ABSTRACT
The effects of pirenzepine, a selective antimuscarinic agent, on both carbachol-stimulated amylase release and \[^{3}H\]N-methyl scopolamine (\[^{3}H\]NMS) binding were studied in isolated mouse pancreatic acini. Pirenzepine at concentrations between 0.1-3.0 \(\mu M\) was found to inhibit carbachol-stimulated amylase release without any appreciable effect on the basal amylase secretion. The inhibition by pirenzepine was rapid and reversible. Biphasic dose-response curves to carbachol for amylase release shifted to the right with an increase in pirenzepine concentration, and \(pA_{2}\) value was estimated to be 6.8. IC\(_{50}\) of pirenzepine for carbachol-stimulated amylase release was 440 nM, and the potency of pirenzepine on the inhibition of amylase release was 2% relative to atropine. The effect of pirenzepine was selective for carbachol; amylase release stimulated by other secretagogues such as cholecystokinin (CCK), bombesin and \(Ca^{2+}\) ionophore A23187, was not affected by pirenzepine. In addition, pirenzepine was found to inhibit the specific binding of \[^{3}H\]NMS, a muscarinic antagonist, to mouse pancreatic acini. The potency of pirenzepine in this action was 1% relative to atropine. The inhibitory effect of pirenzepine on receptor binding was selective for the muscarinic receptor. These results indicate that pirenzepine is a specific but relatively weak antagonist of muscarinic receptor in mouse pancreatic acini.

PIRENZEPINE, 5,11-dihydro-11-[\{(4-methyl-1-piperazinyl)acetyl]-6H-pyrido[2,3-b][1,4]benzodiazepin-6-on-dihydrochloride, is a tricyclic compound and is reported to exert no effect on the central nervous system because of the lack of its lipid solubility (7). The drug was found to distinguish between different subclasses of muscarinic receptors (9-11) and has been widely used in the treatment of peptic ulcer as a selective antimuscarinic agent (3, 13, 17). Hammer et al. (9-11) reported that the affinity of pirenzepine to the muscarinic receptors in exocrine glands such as salivary gland was higher than in heart and smooth muscles, but lower than in sympathetic ganglia. However, few studies have been reported on the effect of pirenzepine on exocrine pancreas (6). In the present study, we have examined the effect of pirenzepine on both carbachol stimulation of amylase release and \[^{3}H\]N-methyl scopolamine (\[^{3}H\]NMS) binding using isolated mouse pancreatic acini.

MATERIALS AND METHODS
Materials
Pirenzepine was a gift from Nippon Boehringer Ingelheim, Hyogo, Japan; synthetic cholecystokinin octapeptide (CCK8) was a gift from Dr M. A. Ondetti, Squibb Institute for Medical Research, Princeton, NJ, U.S.A. The following reagents were purchased: carbamyl choline chloride (carbachol), atropine sulfate, hyaluronidase type-1, soybean trypsin inhibitor from Sigma Chemical, St. Louis, MO, U.S.A.; chromatog-
raphically purified collagenase from Worthington Biochemicals, Freehold, NJ, U.S.A.; bombesin from Protein Research Foundation, Osaka, Japan; minimal Eagle's medium amino acid supplement from Flow Laboratories, McLean, VA, U.S.A.; Ca2+ ionophore A23187 from Calbiochem-Behring Corp., La Jolla, CA, U.S.A.; [3H]N-methyl scopolamine (84.8 Ci/mmol), [125I]-BH-CCK8 (2,200 Ci/mmol) from New England Nuclear, Boston, MA, U.S.A.; INSTAGEL from Packard Instrument, Downers Grove, IL, U.S.A. All other reagents and chemicals were of analytical grade. Krebs-Henseleit bicarbonate (KHB) buffer and N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid (HEPES)-Ringer (HR) buffer containing an essential amino acid supplement and 0.01% soybean trypsin inhibitor, were prepared as previously described by Williams et al. (21).

Preparation of Acini

Mouse pancreatic acini were isolated from male ICR mice fasted overnight by the method of Williams (12, 21). Briefly, pancreata (approximately 1 g) from 5-6 mice were injected with 5 ml KHB buffer containing 0.1 mM Ca2+, purified collagenase (60-75 U/ml), and hyaluronidase (1.8 mg/ml). The tissue was incubated at 37°C with shaking for 50 min and dissociated mechanically through plastic pipettes. Acini were then filtered through nytex mesh, and centrifuged through KHB buffer containing 4% bovine serum albumin. The dispersed acini were washed twice in the same buffer and then once with HR buffer (pH 7.35) containing 0.5% bovine serum albumin and 0.01% soybean trypsin inhibitor. Prior to experiments, the acini were preincubated for 60 min in HR buffer at 37°C.

Measurement of Amylase Release

Amylase release from isolated acini was measured as described previously (12, 21). Briefly, the acini at 0.5-1.0 mg protein/ml were incubated in plastic flasks containing 2 ml of fresh HR buffer. After a 10-min incubation period, pirenzepine or atropine was added, and then secretagogues were added. The incubation continued at 37°C, shaking at 60 cycles/min, and the incubation was terminated at specific time by taking 1 ml aliquots, followed by immediate centrifugation. The pellet was rinsed twice with ice-cold saline, resuspended in 1 ml water, and sonicated with a probe type sonicator. The radioactivity of both lysate (0.5 ml) and the medium (100 µl) were measured in a liquid scintillation spectrometer using INSTAGEL scintillation fluid. Protein content of each pellet was also measured. The radioactivity bound to the acini in the presence of excess amount of atropine (10 µM) was measured to calculate the nonspecific binding, and the amount was subtracted from the total binding to obtain the specific binding.

Measurement of [3H]N-Methyl Scopolamine Binding

[3H]NMS binding experiments were carried out according to the method described by Dehay et al. (6). Briefly, the acini at 0.4-0.6 mg protein/ml were incubated in 2 ml of fresh HR buffer. After a 10-min incubation period, pirenzepine or atropine was added, and then [3H]NMS at a concentration of 1 nM was added. The incubation continued at 37°C, and the incubation was terminated at specific time by taking 0.7 ml aliquots, followed by immediate centrifugation. The pellet was rinsed twice with ice-cold saline, resuspended in 1 ml water, and sonicated with a probe type sonicator. The radioactivity of both lysate and the medium were measured in a liquid scintillation spectrometer using INSTAGEL scintillation fluid. Protein content of each pellet was also measured. The radioactivity bound to the acini in the presence of excess amount of atropine (10 µM) was measured to calculate the nonspecific binding, and the amount was subtracted from the total binding to obtain the specific binding.

Measurement of [125I]-BH-CCK8 Binding

[125I]-BH-CCK8 binding experiments were carried out according to the method by Sankaran et al. (19). The experimental procedures were essentially the same as described in the [3H]NMS binding experiment except for the incubation buffer. In the [125I]-CCK8 binding experiment, the incubation was carried out in the HR buffer supplemented with 0.5 mg/ml bacitracin to minimize the degradation of the tracer. Under this condition, [125I]-CCK8 binding to acini reached maximum at 90 min, and the degradation of [125I]-CCK8 measured by trichloroacetic acid (TCA) method was less than 5%.

RESULTS

Effect of Pirenzepine on Amylase Release

Time course of amylase release from isolated (14). The zero time value of amylase was measured by taking the acinar suspension at the start of the incubation, and was subtracted from values obtained after incubation to obtain amylase release during the incubation. Both the amylase and protein content of the pellet obtained at the start of the incubation were measured. Protein was assayed by the method of Lowry et al. (18) using bovine serum albumin as a standard.

Measurement of [3H]N-Methyl Scopolamine Binding

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Measurement of [125I]-BH-CCK8 Binding

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RESULTS

Effect of Pirenzepine on Amylase Release

Time course of amylase release from isolated

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mouse acini is shown in Fig. 1. Both basal and carbachol-stimulated amylase release was almost linear with time up to 45 min. When 3 μM pirenzepine was added at the start of the incubation, carbachol-stimulated amylase release was markedly reduced. When 3 μM pirenzepine was added after 20 min of incubation, carbachol stimulation of amylase release was rapidly abolished and the slope became almost parallel to that of basal amylase release (Fig. 1). Dose-response curves to carbachol for the stimulation of amylase release were studied in the absence or presence of pirenzepine. As shown in Fig. 2, typical biphasic dose-response curves were obtained which shifted to the right in the presence of pirenzepine. Effect of pirenzepine on basal and carbachol-stimulated amylase release is shown in Fig. 3. Pirenzepine at concentrations between 0.03 and 3.0 μM did not influence the basal amylase release. When amylase release was maximally stimulated by 1 μM carbachol, dose-dependent decrease in amylase release was observed in the presence of pirenzepine. Dose-response curves to antagonists for the inhibition of amylase release are shown in Fig. 4. IC_{50} of pirenzepine for the inhibition of carbachol-stimulated amylase release was 440 nM, and the potency of pirenzepine for the inhibition of amylase release was approximately 2% relative to atropine.

In order to examine the selectivity of this inhibition, we studied the effect of pirenzepine on amylase release induced by various secretagogues. As shown in Fig. 5, the inhibitory effect of pirenzepine was selective for carbachol.

**Effect of Pirenzepine on [^{3}H]NMS Binding**

[^{3}H]NMS binding to isolated acini at 37°C reached maximum at 60 min which continued up to 120 min. When atropine was added at 60 min[^{3}H]NMS binding decreased, indicating that the binding was reversible (Fig. 6). As shown in Fig. 7 pirenzepine and atropine inhibited[^{3}H]NMS binding to acini in a dose-dependent fashion. IC_{50} of pirenzepine for the inhibition of[^{3}H]NMS binding to acini was 1.5 μM, and the potency of pirenzepine in this effect was approximately 1% relative to atropine. The effect of pirenzepine on[^{125}I]-CCK binding to acini was
Fig. 2 Amylase release as a function of various concentrations of carbachol. Acini were incubated for 30 min in the presence of various concentrations of carbachol. Prior to the addition of carbachol, pirenzepine (1 μM or 10 μM) was added. Each value is the mean±SE of triplicate determinations in 4 separate experiments.

Fig. 3 Effect of pirenzepine on basal and carbachol-stimulated amylase release. Acini were incubated for 30 min with various concentrations of pirenzepine plus the indicated concentrations of carbachol. Each value is the mean±SE of triplicate determinations in 4 separate experiments.

also studied. As shown in Table 1, pirenzepine did not inhibit [125I]-CCK binding, indicating that pirenzepine is a selective inhibitor for muscarinic receptor in pancreatic acini.

DISCUSSION
Among them, cholinergic receptors as well as CCK receptors are believed to play a physiologically important role in the action of exocrine pancreas. Atropine, a classical anticholinergic agent, has been used to inhibit the cholinergic function in various tissues. Recently pirenzepine was developed as a selective antimuscarinic agent, and the drug has been widely used in the treatment of peptic ulcer. In order to elucidate the
PIRENZEPINE ON PANCREATIC ACINI

Effect of pirenzepine on the exocrine pancreatic secretion, carbachol-stimulated amylase release and [3H]NMS binding were studied in the presence of various concentrations of pirenzepine using the isolated mouse pancreatic acini.

The present study demonstrates that pirenzepine inhibits carbachol-stimulated amylase release and causes rightward shift of the dose-response curve to carbachol for amylase release. The inhibition by pirenzepine was dose-dependent and competitive. However, the pattern of pirenzepine action with a fixed concentration of carbachol is not always 'inhibitory', when amylase release was plotted as a function of pirenzepine concentration. With a concentration of 10 μM carbachol that was supramaximal for

Fig. 4 Antagonists and the inhibition of amylase release. Acini were incubated for 30 min in the presence of 1 μM carbachol and the indicated concentrations of pirenzepine or atropine. Results are expressed as percentage of amylase release stimulated by 1 μM carbachol. Each value is the mean from 4 separate experiments.

Fig. 5 Effect of pirenzepine on amylase release induced by various secretagogues. Acini were incubated for 30 min in the presence of carbachol (Cch, 1 μM), CCK8 (30 pM), bombesin (BBS, 10 pM) or A23187 (5 μM). Prior to the addition of each secretagogue, pirenzepine (3 μM) was added. Each value is the mean ± SD of triplicate determinations in a representative experiment.
amylase stimulation, increasing concentrations of pirenzepine resulted in the increase of amylase release. The pA2 value estimated by the rightward shift of dose-response curves to carbachol is approximately 6.8. The value seems to be comparable to those obtained in pharmacology studies in various tissues in vitro (1, 4, 10).

The inhibitory effect of pirenzepine on carbachol-stimulated amylase release was rapid and reversible. When the effect of pirenzepine was examined using acini that had been preincubated with 3 μM pirenzepine, the acini were able to respond to carbachol to the same extent as the control acini that had been preincubated without pirenzepine (data not shown).

The effects of carbachol, CCK and bombesin on pancreatic amylase release appears to be mediated by changes in intracellular Ca²⁺ (5, 8, 20). The Ca²⁺ ionophore A23187 allows extracellular Ca²⁺ to enter the cell and mimics these phy-
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Table 1 Effect of Pirenzepine on [3H]NMS Binding and 125I-CCK8 Binding to Mouse Pancreatic Acini

<table>
<thead>
<tr>
<th>Addition</th>
<th>[3H]NMS binding (%-mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.39±0.26</td>
</tr>
<tr>
<td>Pirenzepine (100 µM)</td>
<td>1.05±0.06</td>
</tr>
<tr>
<td>Atropine (3 µM)</td>
<td>0.70±0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Addition</th>
<th>125I-CCK8 binding (%-mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.01±0.36</td>
</tr>
<tr>
<td>Pirenzepine (100 µM)</td>
<td>6.75±0.68</td>
</tr>
<tr>
<td>CCK8 (0.3 µM)</td>
<td>0.38±0.01</td>
</tr>
</tbody>
</table>

Each value is the mean±SE of triplicate determinations from either 7 ([3H]NMS) or 3 (125I-CCK8) separate experiments.

The present study demonstrated that pirenzepine inhibits carbachol-stimulated amylase release without influencing the amylase release induced by other secretagogues such as CCK, bombesin and A23187, indicating that pirenzepine inhibition is selective for the cholinergic agent. Recently Dehaye et al. (6) reported that pirenzepine caused a rightward shift of the dose-response curve for carbachol-stimulated amylase secretion in proportion to the inhibition of [3H]NMS binding in isolated rat pancreatic acini. In their study IC₅₀ of pirenzepine for carbachol-stimulated amylase release was reported to be 4.5 µM, pirenzepine being 150 times less potent than atropine. In the present study in mouse acini, however, IC₅₀ of pirenzepine for carbachol-stimulated amylase secretion was found to be 440 nM, pirenzepine being 50 times less potent than atropine. Although the relative potency of pirenzepine to atropine was found to be weak in mouse acini as well, it should be noted that the concentration of pirenzepine examined in this study can be obtained in plasma after oral administration of pirenzepine (10). Kuntzen et al. reported that amylase output on pancreozymin-secretin test was reduced after intravenous administration of pirenzepine in humans (15).

The binding studies of the muscarinic antagonists, [3H]NMS and [3H]quinuclidinyl benzilate, to rat pancreatic acini have been previously reported to characterize the muscarinic receptors on pancreatic acini (6, 16). In the present study inhibition of [3H]NMS binding by pirenzepine was clearly demonstrated in mouse acini. The pirenzepine inhibition of [3H]NMS binding was dose-dependent. The relative potency of pirenzepine to atropine in the inhibition of [3H]NMS binding was almost comparable to that obtained in the inhibition of amylase secretion. Furthermore, the inhibitory effect of pirenzepine on receptor binding was selective, and the drug had no effect on 125I-CCK8 binding to mouse acini.

In conclusion, we have shown that pirenzepine is a specific but relatively weak antagonist of the muscarinic receptor in mouse pancreatic acini.

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carinic receptors. Nature 283, 90–92