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Muscarinic Receptor Heterogeneity Revealed by Interaction with Bretylium Tosylate

Different Ligand-Receptor Conformations versus Different Receptor Subclasses[1]

GABRIEL SCHREIBER AND MORDECHAI SOKOLOVSKY

Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, 69978 Tel Aviv, Israel

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SUMMARY

The interaction of the antiarrhythmic drug, bretylium tosylate, with the muscarinic receptor in tissue homogenates from regions of rat brain and heart and from submandibular gland and ilial wall was investigated. Competition binding experiments were carried out using the highly specific tritiated antagonist *N*-methyl-4-piperidyl benzilate. Bretylium displayed heterogeneous binding characteristics. The binding of the drug to neural and glandular preparations was found to be best fitted by a one-site model in each case. On the other hand, in the case of muscle preparations (heart and ileum), a two-site model yielded a significantly better fit for the binding data than that given by a single site model. High affinity sites for the drug were detected in the muscle tissue only, with equilibrium binding constants of 0.24 ± 0.12, 0.97 ± 0.27, and 0.57 ± 0.41 μM for the atrium, ventricle, and ileum, respectively. The low affinity binding constants in the muscle tissues were similar (~10 μM) to those in the neural and glandular tissues examined, namely, the cortex, the hippocampus, the medulla pons, and the submandibular gland. The drug had no effect on agonist-binding characteristics. The heterogeneous binding of bretylium is compared to that of another nonclassical antagonist, pirenzepine. The results are discussed in relation to two alternative hypotheses put forward to account for antagonist heterogeneity in binding, the one involving ligand-receptor conformations and the other receptor subclasses.

INTRODUCTION

The binding of classical antagonists to the muscarinic receptor in various brain regions and peripheral tissues of the rat shows only small variations in equilibrium binding constants and yields a simple binding isotherm, indicating homogeneity of the receptor population (1–3). The heterogeneity displayed in studies of agonist binding may reflect modulation of a single recognition site and cannot be taken as conclusive evidence for the existence of different receptor subtypes (4, 5).

Pirenzepine, a nonclassical muscarinic antagonist, was one of the first drugs shown to display heterogeneity of antagonist binding (6), a finding which suggested the possible existence of predetermined subclasses of muscarinic receptors in different tissues (6, 7). However, the interaction of antagonists with the muscarinic receptor is known to induce changes in the receptor; for example, studies have demonstrated isomerization of the receptor-antagonist complex (8–10) as well as cooperative interactions between antagonist-occupied receptors (11, 12).

In the present study, we show that bretylium tosylate, an effective antiarrhythmic drug used in the treatment of refractory ventricular arrhythmias (13), exerts nonclassical antagonist effects in the muscarinic cholinergic system.[2] It was therefore of interest to compare the effects of bretylium to those of pirenzepine, and to relate them to the postulated existence of M_1 and M_2 subclasses of muscarinic receptors (for review, see Ref. 14).

MATERIALS AND METHODS

Materials. [^{3}H]4NMPB,[3] 69.7 Ci/mmol, was prepared by catalytic tritium exchange as described elsewhere (15). Its purity was >97%. Bretylium tosylate was the product of the Wellcome Foundation Ltd., London.

Methods. Adult male rats of the CD strain were obtained from

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[2] The antimuscarinic effects of bretylium as a possible explanation of its antiarrhythmic action are discussed elsewhere (G. Schreiber, M. Friedman, and M. Sokolovsky, *Circ. Res.*, **55**: in press 1984), along with its antagonistic effect on the negative inotropic responses of driven isolated left atria to acetylcholine.

[3] The abbreviations and trivial name used are: 4NMPB, *N*-methyl-4-piperidyl benzilate; bretylium tosylate, 2-bromo-*N*-ethyl-*N*,*N*-dimethylbenzenemethanaminium *p*-toluenesulfonate; 4DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide.

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Levinstein's Farm (Yokneam, Israel), and maintained in an air-conditioned room at 24 ± 2° for 14 hr (0500–1900) under fluorescent illumination and in darkness for 10 hr. Food from Assia Maabarot Ltd. (Tel Aviv, Israel) and water were supplied *ad libitum*. Rats aged 3–4 months and weighing 190–250 g were decapitated (between 1000 and 1200 hr) and the desired organs rapidly were removed. The hippocampus, medulla pons, and cortex were dissected out in a cold room after identification with the aid of a stereotaxic atlas.

Binding assay. The small intestine was washed with cold modified Krebs buffer (see below) to remove its intraluminal contents. Atria, ventricles, submandibular glands, and ileum were each cut up finely with scissors, mixed with 9 volumes of 0.32 M sucrose, homogenized at setting 7 on an Ultra-Turrax (Ika-Werk Instruments) with three 15-sec bursts separated by 30-sec pauses, and then filtered through three layers of cheesecloth and centrifuged twice in a hypotonic 0.1 M sucrose solution (17,000 rpm, 15 min). Brain regions were homogenized as previously described in detail (3) to yield a 3% homogenate (w/v). Homogenates prepared from the medulla-pons (three rats), the cortex (one rat), the hippocampus (one rat), the atrium and ventricle (six rats each), the submandibular glands (two rats), and the ileum (two rats) were used for binding assays, as follows. Tissue preparation (50 μl) was incubated for 40 min at 25° in 2 ml of modified Krebs-Henseleit solution (25 mM Tris-HCl, 118 mM NaCl, 4.69 mM KCl, 1.9 mM $CaCl_2$, 0.54 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 11.1 mM glucose), pH 7.4, containing varying amounts of bretylium tosylate and the labeled ligand. Incubation for twice this time, 80 min, yielded similar results.

The effect of bretylium on the binding of the agonist oxotremorine was investigated by means of agonist/^{3}H-labeled antagonist competition experiments. Tissue preparation was incubated in the same buffer in a series of test tubes containing different agonist concentrations, 2 nM of the labeled antagonist, and a fixed concentration of bretylium. After incubation for 40 min, ice-cold Krebs solution (3 ml) was added and the contents were passed rapidly by suction through a glass filter (Whatman GF/C, 25-mm diameter). The filters were washed three times in 3 ml of ice-cold Krebs solution. All procedures were completed within less than 10 sec. Binding assays were performed in triplicate, together with assays of triplicate control samples containing 5×10^{-5} M unlabeled atropine. The filters were placed in vials containing 4 ml of scintillation liquid (Hydro-Luma, Lumac Systems Inc., Titusville, FL) and were maintained at 25° for 30 min; the radioactivity was then measured by liquid scintillation spectrometry (Packard Tri-Carb 300) with a counting efficiency of 40–45%. Protein was determined by the method of Lowry *et al.* (16) using bovine serum albumin as a standard.

Specific binding was defined as the total binding minus the nonspecific binding, i.e., binding in the presence of 5×10^{-5} M unlabeled atropine. Direct binding studies and competition experiments were carried out as described in detail in previous reports (3, 17).

Data analysis. The competition curves (Fig. 1) were analyzed by a nonlinear least squares curve-fitting procedure using a model for either one or two binding sites. Theoretical competition curves were fitted to the experimental data points using the nonlinear least squares regression computer program BMDPAR (November 1978 revision), developed at the Health Science Computing Faculty (University of California, Los Angeles, CA).

The goodness of fit was evaluated by comparison of the predictive error given by the weighted sum of squared residuals, with the experimental error. The criterion for rejecting the one-site model in the cardiac and glandular preparations was a predictive error, which was significantly ($p < 0.01$, F test) greater than the experimental error.

Student's t test was employed to evaluate whether the deviation of the n_H values in cardiac and glandular preparations from unity is significant.

RESULTS

The interaction of bretylium with the muscarinic receptor was verified by competition binding studies. The extent of bretylium binding was determined by measuring inhibition of the receptor-specific binding of [^{3}H]4NMPB. Inhibition-concentration curves for the drug binding in preparations from brain regions (cerebral cortex, medulla pons, hippocampus) and from various peripheral tissues (submandibular gland, small intestine, and atrium) are shown in Fig. 1. Bretylium, unlike the classical antagonists, displays variations in binding characteristics in the various tissues. The inhibition-concentration curves in muscle tissues (heart and the small intestine) are flattened, have lower IC_{50} values than those of neural and glandular tissues, and have n_H values significantly lower than unity (Table 1). Representative Scatchard plots of the data (Fig. 1, *inset*) clearly illustrate the difference between muscle tissues (*curvilinear lines*) and the other tissues (*straight lines*).

The shape of the inhibition curves in muscle tissues can be explained by assuming heterogeneity of binding of bretylium in these tissues. Nonlinear least squares regression analysis shows that the presence of two populations of bretylium-receptor complexes is needed to adequately account for the flattened curves. Table 2 records the dissociation constants, as determined by the computer analysis, for the binding of bretylium to the various tissue preparations. In contrast to neural and glandular tissues where only low affinity sites are observed, muscle tissues (heart and intestine) possess specific sites for the binding of bretylium that are 1 order of magnitude higher in affinity. The low affinity sites in the heart and the intestine have dissociation constants similar to those in neural and glandular tissue. The nonclassical antimuscarinic drug pirenzepine has been found to display binding heterogeneity to muscarinic receptors that correlates well with the drug's pharmacological activity (6). In order to compare the heterogeneity in the binding of bretylium to muscarinic receptors to that of pirenzepine, competition binding experiments using pirenzepine and the same radioligand, [^{3}H]-4NMPB, were conducted under the same conditions used to determine bretylium binding. The $IC_{50\,cor}$ values for pirenzepine as well as its n_H values in the various tissue preparations are given in Table 1. Pirenzepine-binding characteristics found under the present conditions are similar to those obtained by Hammer *et al.* (6) (see Table 1).

The possibility of linkage between the heterogeneous binding of agonist and of antagonist was investigated by monitoring the binding properties of agonist in the presence of low bretylium concentration. The results showed that bretylium exerts no change in agonist binding properties either in central or in peripheral tissues (Fig. 2).

DISCUSSION

The equilibrium binding constants of the classical antagonists and the shape of their binding curves have shown only small variations in different peripheral tissues and brain regions. Using the nonclassical antimuscarinic drug pirenzepine, Hammer *et al.* (6) reported binding heterogeneity that correlated well with the drug's pharmacological activity. The present study demonstrates that bretylium interacts directly with the cholinergic parasympathetic system and displays nonclassical

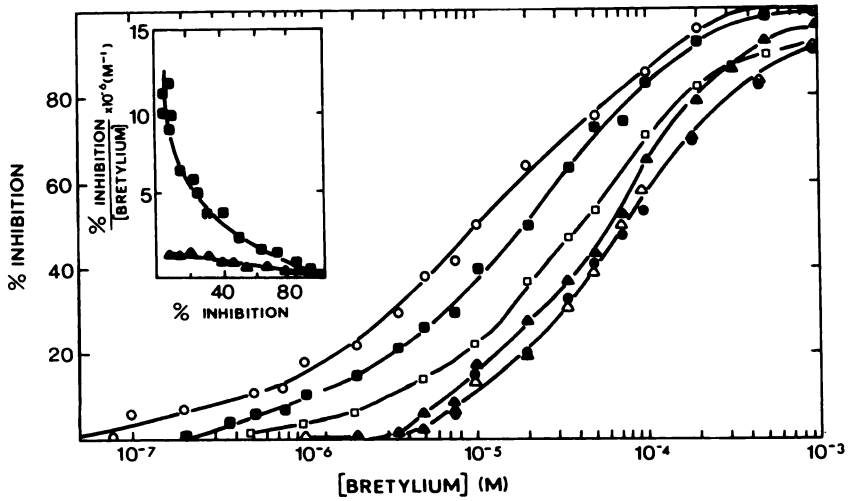

FIG. 1. *Binding of 2 nM [³H]4NMPB to homogenates of different brain regions and cardiac, glandular and intestinal tissues in the presence of various concentrations of bretylium tosylate.*

Each point is the mean of 4–6 experiments whose standard error was less than 5%. Each experiment was performed in triplicate. Total receptor concentrations were 250 pmol/g of protein for the atrium, 1000 pmol/g of protein for both the cortex and the hippocampus, 210 pmol/g of protein for both the submandibular gland and the small intestine, and 300 pmol/g of protein for the medulla-pons. The solid lines are the best fit of the data obtained by computer analysis using nonlinear regression curve-fitting. Cortex (●); hippocampus (△); submandibular gland (▲); small intestine (■); atrium (○); medulla-pons (□). Inset, representative plots of the displacement of [³H]4NMPB by various concentrations of bretylium tosylate shown.

TABLE 1

Heterogeneity of binding to various tissues: comparison between bretylium and pirenzepine

IC_{50} is the concentration of bretylium causing 50% reduction in binding of [³H]4NMPB under the experimental conditions described in Materials and Methods and in Fig. 1. The $IC_{50\,cor}$ values were calculated according to $IC_{50\,cor} = IC_{50}/(1 + [C/K_D])$, where K_D and $[C]$ represent the dissociation constant and the concentration of [³H]4NMPB, respectively. n_H = slopes of Hill plots. K_D values for [³H]4NMPB binding to the cortex, hippocampus, and medulla-pons were 0.4 nM each, to the atrium and ventricle were both 0.8 nM, and to the ileum and the submandibular gland were 0.6 nM each.

Region	Bretylium		Pirenzepine[a]		Pirenzepine[b]	
	$IC_{50\,cor}$	n_H	$IC_{50\,cor}$	n_H	$IC_{50\,cor}$	n_H
Hippocampus	1.8×10^{-5}	~1	3.4×10^{-8}	0.80	4.0×10^{-8}	0.80[c]
Cortex	1.8×10^{-5}	~1	5.1×10^{-8}	0.73	6.0×10^{-8}	0.79[c]
Medulla-pons	1.3×10^{-5}	~1	4.1×10^{-7}	~1	4.2×10^{-7}	~1
Atrium	2.8×10^{-6}	0.79[c]	8.3×10^{-7}	~1	8.5×10^{-7}	~1
Ventricle	4.6×10^{-6}	0.67[c]			4.3×10^{-7}	~1
Ileum	2.7×10^{-6}	0.80[c]	7.9×10^{-7}	~1	6.9×10^{-7}	~1
Submandibular gland	1.1×10^{-5}	~1	1.3×10^{-7}	0.78	1.2×10^{-7}	0.79[c]

[a] Data taken from Hammer *et al.* (6).

[b] Experiments of the type conducted by Hammer *et al.* (6) repeated under the same conditions used for bretylium-binding experiments.

[c] Significantly different from 1 ($p < 0.05$).

antagonistic effects on the muscarinic receptor. It is thus interesting to compare these two drugs. Table 1 presents a comparison between tissue diversities with respect to the binding characteristics (expressed in IC_{50} and n_H values) of bretylium and pirenzepine. Pirenzepine displayed its highest affinity in neural tissue (hippocampus and cortex), where it also shows flattened binding curves with n_H values of less than 1. Its affinity was lowest in muscle tissue (cardiac atrium, ileal wall), where n_H values of ~1 were recorded. A mirror image was observed with bretylium binding, which yielded high affinities in muscle tissue (atrium, ventricle, ileum) with n_H values significantly lower than 1, and low affinities in neural tissues, where n_H values are equal to 1.

On the basis of the heterogeneity in the binding of pirenzepine and several other antagonists (i.e., 4DAMP, gallamine), several authors have suggested that muscarinic receptors can be divided into at least three subclasses (for review, see Ref. 14). Bretylium could be included in this subclassification scheme according to its heterogeneous binding properties, which are reciprocal to those of pirenzipine.

However, a detailed analysis of the results raises certain questions concerning the scheme. (i) This type of classification would rule out the coexistence in the same tissue of a homogeneous binding site population for one nonclassical antagonist and a heterogeneous population for another. Heart and smooth muscle were each found to contain a homogeneous, low affinity population of pirenzepine-binding sites (6). However, two populations

TABLE 2

Proportion of high affinity sites and dissociation equilibrium constants for bretylium

The binding parameters ± standard deviation were calculated by the nonlinear regression procedure for one- and two-site models (17). α denotes the proportion of high affinity sites for bretylium. K_H and K_L are the equilibrium dissociation constants of the high and low affinity binding sites, respectively. The average values of the binding characteristics were determined in 4–6 experiments, each performed in triplicate.

Region	α	K_H (μM)	K_L (μM)
Hippocampus	0		14.6 ± 0.65
Cortex	0		13.7 ± 0.48
Medulla-pons	0		8.7 ± 0.36
Submandibular gland	0		10.3 ± 0.47
Atrium	0.22 ± 0.06	0.24 ± 0.12	6.1 ± 1.02
Ventricle	0.35 ± 0.07	0.97 ± 0.27	10.4 ± 1.53
Ileum	0.30 ± 0.1	0.57 ± 0.41	12.8 ± 3.41

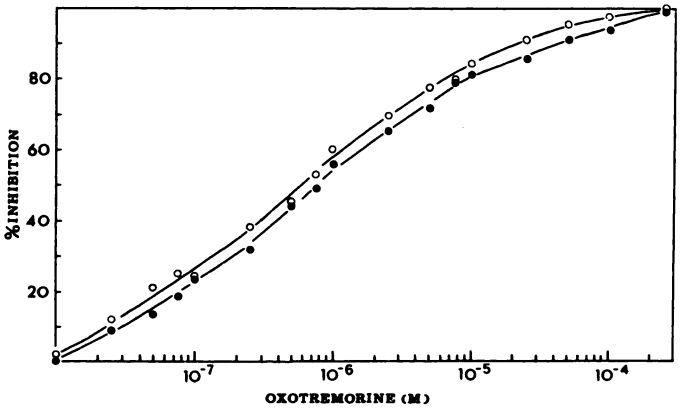

FIG. 2. *Binding of 2 nM [^{3}H]4NMPB to homogenates of medulla-pons in the presence of various concentrations of oxotremorine in the absence (○) or presence of 2 × 10^{-5} M bretylium (●).*

of sites with a difference of at least 1 order of magnitude in their K_d values were shown to exist in muscle tissue (atrium, ventricle, ileum) for the binding of bretylium (see Table 2). Moreover, neural tissue contains a homogeneous population of low affinity binding sites for bretylium, while for pirenzepine both high and low affinity binding sites are observed. (ii) It was proposed (14) that a correlation exists in neural tissue between the receptors classified as high affinity for pirenzepine and as low affinity for agonists. If the division into subclasses is generally applicable, a similar association should also hold for bretylium. However, no such correlation was found between the binding of bretylium and of agonist (Fig. 2).

These results cannot be explained by assuming the existence of predetermined neural and cardiac subclasses of muscarinic receptors. One possibility is that more than two types of receptors exist both in neural and in cardiac tissues, and that both pirenzepine and bretylium can distinguish between the different subclasses, thus yielding completely different binding patterns. The weakness of this explanation is that it proposes the existence of a large number of receptor subclasses.

We propose an alternative hypothesis, which has been already presented previously (for review, see Refs. 19, 20 and 25) and which assumes that the different receptor subtypes are at least partially induced by the ligand which binds to the receptor. According to this assumption, all muscarinic receptors are identical regardless of the tissues or regions in which they exist; site heterogeneity would then simply reflect interconvertibility of binding sites which is affected both by the membrane milieu as well as by the specific properties of the binding ligand. Such phenomena have indeed been observed in the muscarinic system. (i) Changes in ionic strength and buffer composition alter the affinity of antagonists towards the muscarinic receptor in several synaptosomal preparations (21, 22) and in myocardial tissue (21). (ii) Variations in the Ca^{2+} dependency of muscarinic antagonist binding were demonstrated in the adenohypophysis of female rats at difference stages of the estrous cycle (20, 23). (iii) Ligand competition experiments in rat adenohypophysis demonstrate site-site interactions between the antagonist-occupied receptors (11, 12). (iv) Isomerization of the antagonist-receptor complex (8–10) reflects changes induced in the receptors by antagonist binding. Moreover, different muscarinic antagonists (e.g., tropates versus benzilates) induce different ligand-receptor complex conformations (3). (v) Muscarinic sites may also interact with other binding sites. Such interactions may be involved in the cooperative effects exerted on muscarinic receptors by the antiestrogenic drug clomiphene (24), by bispyridinium oximes (25), and by the neuromuscular blocking agent gallamine (26). In the case of gallamine (and of pancuronium), it was already suggested that receptor heterogeneity had been induced, rather than detected, by the actions of the drug (27).

In conclusion, on the basis of the available data, we favor the assumption that heterogeneity of muscarinic receptors is due mainly to ligand-induced conformational changes, which depend in turn, at least partially, on the microenvironment of the receptor.

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Send reprint requests to: Mordechai Sokolovsky, Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, 69978 Tel Aviv, Israel.