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Stable Allosteric Binding of m1-Toxin to m1 Muscarinic Receptors

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SUMMARY

m1-Toxin was found to slow the dissociation of [^{3}H]*N*-methylscopolamine (NMS) and [^{3}H]pirenzepine from m1 muscarinic receptors expressed in the membranes of Chinese hamster ovary cells. When toxin-NMS-receptor complexes were formed in membranes and then dissolved in digitonin, or when these complexes were formed in solution, the toxin completely stopped the dissociation of [^{3}H]NMS for 6 hr at 25°C. Toxin-receptor complexes formed in membranes or in solution were also highly stable in solution at 25°, as shown by the ability of the toxin to prevent the binding of [^{3}H]quinuclidinyl benzilate (QNB). [^{3}H]QNB-receptor complexes were equally stable, whereas unliganded soluble receptors lost most of their ability to bind QNB within an hour. Toxin-receptor complexes could be partially dissociated by incubation at 37° in the presence of digitonin and [^{3}H]QNB, and the freed receptors were then labeled. These results demonstrate that m1-toxin binds allosterically and pseudoirreversibly to m1 receptors, and that the toxin can stabilize the outward-facing pocket of m1 receptors which contains and binds competitive antagonists. The allosteric nature of the binding of m1-toxin should prove to be useful for such unusual purposes as stabilizing the binding of readily reversible and/or nonselective ligands specifically to m1 receptors, for purifying labeled or unlabeled receptors by affinity techniques which recognize the toxin, for recognizing receptors with genetically or biochemically altered primary binding sites, and for stabilization of the native conformation of m1 receptors for structural studies.

m1-Toxin is a newly isolated antimuscarinic toxin from the venom of the Eastern green mamba, *Dendroaspis angusticeps*; it has a molecular mass of 7361 daltons (1). The toxin binds to genetically defined m1 muscarinic receptors in membranes in less than a minute, even when subsaturating amounts are used at 4°, and it remains bound for at least 8 hr at 25° (1, 2). Bound toxin blocks the binding of [^{3}H]antagonists to human and rat m1 receptors in living tissues, on cells, in membranes, and in solution, and it blocks the ability of the agonist, carbachol, to promote the turnover of phosphoinositides in brain slices (2). At the present time, m1-toxin is the most specific ligand known for the extracellular face of m1 receptors: amounts that are just sufficient to fully block m1 receptors can be used at concentrations that have no antagonist effect on m4 receptors, and after the use of higher concentrations, a partial blockade of m4 receptors can be reversed by allowing the toxin to dissociate with a half-time of about 20 min at 25°. Even high concentrations of the toxin do not antagonize m2, m3, or m5 receptors in membranes or in solution (1, 2).

In the course of studying the properties of m1-toxin, we have noted that it blocks the dissociation as well as the association of [^{3}H]antagonists, indicating that the toxin can bind allosterically to receptors that already have a ligand bound to their primary binding site. Many other compounds bind allosterically to muscarinic receptors and can slow either the association or the dissociation of [^{3}H]antagonists; these compounds include polyquaternary drugs like gallamine, and the tertiary amine, tetrahydroaminoacridine (3, 4). Tetrahydroaminoacridine can completely stop the dissociation of *N*-methylscopolamine (NMS) from m1 receptors at a concentration of 0.3 mM (4). However, none of these other compounds stabilizes m1 receptors specifically or pseudoirreversibly like m1-toxin, and few have been studied for their effects on soluble receptors. Since the allosteric binding of m1-toxin is likely to prove particularly useful for the recognition, stabilization, and purification of soluble m1 receptors, this paper emphasizes experiments which demonstrate that m1-toxin stabilizes the binding of [^{3}H]NMS to soluble m1 receptors for many hours at 25°.

Methods

Radioligands. [*N-methyl*-^{3}H]-Methscopolamine ([^{3}H]NMS; 75 Ci/mmol), *L*-[benzylic-4,4′-^{3}H(*N*)]-quinuclidinyl benzilate ([^{3}H]QNB; 45 Ci/mmol), and [*N-methyl*-^{3}H]pirenzepine (70 Ci/mmol) were purchased from Dupont-NEN Products, Boston, MA.

Preparation of cell membranes. Chinese hamster ovary (CHO)

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ABBREVIATIONS: NMS, *N*-methyl-scopolamine; [^{3}H]NMS, [*N-methyl*-^{3}H]-methscopolamine; QNB, quinuclidinyl benzilate; [^{3}H]QNB, *L*-[benzylic-4,4′-^{3}H(*N*)]-quinuclidinyl benzilate; CHO, Chinese hamster ovary.

cells containing cloned human m1 muscarinic receptors were grown and harvested as described by Max *et al.* (1). Membranes were prepared from CHO cells by homogenization and centrifugation in 50 mM sodium phosphate buffer, pH 7.4, containing 10 mM Na_3 EDTA and 0.1 mM freshly prepared phenylmethylsulfonyl fluoride (5). They were resuspended in 50 mM sodium phosphate buffer at pH 7.4 containing 1.0 mM EDTA (phosphate-EDTA buffer) for assays using [^{3}H]NMS or [^{3}H]QNB, and in 20 mM Tris-HCl buffer at pH 7.4 containing 1.0 nM $MnCl_2$ (Tris-Mn buffer) for assays using [^{3}H]pirenzepine; twenty ml of buffer were used for the membranes from each gram of sedimented cells (5). All assays in which receptors were labeled with a [^{3}H]-antagonist in membranes were performed with freshly prepared membranes from 5 mg of cells (0.1 ml); these cells had 0.2–0.4 pmol of m1 receptors. Receptors were dissolved by stirring membranes for an hour in ice-cold phosphate-EDTA buffer containing 1% digitonin (20 ml/g of sedimented cells), followed by centrifugation at 38,000 × g_{max} for 10 min. Assays in which receptors were labeled with an antagonist in solution were performed with freshly prepared protein from 10 mg of cells (0.2 ml).

Preparation and assay of m1-toxin. m1-Toxin was purified from the venom of *D. angusticeps* as described by Max *et al.* (1). The antimuscarinic activity of the toxin was assessed in phosphate-EDTA buffer at 25° with a two-step assay involving preincubation of CHO cell membranes (0.1 ml) with various amounts of m1-toxin (in 0.1 ml), followed by the measurement of residual receptors with 10 ml of 0.1 nM [^{3}H]NMS (2). The minimum amount of toxin necessary to block 95% of the m1 receptors in membranes from 5 mg of CHO cells was defined as 1 unit of toxin. Because of considerable inactivation of the toxin during its preparation, 1 unit of active toxin usually amounted to a 20-fold molar excess of toxin protein (about 6 pmol or 0.05 μg) over m1 receptors.

Experiments beginning with membrane-bound receptors. Receptors in membranes were labeled by incubation with 1.0 nM [^{3}H] NMS, 1.0 nM [^{3}H]QNB or 5.0 nM [^{3}H]pirenzepine for 30 min at 25°, using 1.0 ml for the membranes from each 5 mg of cells. Some membranes were treated with m1-toxin (1 unit in 0.2 ml buffer per 5 mg of cells) for 20 min at 25°. After treatment, membranes were sedimented by centrifugation at 38,000 × g_{max} for 10 min in order to discard unbound radioligand and/or toxin. For dissociation rate experiments, membranes were then resuspended in 10 ml of buffer per assay and incubated with 1.0 μM (±) QNB for 1 to 7 hr at 25° to assess the rate of dissociation of the radioligand. Membranes were collected on glass fiber filters, rinsed with ice-cold buffer, and dried, and radioactivity was counted by liquid scintillation at an efficiency of 48–52% (2). In other experiments, receptors treated with [^{3}H]NMS and m1-toxin in membranes were sedimented, dissolved in digitonin, and incubated with 1.0 μM (±) QNB for 1 to 6 hr to assess the stability of toxin-NMS-receptor complexes after solubilization. In another set of experiments, membranes treated only with m1-toxin were sedimented, dissolved, and assayed at several temperatures with 1.0 nM [^{3}H]QNB (1 ml for the protein from 10 mg of cells) to assess the stability of toxin-receptor complexes during and after solubilization. Finally, the recovery of receptors labeled with [^{3}H] QNB alone was assessed after solubilization by comparing the amount of bound QNB in membranes and in solution. Soluble receptor protein was either collected on glass fiber filters soaked in 0.3% polyethylenimine (Sigma Chemical Co., St. Loius, MO) (6), or was precipitated on ice with protamine sulfate and polyethylene glycol and recovered on filters (7). [^{3}H]NMS was used as the ligand for dissociation rate experiments because it dissociates more rapidly than QNB, and [^{3}H] QNB was used as the ligand when maximum receptor stability or recovery was the issue in question.

Experiments beginning with soluble receptors. Receptors in digitonin were labeled at 25° with 1.0 nM [^{3}H]NMS or 1.0 nM [^{3}H] QNB (0.4–1.0 ml for the protein from 10 mg of cells) for 30 min, or with m1-toxin (2 units for the protein from 10 mg of cells in 0.4 ml) for 20 min, or both, in this sequence. For studies of the rates of dissociation of [^{3}H]NMS from NMS-receptor and toxin-NMS-receptor complexes in solution, the receptors were then diluted with 1.0 μM (±) QNB (to 1 ml per assay) and incubated at 25° for 1 to 6 hr to measure the amount of radioligand retained. For studies of the stability of [^{3}H] QNB- and toxin-liganded receptors in solution, receptors were incubated at 25° for 1 to 6 hr with 1.0 nM [^{3}H]QNB (1 ml for the protein from 10 mg of cells) to measure the number of receptors still capable of binding an antagonist. For studies of the stability of unliganded receptors, soluble protein was incubated in digitonin for 5 to 80 min at 25° before the receptors were labeled with QNB.

For assays of both soluble and membrane-bound receptors, nonspecific binding was assessed in the presence of 1.0 μM (±) QNB.

Radio-iodination of m1-toxin. Multiple attempts were made to label m1-toxin with iodine or ^{125}I, using three methods: chloramine-T (Iodo-Beads or Iodo-Gen; Pierce Co., Rockford, IL), lactoperoxidase (Enzymobeads; Biorad Labs, Richmond, CA), or the Bolton-Hunter reagent (Sulfo-SHPP; Pierce Co.) (8). For the experiment discussed in the Results, 0.6 μg of pure m1-toxin was incubated for 2 min at 22° with one Iodo-Bead and 0.1 μg of NaI in 0.6 ml of 0.1 M sodium phosphate buffer at pH 7.0. The blocking activity of the iodinated toxin was then assessed by the two-step assay used to quantify the activity of unlabeled toxin (1, 2).

Results

The experiments in this paper required the sequential treatment of m1 receptors with a radioactive antagonist and one or more of the following: m1-toxin, 1 μM (±) QNB, and digitonin, with changes in the order of treatment as necessary to examine the stability of unliganded receptors or complexes of receptors with antagonist, toxin, or both, in membranes and in solution.

Fig. 1 shows the rates of dissociation of [^{3}H]NMS from NMS-receptor and toxin-NMS-receptor complexes formed in solution in digitonin and then exposed to 1.0 μM nonradioactive QNB. Without the toxin, the radioligand dissociated with a half-time of 47 min at 25°, whereas in the presence of the toxin, dissociation was negligible for 6 hr. It is evident from these results that

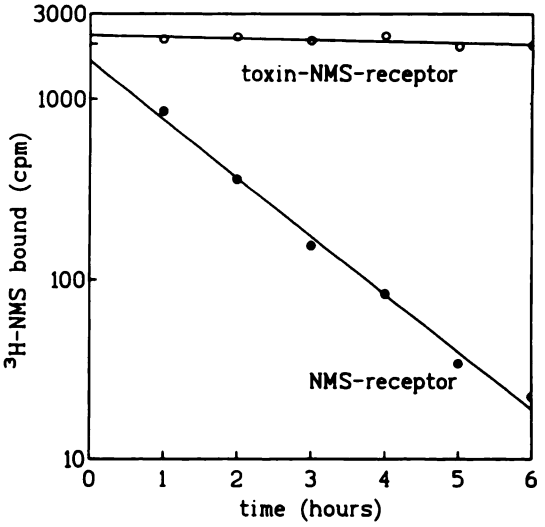

Fig. 1. Rates of dissociation of [^{3}H]NMS from NMS-receptor and toxin-NMS-receptor complexes formed in solution. Soluble m1 receptors from 10 mg of CHO cells were exposed in sequence at 25° to [^{3}H]NMS, buffer or toxin, and then to 1.0 μM nonradioactive QNB to prevent the rebinding of dissociated NMS. In this and subsequent figures (unless otherwise noted), points are mean values from sextuplicate assays, nonspecific binding has been subtracted, standard deviation values were less than 8% of the means, lines are linear regressions, and rates are given in the text. Note that the dissociation of NMS was stopped in the presence of m1-toxin.

m1-toxin and NMS must bind simultaneously to different sites on m1 receptors, that the toxin must remain bound for at least 6 hr to prevent the dissociation of NMS, that m1-toxin stabilizes the binding site of receptors for NMS, and that the stable allosteric binding of the toxin is not affected by the simultaneous presence of a high concentration of QNB.

Fig. 2 shows the stability of unliganded receptors and [^{3}H]QNB-receptor and toxin-receptor complexes formed in solution, and then studied in the presence of 1.0 nM [^{3}H]QNB at 25°. Unliganded receptors lost most of their ability to bind QNB within an hour. [^{3}H]QNB-receptor complexes were completely stable between 1 and 6 hr in solution, confirming the ability of antagonists to stabilize m1 receptors (9, 10). There was negligible binding of [^{3}H]QNB to receptors exposed first to m1-toxin. Evidence that the toxin does not damage the ability of m1 receptors to bind [^{3}H]QNB is given below.

Fig. 3 demonstrates the ability of m1-toxin to slow the dissociation of [^{3}H]NMS from m1 receptors in CHO cell membranes. Unexpectedly, the rate of dissociation of NMS from toxin-NMS-receptor complexes ($t_{1/2}$ = 222 min) was faster in membranes (Fig. 3) than in solution (Fig. 1), although the rate for NMS-receptor complexes was slightly slower in membranes ($t_{1/2}$ = 102 min). The data in Fig. 3 do not demonstrate whether NMS can dissociate from toxin-NMS-receptor complexes in membranes or whether the toxin dissociates before NMS. Experiments with labeled toxin as well as labeled antagonists will be necessary to resolve this issue.

Fig. 4 shows that m1-toxin also slows the dissociation of [^{3}H]pirenzepine from m1 receptors in CHO cell membranes. The $t_{1/2}$ values without and with the toxin were 36 and 124 min, respectively. Thus m1-toxin can bind allosterically to m1 receptors containing a nonquaternary ligand. We considered the possibility that 2% glutaraldehyde might fix toxin-pirenzepine-receptor complexes and thereby trap this normally reversible radioligand on the receptors. Unfortunately, the treatment of membranes with glutaraldehyde caused a very rapid dissociation of [^{3}H]pirenzepine from toxin-pirenzepine-receptor complexes (not shown).

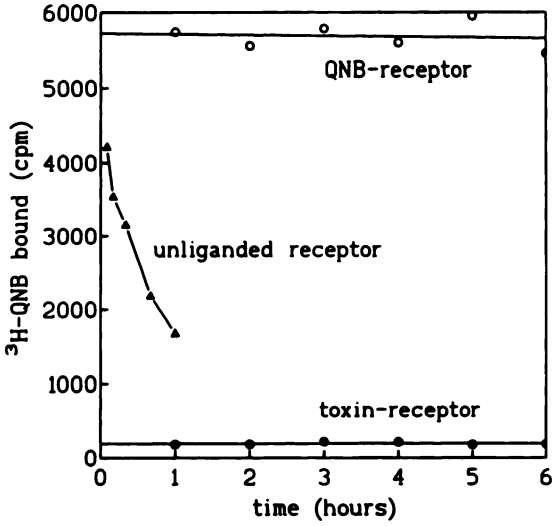

Fig. 2. Stability of unliganded receptors and [^{3}H]QNB-receptor and toxin-receptor complexes formed in solution. Soluble receptors from 10 mg of cells were exposed in sequence at 25° to buffer or toxin, and then to 1.0 nM [^{3}H]QNB to label all remaining receptors capable of binding QNB. "Unliganded" receptors were incubated for 5 to 60 min before exposure to [^{3}H]QNB; the line for these receptors is drawn point to point. Note the rapid loss of unliganded receptors, the stability of QNB-labeled receptors, and the absence of QNB-binding in the presence of the toxin.

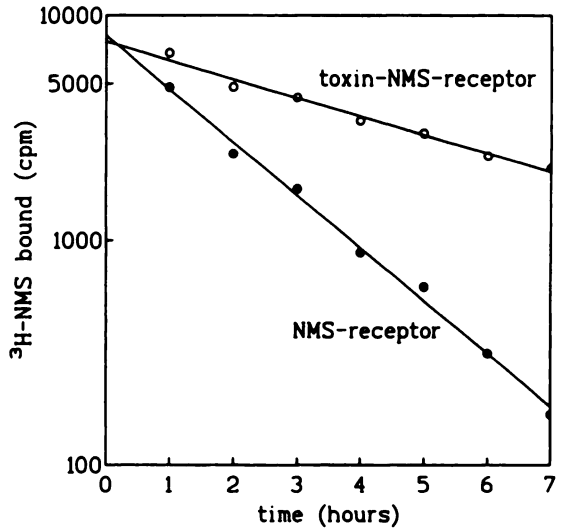

Fig. 3. Rates of dissociation of [^{3}H]NMS from NMS-receptor and toxin-NMS-receptor complexes in CHO cell membranes. Membranes from 5 mg of cells were exposed in sequence at 25° to [^{3}H]NMS, buffer or toxin, and then to 1.0 μM QNB. Compare these results found in membranes with those in Fig. 1, which were obtained with soluble receptors.

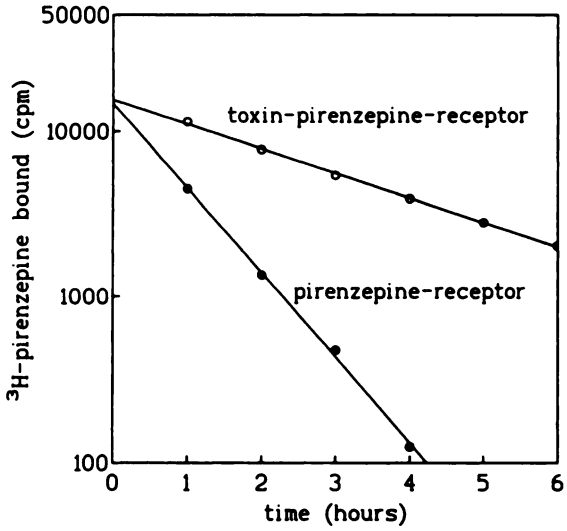

Fig. 4. Rates of dissociation of [^{3}H]pirenzepine from pirenzepine-receptor and toxin-pirenzepine-receptor complexes in CHO cell membranes. Membranes from 5 mg of cells were exposed in sequence at 25° to the radioligand, buffer or toxin, and then to 1.0 μM QNB.

The next question asked was whether m1-toxin is capable of stabilizing m1 receptors during as well as after their solubilization, as might be expected. Toxin-NMS-receptor complexes formed in membranes were found to be completely stable after solubilization at 25° for 6 hr (not shown). Thus the continuing presence of free m1-toxin (as was the case for the results in Fig. 1) is not necessary for the stabilization of these complexes. The recovery of receptors under our solubilization conditions was about 85%.

Fig. 5 shows that toxin-receptor complexes formed in membranes could be partially dissociated after solubilization at 4° in the absence of free toxin, by incubation with [^{3}H]QNB at

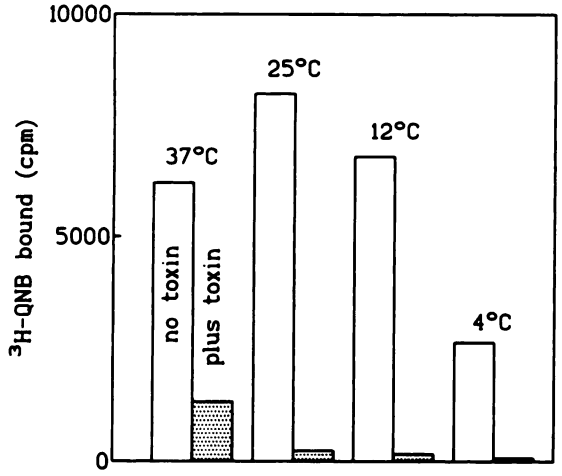

Fig. 5. Partial dissociation of toxin-receptor complexes. Unliganded receptors and toxin-receptor complexes in CHO cell membranes were dissolved in digitonin, and then assayed with 1.0 nM [^{3}H]QNB at the temperatures noted. Note that some of the receptors treated with m1-toxin in membranes (stippled bars) were able to bind QNB at 37° after solubilization.

37°. Toxin-receptor complexes assayed at lower temperatures (4–25°) did not dissociate significantly. The fact that some toxin-receptor complexes can dissociate at 37°, leaving receptors capable of binding [^{3}H]QNB, means that the toxin does not damage m1 receptors. In other experiments, we have shown that toxin-receptor complexes can remain stable in solution for many hours at 25°, but still bind QNB when warmed to 37° (not shown).

Many of the experiments in this study would have been facilitated by the availability of radioactive m1-toxin, to examine directly the formation and stability of toxin-receptor and toxin-antagonist-receptor complexes. It was particularly difficult to assess the recovery of toxin-receptor complexes during solubilization. Unfortunately, all attempts to label m1-toxin with iodine have resulted in marked losses of the blocking activity of the toxin (11). For example, we found that after m1-toxin was exposed to nonradioactive iodine in the presence of an Iodo-Bead, amounts of the toxin up to 2 units had no effect on the binding of [^{3}H]NMS to m1 receptors in CHO cell membranes (not shown). By comparison, both Jerusalinsky *et al.* (12) and Adem *et al.* (13) were able to radio-iodinate two other, reversible, antimuscarinic toxins from the venom of the green mamba, and one of these (MTX2) was shown to retain blocking activity.

Discussion

The present studies demonstrate that m1-toxin binds to m1 muscarinic receptors in membranes or in solution whether or not the primary binding site of each receptor is occupied by an antagonist. The ability of the toxin to prevent the association of [^{3}H]QNB with m1 receptors or the dissociation of [^{3}H]NMS from m1 receptors in solution for many hours at room temperature shows that the toxin binds to m1 receptors with exceptionally high affinity and that it stabilizes the receptor pocket which binds competitive ligands. Both QNB and m1-toxin were capable of preventing the losses of receptors which occur soon after receptor solubilization, and it is logical to suppose that the combination of both ligands provides the most optimum way known at this time to stabilize the native state of these receptors.

The manner in which m1-toxin binds to m1 receptors can be partially surmised from the present results and prior data. Current models of the structure of m1 receptors are based on the known three-dimensional structure of bacteriorhodopsin, and suggest that m1 receptors are composed of a ring of seven transmembrane regions surrounding a water-filled pocket that extends from the extracellular fluid toward the center of the membrane. Muscarinic and competitive antimuscarinic compounds are believed to lie within this pocket and to interact with amino acids within the plane of the lipid membrane (14, 15 and references therein). The fact that the toxin can bind to receptors which have already bound an antagonist implies that the toxin does not project much into the water-filled pocket which holds traditional antagonists. It is also known that most of the amino acids in the transmembrane regions of the five known muscarinic receptors are identical or conserved; they are, therefore, unlikely candidates for specific interactions between m1-toxin and m1 receptors. In fact, only 6 of the 155 putative transmembrane amino acids in rat m1 muscarinic receptors are unique to m1 receptors or are not conserved between m1 and m2-m5 receptors (14), and of these, only alanine-26 and threonine-31 in the first transmembrane domain are close enough to the membrane surface to be likely to interact with m1-toxin; the other four are alanine-38, threonine-41, cysteine-205, and threonine-206. In contrast, 13 of the 77 extramembranous amino acids of rat m1 receptors are unique to this receptor or are not conserved (14; see Fig. 6); some of these amino acids are probably critical for the selective binding of m1-toxin to m1 receptors. Eight of these 13 are on the amino-terminal end of the molecule, none are in outer loop 1, two are in outer loop 2, and three are in outer loop 3. Because

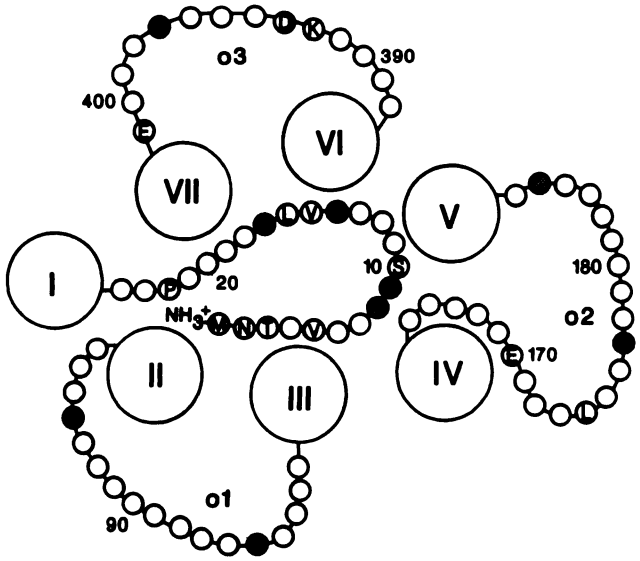

Fig. 6. Extramembranous amino acids in m1 muscarinic receptors which may be responsible for the specific binding of m1-toxin. The large circles depict the seven putative transmembrane regions of rat m1 receptors, and the small circles the 77 putative extramembranous amino acids (14). The lettered amino acids are either unique to m1 receptors or are not conserved in m2-m5 receptors; these are the most likely sites for the specific binding of m1-toxin. Residues that are identical or conserved between m1 and m4 receptors are shown as solid and stippled circles, respectively; some of these may be important for the reversible binding of m1-toxin to m4 receptors.

binding of the toxin to the amino-terminal end of the receptor plus outer loops 2 and/or 3 would be more likely to stabilize the primary binding site than interactions between only the toxin and the terminal end of the receptor, we suspect multiple points of interaction, and that the toxin effectively caps the whole receptor pocket. The very rapid binding of m1-toxin to m1 receptors, even on ice (1), implies that the fit between the opposing loops of the toxin and receptor is excellent and that it requires very little alteration for optimum interaction. It is therefore likely that m1-toxin stabilizes a conformation of m1 receptors that is very close to the resting, unliganded state.

Although some of the amino acids that are unique to m1 receptors must be important for specific toxin binding, the residues noted above are not present in m4 receptors (14) and cannot account for the reversible binding of m1-toxin to m4 receptors (1). Fig. 6 shows three amino acids that are identical in m1 and m4 receptors, and six others that are conserved, although none of these are found only in m1 and m4 receptors (14). It is therefore likely that there are combinations of extramembranous amino acids, including some conserved residues that are not identified in Fig. 6, which are important for the binding of m1-toxin to m4 receptors, and perhaps therefore also to m1 receptors.

The usefulness of m1-toxin depends primarily upon its ability to bind to and block m1 muscarinic receptors with very high affinity and specificity. The fact that the toxin diffuses readily in intact tissue and acts on the extracellular face of these receptors (1, 2) makes it useful for physiological and anatomical studies of surface receptors inserted in neuronal membranes (in distinction to receptors at sites of synthesis or passage along axons). The fact that the toxin is a protein also allows it to be fixed after its binding for very high resolution microscopy. In addition to these points, the fact that m1-toxin binds allosterically has at least four other advantages for various studies. One already noted is the ability of the toxin to stabilize the native conformation of m1 receptors. This should prove useful for structural studies of these receptors by techniques like x-ray crystallography. Second, the toxin can help to stabilize the binding of other ligands which are not as specific and/or long acting. For example, [^{3}H]pirenzepine can be held on m1 receptors with m1-toxin, while the ligand dissociates from m4 receptors. Third, the toxin provides an attachment site on toxin-receptor complexes for affinity chromatography, antibodies, *etc.* Lastly, the toxin permits the specific recognition of m1 receptors regardless of the status of their primary binding sites. This can or may permit the purification of receptors already labeled with an antagonist or with a site-directed agent (*e.g.*, *N*-ethylmaleimide, acetylethylcholine mustard), studies of receptors which are unable to bind competitive ligands (*e.g.*, after phosphorylation), and even studies of receptors with genetically altered primary binding sites.

Acknowledgments

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