Interaction of Bretylium Tosylate with Rat Cardiac Muscarinic Receptors
Possible Pharmacological Relevance to Antiarrhythmic Action

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SUMMARY. The interaction of the antifibrillatory antiarrhythmic drug, bretylium tosylate, with the muscarinic receptor in tissue homogenates from regions of rat brain and heart was investigated. Competition-binding experiments were carried out with the highly specific tritiated antagonist N-methyl-4-piperidyl benzilate. Bretylium tosylate competitively displaced the labeled antagonist from the muscarinic receptor. The binding of the drug to the two brain preparations was found to be best fitted by a one-site model in each case. On the other hand, in the case of both heart preparations, a two-site model yielded a significantly better fit for the binding data than that given by a single-site model. The low affinity-binding constants in the atrium and the ventricle were similar (—10 μM) to those in the brain regions examined, namely, the cortex and the medulla-pons. Sites with relatively higher affinity for the drug were detected in the heart only, with equilibrium-binding constants of 0.24 ± 0.12 μM and 0.97 ± 0.27 μM for the atrium and the ventricle, respectively. The drug also exerted anti-acetylcholine activity (Ki = 14 ± 2 μM) measured physiologically in the guinea pig atrium, which correlated well with the concentration of the drug observed to be efficacious clinically (~10 μM). (Circ Res 55: 653-659, 1984)

Bretylium Tosylate* (scheme 1), which was first introduced as an antihypertensive agent, but later rejected because of rapid development of tolerance, is an effective antiarrhythmic drug, recommended for treatment of refractory ventricular arrhythmias (Kumana and Hamer, 1979). The drug has profound effects on the sympathetic nervous system: it is taken up and concentrated in adrenergic nerve terminals (Iversen, 1973), initially releasing norepinephrine (Gilmore and Siegel, 1962), and, later, preventing its release (Boura and Green, 1959). Bacoaner (1966) reported that bretylium tosylate increased the current threshold for electrical induction of ventricular fibrillation in dogs. Subsequent experimental (Logic, 1973; Kniffen et al., 1975) and clinical (Bernstein and Koch-Weser, 1972; Sanna and Arcidiacono, 1978; Haynes et al., 1981) evaluations of the drug have confirmed its unique antifibrillatory action, while also demonstrating that it exerts various direct electrophysiological effects (Wit et al., 1970; Bigger et al., 1971; Cardinal and Sasyniuk, 1978). It was assumed that the drug did not affect the cardiac muscarinic cholinergic receptors (Bigger and Hoffman, 1980).

In view of the diversity of the effects of bretylium tosylate and its complex interaction with the sympathetic nervous system, it was of interest to evaluate any effect it might exert directly on the para-

* 2-Bromo-N-ethyl-N,N-dimethylbenzenemethanaminium p-toluen sulfonate.

Methods

Materials

[3H]-4NMPB, 69.7 Ci/mmol, was prepared by catalytic tritium exchange as described elsewhere (Kalir et al., 1978). Its purity was >97%. Bretylium tosylate was the product of the Wellcome Foundation Ltd.

Methods

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Br

\[-N^+\cdot C_2H_5\cdot H_3C\cdot C_6H_4\cdot SO_3^-\]

\[CH_3\]

Bretylium Tosylate

Scheme 1

sympathetic system. We have investigated the pos-
sible interaction of bretylium tosylate with musca-

rinic receptors in competition experiments using the
highly specific tritiated antagonist, N-methyl-4-pi-
peridyl benzilate ([3H]-4NMPB) (Kloog and Soko-
kovsky, 1978), in homogenates from various regions
of rat brain and heart. Our findings indicate that
bretylium tosylate interacts directly and competi-
tively with muscarinic receptors in the heart.

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Adult male rats of the CD strain, obtained from Levin-
stein's Farm, were maintained in an air-conditioned room
at 24 ± 2°C for 14 hour (5 a.m. to 7 p.m.) under fluorescent illumination, and in darkness for 10 hours. Food from Assia Maabarot Ltd. and water were supplied ad libitum. Rats aged 3–4 months and weighing 190–250 g were decapitated (between 10 a.m. and noon), and their brains or hearts were rapidly removed. The medulla-pons and the cortex were dissected out in a cold room after identification with the aid of a stereotaxic atlas (DeGroot, 1972).

**Binding Assay**

Atria and ventricles were 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-second bursts separated by 30-second pauses, and then filtered through three layers of cheesecloth and centrifuged twice in hypotonic sucrose solution (17,000 rpm, 15 minutes). Brain regions were homogenized, as previously described in detail (Kloog et al., 1979), to yield a 3% homogenate (wt/vol). Homogenates prepared from the medulla-pons (three rats for each experiment), the cortex (one rat for each experiment), and the atrium and ventricle (six rats each, for each experiment) were used for binding assays as follows: tissue preparation (50 μl) was incubated at 25°C in 2 ml of modified Krebs-Henseleit solution (25 mM Tris-HCl, 118 mM NaCl, 4.69 mM KCl, 1.9 mM CaCl₂, 0.54 mM MgCl₂, 1.0 mM NaH₂PO₄, 11.1 mM glucose), pH 7.4, containing varying amounts of the labeled ligand. After incubation for 40 minutes, 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 in diameter). The filters were washed three times in 3 ml of ice-cold Krebs solution. All procedures were completed within less than 10 seconds. Binding assays were performed in triplicate, together with triplicate control samples containing 5 × 10⁻⁵ M unlabeled atropine. Dose ratios and inhibition constants were calculated from three sets of experiments, were: Kᵰ = 0.8 ± 0.1 nM and Rᵰ = 250 ± 20 pmol/gr protein, respectively.

**Results**

A representative [³H]-4NMPB saturation binding curve to homogenates from rat atrium is shown in Figure 2. The Scatchard representation (see inset) yields a straight line. The dissociation equilibrium constant and the total binding capacity, calculated from three sets of experiments, were: Kᵰ = 0.8 ± 0.1 nM and Rᵰ = 250 ± 20 pmol/gr protein, respectively
To estimate affinities for bretylium tosylate from competitive experiments and statistically verify the possible existence of two muscarinic receptor binding sites for bretylium tosylate in the heart, we performed a nonlinear regression analysis. The data were analyzed using a model for either one or two binding sites, as described previously (Avissar et al., 1981). The binding of bretylium tosylate to the two brain preparations was found to be best fitted by a one-site model, in each case. On the other hand, in heart preparations, the data could be best described by a two-site model.

The possible interaction of bretylium tosylate with the muscarinic receptor was tested by means of competition experiments with [3H]-4NMPB (2 nM). This concentration was selected because it produces approximately 80% occupancy of the sites (Fig. 2). Figure 1 shows the results of competition experiments, with homogenates from brain tissue (cortex and medulla-pons), as well as from cardiac tissue (atrium and ventricle). Bretylium tosylate inhibited the binding of [3H]-4NMPB, thus indicating that it may act as an anticholinergic drug. The behavior of the drug was clearly different in the two types of tissues; this was evident from the variation in its IC50 values by a factor of 5-10 (Table 1), as well as from the differences in shape of the binding curves obtained with the brain and the heart. In cardiac tissue, the binding curves, unlike those for the brain, were flattened, and yielded Hill slopes of significantly less than unity (Table 1). The flattened curves and the different IC50 values can be explained by assuming the presence of multiple muscarinic binding sites in the heart characterized by different affinities for bretylium tosylate. Scatchard representation of the data (Fig. 3) does indeed appear to confirm the complex nature of displacement of [3H]-4NMPB by bretylium tosylate in heart homogenates. Whereas in both the cortex and the medulla-pons a straight line was obtained, the data for both the atrium and the ventricle yielded curvilinear lines deviating markedly from linearity.

**Table 1**

<table>
<thead>
<tr>
<th>Region</th>
<th>IC50 (nM)</th>
<th>nH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>(1.8 ± 0.08) × 10^{-5}</td>
<td>0.96 ± 0.06</td>
</tr>
<tr>
<td>Medulla-pons</td>
<td>(1.3 ± 0.06) × 10^{-5}</td>
<td>0.98 ± 0.05</td>
</tr>
<tr>
<td>Atrium</td>
<td>(2.8 ± 0.11) × 10^{-4}†</td>
<td>0.79 ± 0.06‡</td>
</tr>
<tr>
<td>Ventricle</td>
<td>(4.6 ± 0.20) × 10^{-4}‡</td>
<td>0.67 ± 0.04‡</td>
</tr>
</tbody>
</table>

*IC50 is the concentration of bretylium tosylate causing 50% reduction in binding of [3H]-4NMPB under the experimental conditions described in Methods and in Figure 2. The IC50 values were calculated according to IC50 = IC50/(1 + [C]/Kd), where Kd and [C] represent the dissociation constant and the concentration of [3H]-4NMPB, respectively. nH = slopes of Hill plots. Kd values for [3H]-4NMPB binding to cortex and medulla-pons preparations were 0.4 and 0.8 nM, respectively (Kloog et al., 1979). Values for the atrium and ventricle were 0.8 nM (Fig. 1). The average values of the binding parameters were determined from the values obtained separately in four to six independent experiments, each performed in triplicate. The SE values were calculated from the parameter values obtained at the separate experiments.

† The values given for the cardiac preparations were significantly below the values given for the brain preparations (P < 0.05, Student's t-test).

‡ Significantly different from 1 (P < 0.05, Student's t-test).

Also, significantly different from the nH values given for the brain (P < 0.05, Student's t-test).
FIGURE 3. Scatchard analysis of the displacement of \[^{3}H\]-4NMPB by various concentrations of bretylium tosylate in different brain and heart regions.

TABLE 2

<table>
<thead>
<tr>
<th>Region</th>
<th>(\alpha)</th>
<th>(K_H (\mu M))</th>
<th>(K_L (\mu M))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>0</td>
<td>13.7 ± 0.48</td>
<td></td>
</tr>
<tr>
<td>Medulla-pons</td>
<td>0</td>
<td>8.7 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>Atrium</td>
<td>0.22 ± 0.06</td>
<td>0.24 ± 0.12</td>
<td>6.1 ± 1.02</td>
</tr>
<tr>
<td>Ventricle</td>
<td>0.35 ± 0.07</td>
<td>0.97 ± 0.27</td>
<td>10.4 ± 1.53</td>
</tr>
</tbody>
</table>

*The binding parameters were calculated by the nonlinear regression procedure for one- and two-site models (Avissar et al., 1981); \(\alpha\) denotes the proportion of sites with relatively higher affinity for bretylium tosylate. \(K_H\) and \(K_L\) are the equilibrium dissociation constants for the higher and lower affinity binding sites, respectively. The average values \(\pm\) so of the binding parameters were calculated from the separately determined parameters obtained in four to six experiments, each performed in triplicate.

To elucidate the mechanism of the drug's effect on the muscarinic receptors, competition experiments were conducted with bretylium tosylate and various concentrations of \[^{3}H\]-4NMPB. The resulting double reciprocal plots (Fig. 4) clearly indicate the purely competitive nature of the interaction in the heart, as well as in the brain. Purely competitive behavior on the part of bretylium tosylate would be expected to yield straight lines which satisfy the equation,

\[
\frac{R_T}{R_{A_n}} = 1 + \left(\frac{1}{[A_n]}\right) \left(\frac{[B]K_D}{K_i} + K_D\right),
\]

where \(R_T\) and \(R_{A_n}\) are the concentrations of total receptor and of the receptor-antagonist complex, respectively, \(B\) is bretylium tosylate concentration, and \(K_D\) and \(K_i\) are the dissociation equilibrium constants for \[^{3}H\]-4NMPB and bretylium tosylate, respectively. This equation predicts an intersection of the ordinate at 1, which was indeed the experimental finding.

The dissociation equilibrium constants of bretylium tosylate presented in Table 2 were obtained by nonlinear regression analysis of the competition curves shown in Figure 1. Since such curves are obtained with only one concentration of \[^{3}H\]-4NMPB, it was necessary to verify the results for other concentrations as well. This is shown in the insets to Figure 4, where the slopes of the lines of the double reciprocal plots obtained for the cortex (Fig. 4A) and the ventricle (Fig. 4B) are depicted as functions of bretylium tosylate concentration. In the presence of a single binding site for bretylium tosylate, such a presentation would be expected to yield a straight line intersecting the ordinate at \(K_D\) with a slope of \(K_D/K_i\). The \(K_i\) value, \(1.20 \times 10^{-5} M\), calculated from the slope of the line, is in accord with the value of \(1.37 \times 10^{-5} M\) for \(K_L\) of bretylium tosylate in the brain, shown in Table 2. The existence in the heart of two sites with different \(K_i\) values for bretylium tosylate is also supported by the curvature of the line obtained (Fig. 4B, inset).

In driven isolated left atria, bretylium tosylate (1.4–0.14 \(\mu M\)) inhibited the negative inotropic responses to acetylcholine. At the concentration range
investigated, bretylium tosylate by itself did not induce any inotropic effects on the responses of the atria to the electric stimulation. A parallel shift of the dose-response curves of acetylcholine in the presence of different concentrations of bretylium tosylate was obtained. Schild plot (Fig. 5) of the inhibitory effect curves yielded a straight line with a slope of $\sim 45^\circ$, indicating competitive antagonism, with an inhibition constant $K_i = 14 \pm 2 \mu M$.

**Discussion**

The present study demonstrates the ability of bretylium tosylate to interact directly with the muscarinic parasympathetic system. Bretylium tosylate was able to displace the muscarinic antagonist 4-NMNPB from its receptor-ligand complexes in various brain regions, as well as in the heart. Specific sites with relatively higher affinity for bretylium tosylate were detected among the muscarinic receptors in the heart. Their dissociation equilibrium constants, which were in the range of 0.3–1.0 $\mu M$, were 10 to 25 times lower than those of the corresponding low affinity binding sites (see Table 2); the latter were generally similar for all the brain and heart preparations examined (Table 2). The finding that bretylium tosylate also interacts with brain muscarinic receptors is of interest, since it demonstrates that the interaction of the drug with muscarinic receptors is not a unique feature of these receptors in the heart. However, the effect on the brain is probably without physiological or therapeutic significance, since bretylium tosylate being a quaternary amine, does not cross the blood-brain barrier. The anticholinergic effect of the drug was verified physiologically with the guinea pig atrium preparation. The $K_i$ value obtained, 14 $\mu M$, was in good correlation with the low affinity binding constants found in the compe-
tion-binding experiments in the cardiac tissue. The minimum effective plasma concentration of the drug is in the range of 2.6–5.6 μM, with an average value of 4.1 ± 1.1 μM (Woosley et al., 1982). The usual plasma concentration is in the range of 5.9–12.3 μM, with an average value of 9.1 ± 1.9 μM. The average values of the usual plasma concentration are in agreement with the low affinity binding constants found in the heart, as well as with the Kd value obtained with the guinea pig atrium preparation.

The equilibrium binding constants of the classic antagonists to muscarinic receptors in subcellular preparations have shown only small variations in different peripheral tissues and in different brain regions. Using the antimuscarinic drug pirenzepine, Hammer et al. (1980) reported binding heterogeneity that correlated well with the drug’s pharmacological activity. Table 1 shows the diversities in the binding (expressed in IC50 and nH values) of bretylium tosylate to brain and heart regions. The appearance of relatively higher affinity sites for bretylium tosylate specific for the heart tissue is also expressed in Hill coefficients, which are significantly less than one. Interestingly, although pirenzepine has a higher affinity and lower Hill coefficient value in the cortex than in the atrium (Hammer et al., 1980), bretylium tosylate shows higher affinity and lower nH value in the atrium than in the brain.

Bretylium tosylate is a quaternary ammonium compound. Another quaternary ammonium compound, gallamine, was found to have higher affinity to cardiac muscarinic receptors than to brain receptors. This drug, together with pirenzepine and other selective antagonists (Hammer and Giachetti, 1984), are used for subclassification of muscarinic receptors. However, data from both functional and binding studies indicate that the effect of gallamine in the heart is noncompetitive (Clark and Mitchelson, 1976; Stockton et al., 1983), whereas the effect of bretylium tosylate is competitive (Figs. 4B and 5).

Because of the complex pharmacological actions of bretylium tosylate, the mechanism of its antiarrhythmic effects has been the subject of frequent speculation. Some have suggested that the effects of the drug result from its adrenergic blocking action, since this inhibits catecholamine release (Papp and Vaughan Williams, 1969). Others have assumed that the immediate antiarrhythmic action was caused by a transient increase in sympathetic tone due to early release of catecholamines (Mason et al., 1976) analogous to the action of isoprenaline in torsade de pointes. Still others have reported direct electrophysiological effects of bretylium tosylate, i.e., an increase in duration of action potential and in effective refractory period, independent of its effects on the adrenergic nervous system (Wit et al., 1970; Bigger and Jaffe, 1971; Cardinal and Sasyniuk, 1978). However, there are confusing variations among the reported electrophysiological effects, which cannot easily be rationalized.

It is of interest to note that the first report on the antifibrillatory action of bretylium tosylate by Leveque (1965) was based on his special method of inducing arrhythmia, namely, through acetylcholine-induced atrial fibrillation. It is likely that parasympathetic influences are intimately involved in the induction and facilitation of a number of arrhythmias (Higgins et al., 1973). Parasympathetic activity may induce arrhythmia by depressing the activity of the sinus pacemaker cells, by blocking or slowing atrioventricular conduction, or by promoting nonuniform repolarization of adjacent regions of the myocardium (Higgins et al., 1973). Alessi et al. (1958) demonstrated, in the canine heart, that both direct and reflex vagal stimulation increases the nonuniformity of duration of the refractory period of various atrial sites, an action which favors the development of atrial fibrillation and other arrhythmias. However, it was assumed that bretylium tosylate had no effect on the muscarinic receptors of the heart (Bigger and Hoffman, 1980).

Namm et al. (1976) have determined the uptake of bretylium tosylate and norepinephrine by perfused hearts from normal and immunosympathectomized rats. At a concentration of 0.1–1.0 μM, the uptake of bretylium tosylate by the ventricles was reduced by only 10% following sympathectomy, compared to a reduction of 80% in norepinephrine uptake. Interestingly, this range of bretylium tosylate concentrations at which its uptake was independent of the sympathetic nervous system was close to the value of the high affinity binding constant for bretylium tosylate found in the present study (see Table 2). However, the total direct binding of radiolabeled bretylium tosylate to heart plasma membranes of immunosympathectomized rats was much higher (50 nmol/g) than the total concentration of muscarinic receptors in the ventricles found in this and other studies (Fields et al., 1978) (~50 pmol/g). These relatively high total amounts of directly bound drug suggest that bretylium tosylate is also bound to other nonspecific sites which are distinct from the adrenergic nerve terminals, as well as from the muscarinic receptor.

In view of the known antiarrhythmic action of bretylium tosylate and the involvement of the parasympathetic muscarinic system in the induction and facilitation of cardiac arrhythmias, the present finding of a direct interaction between the drug and the muscarinic receptor in the heart may be relevant, at least in part, to the therapeutic effects of the drug.

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