Interaction of Arrestins with Intracellular Domains of Muscarinic and $\alpha_2$-Adrenergic Receptors

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The intracellular domains of G-protein-coupled receptors provide sites for interaction with key proteins involved in signal initiation and termination. As an initial approach to identify proteins interacting with these receptors and the receptor motifs required for such interactions, we used intracellular subdomains of G-protein-coupled receptors as probes to screen brain cytosol proteins. Peptides from the third intracellular loop ($i_3$) of the $M_2$-muscarinic receptor (MR) (His$^{206}$-Arg$^{387}$), $M_3$-MR (Gly$^{308}$-Leu$^{497}$), or $\alpha_{2A/D}$-adrenergic receptor (AR) (Lys$^{224}$-Phe$^{374}$) were generated in bacteria as glutathione S-transferase (GST) fusion proteins, bound to glutathione-Sepharose and used as affinity matrices to detect interacting proteins in fractionated bovine brain cytosol. Bound proteins were identified by immunoblotting following SDS-polyacrylamide gel electrophoresis. Brain arrestins bound to the GST-$M_2$ fusion protein, but not to the control GST peptide or $i_3$ peptides derived from the $\alpha_{2A/D}$-AR and $M_3$-MR. However, each of the receptor subdomains bound purified $\beta$-arrestin and arrestin-3. The interaction of the $M_2$-MR and $M_3$-MR $i_3$ peptides with arrestins was further investigated. The $M_3$-MR $i_3$ peptide bound in vitro translated [$^{3}H$]$\beta$-arrestin and [$^{3}H$]arrestin-3, but did not interact with in vitro translated or purified visual arrestin. The properties and specificity of the interaction of in vitro translated [$^{3}H$]$\beta$-arrestin, [$^{3}H$]visual arrestin, and [$^{3}H$]arrestin/visual arrestin chimeras with the $M_2$-MR $i_3$ peptide were similar to those observed with the intact purified $M_3$-MR that was phosphorylated and/or activated by agonist. Subsequent binding site localization studies indicated that the interaction of $\beta$-arrestin with the $M_2$-MR $i_3$ peptide required both the amino (Gly$^{308}$-Leu$^{388}$) and carboxyl portions (Lys$^{425}$-Leu$^{497}$) of the receptor subdomain. In contrast, the carboxyl region of the $M_3$-MR $i_3$ peptide was sufficient for its interaction with arrestin-3.

G-protein-coupled receptors possess a characteristic seven segments of hydrophobic amino acids that likely serve as membrane spans to form a core motif important for ligand recognition. The interaction of agonist with the receptor initiates an ill-defined conformational adjustment in this core motif, which is propagated to intracellular domains of the receptor resulting in the activation of G-protein and the initiation of intracellular signaling events. For most members of the superfamily of G-protein-coupled receptors, the third intracellular ($i_3$) loop and the carboxyl-terminal tail of the receptor are key sites for signal initiation and termination, and these receptor domains also exhibit the greatest variability in size among different subfamilies of these receptors. The largest $i_3$ domains (100–240 amino acids) are found in receptors coupled to the $G_{i/o}$ and/or $G_{q}$ family of G-proteins (i.e., muscarinic, $\alpha$-adrenergic), whereas shorter $i_3$ loops are found in the photoreceptor rhodopsin or $\beta$-adrenergic receptors (20–50 amino acids). During the process of signal initiation and termination, several proteins interact with the receptor. The interaction of arrestins with G-protein-coupled receptors is a key component of signal termination (1–5).

The arrestin family consists of visual arrestin, $\beta$-arrestin, arrestin-3, and a cone-specific arrestin termed C- or X-arrestin (6–11). In vertebrates, visual arrestin interacts with phosphorylated rhodopsin in rod cells to terminate signal propagation by interfering with receptor coupling to transducin. $\beta$-Arrestin and arrestin-3 are widely expressed and parallel the role of visual arrestin in terms of signal termination for G-protein-coupled receptors other than rhodopsin. The affinity of arrestin binding to G-protein-coupled receptors is increased by receptor phosphorylation and/or activation by agonist. Receptors of this class are phosphorylated to varying degrees by protein kinase A and C as well as kinases specific for the activated conformation of the receptor (G-protein-coupled receptor kinases). The phosphorylation of the receptor by G-protein-coupled receptor kinases and subsequent arrestin binding are intimately associated with receptor desensitization and sequestration (12–14). Resensitization of the receptor protein involves dissociation of bound arrestin and receptor dephosphorylation.

The interaction of receptors with G-proteins, protein kinases, arrestins, and additional entities controlling receptor trafficking apparently involves discrete motifs in cytoplasmic domains of the receptor. The associations of these proteins with the receptor likely occur within a signal transduction complex that may also include various effector molecules and other proteins that influence signaling specificity/efficiency. To define receptor subdomains important for protein interactions and to begin to identify the components of a signal transduction complex for G-protein-coupled receptor subtypes, we generated peptides from the $i_3$ loop of the $M_2$-muscarinic receptor (MR), $M_3$-MR and $\alpha_{2A/D}$-adrenergic receptor (AR) to use as probes to detect...
interacting proteins in bovine brain cytosol. In the present report, we determined the interaction of the i3 loop with cytosolic proteins involved in receptor regulation.

EXPERIMENTAL PROCEDURES

Materials—Radiolabeled arrestins were in vitro translated as described previously (15). Bovine β-arrestin, visual arrestin, and arrestin-3 were also expressed in BL21 cells and purified to homogeneity by successive chromatography on heparin- and Q-Sepharose (13). Antibodies to protein kinase C isoforms were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and phosphatase 2A/C antibody (Biotechnology, Inc. Santa Cruz, CA), and phosphatase 2A/C antibody was obtained from Sigma. Monoclonal antibody mAB4C1, which recognizes the epitope DGVVLD in visual arrestin, β-arrestin, and arrestin-3, was generously provided by Dr. L. Donoso (Wills Eye Hospital, Philadelphia, PA). Glutathione-Sepharose 4B was purchased from Pharmacia Biotech Inc. Polyvinylidene difluoride membranes were obtained from Gelman Sciences (Ann Arbor, MI).

Fractionation of Brain Cytosolic Proteins—Bovine brain cytosolic proteins in buffer A (10 mM Tris-HCl, pH 7.5, 0.5 mM phenylmethylsulfonyl fluoride) containing 250 mM sucrose were precipitated with 40% ammonium sulfate and pelleted by centrifugation (100,000 × g, 45 min). The precipitated proteins were resuspended in a minimal volume of 0.1 mM Tris-HCl, pH 8.0, followed by extensive dialysis (4 liters of buffer A; 4 liters of buffer B) containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 2 mM β-mercaptoethanol. The supernatant from the 40% ammonium sulfate precipitate was brought to 90% ammonium sulfate to precipitate additional proteins and subsequently processed as described for the 40% ammonium sulfate precipitate. The dialyzed solutions were clarified by centrifugation and applied to an anion-exchange resin (DEAE-Biogel A) equilibrated with buffer B. The column was washed with buffer B and proteins eluted sequentially with buffer B containing 100, 250, and 500 mM NaCl. Eluted proteins were desalted by dialysis, concentrated by lyophilization, and stored at −70 °C.

Plasmid Constructions and Expression of GST Fusion Proteins—The M2-MR i3 construct was obtained from Dr. Barry Wolfe (Department of Physiology, Georgetown University Medical Center, Washington, DC) and encoded the peptide Gly308–Leu497. The M2-MR and M3-MR peptides was determined in a similar manner. Arrestin binding to the specific antibodies. The interactions of purified arrestins with the i3 loops of the VI membrane span. To determine the interaction of these receptor-derived peptides with cytosolic proteins, we first fractionated bovine brain cytosol to enrich for potential interacting proteins.

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Interaction of Receptor Subdomain Probes with Brain Cytosol Proteins—The third intracellular loop of the M2-MR, M3-MR, and the a2AR agonist consists of 180 (His208–Arg387), 238 (Arg253–Gln490), and 157 (Arg218–Phe374) amino acids, respectively. The putative first and second intracellular loops of the three receptors are similar in size ~11–12 amino acids, 1st loop; ~19 amino acids, 2nd loop) and the carboxyl-terminal tails of the M2-MR, M3-MR, and a2AR are ~20, 44, and 20 amino acids in length, respectively. As an initial attempt to define proteins that may interact with the intracellular domains of these G-protein-coupled receptors, we focused on the i3 loop as it is the largest intracellular domain in this receptor group. The juxtamembrane segments of the i3 domain are of critical importance for receptor coupling to G-protein, whereas other segments participate in receptor phosphorylation, receptor trafficking, and other aspects of signal propagation.

The M2-MR (His208–Arg387), M3-MR (Gly253–Leu490), and a2AR (Arg218–Phe374) i3 peptides were expressed in bacteria as a GST fusion protein and used to generate an affinity matrix by saturating a glutathione-Sepharose resin with the fusion protein (Fig. 1). The M2-MR peptide corresponded to the entire i3 loop of the receptor. The M3-MR i3 peptide began 45 amino acids downstream of the amino terminus of the i3 loop and terminated seven amino acids into the VI membrane span. The a2AR i3 peptide began six amino acids downstream of the amino terminus of the i3 loop and terminated at the beginning of the VI membrane span.

FIG. 1. Generation of receptor subdomain probes. Peptides corresponding to segments of the third intracellular loop of the human M2-MR (180 amino acids), rat M3-MR (190 amino acids), and rat a2AR (151 amino acids) (A) were generated as GST fusion proteins in bacteria and purified as described under “Experimental Procedures”. In A, the black segments correspond to the putative fifth and sixth membrane spans of the receptor. B, the purified fusion proteins were electrophoresed on denaturing polyacrylamide gels (10%) and visualized by Coomassie Blue stain of the proteins. The calculated molecular weights of GST and the M2-MR, M3-MR, and a2AR GST fusion proteins were 28,146, 47,864, 49,796, and 43,343, respectively, including adapter amino acids. The arrows indicate the migration of GST and each of the GST fusion proteins. The numbers to the left of the gel indicate the migration of standards of known molecular weight × 10−3.
In the first series of experiments, we determined the interaction of receptor subdomains with brain arrestins. Bovine brain cytosol was fractionated by ammonium sulfate precipitation and ion exchange chromatography as described under "Experimental Procedures." The fractionation of arrestins was determined by immunoblot of a membrane transfer of the crude and fractionated brain cytosol following SDS-PAGE (Fig. 2A, left panel). The cytosol fraction (100 μg of protein) enriched for arrestin was then incubated with control GST, M2-MR, M3-MR, and α2A/R AR affinity matrices (~5 μg of protein) and retained proteins visualized by immunoblotting (Fig. 2A, right panel). The specificity of arrestin binding to the M3-MR peptide was further investigated by determining the interaction of the peptide with other cytosolic proteins. The distribution of protein kinase C isoforms, phosphatase 2A, and the actin-binding protein gelsolin in the fractionated bovine brain cytosol was determined by immunoblotting (Fig. 3, left panel). Two protein kinase C isoforms fractionated in the 250 mM NaCl elution of the 90% ammonium sulfate precipitate. The phosphatase 2A immunoreactive species was identified in the 250 mM NaCl elution of the 40% ammonium sulfate precipitate. Gelsolin was enriched in the 100 mM NaCl elution of the 40% ammonium sulfate precipitate.

**Fig. 2. Interaction of the M2-MR, M3-MR, and α2A/R AR third intracellular loop peptides with arrestins in preparations of bovine brain cytosol.** Brain cytosol was fractionated by ammonium sulfate precipitation and ion exchange chromatography as described under "Experimental Procedures." The fractionation of arrestins was determined by immunoblot of a membrane transfer of the crude and fractionated brain cytosol following SDS-PAGE (A, left panel). The cytosol fraction (100 μg of protein) enriched for arrestin was then incubated with control GST, M2-MR, M3-MR, and α2A/R AR affinity matrices (~5 μg of protein) and retained proteins visualized by immunoblotting (A, right panel). The specificity of arrestin binding to the M3-MR peptide was further investigated by determining the interaction of the peptide with other cytosolic proteins. The distribution of protein kinase C isoforms, phosphatase 2A, and the actin-binding protein gelsolin in the fractionated bovine brain cytosol was determined by immunoblotting (Fig. 3, left panel). Two protein kinase C isoforms fractionated in the 250 mM NaCl elution of the 90% ammonium sulfate precipitate. The phosphatase 2A immunoreactive species was identified in the 250 mM NaCl elution of the 40% ammonium sulfate precipitate. Gelsolin was enriched in the 100 mM NaCl elution of the 40% ammonium sulfate precipitate. The appropriate fraction was then incubated with the M2-MR or the α2A/R AR i3 affinity matrix and processed as described for arrestins. Although the M2-MR affinity matrix adsorbed brain arrestins (Fig. 2), neither the M3-MR or the α2A/R AR i3 peptides interacted with the protein kinase C isoforms, phosphatase 2A, or gelsolin (Fig. 3, right panel).

**Fig. 3. Interaction of brain cytosol proteins with the M2-MR and α2A/R AR third intracellular loop peptide.** Brain cytosol was fractionated as described under "Experimental Procedures," and the fractions containing protein kinase C isoforms (PKC), phosphatase 2A (PTP2A), or gelsolin were determined by immunoblots of membrane transfers of the crude and fractionated brain cytosol following SDS-PAGE (left panels). Anti-sera dilutions: protein kinase C, 1:750; phosphatase 2A, 1:500; gelsolin, 1:1000. Cytosol fractions (100 μg of protein) enriched for the proteins of interest were then incubated with control GST, M2-MR, and α2A/R AR affinity matrices (~5 μg of protein) and retained proteins visualized by immunoblotting (right panels). "Fractionated cytosol" in the right panels refers to the fraction enriched for the particular protein as determined in the series of panels on the left.

**Interaction of the i3 Peptides with Arrestins—**The interaction of arrestins with the i3 peptides was investigated in more detail to define issues of arrestin selectivity and sites of arrestin association. In the first series of experiments, we evaluated arrestin binding to the M2-MR using radiolabeled arrestins. Increasing concentrations of radiolabeled β-arrestin, arrestin-3, and visual arrestin were incubated with the M2-MR or the control GST resin. Both [3H]β-arrestin and [3H]arrestin-3 exhibited specific binding to the M2-MR peptide relative to the binding of the arrestins to the control GST peptide (Fig. 4A). In contrast, the binding of [3H]visual arrestin to the M2-MR matrix was only slightly increased above that observed with the GST peptide itself, consistent with the lower affinity of visual arrestin at G-protein-coupled receptors other than rhodopsin. Interestingly, the affinities exhibited by [3H]β-arrestin and [3H]arrestin-3 (0.5–1.0 nM) are in the range of the Kd for arrestin binding to purified β2-AR and M2-MR (15). The selectivity of...
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FIG. 4. Interaction of the M3-MR with visual arrestin (v-arr), β-arrestin (β-arr), and arrestin-3 (arr-3). A, the M3-MR i3 peptide or GST-substituted resins (∼2.5 μg of protein) were incubated with increasing concentrations of radiolabeled arrestins and processed as described under “Experimental Procedures.” Arrestin binding to the GST control resin at each arrestin concentration was subtracted from that observed with the M3-MR matrix to generate the values shown. Data are representative of three different experiments using different batches of arrestins and fusion proteins. In B, the M3-MR i3 peptide or GST-substituted resins (∼5 μg of protein) were incubated with purified β-arrestin (50 ng) or visual arrestin (50 ng) and the samples were processed as described under “Experimental Procedures.” Arrestins retained by the substituted resins were identified by immunoblottling using the monoclonal antibody mAbF4C1, which recognizes the epitope DGVVLVD present in visual arrestin, β-arrestin, and arrestin-3. The first two lanes indicate the signal detected with 25 ng of each arrestin.

The interaction of arrestins with the intact purified M3-MR was previously characterized using in vitro translated arrestins and β-arrestin/visual arrestin chimeras (15). We thus compared the interaction of arrestins with the M3-MR i3 peptide and the intact receptor relative to the influence of ionic strength and the selectivity of binding for the different arrestins. As observed for the intact purified receptor that was phosphorylated and/or activated by agonist (15), [3H]β-arrestin binding to the i3 peptide was first increased and then decreased with increasing ionic strength of the incubation buffer (Fig. 6A). Previous studies using a series of [3H]β-arrestin/visual arrestin chimeras indicated that the selectivity of β-arrestin and visual arrestin binding to the intact purified M3-MR and the β2-adrenergic receptor involved specific domains of the two arrestins (15). To determine if this selectivity of arrestin binding was maintained with the M3-MR i3 peptide, we evaluated the binding of two [3H]β-arrestin/visual arrestin chimeras. The BBBA chimera consists of amino acids 1–340 of β-arrestin and amino acids 346–404 of visual arrestin. The ABB chimera consists of amino acids 1–213 of visual arrestin and the BBBA chimera is 208–418 of β-arrestin. The binding of BBBA to the intact M3-MR was similar or greater than that observed with β-arrestin, whereas the binding of ABB was intermediate between β-arrestin and visual arrestin (15). The relative binding of [3H]β-arrestin, [3H]visual arrestin, and the [3H]β-arrestin/visual arrestin chimeras to the M3-MR i3 peptide, as well as the M3-MR peptide, was similar to that observed with the intact purified M3-MR (Fig. 6B).

Binding of Brain Arrestins, β-Arrestin, and Arrestin-3 to Subdomains of the M3-MR i3 Loop—The next series of experiments were designed to localize subdomains of the M3-MR i3 loop required for interaction with arrestins. The M3-MR i3 peptide (M3-I) was divided into three segments: an amino-terminal region (M3-II, Gly389–Gln398), the carboxyl-terminal region (M3-III, Val390–Leu397), and a middle portion (Lys389–Thr424) (Fig. 7A). The M3 peptide possesses a concentrated negative charge in the amino-terminal third and a concentrated positive charge in the carboxyl-terminal third of the peptide. The contribution of the middle portion was determined

2 Due to differences in the degree of resin substitution for various fusion proteins, variable membrane transfer efficiencies, and relative signal intensities, it is difficult to accurately determine the affinity of arrestin binding to the resins. However, data obtained thus far indicate no dramatic differences in the affinity of the purified arrestins for the three i3 peptides.
using construct M3-IV in which this segment was deleted and the peptide Gly308-Leu368 was fused to the peptide Lys369-Thr424, black segment in M3-IV) was deleted and peptide Gly308-Leu368 was fused to Lys425-Leu506. The GST fusion proteins M3-I to M3-IV were electro-
phoresed on denaturing polyacrylamide gels and stained with Coomas-
see Blue. B. GST or the M3-I to M3-IV resins (~5 μg of protein) were
incubated with 100 μg of bovine brain cytosol fraction enriched for
arrestins (cytosol), 50 ng of purified β-arrestin or arrestin-3 and proc-
essed for immunoblotting as described in the legend to Fig. 2. Similar
results were obtained in three to five experiments using different
batches of fusion protein.

\[3\] Based on the relative migration of β-arrestin and arrestin-3 in 10% denaturing polyacrylamide gels and the comigration of the partially purified brain arrestin with β-arrestin (Fig. 5), the arrestin identified in the 100 mM NaCl elution of the 90% ammonium sulfate precipitate is predominantly β-arrestin (G. Wu and S. M. Lanier, unpublished data).

**Fig. 6.** Comparison of the properties of arrestin interaction with the M3-MR i3 peptide and the intact purified M3-MR receptor. In A the M3-MR i3 peptide or GST-substituted resins (~2.5 μg of protein) were incubated with in vitro translated [3H]β-arrestin (0.5 nM, 1062 dpm/fmol) in the presence of increasing concentrations of potassium acetate (50–500 mM) and processed as described under “Experimental Procedures.” Arrestin binding to the GST control resin at each salt concentration was subtracted from that observed with the M3-MR i3 peptide matrix to generate the values shown (M3,i3, filled squares). The data are expressed as the percent of specific [3H]β-arrestin binding obtained with incubation buffer containing 50 mM potassium acetate at which [3H]β-arrestin binding to the GST control and M3-MR i3 peptide matrix was 1316 ± 84 dpm (1.24 ± 0.17 fmol) and 5280 ± 392 dpm (4.97 ± 0.37 fmol), respectively. Data are presented as the mean ± S.E. of three experiments. The data presented with the purified M3-muscarinic receptor (M3-MR, open squares) were adapted from Ref. 15 and represent the data obtained with the phosphorylated and agonist-acti-
vated receptor. In B, the M3-MR i3 and M3-MR i3 peptides or GST-
substituted resin (~2.5 μg of protein) were incubated with in vitro translated [3H]β-arrestin, [3H]visual arrestin, or the [3H]β-arrestin/visual arrestin chimeras BBBA or AABB under standard incubation conditions. The concentration of each radiolabeled arrestin was 1.0 nM. The binding of the different arrestins to the GST control resin was subtracted from that observed with the M3-MR i3 or M3-MR i3 peptide matrix to generate the values shown. The data are expressed as the percent of specific binding of [3H]β-arrestin. [3H]β-arrestin binding to the M3-MR and M3-MR matrix was 7940 ± 556 dpm (GST control, 2284 ± 466 dpm) and 9250 ± 1156 dpm (GST control, 3204 ± 1824 dpm), respectively. Data are presented as the mean ± S.E. of three experiments. The data presented with the purified M3-muscarinic receptor (M3-MR) were adapted from Ref. 15 and represent the data obtained with the phosphorylated and agonist-activated receptor.

**DISCUSSION**

As is apparently the case with most complex signal processing systems, G-protein-coupled receptors likely operate within a signal transduction complex that is either preexisting or is generated by the biological stimuli. The components of such a signaling complex are unclear but might include proteins that influence events at the receptor-G-protein or G-protein-effector interface or contribute to the formation of the signal transduction complex itself (Ref. 17 and 18, and references therein). As part of an effort to identify components of this signal transduc-
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The demonstration that the experimental approach using receptor subdomains as probes for receptor-associated proteins resulted in the detection of protein-protein interactions of clear biological relevance underscores the potential utility of the system to identify additional interacting proteins that may contribute to the formation of a signal transduction complex. Such interacting proteins may play an important role in directing the receptor-initiated signal to a specific effector pathway. In contrast to signaling events in the visual system where the components are localized and the interactions between the individual molecules are relatively specific, other G-protein-coupled receptors appear capable of serving as a docking site for arrestin independent of receptor phosphorylation. Indeed, the properties of arrestin binding to the M2-MR i3 peptide appeared similar to those exhibited by the purified receptor protein that was phosphorylated and/or activated by agonist. The binding of β-arrestin and arrestin-3 to the i3 peptides may also relate to the agonist-induced conformational changes responsible for initiating the signaling cascade. In the absence of agonist, the regions of the receptor involved in G-protein activation are stabilized in a conformation that acts as a “brake” on signal initiation (25). Such a conformational “brake” may be released by discrete mutations in the i3 loop of some receptors, such that the receptor becomes constitutively active (26). An analogous situation may occur when the i3 loop is separated from the conformational restrictions imposed by membrane spans of the receptor and assumes an “activated and/or accessible conformation” that is recognized by arrestin.

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coupled receptors operate in diverse cell types and couple to multiple G-proteins and effectors. Perhaps, such receptors have evolved larger i3 loops to maintain the fidelity of the signaling system by providing sites for interaction with additional accessory proteins that influence receptor trafficking and/or signaling specificity and efficiency.

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