Design, Synthesis, and Biological Activity of Methoctramine-Related Polyamines as Putative G_i Protein Activators

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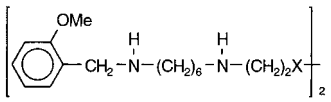
Received August 2, 2001

Abstract: The universal template approach provided a prospect of modifying methoctramine (**2**) structure. Thus, polyamines **3**−**7** were designed in which the flexibility of the diaminohexane spacer of **2** was replaced by a bipiperidinyl moiety. In electrically stimulated guinea pig left atria, these novel polyamines, unlike prototype **2**, displayed a potent intrinsic activity, which was in contrast with the muscarinic antagonism shown in binding studies by some of them (**3** and **4**) and was inhibited by benzalkonium chloride, an inhibitor of G_i proteins.

Introduction. It has been suggested that a polymethylene tetraamine backbone may represent a universal template on which suitable pharmacophores can be inserted to achieve selectivity for any given receptor.[1,2] Accordingly, we have demonstrated that benextramine (**1**) (Chart 1), developed as an irreversible (nonequilibrium in the kinetic sense) antagonist at both α_1 and α_2-adrenoreceptors,[3] could be used as a lead compound for the design of polyamines to achieve specific recognition of muscarinic receptors. This research led to the discovery of methoctramine (**2**),[4] which is widely used as a pharmacological tool for muscarinic receptor subtype characterization (Chart 1).[5] In turn, appropriate structural modifications performed on the structure of **2** have allowed us to obtain new polyamines endowed with high affinity and selectivity for muscarinic receptor subtypes[6] and nicotinic receptors as well.[7] Furthermore, using **1** as the focus, polyamines have been designed to achieve specific recognition for different biological targets such as neuropeptide Y receptors[8] and acetylcholinesterase.[9]

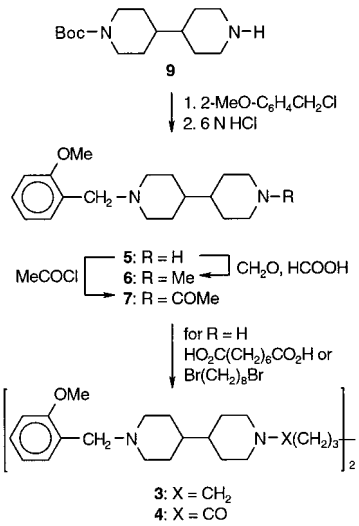
Tetraamine **2** can assume many low-energy conformations in an aqueous environment because of its flexible polymethylene chain. Therefore, more rigid analogues are needed to determine whether flexibility is an important determinant of potency with respect to muscarinic receptors. In this regard, we have demonstrated that changes in flexibility may enable one to design polyamine-containing compounds with specificity for nicotinic receptors over muscarinic receptors.[7]

Chart 1



Benextramine (**1**): X = S
Methoctramine (**2**): X = $(CH_2)_2$

Scheme 1[a]



[a] Boc = Me_3COCO-.

To further investigate the effect of reduction in flexibility, we have synthesized tetraamine **3** in which the diaminohexane spacer of **2** has been replaced by a bipiperidinyl moiety. Since we have already verified that the inner amine functions of tetraamines can be transformed into amide groups without affecting the affinity toward muscarinic receptors,[2] we have also investigated the corresponding diamine diamide **4**.

Polyamines **2**, **3**, and **4** are potent competitive antagonists at muscarinic receptors. However, polyamines **3** and **4**, but not **2**, display a potent inotropic effect in atrial preparations. It is well known that a negative inotropic response in the atrium is typical for muscarinic agonists but not for antagonists, which do not possess intrinsic activity. Such an effect is clearly in contrast with the competitive antagonism observed in binding assays on cloned muscarinic receptors. In an attempt to explain this unexpected biological behavior of polyamines **3** and **4**, we investigated also compounds **5**−**7**, obtained by splitting **3** and **4** into two halves. The rationale for this choice stands on the observation that the diamines obtained by performing a similar modification on the structure of prototype **2** produced very weak antagonists toward muscarinic M_2 receptors.[6] Consequently, we hoped that **5**−**7** would retain the intrinsic activity of parent compounds **3** and **4** in atrial tissue while loosing affinity for muscarinic receptors.

Chemistry. The compounds used in this investigation were synthesized by standard procedures as shown in Scheme 1.[10]

Diamine **5** was obtained by reaction of **9** with 2-methoxybenzyl chloride followed by removal of the protecting group. Diamine **6** and tetraamine **3** were obtained by

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10.1021/jm0155594 CCC: $20.00
Published on Web 10/27/2001

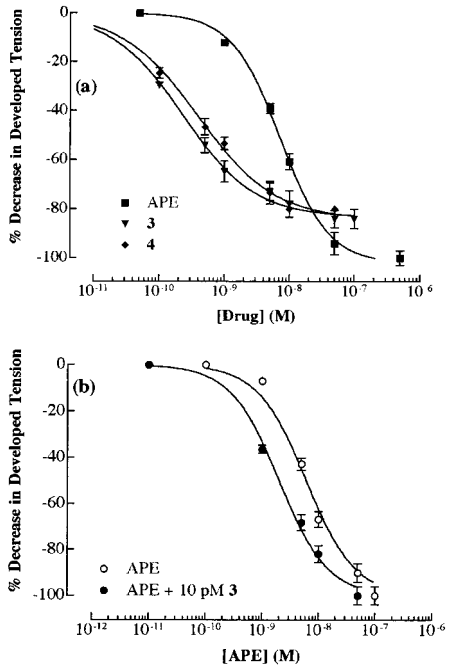

Figure 1. Cumulative concentration−response curves (a) for APE, **3**, and **4** and (b) for APE in the absence and presence of **3** in electrically paced (1 Hz) guinea pig left atria. Each point is the mean ± SE of four experiments.

alkylation of **5** with formaldehyde/formic acid and 1,8-dibromooctane, respectively. Amine amide **7** and diamine diamide **4** were synthesized by amidation of **5** with acetyl chloride and suberic acid, respectively.

Biology. Functional activity of the compounds under investigation was determined in driven guinea pig and rat left atria (1 Hz). Tetraamine **3** was investigated also in left atria from rats pretreated with pertussis toxin. These methods have been described in detail earlier.[6,10] To allow comparison of the results, arecaidine propargyl ester (APE), a muscarinic agonist, was used as a positive control. The biological results are expressed as pEC_{50} values.

The muscarinic receptor subtype selectivity was assessed by employing receptor binding assays in CHO-K1 cells expressing human cloned muscarinic M_1−M_5 receptors as reported previously.[11] Methoctramine (**2**) was used as standard.

Results and Discussion. Polyamines **3** and **4** and their truncated analogues **5**−**7**, unlike **2**, did not behave as muscarinic antagonists in atrial preparations but showed an unexpected intrinsic activity. These novel polyamines displayed potent negative inotropic effects that were even more pronounced than the inotropic responses elicited by APE as revealed by their pEC_{50} values (Figure 1a, Table 1). Furthermore, the onset was definitely different for **3**−**7** in comparison to APE because the maximum effect elicited by the addition of any concentration of drug was reached after at least 30 min incubation of polyamine and after only a few seconds following APE addition. Similar results were obtained in tissues from rat (not shown). A slow onset of action was reported also by others for unexpected muscarinic agonists with an unusual chemical structure.[12] Apparently, these novel polyamines behaved like partial muscarinic agonists because their responses were within 70−85% of the maximal response elicited by APE. Interestingly, concentration−response curves of **3** and **4** were shallower than that of APE as revealed by their Hill slope values (0.72 ± 0.05 and 0.70 ± 0.05 for **3** and **4**, respectively) that were lower than unity (Table 1). Moreover, the effects produced by these compounds, unlike APE, could not be reversed following extensive washing (up to 3 h) of tissues (data not shown). This prevented the study of the effect of **3** or **4** and muscarinic antagonists on the same preparation. To circumvent this problem, one tissue was incubated with the test antagonist whereas another tissue was used as control. After the incubation period, concentration−response curves to **3** were obtained on both preparations, allowing calculation of the EC_{50} value in the presence and the absence of the antagonist. The affinity values obtained for atropine, a nonselective muscarinic antagonist, and tripitramine, a selective muscarinic M_2 receptor antagonist, were totally different from the affinity observed using APE as agonist (Table 2). This finding clearly suggests that **3** hardly interacts with the site where APE and the two antagonists bind.

To clarify the site of action of these novel polyamines we performed binding assays in CHO-K1 cells expressing human cloned muscarinic M_1-M_5 receptors. It turned out that both **3** and **4** are effective ligands for muscarinic receptor subtypes whereas diamines **5** and **6** were not able to displace [^{3}H]NMS, even at relavtively high concentrations (Table 3). To verify whether **3** and **4** are muscarinic agonists the increase of GTPγS-binding in cell membranes from CHO-K1 cells transfected with human cloned muscarinic M_2 and M_4 receptors was investigated. Both compounds behaved as antagonists in the dose range that was examined (0.1 nM−100 μM), whereas carbachol, a muscarinic agonist, showed the expected increase in GTPγS-binding (Figure 2a). To detect an allosteric agonism of these substances, a concentration−response curve to carbachol was determined in the absence and in the presence of a fixed

Table 1. Negative Inotropic Responses, Expressed as pEC_{50} Values, Induced by Polyamines **3**−**7**, APE, and **8** in Electrically Paced (1 Hz) Guinea Pig Left Atria

no.	pEC_{50}[a]	slope	relative efficacy (APE = 100)
APE	8.15 ± 0.03	1.15 ± 0.09	100
3	9.65 ± 0.04	0.72 ± 0.05[b]	84
4	9.40 ± 0.03	0.70 ± 0.05[b]	85
5	9.28 ± 0.06	0.75 ± 0.08	75
6	9.22 ± 0.03	1.15 ± 0.09	70
7	8.89 ± 0.05	0.80 ± 0.07	79
8[c]	9.47 ± 0.03	0.98 ± 0.05	82

[a] $pEC_{50} = -\log EC_{50}$. EC_{50} values are the means ± SE of at least four independent experiments and were calculated by a nonlinear regression curve-fitting computer program.[22] [b] Significantly different from unity ($p < 0.01$). [c] *N*-Dodecyl lysine amide.[21]

Table 2. Antagonist Affinities, Expressed as pK_B Values, of Atropine and Tripitramine in Electrically Paced (1 Hz) Guinea Pig Left Atria Using APE, **3**, and **8** as Agonists

	pK_B[a]		
compound	APE	**3**	**8**[b]
atropine	9.15 ± 0.11	7.18 ± 0.13	7.45 ± 0.09
tripitramine	9.61 ± 0.06	7.32 ± 0.04	

[a] The dissociation constants, expressed as pK_B values ± SE of four experiments, were calculated at one antagonist concentration (0.1 μM; 1 h incubation) by the equation $pK_B = -\log \{[\text{antagonist}]/(DR - 1)\}$.[22] [b] *N*-Dodecyl lysine amide.[21]

Table 3. Affinity Estimates, Expressed as K_i (nM) Values, of Polyamines **3**−**7** and **8** for the Five Human Cloned Muscarinic Receptor Subtypes Expressed in CHO-K1 Cells

	K_i,[a] (nM)				
no.	M_1	M_2	M_3	M_4	M_5
2[b]	49.8 ± 6.2	14.3 ± 2.2	277 ± 27	38.0 ± 3.5	313 ± 22
3	19.8 ± 9.2	8.6 ± 1.1	422 ± 112	15.3 ± 7.5	105 ± 26
4	108 ± 7.8	9.2 ± 1.0	97.2 ± 5.6	16.6 ± 3.3	183 ± 80
5[c]	22%	49%	12%	28%	29%
6[c]	17%	35%	12%	25%	25%
8[c,d]	17%	13%	17%	0%	24%

[a] K_i values are the means ± SE of two to three experiments, each performed in triplicate, and were calculated from IC_{50} values with the equation of Cheng and Prusoff.[23] [b] Data taken from ref 18. [c] Results are the percent reduction of binding of the radiolabeled ligand with the test compound at a concentration of 10 μM. [d] *N*-Dodecyl lysine amide.[21]

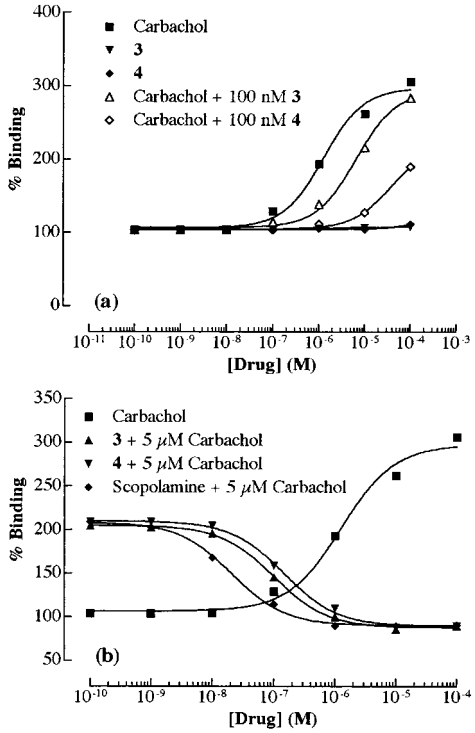

Figure 2. Effect of carbachol, **3**, and **4** on GTPγS binding of CHO-K1 cells expressing human M_2 mAChR. (a) GTPγS binding in the presence of increasing concentrations of carbachol (■), **3** (▼), or **4** (◆) and of carbachol in the presence of 100 nM **3** (△) or 100 nM **4** (◇). (b) GTPγS binding in the presence of increasing concentrations of carbachol (■) and of **3** (▲), **4** (▼), or scopolamine (◆) in the presence of a fixed concentration of carbachol (5 μM). Data represent mean values from duplicate determinations.

concentration (that was more than 10 times higher than the observed K_i values) of **3** and **4**. A rightward shift of the concentration−response curve to carbachol was observed in both cases, confirming that **3** and **4** behave like competitive antagonists (Figure 2a). To exclude a potential allosteric agonism at other concentrations of **3** and **4**, we combined a fixed concentration of carbachol (5 μM) with increasing concentrations of **3** and **4** (0.1 nM−100 μM). It was observed that both compounds were antagonists, because they were only able to inhibit the carbachol stimulated GTPγS-binding. Scopolamine was used as a reference compound and showed the same behavior of **3** and **4** (Figure 2b).

Taken together, the functional studies in cloned receptors suggest clearly that **3** and **4** are muscarinic antagonists rather than agonists. Consequently, the negative inotropic effect observed in atrial preparations remains to be explained. Thus, our attention was focused on other targets, among which we considered the possibility that **3** and **4** could interact directly with G_i proteins, triggering the negative inotropic response without the activation of the muscarinic M_2 receptor to which they are coupled. In other words, **3** and **4** might behave as G_i protein activators. This view is in agreement with the finding that polyamines are able to interact with G proteins.[13−15] The prototype methoctramine (**2**) was found to activate G_i proteins in mast cells, while inhibiting G_i protein in pig atrial membrane preparation enriched in muscarinic M_2 receptors.[16,17] Since it is known that pertussis toxin uncouples muscarinic receptors from inhibiting adenylate cyclase by alkylating a cysteine residue near the carboxy terminus of G_i and G_o α subunits,[18] we have investigated the inotropic effect elicited by **3** in rat atrial tissue pretreated with pertussis toxin. It turned out that **3** was devoid of activity in atrial preparations from pretreated animals with pertussis toxin (not shown), as one would expect if **3** interacts directly with G_i proteins to produce its effects. This finding also rules out the possibility that **3** might interact with a different target, such as a K^+ channel, which is connected to muscarinic M_2 receptors and, consequently, to G_i proteins in atria.[19] Clearly, if **3** was able to activate directly K^+ channels, pretreatment with pertussis toxin should not affect its negative inotropic effect in atrial tissue.

To gain further insight into the potential target of our compounds, we used benzalkonium chloride, an inhibitor of G_i proteins,[20] to antagonize the effects produced by **3** and APE (see Supporting Information). The inotropic effect induced by APE was not antagonized by benzalkonium chloride whereas the responses elicited by **3** were noncompetitively blocked with an IC_{50} value of 22.9 ± 0.6 μM in agreement with the hypothesis that **3** interacts with G_i proteins. However, if **3** is an activator of G_i proteins, an intriguing question arises: Why does a compound that directly activates G_i proteins not increase GTPγS binding (see Figure 2)? A possible explanation could be that **3** binds to muscarinic M_2 receptors with high affinity as well (Table 3). The interaction of **3** with the receptor would stabilize the complex between GDP and α, β, and γ subunits of G_i proteins, preventing the dissociation of the α subunit from the receptor and from $\beta\gamma$ dimer and, as a consequence, the regulatory cycle of G_i proteins. This reasoning may find support in the observation that **3** produced a concentration−response curve with a Hill slope value significantly lower than unity, which might be the result of a negative cooperativity by way of a dual mode of action of **3**, that is, antagonism at the receptor and "agonism" at the G_i protein. The interaction of **3** with the receptor would induce a conformational change in G_i protein structure, decreasing its affinity for the binding site on the G_i protein. Another piece of evidence supporting the view that **3** may be a G_i protein activator came from the observation that pretreatment of guinea pig left atria with 10 pM **3** (a concentration 1000-fold lower than the observed K_i value at muscarinic M_2 receptors) caused a significant leftward shift of the concentration−response curve to APE (Figure 1b).

Clearly, tetraamine **3**, by interacting with G_i proteins, would stabilize, by way of a conformational change, the activated state of the receptor thus increasing the affinity of APE for its site.

To test the hypothesis that **3** is a G_i protein activator we have investigated (a) diamines **5** and **6** because they displayed a very weak, if any, affinity for muscarinic M_2 receptors while having a potent inotropic effect in atrium (Tables 1 and 3) and (b) *N*-dodecyl lysine amide (**8**) because it was reported to be a G_i protein activator.[21] Interestingly, **8** showed a potent intrinsic activity comparable to that of both **5** and **6** and **3** as well (Table 1); this effect was antagonized by benzalkonium chloride ($IC_{50} = 35.7 \pm 1.1$ μM) and the muscarinic antagonists atropine and tripitramine in a manner similar to that observed for **3** (Figure 3 and Table 2). Furthermore, compounds **5**, **6**, and **8**, unlike **3** and **4**, produced concentration−response curves with Hill slope values not significantly different from unity (Table 1), suggesting that the lack of interaction with muscarinic M_2 receptors did not affect the interaction with G_i proteins. This reasoning rationalizes rather well the results obtained in functional assays. However, it does not give an answer to the question of why **5** and **8**, which have almost no affinity for muscarinic receptors in CHO-K1 cells (Table 3), behaved like **3** rather than to give a synergistic activation of the carbachol signal in GTPγS binding assays (not shown), as one would expect if they are supposed to interact directly with G_i proteins. Work is in progress to gain a better understanding of the intriguing trends noted above.

In conclusion, although methoctramine (**2**) is a potent muscarinic M_2 receptor antagonist in both functional and binding assays, its constrained analogues **3** and **4** were not muscarinic antagonists in isolated atria. They displayed a potent intrinsic activity similar to that of **8**, a G_i protein activator, but this effect was different from that of APE, a muscarinic agonist. It is also clear that four basic nitrogen atoms are not required for optimum activity in atria as diamine diamide **4** was only slightly less potent than tetraamine **3**. Furthermore, a tetraamine or a diamine diamide backbone is not necessary for activity in atria as diamines **5** and **6** and amine amide **7**, obtained by truncating in two halves **3** and **4**, respectively, were almost as active as or slightly less potent than their parent compounds. Interestingly, diamines **5** and **6** were almost inactive as muscarinic antagonists. Consequently, appropriate structural modification of methoctramine structure can afford compounds endowed with affinity for muscarinic receptors or for different biological targets such as, in the present case, G_i proteins.

Acknowledgment. This research was supported by grants from the University of Bologna, the European Community (BMH4-CT97-2395), and MURST.

Supporting Information Available: Synthesis of compounds **3**−**7** and effects of benzalkonium chloride antagonism are available free of charge via the Internet at http://pubs.acs.org.

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JM0155594