Pharmacological Profile of KSG-504, a New Cholecystokinin-A-Receptor Antagonist

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ABSTRACT—Pharmacological effects of KSG-504, a newly synthesized compound, on the response induced by exogenous CCK-8 were investigated. KSG-504 inhibited 125I-CCK-8 binding to both rat pancreas and cerebral cortex with IC50 values of $2.0 \times 10^{-7}$ M and $8.0 \times 10^{-5}$ M, respectively. The selectivity ratio of KSG-504 for pancreatic CCK receptor (CCK-A) was estimated as 400. In the isolated pancreatic acini of rats, KSG-504 caused a parallel rightward shift of the concentration-response curve for CCK-8-stimulated amylase release with no change in its maximal response, indicating a competitive antagonism of the drug for the CCK-A receptor (Schild plot analysis; slope = 0.927, $pA_2 = 6.9$). In addition, KSG-504 produced a significant inhibition of CCK-8-induced pancreatic amylase secretion when administered intravenously or intraduodenally to rats (ED50: 52 pg/kg/min by the i.v. route and 12.1 mg/kg by the i.d. route). KSG-504 had equipotent inhibitory effects on both CCK-8-stimulated pancreatic secretion and gallbladder contraction in dogs with ED50 values of 0.98 and 0.84 mg/kg, respectively. KSG-504 also inhibited the CCK-8-induced contraction of isolated guinea pig ileum in a concentration-dependent manner (IC50 = $3.0 \times 10^{-6}$ M). These results demonstrate that KSG-504 is a competitive and selective CCK-A-receptor antagonist that is effective in vivo after oral administration.

Keywords: KSG-504, CCK-A-receptor antagonist, Non-peptide antagonist, Pancreatic secretion, Gallbladder contraction

Cholecystokinin (CCK) is a peptide hormone found in the gastrointestinal tract and the central nervous system. Two types of CCK receptors have been recognized in alimentary (CCK-A) and brain (CCK-B) tissues (1). In the periphery, CCK is released from duodenal I cells (2) into the systemic circulation when the digested protein or lipid enters into the duodenum, and thus plays an important role in the regulation of pancreatic secretion (3), gallbladder contraction (4) and gut motility (5) via the CCK-A receptor. CCK stimulates both exocrine secretion of digestive enzyme from acinar cells and endocrine secretion of insulin from ß-cells (6) of the pancreas. Pancreatic cell growth (7, 8) is also controlled by CCK. From a pathophysiological point of view, it is suggested that endogenous CCK may be closely related to the etiology and development of pancreatitis, because CCK and its C-terminal analogue caerulein have been reported to produce acute pancreatitis (9, 10) or to worsen it (11) in experimental animals, and because pancreatitis patients frequently suffer from accompanying hypercholecystokinemia (12). It has also been reported that CCK may be implicated in diseases such as acute cholecystitis, biliary dyskinesia (13) and irritable bowel syndrome (14).

In recent years, several CCK-A-receptor antagonists have been developed and used for evaluating the physiological and pathophysiological roles of CCK. These antagonists are currently classified into the following types based on their chemical structures: 1) cyclic nucleotides such as dibutyryl cyclic GMP (15); 2) C-terminal CCK analogues such as CCK-27-32-amide (16); 3) amino acid derivatives such as lorglumide (17), loxiglumide (18) and benzotript (19); and 4) benzodiazepine derivatives such as asperlicin (20) and L-364,718 (21).

KSG-504 ((S)-arginium (R)-4-[N-(3-methoxypropyl)-N-pentylcarbamoyl]-5-(2-naphthylsulfonfonyl) pentanoate monohydrate) (Fig. 1) is a new non-peptide compound synthesized by Kissei Pharmaceutical Co., Ltd. In this study, we characterized the pharmacological effects of KSG-504 on exogenous CCK-induced responses in various organs and animal species.
MATERIALS AND METHODS

Drugs and chemicals
KSG-504 was synthesized by Kissei Pharmaceutical Co., Ltd. (Matsumoto). Other drugs and chemicals were obtained from the following commercial sources: 125I-CCK-8 (New England Nuclear, Boston, MA, USA); CCK-8 (Peptide Institute Inc., Minoh); collagenase, bacitracin and cycloheximide (Sigma, St. Louis, MO, USA); dithiothreitol and bovine serum albumin F-V (Nacalai Tesque, Kyoto); pentobarbital sodium (Dainippon Pharm, Osaka); and urethane (Kantoh Chem, Tokyo).

Animals
Male Wistar rats (200–250 g), male Japanese White rabbits (2.3–3.2 kg), mongrel dogs of either sex (5–13 kg) and male Hartley guinea pigs (350–450 g) were used after an overnight fast with free access to water.

Radioisotopes binding to CCK receptors
For the CCK-A receptor binding assay, pancreatic homogenate was prepared according to the method of Chang et al. (22), with minor modifications. Pancreas was isolated from the rat, homogenized in 50 volumes of 50 mM Tris-HCl buffer (pH 7.7 at 25°C) for 10 sec by an Ultradisperser (Janke and Kunkel GMBH, Staufen, Germany) and centrifuged at 50,000 × g for 10 min. The pellet was resuspended in the original volume of fresh Tris-HCl buffer and centrifuged as above. The final pellet was resuspended in 30 volumes of binding assay buffer (50 mM Tris-HCl, 5 mM MgCl2, 5 mM dithiothreitol, 0.14 mg/ml of bacitracin and 2 mg/ml of bovine serum albumin) using a glass homogenizer. Ten microliters of 125I-CCK-8 (30 pM final concentration) and KSG-504 were added to duplicate tubes containing 980 μl of tissue membrane (1 mg of original wet wt./ml). After incubation at 37°C for 10 min, the incubation was terminated by filtration under reduced pressure through a glass fiber filter (GF/B; Whatman, Maidstone, England), and then the filter was washed four times with 4 ml of ice-cold Tris-HCl buffer. The radioactivity trapped on the filter was counted in a gamma-counter (Auto-Gamma 5650; Packard, Downers Grove, IL, USA).

For the CCK-B receptor binding assay, the cerebrocortical microsomal fraction was used as the receptor source. The cerebral cortex of the rat was homogenized in 10 volumes of 50 mM Tris-HCl buffer (pH 7.7 at 25°C) for 10 sec and centrifuged at 8,000 × g for 8 min. The supernatant obtained was recentrifuged at 105,000 × g for 1 hr. The final pellet was resuspended in binding assay buffer and stocked at −40°C. Protein concentration was determined by the method of Lowry et al. (23). Five microliters of 125I-CCK-8 (100 pM final concentration) and KSG-504 were added to duplicate tubes containing 490 μl of microsomal fraction (1.5 mg of protein/ml). After incubation at 25°C for 2 hr, the incubation was terminated by filtration as described above.

Non-specific binding was defined as the radioactivity bound in the presence of 10−6 M of CCK-8 in both receptor binding assays.

Amylase release from isolated pancreatic acini of rats
Rat pancreatic acini were prepared by the method of Otsuki and Williams (24). After isolating the rat pancreas, 5 ml of Krebs-Henseleit buffer (pH 7.35 at 25°C) containing 120 unit/ml of collagenase was injected into the tissue. The tissue was incubated at 37°C for 30 min. Acini were then mechanically dissociated by forceful pipetting and purified by filtration through a nylon mesh (150 μm), and centrifuged at 50 × g for 4 min through Krebs-Henseleit buffer containing 4% bovine serum albumin. The isolated acini were preincubated at 37°C for 30 min with 10 mM Heps-Ringer buffer (pH 7.35 at 25°C) containing cycloheximide (300 μg/ml at a final concentration). Then the acini suspension (total 1 ml) gassed with 100% O2 was incubated at 37°C for 30 min with KSG-504 and CCK-8, and amylase activity in the medium was measured.

Pancreatic secretion in anesthetized rats
The rats were anesthetized with urethane (1.5 g/kg, s.c.), and the abdomen was incised. The common bile duct was ligated proximal to the pancreas below the hilum of the liver, and a polyethylene tube (PE-10; Becton Dickinson, Parsippany, NJ, USA) was inserted into the bile duct above the ligature. Another polyethylene tube was also inserted into the pancreatic duct at its entrance to the duodenum. Pancreatic juice volume and its amylase activity were measured after collecting the pure pancreatic juice every 30 min. CCK-8, dissolved in physiological saline containing 1% bovine serum albumin, was infused at a dose of 5 ng/kg/min or injected at 1 μg/kg intravenously. In the former experiment, intravenous infusion of

Fig. 1. Chemical structure of KSG-504, (S)-arginine (R)-4-[N-(3-methoxypropyl)-N-pentylcarbamoyl]-5-(2-naphthylsulfonyl) pentanoate monohydrate.
KSG-504 was initiated 90 min after the start of CCK-8 infusion. In the latter, the drug was administered intraduodenally 10 min or intravenously 1 min before CCK-8 injection.

Pancreatic secretion in anesthetized rabbits

The rabbits were anesthetized with urethane (1.2 g/kg, s.c.), and then the abdomen was incised. A polyethylene tube (PE-10) was inserted into a common pancreatic duct. Pancreatic juice was collected every 30 min, and its volume and amylase activity were measured. CCK-8 was injected intravenously at 2 μg/kg 1 min after the administration of KSG-504.

Pancreatic secretion and gallbladder contraction in anesthetized dogs

The dogs were anesthetized with pentobarbital sodium (30 mg/kg, i.v.), and then the abdomen was opened by midline incision. A polyethylene tube (PE-50) was inserted into a common pancreatic duct. Pancreatic juice was collected every 20 min, and its volume and amylase activity were measured.

An additional polyethylene tube (No. 6; Hibiki, Tokyo) was inserted into the gallbladder through a small incision at its bottom, and the gallbladder was perfused with warmed saline at a flow rate of 0.2 ml/min. The perfusion pressure was continuously measured by a pressure transducer (1829; San-ei, Tokyo) placed between the pump and the cannula, and the response was recorded on a pen-writing oscillograph (Rectigraph 8K, San-ei). CCK-8 was injected intravenously at 0.1 pg/kg 1 min after the administration of KSG-504.

Isolated ileal contraction in guinea pigs

The ileums of the guinea pigs were isolated and suspended in a Magnus bath containing Tyrode solution (95% O₂ + 5% CO₂, 30°C). Ileal contraction induced by 10⁻⁸ M of CCK-8 was measured with a force-displacement transducer (SB-1T; Nihon Kohden, Tokyo) and recorded on a pen-writing oscillograph (Rectigraph 8S, San-ei). KSG-504 was added to the bath 1 min before the application of CCK-8.

Determination of pancreatic amylase activity

The amylase concentration in the medium or pancreatic juice was determined by the CM-amylase DEX method (Amylase-B Test Wako; Wako Pure Chemical, Osaka) after an appropriate dilution, and the amylase output was calculated.

Data analysis

The results obtained were expressed as the mean ± S.E. Statistical significance was determined by Student’s t-test.

RESULTS

Radioligand binding to CCK receptors

¹²⁵I-CCK-8 showed saturable binding to rat pancreatic homogenate with a Kd value of 0.19 nM. KSG-504 at concentrations over 10⁻⁸ M produced a concentration-dependent inhibition of ¹²⁵I-CCK-8 specific binding to rat pancreas (Fig. 2), and the IC₅₀ value was (2.0±0.1) x 10⁻⁷ M. KSG-504 inhibited the ¹²⁵I-CCK-8 specific binding to rat cerebral cortex with an IC₅₀ value of (8.0±2.7) x 10⁻⁵ M, which was 400 times as high as that for the pancreas.

Amylase release from isolated pancreatic acini of rats

KSG-504 at a concentration of 5 x 10⁻³ M alone did not produce acinar cell damage during the 30-min incubation period, judging by the trypan blue exclusion test.

When isolated rat pancreatic acini were incubated with CCK-8, concentration-dependent amylase release was observed. The maximal amylase release was obtained at 10⁻¹⁰ M of CCK-8. KSG-504 caused a parallel rightward shift of the concentration-response curve for CCK-8, but did not alter the maximal amylase release from the acini (Fig. 3A).

From the Schild plots analysis for the inhibition of CCK-8-stimulated amylase release by KSG-504 (0.3–3 μM), a slope of 0.927 and a pA₂ value of 6.9±0.1 were calculated (Fig. 3B).

Pancreatic secretion in anesthetized rats

After the start of CCK-8 infusion, pancreatic amylase secretion increased gradually from 70±12 IU/30 min to the maximal level of 1021±151 IU/30 min (n=10) at 90
min of the infusion and lasted throughout the experiment. When KSG-504 was infused intravenously 90 min after the start of CCK-8 infusion, pancreatic amylase secretion was significantly inhibited in a dose-dependent manner. The ED$_{50}$ value of KSG-504 was calculated to be 52 pg/kg/min at 60 min after the drug infusion (Fig. 4).

Intravenous injection of CCK-8 (1 pg/kg) produced a 11.5-fold increase in pancreatic amylase secretion at 30 min after the injection. When KSG-504 was injected intravenously, a significant inhibition of pancreatic secretion was observed (ED$_{50}$=0.54 mg/kg).

Intraduodenal administration of KSG-504 also caused a dose-dependent inhibition of the pancreatic amylase secretion induced by CCK-8 injection (1 pg/kg, i.v.), with an ED$_{50}$ value of 12.1 mg/kg (Fig. 5).
Pancreatic secretion in anesthetized rabbits

After intravenous injection of 2 μg/kg of CCK-8, pancreatic amylase secretion increased from 94±19 IU/30 min to 1047±88 IU/30 min (n=17). When KSG-504 was injected intravenously 1 min before CCK-8 injection, pancreatic amylase secretion was inhibited dose-dependently, with an ED50 value of 1.1 mg/kg.

Pancreatic secretion and gallbladder contraction in anesthetized dogs

The effect of KSG-504 on CCK-8-induced pancreatic secretion and gallbladder contraction was examined simultaneously in the same dog. After intravenous injection of 0.1 μg/kg of CCK-8, pancreatic amylase secretion increased from 314±74 IU/20 min to 4802±679 IU/20 min (n=10). When KSG-504 was injected intravenously 1 min before CCK-8 injection, pancreatic amylase secretion was inhibited dose-dependently (Fig. 6A).

Intragallbladder pressure was increased from 9.9±1.3 cmH2O to 21.9±1.6 cmH2O (n=10) by CCK-8 injection, and this change was antagonized by KSG-504 pretreatment (Fig. 6B). The ED50 values of KSG-504 were 0.98 mg/kg and 0.84 mg/kg for the pancreas and gallbladder, respectively.

Isolated ileal contraction of guinea pigs

When 10⁻⁸ M of CCK-8 was applied to isolated guinea pig ileum, phasic contraction (2.54±0.17 g, n=6) was induced. KSG-504 at concentrations of 10⁻⁶ M or higher inhibited the contraction significantly (Fig. 7). The IC₅₀ value of KSG-504 was (3.0±0.6) x 10⁻⁶ M.

DISCUSSION

In the present study, we evaluated the effect of KSG-504 on exogenous CCK-induced responses both in vitro and in vivo and characterized the effect pharmacologically.

In the first experiment, selectivity of KSG-504 on CCK-A and CCK-B receptors was examined by a radioligand binding assay. The Kₐ value of ¹²⁵I-CCK-8 binding to the pancreatic membrane preparation of rats was 0.19 nM,
which is comparable to the value reported by Chang et al. (about 0.10 nM) (22). KSG-504 inhibited the $^{125}$I-CCK-8 binding to the same preparation with the IC$_{50}$ value of $(2.0 \pm 0.1) \times 10^{-7}$ M. KSG-504 also produced an inhibition of $^{125}$I-CCK-8 binding to the cerebrocortical membrane preparation of rats, but the inhibition was significant only after treatment with $10^{-5}$ M of the drug. From the IC$_{50}$ values of this drug, we confirmed that KSG-504 was 400 times more selective for CCK-A receptors than for CCK-B receptors. The selectivity of KSG-504 for CCK-A receptors is apparently superior to that of another CCK-A receptor antagonist, loxiglumide, which has been reported to possess about 30 times higher selectivity for CCK-A receptors compared to CCK-B receptors (18).

In the next experiment, we examined the effect of KSG-504 on CCK-8-stimulated amylase release from isolated pancreatic acini to clarify whether the inhibition of $^{125}$I-CCK-8 binding to pancreatic membrane preparation by KSG-504 was due to its CCK-antagonistic effect. KSG-504 itself did not stimulate amylase release from isolated rat acini, which indicates that this drug lacks CCK-like agonistic activity. In addition, KSG-504 produced a parallel rightward shift of the concentration-response curve for CCK-8-stimulated amylase release without affecting its maximal amylase release. Schild plot analysis of the data in which the slope of the regression line was calculated as 0.927 demonstrates that the antagonism of KSG-504 against the CCK-A receptor is competitive in nature.

Anticholecystokinin effect of KSG-504 on the exocrine pancreas was also examined in vivo in several animal species including rats, rabbits and dogs. Intravenous infusion of KSG-504 caused a dose-dependent inhibition of CCK-8-induced pancreatic amylase secretion in rats with an ED$_{50}$ value of 52 $\mu$g/kg/min. Recently, Moriyoshi et al. (25) have reported that ED$_{50}$ value of KSG-504 in inhibiting CCK-8-stimulated pancreatic amylase secretion in rats was about 8 $\mu$g/kg/min, when exocrine pancreatic secretion was stimulated with a physiological dose of CCK-8 (1 ng/kg/min). The difference in efficacy of the drug may be due to the dose of CCK-8 used because we stimulated pancreatic amylase secretion with a pharmacological dose of CCK-8 (5 ng/kg/min) that produced a submaximal pancreatic secretion. There were no species differences in suppressing CCK-8-induced pancreatic amylase secretion after bolus i.v. injection of KSG-504. The inhibitions were observed in the same dose ranges in all species tested (ED$_{50}$=0.54-1.1 mg/kg).

Moreover, KSG-504 inhibited CCK-8-induced pancreatic amylase secretion when administered intraduodenally to the rat, but the inhibitory effect of intraduodenal KSG-504 was about one twentieth that of intravenous KSG-504, based on their ED$_{50}$ values. It seems likely that the differences in potency of KSG-504 may have resulted from its relatively high first pass effect and/or from its different pretreatment timing. We have confirmed that KSG-504 was immediately excreted to the bile when administered intraduodenally to the rat.

CCK is known to play a physiological role in regulating gallbladder emptying and gastrointestinal motility through CCK-A receptors. Although KSG-504 inhibited the CCK-8-induced pancreatic amylase secretion both in vitro and in vivo and also inhibited the contraction in canine gallbladder and guinea pig ileum, sensitivities to the drug were apparently different among various organs. KSG-504 showed equipotent inhibition of CCK-8-induced pancreatic amylase secretion and gallbladder contraction in the same dog. However, the inhibitory effect of KSG-504 on ileal contraction induced by CCK-8 was less potent than on the others. We have previously obtained the IC$_{50}$ value of $1.9 \times 10^{-6}$ M for KSG-504 in the inhibition of amylase release from rat pancreatic acini when it was stimulated with $10^{-10}$ M of CCK-8 which produced a submaximal amylase release from the acini. This IC$_{50}$ value of KSG-504 was comparable to that for the inhibition of ileal contraction induced by a submaximal dose of CCK-8 ($10^{-8}$ M).

The present study demonstrates that KSG-504 is a potent, competitive, specific and orally available CCK-A antagonist. Furthermore, we found in the preliminary experiment in the rabbit that KSG-504 could not enter into brain from the peripheral circulation even when a high dose of the drug, estimated to be 10-fold the ED$_{50}$, was infused intravenously for two hr. The concentration of KSG-504 in the cerebrospinal fluid at the cessation of infusion was below the determination limit (50 ng/ml). From these results, it will be assumed that the action of KSG-504 may be restricted for peripheral CCK-A receptors. Recently, it was reported that KSG-504 has protective and therapeutic effects on experimental pancreatitis models such as caerulein-induced pancreatitis (26) and duodenal loop-induced pancreatitis (27) which was accompanied by increased endogenous CCK. Further investigations are necessary to determine the clinical utility of KSG-504 for the treatment of CCK-related diseases and disorders.

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