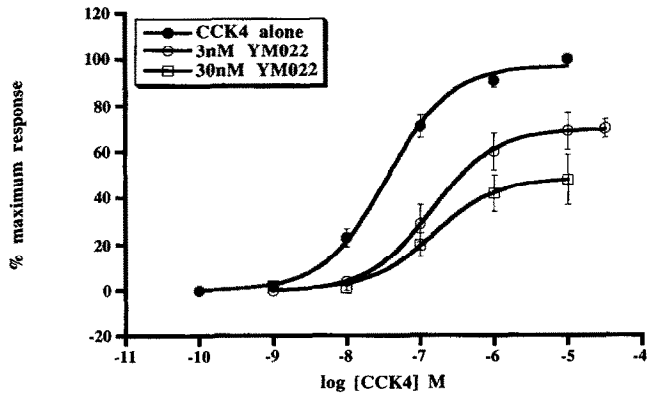

FIG. 2. Log concentration–response curve for CCK-4-stimulated Ca^{2+} mobilization in hCCK-B.CHO cells in the absence and presence of YM022. Results are expressed as a percentage of the maximum response to 10 μM CCK-4 and represent means ± SEM from 3 independent experiments.

Microphysiometer

Figure 4 demonstrates the effect of 30 nM YM022 (panel a) and 30 nM L-365,260 (panel b) on CCK-4-stimulated extracellular acidification in hCCK-B.CHO cells. Both YM022 and L-365,260 inhibited the response to 30 nM CCK-4 by 59 and 67%, respectively. However, Fig. 4 illustrates the irreversible nature of YM022, whilst the antagonist activity of L-365,260 was fully reversible.

DISCUSSION

The recently developed CCK-B receptor antagonist YM022 represents another in the series of molecules incorporating

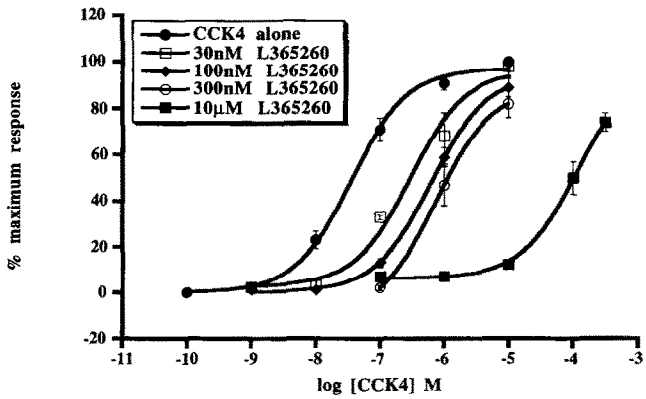

FIG. 3. Log concentration–response curve for CCK-4-stimulated Ca^{2+} mobilization in hCCK-B.CHO cells in the absence and presence of L-365,260. See legend to Fig. 2 for other details.

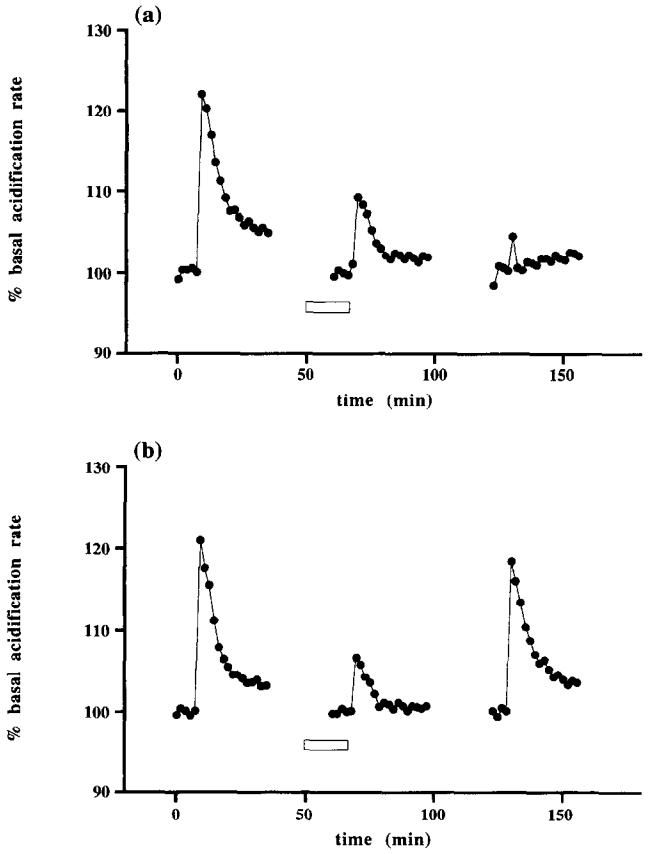

FIG. 4. Effect of 30 nM YM022 (a) and 30 nM L-365,260 (b) on the stimulation of extracellular acidification in hCCK-B. CHO cells in response to 30 nM CCK-4. Three consecutive CCK-4 stimulations were introduced for a 30-sec duration with a 1-hr wash in between. The bar indicates the presence of antagonist. Profiles from representative experiments are presented.

the benzodiazepine template, of which L-365,260 represents one of the earliest described members. YM022 had considerably greater potency for the CCK-B receptor subtype than its structural analog L-365,260 and also exhibited a much higher degree of selectivity for the human CCK-B receptor relative to the type-A receptor. Additionally, this study revealed a greater degree of selectivity exhibited by YM022 for the human CCK-B receptor (6000-fold) relative to the A-type receptor when compared with the selectivity ratio of 1000 reported for the rat CCK-B receptor [9].

Both YM022 and L-365,260 exhibited CCK-B receptor antagonist activity in the two functional assays described here although there is a clear distinction between the functional activity of the two molecules. The suppression of the maximum response to CCK-4 in the Ca^{2+}-mobilization assay in the presence of YM022 suggested a non-competitive mode of antagonism. In contrast, L-365,260 exhibited properties consistent with competitive antagonism in this system, producing a rightward shift in the concentration–response curve to CCK-4, with no reduction in the maximum response to CCK-4.

Further evaluation of the antagonist activity of YM022, measuring changes in CCK-4-stimulated extracellular acidification with the microphysiometer, revealed the irreversible antagonist activity of this molecule. Thus, following removal of YM022 and a 1-hr washout, the agonist response observed for CCK-4 in this assay was not restored. The irreversible antagonism observed for YM022 using the microphysiometer would account for the non-competitive profile exhibited in the Ca^{2+} assay. In the case of L-365,260, the response to CCK-4 measured in the microphysiometer was restored completely following removal and washout, an effect consistent with the competitive antagonist profile produced in the Ca^{2+} assay.

Based on the structural similarity of these two molecules, it would be reasonable to predict that they share a common site of action at the receptor level. However, the introduction of an aromatic ketone group in YM022, which distinguishes this molecule from its structural analogue L-365,260, clearly influences the dissociation of this molecule from the receptor. Taken together, the structural similarity of the two molecules coupled with the competitive antagonism exhibited by L-365,260 suggest that YM022 is best described as an irreversible competitive antagonist of the CCK-B receptor subtype.

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