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MUSCARINIC RECEPTORS IN ISOLATED GUINEA PIG PANCREATIC DUCTS

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Abstract—Biochemical and pharmacological characteristics of muscarinic cholinergic receptors in isolated guinea pig pancreatic ducts were determined in the present study. Duct homogenates bound 6.82 ± 0.69 fmol of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS)/μg of DNA with a K_d of 0.73 ± 0.05 nM. The density of [^{3}H]NMS binding sites in the excretory ducts was seven times greater than that in acini from the same pancreases. Competition binding studies with atropine, pirenzepine, 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*] [1,4]benzodiazepine-6-one (AF-DX 116), and 4-diphenylacetoxy-*N*-methyl piperidine methiodide (4-DAMP) indicated that both M2 and M3 subtypes of muscarinic receptors are present in these preparations of isolated pancreatic ducts. Electrophoretic analysis of [^{3}H]propylbenzilylcholine mustard-labeled unreduced and reduced duct muscarinic receptors provided molecular mass estimates of 62.6 ± 2.5 and 58.0 ± 1.6 kDa, respectively. Deglycosylation of ductal muscarinic receptors with *N*-glycanase decreased their apparent molecular mass by approximately 4 kDa. These results demonstrate that isolated pancreatic ducts express both M2 and M3 muscarinic receptors, with the former subtype predominating.

While secretin appears to be the chief hormonal regulator of postprandial fluid and bicarbonate ion secretion by the excretory duct epithelium of the mammalian exocrine pancreas [1], a number of studies have demonstrated that its stimulatory effects can be inhibited dramatically by systemic infusion of atropine, a classical muscarinic cholinergic antagonist [2–4]. This seemingly paradoxical observation has been interpreted as suggesting that cholinergic input from the parasympathetic nervous system provides an underlying "tone" that conditions the response of the pancreatic duct epithelium to secretin [5]. However, the molecular events that underlie cholinergic potentiation of secretin-stimulated bicarbonate secretin have not been investigated. Although mechanisms of cholinergic signal transduction in pancreatic acinar cells have been studied extensively [6, 7], the first direct demonstrations of physiological responsiveness of pancreatic ductal epithelial cells to cholinergic agonists have been reported only recently. In studies in our laboratory, the cholinergic agonist carbachol caused a transient increase in intracellular free calcium levels in isolated, fura-2-loaded, rat and guinea pig interlobular duct epithelial cells [8], and the degranulation of goblet cells in isolated segments of guinea pig main pancreatic duct [9]. Evans *et al.* [10] also have demonstrated recently that acetylcholine stimulates fluid secretion from isolated rat pancreatic ducts.

The biochemical and pharmacological characteristics of muscarinic cholinergic receptors expressed by pancreatic acinar cells have been investigated by several laboratories. Competitive binding assays utilizing selective muscarinic antagonists have indicated that pancreatic acinar cells express primarily the M3 subtype of the five-subtype muscarinic receptor family [11–13]. Labeling of muscarinic receptors in isolated rat pancreatic acini with [^{3}H]propylbenzilylcholine mustard ([^{3}H]-PrBCM†), an irreversible muscarinic antagonist, has yielded a molecular mass estimate of 118 kDa for these receptors [14]. By contrast, nothing is known of the properties of muscarinic cholinergic receptors in the pancreatic duct system. Therefore, the present study was undertaken to characterize ductal muscarinic receptors and to compare their properties with those of muscarinic receptors in pancreatic acinar cells. Our results indicate that both M2 and M3 muscarinic receptors are present in isolated guinea pig pancreatic ducts.

MATERIALS AND METHODS

Chemicals. Atropine sulfate, bovine serum albumin (BSA, fraction V), carbamylcholine chloride (carbachol), cholic acid, digitonin, glutamine, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), imidazole, *N*-acetylglucosamine, Nonidet P-40, and soybean trypsin inhibitor (SBTI, type I) were purchased from the Sigma Chemical Co. (St. Louis, MO). Purified collagenase (CLSPA, 783 U/mg) was obtained from Worthington Biochemicals (Freehold, NJ), *N*-glycanase from the Genzyme Corp. (Cambridge, MA), and *Triticum vulgans* wheat germ agglutinin (WGA) lectin-agarose from ICN Biochemicals (Cleveland, OH). 4-Diphenyl-

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† Abbreviations: AF-DX 116, 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*] [1,4]benzodiazepine-6-one; BSA, bovine serum albumin; 4-DAMP, 4-diphenylacetoxy-*N*-methyl piperidine methiodide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; NMS, *N*-methylscopolamine; PrBCM, propylbenzilylcholine mustard; SBTI, soybean trypsin inhibitor; and WGA, wheat germ agglutinin.

acetoxy-*N*-methyl piperidine methiodide (4-DAMP) and pirenzepine dihydrochloride were purchased from Research Biochemicals (Natick, MA). Minimal Eagle's medium amino acids were obtained from Gibco BRL (Gaithersburg, MD). 11-[[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*] [1,4]benzodiazepine-6-one (AF-DX 116) was a gift from Boehringer-Ingelheim Pharmaceuticals (Ridgefield, CT). [*N-methyl*-^{3}H]-Scopolamine methyl chloride ([^{3}H]NMS) (78.9 Ci/mmol) and [*propyl*-2,3-^{3}H]PrBCM hydrochloride (57.7 Ci/mmol) were purchased from Du Pont-New England Nuclear (Boston, MA).

Animals. Male outbred guinea pigs (250–300 g) were obtained from the Michigan Department of Public Health (Lansing, MI). Animals were maintained on a standard laboratory diet and water and were fasted for 12–14 hr before use.

Preparation of isolated pancreatic acini and ducts. Pancreatic acini and segments of the excretory duct system were isolated by collagenase digestion and manual selection as described previously [14, 15] and were suspended in a Ringer's solution consisting of (in mM): 118 NaCl, 4.7 KCl, 1.0 Na_2HPO_4, 1.1 $MgCl_2$, 5.5 glucose, 1.3 $CaCl_2$, 2.0 glutamine, and 10.0 Hepes. This solution was adjusted to pH 7.4 with 2.0 N NaOH and enriched with minimal Eagle's medium amino acids, 0.1% BSA, and 0.01% SBTI.

Quantitation and subtype analysis of ductal muscarinic receptors. To quantitate muscarinic receptors, acini and segments of main and interlobular ducts were separately disrupted in chilled 50 mM sodium phosphate/5 mM $MgCl_2$ (pH 7.4) with 15–20 strokes of a ground glass homogenizer. Aliquots of these homogenates were assayed for [^{3}H]NMS binding in a total volume of 2.0 mL of the same buffer with concentrations of the labeled antagonist from 50 pM to 1.6 nM. Atropine (50 μM) was included in duplicate samples for determination of non-specific binding. Incubations were carried out for 120 min at 37° and terminated by pouring solutions over Whatman GF/B glass fiber filters mounted in a vacuum filtering manifold. Each filter was rinsed three times in rapid succession with 5 mL of cold incubation buffer and placed in a scintillation vial. Filters were extracted and counted at an efficiency of 35–50% by standard liquid scintillation spectrometry procedures. Specific binding of [^{3}H]NMS was related to DNA content in each sample, determined by the diphenylamine procedure [16]. Scatchard analysis of results from saturation binding studies was carried out using the InStat linear regression program (GraphPad, San Diego, CA).

To determine the affinity of pancreatic ductal muscarinic receptors for subtype-selective muscarinic antagonists, aliquots of ductal homogenate were incubated as above with 0.5 nM [^{3}H]NMS and concentrations of AF-DX 116, atropine, 4-DAMP, and pirenzepine from 0.1 nM to 1.0 mM. Reactions were terminated and samples counted as described above. Constants for inhibition of binding of [^{3}H]-NMS by the three unlabeled antagonists (K_is) were calculated from inhibition curves using the equation of Cheng and Prusoff [17].

Determination of molecular size of pancreatic acinar and ductal muscarinic receptors. Pancreatic duct and acinar muscarinic receptors were covalently labeled by incubation of isolated acini and duct segments in enriched Ringer's solution containing 40–50 nM [^{3}H]PrBCM for 90 min at 23°. After labeling, duct fragments were rinsed several times in Ringer's solution and then homogenized as above. Homogenates were combined with a sodium dodecyl sulfate-containing sample buffer, heated to 95° for 5 min, cooled, and electrophoresed on linear 7.5% polyacrylamide gels as described previously [14]. In some instances, protein in samples was reduced by addition of 2% 2-mercaptoethanol after heating. Apparent molecular masses of the labeled receptors were determined from the position of peaks of radioactivity on gels relative to the positions of molecular weight standards.

To determine the contribution of glycosylation to the molecular mass of ductal muscarinic receptors, isolated pancreatic duct segments were labeled with [^{3}H]PrBCM as above, rinsed, and homogenized in 0.5 mL of 50 mM sodium phosphate (pH 7.8). After homogenization, two 0.2-mL aliquots were separated and to each was added 0.1% sodium dodecyl sulfate and 1.0% Nonidet P-40. After vortexing, samples were incubated for 10 min at 23°, and then 2 units of *N*-glycanase were added to one sample. Both samples were incubated for 24 hr at 37°, then electrophoresed, and analyzed as described above.

Lectin chromatography of pancreatic ductal muscarinic receptors. Duct segments were labeled with [^{3}H]PrBCM as above and homogenized in 2.0 mL of cold 50 mM imidazole/2 mM EDTA (pH 7.4). These homogenates were each combined with 2.0 mL of a detergent solution consisting of 2% digitonin and 0.4% cholic acid and incubated with gentle mixing for 60 min at 23°. After detergent extraction, solutions were centrifuged for 5 min at 17,000 *g* and the supernatant was diluted to 8.0 mL in a chromatography buffer consisting of 25 mM imidazole, 1 mM EDTA, 0.4% digitonin, and 0.08% cholic acid. Detergent solutions containing solubilized, [^{3}H]PrBCM-labeled ductal muscarinic receptors were applied to a 2.0-mL WGA-agarose column at a flow rate of approximately 0.4 mL/min. After sample addition, the column was rinsed with 20 mL of the above chromatography buffer and then with successive 10-mL aliquots of the same buffer containing 10, 100 and 300 mM *N*-acetyl-glucosamine. All chromatography steps were carried out at 4°. Successive 1.5-mL fractions of the eluate were collected in scintillation vials and counted. Lectin chromatography of both intact and *N*-glycanase-treated ductal muscarinic receptors was carried out.

RESULTS

Quantitation of pancreatic duct muscarinic receptors. Saturation binding studies using [^{3}H]NMS were carried out on homogenates of each of four preparations of guinea pig pancreatic duct segments and acini. Scatchard analysis (Fig. 1) gave mean values for K_d and B_{max} of 0.73 ± 0.05 nM and 6.82 ± 0.69 fmol/μg of DNA, respectively, for ductal homogenates and 0.62 ± 0.07 nM and 0.94 ±

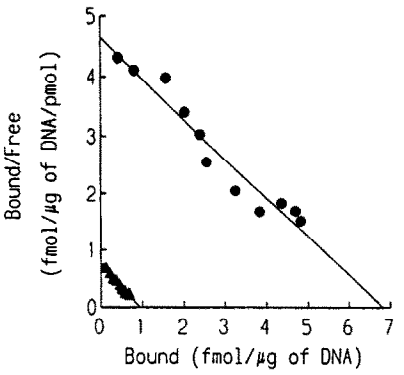

Fig. 1. Scatchard plot of [^{3}H]NMS binding to guinea pig pancreatic acinar (▲) and ductal (●) homogenates. The maximal binding level (B_{max}) was 6.82 fmol/μg of DNA and the dissociation constant (K_d) was 0.734 nM for ducts. Corresponding values for acini were 0.94 fmol/μg of DNA and 0.622 nM. Results are the means of four experiments.

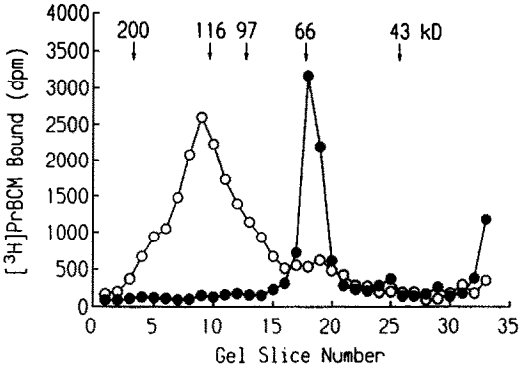

Fig. 3. Electrophoretic profile of [^{3}H]PrBCM-labeled muscarinic receptors of isolated guinea pig pancreatic acini (○) and ducts (●). Acinar and ductal muscarinic receptors were labeled by exposure to 50 nM [^{3}H]PrBCM for 90 min at 23° prior to detergent solubilization and electrophoresis. Arrows indicate positions of molecular weight standards. Shown are the results of a single experiment, representative of at least seven.

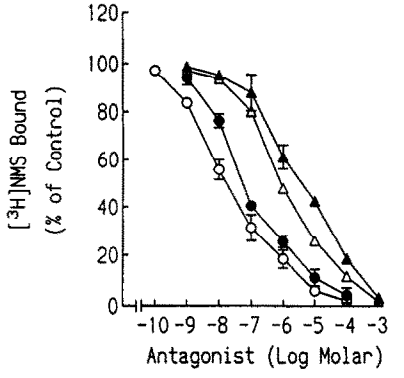

Fig. 2. Antagonist inhibition of [^{3}H]NMS binding to guinea pig pancreatic ductal homogenates. Samples were incubated for 120 min at 37° with 0.5 nM [^{3}H]NMS and the indicated concentrations of atropine (○), 4-DAMP (●), AF-DX 116 (△) and pirenzepine (▲). The control value (100%) was 2.5 fmol of [^{3}H]NMS bound/μg of DNA. Results are the means ± SEM of four experiments.

0.17 fmol/μg of DNA for homogenates of acini. K_d values for [^{3}H]NMS binding to ducts and acini were not significantly different ($P > 0.05$). By contrast, the density of [^{3}H]NMS in isolated duct segments was roughly seven times as great as in acini.

Subtype analysis of ductal muscarinic receptors. Results of competition binding studies with [^{3}H]-NMS and four unlabeled muscarinic antagonists, three of them putatively subtype-selective, are shown in Fig. 2. Of the four antagonists tested, atropine, which is not subtype-selective, was the most potent inhibitor of [^{3}H]NMS binding, with a calculated K_i of 12.4 ± 2.5 nM. The M3-selective muscarinic antagonist 4-DAMP was almost as potent as atropine, with a calculated K_i of 22.1 ± 3.1 nM. The M2 subtype-selective antagonist AF-DX 116 was substantially less potent, with a demonstrated K_i of 0.40 ± 0.04 μM and pirenzepine, an M1-selective antagonist, was the least potent of the antagonists tested, with a K_i of 3.33 ± 0.41 μM. Hill coefficients for each of these competitive binding curves were substantially less than one.

Molecular size and carbohydrate content of ductal muscarinic receptors. [^{3}H]PrBCM was used to covalently label muscarinic receptors of isolated acini and duct segments (Fig. 3). Labeled, non-reduced muscarinic receptors from acini migrated on polyacrylamide gels as a broad peak with a mean molecular mass of 133 ± 9 kDa (N = 13). By contrast, muscarinic receptors in acutely isolated pancreatic duct segments had a mean molecular mass of 62.6 ± 2.5 kDa (N = 7) and the labeled peaks on gels were much narrower. In these studies, receptor proteins were electrophoresed without prior reduction because in earlier studies we found that reduction of [^{3}H]PrBCM-labeled pancreatic acinar cell receptors caused formation of high molecular mass aggregates. This phenomenon has been observed by others [18]. However, in the present studies we found that reduction of [^{3}H]PrBCM-labeled pancreatic duct muscarinic receptors with 2-mercaptoethanol did not cause aggregation, but reduced the apparent molecular mass of the labeled protein slightly to 58.0 ± 1.6 kDa (N = 5).

In four experiments, non-reduced, [^{3}H]PrBCM-labeled ductal muscarinic receptors were digested for 24 hr with the deglycosylating enzyme *N*-glycanase (Fig. 4). Receptors in undigested controls averaged 62.9 ± 2.7 kDa, while those incubated with *N*-glycanase had a molecular mass of 58.9 ± 2.6 kDa. While this difference was not statistically significant due to variability inherent in the electrophoretic determination of molecular masses from one preparation to another, in each of the four experiments carried out, *N*-glycanase reduced the molecular mass of the [^{3}H]PrBCM-labeled protein by approximately 4 kDa. These results indicate that

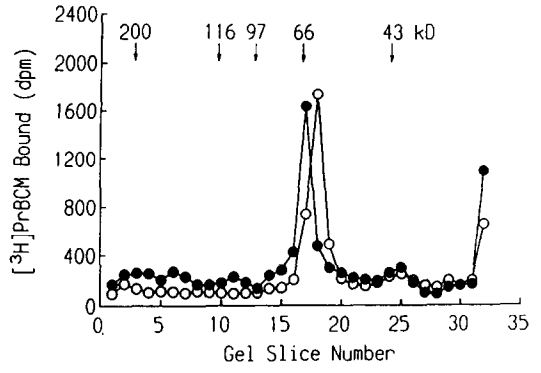

Fig. 4. Effect of *N*-glycanase digestion on molecular mass of [^{3}H]PrBCM-labeled muscarinic receptors of pancreatic ducts. Duct segments labeled as in Fig. 3 were solubilized in sodium dodecyl sulfate and Nonidet P-40 and incubated at 37° for 24 hr in the absence (●) and presence (○) of 10 U/mL of *N*-glycanase prior to electrophoresis. Arrows indicate positions of protein standards of known molecular weight. Shown are the results of a single experiment, representative of four.

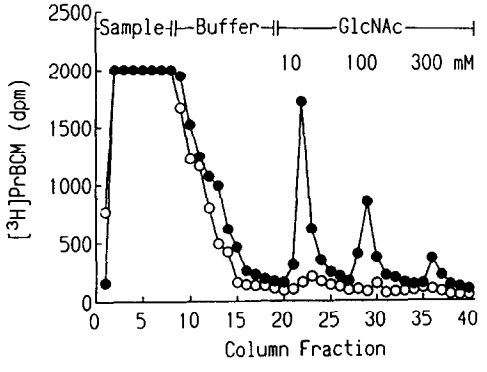

Fig. 5. Lectin chromatography of intact and enzymatically deglycosylated guinea pig pancreatic ductal muscarinic receptors. [^{3}H]PrBCM-labeled receptors were solubilized in digitonin/cholate both before (●) and after (○) *N*-glycanase treatment and applied to a WGA-agarose column. Labeled glycoproteins were eluted from the column with successive 10-mL aliquots of 10, 100 and 300 mM *N*-acetylglucosamine (GlcNAc). All steps were carried out at 4°. Shown are the results of a single experiment, representative of two.

if muscarinic receptors in guinea pig pancreatic ducts are in fact glycosylated, they must contain only a small content of carbohydrate.

To determine whether *N*-glycanase treatment had fully deglycosylated the ductal muscarinic receptors, preparations of [^{3}H]PrBCM-labeled receptors were solubilized in digitonin/cholate and chromatographed on a WGA-agarose column (Fig. 5). When control samples were applied to the WGA-agarose column, a large amount of free [^{3}H]PrBCM was eluted with the column buffer rinse. In the control preparations, successive rinses with buffer containing 10, 100 and 300 mM *N*-acetylglucosamine after elution of free [^{3}H]PrBCM resulted in elution of three successively smaller peaks of radioactivity. In preparations treated with *N*-glycanase, a large amount of free [^{3}H]PrBCM was eluted in the initial buffer rinse, but addition of *N*-acetylglucosamine caused only a very small elution of radioactivity from the column. These results confirm that the native ductal muscarinic receptor contains complex carbohydrates and that 24-hr treatment with *N*-glycanase was successful in deglycosylating virtually all of the receptor protein.

[^{3}H]PrBCM-labeled acinar cell membranes also were treated with *N*-glycanase in several experiments. However, in all cases after a 24-hr incubation no substantial peaks of radioactivity at molecular masses greater than 40 kDa were seen on gels, indicating that proteolytic degradation of the receptors had occurred. Inclusion in the incubation buffer of several protease inhibitors including benzamidine, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and ethylene glycol bis (β-aminoethyl ether)*N,N,N'N'*-tetraacetic acid (EGTA) failed to prevent this degradation. Heating and 2-mercaptoethanol reduction of acinar membrane samples prior to digestion with *N*-glycanase also was carried out in several experiments in an attempt to inactivate endogenous proteases. However, this treatment uniformly caused radiolabeled acinar proteins to aggregate into high molecular mass complexes.

DISCUSSION

The present study constitutes the first examination of the structural and functional characteristics of muscarinic acetylcholine receptors in the pancreatic duct system. Results of saturation binding studies presented in Fig. 1 indicate that the cellular expression of muscarinic receptors in acutely isolated guinea pig pancreatic ducts was 7-fold greater than in isolated acini. The low level of [^{3}H]NMS binding to acinar homogenates in the present study did not appear to result from proteolytic degradation, since the B_{max} determined, 0.94 fmol of [^{3}H]NMS/μg of DNA, was not significantly different from the value of 0.91 fmol/μg of DNA previously shown [19] for [^{3}H]NMS binding to viable isolated guinea pig pancreatic acini. The abundance of muscarinic receptors in isolated duct segments was not entirely unexpected, since previous studies by ourselves and others [8–10] have shown that both the bicarbonate-secreting principal epithelial cells and mucus-secreting goblet cells of the duct system are physiologically responsive to acetylcholine and its analogs.

Competitive binding studies with subtype-selective muscarinic antagonists in the present work suggest that at least two receptor subtypes may be present in the isolated ducts. The order of affinity of muscarinic receptors in duct homogenates for the antagonists tested was: atropine > 4-DAMP > AF-DX 116 > pirenzepine (Fig. 2). While muscarinic receptors of the M3 subtype, including those expressed by pancreatic acini [11–13], charac-

teristically have a high affinity for 4-DAMP they also display a higher affinity for pirenzepine than for AF-DX 116, an M2 subtype-selective antagonist [20]. By contrast, in the present studies, the affinity of muscarinic receptors in duct homogenates for AF-DX 116 was found to be 9-fold greater than the affinity for pirenzepine. The profile of relative binding affinities seen in Fig. 2 thus seems to represent a combination of the properties of M3 and M2 muscarinic receptors. The competitive binding curves for all three of the subtype-selective antagonists tested also were broad, with Hill coefficients ranging from 0.5 to 0.7, an observation consistent with the presence of at least two receptor subtypes.

The predominant form of muscarinic receptor present in isolated guinea pig pancreatic duct segments had an apparent molecular mass of 62–63 kDa prior to reduction of disulfide bonds and 58 kDa after reduction with 2-mercaptoethanol. Treatment of [^{3}H]PrBCM-labeled receptor protein with *N*-glycanase further reduced this apparent molecular mass by approximately 4 kDa (Fig. 4), giving a minimum estimate for the deglycosylated receptor protein of 54 kDa. This is very close to the estimate of 51.7 kDa for the M2 muscarinic receptor subtype calculated from its amino acid sequence [21], but is much smaller than the calculated mass of 66.1 kDa for the M3 muscarinic receptor.

During the course of these studies, we saw no evidence for the presence of the high molecular mass form of muscarinic receptor expressed by acinar cells in the pancreatic duct preparations. The absence of these larger forms indicates that the duct preparations were not contaminated with acini and that M3 muscarinic receptors, if present in the isolated ducts, are glycosylated to a much smaller extent than those in acinar cells. The present studies thus have confirmed the presence of muscarinic receptors in isolated pancreatic ducts and have provided the first information on their level of expression and structural characteristics. However, the cellular location of these receptors remains to be established. Isolated duct segments consist of a hollow tube formed of a single layer of epithelial cells surrounded by a thin sheath of connective tissue [15]. The epithelium is composed primarily of bicarbonate-secreting cells, but also contains a substantial number of goblet cells. As noted previously, both cell types have been shown to be physiologically responsive to cholinergic agonists and are therefore likely sites of muscarinic receptor expression. The thin band of connective tissue that surrounds the epithelium contains fibroblasts and occasionally small blood vessels and may also contain nerve terminals. While fibroblasts would not be expected to express muscarinic receptors, nerve endings and endothelial and smooth muscle cells of vascular elements may constitute additional sites of receptor expression. Further studies using *in situ* hybridization techniques and subtype-specific nucleic acid probes will be required to delineate the cellular distribution of muscarinic receptor subtypes in the isolated pancreatic ducts.

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