Characterization of Muscarinic Cholinergic Receptors on Rat Pancreatic Acini by N-[^3]H]Methylscopolamine Binding

THEIR RELATIONSHIP WITH CALCIUM 45 EFFLUX AND AMYLASE SECRETION*

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N-[^3]H]Methylscopolamine (NMS) binding, amylase secretion, and 45Ca efflux from dispersed rat pancreatic acini were investigated in parallel, in the presence or absence of 4 muscarinic agonists and 3 muscarinic antagonists. Scatchard analysis of [^3]H]NMS saturation isotherms gave a $K_D$ of 0.9 nm and an average binding capacity of 24,000 sites per cell. Binding competition curves with the antagonists atropine, dextemizide, and NMS gave $K_D$ values of 3.5, 3.5, and 0.5 nm, respectively. With the 3 full agonists oxotremorine, muscarine, and carbamylcholine, the receptor population could be divided into two classes of binding sites: a minor one (15%) with high affinity ($K_P = 20-35$ nm) and a major one (85%) with low affinity ($K_P = 3-65$ nm). There was a receptor reserve of about 50% with respect to carbamylcholine-stimulated amylase secretion. Further analysis of dose-effect curves suggests that low affinity binding sites were involved in the secretory response to muscarinic stimulation. Pilocarpine, like muscarinic antagonists, recognized all binding sites with the same affinity but acted as a partial agonist on amylase secretion and 45Ca efflux.

In pancreatic acinar cells, secretagogues may increase enzyme secretion through two functionally distinct mechanisms. On the one hand, acetylcholine, pancreozymin-cholecystokinin, and peptides of the bombesin family mobilize cellular calcium after binding to their respective receptors, and a stimulation of enzyme secretion follows. These secretagogues also increase phosphatidylinositol turnover, but the relationship between this effect and emiocytosis is unclear. On the other hand, secretin and vasoactive intestinal peptide stimulate membrane adenylate cyclase after binding to their own specific receptors. The resulting increase in cyclic AMP activates cyclic AMP-dependent protein kinase(s), a process that also leads to secretion (for review, see Ref. 1).

While several of the aforementioned receptors have already been characterized in intact rodent pancreatic acini by direct binding studies, muscarinic cholinergic receptors were identified up to now by the ability of muscarinic antagonists to inhibit competitively the action of muscarinic agonists. Only one recent characterization of muscarinic receptors was achieved in pancreatic acini with [^3]H]quinuclidinyl benzilate binding (2), despite the inconvenience of high nonspecific labeling (1) and low dissociation rate (2) observed with this radioligand. In the present study, the binding to rat pancreatic acini of another muscarinic antagonist, N-[^3]H]methylscopolamine, with much less nonspecific binding (3), was examined in the presence of 3 muscarinic agonists, of the partial agonist pilocarpine, and of 3 muscarinic antagonists. A comparison of muscarinic agonist binding with amylase release suggests that low affinity binding sites were largely if not exclusively involved in this biological effect.

EXPERIMENTAL PROCEDURES

Materials

N-[^3]H]Scopolamine methyl chloride (specific radioactivity, 53.5 Ci/mmol) was obtained from New England Nuclear (Draceh, F.R.G.). 4Ca chloride (23 mCi/mg of Ca) and [^3]H]Sucrose (10 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, Bucks, England). Oxotremorine sesquifumarate and isotubymethyloxanthine were obtained from Astra (Beere, Belgium), atropine and muscaraine from Sigma, and pilocarpine and carbamylcholine from Peders (Brussels, Belgium). Scopolamine methyl bromide was from Merck (Darmstadt, F.R.G.). Propylbenzilycholine mustard was from the Radiochemical Centre (Amersham, Bucks, England). Dextemizide and levitiride were generous gifts from Janssen Pharmaceutica (Beere, Belgium) and Trasylol a generous gift from Bayer (Brussels, Belgium). Isoubutylphthalate (d = 1.042 g/liter) was from BDH Chemicals Ltd. (Poole, England). Purified collagenase CLSPA was from Worthington.

Methods

Incubation of Rat Pancreatic Acini—Rat pancreatic acini were prepared from male Wistar albino rats (130-150 g) fed ad libitum on standard chow according to the procedure of Peikin et al. (4). Before aliquoting, dispersed acini from one pancreas were suspended at 20°C in 20 ml of an incubation medium consisting of 24.5 mM Hepes (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM NaHPO₄, 1.0 mM MgCl₂, 0.5 mM CaCl₂, 11.5 mM glucose, 5 mM sodium fumarate, 5 mM sodium glutamate, 3 mM sodium pyruvate, 2 mM glutamine, 1% (w/v) amino acid mixture BME without 1-glutamine (Gibco Europe, Uxbridge, Middexsec, England), 0.5 mM isoubutymethyloxanthine, 1% (w/v) albumin, and 0.01% Trasylol (500 kallikrein inhibitor units/ml).

Assay of [^3]H]NMS Binding—In routine assays, 0.5 ml aliquots of

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pancreatic acini (0.4 to 0.5 mg of protein) were incubated at 37 °C in the standard incubation solution with 1 nM [³H]NMS. At the end of the incubation, 200-μl samples were taken in duplicate and layered above 100 μl of di-n-butylphthalate. The acini were sedimented through the dense layer by a 15-s centrifugation in a Beckman 152 microfuge. The microfuge tube was frozen in liquid nitrogen and cut through the dense layer. The acini in the lower part were digested at 70 °C for 3 h with 1 ml of Lumasolve (Lumac, Schaesberg, The Netherlands). After adding 100 μl of 6 M HCl to decrease chemiluminescence, 8 ml of Aquasolve 2 (New England Nuclear, Dreieich, F.R.G.) were added, and the radioactivity was counted in a 7500 Beckman liquid scintillation spectrometer. The results were corrected for the volume of incubation medium accompanying the total through the dense layer. This was estimated by incubating acini in the presence of 1 nM [³H]NMS (10 μCi/ml). The volume of the extracellular medium trapped under these conditions was 0.36 ± 0.08 μl out of a total initial volume of 200 μl and was not modified in the presence of muscarinic agonists and antagonists.

Non-specific binding was defined as tracer binding in the presence of 10 μM atropine. This antagonist concentration also prevented agonist-stimulated anyslyte output and [³Ca] efflux without affecting basal activities. Similar inhibition effects on the three parameters were in fact exerted by atropine tested in the 10⁻¹ to 10⁻⁴ M concentration range (not shown).

**Dissociation Kinetics**—Rat pancreatic acini (5-6 mg of acinar protein) were preincubated for 1 h at 37 °C in 5 ml of standard medium in the presence of 3 nM [³H]NMS and in the presence or absence of 10 μM atropine. Aliquots containing 60 μl of water (control) or atropine (allowing a final 10 μM concentration of the unlabeled antagonist) were then added. At appropriate time intervals, duplicate 200-μl aliquots were collected as described above. Results were expressed as percentage of control per cent.

**Scatchard Plots**—Rat pancreatic acini were incubated as indicated above in the presence of increasing concentrations of [³H]NMS (0.1 to 10 nM) and in the presence or absence of 10 μM atropine. Specific binding was measured at 10, 20, 50, 90, 90, and 120 min. The saturation isotherms at each time were analyzed according to Scatchard (5).

**Amylase Secretion**—At the end of the incubation period (1 h usually), an aliquot of the suspended acini was centrifuged for 15 s in a Beckman 152 microfuge. α-Amylase activity was determined in the supernatant (6). Controls taken at the beginning of each incubation period, for determining the amylase content of the medium at time zero, allowed appropriate corrections.

**Estimation of the Degree of Receptor Reserve with Respect to Carbachol stimulation**—At the end of the preincubation, the acini were centrifuged for 1 min at 200 x g and resuspended in fresh incubation medium in the absence of PrBCM. [³H]NMS specific binding and amylase release with various concentrations of carbachol were achieved after 60 min as previously described. The fraction q of receptors remaining free after preincubation was derived 1) from direct binding data and 2) from a comparison of the control secretory curve of carbachol with that obtained after preincubation with PrBCM. The fraction of receptors remaining free and the affinity of the stimulatory agent for receptors were derived from the equation of Furchgott and Bursztyn (9),

\[
\frac{1}{A} = \frac{1}{q} \times \frac{1}{A^*} \times \frac{1-q}{k_D}
\]

where A and A* are pairs of carbachol concentrations giving the same secretory response before and after PrBCM treatment, q is the fraction of free muscarinic receptors, and k_D is the dissociation constant of carbachol for receptors involved in secretion.

**Study of Negative Cooperativity Interactions**—Negative cooperativity interactions were tested as described by Birdsall et al. (10). Briefly, when the binding of an agonist to a receptor is characterized by flattened displacement curves and a n_H < 1, negative cooperativity interactions can be tested by blocking an increasing fraction of these receptors. Such interactions will then be weakened with a steepening of the displacement curve and a n_H tending to 1. In practice, acini were preincubated with various concentrations of 10⁻⁸ to 10⁻¹ M PrBCM for 15 min at 37 °C, washed, and resuspended in fresh incubation medium. [³H]NMS binding was then performed in the presence of various concentrations of carbachol.

**Calcium Efflux**—[³Ca] efflux was included (2.5 μCi/ml) in the media used for preparing and washing the acini (4), i.e. for a 75-min period. When testing the muscarinic agonists, the acini were resuspended in a final unlabeled incubation medium and incubated for 5 min with the agonists. When testing the inhibitory effects of muscarinic antagonists on carbachol-stimulated [³Ca] efflux, the preloadeed acini were preincubated for 1 h with [³Ca] efflux in the presence of the antagonists and then incubated for 5 min in an unlabeled incubation medium with the antagonists and 3.2 μM carbachol. This preincubation did not modify the total [³Ca] content of the acini. After the incubation, an aliquot of the suspension was centrifuged at a Beckman 152 centrifuge for 15 s. The radioactivity in the supernatant was counted. Controls taken at the beginning of each incubation period, for determining the [³Ca] efflux remaining linear during that period, indicating no significant reuptake of the released [³Ca].

**Protein Determination**—Protein determination was performed according to Lowry et al. (11) using bovine serum albumin as a standard.

**Analysis of the Data—Dose-effect curves of inhibition of [³H]NMS binding by muscarinic agonists were analyzed by computer fitting to a model of two classes of binding sites (12).**

The goodness of fit of the two-site model was tested by comparison with a one-site model, using the method of Munson and Rodbard (13). Expected Y values were computed manually with a TI-59 using the kinetic program developed by Thakur et al. (14) for a two-site model and a simple linear regression for the one-site model. Residual sums of unweighted squares of deviations of the experimental points to the fitted curves were used for the F test.

**RESULTS**

**General Characteristics of [³H]NMS Binding**—After a 1-h incubation period with 1 nM [³H]NMS, specific binding to dispersed pancreatic acini was proportional to the amount of acini incubated in the 0.1-2.5 μg of acinar protein/ml range (not shown) so that a 0.4-0.5 μg of protein/ml concentration was generally utilized in further experiments. Under these conditions, the binding of 1 nM [³H]NMS at 37 °C was maximal by 60 min, did not change significantly from 60 to 90 min, and corresponded to about 11% of the tracer added (Fig. 1). Adding 1 μM atropine decreased this binding to 1.2% of the total tracer added with higher concentrations of atropine causing no further decrease.

When tested after 60 min (i.e. at equilibrium) specific [³H]NMS binding reached saturation at a 2.5 nM ligand concentration while nonspecific binding remained proportional to
ligand concentration in the 0.1-10 nM [3H]NMS concentration range (Fig. 2A).

After 1 h of incubation, [3H]NMS bound with an apparent
Kd of 0.9 ± 0.2 nM (n = 5) and with longer incubation periods no further decrease of the Kd value was observed (not shown), suggesting true binding equilibrium after 1 h. There was no evidence for heterogeneity among muscarinic antagonist binding sites, and the receptor concentration was 140 ± 15 fmol/mg of protein (Fig. 2B).

To examine the reversibility of the binding reaction, acini were preincubated with 3 nM [3H]NMS for 1 h at 37 °C, then incubated in the presence of 10 μM atropine (Fig. 3). The halftime of dissociation was 21.5 ± 3 min (n = 3), corresponding to an average Kd value of 0.032 min⁻¹. Reducing the incubation temperature from 37 to 4 °C reduced the rate of dissociation 16-fold (to 6% after 2 h; not shown). Finally, acini were incubated in a large incubation medium as described under “Experimental Procedures,” in order to prevent ligand reassociation. Dilution only or dilution in the presence of 10 μM oxotremorine allowed dissociation rates similar to those observed in the previous experiments (not shown) suggesting that negative cooperativity was not manifest in the dissociation kinetics.

Specificity of Muscarinic Receptors—0.1 μM d-tubocurarine, 1 μM hexamethonium, 1 μM secretin, 1 μM vasoactive intestinal peptide, 1 μM glucagon, 0.5 μM somatostatin, 20 nM insulin, 3 μM physalaemin, 3 μM leuline-Askophilamin, 10 μM leucine-Askophenalin, 10 μM bombesin nonapeptide, 10 μM COOH-terminal octapeptide of cholecystokinin, 10 μM caserulinal, and 1 mM dibutyryl cyclic GMP did not inhibit [3H]NMS binding (not shown).

Muscarinic Antagonists—Dexetimide was at least 1000 times more potent than levitimide (the inactive stereoisomer) in displacing [3H]NMS. Competition curves with unlabeled atropine, dexetimide, and NMS, and the derived values of the Hill coefficient indicated that these antagonists bound to a single class of receptors (Fig. 4 and Table I). When correcting for tracer concentration (15), the following Kd values were obtained: 3.5 nM for atropine, 3.5 nM for dexetimide, and 0.5 nM for NMS (Table I).

These antagonists were also tested for their capacity to inhibit amylase secretion and ⁴⁰Ca efflux stimulated by 3.2 μM carbacholcholine. In order to attain equilibrium, acini were first preincubated for 1 h in the presence of the antagonists and under conditions identical with those used for the binding assays. They were then incubated in the combined presence...
Muscarinic Receptors in Rat Pancreas

TABLE I

| Parameter                  | Binding constants | pA<sub>2</sub> | Amlyase secretion |
|----------------------------|-------------------|---------------|-------------------|
|                            | K<sub>i</sub>     | n Hill        | [^H]NMS efflux     |
| Atropine                   | 3.5 ± 0.7         | 1.1 ± 0.03    | 8.6 ± 0.17        |
| Dextemetide                | 3.5 ± 0.9         | 1.1 ± 0.02    | 8.6 ± 0.24        |
| N-Methyl-scopolamine       | 0.5 ± 0.3         | 1.08 ± 0.05   | 9.45 ± 0.12       |

The K<sub>i</sub> values for binding were calculated according to Cheng and Prusoff (15) and are the means ± S.E. of 4 experiments.

Values of K<sub>i</sub> and Hill coefficients for antagonist competition with [^H]NMS binding and of pA<sub>2</sub> for antagonist inhibition of stimulated <sup>45</sup>Ca efflux and amlyase release

Fig. 5. Dose-effect curves of inhibition of carbachyamoline-stimulated amlyase release (A) and <sup>45</sup>Ca efflux (B) from rat pancreatic acini by 4 muscarinic antagonists. A, rat pancreatic acini were incubated for 1 h at 37 °C in the presence of the indicated concentrations of atropine (●), dextemetide (■), NMS (○), or levitimid (○). 3.2 μM carbachyamoline was then added and the incubation continued for 15 min. Amlyase secretion was measured as described under "Methods." Results are expressed as percentage S of maximal amlyase secretion with 3.2 μM carbachyamoline S<sub>max</sub>. B, rat pancreatic acini were labeled with <sup>45</sup>Ca during their isolation (see under "Methods"). They were then preincubated for 1 h in the presence of <sup>45</sup>Ca and of the indicated concentrations of the 4 antagonists (see A). <sup>45</sup>Ca efflux was measured for 5 min in unlabeled medium added with 3.2 μM carbachyamoline and the antagonists. Results are expressed as percentage E of maximal <sup>45</sup>Ca efflux with 3.2 μM carbachyamoline E<sub>max</sub>. Each value was determined in duplicate. This experiment is representative of 3 others.

The proportion of high affinity binding sites was 14% of the total number of binding sites for oxotremorine (F = 4.82; df 2, 89; p < 0.05), 15% for muscarine (F = 5.54; df 2, 7; p < 0.05), and 16% for carbachyamoline (F = 7.11; df 2, 12; p < 0.01). By contrast, pilocarpine recognized only one class of sites.

The dose-effect curves describing the stimulation of amlyase release caused by oxotremorine, muscarine, and carbachyamoline were parallel and similar in that, as the concentration of these agents was increased, amlyase release increased, became maximal, and then decreased. These 3 agonists had the same efficacy in terms of maximal amlyase secretion (Fig. 6A). The potency to stimulate amlyase release (expressed as EC<sub>50</sub>) decreased in the following order: oxotremorine 0.2 μM, muscarine 0.6 μM, and carbachyamoline 1 μM (Fig. 6A and Table II).

The potency of the 3 full muscarinic agonists to stimulate <sup>45</sup>Ca efflux, after a 5-min incubation period, was the following when expressed as EC<sub>50</sub>:oxotremorine 0.2 μM, muscarine 1.5 μM, and carbachyamoline 2 μM (Fig. 6B and Table II).

The dose-effect curve of inhibition of [^H]NMS binding by the partial agonist pilocarpine (10) was steep as reflected by a Hill coefficient of 0.97 (Fig. 4 and Table II) and that describing pilocarpine stimulation of amlyase release was shallower than the dose-effect curves of full agonists, with a lower maximal release (Fig. 6A). Finally, pilocarpine exerted only a limited effect on <sup>45</sup>Ca efflux (Fig. 6B). Additional experiments with 10 and 100 μM pilocarpine demonstrated the capacity of this partial agonist to inhibit competitively carbachyamoline-stimulated <sup>45</sup>Ca efflux (Fig. 7) with a K<sub>i</sub> of 3.6 μM suggesting that pilocarpine and carbachyamoline were acting on the same receptors.

The existence of a receptor reserve for carbachyamoline—the existence of a receptor's reserve for carbachyamoline was tested using the technique of Ariens et al. (7) in an effort to relate amlyase secretion to a given type of binding sites. Preincubation with 18 nM PrBCM for 15 min displaced the dose-response curve for carbachyamoline to the right without affecting the maximal response (Fig. 8A). The fraction of receptors blocked by this pretreatment was 47% as estimated by [^H]NMS binding and 49% when applying the equation of Furchgott and Bursztyn to amlyase secretion (9) (see under "Methods"). Preincubation with higher concentrations of PrBCM blocked a greater proportion of receptors and led to a decrease in the maximal secretory response to carbachyamoline. This progressive decrease in the fraction (q) of free receptors, derived from data on both [^H]NMS binding and amlyase secretion, was plotted in Fig. 8B as −ln q versus the concentration of PrBCM; the two series of values yielded straight lines and were comparable within 10%.

This shows that PrBCM acted at the receptor level (16, 17). Besides, PrBCM did not selectively block one population of sites since a PrBCM treatment did not modify the IC<sub>50</sub> and n<sub>H</sub> values of the dose-effect curves of inhibition of [^H]NMS by carbachyamoline (Table III). According to Birdsall et al. (10), these results also do exclude negative cooperative interactions between muscarinic receptors (see under "Methods").

DISCUSSION

The binding of the labeled antagonist [^H]NMS to rat pancreatic acini at 37 °C was saturable, stereospecific, of high affinity, and exhibited the pharmacological properties of a muscarinic agent. The ratio of specific over nonspecific [^H]NMS binding was high: 10.2 ± 0.8. This binding of [^H]NMS was reversible and, at equilibrium, muscarinic receptors behaved as a homogeneous population with respect to [^H]NMS.

Characteristic of the model was a model of two classes of binding sites, whose K<sub>i</sub> values are listed in Table II. The proportion of high affinity binding sites was 14% of the total number of binding sites for oxotremorine (F = 4.82; df 2, 89; p < 0.05), 15% for muscarine (F = 5.54; df 2, 7; p < 0.05), and 16% for carbachyamoline (F = 7.11; df 2, 12; p < 0.01). By contrast, pilocarpine recognized only one class of sites.

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The existence of a receptor reserve for carbachyamoline—the existence of a receptor's reserve for carbachyamoline was tested using the technique of Ariens et al. (7) in an effort to relate amlyase secretion to a given type of binding sites. Preincubation with 15 μM PrBCM for 15 min displaced the dose-response curve for carbachyamoline to the right without affecting the maximal response (Fig. 8A). The fraction of receptors blocked by this pretreatment was 47% as estimated by [^H]NMS binding and 49% when applying the equation of Furchgott and Bursztyn to amlyase secretion (9) (see under "Methods"). Preincubation with higher concentrations of PrBCM blocked a greater proportion of receptors and led to a decrease in the maximal secretory response to carbachyamoline. This progressive decrease in the fraction (q) of free receptors, derived from data on both [^H]NMS binding and amlyase secretion, was plotted in Fig. 8B as −ln q versus the concentration of PrBCM; the two series of values yielded straight lines and were comparable within 10%.

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Muscarinic Receptors in Rat Pancreas

### Table II
Values of binding constants for agonist competition with \[^{3}H\]NMS binding and of EC\(_{50}\) for agonist stimulation of \[^{45}Ca\] efflux and amylase release

| Muscarinic agonist | IC\(_{50}\) \(^\mu\)M | \[^{3}H\]NMS binding \(n_{Hi}^{b}\) | High-affinity site \(K_{D}^{a}\) M | Low-affinity site \(K_{D}^{b}\) M | \[^{45}Ca\] Efflux \(EC_{50}\) \(\mu\)M | Amylase secretion \(\mu\)M |
|------------------|---------------------|---------------------|-----------------------------|-----------------------------|---------------------|-----------------------------|
| Oxotremorine     | 4 ± 2               | 3                    | 0.84 ± 0.05                 | 35 ± 20                     | 0.6 ± 0.2           | 0.2 ± 0.1                   |
| Muscarine        | 36 ± 23             | 26                   | 0.64 ± 0.07                 | 20 ± 12                     | 1.5 ± 0.4           | 0.6 ± 0.1                   |
| Carbamylcholine  | 75 ± 35             | 54                   | 0.66 ± 0.08                 | 35 ± 19                     | 2.0 ± 0.3           | 1.0 ± 0.1                   |
| Pilocarpine      | 7 ± 2               | 100                  | 1.00 ± 0.02                 | 65 ± 25                     | 4.0 ± 0.6           | 5.0 ± 0.3                   |

\(^{a}\) [L\(_{50}\)] is the equilibrium concentration of free cholinergic drug at 50% saturation of its binding to receptor, calculated from competition date, according to Burgermeister et al. (3).

The \(K_{D}\) for high and low affinity sites were calculated according to Minneman et al. (12), then corrected according to Cheng and Prusoff (15).

#### Fig. 6
Dose-effect curves of stimulation of amylase release (A) and of \[^{45}Ca\] efflux (B) from rat pancreatic acini by 4 muscarinic agonists. A, rat pancreatic acini were incubated for 1 h at 37 °C in the presence of the indicated concentrations of oxotremorine ( ), carbamylcholine ( ), muscarine ( ), or pilocarpine ( ). Amylase release was measured as described under “Methods.” Values are expressed as percentage of total initial "Ca content-5 min". Each value was determined in duplicate. This experiment is representative of 3 to 5 experiments.

binding. It is recognized that, as in brain, such linear Scatchard plots obtained at equilibrium with rat acini might correspond to complex association and dissociation kinetics (e.g., a fast binding step followed by slow isomerization of the receptor-ligand complex (18)). The \(K_{D}\) value of 0.9 nM (Fig. 2) determined according to Scatchard (5) was comparable to the \(K_{D}\) of 0.5 nM for the displacement of \[^{3}H\]NMS by unlabeled NMS (Table I) indicating similar binding properties for the labeled and unlabeled ligand.

The number of binding sites was 140 fmol/mg of protein, which corresponds to an average number of 24,000 binding sites per cell when related to the protein and DNA contents of the rat pancreas. Working with \[^{3}H\]QNB, Larose et al. (2) observed a binding capacity of 2500 fmol/mg of DNA, equivalent to 35 fmol/mg of protein. This discrepancy might be explained, at least in part, by the use of different antagonists. The advantages of using \[^{3}H\]NMS rather than \[^{3}H\]QNB as a radiolabeled muscarinic ligand were 2-fold: 1) a much lower nonspecific binding; 2) the good reversibility of binding due to a 15-fold higher \(K_{D}\) value with \[^{3}H\]NMS as compared to \[^{3}H\]QNB (see above and Ref. 2).

Inhibition of \[^{3}H\]NMS binding by atropine and dextemide was comparable, with a Hill coefficient close to 1, suggesting that muscarinic antagonists bound to a single class of receptors. The \(K_{D}\) of NMS, atropine, and dextemide were in the nanomolar range. In many tissues, the affinity of muscarinic binding sites for antagonists is high (19) in contrast to the much lower affinity for agonists (Table II and Ref. 20).

The relationship between antagonist binding and the inhibition of carbamylcholine-stimulated amylase release and \[^{45}Ca\] efflux (Table I) suggests that \[^{3}H\]NMS binding sites were involved in biological responses, since the order of potency of the antagonists for binding and for inhibiting stimulated amylase secretion and \[^{45}Ca\] efflux was the same (Table I).

Competition curves of \[^{3}H\]NMS with agonists developed on more than two logarithms (Fig. 4). Similar flattened agonist/[^{3}H\]antagonist competition curves have been documented for muscarinic receptors in membranes from brain, heart, smooth muscle, and cloned neuroblastoma cells, as well as in intact smooth muscle cells from rat and guinea pig ileum and in fragments of rat brain (10, 21).

These flattened competition curves could be explained by
with resulting receptor heterogeneity (23). However, data mechanism is also unlikely to account for the flattened displacement generated by these 2 models are also fitted by curves describing dissociation kinetics of [3H]NMS were similar in the absence of receptors by PrBCM did not steepen the carbamylcholine a decrease in their affinity (22). In the present case, this tive cooperativity interactions among binding sites leading to mechanism is unlikely considering that 1) random alkylation al. (12). This experiment is representative of four others. PrBCM (0-56 nM) and various concentrations of carbamyl­ presence of 1 [3H]NMS and various concentrations of carbamyl­ choline. Acini were preincubated for 15 min with PrBCM (0-56 nM), then centrifuged, resuspended in fresh medium, and incubated in the presence of 1 nM [3H]NMS and various concentrations of carbamyl­ choline for 1 h (see under “Methods”). The proportions of high and low affinity binding sites were estimated according to Minneman et al. (12). This experiment is representative of four others.

**TABLE III**

Effect of a pretreatment of rat pancreatic acini with propylbenzylcholine mustard on dose-effect curves of inhibition of [3H]NMS binding by carbamylcholine

| PrBCM concentration (nM) | Occlusion of binding sites | Inhibition of specific [3H]NMS binding (IC50, nM) | Proportions of binding sites | High affinity affinity | Low affinity affinity |
|-------------------------|-------------------------|-----------------------------|----------------------------|----------------------|----------------------|
|                         | 1 | 10 | 0.1 | 1 | 1 | 10 | 0.1 | 1 | 1 | 10 | 0.1 | 1 | 1 | 1 |
| 0 | 0 | 10 | 32 | 52 | 73 | 75 | 0.61 | 13 | 87 |
| 18 | 47 | 15 | 38 | 61 | 84 | 50 | 0.67 | 17 | 83 |
| 32 | 59 | 5 | 24 | 48 | 72 | 113 | 0.68 | 14 | 86 |
| 56 | 90 | 6 | 27 | 50 | 71 | 100 | 0.63 | 15 | 82 |

*Carbamylcholine concentrations.*

3 mechanisms. First, muscarinic agonists might induce negative cooperativity interactions among binding sites leading to a decrease in their affinity (22). In the present case, this mechanism is unlikely considering that 1) random alkylation of receptors by PrBCM did not steepen the carbamylcholine inhibition curve of [3H]NMS binding (Table III) (10) and 2) dissociation kinetics of [3H]NMS were similar in the absence of presence or agonists and antagonists (data not shown).

Second, a subheterogeneity among muscarinic receptors might reflect bimolecular dissociation or two-step interactions with resulting receptor heterogeneity (23). However, data generated by these 2 models are also fitted by curves describing apparent negative cooperativity (23), so that this mechanism is also unlikely to account for the flattened displacement curves by agonists.

Third, 2 or more classes of receptors coexist, with structural differences at the level of binding sites, that show distinct affinities for agonists and a similar high affinity for antagonists. To consider the last model more quantitatively, we analyzed agonist [3H]NMS competition curves according to a "two classes of binding sites" model (12) in order to categorize the subtyping. The data were compatible with the presence of about 15% of high affinity binding sites and about 85% of low affinity sites for oxtremorine, carbamylcholine, and muscarine corresponding to approximately 3,500 and 20,500 sites per cell, respectively, on an average. Pancreatic acini contain no more than 10% of centro-acinar and duct cells and a large majority of amylase-secreting acinar cells (24). On this ground, it cannot be excluded that the two classes of muscarinic binding sites were located on different cell types. A minority of high affinity muscarinic binding sites and a larger proportion of low affinity muscarinic binding sites are similarly present in brain and smooth muscle (10).

To delineate the biochemical significance of the binding data, it is of interest to note that dose-effect curves of agonist stimulation of amylase release developed within a narrow concentration range (Fig. 6), suggesting that each agonist interacted with no more than one class of muscarinic binding sites.

The EC50 of carbamylcholine for stimulation of amylase secretion was 1 μM, while the [L50] of its binding to muscarinic receptors was 54 μM (Table II). This high efficacy of carba­mylcholine suggested the presence of a receptor reserve.

To approach this problem, we first noticed that the pre­treatment of acini with PrBCM was unable to modify the proportions of high and low affinity binding sites (Table III) and that the estimation of the total number of receptors blocked by PrBCM was similar when tested by [3H]NMS binding (Table III) and by the method of Furchgott and Bursztyn (9) as applied to amylase secretion (Fig. 8). This parallelism indicated that PrBCM could be used as an irreversible blocker of muscarinic receptors having no further action on effector mechanism(s) (16). Maximal carbamylcholine response remained possible even when 47% of the receptors were blocked by PrBCM but the efficacy of carbamylcholine started to decrease when 59% of the receptors were not available anymore (Fig. 8A). From 47 to 59% of the muscarinic receptors were, therefore, reserve receptors with respect to carbamylcholine. The "true" affinity constant Kd of the car­bamylcholine effect on amylase secretion was estimated to be 10 μM when plotting 50% values of maximal effects (Fig. 8A) against the log of carbamylcholine concentration (25). Besides, the Kd of carbamylcholine for receptors involved in secretion was estimated according to Furchgott and Bursztyn (9) (see under "Methods") using secretory curves under control conditions and after the blockade of various fractions of receptors. This Kd was found to be 8-14 μM. Since the Kd of carbamylcholine for high affinity and low affinity binding sites were, respectively, 35 nM and 85 μM (Table II) the secretion of amylase in response to carbachol correlated with the occupancy of low affinity sites, although the participation of high affinity sites cannot be excluded. Similarly, Birdsell et al. (10) and Halvorsen and Nathanson (26) have reported that smooth muscle and heart responsiveness to muscarinic agonists was best correlated with the occupancy of low affinity receptor sites.

The EC50 values for pilocarpine binding and resulting amylase secretion were similar (Table II), suggesting that this agent reacted with a single class of binding sites among which there was no receptor reserve. This is in line with the current hypothesis that the degree of sparseness is a function of the ligand bound to the receptor (17). As discussed before, be-
Muscarinic Receptors in Rat Pancreas

tween 47 and 59% of the receptors were spare receptors when carbamylcholine was used; with respect to pilocarpine, there was no spareness and, furthermore, pilocarpine was unable to fully stimulate amylase secretion (Fig. 6A) and had hardly any effect on $^{45}$Ca efflux (Fig. 6B). This ligand was capable, however, to inhibit competitively the carbamylcholine stimulation of $^{45}$Ca efflux, showing, therefore, binding to genuine muscarinic receptors but with no efficient coupling to effector mechanism(s). Thus, pilocarpine met the theoretical requirements for being a partial agonist (7).

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