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β_1-Adrenergic Receptor Association with PSD-95

INHIBITION OF RECEPTOR INTERNALIZATION AND FACILITATION OF β_1-ADRENERGIC RECEPTOR INTERACTION WITH *N*-METHYL-D-ASPARTATE RECEPTORS*

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The β_1-adrenergic receptor (β_1AR) is the most abundant subtype of β-adrenergic receptor in the mammalian brain and is known to potently regulate synaptic plasticity. To search for potential neuronal β_1AR-interacting proteins, we screened a rat brain cDNA library using the β_1AR carboxyl terminus (β_1AR-CT) as bait in the yeast two-hybrid system. These screens identified PSD-95, a multiple PDZ domain-containing scaffolding protein, as a specific binding partner of the β_1AR-CT. This interaction was confirmed by *in vitro* fusion protein pull-down and blot overlay experiments, which demonstrated that the β_1AR-CT binds specifically to the third PDZ domain of PSD-95. Furthermore, the full-length β_1AR associates with PSD-95 in cells, as determined by co-immunoprecipitation experiments and immunofluorescence co-localization studies. The interaction between β_1AR and PSD-95 is mediated by the last few amino acids of the β_1AR, and mutation of the β_1AR carboxyl terminus eliminated the binding and disrupted the co-localization of the β_1AR and PSD-95 in cells. Agonist-induced internalization of the β_1AR in HEK-293 cells was markedly attenuated by PSD-95 co-expression, whereas co-expression of PSD-95 has no significant effect on either desensitization of the β_1AR or β_1AR-induced cAMP accumulation. Furthermore, PSD-95 facilitated the formation of a complex between the β_1AR and *N*-methyl-D-aspartate receptors, as assessed by co-immunoprecipitation. These data reveal that PSD-95 is a specific β_1AR binding partner that modulates β_1AR function and facilitates physical association of the β_1AR with synaptic proteins, such as the *N*-methyl-D-aspartate receptors, which are known to be regulated by β_1AR stimulation.

β-Adrenergic receptors (β_1AR, β_2AR, and β_3AR)[1] are heptahelical G-protein-coupled receptors that mediate physiological responses to the hormone epinephrine and the neurotransmitter norepinephrine. β_1AR and β_3AR exhibit high affinity for both epinephrine and norepinephrine, whereas β_2AR binds with high affinity only to epinephrine. The tissue distributions of the three receptors are distinct: β_2AR is highly expressed in many tissues, β_3AR is expressed at high levels only in adipose tissue, and β_1AR is expressed at high levels in the heart and brain and lower levels elsewhere (1). In the brain, β_1AR exhibits a predominantly neuronal expression pattern, whereas β_2AR is expressed mainly in glial cells (2–4). β_1AR is thus considered to be the "synaptic" β-adrenergic receptor, because electrophysiological experiments with specific antagonists demonstrate that β-adrenergic modulation of hippocampal neuronal activity exhibits a β_1AR-like pharmacological profile (5–7).

Noradrenergic stimulation of synaptic β_1-adrenergic receptors is known to potently regulate memory formation and synaptic plasticity. Emotionally charged events often lead to the creation of vivid memories (8), and the formation of such emotional memories is due in large part to a surge in noradrenaline release and consequent stimulation of brain β-adrenergic receptors (8–13). The powerful effects of β-adrenergic stimulation on memory formation correlate well with electrophysiological experiments demonstrating profound effects of β-adrenergic stimulation on the development of long term potentiation (LTP), an enhancement of synaptic responses that underlies some forms of memory (14). In both the hippocampus (15–22) and the amygdala (23–25), two brain regions known to play key roles in the formation of emotional memories, LTP is markedly enhanced by β-adrenergic stimulation. This β-adrenergic modulation of LTP is mediated exclusively by β_1-adrenergic receptors, because hippocampal slices prepared from mice lacking β_1-adrenergic receptors do not exhibit β-adrenergic modulation of LTP (21). One likely mechanism by which β_1-adrenergic receptor stimulation promotes LTP induction is through enhancement of NMDA receptor activity (26–28), because intense activation of NMDA receptors is required for the development of most forms of LTP (14).

Electron microscopic examinations of brain slices reveal that β_1-adrenergic receptors are concentrated in the post-synaptic density (PSD) (29, 30). The molecular mechanisms by which β_1AR might be specifically targeted to the PSD, however, are completely unknown. Several key PSD components, such as NMDA receptors, are known to be clustered at synaptic sites through interactions with PDZ domain-containing scaffolding

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[1] The abbreviations used are: β_1AR, β_1-adrenergic receptor; β_2AR, β_2-adrenergic receptor; β_3AR, β_3-adrenergic receptor; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; LTP, long term potentiation; NHERF, Na^+/H^+-exchanger regulatory factor; NMDA, *N*-methyl-D-aspartate; NR2, NMDA receptor subunit 2; PDZ, PSD-95/Dlg/ZO-1 homology domain; PSD-95, post-synaptic density protein 95; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

proteins (31–34). PDZ domains, named for the first three proteins in which they were discovered (the post-synaptic density protein PSD-95, the *Drosophila* tumor suppressor protein Dlg, and the tight junction protein ZO-1), bind to their target proteins through specific carboxyl-terminal motifs (31, 35). The β_2-adrenergic receptor is known to associate via its carboxyl terminus with a family of PDZ domain-containing proteins, the Na^+/H^+ exchanger regulatory factors (NHERF) (36, 37). This interaction allows for a specialized form of β_2-adrenergic regulation of Na^+/H^+ exchange (36) and also modulates β_2AR endocytic sorting (38). NHERF binds to a DSLL motif at the carboxyl terminus of β_2AR (36, 37). The β_1AR does not contain this motif, but rather terminates in an ESKV motif that might plausibly mediate binding to PDZ domain-containing proteins other than NHERF. Thus, we used the β_1AR carboxyl terminus as bait in yeast two-hybrid screens of a rat brain library to identify potential β_1AR-interacting proteins that might play a role in β_1AR localization and regulation.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—Mammalian expression plasmids pcDNA3/Flag-β_1AR and pcDNA3/Flag-β_2AR were described before (39, 40). β_1AR-CT (the carboxyl-terminal 99 amino acids of the human β_1AR) and β_2AR-CT (the carboxyl-terminal 86 amino acids of the human β_2AR) were amplified by PCR and then subcloned into pGEX-4T using the *Eco*RI and *Sal*I restriction sites for GST fusion protein expression. GST-β_1AR-loop 3 (corresponding to amino acids 247–321 of the human β_1AR) and GST-β_2AR-loop 3 (corresponding to amino acids 222–272 of the human β_2AR) constructs were described previously (40). Point mutations of both β_1AR-CT and full-length Flag-β_1AR constructs (V477A, S475D, S475A, and S473A) were introduced by PCR and verified by sequencing. PSD-95 PDZ1+2 (corresponding to amino acids 59–303 of rat PSD-95) and PSD-95 PDZ3 (corresponding to amino acids 307–446 of rat PSD-95) were amplified by PCR and inserted into pET-30A (Novagen) at the *Eco*RI and *Xho*I restriction sites for expression as hexahistidine- and S-tagged fusion proteins. Plasmids GW1/myc-PSD-95, GW1/NR1, and GW1/NMDA2B were generous gifts from Morgan Sheng (Harvard Medical School). NR2A-CT-GST fusion protein (the final 200 amino acids of rat NR2A fused to GST) was a generous gift from Fang Zheng (Emory University School of Medicine).

Polyclonal (A-14) and monoclonal (9E10) anti-myc antibodies and the monoclonal anti-GST antibody were from Santa Cruz Biotechnologies. Monoclonal anti-hemagglutinin 12CA5 antibody was from Roche Molecular Biochemicals. Rabbit anti-NMDA2B was a gift from Morgan Sheng. Anti-Flag M2 antibody, anti-mouse IgG FITC conjugate, and Anti-Flag M2 affinity gel were from Sigma. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG secondary antibodies were from Amersham Pharmacia Biotech. Anti-rabbit and anti-mouse IgG Texas Red conjugates were from Calbiochem.

Yeast Two-hybrid Screening—The β_1AR-CT (amino acids 381–end) was amplified by PCR and cloned into the *Eco*RI and *Pst*I restriction sites of the yeast two-hybrid vector pAS2-1. This construct (pAS2-1/β_1AR-CT) was used as bait to screen a rat brain cDNA library (CLONTECH). Plasmid pAS2-1/β_1AR-CT and the rat brain library cDNAs (in the vector pGAD-10) were co-transformed into yeast strain PJ69-4A using a standard yeast transformation protocol. Yeast were plated on selective medium (SD-Leu/Trp/His, +2 mM 3-aminotriazole) and allowed to grow for 4–6 days at 30 °C. Positive colonies were then restreaked on selective medium (SD-Leu/Trp/Ade or SD-Leu/Trp/His) plates. Plasmids were rescued from positive colonies that exhibited positive growth on both-His and-Ade plates and were then transformed into bacterial DH5α cells. Purified bacterial DNA was screened by restriction digestion. Plasmids with unique restriction digest patterns were subjected to further automatic sequence analysis. Sequence information was analyzed online using the BLAST search program at the National Center for Biotechnology Information. To further confirm positive interactions, isolated library cDNAs were co-transformed back into yeast together with a bait plasmid, either pAS2-1/β_1AR-CT, empty vector pAS2-1, or other test plasmids as indicated in the figures. Yeast were then subjected to growth tests on selective plates.

Cell Culture, Transfection, and Harvest—All tissue culture medium and related reagents were purchased from Life Technologies. COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplied with 10% fetal bovine serum and penicillin/streptomycin in 5% CO_2 incubator. Cells in 100-mm dishes were transfected with 20 μl of LipofectAMINE (at 4:1 ratio to DNA) according to the manufacturer's protocol. HEK-293 cells were maintained in the same condition as that of COS-7 cells except minimal essential medium was used. HEK-293 cells were transfected with a modified calcium phosphate method. After transfection, cells were grown 36–48 h before harvesting.

Whole cell extracts were prepared by lysing cells in 1 ml of lysis buffer (20 mM HEPES, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, and protease inhibitors) on ice for 30 min. The lysates were clarified by centrifugation at 21,000 $\times$ *g* for 12 min at 4 °C. The clarified supernatants were then used in all the following *in vitro* GST pull-down or cellular co-immunoprecipitation experiments. 50 μl of each supernatant was diluted into an equal amount of 3$\times$ sample buffer and served as whole cell extract.

GST Fusion Protein Pull-down and Cellular Immunoprecipitations—GST fusion proteins were purified from bacteria using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer's protocol, and resuspended in PBS containing 0.5% Nonidet P-40 and protease inhibitors. Equal amounts of GST fusion proteins (conjugated on beads) were incubated with 1 ml of clarified whole cell extracts from COS-7 cells over-expressing myc-PSD-95. After incubation at 4 °C with gentle rotation for 4 h, beads were extensively washed with ice-cold PBS containing 0.5% Nonidet P-40. Proteins were eluted from beads with 3$\times$ SDS sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. PSD-95 was detected via Western blotting with a rabbit anti-myc antibody (1:1000) followed by HRP-conjugated anti-rabbit IgG secondary antibody (1:2000). Bands were visualized via chemiluminescence using the ECL kit from Amersham Pharmacia Biotech.

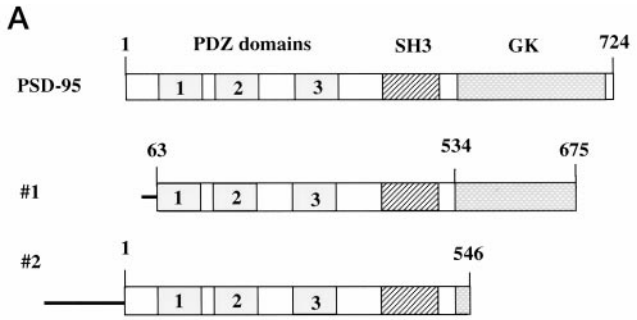
For immunoprecipitation experiments, HEK-293 cells or COS-7 cells were transiently transfected with appropriate plasmid combinations as indicated in the figure legends. 1 ml of clarified cell extract was incubated with 25 μl of anti-Flag M2 affinity gel slurry at 4 °C with gentle rotation for 4 h. Beads were washed four to five times with ice-cold lysis buffer, and the bound proteins were eluted with 3$\times$ SDS-PAGE sample buffer. Immunoprecipitated complexes were resolved on SDS-PAGE and subjected to Western blot analysis as described above.

Overlay Assay—The binding of receptor carboxyl terminus GST fusion proteins to hexahistidine-tagged PDZ domain fusion proteins was assayed via a far-Western blot overlay technique. The His_6-tagged PDZ domains (2 μg per lane) were run on 4–20% SDS-PAGE gels, blotted, and overlaid with the receptor CT-GST fusion proteins (50 nM final concentration) in 2% milk and 0.1% Tween 20 in PBS ("blot buffer") for 1 h at room temperature. The blots were then washed three times with blot buffer and incubated for 1 h at room temperature with a monoclonal anti-GST antibody. The blots were then washed again three times with blot buffer and incubated with an HRP-conjugated anti-mouse IgG secondary antibody in blot buffer. Following three final washes with blot buffer, the binding of the GST fusion proteins was visualized via chemiluminescence as described above.

Receptor Internalization and Adenylyl Cyclase Assays—For receptor internalization assays, HEK-293 cells in 100-mm dishes were transiently transfected with pcDNA3/Flag-β_1AR or pcDNA3/Flag-β_2AR in the absence and presence of GW1/myc-PSD-95. One day after transfection, cells were split into poly-lysine-coated 6-well plates (Biocoat) and grown overnight at 37 °C. Cells were serum-starved for 1 h before stimulation with 10 μM isoproterenol for 30 min at 37 °C. Cells were placed on ice, and Flag-tagged receptors were detected with anti-Flag M2 antibody followed by FITC-conjugated anti-mouse IgG as described previously (40). Receptor internalization was defined as the percentage of agonist-stimulated loss of surface receptors, as measured by cell flow cytometry.

For whole cell cAMP accumulation assays, HEK-293 cells in 100-mm dishes were transiently transfected with pcDNA3/Flag-β_1AR in the absence and presence of GW1/myc-PSD-95. One day after transfection, cells were split into 12-well poly-lysine-coated plates and then labeled with modified essential medium supplement with 5% fetal bovine serum and 2 μCi/ml [^{3}H]adenine for 4 h to overnight at 37 °C incubator. Cells were serum-starved for 30 min and then stimulated with various concentrations of dobutamine or 10 μM forskolin for 10 min. The cAMP accumulation was quantitated by chromatography and expressed as the percentage incorporation of ^{3}H into cAMP as described previously (40). For the receptor desensitization experiments, cells were stimulated with (desensitized cells) or without (control cells) 1 μM isoproterenol for 20 min. The membrane preparations and membrane adenylyl cyclase activity assays were performed according to the method of Freedman *et al.* (39).

Immunofluorescence Microscopy—HEK-293 cells were transfected with pcDNA 3/Flag-β_1AR (or V477A mutant) and/or GW1/myc-PSD-95



B

Baits	Preys	Growth on Selective Medium		
	pGAD-10/PSD-95	-LW	-LWH	-LWA
pAS2-1/β_1-CT		+++	++	+
pAS2-1/β_2-CT	Clone #1	+++	–	–
pAS2-1		+++	–	–
pAS2-1/β_1-CT		+++	+	+
pAS2-1/β_2-CT	Clone #2	+++	–	–
pAS2-1		+++	–	–

FIG. 1. **PSD-95 is identified as an interacting partner of β_1-adrenergic receptor carboxyl terminus in yeast two-hybrid screens.** *A*, domain structures of the PSD-95 and two isolated clones. The β_1-adrenergic receptor carboxyl terminus was used as bait to screen rat brain cDNA library in a yeast two-hybrid system. Two positive clones (*#1* and *#2*) were identified encoding partial sequence of rat PSD-95 plus 5′-untranslated region. The domain structure of these two isolated clones is shown for comparison with that of full-length PSD-95 (*B*). The interaction between PSD-95 and β_1AR-CT in the yeast two-hybrid system is specific. Yeast strain PJ69-4A was co-transformed with bait DNA (pAS2-1, pAS2-1/β_1AR-CT, or pAS2-1/β_1AR-CT) together with isolated pGAD-10/PSD-95 (either clone *#1* or *#2*). Yeast colonies were subjected to growth tests on selective medium *-LW* (SD-Leu/Trp), *-LWH* (SD-Leu/Trp/His), or *-LWA* (SD-Leu/Trp/Ade). The yeast growth rates were rated from fast (+++) to slow (+) to no growth (−).

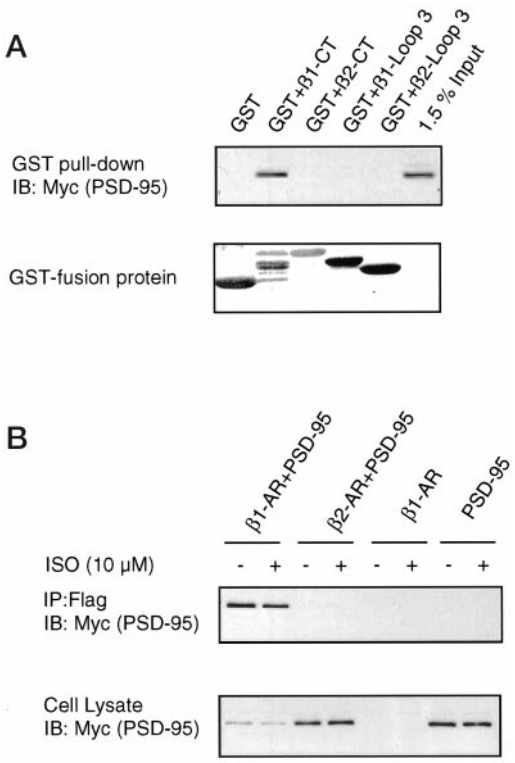


FIG. 2. **PSD-95 associates specifically with the β_1-adrenergic receptor both *in vitro* and in cells.** *A*, PSD-95 binds to the GST-β_1AR-CT *in vitro*. Equal amounts of various GST fusion proteins were adsorbed to glutathione-Sepharose 4B beads and then incubated with cell lysates transfected with myc-PSD95 as described under "Experimental Procedures." PSD-95 bound to the GST fusion proteins was resolved on SDS-PAGE and detected with anti-myc antibody (*upper panel*). A Ponceau S-stained nitrocellulose membrane showing total loading of the GST fusion proteins is shown in the *bottom panel*. A diluted sample representing 1.5% of the total starting input lysate was run in the last lane as a reference. The data shown are representative of four similar experiments. *B*, cellular PSD-95 co-immunoprecipitates with β_1AR. HEK-293 cells were transiently transfected with pcDNA3/Flag-β_1AR, pcDNA3/FL-β_2AR, and/or GW1/myc-PSD-95. Forty-eight hours post-transfection, cells were treated with serum-free medium for 1 h and then incubated either with (+) or without (−) 10 μM isoproterenol (*ISO*) for 10 min. Immunoprecipitation was performed as described under "Experimental Procedures." PSD-95 bound to the beads was resolved on SDS-PAGE and Western blotted with an anti-myc antibody (*upper panel*). Expression of PSD-95 in the cell lysates is shown in the *bottom panel*.

and were split into 30-mm dishes with glass bottoms (MatTek) designed for microscopy. Cells were washed with minimal essential medium and then incubated with anti-Flag M2 monoclonal antibody (1:500) for 1 h at 37 °C. After two brief washes with PBS, the cells were incubated with a FITC-conjugated anti-mouse IgG (1:200) for 1 h at 37 °C. After two washes with PBS, cells were fixed with 4% formaldehyde (in PBS) for 10 min at room temperature and then permeabilized with PBS containing 0.1% Triton X-100. The cells were then incubated with a rabbit polyclonal anti-myc (A-14) (1:250) followed by a Texas Red-conjugated anti-rabbit IgG secondary antibody (1:200). FITC-labeled receptor and Texas Red-labeled PSD-95 were visualized with a Zeiss LSM-410 laser confocal microscope.

RESULTS

PSD-95 Is Identified as a Binding Partner of β_1AR-CT—To search for potential binding partners of the β_1-adrenergic receptor, we used the β_1AR carboxyl terminus (β_1AR-CT) as bait in yeast two-hybrid screens of a rat brain cDNA library. From a total of 11 million independent colonies screened, eight positives were obtained and subjected to further sequence analysis. Two of these positives yielded sequences encoding rat PSD-95, a multiple PDZ domain-containing protein. Both sequences contained partial PSD-95 sequence (Fig. 1*A*). One clone (*#1*) was found to encode a nearly full-length version of PSD-95 plus a long 5′-untranslated region, whereas another clone (*#2*) represents a truncated or alternate splicing isoform of PSD-95.

The specificity of the interaction between PSD-95 and the β_1AR-CT was confirmed by further work with the yeast two-hybrid system. PSD-95 clones were transformed back into yeast strain PJ69-4A together with the bait plasmids pAS2-1, pAS2-1/β_1AR-CT, or pAS2-1/β_2AR-CT. Transformed yeast containing both bait and PSD-95 clones were then subjected to growth tests on selective medium. As shown in Fig. 1*B*, only yeast containing both PSD-95 and β_1AR-CT were able to grow on both −His and −Ade plates. Moreover, this interaction between β_1AR-CT and PSD-95 is specific, because neither empty vector pAS2-1 nor pAS2-1/β_2AR-CT could support yeast growth under the same conditions when co-transformed with PSD-95.

Fusion protein pull-down experiments were employed to study the interaction between β_1AR-CT and PSD-95. Equal amounts of GST fusion proteins, including GST alone as well as GST fused to β_1AR-CT, β_2AR-CT, β_1AR-loop 3, and β_2AR-loop 3, were absorbed onto glutathione-Sepharose 4B beads and then incubated with cell extracts from COS-7 cells that had been transfected with myc-PSD-95. PSD-95 bound to beads was detected by Western blotting using rabbit polyclonal anti-myc antibody. As shown in Fig. 2*A*, only GST-β_1AR-CT pulled down

PSD-95 from the cell lysates, whereas neither GST alone nor other GST fusion proteins (GST-β_2AR-CT, GST-β_1AR-loop 3, and GST-β_2AR-loop 3) were able to pull down PSD-95 from the cell lysates under the same conditions.

To determine whether the full-length β_1AR associates with PSD-95 in a cellular context, we expressed both Flag-β_1AR and myc-PSD-95 in HEK-293 cells. Immunoprecipitation of β_1AR followed by Western blotting for PSD-95 revealed robust co-immunoprecipitation of a PSD-95·β_1AR complex (Fig. 2*B*). Treatment of the cells with the β-adrenergic agonist isoproterenol had no significant effect on this association. Consistent with the fusion protein pull-down results, PSD-95 did not detectably co-immunoprecipitate with full-length Flag-β_2AR when the two proteins were expressed together in HEK-293 cells. Thus, the yeast two-hybrid studies, the *in vitro* fusion protein pull-down experiments, and the cellular co-immunoprecipitation experiments all demonstrated that β_1AR interacts specifically with PSD-95.

The Carboxyl-terminal ESKV Motif in the β_1-Adrenergic Receptor Specifically Binds to the Third PDZ Domain of PSD-95—PSD-95 contains three PDZ domains, protein-protein interaction modules known to bind to specific motifs at the carboxyl termini of target proteins (31, 35). The carboxyl terminus of β_1AR contains an ESKV-carboxyl motif, which is a likely site of interaction with PSD-95 via a PDZ domain-mediated interaction. To examine the structural determinants of the β_1AR·PSD-95 interaction, we individually mutated the last five residues of β_1AR-CT to alanine and expressed these mutants as GST fusion proteins. As shown in Fig. 3*A*, mutation of Val at position 0, Ser at the −2 position, or Glu at the −3 position resulted in complete elimination of β_1AR-CT binding to PSD-95. In contrast, mutation to Ala at positions −1 or −4 had little effect on the β_1AR-CT·PSD-95 interaction. These results reveal that three of the last four residues of β_1AR-CT are the critical determinants for association with PSD-95.

Based on the findings with the mutant β_1AR-CT fusion proteins, we next constructed several mutant versions of the full-length β_1AR to examine the structural determinants of the β_1AR·PSD-95 interaction in cells. As shown in Fig. 3*B*, PSD-95 co-immunoprecipitated with wild-type Flag-β_1AR when the two proteins were co-expressed in COS-7 cells, consistent with our earlier results. However, when these studies were repeated with a mutant version of β_1AR, which has the last residue changed to Ala (V477A), no co-immunoprecipitation of PSD-95 was observed. Similarly, no PSD-95 co-immunoprecipitation could be detected when the key serine residue at −2 of β_1AR was mutated to either Ala (S475A) or Asp (S475D). In contrast, PSD-95 co-immunoprecipitation with a mutant β_1AR bearing a change of the Ser at −4 to Ala (S473A) was reduced only slightly relative to the level of PSD-95 co-immunoprecipitation observed with the wild-type β_1AR. These data further support the conclusion that several of the last few residues of β_1AR are critical determinants for the interaction with PSD-95.

To determine the region of PSD-95 responsible for binding to β_1AR-CT, we prepared His_6-tagged fusion proteins corresponding to the first two PDZ domains (PDZ1+2) and the third PDZ domain (PDZ3) of PSD-95. These fusion proteins were examined in blot overlay studies, along with His_6-tagged fusion proteins corresponding to the PDZ1 and PDZ2 regions of NHERF, for their ability to bind either β_1AR-CT, β_2AR-CT, or the carboxyl terminus of the NMDA receptor NR2A subunit (NR2A-CT). As shown in Fig. 4, β_1AR bound specifically to PSD-95 PDZ3 but did not detectably bind to the other PDZ domains. In contrast, β_2AR-CT did not bind at all to the PSD-95 PDZ domains, but instead bound avidly to NHERF PDZ1, consistent with previous findings (36, 37). The NR2A-CT

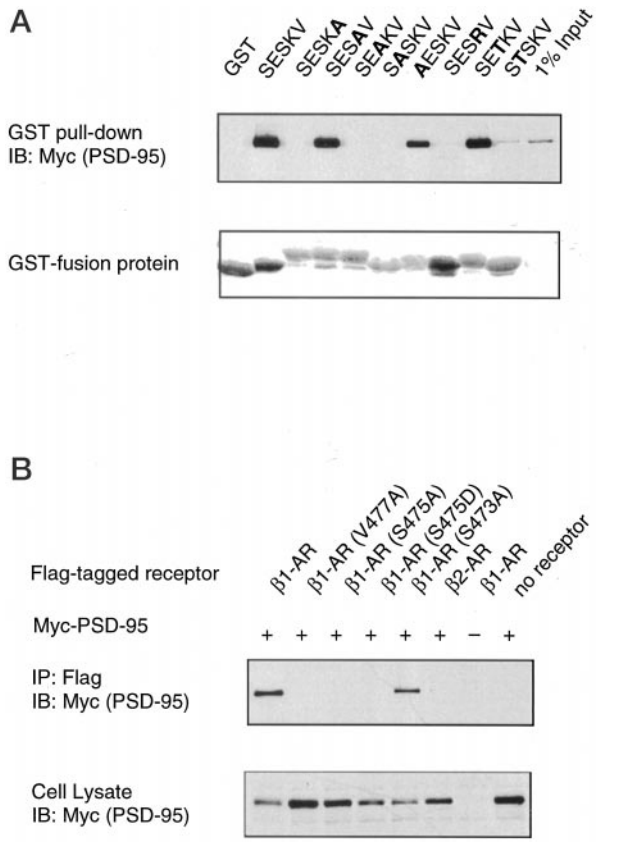


FIG. 3. **The ESKV motif in the β_1-adrenergic receptor mediates the interaction with PSD-95.** *A*, point mutants of β_1AR-CT bind differentially to PSD-95 *in vitro*. Wild-type GST-β_1AR-CT (denoted by the last five amino acids, SESKV) and point mutants (with mutations indicated in *boldface*) were purified and adsorbed in equal amounts onto glutathione-Sepharose 4B beads that were then incubated with cell lysates containing myc-PSD-95. Any myc-PSD-95 that was pulled down by the beads was detected with an anti-myc antibody (*upper panel*). The total amount of GST fusion protein in each lane was assessed by Ponceau S staining, as shown in the *lower panel*. *B*, cellular PSD-95 differentially co-immunoprecipitates with point mutants of the full-length β_1AR. Full-length Flag-tagged wild-type receptors (β_1AR or β_2AR) or various β_1AR mutants (V477A, S475A, S475D, and S473A) were transfected into COS-7 cells co-expressing myc-tagged PSD-95. Flag-tagged receptors were immunoprecipitated by anti-Flag M2 beads and resolved on SDS-PAGE. PSD-95 bound to the beads (*upper*) and PSD-95 in the total starting lysate (*bottom*) was blotted with an anti-myc antibody.

bound well only to PSD-95 PDZ1+2, as previously reported (31, 32). These findings demonstrate that β_1AR-CT binds specifically to the third PDZ domain of PSD-95 but not to the first two PDZ domains of PSD-95 or to NHERF PDZ1, a PDZ domain that binds with high affinity to β_2AR-CT.

PSD-95 Colocalizes with the β_1-Adrenergic Receptor in HEK-293 Cells—To study the subcellular localization of β_1-adrenergic receptors and PSD-95, as well as their potential for co-localization and mutual regulation, fluorescence microscopy studies were performed on HEK-293 cells transiently transfected with Flag-β_1AR and myc-PSD-95. The Flag-tagged receptors were labeled with a FITC-conjugated antibody, myc-tagged PSD-95 was labeled with Texas Red-conjugated antibody, and both proteins were visualized using laser confocal microscopy. Flag-tagged β_1-adrenergic receptors were located exclusively at the plasma membrane, as expected, forming a smooth rim around the cell (Fig. 5*B*). In cells transfected with PSD-95 alone, PSD-95 was distributed evenly in the cytosol (Fig. 5*A*). However, when co-expressed with β_1-adrenergic receptors, a large fraction of PSD-95 was recruited to the plasma membrane and formed a bright outline at the cell

surface (Fig. 5*C*), exhibiting excellent co-localization with the β_1-adrenergic receptors (Fig. 5*E*). In cells co-expressing PSD-95 along with the β_1AR mutant (V477A), the mutant receptors were still distributed along the cell surface (Fig. 5*G*), but PSD-95 remained uniformly distributed throughout the cytoplasm (Fig. 5*F*), similar to the distribution observed in cells expressing PSD-95 alone. Thus, PSD-95 did not exhibit co-localization with the V477A mutant β_1AR (Fig. 5*H*).

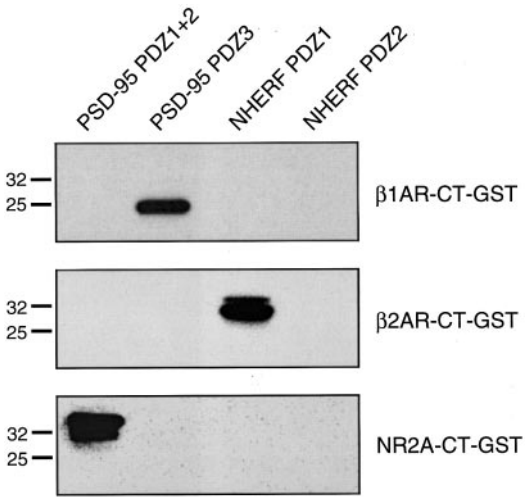


FIG. 4. **The β_1-adrenergic receptor carboxyl terminus specifically binds to the third PDZ domain of PSD-95.** Hexahistidine-tagged fusion proteins corresponding to the first two PDZ domains of PSD-95 (*PSD-95 PDZ1+2*), the third PDZ domain of PSD-95 (*PSD-95 PDZ3*), the first PDZ domain of NHERF (*NHERF PDZ1*), and the second PDZ domain of NHERF (*NHERF PDZ2*) were run on SDS-PAGE, transferred to nitrocellulose, and overlaid with a 50 nM concentration of either β_1AR-CT, β_2AR-CT, or NR2A-CT expressed as GST fusion proteins. Binding of the GST fusion proteins was visualized via a far-Western blot approach using an anti-GST antibody. The positions of molecular mass standards (in kDa) are shown on the *left*. These data are representative of five similar experiments.

PSD-95 Decreases β_1-Adrenergic Receptor Internalization While Having No Effect on Either Receptor-induced cAMP Accumulation or Receptor Desensitization—Agonist-induced receptor internalization, a process in which activated receptors move from the cell surface into intracellular vesicles, is a common feature of G-protein-coupled receptors and is important for receptor regulation (41). Like most G-protein-coupled receptors, the β_1-adrenergic receptor undergoes agonist-dependent internalization but to a lesser extent than does the β_2-receptor (40, 42, 43). Because PSD-95 selectively binds to β_1AR, we postulated that co-expression of PSD-95 might affect β_1-adrenergic receptor internalization. Accordingly, we examined the effect of PSD-95 on β_1AR and β_2AR internalization in HEK-293 cells transiently transfected with either β_1AR or β_2AR in the absence and presence of PSD-95 co-expression. Upon agonist stimulation in the absence of PSD-95, β_1AR exhibited 18% internalization and β_2AR exhibited 30% internalization. Co-expression of PSD-95 reduced the level of β_1AR internalization by approximately half, to about 10%, while having no effect on β_2AR internalization (Fig. 6*A*).

We next examined the effect of PSD-95 on whole cell cAMP accumulation mediated by β_1-adrenergic receptors. HEK-293 cells were transiently transfected with wild-type or mutant β1-AR (V477A) in the absence and presence of PSD-95 co-expression. Agonist dose-response curves of the cAMP accumulation induced by the selective β_1AR agonist dobutamine are shown in Fig. 6*B*. PSD-95 expression had no effect on whole cell cAMP accumulation, changing neither the maximal level of cAMP accumulation nor the EC_{50}. Whole cell cAMP accumulation produced by the mutant β_1AR (V477A), also was not significantly different from that produced by the wild-type β1 adrenergic receptor. We also examined the effect of PSD-95 on the desensitization of β_1AR caused by exposing cells to isoproterenol, by measuring the membrane adenylyl cyclase activity. Co-expression of PSD-95 with β_1AR had no significant effect on the agonist-induced adenylyl cyclase response (data not shown).

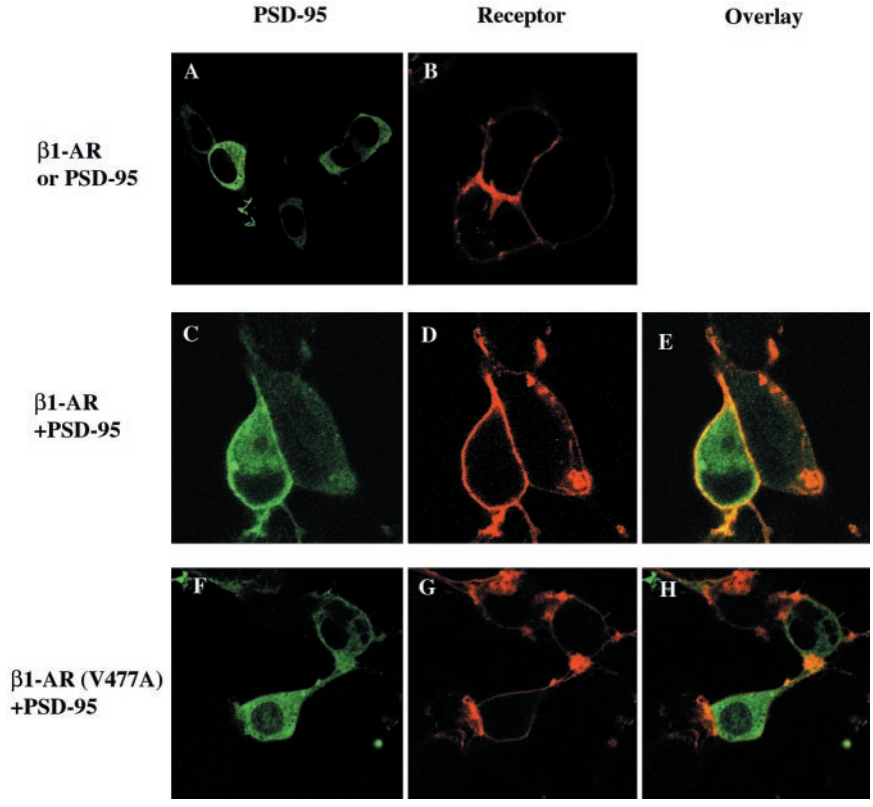


FIG. 5. **PSD-95 co-localizes with wild-type β_1-adrenergic receptor but not the V477A mutant receptor in HEK-293 cells.** Cells were transiently transfected with myc-tagged PSD-95 alone (*A*), Flag-tagged β_1AR alone (*B*), Flag-β_1AR and myc-PSD-95 (*C–E*), or Flag-β_1AR mutant (V477A) and myc-PSD-95 (*F–H*). Flag-tagged receptors were first labeled with mouse anti-Flag M2 antibody followed by Texas Red-conjugated anti-mouse IgG (*B*, *D*, *G*). After fixation and permeabilization, cells were stained with rabbit anti-myc antibody (A-14) followed by FITC-conjugated anti-rabbit IgG (*A*, *C*, *F*). Overlaid images are shown on the *right* (*E*, *H*), with *yellow* indicating co-localization of β_1AR and PSD-95.

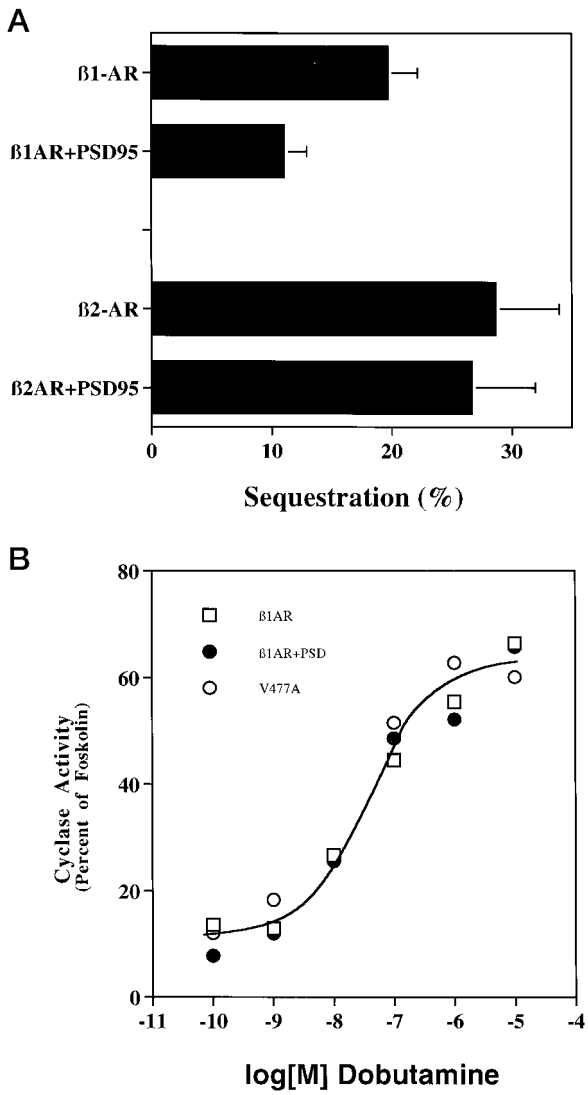


FIG. 6. **PSD-95 inhibits β_1-adrenergic receptor internalization but has no effect on receptor-mediated cAMP accumulation.** *A*, PSD-95 inhibits receptor internalization. HEK-293 cells were transiently transfected with pcDNA3/Flag-β_1AR, pcDNA3/FL-β_2AR, and/or GW1/myc-PSD95. Forty-eight hours after transfection, cells were treated with serum-free medium for 30 min and then incubated with/without 10 μM isoproterenol for 30 min at 37 °C. Cell surface receptors were labeled with a FITC-conjugated antibody, and the cells were harvested and subjected to fluorescence-activated cell sorting analysis. Receptor internalization is presented as a percentage loss of cell surface receptors upon agonist stimulation relative to matched controls. *Bars* and *error bars* represent mean ± S.E. for four to five independent experiments. *B*, PSD-95 does not alter whole cell cAMP accumulation induced by β_1AR stimulation. HEK-293 cells were transiently transfected with pcDNA3/Flag-β_1AR (□) DNA3/Flag-β_1AR (V477A) (○), or pcDNA3/Flag-β_1AR plus GW1/myc-PSD-95 (●). Forty-eight hours after transfection, cells were treated with serum-free medium for 30 min and then stimulated with β_1AR-selective agonist dobutamine (at the concentrations indicated) or 50 μM forskolin for 10 min at 37 °C. Whole cell cAMP accumulation was determined by chromatography and is presented as percentage conversion of [^{3}H]adenine into [^{3}H]cAMP. Dobutamine-induced cAMP accumulation was normalized to that induced by 50 μM forskolin.

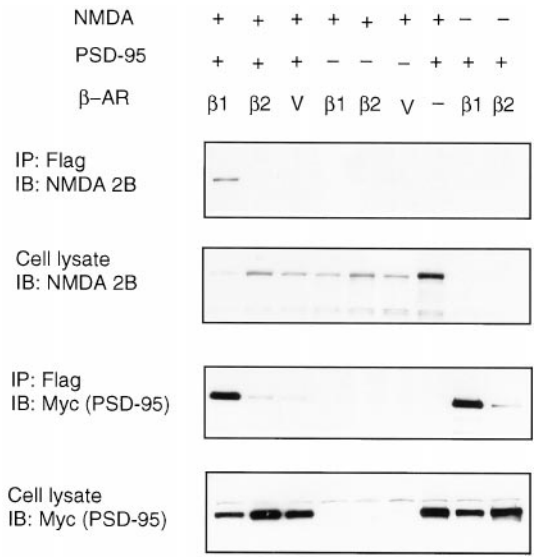


FIG. 7. **PSD-95 mediates association of the β_1-adrenergic receptor with NMDA receptors.** COS-7 cells were transiently transfected with different combination of plasmids as indicated in the figure. *NMDA* represents co-expression of two subunits of the NMDA receptor (NR1 and NR2B). Flag-tagged wild-type β_1AR (*β1*), V477A mutant (*V*), or wild-type β_2AR (*β2*) were all expressed in the absence and presence of co-expression of myc-tagged PSD-95. Cells were grown in modified Eagle's medium supplied with 10% bovine serum and 0.5 mM ketamine to inhibit NMDA receptor activity. Flag-tagged receptors were immunoprecipitated by anti-Flag M2 antibody as described under "Experimental Procedures," and NR2B in the immunoprecipitated samples was detected with rabbit anti-NR2B antiserum. Expression of NR2B in the whole cell lysates is shown for comparison. myc-PSD-95 present in the immunoprecipitated samples and whole cell lysate was detected with an anti-myc antibody.

PSD-95 Facilitates the Formation of a Cellular Complex between β_1-Adrenergic Receptors and NMDA Receptors—A primary role of PDZ domain-containing scaffold proteins like PSD-95 is to facilitate the formation of intracellular signaling complexes. We therefore examined the possibility that cellular PSD-95 might be able to physically link β_1AR to NMDA-type glutamate receptors, because β_1AR can regulate neuronal NMDA receptors (26–28) and NMDA receptors are known to associate with PSD-95 (31, 32). When β_1AR and the NMDA receptor (both NR1 and NR2B subunits) were co-expressed in COS-7 cells in the absence of PSD-95, immunoprecipitation of β_1AR did not result in any evident co-immunoprecipitation of NR2B (Fig. 7). When PSD-95 was co-expressed, however, NR2B readily co-immunoprecipitated with β_1AR. This PSD-95-dependent co-immunoprecipitation was not observed when the studies were performed with the V477A mutant β_1AR in place of the wild-type receptor, nor was any co-immunoprecipitation of NR2B observed with β_2AR under any conditions. These data demonstrate that PSD-95 can facilitate the specific assembly of a cellular complex containing β_1-adrenergic receptors and NMDA receptors.

DISCUSSION

The data presented here identify PSD-95 as a novel binding partner for the β_1-adrenergic receptor. PSD-95 was originally identified as a protein tightly associated with the post-synaptic density (44). The interaction of β_1AR with PSD-95 reported here may help to explain the localization of β_1AR to synapses (29, 30) and the potent ability of β_1AR to regulate synaptic plasticity (15–25). One specific mechanism by which β_1AR is known to modulate synaptic activity is through potentiation of NMDA receptor function (26–28). As shown here, PSD-95 binds to NMDA receptors and to β_1AR through distinct domains and facilitates the physical association of the two receptors in cells.

The interaction between β_1AR and PSD-95 is highly specific. β_1AR-CT binds avidly to the third PDZ domain of PSD-95 but does not bind at all to the first PDZ domain of NHERF, which binds with high affinity to β_2AR-CT (36, 37). β_1AR-CT also does not detectably bind to the first and second PDZ domains of

PSD-95, which bind well to the carboxyl termini of NMDA receptor NR2 subunits (31–34) and Kv1.4 potassium channels (35). Several other proteins have been reported to bind to the third PDZ domain of PSD-95, such as neuroligin (45), CRIPT (46), and citron (47). Although these proteins may compete with β_1AR for binding to PDZ3 of PSD-95 in neurons, β_1AR is likely to be a preferred PSD-95 cellular binding partner, because peptide library screening studies have shown that the motif ESKV (identical to that found at the β_1AR carboxyl terminus) is the ideal motif for association with PSD-95 PDZ3 (48).

β_1AR and β_2AR share 54% sequence identity and couple primarily to the same G-protein (1), yet they are known to exert differential effects on various cellular processes. For example, in the kidney proximal tubule, β_2AR is known to regulate Na^+/H^+ exchange in a manner opposite that of other $G\alpha_s$-coupled receptors (49, 50), and this unusual regulation is explained at least in part by the ability of β_2AR to couple to NHERF (36). In cardiac myocytes, β_1AR and β_2AR are expressed at comparable levels and induce similar rises in cellular cyclic AMP levels when stimulated, yet they exhibit differential effects on regulation of cellular calcium levels and cell contractility (51, 52). Targeted deletion of the genes for β_1AR and β_2AR yields mice with significantly different phenotypes (53, 54), providing further evidence for differential cellular actions of the two receptors. These functional differences between β_1AR and β_2AR are presumably due to differences in their coupling to intracellular proteins that alter receptor localization, modulate receptor function, and/or mediate receptor signaling. The differential binding reported here of β_1AR and β_2AR to PSD-95 and NHERF, respectively, represents an important step toward understanding the molecular basis of functional differences between these two closely related receptor subtypes.

One specific functional difference between β_1AR and β_2AR that has been repeatedly observed is a difference in the rate of receptor internalization in response to agonist stimulation (40, 42, 43). In the present report, we have demonstrated that PSD-95 expression markedly slows the rate of agonist-induced β_1AR internalization while having no effect on β_2AR internalization. In a previous study (40), we found that cellular expression of another β_1AR-interacting protein, endophilin 1, markedly enhances β_1AR internalization and thus exerts the opposite effect of PSD-95 expression. Endophilin 1 binds to a proline-rich stretch of the third intracellular loop of β_1AR (40), whereas PSD-95 binds to the most distal portion of the β_1AR carboxyl terminus. Thus, the two proteins could potentially bind to and regulate β_1AR simultaneously in certain cells, and the net effect of these interactions on receptor internalization would likely be dependent upon the relative cellular expression levels of these β_1AR-associated proteins. This regulation of β_1AR internalization by multiple proteins may explain why the rate of β_1AR internalization differs from that of β_2AR, and also may explain why the rate of β_1AR internalization varies significantly among cell types (40, 42, 43).

Our finding that PSD-95 inhibits β_1AR internalization parallels previous observations that PSD-95 inhibits internalization of the potassium channel Kv1.4 (55). One potential mechanistic explanation for this PSD-95-mediated suppression of transmembrane protein internalization is that PSD-95 binds to many intracellular proteins and also to itself (56) to form large intracellular protein complexes that are resistant to internalization. A second potential explanation is that PSD-95 is able to directly tether associated proteins to the plasma membrane due to lipid modifications of PSD-95 that help anchor it to cell membranes (57). This latter idea is supported by the observation that Kv1.4 internalization was not inhibited but instead was actually enhanced by cellular expression of a mutant version of PSD-95, which could not be palmitoylated (55). A third potential explanation for the effect of PSD-95 on β_1AR internalization is that binding of PSD-95 to the receptor prevents other proteins important for receptor internalization from binding; such proteins might include G-protein-coupled receptor kinases and β-arrestins (1). However, the association of PSD-95 with β_1AR clearly has little or no effect on β_1AR coupling to G-proteins, because we observed no significant effect of PSD-95 expression on β_1AR-induced cAMP accumulation. This finding is in agreement with previous observations that the carboxyl-terminal regions of both β_1AR and β_2AR can influence the receptors' rates of desensitization but not their G-protein-coupling efficacies (58).

The data shown here demonstrate that PSD-95 can facilitate the physical association of β_1-adrenergic receptors and NMDA receptors in cells. One important role of PDZ domain-containing scaffold proteins is to organize intracellular signaling cascades, with perhaps the most striking example to date being the *Drosophila* InaD protein, which contains seven PDZ domains and associates with multiple signaling proteins to increase the efficiency of visual system signal transduction (59). In the case of PSD-95, it has been shown that NMDA receptor modulation of nitric oxide-induced neurotoxicity is promoted by physical linkage of NMDA receptors to neuronal nitric oxide synthase by PSD-95 (60). NMDA receptor currents in neurons are known to be potentiated by β_1AR stimulation (26–28), and this physiological effect may be promoted by physical linkage of the β_1AR to NMDA receptors through the joint association of the two receptors with PSD-95.

Stimulation of β_1-adrenergic receptors in the brain is known to profoundly regulate synaptic plasticity (15–25) and to be important for the formation of emotionally charged "flashbulb" memories (8–13). It has not been clear why, of all the myriad ways by which cAMP levels in neurons may be elevated, stimulation of β_1-adrenergic receptors should receive such unique attention as a physiologically important means of modulating synaptic plasticity. Our data demonstrate that β_1-adrenergic receptors associate specifically with the synaptic protein PSD-95, thus providing a molecular mechanism by which β_1-adrenergic receptors may be localized to synapses (29, 30). Even more specifically, this association provides a mechanism by which β_1-adrenergic receptors may be directly coupled to NMDA receptors and other synaptic components involved in the development of synaptic plasticity.

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