Paired Activation of Two Components within Muscarinic M₃ Receptor Dimers Is Required for Recruitment of β-Arrestin-1 to the Plasma Membrane*

Francesca Novi†, Laura Stanasila§, Franco Giorgi‡, Giovanni U. Corsini‡, Susanna Cotecchia§, and Roberto Maggio¶*

From the ‡Department of Neurosciences, University of Pisa, 56100 Pisa, Italy and §Department of Pharmacology and Toxicology, University of Lausanne, 1005 Lausanne, Switzerland

β-Arrestins regulate the functioning of G protein-coupled receptors in a variety of cellular processes including receptor-mediated endocytosis and activation of signaling molecules such as ERK. A key event in these processes is the G protein-coupled receptor-mediated recruitment of β-arrestins to the plasma membrane. However, despite extensive knowledge in this field, it is still disputable whether activation of signaling pathways via β-arrestin recruitment entails paired activation of receptor dimers. To address this question, we investigated the ability of different muscarinic receptor dimers to recruit β-arrestin-1 using both co-immunoprecipitation and fluorescence microscopy in COS-7 cells. Experimentally, we first made use of a mutated muscarinic M₃ receptor, which is deleted in most of the third intracellular loop (M₃-short). Although still capable of partially precipitate with β-arrestin-1, this receptor loses almost completely the ability to recruit β-arrestin-1 following carbachol stimulation in COS-7 cells. Subsequently, M₃-short was co-expressed with the M₁ receptor. Under these conditions, the M₃/M₁-short heterodimer could not recruit β-arrestin-1 to the plasma membrane, even though the control M₃/M₁ homodimer could. We next tested the ability of chimeric adrenergic muscarinic α₂/M₃ and M₁/α₂ heterodimeric receptors to co-immunoprecipitate with β-arrestin-1 following stimulation with adrenergic and muscarinic agonists. β-Arrestin-1 co-immunoprecipitation could be induced only when carbachol or clonidine were given together and not when the two agonists were supplied separately. Finally, we tested the reciprocal influence that each receptor may exert on the M₃/M₁ heterodimer to recruit β-arrestin-1. Remarkably, we observed that M₃/M₁ heterodimers recruit significantly greater amounts of β-arrestin-1 than their respective M₃/M₃ or M₁/M₁ homodimers. Altogether, these findings provide strong evidence in favor of the view that binding of β-arrestin-1 to muscarinic M₃ receptors requires paired stimulation of two receptor components within the same receptor dimer.

Over the past several years, receptor dimerization has become an established concept in the field of G protein-coupled receptors (1, 2). Although the mechanism(s) by which receptors may undergo dimerization has yet to be elucidated in detail, it is becoming ever more clear that the phenomenon is playing a key role in receptor maturation, G protein coupling, and downstream signaling besides regulating such processes as internalization and desensitization. One of the most critical issues in receptor homodimerization and heterodimerization is whether signal transduction may require pair activation of receptor dimers. Many of the recent reports on G protein coupling consider separate stimulation of receptor dimers to be sufficient for activating G proteins in co-transfected cells (3–7). Accordingly, heterodimerization would not necessarily interfere with G protein coupling and the receptor monomer should be sufficient by itself to activate G protein. This conclusion is also supported by the observation that heterodimerization between receptors that bind to distinct G proteins quite often leaves their coupling selectivity unaltered. For instance, β₂ adrenergic receptors that couple with stimulatory G proteins or δ and κ opioid receptors that couple with inhibitory G proteins both form heteromeric complexes but their heterodimerization does not significantly alter ligand binding or their coupling properties (8).

However, this interpretation does not take into account the possibility that simultaneous activation of both receptors with selective agonists presupposes a different pharmacology from that foreseeable for two receptors activated separately (6). In addition, the above conclusion is contradicted by a number of recent evidence. For instance, Baneres and Parello (9) have been able to demonstrate unambiguously with a combination of mass spectrometry and neutron scattering in solution that only one G protein trimer binds to a leukotriene B₄ (LTB₄) receptor BLT1 dimer (2xBLT1.LTB₄), thus forming a stoichiometrically defined (2xBLT1.LTB₄)Gα₁β₂δ₂ pentameric assembly. They suggested that receptor dimerization may be crucial for LTB₄-induced signaling. Similar conclusions have been reached in a recent paper by Chinault et al. (10) who demonstrated that yeast α-factor receptor oligomerization is required for G protein activation. These two latter papers point to the receptor dimer as the minimal structural configuration necessary to sustain receptor functioning. In line with these findings, we have recently demonstrated that paired activation of two receptor monomers within muscarinic M₃ dimers is required to stimulate ERK1/2 phosphorylation. In fact, a mutant muscarinic M₃ receptor (M₃-short), which by itself cannot stimulate ERK1/2 phosphorylation, reduces by a large extent the ability of M₃ to

The abbreviations used are: LTB₄, leukotriene B₄; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; 3HA-M₃, 3×HA N-terminal tagged M₃ receptor; M₃-short, mutant muscarinic M₃ receptor; GFP, green fluorescence protein.

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† To whom correspondence should be addressed: Dept. of Neurosciences, University of Pisa, Via Roma 55, 56100 Pisa, Italy. Tel.: 39-050-2218707; Fax: 39-050-2218717; E-mail: r.maggio@drugs.med.unipi.it.

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activate ERK1/2 (11). However, the most compelling evidence supporting the requirement for receptor dimerization in ERK1/2 phosphorylation was obtained with chimeric α2βM3 and M3/αβ receptors (12, 13). We demonstrated that both adrenergic and muscarinic components of the α2βM3 and M3/αβ chimeric receptor heterodimers must be activated for ERK1/2 to be phosphorylated (11). In the same study, we provided additional evidence that activation of ERK1/2 by M3 receptors in COS-7 cells occurs independently of G protein activation. Because β-arrestins may activate mitogen-activated protein kinase by recruiting signaling molecules into a complex with agonist-occupied receptors (14), we suggested that activation of ERK1/2 via M3 receptors may be due to the recruitment of β-arrestin to the site of the activated receptor.

The combined evidence that M3 receptors might activate ERK1/2 via β-arrestin and that ERK1/2 activation is dependent upon receptor dimerization strongly suggests that the binding of M3 receptors to β-arrestin depends on both receptor components of the muscarinic M3 dimers. To test this hypothesis, we have extensively characterized the binding of β-arrestin-1 to a number of muscarinic M3 receptor dimers using co-immunoprecipitation and fluorescence microscopy in COS-7. Our findings provide strong evidence that the binding of β-arrestin-1 to muscarinic M3 receptor dimers requires a paired activation of two receptor components within the same receptor dimer.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents—**N-[3H]Methylscopolamine (83 Ci/mmol) and [3H]adenine (32 Ci/mmol) were from PerkinElmer Life Sciences. M60-[3H]inositol (23 Ci/mmol) was from Amersham Biosciences. Tissue culture media and sera were from Sigma and Celbio. Carbachol, clonidine, and atropine were from Sigma. Triptiramine was kindly provided by Dr. Carlo Melchiorre (University of Bologna).

**Eukaryotic Expression Vectors—**Construction of M3-short and chimeric adrenergic/muscarinic α2βM3 and M3/αβ receptors has been described elsewhere (12, 13). In particular, M3-short was obtained by deleting 196 amino acids from the third cytoplasmic (i3) loop of M3 (the remaining i3 loop was 43 amino acids long). Chimeric adrenergic/muscarinic α2βM3 and M3/αβ receptors were obtained by exchanging the last two transmembrane domains between the adrenergic ε2β2 and the muscarinic M3 receptor. In both chimeras, the third cytoplasmic loop was from the M3 receptor. A schematic representation of the receptor mutants used in this study is shown in Fig. 1. For co-immunoprecipitation experiments, we used a triple HA N-terminal tagged human M3 receptor inserted in a pcDNA3 vector from Guthrie cDNA Resource Center and a c-Myc N-terminal tagged human M2 receptor kindly provided by Dr. Tatsuya Haga. The chimeric M3-short was obtained by cutting out 196 amino acids from the i3 loop of the M3 receptor (black strip). The α2βM3 and M3/αβ chimeras were obtained by exchanging the last two transmembrane domains between the muscarinic M3 and the adrenergic α2c receptor (green strip). In both chimeras, the i3 loop was from the M3 receptor.

**Membrane Preparation and Binding Assay—**On day 1, COS-7 cells were transfected with the plasmids(s) of interest. 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, cells were scraped off the plate and centrifuged at 17,000 rpm 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, 155 mM NaCl, 0.01 mg/ml bovine serum albumin). Binding was carried out at +30 °C in a final volume of 1 ml, and atropine 1 µM was used to define nonspecific binding. The bound ligand was separated from the unbound ligand using glass-fiber filters (Whatmann, GF/B) with a Brandel cell harvester, and the filters were counted with a scintillation β-counter.

**Phosphatidylinositol Breakdown Assay—**Transfected COS-7 cells were incubated with myo-[3H]inositol (3 µCi/ml) for 48 h. Immediately prior to the experiment, the cells were washed twice with phosphate-buffered saline and incubated for 15 min in Eagle’s minimal essential medium containing 10 mM LiCl and 20 mM HEPES. The medium was then replaced by 0.25 ml of the same medium containing the experimental agonist. After a 1-h incubation at +25 °C, the reaction was arrested by the addition of 0.75 ml of 7.5% (w/v) ice-cold trichloroacetic acid followed by a 30-min incubation on ice. The trichloroacetic acid was extracted with water-saturated diethyl ether (3 × 4 ml), and the levels of inositol monophosphate were determined by anion exchange chromatography.

**Adenyl Cyclase Assay—**COS-7 cells were transfected with the plasmid containing the receptor of interest plus the adenyl cyclase V. 24 h after transfection, the cells were trypsinized and recultured in 24-well plates, and after an additional 24 h, they were assayed for adenyl cyclase activity. The cells in the 24-well plates were incubated for 2 h with 0.25 ml/well fresh growth medium containing 5 µCi/ml [3H]adenine, and this medium was replaced with 0.5 ml/well DMEM containing 20 mM HEPES, pH 7.4, 0.1 mg/ml bovine serum albumin, and then with a mixture containing 1 mM PDBu (200 µmol/l), 100 µM o-phosphatidylserine (0.3 µmol/l) and RO-20-1724 (0.5 mM). Adenyl 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 was terminated by the addition of perchloric acid containing 0.1 mM unlabeled cAMP followed by neutralization with KOH. The amount of [3H]cAMP formed was determined by a two-step column purification procedure.

**Immunoprecipitation and Western Blotting—**For immunoprecipitation, confluent cells were treated with the selected compound 3 days after transfection. Following incubation, the reaction was stopped by washing the cells twice with ice-cold sterile 0.9% NaCl. Cells were then scraped with 300 µl of buffer solution A (0.2% digitonin, 1 mM EDTA in phosphate-buffered saline) containing maximum 1 mM protease inhibitors (1.7 µg/ml aprotinin, 4 µg/ml leupeptin, 0.01 µg/ml antipoin, 500 µg/ml phenylmethylsulfonyl fluoride). Lysed cells were incubated on ice for 20 min and then centrifuged at 6000 rpm for 10 min at +4 °C, and the supernatant was recovered for immunoprecipitation. The amount of proteins was assayed with the protein assay kit of Bio-Rad, and aliquots of 80 µg of proteins (if not otherwise specified) were used for immunoprecipitation.

To eliminate proteins nonspecifically bound to magnetic beads, the cell extract was preexposed to 25 µl of protein G magnetic beads (New England Biolab) for 1 h at +4 °C. A magnetic field was applied to the side of the tube, and the supernatant was recovered and transferred to a clean 1.5-ml microcentrifuge tube. For immunoprecipitation, 5 µg of a monoclonal anti-HA (Roche Applied Science) or an anti-c-Myc antibody (Sigma) was added to the tube and the suspension was incubated at +4 °C for 1 h. At the end of this incubation period, 25 µl of protein G magnetic beads were added and incubated at +4 °C for an additional hour. A magnetic field was applied to pull beads to the side of the tube, and the supernatant was carefully removed. The magnetic beads were washed with 500 µl of buffer solution B (0.1% Niaprox, 0.2 µM NaCl, 1 mM EDTA, 50 mM Tris, pH 7.5) including the protease inhibitor mix. The bead pellets were recovered by applying a magnetic field, and they were resuspended in 30 µl of Laemmli buffer and incubated at +70 °C for 5 min. A magnetic field was applied to the sample, and the supernatant was recovered and loaded on 10% SDS-polyacrylamide gel. Proteins were run at 90 V for 2–3 h and transferred to the nitrocellulose membrane for 1 h at 100 V. Co-immunoprecipitated proteins were determined by immunoblotting with specific antibodies such as polyclonal anti-β-arrestin-1 and monoclonal anti-HA.
restin-1 (Santa Cruz Biotechnology), polyclonal anti-i3-loop M3 (Santa Cruz Biotechnology), monoclonal anti-FLAG (Sigma), and monoclonal anti-c-Myc antibody. Data from separate experiments were digitized on a flatbed scanner and analyzed with a Kodak Scientific Imaging Systems software.

Immunocytochemistry—COS-7 cells were transfected using the DEAEdextran chloroquine method on 100-mm plates, and the day after transfection, they were split and seeded on coverslips previously placed on 6-well plates at a confluence of 2 × 10^6 cells/well. Three days after transfection, they were treated with 100 mM carbachol and then processed for immunocytochemistry. Cells were fixed for 10 min in 3.7% paraformaldehyde, permeabilized for 5 min with 0.2% Triton X-100, and then incubated in 1% BSA for 1 h to reduce background staining. They were then exposed to a rabbit polyclonal anti-HA antibody (Santa Cruz Biotechnology) for 1 h at a working dilution of 1/100 followed by another incubation with rhodamine-labeled donkey anti-rabbit secondary antibody (Jackson Immunoresearch) at a working dilution of 1/100. Coverslips were washed with phosphate-buffered saline and mounted on slides using Prolong Antifade kit (Molecular Probe). The coverslips were analyzed with a Zeiss Axiosplan fluorescence microscope using ×63/1.25 oil Zeiss objective.

RESULTS

To characterize the expression of endogenous β-arrestin-1 in COS-7 cells, protein extracts from mock-transfected COS-7 cells and from cells transfected with a C-terminal tagged FLAG-β-arrestin-1 were immunoblotted with an antibody directed against the C-terminus of β-arrestin-1. In mock-transfected COS-7 cells, a band corresponding to the molecular mass of β-arrestin-1 (55 kDa) was detected on the blot (Fig. 2A). In COS-7 cells transfected with FLAG-β-arrestin-1, the same antibody recognized a broad band corresponding to the FLAG-β-arrestin-1. In COS-7 cells transfected with FLAG-β-arrestin-1, the anti-FLAG antibody recognized the same band as the anti-β-arrestin-1 antibody, whereas it did not detect any signal in mock-transfected COS-7 cells (Fig. 2B).

To investigate the nature of receptor-arrestin interaction, co-immunoprecipitation experiments were performed in COS-7 cells co-expressing β-arrestin-1 and the 3HA-N-terminal tagged M3 receptor (3HA-M3). The 3HA-M3 was immunoprecipitated with an anti-HA antibody, and the amount of β-arrestin-1 pulled down was normalized to the amount of the immunoprecipitated receptor. The same amount of immunoprecipitated 3HA-M3 (and in some experiments M3) obtained with an antibody directed against the third cytoplasmic loop could be reproducibly recovered in different experiments. In cells transfected with 3HA-M3 or M3 (results not shown), this antibody recognizes two bands that most likely correspond to the monomeric and dimeric forms of the muscarinic receptor (Fig. 2C).

The amount of β-arrestin-1 co-immunoprecipitated with the 3HA-M3 receptor in the absence of agonist (basal) was usually very low or undetectable. In contrast, stimulation of 3HA-M3 receptors with 100 μM carbachol induced a time- and concentration-dependent increase in the co-immunoprecipitated β-arrestin-1 complex, which then reached its maximum by ~20 min (Fig. 3). A dose-response curve of carbachol-induced β-arrestin-1 co-immunoprecipitation was obtained by incubating cells transfected with 3HA-M3 receptors for 10 min at the different concentrations of the agonist. The EC50 was between 10 and 100 μM, and no further increase in β-arrestin-1 co-immunoprecipitation was observed up to 1 μM (Fig. 4).

It has been shown that the phosphorylated i3 loop of M3 receptors is important for binding β-arrestin-1 (15). Because deletion of the i3 loop is expected to impair carbachol-induced stimulation of β-arrestin-1 binding, we tested the ability of a mutated muscarinic receptor in which 196 amino acids of the i3 loop were deleted (M3-short) to co-immunoprecipitate with β-arrestin-1. For this purpose, we used the N-terminal tagged HA-M3-short. As predicted, stimulation of this receptor with 100 μM carbachol did not increase the amount of co-immunoprecipitated β-arrestin-1 compared with the basal value (Fig. 5). Nonetheless, the basal amount of β-arrestin-1 that co-immunoprecipitated with the HA-M3-short receptor was significantly higher than that co-immunoprecipitated with the wild type receptor. This suggests that, in addition to the i3 loop, other segments of the M3 receptor can also be involved in β-arrestin-1 binding.

We have previously shown that the muscarinic M3-short receptor interacts with the wild type M3 receptor to form heterodimers (11). We calculated that up to 76.6% M3 receptor expressed could be co-immunoprecipitated with M3-short in COS-7 cells co-transfected with 2 μg of M3-short and 1 μg of M3. In the same paper, we showed that M3-short impairs the ability of M3 to stimulate ERK1/2 phosphorylation in the heterodimer. Here, we tested whether the M3-short could interfere with the ability of 3HA-M3 to co-immunoprecipitate with β-arrestin-1. As shown in Fig. 5, the expression of M3-short almost entirely prevents carbachol-dependent co-immunoprecipitation of β-arrestin-1 with the M3 receptor. This result was not due to potential problems linked to co-transfection, because overexpression of wild type M3 did not impair the amount of β-arrestin-1 co-immunoprecipitated with the 3HA-M3 (Fig. 5).

These findings strongly suggest that M3-short interferes with the ability of 3HA-M3 to recruit β-arrestin-1. To test this hypothesis, we verified whether β-arrestin-1 and 3HA-M3 could co-localize following muscarinic receptor activation in living cells. For this purpose, β-arrestin-1 C-terminal tagged with GFP (β-arrestin-1-GFP) and the 3HA-M3 receptor were...
can interact physically, 3HA-M3 receptors were co-expressed in cells transfected with 3HA-M3 and 3HA-M2. COS-7 cells were transfected with 1 µg of 3HA-M3 (upper blot) or with 1 µg of 3HA-M3 + 2 µg of M2 plasmid DNA (lower blot). Confluent monolayers of COS-7 cells were stimulated with carbachol (100 µM) for the time indicated, and incubation was terminated by adding ice-cold NaCl (0.9%). Cells were extracted in digitonin, and aliquots of 60-µg proteins were incubated with an anti-HA antibody and processed for immunoprecipitation (IP) with protein G magnetic beads (see “Experimental Procedures”). The amount of β-arrestin-1 co-immunoprecipitated was detected with an antibody directed against the C-terminal end of β-arrestin-1 and was normalized for the amount of 3HA-M3 receptor immunoprecipitated. The blots are representative of four experiments. Bar graph shows the means ± S.E. of all of the experiments. *, significantly different from cells transfected with 3HA-M3 (paired Student’s two-tailed t test, p < 0.05). IB, immunoblot.

Interestingly, the time course of carbachol-induced β-arrestin-1 co-immunoprecipitation in cells transfected with 3HA-M3 and 3HA-M3 + M2, contrasted with that of cells transfected with 3HA-M3 alone (Fig. 3). The time course of carbachol-induced β-arrestin-1 co-immunoprecipitation in cells transfected with 3HA-M3 and 3HA-M3 + M2. COS-7 cells were transfected with 1 µg of 3HA-M3 (upper blot) or with 1 µg of 3HA-M3 + 2 µg of M2 plasmid DNA (lower blot). Confluent monolayers of COS-7 cells were stimulated with different concentrations of carbachol for 10 min, and incubation was terminated by adding ice-cold NaCl (0.9%). Cells were extracted in digitonin, and aliquots of 60-µg proteins were incubated with an anti-HA antibody and processed for immunoprecipitation (IP) with protein G magnetic beads (see “Experimental Procedures”). The amount of β-arrestin-1 co-immunoprecipitated was detected with an antibody directed against the C-terminal end of β-arrestin-1 and was normalized for the amount of 3HA-M3 receptor immunoprecipitated. The blots are representative of four experiments. Bar graph shows the means ± S.E. of all of the experiments. *, significantly different from cells transfected with 3HA-M3 (paired Student’s two-tailed t test, p < 0.05). IB, immunoblot.

To further test whether recruitment of β-arrestin-1 does indeed require two fully functional receptors within a dimer, β-arrestin-1 was allowed to interact with a dimer formed by muscarinic M2 and M3 receptors. Using a pharmacological approach, we had previously suggested that M2 and M3 receptors can form heterodimers (16). In a recent work, Hornigold et al. (17) have provided evidence suggesting that both second messengers and extracellular signal-regulated kinases may be regulated via a cross-talk between M2 and M3 muscarinic acetylcholine receptors. To determine whether M2 and M3 receptors can interact physically, 3HA-M2 receptors were co-expressed with Myc-M2 receptors. Under these conditions (Fig. 7), the Myc-M2 receptor (panel A, lane 3) can be immunoprecipitated with anti-HA antibodies and the 3HA-M3 receptor (panel B, lane 3) can be immunoprecipitated with anti-Myc antibodies. Contrary to our data, Zeng and Wess (18) reported no co-immunoprecipitation between the muscarinic M2 and M3 receptors. We do not have any explanation for this discrepancy.

It is interesting to note that, when transfected alone, the M3 receptor migrates both as a dimer and a monomer in polyacrylamide gels (Figs. 2C and 7B), whereas when co-immunoprecipitated with M2, it can only be detected in a monomeric form and not in a M3/M2 heterodimeric form (Fig. 7B). The observation that receptor dimers may persist in denaturing gels suggests the possibility that the two-receptor monomers might be interacting through disulfide bridges. As a matter of fact, Zeng and Wess (18) have previously shown that M2/M3 homodimers are stabilized by disulfide bonds and that modification of Cys140 and/or Cys220 in the receptor greatly reduces the amount of M3 detectable in the high molecular weight dimeric form. A likely explanation for the apparent discrepancy with our results may be that M2 and M3 do not interact through disulfide bonds such that the heterodimer may break apart upon running in denaturing gels.

Interestingly, the time course of carbachol-induced β-arrestin-1 co-immunoprecipitation is clearly left-shifted in cells co-expressing the 3HA-M3 and M2 receptors compared with that...
Inhibition of carbachol induced 3HA-M3 recruitment of β-arrestin-1 by the M3-short receptors. COS-7 cells were co-transfected with 1-μg DNA of 3HA-M3 and 2-μg DNA of the M3-short or M3, or pCD plasmid. An additional control was transfected with 2-μg DNA of the HA-M3-short receptor. Confluent monolayers of COS-7 cells were stimulated with carbachol (100 μM) for 10 min, and incubation was terminated by adding ice-cold NaCl (0.9%). Cells were extracted in digitonin, and aliquots of 60-μg proteins were incubated with an anti-HA antibody and processed for immunoprecipitation (IP) with protein G magnetic beads (see “Experimental Procedures”). The amount of β-arrestin-1 co-immunoprecipitated was detected with an antibody directed against the C-terminal end of β-arrestin-1 and was normalized for the amount of 3HA-M3 receptor immunoprecipitated. The blots are representative of five experiments. Bar graph shows the means ± S.E. of all of the experiments. *, significantly different from control (paired Student’s two-tailed t test, p < 0.05). IB, immunoblot.

Carbachol stimulation of β-arrestin-1-GFP translocation in COS-7 cells transfected with 3HA-M3 or with 3HA-M3 and M3-short. COS-7 cells were co-transfected with 1 μg of 3HA-M3 and 1 μg of β-arrestin-1-GFP (upper two rows of panels) or with 1 μg of 3HA-M3, 2 μg of M3-short, and 1 μg of β-arrestin-1-GFP (bottom two rows of panels). Cells were stimulated with 100 μM carbachol for 30 min, fixed in formalin, and then permeabilized with Triton X-100. In order to recognize the 3HA-M3 receptor, cells were exposed to a primary anti-HA antibody and then to a secondary antibody labeled with rhodamine. The red fluorescence (rhodamine) and the GFP images were acquired with a fluorescence microscope. The two images were merged with Image software running on a MacIntosh computer. After 30 min of carbachol incubation, it was possible to see a clear co-localization of β-arrestin-1 and 3HA-M3 in endocytotic vesicles of cells transfected with 3HA-M3. The co-localization of β-arrestin-1 and 3HA-M3 is prevented by the M3-short receptor.

Altogether these findings suggest that M3 receptors heterodimerization enhances the ability of each receptor monomer to recruit β-arrestin-1. This conclusion is further corroborated by the observations that carbachol stimulation in COS-7 cells co-expressing 3HA-M3 and M3 receptors causes β-arrestin-GFP to become redistributed within the cell. As is shown in Fig. 10, recruitment of β-arrestin-1-GFP in these cells is more rapid than in cells transfected solely with 3HA-M3. Following 15 min of carbachol stimulation and even more so at a 30-min interval, a large number of endocytotic vesicles appear labeled by both red and green fluorescence, indicating that β-arrestin-1 and 3HA-M3 receptors co-localize along the plasma membrane (Fig. 9, Merge). To provide further support to the view that M3 receptor may act by enhancing β-arrestin-1 binding to the heterodimeric M3/3HA-M3 receptor, co-immunoprecipitation was performed in the presence of the selective M3 antagonist triptiramine (20). By N-[3H]methylscopolamine displacement binding analysis, we estimated that, in the presence of 4 nM triptiramine, ~96% M3 receptors and only 9% 3HA-M3 receptors are saturated by the antagonist (Fig. 10A). Furthermore, functional experiments indicated that, in COS-7 cells co-transfected with M3 and 3HA-M3, 4 nM triptiramine prevented the carbachol-stimulated M3 inhibition of adenylyl cyclase, whereas it did not prevent the M3-induced phosphatidylinositol hydrolysis (Fig. 10B). Thus, cells co-expressing the 3HA-M3 and M3 were stimulated with carbachol for 10 min in the presence and absence of 4 nM triptiramine. As it can be seen in Fig. 10C, triptiramine largely reduced the co-immunoprecipitation of β-arrestin-1 in cells co-expressing 3HA-M3 and M3, whereas it did not affect...
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β-arrestin-1 co-immunoprecipitation in cells co-expressing 3HA-M₃ and M₂ receptors.

In previous work, we demonstrated that the co-expression of chimeric adrenergic/muscarinic α₁/α₂ and M₃/α₁ receptors could rescue the binding of muscarinic and adrenergic ligands (12, 13). In both chimeric receptors, the i3 loop was from the muscarinic M₂ receptor. This explains why the activation of both the cholinergic or the adrenergic halves of the reconstituted receptor stimulates phosphatidylinositol hydrolysis (11). The chimeric α₁/α₂-M₃ and M₂/α₁ receptors are a good tool for testing whether the paired stimulation of a dimer is required to recruit β-arrestin-1 to the plasma membrane, because the only functional form is a dimer. For this purpose, the N-terminal tagged HA-M₃/α₂ receptor (11) was co-expressed in COS-7 cells with the other α₁/α₂ chimeras. Because through binding analysis the estimated amount of the two chimeras that fold correctly and form heterodimers is very low (30–70 fmol mg proteins, ~5% of the wild type 3HA-M₃ receptor), to magnify the amount of translocated β-arrestin-1, the receptors were stimulated with the agonists for 30 min. Furthermore, for co-immunoprecipitation, we used five times the amount of proteins that we used with the other receptors. As shown in Fig. 11, β-arrestin-1 co-immunoprecipitation was clearly detected only when the adrenergic and the muscarinic receptor components of the heterodimer were stimulated simultaneously with carbachol and clonidine. No co-immunoprecipitation was observed when cells were stimulated with either carbachol or clonidine alone. These results further support the hypothesis that both components of the dimer need to be activated to recruit β-arrestin-1 to the plasma membrane.

FIG. 7. Co-immunoprecipitation of muscarinic M₂ and M₃ receptors. Myc-M₂ and 3HA-M₃ receptors were transfected either singly or together in COS-7 cells. Complexes were immunoprecipitated (IP) with anti-HA antibodies and detected with anti-Myc-antibodies (panel A) or were immunoprecipitated with anti-Myc antibodies and detected with anti-HA-antibodies (panel B). The position of Myc-M₂ and 3HA-M₃ on the blots are indicated. The first line of each blot just shows the position of authentic Myc-tagged M₂ receptor (panel A) or authentic 3HA-tagged M₃ receptor (panel B). IB, immunoblot.

FIG. 8. Lack of co-immunoprecipitation of β-arrestin-1 in COS-7 cell transfected with 2 μg of Myc-M₂ plasmid DNA and stimulated with carbachol. In panel A, confluent monolayers of COS-7 cells were stimulated for different times and with different concentration of carbachol. Incubation was terminated by adding ice-cold NaCl (0.9%). Cells were extracted in digitonin, and aliquots of 60-μg proteins were incubated with an anti-Myc antibody and processed for immunoprecipitation (IP) with protein G magnetic beads (see “Experimental Procedures”). The amount of β-arrestin-1 co-immunoprecipitated was detected with an antibody directed against the C-terminal end of β-arrestin-1. In any of the conditions, it was possible to detect β-arrestin-1 co-immunoprecipitation. As a control, COS-7 cells were also co-transfected with Myc-M₂ and M₃. In this case, a clear co-immunoprecipitation of β-arrestin-1 could be detected on the blot after stimulation with 100 μM carbachol for 5 min. In panel B, COS-7 cells were transfected with Myc-M₂ and FLAG-β-arrestin-1 and stimulated with 100 μM carbachol. Cells were processed as described above, and the amount of β-arrestin-1 co-immunoprecipitated was detected with an anti-FLAG antibody. As it can be seen in the third line, FLAG-β-arrestin-1 could be detected on the blot after stimulation with carbachol. The blots are representative of five (A) and three (B) experiments. IB, immunoblot; Carb., carbachol.

FIG. 9. Carbachol stimulation of β-arrestin-1-GFP translocation in COS-7 cells transfected with 3HA-M₃ and M₂. COS-7 cells were co-transfected with 1 μg of 3HA-M₃, 2 μg of M₂, and 1 μg of β-arrestin-1-GFP. Cells were stimulated with 100 μM carbachol for the times indicated, fixed in formalin, and then permeabilized in Triton X-100. In order to recognize the 3HA-M₂ receptor, cells were exposed to a primary anti-HA antibody and then to a secondary antibody labeled with rhodamine. The red fluorescence (rhodamine) and the GFP images were acquired with a fluorescence microscope. The two images were merged with Image Photoshop software running on a Macintosh computer. After 15 min of carbachol stimulation, it was possible to observe a clear co-localization of β-arrestin-1 with 3HA-M₃ in endocytotic vesicles that increased significantly at 30 min.

DISCUSSION

In this study, we provide strong evidence that both components of muscarinic receptor dimers are required to fully recruit β-arrestin-1 and that this might have important functional implications on activation of the ERK1/2 pathway.

The main finding in support of this evidence is that a receptor dimer formed by the wild type M₃ receptor and its M₃-short
Recruitment of β-Arrestin by M₂ Dimers

![Graph](https://example.com/graph.png)

**Panel A.** The concentration of tripitramine at 4 nM saturated M₂ and M₃ receptors. COS-7 cells were co-transfected with 2 µg of M₂ and 1 µg of 3HA-M₃ plasmid DNA and stimulated with 100 µM carbachol in the presence or absence of tripitramine for 10 min and incubation was terminated by adding ice-cold NaCl (0.9%).

**Panel B.** The interaction with β-arrestin-1 observed in co-immunoprecipitation experiments displays slightly faster kinetics than in fluorescence microscopy. This might be due to the fact that, under the conditions of fluorescent microscopy used in our experiments, a distinct signal can only be seen when the receptor and β-arrestin-1 are accumulated in the defined part of the cells (clathrin-coated pits). It is likely that at earlier times the translocation of β-arrestin-1 to the plasma membrane could not be appreciated.

In contrast, carbachol did not induce co-immunoprecipitation of β-arrestin-1 with the M₂-short receptor mutant lacking most of its i3 loop. This is consistent with the fact that the phosphorylated i3 loop of the receptor plays an important role in this interaction. Surprisingly, the amount of β-arrestin-1 co-immunoprecipitated with the receptor under basal conditions was significantly increased. This suggests that, even though the deletion of the i3 loop abolished the agonist dependence of β-arrestin-1 binding to M₂, other parts of the receptor can interact with β-arrestin-1. This would be consistent with the finding that mutations in the highly conserved DRY region of the N-formyl peptide receptor (22) and of the α₁b adrenergic receptor (23) can impair β-arrestin-1 binding. It is also possible...

**Panel C.** The blots are representative of three experiments. Bar graph shows the means ± S.E. of all experiments. *p < 0.05). IB, immunoblot; IP assay, inositol phosphate breakdown assay; Carb., carbachol; Tripit., tripitramine.

**Fig. 10.** Effect of the selective antagonist tripitramine on carbachol-stimulated β-arrestin-1 co-immunoprecipitation in cells co-transfected with 3HA-M₃ + M₂ and 3HA-M₃ + M₂. Panel A, in a preliminary experiment, we determined the concentration of the antagonist tripitramine that saturates ~90% M₂ receptors in COS-7 cells co-transfected with 2 µg of M₂ and 1 µg of 3HA-M₃ plasmid DNA. The values of the tripitramine concentration were modified for the occupancy of the radioligand N-[³H]methylscopolamine (N-[³H]NMS, 1 nM) to the muscarinic M₂ and M₃ receptors. As shown by the dashed line, tripitramine at a concentration of 4 nM saturated ~96% M₂ receptor and only 9% M₃ receptor. In the same experiment, we calculated that the amount of M₂ receptor expressed in the cells was ~2.2-fold the amount of M₃. Panel B, we also tested the influence of tripitramine at the concentration of 4 nM on the functional activity of M₂ and M₃ mutant, which lacks the ability of recruiting β-arrestin-1, was impaired in its ability to interact with β-arrestin-1. Since we and others (11, 21) have previously shown that the M₂-short was unable to activate the ERK1/2 pathway, this finding strongly supports the hypothesis that ERK1/2 activation requires the recruitment of β-arrestin-1 by the paired activation of the two-receptor components of the muscarinic M₂ dimers.

The interaction of β-arrestin-1 with the wild type M₂ receptor and its M₂-short mutant was explored both by co-immunoprecipitation and fluorescence microscopy experiments in COS-7 cells co-expressing the different proteins. The muscarinic M₃ receptor was able to co-immunoprecipitate with β-arrestin-1 after agonist stimulation in a time- and concentration-dependent manner. In co-immunoprecipitation experiments, the interaction with β-arrestin-1 could be already observed on the blots after 5 min of stimulation with carbachol. In fluorescence microscopy, the co-localization of the M₂ and β-arrestin-1 was clearly observed after 30 min of carbachol stimulation. Thus, the recruitment of β-arrestin-1 observed in co-immunoprecipitation experiments displays slightly faster kinetics than in fluorescence microscopy. This might be due to the fact that, under the conditions of fluorescent microscopy used in our experiments, a distinct signal can only be seen when the receptor and β-arrestin-1 are accumulated in the defined part of the cells (clathrin-coated pits). It is likely that at earlier times the translocation of β-arrestin-1 to the plasma membrane could not be appreciated.

In contrast, carbachol did not induce co-immunoprecipitation of β-arrestin-1 with the M₂-short receptor mutant lacking most of its i3 loop. This is consistent with the fact that the phosphorylated i3 loop of the receptor plays an important role in this interaction. Surprisingly, the amount of β-arrestin-1 co-immunoprecipitated with the receptor under basal conditions was significantly increased. This suggests that, even though the deletion of the i3 loop abolished the agonist dependence of β-arrestin-1 binding to M₂, other parts of the receptor can interact with β-arrestin-1. This would be consistent with the finding that mutations in the highly conserved DRY region of the N-formyl peptide receptor (22) and of the α₁b adrenergic receptor (23) can impair β-arrestin-1 binding. It is also possible...
that the deletion of the bulky i3 loop in the M3-short allows the binding of β-arrestin-1 to other parts of the receptor. The functional implications of this interaction remain to be investigated.

Because the receptor dimer formed by the wild type M3 receptor and its M3-short mutant, which lacks the ability of recruiting β-arrestin, was impaired in its ability to interact with β-arrestin-1, this finding strongly suggests that the interaction of β-arrestin-1 with M3 dimers requires the activation of both monomeric components within the dimers.

These findings can have different mechanistic interpretations. One possibility is that M3-short modifies the structure of wild type M3 in such a way that β-arrestin-1 binding is precluded. Nevertheless, we consider this possibility unlikely since we have shown previously (13) that co-expression of the M3-short does not alter M3 binding and function. This finding suggests that the overall structure of the M3-short receptor, the mechanism of activation, and probably its dimerization interface are not altered. Two alternative hypotheses are the most likely to interpret our data. One possibility is that a β-arrestin monomer requires two full-length i3 loops to stably bind to the receptor, thus preferentially interacting with receptor dimers. If this is the case, heterodimeric M3/M3-short receptors will provide only one i3 loop, thus resulting in lower β-arrestin binding. This hypothesis would be consistent with the results from a recent study on the organization of rhodopsin in native membranes (24). Arrestin, the cognate β-arrestin in the visual system, has a bipartite structure of two structurally homologous seven-stranded β-sandwiches forming two putative rhodopsin-binding groves that are separated by 3.8 nm (25, 26). The positive charge arrangement of the surface of the rhodopsin dimer matches the negative charges on arrestin. Thus, Liang et al. (24) speculate that one arrestin monomer is likely to bind one rhodospin dimer.

The second hypothesis is that a β-arrestin-1 dimer binds to the receptor dimer. This could occur in two ways. (i) A preformed β-arrestin-1 dimer binds all at once to the receptor dimer, or (ii) two β-arrestin-1 molecules bind sequentially to the receptor dimer with the second one stabilizing the complex. In both cases, heterodimeric M3/M3-short receptors would have only one arm able to bind the β-arrestin-1 dimer and the receptor/β-arrestin-1 complex would be much weaker.

The idea of dimeric β-arrestin-1 as a functional unit comes from different observation. Monomeric and dimeric forms of visual arrestin are at equilibrium under physiological conditions (27). Dimerization has been demonstrated also for β-arrestin. β-Arrestin-(1–382), a C-tail truncation mutant of bovine β-arrestin, exists as a mixture of monomeric and dimeric species (28). It was shown that β-arrestin forms a tail-to-tail dimer with two C-tail domains facing each other (29–32). Taking together these data, Han et al. (28) propose a mechanistic model of β-arrestin-receptor interaction in which the initial binding of the first β-arrestin to the receptor is followed by the displacement of its terminal C-tail and dimerization with another molecule of β-arrestin. They speculate that β-arrestin dimerization may help β-arrestin-receptor complexes fit better with the internalization machinery of the coated pits. Furthermore, they left open the possibility that dimerization of β-arrestin could play a role as scaffold for mitogen-activated protein kinase given that complexes containing β-arrestin and mitogen-activated protein kinase are large in size (33, 34).

The experiments with the M3-short receptor mutant indicate that two fully active receptors are necessary for β-arrestin-1 binding but do not suggest whether the two receptors monomers must be activated simultaneously or not. A clear answer to this question comes from the experiments using muscarinic/adrenergic chimeric α2/M3 and M3/α2 receptors. These receptors expressed alone are not functional and do not bind either muscarinic or adrenergic ligands, whereas the only functional form is the α2/M3-M3/α2 heterodimer (12). We demonstrated that the recruitment of β-arrestin-1 by this chimeric α2/M3-M3/α2 receptor heterodimer requires the paired activation of both the muscarinic and adrenergic components. In fact, no β-arrestin-1 co-immunoprecipitation was observed when cells co-expressing the chimeric receptors were stimulated with carbachol or clonidine alone (paired Student’s two-tailed t test, p < 0.05). IB, immunoblot.

![Figure 11](http://www.jbc.org/)

**FIG. 11.** Paired stimulation with carbachol and clonidine of chimeric muscarinic/adrenergic α2/M3 and HA-M3/α2 receptors induced β-arrestin-1 co-immunoprecipitation. COS-7 cells were co-transfected with the chimeric α2/M3 and HA-M3/α2 receptors (2 μg of DNA each). Confluent monolayers of COS-7 cells were stimulated with carbachol (100 μM), clonidine (100 μM), or a mixture of the two for 30 min. Incubation was terminated by adding ice-cold NaCl (0.9%). Cells were extracted in digitonin, and aliquots of 300-μg proteins were incubated with an anti-HA antibody and processed for immunoprecipitation (IP) with protein G magnetic beads (see "Experimental Procedures"). The amount of β-arrestin-1 co-immunoprecipitated was detected with an antibody directed against the C-terminal end of β-arrestin-1. Only in cells treated with both agonists (carbachol and clonidine), it was possible to see β-arrestin-1 co-immunoprecipitation. The blot is representative of four experiments. Bar graph shows the means ± S.E. of all of the experiments. The amount of β-arrestin-1 co-immunoprecipitated was normalized for the amount of the α2/M3 and HA-M3/α2 receptors immunoprecipitated that were detected with the anti-i3 loop M3 antibody, * significantly different from clonidine and carbachol alone (paired Student’s two-tailed t test, p < 0.05). IB, immunoblot.
Recruitment of β-Arrestin by M₃ Dimers

The binding of β-arrrestin 1 probably correlates with ERK1/2 activation since Hornigold et al. (17) have shown that, when the muscarinic M₂ and M₃ receptors are co-expressed in Chinese hamster ovary cells, these receptors work synergistically to activate the ERK pathway. We also found that a selective antagonist of the M₂ receptor markedly reduced the carbachol-activated ERK pathway. We also found that a selective antagonist of the M₂ receptor markedly reduced the Erk activation since Hornigold et al. (17) have shown that, when the muscarinic M₂ and M₃ receptors are co-expressed in Chinese hamster ovary cells, these receptors work synergistically to activate the ERK pathway. We also found that a selective antagonist of the M₂ receptor markedly reduced the carbachol-induced co-immunoprecipitation of β-arrrestin 1 by the muscarinic M₂/M₃ heterodimer. This result indicates that, as has been observed with the homodimeric M₂/M₃, the stimulation of both receptor components in the heterodimer is an essential requirement for β-arrrestin 1 binding. In conclusion, our findings demonstrate that the recruitment of β-arrrestin 1 by homodimeric M₂/M₂ and heterodimeric M₂/M₃ muscarinic receptors as well as heterodimeric muscarinic/adrenergic chimeric receptors requires the paired activation of the single receptor components within the dimer. We propose that this phenomenon is mechanistically correlated to the activation of the ERK1/2 pathway. This is, to our knowledge, the first clear evidence that β-arrrestin 1 binding requires dimerization of G protein-coupled receptors and provides some mechanistic hypothesis regarding receptor-β-arrestin interaction that should be investigated in future studies. A crucial issue to address will be whether β-arrrestin binds to G protein-coupled receptor dimers in its monomeric or dimeric form. Unraveling the molecular mechanisms of receptor-β-arrestin interaction might have important implications for the further understanding the endocytic and signaling processes of G protein-coupled receptors.

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Paired Activation of Two Components within Muscarinic M₃ Receptor Dimers Is Required for Recruitment of β-Arrestin-1 to the Plasma Membrane
Francesca Novi, Laura Stanasila, Franco Giorgi, Giovanni U. Corsini, Susanna Cotecchia and Roberto Maggio

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