ELSEVIER

European Journal of Pharmacology 314 (1996) 385–392

ejp

Evidence for a multiple binding mode of bispyridinium-type allosteric modulators of muscarinic receptors

Evi Kostenis [b], Hector M. Botero Cid [c], Ulrike Holzgrabe [c], Klaus Mohr [a,*]

[a] *Pharmacology and Toxicology, Institute of Pharmacy, University of Bonn, An der Immenburg 4, 53121 Bonn, Germany*
[b] *Laboratory of Bioorganic Chemistry, National Institutes of Health, Bethesda, MD 20892, USA*
[c] *Pharmaceutical Chemistry, Institute of Pharmacy, University of Bonn, Kreuzbergweg 26, 53115 Bonn, Germany*

Received 18 June 1996; accepted 16 July 1996

Abstract

The ligand binding properties of muscarinic receptors can be modulated by allosterically acting compounds. Here, a set of novel bispyridinium-type compounds was investigated which were designed to study structure-activity relationships and to provide more insight into the molecular events underlying the allosteric delay of the dissociation of [^{3}H]*N*-methylscopolamine from muscarinic M_2 receptors in porcine cardiac membranes. The parent compound, a non-substituted bispyridinium oxime, displayed a weak allosteric potency and was unable to prevent radioligand dissociation at maximum concentrations. Introduction of either a phthalimidomethyl-moiety or a dichlorobenzyl-moiety at one end of the parent compound led to a considerable increase of the allosteric activity with regard to both the potency and the maximum effect. In these unilaterally ring-substituted bispyridiniums, homologous contralateral non-aromatic modifications were accompanied by divergent potency shifts depending on whether the unilateral ring was phthalimidomethyl or dichlorobenzyl. The findings point to a multiple binding mode of bispyridinium compounds at M_2 receptors in the [^{3}H]*N*-methylscopolamine-occupied state, i.e., different orientations of the compounds at the allosteric binding area or even an interaction with distinct allosteric recognition sites.

Keywords: Muscarinic receptor; Allosteric modulation; Structure-activity relationship; Heart porcine

1. Introduction

Ligand binding to muscarinic receptors may be affected allosterically, especially in case of the M_2-subtype (e.g., Tuček and Proška, 1995; Lazareno and Birdsall, 1995). Allosteric modulators are commonly found to inhibit the association of ligands and to retard ligand dissociation. Whereas the former effect is also seen with conventional competitors of ligand binding, an alteration of the decay characteristics of a ligand-receptor complex can only be elicited via a site apart from the ligand binding site. Thus, an altered ligand dissociation is unambiguously indicative of an allosteric action.

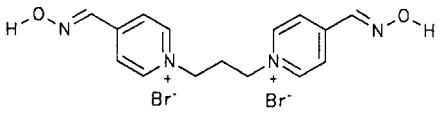
Various drugs from different pharmacological groups have been shown to be capable of retarding the dissociation of radiolabelled antagonists from muscarinic receptors. Most of the compounds available so far are rather heterogeneous with respect to their chemical structure and have another predominant pharmacological action, such as the often applied research tools gallamine and alcuronium (e.g., Proška and Tuček, 1995). A common structural feature are quaternary or tertiary nitrogens protonated at the pH of the assay conditions (Lee and El-Fakahany, 1991). The structural heterogeneity among the compounds raises concern about the specificity of the allosteric effect and impedes identification of essential structural components. With regard to specificity, Ellis and Seidenberg (1992) demonstrated that the allosteric effects of gallamine and tacrine on the dissociation of [^{3}H]*N*-methylscopolamine from M_2 receptors can be antagonized by obidoxime, which led to the concept of a common allosteric site. Furthermore, evidence was provided for a competitive interaction between the modulators gallamine on the one hand and *d*-tubocurarine (Waelbroeck, 1994), alcuronium or the structurally closely related strychnine (Proška and Tuček, 1995) on the other hand.

With regard to the identification of essential structural components, systematic investigation of structure-activity relationships with closely related compounds should be a

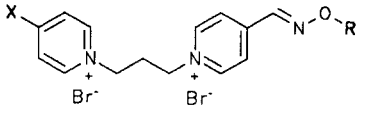
* Corresponding author. Tel.: (49-228) 73-9103; Fax: (49-228) 73-9215.

PII S0014-2999(96)00568-7

useful strategy. Structure-activity relationships may also provide insight into the molecular interaction between allosteric ligands and the receptor protein. So far, systematic structure-activity relationships have been investigated in bispyridinium-type modulators (Botero Cid et al., 1994; Gasteiger et al., 1995) and hexamethonium (alkane-bis-ammonium)-type compounds (Kostenis et al., 1994). Both series contain molecules which belong to the potent al-

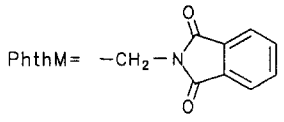


TMB4 EC_{50}= 1865 μM
n_H= 1.95



X	R = PhthM	R = DCB
H-O-N=CH-	3a EC_{50}= 4.9 ± 0.4 μM; n_H= 1.3 ± 0.1; n= 4	4a EC_{50}= 11.5 ± 0.6 μM; n_H= 1.3 ± 0.2; n= 4
H_3C-O-N=CH-	3b EC_{50}= 6.9 ± 1.4 μM; n_H= 1.3 ± 0.4; n= 6	4b EC_{50}= 4.5 ± 1.0 μM; n_H= 1.2 ± 0.4; n= 4
$(H_3C)_2N$-	3c EC_{50}= 2.9 ± 0.1 μM; n_H= 1.1 ± 0.1; n= 5	4c EC_{50}= 4.2 ± 0.3 μM; n_H= 1.4 ± 0.2; n= 4
DCB-O-N=CH-	3d EC_{50}= 1.3 ± 0.1 μM; n_H= 1.8 ± 0.2; n= 8	4d (DUO 3) EC_{50}= 4.2 ± 0.6 μM; n_H= 1.7 ± 0.2; n= 6
PhthM-O-N=CH-	3e (WDUO) EC_{50}= 0.5 ± 0.05 μM; n_H= 1.4 ± 0.1; n= 5	3d

Significance: 4a vs. 3a: *; 4b vs. 3b: ns; 4c vs. 3c: ns; 4d vs. 3d: ***; 3b vs. 3a: **; 3c vs. 3b: ***; 3d vs. 3b: **; 3e vs. 3d: **; 4b vs. 4a: *; 4c vs. 4b: ns; 4d vs. 4c: ***

PhthM= 

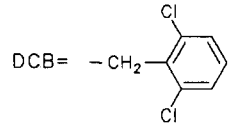
DCB= 

Fig. 1. Structural formulae of the test compounds; EC_{50} values and Hill coefficients n_H of the concentration-effect curves for the allosteric retardation of the dissociation of [^{3}H]*N*-methylscopolamine from porcine muscarinic M_2 receptors. Data for compounds 4a–d have been reported in Botero Cid et al. (1994). For 3e and 4d, the names WDUO and DUO3, respectively, have previously been used (Bejeuhr et al., 1992; Buschendorf et al., 1990). EC_{50} = concentration reducing the rate of radioligand dissociation to 50% of the control value, n = number of concentration-effect curves evaluated. Asterisks indicate the extent of statistical significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant). Due to the large number of possible comparisons, statistical tests were performed only for comparisons of interest.

losteric modulators (Tränkle et al., 1994). Furthermore, the weakly active modulator obidoxime which has been used as a tool to check for competitive interactions at the putative allosteric site (Ellis and Seidenberg, 1992) also represents a bispyridinium-type compound.

Comparison of the structure-activity relationships previously found in bispyridinium- and hexamethonium-type model substances suggested the compounds to adopt differing orientations at the putative allosteric binding area in the [^{3}H]*N*-methylscopolamine-occupied state of the receptor protein (Kostenis et al., 1994), despite considerable similarity between the model compounds with respect to the molecular shape and the charge distribution (Bejeuhr et al., 1992). However, the two series of compounds differed in both the bisquaternary middle chain and the attached substituents.

In order to define more precisely which structural elements are essential for the interaction with the [^{3}H]*N*-methylscopolamine-receptor complex, we aimed to compare the structure-activity relationships in two homologous series of bispyridinium derivatives containing as the unilateral substituent at one end of the middle chain either a phthalimidomethyl (PhthM) or a dichlorobenzyl (DCB) moiety (indicated as residue R in Fig. 1). For this purpose, the PhthM compounds were synthesized in order to allow for a comparison with the DCB compounds, the effects of which have been reported before (Botero Cid et al., 1994). The bispyridinium oxime TMB4 (structural formula in Fig. 1) was included which can be regarded as the parent compound of all presented derivatives. It was hypothesized that if the orientation of the compounds within the allosteric site is mainly influenced by the bispyridinium middle chain but not by the type of unilateral ring substituent, homologous structural modifications on the opposite side (residue X in Fig. 1) should yield parallel potency shifts in the two series of compounds.

2. Materials and methods

2.1. Preparation of porcine cardiac homogenates

The procedure has been described previously in detail (Jepsen et al., 1988; Botero Cid et al., 1994). All preparation steps were carried out at an ambient temperature of 4°C. Pieces of the ventricular wall of procine hearts obtained from the local slaughter-house were minced and rinsed with 0.32 M sucrose solution in order to be cleaned of blood. The tissue was homogenized in 0.32 M sucrose solution by means of a Waring Blendor (New Hartford, CT, USA) and a Potter Elvejhem homogenizer. The homogenate was centrifuged at $300 \times g$ for 10 min and the resulting supernatant was centrifuged at $80\,000 \times g$ for 40 min. The pellet was resuspended in 50 mM Tris buffer, pH 7.4. 1-ml aliquots of the resuspension were filled in plastic vials (Eppendorf, Hamburg, Germany) and shock-frozen with liquid nitrogen. The homogenate was stored at −20°C until use in the radioligand binding experiments. Protein content was determined according to Lowry et al. (1951) with human serum albumin as the standard.

2.2. Radioligand binding assays

In order to evaluate the effect of the test compounds on the rate of dissociation of [^{3}H]*N*-methylscopolamine, two procedures were applied as previously described in more detail (Botero Cid et al., 1994). In the 'two-point-kinetic experiments', the membranes were incubated together with 0.2 nM [^{3}H]*N*-methylscopolamine for 30 min in a medium composed of 3 mM $MgHPO_4$, 50 mM Tris, pH 7.3 at 37°C. Measurements were made in quadruplicate. Binding of [^{3}H]*N*-methylscopolamine was measured before and 4 min after revealing the dissociation of [^{3}H]*N*-methylscopolamine by addition of 1 μM atropine. The test compounds or an aliquot of the solvent were applied together with atropine. The level of the non-specific binding of [^{3}H]*N*-methylscopolamine was determined in the presence of 1 μM atropine and amounted to less than 10% of the total. Dissociation was terminated by filtration through glass fibre filters (Schleicher and Schüll, Dassel, Germany) under suction of a vacuum pump. The radioactivity on the filters was quantified by liquid scintillation counting.

In the 'complete-kinetic experiments', the same procedure was applied but the incubation volume was increased to about 20 ml in order to allow for the removal of various 1-ml aliquots over a total time period of 2 h.

To check for an effect of the test compounds on the equilibrium binding of [^{3}H]*N*-methylscopolamine, membranes were incubated under the above-mentioned conditions with the radioligand for 2 h in the absence and in the presence of the EC_{50} concentrations for the allosteric effect. Under control conditions, the equilibrium binding of [^{3}H]*N*-methylscopolamine was characterized by a $K_d = 0.6 \pm 0.1$ nM and a $B_{max} = 122 \pm 20$ fmol/mg protein (means ± S.D., $n = 3$).

2.3. Statistics / data analysis

A monoexponential decay equation was fitted to the results of the complete-kinetic experiments. Concentration-effect curves for the allosteric effect were calculated according to a four parameter logistic equation (Inplot Software; GraphPad, San Diego, CA, USA).

Statistical data evaluation was performed with the Mann-Whitney non-parametric test (Instat Software; GraphPad). A P value of < 0.05 was taken as the criterion for statistical significance.

2.4. Materials

[^{3}H]*N*-methylscopolamine (specific activity 81.5 Ci/mmol) was obtained from NEN-Dupont (Homburg,

Germany). *N*-methylscopolamine-bromide and atropine-sulfate were purchased from Sigma (Deisenhofen, Germany). The synthesis of the dichlorobenzyl (DCB)-substituted bispyridinium compounds (designated here as '4') is described in Botero Cid et al. (1994), the synthesis of WDUO ('3e') is reported in Bejeuhr et al. (1992).

2.5. Synthesis of the novel phthalimidomethyl containing bispyridinium compounds

The synthesis pathway leading to compounds 3a–d (Fig. 1) is analogous to previously published procedures. An oxime ether is formed by conversion of pyridine-4-carboxaldoxime and phthalimidomethyl (PhthM) bromide in the presence of sodium methanolate (Bejeuhr et al., 1992). In order to obtain 3a, the oxime ether is monoalkylated with dibromopropane and subsequently connected with a second pyridine-4-carboxaldoxime. 3b–d are formed by conversion of the oxime ether with the respective N-alkylated pyridinium parts the synthesis of which has already been described (Botero Cid et al., 1994). The analytical and spectroscopic (IR, NMR) data were in accordance with the chemical structures. These data as well as details of the synthesis are available upon request.

3. Results

The procedure to evaluate the allosteric effect of the test compounds on the dissociation of [^{3}H]*N*-methylscopolamine from porcine cardiac membranes is illustrated in Fig. 2 by means of representative experimental data for compound 3c. The dissociation curves were monophasic both

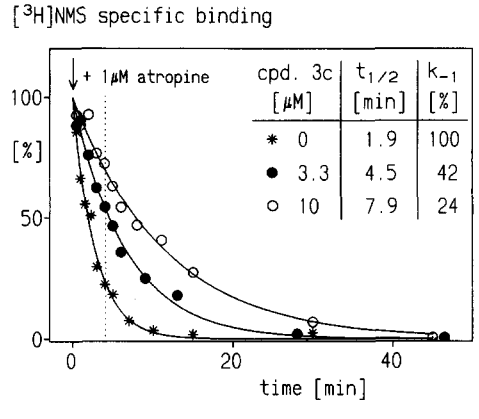


Fig. 2. Dissociation of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS, 0.2 nM) from porcine cardiac membranes under the control condition (asterisks) and in the presence of the indicated concentrations of compound 3c (complete kinetic experiments). Radioligand dissociation was revealed by addition of 1 µM atropine; the test compounds were added together with atropine. Curve-fitting was done by nonlinear regression analysis and led to the half-life time $t_{1/2}$ and the apparent rate constant of dissociation k_{-1}, respectively (inset). The dotted line indicates the level of [^{3}H]*N*-methylscopolamine residual binding 4 min after the start of the dissociation.

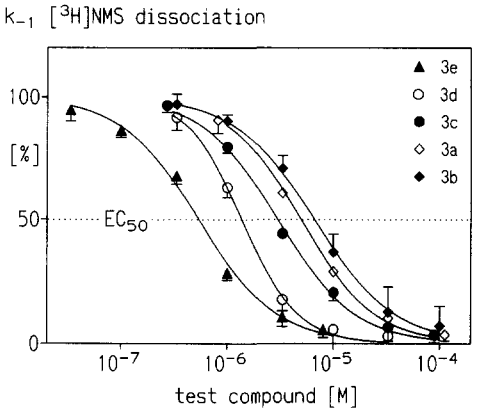


Fig. 3. Concentration-effect curves for the allosteric reduction by the indicated compounds of the apparent rate constant k_{-1} of the dissociation of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS, 0.2 nM), expressed as a percentage of the control value in the absence of a test compound. The curves are based on data from two-point-kinetic experiments measuring the starting level of [^{3}H]*N*-methylscopolamine binding and its residual binding 4 min after start of the dissociation (for details, see text). Indicated are means ± S.D. of $n = 4$–8 separate experiments, each performed as quadruplicate determinations; scatter bars are not shown where they do not exceed the symbols. The EC_{50} values (concentrations retarding the rate of radioligand dissociation to half of the control) and the slope factors n_H of the curves are compiled in Fig. 1.

under the control condition and in the presence of compound 3c and could, thus, be characterized by the half life time $t_{1/2}$ and the apparent rate constant of dissociation k_{-1}, respectively. Compound 3c retarded concentration-dependently the dissociation of [^{3}H]*N*-methylscopolamine, which indicated an interaction of the modulator with the radioligand-receptor complex. As the time course of dissociation was monophasic, measurement of [^{3}H]*N*-methylscopolamine binding at the starting level and residual binding at an appropriate time interval was sufficient to evaluate the concentration-dependent decline of k_{-1}. This fact was utilized to quantify the allosteric potency by means of the two-point-kinetic type of experiments, the principles of which are discussed in detail elsewhere (Kostenis and Mohr, 1996). The time interval (4 min) after which dissociation was terminated in the two-point-kinetic experiments is indicated by a dotted line in Fig. 2. During this interval, approximately 75% of the bound [^{3}H]NMS dissociated from the receptors in the absence of a test compound. Applying a monoexponential decay equation $B_t = B_0\ e^{-k_{-1} \cdot t}$ allowed to derive the apparent rate constant of dissociation k_{-1} from the residual binding data (B_t, 4 min value of residual binding; B_0, starting level of binding; t, 4 min). The resulting curve for the effect of compound 3c on the apparent rate constant of dissociation k_{-1} is included in Fig. 3. The allosteric effects of the other test compounds on the dissociation of [^{3}H]*N*-methylscopolamine were likewise measured. Since monophasicity of dissociation is a prerequisite for the procedure, it was checked for each test compound by means of 'complete kinetic experiments' (cf., Fig. 2) at selected concentrations

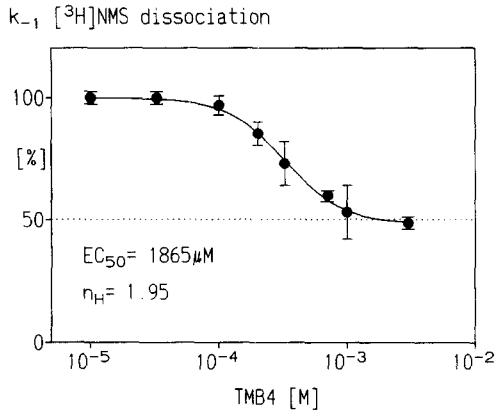


Fig. 4. Concentration-effect curve for the allosteric effect of TMB4 on the apparent rate constant k_{-1} of the dissociation of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS, 0.2 nM), expressed as percent of the control value. Data obtained by two-point-kinetic experiments. Indicated are means ± S.D. of $n = 2–5$ experiments. Error bars are not shown where they do not exceed the symbols.

reducing k_{-1} to about 50% and about 10% of the control value that dissociation remained monophasic (data not shown).

The concentration-effect curves displayed in Fig. 3 reveal that the compounds at the highest applied concentrations have in common the ability to nearly prevent the dissociation of [^{3}H]*N*-methylscopolamine. The concentration-effect curves were characterized by the EC_{50} value (concentration reducing dissociation rate to 50% of the control value) and the slope factor n_H; these values are compiled in Fig. 1. In contrast to the uni- and bilaterally substituted derivatives, the parent compound TMB4 revealed only a weak allosteric activity to retard the dissociation of [^{3}H]*N*-methylscopolamine (Fig. 4). The rate of

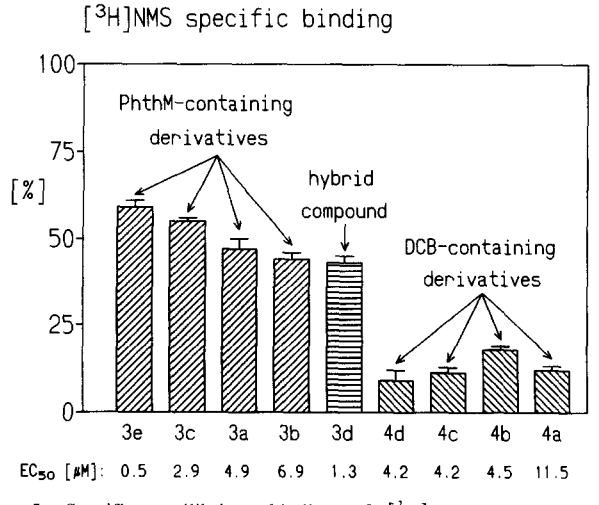


Fig. 5. Specific equilibrium binding of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS, 0.2 nM) in the presence of the indicated compounds at their EC_{50} concentrations to retard radioligand dissociation. Indicated are means ± S.D. of $n = 3–5$ experiments; the data for 4a–d were taken from Botero Cid et al. (1994).

dissociation was reduced to 50% of the control value at a concentration of $EC_{50} = 1865$ µM. At maximum concentrations, TMB4 was only capable of slowing the rate of [^{3}H]*N*-methylscopolamine dissociation to slightly less than 50% of the control value.

In order to check for an effect of the compounds on [^{3}H]*N*-methylscopolamine equilibrium binding, they were applied in the EC_{50} concentrations for the allosteric effect on radioligand dissociation (Fig. 5). The compounds containing at least one PhthM moiety (3a–e) depressed [^{3}H]*N*-methylscopolamine equilibrium binding to a lesser extent than did the DCB-containing derivatives 4a–d. It should be noted that the effect of the hybrid compound 3d – containing both a PhthM and a DCB moiety – was of similar magnitude as in the PhthM series of compounds.

4. Discussion

In case of various G-protein-coupled receptors, ligand binding has been shown to be sensitive towards allosteric modulation, e.g., adrenergic α_2 receptors (Nunnari et al., 1987), adenosine A_1 receptors (Bruns and Fergus, 1990), dopamine D_2 receptors (Baures et al., 1994; Hoare and Strange, 1995); so far this phenomenon has been most extensively studied with muscarinic receptors, especially the M_2-subtype. Both, the association and the dissociation of a ligand may be altered by the allosteric modulator. The interplay between these actions is reflected by the effect of the modulator on the equilibrium binding of the ligand.

The events underlying an allosteric modulation on the molecular level are still unresolved. In general, the allosteric modulator may alter the ligand binding characteristics of the receptor by affecting the overall conformation of the receptor protein including the ligand binding site or by interacting directly with the ligand 'binding pocket'. Moreover, it is not clear whether the effect of an allosteric modulator on ligand association, which occurs at the free, non-liganded receptor, is mediated via another site than the effect on ligand dissociation, which takes place at a receptor occupied by the ligand. Several lines of evidence suggest, however, that different sites are involved: Structural requirements of the two effects have been found divergent with regard to both the structure of the modulators (e.g., differing stereoselectivities: Gerry et al., 1987; Waelbroeck, 1994; Gao and Liu, 1995) and the structure of the receptor protein (Leppik et al., 1994; Jakubík and Tuček, 1995). In the present study, we focused on the allosteric site of muscarinic M_2 receptors relevant for the delay of ligand dissociation.

The actions of allosteric modulators on ligand dissociation are known to depend on the type of radioligand under investigation (e.g., Lee and El-Fakahany, 1991; Tuček and Proška, 1995). Here, [^{3}H]*N*-methylscopolamine was chosen, because it is the commonly applied radioligand in this field of research.

All test compounds were found capable of retarding [^{3}H]*N*-methylscopolamine dissociation. The time course of dissociation remained monophasic in the presence of the test compounds. This finding is likely to indicate that the binding kinetics of the modulator at the receptor are considerably faster than the kinetics of the radioligand (Lazareno and Birdsall, 1995). This conclusion appears reasonable taking into account that a low binding affinity often results from fast dissociation kinetics and that the effect of the test compounds and, thus, probably their binding occurred at about 1000-fold higher concentrations compared with the binding of [^{3}H]*N*-methylscopolamine.

The EC_{50} value, i.e., the concentration reducing the probability of desintegration of the [^{3}H]*N*-methylscopolamine-receptor complex by 50%, is used here as a measure of the potency to allosterically stabilize the radioligand-receptor complexes. Except in case of TMB4, the EC_{50} is equivalent to the inflection point of the concentration effect curves and might represent a measure for the binding affinity of the modulator to the site mediating the retardation of radioligand dissociation.

The allosteric potency of the parent compound, i.e., the bisoxime TMB4, is low (Fig. 4; EC_{50} = 1865 μM; inflection point of the curve at 333 μM). Furthermore, TMB4 slowed [^{3}H]*N*-methylscopolamine dissociation at maximum to slightly less than 50% of the control value; this may be termed a submaximum allosteric efficacy. Unilateral introduction of a PhthM or a DCB substituent leads to a considerable increase of both the allosteric potency and the allosteric efficacy (cf., Fig. 1). This finding indicates that the lateral substituent dominates over the bispyridinium middle chain with regard to the allosteric activity.

The PhthM-containing compound 3a (EC_{50} = 4.9 μM) surpasses in potency the DCB-containing derivative 4a (EC_{50} = 11.5 μM) by a factor of two. If the orientation of the PhthM and the DCB compounds within the allosteric binding area of the receptor protein were identical, the higher potency would point to a better fit of PhthM at the putative allosteric site.

In case of an identical orientation of the PhthM- and the DCB compounds at the allosteric site, analogous modifications at the opposite end of the respective structures should induce similar potency shifts in each group of compounds. However, some of the experimental results are not in line with this prediction (cf., Fig. 1): for instance, contralateral substitution of the oxime proton by methyl decreases the allosteric potency slightly but significantly in the PhthM-containing derivatives (3b vs. 3a) whereas the potency is increased in the DCB compounds (4b vs. 4a). A set of alterations of the methyloxime moiety significantly alters the allosteric potency in the PhthM compounds (3b vs. 3c vs. 3d), but does not affect the potency in the DCB series (4b vs. 4c vs. 4d). These observations lead to the conclusion that the PhthM-substituted bispyridinium compounds may not have the same orientation within the allosteric binding area compared with the DCB-substituted agents. It appears as if the PhthM ring may guide a bispyridinium compound into another location than does the DCB ring.

The dominating role of the PhthM ring for the binding affinity may explain that the contralateral modifications in the set 3a–d were accompanied by a rather limited, though significant, potency shift: Compared with the PhthM-containing side of the molecules the opposite end apparently contributes only to a minor extent to the overall binding affinity of the molecule. Nevertheless, this end is obviously also involved in attaching to the receptor protein and may, thus, serve as a probe for the respective part of the binding site.

Considering the subset of bispyridinium compounds with bilateral ring substitution, a different pattern of potency shifts is encountered. Introduction of DCB into the PhthM oxime 3a and the DCB oxime 4a, respectively, augments the potency in both cases (3d vs. 3a, 4d vs. 4a). Furthermore, the steepness of the concentration-effect curve (cf., the Hill coefficients n_H in Fig. 1) is increased to the same extent. A similarly high steepness has been observed with tacrine for the delay of radioligand dissociation from M_1 receptors (Potter et al., 1989; Mohr and Tränkle, 1994) and from M_2 receptors (Ellis and Seidenberg, 1992); so far, however, an explanation for this phenomenon is not possible. The results obtained with the bilaterally ring substituted compounds may be compatible with, but do not prove, an identical orientation within the allosteric binding area.

In any case, taken together our findings lead us to the idea that bispyridinium compounds may attach in more than one orientation to the site(s) mediating the allosteric delay of [^{3}H]*N*-methylscopolamine dissociation. This conclusion is in line with the concept of a multiple binding mode, i.e., seemingly minor structural modifications result in an altered orientation of a ligand at the binding site(s) of a receptor or an enzyme (for examples, see Kubinyi, 1993, 1995; Folkers and Michael, 1994). It may be imagined that the compounds utilize different points of attachment within a common binding area for allosteric modulators on the M_2 receptor protein. However, the structure-activity relationships might as well imply the presence of topographically distinct sites of action. Thus, the findings are not readily compatible with the model of a common site for allosteric modulators to delay radioligand dissociation from M_2 receptors.

The concept of a multiple binding mode may offer an explanation for the submaximum allosteric efficacy found with TMB4. Its binding affinity appears to be low, and high concentrations have to be applied to obtain an allosteric effect. Since TMB4 does not contain a ring substituent, the molecule may not be preferentially attracted into a distinct orientation at the allosteric binding area. As the aminoacid sequence of the muscarinic M_2 receptor protein contains several negatively charged residues which could serve as points of attachment for positively charged ligands (Kubo et al., 1986; Wess, 1993), it is conceivable

that TMB4 may bind to the receptor protein in various orientations. If not all of the possible orientations result in an allosteric delay of radioligand dissociation and if TMB4 bound in one orientation interferes with binding in other orientations, a maximum occupancy of the muscarinic M_2 receptors by TMB4 will lead to an allosteric effect below maximum. To the best of our knowledge, the allosteric modulators with partial intrinsic activity known so far share a low allosteric potency (Ellis and Seidenberg, 1992; Tränkle and Mohr, 1994). However, at present this idea is merely speculative and we are well aware that there are other possible explanations for the phenomenon of a sub-maximum allosteric efficacy.

The experiments to check for an effect of the compounds on the equilibrium binding of [^{3}H]*N*-methylscopolamine (Fig. 5) had a screening type character, but nevertheless allow some tentative conclusions. The allosteric delay of [^{3}H]*N*-methylscopolamine dissociation would per se increase the binding affinity of the radioligand. Since the compounds actually reduced [^{3}H]*N*-methylscopolamine equilibrium binding (cf., Fig. 5), their inhibitive effect on radioligand association has to exceed the retarding action on radioligand dissociation. The PhthM-containing compounds reduced equilibrium binding less than did the DCB-containing derivatives at concentrations being equieffective with regard to the effect on ligand dissociation. Thus, the inhibition of radioligand association is, relative to the allosteric effect on dissociation, more pronounced in the DCB-containing derivatives than in the PhthM compounds. This implies that the structure-activity relationships for the effects on radioligand association and dissociation do not correspond. Apparently, the binding site mediating the effect on ligand association has divergent properties compared with the allosteric site mediating the effect on radioligand dissociation. The former site may consist of the radioligand binding site, or of another site accessible for the modulator when the receptor protein is not occupied by the radioligand, or of the above-mentioned allosteric site in a state differing from the state involved in the delay of radioligand dissociation.

In any case, also with respect to the interference with radioligand association the lateral ring moieties appear to predominate over the bispyridinium middle chain: the hybrid compound 3d containing both PhthM and DCB behaves like a PhthM compound, suggesting that PhthM is also superior to DCB in determining the mode of interaction with the site mediating the inhibition of radioligand association.

In conclusion, the structure-activity data provide evidence that bispyridinium-type allosteric modulators – though structurally closely related – may interact in more than one orientation with the muscarinic M_2 receptor in the [^{3}H]*N*-methylscopolamine-occupied state. Future investigations will have to clarify whether topographically distinct sites or a common binding area for allosteric modulators are involved in this phenomenon.

Acknowledgements

It is gratefully indicated that the work was supported by the Fond der Chemischen Industrie (Germany) and by the KAAD (grant to H.M.B.C.).

References

Baures, P.W., W.H. Ojala, W.B. Gleason, R.K. Mishra and R.L. Johnson, 1994, Design, synthesis, X-ray analysis, and dopamine receptor-modulating activity of mimics of the 'C5' hydrogen-bonded conformation in the peptidomimetic 2-oxo-3(*R*)-[2(*S*)-pyrrolidinylcarbonyl)amino]-1-pyrrolidineacetamide, J. Med. Chem. 37, 3677.

Bejeuhr, G., U. Holzgrabe, K. Mohr, U. Sürig and A. Von Petersenn, 1992, Molecular modelling and synthesis of potent stabilizers of antagonist binding to M-cholinoceptors, Pharm. Pharmacol. Lett. 2, 100.

Botero Cid, M.H., U. Holzgrabe, E. Kostenis, K. Mohr and C. Tränkle, 1994, Search for the pharmacophore of bispyridinium-type allosteric modulators of muscarinic receptors, J. Med. Chem. 37, 1439.

Bruns, R.F. and J.H. Fergus, 1990, Allosteric enhancement of adenosine A_1 receptor binding and function by 2-amino-3-benzoylthiophenes 38, 939.

Buschendorf, K., K. Mohr and J. Schnekenburger, 1990, Comparison between the allosteric and the competitive action of a series of ligands at mACh-receptors, Naunyn-Schmiedeberg's Arch. Pharmacol. 341 (Suppl.), R80.

Ellis, J. and M. Seidenberg, 1992, Two allosteric modulators interact at a common site on cardiac muscarinic receptors, Mol. Pharmacol. 42, 638.

Folkers, G. and M. Michael, 1994, Pharmacodynamics of opioids: recent developments, Pharm. Acta Helv. 69, 61.

Gao, Z. and C. Liu, 1995, Competitive and allosteric binding of 2 α-DHET and its optical isomers to rat cardiac muscarinic receptors, Eur. J. Pharmacol. 289, 369.

Gasteiger, J., U. Holzgrabe, E. Kostenis, K. Mohr, U. Sürig and M. Wagener, 1995, Variation of the oxime function of bispyridinium-type allosteric modulators of M_2-cholinoceptors, Pharmazie 50, 99.

Gerry, R.H., B. Rauch, R.A. Colvin, P.N. Adler and F.C. Messineo, 1987, Verapamil interaction with the muscarinic receptor: stereoselectivity at two sites, Biochem. Pharmacol. 36, 2951.

Hoare, S.R.J. and P.G. Strange, 1995, Allosteric regulation of the rat D_2 dopamine receptor, Biochem. Soc. Transact. 23, 92S.

Jakubík, J. and S. Tuček, 1995, Positive allosteric interactions on cardiac muscarinic receptors: effects of chemical modifications of disulphide and carboxyl groups, Eur. J. Pharmacol. 289, 311.

Jepsen, K., H. Lüllmann, K. Mohr and J. Pfeffer, 1988, Allosteric stabilization of [^{3}H]*N*-methylscopolamine-binding in guinea pig myocardium by an antidote against organophosphate intoxication, Pharmacol. Toxicol. 63, 163.

Kostenis, E., U. Holzgrabe and K. Mohr, 1994, Allosteric effect on muscarinic M_2-receptors of derivatives of the alkane-bis-ammonium compound W84 – comparison with bispyridinium-type allosteric modulators, Eur. J. Med. Chem. 29, 947.

Kostenis, E. and K. Mohr, 1996, Two-point-kinetic experiments to quantify allosteric effects on radioligand dissociation, Trends Pharmacol. Sci. 17, 280.

Kubinyi, H., 1993, Comparative molecular field analysis, in: QSAR: Hansch Analysis and Related Approaches, Vol. 1, eds. R. Mannhold, P. Kroogsgard-Larsen and H. Timmermann (VCH, Weinheim) p. 163.

Kubinyi, H., 1995, Lock and key in the real world: concluding remarks, Pharm. Acta Helv. 69, 259.

Kubo, T., A. Maeda, K. Sugimoto, I. Akiba, A. Mikami, H. Takahashi, T. Haga, K. Haga, A. Ichiyama, K. Kangawa, H. Matsuo, T. Hirose and

S. Numa, 1986, Primary structure of porcine cardiac muscarinic acetylcholine receptor deduced from the cDNA sequence, FEBS Lett. 209, 367.

Lazareno, S. and N.J.M. Birdsall, 1995, Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors, Mol. Pharmacol. 48, 362.

Lee, N.H. and E.E. El-Fakahany, 1991, Allosteric antagonists of the muscarinic acetylcholine receptor, Biochem. Pharmacol. 42, 199.

Leppik, R.A., R.C. Miller, M. Eck and J.-L. Paquet, 1994, Role of acidic amino acids in the allosteric modulation by gallamine of antagonist binding at the m2 muscarinic acetylcholine receptor, Mol. Pharmacol. 45, 983.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193, 265.

Mohr, K. and C. Tränkle, 1994, Allosteric effects of the alkane-bis-ammonium compound W84 and of tacrine on [^{3}H]pirenzepine binding at M_1-receptors in rat cerebral cortex, Pharmacol. Toxicol. 75, 391.

Nunnari, J.M., M.G. Repaske, S. Brandon, E.J. Cragoe Jr. and L.E. Limbird, 1987, Regulation of porcine brain α_2-adrenergic receptors by Na^+, H^+ and inhibitiors of Na^+/H^+ exchange, J. Biol. Chem. 262, 12387.

Potter, L.T., C.A. Ferrendelli, H.E. Hancett and M.A. Hollifield, 1989, Tetrahydroaminoacridine and other allosteric antagonists of hippocampal M_1 muscarinic receptors, Mol. Pharmacol. 35, 652.

Proška, J. and S. Tuček, 1995, Competition between positive and negative allosteric effectors on muscarinic receptors, Mol. Pharmacol. 48, 696.

Tränkle, C., E. Kostenis, U. Holzgrabe and K. Mohr, 1994, Ranking of allosteric modulators of M_2-cholinoceptors, Naunyn-Schmiedeberg's Arch. Pharmacol. 302, R76.

Tränkle, C. and K. Mohr, 1994, Hexamethonium and obidoxime antagonize the allosteric actions of alcuronium, W84, W-DUO3 and gallamine at cardiac M_2-receptors, Life Sci. 56, 16.

Tuček, S. and J. Proška, 1995, Allosteric modulation of muscarinic acetylcholine receptors, Trends Pharmacol. Sci. 16, 205.

Waelbroeck, M., 1994, Identification of drugs competing with d-tubocurarine for an allosteric site on cardiac muscarinic receptors, Mol. Pharmacol. 46, 685.

Wess, J., 1993, Molecular basis of muscarinic acetylcholine receptor function, Trends Pharmacol. Sci. 14, 308.