Effect of T-0632, a Cholecystokinin$_A$ Receptor Antagonist, on Experimental Acute Pancreatitis

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ABSTRACT—Effects of a new cholecystokinin (CCK)$_A$-receptor antagonist, T-0632 [sodium (S)-1-(2-fluorophenyl)-2,3-dihydro-3-[(3-isoquinolinylcarbonyl)amino]-6-methoxy-2-oxo-1H-indole-3-propanoate], on caerulein-induced and pancreatic duct ligation-induced pancreatitis models were studied and compared with the CCK$_A$-receptor antagonist loxiglumide and the orally active protease inhibitor camostate, respectively. In rats, orally administered T-0632 potently prevented the caerulein-induced increases in pancreatic digestive enzymes in plasma and suppressed the histological changes in the pancreas. The estimated ED$_{50}$ values of T-0632 and loxiglumide were 0.0092 and 8.9 mg/kg, respectively. In dogs, T-0632 (0.1, 1 mg/kg, i.d.) prevented the caerulein-induced increase in plasma amylase activity in a dose-dependent manner. Loxiglumide (100 mg/kg, i.d.) did not show any preventive effects. In pancreatic duct ligation (6 hr)-induced pancreatitis of the rat, T-0632 (0.001–0.1 mg/kg, p.o.) partially prevented both the increase in plasma amylase activity and the histological changes in the pancreas, whereas camostate (10, 100 mg/kg, p.o.) did not show any preventive effects. In pancreatic duct ligation (3 hr)-induced pancreatitis, caerulein injection (1 μg/kg, s.c.) caused a further increase in plasma amylase activity, and T-0632 (0.01, 0.1 mg/kg, p.o.) dose-dependently decreased the aggravation by caerulein. We conclude that T-0632 showed preventive effects on all of these pancreatitis models by oral or intraduodenal administration. These results suggest that CCK plays an important role in progression and aggravation of acute pancreatitis, and T-0632 may have a therapeutic value in these disease states.

Keywords: Cholecystokinin (CCK)$_A$ receptor, Pancreatitis, Caerulein, Pancreatic duct ligation, T-0632

Although the pathogenesis of acute pancreatitis is not fully understood, the key event is considered to be the inappropriate release and intrapancreatic activation of pancreatic proenzymes (1). Cholecystokinin (CCK) is known to stimulate secretion of the pancreatic digestive enzymes via the CCK$_A$ receptor (2). Therefore, CCK is considered to play an important role in progression and aggravation of acute pancreatitis.

Experimental models of acute pancreatitis have been produced by various methods in several animal species (3, 4). Pancreatitis induced by supramaximal doses of caerulein, an analog of CCK, is regarded as a good model of edematous pancreatitis (5). In this model, CCK$_A$-receptor antagonists have been reported to be effective in several animal species (6–8). Pancreatic duct ligation-induced pancreatitis is considered to be a model of human pancreatitis caused by gallstones or pancreatic stones (3, 4). This model is known to cause acute pancreatitis with edematous and necrotic changes in the pancreas in the early stage, followed by atrophic and fibrous changes with acinar cell regeneration several weeks after the ligation (9, 10). The contribution of CCK to this model is not clearly known because the effects of CCK$_A$-receptor antagonists on this model have been controversial (10–12).

Recently, we found T-0632 [sodium (S)-1-(2-fluorophenyl)-2,3-dihydro-3-[(3-isoquinolinylcarbonyl)amino]-6-methoxy-2-oxo-1H-indole-3-propanoate] to be a novel nonpeptide and water-soluble CCK$_A$-receptor antagonist (13). The compound showed a potent CCK$_A$-receptor antagonistic action in both in vitro and in vivo studies (13, 14). In the present report, effects of T-0632 on two experimental acute pancreatitis models, caerulein- and pancreatic duct ligation-induced pancreatitis, were studied and compared with loxiglumide, a representative CCK$_A$-receptor antagonist, and camostate, an orally active protease inhibitor, respectively.
MATERIALS AND METHODS

Animals
Male Wistar rats weighing 180–230 g, Male Sprague-Dawley rats weighing 180–210 g and female beagle dogs weighing 7–12 kg were used. They were deprived of food for 24 hr with free access to tap water before the experiment.

Caerulein-induced acute pancreatitis in rats
Caerulein-induced acute pancreatitis was produced in rats (Wistar) by subcutaneous injections of caerulein (20 μg/kg, 4 times) at hourly intervals over 3 hr according to Tani et al. (5). T-0632 and loxiglumide were administered by the oral route 30 min before the first caerulein injection. Three hours after the last caerulein injection, blood was collected and the pancreas was removed under ether anesthesia. Pancreatic tissues were fixed in 10% formaldehyde and stained with hematoxylin and eosin for light microscopic examination. Amylase and lipase activities in plasma were measured with commercially available kits, α-Amylase® and Lipase® (Boehringer Mannheim Yamanouchi, Tokyo), respectively. Trypsin activity in plasma was measured according to the method described by Kawabata et al. (15) with minor modifications. In brief, 20 μl of plasma was added to a 980 μl buffer-substrate solution (51.5 mM Tris, 155 mM NaCl, 1.03 mM CaCl2, 0.103 mg/ml bovine serum albumin, 103 μM Boc-Gln-Ala-Arg-MCA) and incubated at 37°C. After 10 min, 1 ml of 30% acetic acid was added, and released 7-amino-4-methyl-coumarin (AMC) was determined fluorometrically with excitation at 380 nm and emission at 440 nm. Trypsin activity was expressed as the rate of AMC release (nmol/min/ml) from the substrate (Boc-Gln-Ala-Arg-MCA).

Caerulein-induced acute pancreatitis in dogs
Caerulein-induced acute pancreatitis in dogs was produced according to the method of McEntee et al. (16) with minor modifications. Dogs were anesthetized by an initial bolus injection (30 mg/kg, i.v.) followed by continuous intravenous infusion (5.5 mg/kg/hr) of sodium pentobarbitone. Positive pressure ventilation was maintained with a respirator through an endotracheal tube. The left femoral artery was cannulated and connected to a pressure transducer for monitoring the blood pressure. The right femoral vein was cannulated for infusion of caerulein. The duodenum was cannulated for administration of T-0632 and loxiglumide. One hour after the operation, T-0632 or loxiglumide was administered intraduodenally. Caerulein (10 μg/kg/hr) was infused for 5 hr through the femoral vein from 1 hr after the drug administration. Blood was collected at 30-min intervals, and amylase activity in plasma was measured as described above.

Pancreatic duct ligation-induced acute pancreatitis in rats
Pancreatic duct ligation-induced pancreatitis in rats (Sprague-Dawley) was produced according to the method of Murayama et al. (9) with minor modifications. Under ether anesthesia, the abdomen was incised and the hepatic bile duct and the common bile-pancreatic duct were ligated, and then the abdomen was closed. Six hours later, the animals were sacrificed with ether, and blood was collected and the pancreas was removed. Pancreatic tissues were fixed as described above. For the aggravation study with caerulein, caerulein (1 μg/kg, s.c.) was injected immediately after the hepatic bile duct and common bile-pancreatic duct ligation. Three hours later, the animals were anesthetized with ether, and blood was collected. T-0632 or camostate was administered by the oral route 30 min before the hepatic bile duct and common bile-pancreatic duct ligation. Amylase activity in plasma was measured as described above.

Drugs
T-0632, loxiglumide [D,L-4-(3,4-dichlorobenzoylamino)-5-(N3-methoxypropyl-pentylamino)-5-oxopentanoic acid] and camostate [N,N-dimethyl-carbamoylmethyl-4-(4-guanidinobenzoyloxy)phenylacetate methanesulfonate] were synthesized at the Lead Optimization Research Laboratory of Tanabe Seiyaku Co., Ltd. Caerulein was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo). Boc-Gln-Ala-Arg-MCA and AMC were purchased from Peptide Institute (Osaka). All other chemicals used were of the reagent grade. Drugs were dissolved or suspended in 1% Tween 80 (polyoxyethylene sorbitan monooleate) for oral and intraduodenal administration.

Statistics
Regression analysis was used for estimation of ED₅₀ values. Data were analyzed by Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. P values of <5% were considered significant.

RESULTS

Caerulein-induced acute pancreatitis in rats
Subcutaneous injections of caerulein (20 μg/kg, 4 times) caused an about 10-fold increase in plasma amylase activity compared with the basal level (saline injection). T-0632 (0.01, 0.1 mg/kg, p.o.) and loxiglumide (1–100 mg/kg, p.o.) prevented the caerulein-induced increase in plasma amylase activity in dose-dependent manners with the estimated ED₅₀ values of 0.0092 and 8.9...
Fig. 1. Effects of orally administered T-0632 (A) and loxiglumide (B) on the increase in plasma amylase activity after injection of caerulein (20 μg/kg, s.c., 4 times at hourly intervals) in rats. Drugs were administered 30 min before the first caerulein injection. The data represent the mean ± S.E.M. of 5 to 6 rats. *P < 0.05, **P < 0.01, compared with the caerulein plus vehicle group.

Fig. 2. Effects of T-0632 on caerulein-induced histological alterations in rat pancreas. A: saline plus vehicle-treated group. B: caerulein plus vehicle-treated group. C: caerulein plus T-0632 (0.1 mg/kg)-treated group. Sections were stained with hematoxylin and eosin and evaluated by light microscopy. (IL, islets of Langerhans; × 90)
mg/kg, respectively (Fig. 1: A and B). Plasma lipase and trypsin activities were also increased by the injection of caerulein from 0.38±0.02 I.U./ml to 28.9±2.7 I.U./ml and from 0.67±0.04 nmol/min/ml to 9.4±0.6 nmol/min/ml, respectively. T-0632 (0.1 mg/kg, p.o.) completely prevented the increase in plasma lipase and trypsin activities to the basal levels of 0.48±0.05 I.U./ml and 0.78±0.06 nmol/min/ml, respectively.

Histological changes in the pancreas in caerulein-induced acute pancreatitis were also investigated by light microscopy. The saline plus vehicle-treated (control) group exhibited the normal architecture of the pancreas (Fig. 2A). Remarkable interstitial edematous changes with infiltration of neutrophils were seen in the caerulein plus vehicle-treated group (Fig. 2B). In the caerulein plus T-0632 (0.1 mg/kg)-treated group, interstitial edema and cellular inflammatory infiltration were of lesser degrees than those in the caerulein plus vehicle-treated group (Fig. 2C).

**Caerulein-induced acute pancreatitis in dogs**

Intravenous infusion of caerulein (10 μg/kg/hr) caused time-dependent increases in the plasma amylase activity of dogs. Five hours after the start of infusion, the plasma amylase activity was increased from the basal level of 1.1±0.09 I.U./ml to 41.0±3.4 I.U./ml (Fig. 3). T-0632 (0.1, 1 mg/kg, i.d.) prevented the caerulein-induced increase in plasma amylase activity in a dose-dependent manner, whereas loxiglumide (100 mg/kg, i.d.) did not show any preventive effects (Fig. 3).

**Pancreatic duct ligation-induced acute pancreatitis in rats**

The plasma amylase activity in the hepatic bile duct only-ligated (BDL) group was not significantly different from that of the sham operation group (2.9±0.3 I.U./ml). Both hepatic bile duct and common bile-pancreatic duct ligation (PBDL) caused a time-dependent increase in plasma amylase activity (Fig. 4). T-0632 (0.001–0.1 mg/kg, p.o.) prevented the PBDL (6 hr)-induced increase in plasma amylase activity in a dose-dependent manner with a maximum inhibition of 70% at the dose of 0.1 mg/kg. Camostate (10, 100 mg/kg, p.o.) did not show any preventive effects (Fig. 5).

Histological changes in the pancreas were also investigated by light microscopy. Slight interstitial edema with slight infiltration of neutrophils was seen in the BDL group (Fig. 6A). More intensive interstitial edema and cellular inflammatory infiltration were seen in the PBDL group, and necrosis of acinar cells was also observed (Fig. 6B). In the PBDL plus T-0632 (0.1 mg/kg) group, the
Fig. 4. Time courses of plasma amylase activity in hepatic bile duct-ligated (BDL, open columns) and hepatic bile duct and common bile-pancreatic duct-ligated (PBDL, hatched columns) rats. The data represent the mean ± S.E.M. of 4 to 6 rats. Significant differences (*P < 0.05, **P < 0.01) between the BDL and the PBDL groups at the respective times.

Fig. 5. Effects of orally administered T-0632 and camostate on pancreatic duct ligation (6 hr)-induced pancreatitis in rats. BDL: hepatic bile duct ligation. PBDL: hepatic bile duct and common bile-pancreatic duct ligation. Drugs were administered 30 min before the ligation. The data represent the mean ± S.E.M. of 9 rats. *P < 0.05, **P < 0.01, compared with the PBDL plus vehicle group.
histological changes were of lesser degrees than those in the PBDL group (Fig. 6C).

Administration of caerulein (1 μg/kg, s.c.) to the PBDL (3 hr) treatment group caused a further increase in plasma amylase activity (Fig. 7). In contrast, the same dosage of caerulein in the BDL treatment group did not cause a change in plasma amylase activity. T-0632 (0.01, 0.1 mg/kg) dose-dependently inhibited the increase in
plasma amylase activity caused by the above pancreatitis aggravation by caerulein (Fig. 7).

DISCUSSION

In rats, T-0632 potently prevented the caerulein-induced increases in the pancreatic digestive enzymes in plasma and suppressed the histological changes in the pancreas, suggesting that T-0632 is orally effective against caerulein-induced acute pancreatitis. The preventive effect of T-0632 on caerulein-induced acute pancreatitis was about 1000-fold more potent than that of loxiglumide. The efficacy of loxiglumide on caerulein-induced acute pancreatitis was compatible with what had been previously reported (17). In dogs, T-0632 (0.1, 1 mg/kg, i.d.) dose-dependently prevented caerulein-induced acute pancreatitis. However, loxiglumide lacked preventive efficacy even at a dose of 100 mg/kg. These results suggest that T-0632 may have a potent preventive action on caerulein-induced acute pancreatitis compared with loxiglumide. We have previously reported that the antagonistic action of T-0632 for the CCKA receptor was more potent than that of loxiglumide in both in vitro and in vivo studies (13, 14). Moreover, the duration of action of T-0632 has been reported to be longer than that of loxiglumide in both in vitro and in vivo studies (14). The potent preventive effect of T-0632 on caerulein-induced acute pancreatitis may be explained by the higher affinity to the CCKA receptor and a longer duration of action of T-0632 compared with loxiglumide.

Pancreatic duct ligation caused a time-dependent increase in plasma amylase activity as reported by other investigators (18). In 6-hr pancreatic duct ligation-induced pancreatitis, administration of T-0632 partially prevented both the increase in plasma amylase activity and the histological changes in the pancreas. Partially preventive effects of CCKA-receptor antagonists in this model have also been reported by others (10–12). On the other hand, CCKA-receptor antagonists have been reported to have no preventive effect on long-term pancreatic duct ligation-induced pancreatitis (10). These results suggest that CCK may play a role in the pathogenesis of the acute pancreatitis model only in the early phase.

To confirm the contribution of CCK to this model, we examined the effect of caerulein on the early phase of pancreatic duct ligation (3 hr)-induced pancreatitis. The dose of caerulein used in this study was lower than the dose that caused acute pancreatitis. In fact, BDL plus caerulein caused no increase in plasma amylase activity compared with BDL only. Caerulein administration to the PBDL group caused a further increase in plasma amylase activity and T-0632 reduced this change. These results suggest that CCK may work as an aggravating factor in the progression of the acute pancreatitis. Recent studies have also suggested that CCK, even at physiological concentrations, may contribute to the pathogenesis in various experimental acute pancreatitis models (19–23).

Protease inhibitors, including camostate, have been reported to have preventive effects in several models of acute pancreatitis such as caerulein-, trypsin-, taurocholate- and CDE (choline-deficient ethionine-supplemented) diet-induced pancreatitis (24–27). However, camostate did not show any preventive effect on pancreatic duct ligation-induced pancreatitis. Although the reason is unclear at present, the activation of pancreatic protease may not be involved in this acute pancreatitis model.

In Japan, loxiglumide is in clinical trials to investigate its potential therapeutic value in acute and chronic pancreatitis (28). On the other hand, CCKA-receptor antagonists have been suggested to have a potential hazard of gallstone formation through gallbladder stasis (29–31). T-0632 showed good selectivity for the pancreatic CCKA receptor over that of the gallbladder in both in vitro and in vivo studies and good reversibility of its effect on the gallbladder in an in vitro study (13, 14). Therefore, T-0632 might have a therapeutic advantage in treating pancreatitis with little side effect on the gallbladder.

In the present studies, we evaluated the effect of T-0632 on the caerulein-induced and pancreatic duct ligation-induced pancreatitis models. In both pancreatitis models, T-0632 showed preventive effects by oral or intraduodenal administration. These results suggest that CCK plays an important role in the progression and aggravation of acute pancreatitis, and T-0632 may have a therapeutic potential in these disease states.

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