Regulated Interactions of the α2A Adrenergic Receptor with Spinophilin, 14-3-3ζ, and Arrestin 3*

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The present studies demonstrate that no single stretch of sequence in the third intracellular (3i) loop of the α2A adrenergic receptor (α2A-AR) can fully account for its previously described interactions with spinophilin (Richman, J. G., Brady, A. E., Wang, Q., Hensel, J. J., Colbran, R. J., and Limbird, L. E. (2001) J. Biol. Chem. 276, 15003–15008), 14-3-3ζ (Prezeau, L., Richman, J. G., Edwards, S. W., and Limbird, L. E. (1999) J. Biol. Chem. 274, 13462–13469), and arrestin 3 (Wu, G., Krupnick, J. G., Benovic, J. L., and Lanier, S. M. (1997) J. Biol. Chem. 272, 17836–17842), suggesting that a three-dimensional surface, rather than a linear sequence, provides the basis for these interactions as proposed for 3i loop tethering of the α2A-AR to the basolateral surface of Madin-Darby canine kidney cells (Edwards, S. W., and Limbird, L. E. (1999) J. Biol. Chem. 274, 16331–16336). Sequences at the extreme N-terminal and C-terminal ends of the 3i loop are critical for interaction with spinophilin but not for interaction with 14-3-3ζ or arrestin 3, for which the C-terminal half of the loop is more important. Competition binding for 35S-labeled α2A-AR 3i loop binding to glutathione S-transferase (GST)-spinophilin amino acids 151–444 revealed a relative affinity of spinophilin ≈ arrestin > 14-3-3ζ for the unphosphorylated α2A-AR 3i loop. Agonist occupancy of the α2A-AR increases receptor association with spinophilin, and arrestin 3 appears to compete for this enrichment. However, when the G protein-coupled receptor kinase 2 substrate sequence was deleted from the 3i loop, arrestin 3 could not compete for the agonist-enriched binding of spinophilin to the mutant α2A-AR. These data are consistent with a model where sequential or competitive interactions among spinophilin, arrestin, and/or 14-3-3ζ play a role in α2A-AR functions.

The three α2 adrenergic receptor (AR)1 subtypes (α2A-, α2B-, and α2C-AR) mediate their responses via coupling to GTP-binding proteins of the G/Gi family. In native cells, these receptors mediate inhibition of adenylyl cyclase, activation of receptor-operated K+ currents, suppression of voltage-gated Ca2+ currents, and activation of mitogen-activated protein kinase activity (1–3). In heterologous systems, the α2A-AR also has been demonstrated to activate phospholipase D (4), phospholipase C (5), and phospholipase A2 (6–8).

The α2A-ARs often mediate their responses in highly polarized cells, such as epithelial cells from the kidney and intestine or neurons of the peripheral and central nervous system (9). We have studied the polarization of the α2A-AR subtypes extensively in Madin-Darby canine kidney (MDCKII) cells as a model system. In these studies, we demonstrated that basolateral targeting of the α2A-AR required sequences embedded in or near the bilayer, whereas retention on that surface involved the third intracellular (3i) loop (10). These findings suggested the possibility that the 3i loop may interact with proteins underlying the basolateral surface to extend its half-life there.

In studies intended to identify 3i loop-interacting proteins, we demonstrated that the 3i loop interacts with 14-3-3ζ (11) as well as with spinophilin (12). Receptor interactions with spinophilin, in particular, are enhanced by agonist occupancy of the receptor (12). Morphological studies established that both 14-3-3ζ and spinophilin (12–14) are enriched at the basolateral surface of MDCKII cells, although these proteins also are expressed widely throughout the cytoplasm.

To further understand the role of receptor interactions with spinophilin and/or 14-3-3ζ proteins, we sought to identify the particular regions of the α2A-AR 3i loop sequence involved in the protein-protein contacts. The present studies examine the basis for α2A-AR 3i loop interactions with spinophilin, 14-3-3ζ proteins, and arrestin 3 (also known as β-arrestin 2), another molecule known to interact with the α2A-AR 3i loop (15, 16). We also explored the functional relevance of these interactions by assessing the ability of spinophilin or arrestin to compete for receptor interactions upon agonist occupancy. The data obtained are consistent with a regulatory cycle where agonist-regulated interactions of these proteins occur, perhaps relevant for α2A-AR-mediated functions and/or localization.

EXPERIMENTAL PROCEDURES

Materials—The pGEMEX-2 vector and TNT in vitro translation kit were from Promega (Madison, WI). [35S]Methionine (1000 Ci/mmol) was purchased from PerkinElmer Life Sciences. Centricon-10 concentrating filters were purchased from Amicon (Beverly, MA). Dodecyl-β-D-maltoside (DjM) was from Calbiochem (Darmstadt, Germany). Cholesterol hemisuccinate (CHS) was from Sigma. Anti-HA rat monoclonal antibody was from Roche Molecular Biochemicals. Anti-HA mouse monoclonal antibody (HA.11) was from CRP (BabCO, Denver, PA). Immobilized protein G-agarose was from Pierce. All other chemicals were reagent-grade from Fisher Chemicals (Pittsburgh, PA).

Synthesis of Radio-labeled Wild Type and Mutant α2A-AR 3i Loops—Four methionines were inserted via PCR into the N terminus of the porcine α2A-AR 3i loop (amino acids 218–377 (12)), and the resulting

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‡ The abbreviations used are: AR, adrenergic receptor; 3i loop, third intracellular loop; CHS, cholesterol hemisuccinate; HA, hemagglutinin; WT, wild type; aa, amino acid; GST, glutathione-S-transferase; DjM, dodecyl-β-maltoside; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; GRK, G protein-coupled receptor kinase; Sp, spinophilin (neurabin II).

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product was subcloned into the modified pGEMEX2 vector in which the sequence encoding viral coat protein gene 10 was deleted. The resulting construct, referred to as (Met)\textsubscript{4}-\textalpha\textsubscript{2}-\textalpha\textsubscript{3}i3 (structure WT in Fig. 3), was verified by DNA sequencing. Deletions and truncations were introduced into (Met)\textsubscript{1}–\textalpha\textsubscript{2}-\textalpha\textsubscript{3}i3 through PCR and/or restriction enzyme digestion and religation of corresponding fragments; all constructs were verified by DNA sequencing. The resulting 3i loop structures (designated 1–8) each carry a deletion as follows: 1, \Delta\textalpha\textsubscript{aa} 252–257; 2, \Delta\textalpha\textsubscript{aa} 268–285; 3, \Delta\textalpha\textsubscript{aa} 286–303; 4, \Delta\textalpha\textsubscript{aa} 304–314; 5, \Delta\textalpha\textsubscript{aa} 315–326; 6, \Delta\textalpha\textsubscript{aa} 327–340; 7, \Delta\textalpha\textsubscript{aa} 331–343; 8, \Delta\textalpha\textsubscript{aa} 351–377. Structures 9 and 10 represent the N- (\aa\textsubscript{aa} 218–294) and C- (\aa\textsubscript{aa} 294–377) terminal half of the loop, respectively. Structures 11–13 each carry a deletion at the origin of the N-terminal half of the loop: 11, \Delta\textalpha\textsubscript{aa} 218–231; 12, \Delta\textalpha\textsubscript{aa} 218–267; 13, \Delta\textalpha\textsubscript{aa} 218–285. Structures 14 and 15 contain \aa\textsubscript{aa} 394–350 and aa 232–359, respectively. Structure 15 contains aa 218–350 with a deletion of aa 252–267.

Wild type and mutant 3i loops were transcribed, translated, and \textsuperscript{35}S-labeled using the Promega transcription and translation-coupled (TNT) rabbit reticulocyte lysate kit as described previously (11). Following each synthesis, products were analyzed and quantitated by 12% SDS-PAGE and autoradiography. The band representing each probe was cut out of the dried gel and counted to determine cpm/albumin of product attributable to \textsuperscript{35}S-labeled 3i loops. GST pull-down assays were performed such that each incubation contained an equivalent amount of \textsuperscript{35}S-labeled wild type or mutant 3i loop.

Preparation of GST-\textalpha\textsubscript{2}-\textalpha\textsubscript{3}i3 Loops—A DNA fragment encoding the \textalpha\textsubscript{2}-\textalpha\textsubscript{3}i3 loop amino acids 239–370, representing the NotI/Eco47III fragment from the cDNA encoding the porcine \textalpha\textsubscript{2}-\textalpha\textsubscript{3}AR, was blunted and then subcloned into the pGEX2Smal site in frame with the DNA encoding GST; the resulting construct is referred to as GST-\textalpha\textsubscript{2}-\textalpha\textsubscript{3}i3 short (GST-\textalpha\textsubscript{2}s, structure WT, in Fig. 4). Deletions and truncations were introduced into GST-\textalpha\textsubscript{2}-\textalpha\textsubscript{3}i3 through PCR and/or restriction enzyme digestion and religation of corresponding fragments. Resulting structures of the GST-3i loop, numbered 17–25, each carry a deletion as follows: 17, \Delta\textalpha\textsubscript{aa} 252–267; 18, \Delta\textalpha\textsubscript{aa} 268–285; 19, \Delta\textalpha\textsubscript{aa} 286–303; 20, \Delta\textalpha\textsubscript{aa} 304–314; 21, \Delta\textalpha\textsubscript{aa} 315–326; 22, \Delta\textalpha\textsubscript{aa} 327–340; 23, \Delta\textalpha\textsubscript{aa} 331–343; 24, \Delta\textalpha\textsubscript{aa} 344–357; 25, \Delta\textalpha\textsubscript{aa} 358–370. Structures 26 and 27 contain aa 229–294 and aa 294–370 of the 3i loop fused with GST, respectively. The full-length \textalpha\textsubscript{2}-\textalpha\textsubscript{3}AR 3i loop (aa 218–377) was fused with GST by subcloning the EcoRI/HindIII (blunt) fragment from (Met)\textsubscript{4}-\textalpha\textsubscript{2}-\textalpha\textsubscript{3}i3 into pGEX2T/EcoRI (blunt) vector; the resulting construct is referred to as GST-\textalpha\textsubscript{2}-\textalpha\textsubscript{3}i3 long (GST-\textalpha\textsubscript{2}l, structure WT, in Fig. 4). The N-terminal half of the 3i loop (aa 218–294) was fused with GST by subcloning the EcoRI/HindIII (blunt) fragment from structure 9 into pGEX2T/EcoRI (blunt) vector; the resulting structure is referred to as structure 28. In all studies where GST fusion proteins were employed, we stained the same SDS-PAGE gel as evaluated with autoradiography with Coomassie Blue to confirm that equivalent amounts were present in all incubations.

Preparation of GST-Spinophilin—(151–444) Fusion Protein—GST-spinophilin fusion protein was generated with spinophilin amino acid regions 151–444 (referred to as GST-Sp151–444) and expressed in DH5\textalpha\text sub or BL21 cells as described previously (12). Briefly, bacteria were grown at 37 °C to an \textalpha_{OD}\textsubscript{600} of 0.6. GST or GST fusion protein expression was initiated with the addition of 1 mM isopropyl-\textbeta\textsubscript{-}D-thiogalactopyranosyl-

The ability of GST-\textalpha\textsubscript{2}-\textalpha\textsubscript{3}AR to increase association of a particular protein with the \textalpha\textsubscript{2}-\textalpha\textsubscript{3}AR or an \textalpha\textsubscript{2}-\textalpha\textsubscript{3}AR-enriched complex was assessed using co-immunoprecipitation strategies. COS-M6 cells transiently transfected with \textalpha\textsubscript{2}-\textalpha\textsubscript{3}AR or a GST-\textalpha\textsubscript{2}-\textalpha\textsubscript{3}AR Loops—GST or GST fusion proteins expressed from a 25-ml culture of DH5\textalpha\text sub bacteria transformed with wild type or mutant GST-3i loop constructs (estimated as 25 \textmu\textsubscript{g} per culture) were incubated with 100 \textmu\textsubscript{g} of GST-agarose (1:1 slurry equilibrated with TTB buffer) for 2 h at 4 °C. Equal amounts of \textsuperscript{35}S-labeled wild type or mutant 3i loops were added to each incubation and rotated for another 2 h at 4 °C. After collection by centrifugation, the resin was washed three times with 1 ml of TTB. Bound wild type or mutant 3i loops were eluted, resolved, and quantitated as above.
tagged spinophilin (12), and/or GFP-arrestin 3 (a generous gift from Dr. Mark Caron at Duke University); protein (0.2, 0.6, 6, and 6 µg per 10-cm plate, respectively), were serum-starved overnight and treated with 100 µM epinephrine (agonist-treated) or 1 µM yohimbine (antagonist-treated) for 5 min at 37 °C in the presence of 1 µM propranolol to block epinephrine effects on tract endogenous β-adrenergic receptors. The incubation was terminated by placing the culture dishes on ice, aspirating the incubation medium, and washing it once with ice-cold PBS containing 1 mM MgCl₂ and 0.5 mM CaCl₂. Cells were then scraped into ice-cold lysis buffer containing 15 mM Hepes, pH 7.6, 5 mM EGTA, and 5 mM EDTA. Then washed three times with 1 ml of PBS lysis buffer, containing 20 mM Hepes, pH 7.6, 25 mM glycyglycine, and 5 mM EGTA with the following protease inhibitors: 100 µM PMSF, 10 µg/ml leupeptin, 5 µg/ml aprotinin, and 1 µg/ml soybean trypsin inhibitor, by 10 passage through a 25-gauge needle on a 1-ml syringe. The extract was centrifuged at 100,000 × g for 30 min at 4 °C. The supernatant was defined as the solubilized membrane preparation.

Typically, 0.4 ml of detergent extract was precleared with 25 µl of a 1:1 protein G-agarose/Dj/M/CHS lysis buffer slurry and incubated with a 1:50 dilution of rat anti-HA antibody overnight at 4 °C before addition of 25 µl of a 1:1 slurry of protein G-agarose for 2 h. The protein G-agarose was pre-equilibrated in Dj/M/CHS lysis buffer (see above) also containing 0.2% bovine serum albumin to block nonspecific binding of the protein. The protein G-agarose-rat anti-HA-α₂-AR complex was then washed three times with 1 ml of Dj/M/CHS wash buffer (0.5 mg/ml DjM, 0.1 mg/ml CHS, 20 mM Hepes, 25 mM glycyglycine, 5 mM EDTA). Bound protein was eluted in 1× Laemmli buffer by a 15-s water bath sonication and 5 min of incubation at 70 °C and separated on 10% SDS-PAGE. Western blot analyses were performed following ECL Western blotting protocols (Amersham Biosciences).

Intact Cell Receptor Phosphorylation—COS-M6 cells grown in 12.5-cm² flasks were transiently transfected with cDNA encoding wild type HA-α₂-AR or a mutant HA-α₂-AR (HEEESSSS) (10). Cells were labeled with 0.1 µCi/ml [35S]Met/leucine for 1 h following incubation in phosphate-free, serum-free Dulbecco’s modified Eagle medium for 2 h at 37 °C. Cells were then treated with 100 µM epinephrine (agonist-treated) or 1 µM yohimbine (antagonist-treated) for 5 min at 37 °C in the presence of 1 µM propranolol to block epinephrine effects on tract endogenous β-adrenergic receptors. The incubation was terminated by placing the culture dishes on ice, aspirating the incubation medium, and washing the dishes twice with ice-cold PBS containing 2 mM Na₂VO₄. Cells were then scraped into 400 µl of PBS lysis buffer containing 1% Triton X-100, 0.05% SDS, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 10 mM sodium pyrophosphate, and protease inhibitors, passed ten times through a 20-gauge needle on a 1-ml syringe, and rotated end over end for 30 min at 4 °C. The lysate was centrifuged at 100,000 × g for 30 min at 4 °C; the resulting supernatant was subjected to immuno precipitation with rat anti-HA antibody and 15 µl of a 1:1 slurry of protein G-agarose/PBS lysis buffer. Immunoprecipitates were washed three times with 1 ml of PBS lysis buffer, eluted in 1× Laemmli buffer by a 15-s water bath sonication and 5 min of incubation at 70 °C, separated on 10% SDS-PAGE, and analyzed by autoradiography.

RESULTS AND DISCUSSION

Interaction of the α₂AR 3i Loop with Spinophilin—Spinophilin (neurabin II) is a multidomain protein with an N-terminal actin-binding domain, a receptor-interaction domain and of the GST fusion protein that was used in pull-down assays to evaluate the saturability of binding. B, interaction of the α₂AR 3i loop with GST-Sp151–444 (upper panel) versus GST (lower panel) in GSH-agarose pull-down assays. The “binding” panels represent the amount of 35S-labeled α₂AR 3i loop pulled down by GSH-agarose in the presence of GST-spinophilin versus GST, respectively, quantitated by cutting and counting after autoradiography of the gel. The “input” panels show increasing amounts of GST-Sp151–444 (upper panel) and GST (lower panel) added to incubation (stained by Coomassie Blue). The “1/30 input” lane represents 1/30 of the total amount of 35S-labeled α₂AR 3i loop added in each reaction.

Fig. 1. Specific interaction of spinophilin with the α₂AR 3i loops. A, schematic diagram of the predicted spinophilin domain structure and of the GST fusion protein that was used in pull-down assays to evaluate the saturability of binding. B, interaction of the α₂AR 3i loop with GST-Sp151–444 (upper panel) versus GST (lower panel) in GSH-agarose pull-down assays. The “binding” panels represent the amount of 35S-labeled α₂AR 3i loop pulled down by GSH-agarose in the presence of GST-spinophilin versus GST, respectively, quantitated by cutting and counting after autoradiography of the gel. The “input” panels show increasing amounts of GST-Sp151–444 (upper panel) and GST (lower panel) added to incubation (stained by Coomassie Blue). The “1/30 input” lane represents 1/30 of the total amount of 35S-labeled α₂AR 3i loop added in each reaction.

We have shown previously that not only spinophilin (12), but also 14-3-3ζ (11) interacts with the α₂AR 3i loop. Others have demonstrated the interaction of arrestin 3 (also known as β-arrestin 2) with the 3i loop of the α₂AR (15, 16). To evaluate the relative affinity of the 3i loop for spinophilin versus 14-3-3ζ or arrestin 3, we examined the ability of in vitro translated and unlabeled 14-3-3ζ, spinophilin, or arrestin 3 to “usurp” the 35S-labeled 3i loop ligand available for binding to GST-Sp151–444 (Fig. 2A). We utilized this approach because, unlike for GST-Sp151–444, we were unable to synthesize large quantities of either GST-14-3-3ζ or GST-arrestin 3, and thus could not perform competition binding experiments in the traditional fashion. As shown in Fig. 2B, the presence of in vitro translated, unlabeled (Met)₅-spinophilin-(151–444), 14-3-3ζ, and arrestin 3 decreases availability of the 35S-labeled 3i loop for GST-Sp151–444 binding. Spinophilin-(151–444) and arrestin 3 have a relatively equal affinity in doing so, which is 10-fold more sensitive than inhibition of 35S-labeled 3i loop binding by 14-3-3ζ (Fig. 2C). Under the conditions of this experiment, the 3i loop is not phosphorylated, which has implications for the interpretations of findings to be discussed in Fig. 6, A and C. It is also interesting to note that spinophilin competes for 80% of 35S-labeled α₂AR 3i loop binding, whereas arrestin 3 and 14-3-3ζ compete for only ~50% of binding. The possible significance of this finding is addressed later.

Spinophilin Interacts with Multiple Non-contiguous Regions of the 3i Loop of the α₂AR—To identify the region, or regions, within the 3i loop of the α₂AR responsible for interactions...
of the deleted sequences are required for the 3i loop-spinophilin interaction. Similarly, deletion of either the entire amino-half (structure 10) or the carboxyl-half (structure 9) of the 3i loop also does not eliminate its interaction with spinophilin (Fig. 3B, lower left panel). These data suggest that no single stretch of sequence can account for α2AR 3i loop interaction with spinophilin; we interpret these observations to suggest that a folded surface formed by multiple non-contiguous regions of the 3i loop interacts with spinophilin. Interestingly, we have similarly found that multiple, non-contiguous regions of the α2AR-3i loop are involved in stabilizing the α2AR on the basolateral surface (21).

Since the 3i loops of all three α2AR subtypes interact with spinophilin (12) and amino acid sequences among these loops are very divergent, we performed a sequence homology search among the 3i loops of the three 2-AR subtypes. Five categories and deletions within the 3i loop, shown schematically in Fig. 3A. The structure designated WT represents the entire α2AR-3i loop (aa 218–377) as reported previously (20). Structures 1 through 8 illustrate the deleted regions throughout the 3i loop, with the GST-Sp151–444 fusion protein pull-down assays were performed with 5 μg of GST-Sp151–444 (estimated concentration = 7.2 × 10^{-7} M) and 6000 cpm (estimated concentration = 9.2 × 10^{-12} M) of 35S-labeled α2AR-3i loop in the presence of increasing amounts (0–16 μl) of in vitro translated Sp151–444, 14-3-3ζ, or arrestin 3 (see “Experimental Procedures”). A, scheme of the experimental design. B, the autoradiography of 35S-labeled 3i loop bound to GST-Sp151–444 in the presence of different amounts of in vitro translated Sp151–444, 14-3-3ζ, or arrestin 3 from one representative experiment. C, representation of mean data ± S.E. from three independent experiments. The concentration of each unlabeled in vitro translated protein was estimated by determining the concentration of the same protein radiolabeled in parallel under identical conditions, assuming that both reactions would proceed at comparable efficiency. The EC_{50} value for depletion of 9.2 × 10^{-12} M 35S-labeled 3i loop available for GST-Sp151–444 is 1.1 × 10^{-10} M for in vitro translated (Met)_{4}–spinophilin, 4.2 × 10^{-10} M for in vitro translated 14-3-3ζ, and 5.4 × 10^{-11} M for in vitro translated arrestin 3. These EC_{50} values, however, are not thermodynamic binding constants as the 35S-labeled 3i loop was not in excess of the GST-Sp151–444 fusion protein and relative binding affinity was assessed indirectly by examining the ability of unlabeled in vitro translated proteins to deplete radioligand (35S-labeled 3i loop) availability.

with spinophilin-(151–444), we constructed a variety of truncations and deletions within the 3i loop, shown schematically in Fig. 3A. The structure designated WT represents the entire α2AR-3i loop (aa 218–377) as reported previously (20). Structures 1 through 8 illustrate the deleted regions throughout the α2AR-3i loop (defined in detail under “Experimental Procedures”), many of which represent computer-predicted regions of defined secondary structure, such as the predicted amphipathic helices encoded by regions deleted in structures 3 and 6. As demonstrated in Fig. 3B, however, each of these eight different 3i loop structures are as capable as the WT loop in interacting with the GST-Sp151–444 fusion protein, indicating that none of the deleted sequences are required for the 3i loop-spinophilin interaction. Similarly, deletion of either the entire amino-half (structure 10) or the carboxyl-half (structure 9) of the 3i loop also does not eliminate its interaction with spinophilin (Fig. 3B, lower left panel). These data suggest that no single stretch of sequence can account for α2AR 3i loop interaction with spinophilin; we interpret these observations to suggest that a folded surface formed by multiple non-contiguous regions of the 3i loop interacts with spinophilin. Interestingly, we have similarly found that multiple, non-contiguous regions of the α2AR-3i loop are involved in stabilizing the α2AR on the basolateral surface (21).

Since the 3i loops of all three α2AR subtypes interact with spinophilin (12) and amino acid sequences among these loops are very divergent, we performed a sequence homology search among the 3i loops of the three α2AR subtypes. Five homologous regions were revealed (cf. Table I), three at the N terminus (denoted as sequences a, b, and c) and two at the C terminus (denoted as sequences d and e). These regions represent homologous sequences among all three α2AR subtypes. B, GST fusion protein pull-down assays were performed as described in the Materials and Methods. The amounts of 35S-labeled wild type or mutant 3i loops retained in the GSH-agarose–GST-Sp151–444 pellet were visualized by autoradiography. No 35S-labeled wild type or mutant 3i loop was pulled down by GSH-agarose interacting with GST alone (data not shown).
Homologous sequences among the 3i loops of the three α2-AR subtypes

| a | b | c | d | e |
|---|---|---|---|---|
| α2A-AR | RIVQAARKRTR | ALPGGAER | RVGAAEAP | RVRGRQVRREKRPTFF |
| α2B-AR | RIVVARKSHC | ALPRSGGQG | GLAAEQG | GATAEGG |
| α2C-AR | RIVVRKLRTR | ALRGGRRR | RHEGEGT | RRRTQLRREKRPTFF |

Homologous regions are defined as a to e from the N terminus to the C terminus. Amino acid residues that are identical among all three subtypes are bold and underlined; residues that are the same between two subtypes are bold.

structure 25 has virtually no binding to spinophilin. We also noticed that structure 26 (aa 239–294), analogous to structure 11 in Fig. 3, does not interact with either 14-3-3ζ or arrestin 3 (Fig. 4, B and C), just as structure 11 does not bind to spinophilin (Fig. 3B). We further asked if the entire N-terminal half of the 3i loop (aa 218–294, structure 28 in Fig. 4D, analogous to structure 9 in Fig. 3) could interact with 14-3-3ζ and arrestin 3. Addition of sequences from aa 218–238 of the 3i loop only partially restores the loop interaction with 14-3-3ζ and arrestin 3 (Fig. 4D), which is different from the ability of spinophilin to bind to structure 9 (an analogue of structure 28) almost as well as the WT structure containing the entire loop (see left lanes in bottom panel of Fig. 3B). We further confirmed the interaction of structure 28 with radiolabeled Sp151–444 in Fig. 4E, which is comparable to that of structure WT1 with spinophilin.

Thus, although multiple noncontiguous regions of the loop are needed for binding to these three regulatory proteins, differential interactions of the 3i loop are evident with spinophilin versus 14-3-3ζ and arrestin 3. Spinophilin interacts with the N- (aa 218–294) and C-terminal half (aa 294–377) of the loop with relatively similar effectiveness, just slightly lower than the binding detectable with the entire loop (aa 218–377, Figs. 3B and 4E). However, the C-terminal half of the loop interacts with 14-3-3ζ and arrestin 3 as well as the entire loop, and both are about twice as effective in binding 14-3-3ζ and arrestin 3 as the N-terminal half of the loop (Fig. 4, B–D). Moreover, deletion of the extreme end sequences of the loop containing sequences a and e does not affect the loop interactions with 14-3-3ζ and arrestin 3 (see structure 25 in Fig. 4, B and C), but eliminates interaction with spinophilin to less than 4% of control values (see structure 16 in Fig. 3B and structure 25 in Fig. 4E). An important implication of these findings (summarized in Fig. 4F) is that spinophilin, via N-terminal sequences of an appropriately folded loop, and arrestin (or 14-3-3ζ), via regions in the C terminus of the loop, may be able to simultaneously interact with the α2A-AR. This interpretation also is consistent with findings in Fig. 2C, where arrestin 3 and 14-3-3ζ compete for only 50% of 35S-labeled 3i loop binding to GST-spinophilin.

Regulated Interactions of α2A-AR with Spinophilin and Arrestin 3—Our data suggest that the α2A-AR interacts with spinophilin, arrestin 3, and 14-3-3ζ through multiple regions of the 3i loop (Figs. 3 and 4) and that arrestin 3 and 14-3-3ζ compete for spinophilin interaction with the loop in vitro (Fig. 2). It was therefore of interest to determine in the context of intact cells whether these interactions occur independently of one another, e.g., in different target cells or in different subdomains of a single cell, or whether they occur as part of a regulatory cycle. Extant data suggest that a regulatory cycle depicted schematically in Fig. 5 is possible. Based on existing data, it is reasonable to postulate that the α2A-AR interacts with 14-3-3ζ when the receptor is in an inactive state. Interactions of α2A-AR with 14-3-3ζ are competed for by a phosphorylated Raf peptide but not by its non-phosphorylated counterpart (11), and agonist activation of α2A-AR has been demonstrated to activate the Ras/Raf cascade in a variety of target cells (11, 22, 23). Agonist occupancy may favor at least a transient interaction or more stable association with spinophilin since ag-

We also evaluated the regions of the 3i loop critical for interactions of 14-3-3ζ and arrestin 3. The wild type 3i loop containing aa 232–371 (WT3) and loops with incremental deletions were fused with GST (Fig. 4A) and examined for the ability to interact with radiolabeled 14-3-3ζ and arrestin 3. As shown in Fig. 4, B and C, incremental deletions across the 3i loop do not perturb the binding of either 14-3-3ζ or arrestin 3, indicating that interactions with these two proteins, as with spinophilin, also involve multiple, non-contiguous regions. Of interest is the observation that structure 25 (aa 239–359, Fig. 4A), which is an analogue of structure 16 (aa 232–359) in Fig. 3, interacts with both 14-3-3ζ and arrestin 3 very well (Fig. 4, B and C) even though structure 16 has virtually no detectable binding to spinophilin (Fig. 3B).

We had exploited subtly different experimental strategies to measure binding of the α2A-AR 3i loop to spinophilin (Fig. 3) versus binding to 14-3-3ζ or arrestin. This is because 35S-labeled Sp151–444 manifests some binding to GST alone, confounding studies using radiolabeled spinophilin to GST-3i loop structures. Nonetheless, as shown in Fig. 4E, we can directly compare the regions. To rule out the possibility that different experimental approaches led to our observed differences in requirements for binding of the 3i loop to 14-3-3ζ or arrestin 3 compared with spinophilin, we tested the interaction of structure 25 with radiolabeled Sp151–444. As shown in Fig. 4E, 3 D. Mochly-Rosen and Q. Wang, unpublished findings.


**a2-AR 3i Loop Interactions with Spinophilin, 14-3-3z, and Arrestin 3**

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**FIG. 5.** Schematic diagram for interactions of spinophilin, 14-3-3z, and arrestin 3 with the 3i loop of the a2A-AR. Data previously published from our laboratory (11, 12) and others (23) suggest a possible regulatory cycle for these interactions. The interactions of the a2A-AR with 14-3-3z can be disrupted by phosphorylated Raf peptides (11), a consequence of a2A-AR activation of the Ras cascade (22, 29). Agonist occupancy of the a2-AR appears to stabilize agonist interaction with spinophilin (12). This interaction may be transient since phosphorylation of Ser296, Ser299 by GRK leads to arrestin 3 interaction with the receptor and presumed hyperphosphorylation of the receptor-arrestin complex. Furthermore, as shown in Fig. 6, arrestin 3 competes for spinophilin binding to the phosphorylated receptor, but not a mutant a2-AR (ΔLEESSSS) that lacks the GRK sites for phosphorylation of the receptor. Both agonist dissociation from the receptor and phosphatase-catalyzed dephosphorylation may favor interactions of the a2-AR with 14-3-3z, although we have no direct evidence to affirm or refute this aspect of the regulatory cycle.

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Agonist treatment of cells increases the amount of spinophilin that can be detected in immunololates of the a2A-AR (12). Agonist-elicited phosphorylation of the a2A-AR via G protein-coupled receptor kinases (GRKs) may favor the interaction of the a2A-AR with arrestin over spinophilin since a2A-AR interaction with arrestin 3 is favored by phosphorylation of the receptor at Ser296–Ser299 in the 3i loop (24). In addition, we have shown in vitro that the interactions of the a2A-AR 3i loop with spinophilin, 14-3-3z, and arrestin 3 are capable of competing with each other (Fig. 2). To test the possibility that, in fact, these interactions are competitive with one another in the context of a cell, rather than parallel and functionally nonrelated interactions, we examined the ability of arrestin 3 and spinophilin to mutually influence each other’s interactions with the a2A-AR.

We examined the ability of arrestin 3 to influence spinophilin interaction with the a2A-AR in COS cells, taking advantage of the relatively low level of expression of endogenous arrestins in this cell line. As shown in Fig. 6A, agonist occupancy of the a2A-AR indeed enriches the amount of exogenously expressed Myc-tagged spinophilin in the HA-a2A-AR immunosolubilized complex by 1.95-fold (Fig. 6A, lane 2 versus lane 1, p < 0.05), as expected from our previous finding (12). However, this increased association of spinophilin is prevented, or masked, when arrestin 3 is exogenously overexpressed in COS-M6 cells (Fig. 6A, lane 4 versus lane 3). Even the basal interaction of Myc-tagged spinophilin with HA-a2A-AR before agonist treatment is decreased to 78% of the control values (Fig. 6A, lane 3 versus lane 1, p < 0.05). These data are consistent with the interpretation that spinophilin and arrestin 3 are capable of interacting with the same pool of a2A-AR in the context of the cell.

From extant data in the literature we know that arrestin 3 favors binding to GRK-phosphorylated receptors following receptor activation (25–27). We asked whether arrestin 3 could still prevent agonist-simulated spinophilin binding to the a2A-AR when the receptor cannot be phosphorylated by GRK. The a2A-AR is a substrate for GRK2 at the LEESSSSS sequence in the N-terminal half of the 3i loop (28). We tested the ability of arrestin 3 to compete for spinophilin binding to a mutant a2A-AR with the LEESSSSS sequence deleted (10), which as shown in Fig. 6B can not be effectively phosphorylated follow-
spinothin in the receptor-immunoisolated complex (Fig. 6C, lane 2 versus lane 1), but to an even stronger extent (4.43-fold in Fig. 6C versus 1.95-fold in Fig. 6A). However, exogenously expressed arrestin 3 does not decrease either the basal association (before agonist treatment, Fig. 6C, lane 3 versus lane 1) or enhanced association (following agonist occupancy, Fig. 6C, lane 4 versus lane 3) of spinophilin to the ΔLEESSSS α2A-AR receptor. These data suggest that receptor phosphorylation increases the probability of arrestin 3 competition for spinophilin binding to the α2A-AR. This is likely a consequence of the known increase in affinity of arrestin for GRK-phosphorylated receptors (25–27), perhaps due to the creation of another contact site for arrestin stabilization upon phosphate incorporation into the tetra-serine LELESSSS sequence.

In summary, the present data suggest that a surface presented by non-contiguous sequences within the predicted third intracellular loop of the α2A-AR interacts with spinophilin, arrestin 3, and 14-3-3. This surface interchangeably interacts with these three proteins in an agonist-regulated fashion, likely due to agonist-induced alterations in the structure of the 3i loop; however, GRK phosphorylation of the α2A-AR 3i loop preferentially drives the interaction with arrestin 3. Time-resolved fluorescence studies will likely be required to more definitively outline the regulatory cycle and its functional consequences.

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