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Atropine dissociates complexes of muscarinic acetylcholine receptor and guanine nucleotide-binding protein in heart membranes

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Complexes of muscarinic acetylcholine receptor and guanine nucleotide-binding protein (G protein) are formed in the presence of the agonist carbachol. The complexes remain stable after removal of agonist, and survive subsequent solubilization from cardiac membranes and purification. Dissociation of the receptor from the G protein occurs when the antagonist atropine is added following removal of agonist. This is the first direct demonstration of destabilization of receptor–G protein complexes by the binding of an antagonist.

Muscarinic acetylcholine receptor; G protein; Atropine

1. INTRODUCTION

It has been shown that agonists promote the formation of stable complexes of muscarinic acetylcholine receptor (mAChR) and G protein in rat cardiac membranes [1]. Antagonists do not produce this effect. However, it is unclear whether receptor occupancy by antagonists is passive with regard to mAChR-G protein interaction. That is, can antagonists bind to a mAChR associated with a G protein and yet not disrupt the protein-protein interaction? In this study, a method is presented to address this question. It is based on the ability to remove bound agonist from receptor with concentrations of GTP that do not disrupt the mAChR–G protein complex. With the ligand binding site unoccupied, effects of added antagonist on mAChR-G protein complex stability can be monitored.

2. MATERIALS AND METHODS

Rat hearts were obtained from Pel Freeze and membranes prepared as described [1]. Membranes were resuspended in 10 mM Tris, 1 mM EDTA, 3 mM $MgCl_2$ and incubated with 100 nM carbachol (Sigma) for 30 min at 4°C. GTP (0.1 mM) was added where indicated and samples incubated for an additional 10 min at 4°C. Samples were then diluted 10-fold with 10 mM Tris, 1 mM EDTA in the presence or absence of 10 μM atropine (Sigma) and membranes collected by centrifugation. Solubilization of mAChRs, purification by wheat germ agglutinin chromatography, immunoprecipitation using 31-1D1 anti-receptor antibody [2,3], and Western blotting were as described previously [1]. [^{3}H]NMS (Amersham) and [^{3}H]oxo-M (Dupont/NEN) binding to heart membranes was performed as described [1].

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3. RESULTS

The monoclonal antibody, 31-1D1, used in this study quantitatively (>90%) precipitates muscarinic receptors solubilized from porcine or rat heart [2,3], or from cells transfected with cDNA coding for the m_2 muscarinic receptor (B.B. Wolfe, personal communication). When cardiac membranes are incubated with carbachol to activate mAChRs, this antibody can be used to precipitate solubilized muscarinic receptors and associated G proteins [1]. G proteins present in the immunoprecipitated complexes are identified by Western blotting using antibodies specific for G protein alpha subunits [4]. It was previously shown [1] that low concentrations of GTP (0.1 mM) could fully reverse agonist binding to membrane-bound mAChRs, but did not reverse the mAChR–G protein interaction. This observation forms the basis of the current experiments.

Formation of stable mAChR-G protein complexes was produced by incubation of cardiac membranes with the agonist carbachol (100 nM). Agonist was then removed from the complex using a limiting concentration of GTP (0.1 mM), followed by centrifugation and resuspension of membranes in buffer lacking agonist or GTP. To confirm the removal of agonist, [^{3}H]NMS binding was indistinguishable using membranes treated with carbachol, followed by 0.1 mM GTP (Fig. 1B), or control membranes not initially exposed to agonist and GTP (Fig. 1A). [^{3}H]NMS binding was inhibited by approximately 50% when 100 nM carbachol was included in the binding assay (not shown). A comparison of [^{3}H]oxo-M binding to control (Fig. 1A) or treated (Fig. 1B) membranes suggests that the GTP concentration is reduced under these conditions to levels which do not interfere with high-affinity agonist binding.

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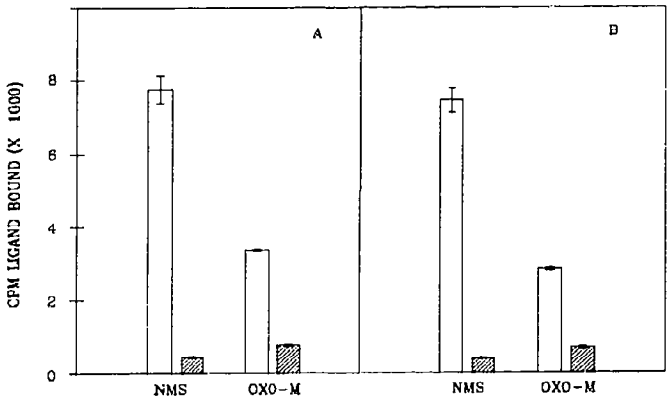

Fig. 1. Binding of [^{3}H]NMS and [^{3}H]Oxo-M to cardiac membranes. (A) control membranes; (B) membranes incubated with 100 nM carbachol, followed by 0.1 mM GTP. Radioligands were added after centrifugation of membranes and resuspension in buffer lacking carbachol or GTP. Binding of radioligands (mean ± SEM, $n = 3$) in the presence ■ or absence □ of 1 μM atropine.

Detection of the mAChR-G protein complexes formed under these experimental conditions is shown in Fig. 2. Thus, lane 2 in Fig. 2 shows Gα subunits that co-purified with mACh receptors incubated with carbachol. No Gα subunits were detected when atropine alone was used (lane 6). When membranes were incubated with agonist to induce formation of mAChR-G protein complexes, and the agonist removed by addition of 0.1 mM GTP, the mAChR-G protein complexes initially formed remained present (lane 3). However, if the antagonist atropine (10 μM) was added during (not shown) or subsequent to incubation with GTP, the levels of mAChR-G protein complex were reduced to background levels (lane 4). If atropine was added to carbachol-treated samples without incubation with GTP, no reduction in the levels of mAChR-G protein complex was observed (lane 5). These results suggest that the mAChR ligand binding site must be cleared of bound agonist before atropine can bind to and destabilize the mAChR-G protein complex. These results also confirm the ligand binding experiments (Fig. 1) showing that the agonist was removed by incubation with GTP.

The above experiments demonstrated the ability of atropine to destabilize mAChR-G protein complexes in the absence of agonist. This effect of atropine was also observed in the presence of agonist when GTP (0.1 mM) was incluced in the incubation. In these experiments, membranes were incubated with a concentration of atropine (10 nM) sufficient to saturate all receptors, based on 100% inhibition of [^{3}H]NMS binding (not shown). At the same concentration of NMS, 10 μM carbachol produced full occupancy of high-affinity agonist binding sites (Fig. 3A and B). Under these conditions, the high-affinity agonist binding was reversed by addition of 0.1 mM GTP (Fig. 3). In membranes incubated

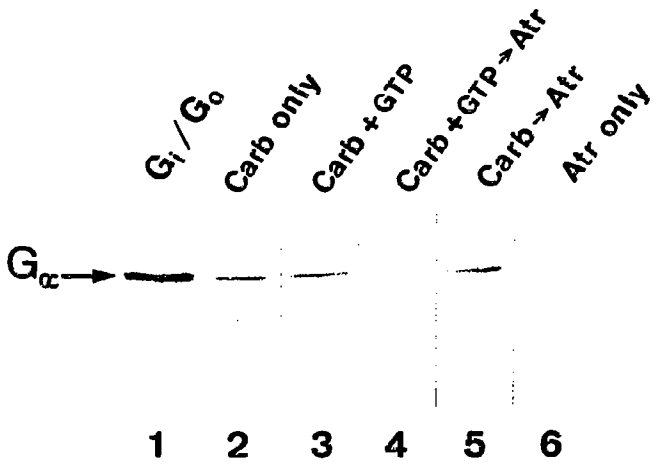

Fig. 2. Reversal of mAChR-G protein complexes by atropine. Western blot shows Gα subunits that co-purified with mAChRs solubilized from cardiac membranes treated as indicated above each lane. Lane 1 shows Gα subunits partially purified from rat brain [1]. Resolution of $G_{\alpha 41}$ and $G_{\alpha 39}$ subunits is not achieved under these conditions. Carb = 100 nM carbachol; Atr = 10 μM atropine; GTP = 0.1 mM.

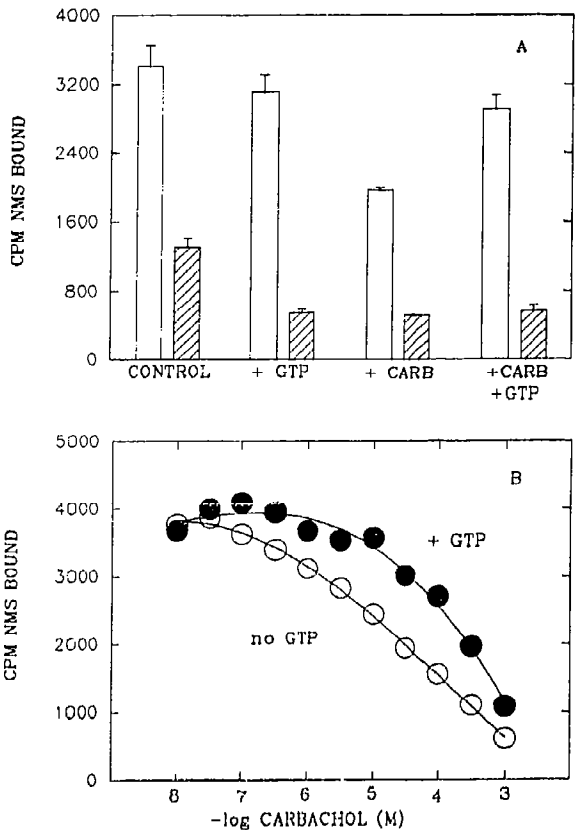

Fig. 3. A. Membranes were incubated for 30 min on ice with 10 nM [^{3}H]NMS plus 10 μM carbachol or 0.1 mM GTP where indicated. □ total binding; ■ plus 1 μM atropine (mean ± SEM, $n = 3$). B. Membranes were incubated with 10 nM [^{3}H]NMS plus various concentrations of carbachol for 30 min on ice. Points are the average of duplicate determinations.

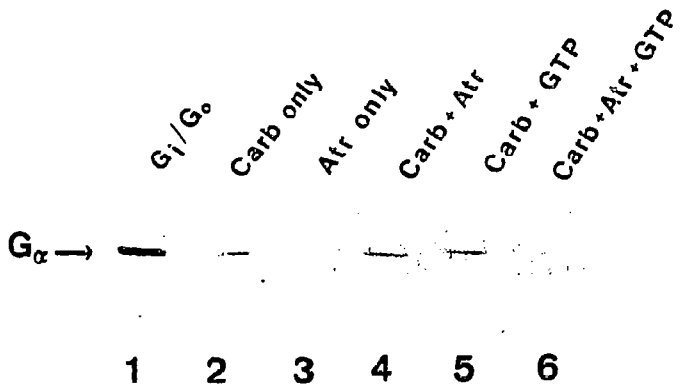

Fig. 4. Reversal of mAChR–G protein complexes by atropine in the presence of agonist. Western blot shows Gα subunits that co-purified with mAChRs treated as indicated above each lane. Carb = 100 nM carbachol; Atr = 10 μM atropine; GTP = 0.1 mM.

under identical experimental conditions (Fig. 4), carbachol alone (10 μM) or in the presence of 10 nM atropine supported mAChR–G protein interaction (lanes 2 and 4). GTP alone did not reverse this effect of carbachol (lane 5). In contrast, in the presence of 10 nM atropine in addition to carbachol and GTP, no mAChR–G protein complexes were detected (lane 6).

4. DISCUSSION

The ability of mAChR agonists to promote formation of mAChR–G protein complexes in cardiac membranes has been described previously [1], and documented again in this report. The complexes thus formed are refractory to additions of low concentrations of GTP and GDP which are sufficient to remove bound agonist, and therefore do not appear to require the continuing presence of agonist for the proteins to remain in association. We now document that the muscarinic antagonist atropine dissociates complexes of mAChR and G protein initially induced by agonist. Regarding the effects of GTP on muscarinic agonist binding, and the use of this parameter as an index of receptor–G protein interaction, we have found that low (10 and 100 nM) concentrations of carbachol or [^{3}H]oxo-M support levels of mAChR–G protein complex formation which are equivalent in the presence or absence of 0.1 mM GTP. Using direct binding of [^{3}H]oxo-M or competition of carbachol for [^{3}H]NMS binding to cardiac membranes, it can be shown that this same concentration of GTP fully inhibits high-affinity agonist binding to the muscarinic receptor [1]. In the case of the competition binding experiments, and depending on the levels of [^{3}H]-antagonist used as a tracer, complexes of mAChR–G protein formed by carbachol under these conditions appear to be reversed by binding of antagonist present during the incubation. Thus, using radiolabelled agonist, no inference could be made about the effect of GTP on the presence or absence of mAChR–G protein complexes, while competition binding using high concentrations of radiolabelled antagonist would provide a more accurate estimate of the existence of R–G complexes.

In summary, this report documents the reversal of muscarinic receptor–G protein complexes by antagonist binding. Further studies using this approach will clarify other parameters associated with this protein–protein interaction.

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