Telenzepine-sensitive muscarinic receptors on rat pancreatic acinar cells

GEORGE A. SCHEELE,3 AND FRED S. GORELICK

AUBRY STOCH,1 STEVE RHEE,1 MICHAEL H. NATHANSON,1 GEORGE A. SCHEELE,3 AND FRED S. GORELICK1

Departments of Surgery, Medicine, and Cell Biology, Connecticut Health Care Department of Veterans Affairs, West Haven, and Yale University School of Medicine; New Haven, Connecticut 06516; Department of Visceral and Transplantation Surgery, University of Bern, 3010 Bern, Switzerland; and AlphaGene, Inc., Charlestown, Massachusetts 02129

Schmid, Stefan W., Irvin M. Modlin, Laura H. Tang, Aubry Stoch, Steve Rhee, Michael H. Nathanson, George A. Scheele, and Fred S. Gorelick. Telenzepine-sensitive muscarinic receptors on rat pancreatic acinar cells. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G734–G741, 1998.—To identify the muscarinic subtype present on the rat pancreatic acinar cell, we examined the effects of different muscarinic receptor antagonists on amylase secretion and proteolytic zymogen processing in isolated rat pancreatic acini. Maximal zymogen processing required a concentration of carbachol 10- to 100-fold greater (10–3 M) than that required for maximal amylase secretion (10–5 M). Although both secretion and conversion were inhibited by the M3 antagonist 4-diphenylacetoxy-N-methyl-piperidine (4-DAMP) (50% inhibition ~6 × 10–7 M and 1 × 10–8 M, respectively), the most potent inhibitor was the M1 antagonist telenzepine (50% inhibition ~5 × 10–10 M and 1 × 10–11 M, respectively). Pirenzepine, another M1 antagonist, and the M2 antagonist methoctramine did not reduce amylase secretion or zymogen processing in concentrations up to 1 × 10–5 M. Analysis of acinar cell muscarinic receptor by PCR revealed expression of both m1 and m3 subtypes. The pancreatic acinar cell has a distinct pattern of muscarinic antagonist sensitivity (telenzepine > 4-DAMP > pirenzepine) with respect to both amylase secretion and zymogen conversion.

MATERIALS AND METHODS

Preparation of isolated pancreatic acini. Pancreatic acini were prepared in accordance with procedures outlined in The National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Department of Veterans Affairs Animal Studies Subcommittee. Pancreata harvested from fasted male Sprague-Dawley rats (weighing ~100 g) were subjected to collagenase digestion and mechanical dispersion and filtered through Nytex 200 gauze as previously described (15). For the preparation of small groups of acinar cells (mini-acini), acinar preparations were incubated for an additional 10 min in medium with 2 mM EGTA and no Ca2+, washed into standard medium, and filtered through Nytex 20 gauze. Acini were then incubated in plates with 24 wells containing incubation buffer consisting of (in generating a maximal secretory response) of CCK or cholinergic agonists generate acute pancreatitis (9) and zymogen activation (18). Likewise, hyperstimulation of isolated pancreatic acini by CCK leads to the rapid activation of proteases and enhanced zymogen processing (15). The protease activation observed in isolated pancreatic acini may be relevant to the pathogenesis of acute pancreatitis.

The identification of the muscarinic receptors on the pancreatic acinar cell is relevant to pancreatic physiology and pathology. Muscarinic antagonists are most often used to establish the class of muscarinic receptors; based on the relative efficiency of these agents, the acinar cell receptor has been designated an M3 subtype (11, 12, 28), whereas more proximal cholinergic pathways that affect the pancreas appear to be regulated by M1 receptors (26). Multiple subtypes of muscarinic receptor may be present on a single cell (5) and may potentially couple to distinct signaling pathways. Preliminary studies from our laboratory suggest that zymogen secretion and intracellular zymogen processing may be activated by different signaling pathways. Thus different muscarinic receptors on the acinar cell might mediate these distinct cellular responses. In this study we examined the effects of muscarinic antagonists on carbachol (CCh)-induced secretion and zymogen processing. Our results demonstrate that both responses are potently inhibited by the M1 antagonist telenzepine. Similar inhibition is observed with the M3 antagonist 4-diphenylacetoxy-N-methyl-piperidine (4-DAMP), but at a concentration about 1,000-fold greater. Thus telenzepine-sensitive muscarinic receptors regulate both secretion and zymogen processing in the pancreatic acinar cell.

NEUROHUMORAL STIMULATION OF THE EXOCRINE PANCREAS generates a number of physiological responses, including enhanced zymogen secretion. Recent studies suggest that cholinergic pathways may have a more important role in regulating pancreatic secretion than previously appreciated. Thus the primary action of CCK released by the intestinal nutrients may be to stimulate the cholinergic release of acetylcholine (ACh) (1, 16). The neurotransmitter then acts on muscarinic ACh receptors (mACHR) to stimulate acinar cell secretion. Cholinergic pathways may play a role in physiological processes such as the proteolytic processing of zymogens to active forms.

Cholinergic stimulation may contribute to the pathogenesis of pancreatitis such as that induced by the bite of the Tityus serrulatus scorpion (22), alcohol-associated disease, and cholinesterase inhibitors (17). A key step for initiating many forms of pancreatitis appears to be the pathological intracellular activation of digestive zymogens (15). In vivo studies that use supramaximal concentrations (10- to 100-fold greater than that
mM) 25 HEPES, 98 NaCl, 4.8 KCl, 2 CaCl₂, and 1.2 MgCl₂, and 0.2% bovine serum albumin, and 0.1 mg/ml soybean trypsin inhibitor (pH 7.4), under continuous oxygenation at 37°C for 1 h before treatment.

Exposure to secretagogues. After the equilibration period, acini were treated with CCK-8 (Squibb Diagnostics, New Brunswick, NJ) or carbachol chloride (CCh; Sigma, St. Louis, MO), using the doses and duration of treatment indicated. Acini exposed to incubation medium alone served as unstimulated controls.

Receptor specificity. For each individual experiment, acini were pretreated with either atropine (nonselective muscarinic antagonist) (Sigma), pirenzepine (M1-selective receptor antagonist) (Sigma), telenzepine dihydrochloride (telenzepine; M1-selective receptor antagonist; provided by Byk Gulden, Konstanz, Germany), methoctramine (M2-selective receptor antagonist; gift from Dr. G. Makhlof, Richmond, VA), or 4-DAMP (M3-selective receptor antagonist; gift from Dr. R. Goyal, Boston, MA) for 15 min before stimulation with CCh.

Determination of amylase activity. After pretreatment with antagonists and exposure to secretagogues, amylase activity was measured in the incubation medium with the use of a Phadebas amylase assay kit (Pharmacia Diagnostics, Piscataway, NJ), and secretion was expressed as a ratio in comparison to the unstimulated control. Because preliminary experiments had revealed a uniform distribution of acini in the tubes (∼5% in total amylase content), the percentage of released amylase in comparison to the total content measured after sonication of the cells was not regularly measured. However, during a 30-min incubation, basal amylase release ranged from 3% to 6% and stimulated release from 12% to 18%. Preparations exhibiting less than a 2.5-fold increase in amylase release over the unstimulated condition were excluded from analysis.

Detection of zymogen proteolysis. At the conclusion of the incubation period, proteins were solubilized by boiling in Laemmli sample buffer containing β-mercaptoethanol (10%) and subjected to SDS-PAGE (7.5%) as described previously (13). Separated proteins were electrophoretically transferred to Immobilon-P transfer membranes (Millipore/Continental Water Systems, Bedford, MA) and immunoblotted with a rabbit polyclonal antiserum that binds with comparable affinity to the zymogen procarboxypeptidase A1 (PCA1), and the active form of PCA1, carboxypeptidase A1 (CA1), as described previously (15). Labeled proteins were detected using 125I-labeled goat anti-rabbit IgG (ICN Biochemicals, Irvine, CA) followed by autoradiography. The radioactive bands were excised from the membrane, and the amount of labeling was quantitated by measuring gamma emissions. The amount of immunoreactivity of the active enzyme form under various treatment conditions was calculated as a ratio in comparison to unstimulated control and expressed as relative conversion.

The percentage of PCA1 relative to CA1 has been estimated in previous studies; in the basal state 1–4% may be present in active form, and the value increases from 6% to 14% in the active form of PCA1, carboxypeptidase A1 (CA1), as described above and then loaded with the Ca²⁺-sensitive fluorescent dye fluo 3-AM (8.8 mM) for 30 min at room temperature in Liebovitz L-15 medium containing 10% fetal calf serum (20). The fluo 3-loaded cells were transferred to a perfusion chamber on the stage of a Bio-Rad MRC-600 confocal microscope and then continuously perfused at 37°C with a HEPES-based buffer solution. The fluo 3 was excited with the 488-nm line of a krypton-argon laser, and emission signals above 515 nm were collected. Small (2–10 cells) clusters of acini were identified to ensure that there was no associated nerve tissue, and then the cells were examined in the line-scanning mode (20) during either 1) stimulation with CCh (10⁻⁵ M) or 2) pretreatment with telenzepine (10⁻⁸ M) followed by serial stimulation with CCh (10⁻⁵ M). The spatial resolution was 0.26 mm²/pixel so that fluorescence signals from neighboring cells could readily be distinguished, and signals were collected at a rate of 3–5/s.

Preparation of mRNA and cDNA from isolated pancreatic acini and brain. Rats were killed, and their brains were immediately removed and homogenized in liquid nitrogen. Freshly prepared pancreatic acini or mini-acini from young rats (~100 g) were immediately resuspended in lysis buffer (from FastTrack kit; see below) and subjected to mRNA preparation using the FastTrack mRNA isolation kit (Invitrogen, San Diego, CA). Approximately 2–3 µg of mRNA were obtained from one of the standard acinar preparations and 1–2 µg was obtained from mini-acini. The first-strand cDNA was then transcribed from 0.4–0.8 µg mRNA, using reverse transcriptase (Superscript kit; Life Technologies, Gaithersburg, MD). To eliminate the contamination of genomic DNA in the mRNA preparation, a reaction without reverse transcriptase was always included in the first-strand cDNA synthesis, which was subsequently evaluated in the gene-specific PCR amplification.

PCR and Southern blotting. Approximately 10% of the resulting cDNA (obtained from 20–40 ng original mRNA) was amplified by gene-specific PCR for 30 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min), using an M) Research PTC-200 thermal cycler. The following rat mAChR-specific primers were used to direct synthesis of the muscarinic receptor subtypes in PCR reactions (30): m1, upper primer, 5′-GCACAGGGCACCCACAGCAGC-3′, coding for the termini 1073–1093, lower primer, 5′-AGAGCACGACGACGCGAACG-3′, coding for 1445–1425; m2, upper primer, 5′-GCCACAGCACTGACCTACC-3′, coding for 826–846, lower primer, 5′-TCTGACCCGACGCACCACTA-3′, coding for 1508–1488; m3, upper primer, 5′-GTCTGCTGTGGTGCTCATTCCCT-3′, coding for 1318–1438, lower primer, 5′-GCTGCTGCTGTGGTCTTGCCTG-3′, coding for 1751–1731. PCR products had a size of 373 bp (m1), 683 bp (m2), and 434 bp (m3). They were separated by electrophoresis on 1% agarose gels containing ethidium bromide and photographed under fluorescent illumination. The PCR products were then transferred to nylon membranes (Amersham), and the nucleic acids were fixed by ultraviolet cross-linking. For Southern blot analysis the following internal sequence probes were used for hybridization: m1, 5′-CACGAAACCTCTGACCTACCC-3′, coding for the termini 1073–1093; m2, 5′-GGCTCCACGG-GCTGCTGCTGCT-3′, coding for 1318–1438; m3, 5′-GTCTGCTGCTGTGGTCTTGCCTG-3′, coding for 1751–1731. The membranes were then washed four times in a solution of 2× or 0.5× SSC and 0.1% SDS at room temperature. After 1-h preincubation in a 1% blocking solution, the
membrane was exposed to anti-DIG Fab fragments conjugated to alkaline phosphatase (working concn 75 mU/ml) for 30 min. The membranes were then washed in 100 mM maleic acid, 150 mM NaCl, and 0.3% Tween 20. Positively hybridized nucleic acids on the membrane were detected with the chemiluminescent alkaline phosphatase substrate disodium 3'-4-methoxy-3',4'-diotexane-3,2'-[5'-chloro]tricyclo(3.3.1.13,7)decan]-4-yl(phenyl phosphate (Boehringer Mannheim), using a concentration of 250 mM in 100 mM Tris·HCl and 100 mM NaCl (pH 9.5). After incubation for 15 min at 37°C, DNA bands were revealed by autoradiography. The same membrane was then stripped twice with alkaline probe-stripping buffer (0.2 N NaOH and 0.1% SDS) for 10 min at 37°C before it was reprobed with a second or a third gene-specific probe.

Statistical analysis. Data are expressed as means ± SE, and differences between individual means are assessed by unpaired Student's t-test. The significance level was set at P < 0.05.

RESULTS

Dose and time dependence of CCh-induced zymogen conversion. Secretion of amylase reached a maximum level at $10^{-5}$ M CCh and decreased at higher doses (Fig. 1A), as reported previously (25). The addition of CCh ($10^{-6}$ to $10^{-3}$ M) resulted in a concentration-dependent increase of CA1 (Fig. 1B); zymogen conversion was detected after 10 min of treatment, reached a maximum level at 30 min, and decreased after 60 min (Fig. 1C).

The conversion of procarboxypeptidase B2 and chymotrypsinogen 2 was also stimulated by high concentrations ($10^{-3}$ M) of CCh and CCK ($10^{-7}$ M) (10). Although the selective proteolysis of each of thesezymogens in response to neurohumoral stimulation was qualitatively similar, the signal for PCA1 conversion was more easily quantitated than the signal generated by the other zymogens. Thus the conversion of PCA1 to CA1 has been used as the marker for selective zymogen proteolysis.

Effects of cholinergic antagonists. To define the class of cholinergic receptor responsible for mediating this CCh effect, acini were exposed to atropine, a nonselective muscarinic antagonist. This pretreatment caused a dose-dependent inhibition of amylase secretion, with an approximate IC$_{50}$ of $1 \times 10^{-9}$ M (Fig. 2). Likewise, atropine in concentrations of $10^{-6}$ to $10^{-4}$ M eliminated the enzyme conversion stimulated by high doses of $10^{-4}$ M CCh (normalized mean values ± SE for $10^{-4}$ M CCh = 4.4 ± 0.4, and for atropine: $10^{-6}$ M = 1.6 ± 0.2, $10^{-5}$ M = 1.3 ± 0.1, and $10^{-4}$ M = 1.3 ± 0.1; n = 7). To examine the subtype of muscarinic receptors responsible for mediating this conversion, acini were incubated with selective muscarinic antagonists (2) before the addition of CCh. Preincubation with pirenzepine ($10^{-8}$ to $10^{-5}$ M), an M1-selective antagonist, did not significantly reduce amylase secretion or PCA1 conversion (Fig. 3). Indeed, there was some increase in both the secretory response and zymogen conversion in the presence of pirenzepine (Fig. 3).

Although M2 receptors have not been previously detected on pancreatic acini, the ability of methoctra-
mine, an M2-selective antagonist, to inhibit this conversion was tested. Methoctramine in doses of $10^{-9}$ to $10^{-6}$ M failed to inhibit the conversion (normalized mean values ± SE for $10^{-3}$ M CCh = 1.9 ± 0.2, and for methoctramine: $10^{-6}$ M = 1.8 ± 0.2, $10^{-5}$ M = 1.7 ± 0.2, and $10^{-4}$ M = 1.6 ± 0.2; $n = 4$). However, pretreatment with 4-DAMP, an M3-selective antagonist, resulted in concentration-dependent inhibition of CCh-induced secretion (Fig. 4). Similar to pirenzepine, low concentrations of 4-DAMP tended to increase both secretion and zymogen conversion.

Pretreatment of acini with the M1 antagonist telenzepine unexpectedly resulted in the inhibition of both secretion and zymogen conversion. Telenzepine was 1,000 times more potent than 4-DAMP in decreasing CCh-stimulated amylase secretion (Fig. 5). The estimated telenzepine concentration required to inhibit 50% of the CCh-induced amylase release was $5 \times 10^{-10}$ M. Similarly, 50% zymogen conversion was inhibited by a concentration of $1 \times 10^{-11}$ M telenzepine. In control studies telenzepine ($10^{-12}$ to $10^{-8}$ M) did not affect basal or CCK ($5 \times 10^{-10}$)-induced secretion.

Telenzepine is thought to affect pancreatic secretion primarily by acting on neural pathways. To exclude any...
possible effect of CCh on neural elements in the acinar preparation, two studies were performed. First, before stimulation, isolated acini were treated with tetrodotoxin, an inhibitor of neural transmission. However, this compound \((10^{-11} \text{ to } 10^{-5} \text{ M})\) changed neither the secretory response nor zymogen processing in acini stimulated by CCh (not shown). Second, a preparation of mini-acini containing 2–10 cells was generated. These small groups of acini appear to be devoid of neural elements when examined by electron microscopy (not shown). Because these preparations exhibit a poor secretory response compared with whole acini, the effects of CCh on \([\text{Ca}^{2+}]_{i}\) in individual acinar cells were measured (Fig. 6). Every cell \((n = 23)\) stimulated with CCh responded with a rapid, sustained increase in \([\text{Ca}^{2+}]_{i}\), (Fig. 6A), as has been described previously (19). In contrast, no cell \((n = 21)\) pretreated with telenzepine responded to CCh (Fig. 6B). The telenzepine effect was specific for CCh; 20 (95%) of the telenzepine-treated cells displayed an increase in \([\text{Ca}^{2+}]_{i}\) in response to subsequent stimulation with CCK. These findings confirm that telenzepine is acting directly on acinar cells and not on nerves.

PCR and Southern blot analysis. cDNA from rat brain (amplified from 2–4 ng of original mRNA) was utilized as a positive control in PCR, since it is known to express all five subtypes of mAChR. As shown in Fig. 7, a single band of ~370 bp, ~680 bp, or ~430 bp, which were the predicted sizes corresponding to m1, m2, and m3, respectively, was amplified in each reaction using m1, m2, and m3 primer pairs. However, pancreatic acinar cell cDNA (amplified from 20–40 ng of original mRNA of either the standard acinar cell preparation or mini-acini) revealed only the muscarinic subtypes m1 and m3; no m2 DNA was detected. To verify the specificity of individual PCR products, Southern blot analysis with specific internal sequences was performed. These results confirmed that PCR products from brain tissue and pancreatic acinar cells were specific mAChR subtypes.
that described for PCA1 (10). Saluja et al. (24) found have reported that procarboxypeptidase B and chymo-
gerater than that required for maximal secretion. We ing is elicited by a dose that is approximately 100-fold concentration dependent. The highest level of process-
CCK (15), the effect of CCh on zymogen processing is tion of the pharmacological effects of selective receptor receptors has to date been largely based on the evalua-
Although five classes of muscarinic receptors in the brain have been cloned (3, 4), the classification of these receptors has to date been largely based on the evaluation of the pharmacological effects of selective receptor antagonists. Although early reports classified the pancreatic acinar cell muscarinic receptor as the M2 type, later studies noted that cholinergic secretion was mediated by a 4-DAMP-sensitive M3 acinar cell receptor (11, 12, 28). Our present pharmacological studies are in agreement with previous reports in regard to the presence of a 4-DAMP-sensitive muscarinic receptor on the pancreatic acinar cell. Thus secretion and limited proteolytic conversion were inhibited by 4-DAMP but not by equivalent doses of pirenzepine or methoctra-
mine. However, the finding that telenzepine, a putative M1-selective antagonist, was a potent inhibitor of CCh-induced enzyme secretion and zymogen processing in an acinar cell preparation was unexpected. Telenzepine was initially described as a selective M1 inhibitor and evaluated as a therapeutic agent for peptic ulcer dis-
ease. In this respect it proved to be 4-10 times more potent than the selective M1 antagonist pirenzepine in the inhibition of gastric acid secretion (8). In addition, telenzepine was noted to inhibit pancreatic bicarbonate and protein output in dogs (26, 27) and to completely block cholinergically stimulated pancreatic secretion in humans (21). Because the acinar cell was previously thought to lack an M1 muscarinic receptor, the inhibi-
tory effects of telenzepine on pancreatic secretion have usually been ascribed to an indirect effect on neural pathways.

The absence of an effect of tetrodotoxin on CCh-
induced secretion and the ability of telenzepine to block a CCh-induced increase in [Ca$^{2+}$], in small groups of 2-10 acinar cells lacking neural innervation suggest that it is very unlikely that CCh is acting on neural pathways in the acinar preparation and confirm that telenzepine is directly blocking receptors on the acinar cell.

Similar to other systems (29), it appears that the rat pancreatic acinar cell may express both the M1 and M3 receptor subtype. Therefore, it was possible that the two responses to CCh, secretion and zymogen processing, might be regulated by activation of specific receptors. However, the muscarinic antagonists demonstrated a similar pattern for both secretion and zymogen processing. These findings suggest that the same muscarinic receptor subtype is mediating both of these responses but that it may couple to distinct signaling pathways depending on the concentration of agonist. This conclusion is supported by the observation that concentration-dependent changes in the pattern of agonist-induced Ca$^{2+}$ signaling are a recognized prop-
terty of many G-protein linked receptors (19).

The potent effects of telenzepine on rat pancreatic acinar cell muscarinic receptor were surprising. For receptors characterized as the M1 subtype, the usual rank order of potency is telenzepine > pirenzepine > 4-DAMP; for the M3 subtype the rank order of potency is 4-DAMP > telenzepine > pirenzepine (14). Comparative studies in the rat parotid gland have demonstrated that 4-DAMP was 100-fold more potent for displacing [H]quinuclidinyl benzilate binding and 500-fold more potent in blocking the generation of intracellular Ca$^{2+}$ than pirenzepine (6). Similarly, in preliminary studies we found that the rank order of antagonists in rat parotid gland, designated as M3 receptor subtype, was 4-DAMP > telenzepine > pirenzepine for inhibiting CCh-stimulated amylase release (Schmid and Rhee, unpublished observations). These findings are consist-
tent with previous data obtained from studies of rat submandibular gland (14) but clearly differ from data obtained from studies of the pancreas and help to exclude a trivial explanation for the dramatic effects of telenzepine on the rat pancreatic acinar cell.

All antagonists (except telenzepine) enhanced the effect of CCh at low concentrations. The reason for this phenomenon is unclear. Because secretion is a biphasic response (decreasing after a peak level is attained), an enhanced response might have been obtained by competition with the CCh if the agonist had not been used at
the maximum secretory concentration. However, this would not explain the similarly enhanced response for conversion, which is monophasic, nor would it explain the lack of an effect by telenzepine. It is possible that the enhanced responses are affected by antagonists: a recent study has shown that some components of receptor signaling may be generated by an antagonist (23).

The reason for the distinct sensitivity of the pancreatic muscarinic receptor to telenzepine is uncertain. One possibility is that a distinct form of muscarinic receptor is present in the rat pancreatic acinar cell. Another explanation is that the sensitivity of a receptor to an antagonist may be tissue specific. For example, when muscarinic receptors were transfected into CHO cells, the M3 antagonist 4-DAMP was found to have a similar affinity for M1 and M3 subtypes (7). Thus the rank order sensitivity of a receptor to antagonists may change in a tissue-specific manner.

To provide information on the molecular identity of acinar cell muscarinic receptors, we performed PCR analysis and demonstrated that message for both the m1 and m3 subtypes is present in the acinar cells. An equal amount of cDNA was used in both reactions, but amplification of m1 and m3 was carried out with different PCR conditions. Although the quantity of m1 and m3 mRNA in the pancreatic acini cannot be directly compared, the message for both receptors appears to be present in the acinar cell.

These molecular data are consistent with the presence of m1 and m3 muscarinic receptor subtypes on the pancreatic acinar cell. Additional studies will be required to determine the identity of the muscarinic receptor on the acinar cell responsible for its sensitivity to telenzepine.

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Address for reprint requests: I. M. Modlin, GI Research Laboratory, Bldg. 27, Connecticut Health Care VA, West Haven, CT 06516.

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