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Divergent Modes of Action among Cationic Allosteric Modulators of Muscarinic M_2 Receptors

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SUMMARY

We tested the hypothesis that structurally related modulators of ligand binding to muscarinic M_2 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 phthalimidoethyl-substituted hexamethonium compound (W84), 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 [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) from porcine heart muscarinic receptors was measured in 5 mM sodium/potassium phosphate buffer (4 mM Na_2HPO_4 and 1 mM KH_2PO_4, pH 7.4) at 23° (control $t_{1/2} \approx 4$ min). The concentration-effect curve of obidoxime, which has a weak potency and submaximal efficacy to allosterically retard [^{3}H]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 [^{3}H]NMS dissociation were shifted to the right in the presence of obidoxime, yet to a different extent. For WDuo3, W84, alcuronium, and gallamine, the shift induced by increasing concentrations of obidoxime was compatible with a competitive interplay. The pK_b values of obidoxime against these modulators lay in a narrow range from pK_b = 4.70 with gallamine to pK_b = 4.16 with WDuo3. In contrast, the ability of obidoxime to shift the concentration-effect curve of Duo3 was weak (pA_2 = 3.00) and not compatible with a competitive interplay. In conclusion, cationic allosteric modulators may stabilize [^{3}H]NMS binding to M_2 receptors by divergent modes of allosteric action. The findings suggest that the M_2 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 M_2 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 sterical 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 M_2 receptors that are occupied by [^{3}H]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 M_2 receptors: Ellis and Seidenberg (6) demonstrated that the allosteric delay of the dissociation of [^{3}H]NMS from M_2 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 [^{3}H]NMS. Proška and Tuček (8) could predict the effects of combinations of alcuronium and gallamine, which elevate and reduce [^{3}H]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 M_2 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; IP_H, high affinity component of the fit; IP_L, low affinity component of the fit.

that differed only in the aromatic ring introduced at one end of the molecules. The structure-activity relationships for the allosteric delay of [^{3}H]NMS dissociation from porcine M_2 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 M_2 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-(phthalimidomethoxyimino-methyl)-pyridinium-1-yl]propane dibromide] and the dichlorobenzyl-containing Duo3 [4,4′-bis-[(2,6-dichloro-benzyloxy-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 C_7/3′-phth (12) represent potent allosteric modulators of muscarinic M_2 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 M_2 receptors (5). The effect of the test compounds on the dissociation of [^{3}H]NMS from porcine cardiac M_2 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. [^{3}H]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 ×

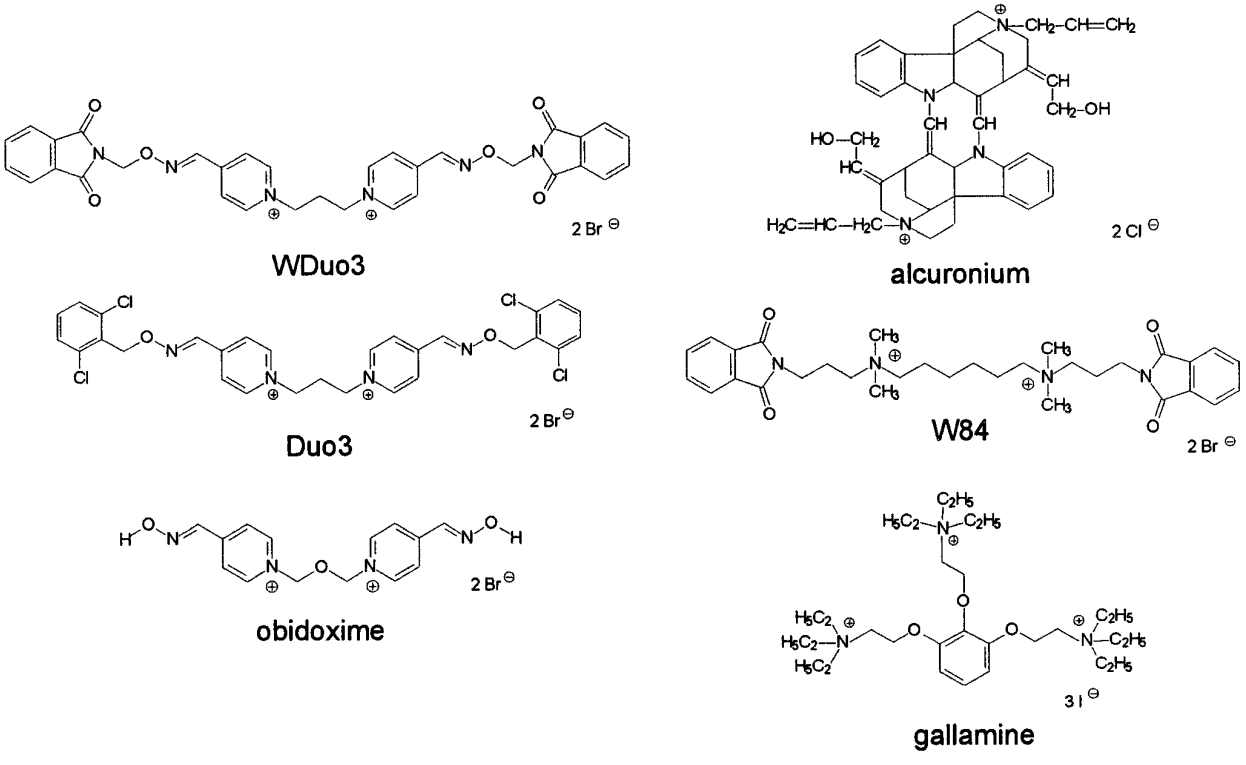

Fig. 1. Structural formulas of the applied allosteric modulators of ligand binding at porcine muscarinic M_2 receptors.

g [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 Na_2HPO_4 and 1 mM KH_2PO_4, 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 [^{3}H]NMS in 5 mM sodium/potassium phosphate buffer, pH 7.4, at 23°. Nonspecific binding was measured in the presence of 1 μM atropine. Specific binding of [^{3}H]NMS under control conditions was characterized by values of K_D = 0.36 ± 0.04 nM and B_{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 preincubated for 30 min before the time course of [^{3}H]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 [^{3}H]NMS either after a 2-hr preincubation 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 [^{3}H]NMS dissociation starts to develop, and at 1000 μM, which was a typical concentration in the antagonist experiments. At 1000 μM obidoxime under preincubation conditions, it was necessary to elevate the [^{3}H]NMS concentration by ~30-fold (to 2.4 nM [^{3}H]NMS plus 4.3 nM unlabeled NMS) to counteract the inhibitory effect of obidoxime on the equilibrium binding of

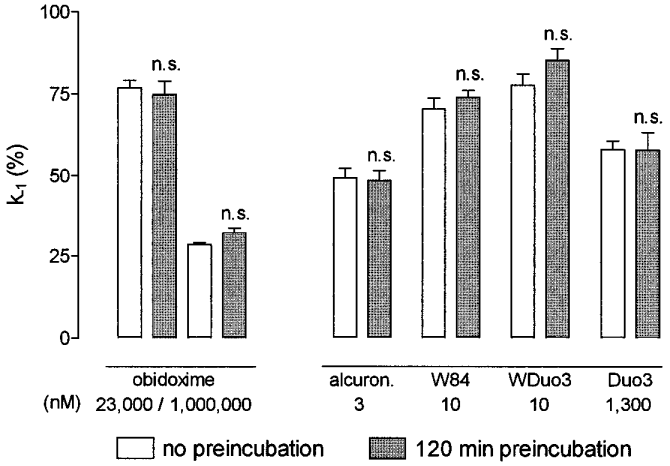

Fig. 2. Effect of the modulators at the indicated concentrations on the dissociation of [^{3}H]NMS (0.2 nM) depending on whether the modulators were applied together with atropine to start the [^{3}H]NMS dissociation phase (*open bars*) or 120 min before measurement of [^{3}H]NMS dissociation (*shaded bars*). k_{-1}, apparent rate constant of [^{3}H]NMS dissociation as a percentage of the control in the absence of a test compound (mean ± standard error; two to seven experiments). To compensate for the inhibitory effect on [^{3}H]NMS equilibrium binding exerted by 1 mM obidoxime in the preincubation experiments, the concentration of [^{3}H]NMS was elevated to 6.7 nM for this condition (for details, see text). *n.s,* not significant (*t* test, $p > 0.05$).

[^{3}H]NMS, which was depressed to 5 ± 1% and 26 ± 4% of the control value at 0.2 and 6.7 nM [^{3}H]NMS, respectively (mean ± standard error; three or four experiments). As illustrated in Fig. 2, preincubation did not enhance the effect of obidoxime. In addition, with alcuronium, W84, and WDuo3, which are compounds previously suspected to have a delayed onset of action (5), preincubation 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 preincubation 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 WDuo3, which do not depress equilibrium binding of [^{3}H]NMS, were obtained under 2-hr preincubation 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 Schüell, 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 monoexponential 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_{-1} was based on a four-parameter logistic function, except for obidoxime, for which a two-site fit was used. The parameters IP and n_H were variables ($n_H = -1$ in the two-site fit); the upper plateau of the curve was the control value of k_{-1} and was set 100%. With regard to the lower plateau of the curve, we tested (partial F test) whether k_{-1} = variable yielded a better fit than k_{-1} = 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_{-1} = 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 [^{3}H]NMS dissociation to half of the control) and the B value (the concentration of obidoxime):

$$pEC_{0.5} = -\log([B] + 10^{-pK_b}) - \log c$$

(equivalent to a Schild plot with a slope of unity)

$$pEC_{0.5} = -\log([B]^n + 10^{-pK_b}) - \log c$$

(equivalent to a Schild plot with a slope deviating from unity)

$$pEC_{0.5} = -\log\{[B](1 + n[B]/10^{-pK_b}) + 10^{-pK_b}\} - \log c$$

(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 WDuo3 on the time course of [^{3}H]NMS dissociation from porcine heart M_2 receptors are illustrated in Fig. 3. Dissociation of [^{3}H]NMS was monophasic both under control condi-

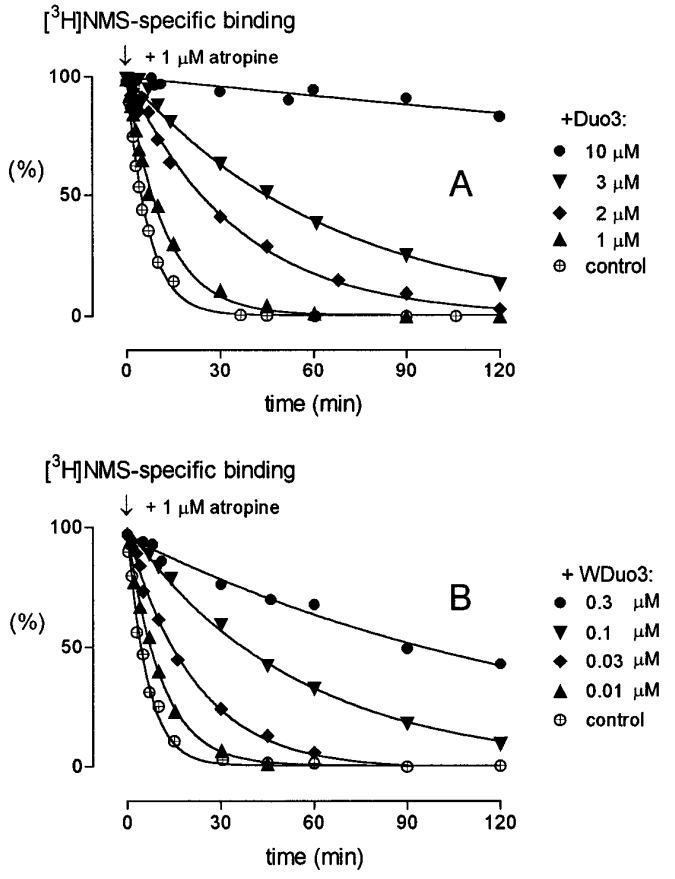

Fig. 3. Monoexponential curve fitting showing time course of dissociation of [^{3}H]NMS from porcine M_2 receptors under control conditions and in the presence of the indicated concentrations of (A) Duo3 and (B) WDuo3 in representative experiments. Dissociation was measured after the addition of 1 μM atropine. As outlined previously (5), Duo3 was applied with atropine, and WDuo3 was added 2 hr before atropine.

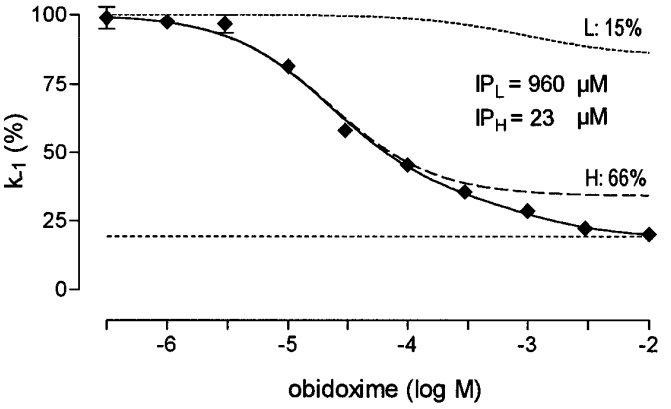

Fig. 4. Concentration-dependent effect of obidoxime on the apparent rate constant k_{-1} of [^{3}H]NMS dissociation as a percentage of the control determined in the absence of obidoxime. *Data points* are from complete dissociation curves (mean ± standard error; 4–41 experiments). *Error bars,* not shown when they do not exceed the symbols. Curve fitting was based on a two-site model. *Horizontal dashed line*, 18.5% (bottom of the curve). *Sigmoid dashed lines*, IP_H and IP_L; respective IPs and capacities are indicated.

tions 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 ($k_{-1} = \ln 2/t_{1/2}$). Under control conditions, $t_{1/2}$ = 4.2 ± 0.1 min (mean ± 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 ($EC_{0.5}$). A compilation of these values has been reported for a number of allosteric modulators of M_2 receptors, including the compounds applied in the current study (5). The potency of Duo3 ($EC_{0.5}$ = 1.3 μM, $n_H = -2.6$) is considerably lower than that of WDuo3 ($EC_{0.5}$ = 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, [^{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 [^{3}H]NMS dissociation; Ellis and Seidenberg (6) reported a minimum level of ~40%. Details of this curve are discussed below.

Fig. 5 illustrates the interference of a high concentration of obidoxime (1000 μM) with Duo3 (Fig. 5A) and WDuo3 (Fig. 5B). The latter compounds were applied in concentrations that in the absence of obidoxime induced a similar allosteric delay of [^{3}H]NMS dissociation. When the compounds were combined with obidoxime, [^{3}H]NMS dissociation was less retarded, which indicates an antagonistic action of obidoxime. However, the leftward shift of the [^{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 [^{3}H]NMS dissociation induced by obidoxime itself, the curves start at k_{-1} values of ~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 [^{3}H]NMS dissociation of ≈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

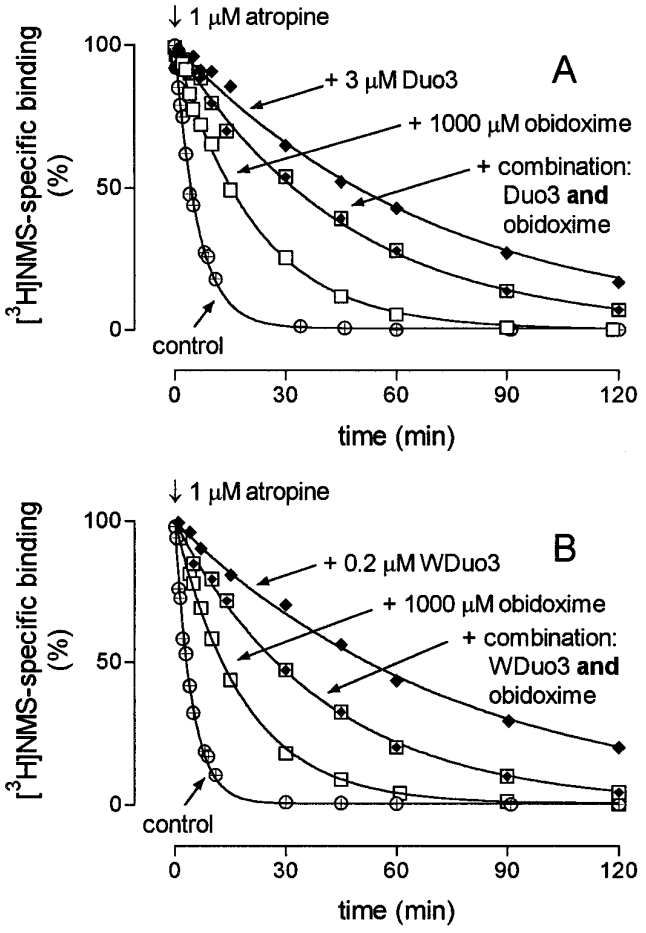

Fig. 5. Monoexponential curve fitting showing the effects of obidoxime (1000 μM) on the allosteric delay of [^{3}H]NMS dissociation by (A) Duo3 (3 μM) and (B) WDuo3 (0.2 μM). Results are representative, with each set obtained from one experiment.

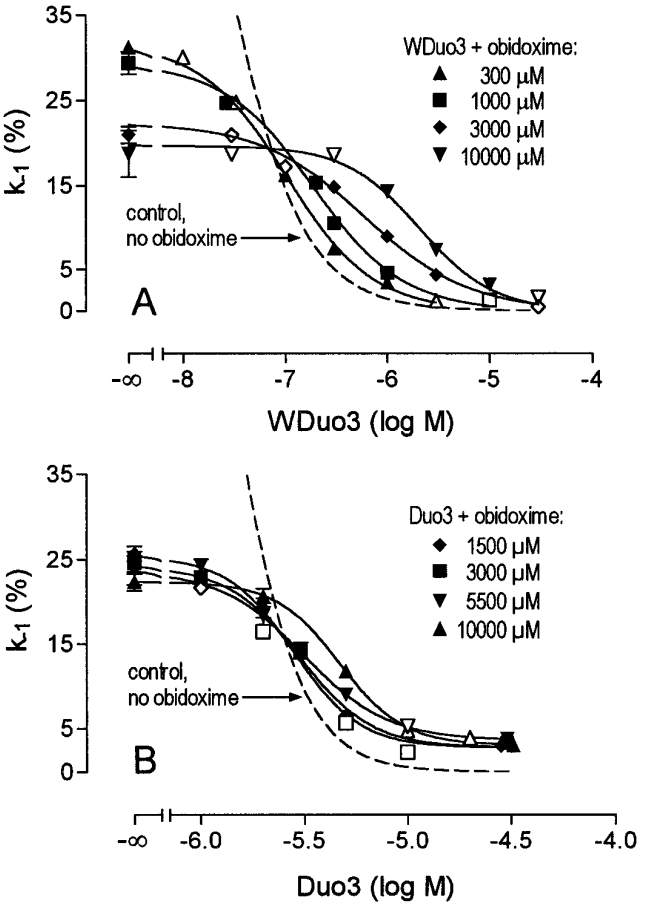

Fig. 6. Concentration effect curves for the allosteric delay of [^{3}H]NMS dissociation by (A) WDuo3 and (B) Duo3 measured in the presence of the indicated concentrations of obidoxime. k_{-1} is a percentage of the value in the absence of any compound. Data are mean ± standard error values from two to five experiments (*filled symbols*) or single values derived from complete dissociation curves (*open symbols*). *Dashed lines*, respective segments of the control curves displayed in Fig. 7. (Note the different scale of the *abscissa*.)

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 [^{3}H]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 ± 0.2% (mean ± 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, $k_{-1,\min}$ under control conditions was 4% (5) but reached 0% in the presence of 300 and 1000 μM 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 μM obidoxime in the preincubation phase before the addition of 10 μM Duo3 at the start of the [^{3}H]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 μM), W84 (0.3 μM), and alcuronium (0.1 μM) before the start of dissociation with 1 μM atropine plus 1000 μM 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

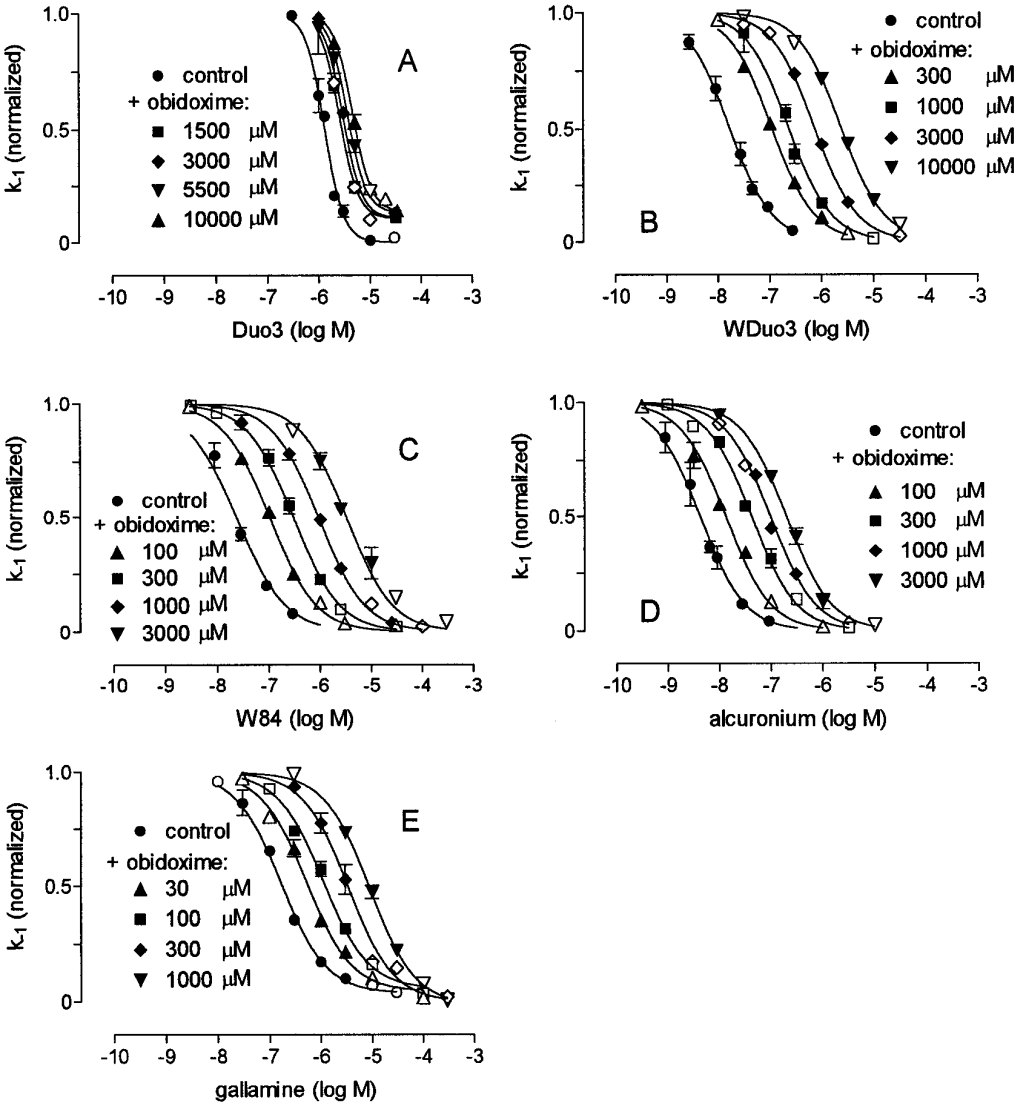

Fig. 7. Normalized concentration-effect curves for the allosteric delay of [^{3}H]NMS dissociation induced by the test compounds (A) Duo3, (B) WDuo3, (C) W84, (D) alcuronium, and (E) gallamine under control conditions and in the presence of the indicated concentrations of obidoxime. k_{-1} (normalized), apparent rate constant of [^{3}H]NMS dissociation normalized to compensate for the obidoxime-induced delay of [^{3}H]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.

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 action of obidoxime in greater detail. We used a different approach than Ellis and Seidenberg (6), who based curve fitting to the experimental combination data on the assumption of a competitive interplay between gallamine and obidoxime. Both compounds were assumed to bind to the allosteric site according to a Langmuir adsorption isotherm. The values of the parameters "affinity" and "maximal allosteric effect on radioligand dissociation" thus extracted from the combination data for both compounds were in excellent agreement with the respective values derived from the concentration-effect curves of gallamine and obidoxime alone (6). With our test compound Duo3, however, the concentration-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.

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 WDuo3, W84, alcuronium, and gallamine. Only with Duo3 was a significantly better fit obtained using a model for a mechanism not compatible with a competitive

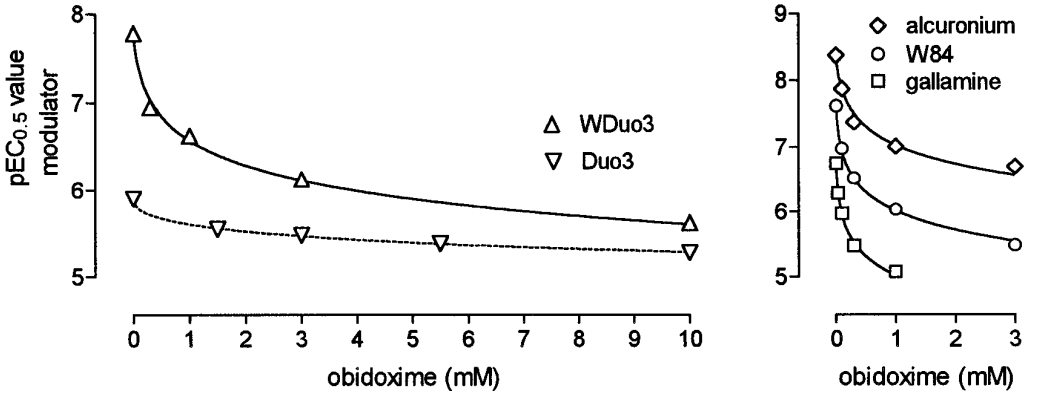

Fig. 8. Analysis of the obidoxime-induced shift in the concentration-effect curves of the indicated allosteric modulators according to Lew and Angus (16). $pEC_{0.5}$ values are derived from Fig. 7 and represent log concentrations at which the normalized k_{-1} is reduced to half of the value k_{-1} = 1.0 in the absence (Fig. 7, *Control*) or presence of obidoxime, respectively. *Curves,* obtained through nonlinear regression analysis by the application of equations that represent either a competitive mechanism (*solid lines*) or an interplay not compatible with a competitive mechanism (*dashed line with Duo3*). For details, see text.

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 $EC_{0.5}$ 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 [^{3}H]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,

TABLE 1

Potency of obidoxime to antagonize the delay of [^{3}H]NMS dissociation by the indicated allosteric modulators

The concentrations of obidoxime are given at which the curves for the allosteric action of the respective modulators are shifted to the right by a factor of 2 (i.e., pK_b values for interactions compatible with a competitive model, pA_2 values for the interplay with Duo3 that was not compatible with a competitive mechanism; $pA_2 = pK_b/n$; see eq. 2 in Data Analysis).[2] Values are mean ± standard error, derived from five experiments. $EC_{0.5}$ values are derived according to Lew and Angus (16). Values were compared using an unpaired two-sample *t* test.

Modulator	Obidoxime
	pK_b
Gallamine	4.70 ± 0.06
W84	4.61 ± 0.05
Alcuronium	4.30 ± 0.14[b]
WDuo3	4.16 ± 0.07
Duo3	3.00 ± 0.08[d]

N.S. (gallamine vs. W84); a (W84 vs. alcuronium); N.S. (alcuronium vs. WDuo3); b (gallamine vs. alcuronium); b (W84 vs. WDuo3); c (alcuronium vs. Duo3); N.S. (WDuo3 vs. Duo3)

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.
N.S., not significant.
[d] Value is pA_2.

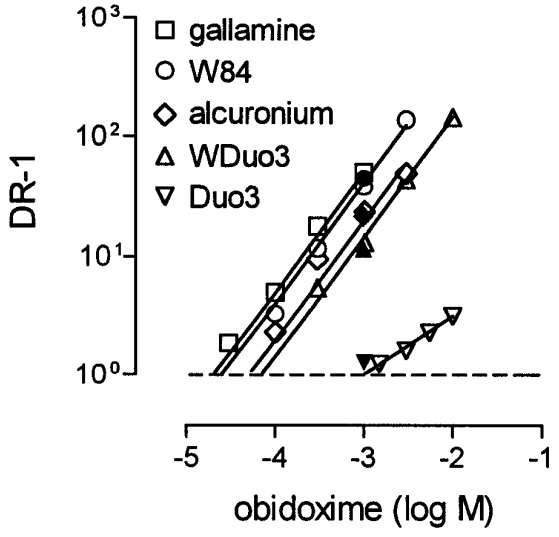

Fig. 9. Result of the analysis according to Lew and Angus displayed in the form of a Schild plot. The obidoxime-induced curve shifts as displayed in Fig. 7 are expressed as dose ratios (*DR*) ($EC_{0.5,obidoxime}/EC_{0.5,control}$; *open symbols*). The lines were generated according to the results of the Lew and Angus analysis (see legend to Fig. 8). *Filled symbols*, results of control experiments with a modified order of addition of the test compounds (i.e., preincubation with obidoxime before the addition of Duo3 at the start of the [^{3}H]NMS dissociation phase and preincubation with W84, alcuronium, and WDuo3 before the addition of obidoxime at the start of [^{3}H]NMS dissociation). For details, see text.

[2] M. J. Lew, personal communication.

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 [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]NMS dissociation depends rather weakly on the ionic composition of the incubation medium. In a buffer containing 3 mM $MgHPO_4$ 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[1] revealed that Duo4, a derivative of Duo3 containing a middle chain of four methylenes instead of three, stabilized [^{3}H]NMS/receptor complexes in contracting guinea pig atria (modified Tyrode's solution) with the same potency as in guinea pig heart homogenates (3 mM $MgHPO_4$, 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 M_2 receptor protein because the bisquaternary compounds are highly unlikely to pass cell membranes. For the bisquaternary alcuronium, it has been shown by Jakubík *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 polyanionic heparin found in CHO cell homogenates was not seen in intact cells. Gerstin *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 [^{3}H]NMS-occupied M_2 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 [^{3}H]NMS dissociation is composed of two components. The underlying binding sites would differ in their binding affinity for obidoxime (see Fig. 4: IP_H = 23 μM and IP_L = 960 μM) and the maximum reduction of the dissociation rate ($\Delta k_{-1,H}$ = 66% and $\Delta k_{-1,L}$ = 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 pIP_H (4.64) corresponds with the range of pK_b (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 pIP_L (3.02) corresponds with the pA_2 (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 [^{3}H]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 M_2 receptor protein.

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