Use of Antimuscarinic Toxins To Facilitate Studies of Striatal m4 Muscarinic Receptors

SHERRY L. PURKERSON and LINCOLN T. POTTER
Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine, Miami, Florida
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ABSTRACT
Striatal m4 muscarinic receptors are important because their blockade controls movement, and they are preferentially located on striatal neurons that project to the internal globus pallidus. The following studies were performed in vitro to provide a basis for using antimuscarinic toxins to study the effects of selective m4 blockade on movement in vivo. Because m4-toxin has limited selectivity alone (102-fold higher affinity for m4 than m1 receptors), m1-toxin was used first to occlude m1 receptors selectively, fully and irreversibly. It blocked 42% of the sites for 1.0 nM [3H]-N-methylscopolamine in rat striatal membranes and 43% in sections of cat striatum. m4-Toxin blocked 88% of the residual, non-m1 sites in membranes, showing 64 pmol m4 receptors/g tissue. In comparison, AFDX-116, biperiden, clozapine, gallamine, hexahydropifenidol, himbacine, R(1+)hyoscyamine, methocramine, pirenzepine, sila hexocyclium, trihexyphenidyl and tripitramine did not distinguish m4 from other non-m1 receptors. [3H]-Pirenzepine dissociated twice as rapidly from non-m1 as m1 receptors. Autoradiography was used to test the idea that m4 receptors are localized preferentially in the striosomes of the cat striatum. Non-m1 receptors were distributed equally in striosomes and matrix, indicating that striatal neurons with m4 receptors are in both compartments. Thus m1-toxin facilitates studies of m4 receptors by occluding m1 receptors, and m4-toxin is a selective antagonist for residual m4 receptors.

The rational development of new drugs for the treatment of hypo- and hyper-kinetic disorders of the striatum depends upon detailed knowledge of the neural circuits passing through the striatum, the target proteins (e.g., receptors) that control each circuit, and the availability of ligands that can be used to test the effects of activating or blocking these receptors on movement. In recent years, scientists interested in Parkinson’s and Huntington’s diseases have learned a great deal about the microcircuitry of the striatum, the functioning of this circuitry in health and disease, and the ability of ligands for dopaminergic and muscarinic receptors to control two sets of glutamate-activated, GABAergic striatal projection neurons (for reviews see DeLong, 1990; Bolam and Bennett, 1995; Potter and Purkerson, 1995). It is clear that nonselective dopaminergic agonists and nonselective muscarinic antagonists are useful for treating Parkinson’s disease, that D2-selective antagonists cause Parkinson-like syndromes, and that D2-selective antagonists are effective in early Huntington’s disease. Because dopamine acts on several subtypes of striatal dopamine receptors, there have been intense efforts to determine whether different dopamine receptors control each set of striatal output neurons (e.g., Ariano et al., 1995; Le Moine and Bloch, 1995), with the hope that drugs selective for specific receptors can be used both as research tools to understand the functions of each circuit, and to improve movement. There are parallel and equally interesting questions about the m1 and m4 muscarinic receptors that regulate striatal output neurons (Hersch et al., 1994; Potter and Purkerson, 1995). But there are no m4-selective, competitive agonists or antagonists that can be used to establish the effects of activating or blocking m4 receptors on movement. Two toxins, m1-toxin and m4-toxin, have the requisite selectivity for distinguishing m1 and m4 receptors, both are effective after intracerebral injection, and m1-toxin binds irreversibly at 37°C (see below). But the effects of these toxins on striatal muscarinic receptors have not been adequately established in vitro. Our studies were performed to provide a solid basis for using toxins for studies of selective m4-blockade in vitro and in vivo. We established that m1-toxin occludes m1 receptors in striatal membranes and tissue slices, that m4-toxin distinguishes between the different, residual non-m1 receptors (88% m4 receptors) and that neurons with non-m1 receptors originate in both the striosomes and matrix of the striatum. Thus antimuscarinic toxins can facilitate pharmacological studies of striatal neurons that have m1 and m4 receptors.

ABBREVIATIONS: CHO cells, Chinese hamster ovary cells; NMS, N-methylscopolamine; QNB, quinuclidinyl benzilate; GABA, γ-aminobutyric acid; nH, Hill coefficient.

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The striatum contains more choline acetyltransferase, acetylcholine and acetylcholinesterase than other tissues (Graybiel and Ragsdale, 1983), implying that acetylcholine plays an unusually important role in controlling the functions of this tissue. The concentration of m4 receptors, and the ratio of m4 to other subtypes of muscarinic receptors, are higher in the striatum than in any other tissue, and striatal cells express primarily m4 and m1 receptors (Waelbroeck et al., 1990; Levey et al., 1991; Hersch et al., 1994). Studies by in situ hybridization and immunocytochemistry indicate that the next most prevalent receptors are m2 receptors, but the levels of mRNA for m2, m3 and m5 receptors, and the levels of m2 receptor protein, are exceptionally low (Weiner et al., 1990; Bernard et al., 1992; Hersch et al., 1994; Wei et al., 1994). Only half of the output neurons of the striatum have m4 receptors (see below), and m4 and m1 receptors modulate different calcium currents and different second messenger systems in output neurons (Surmeier et al., 1995). Antagonists that block m4 and m1 receptors nonspecifically, e.g., benzotropine, biperiden and trihexyphenidyl (Dörje et al., 1991; Bolden et al., 1992), are useful for treating Parkinsonian syndromes due to dopamine deficiency or due to the antagonism of dopamine receptors. These observations indicate that the activation of m4 receptors controls movement, and that new m4-selective drugs should prove useful for the treatment of movement disorders. It is therefore important to have receptor ligands that allow studies of the functional roles of m4 receptors in the striatum.

It is also important to know where m4-selective drugs can act in the striatum. Almost all of the m1 and m4 muscarinic receptors in the striatum lie on medium spiny GABAergic projection neurons (Hersch et al., 1994). Studies by in situ hybridization and immunohistochemistry show that m1 receptors are present on almost all of these projection neurons, whereas m4 receptors are prevalent on half as many (Weiner et al., 1990; Bernard et al., 1992; Hersch et al., 1994). Projection neurons are present in two distinct neural circuits. Neurons in the “direct” pathway contain substance P and dynorphin as well as GABA, and project to the internal segment of the globus pallidus (Bolam and Bennett, 1995). They are believed to be underactive in Parkinsonism and overactive in hyperkinetic disorders (DeLong, 1990). Neurons in the “indirect” pathway contain enkephalin as well as GABA, and project to the external segment of the globus pallidus. They are believed to be overactive in Parkinsonism, and they degenerate early in Huntington’s disease (DeLong, 1990). Projection neurons are also localized macroscopically in two separate compartments, the striosomes and matrix, which are well defined in the human and cat striatum. Because almost all projection neurons have m1 receptors, neurons with m1 receptors must be present in both pathways and both compartments. But the location of neurons with m4 receptors is not clear. Studies by in situ hybridization show that mRNA for m4 receptors is present primarily in the substance P-containing neurons of the direct pathway (Weiner et al., 1990; Bernard et al., 1992). There is separate evidence that substance P is concentrated in striosomes and that enkephalin is concentrated in the matrix (Graybiel, 1990). Taken together, these observations suggest that neurons with m4 receptors may be preferentially located in the striosomes. To test this idea, we used autoradiography to examine the localization of non-m1 (88% m4) muscarinic receptors in the striosomes and matrix of the adult cat striatum.

The key problem with working with striatal muscarinic receptors has been the lack of ligands that can distinguish the different subtypes of muscarinic receptors, especially m1 and m4 receptors. The agonist, McNeil A-343, has higher affinity and efficacy at m4 than other muscarinic receptors (Lazareno et al., 1993), but the differences are insufficient to permit selective m4-activation. The antagonist, himbacine, shows only 10-fold higher affinity for m4 than m1 receptors, and the same affinity for m4 and m2 receptors (Dörje et al., 1991). Pirenzepine and guanypirenzepine show 6- and 17-fold higher affinity for m1 than m4 receptors, respectively (Buckley et al., 1989; Dörje et al., 1991; Ferrari-DeLeo et al., 1994), but are incapable of blocking most m1 receptors without causing a high degree of m4 blockade. Some information about the effects of agonists on mixed m4 and m2 receptors in the striatum has been obtained from biochemical studies of receptors coupled to the inhibition of adenylate cyclase (McKinney et al., 1991) and from physiological studies of receptors mechanisms sensitive to pertussis toxin (Surmeier et al., 1995). It is obvious that more specific ligands are needed to study striatal m1 and m4 receptors, particularly in vivo.

m1-Toxin (Max et al., 1993a, b, c) and m4-toxin (Max et al., 1993d; Liang et al., 1996) are the most promising selective antagonists for further studies of the striatum. m1-Toxin can block m1 muscarinic receptors selectively and fully in membranes and tissues, with no effect on m2-m5 receptors (Max et al., 1993a, b, c; Carsi-Gabrenas, 1997). m4-Toxin is known to bind reversibly with 102-fold higher affinity to m4 than m1 receptors (Max et al., 1993d; Liang et al., 1996), and it shows more than 500-fold higher affinity for m4 than m2, m3 or m5 receptors (Jolkkonen, 1996; “MT3” = m4-toxin). Hence m4-toxin can be used after m1-toxin to distinguish m4 receptors from other non-m1 receptors. Both toxins have been shown to be useful for physiological studies in vitro (Surmeier et al., 1995; Cuevas et al., 1997; Marino et al., 1997), and m1-toxin (Liang JS, Santiago MP and Potter LT, unpublished data), MT1 (Jerusalinsky and Harvey, 1995) and m4-toxin (Wang et al., 1997) have also been used in vivo.

m1-Toxin is of particular interest for studies in vivo because its action is irreversible at 37°C (Carsi-Gabrenas, 1997).

Methods

H-NMS (79.5 Ci/mmol) and [N-methyl-3H]pirenzepine (83.1 Ci/mol) were purchased from Du Pont-New England Nuclear Products (Boston, MA). Trihexyphenidyl, gallamine and scopolamine methyl bromide (NMS) were from Sigma Chemical Co. (St. Louis, MO), methoctramine and McN-A-343 from Research Biochemicals Inc. (Natick, MA), biperiden from Knoll Pharmaceutical Co. (Mount Olive, NJ) and pirenzepine from Boehringer Pharmaceuticals (Ridgefield, CT). The following were gifts: AP-DX 116 from Dr. K. Thomae, Boehringer Ingelheim, Germany; himbacine from Dr. W. C. Taylor, University of Sydney, Australia; R-(+)-hyoscymamine from Dr. M. Baldini, University of Florence, Italy; hexahydrodifenidol and silahexocyclium from Dr. G. Lambrecht, University of Frankfurt, Germany; and triptiramine from Dr. C. Melchiorre, University of Bologna, Italy. Antimuscarinic toxins were purified from the venom of the green mamba, Dendroaspis angusticeps, as described by Max et al. (1993a), Liang et al. (1996) and Carsi-Gabrenas (1997).

Striatal tissue was obtained from Sprague-Dawley male rats weighing 200 to 250 g and from domestic shorthair cats. Rats were anesthetized with diethyl ether and decapitated. Cats were anesthe-
tized with pentobarbital and exsanguinated. Brains were removed immediately to ice and dissected from their dorsal aspect. Each cerebral cortex was reflected laterally to expose the hippocampus and striatum, and the former was removed to expose the striatum fully. Striatal tissue was separated from the surrounding cortex with a smooth probe. About 0.1 g was obtained from each rat. Membranes were prepared as described by Potter et al. (1984) and were suspended in 20 mM Tris-HCl buffer containing 1.0 mM MnCl₂ at pH 7.4 (‘Tris-Mn buffer’) or in 50 mM sodium phosphate buffer at pH 7.4 containing 1.0 mM EDTA (‘phosphate-EDTA buffer’), using 50 μl of original tissue. Assays were carried out with fresh membranes from 2.0 mg of tissue. Most assays were carried out in Tris-Mn buffer because the Kᵦᵣ for ³H-pirenzepine is relatively low in this hypotonic buffer, and because we wanted to use this buffer for studies of the binding of agonists to striatal m4 receptors (Potter et al., 1988; Potter and Ferrendelli, 1989; Potter and Purkerson, 1995). Manganese ions have been shown in these prior studies to facilitate the binding of agonists. m1-Toxin and m4-toxin have been shown to be equally effective in hypotonic and physiological media (Max et al., 1993b; Liang et al., 1995).

CHO cells expressing human m4 muscarinic receptors were grown and harvested as described by Max et al. (1993a). Their membranes were resuspended in Tris-Mn buffer, using 20 μl/g of sedimented cells. Assays were carried out with fresh membranes from 5.0 mg of cells.

Binding assays involved the incubation of membranes with toxins, ³H-NMS or ³H-pirenzepine, and various antagonists, followed by the collection and rinsing of membranes on glass fiber filters (Potter et al., 1984). Nonspecific binding was determined in the presence of 1.0 μM (±) QNB. Filters were dried in an oven, immersed in 4 ml of Cytoscent ES (ICN Biomedicals, Costa Mesa, CA) and counted by liquid scintillation at an efficiency of 50 ± 1%. Binding curves were fitted to one- and two-site binding models using an iterative, nonlinear, least-squares, curve-fitting program, and an F-test was used to choose the better fit (GraphPad Prism). Separate fits to binding curves with variable slope were used to estimate Hill coefficients (nₒ).

Because m1-toxin binds irreversibly at 25 and 37°C (Max et al., 1993a, b, c; Carsi-Gabrenas, 1997), it can bind to receptors that have already bound an antagonist (Max et al., 1993b), and because it is obtainable only in sub-milligram amounts, m1 receptors were blocked before the use of radioligands by incubating membranes with a small volume of m1-toxin. To estimate the concentration of m1-toxin necessary to occlude rat striatal m1 receptors, membranes from 2.0 mg of tissue were incubated with increasing amounts of m1-toxin in 0.2 ml of Tris-Mn buffer at 25°C for 20 min, and then additionally with 4.8 ml of 1.0 nM ³H-pirenzepine for 45 min. The concentration of m1-toxin required to reduce total binding to a stable level near nonspecific binding was determined (see “Results”), and found to be the same as the concentration necessary to block pure m1 receptors from CHO cells (1.5 μg/ml = about 200 nM; Carsi-Gabrenas, 1997). These experiments were repeated using 0.8 ml of 1.2 nM ³H-NMS instead of pirenzepine, to determine the fraction of total receptors blocked by m1-toxin. For all subsequent experiments with non-m1 receptors in rat striatal membranes, membranes were first treated with the concentration of m1-toxin necessary to fully block m1 receptors.

Because m4-toxin binds reversibly, the percentage of rat striatal receptors of the m4 subtype was estimated by co-incubating various concentrations of m4-toxin with membranes (already treated with m1-toxin) and 1.0 nM ³H-NMS in 1.0 ml of Tris-Mn buffer at 25°C for 2 hr. The concentrations used for inhibition curves were limited to 100 μg/ml because m4-toxin is not very abundant in the venom of the green mamba (≈2 mg/g dried venom; Liang et al., 1996).

The ability of various competitive antagonists to block non-m1 rat striatal muscarinic receptors was determined by incubating multiple concentrations of each antagonist with membranes from 2.0 mg of rat striatum (treated with m1-toxin), in 5.0 ml of 1.0 nM ³H-NMS at 25°C for 45 min. The ability of some antagonists to block pure m4 receptors was determined in the same way using membranes from 5.0 mg of CHO cells expressing only m4 receptors.

The dissociation of ³H-NMS from non-m1 receptors in rat striatal membranes was determined in phosphate-EDTA buffer because dissociation is faster in this buffer than in Tris-Mn buffer (Potter et al., 1984). Membranes treated with m1-toxin were incubated with 1.0 nM ³H-NMS (0.625 ml/mg tissue) for one hour. Each suspension was then brought to 1.0 μM (±) QNB to permit studies of radioligand dissociation. Samples (1.0 ml containing membranes from 1.6 mg of tissue) were taken during continued incubation at 25°C, and the exponential decay of ³H-NMS binding was estimated (GraphPad Prism).

The rates of dissociation of ³H-pirenzepine from m1 and non-m1 rat striatal receptors were determined in Tris-Mn buffer. Receptors in membranes not treated with m1-toxin were labeled with 2.0 nM ³H-pirenzepine (1.25 ml/mg tissue), and non-m1 receptors were labeled with 40 nM ³H-pirenzepine (0.05 ml/mg tissue), for 1 hr in each case. Membranes were sedimented by centrifugation at 38,000 × g for 15 min and resuspended in buffer containing 1.0 μM (±) QNB. Samples of membranes were taken during continued incubation at 25°C, and at 3.0 hr to estimate nonspecific binding. Sedimentation of the membranes before the measurement of ligand dissociation was necessary to diminish the level of nonspecific binding when 40 nM ³H-pirenzepine was used.

Sections of fresh cat striatum 100-μ thick and weighing approximately 7.0 mg were prepared at 4°C with a vibrotome and studied in oxygenated Krebs-phosphate buffer at 25°C. They were incubated in sequence in: 2.0 ml of buffer containing various concentrations of m1-toxin for 20 min, 2.0 ml of 1.0 nM ³H-NMS or 2.0 ml of 2.0 nM ³H-pirenzepine for 45 min, and then several changes of ice-cold buffer during 30 min. The slices were then homogenized and membranes collected for radioassay. The concentration of m1-toxin necessary to reduce the binding of ³H-pirenzepine to nonspecific levels was the same as that required for rat tissue.

For histochemistry and autoradiography, single hemispheres of the cat brain were frozen in 2-methylbutane at -30°C and sectioned with a cryostat. Serial coronal sections 20-μ thick were mounted on gelatin-coated slides. Some sections were fixed in 3% fresh glutaraldehyde and stained for acetylcholinesterase by the method of Geschwind and Blackstad (1971). Most were transferred to Tris-Mn buffer ± the amount of m1-toxin necessary to block m1 receptors in the vibrotome sections for 30 min at 25°C, then to 1.0 nM ³H-NMS ± 1.0 μM (±) QNB for 60 min at 25°C, and finally to ice-cold buffer alone for 15 min. Sections were then dried and exposed to LKB Ultratime (Kodak) for 3 wk at room temperature, and the film was developed for study (Mash and Potter, 1986).

Results

Figure 1 shows the effect of m1-toxin on the binding of ³H-antagonists to muscarinic receptors in membranes from the rat striatum. The concentration of m1-toxin necessary to block almost all of the binding sites for 1.0 nM ³H-pirenzepine was 1.5 μg/ml, and the same concentration blocked 51% of the total binding sites for 1.0 nM ³H-NMS (50 pmol of a total of 119 pmol/g tissue). Thus 42% of the muscarinic receptors in the rat striatum are m1 receptors.

The effect of m4-toxin on the striatal receptors remaining after the use of m1-toxin is shown in figure 2. The inhibition curve was best fit with a one-site binding model. The maximum blockade produced by m4-toxin indicated that 88% of the non-m1 sites (≈51% of the total receptors) were m4 receptors. Thus about 12% of the non-m1 receptors (≈7% of the total receptors) must be m2, m3 and/or m5 receptors.

The dissociation of ³H-NMS from non-m1 striatal receptors...
was at least biphasic and included a slow component accounting for about 83% of the non-m1 receptors (54% of total receptors) (fig. 3).

Rat striatal receptors not blocked by m1-toxin were studied further with 13 antagonists that interact reversibly with m2-m5 receptors, in order to find out whether any of these agents could discriminate between m4 and other non-m1 receptors (figs. 4 and 5, and other data not shown). Binding data are summarized in table 1. Twelve of these antagonists (all but NMS) have been considered useful for distinguishing muscarinic receptor subtypes. Each curve was fitted best with a one-site binding model showing $r^2$ and $n_H$ values close to one, and each antagonist blocked all the non-m1 receptors.

Thus none of these antagonists distinguished subpopulations among non-m1 receptors. Four IC$_{50}$ values were determined in parallel using pure cloned m4 receptors, and found to be very similar to the IC$_{50}$ values for non-m1 receptors.

The rates of dissociation of $^3$H-pirenzepine from m1 and non-m1 (primarily m4) striatal receptors were measured directly as shown in figure 6. Pirenzepine dissociated twice as rapidly from non-m1 receptors as from m1 receptors.

Studies of the ability of m1-toxin to block muscarinic receptors in Vibratome sections of the adult cat striatum showed that m1-toxin readily blocked sites labeled with 2.0

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Fig. 1. Effect of m1-toxin on the binding of $^3$H-antagonists to muscarinic receptors in membranes from the rat striatum. Membranes were incubated first with m1-toxin in 0.2 ml of buffer, and residual non-m1 muscarinic receptors were then labeled with $^3$H-NMS or $^3$H-pirenzepine. Because m1-toxin binds irreversibly, toxin concentrations are given during the preincubation step. Points are mean values from sextuplicate assays $\pm$ 1.0 $\mu$M QNB for blanks. The S.E.M. for each point was less than 8% of the mean. Total specific binding for NMS and pirenzepine were 21,040 and 1,090 cpm, respectively. Lines are drawn point-to-point. The data show that 1.5 $\mu$g/ml of m1-toxin (about 200 nM) blocked almost all of the binding of 1.0 nM $^3$H-pirenzepine (which is selective for m1 receptors), and 42% of the binding of 1.0 nM $^3$H-NMS (which is nonselective for m1-m5 receptors). Mean values from five experiments were 42 $\pm$ 3%.

Fig. 2. Effect of m4-toxin on the binding of 1.0 nM $^3$H-NMS to rat striatal receptors remaining after the blockade of m1 receptors with m1-toxin. The first nine points are mean values from triplicate assays in one experiment. The S.E.M. for each point was less than 10% of the mean. The last point is the mean of two determinations from separate experiments. The curve was fit best to a one-site binding model and had an $r^2$ = 0.999. The extrapolated bottom of the inhibition curve indicates that m4-toxin blocked 88% of the non-m1 receptors.

Fig. 3. Dissociation of $^3$H-NMS from rat striatal membranes after blockade of m1 receptors with m1-toxin. Membranes were labeled with 1.0 nM $^3$H-NMS, and the dissociation of this ligand was then studied in the presence of 1.0 $\mu$M (±) QNB. The first and last three points were carried out in sextuplicate, and the other points were carried out in duplicate. The line is an exponential decay curve ($r^2$ = 0.989) fit to one faster ($t_{1/2}$ = 0.3 min; 17% total) and one slower-dissociating component ($t_{1/2}$ = 31 min; 83% of total). Nonspecific binding (determined at 3 hr) has been subtracted.

Fig. 4. Competition between three reversible antagonists and 1.0 nM $^3$H-NMS for non-m1 receptors in rat striatal membranes. Points are mean values from sextuplicate assays; the S.E.M. was less than 9% of the mean, and within the points shown. Curves were fit best to a one-site binding model (mean $r^2$ = 0.999). IC$_{50}$ values and Hill coefficients are summarized in table 1.
approximately 51% of the total binding sites for 1.0 nM 3H-NMS in rat striatal membranes. Points are mean values from sextuplicate assays; the S.E.M. was less than 10% of the mean, and within the points shown. Curves were fit best to a one-site binding model (mean $r^2 = 0.999$). IC50 values and Hill coefficients are summarized in Table 1.

nM 3H-pirenzepine, and 43% of the sites labeled with 1.0 nM 3H-NMS (Purkerson, 1995; not shown). Figure 7 shows the localization of 3H-NMS binding in this tissue before and after the treatment of cryostat sections with m1-toxin. In each case binding was nearly uniform over the caudate and putamen. In contrast, parallel sections showed matrix rich in acetylcholinesterase and esterase-poor striosomes (Purkerson, 1995), in confirmation of prior data (Graybiel and Ragsdale, 1983).

Discussion

Our studies show that 42% of the binding sites for 1.0 nM 3H-NMS in the rat striatum can be blocked readily by m1-toxin. Thus approximately 42% of the muscarinic receptors in the rat striatum are m1 receptors. (Small corrections to the levels of receptors based on their degree of saturation with 3H-NMS are noted below.) This value is considerably higher than the 30% value found in immunoprecipitation studies (review: Levey, 1993). The disparity may be due to differences in the dissection of the striatum, or to difficulties in quantifying m1 receptors by immunoprecipitation (Li et al., 1991).

The use of m4-toxin after m1-toxin showed that approximately 51% of the total binding sites for 1.0 nM 3H-NMS in rat striatal membranes are m4 receptors. This result is in accord with the 46% value calculated by Waeltbroeck et al. (1990) on the basis of inhibition data with himbacine and methoctramine. Neither set of results correlates well with the 29% value found by immunoprecipitation (Levey, 1993).

After the treatment of striatal membranes with m1-toxin, 3H-NMS dissociated slowly from about 83% of the labeled non-m1 sites. This value is similar to the percentage of non-m1 sites blocked by m4-toxin, but cannot be used alone to identify m4 receptors in the striatum, since NMS dissociates as slowly from m3 as m4 receptors (Ferrari-Dileo et al., 1994).

Twelve antagonists that have been used previously to study muscarinic receptor subtypes (all but NMS in Table 1) yielded a one-site inhibition curve with complete blockade of striatal non-m1 receptors, whereas m4-toxin disclosed heterogeneity (88% m4 and 12% other receptors). We conclude that m4-toxin is the most useful antagonist for distinguishing between m4 and other non-m1 receptors.

Under the assay conditions used in this study, m2 receptors have a $K_i$ of 0.125 nM for 3H-NMS (Potter et al., 1991), and cloned m4 receptors have an estimated $K_i$ of 0.054 nM (Table 1). One nM radioligand was chosen to achieve nearly equal saturation of these receptors (95% for m4, 89% for m2), and comparable labeling of m1, m3 and m5 receptors. When corrected for this degree of saturation, the $B_{max}$ value for rat striatal m4 receptors is about 64 pmol/g tissue, based on the population of sites blocked with m4-toxin.

Waeltbroeck and others have used the different rates of dissociation of 3H-NMS and other ligands to achieve selective labeling of m4 and other muscarinic receptors (Waeltbroeck et al., 1990; Flynn and Mash, 1993; Ferrari-Dileo et al., 1994). In our study, 3H-pirenzepine dissociated twice as rapidly from non-m1 (primarily m4) receptors as from m1 receptors. These are the first direct studies of the dissociation of pirenzepine from m4 receptors in a brain tissue. Although m4 receptors are not easy to label with 3H-pirenzepine, the duration of its retention on m4 receptors after labeling is long enough to permit autoradiographic studies of m4 receptors in tissues treated with m1-toxin (Max et al., 1993a).

We tested the idea that neurons with m4 receptors are localized preferentially in striosomes for three reasons: 1) the neuronal input to striosomes and matrix is different (Bolam and Bennett, 1995), 2) segregation of m4 receptors in striosomes might affect recordings from micropipettes inserted blindly into the striatum and 3) changes in the relative levels of muscarinic receptors in the striosomes vs. matrix might be useful anatomical measures of the degree of m4 blockade achieved in an experiment conducted in vivo. The data in figure 7 showed that non-m1 receptors are distributed equally in the striosomes and matrix. Thus projection neurons with m4 receptors must have their cell bodies in both.
compartmental movements of such rats. If m4 blockade helps, then drug selective blockade of striatal m4 receptors could help the disease. A relatively simple way to test m4 blockade has been to use drugs that improve the rotational movement of rats with 6-hydroxydopamine lesions of the internal globus pallidus.

Data mean IC50 values from figures 4 and 5 and other data not shown. Each experiment was carried out in sextuplicate; n = the number of experiments, and n was 1 unless otherwise noted. The primary point of these experiments was to discover whether or not the antagonist distinguished subpopulations of non-m1 receptors. Ki values for m4 receptors are from the literature: ref 1 = Buckley et al., 1988; ref 2 = Bolden et al., 1992; ref 3 = Bolden et al., 1991; ref 4 = Dong et al., 1995; ref 5 = Dejer et al., 1991; ref 6 = Maggio et al., 1994. Ki values for striatal non-m1 receptors were estimated using the Cheng-Prusoff equation, based on the published Kd value for 3H-NMS given for pure rat receptors (Buckley et al., 1989).

Fig. 7. Autoradiographic localization of 3H-NMS in cryostat sections of the cat striatum ± m1-toxin. Sections were labeled with 1.0 nM 3H-NMS. Note uniform labeling of the bulk of the striatum before (left) and after (right) irreversible blockade of m1 receptors with m1-toxin, with no suggestion of concentration of receptors in the striosomes or matrix compartments of this tissue. The bar is 4.0 mm, and the arrowheads point to dense labeling of the islands of Calleja. Sections prepared with 1.0 μM QNB as well as 1.0 nM 3H-NMS were completely blank (not shown). The sections shown were one pair among many processed; all showed the same result.

It has been demonstrated that the nonspecific blockade of muscarinic receptors with systemically administered scopolamine improves the rotational movement of rats with 6-hydroxydopamine lesions in one substantia nigra (Morelli et al., 1993). Our primary reason for conducting the present experiments was to develop methods that could test the idea that selective blockade of striatal m4 receptors could help the movements of such rats. If m4 blockade helps, then drug companies will presumably be very interested in developing m4-selective antagonists for the treatment of Parkinson’s disease. A relatively simple way to test m4 blockade has emerged from our finding that most non-m1 striatal receptors are m4 receptors. We believe that complete, stable and bilateral m1 blockade can be accomplished by the bilateral intracerebral infusion of m1-toxin, in large part because of the irreversible binding of the toxin at 37°C. The fact that most striatal receptors are then m4 receptors suggests that any nonselective muscarinic antagonist will act in the striatum primarily on m4 receptors. We conclude that systemic scopolamine can probably be used after m1-toxin in vivo to control GABAergic striatal projection neurons in the direct pathway.

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Send reprint requests to: Dr. Lincoln T. Potter, Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine, P.O. Box 016189, Miami, FL 33101.