An Unusual Form of the Association Binding Kinetics of N-[^3]H]Methylscopolamine to the Split Muscarinic M2trunk/M2tail Receptor

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ABSTRACT
The muscarinic M2 receptor was split at the third cytoplasmic loop into two fragments: the one containing the first five transmembrane regions and the N-terminal part of the third cytoplasmic loop was named M2trunk, while the other, which contained the last two transmembrane regions and the C-terminal part of the third cytoplasmic loop, was named M2tail. As seen in many other G protein-coupled receptors, when these two fragments were transfected together in COS-7 cells they rescued the pharmacological profile and the functional activity of the wild-type M2 receptor. Conversely, N-[^3]H]methylscopolamine ([^3]H]NMS) association binding experiments showed a substantial difference between the wild-type M2 and the split M2trunk/M2tail receptors. The progression of the association binding kinetic of the M2trunk/M2tail receptor was strictly dependent upon the amount of the fragment DNA transfected. When the amount of transfected DNA was 4 μg/plate and the B\textsubscript{max} of [^3]H]NMS at equilibrium was around 200 fmol/mg protein the form of the association was that of classical saturation, but when the amount of transfected DNA was lower the [^3]H]NMS association reached a maximum binding point and then declined to a lower equilibrium binding level. The form of the association was temperature-dependent: as the temperature was lowered, the maximum binding point tended to be higher. We suggest that this peculiar form of the [^3]H]NMS association binding to the muscarinic M2trunk/M2tail receptor is attributable to a less stable interaction between the trunk and the tail fragments of the split receptor.

The G protein-coupled receptors (GPCRs) are seven-transmembrane domain proteins that mediate a variety of signaling processes. Several reports have illustrated the capability of GPCRs to be split into fragments and, depending on where the receptor is split, it is possible for the mixture of the coexpressed fragments to show some or all the properties of the wild-type receptor. Typically, binding may be observed if the receptor is cut at extracellular loop 2 or at intracellular loops 2 and 3 (Schöneberg et al., 1995). However, full activity on coexpression, namely binding and G protein activation, has been observed only when the receptor is split at the intracellular loop 3, between helices 5 and 6, as shown for the rhodopsin (Ridge et al., 1996), α\textsubscript{2}-adrenergic (Kobilka et al., 1988), M\textsubscript{1}- and M\textsubscript{2}-muscarinic (Maggio et al., 1993), vasoressin V\textsubscript{2} (Schöneberg et al., 1996), gonadotropin-releasing hormone (Gudermann et al., 1997), neurokinin NK\textsubscript{1} (Nielsen et al., 1998), and dopamine D\textsubscript{2} and D\textsubscript{3} receptors (Scarselli et al., 2000, 2001). The individual fragments are not usually active, although an exception to this rule is seen in the chemokine receptors CCR5 and CXCR4, in which five-transmembrane domains appear to meet the minimum structural requirements for a functional GPCR (Ling et al., 1999).

A detailed study by Schöneberg et al. (1995) has shown that fragments can reach the plasma membrane individually, demonstrating that they can function as autonomous folding domains. This makes the association of the two fragments in the plasma membrane possible, although it does not exclude that they can meet earlier during the maturation process in the intracellular compartments. Jakubik and Wess (1999), using a sandwich enzyme-linked immunosorbent assay, demonstrated that muscarinic agonists and antagonists or allosteric ligands lead to a significant increase in the efficiency with which M\textsubscript{2} receptor fragments associate. They postulate that ligands can act as “anchors” between the N- (M\textsubscript{2}trunk) and C-terminal (M\textsubscript{2}tail) fragments. In line with this view, they showed that tetramethylammonium, a rather small positively charged ammonium compound, failed to promote the interaction between the M\textsubscript{2}trunk and M\textsubscript{2}tail polypeptides. Mutagenesis studies suggest that tetramethylammonium...
nium interacts with a conserved aspartate in transmembrane region III on the M₃ receptor protein (Asp-147 in M₃trunk) and that tetramethylammonium binding does not critically depend on residues located on transmembrane regions VI and VII (which are contained in M₃tail).

Gouldson et al. (1997), on the basis of computational studies applied to the problem of docking adrenergic ligands into a model of the β₂-adrenergic receptor, proposed a dynamic interaction between receptor fragments. The GPCR heptahelical bundle is a compact structure, and so there is not much space left free for docking adrenergic ligands using interactive molecular graphics. One solution they propose for solving this problem involves a three-stage docking process. In the first stage, the B domain (containing transmembrane regions VI and VII) is moved away from the A domain (containing transmembrane regions I to V). In the second stage, the ligand is docked against the A domain, and in the third stage the B domain is allowed to move back to the A domain during the course of molecular dynamic simulation to generate the final docked conformation.

In this study, using kinetic binding experiments, we observed a peculiar form of the association binding of N-[³H]methylscopolamine (³H[NMS]) to the split muscarinic M₃trunk/M₃tail receptor; we suggest that these results are attributable to a less stable interaction between the trunk and the tail fragments of the split receptor compared to the wild-type muscarinic M₂ receptor.

Materials and Methods

Reagents. N-[³H]methylscopolamine (83 Ci/μmol) was from PerkinElmer Life Sciences (Boston, MA); [³H]quinuclidinylbenzilate ([³H]QNB) (43 Ci/μmol) was from Amersham Biosciences Inc. (Piscataway, NJ); forskolin, gallamine, carbachol, glutaraldehyde, soybean trypsin inhibitor, digitonin, and Sephadex G-50 columns were from Sigma-Aldrich (St. Louis, MO); Tissue culture media and sera were from Sigma-Aldrich and Invitrogen (Carlsbad, CA).

Plasmids and Preparation of Mutant Muscarinic Receptor Constructs. We used the human M₂ muscarinic wild-type receptor inserted in a pCD plasmid (Bonner et al., 1987). The construction of the M₂trunk and M₂tail fragments has been described previously (Maggio et al., 1993). In particular, the M₂trunk is truncated after Ser-283, and the tail fragments of the split receptor compared to the wild-type muscarinic M₂ receptor.

Cell Cultures and Transfection. COS-7 cells were incubated at +37°C in a humidified atmosphere (containing 5% CO₂) and grown in Eagle's medium as modified by Dulbecco, which was supplemented with 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded at a density of 2 × 10⁵/100-mm dish and, 24 h later, they were transiently transfected with the plasmid DNA by the DEAE-dextran chloroquine method (Cullen, 1987). The total amount of DNA used for each transfection was brought to 4 μg by adding an appropriate amount of vector DNA.

Membrane Preparation and Binding Assay. COS-7 cells were transfected with the wild-type M₂ or the split M₂trunk/M₂tail receptor. Three days after transfection, confluent plates of cells were lysed by replacing the medium with ice-cold hypotonic buffer (1 mM Na-HEPES, 2 mM EDTA). After 20 min the cells were scraped off the plate and centrifuged at 17,000g for 20 min at +4°C. The lysed cell pellet was homogenized with a Polytron homogenizer in ice-cold binding buffer (50 mM Tris-HCl pH 7.4, 15 mM NaCl, 0.01 mg/ml bovine serum albumin). Membranes were kept on ice and warmed up to 37°C for 30 min. After incubation at 37°C for 15 min before the assay. Binding of [³H]NMS and [³H]QNB was carried out at +30°C in a final volume of 1 ml. Atropine 1 μM was used to define nonspecific binding. The bound ligand was separated from the unbound ligand using glass-fiber filters (Whatman, GF/B) with a Brandel Cell Harvester, and the filters were counted with a scintillation β-counter. Assay binding experiments were performed adding, at different times, 200 μl of [³H]NMS 2.5 nM (final concentration in the sample 500 pM) or 200 μl of [³H]QNB 5 nM (final concentration in the sample 1 nM). To avoid significant dilution of the radioligand concentration, the amount of membranes added to each sample was adjusted to give a total receptor binding (specific + nonspecific) of a maximum of 2.5% of the total radioligand added (about 2,900 dpm against 91,000 dpm).

Solubilized Receptor Fragments and Binding Assay on Solubilized Receptors. Cells were scraped off the plate into ice-cold buffer (buffer A) containing 20 mM KH₂PO₄, 20 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 0.02% (w/v) sodium azide, 1 mM benzamidine, 2 μg/ml pepstatin A, 0.2 μg/ml leupeptin, and 200 μg/ml bacitracin, pH 7.6. They were homogenized in a Polytron homogenizer for 30 s and spun down in a centrifuge for 40 min at +4°C and 100,000g. This step was then repeated in a slightly different buffer (buffer B): 20 mM KH₂PO₄, 20 mM NaCl, 1 mM EDTA, and 0.1 mM PMSF, pH 7.4. Protein content was assessed by the method of Lowry and the relative concentration was adjusted to 5 mg of protein/ml. Detergents were added in buffer B to a final concentration of 1% digitonin and 0.06% sodium cholate. The suspension was shaken on a horizontal shaker for 40 min at +4°C and then centrifuged for 40 min at +4°C and 100,000g. The supernatant fraction was stored on ice until required for the binding assay. Binding was carried out in a final volume of 55 μL/μl of soluble receptor preparation and 50 μl of [³H]NMS 10 nM in buffer E (20 mM KH₂PO₄, 20 mM NaCl, 1 mM EDTA, 5 mM MgSO₄, 0.1 mM PMSF, pH 7.4) containing 0.3% digitonin and 0.02% sodium cholate. The reaction was carried out at +30°C in Eppendorf microfuge tubes. At the end of the incubation period, 50-μl aliquots were loaded in Sephadex G-50 fine (0.8 × 6.5 cm) columns to separate the bound ligand from the unbound one. The fraction of the radioligand bound to the receptor recovered from the column was a maximum of 1.6% of the total radioligand added to each sample (about 1,500 dpm against 91,000 dpm).

Adenylyl Cyclase Assay. COS-7 cells were transfected with the wild-type M₂ or the split M₂trunk/M₂tail receptor plus the adenylyl cyclase V. Twenty-four hours after transfection, the cells were trypsinized and re-cultured in 24-well plates and, after an additional 24 h, the cells were assayed for adenylyl cyclase activity. The assay was performed in triplicate as described by Avidor-Reiss et al. (1995). In brief, the cells in the 24-well plates were incubated for 2 h with 0.25 ml/well of fresh growth medium containing 5 μCi/ml [³H]adenine, and this medium was replaced with 0.5 ml/well of Dulbecco’s modified Eagle’s medium containing 20 mM HEPES, pH 7.4, 0.1 mg bovine serum albumin, and the phosphodiesterase inhibitors 1-methyl-3-isobutylxanthine (0.5 mM) and RO-20-1724 (0.5 mM). Adenylyl cyclase activity was stimulated by the addition of 1 μM forskolin in the presence or absence of carbachol. After 10 min of incubation at +30°C, the medium was removed and the reaction terminated by the addition of perchloric acid containing 0.1 mM unlabeled CAMP followed by neutralization with KOH. The amount of [³H]AMP formed was determined by a two-step column separation procedure, as described by Avidor-Reiss et al. (1995).

Analysis of the Data. Saturation binding, kinetic association, and dissociation binding data were fitted (whenever possible) by using canonical equations for this type of experiment (Williams and Leffkowitz, 1978). Displacement experiments of [³H]NMS by the allosteric ligand gallamine were fitted using the equation described by Lazareno and Birdsall (1995). In association experiments where it was not possible to fit the data with an exponential growth curve, to
make the progression of the $[^3H]$NMS binding more evident throughout time we arbitrarily fitted the data to the equation $y = A_1 \cdot [1 - \exp(-xt)] + A_2 \cdot [1 - \exp(-xt_2)] + A_3 \cdot [1 - \exp(-xt_3)]$. All parameters were estimated by nonlinear regression, and values at successive interactions of the fitting procedure were adjusted according to the Marquardt (1963) algorithm.

### Results

In a first set of experiments, we compared the affinity of $[^3H]$NMS to the wild-type $M_2$ receptor with that of the split $M_{2trunk}/M_{2tail}$ receptor (Fig. 1). As mentioned under the Materials and Methods section, to keep the ratio between the two fragments stable throughout the experiments we used (when not otherwise specified) a plasmid that contained the two transcriptional units of $M_{2trunk}$ and $M_{2tail}$. Equilibrium binding experiments showed that $[^3H]$NMS binds to the wild-type and to the split muscarinic $M_2$ receptors with the same affinity (Table 1). In terms of function, the inhibition of the forskolin-stimulated cAMP accumulation by carbachol gave comparable $K_{D}$ values for the $M_2$ and the $M_{2trunk}/M_{2tail}$ receptors, although the extent of the inhibition was slightly different (Table 1). A substantial difference was observed, however, in the $B_{max}$. With the same amount of DNA (4 μg) the number of binding sites for the split $M_{2trunk}/M_{2tail}$ receptor compared to the wild-type $M_2$ receptor was, on average, 1:7 (Table 1).

To determine whether the binding kinetic of $[^3H]$NMS to the split and the wild-type $M_2$ receptor was similar, we measured the association and dissociation rate constants of this compound. Dissociation was started after a 2-h preincubation with 500 pM $[^3H]$NMS by diluting the tissue 100-fold in binding buffer containing 1 μM atropine. As can be seen in Fig. 2A, the normalized dissociation binding data of the $M_2$ receptor were fitted by a mono-exponential decay curve that gives a $k_{off}$ value of 0.29 ± 0.02 min$^{-1}$ (Table 2). $[^3H]$NMS dissociation experiments from the wild-type muscarinic $M_2$ receptors were performed also in the presence of three different concentrations of cold NMS, 1 μM, 0.1 mM, and 1 mM. Figure 2C shows that, at the concentration of 1 mM cold NMS, the rate of dissociation of $[^3H]$NMS from the $M_2$ receptor decreased.

Similar results were obtained with the split $M_{2trunk}/M_{2tail}$ receptor. Dissociation binding data were fitted by a mono-exponential decay curve (Fig. 2B and Table 2), and the $k_{off}$ was very close to that of the $M_2$ receptor (0.28 ± 0.03 min$^{-1}$). Again, high concentrations of cold NMS decreased the rate of dissociation of $[^3H]$NMS from the split $M_{2trunk}/M_{2tail}$ receptor (Fig. 2D). There was no difference in the percentage of $[^3H]$NMS dissociation between the two receptors in all the conditions tested.

The association kinetic was substantially different between the two receptors. The association binding data of $[^3H]$NMS (500 pM) to the wild-type receptor, no matter how much DNA was transfected, had the form of typical saturation (Fig. 3A) and were well fitted by a mono-exponential association curve (the actual amount of binding at equilibrium, in fmol/mg protein, is given in Table 3). The association rate constant ($k_{on}$) calculated was $14.5 \times 10^{6} \pm 2.87 \times 10^{6}$ min$^{-1}$ M$^{-1}$, and the $k_{off}/k_{on}$ ratio was well in agreement with the value of the $K_{D}$ calculated at equilibrium (Table 2). The progression of the association binding data of $[^3H]$NMS to the split $M_{2trunk}/M_{2tail}$ receptor was strictly dependent upon the amount of DNA transfected, and therefore on the number of fragments expressed on the membrane. When the amount of transfected DNA was 4 μg per plate, and the number of receptors at equilibrium was around 200 fmol/mg protein, the association binding data usually described a normal saturation curve (Fig. 3B) and were well fitted by a mono-exponential association curve. The $k_{off}$ was not significantly different from that of the wild-type receptor (Table 2). Again, the $k_{off}/k_{on}$ ratio agreed well with the $K_{D}$ of the split $M_{2trunk}/M_{2tail}$ receptor calculated at equilibrium. When the concentration of transfected DNA was progressively lowered, the binding kinetic changed proportionally: in particular, the association binding data reached a maximum after 2 to 10 min and thereafter they started to decline, reaching equilibrium at lower values of binding (Fig. 3B). The actual amount of binding at the top of the curve and at equilibrium, in fmol/mg protein, is given in Table 3. In the same experiments with very low levels of transfected DNA (0.25 μg), we found that $[^3H]$NMS binding was detectable at the beginning but that it then decreased to undetectable levels. To make the progression of the $[^3H]$NMS binding more evident, these atypical association data were arbi-

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**Fig. 1.** Schematic representation of wild-type muscarinic $M_2$ receptor and the derived receptor fragments. The truncated $M_2$ receptor ($M_{2trunk}$) contains an in-frame STOP codon after Ser-283, while the tail fragment ($M_{2tail}$) contains a START codon before Leu-281.

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**TABLE 1**

Parameters of $[^3H]$NMS and $[^3H]$QNB saturation binding and carbachol inhibition of forskolin-stimulated cAMP accumulation in COS-7 cells transiently transfected with $M_{2}$ and $M_{2trunk}/M_{2tail}$ receptors

<table>
<thead>
<tr>
<th>Binding data</th>
<th>$K_{D}$</th>
<th>$B_{max}$</th>
<th>$K_{D}$</th>
<th>$B_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^3H]$NMS</td>
<td>164 ± 25.9</td>
<td>1414 ± 151</td>
<td>136 ± 22.4</td>
<td>195 ± 26</td>
</tr>
<tr>
<td>$[^3H]$QNB</td>
<td>37.2 ± 3.93</td>
<td>1823 ± 215</td>
<td>33.3 ± 3.7</td>
<td>280 ± 32</td>
</tr>
<tr>
<td>cAMP assay</td>
<td>EC$_{50}$ (nM)</td>
<td>% inhibition</td>
<td>EC$_{50}$ (nM)</td>
<td>% inhibition</td>
</tr>
<tr>
<td>Carbachol</td>
<td>26.6 ± 3.7</td>
<td>50.1 ± 1.1</td>
<td>59.8 ± 7.3</td>
<td>40.8 ± 3.2</td>
</tr>
</tbody>
</table>

For radioligand binding assays, COS-7 cells were transiently transfected with 4 μg of DNA. For functional assays, COS-7 cells were transiently transfected with 2 μg of receptor DNA and 2 μg of adenyllyl cyclase V. These data represent the mean ± S.E. of two experiments, each performed in triplicate.
In our experiments, we exposed the tissue to [3H]NMS for 1 hour; then, taking advantage of its rapid dissociation kinetic, we performed parallel experiments using two different concentrations of [3H]NMS, 500 pM and 5 nM. Figure 2 shows that the higher concentration of [3H]NMS did not modify the progression of the kinetic, but only changed the values of the binding maximum and of the binding at equilibrium.

To determine whether the decrease in binding was due to the modified affinity of the receptor for [3H]NMS during the course of the association, we did parallel experiments using two different concentrations of [3H]NMS, 500 pM and 5 nM. Figure 5 shows that the higher concentration of [3H]NMS did not modify the progression of the kinetic, but only changed the values of the binding maximum and of the binding at equilibrium.

Next, we tested to see whether the loss of [3H]NMS binding during the association experiment was reversible or not. In one set of experiments, we exposed the tissue to [3H]NMS for 1 hour; then, taking advantage of its rapid dissociation kinetic, [3H]NMS was washed off the tissue with two 15-minute centrifugations at +4°C. The tissue was then re-exposed to 1 μM atropine (panels A and B) or unlabeled NMS at two different concentrations of 0.1 and 1 mM (panels C and D) were added. Ordinate, percentage of binding before the start of dissociation. Values are the average of triplicate determinations, with error bars corresponding to S.E. (panels A and B) of a representative of three experiments.

We reported the values of the association in a bar graph after 7 min for the maximum binding point and after 60 min for the binding at equilibrium. To set the percentage of binding loss, we performed an [3H]NMS association binding curve in a control tissue without any treatment. After 60 min of exposure to [3H]NMS, there was an approximately 40% decrease in binding with respect to the binding measured at 7 min; the absolute value of the binding maximum was 134 ± 7.7 fmol/mg protein (Fig. 6). In the tissue pretreated for 1 hour with [3H]NMS and then washed, the value of [3H]NMS binding measured after 7 min was 122 ± 10 fmol/mg of protein.
We studied how the allosteric ligand gallamine changes the binding kinetic of [3H]NMS to the M2<sub>trunk</sub>/M2<sub>tail</sub> receptor in comparison to M2. Initially, to estimate the affinity of gallamine (K<sub>Dal</sub>) for the allosteric site of the two receptors and its cooperativity with [3H]NMS, we performed inhibition experiments against two concentrations of [3H]NMS, 0.2 and 1 nM. Thereafter, we fitted the data and we calculated the different parameters applying the methods of Lazareno and Lazareno.

Changes in temperature modify the binding kinetic; therefore, we tested to see how this parameter could influence the progression of the kinetic. We performed parallel experiments at +30, +23, +10, and +4°C. The decrease in temperature determined an increase in the maximum binding point and a shift in the time at which this value was reached (Fig. 8).

In one set of experiments performed to exclude that this phenomenon could be related in some way to the binding of the split receptor to the G protein, we preincubated the tissue with 100 μM GTPγS to dissociate the receptor from the G protein and then we performed the association experiment. GTPγS did not modify the kinetic of the [3H]NMS association binding (Fig. 7).

In the experiments performed in panel A, the cells were transfected with a plasmid containing the two transcriptional units of M2<sub>trunk</sub> and M2<sub>tail</sub>, while in the experiment performed in panel C the cells were transfected with two individual plasmids, one containing the M2<sub>trunk</sub> and the other the M2<sub>tail</sub> receptor fragment. In these experiments, we kept the amount of the M2<sub>trunk</sub> fragment DNA fixed at 1 μg, and we varied the amount of the M2<sub>tail</sub> fragment DNA. The amount of DNA transfected is shown above each kinetic. Experiments were performed at +30°C with 1 nM [3H]NMS. Ordinate, percentage of the binding at equilibrium in COS-7 cells transfected with 4 μg of M2<sub>trunk</sub>/M2<sub>tail</sub> DNA. The association curve of [3H]QNB to the M<sub>2</sub> receptor was normalized to the value of the binding at equilibrium in COS-7 cells transfected with 2 μg of M2<sub>trunk</sub>/M2<sub>tail</sub>. The actual value of binding at equilibrium in cells transfected with 4 μg of M<sub>2</sub> and M2<sub>trunk</sub>/M2<sub>tail</sub> DNA was, respectively, 1921 ± 186 and 253 ± 27.3 fmol/mg protein. Values are the average of triplicate determinations of a representative of three experiments. The data were fitted by a mono-exponential association curve.

### Table 3

<table>
<thead>
<tr>
<th>DNA Transfected</th>
<th>M&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;trunk&lt;/sup&gt;/M&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;tail&lt;/sup&gt; DNA Transfected</th>
<th>M&lt;sub&gt;2&lt;/sub&gt; Binding at Equilibrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>μg</td>
<td>Binding at the Top of the Curve</td>
<td>Binding at Equilibrium</td>
</tr>
<tr>
<td>4</td>
<td>196</td>
<td>196</td>
</tr>
<tr>
<td>3</td>
<td>160</td>
<td>131</td>
</tr>
<tr>
<td>2</td>
<td>131</td>
<td>87</td>
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<tr>
<td>1</td>
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<td>56</td>
</tr>
<tr>
<td>0.5</td>
<td>88</td>
<td>13</td>
</tr>
<tr>
<td>0.25</td>
<td>25</td>
<td>N.D.</td>
</tr>
<tr>
<td>0.05</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined.
Birdsall (1995). Table 4 shows that the affinity and the cooperativity of gallamine with [3H]NMS did not change substantially between the split muscarinic M<sub>2trunk</sub>/M<sub>2tail</sub> and the wild-type M<sub>2</sub> receptor. Comparable results were obtained when COS-7 cells were transfected with two different concentrations of plasmid DNA, 2 and 4 µg, both for the M<sub>2</sub> and the M<sub>2trunk</sub>/M<sub>2tail</sub> receptor.

We then calculated the effect of gallamine on the dissociation of [3H]NMS from the M<sub>2trunk</sub>/M<sub>2tail</sub> and the M<sub>2</sub> receptors. Membranes were incubated with 500 pM [3H]NMS for 2 h, then dissociation was initiated by 100-fold dilution and by the addition of binding buffer containing atropine (1 µM) and various concentrations of gallamine. The effect of gallamine on the [3H]NMS off-rates is shown in Fig. 9. This figure shows how the ratio changes between the <i>k</i><sub>off</sub> in the presence of gallamine and the <i>k</i><sub>off</sub> in the absence of gallamine (<i>k</i><sub>0</sub>), and with increasing concentrations of gallamine. The curves obtained by interpolating the data with the Hill equation were identical, and the Hill slopes were 1.13 ± 0.10 and
Inhibition by gallamine of \[^3H\]NMS dissociation from \(M_2\) and \(M_{2\text{trunk}}/M_{2\text{tail}}\) receptors. \[^3H\]NMS (500 pM) was equilibrated at \(+30^\circ\text{C}\) for 2 h with membranes containing the \(M_2\) or the \(M_{2\text{trunk}}/M_{2\text{tail}}\) receptor, and then atropine (1 \(\mu\text{M}\)) and gallamine (at different concentrations) were added at time 0. The \(k_{\text{off}}\) values calculated for the off-rates in the presence of gallamine are expressed as a ratio of \(k_{\text{off}}\) to \(k_{\text{off}}^0\) (where \(k_{\text{off}}\) is the \(k_{\text{off}}\) value in the absence of gallamine).

1.11 \pm 0.15, respectively, for the \(M_{2\text{trunk}}/M_{2\text{tail}}\) and the \(M_2\) receptor. Gallamine at a concentration of 100 \(\mu\text{M}\) virtually blocks the dissociation of \[^3H\]NMS from both the wild-type and the split receptor.

To determine whether cross-linking agents can modify the association binding kinetic of the \(M_{2\text{trunk}}/M_{2\text{tail}}\) receptor, we performed \[^3H\]NMS association binding in the presence of the alkylating agent glutaraldehyde. The cells were exposed to increasing concentrations of glutaraldehyde (0.1, 0.5, and 1%) for 60 s; the glutaraldehyde was then removed by repeated washing and cells processed for membrane preparation. We began to see an effect of the glutaraldehyde at the 0.5% concentration but, in contrast to what we were expecting, glutaraldehyde tended to increase the ratio between the maximum binding point and the equilibrium binding point and to decrease the total amount of receptor binding; a representative experiment with 1% glutaraldehyde is shown in Fig. 10A. These results could be interpreted as glutaraldehyde altering the binding site of the receptor for \[^3H\]NMS. To prevent this effect, we saturated receptors with 1 \(\mu\text{M}\) NMS and then we exposed cells to glutaraldehyde, always in the presence of a saturating concentration of NMS. Thereafter, we removed both glutaraldehyde and NMS by repeated washing. The results were similar to the previous ones shown in Fig. 10A.

We also performed experiments with the wild-type \(M_2\) receptor. We treated the cells with 0.5 or 1% glutaraldehyde as described above. In this case, the glutaraldehyde did not change the profile of the \[^3H\]NMS association kinetic to the wild-type \(M_2\) receptor, but it considerably decreased the binding at equilibrium (Fig. 10B). These results were not due to a change in affinity, since the \(K_m\) values calculated in 1% glutaraldehyde-treated cells and in nontreated cells were not significantly different (control, \(K_m = 182 \pm 16.4\) pM; glutaraldehyde, \(K_m = 158 \pm 21.8\) pM).

Preliminary experiments were performed with solubilized receptors as well to see if we could reproduce the same association binding profile we saw in membranes. Cells were transfected with different amounts of \(M_{2\text{trunk}}/M_{2\text{tail}}\) receptor (4, 2, 1, and 0.25 \(\mu\text{g}\)), and then the receptor fragments were solubilized in sodium cholate-digitonin (as described under Materials and Methods). Solubilized receptors were exposed for different lengths of time to 9.1 nM \[^3H\]NMS at \(+30^\circ\text{C}\) and at the end the reaction was stopped by loading the samples in Sephadex G-50 columns to separate the bound ligand from the unbound one. We used a higher concentration of \[^3H\]NMS because the affinity of the radioligand for the solubilized \(M_{2\text{trunk}}/M_{2\text{tail}}\) receptors (\(K_m\) = 1.95 \pm 0.16 nM) was reduced compared to the receptors in the membranes. As shown in Fig. 11, the association binding curves of solubilized receptors showed a normal saturation form, no matter what amount of DNA had been originally transfected (parallel experiments performed on the same membranes from which the receptor fragments were extracted gave the usual pattern described above).

In a final experiment, we tested the ability of a fragmented \(M_{2\text{trunk}}/M_{2\text{tail}}\) receptor to bind \[^3H\]NMS. We have previously published that this fragmented receptor is not able to bind \[^3H\]NMS in equilibrium binding experiments (Maggio et al., 1993). This result was different from that observed with an analogous chimeric receptor, in which the first five trans-
membrane regions of the $M_3$ muscarinic receptor were joined to the last two transmembrane regions of the $M_2$ muscarinic receptor (Wess et al., 1990). As may be seen in Fig. 12, in equilibrium in COS-7 cells transfected with 4 g of the split $M_3$trunk/$M_2$tail receptor, with the exception of $A_2$ that membranes pretreated with [3H]NMS for 2 h, and then washed by repeated centrifugations, fully recovered the maximum binding point of [3H]NMS observed in the first association curve. The reversibility of the binding loss excludes the possibility that this phenomenon might be due to degradation of the split $M_3$trunk/$M_2$tail receptor.

Discussion

Five observations appear to be the most important from a conceptual point of view.

1. Equilibrium binding and functional experiments do not show any significant differences between the wild-type $M_2$ and the split $M_3$trunk/$M_2$tail receptor, with the exception of a significant difference in the $B_{\text{max}}$. This supports the view that the two fragments can fold independently of one another and can interact with each other, forming a complex with the pharmacological profile and the function of the wild-type receptor. The difference in $B_{\text{max}}$ that is usually observed when receptor fragments are compared to wild-type receptors has been attributed to the reduced trafficking of the fragments to the plasma membrane (Schöneberg et al., 1995). Nevertheless, as we shall mention below, it could also be due to the fact that part of the fragments are not in a complex, but remain free within the membrane.

2. Dissociation binding curves of [3H]NMS from the wild-type and the split muscarinic receptor are practically identical; on the contrary, association binding curves are substantially different. The association curve of [3H]NMS to the wild-type $M_2$ receptor has a normal saturation form, and the equilibrium is reached shortly after the addition of the radioligand. The association binding kinetic of [3H]NMS to the split $M_3$trunk/$M_2$tail receptor reaches a maximum binding point and then starts to decline to a lower equilibrium binding level. The ratio between the higher binding point and the equilibrium binding level is inversely correlated with the amount of DNA transfected in COS-7 cells, hence with the amount of fragments expressed on the membrane. The less there is of the DNA being transfected, the higher the ratio. From these results, it would appear that in equilibrium binding experiments the potential number of receptors that can be formed by the interacting fragments might be underestimated; therefore, in this case the $B_{\text{max}}$ would not be a true parameter that expresses the total amount of proteins present in the membrane.

3. Increasing the concentration of [3H]NMS from 0.5 to 5 nM does not modify the progression of the association binding kinetic. This indicates that the binding loss cannot be explained with a change in affinity in the range of the [3H]NMS concentrations we used, but we may postulate that the dissociation of [3H]NMS from the split $M_3$trunk/$M_2$tail receptor complex derives from a more significant modification in the receptor site.

4. The binding loss observed in the [3H]NMS association experiments was reversible. This is indicated by the fact that membranes pretreated with [3H]NMS for 2 h, and then washed by repeated centrifugations, fully recovered the maximum binding point of [3H]NMS observed in the first association curve. The reversibility of the binding loss excludes the possibility that this phenomenon might be due to degradation of the split $M_3$trunk/$M_2$tail receptor.

5. The allosteric site of the receptor is not altered in the split muscarinic $M_3$trunk/$M_2$tail receptor complex, as indicated by the fact that gallamine retains the same affinity for its binding site and has the same allosteric effect on [3H]NMS binding. As seen for the wild-type muscarinic $M_2$ receptor, high concentrations of gallamine completely suppress the dissociation of [3H]NMS from the $M_3$trunk/$M_2$tail receptor complex. Furthermore, high concentrations of NMS slow down the dissociation of [3H]NMS from the split $M_3$trunk/$M_2$tail receptor similarly to the wild-type muscarinic $M_2$ receptor, indicating that a low-affinity site recognized by NMS allosterically inhibits the dissociation of [3H]NMS from its canonical binding site (Jakubik et al., 2000).
All these considerations indicate that, once the binding of \[^{3}H\]NMS to the split receptor reaches equilibrium from a pharmacological point of view, it becomes indistinguishable from the wild-type M2 receptor. Then it is obvious that something occurs in the initial phase of the binding to make these two receptors different.

In view of the fact that the most important difference between the M2 and the M2\(_{trunk}\)/M2\(_{tail}\) receptor is the split third cytoplasmic loop, it is likely that a less stable interaction among the fragments composing the M2\(_{trunk}\)/M2\(_{tail}\) receptor exists. If we assume that the trunk and tail fragments interact dynamically, in the wild-type receptor, where the two domains are linked by the third cytoplasmic loop, their dissociation should be readily reversible, while in the split receptor it would free the fragments. Although we do not know yet how to apply this concept to a working model, two facts suggest that the phenomenon we observed with the M2\(_{trunk}\)/M2\(_{tail}\) receptor has a dynamic nature: 1) the binding loss observed in the \[^{3}H\]NMS association experiments is reverted by washing off the radioligand from the membrane; 2) changes in temperature modify the form of the \[^{3}H\]NMS association curve substantially. With the same amount of receptor fragments transfected on the membrane, the maximum binding point was reached slowly at +4°C but was considerably higher compared to that reached at +30°C.

Furthermore, if we presume the M2\(_{trunk}\)/M2\(_{tail}\) complex to be stable and that no association or dissociation of the fragments will occur during the binding assay, the percentage of binding loss compared to the maximal binding should be constant. Conversely, we have observed that this ratio changes considerably, and that this was particularly evident when we kept the amount of M2\(_{trunk}\) DNA transfected constant and changed the amount of the M2\(_{tail}\) DNA. If the diffusion of these fragments becomes a limiting factor for their re-assembly, then increasing their concentration should overcome this disadvantage.

As we mentioned in the Introduction, Gouldson et al. (1997) proposed a dynamic interaction between the “trunk” and “tail” domain of the receptor, having applied computational studies to the problem of docking adrenergic ligands into a model of the β2-adrenergic receptor. Using molecular modeling, they showed that the N terminus and the three extracellular loops create a tight canopy over the receptor, but it is not clear how a ligand can bind unless there is a substantial breathing of both the extracellular loops and the helical domains. Indeed, Kamiya and Reynolds (1999), using Brownian dynamic simulations of the extracellular loops of the adrenergic receptor, have shown that movement of the loops alone may not be sufficient to permit binding of all but the smallest of ligands. Thus, the domain movement may allow ligands to enter the receptor.

To substantiate the idea of there being a dynamic interaction between the two muscarinic receptor fragments, we started experiments with the cross-linking agent glutaraldehyde. The idea was that cross-linking agents stabilizing the M2\(_{trunk}\)/M2\(_{tail}\) complex could prevent the loss of \[^{3}H\]NMS binding in association binding experiments. Disappointingly, the experiments with glutaraldehyde did not suffice to shed light on the phenomenon. Despite our conviction, glutaraldehyde accentuated the phenomenon. We also tried to protect the binding site of the receptor with cold NMS given 30 min before the glutaraldehyde, but the results did not change. The likely explanation is that the cross-linking agent substantially alters the normal conformation of the receptor fragments and consequently the \[^{3}H\]NMS binding site. This interpretation can find corroboration also in the fact that the same experiment performed with the wild-type M2 receptor resulted in a net loss of binding sites, without any change in the kinetic or in the affinity of \[^{3}H\]NMS for the receptor.

Any influence of G protein-coupling in this phenomenon has been excluded, due to the fact that GTPγS did not influence the \[^{3}H\]NMS association binding to the split muscarinic M2\(_{trunk}\)/M2\(_{tail}\) receptor. If anything, we can postulate that other proteins, like regulators of G protein signaling (RGS), for example, might be responsible in some way for our results.

To gain deeper insight into the mechanism of \[^{3}H\]NMS association binding to the split M2\(_{trunk}\)/M2\(_{tail}\) receptor, we performed preliminary experiments with solubilized receptor fragments. Again, in contrast to what we expected, solubilized receptors did not reveal the phenomenon seen in membranes, no matter what amount of M2\(_{trunk}\)/M2\(_{tail}\) DNA was originally transfected in the cells. At present, we have no feasible explanation for this discrepancy.

A final point that must still be discussed is why we were unable to reproduce the peculiar form of the \[^{3}H\]NMS association binding curve with \[^{3}H\]QNB. This difference would suggest that the phenomenon might depend upon the radioligand used. The kinetic of association and dissociation of \[^{3}H\]QNB from the M2 and M2\(_{trunk}\)/M2\(_{tail}\) receptors is much slower compared to \[^{3}H\]NMS. The binding of \[^{3}H\]NMS reaches the equilibrium point in about 5 min; then, if the underlying phenomenon that leads to the decrease in binding is slow, it can be easily observed. At a concentration of 1 nM, \[^{3}H\]QNB reaches equilibrium in about 40 min, so it is likely that the gradual loss in binding could be quenched by the slow association kinetic. This problem could have been bypassed by increasing the concentration of the radioligand. Unfortunately, we could not raise the concentrations of \[^{3}H\]QNB much more, because the increase in the nonspecific binding made the data too variable and did not allow clear measurement of the low specific binding of \[^{3}H\]QNB to the M2\(_{trunk}\)/M2\(_{tail}\) receptor.

As pointed out by one of the anonymous referees, we must consider having an alternative explanation to account for the difference between \[^{3}H\]NMS and \[^{3}H\]QNB. \[^{3}H\]NMS has been known to contain nonlabeled impurities that compete with \[^{3}H\]NMS to inhibit binding (Lazareno and Birdsall, 2000; Sum et al., 2001). The result is that the estimated B\(_{max}\) value of the quaternary ligand is usually lower than that of the tertiary ligand \[^{3}H\]QNB (in our case 77.6 and 69.6% for M2 and M2\(_{trunk}\)/M2\(_{tail}\), respectively). If \[^{3}H\]NMS binds rapidly and is then slowly competed off the receptor by a contaminant, this could explain our kinetics.

Nevertheless, this view does not explain why we did not see the same results with the M2 receptor, even when it was expressed at a very low level. To stay with this interpretation, we have to assume that the contaminant binds differently to the M2 and the M2\(_{trunk}\)/M2\(_{tail}\) receptor, and all our results suggest that the two receptors behave the same at equilibrium.

Furthermore, it does not justify the importance of the titer of the M2\(_{trunk}\)/M2\(_{tail}\) DNA transfected in this phenomenon. A high number of receptors that dilute the concentration of
[3H]NMS and make its binding less susceptible to competition by a putative nonlabeled contaminant could probably explain the change in the shape of the curve with the DNA title; the less the receptor is expressed, the lower is the dilution of the radioligand and the nonlabeled contaminant. Nevertheless, the concentration of the receptor in our experiments was a maximum of 2.5% of the total [3H]NMS added, therefore the dilution of the radioligand was negligible.

The kinetic phenomena that we have described for the Mstrunk/Mtail receptor explains a result we obtained in the past with another split chimeric receptor constituted by the Mstrunk and the Mtail fragments (Maggio et al., 1993). In contrast to the results obtained with a chimeric receptor, in which the first five transmembrane regions of the M3 receptor were linked to the last two transmembrane regions of the M2 receptor (Wess et al., 1990), the split Mstrunk/Mtail receptor was unable to bind the radioligand [3H]NMS. This led us to postulate that for some reason the fragments change conformation when expressed as two separate proteins, while in the chimeric receptor they adapt to each other. In association binding experiments we have now shown that [3H]NMS binding is indeed present for the slip Mstrunk/Mtail receptor, but it can be observed only for a brief period of time after the addition of the radioligand, and afterward it fades away to undetectable levels.

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References


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