Role of Receptor Protein and Membrane Lipids in Xanomeline Wash-Resistant Binding to Muscarinic M₁ Receptors

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ABSTRACT
Xanomeline is a novel agonist functionally selective for muscarinic receptors of the M₁ subtype. It binds to this receptor in two modes, reversible and quasi-irreversible (wash-resistant). We investigated the unknown mechanism of the wash-resistant binding in experiments with muscarinic M₁ receptors expressed in transfected Chinese hamster ovary cells. Xanomeline’s structure consists of two heterocycles and an hexyl side chain. We compared the wash-resistant binding of xanomeline and its analogs with shorter O-alkyl side chains. For the wash-resistant binding to occur, the O-alkyl chain had to be at least O-butyl or longer. Accumulation of inositol phosphates was enhanced in washed cells that had been preexposed to xanomeline or its pentyl analog, whereas the agonistic effects of the methyl, propyl, and butyl analogs were abolished by washing. Only the reversible binding of xanomeline was detected on purified soluble receptors, but both binding modes occurred on purified receptors reconstituted into liposomes and exposed to xanomeline only after reconstitution. The wash-resistant binding did not occur if the exposure of purified receptors or liposomes alone to xanomeline, followed by washing, preceded reconstitution. Simultaneous presence of receptors and their lipid environment is therefore essential for the wash-resistant binding to take place. We suggest that the wash-resistant binding of xanomeline involves interhelical penetration of the M₁ muscarinic receptor by xanomeline’s O-alkyl chain and its interaction with membrane lipids surrounding the receptor.

Molecular Mechanistic Interpretation

Muscarinic receptors of the M₁ subtype represent the prevailing species of muscarinic receptors in the brain cortex and hippocampus and are believed to play important roles in higher nervous functions. The discovery that cholinergic innervation of the brain cortex is severely impaired in patients with Alzheimer’s disease has stimulated intensive search for muscarinic agonists selective for the M₁ subtype of muscarinic receptors to reverse memory deficit in this debilitating disease. One of the outcomes of this search has been the discovery of xanomeline [3(3-hexyloxy-1,2,5-thiadiazol-4-yl)1,2,5,6-tetrahydro-1-methylpyridine; Fig. 1], described to act as a functionally selective M₁ receptor agonist (Ward et al., 1995; Bymaster et al., 1998). The binding of xanomeline to the M₁ receptors differs from that of other muscarinic receptor agonists and antagonists in that a significant fraction of binding and receptor activation is resistant to washing (Christopoulos et al., 1998, 1999, 2000; Jakubík et al., 2002). At present, the mechanism of the wash-resistant binding of xanomeline remains unclear. The wash-resistant binding of xanomeline is a phenomenon of great importance. Understanding of its underlying molecular mechanism may provide a key to the synthesis of muscarinic ligands (both agonists and antagonists) with extremely long duration of action. To probe these mechanisms, we have investigated three aspects of the wash-resistant binding: 1) its temperature dependence, 2) its dependence on the nature of the O-alkyl group in the molecule of xanomeline and its analogs, and 3) its dependence on the presence of membrane lipids. Our findings demonstrate an important role of the O-alkyl group in xanomeline wash-resistant binding and receptor activation and necessity of the cell membrane lipid environment for xanomeline wash-resistant binding. We also offer a molecular mechanistic interpretation of the data.

Materials and Methods

Reagents. [3H]-N-Methylscopolamine ([3H]NMS), [3H]quinuclidinyl benzilate ([3H]QNB), (±)-[3H]4-(3-tertiarybutylamino-2-hydroxypropoxy)-benzimidazole-2-on hydrochloride ([3H]CGP-12177), and...
Mild trypsinization 7 days after subculturing and washed twice by growing in plastic dishes in Dulbecco’s modified Eagle’s medium with 10% bovine calf serum and 0.005% geneticin. They were harvested by mild trypsinization 7 days after subculturing and washed twice by centrifugation (3 min at 300g) and resuspension in a HEPES medium (110 mM NaCl, 5.3 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 25 mM glucose, 20 mM HEPES, 58 mM sucrose, with pH adjusted to 7.4 and osmolarity to 340 mosM). Membranes were prepared by homogenization with a Polytron PT3000 homogenizer and centrifugation at 30,000g for 20 min. Pellets were kept frozen at −20°C.

**Receptor Purification.** Receptors were purified by ABT-agrose affinity chromatography as described previously (Jakubík et al., 1998), with two modifications: 1) CHO cells expressing human muscarinic M1 receptors (kindly provided by Dr. M. Brann, Acadia Pharmaceuticals, Inc., San Diego, CA) and on membranes and purified receptors prepared from these cells. Cells were grown in plastic dishes in Dulbecco’s modified Eagle’s medium with 10% bovine serum and 0.005% geneticin. They were harvested by mild trypsinization 7 days after subculturing and washed twice by centrifugation (3 min at 300g) and resuspension in a HEPES medium (110 mM NaCl, 5.3 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 25 mM glucose, 20 mM HEPES, 58 mM sucrose, with pH adjusted to 7.4 and osmolarity to 340 mosM). Membranes were prepared by homogenization with a Polytron PT3000 homogenizer and centrifugation at 30,000g for 20 min. Pellets were kept frozen at −20°C.

**Measurement of Radioligand Binding.** Measurement of radioligand binding to muscarinic receptors was performed essentially as described previously (Jakubik et al., 1995). Membranes from 300,000 to 600,000 cells were incubated at 37°C in a final incubation volume of 0.8 ml. The composition of the incubation medium was the same as that of the homogenization medium (see above), with added ligands as indicated. The incubation was terminated by filtration through GF/C glass fiber filters using a cell harvester (Brandel Inc., Gaithersburg, MD), and the radioactivity retained on the filters was measured by liquid scintillation spectrometry. In experiments with purified receptors or liposomes, 50 to 100 pmol of purified receptors or purified receptors reconstituted in liposomes were incubated with 100 pM [3H]QNB (purified receptors) or 500 pM [3H]QNB (solubilated receptors) for 2 h at 25°C. The final incubation volume was 0.8 ml for reconstituted receptors and 0.2 ml for purified receptors. The incubation was terminated by filtration through GF/C glass fiber filters (reconstituted receptors) or gel filtration through 2 ml of Sephadex G-50 columns (purified receptors). Atropine (5 μM) was applied to determine nonspecific binding of [3H]QNB.

Measurement of the Binding of Xanomeline. The binding of xanomeline was determined indirectly according to its ability to diminish the binding of the muscarinic antagonists (Jakubík et al., 2002) [3H]NMS (in experiments on native membranes) or [3H]QNB (in experiments with purified and reconstituted receptors). Two kinds of binding experiments were performed in principle. In one type of experiments, xanomeline or its analogs were present in the incubation buffer and the membranes were incubated with [3H]NMS or [3H]QNB. In these experiments, the observed loss of radioligand binding (compared with control incubations without xanomeline) was due to both the wash-resistant and the reversibly bound xanomeline. In the second type of experiments, membranes were first pretreated with xanomeline or its analogs in the absence of the radioligand then washed to remove the free and reversibly bound drug (paradigm of preexposure). After the washing, membranes were incubated with the radioligand at 37°C. In this type of experiment, the loss of radioligand binding was only due to the wash-resistant bound xanomeline. Membranes incubated in the absence of xanomeline or its analogs were sham-treated (washed and centrifuged) in parallel with drug-pretreated samples.

**Accumulation of Inositol Phosphates Induced by Xanomeline.** The method was adapted from Berridge et al. (1982) with modifications according to Wang and El-Fakahany (1993) and Jakubík et al. (1995). Cells (3 × 10−4–4 × 105/tube; 0.8-ml incubation volume) were preincubated for 60 min at 37°C with [3H]myo-inositol at a final concentration of 10 μCi/ml, and this was followed by washing, 10-min incubation with 10 mM LiCl, and 60-min incubation with xanomeline or its analogs. Incubation was stopped by a mixture of chloroform/methanol/HC1 (2:1:0.25). Labeled inositol mono-, bis-, and tris-phosphates were separated together from myo-inositol and other labeled compounds by ion-exchange chromatography (Berridge et al., 1982), collected, and measured by liquid
scintillation spectrometry. Pretreatment with xanomeline or its analogs (paradigm of preexposure) was done concurrently with loading cells with [3H]myo-inositol.

**Treatment of Data.** Data were processed and analyzed with open source software OpenOffice 6.0.41 (OpenOffice Foundation, www.openoffice.org) and Grace 5.1.6 (Grace Development Team, www.plasma-gate.weizmann.ac.il/Grace) on Mandrake distribution of Linux (www.mandrake.com).

**Results**

**Dependence of Xanomeline Wash-Resistant Binding on Temperature.** To obtain information on the physico-chemical nature of the wash-resistant binding of xanomeline, we preincubated membranes with 0.1 μM to 1 mM xanomeline at temperatures ranging from 4°C to 37°C. After 24 h at 4°C or 15°C, or after 1 h at 25°C or 37°C, membranes were centrifuged and washed three times with HEPES medium. Incubations with xanomeline at 4°C or 15°C had to be prolonged to 24 h to allow xanomeline wash-resistant binding to approach equilibrium. Then they were incubated with 100 pM [3H]NMS for 1 h at 37°C in all cases to avoid influence of temperature on [3H]NMS binding. Interestingly, lowering the pretreatment temperature markedly diminished the affinity of the wash-resistant binding of xanomeline (Fig. 2). Nonlinear regression of data yielded pEC50 values of 5.6 ± 0.1, 5.3 ± 0.1, 4.8 ± 0.1, and 4.6 ± 0.1 (mean ± S.E.M., n = 3) at 37°C, 25°C, 15°C, and 4°C, respectively. This type of temperature dependence suggests a hydrophobic nature of xanomeline wash-resistant interaction with the receptor.

The decrement in [3H]NMS binding was due to increase in $K_d$, and for pretreatment in 25°C and 37°C also decrease in $B_{max}$ and as shown in Table 1.

**Wash-Resistant Binding of Xanomeline Analog.** We compared how the binding of [3H]NMS to membranes is affected by xanomeline and its four analogs with shorter alkoxy side chains (Fig. 3). Binding of [3H]NMS was measured both in the continued presence of increasing concentrations of xanomeline or its analogs, and after the membranes had been pretreated with increasing concentrations of the compounds and subjected to the washing procedure. Shortening the hexyloxy side chain of xanomeline to either pentyl or butyl did not markedly change the binding affinities of the compounds when they were continuously present during the binding experiment (Fig. 3; Table 2). In contrast, affinities of the propyl and methyl analogs were 0.5 and 1 order of magnitude lower, respectively, compared with xanomeline in this experimental paradigm (Fig. 3; Table 2). In experiments designed to measure the wash-resistant component of ligand binding, xanomeline and its pentyl analog displayed approximately 2 orders of magnitude lower affinities than those measured in the absence of washing. This is consistent with previous observations (Christopoulos et al., 1999, 2000). The difference between affinities determined in the two experimental paradigms was markedly larger in the case of the butyl analog (approximately 3.5 orders of magnitude). Pretreatment of membranes with the 0.1 mM of the butyl-analog increased the equilibrium dissociation constant for [3H]NMS from 0.39 ± 0.03 to 0.57 ± 0.04 nM and decreased maximum binding capacity from 105 ± 11 to 83 ± 7

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**TABLE 1**

Values of apparent equilibrium dissociation constants ($K_d$) and maximum binding capacities ($B_{max}$) for [3H]NMS

<table>
<thead>
<tr>
<th>Temperature</th>
<th>$K_d$ (nM)</th>
<th>$B_{max}$ (fmol/10^6 cells)</th>
</tr>
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<tbody>
<tr>
<td>Sham-treated</td>
<td>0.38 ± 0.02</td>
<td>108 ± 6</td>
</tr>
<tr>
<td>4°C</td>
<td>0.55 ± 0.06*</td>
<td>98 ± 5</td>
</tr>
<tr>
<td>15°C</td>
<td>0.62 ± 0.07*</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>25°C</td>
<td>0.84 ± 0.10*</td>
<td>83 ± 4*</td>
</tr>
<tr>
<td>37°C</td>
<td>1.24 ± 0.15*</td>
<td>65 ± 4*</td>
</tr>
</tbody>
</table>

* Significantly different from sham-treated (p < 0.05, one-way ANOVA with Tukey’s multiple comparison test). For more statistical analysis, see Results.
fml/million cells (mean ± S.E.M., n = 4, p < 0.05, Student’s two-tailed test; data not shown). In contrast, the propyl and methyl analogs did not show any detectable wash-resistant binding (Fig. 3; Table 2).

**Accumulation of Inositol Phosphates Induced by Xanomeline and Its Analogs.** To test whether the agonistic potency and efficacy of xanomeline and its analogs depend on the nature of their alkoxy chains, accumulation of inositol phosphates was measured in the presence of increasing concentrations of xanomeline or its analogs, or in cells that had been preexposed to these agents and then extensively washed, without further addition of agonists (preincubation/washing paradigm). Xanomeline and tested analogs exerted marked agonistic effects when continuously present. The length of the alkoxy chain correlated positively with ligand potency and efficacy (Fig. 4; Table 2). The maximum stimulation of the accumulation of inositol phosphates induced by the pentyl analog was close in magnitude to that of xanomeline in both experimental paradigms. Apparent potencies of xanomeline and its pentyl analog were approximately 3 or- 

\[ \text{potency and efficacy of xanomeline and its analogs depend on} \]

\[ \text{binding (Fig. 3; Table 2).} \]

\[ \text{Potency and/or efficacy of xanomeline and its analogs in inhibiting} \]

\[ [\text{H}]NMS binding or stimulating the accumulation of inositol phosphates at M_1\text{ muscarinic receptors expressed in CHO cells} \]

Xanomeline or its analogs were either present during incubation or the paradigm of preincubation followed by washing before measurements of radioligand binding or accumulation of inositol phosphates was used. pIC \(_{50}\) values were obtained by nonlinear regression analysis of radioligand binding data in Fig. 3. pEC \(_{50}\) and \( E_{\text{max}} \) values were obtained by nonlinear regression analysis of inositol phosphates accumulation data in Fig. 4. Data are means ± S.E.M. of three independent experiments.

<table>
<thead>
<tr>
<th>Presence</th>
<th>Prelabeling/Washing</th>
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<tbody>
<tr>
<td></td>
<td>pIC (_{50})</td>
</tr>
<tr>
<td>Xanomeline</td>
<td>7.8 ± 0.1</td>
</tr>
<tr>
<td>Pentyl analog</td>
<td>7.8 ± 0.1</td>
</tr>
<tr>
<td>Butyl analog</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>Propyl analog</td>
<td>7.2 ± 0.1*</td>
</tr>
<tr>
<td>Methyl analog</td>
<td>6.7 ± 0.2*</td>
</tr>
</tbody>
</table>

N.C., did not converge; N.I., no inhibition; N.R., no response.

* Significantly different from xanomeline (p < 0.05, one-way ANOVA with Tukey’s multiple comparison test). For more statistical analysis, see Results.

The methyl and propyl analogs became completely lost after washing, confirming that these analogs did not bind to receptors in a wash-resistant manner. Interestingly, the butyl analog failed to show wash-resistant receptor activation, in spite of its ability to bind to the receptor after washing (see above).

**Competition for Binding between Xanomeline and Its Analogs.** At first glance, data shown in Fig. 3 suggest that the methyl and propyl analogs of xanomeline do not bind to M\(_1\) receptors in a wash-resistant mode. However, another possible interpretation is that they could continue to bind to the receptor after washing, but their limited length makes their active head group unable to reach the receptor primary binding pocket and interfere with the binding of \([\text{H}]NMS\). To distinguish between these two possibilities, we tested whether, after washing, they would interfere with subsequent wash-resistant binding of xanomeline. Membranes were preincubated for 1 h in the absence or presence of the methyl, propyl, or butyl analogs of xanomeline (1, 1, and 0.1 mM, respectively), thoroughly washed, incubated with increasing concentrations of xanomeline for 1 h, and washed again. Finally, they were incubated with \([\text{H}]NMS\) for 1 h. Neither the propyl nor the methyl analogs affected subsequent xanomeline wash-resistant binding (Fig. 5). In contrast, the butyl analog diminished the potency of xanomeline in eliciting wash-resistant inhibition of \([\text{H}]NMS\) binding without affecting the maximal effect of xanomeline (Fig. 5). The apparent competition of the butyl analog with xanomeline suggests that their wash-resistant binding takes place at a common saturable site(s).

**Binding of Xanomeline to Purified Soluble Receptors and Receptors Reconstituted into Liposomes.** To test the role of the receptor’s lipid surroundings, the binding of xanomeline to soluble purified M\(_1\) muscarinic receptors (Fig. 6) and purified receptors reconstituted into liposomes (Fig. 7) was investigated. In experiments with purified receptors, a radioligand with slow dissociation (\([\text{H}]QNB\)) was used because \([\text{H}]NMS\) is not suitable for gel filtration. Although the equilibrium dissociation constant of \([\text{H}]QNB\) was 64 ± 3 pM in membranes, it was 345 ± 25 and 72 ± 5 pM in case of soluble purified receptors and purified receptors reconstituted in liposomes, respectively (mean ± S.E.M., n = 3; data not shown). Xanomeline present simultaneously with \([\text{H}]QNB\) during 2-h incubation at 25°C diminished \([\text{H}]QNB\) binding to soluble purified receptors with IC \(_{50}\) value in the submicromolar range (Fig. 6, filled symbols). On the other hand, pretreatment of soluble purified receptors with xa-
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Our finding that the alkyloxy side chain of xanomeline and its analogs is required for wash-resistant effects is supported both by binding studies and measurements of accumulation of inositol phosphates. Although both the pentyl and butyl analogs elicited wash-resistant binding to the M1 muscarinic

treated with xanomeline before reconstitution and then washed, reconstituted, and incubated with [3H]QNB, the binding of [3H]QNB to receptors in such reconstituted liposomes was unaffected by the preceding exposure to xanomeline (Fig. 7, open squares and triangles for purified receptors pretreated with xanomeline before reconstitution and then washed, reconstituted, and incubated with [3H]QNB). Alternatively, either soluble purified receptors without liposomes (open squares) or liposomes without receptors (open triangles) were pretreated with xanomeline at the concentrations indicated on the abscissa for 1 h, washed through the gel-filter, and only then reconstituted into liposomes and subsequently incubated with [3H]QNB in the absence of xanomeline. Data are means ± S.E.M. of three independent experiments performed in triplicates. Counts in control samples in independent experiments ranged from 2400 to 4800 dpm. In individual experiments counts of control samples of soluble purified receptors and reconstituted receptors were close to each other.


treated with xanomeline at the concentrations indicated on the abscissa followed by washing did not affect [3H]QNB binding during subsequent 2-h incubation (Fig. 6, open symbols). Purified receptors did regain the ability to bind xanomeline in the wash-resistant mode after reconstitution into liposomes. Xanomeline present simultaneously with [3H]QNB during 2-h incubation diminished [3H]QNB binding to receptors reconstituted in liposomes with a pIC50 value of 7.3 ± 0.1 (mean ± S.E.M., n = 3) (Fig. 7, filled circles). On receptors reconstituted into liposomes and treated with xanomeline only after reconstitution but washed before the incubation with [3H]QNB, the pIC50 value for the residual inhibitory effect of xanomeline on [3H]QNB binding was 5.6 ± 0.2 (mean ± S.E.M., n = 3) (Fig. 7, open circles). However, if either the soluble purified receptors or empty liposomes were pre-

Discussion

Fig. 5. Effects of pretreatment with xanomeline analogs on xanomeline wash-resistant binding. CHO cell membranes were preincubated for 1 h in the absence or presence of the methyl, propyl, or butyl derivatives of xanomeline (1, 1, and 0.1 mM, respectively) and washed. Membranes were then incubated with increasing concentrations of xanomeline for 1 h, followed by another series of washing. Finally, samples were incubated with [3H]NMS for 1 h at 37°C. [3H]NMS binding is expressed in dpm per tube. Data are means ± S.E.M. of three individual experiments performed in triplicate. R, (3(3-oxy-1,2,5-thiadiaz-4-yl)-1,2,5,6-tetrahydron-1-methylpyridine.

Fig. 6. Effects of xanomeline on [3H]QNB binding to purified soluble M1 muscarinic receptors. Binding of [3H]QNB was measured in the presence of increasing concentrations of xanomeline (closed squares). Alternatively, purified receptors were pretreated for 1 h with increasing concentrations of xanomeline, followed by washing through gel-filter and then incubated with [3H]QNB for 2 h at 25°C (open squares). [3H]QNB binding is expressed as percentage of binding to receptors not treated with xanomeline. Data are means ± S.E.M. of three individual experiments performed in pentaplicate.

Specificity of Xanomeline Wash-Resistant Effects to Muscarinic Receptors. CHO cells express endogenous β-adrenergic receptors as determined by [3H]CGP-12177 binding (Kd = 0.28 ± 0.03 nM, Bmax = 31 ± 6 fmol/million cells, mean ± S.E.M., n = 3). Xanomeline present simultaneously at 1 mM during incubation with 1 nM [3H]CGP-12177 decreased ligand binding to 72 ± 7 and 75 ± 8%; two independent experiments performed in triplicate. However, preincubation of cells with 1 mM xanomeline followed by washing did not alter subsequent binding of 1 nM [3H]CGP-12177 (99 ± 8 and 97 ± 9% of control binding; two independent experiments performed in triplicate). Therefore, wash-resistant effects of xanomeline are specific to muscarinic receptors.

Fig. 7. Effects of xanomeline on [3H]QNB binding to purified M1 muscarinic receptors reconstituted into liposomes. Liposomes with reconstituted receptors were incubated with 100 pM [3H]QNB in the presence of increasing concentrations of xanomeline (closed circles). Alternatively, liposomes with reconstituted receptors had been pretreated with xanomeline at the concentrations indicated on the abscissa for 1 h, followed by washing through gel-filter, and subsequently incubated with [3H]QNB (open circles). Alternatively, either soluble purified receptors without liposomes (open squares) or liposomes without receptors (open triangles) were pretreated with xanomeline at the concentrations indicated on the abscissa for 1 h, washed through the gel-filter, and only then reconstituted into liposomes and subsequently incubated with [3H]QNB in the absence of xanomeline. Data are means ± S.E.M. of three individual experiments performed in triplicates. Counts in control samples in independent experiments ranged from 2400 to 4800 dpm. In individual experiments counts of control samples of soluble purified receptors and reconstituted receptors were close to each other.
receptor, only the former exhibited wash-resistant receptor activation. At present, we do not have satisfactory explanation for this discrepancy. Wash-resistant binding of the butyl analog seems to take place at the same receptor domain as xanomeline. Analogs with shorter side chains did not exhibit wash-resistant effects on the binding of either xanomeline or [3H]NMS. Thus, these analogs are incapable of attaching to the M1 receptor in a wash-resistant manner.

The fact that xanomeline does not exert wash-resistant binding to solubilized and purified receptors before reconstitution (Fig. 6) means that lipids are required for this interaction. Regaining of xanomeline wash-resistant binding upon reconstitution of solubilized and purified receptors into artificial lipid vesicles (Fig. 7) suggests that a lipid environment is a prerequisite for xanomeline wash-resistant binding. Thus, the xanomeline hydrophobic chain might interact directly with membrane lipids surrounding the receptor in a receptor-dependent manner. However, the possibility of xanomeline partitioning in the lipid bilayer in a receptor-independent manner was excluded. There is no xanomeline wash-resistant binding to reconstituted receptors when only liposomes were pretreated with xanomeline and then washed, before reconstitution (Fig. 7). Together, xanomeline wash-resistant binding requires both the receptor protein and adjacent membrane lipids. An alternative interpretation of the requirement of receptor reconstitution of solubilized receptors for xanomeline wash-resistant binding is the lack of appropriate receptor conformation in the soluble state. Thus, accessibility of the site of xanomeline wash-resistant attachment on the receptor might be obliterated.

It is possible that xanomeline at concentrations as high as 1 mM might act as a detergent due to its amphipathic properties (polar heterocycles and hydrophobic O-alkyl side chain). This would lead to perturbation of receptor-lipid interactions and decreased radioligand binding to the muscarinic receptors. However, this is very unlikely due to the following reasons. As discussed above, the wash-resistant binding is not simply the result of xanomeline partitioning in the membrane, a common property of detergents. Second, xanomeline fully stimulates accumulation of inositol phosphates in a wash-resistant manner. Conservation of receptor function would not be expected in presence of a detergent that would hamper interactions between the receptor and other cellular components necessary for receptor signaling. Last, but not least, pretreatment of membranes of CHO cells with 1 mM xanomeline followed by washing does not affect binding of [3H]CGP-12177 to endogenous β-adrenergic receptors. Such specificity of the wash-resistant effects of xanomeline to muscarinic receptors do not conform to a generalized detergent-like mechanism of action.

Together, these observations offer a consistent mechanistic explanation for the wash-resistant association of xanomeline with M1 muscarinic receptors. Apparently, the wash-resistant association of xanomeline involves its alkyloxy side chain and depends on interaction not only with the receptor protein but also with lipids surrounding the receptor. Both the receptor protein and surrounding membrane lipids must be present simultaneously for wash-resistant binding to occur. Data obtained on empty liposomes make it clear that the phenomenon of wash-resistant binding of xanomeline is not a consequence of simple partitioning of xanomeline into membrane lipids.

One likely scenario is that xanomeline enters the binding pocket of the receptor and associates with the receptor protein in a position that enables its alkyloxy side chain to penetrate between the receptor’s transmembrane segments and interact hydrophobically with the lipids surrounding the receptor. The computed length of the hexyloxy arm of xanomeline (0.79 nm) is indeed a bit longer than the diameter of a regular peptidic α-helix (0.68 nm). What remains unclear is the spatial relation between the classical binding sites of muscarinic receptors (with which xanomeline interacts in a competitive reversible manner) and the binding domain(s) involved in the wash-resistant binding. The two binding domains of xanomeline are not identical, because the wash-resistant binding cannot be prevented by atropine (Jakubik et al., 2002). Furthermore, interaction between ligand binding to the classical receptor binding site and wash-resistant bound xanomeline seems to be allosteric in nature (Jakubik et al., 2002). Regardless, however, clarification of the role of the hydrophobic side chain in the molecule of xanomeline and of the lipid surroundings of the M1 receptor in the long-lasting, wash-resistant binding of xanomeline may prove extremely useful for the design of new agonists and antagonists with a prolonged duration of action.

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References


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