Elevation of Ligand Binding to Muscarinic M_2 Acetylcholine Receptors by Bis(ammonio)alkane-Type Allosteric Modulators

Alexandra Raasch,[†] Olaf Scharfenstein,[‡] Christian Tränkle,[†] Ulrike Holzgrabe,[‡] and Klaus Mohr*[,†]

Pharmacology and Toxicology, Institute of Pharmacy, University of Bonn, An der Immenburg 4, 53121 Bonn, Germany, and Pharmaceutical Chemistry, Institute of Pharmacy, University of Würzburg, Am Hubland, 97074 Würzburg, Germany

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Bis(ammonio)alkane-type compounds are archetypal muscarinic allosteric modulators. Phthalimido-substituted hexane-bis-ammonium agents were methylated in the phthalimide moieties and the lateral propyl side chains. All compounds retarded allosterically the dissociation of the orthosteric ligand [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) from porcine heart M_2 receptors. [^{3}H]NMS equilibrium binding was reduced, left unaltered, or elevated, depending on the degree and position of methylation. This is the first time that an allosteric elevation of ligand binding is demonstrated for bis(ammonio)alkane-type compounds.

Introduction

With W84 (**1a**) as a lead compound, we have previously developed a number of bis(ammonio)alkane-type allosteric agents with increased affinity for the allosteric site in *N*-methylscopolamine (NMS) occupied and in free muscarinic M_2 acetylcholine receptors.[1−4] The methylphthalimido derivative dimethyl-W84 (**3a**) has, under conditions of low ionic strength and in the absence of divalent cations, a top position among allosteric agents with respect to the affinity for NMS-occupied receptors and was introduced as the first (and still only) radioligand for the common allosteric site.[1] An allosteric elevation of NMS binding is reported for some compounds such as the common site allosteric agent alcuronium.[5] Yet, with regard to the bis(ammonio)alkane-type agents, even **3a** maintained a weak inhibitive effect on NMS binding despite its high affinity for the allosteric site of NMS-occupied M_2 receptors.[1] Here, however, we report that an introduction of methyl groups into the propyl side chain of **1a** and its derivatives may lead to allosteric agents that elevate orthosteric ligand binding in muscarinic M_2 receptors. These findings demonstrate that bis(ammonio)alkane-type agents may serve as lead structures for the development of allosteric enhancers of ligand binding to muscarinic receptors.

Synthesis

Because of the availability of 3-(*N*,*N*-dimethylamino)-propylphthalimide and the corresponding methylphthalimides **4** and **5**, **1a** and **3a** could be obtained by conversion of two molecules of the phthalimide derivatives with dibromohexane.[1] To achieve the phthalimides **6** and **7**, phthalic acid anhydride and methylphthalic acid anhydride, respectively, were refluxed in the presence of 1,3-diamino-*N*,*N*-2,2-tetramethylpropane in toluene using a water separator. Conversion of two molecules of **6** and **7**, respectively, with dibromohexane gave the symmetrical compounds **1c** and **3c**. To obtain the nonsymmetrical compounds, the phthalimides **4**, **6**, and **7** first have to be alkylated by using an excess of dibromohexane without any reaction solvent[3] to achieve **8**, **9**, and **10**, respectively. Alkylation of **6** with **8**, **5** with **9**, **4** with **10**, and **5** with **10** by refluxing in acetonitrile gave **1b**, **2c**, **2b**, and **3b**, respectively (see Scheme 1). All bis(ammonio)alkane-type compounds **1**−**3** were obtained in satisfactory yields. The identities were established by one- and two-dimensional ^{1}H NMR (Supporting Information) and ^{13}C NMR spectroscopical experiments. The phthalimido moiety of the compounds tends to hydrolyze to give amido derivatives. However, the stability of all compounds was found to be high ($t_{1/2}$ > 36 h). The log *P* value characterizes the compounds to be slightly hydrophilic. Lipophilicity does not correlate with the allosteric potency of the compounds.

Pharmacology

The allosteric interaction of the test compounds with muscarinic M_2 receptors was studied as described previously[6] in homogenates of porcine heart ventricles with [^{3}H]*N*-methylscopolamine as the ligand for the orthosteric receptor site. The concentration of the test compounds that inhibits the dissociation of [^{3}H]NMS half-maximally, $EC_{50,diss}$, can be taken as a measure of the equilibrium dissociation constant of allosteric modulator binding to [^{3}H]NMS-occupied receptors.[1] Experiments to measure the effect of the allosteric test compounds on the equilibrium binding of [^{3}H]NMS yielded the cooperativity factor α and pK_A, i.e., the negative log value of the equilibrium dissociation constant K_A of allosteric agent binding to ligand-free receptors. If the allosteric agent enhances [^{3}H]NMS binding, there is a positive cooperativity between the allosteric and the orthosteric compound, and according to Ehlert,[7] the cooperativity factor amounts to $\alpha < 1$. In the case of negative cooperativity, [^{3}H]NMS binding is lowered by the allosteric agent and $\alpha > 1$. Neutral cooperativity means that [^{3}H]NMS binding is unchanged despite allosteric agent binding to the receptors, $\alpha = 1$.

* To whom correspondence should be addressed. Phone: + 228-73-9103. Fax: + 228-73-9215. E-mail: k.mohr@uni-bonn.de.
[†] University of Bonn.
[‡] University of Würzburg.

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Scheme 1. Synthesis Pathway of the Compounds Studied

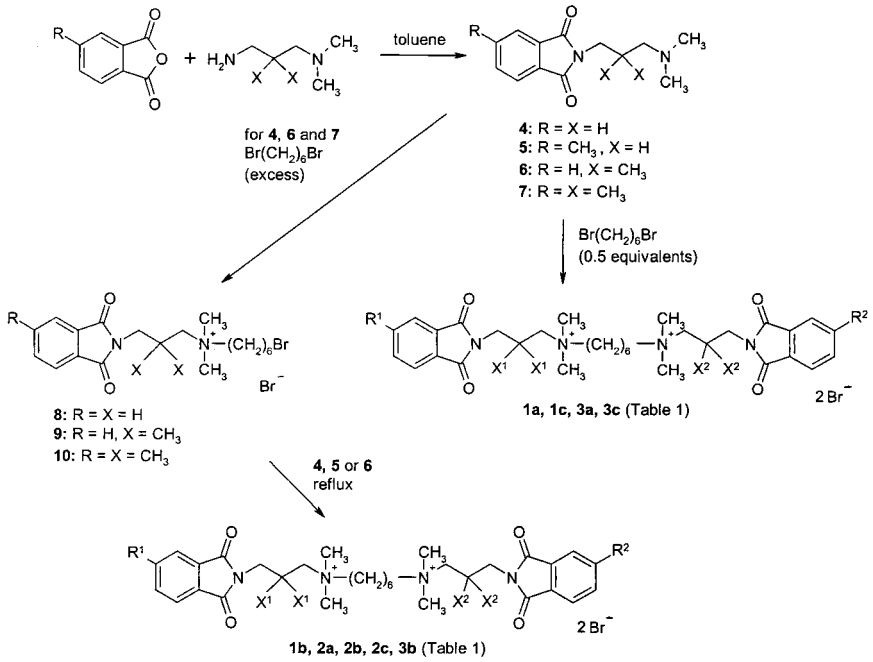
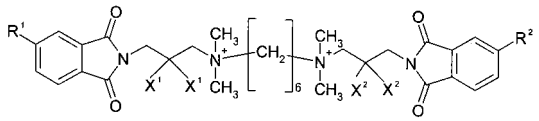


Table 1. Parameters Characterizing the Allosteric Interaction of the Indicated Test Compounds with [^{3}H]*N*-methylscopolamine at Porcine Heart M_2 Receptors (Mean Values ± SEM from Three Independent Experiments; For Details See Text)



compd	R^1	R^2	X^1	X^2	pK_A	log α	α	p(αK_A)	p$EC_{50,diss}$	p(αK_A)/p$EC_{50,diss}$
1a	H	H	H	H	6.53 ± 0.02	0.48 ± 0.02	3.02	6.05	6.00 ± 0.07	1.01
1b	H	H	CH_3	H	6.90 ± 0.03[a,b]	0.04 ± 0.03[a]	1.10	6.86	6.87 ± 0.04[a]	1[b]
1c	H	H	CH_3	CH_3	7.08 ± 0.09[a]	0.21 ± 0.02[a]	1.62	6.87	6.75 ± 0.04[a]	1.02
2a	CH_3	H	H	H	6.89 ± 0.18	0.43 ± 0.01	2.69	6.46	6.49 ± 0.04[a]	1.00
2b	CH_3	H	CH_3	H	7.26 ± 0.03[a]	−0.19 ± 0.02[a,c]	0.65	7.45	7.26 ± 0.02[a]	1.03
2c	CH_3	H	H	CH_3	7.20 ± 0.22	0.13 ± 0.02[a]	1.35	7.07	7.15 ± 0.11[a]	0.99
3a	CH_3	CH_3	H	H	7.08 ± 0.10[a,d]	0.33[a,d]	2.14	6.75	6.77 ± 0.03[a,d]	1.00
3b	CH_3	CH_3	CH_3	H	7.41 ± 0.02[a,b]	−0.03 ± 0.02[a]	0.93	7.44	7.44 ± 0.06[a]	1[b]
3c	CH_3	CH_3	CH_3	CH_3	7.21 ± 0.11[a]	−0.17 ± 0.02[a,c]	0.68	7.38	7.33 ± 0.10[a]	1.01

[a] Significantly different from the parameter value of the parent compound **1a**; $P < 0.05$. [b] Curve-fitting under the assumption that p(αK_A)/p$EC_{50,diss}$ = 1. [c] Significantly different from log α of compound **3b**; $P < 0.05$. [d] Data taken from ref 13.

Results

All test compounds reduced the apparent rate constant k_{-1} of [^{3}H]NMS dissociation and displayed concentration/effect curves with the slope factors not different from unity and bottom plateaus not significantly different from $k_{-1} = 0$. The curves (available as Supporting Information) reflect binding of the allosteric agents to [^{3}H]NMS-occupied receptors. $EC_{50,diss}$ values are listed in Table 1. As described in the Experimental Section, the $EC_{50,diss}$ values were used to find out for which concentrations of the allosteric agents [^{3}H]NMS equilibrium binding could be attained within an incubation time of up to 180 min. **1a** displayed the expected[8] submaximal inhibition of [^{3}H]NMS equilibrium binding, which is indicative of a negative cooperativity between the allosteric and the orthosteric ligand, α = 3.02 (Table 1). **1b** had almost no effect on [^{3}H]NMS equilibrium binding. Since the dissociation experiments proved that there is an interaction with the receptor, it can be concluded that receptor occupation of this agent does not affect the binding affinity of [^{3}H]NMS. The cooperativity factor α = 1.10 is close to unity, which indicates nearly neutral cooperativity between this allosteric agent and [^{3}H]NMS. **2b** enhanced [^{3}H]NMS binding. This indicates positive cooperativity; α = 0.65 means that the receptor-bound allosteric agent increases the binding affinity of [^{3}H]NMS almost 2-fold. The cooperativity factors for all test compounds are compiled in Table 1. Because α values are known to be log-normally distributed,[9] log α values were used for computing mean values ± SEM and for statistical testing.

If an allosteric agent affects the equilibrium binding of the orthosteric radioligand, allosteric curve fitting also yields the equilibrium dissociation constant K_A for the interaction of the allosteric agent with ligand-free receptors. The product αK_A represents the equilibrium dissociation constant of alloster binding to [^{3}H]NMS-occupied receptors.[7] As mentioned above, the dissociation experiments also provide a measure of this binding constant, i.e., $EC_{50,diss}$. To check whether the independent experimental approaches yielded consistent findings, the ratio p(αK_A)/p$EC_{50,diss}$ was computed, which should amount to unity. As can be seen in Table 1, this was the case. If an allosteric agent does not affect

equilibrium binding, as found with **1b** and **3b**, curve-fitting with α and K_A as variable parameters does not work. In that instance, we replaced K_A by $EC_{50,diss}/\alpha$ (since $\alpha K_A/EC_{50,diss} = 1$, as mentioned earlier). Because $EC_{50,diss}$ was known, the remaining variable α could be obtained from the curve fit and K_A was then derived from $EC_{50,diss}/\alpha$.

Discussion

In the present study we show for the first time that allosteric enhancers of muscarinic ligand binding may be derived from bis(ammonio)alkane-type agents by rather small structural modifications. The parent compound **1a** has an inhibitive effect on the binding of NMS, which indicates negative cooperativity between these ligands. A seemingly minor structural modification, i.e., the introduction of methyl groups in two positions (**2b**), was sufficient to elicit positive cooperativity with the orthosteric ligand (Table 1). Compared with **1a**, methylation generally tended to increase binding affinity both at the ligand free receptor (see pK_A in Table 1) and at the NMS occupied receptor (see $EC_{50,diss}$ in Table 1). With respect to positive cooperativity ($\alpha < 1$), however, it is pivotal that the affinity of the allosteric agent for the orthoster-occupied receptor is higher than for the ligand-free receptor. This is because binding of the allosteric agent to the orthoster-occupied receptor inhibits orthoster dissociation and, thereby, promotes orthoster equilibrium binding. Binding of the allosteric agent to unoccupied receptors inhibits the orthosteric ligand association and, thus, tends to diminish orthoster equilibrium binding. With regard to allosteric agent binding to NMS-occupied receptors, there is a strict dependence on the position of methylation (Table 1). Introduction of a methyl group into the phthalimide moiety, either on one (**2a**) or on both sides of the molecule (**3a**), hardly reduces the extent of negative cooperativity compared with the parent compound **1a**. Methylation of the propyl chains can induce a more pronounced diminution of negative cooperativity. Remarkably, methylation in one of the propyl chains allows for neutral cooperativity (**1b**), whereas methylation of both propyl chains still goes with negative cooperativity (**1c**). A combination of both phthalimide ring methylation and propyl chain methylation may evoke positive cooperativity (**2b**, **3c**) but not in all cases (**2c**, **3b**). Comparison of **2b** and **2c** indicates that propyl chain methylation and phthalimide methylation on the same side of the molecule induce positive cooperativity, whereas these methylations carried out on opposite sides do not. In fact, phthalimide plus propyl methylations on one side of the molecule (**2b**) yield a level of positive cooperativity that is not overcome by additional methylations on the other side of the molecule (**3b**, **3c**).

The effect of an allosteric modulator on orthosteric ligand binding is known to depend on the type of orthosteric ligand.[10] Future investigations will reveal how much bis(ammonio)alkane-type compounds also elevate the binding of muscarinic agonists such as acetylcholine.

Conclusion

The present study shows for the first time that methylation in appropriate positions of phthalimidopropyl-substituted bis(ammonio)alkane-type compounds endows these agents with the property to elevate allosterically the binding of an orthosteric ligand to muscarinic receptors.

Experimental Section

Melting points (Supporting Information) were determined with a Dr. Tottoli and a Gallenkamp melting point apparatus (Büchi, Switzerland) and were not corrected. ^{1}H NMR spectra were recorded on a Varian XL 300 instrument (^{1}H 299.956 MHz; Supporting Information). IR spectra (Supporting Information), recorded as KBr disks, were obtained using a Perkin-Elmer 298 spectrometer, and UV spectra were recorded on a Hewlett-Packard 8452A diode array spectrometer. Dry solvents were used throughout. **1a** was synthesized according to ref 2, **2a** according to ref 3, and **3a** according to ref 1, **4** was purchased from Aldrich (Steinheim, Germany), and **5** was synthesized according to ref 1. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical value (Supporting Information).

Synthesis of 2-[3-(*N*,*N*-Dimethylamino)-2,2-dimethylpropyl]isoindole-1,3-dione (6). Phthalic acid anhydride (7.41 g, 50 mmol) was dissolved in toluene (100 mL) at 80 °C, then 1,3-diamino-*N*,*N*-2,2-tetramethylpropane (6.51 g, 50 mmol) was added dropwise, and the resulting solution was refluxed using a water separator. After about 3 h, the solvent was evaporated, affording an oil that was crystallized from ethanol and a few drops of petroleum ether to give **6**: 63% yield, mp 75−78 °C.

Synthesis of 2-[3-(*N*,*N*-Dimethylamino)-2,2-dimethylpropyl]-5-methylisoindole-1,3-dione (7). 4-Methylphthalic acid anhydride (6.49 g, 40 mmol) was dissolved in toluene (100 mL), then 1,3-diamino-*N*,*N*-2,2-tetramethylpropane (5.21 g, 40 mmol) was added dropwise, and the resulting solution was refluxed using a water separator. After 4 h, the solvent was evaporated, affording the product that was purified by means of column chromatography (silica gel; eluent CH_2Cl_2/MeOH = 5:1, $R_f = 0.65$) to give **7**: 68% yield, mp 81−83 °C.

General Procedure for the Synthesis of (6-Bromohexyl)[3-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)propyl]dimethylammonium Bromide (8), (6-Bromohexyl)[3-(1,3-dioxo-1,3-dihydroisoindol-2-yl)-2,2-dimethylpropyl]dimethylammonium Bromide (9), and (6-Bromohexyl)[3-(1,3-dioxo-1,3-dihydro-5-methylisoindol-2-yl)-2,2-dimethylpropyl]dimethylammonium Bromide (10). A mixture of the corresponding amines (0.01 mol), **4**, **6**, and **7**, respectively, and a 3-fold excess of dibromohexane was allowed to stand at least 5 days at ambient temperature until a precipitate appeared. The crystals were filtrated, washed with ethyl acetate, recrystallized from 2-propanol/ethanol (5:1) to give **8** (yield 63%; mp 189−90 °C), recrystallized from acetone and petroleum ether to give **9** (yield 35%; mp 157−69 °C (dec)), and recrystallized from 2-propanol/ethanol (5:1) and a few drops of diethyl ether to give **10** (yield 10%; mp 131−133 °C).

Synthesis of 2-{3-[1-(6-{1,1-Dimethyl-1-[3-(1,3-dioxo-1,3-dihydroisoindol-2-yl)-2,2-dimethylpropyl]ammonio}-hexyl)-1,1-dimethylammonio]-2,2-dimethylpropyl}-1,3-isoindolindione Dibromide (1c) and 2-{3-[1-(6-{1,1-Dimethyl-1-[3-(1,3-dioxo-1,3-dihydro-5-methylisoindol-2-yl)-2,2-dimethylpropyl]ammonio}hexyl)-1,1-dimethylammonio]-2,2-dimethylpropyl}-5-methyl-1,3-isoindolindione Dibromide (3c). Compounds **6** and **7** (10 mmol), respectively, and dibromohexane (5 mmol) were dissolved in acetonitrile (100 mL), and a catalytic amount of a mixture of KI and K_2CO_3 (1:1) was added. The reaction solution was refluxed for 3 days (TLC control, silica gel, mobile phase = CH_3OH/0.2 M NH_4NO_3 solution (aqueous) = 3:2). The obtained precipitate was filtered, and the filtrate was recrystallized several times from a solvent mixture composed of ethanol and a few drops of petroleum ether to give **1c** (yield 60%) and **3c** (yield 61%), respectively.

General Procedure for the Synthesis of 2-{3-[1-(6-{1,1-Dimethyl-1-[3-(1,3-dioxo-1,3-dihydroisoindol-2-yl)-2,2-dimethylpropyl]ammonio}hexyl)-1,1-dimethylammonio]-propyl}-1,3-isoindolindione Dibromide (1b), 2-{3-[1-(6-{1,1-Dimethyl-1-[3-(1,3-dioxo-1,3-dihydro-5-methylisoindol-2-yl)-2,2-dimethylpropyl]ammonio}hexyl)-1,1-dimethylammonio]propyl}-1,3-isoindolindione Dibromide (2b), 2-{3-[1-(6-{1,1-Dimethyl-1-[3-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-2,2-dimethylpropyl]ammonio}hexyl)-1,1-dimethylammonio]propyl}-5-methyl-1,3-isoindolindione Dibromide (2c), and 2-{3-[1-(6-{1,1-Dimethyl-1-[3-(1,3-dioxo-1,3-dihydro-5-methylisoindol-2-yl)-2,2-dimeth ylpropyl]ammonio}hexyl)-1,1-dimethylammonio]-propyl}-5-methyl-1,3-isoindolindione Dibromide (3b). Equimolar amounts of **8**, **9**, and **10** (1 mmol), respectively, and the corresponding dimethylaminopropylimides **6**, **5**, and **4** were dissolved in acetonitrile (100 mL), and a catalytic amount of a mixture of KI and K_2CO_3 (1:1) was added. The mixture was refluxed for 2−4 days (TLC control, silica gel, mobile phase = CH_3OH/0.2 M NH_4NO_3 solution (aqueous) = 3:2). The obtained precipitate was filtered and washed several times with hot acetonitrile to give about 60% of each compound.

Stability. Tris-Mg buffer consisted of 3.6 mM $MgHPO_4$, 50 mM Tris (pH 7.3 attained by using HCl), and 500 mL of distilled water. A 10^{-4} M solution of each compound was prepared in Tris buffer; the absorption spectra of these solutions were recorded between 220 and 800 nm every 15 min for a period of 24 h. The decrease in the absorption at the maximum wavelength at 300 nm was registered. Nonlinear regression analysis (GraphPad software) yielded the half-life $t_{1/2}$ of the hydrolysis. The half-lives were found to be higher than 36 h.

Octanol/Buffer Partition Coefficient. A solution of each compound (10^{-5} M) was prepared in phosphate buffer (pH 7.4, 0.078 M), saturated with 1-octanol, and the absorbance was measured at the maximum wavelength (222−228 nm). This solution (1 mL) was shaken for 4 h with 1-octanol (1 mL) saturated with buffer. After centrifugation, the aqueous layer was separated and again the absorption measured. From the differences of the absorptions, the octanol/buffer partition coefficient was calculated, and as can be seen from the log *P* values, the compounds were found to be slightly hydrophilic: **1b** = −0.72; **1c** = −0.82; **2b** = −0.33; **2c** = −0.63; **3b** = −0.79; **3c** = −0.39.

Radioligand Binding Studies. Porcine cardiac homogenates were prepared as described previously.[1] Binding of [^{3}H]-*N*-methylscopolamine ([^{3}H]NMS) (0.2 nM; specific activity 70.0−83.5 Ci/mmol; Perkin-Elmer Life Sciences, Boston, MA) was measured in 45 mM Tris-HCl and 2.6 mM $MgHPO_4$: pH 7.3, 37 °C. Nonspecific [^{3}H]NMS binding was marked by 1 μM atropine and was less than 5% of the total binding. Membranes were separated by vacuum filtration after 2−3 h of incubation. Radioactivity was determined by liquid scintillation counting. The pK_D of NMS binding amounted to 9.50 ± 0.08 (mean ± SEM, n = 4). [^{3}H]NMS dissociation was monophasic ($t_{1/2,control}$ = 3.0 ± 0.1 min; mean ± SEM, n = 31). After 30 min of preincubating the membranes with [^{3}H]NMS, its dissociation was made visible by addition of 1 μM atropine alone or in combination with a test compound. Two-point kinetic experiments[11] were applied with measurements of [^{3}H]-NMS binding at t = 0 and t = 10 min. To investigate [^{3}H]-NMS equilibrium binding, the appropriate time of incubation was determined according to Lazareno and Birdsall[12] (eq 31 therein). For the time needed for equilibrium binding, we took 5 half-lives. Equilibrium binding data were analyzed according to Ehlert[7] as described previously (eq 3 in ref 8). For nonlinear regression analysis, we used the software Prism 3.0, Graph Pad. Two-tailed *t* tests with Welch correction when necessary were carried out with the Instat software (version 1.11a, GraphPad, San Diego, CA).

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Supporting Information Available: Analytical data, ^{1}H NMR data, concentration/effect-curves for the inhibition of [^{3}H]NMS dissociation by representative test compounds, and concentration/effect curves for the effect of representative test compounds on [^{3}H]NMS equilibrium binding. This material is available free of charge via the Internet at http://pubs.acs.org.

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