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Muscarinic Receptor Coupling to Intracellular Calcium Release in Rat Pancreatic Acinar Carcinoma[1]

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ABSTRACT

Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cholinergic receptor protein affinity labeled with the muscarinic antagonist [^{3}H]propylbenzilylcholine mustard revealed a major polypeptide with molecular weight of 80,000–83,000 in both acinar carcinoma and normal acinar cells of rat pancreas. Muscarinic receptor protein is therefore conserved in pancreatic acinar carcinoma. A small but significant difference was detected in the affinity of carcinoma cell receptors (K_d ~ 0.6 nM) and normal cell receptors (K_d ~ 0.3 nM) for reversible binding of the muscarinic antagonist drug, *N*-methylscopolamine. In addition, carcinoma cell muscarinic receptors displayed homogeneous binding of the agonist drugs carbamylcholine (K_d ~ 31 μM) and oxotremorine (K_d ~ 4 μM), whereas normal cell receptors demonstrated heterogeneous binding, with a minor receptor population showing high affinity binding for carbamylcholine (K_d ~ 3 μM) and oxotremorine (K_d ~ 160 nM), and a major population showing low affinity binding for carbamylcholine (K_d ~ 110 μM) and oxotremorine (K_d ~ 18 μM). Both carcinoma and normal cells exhibited concentration-dependent carbamylcholine-stimulated increases in cytosolic free Ca^{2+}, as measured by $^{45}Ca^{2+}$ outflux assay and intracellular quin 2 fluorescence. However, carcinoma cells were observed to be more sensitive to Ca^{2+} mobilizing actions of submaximal carbamylcholine concentrations, demonstrating 50% maximal stimulation of intracellular Ca^{2+} release at a carbamylcholine concentration (~0.4 μM) approximately one order of magnitude below that seen for normal cells. These results indicate altered muscarinic receptor coupling to intracellular Ca^{2+} release in acinar carcinoma cells, which manifests as a single activated receptor state for agonist binding, and increased sensitivity of Ca^{2+} release in response to muscarinic receptor stimulation.

INTRODUCTION

The concentration of cytosolic free Ca^{2+} in acinar cells of rat pancreas is maintained by a nonmitochondrial, Mg^{2+}- and ATP-dependent pool (1, 2), which is at least partly located within cisternae of the rough endoplasmic reticulum (1). Acinar cells regulate their cytosolic free Ca^{2+} to a narrow physiological concentration range (~10^{-7} mol/liter) by release from or uptake of Ca^{2+} into the nonmitochondrial membrane-bound Ca^{2+} pools (2). Binding of agonist drugs to cholinergic muscarinic receptors stimulates Ca^{2+} release from membrane-bound stores (2), causing a rise in intracellular free Ca^{2+} concentration of pancreatic acinar cells (3). Consequently, muscarinic cholinergic receptors modulate Ca^{2+}-directed functions of pancreatic acinar cells, including protein phosphorylation (4, 5), protein synthesis (6, 7), and protein secretion (8). Plasmalemma muscarinic receptors of dispersed pancreatic acinar cells are composed of a major protein component with a molecular weight of ~87,000 (9), and thereby resemble muscarinic receptors isolated from brain synaptosome fractions, cardiac muscle, ileum smooth muscle, parotid gland, and *Drosophila melanogaster* heads (9–11). Agonist drugs bind by simple mass action to muscarinic receptor protein solubilized from cell membranes with a single dissociation constant of binding in the micromolar range (12, 13). However, for muscarinic receptors in intact cells, including pancreatic acinar cells, agonist drugs appear to interact with two classes of binding sites. One class consists of high-affinity binding sites with K_d values ranging from nanomolar to low micromolar concentrations, and another class of low-affinity sites with K_d values in the micromolar range (14, 15). It appears that muscarinic receptor protein in intact cells undergoes conformational change, perhaps biphasic in nature, upon high- and low-affinity agonist binding. The conformational change directly couples muscarinic receptor protein to the Ca^{2+} release pathway in acinar cells, with generation of an activated receptor system (16, 17). In contrast, antagonist drugs are thought to bind to inactive ground state receptors in which the muscarinic receptor protein is not functionally linked to intracellular Ca^{2+} release. Antagonists bind to ground state receptors of intact cells by simple mass action with a single K_d value.

Pancreatic acinar carcinoma cells secrete protein in response to the muscarinic agonist carbamylcholine (18, 19), and more recently our laboratory has demonstrated a full complement in carcinoma cells of muscarinic receptors which bind the antagonist NMS[3] equivalent in membrane density to muscarinic receptors in normal acinar cells (20). To assess modes of muscarinic receptor linkage with intracellular Ca^{2+} release in pancreatic acinar carcinoma, muscarinic receptor proteins of carcinoma and normal cells were characterized by affinity labeling with [^{3}H]PrBCM and SDS-PAGE, antagonist and agonist drug interactions with carcinoma and normal cell receptors were quantitatively compared by competition binding assay, and receptor activation of Ca^{2+} release from intracellular stores in carcinoma and normal cells was followed by $^{45}Ca^{2+}$ outflux assay and quin 2 fluorescence measurements. Our results indicate that muscarinic receptor protein is conserved in acinar carcinoma, but that muscarinic agonists bind in homogeneous fashion to a single-affinity receptor state in carcinoma cells, in contrast to heterogeneous binding to high- and low-affinity receptor states in normal acinar cells. In addition, acinar carcinoma cells are more sensitive to muscarinic stimulation of intracellular Ca^{2+} release than normal acinar cells. These results indicate that muscarinic receptor linkage to cellular Ca^{2+} responses is altered in pancreatic acinar carcinoma. This may be due to change in structures of receptor protein, and/or perturbation in the energetics of component proteins active in post-receptor transduction of Ca^{2+} release. A preliminary report of this study has appeared elsewhere (21).

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[3] The abbreviations used are: NMS, *N*-methylscopolamine; [^{3}H]PrBCM, [^{3}H]propylbenzilylcholine mustard; [^{3}H]NMS, *N*-[*methyl*-^{3}H]scopolamine; CCK-8, cholecystokinin octapeptide; KRH buffer, Krebs-Ringer-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid buffer; BF-KRH buffer, bovine serum albumin-free KRH buffer; SHB buffer, 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid buffer, pH 7.4, containing 0.3 M sucrose and 1.0 mM benzamidine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; K_d, dissociation constant of binding; n_H, Hill coefficient; EC_{50}, effective agonist concentration yielding 50% maximal response.

MATERIALS AND METHODS

Materials

Pancreatic agonist and antagonist drugs utilized in these experiments were purchased as follows: carbamylcholine chloride, oxotremorine, NMS, atropine sulfate, and *d*-tubocurarine from the Sigma Chemical Co., St. Louis, MO; CCK-8 from E. R. Squibb & Sons, Inc., Princeton, NJ. Radiolabeled NMS was obtained as [^{3}H]NMS (79.6 Ci/mmol) from the Amersham Corp., Arlington Heights, IL, radiolabeled PrBCM as [*propyl*-2,3-^{3}H]PrBCM (43.0 Ci/mmol), and $^{45}Ca^{2+}$ as $^{45}CaCl_2$ (30.5 mCi/mg) from New England Nuclear, Boston, MA. Quin 2-tetra-(acetoxymethyl)ester was purchased from the Sigma Chemical Co. Collagenase (type III) was purchased from Worthington Biochemical Corp., Freehold, NJ, and was further purified by cation exchange chromatography on SP-Sephadex C50 (Pharmacia Fine Chemicals, Freehold, NJ) (22). Collagenase activity of the purified enzyme was determined using the synthetic substrate *N*-carbobenzoxyglycylprolyl-glycylglycylprolylalanine (Sigma), according to the technique of Soberano and Schoellman (23). The KRH buffer utilized in these experiments contained 98.0 mM NaCl, 6.0 mM KCl, 2.5 mM Na_2HPO_4, 2.0 mM $CaCl_2$, 11.5 mM glucose, 0.01% (w/v) soybean trypsin inhibitor (Sigma), 0.2% (w/v) fraction V bovine serum albumin (Sigma), 1.0% (v/v) complete amino acid mixture (Kansas City Biologicals, Lenexa, KS), 2.0 mM glutamine (Kansas City Biologicals), antibiotics (100 units penicillin/ml, 100 μg streptomycin/ml), and 24.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4.

Methods

Preparation of Normal Pancreatic Acinar Cells and Acinar Carcinoma Cells. Normal pancreas was obtained from male F344 rats (150–200 g). The nafenopin-induced acinar carcinoma was maintained as a transplantable tumor in male F344 rats (24). Normal pancreas and acinar carcinoma were dissociated into single cells as described previously (20). Briefly, carcinoma fragments obtained by mechanical disruption of tumor or normal pancreas were incubated for 20 min at 37°C with collagenase (200 units/ml) in KRH buffer, followed by two 5-min incubations with 2 mM EDTA in Ca^{2+}- and Mg^{2+}-free KRH buffer, and incubated again with collagenase for 30–45 min. Cell preparations were sheared by passage through a serological pipet (TD Kimax), and filtered through 60-μm Nitex nylon mesh. The viability of normal acinar cells and acinar carcinoma cells obtained by this procedure was ≥95% as determined by trypan blue exclusion.

Preparation of Membrane Fractions from Normal Pancreatic Acinar Cells and Acinar Carcinoma Cells. Membrane fractions were prepared from collagenase-EDTA-dissociated normal pancreatic acinar cells and acinar carcinoma cells using a modification of the procedure of Ponappa *et al.* (25). Normal acinar or carcinoma cells were homogenized in 10 volumes of chilled SHB buffer using a Potter-Elvehjem homogenizer. The homogenate was centrifuged in a Sorvall RC-2B centrifuge at 450 × *g* for 5 s, and the postnuclear supernatant was collected for recentrifugation at 1,000 × *g* for 10 min. The resultant membrane pellet was washed twice by suspension in chilled buffer followed by centrifugation, and stored frozen at −70°C. To isolate plasma membrane-enriched fractions from carcinoma cells (25), a membrane pellet obtained as described above was suspended in 1.35 M sucrose solution containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, and 1.0 mM benzamidine, placed in 12.7- x 50-mm ultracentrifuge tubes, overlayed with SHB buffer, and centrifuged at 192,000 × *g* for 60 min. The plasma membrane-enriched fraction was collected at the 0.3 M-1.35 M sucrose interface, diluted with SHB buffer solution, and centrifuged at 100,000 × *g* for 60 min. The resultant pellet was suspended in SHB buffer, and stored frozen at −70°C.

Muscarinic Receptor Affinity Labeling and SDS-PAGE. Covalent binding of [^{3}H]PrBCM to membranes of normal and carcinoma cells was performed essentially under the conditions described by Hootman *et al.* (9). Membrane preparations from normal and tumor cells were thawed, diluted with chilled BF-KRH buffer, and pelleted by centrifugation at 100,000 × *g* for 60 min at 4°C in a Beckman L5-75 ultracentrifuge using a TI50 rotor. The membranes were resuspended (0.5 to 1.0 mg protein/ml) in 10 ml volumes of chilled BF-KRH buffer by gentle homogenization, preincubated at 30°C without or with 0.1 mM atropine, and incubation was continued at 30°C for 30 min in the presence of 5 nM cyclized [^{3}H]PrBCM. [^{3}H]PrBCM-labeled membranes were then separated from the incubation medium by centrifugation at 100,000 × *g* for 60 min at 4°C, washed twice in chilled BF-KRH buffer, resuspended in buffer, and a sample was removed for protein concentration determination by the method of Lowry *et al.* (26). The remaining labeled membrane suspension was solubilized by equal volume addition of 95°C sample buffer containing 0.125 M Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol, and 0.002% bromophenol blue, and boiled for 5 min. [^{3}H]PrBCM-muscarinic receptor complexes were analyzed on 7.5% polyacrylamide gels in sodium dodecyl sulfate according to the method of Laemmli (27). Standard proteins (Bio-Rad) were analyzed on each gel. Lanes containing the standard proteins were removed after electrophoresis, and were stained with 0.2% Coomassie brilliant blue in 20% trichloroacetic acid for molecular weight estimates. The lanes containing muscarinic receptor protein were cut into 3-mm slices, each slice was dried overnight at 24°C, and digested by incubation at 55°C in solution containing 99 volumes of 30% H_2O_2 and 1 volume of 28% NH_4OH. Ready-Solv MP scintillation fluid (Beckman) was added to each gel slice digestate and the radioactivity was measured by scintillation counting.

[^{3}H]NMS Competition Binding Assay. Prior to measurement of antagonist or agonist binding, freshly dissociated normal acinar cells or acinar carcinoma cells were maintained for 1–2 h at 37°C in Parsa's defined medium (28), to allow recovery from membrane damage resulting from the dissociation procedure (20). Cells (0.5–5.0 × 10^6/ml) were then incubated at 37°C under O_2 in KRH buffer containing 0.1 nM [^{3}H]NMS in the absence and presence of 0.1 mM atropine, and with increasing concentrations of unlabeled antagonist or agonist drug. Kinetic measurements indicate that maximal specific [^{3}H]NMS binding to carcinoma cells occurs within 30 min of incubation (20). A 60-min incubation period was thus utilized to assure conditions of equilibrium binding. Approximately one-fifth of muscarinic receptors in normal acinar or acinar carcinoma cells are occupied by [^{3}H]NMS under these binding conditions (20). Following the 60-min incubation, 1.0-ml aliquots of cell suspension were obtained in triplicate, and cells were captured by vacuum filtration on Whatman GF/B filters. Filters containing trapped cells with bound [^{3}H]NMS were washed 3 times with chilled (4°C) KRH buffer, dried in counting vials overnight, extracted in Ready-Solv MP scintillation fluid, and the radioactivity was measured by liquid scintillation counting in a Beckman LS-9000 spectrometer. Cell numbers used in calculation of binding parameters were obtained by determination of absorbance at 260 nm of cells digested overnight in 1.0 N NaOH (20). Antagonist binding data obtained with normal and carcinoma cells, or agonist binding data obtained with carcinoma cells were treated by linear regression analysis. Agonist binding data for normal cells were subjected to a computer-fitting model of low- and high-affinity binding sites, using an iterative method based on least-squares analysis.

Assay of $^{45}Ca^{2+}$ Outflux. To measure muscarinic agonist stimulation of $^{45}Ca^{2+}$ outflux, normal pancreatic acinar cells and acinar carcinoma cells were labeled in KRH buffer by incubation for 60 min at 37°C with $^{45}Ca^{2+}$ (4 μCi $^{45}CaCl_2$/ml). Labeled cells were rapidly washed 4 times by centrifugation (200 × *g*; 1 min) in chilled (4°C) KRH buffer containing no $^{45}CaCl_2$, and were then suspended in KRH buffer at 37°C, with or without the muscarinic agonists carbamylcholine or oxotremorine. To demonstrate muscarinic antagonist inhibition of $^{45}Ca^{2+}$ outflux, appropriate NMS concentrations were included in the KRH buffer. To measure $^{45}Ca^{2+}$ outflux, 300-μl aliquots of cell suspension were obtained in triplicate, and the cells were pelleted by centrifugation (10,000 × *g*; 15 s) in a Beckman microfuge. Supernatant was immediately aspirated from the microfuge tubes, cell pellets in tips of the tubes were dispersed in Ready-Solv MP scintillation fluid, and the amount of $^{45}Ca^{2+}$ present in the cells was measured by scintillation counting.

Measurement of Cytosolic Ca^{2+} Concentration in Quin 2-loaded Cells. Quin 2 loading of normal pancreatic acinar cells and acinar carcinoma

cells was carried out by a modification of the method of Tsien *et al.* (29). Cells were suspended in 10–15 ml of KRH buffer (5×10^6 cells/ml), prewarmed at 37°C for 10 min, and loaded by incubation for 20 min at 37°C under an O_2 gas phase with 50 μM quin 2-tetra-(acetoxymethyl)ester. The cells were then washed by centrifugation at $50 \times g$ for 5 min, resuspended in 50–75 ml KRH buffer, and maintained at room temperature. Intracellular quin 2 concentration obtained under these reaction conditions was approximately 0.1 mM. Prior to each fluorescence measurement, cell suspensions were centrifuged at $850 \times g$ for 2 min, resuspended in KRH buffer, prewarmed at 37°C for 5 min, and then placed in a 1-cm^2 quartz cuvette. Fluorescence changes were measured using an Aminco-Bowman spectrofluorometer with an excitation wavelength of 330 nm and emission wavelength of 510 nm. Cell suspensions were continuously stirred, and maintained at 37°C in the absence and presence of carbamylcholine during fluorescence measurements. Following completion of measurements, cells were lysed with 50 μM digitonin, and the fluorescence of quin 2 in the presence and absence of Ca^{2+} was determined.

RESULTS

Identification of Muscarinic Receptor Protein in Acinar Carcinoma Membranes by Affinity-labeling and SDS-PAGE. Muscarinic receptors in acinar carcinoma cell membranes were affinity labeled with the covalent ligand [^{3}H]PrBCM, and analyzed by SDS-PAGE (Fig. 1). The apparent molecular weight of the major radiolabeled peak was 82,400 ± 1,800 (SE; n = 5). A similar major protein peak at M_r 80,100 was detected using a plasma membrane-enriched fraction prepared from carcinoma cells for [^{3}H]PrBCM labeling and SDS-PAGE. In some, but not all gels, two minor proteins of lower molecular weight and a protein of higher molecular weight were also observed. Absence of the minor peaks in the presence of 0.1 mM atropine indicates that they represent some portion of the antagonist binding site. Results with normal acinar cell membranes obtained by SDS-PAGE analysis of [^{3}H]PrBCM-labeled receptors were similar, with a major peak at M_r 80,100 ± 4,500 (n = 3), and minor peaks at M_r 120,000, 45,000, and 30,000. There is some evidence that the native protein species labeled with [^{3}H]PrBCM in pancreatic acini is the large protein at M_r 120,000 (9). It has been suggested that this large protein is labile, and is thus partially degraded during cellular subfractionation (9), perhaps by endogenous proteases of the cells. However, our values for the molecular weight of the major muscarinic receptor protein in acinar carcinoma and normal acinar cell membranes are comparable to values reported by other laboratories for receptor protein from brain, cardiac and ileum smooth muscle, parotid glands, and *Drosophila* heads (9–11). These results indicate that muscarinic receptor protein is conserved in membranes of acinar carcinoma cells.

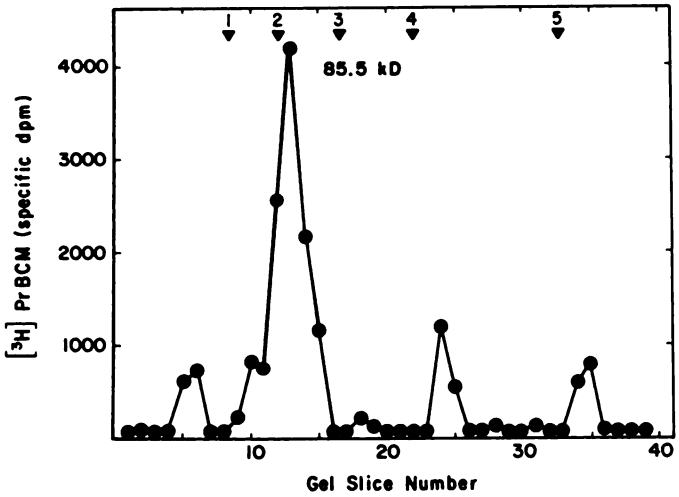

Fig. 1. SDS-PAGE profile of [^{3}H]PrBCM-labeled acinar carcinoma cell membranes. Acinar carcinoma cell membranes were incubated for 30 min at 30°C with 5 nM [^{3}H]PrBCM in the presence of 0.1 mM atropine to measure nonspecific [^{3}H]PrBCM labeling and in the absence of atropine to measure total [^{3}H]PrBCM labeling. Membranes were washed free of buffer containing [^{3}H]PrBCM, and [^{3}H]PrBCM-muscarinic receptor complexes were solubilized with hot 4% sodium dodecyl sulfate and then electrophoresed in a 7.5% polyacrylamide gel. The gel was cut into 3.0-mm slices and radioactivity in each slice was determined by liquid scintillation counting. Specific labeling of muscarinic receptors by [^{3}H]-PrBCM was calculated as the difference between total and nonspecific labeling and reported on the *ordinate* as specific dpm. Protein standards run with each gel were: β-galactosidase, M_r 116,250 (1); phosphorylase *b*, M_r 92,500 (2); bovine serum albumin, M_r 66,200 (3); ovalbumin, M_r 45,000 (4); and soybean trypsin inhibitor, M_r 21,500 (5).

Comparison of Antagonist and Agonist Binding to Muscarinic Receptors of Acinar Carcinoma and Normal Pancreatic Acinar Cells. Competition binding assay was utilized to compare antagonist and agonist drug interactions with muscarinic receptors in normal and neoplastic acinar cells. The radiolabeled antagonist [^{3}H]NMS served as a probe to determine the percentage of muscarinic receptors occupied by the unlabeled antagonist NMS, or the unlabeled agonists carbamylcholine and oxotremorine. Final K_d values obtained by Scatchard analysis of unlabeled antagonist and agonist drug binding were corrected for [^{3}H]NMS binding affinity and concentration, according to the method of Minneman *et al.* (30).

Receptor occupancy curves generated for NMS binding to normal and carcinoma cells were steep (Fig. 2*A*), and Hill plot analyses were linear (data not shown) with values for n_H around unity (Table 1), indicating a single class of NMS receptors in normal acinar and acinar carcinoma cells. Scatchard plots of the NMS receptor occupancy curves were also linear (Fig. 3*A*), and values of 0.29 ± 0.07 and 0.58 ± 0.01 nM (n = 3) (Table 1) were calculated for the K_d of NMS binding to normal and carcinoma cells. The K_d values of NMS binding to normal and carcinoma cells given in Table 1 are similar to our previously reported values derived from direct [^{3}H]NMS binding assay (20), and our K_d for binding to normal cells is close to values of 0.50 ± 0.30 and 0.90 ± 0.20 nM measured by Dehaye *et al.* (15) with competition and direct binding assay, respectively. The good agreement between results from direct and competition binding assay indicates similar binding properties for labeled and unlabeled muscarinic drug in these assays. In the

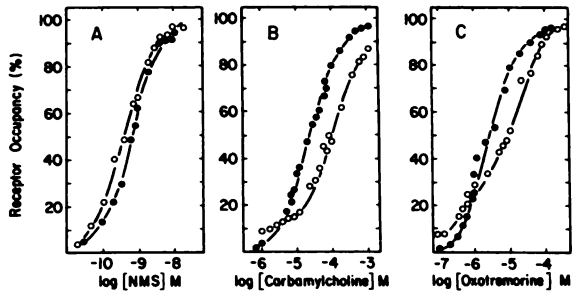

Fig. 2. Concentration dependence of NMS (*A*), carbamylcholine (*B*), and oxotremorine (*C*) binding to normal pancreatic acinar cells (○) and acinar carcinoma cells (●). Normal acinar cells or carcinoma cells were incubated at 37°C for 60 min in KRH buffer containing 0.1 mM [^{3}H]NMS without and with 0.1 mM atropine, and with concentrations indicated on the abscissa of unlabeled NMS, carbamylcholine, or oxotremorine. Total [^{3}H]NMS binding was corrected for nonspecific binding by subtracting the amount of [^{3}H]NMS bound in the presence of 0.1 mM atropine. Specific [^{3}H]NMS binding to normal and carcinoma cells was ≥90% of total [^{3}H]NMS binding. Muscarinic receptor occupancy by NMS, carbamylcholine, or oxotremorine is reported on the ordinate as the percentage of specific [^{3}H]NMS binding blocked by the presence of unlabeled antagonist or agonist drug. *Points*, mean of three replicate values obtained in a single experiment. This experiment is representative of three separate experiments.

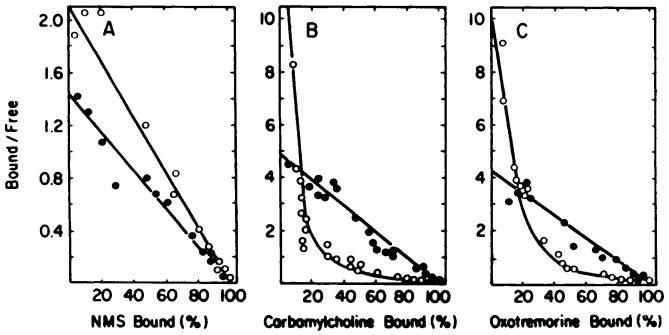

Fig. 3. Scatchard plot analysis for the binding of NMS (*A*), carbamylcholine (*B*), and oxotremorine (*C*) to normal pancreatic acinar cells (○) and acinar carcinoma cells (●). Ligand binding to cells is presented according to a modification of the method of Scatchard (30). *Abscissa*, percentage of total muscarinic receptors bound by NMS, carbamylcholine, or oxotremorine. Correct values for the ratio bound/free (*ordinate*) can be obtained by multiplication of reported values by 10^{-11}, 10^{-6}, and 10^{-7} for *A*, *B*, and *C*, respectively. The analysis reported here utilizes data shown in Fig. 2, and is representative of analysis performed with data from three separate experiments. Linear plots were obtained by linear regression analysis. Linear components of the biphasic curves were obtained by computer fitting to a model of high- and low-affinity binding sites using an iterative method based on least-squares analysis.

Table 1 *Binding of antagonist and agonist drugs to muscarinic receptors in normal acinar cells and acinar carcinoma cells of rat pancreas*[a]

Muscarinic drug	Normal cells		Carcinoma cells	
	n_H[b]	K_d (M)[c]	n_H	K_d (M)
NMS	0.92 ± 0.01	2.9 ± 0.7 ($\times 10^{-10}$)[d]	0.90 ± 0.09	5.8 ± 0.1 ($\times 10^{-10}$)
Carbamylcholine	0.63 ± 0.03	2.6 ± 0.4 ($\times 10^{-6}$) 1.1 ± 0.1 ($\times 10^{-4}$)	0.86 ± 0.02	3.1 ± 0.8 ($\times 10^{-5}$)
Oxotremorine	0.71 ± 0.01	1.6 ± 1.1 ($\times 10^{-7}$) 1.8 ± 0.3 ($\times 10^{-5}$)	0.90 ± 0.01	3.7 ± 1.1 ($\times 10^{-6}$)

[a] Reported values represent the mean ± SE obtained from three separate experiments.
[b] Hill coefficient obtained from Hill plot analysis, in which abscissa = log [muscarinic drug] M; and ordinate = $y/1 - y$, where y = fractional receptor occupancy.
[c] Dissociation constant obtained from Scatchard plot analysis and then corrected according to Minneman *et al.* (30).
[d] Comparison by Student's *t* test indicates significance difference between carcinoma and normal cells ($P < 0.01$).

present group of experiments, there was little variability in K_d values obtained from competition binding measurements, and the K_d of NMS binding to carcinoma cells detected in separate experiments (0.573, 0.583, and 0.577 nM) was consistently different from the K_d of NMS binding to normal cells (0.311, 0.160, and 0.402 nM). Thus, although the difference is small, the affinity of carcinoma cell muscarinic receptors for NMS appears to be less than the affinity of normal cell receptors (Table 1).

Receptor occupancy curves for the binding of the agonist drugs carbamylcholine (Fig. 2*B*) and oxotremorine (Fig. 2*C*) to normal acinar cells were shallow as compared to the occupancy curve obtained with the antagonist NMS, showing binding over a larger ligand concentration range. Hill plot analysis using the data reported in Fig. 2, *B* and *C*, yielded n_H values of 0.66 and 0.69, respectively. Scatchard transforms were curvilinear (Fig. 3, *B* and *C*), and analysis by computer fitting to a 2-site binding model confirmed the presence of high- and low-affinity muscarinic binding, with $K_d = 2.6 \pm 0.4$ μM and 160 ± 110 nM ($n = 3$) for high-affinity carbamylcholine or oxotremorine binding, and $K_d = 110 \pm 10$ and 18 ± 3 μM for low-affinity carbamylcholine or oxotremorine binding (Table 1). The more avid binding of oxotremorine to normal pancreatic acinar cells noted here has also been observed with neural muscarinic receptors (31). Values observed by our laboratory for the K_d of high- and low-affinity oxotremorine binding to normal acinar cells are similar to values given by Dehaye *et al.* (15) (35 ± 19 nM and 65 ± 25 μM, respectively), and our K_d value for low-affinity carbamylcholine binding to normal cells is also close to that of Dehaye *et al.* (15) (65 ± 25 μM), as well as to the K_d reported by Larose *et al.* (14) (34 μM). However, the K_d for high-affinity carbamylcholine binding to normal cells calculated from our competition binding data is two orders of magnitude larger than that reported by Dehaye *et al.* (15) (35 ± 19 nM). Larose *et al.* (14) obtained a K_d value of 0.24 μM for high-affinity carbamylcholine binding to rat pancreatic acinar cells. The reason for these discrepancies is unclear, but K_d values obtained by all three laboratories (Table 1) (14, 15) are in the nanomolar to low micromolar range characteristic of high-affinity muscarinic agonist binding. Also, composite values for the Hill coefficient (n_H) obtained from separate experiments for carbamylcholine and oxotremorine binding to normal acinar cells are less than unity (Table 1), and in agreement with values reported by others, which are $n_H = 0.66 \pm 0.03$ and 0.84 ± 0.05 for carbamylcholine and oxotremorine, respectively (15). Finally, apparent high-affinity receptors constituted 18 ± 3% of total muscarinic receptors for carbamylcholine, and 26 ± 10% of total muscarinic receptors for oxotremorine in normal pancreatic acinar cells. Our values are close to values of 16, 14, and 22% of total muscarinic receptors available for high-affinity agonist binding, reported by other laboratories (14, 15). In summary, high- and low-affinity agonist drug interactions with muscarinic receptors are well-documented characteristics of normal pancreatic acinar cells.

Distinctly different receptor occupancy curves were obtained for agonist drug binding to acinar carcinoma cells (Fig. 2, *B* and *C*). Agonist binding resembled antagonist binding, in that receptor occupancy curves were steep, and Hill plot analysis of the data in Fig. 2, *B* and *C*, yielded n_H values of 0.89 and 0.90 for carbamylcholine and oxotremorine, respectively. Scatchard plots for carbamylcholine (Fig. 3*B*) and oxotremorine binding (Fig. 3*C*) were linear. Composite results obtained from separate experiments confirmed values for n_H close to unity, and homogeneous agonist binding by Scatchard analysis with $K_d = 31 \pm 8$ and 3.7 ± 1.1 μM ($n = 3$) for carbamylcholine and oxotremorine, respectively (Table 1). These results indicate that muscarinic receptors of acinar carcinoma cells exist in a single affinity state for binding of agonist drugs.

The specificity of our [^{3}H]NMS competition binding assay for muscarinic receptor activity was checked by testing the

Table 2 *N-Methylscopolamine inhibition of carbamylcholine- and oxotremorine-stimulated $^{45}Ca^{2+}$ outflux from acinar carcinoma cells*[a]

Acinar carcinoma cells were labeled with $^{45}Ca^{2+}$ by incubation for 60 min at 37°C in KRH buffer containing $^{45}Ca^{2+}$ and the indicated concentrations of NMS. The cells were then washed, and $^{45}Ca^{2+}$ outflux was measured as the decrease in cellular $^{45}Ca^{2+}$ content after a 6-min incubation in buffer containing NMS plus 10 μM carbamylcholine or oxotremorine. Results of NMS inhibition are expressed as the percentage of maximal $^{45}Ca^{2+}$ outflux stimulated by each agonist drug in absence of NMS.

NMS (nM)	Carbamylcholine (%)	Oxotremorine (%)
0.05	97.7 ± 5.2	94.3 ± 3.8
0.10	64.5 ± 17.1	60.8 ± 2.2
0.50	34.3 ± 2.1	31.1 ± 6.9
1.00	9.3 ± 6.4	27.0 ± 14.4
5.00	7.5 ± 7.5	16.6 ± 4.8

[a] Reported values represent the mean ± SE obtained from three separate experiments.

nicotinic antagonist *d*-tubocurarine and the cholecystokinin octapeptide agonist CCK-8 for binding activity. Neither *d*-tubocurarine (0.1 nM–0.1 μM) nor CCK-8 (1.0 pM–0.1 μM) demonstrated detectable blocking activity toward [^{3}H]NMS with acinar carcinoma cells in the competition binding assay.

Comparison of Muscarinic Agonist-stimulated $^{45}Ca^{2+}$ Outflux from Acinar Carcinoma and Normal Acinar Cells. Muscarinic receptor function in acinar carcinoma cells was evaluated by measuring rates of agonist-stimulated cellular $^{45}Ca^{2+}$ outflux. Binding of muscarinic agonist drugs to plasmalemma receptors in pancreatic acinar cells induces release of Ca^{2+} from intracellular membrane-bound pools, resulting in increased cytosolic free Ca^{2+} (2, 3). The increased intracellular free Ca^{2+} stimulates plasma membrane Ca^{2+}-ATPase, which extrudes Ca^{2+} from the cell (32, 33). Receptor-mediated Ca^{2+} mobilization in acinar cells can thus be followed by measuring rates of $^{45}Ca^{2+}$ outflux. Exposure of $^{45}Ca^{2+}$-labeled carcinoma cells to 10 μM carbamylcholine or oxotremorine stimulated a brisk cellular outflux of $^{45}Ca^{2+}$ (data not shown). During a 6-min incubation of carcinoma cells with agonist, 10 μM carbamylcholine or oxotremorine stimulated outflux of 40.3 ± 3.4% (n = 4) and 39.3 ± 4.7% (n = 4), respectively, of total cellular $^{45}Ca^{2+}$ content, which represented an increase in $^{45}Ca^{2+}$ outflux of 299 and 265% above basal (nonstimulated) outflux. NMS antagonized agonist-stimulated $^{45}Ca^{2+}$ outflux (Table 2), and values of 0.28 and 0.18 nM were calculated for the half-maximal inhibitory concentration of NMS antagonism of carbamylcholine- or oxotremorine-stimulated Ca^{2+} outflux. These half-maximal inhibitory concentration values are comparable to K_d values obtained for equilibrium binding of [^{3}H]NMS to acinar carcinoma cells (Table 1). Identical NMS antagonism has been previously described for agonist-induced $^{45}Ca^{2+}$ outflux in normal pancreatic acinar cells (15). These results show that carcinoma and normal cell receptors detected by [^{3}H]NMS competition binding assay are biologically functional muscarinic cholinergic receptors.

Normal and carcinoma cells displayed similar time courses of $^{45}Ca^{2+}$ outflux in response to a maximal stimulatory concentration of carbamylcholine (Fig. 4, *inset*). The response in both cell types was immediate, and plateaued 6 min after agonist exposure. $^{45}Ca^{2+}$ outflux from both carcinoma and normal cells was stimulated in concentration-dependent fashion by carbamylcholine (Fig. 4). However, significant differences in $^{45}Ca^{2+}$ outflux between carcinoma and normal cells were observed at 10^{-7} M ($P < 0.025$), 10^{-6} M ($P < 0.001$), and 10^{-5} M ($P < 0.0025$) carbamylcholine. The calculated EC_{50} for carbamylcholine-stimulated $^{45}Ca^{2+}$ outflux from carcinoma cells, 0.40 μM, was one order of magnitude below the EC_{50} value of 4.0 μM for normal cells. Thus, carcinoma cells are more sensitive than normal cells to muscarinic stimulation by submaximal agonist drug concentrations.

Comparison of Muscarinic Agonist-stimulated Intracellular Ca^{2+} Release from Acinar Carcinoma and Normal Acinar Cells. To directly measure intracellular Ca^{2+} release, carcinoma and normal cells were loaded with the fluorescent dye quin 2, and the effect of carbamylcholine on cytosolic free Ca^{2+} concentrations was followed by spectrofluorimetry (Fig. 5). Under basal conditions, cytosolic free Ca^{2+} concentration of carcinoma and normal cells was 179 ± 6 nM (n = 25) and 207 ± 8 nM (n = 24), respectively. These values are comparable to those reported by other laboratories for normal pancreatic acinar cells (3), lymphocytes (29), hepatocytes (34), and platelets (35). Immediately following carbamylcholine addition, marked increase in cytosolic free Ca^{2+} concentration occurred, followed by steady

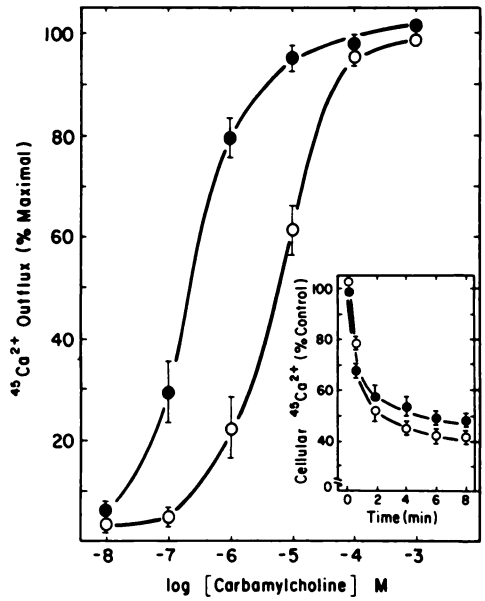

Fig. 4. Concentration dependence of carbamylcholine-stimulated $^{45}Ca^{2+}$ outflux from normal pancreatic acinar cells (○) and acinar carcinoma cells (●). Normal cells and acinar carcinoma cells were preincubated at 37°C for 60 min in KRH buffer containing $^{45}Ca^{2+}$. The cells were washed free of buffer $^{45}Ca^{2+}$ by rapid centrifugation, and incubated for 6 min at 37°C in KRH buffer without and with carbamylcholine concentrations indicated on the abscissa. The amount of $^{45}Ca^{2+}$ released by cells in response to each agonist concentration was calculated as the difference in cellular $^{45}Ca^{2+}$ concentration between unstimulated control cells at t = 0 min and stimulated cells at t = 6 min. Results are expressed on the ordinate as a percentage of the maximal amount of $^{45}Ca^{2+}$ outflux obtained from stimulation with 1 mM carbamylcholine. *Points*, mean value obtained from three separate experiments; *bars*, SE. *Inset*: time course of carbamylcholine-stimulated $^{45}Ca^{2+}$ outflux from normal pancreatic acinar cells (○) and acinar carcinoma cells (●). Normal cells and acinar carcinoma cells were preincubated at 37°C for 60 min in KRH buffer containing $^{45}Ca^{2+}$. The cells were washed free of buffer $^{45}Ca^{2+}$, and suspended in KRH buffer at 37°C without and with 1 mM carbamylcholine. Stimulated outflux of $^{45}Ca^{2+}$ was followed by measuring decrease in cellular $^{45}Ca^{2+}$ concentration at times indicated on the abscissa. $^{45}Ca^{2+}$ concentration of agonist-stimulated cells is expressed on the ordinate as the percentage of $^{45}Ca^{2+}$ concentration of unstimulated cells at t = 0. *Points*, mean value obtained from three separate experiments; *bars*, SE.

decrease during the subsequent 2 min, reflecting the Ca^{2+} outflux response (Fig. 4).

The dose dependence of increases in cytosolic free Ca^{2+} (Fig. 6) closely parallels the dose dependence for $^{45}Ca^{2+}$ outflux (Fig. 4). Increase in cytosolic Ca^{2+} to concentrations of 1932 and 2151 nM seen in the tracings of Fig. 5 for carcinoma and normal cells treated with 10^{-3} M carbamylcholine corresponds to 967 and 828% increases above basal Ca^{2+} concentrations. Composite results revealed 902 ± 33% (n = 3) and 848 ± 18% (n = 3) increases for carcinoma and normal cells maximally stimulated with 10^{-3} M carbamylcholine. However, greater sensitivity of carcinoma cells to submaximal agonist concentrations is clearly seen in the tracings of Fig. 5, where 10^{-6} M carbamylcholine increased cytosolic Ca^{2+} concentration to 1201 nM in carcinoma cells, in contrast to 838 nM in normal cells. The tracings in Fig. 5 show a 595 and 392% increase stimulated by 10^{-6} M carbamylcholine over basal cytosolic Ca^{2+} concentrations in carcinoma and normal cells, respectively, and these tracings are representative of the composite values 583 ± 14% (n = 4) and 298 ± 51% (n = 3). The dose-effect curve for agonist stimulation (Fig. 6) indicates significant differences between carcinoma and normal cells at 10^{-7} M ($P < 0.005$), 5×10^{-6} M ($P < 0.005$), 10^{-6} M ($P < 0.01$), and 10^{-5} M ($P < 0.0025$) carbamylcholine. Values of EC_{50} for Ca^{2+} release measured by quin 2 fluorescence in carcinoma and normal cells were 0.8 and 5.0 μM carbamylcholine, respectively. In conclusion then, results obtained using quin 2 fluorescence confirm increased sensitivity of carcinoma

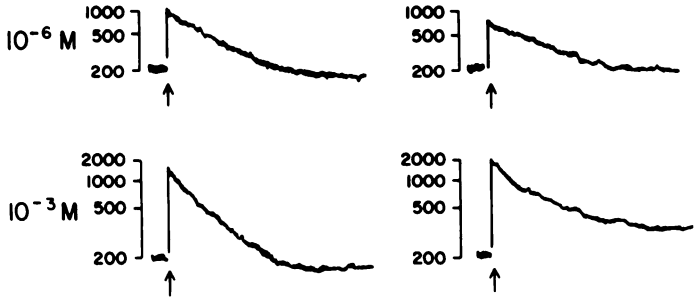

Fig. 5. Carbamylcholine-stimulated increase in cytosolic Ca^{2+} concentration in acinar carcinoma cells (*left tracing*) and normal acinar cells (*right tracing*). Normal acinar cells and acinar carcinoma cells were preincubated for 20 min at 37°C in KRH buffer containing quin 2-tetra(acetoxymethyl)ester, washed free of buffer quin 2-tetra(acetoxymethyl)ester, and then maintained in KRH buffer at room temperature until used. Fluorescence measurements were taken of cells freshly resuspended in KRH buffer, continuously stirred, and maintained at 37°C in the presence of 10^{-6} or 10^{-3} M carbamylcholine added at points indicated by the *arrows*. Fluorescence changes were measured continuously for 2 min. Cytosolic nM concentrations of free Ca^{2+} are indicated at the left of each tracing, and were calculated according to the equation

$$[Ca^{2+}]_C = \frac{K_d(F - F_{min})}{F_{max} - F}$$

in which K_d is the calculated dissociation constant of quin 2 complexed with Ca^{2+} in the presence of 1 mM Mg^{2+} (K_d = 189.5 nM), F is the fluorescence signal of intact quin 2-loaded cells, F_{max} is the fluorescence of quin 2 in the presence of a saturating concentration of Ca^{2+} (1.25 mM), and F_{min} is the fluorescence of quin 2 in the presence of 2.0 mM EDTA. F_{max} and F_{min} were measured following solubilization of cells with 50 μM digitonin which released all intracellular quin 2 into the buffer. Each tracing shown is representative of at least three tracings obtained with different cell preparations.

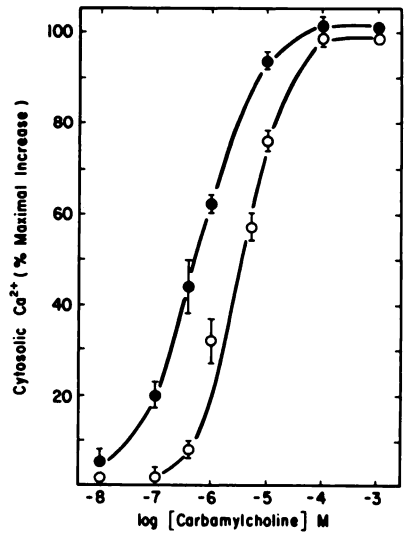

Fig. 6. Concentration dependence of carbamylcholine-stimulated increase in cytosolic Ca^{2+} concentration in normal pancreatic acinar cells (○) and acinar carcinoma cells (●). Normal acinar cells and acinar carcinoma cells were preincubated for 20 min at 37°C in KRH buffer containing quin 2-tetra(acetoxymethyl)ester, washed free of buffer quin 2-tetra(acetoxymethyl)ester, and maintained in KRH at room temperature. Fluorescence measurements were taken of cells in the absence and presence of carbamylcholine concentrations indicated on the abscissa and concentrations of free cytosolic Ca^{2+} calculated as described in the legend of Fig. 5. Responsiveness to carbamylcholine was evaluated as percentage of net increase in cytosolic Ca^{2+} concentration calculated according to the equation:

$$\%\ \text{net increase of } [Ca^{2+}]_C = \frac{[Ca^{2+}]_{SC} - [Ca^{2+}]_{BC}}{[Ca^{2+}]_{BC}} \times 100\%$$

in which $[Ca^{2+}]_{SC}$ is the cytosolic Ca^{2+} concentration corresponding to peak fluorescence after carbamylcholine stimulation, and $[Ca^{2+}]_{BC}$ is the basal cytosolic Ca^{2+} concentration corresponding to fluorescence of cells prior to stimulation. Results are expressed on the *ordinate* as a percentage of the maximal net increase in cytosolic Ca^{2+} concentration obtained after stimulation with 1 mM carbamylcholine. *Points*, mean values obtained from at least three separate experiments; *bars*, SE.

cells to stimulation of Ca^{2+} release from intracellular stores by submaximal carbamylcholine concentrations.

Carbamylcholine Binding and Dose-response Curves for Stimulation of Ca^{2+} Release and Protein Secretion in Acinar Carcinoma and Normal Acinar Cells. Maximum stimulation of Ca^{2+} release in normal acinar cells was obtained at a carbamylcholine concentration of 10^{-4} M, as measured both by $^{45}Ca^{2+}$ outflux (Fig. 4) and quin 2 fluorescence (Fig. 6). Only 50% of muscarinic receptors in normal cells are occupied at 10^{-4} M carbamylcholine (Fig. 2*B*), indicating the presence of spare receptors for stimulation of Ca^{2+} release. Using irreversible PrBCM blocking of muscarinic receptors, it has been estimated that between 47 and 59% of muscarinic receptors in normal acinar cells are spare receptors for carbamylcholine stimulation (15). Thus, results of carbamylcholine receptor binding, concentration-dependent agonist-stimulated Ca^{2+} release, and irreversible receptor-blocking experiments consistently indicate a large excess of muscarinic receptors in normal acinar cells. In comparable analysis of acinar carcinoma cells, maximum Ca^{2+} release was stimulated by 10^{-5} M carbamylcholine (Figs. 4 and 6), where only approximately 40% of tumor cell muscarinic receptors were occupied (Fig. 2*B*). Thus, carcinoma cells resemble normal cells by presence of approximately one-half of total muscarinic receptors as spare receptors.

At threshold concentrations of carbamylcholine for stimulation of Ca^{2+} release in normal cells (~10^{-6} M) (Figs. 4 and 6), only very low levels of agonist binding were detectable by the [^{3}H]NMS competition assay. Close examination of the carbamylcholine inhibition curve of [^{3}H]NMS binding obtained by Dehaye *et al.* indicates an identical result (see Fig. 4 of Ref. 15). Therefore, inability to accurately measure agonist binding at threshold stimulatory concentrations is a limitation of the [^{3}H]NMS competition assay. This likely results from two factors, first the use of high-affinity antagonist as the radioactive trace label in concert with relatively lower-affinity nonradioactive agonists, and second, the abundance of spare muscarinic receptors in the plasmalemma of pancreatic acinar cells.

Finally, dose-response curves for stimulation of intracellular Ca^{2+} release and protein secretion (18) fall in the same carbamylcholine concentration range for both carcinoma and normal cells (18). Utilizing data previously reported by our laboratory (18), an EC_{50} value of 0.16 μM was calculated for carbamylcholine-stimulated protein secretion by carcinoma cells, and 0.74 μM for normal cells. The EC_{50} values for protein secretion are lower than EC_{50} values for stimulation of Ca^{2+} release from carcinoma (~0.4 μM) and normal cells (~4.0 μM). This indicates that carbamylcholine stimulates more Ca^{2+} release than required for maximal stimulation of enzyme secretion. An identical result has been obtained by other investigators (8) with normal acinar cells. Finally, the greater potency of carbamylcholine for stimulation of protein secretion by carcinoma cells is another indication of the greater sensitivity of carcinoma cells to muscarinic stimulation.

DISCUSSION

Muscarinic receptor protein is highly conserved in different cholinergic-responsive cell types of various species. Common patterns of immunoprecipitation of [^{3}H]PrBCM affinity-labeled protein with monoclonal antibody for muscarinic receptors (11), tryptic fragments of similar molecular mass (10), and monomeric protein with molecular weight of 70,000 to 87,000 (9–13, 36, 37), obtained with muscarinic receptors from sub-

stantially different tissues and species, all indicate structural homology in muscarinic receptor protein from diverse biological sources. Although one laboratory has described a larger receptor protein (M_r ~ 105,000–118,000) in [^{3}H]PrBCM-labeled pancreatic acini (9), the major muscarinic protein both in intact acinar cells (M_r ~ 81,000) (9) and in isolated membrane preparations of pancreatic acinar cells (M_r ~ 80,000) demonstrates a molecular size similar to that observed for receptor protein in other cell types (9–11, 13). According to the criterion of molecular size, muscarinic protein with molecular weight of ~82,000 is thus fully expressed in the plasmalemma of pancreatic acinar carcinoma cells. The number of muscarinic receptors present in membranes of normal and carcinoma cells is in excess of that required for stimulation of intracellular Ca^{2+} release (spare receptors constitute approximately 50% of total receptors), and direct assay of [^{3}H]NMS binding has revealed an identical plasmalemma density (~25 receptors/μm^2) of muscarinic receptors in acinar carcinoma and normal acinar cells (20). Also, an equivalent number of muscarinic receptors is present in poorly differentiated carcinoma cells deficient in zymogen granules and protein secretion, as compared to well-differentiated cells which contain granules and secrete protein (20). Clearly, the expression of muscarinic receptor protein is a stable phenotypic property of acinar carcinoma cells, and appears to occur independently of neoplastic zymogen cytodifferentiation. Data from SDS-PAGE of affinity-labeled receptors and direct [^{3}H]NMS binding assay (20) indicate structural homology in muscarinic receptor protein of carcinoma and normal cells. However, the small but reproducible difference detected for the K_d of NMS binding to carcinoma cells utilizing competition binding assay suggests variation in amino acid structures and/or posttranslational modification of receptor protein(s), or alteration in membrane glycolipid composition in the vicinity of muscarinic receptors.

Antagonist and agonist binding properties of muscarinic cholinergic receptors have been extensively studied in normal mammalian cells and membrane preparations (31, 38). Antagonists characteristically demonstrate homogeneous binding to muscarinic receptors. The antagonist drug NMS demonstrated homogeneous binding to both normal and carcinoma cells of rat pancreas, with steep receptor occupancy curves which occurred over a 100-fold range of NMS concentration, Hill coefficients of ~1.0, and linear Scatchard plots. Agonist drugs demonstrate heterogeneous binding to muscarinic receptors in intact normal cells and membrane fragments. Heterogeneous binding of muscarinic agonists to normal acinar cells of rat pancreas was observed in the present study, with shallow receptor occupancy curves which developed over a 1000-fold range of agonist concentration, Hill coefficients of ~0.6 − 0.8, and the appearance of two separate linear segments in Scatchard plots of binding data. Binding of antagonist and agonist drugs is competitive and mutually exclusive, both to muscarinic receptors in intact cell membranes, and to isolated receptor protein purified from membranes. Thus, a single receptor protein species appears to bind both muscarinic antagonists and agonists. To account for the paradoxical situation of a single polypeptide species which exhibits heterogeneous agonist binding but homogeneous antagonist binding, two major factors must be considered. The first factor involves inherent properties of the muscarinic drugs, and the second factor involves intrinsic properties or interactions of plasmalemma muscarinic receptors. Heterogeneous binding of agonist drugs could be due to monomer-oligomer equilibrium with different binding properties for monomer and oligomer, or multiple conformations of agonist drug. However, binding of agonist drugs to carcinoma cells, when measured under conditions identical to those utilized for normal acinar cells, was homogeneous with steep receptor occupancy curves, values for n_H around unity, and linear Scatchard plots. It is unlikely, therefore, that inherent properties of agonist drugs are responsible for heterogeneous binding to normal cells. Heterogeneous agonist binding must thus reflect intrinsic properties of muscarinic receptors, and could result from negative cooperativity interactions between muscarinic receptors, or the presence of two noninteracting receptor populations with different affinities for binding of agonist drugs. Negative cooperativity has been tested by blocking an increasing fraction of muscarinic receptors with PrBCM, and then measuring carbamylcholine binding by competition displacement assay with [^{3}H]NMS (15). Even when 90% of muscarinic receptors in normal pancreatic acinar cells were occluded with PrBCM, carbamylcholine binding to the normal acinar cells remained heterogeneous, with unchanged values for n_H. Negative cooperativity can thus be excluded as a mechanism for heterogeneous agonist binding to normal cells. Two-site model analysis of muscarinic agonist dose-dependent binding curves, in this and previous studies (15), are fully compatible with the presence of two receptor populations in normal pancreatic acinar cells, a minor receptor population with high-affinity agonist binding sites, and a major receptor population with low-affinity binding sites. Consequently, a two-site mechanism of receptor binding best explains heterogeneous interactions of agonist drugs with normal acinar cells.

To explain why antagonist drugs interact in homogeneous fashion with muscarinic receptors, while agonist drugs bind to two receptor classes, Birdsall *et al.* (17) have proposed a model of multiple state receptor-effector coupling. This model is comprised of a single receptor protein, R, and an effector system, E, which are functionally uncoupled in an inactive ground state, R·E. A conformational change in receptor protein, R → R*, is directly linked to change in the effector system, E → E*, with generation of the activated receptor-effector system R*E*. R·E and R*E* exist in equilibrium, with R·E as the dominant form when the cell is in the basal (nonstimulated) state. Antagonists (Ant) bind selectively to R, forming an inactive complex Ant-R·E, with no stimulation of the effector system. Agonists (Agt) bind to R* to form an activated complex Agt-R*E*, with increased conversion of R to R*, and stimulation of the effector system. Different states of conformational coupling between R* and E* exist for muscarinic receptors, defined here as R_H*E* and R_L*E*, which can be identified by high- and low-affinity agonist binding.

Approximately 15–25% of receptors in normal acinar cells exist in the high agonist affinity state, R_H*E*, and 75–85% in the low agonist-affinity state, R_L*E*. In contrast to normal cells, carcinoma cell muscarinic receptors do not conform to the Birdsall model of homogeneous antagonist binding and heterogeneous agonist binding. Carcinoma cells bind both antagonists and agonists in uniform, homogeneous fashion. It is clear that muscarinic receptors in acinar carcinoma cells are functionally coupled to the Ca^{2+}-mobilizing effector system and protein secretion (18, 19). However, carcinoma cell receptors possess only a single affinity state for agonist binding, whose K_d is different from the K_d of affinity states for agonist binding in normal cells (Table 1). Our results also demonstrate increased sensitivity of acinar carcinoma cells toward stimulation of intracellular Ca^{2+} release by submaximal agonist concentrations (Figs. 4–6). Increased sensitivity to Ca^{2+}-mobilizing muscarinic agonists indicates alteration in receptor coupling, which

favors formation of an activated effector system upon agonist binding. Our results suggest, therefore, that the activated receptor-effector system in acinar carcinoma cells exists in a single state, designated R_{AC}*E*, whose linkage is altered in such a way as to render tumor cells more sensitive to muscarinic agonist-stimulated Ca^{2+} release. The affinity of R_{AC}* for agonist binding differs from that of the normal receptor states, R_H* and R_L*.

Two possible mechanisms could account for existence of a unique R_{AC}*E* complex in acinar carcinoma cells. The first mechanism is alteration in receptor structure resulting from neoplastic transformation of acinar cells. According to the Birdsall model, any change in the energetics of receptor conformational change, R → R*, will also change the energetics of effector activation, E → E*, as a consequence of conformational coupling between receptor and effector. When compared to normal cells, carcinoma cells demonstrated a small but significant change in affinity for antagonist binding. High-affinity antagonist binding mostly reflects the close structural complementarity between antagonist drug and receptor protein. Thus, the difference in NMS binding suggests alteration in muscarinic receptor structures.

The second mechanism is alteration in the energetics of signal flow through postreceptor proteins of the effector system, so that equilibrium favors formation of the activated state E*. There is strong evidence that the polyphosphatidylinositol pathway is coupled to muscarinic receptors in pancreatic acinar cells for release of intracellular Ca^{2+}. Binding of carbamylcholine to muscarinic receptors in normal pancreatic acinar cells stimulates rapid hydrolysis of polyphosphatidylinositols, primarily phosphatidylinositol 4,5-bisphosphate, to form diacylglycerol and inositol trisphosphate (39, 40). Diacylglycerol serves to activate Ca^{2+}- and phospholipid-dependent protein kinase C (41), whereas inositol trisphosphate is released into the cytosol, where it appears to trigger release of Ca^{2+} sequestered in the endoplasmic reticulum (42, 43). Alteration in the effector system of acinar carcinoma cells could reside in any or all component proteins of the polyphosphatidylinositol signal transduction system.

At this time the specific molecular mechanisms responsible for altered muscarinic receptor coupling in acinar carcinoma cells remain to be identified. A striking concordance exists between the stimulation of membrane metabolism of phosphatidylinositol by muscarinic agonists, and regulation of mammalian cell growth by phosphatidylinositol hydrolysis (44). The muscarinic receptor signal transduction system of acinar carcinoma cells thus provides an excellent model to define molecular components of muscarinic receptor-effector activation, and their relationships to the growth of neoplastic pancreatic acinar cells.

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