Divergent Modes of Action among Cationic Allosteric Modulators of Muscarinic M2 Receptors

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SUMMARY
We tested the hypothesis that structurally related modulators of ligand binding to muscarinic M2 receptors may not use a common recognition site. The applied test compounds are potent allosteric modulators [i.e., two bispyridinium model compounds substituted symmetrically either with phthalimidomethyl (WDuo3) or dichlorobenzyl (Duo3), a phthalimidioethyl-substituted hexamethonium compound (WB4), alcuronium, and, for sake of comparison, gallamine]. As introduced by Ellis and Seidenberg as a tool to check for a common allosteric site [Mol. Pharmacol. 42:638–641 (1992)], obidoxime was used to antagonize the actions of the test compounds. The allosteric delay of the dissociation of [3H]N-methylscopolamine ([3H]NMS) from porcine heart muscarinic receptors was measured in 5 mM sodium/potassium phosphate buffer (4 mM Na2HPO4 and 1 mM KH2PO4, pH 7.4) at 23° (control t1/2 ≈ 4 min). The concentration-effect curve of obidoxime, which has a weak potency and submaximal efficacy to allosterically retard [3H]NMS dissociation, was better described with a two-site model than with a one-site model. The concentration-effect curves of the test compounds for the allosteric delay of [3H]NMS dissociation were shifted to the right in the presence of obidoxime, yet to a different extent. For WDuo3, WB4, alcuronium, and gallamine, the shift induced by increasing concentrations of obidoxime was compatible with a competitive interplay. The pKd values of obidoxime against these modulators lay in a narrow range from pKd = 4.70 with gallamine to pKd = 4.16 with WDuo3. In contrast, the ability of obidoxime to shift the concentration-effect curve of Duo3 was weak (pA2 = 3.00) and not compatible with a competitive interplay. In conclusion, cationic allosteric modulators may stabilize [3H]NMS binding to M2 receptors by divergent modes of allosteric action. The findings suggest that the M2 receptor protein contains more than one allosteric recognition site on its extracellular face.

Ligand binding to muscarinic receptors may be subject to allosteric modulation (1), especially in case of muscarinic M2 receptors (2–4). The allosteric modulators known to date affect both events underlying ligand binding to muscarinic receptors (i.e., ligand association and ligand dissociation). The effect on ligand dissociation results from an interaction of the modulator with the ligand-occupied receptor and is thus indicative of an interaction with a recognition site distinct from the ligand binding site. The term “allosteric site” does not indicate whether binding of a modulator induces a conformational change of the receptor protein or imposes a steric hindrance on ligand association and dissociation. The effect on ligand association, which to the best of our knowledge is inhibitive with all known allosteric modulators, can result from binding to the allosteric site, the ligand binding site, or another site accessible to the modulator in the absence of a bound ligand. In these cases, the modulator interacts with the free receptor. In the current study, we focus on the interaction of allosteric modulators with M2 receptors that are occupied by [3H]NMS.

Most of the allosteric agents described to date contain at least one positively charged nitrogen at pH 7. Otherwise, the compounds are rather heterogeneous both in chemical structure and in the characteristics of interaction with ligand binding (5). Nevertheless, there is evidence for a common allosteric site on muscarinic M2 receptors: Ellis and Seidenberg (6) demonstrated that the allosteric delay of the dissociation of [3H]NMS from M2 receptors induced by gallamine is antagonized by obidoxime in a competitive fashion. The allosteric actions of tacrine and the agent 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate are also inhibited by obidoxime, but the type of antagonism has not been analyzed (6). Waelbroeck (7) observed a competitive interplay between the effects of the modulators d-tubocurarine and gallamine with regard to the equilibrium binding of [3H]NMS. Proska and Tuček (8) could predict the effects of combinations of alcuronium and gallamine, which elevate and reduce [3H]NMS equilibrium binding, respectively, on the basis of a model for a competitive interaction.

On the other hand, structure-activity relationships in bispyridinium-type model compounds, which are potent allosteric modulators of muscarinic M2 receptors (5, 9), were not readily compatible with the common-site model (10); two structurally homologous sets of compounds were compared.

ABBREVIATIONS: NMS, N-methyl-scopolamine; IP, inflection point; IP50, high affinity component of the fit; IP0, low affinity component of the fit.

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that differed only in the aromatic ring introduced at one end of the molecules. The structure-activity relationships for the allosteric delay of [3H]NMS dissociation from porcine M₂ receptors were divergent, depending on whether the aromatic substituent was phthalimidomethyl or dichlorobenzyl. These findings were interpreted to indicate a multiple binding mode of bisquaternary allosteric modulators (10).

The determination of whether the M₂ receptor protein contains more than one allosteric recognition site for structurally closely related compounds might be pivotal for an understanding of allosteric phenomena on the molecular level and for the development of new modulators with improved properties. Currently, a radioligand is not available to label an allosteric recognition site, and thus, direct competition experiments with unlabeled modulators are not possible. Therefore, we applied the experimental approach introduced by Ellis and Seidenberg (6) and used obidoxime as a probe to antagonize the allosteric actions of two bispyridinium model compounds (Fig. 1): the phthalimidomethyl-containing WDuo3 [1,3-bis{4-(phthalimidomethyloximino-methyl)-pyridinium-1-yl}propane dibromide] and the dichlorobenzyl-containing Duo3 [4,4'-bis({2,6-dichloro-benzoxyl-imino)methyl]-1,1'-propane-1,3-diyl-bis-pyridinium dibromide]. According to the hypothesis that these compounds interact with distinct sites, obidoxime should interfere differently with the allosteric actions of both compounds.

The above-mentioned structure-activity relationships also imply that in contrast to the middle chain, the aromatic substituents are decisive for the allosteric activity (10). Therefore, a phthalimido-substituted compound containing an alkane-bis-ammonium middle chain instead of bispyridinium was included: W84 [hexane-1,6-bis(dimethyl-3'-phthalimidopropyl-ammonium bromide)] (Fig. 1). W84 (11) and its heptane analogue C₇/₃-phth (12) represent potent allosteric modulators of muscarinic M₂ receptors (5). With the assumption of a common site for phthalimido-containing agents, the sensitivities of W84 and WDuo3 toward an antagonistic effect of obidoxime should be similar. To determine whether the sites used by our model compounds are related to the sites of action of gallamine (6) and alcuronium, these modulators were included. Alcuronium is the most potent known modulator of M₂ receptors (5). The effect of the test compounds on the dissociation of [3H]NMS from porcine cardiac M₂ receptors was measured under the conditions applied by Ellis and Seidenberg in their pioneering work (6). The results indicate that Duo3 stands out as being much less sensitive to obidoxime and as being antagonized by obidoxime in a manner not compatible with a competitive interplay.

**Experimental Procedures**

**Materials.** [3H]NMS (specific activity, 84.0 Ci/mmol) was purchased from DuPont-New England Nuclear (Bad Homburg, Germany). Obidoxime dichloride and alcuronium dichloride were generously provided by Merck KG (Darmstadt, Germany) and Hoffmann-La Roche AG (Grenzach-Wyhlen, Germany), respectively. Atropine sulfate, (−)-scopolamine methylbromide, and gallamine triethiodide were obtained from Sigma Chemical (Munich, Germany). The bisquaternary compounds Duo3 (9) and WDuo3 (13) were synthesized and generously provided by Prof. Dr. Ulrike Holzgrabe and her coworkers (Department of Pharmaceutical Chemistry, Institute of Pharmacy, University of Bonn, Germany). W84 was synthesized by Dr. Joachim Pfeffer (Department of Pharmacology, University of Kiel, Germany) according to the procedure of Wassermann (14).

**Homogenate preparation.** Porcine cardiac membranes were prepared as described previously (5). Ventricular tissue (40 g) of porcine hearts obtained from the local slaughterhouse was homogenized in a 0.32 M sucrose solution and centrifuged for 11 min at 300 × g.

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**Fig. 1.** Structural formulas of the applied allosteric modulators of ligand binding at porcine muscarinic M₂ receptors.
Phase (2,000 rpm in a Beckman rotor model 35 (Beckman Instruments, Palo Alto, CA). The supernatant was centrifuged for 41 min at 80,000 × g (32,000 rpm in a Beckman rotor model 35). The pellet was resuspended in sodium/potassium phosphate buffer (4 mM Na₂HPO₄ and 1 mM KH₂PO₄, pH 7.4 (4 ml/g of original tissue, wet weight)). All preparation steps were carried out at an ambient temperature of 4°. Finally, aliquots of 0.5 ml were frozen in liquid nitrogen and stored at −80°. Protein content was determined according to the method of Lowry et al. (15) with human serum albumin as a standard. The protein content ranged from 3.1 to 4.9 mg/ml membrane suspension.

Radioligand binding assays. Cardiac membranes at a protein concentration of 260–400 µg/ml were incubated with 0.2 nM [³H]NMS in 5 mM sodium/potassium phosphate buffer, pH 7.4, at 23°. Non-specific binding was measured in the presence of 1 µM atropine. Specific binding of [³H]NMS under control conditions was characterized by values of $K_p = 0.36 ± 0.04$ nM and $B_{\text{max}} = 158 ± 28$ fmol/mg of protein (mean ± standard error; four experiments). To measure the kinetics of radioligand dissociation, the assays were prepared in a larger volume, and 1-ml aliquots were drawn at various time intervals. Radioligand and membranes were pre-incubated for 30 min before the time course of [³H]NMS dissociation was measured by the addition of 1 µM atropine. Test compounds were applied together with atropine, either alone or combined with obidoxime. Stock solutions containing the respective additions were prepared before the experiments, and special care was taken to allow rapid mixing with the reaction medium on addition to the assay.

Control experiments were performed to check whether the equilibrium effect of the test compounds was attained under these conditions. Fig. 2 illustrates the effect of the compounds on the dissociation of 0.2 nM [³H]NMS either after a 2-hr pre-incubation with the membranes and the radioligand (Fig. 2, shaded bars) or on simultaneous addition with atropine at the beginning of the dissociation phase (Fig. 2, open bars). Obidoxime was applied at a low concentration, 23 µM, at which the effect on [³H]NMS dissociation starts to develop, and at 1000 µM, which was a typical concentration in the antagonist experiments. At 1000 µM obidoxime under pre-incubation conditions, it was necessary to elevate the [³H]NMS concentration by 30-fold (to 2.4 nM [³H]NMS plus 4.3 nM unlabeled NMS) to counteract the inhibitory effect of obidoxime on the equilibrium binding of [³H]NMS, which was depressed to 5 ± 1% and 26 ± 4% of the control value at 0.2 and 6.7 nM [³H]NMS, respectively (mean ± standard error; three or four experiments). As illustrated in Fig. 2, pre-incubation did not enhance the effect of obidoxime. In addition, with alcuronium, W84, and W Duo3, which are compounds previously suspected to have a delayed onset of action (5), pre-incubation was dispensable under the conditions of the current study (Fig. 2). Likewise, the effect of Duo3 was present immediately on addition to the assay (Fig. 2). For gallamine, it has been previously shown that pre-incubation is not necessary (5). Obviously, the receptor kinetics of the modulators are much faster than the kinetics of the radioligand.

Nevertheless, the control curves measured in the absence of obidoxime for alcuronium, W84, and W Duo3, which do not depress equilibrium binding of [³H]NMS, were obtained under 2-hr pre-incubation conditions (5).

To terminate the incubation, 1-ml aliquots of the reaction medium were subjected to rapid filtration (glass-fiber filters No. 6; Schleicher and Schüll, Dassel, Germany). Filters were washed twice with 5 ml of ice-cold incubation buffer and placed into scintillation vials. After the addition of 5 ml of Ready Protein (Beckman), radioactivity was determined by liquid scintillation counting in a Beckman counter model LS 6000 at a counting efficiency of 53%.

Data analysis. The data from individual experiments were analyzed separately by nonlinear regression analysis with Prism (Version 2.01; GraphPAD, San Diego, CA). Curve fitting to the dissociation data was based on a monoeponential decay equation; biexponential curve fitting did not yield better results (partial $F$ test, $p > 0.05$, data not shown). Curve fitting to obtain concentration-effect curves for the reduction in the apparent rate constant of dissociation $k_-$ was based on a four-parameter logistic function, except for obidoxime, for which a two-site fit was used. The parameters $I$ and $n$ were variables ($n_{I} = -1$ in the two-site fit); the upper plateau of the curve was the control value of $k_-$ and was set 100%. With regard to the lower plateau of the curve, we tested (partial $F$ test) whether $k_-$ variable yielded a better fit than $k_-$ = 0%: this was found for the individual curves of obidoxime and gallamine and for the combination curves of Duo3 with obidoxime (for details, see Results). Consequently, these curves were fitted with a variable value of the lower plateau. In all other cases, the lower plateau of the curve was fixed at $k_- = 0%$. The rightward shift of the concentration-effect curves of the modulators induced by obidoxime was analyzed according to Lew and Angus (16). We tested which of the following equations yielded a sufficient description of the relationship between the $EC_{0.5}$ value (concentration reducing the normalized rate constant of [³H]NMS dissociation to half of the control) and the $B$ value (the concentration of obidoxime): $pEC_{0.5} = -\log([B] + 10^{nK_b}) - \log c$ (equivalent to a Schild plot with a slope of unity) $pEC_{0.5} = -\log([B]^{n} + 10^{nK_b}) - \log c$ (equivalent to a Schild plot with a slope deviating from unity) $pEC_{0.5} = -\log([B](1 + n[B]/10^{nK_b}) + 10^{nK_b}) - \log c$ (equivalent to a nonlinear Schild plot)

Fits obtained by nonlinear regression analysis were statistically compared with the use of a partial $F$ test. A value of $p < 0.05$ was taken as the criterion for significance.

Results

The effects of the bispyridinium compounds Duo3 and W Duo3 on the time course of [³H]NMS dissociation from porcine heart M₂ receptors are illustrated in Fig. 3. Dissociation of [³H]NMS was monophasic both under control condi-
Under control conditions, (EC\textsubscript{0.5} pounds applied in the current study (5). The potency of Duo3
and in the presence of the indicated concentrations of (A) Duo3 and (B) WDuo3 in representative experiments. Dissociation was measured after the addition of 1 \mu M atropine. As outlined previously (5), Duo3 was applied with atropine, and WDuo3 was added 2 hr before atropine.

Fig. 3. Monoexponential curve fitting showing time course of dissociation of [\textsuperscript{3}H]NMS from porcine M\textsubscript{2} receptors under control conditions and in the presence of the test compounds. The time course of dissociation could thus be characterized by \( t_{1/2} \) or the apparent rate constant of dissociation (\( k_{-1} = \ln2/t_{1/2} \)). Under control conditions, \( t_{1/2} = 4.2 \pm 0.1 \text{ min (mean} \pm \text{ standard error); 97 experiments). [The concentration-effect curves for the reduction in \( k_{-1} \) by Duo3 and WDuo3 are shown in Fig. 7, A and B, respectively (Control)]. The S-shaped curves can be described with the use of IP, the slope factor \( n_H \), and the minimum level \( k_{-1,min} \). If \( k_{-1,min} \) = 0, the IP indicates the concentration of the test compound at which \( k_{-1} \) is reduced to 50\% of the control value (EC\textsubscript{50}). A compilation of these values has been reported for a number of allosteric modulators of M\textsubscript{2} receptors, including the compounds applied in the current study (5). The potency of Duo3 (EC\textsubscript{0.5} = 1.3 \mu M, \( n_H = -2.6 \)) is considerably lower than that of WDuo3 (EC\textsubscript{0.5} = 17 nM, \( n_H = -1.1 \)) under the conditions applied here, and the concentration-effect relationship is remarkably steep (\( n_H \) significantly different from unity, partial \( F \) test, \( p < 0.0001 \)). In addition, with the other test compounds, [\textsuperscript{3}H]NMS dissociation remained monophasic (5). (The concentration-effect curves for W84, alcuronium, and gallamine are shown in Fig. 7.) The concentration-effect relationship of obidoxime is illustrated in Fig. 4. In general, it resembles the curve reported by Ellis and Seidenberg (6).

However, with our data, a one-site model did not yield a satisfying fit. A two-site fit gave a significantly better result (partial \( F \) test, \( p < 0.02 \)). The curve leveled off at \( k_{-1,min} \approx 19\% \), indicating a submaximal efficacy of obidoxime in retarding [\textsuperscript{3}H]NMS dissociation. When the compounds were combined with obidoxime, [\textsuperscript{3}H]NMS dissociation was less retarded, which indicates an antagonistic action of obidoxime. However, the leftward shift of the [\textsuperscript{3}H]NMS dissociation curve for the combination seems to be smaller with Duo3 than with WDuo3, suggesting a weaker antagonistic action of obidoxime against Duo3.

We characterized the concentration dependency of the antagonistic action of obidoxime against the test compounds in greater detail. The experimental setup is illustrated in Fig. 6. Concentration-effect curves for the allosteric effect of WDuo3 were determined in the presence of selected concentrations of obidoxime (Fig. 6A). Due to the effect on [\textsuperscript{3}H]NMS dissociation induced by obidoxime itself, the curves start at \( k_{-1} \) values of \( \sim 25\% \) of the control value. On the first glance, the remaining experimental range of \( k_{-1} \) from 25\% to 0\% seems to be rather small for reliable experimental results. However, \( k_{-1} = 25\% \) is equivalent to a \( t_{1/2} \) of [\textsuperscript{3}H]NMS dissociation of \( \approx 16 \) min, and in terms of \( t_{1/2} \) values, the scale is open to infinity. For example, \( k_{-1} = 5\% \) means that \( t_{1/2} \approx 80 \) min, which can reliably be discriminated by experimentation from \( t_{1/2} \approx 16 \) min.

The IPs of the concentration-effect curves of WDuo3 are shifted to the right depending on the concentration of obidoxime. To facilitate a comparison of the concentration-effect curves of WDuo3, the curves were normalized. A value of \( k_{-1} \) (normalized) = 1.0 was assigned to the starting level of the curves. The transformed curves are shown in Fig. 7B. According to Lew and Angus (16), the curve-fitting procedure includes determination of whether the use of individual \( n_H \)
values yields a significantly better result for a set of curves than the use of the mean slope factor. This was not the case for the set of curves shown in Fig. 7 (partial $F$ test, $p > 0.05$, data not shown). Thus, it is concluded that obidoxime induced a parallel rightward shift of the WDuo3 concentration-effect curve.

The concentration-effect curves of Duo3 in the presence of obidoxime are displayed in Fig. 6B. Obidoxime induced a slight rightward shift. This is especially evident from the normalized curves shown in Fig. 7A, because the log(concentration) axis is identically scaled for all test compounds in this figure.

The maximum retardation of [3H]NMS dissociation that could be attained by Duo3 (Fig. 6B) was slightly but significantly (partial $F$ test, $p < 0.05$) reduced by obidoxime to a level of $k_{1} = 3.2 \pm 0.2\%$ (mean $\pm$ standard error; four experiments). This finding is amplified with the normalized curves of Fig. 7A.

Analogously, the interplay of obidoxime with W84, alcuronium, and gallamine was measured; the results are compiled in Fig. 7. In addition, for these compounds, the curves were shifted in a parallel fashion. With gallamine, $k_{1,\text{min}}$ under control conditions was $4\%$ (5) but reached $0\%$ in the presence of $300$ and $1000\mu M$ obidoxime, respectively.

To exclude the possibility that another order of addition of the test compounds might alter the antagonistic effect observed with obidoxime, the following control experiments were done. First, it was tested whether the weak antagonistic action of obidoxime against Duo3 could be augmented by the application of $1000\mu M$ obidoxime in the preincubation phase before the addition of $10\mu M$ Duo3 at the start of the [3H]NMS dissociation phase. This was not the case. To illustrate the finding, $k_{1}$ (normalized) was converted into a shift factor with the assumption of a parallel curve shift as depicted in Fig. 7A for the Duo3/obidoxime combination curves. (This shift factor is shown in Fig. 9.) Second, it was checked whether preincubation with the modulators WDuo3 ($0.3\mu M$), W84 ($0.3\mu M$), and alcuronium ($0.1\mu M$) before the start of dissociation with $1\mu M$ atropine plus $1000\mu M$ obidoxime would promote the action of these modulators and reduce the antagonistic power of obidoxime, respectively. Again, this was not the case, as illustrated by the respective shift factors (see Fig. 9).

**Discussion**

In the case of competitive antagonism, the receptor affinity of a given antagonist is constant and independent of the
competing ligand (17). In the current study, we applied an experimental approach introduced by Ellis and Seidenberg (6) and used obidoxime to antagonize the allosteric actions of various bisquaternary compounds and of the terquaternary gallamine. If all tested modulators act on a common site, the antagonistic activity of obidoxime should not depend on the modulator under investigation.

However, even on a descriptive level (see Fig. 7), it is evident that Duo3 is distinctive in its low sensitivity toward the antagonistic action of obidoxime compared with the other test compounds. Nevertheless, we attempted to quantify the antagonistic activity of obidoxime should not depend on the modulator under investigation.

Fig. 7. Normalized concentration-effect curves for the allosteric delay of \([3H]\)NMS dissociation induced by the test compounds (A) Duo3, (B) W Duo3, (C) W84, (D) alcuronium, and (E) gallamine under control conditions and in the presence of the indicated concentrations of obidoxime. k_−1 (normalized), apparent rate constant of \([3H]\)NMS dissociation normalized to compensate for the obidoxime-induced delay of \([3H]\)NMS dissociation (i.e., k_−1 observed in the presence of obidoxime alone was set at k_−1 = 1.0). Data are (filled symbols) mean ± standard error values from two to five experiments or (open symbols) single values derived from complete dissociation experiments. Error bars, not shown when they do not exceed the symbols. Sets of curves could be fitted using mean slope factors; for details, see text.

The dependency of the pEC_{0.5} values of the test compounds on the concentration of obidoxime is plotted in Fig. 8 according to Lew and Angus (16). The EC_{0.5} is the concentration of a test compound at which the normalized k_−1 = 0.5. Duo3 was included in the analysis, although, in a strict sense, the prerequisite of a parallel shift is not unambiguously given because of the slight but significant elevation of the bottom level of the Duo3 curves seen in the presence of obidoxime (Fig. 6B). Curve fitting on the basis of three equations that describe different forms of antagonism (for details, see Data Analysis) revealed that a competitive model (eq. 1) was adequate for W Duo3, W84, alcuronium, and gallamine. Only with Duo3 was a significantly better fit obtained using a model for a mechanism not compatible with a competitive effect relationship was remarkably steep (n_H = −2.6). On the molecular level, the events that may underlie this phenomenon are still obscure (18, 19). Furthermore, in our study, the concentration-effect curve of obidoxime was more complex than expected for an interaction with a single site. Therefore, we analyzed the extent by which obidoxime shifted the concentration-effect curves of the modulators to the right because this approach does not require predictions of the binding characteristics.
interplay (eq. 2). For the sake of comparison and to illustrate the antagonistic action of obidoxime in the more familiar Schild plot, dose ratios were derived from the observed EC0.5 values and plotted according to Schild (Fig. 9); the lines, however, were generated using the result of the Lew and Angus analysis. The plot illustrates that the antagonistic effect of obidoxime against Duo3 is particular with regard to the low potency of obidoxime and the shallow incline of its antagonistic action with increasing concentrations. The plots for gallamine, W84, alcuronium, and WDUO3 are very similar with respect to the antagonistic potency of obidoxime and the slope of unity, suggesting a competitive interplay. However, according to statistical testing, the $pK_b$ of obidoxime is not independent of the modulator under investigation (Table 1). For example, the $pK_b$ of obidoxime versus gallamine is significantly different from the $pK_b$ of obidoxime versus alcuronium or WDUO3. Yet, one hesitates to conclude that different sites of interaction are involved. First, a comparison of the obidoxime sensitivities of the modulators ranked in Table 1 reveals that there are no significant differences between compounds next to each other: gallamine versus W84, W84 versus alcuronium, and alcuronium versus WDUO3. This reflects that the sensitivities of the modulators toward obidoxime lie in a narrow range. Second, Proška and Tuček (8), applying equilibrium binding measurements with the radioligand $[^3H]$NMS, demonstrated that the interplay between gallamine and alcuronium was compatible with a competitive model. Therefore, we favor the assumption that the differences in sensitivity, although statistically significant, are not biologically relevant in the sense of distinct recognition sites and hypothesize that these modulators interact with the common site proposed by Ellis and Seidenberg (6). However, Duo3 is clearly different from the other modulators with regard to the weak antagonistic effect of obidoxime, significantly different from the $pK_b$ of obidoxime versus alcuronium or WDUO3. Yet, one hesitates to conclude that different sites of interaction are involved. First, a comparison of the obidoxime sensitivities of the modulators ranked in Table 1 reveals that there are no significant differences between compounds next to each other: gallamine versus W84, W84 versus alcuronium, and alcuronium versus WDUO3. This reflects that the sensitivities of the modulators toward obidoxime lie in a narrow range. Second, Proška and Tuček (8), applying equilibrium binding measurements with the radioligand $[^3H]$NMS, demonstrated that the interplay between gallamine and alcuronium was compatible with a competitive model. 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which is not compatible with a competitive mechanism. Compared with the majority of allosteric modulators (5), Duo3 also differs in the high steepness of its concentration-effect curve for the allosteric delay of [3H]NMS dissociation (see Fig. 7). Furthermore, the sensitivity of the allosteric effect on the ionic composition of the incubation buffer is considerably smaller with Duo3 than with WDUO3, W84, alcuronium, or gallamine (5). These observations suggest that Duo3 affects [3H]NMS/receptor complexes allosterically by a distinct molecular mode of allosteric action. From a structural point of view, this conclusion was not anticipated because molecular modeling revealed rather close similarities between the test compounds in charge distribution and molecular shape (13, 20, 21). Our results are compatible with the hypothesis derived from structure-activity relationship investigations (10) that bispyridinium-type allosteric modulators may be guided into separate locations at the [3H]NMS-occupied receptor depending on the lateral substituent (i.e., dichlorobenzyl, as in Duo3, or phthalimidomethyl, as in WDUO3). However, the possibility cannot be excluded that compared with the other test compounds, Duo3 binds at the same binding location of the receptor protein, preferring, however, a distinct conformational state.

Although the molecular events underlying the particular action of Duo3 await clarification, the compound may serve as a lead structure for an alternative way to find allosteric modulators with improved properties and therapeutic perspectives.

The allosteric effect of Duo3 on [3H]NMS dissociation depends rather weakly on the ionic composition of the incubation medium. In a buffer containing 3 mM MgHPO4 and 50 mM Tris-HCl, pH 7.3, the allosteric potency was only 3-fold lower than that of the current assay conditions (~5-fold lower ionic strength and absence of divalent cations). For alcuronium, WDUO3, W84, and gallamine, the corresponding loss of potency was 14—89-fold (5). Furthermore, preliminary experiments revealed that Duo4, a derivative of Duo3 containing a middle chain of four methylene instead of three, stabilized [3H]NMS/receptor complexes in contracting guinea pig atria (modified Tyrode’s solution) with the same potency as in guinea pig heart homogenates (3 mM MgHPO4, 50 mM Tris-HCl, pH 7.3). Other compounds that are sensitive toward the ionic composition of the incubation medium (5), such as W84 and WDUO3, were found to decline in allosteric potency under organ bath conditions by a factor of ~10 (11, 22).

The experiments carried out in intact beating guinea pig atria reveal that the allosteric site of action of Duo3, WDUO3, and W84 is probably located on the extracellular face of the M2 receptor protein because the bisquaternary compounds are highly unlikely to pass cell membranes. For the bisquaternary alcuronium, it has been shown by Jakubik et al. (23) that an allosteric effect can be elicited in intact cells. In contrast, Wang et al. (24) reported that the allosteric effect of the polyamionic heparin found in CHO cell homogenates was not seen in intact cells. Gerstén et al. (25) provided evidence that heparin interferes with the receptor/G protein coupling.

The bisquaternary obidoxime was able to antagonize the allosteric effect of Duo3, although the action of Duo3 was apparently not mediated via the “obidoxime-sensitive common site.” The concentration-effect curve for the allosteric action of obidoxime itself was better fitted by a two-site model than by a one-site model. It is speculative to assume a two-site model but not unrealistic; results of recent structure-activity relationship investigations suggested that bispyridinium-type modulators may interact in more than one orientation with the [3H]NMS-occupied M2 receptor and that the type of lateral aromatic substituent may guide the compounds into a distinct localization (10). Because obidoxime is a bispyridinium that lacks lateral aromatic substituents, it seems justified to assume that there is more than one binding site for obidoxime. The two-site model implies that the allosteric effect of obidoxime on [3H]NMS dissociation is composed of two components. The underlying binding sites would differ in their binding affinity for obidoxime (see Fig. 4: IPH = 23 µM and IP1 = 960 µM) and the maximum reduction of the dissociation rate (Δk1,−1,H = 66% and Δk1,−1,L = 15%). Furthermore, the model is based on the assumption that both sites can be occupied simultaneously by obidoxime molecules. The IP of the high affinity component IPH (4.64) corresponds with the range of pK1 (4.70—4.16) for the antagonistic action of obidoxime against gallamine, W84, alcuronium, and WDUO3. This supports the notion that the site of interference with the action of the latter test compounds is identical to the site by which obidoxime induces its allosteric effect. The IP of the low affinity component IP1 (3.02) corresponds with the pK2 (3.00) for the antagonistic effect of obidoxime against Duo3. Because the antagonism is not compatible with a competitive interplay, the occupation of the low affinity site by obidoxime would interfere indirectly with the binding of Duo3 or with the transformation of binding into the delay of [3H]NMS dissociation. Although these considerations are rather speculative, they offer a unifying view of both qualities of obidoxime action that we investigated (i.e., its antagonistic action against other modulators and its own allosteric effect).

In conclusion, divergent sensitivities toward the antagonistic tool obidoxime were found for structurally closely related bisquaternary allosteric modulators. This result provides evidence that more than one allosteric recognition site may be present on the extracellular face of the M2 receptor protein.

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References


