Muscarinic Acetylcholine Receptor Structure in Acinar Cells of Mammalian Exocrine Glands*

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Muscarinic acetylcholine receptors are important mediators of secretory events in mammalian exocrine organs such as the pancreas and lacrimal and salivary glands. Occupation of these receptors in these organs by acetylcholine or its synthetic analogues leads to characteristic alterations in the functional status of secretory epithelial cells. Initial events triggered by receptor occupancy include increased turnover of membrane polyphosphoinositides (1), release of Ca\(^{2+}\) from intracellular stores (2), and changes in flux rates across cell plasma membranes for both monovalent and divalent ions (3). How interaction of acetylcholine with its membrane receptors gives rise to these early events and ultimately protein and fluid secretion remains largely unknown. To a large extent this results from a lack of knowledge concerning the molecular characteristics of epithelial muscarinic receptors and of the assemblages of enzymes and ion channels with which they are functionally associated in the secretory cell plasma membrane. An initial step in elucidating the molecular topography of the receptor-effector complex, therefore, would be determination of the molecular weight of the receptor and whether or not the receptor exists in the native membrane as a single protein or an assemblage of disulfide-linked subunits.

The recent development of muscarinic antagonists that form covalent associations with the receptor molecule allows such studies. Using tritiated azido derivatives of the potent antimuscarnic, 3-quiniclidinyl benzilate and N-methyl-4-piperidyl benzilate, Amiata et al. (4) labeled muscarinic receptors in a membrane fraction derived from rat brain cortex and analyzed binding by NaDodSO\(_4\)-PAGE. Liquid scintillation counting of gel slices revealed a sharp peak of radioactivity corresponding to \(M_w = 86,000\). Peaks of similar molecular weight (78,000-86,000) were more recently reported by Venter (5), who used the antagonist \(^{[3]}\)Hpropylbenzilylcholine mustard to label receptors in membranes prepared from guinea pig ileal smooth muscle and rat and canine heart and brain. A slightly lower value, \(M_w = 70,000\), was reported for digitonin-solubilized calf forebrain muscarinic receptors, purified by affinity chromatography with Affi-Gel-linked dexetimide before labeling with \(^{[3]}\)HPrBCM (6). By contrast, Hulme et al. (7) recently provided an estimate of \(M_w = 110,000\) for the \(^{[3]}\)H PrBCM pre-labeled muscarinic receptors solubilized from rat brain synaptosomes with 0.3% cholate, 1 MNaCl. In each of these studies, reducing agents such as mercaptoethanol and dithiothreitol had no effect on the molecular weight of labeled peaks, thus indicating that muscarinic receptors consist of a

* This research was supported by Grants AM32994 and AM3708 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: NaDodSO\(_4\), sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HR, HEPES-buffered Ringer's solution; PrBCM, propylbenzilylcholine mustard; QNB, quinuclidinyl benzilate.
Muscarinic Receptor Structure in Exocrine Glands

single polypeptide. However, the range of molecular weights reported (70,000-110,000) suggests that species- and/ or tissue-specific heterogeneity exists among these receptors or that the lower molecular weight peptides reported represent proteolytic fragments of the intact receptor. One way to distinguish among these possibilities is to covalently label the receptors in intact viable cells and denature and solubilize the cells directly without fractionation prior to electrophoretic analysis. Such a protocol should yield a truer estimate of the in situ molecular weight of the receptor, since it is possible that the cellular disruption necessary to prepare subcellular membrane fractions could alter the structure and associations of membrane proteins. Using these methods, we have labeled muscarinic receptors with [3H]PrBCM on dispersed acinar cells or intact acini from rat pancreas and lacrimal and parotid glands, as well as on cultured IM-9 lymphocytes and NG108 neuroblastoma x glial hybrids. On polyacrylamide gels, labeled peaks of several molecular weights up to 118,000 were observed. Receptors in each cell type examined displayed a characteristic molecular size for that cell type that was different from the other cells. These data indicate that substantial heterogeneity in molecular size is a characteristic of muscarinic acetylcholine receptors in living cells.

EXPERIMENTAL PROCEDURES

Materials

[proplyl-2,3,4,5]Prolidylbenzylcholine mustard (33.0 and 43.3 Ci/mmol) and [1-benzyl-4,5,6,7]-[3H]quiniclidinyl benzilate (33.1 Ci/mmol), Omnipruf, and Protosol were purchased from New England Nuclear. Purified collagenase (441 units/mg) was obtained from Cooper Biomedical (Malvern, PA). Crude collagenase (160 units/mg), hyaluronidase (300 units/mg), a-chymotrypsin (62 units/mg), DTT (800 units/mg), papain (24 units/mg), and trypsin inhibitor (type I-S) were obtained from Sigma. Bovine serum albumin (fraction V) was from Miles Laboratories (Elkart, IN). Eagle's minimal essential medium amino acids (50X concentrate) was from Grand Island Biological (Malvern, PA). Eagle's minimal essential medium amino acids, adjusted to pH 7.4 with NaOH, and gassed with 100% CO2 in a shaking water bath, was from BioWhittaker. Crude collagenase (160 units/mg), hyaluronidase (300 units/mg), a-chymotrypsin, papain, DTT, trypsin inhibitor, and trypsin inhibitor (type I-S) were obtained from Sigma. Bovine serum albumin (fraction V) was from Miles Laboratories (Elkart, IN). Eagle's minimal essential medium amino acids (50X concentrate) was from Grand Island Biological (Malvern, PA). Eagle's minimal essential medium amino acids, adjusted to pH 7.4 with NaOH, and gassed with 100% CO2 in a shaking water bath, was from BioWhittaker.

Methods

Preparation of Cells and Acini—Rats were killed by a blow to the head followed by exsanguination. The selected exocrine organs (pancreas, salivary glands, and lacrimal glands) were removed, trimmed of excess adipose and connective tissue, and dissociated to yield either single acinar cells or acini. Singly dispersed cells were prepared as described previously (8, 9). Acini from the three glands were prepared by a modification of the procedure of Schultz et al. (10). After dissection, glands were injected several times with 5 ml of HEPES-buffered Ringer's solution (HR) containing 2% NaCl, 4.7 mM KCl, 1.0 mM CaCl2, 5.5 mM NaHEPES, and 2.0 mM L-glutamine. This medium was enriched with Eagle's minimal essential medium amino acids, adjusted to pH 7.4 with 2.0 N NaOH, and gassed with 100% O2. After injection, the disintegrated acini were minced and incubated in three successive 10-ml volumes of the injection medium for a total of 120 min at 37°C with shaking at 90 cycles/min. At the end of the incubation, tissue pieces were transferred to a polyethylene culture tube, and the medium was replaced with HR containing bovine serum albumin and trypsin inhibitor but no collagenase. Dissociation was effected by pipetting the tissue several times through the bore of a siliconized Pasteur pipette and filtering the resulting suspension through 100 µm nylon mesh. Subcellular debris was removed by centrifugation the acini twice for 3 min at 50 X g and resuspending in HR.

[3H]QNB Binding to Dispersed Acinar Cells—The presence of muscarinic receptors on dispersed acinar cells and cultured cells was ascertained by saturation binding studies with [3H]QNB. Approximately 106 cells were incubated in 5.0-ml aliquots of HR (2 X 105/ml) containing concentrations of [3H]QNB from 10-300 pM for 2 h at 37°C. Duplicate suspensions containing, in addition, 10 µM atropine were co-incubated as controls for estimation of nonspecific binding. Incubations were terminated by pouring the suspensions onto a glass fiber filter in a Packard Tri-Carb liquid scintillation spectrometer. The difference in radioactivity associated with filters holding cells incubated in the absence or presence of atropine was defined as specific [3H]QNB bound. Cells in these suspensions were counted directly using a hemacytometer and binding was expressed as fmol of [3H]QNB/106 cells, which was then converted to muscarinic receptors/cell, assuming a 1:1 correspondence between [3H]QNB binding sites and cellular receptors. Equilibrium dissociation constants (Ka) and maximal binding levels (Bmax) were determined from Scatchard analysis of saturation binding isotherms.

[3H]PrBCM Binding to Acini and Dispersed Acinar Cells—Initial studies were carried out wherein the time and concentration dependences of [3H]PrBCM binding to dispersed rat pancreatic acinar cells were examined. In saturation binding studies, cells (7 X 106/ml) were incubated in 5 ml of HR at 30°C with concentrations of [3H]PrBCM from 0.5-10 nM. [3H]PrBCM was cyclic for 50-60 min in 50 mM NaPO4 (ph 7.4). Atropine (10 µM) was included in duplicate suspensions, and incubations were terminated at 30 min by the addition of 5 ml of cold 0.9% NaCl, and collected on GF/A filters. Filters were rinsed, extracted, and counted as described. From these preliminary studies, standard conditions of incubation for covalent labeling of muscarinic receptors in acini and single acinar cells were chosen. In most instances, cells or acini were preincubated in 10 ml of HR at 30°C with or without 10 µM atropine for 30 min before incubation with 10 µM [3H]PrBCM. Labeling proceeded for 30 min at 30°C, at which time cells were separated from the incubation medium by centrifuging at 50 X g for 5 min. Acinar or cell pellets were rinsed three times by resuspension in 10 ml of HR at 30°C.

NaDodSO4-PAGE of [3H]PrBCM-labeled Acini or Acinar Cells—After rinsing, [3H]PrBCM-labeled cell pellets were resuspended in 0.75 ml of HR, and 0.1 ml was removed for protein determination by a Coomassie blue binding assay (Bio-Rad). The remainder of the suspension was mixed by vortexing with 0.3 ml of 95°C sample buffer containing 3% NaDodSO4, 15% glycerol, 0.05% bromphenol blue, and 1 M Tris-HCl (ph 6.8). The sample was then boiled in boiling water for 5 min. In some instances, mercaptoethanol was added to the heated samples to a final concentration of 2%. Samples were stored frozen at -40°C.

Polyacrylamide gel electrophoresis was carried out by the method of Laemmli (11) with the following modifications. In order to resolve clearly peaks of radioactivity on gels, samples having a total protein content of up to 5 mg were analyzed. To handle this large protein load, slab gels 130 mm wide x 120 mm high x 3 mm thick were used. Special combs were designed for sample loading, having two peripheral and one center slot, each 4 mm wide, for molecular weight standards (Bio-Rad Laboratories). Polyacrylamide gels, 7.5% T/4% C, and 10% T/6% C, were prepared as above. In between these, two slots each 50 mm in width, for samples, were made. The large wells thus constructed allowed placement of up to 1.0 ml of sample on each half of the gel. In all cases, 7.5% resolving gels were used. After electrophoresis, three narrow strips containing the molecular weight standards were removed from the slab and stained with 0.5% Coomassie brilliant blue in 50% trichloroacetic acid. The remaining two sections of the slab were each cut into approximately 40 3-mm slices. Each slice was placed in a scintillation vial and extracted for at least 24 h in Protosol/Omnipruf as above with frequent vortexing before counting.

Preparation of Pancreatic Plasma Membranes and Labeling with [3H]PrBCM—Purified plasma membrane fractions were prepared from rat pancreatic acini by the procedure of Ponappa et al. (12) and frozen at -70°C. Membranes (0.5-1.0 mg of protein) were thawed and centrifuged once at 100,000 X g for 60 min at 4°C and resus-
prepared from rat exocrine pancreas and lacrimal and parotid
and electrophoresed as described above.

RESULTS

Binding of \[^{3}H\]QNB to Dispersed Acinar Cells—In our initial studies, suspensions of dispersed acinar cells were prepared from rat exocrine pancreas and lacrimal and parotid glands by a combination of enzymatic digestion, divalent cation chelation, and mechanical dispersion. Previous studies in our laboratory showed that acinar cells prepared by this technique are responsive to cholinergic secretagogues (8, 9). Viability of acinar cells from the three glands prepared in this fashion was over 90% as assessed by trypan blue exclusion. To quantitate muscarinic receptors in these cells, saturation binding experiments were carried out using the reversible muscarinic antagonist \[^{3}H\]QNB. As shown in Fig. 1, two components of binding were observed. In the presence of 10 \( \mu M \) atropine, binding was linearly dependent on \[^{3}H\]QNB concentration from 10–300 PM. Subtraction of this linear component from total binding measured in the absence of atropine revealed a saturable component. Scatchard plots of the saturable binding curve (inset, Fig. 1) were invariably linear. From these plots, \( K_d \) and \( B_{max} \) for the binding reaction were determined (Table 1). Average numbers of specific \[^{3}H\]QNB binding sites per acinar cell were 21,400 in parotid gland, 25,700 in lacrimal gland, and 26,400 in suspensions derived from the pancreas. All three glands were thus similar in their density of muscarinic receptors. The affinity of \[^{3}H\]QNB for receptors in the three glands was likewise similar. The \( K_d \) values for binding in parotid and lacrimal suspensions, 35.9 and 40.9 PM, respectively, were not statistically different from each other. The \( K_d \) for binding in pancreatic suspensions, 59.5 PM, however, was statistically different from the other two values (\( p < 0.01 \)), although this difference is small enough that it probably does not represent a biologically meaningful variation in antagonist interaction with the receptor. These values for \( K_d \) and \( B_{max} \) are similar to those determined by others for \[^{3}H\]QNB binding to exocrine gland acinar cells (15–17).

\[^{3}H\]PrBCM Binding to Dispersed Pancreatic Acinar Cells—The above studies indicated that acinar cells from each gland retained muscarinic receptors after dispersion. We therefore examined some of the characteristics of \[^{3}H\]PrBCM binding to pancreatic acinar cells, using initially the temperature and \[^{3}H\]PrBCM concentration found suitable by Venter (5) for labeling these receptors in rat brain membrane fractions. At a ligand concentration of 1.0 nM, a constant level of specific binding (total — atropine-insensitive) was attained by 30 min at 30°C (Fig. 2). Nonspecific binding (\(+10 \mu M \) atropine) accounted for approximately half of total binding and slowly increased with time out to 60 min. The concentration dependence of \[^{3}H\]PrBCM binding was also examined (Fig. 3). At \[^{3}H\]PrBCM concentrations from 0.5–5.0 nM, both total ligand binding and binding in the presence of atropine were linearly dependent on the medium concentration of free \[^{3}H\]PrBCM. No evidence of a saturable component was seen. In fact, the atropine-inhibitable component of binding, which supposedly represents specific radioligand binding to muscarinic receptors, was greater \((77 \pm 17 \text{ fmol/}10^6 \text{ cells})\) at 5 nM \[^{3}H\]PrBCM than could be accounted for by \[^{3}H\]QNB binding data, which gave a \( B_{max} \) of 44 ± 4 fmol/10^6 cells for pancreatic acinar cells. Thus, atropine appeared to retard binding to muscarinic receptors on these cells and to a separate cellular component.

![Fig. 1. Binding of \[^{3}H\]QNB to dispersed pancreatic acinar cells as a function of ligand concentration.](image1)

Values represent means ± S.E. of three experiments. MACHR, muscarinic acetylcholine receptors.

<table>
<thead>
<tr>
<th>Gland</th>
<th>( B_{max} )</th>
<th>( K_d )</th>
</tr>
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<tbody>
<tr>
<td>Pancreas</td>
<td>26,400 ± 2,300</td>
<td>59.5 ± 3.0</td>
</tr>
<tr>
<td>Parotid</td>
<td>21,400 ± 1,900</td>
<td>35.9 ± 1.7</td>
</tr>
<tr>
<td>Lacrimal</td>
<td>25,700 ± 4,500</td>
<td>40.0 ± 6.0</td>
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![Fig. 2. Time dependence of labeling of dispersed pancreatic acinar cells with \[^{3}H\]PrBCM.](image2)

Inset, \( \Delta \), time course of atropine-sensitive binding.
In an attempt to determine whether either component could be removed from the cells by further incubation in PrBCM-free medium, we labeled pancreatic acinar cells for 30 min at 30 °C with 5 nM [3H]PrBCM in the absence or presence of 10 μM atropine, pelleted the cells, and resuspended each pellet in 10 ml of HR at 30 °C without either ligand. Samples were removed from each suspension immediately and collected on GF/A filters, rinsed, and counted as described above. Successive samples were taken at intervals to 120 min. The amount of [3H]PrBCM bound to cells both in the presence and absence of atropine did not decrease appreciably during this period, suggesting that the association of the ligand with both atropine-sensitive and -insensitive cellular components was not readily reversible.

NaDodSO4-PAGE of [3H]PrBCM-labeled Acinar Cells, Acini, and Acinar Plasma Membranes—When [3H]PrBCM-labeled intact acinar cells from each of the three glands were solubilized with hot NaDodSO4 and electrophoresed, a single peak of radioactivity was observed (Fig. 4, A–C). These peaks, however, appeared at different molecular weights in each gland (Table II). Apparent molecular weight of the peak observed when solubilized pancreatic acinar cells were applied to gels averaged 87,600 ± 1,800, while that from parotid cells was 78,000 ± 1,400 and that from lacrimal gland cells was 62,800 ± 1,200. Each value was significantly different (p < 0.01) from the other two. In addition, in some but not all gels, one or more subsidiary peaks of lower apparent molecular weight were observed (Fig. 5 and Table II). In gels of [3H] PrBCM-labeled pancreatic acinar cells, small peaks of M, = 61,200 and 41,200 were seen, while in gels from parotid and lacrimal gland cell preparations, single peaks of M, = 40,400 and 47,400, respectively, were resolved. That the peaks in each instance represented at least some portion of the muscarinic receptor was confirmed by their absence when [3H] PrBCM labeling was carried out in the presence of 10 μM atropine.
atropine. Although in most experiments, 10 μM atropine was used to block binding of [3H]PrBCM, in a few experiments we used 1 μM atropine or this lower concentration of the other muscarinic antagonists, QNB and scopolamine. In each case, a covalent association of [3H]PrBCM with high molecular weight components was virtually eliminated. A large number of counts was always present in the last several gel slices which contained material that migrated at or in front of the dye front. Since the molecular weight of bromphenol blue is 670 and that of PrBCM is 412, this radioactivity must represent free [3H]PrBCM or [3H]PrBCM bound to small molecules. Although not shown in Fig. 4, the total dye front radioactivity in gels loaded with cells incubated with atropine was always much less than in duplicate samples labeled in the absence of the competing antagonist. For example, total radioactivity in the dye front in Fig. 4A was 31,800 dpm in the absence of atropine and 20,500 dpm when labeling was carried out in its presence. Dye front radioactivity in Fig. 4, B and C, was 29,400 and 35,700 dpm, respectively, in the absence and 22,100 and 22,500 dpm in the presence of atropine. These differences are of a sufficient magnitude to account for the additional atropine-sensitive [3H]PrBCM binding above that which could be accounted for by binding of the radiolabeled ligand to muscarinic receptors in Fig. 3.

Addition of 2% mercaptoethanol to the sample buffer did not alter the position or number of peaks observed, indicating that each consisted of a single polypeptide and not an assemblage of disulfide-linked subunits. The positions of the peaks were also not altered in any consistent fashion when the amount of cell protein applied to the gels was varied from 0.5–5.6 mg/per sample well. Varying the concentration of [3H]PrBCM from 1–10 nM likewise did not alter the positions of the labeled peaks in cell suspensions from pancreas (Fig. 5) or parotid gland (not shown), although total radioactivity in peaks was increased. In Fig. 5, labeling in gel slices 1–35 amounted to 13.6, 20.5, 34.9, and 69.9 fmol of [3H]PrBCM/mg of protein at 1, 2, 5, and 10 nM, respectively. In this preparation, a ratio of 308 μg of protein/106 cells was calculated from direct cell counts and protein assays. Using this factor, labeling can be expressed as [3H]PrBCM molecules covalently bound/acinic cell. These calculations give values ranging from 2,520 at 1 nM to 12,940 at 10 nM. Assuming that 1 [3H]PrBCM molecule binds to 1 muscarinic receptor, it becomes apparent that even at 10 nM [3H]PrBCM, only about half of the cellular receptors are labeled. Total radioactivity in the dye front (gel slices 36–40) also increased in proportion to [3H]PrBCM concentration.

For the same amount of protein, pancreas samples always gave a somewhat smaller peak than did samples from either parotid or lacrimal gland cells, indicating that the efficiency of labeling in these suspensions was lower. The reason for this decrease is not readily apparent. However, it did not prevent resolving a discrete peak on gels.

Although the apparent molecular weight determined for muscarinic receptors on dispersed pancreas and parotid acinar cells fell within the range reported by others for the receptor in brain and muscle (4–7), that for lacrimal gland cells was significantly lower. However, the value obtained for the receptor on these cells (M0 = 62,800 ± 1,200) closely matched the molecular weight of a major peak generated by limited proteolysis of muscarinic receptors in membrane preparations from brain and from cardiac and skeletal smooth muscle (5). This correspondence suggested that the relatively low molecular weight determined in lacrimal gland cells might result from degradation of muscarinic receptors by the enzymes used to dissociate the gland into single cells or by an endogenous tissue-specific protease released from cells damaged during isolation. To assess this first possibility, we prepared acini rather than single cells from lacrimal glands using purified collagenase which is substantially free of contaminating nonspecific proteolytic activity. When acini were labeled with 5 nM [3H]PrBCM, solubilized, and electrophoresed, a distinctly different gel profile was seen (Fig. 6A). The major peak of radioactivity now was located at M0 = 87,200 ± 2,200 (Table II). Much smaller but still discernible peaks also were observed at 63,100 ± 600 and 45,900 ± 300. Since changing the method of gland dissociation had substantially altered the position of the primary peak of [3H]PrBCM-labeled material from the lacrimal gland, we likewise prepared acini from parotid glands and pancreas by the same procedure and labeled them with [3H]PrBCM (Fig. 6, B and C, and Table II). On gels loaded with solubilized, pancreatic acini, four peaks of radioactivity were usually seen. The major peak was located at M0 = 117,600 ± 1,200. This broad peak overlapped a smaller peak found at 85,700 ± 1,500. Two minor peaks located at 59,000 ± 2,200 and 38,200 ± 1,300 also were present. Three peaks were seen on gels loaded with parotid acini (Fig. 6C and Table II): the first and major peak at M0 = 104,800 ± 2,500, a secondary peak at 74,500 ± 1,200, and a very small peak at 38,900 ± 800. Addition of either 1 μM QNB, scopolamine, or atropine to the [3H]PrBCM-labeled medium abolished all of the peaks (data not shown). By contrast, inclusion of 2% mercaptoethanol in the sample buffer had no effect on peak number or position.

We also tested the possibility of endogenous proteases degrading muscarinic receptors in lacrimal gland by vigorously homogenizing lacrimal glands in HR and incubating [3H]PrBCM-labeled pancreatic acini with this homogenate. However, no alteration in the characteristic gel profile of pancreatic receptors was observed.

Since virtually all covalent labeling of muscarinic receptors carried out heretofore by others has utilized subcellular membrane fractions rather than intact cells, for comparative purposes we prepared and labeled with [3H]PrBCM two prepa-

![Graph](image_url)

**Fig. 5.** Effect of [3H]PrBCM concentration on labeling of proteins in rat pancreatic acinar cells. Cells were incubated for 30 min at 30°C in HR with 1 nM (A), 2 nM (B), 5 nM (C), and 10 nM (D) [3H]PrBCM. Labeled acinar cells were solubilized and electrophoresed as described under "Methods." Sample protein on gels was 2.8 mg. Protein standards are as in the legend to Fig. 4.
Muscarinic Receptor Structure in Exocrine Glands

1. NaDdS04-PAGE profile of [3H]PrBCM-labeled acini from lacrimal gland (A), pancreas (B), and parotid gland (C). Acini were labeled, solubilized, and electrophoresed under conditions identical to those in the legend to Fig. 4. Each profile is representative of a minimum of five experiments. Sample protein loaded on each gel was: A, 1.6 mg; B, 3.5 mg; and C, 4.6 mg. Protein standards are as in the legend to Fig. 4.

2. NaDdS04-PAGE profile of [3H]PrBCM-labeled pancreatic plasma membranes. Membranes were incubated in HR with 5 nM [3H]PrBCM alone (●) or with 10 μM atropine (○) for 30 min at 30 °C. Sample protein loads were 0.32 mg (●) and 0.27 mg (○). Protein standards are as in the legend to previous figures.

3. NaDdS04-PAGE profile of [3H]PrBCM-labeled pancreatic acini incubated after labeling for 40 min at 37 °C in HR containing 10 units/ml of α-chymotrypsin, 100 units/ml of crude collagenase, and 100 units/ml of hyaluronidase. Protein load on the gel was 3.5 mg. Protein standards are as in the legends to previous figures.

In a second preparation, a single peak of M, = 87,000 was resolved. As with intact cells, in both preparations of membranes labeled, total dye front radioactivity was substantially less in samples labeled in the presence of atropine (not shown).

Digestion of [3H]PrBCM-labeled Muscarinic Receptors—
For all three glands, the second peak observed on gels of labeled acini corresponded to the major peak seen when singly dispersed acinar cells were labeled. This correlation indicated that the smaller peak was probably a fragment of the larger intact receptor. To directly test this supposition, we labeled pancreas and parotid acini as usual with [3H]PrBCM and then incubated the acini in HR containing the mixture of crude collagenase, α-chymotrypsin, and hyaluronidase used to prepare single acinar cells for 40 min at 37 °C (time for digestion and temperature were chosen to equal those of the cell dispersion procedure). In each case, the highest molecular weight peak was abolished. In the pancreas preparation (Fig. 8), most of the radioactivity was present in a peak of apparent molecular weight of 88,000 with a much smaller peak also seen at 44,100. The gel profile obtained from parotid acini treated in the same manner exhibited a major peak at M, = 82,300 and a smaller peak at 41,100 (data not shown). These data suggest that the gel peaks observed when single acinar cells are labeled with [3H]PrBCM represent major receptor fragments and that one or more of the proteolytic components of the enzyme mixture can cleave a fragment of roughly 25,000–30,000 from the larger portion of the receptor that bears the antagonist binding site. To determine which enzyme (or enzymes) was responsible for this shift in apparent molecular weight of the receptor, we incubated [3H]PrBCM-labeled pancreatic acini with each of the three enzymes separately. Collagenase and hyaluronidase had no effect on gel profiles. By contrast, digestion with 10 units/ml of α-chymotrypsin for 40 min gave results identical to those in Fig. 8. Increasing the concentration of α-chymotrypsin to 50 units/ml did not further alter peak position.

The presence of multiple peaks of radioactivity on gels of acinar preparations (Table II) indicated that the receptor can be cleaved in several places down to a limit of about 38,000
by proteases acting at the extracellular surface of the plasma membrane. To verify this limit size, we labeled cells prepared from each of the three glands and incubated them at 37°C for 60 min in HR containing concentrations of papain from 0.1–1.0 mg/ml. Substantial shifts in the molecular weight of labeled peaks were not seen at papain concentrations below 0.5 mg/ml. At 1.0 mg/ml, however, in each tissue the usually observed peak was gone and a broad peak with an apparent molecular weight of about 40,000 was present (Fig. 9). Control suspensions co-incubated in the absence of papain still displayed the characteristic peak.

Properties of Muscarinic Receptors in Nonepithelial Cell Types—The heterogeneity of size of proteins labeled with [3H]PrBCM in the three exocrine glands studied prompted us to briefly investigate the size of muscarinic receptors in two cultured cell lines of nonepithelial origin, the IM-9 lymphocyte and NG108 neuroblastoma x glioma cell hybrid. Both cell types bound [3H]QNB, indicating the presence of muscarinic receptors. NG108 cells possessed 13,900 receptors/cell with a high affinity for QNB ($K_d = 12.8\text{ pM}$). IM-9 cells exhibited only 5,200 receptors/cell and a somewhat lower affinity of 99.5 pM. Three preparations of each cell type were labeled with [3H]PrBCM (Fig. 10). In IM-9 preparations, a high molecular weight peak of $M_r = 114,300 \pm 3,700$ and slightly smaller peak of 79,700 ± 2,900 were seen. In NG108 cells, [3H]PrBCM labeling indicated a single broad peak of relatively low molecular weight, $M_r = 72,100 \pm 2,600$.

**DISCUSSION**

Results of the studies presented here indicate that the molecular size of muscarinic acetylcholine receptors as deduced from covalent antagonist labeling of intact cells varies considerably from one cell type to another, even among parenchymal cells of exocrine glands from the same animal. That the [3H]PrBCM-labeled peaks resolved on polyacrylamide gels do in fact represent muscarinic receptors or fragments thereof is confirmed by their disappearance from gels when cells were incubated with [3H]PrBCM together with an excess of a competing muscarinic antagonist, atropine, QNB, or scopolamine. Another observation of interest in these studies was the presence of atropine-inhibitable [3H]PrBCM binding to acinar cells above the level that could be accounted for by binding of the radioligand to the receptor. This labeling component could not be removed readily from the cells by rinsing, but after solubilization of cells in NaDodSO₄ and electrophoresis, the [3H]PrBCM bound to this component migrated at the gel dye front. Either the radioligand was released during detergent solubilization or remained bound to an unidentified cellular component of very low molecular weight. Since this excess of dye front radioactivity also was observed in the pancreatic plasma membrane preparations labeled with [3H]PrBCM, the binding entity apparently is a cell surface component. Other authors (5–7, 13) have either not noted this component of [3H]PrBCM binding or have chosen not to comment on it, although a substantial reduction in dye front radioactivity in the presence of atropine is clearly seen in gel profiles in at least one previous study (13). This interaction of [3H]PrBCM in and of itself does not, however, invalidate identification of the labeled high molecular weight proteins as muscarinic receptors. Rather, these results indicate that neither PrBCM or atropine binds exclusively to the receptor, but that they and possibly other muscarinic ligands as well have some common affinity for other sites on the cell surface. The nature of these sites remains unknown, if they are proteins, as muscarinic receptors are known to be, [3H]PrBCM binding to them is not of a covalent nature.

The variation in receptor size observed in the present study is surprising, since in organs such as the pancreas and lacrimal and parotid glands, the pharmacological characteristics of binding of muscarinic agonists and antagonists are quite similar. Muscarinic antagonists such as [3H]QNB and N-[3H]methylscopolamine bind to receptors on intact cells or cell membrane preparations in a simple mass action fashion. A single class of high affinity binding sites with $K_d$ values in the picomolar range are usually reported (15, 16). For agonists, two classes of binding sites are routinely observed: a high affinity site with reported $K_d$ values ranging from nanomolar to low micromolar concentrations, depending on the agonist, and a low affinity binding site in the micromolar range (15, 18, 19). Another point of similarity among these tissues in this respect is the density of muscarinic receptors. From [3H]
QNB binding studies with dispersed rat parotid gland acinar cells, Putney and Van De Walle (17) derived an estimate of 23,000 receptors/cell. Desai et al. (19) recently estimated an average binding capacity of 24,000 sites/cell for N-[3H]methylxylamine on rat pancreatic acini. These values are nearly identical to those obtained in the present study (Table I). A final point that would lead one to expect a similarity in structure of muscarinic receptors in these tissues is the similarity of initial subcellular events initiated by their occupancy by agonists. In pancreatic acinar cells as well as in those from lacrimal and parotid glands, a primary response to receptor activation is an increase in turnover of plasma membrane phosphoinositides (20-22). This increased breakdown is apparently mediated by activation of membrane-associated phospholipases (21, 23). This similarity in primary effector coupling would seem to suggest that at least that portion of the receptor that communicates its occupancy status, presumably at the cytoplasmic membrane surface, would be conserved across these tissues.

Despite these indications of conservancy, the results presented in Figs. 4 and 5 and Table II demonstrate a striking degree of variation in molecular size of [3H]PrBCM-labeled proteins. Among the three exocrine glands examined, the major protein species labeled in the pancreas is the largest \( M_r = 117,600 \), while the parotid receptor is intermediate \( (104,400) \) and that in the lacrimal gland is smallest \((87,200)\). In each case, a sequence of peaks progressively smaller both in magnitude and molecular weight was seen down to a molecular weight of approximately 40,000 (Fig. 6). The smaller peaks evidently constitute fragments of the intact receptor, since they can be produced by treatment of acinar cells with proteolytic enzymes that cause disappearance of the largest peak. In each tissue, \( \alpha \)-chymotrypsin removes a fragment of \( M_r = 25,000-35,000 \) from the receptor, while papain reduces the \([3H]PrBCM\)-binding portion of the receptor to \( M_r = 35,000-45,000 \). At present we do not know the nature of the cleaved subfragments or whether they are freed from the cell surface following proteolysis or remain in the plasma membrane. Muscarinic receptors from porcine brain and atria have been shown to be glycoproteins (24, 25), and it is conceivable that one or more of these fragments represent the bouquet of glycosyl residues on the receptor. Further studies using purified glycosidases and probes of definitiveness should help resolve these questions. Our studies utilizing purified pancreatic plasma membranes suggest that these receptors are labile and may be partially degraded during cellular subfractionation even in the presence of protease inhibitors. This finding brings into question whether the molecular weights reported for muscarinic receptors by others where covalent ligand binding was carried out on membrane preparations do in fact represent the whole receptor or a receptor fragment. In the only other study to date to compare receptor size in intact cells and membrane fractions, Birdsell et al. (13) labeled guinea pig ileal smooth muscle strips and microsomes with \([3H]PrBCM\) and in both cases determined molecular weights of 80,000. Hence, it seems that fractionation in and of itself does not necessarily result in receptor degradation. However, it seems prudent on the basis of our results to utilize intact cells for labeling whenever possible.

Since our studies have been carried out on intact, viable cells, portions of the receptor extending out from the lipid bilayer of the inner aspect of the plasma membrane would not be expected to be cleaved by enzymes present in the medium bathing the cells. However, in studies using membrane preparations where both sides of the membrane are accessible, Venter (5) recently was able to reduce the \([3H]PrBCM\)-labeled portion of the receptor by limited proteolysis to a molecular weight of 18,000. This small fragment may therefore be the core of the receptor that is buried in the bilayer, and the molecular weight of that portion of the muscarinic receptor exposed at the inner plasma membrane surface must be 20,000–25,000.

The limited studies of muscarinic receptors in cultured cell lines of nonepithelial origin also revealed variation in size. Human lymphocytes have been reported to possess muscarinic receptors (26). We confirmed this for IM-9 lymphocytes by \([3H]QNB\) binding. In three preparations of IM-9 cells, \([3H]PrBCM\) labeled two peaks, a major one with a molecular weight of 110,000–120,000 and a slightly smaller peak of 75,000–85,000. Neuroblastoma \( \times \) glioma (NG108) hybrids, a cell type of neural origin that has been utilized extensively to investigate regulation of muscarinic receptors (27, 28), also bound \([3H]QNB\), but the \([3H]PrBCM\)-labeling profile on gels was distinctly different. In the three preparations examined, single broad peaks of 67,000–76,000 were observed. While these studies on IM-9 and NG108 cells were not extensive, they do point out that the maximal size of the receptor varies in nonepithelial cells as well. Neither of these cultured cell lines had been exposed to exogenous proteases, so it appears that the variation seen represents a real difference in native receptor size between NG108 and IM-9 cells and not a preparatory artifact.

Although in our studies we noted a wide range in molecular size of proteins labeled with \([3H]PrBCM\) \( (M_r = 70,000–120,000) \), no proteins greater than 120,000 were reproducibly labeled in any of the five cell types examined. This contrasts to the data obtained by Avisar et al. (29, 30), who covalently labeled muscarinic receptors in membrane preparations derived from different regions of rat brain. In regions such as hippocampus, which exhibit primarily low affinity agonist binding sites, a single protein of \( M_r = 86,000 \) was labeled. In medulla, where both high and low affinity sites are present, an additional labeled peak at 160,000 was resolved on gels. These authors suggested that this second protein was a dimer of the first and that dimerization was responsible for conversion of low to high affinity states and vice versa. However, pancreatic acinar cells display both high and low affinity binding sites for muscarinic agonists (18, 19), yet we did not observe labeled peaks above 120,000. Since at \([3H]PrBCM\) concentrations below 10 nM, all muscarinic receptors on acinar cells are not labeled (Fig. 5), it remains possible that some of the receptors that escaped labeling were of higher molecular weight. Birdsell et al. (14) and Dehaye et al. (19) have demonstrated, however, that \([3H]PrBCM\) does not discriminate in binding to high and low agonist affinity sites, which leads us to believe that we have covalently labeled a representative proportion of cellular muscarinic receptors. The significance of the observations of Avisar and co-workers to muscarinic receptor function in intact cells, thus, is not clear at present, but deserves further attention.

The currently prevailing view of muscarinic receptor structure, based primarily on covalent affinity labeling of receptors in membranes prepared from central nervous system and muscle tissue (5, 31) is that they are highly conserved proteins that display little if any phylogenetic structural diversity. By contrast, using the same alkylating reagent to label intact cells, we have shown that receptor size varies considerably even among tissues in the same animal. Whether these differences in apparent molecular weight reflect differences in the functional attributes of muscarinic receptors or are in some manner related to the biosynthesis of these receptors remains to be established.
Acknowledgments—We thank Dr. John A. Williams for valuable discussions during the course of this work and critical reading of the manuscript.

REFERENCES