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Pharmacological profile of TP-680, a new cholecystokinin$_A$ receptor antagonist

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1 The pharmacological characteristics of a newly developed serine derivative (**R**)-1-[3-(3-carboxypyridine-2-yl)thio-2-(indol-2-yl)carbonylamino]propionyl-4-diphenylmethyl-piperazine (TP-680), a cholecystokinin type A (CCK_A) receptor antagonist, were studied and compared with those of MK-329 and loxiglumide.

2 TP-680 showed approximately 2 and 22 times greater selectivity for peripheral CCK_A receptors relative to brain CCK (CCK_B) receptors than MK-329 and loxiglumide, respectively, when IC_{50} values for inhibition of [^{125}I]-CCK-8 binding in isolated acini and cerebral cortex were compared.

3 TP-680 was approximately 17 times less potent than MK-329, but was 106 times more potent than loxiglumide in inhibiting 100 pM CCK-8-stimulated amylase release from rat pancreatic acini. The antagonism produced by TP-680 was specific for CCK in that the effects of other receptor secretagogues or agents bypassing receptors were not altered.

4 TP-680 caused a parallel rightward shift of the dose-response curve for CCK-8-stimulated amylase release as did MK-329 and loxiglumide. However, in contrast to MK-329 and loxiglumide, TP-680 suppressed the maximal responses of CCK-8-induced amylase release in a concentration-dependent fashion, indicating that TP-680 is an unsurmountable antagonist.

5 Repeated washing of acini after a 30 min treatment with TP-680 restored the responsiveness but not the sensitivity, causing a residual inhibition on the action of CCK-8.

6 The addition of loxiglumide prior to or together with application of TP-680 protected CCK receptors from unsurmountable and irreversible antagonism by TP-680.

7 Our results indicate that TP-680 is a potent and the most selective CCK_A receptor antagonist for the pancreas reported to date.

Keywords: CCK_A receptor antagonist; isolated pancreatic acini; TP-680; residual inhibition; unsurmountable inhibition

Introduction

Cholecystokinin (CCK) plays an important role in the hormonal control of pancreatic secretion, gallbladder contraction, and gut motility (Williams, 1982). In addition to peripheral tissues, CCK is also found in the brain (Williams, 1982). CCK receptors are classified into type A (peripheral) and type B (central), depending on the structural specificity expressed and the relative affinities for various members of the CCK-gastrin family (Moran *et al.*, 1986). Recently, highly potent and specific antagonists for the peripheral CCK_A receptors have been reported (Makovec *et al.*, 1985; Chang & Lotti, 1986; Akiyama & Otsuki, 1994). MK-329 (formerly termed L-364,718) is considered the most potent and highly specific CCK antagonist and is used to characterize the peripheral CCK_A receptors (Otsuki *et al.*, 1988; 1989; Niederau *et al.*, 1989). Although MK-329 has almost 160 times greater affinity for CCK_A receptors than for CCK_B receptors (Hughes *et al.*, 1990), it is nevertheless a potent CCK_B antagonist and care should be taken when attempting to distinguish between effects mediated by CCK_A or CCK_B receptors with this compound (Woodruff & Hughes, 1991).

Recent clinical and experimental studies have indicated the possible involvement of endogenous CCK in pancreatic and gastrointestinal physiology and pathophysiology (Meyer *et al.*, 1989; Rovati, 1991; Schmidt *et al.*, 1991; Beglinger *et al.*, 1992a,b; Niederau *et al.*, 1992; Tani *et al.*, 1993; Herrington & Adrian, 1995). A number of clinical studies with another CCK receptor antagonist, loxiglumide, suggested that CCK is involved in postprandial pancreatic enzyme secretion (Meyer *et al.*, 1989; Hildebrand *et al.*, 1990; Beglinger *et al.*, 1992a), whereas MK-329 failed to inhibit postprandial pancreatic trypsin output (Cantor *et al.*, 1992). Furthermore, loxiglumide significantly accelerates gastric emptying of test meal in human subjects (Meyer *et al.*, 1989; Fried *et al.*, 1991), whereas MK-329 has little effect on postprandial gastric emptying in man (Liddle *et al.*, 1989; Cantor *et al.*, 1992). The underlying mechanisms of these discrepancies are not clear at present. However, further studies using other CCK_A receptor antagonists may be helpful in elucidating the physiological role of CCK. Thus, the development of CCK_A receptor antagonists with higher selectivity for CCK_A versus CCK_B receptors or with different characteristics is desirable in order to investigate new insights into digestive physiology and pathophysiology, to distinguish further CCK receptor substypes, and to examine the role of CCK in the treatment of gastrointestinal disorders (Meyer *et al.*, 1989; Beglinger *et al.*, 1991; 1992a; Rovati, 1991; Schmidt *et al.*, 1991; Niederau *et al.*, 1992; Tani *et al.*, 1993; Herrington & Adrian, 1995).

A new serin derivative (**R**)-1-[3-(3-carboxypyridine-2-yl)-thio-2-(indol-2-yl)carbonylamino]propionyl-4-diphenylmethylpiperazine (TP-680; Figure 1) has been recently developed in Japan. In the present study, we examined the pharmacological profile of this new compound *in vitro* and compared its properties to the CCK receptor antagonists, MK-329 and loxiglumide.

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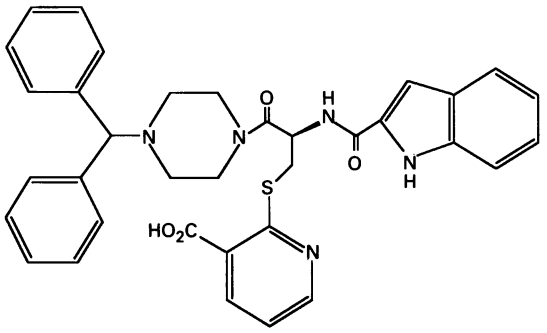

Figure 1 Structure of TP-680 ((R)-1-[3-(3-carboxypyridin-2-yl)thio-2-(indol-2-yl)carboxylamino]propionyl-4-diphenylmethylpiperazine). Molecular weight: 619.74.

Methods

Preparation of cerebral cortex membranes

Membranes from rat cerebral cortex were prepared according to the method described by Saito *et al.* (1980). Briefly, male Wistar rats weighing approximately 250 g were decapitated and their cerebral cortex excised and homogenized in twenty volumes of 0.32 M sucrose at 4°C with a motor-driven Teflon-glass homogenizer (1,000 r.p.m., 10 strokes). The homogenate was centrifuged at 1,000 *g* for 10 min at 4°C. The supernatant was recentrifuged at 42,000 *g* for 15 min at 4°C, and the resulting pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) and centrifuged at 42,000 *g* for 15 min at 4°C. The washing procedure was repeated 3 times. The resulting pellet was suspended in 20 mM HEPES buffer (pH 6.5) containing 5 mM $MgCl_2$, 1.0 mM EGTA, 0.2 mg ml^{-1} bacitracin, 0.36 M NaCl, 15 mM KCl to obtain membrane specimens.

Preparation of isolated pancreatic acini

Rat isolated pancreatic acini were prepared from a male Wistar rat fed *ad libitum*, weighing approximately 250 g, by the method described previously by Otsuki & Williams (1982). The basic medium used to prepare the isolated acini was a modified Krebs-Henseleit bicarbonate buffer (KHB), containing 11.1 mM glucose, 0.1 mg ml^{-1} soybean trypsin inhibitor, and minimal Eagle's medium amino acid supplement, and was gassed with 95% O_2 and 5% CO_2. The preincubation and incubation medium was HR, which was similar to KHB but contained 10 mM HEPES (pH 7.4) as a buffer, and 5 mg ml^{-1} bovine plasma albumin, and was gassed with 100% O_2.

[^{125}I]-CCK-8 binding

The binding of [^{125}I]-CCK to its receptors on isolated pancreatic acini was determined as reported previously (Otsuki *et al.*, 1984). Isolated acini were suspended at a protein concentration of 0.3–0.4 mg ml^{-1} in the HR solution supplemented with 0.5 mg ml^{-1} bacitracin; 4 ml aliquots were distributed into plastic flasks together with [^{125}I]-CCK-8 at a concentration of 10 pM, and various concentrations of TP-680, MK-329, or loxiglumide. Incubation was carried out for 120 min in a 37°C water bath shaking at a rate of 60 min^{-1}. At steady state of binding, triplicate 1.0 ml samples were removed from each flask and bound radioactivity was determined as previously reported (Otsuki *et al.*, 1984). Nonspecific binding was determined by incubating acini with [^{125}I]-CCK-8 in the presence of an excess amount of unlabelled CCK-8 (500 nM).

CCK binding to membranes from cerebral cortex was determined as reported previously (Saito *et al.*, 1982). Freshly prepared membranes (0.2–0.3 mg protein ml^{-1}) were incubated in plastic microcentrifuge tubes in 500 μl of incubation medium with 20 pM [^{125}I]-CCK-8 together with various concentrations of TP-680, MK-329, or loxiglumide. Incubation was carried out for 120 min in a 24°C water bath with constant shaking at 60 times min^{-1}. At a steady state of binding, the tubes were centrifuged at 10,000 *g* for 1 min, and the pellets were rinsed with cold buffer and recentrifuged. This procedure was repeated twice, and then the tips of the tubes were cut off and the radioactivity was determined. Nonspecific binding was determined by adding 1.0 μM CCK-8.

Amylase release

Acini were preincubated for 30 min at 37°C at a density of 1.0–1.5 mg acinar protein ml^{-1} with constant shaking at a rate of 60 min^{-1}. After preincubation, the acini were centrifuged and resuspended in fresh HR at a density of 0.25–0.35 mg acinar protein ml^{-1}. Two-milliliter aliquots were distributed into 25 ml polycarbonate incubation flasks, and secretagogues were added in the presence or absence of the CCK receptor antagonists. The flasks were incubated at 37°C with constant shaking at 60 times min^{-1}.

Four sets of experiments were performed. First, potency in inhibiting amylase release stimulated by a maximal effective concentration of CCK-8 (100 pM) was compared among TP-680, MK-329 and loxiglumide. Acini were incubated with 100 pM CCK-8 in the presence of TP-680 at concentrations ranging from 100 pM to 10 μM. Similar experiments were performed with MK-329 and loxiglumide.

In the second series of experiments, the specificity of the inhibitory action of TP-680 on amylase release was examined. Receptor secretagogues CCK-8 (100 pM), carbamylcholine (1.0 μM), bombesin (100 nM), secretin (10 nM), or VIP (10 nM), or agent bypassing receptors A23187 (1.0 μM), TPA (100 nM), or 8Br-cyclic AMP (100 μM) (Williams & Hootman, 1986) were incubated in the absence or presence of 1.0 μM TP-680.

In the third set of experiments, the mode of the inhibitory action of TP-680 on CCK-8-stimulated amylase release was examined. Acini were divided into two groups and incubated with various concentrations of CCK-8 ranging from 1 pM to 100 nM in the absence or presence of 3–300 nM TP-680. In addition, the effect of TP-680 on the time course of CCK-8-stimulated amylase release was examined. TP-680 at a concentration of 1.0 μM was added from the beginning or 20 min after the start of incubation with 100 pM CCK-8.

Finally, the reversibility of the inhibitory effect of TP-680 was examined. Acini were incubated for 30 min at 37°C with or without 100 nM or 1.0 μM TP-680 and centrifuged. The supernatant was discarded while the precipitate (acini) was resuspended in 10 ml fresh HR. The acinar suspension was centrifuged again. The procedure was repeated for three times before resuspending acini in fresh HR for subsequent amylase study. Amylase release during a second 30 min incubation at 37°C with different concentrations of CCK-8 was determined. Similar experiments were performed with 100 nM MK-329- or 10 μM loxiglumide-pretreated acini. In addition, the effect of loxiglumide on the residual inhibitory action of TP-680 on CCK-8-stimulated amylase release was also examined. Acini were conincubated with 100 μM loxiglumide and 100 nM TP-680 for 30 min at 37°C. They were then washed three times, followed by determination of amylase release during a second 30 min incubation at 37°C with different concentrations of CCK-8.

Amylase activity was measured by the Phadebas amylase test (Ceska *et al.*, 1969). Amylase released into the extracellular medium during a 30 min incubation with various concentrations of secretagogues is expressed as a percentage of the total content of the enzyme present in the acinar pellet at the beginning of the incubation period (Otsuki & Williams, 1982).

Viability of acini

Viability of acini after 30 min incubation with 1.0 μM TP-680 was evaluated by determining LDH release into the incubation

medium and by the dye exclusion test of trypan blue. LDH activity was determined according to the method of Wroblewski & LaDue (1955).

Chemicals

TP680 was synthesized and supplied by Tobishi Pharmaceutical Co., Tokyo, Japan. MK-329 (3(S)-(−)-1,3-dihydro-3-(2-indolecarbonyl-amino)-1-methyl-5-phenyl-2H-[1,4]-benzodiazepine-2-one) was a generous gift from Dr. V.J. Lotti (Merck Sharp and Dohme Research Laboratories, West Point, PA, U.S.A.). Loxiglumide was supplied by Kaken Pharmaceutical Co. (Tokyo). TP-680 and MK-329 were dissolved in 100% dimethylsulphoxide (DMSO), while loxiglumide was dissolved by 0.1 N NaOH. The following were purchased: soybean trypsin inhibitor (type 1-S), carbamylcholine chloride, the calcium ionophore A23187, 8-bromoadenosine 3′,5′-monophosphate (8Br- cyclic AMP), 12-O-tetradecanoylphorbol 13-acetate (TPA), N-2-hydroxyethylpiperazine-N′-2-ethane sulphonic acid (HEPES), and bacitracin from Sigma Chemical Co. (St. Louis, MO, U.S.A.); chromatographically purified collagenase (type CLSPA) from Cooper Biochemical (Malvern, PA, U.S.A.); minimal Eagle's medium amino acid supplement from GIBCO Laboratories (Life Technologies Inc., Grand Island, NY, U.S.A.); bovine plasma albumin (fraction V) from Armour Pharmaceutical Co. (Phoenix, AZ, U.S.A.) and Phadebas amylase test (Amylase Test A) from Shionogi Pharmaceutical Co. (Osaka, Japan). [^{125}I]-Bolton Hunter labelled CCK-8 ([^{125}I]-CCK-8; specific activity, 81.4 TBq mmol^{-1}) was purchased from Du Pont Co. (Biotechnology System, Wilmington, DE, U.S.A.). Synthetic COOH-terminal octapeptide of CCK (CCK-8), synthetic porcine vasoactive intestinal peptide (VIP), synthetic secretin and synthetic bombesin were purchased from Peptide Institute (Protein Research Foundation, Osaka). Synthetic caerulein was obtained from Kyowa Hakko Kogyo (Tokyo). Stock solutions of synthetic peptides were prepared with 0.15M NaCl containing 1% bovine plasma albumin and stored at −80°C. At the time of the experiment, peptides were diluted with HEPES-buffered Ringer solution (HR) containing 0.5% bovine plasma albumin.

Statistics

Data are expressed as mean ± s.e.mean. Regression analysis was used to estimate EC_{50} and IC_{50} values. The mean values were compared by analysis of variance or Wilcoxon rank-sum test. P values <0.05 were considered to indicate statistically significant difference between two groups.

Results

Receptor binding studies

The ability of TP-680, MK-329, and loxiglumide to occupy the CCK receptors on rat isolated pancreatic acini and the biological membranes from rat cerebral cortex was assessed by the displacement of [^{125}I]-CCK-8 by these compounds. TP-680, MK-329 and loxiglumide inhibited [^{125}I]-CCK-8 binding to rat isolated pancreatic acini in a dose-dependent manner (Figure 2a). The calculated IC_{50} values of TP-680, MK-329 and loxiglumide were 1.2 ± 0.04, 0.42 ± 0.07, and 270 ± 70 nM, respectively TP-680 was three times less potent than MK-329 but was 225 times more potent than loxiglumide in inhibiting [^{125}I]-CCK-8 binding to rat isolated pancreatic acini (Table 1).

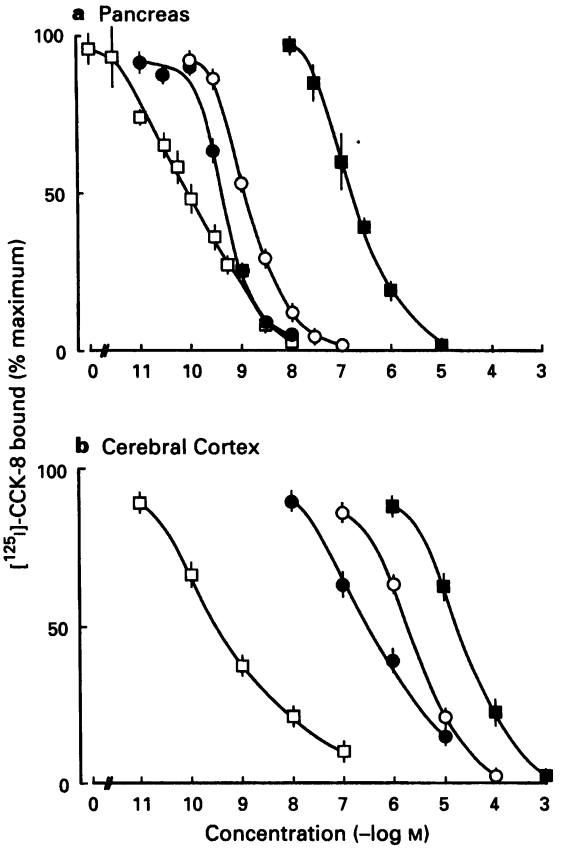

Figure 2 Effects of TP-680 (○), MK-329 (●) and loxiglumide (■) on binding of [^{125}I]-CCK-8 (□) to isolated pancreatic acini (a) and the biological membranes from rat cerebral cortex (b). Binding of [^{125}I]-CCK-8 is expressed as percentage of saturable binding obtained with [^{125}I]-CCK-8 alone. In each experiment, each value was determined in triplicate, and data represent the mean ± s.e.mean of four separate experiments.

In brain receptor binding studies using rat cerebral cortex membranes, CCK receptor antagonists also showed a concentration-dependent effect in displacing [^{125}I]-CCK-8 binding with IC_{50} values of 1,812.5 ± 283.1, 320 ± 54.2, and 18,750 ± 1,974 nM for TP-680, MK-329 and loxiglumide, respectively (Figure 2b and Table 1). Thus, TP-680 bound to rat pancreatic CCK_A receptors 1,510 times more potently than to the rat brain CCK_B receptors, whereas MK-329 and loxiglumide showed 762 and 69 times greater affinity, respectively, for the pancreatic receptors compared with brain receptors (Table 1).

Table 1 Half maximal inhibitory concentration of TP-680, MK-329, and loxiglumide on 100 pM CCK-8-stimulated amylase release, and [^{125}I]-CCK-8 binding to rat isolated pancreatic acini and biological membranes from rat cerebral cortex

	Amylase release (nM)	*[^{125}I]-CCK-8 binding* *Acini* (nM)	*Cortex* (nM)	*Cortex / Acini*
TP-680	57.3 ± 4.5	1.2 ± 0.04	1812.5 ± 283.1	1,510
CCK-8		0.10 ± 0.01	0.35 ± 0.04	3.5
MK-329	3.3 ± 0.1	0.42 ± 0.07	320 ± 54.2	762
Loxiglumide	6066 ± 751	270 ± 70	18750 ± 1973.8	69

In each experiment, each value was determined in triplicate. Data represent the mean ± s.e.mean of 4–6 separate experiments.

Inhibitory potency on amylase release

TP-680, MK-329 and loxiglumide caused a concentration-dependent inhibition of CCK-8-stimulated amylase secretion (Figure 3). Neither DMSO (solvent of TP-680 and MK-329) nor any of the antagonists tested stimulated amylase secretion when present alone (data not shown). Based on data presented in Figure 3, the calculated IC_{50} for TP-680, MK-329, and loxiglumide was estimated at 57.3±4.5, 3.3±0.1, and 6,066± 751 nM, respectively. TP-680 was approximately 17 times less potent than MK-329, but was 106 times more potent than loxiglumide in its ability to inhibit 100 pM CCK-8-stimulated amylase release.

Specificity

The inhibitory action of TP-680 was specific for the CCK receptor. A concentration of 1.0 μM TP-680 that almost completely inhibited the increase in amylase release caused by secretagogues that interact with CCK receptors had no effect on stimulation by carbamylcholine, bombesin, secretin, or VIP, which interact with distinct receptors, or the calcium ionophore A23187, TPA, or 8Br-cyclic AMP, that have a post-receptor mechanism of action (Table 2).

Mode of inhibitory action

Pancreatic acini were incubated with increasing concentrations of CCK-8, with or without various concentrations of the antagonists. TP-680 caused a parallel rightward shift in the entire dose-response curve for CCK-8-stimulated amylase release (Figure 4). In addition, TP-680 significantly suppressed the maximal amylase release (peak response, control; 13.7±0.4%, 100 nM TP-680; 11.7±0.7% $P<0.01$). The magnitude of the shift of the dose-response curve and the reduction in maximal response were proportional to the concentration of TP-680, indicating that TP-680 is an unsurmountable antagonist (Fig-

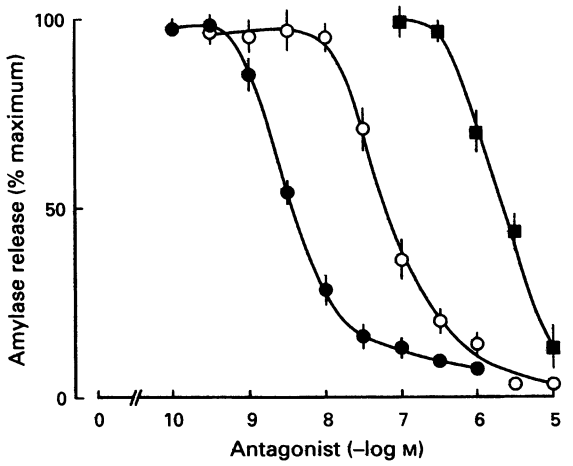

Figure 3 Effect of TP-680 (○), MK-329 (●) and loxiglumide (■) on CCK-8-stimulated amylase release from the isolated pancreatic acini. The acini were incubated for 30 min at 37°C with 100 pM CCK-8 together with various concentrations of TP-680, MK-329 or loxiglumide. In each experiment, each value was determined in triplicate, and data represent the mean±s.e.mean of 4–5 separate experiments.

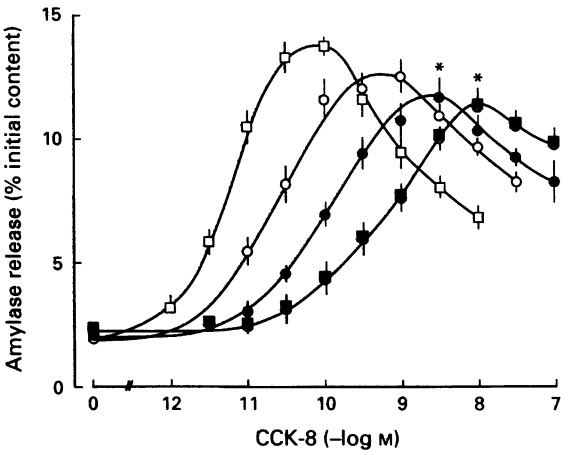

Figure 4 Effect of TP-680 on CCK-8-stimulated amylase release from isolated pancreatic acini. In each experiment, acini were divided into two groups and the release of amylase in response to various concentrations of CCK-8 was determined, in the absence (control, □) or presence of 30 (○), 100 (●) or 300 nM (■) TP-680. In each experiment, each value was determined in triplicate, and data represent the mean±s.e.mean from 4–6 separate experiments.

Table 2 Effect of TP-680 on stimulation of amylase secretion by various secretagogues

Secretagogue		*Amylase release (% of initial content)* TP680 (1.0 μM) *without*	*with*
None		1.7±0.2	1.7±0.2
CCK-8	100 pM	13.8±0.4	3.1±0.1
Caerulein	100 pM	13.8±1.3	3.3±0.6
Carbamylcholine	1 μM	12.9±0.6	12.7±0.6
Bombesin	100 nM	13.1±0.4	13.0±0.8
Secretin	10 nM	5.4±0.6	5.5±0.3
VIP	10 nM	4.2±0.3	4.2±0.2
8Br-cyclic AMP	100 μM	3.2±0.1	3.4±0.2
TPA	100 nM	6.9±0.4	7.5±0.5
A23187	1 μM	4.0±0.5	3.8±0.3

Amylase released into the extracellular medium during a 30 min incubation at 37°C with various pancreatic secretagogues alone or in the presence of 1.0 μM TP-680 was expressed as a percentage of the total content of the enzyme present in the acinar pellet at the beginning of the incubation period. In each experiment, each value was determined in triplicate. Data represent the mean±s.e.mean of 4–6 separate experiments.

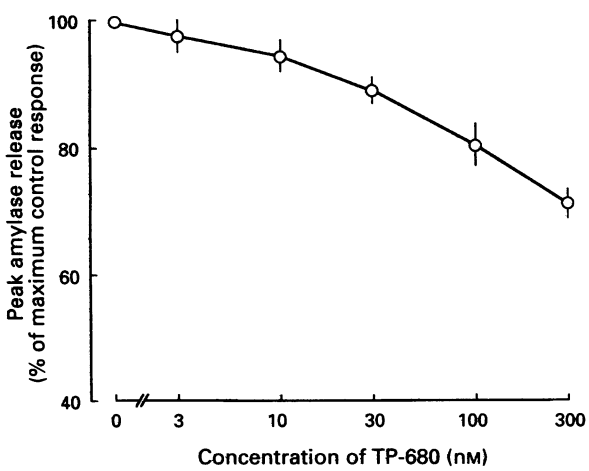

Figure 5 Kinetic analysis of the concentration-dependent decay of CCK-8-stimulated maximum amylase release by TP-680. In each experiment, acini were divided into two groups and amylase release in response to various concentrations of CCK-8 was determined in the absence (control) or presence of 3, 10, 30, 100, or 300 nM TP-680. Each value represents the percentage of the maximal response to CCK-8 in control acini incubated without TP-680. Data represent the mean±s.e.mean of 4–6 separate experiments.

ure 5). Furthermore, the slope of the Schild regression line of TP-680 was 1.69, indicating that its mode of inhibition was not competitive. In contrast, MK-329 and loxiglumide shifted the entire dose-response curve for CCK-8-stimulated amylase release to the right without suppressing the maximal response (data not shown, see Otsuki *et al.*, 1988; 1989). Pretreatment with 100 μM loxiglumide for 15 min prior to the application of TP-680 shifted the entire dose-response curve further to the right, but there was no decrease in the maximal response protecting the CCK receptors from the unsurmountable antagonism by TP-680 (Figure 6).

TP-680 not only prevented but also reversed the stimulation of amylase release caused by CCK-8 (Figure 7). A significant inhibition was seen within 5 min of adding TP-680 (1.0 μM) at the beginning, whereas a significant effect was not observed within 10 min when TP-680 was given 20 min after the addition of 100 pM CCK-8.

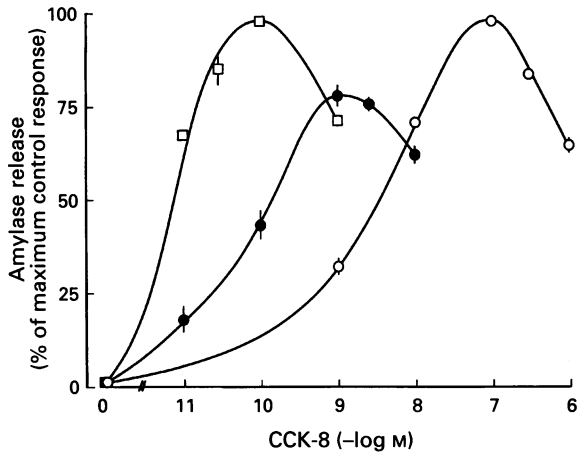

Figure 6 Effect of loxiglumide on the unsurmountable antagonism by TP-680. The acini were incubated for 15 min at 37°C with 100 μM loxiglumide prior to the application of 100 nM TP-680. In each experiment, acini were divided into three groups and amylase release in response to various concentrations of CCK-8 was determined in the absence (control □) or presence of 100 nM TP-680 (●) or 100 nM TP-680 plus 100 μM loxiglumide (○). Each value represents the percentage of the maximal response to CCK-8 in control acini incubated without TP-680. Data represent the mean ± s.e.mean of 4–6 separate experiments.

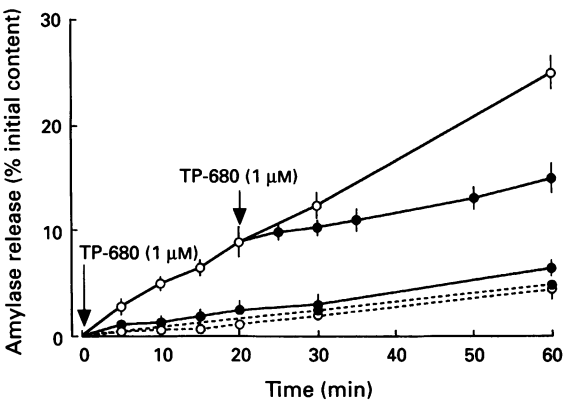

Figure 7 Effect of TP-680 on time course of CCK-8-stimulated amylase release. Acini were incubated with (○—○) or without 100 pM CCK-8. TP-680 (1.0 μM) (●—●) was added at 0 or 20 min; (●- - -●) TP-680 alone; (○- - -○) DMSO alone. Amylase release was determined at indicated times and expressed as percentage of total cellular amylase at beginning of incubation and released into extracellular medium during incubation. In each experiment each value was determined in duplicate, and data represent the mean ± s.e.mean from 4 separate experiments.

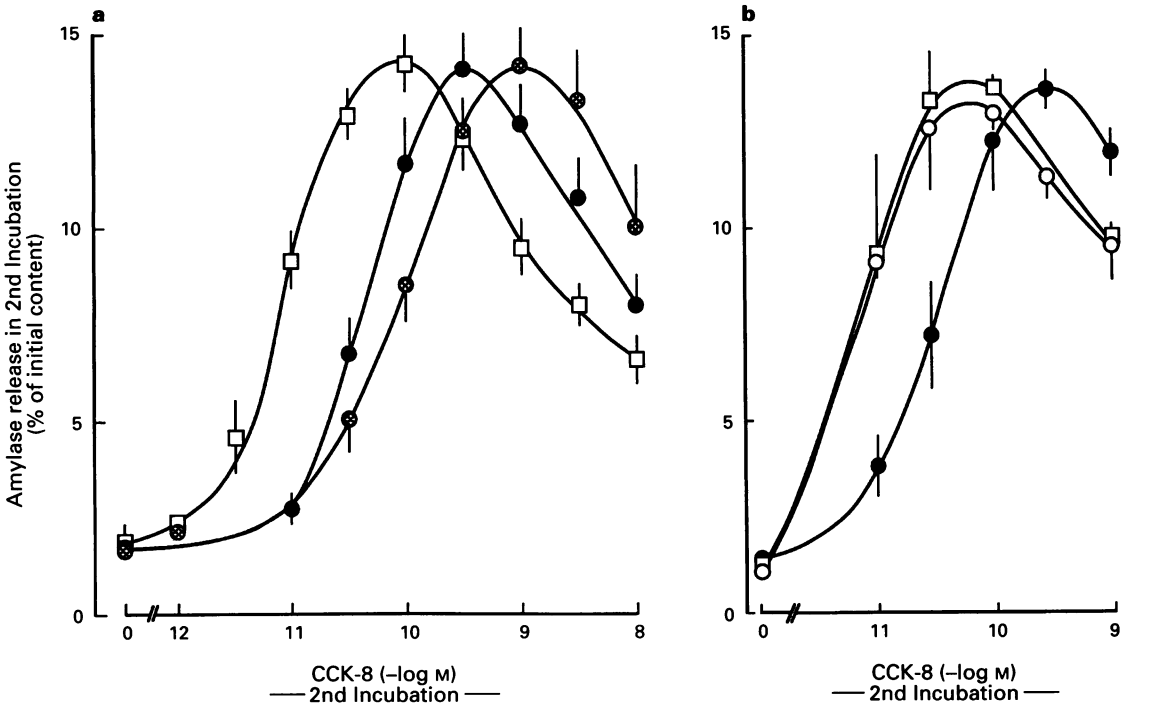

Figure 8 The effect of first incubating acini with TP-680 on the subsequent stimulation of amylase release by CCK-8. (a) The acini were incubated for 30 min at 37°C without (□) or with 100 nM (●) or 1.0 μM TP-680 (⊗) (first incubation). The acini were then washed three times and incubated for 30 min at 37°C with various concentrations of CCK-8 (second incubation). (b) The acini were incubated for 30 min at 37°C without (□) or with 100 nM TP-680 (●) or 100 nM TP-680 plus 100 μM loxiglumide (○) (first incubation). The acini were then washed three times and incubated for 30 min at 37°C with various concentrations of CCK-8 (second incubation). In each experiment, each value was determined in triplicate, and data represent the mean ± s.e.mean of 4 separate experiments.

Reversibility of the inhibitory effects

To examine the reversibility of the anti-CCK effects of TP-680, pancreatic acini were first incubated with 100 nM or 1 μM TP-680 for 30 min at 37°C, washed three times with fresh HR, and then resuspended in fresh HR without TP-680. Subsequent dose-response curve to CCK-8 in TP-680-pretreated acini was still shifted towards 3- (preincubated with 100 nM TP-680) and 10 fold (1.0 μM) higher concentrations compared with the control (Figure 8a). However, the response to CCK-8 was completely recovered and there was no decrease in the maximal response to CCK-8 of the acini pretreated with TP-680 (Figure 8a). Since the anti-CCK effects of loxiglumide was reversible after washing (data not shown, see Otsuki *et al.*, 1988; 1989), acini were pretreated with 100 nM TP-680 in the presence of 100 μM loxiglumide to examine whether the latter can protect CCK receptors from the irreversible antagonism by TP-680. Residual inhibition caused by TP-680 disappeared completely when acini were treated with TP-680 in the presence of loxiglumide (Figure 8b).

Viability of acini

At the highest concentration of TP-680 used in the present study (1.0 μM), all acini remained essentially intact as shown by a lack of significant increase of LDH release into the medium during a 30 min incubation and the exclusion of trypan blue (data not shown).

Discussion

The major finding of our study is that the newly developed serine derivative, TP-680, inhibited [^{125}I]-CCK-8 binding to rat isolated pancreatic acini with IC_{50} value of 1.2 ± 0.04 nM, making it less potent than MK-329, but more potent than loxiglumide. The present study of receptor binding also demonstrated that TP-680 binds to the rat pancreatic acini 1,510 times more potently than to brain CCK receptors. MK-329 and loxiglumide also showed 762 and 69 times, respectively, greater affinity for the pancreatic compared with brain CCK receptors. Thus, TP-680 is the most selective antagonist for peripheral CCK$_A$ receptors so far reported. The selectivity of TP-680 for the CCK receptor was further demonstrated by a lack of significant inhibition against amylase secretion caused by peptides acting on receptors other than CCK, including carbamylcholine, bombesin, secretin and VIP, and by a lack of effect on the amylase-releasing action of agents that bypass receptors, such as the calcium ionophore A23187, TPA, or 8Br-cyclic AMP.

Loxiglumide was found to have 27 times greater affinity for rat isolated pancreatic acini than for mouse cerebral cortex membranes (Setnikar *et al.*, 1987). On the other hand, MK-329 had a 166 fold greater selectivity for the peripheral CCK receptors than for the brain receptors (Hughes *et al.*, 1990). The difference between those studies and the present results are probably due to species differences or to differences in the preparations used for the binding studies. We used rat isolated pancreatic acini and rat cerebral cortex membranes, whereas previous studies used mouse cerebral cortex membranes and rat isolated pancreatic acini (Setnikar *et al.*, 1987) or rat pancreas membranes (Hughes *et al.*, 1990).

TP-680 was approximately 106 times more potent than loxiglumide, but was 17 times less potent than MK-329 in inhibiting 100 pM CCK-8-stimulated amylase release from rat pancreatic acini. Increasing concentrations of TP-680 caused a parallel rightward shift of the entire dose-response curve of CCK-8-stimulated amylase release. However, in contrast to MK-329 and loxiglumide, TP-680 dose-dependently decreased the maximum response of amylase release stimulated by CCK-8. Schild plot analysis of the effect of TP-680 showed that the slope was 1.69. This form of noncompetitive inhibition was termed 'unsurmountable antagonism' by Gaddum *et al.* (1955), which is characterized by its slow onset of action, long-lasting inhibition and reduction of the maximal response. These inhibitory mechanisms are thought to reflect a slow dissociation of the antagonist from its receptors (Mills & Wood, 1989).

The irreversibility of the antagonism exerted by TP-680 was also noteworthy. After the first incubation of the acini with loxiglumide, repeated washing of the acini restored the sensitivity to CCK-8 (Otsuki *et al.*, 1989). In contrast, the inhibitory effect of TP-680 persisted in spite of washing of the acini. In addition, TP-680 caused a dose-dependent residual inhibition of the action of CCK-8. In contrast to the sensitivity, repeated washing of the acini restored their responsiveness to CCK-8 after TP-680 treatment. Thus, TP-680-induced inhibition appears to have a two-step reaction; the first is reversible while the second is irreversible. The altered sensitivity of the acini to CCK-8 stimulation may be explained by tight and persistent occupation of CCK receptors by TP-680. The other possible explanation for the residual inhibition caused by TP-680 is that the compound influences membrane fluidity or mobility and thus changes the effect of CCK on exocrine pancreas or affects a specific protein adjacent to the receptors rather than the receptor itself due to its ability to enter and cross the lipid membrane. Such an effect on acinar cells might have some influence on the cellular mechanisms related to stimulus-secretion coupling in response to the subsequent CCK-8 stimulation in isolated pancreatic acini. This is unlikely, however, since the addition of loxiglumide together with TP-680 protected CCK receptors from the persistent residual antagonism by TP-680. Loxiglumide is a well-characterized hydrophilic CCK$_A$ receptor antagonist that rapidly binds to and dissociates from the CCK$_A$ receptors (Otsuki *et al.*, 1989). These results suggest that TP-680 interacts selectively and specifically with the CCK$_A$ receptor as its antagonist. Considered together, our results suggest that TP-680-induced inhibition is initially competitive but becomes noncompetitive due to its irreversible action at the CCK receptor. Thus, it is possible that TP-680 is not a noncompetitive antagonist for the CCK receptor, but instead, is a competitive and irreversible antagonist that interacts with the receptors.

The present data derived from an *in vitro* rat isolated pancreatic acini study, have demonstrated that the newly synthesized serine derivative, TP-680, is a potent, unsurmountable CCK$_A$ receptor antagonist and binds to CCK$_A$ receptors with greater affinity than MK-329 and loxiglumide. TP-680 appears to bind to CCK receptors on acinar cells in a slowly dissociating state and seems to remain active for a long period of time. Because of its selectivity for peripheral CCK$_A$ receptors and long duration of action, TP-680 may represent an excellent tool for studying the physiological function of CCK and gastrointestinal disorders in which CCK is possibly involved.

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