Proteomic Analysis of $\beta_1$-Adrenergic Receptor Interactions with PDZ Scaffold Proteins

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Many G protein-coupled receptors possess carboxyl-terminal motifs ideal for interaction with PDZ scaffold proteins, which can control receptor trafficking and signaling in a cell-specific manner. To gain a panoramic view of $\beta_1$-adrenergic receptor ($\beta_1$AR) interactions with PDZ scaffolds, the $\beta_1$AR carboxyl terminus was screened against a newly developed proteomic array of PDZ domains. These screens confirmed $\beta_1$AR associations with several previously identified PDZ partners, such as PSD-95, MAGI-2, GIPC, and CAL. Moreover, two novel $\beta_1$AR-interacting proteins, SAP97 and MAGI-3, were also identified. The $\beta_1$AR carboxyl terminus was found to bind specifically to the first PDZ domain of MAGI-3, with the last four amino acids (E-S-K-V) of $\beta_1$AR being the key determinants of the interaction. Full-length $\beta_1$AR robustly associated with full-length MAGI-3 in cells, and this association was abolished by mutation of the $\beta_1$AR terminal valine residue to alanine (V477A), as determined by co-immunoprecipitation experiments and immunofluorescence colocalization studies. MAGI-3 co-expression with $\beta_1$AR profoundly impaired $\beta_1$AR-mediated ERK1/2 activation but had no apparent effect on $\beta_1$AR-mediated cyclic AMP generation or agonist-promoted $\beta_1$AR internalization. These findings revealed that the interaction of MAGI-3 with $\beta_1$AR can selectively regulate specific aspects of receptor signaling. Moreover, the screens of the PDZ domain proteomic array provide a comprehensive view of $\beta_1$AR interactions with PDZ scaffolds, thereby shedding light on the molecular mechanisms by which $\beta_1$AR signaling and trafficking can be regulated in a cell-specific manner.

Cellular responses to a given hormone or neurotransmitter can vary markedly between different types of cells. In many cases, such cellular differences are because of differential expression of receptor subtypes. However, even cells expressing exactly the same receptor subtypes can exhibit distinct responses to a given ligand, because receptors often behave quite differently in distinct cellular environments. The trafficking and signaling properties of G protein-coupled receptors (GPCRs), which comprise the largest family of cell surface receptors, are known to be especially influenced by cellular context. The activity of a GPCR depends not only on the complement of downstream effectors expressed in a given cell but also on the set of expressed G proteins, kinases, and scaffold proteins that directly interact with the receptor to shape its signaling capability.

PDZ scaffolds comprise a key class of GPCR-interacting proteins that can strongly influence receptor trafficking and signaling. The term PDZ is derived from the names of the first three proteins in which these domains were first identified: the post-synaptic density protein PSD-95, the Drosophila protein Discs-large, and the tight junction protein ZO-1. PDZ domains are ~90 amino acids in length and bind to specific carboxyl-terminal motifs on their target proteins. There are three general classes of PDZ domain: Class I domains, which recognize the carboxyl-terminal motif S/T-X-\(\phi\) (where \(\phi\) indicates a hydrophobic amino acid and \(\chi\) indicates any amino acid), Class II domains, which recognize the carboxyl-terminal motif D/E-X-\(\phi\) as their preferred carboxyl-terminal motif (1).

Many GPCRs possess carboxyl-terminal motifs appropriate for interaction with PDZ domains. One subfamily of GPCRs for which there are already several reports of PDZ interactions is the subfamily of $\beta$-adrenergic receptors ($\beta$ARs). This subfamily includes the closely related subtypes $\beta_1$AR, $\beta_2$AR, and $\beta_3$AR and mediates physiological responses to epinephrine and norepinephrine (2). It has been shown that several Class I PDZ proteins associate with the carboxyl terminus (CT) of $\beta_1$AR, including PSD-95 (postsynaptic density-95) (3–5), MAGI-2 (membrane-associated guanylate kinase inverted-2) (4), CNrasGEF (cAMP-dependent guanine nucleotide exchange factor, also known as PDZ-GEF1) (6), GIPC (GAIP-interacting protein, carboxyl terminus) (7), and CAL (cystic fibrosis transmembrane conductance regulator-associated ligand, also known as GOPC or FIG) (8). MAGI-2 and PSD-95 are structurally related PDZ proteins of the membrane-associated guanylate kinase-like (MAGUK) family, but nonetheless they exhibit diurnally opposing effects on agonist-induced $\beta_1$AR internalization; MAGI-2 strongly promotes $\beta_1$AR internalization (4), whereas PSD-95 markedly inhibits it (3, 4). In contrast, CNrasGEF and GIPC have no obvious effects on $\beta_1$AR endocytosis but rather regulate various aspects of $\beta_1$AR signaling (6, 7), whereas the Golgi-associated protein CAL directs $\beta_1$AR anterograde trafficking through the endoplasmic reticulum–Golgi complex to the plasma membrane (8). Interestingly, despite the high degree of overall sequence similarity between $\beta_1$AR and $\beta_2$AR, none of the aforementioned $\beta_1$AR-associated PDZ proteins interact with $\beta_2$AR.
Instead, \( \beta_1 \)-AR has been shown to interact with the \( \text{Na}^+/\text{H}^+ \)-exchanger regulatory factors NHERF-1 (also known as EBPs) and NHERF-2 (also known as E3KARP) (9–11). These interactions mediate regulation of \( \text{Na}^+/\text{H}^+ \) exchange by \( \beta_1 \)-AR (9) and also dictate \( \beta_1 \)-AR postendocytic sorting following agonist-promoted internalization (11).

It has been known for many years that \( \beta_1 \)-AR exhibits differential behavior in distinct cellular environments, with tissue-specific differences in the rate of receptor endocytosis being particularly pronounced (12–15). Such differences in \( \beta_1 \)-AR behavior in distinct cells may be explained in large part by the differential expression of \( \beta_1 \)-AR-interactive proteins such as PDZ scaffolds, which as described above can strongly regulate \( \beta_1 \)-AR trafficking and activity. Thus, it is a point of clear physiological interest to determine the full set of PDZ scaffolds that can associate with \( \beta_1 \)-AR. There are \( \sim \)440 PDZ domains in the human proteome, with \( \sim \)25% of these being probable Class I PDZ domains (1). We have recently prepared the majority of known or probable Class I PDZ domains as hexahistidine-tagged fusion proteins and arranged them on a proteomic array to facilitate the rapid analysis of PDZ domain-mediated interactions (16). Here, we screened this PDZ domain array with the \( \beta_1 \)-AR-CT resulting in the confirmation of several known \( \beta_1 \)-AR/PDZ interactions as well as the elucidation of several novel interactions. Furthermore, one of the novel interactions, the association between \( \beta_1 \)-AR and MAGI-3, was found to occur in a cellular context and to also regulate \( \beta_1 \)-AR signaling activity.

**EXPERIMENTAL PROCEDURES**

**Preparation of Plasmids and Fusion Proteins**—The \( \beta_1 \)-AR and \( \beta_2 \)-AR carboxyl termini (the last 30 amino acids of the human \( \beta_1 \)- and \( \beta_2 \)-adrenergic receptors) were amplified via PCR and subcloned into pGEX-4T-1 for expression as GST fusion proteins. Point mutations of both \( \beta_1 \)-AR-CT and full-length Flag-\( \beta_2 \)-AR construct (V477A) were introduced via PCR and verified by ABI sequencing. The full-length V5/His/MAGI-3 construct in pcDNA3 was kindly provided by Rich Laura (Genentech). The individual PDZ domains of MAGI-3, and all of the other PDZ domains utilized on the PDZ array, were amplified via PCR and inserted into pET30A for expression as His/S-tagged fusion proteins, as described (16).

**PDZ Array Overlay Assay**—Gridded nylon membranes were spotted with His/S-tagged PDZ domain fusion proteins (1 \( \mu \)g/bin), as described (16). The membranes were placed at room temperature for 24 h to dry and then stored at 4 °C for later use. Prior to experiments, membranes were blocked in blot buffer for 30 min at room temperature then overlaid with 100 nM GST fusion proteins in blot buffer for 1 h at room temperature. The arrays were washed three times with blot buffer, and incubated with an horseradish peroxidase-conjugated anti-GST antibody (1:3000, Amersham Biosciences). Interactions of the GST fusion proteins with the various PDZ domains were then visualized via chemiluminescence using the ECL kit (Pierce).

**Blot Overlay Assay**—The binding of receptor carboxyl terminus-GST fusion proteins to His/S-tagged PDZ domain fusion proteins was assayed via a blot overlay technique. The receptor CT-GST fusion proteins (1 \( \mu \)g/lane) were run on 4–20% SDS-polyacrylamide gels (Novex, San Diego, CA), blotted, and overlayed with the His/S-tagged PDZ domains (50 nM final concentration) in blot buffer for 1 h at room temperature. The blots were then washed three times with blot buffer and incubated with S-protein horseradish peroxidase conjugate (Novagen, 1:4000) for 1 h at room temperature and were visualized via chemiluminescence as described above.

**GST Fusion Protein Pull-down Assay**—GST fusion proteins were purified from bacteria using glutathione-Sepharose 4B beads (Sigma) 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 transfected with His/V5-MAGI-3. After incubation at 4 °C with gentle rotation for 4 h, the beads were extensively washed with ice-cold PBS containing 0.5% Nonidet P-40. Proteins were eluted from the beads with SDS-PAGE sample buffer, resolved on SDS-polyacrylamide gels, and transferred to nitrocellulose. MAGI-3 was then detected via Western blotting with the monoclonal anti-V5 antibody followed by a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:2000).

**Cell Culture and Transfection**—All tissue culture medium and related reagents were purchased from Invitrogen. HEK-293 and COS-7 cells were maintained in complete medium (Dulbecco’s modified eagle medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) in a 37 °C/5% CO\(_2\) incubator. For transfections, 1 \( \mu \)g of total DNA was mixed with Lipofectamine 2000 (15 \( \mu \)l) (Invitrogen) and added to 5 ml of complete medium in 10-cm tissue culture plates containing cells at \( \sim \)50–80% confluency. Following a 4-h incubation, fetal bovine serum was added to the medium to 10%, and cells were then harvested after 48 h.

**Western Blotting and Antibodies**—Samples were run on 4–20% SDS-polyacrylamide gels (Invitrogen) for 1 h at 150 V and then transferred to nitrocellulose. The blots were blocked in “blot buffer” (2% nonfat dry milk, 0.1% Tween 20, 50 mM NaCl, pH 7.4) for at least 30 min and then incubated with primary antibody in blot buffer for 1 h at room temperature. The blots were then washed three times with 10 ml of blot buffer each and incubated for 30 min at room temperature with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) in blot buffer. Finally, the blots were washed three more times with 10 ml of blot buffer each and visualized via enzyme-linked chemiluminescence as described above. Horseradish peroxidase-conjugated anti-GST, polyclonal anti-His, and horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG secondary antibodies were purchased from Amersham Biosciences. Monoclonal anti-V5 was from Invitrogen, and monoclonal anti-hemagglutinin (HA) 12CA5 antibody was from Roche Molecular Biochemicals. Polyclonal anti-MAGI-3, anti-FLAG M2 monoclonal antibody, and anti-FLAG M2 affinity gel were from Sigma. Polyclonal anti-\( \beta_1 \)-AR (V-19) was purchased from Santa Cruz.

**Immunoprecipitation**—Cells were harvested in ice-cold solubilization buffer (10 mM HEPES, 50 mM NaCl, 5 mM EDTA, 1 mM benzamidine, 0.5% Triton X-100) and incubated end-over-end at 4 °C for 1 h to achieve solubilization. Particulate matter was removed via centrifugation for 15 min at 15,000 \( \times \) g (4 °C). The solubilizes were then incubated with 30 \( \mu \)l of anti-FLAG M2 affinity gel (for transfected cells) or 30 \( \mu \)l of protein A/G-agarose plus 5 \( \mu \)l of polyclonal anti-\( \beta_1 \)-AR antibody (for brain lysates) for 2 h with end-over-end rotation at 4 °C. After five washes with 1.0 ml of ice-cold solubilization buffer, the immunoprecipitated proteins were eluted from the beads via incubation with SDS-PAGE sample buffer and visualized via Western blot using appropriate antibodies.

**Cell Surface Expression Assay**—Receptor expression in the plasma membrane was quantified as described (17). Briefly, cells were grown in 35-mm dishes and incubated in the absence and presence of agonist. The cells were then rinsed in PBS and fixed with 4% paraformaldehyde in PBS for 30 min and then rinsed three more times in PBS and blocked with blocking buffer (2% nonfat dry milk in PBS) for at least 30 min. The fixed cells were incubated with primary antibody in blocking buffer for 1 h at room temperature. The dishes were subsequently washed three
times with 2 ml of block buffer and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody in blocking buffer. Finally, the dishes were washed three times with 2 ml of blocking buffer and one time with 2 ml of PBS and incubated with 2 ml of ECL reagent for exactly 15 s. The luminescence, which corresponds to the amount of receptor on the cell surface, was determined by using a TD 20/20 luminometer.

**Immunofluorescence Microscopy**—HEK-293 cells were transiently transfected with combinations of Flag-β1AR, Flag-V477A, and/or V5/His-MAGI-3. Forty-eight hours after transfection, cells were washed three times with Dulbecco's PBS and then fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. To visualize the subcellular localization of β1AR and MAGI-3, cells were blocked and permeabilized with a buffer containing 2% bovine serum albumin and 0.04% saponin in PBS ("saponin buffer") for 30 min at room temperature. The cells were then incubated with mouse monoclonal anti-FLAG and rabbit polyclonal anti-His antibodies for 1 h at room temperature. After three washes (1 min) with saponin buffer, the cells were incubated with a rhodamine red-conjugated anti-mouse IgG at 1:200 dilution and fluorescein isothiocyanate-conjugated anti-rabbit IgG at 1:200 dilution (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. After three washes (1 min) with saponin buffer and one wash with PBS, coverslips were mounted and rhodamine red-labeled β1AR and fluorescein isothiocyanate-labeled MAGI-3 were visualized with a Zeiss LSM-410 laser confocal microscope. Multiple control experiments, utilizing either transfected cells in the absence of primary antibody or untransfected cells in the presence of primary antibody, revealed a very low level of background staining, indicating that the primary antibody-dependent immunostaining observed in the transfected cells was specific.

**Phospho-ERK Assay**—Twenty-four hours after transfection, cells were split into 6-well dishes and incubated in serum-free medium overnight prior to experiments. Agonist stimulation was performed at 37 °C in serum-free media for 5 min. The medium was removed, and cells were then harvested in SDS-PAGE sample buffer. The samples were sonicated briefly and analyzed via SDS-PAGE. The levels of p42/44 ERK phosphorylation were visualized by Western blot using an anti-phospho-ERK1/2 antibody (Cell Signaling Technology), whereas the levels of total ERK in the same lysates were assessed using an anti-ERK antibody (Cell Signaling Technology). Immunoreactive bands were visualized via chemiluminescence and quantified using NIH Image 1.62. For each sample, the level of phospho-ERK immunoreactivity was normalized to total ERK immunoreactivity. Variation in total ERK immunoreactivity between samples was typically very small (<10%), so only a small amount of normalization was required in each experiment.

## RESULTS

**Proteomic Analysis of β1AR/PDZ Interactions**—To obtain a panoramic view of the set of PDZ domains that bind to β1AR, a GST fusion protein corresponding to the β1AR-CT was purified and subsequently used in overlay experiments. Individual His/S-tagged fusion proteins of 96 distinct PDZ domains were purified and spotted on a gridded nylon membrane (1 μg of fusion protein/bin) and then overlaid with purified β1AR-CT-GST (100 μl). As shown in Fig. 1, β1AR-CT did not bind to the majority of PDZ domains on the array but rather bound strongly to a handful of specific PDZ domains. These included MAGI-1 PDZ1 (bin A1), MAGI-2 PDZ1 and PDZ2 (A5-A6), PSD-95 PDZ3 (B8), CAL PDZ (B10), and GIPC PDZ (D8), which are PDZ domains that have previously been reported to bind to β1AR-CT (3, 4, 7, 8). The overlays of the PDZ array also revealed β1AR-CT interactions with MAGI-3 PDZ1 (A10) and SAP97 PDZ3 (C3), which have not previously been shown to interact with β1AR. In matching control membranes where GST alone was overlaid onto the array, no binding of GST was detectable (data not shown), demonstrating the low amount of nonspecific binding in these assays. Because the interaction of β1AR-CT with MAGI-3 PDZ1 was consistently the most robust association observed, we decided to characterize this interaction further and assess its functional significance.

**Structural Determinants of the β1AR-CT Interaction with MAGI-3 PDZ1**—The binding of MAGI-3 PDZ1 to various point-mutated versions of the β1AR-CT was examined in overlay experiments (Fig. 2A, lanes 1–6). The wild-type amino acid sequence of β1AR is SESKV. The mutation to Ala of the terminal Val residue, as well as Ser at the −2 position, completely blocked binding to MAGI-3 PDZ1. In addition, the mutation of Glu at the −3 position also impaired the interaction. However, mutation to Ala at the −1 and −4 positions had little effect. To further characterize the structural determinants of this interaction, we mutated the wild-type SESKV to SESKV, SETKV, and STSKV and examined the binding of these mutant fusion proteins to MAGI-3 PDZ1 (Fig. 2A, lanes 7–9). The mutation to Thr at the −3 position and Arg at −1 strongly inhibited binding, but the mutation to Thr at the −2 position had little effect on the interaction. These findings revealed a selective inhibition of binding to MAGI-3 PDZ1 by specific point mutations to three of the last five amino acids of the β1AR-CT.

To examine the binding of the β1AR-CT to full-length MAGI-3 rather than simply the isolated PDZ1 domain, HEK-293 cells were transfected with full-length V5/His-tagged MAGI-3, and pull-down assays were performed using purified GST fusion proteins corresponding to β1AR-CT with the terminal Val residue mutated to either Ala, Ile, Met, or Leu (Fig. 2B). Each of these mutations completely abolished the interaction between MAGI-3 and β1AR-CT, revealing a critical importance of the terminal Val residue in mediating the β1AR/MAGI-3 interaction.

**β1AR and MAGI-3 Associate in Cells**—We next examined the interaction between full-length β1AR and full-length MAGI-3 in a cellular context. HEK-293 cells were transfected with V5/His-MAGI-3 and either Flag-β1AR or Flag-V477A, a mutant receptor with the terminal Val changed to Ala. Co-immunoprecipitation experiments were performed in the presence and absence of stimulation with the β1AR-selective agonist isoproterenol (Fig. 3A). MAGI-3 co-immunoprecipitated with β1AR, and the amount of co-immunoprecipitation did not significantly change when β1AR was stimulated with agonist for 15 min. No co-immunoprecipitation was observed, however, between the V477A mutant receptor and MAGI-3, revealing the importance of the terminal Val residue for the cellular interaction of β1AR and MAGI-3.

In addition to the transfection experiments, we also studied the interactions of full-length endogenous β1AR and MAGI-3 in various native tissues, including brain, heart, and kidney. Solubilized lysates derived from these tissues were incubated with an anti-β1AR antibody linked to protein A/G-agarose beads, and the resultant immunoprecipitates were probed via Western blot for MAGI-3 co-immunoprecipitation using an anti-MAGI-3 antibody. Robust co-immunoprecipitation of MAGI-3 with β1AR was observed from all three tissues (Fig. 3B). These data reveal the existence of a physical complex between β1AR and MAGI-3 in native tissues where neither protein is overexpressed.

The potential co-localization of β1AR and MAGI-3 in cultured cells was examined via immunofluorescence microscopy (Fig. 4). HEK-293 cells were transfected with V5/His-MAGI-3 and/or Flag-β1AR or Flag-V477A. When expressed alone, β1AR and the V477A mutant receptor were both predominantly localized to the plasma membrane (Fig. 4A), whereas MAGI-3 expressed alone was found predominantly in the nucleus, with light cytoplasmic staining also evident (Fig. 4B). Strikingly,
when co-expressed with wild-type $\beta_1$AR, MAGI-3 was found almost exclusively at the plasma membrane where it co-localized well with $\beta_1$AR (Fig. 4, C–E). In contrast, no co-localization was observed when MAGI-3 was co-expressed with V477A (Fig. 4, F–H), as MAGI-3 remained predominantly targeted to the nucleus and exhibited little if any co-localization with the mutant receptor. Stimulation of the cells with isoproterenol (15 min) had no significant effect of the extent of $\beta_1$AR/MAGI-3 co-localization (data not shown). These data confirm the findings from the co-immunoprecipitation experiments that $\beta_1$AR and MAGI-3 can interact in cells and, furthermore, reveal that this
interaction results in a striking redistribution of MAGI-3 from the nucleus to the plasma membrane.

MAGI-3 Modulates βAR-mediated ERK Activation—We next explored the potential functional significance of the βAR/MAGI-3 interaction. Because it has previously been reported that PSD-95 and MAGI-2 strongly regulate βAR agonist-promoted internalization, we studied βAR endocytosis from the cell surface in the absence and presence of MAGI-3 co-expression. However, βAR internalization in response to a 15-min stimulation with isoproterenol (10 μM) was not found to be significantly different when βAR was expressed alone (18 ± 4%) versus when βAR/MAGI-3 were co-expressed (16 ± 5%, n = 4). In further functional studies, no differences were observed in cAMP generation following isoproterenol stimulation of βAR expressed alone (5.6 ± 1.2-fold over untransfected cells) versus βAR/MAGI-3 co-expressed (5.1 ± 1.0-fold over untransfected cells, n = 3). However, as shown in Fig. 5, co-expression with MAGI-3 had a substantial effect on βAR-mediated activation of ERK. Isoproterenol-stimulated ERK activation was more than 6-fold for βAR expressed alone but less than 4-fold when βAR and MAGI-3 were co-expressed (Fig. 5A). In contrast, no significant effect of MAGI-3 co-transfection was observed on the ability of the V477A mutant receptor to couple to ERK activation (Fig. 5B). A quantification of these data is presented in Fig. 5C. The magnitude of the attenuation of βAR-mediated ERK activation by MAGI-3 was comparable at three different time points (Fig. 5D). Western blot analyses revealed that MAGI-3 co-expression had no effect on the total expression levels of βAR and also revealed that wild-type βAR and the V477A mutant receptor were expressed at comparable levels. The stimulation of ERK phosphorylation induced by both wild-type and mutant βAR was completely blocked by pretreatment of the cells with pertussis toxin, which indicates a requirement for βAR coupling to G/i in this signaling pathway (data not shown). These data suggest that the ability of MAGI-3 to modulate βAR coupling to Gi and downstream ERK activation is dependent on the physical interaction between βAR and MAGI-3.

Differential PDZ Domain Interactions of βAR and β2AR—βAR and β2AR exhibit a high degree of sequence similarity, and even their carboxyl-terminal motifs (ESKV for βAR versus DSQLL for β2AR) are roughly similar, featuring acidic residues at the −3 positions, Ser residues at the −2 positions, and hydrophobic residues with long aliphatic side chains at the terminal positions. To examine whether there may be any overlap between βAR and β2AR in their interactions with PDZ domains, overlays of the PDZ domain array were performed with fusion proteins corresponding to the carboxyl termini of both receptors. As described above, βAR-CT interacted with MAGI-1 PDZ1, MAGI-2 PDZ1/2, MAGI-3 PDZ1, PSD-95 PDZ3, SAP97 PDZ3, and GIPC PDZ (shown earlier in Fig. 1). In contrast, overlays of the PDZ domain array with β2AR-CT resulted in a pattern of binding completely distinct from those of the other receptors.
**DISCUSSION**

Interactions between the $\beta_1$-adrenergic receptor and Class I PDZ domain-containing proteins have been uncovered previously via several different techniques. $\beta_1$-AR interactions with PSD-95 (3) and GIPC (7) were identified in yeast two-hybrid screens, whereas associations with MAGI-2 (4), CNrasGEF (6), and CAL (8) were identified via biochemical approaches. To get as comprehensive a view as possible of $\beta_1$-AR/PDZ interactions, we turned to a proteomic approach in which most known or putative Class I PDZ domains were expressed as fusion proteins and arranged in a proteomic array. Screens of this array with $\beta_1$-AR-CT revealed interactions with most of the previously reported PDZ partners of $\beta_1$-AR, as well as associations with two novel partners, SAP97 and MAGI-3. The interaction with MAGI-3 was shown to occur in cells and to exert significant effects on $\beta_1$-AR signaling, thereby validating the use of the proteomic PDZ array as a means of identifying physiologically relevant interactions between PDZ scaffolds and transmembrane proteins, such as $\beta_1$-AR, that possess potential PDZ-interacting motifs. $\beta_1$-AR is known to regulate cellular physiology primarily by coupling to $G_i$, but disruption of $\beta_1$-AR/PDZ interactions in cardiac myocytes has been shown to result in dramatically increased coupling of the receptor to $G_i$ (18). The relevant PDZ interaction underlying this effect has not been conclusively identified, although it has been shown in COS-7 cells that $\beta_1$-AR association with GIPC leads to decreased $G_i$-mediated ERK activation by $\beta_1$-AR (7). Interestingly, our data reported here reveal that MAGI-3 interaction with $\beta_1$-AR also strongly impairs $G_i$-mediated ERK activation by $\beta_1$-AR. These findings reveal that inhibition of $G_i$ coupling to $\beta_1$-AR is not unique to GIPC, but can also be mediated by other PDZ partners of $\beta_1$-AR. It is possible that such alterations in receptor coupling to $G$ proteins may occur through allosteric changes in the accessibility of the $G$ protein binding pocket of the receptor following receptor association with PDZ proteins. MAGI-3 is known to be abundantly expressed in cardiac tissue (19); thus $\beta_1$-AR interaction with MAGI-3 in cardiac myocytes may contribute to the previously observed importance of the $\beta_1$-AR PDZ-interacting motif in modulating $\beta_1$-AR coupling to $G$ proteins in this native cell type (18).

In addition to demonstrating regulation of $\beta_1$-AR by MAGI-3, our studies also revealed a converse regulation of MAGI-3 by $\beta_1$-AR. Specifically, co-expression with $\beta_1$-AR resulted in a striking translocation of MAGI-3 to the plasma membrane, whereas MAGI-3 expressed alone exhibited a predominantly nuclear localization. MAGI-3 was first identified only several years ago (19), and little is known at present about the set of cellular proteins with which MAGI-3 can interact. Given the fact that we and others (20) have observed strong MAGI-3 expression in the nuclei of certain cell types, it is tempting to speculate that MAGI-3 may bind to various partners in the nucleus and play a role in transcriptional regulation, as has been shown to be the case for other PDZ scaffolds (21–24). If MAGI-3 does have a physiological role in the nucleus, then the control of MAGI-3 localization via association with transmembrane proteins such as $\beta_1$-AR may represent a novel and specific mechanism by which such PDZ-interacting transmembrane proteins can influence nuclear function.

Our screens of the PDZ array revealed that $\beta_2$-AR interacts with a narrow set of PDZ partners entirely distinct from the set of PDZ domains bound by $\beta_1$-AR. These findings are consistent with the fact that $\beta_2$-AR has been previously reported to associate with NHERF-1 (9–11) and NHERF-2 (10) but not with any other PDZ partners. Interestingly, mutation of the last amino acid of the $\beta_2$-AR-CT to match the last amino acid of $\beta_1$-AR-CT resulted in a striking change in $\beta_2$-AR/PDZ interactions, with the $\beta_2$-AR-CT now exhibiting strong binding to the NHERF PDZ domains instead of its normal set of PDZ partners. This observation emphasizes the specificity of interactions between GPCRs and their associated PDZ scaffolds. $\beta_1$-AR and $\beta_2$-AR are known to bind the same endogenous ligands and couple to the same $G$ proteins but yet are also known to exert significantly different effects on cellular physi-

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**FIGURE 5.** MAGI-3 specifically inhibits $\beta_1$-AR-stimulated ERK activation. COS-7 cells were transiently transfected with Flag-$\beta_1$-AR, Flag-V477A mutant receptor, and/or V5/His-MAGI-3. Twenty-four to forty-eight hours after transfection, cells were treated with serum-free medium overnight. Serum-starved cells were stimulated with 10 mM isoproterenol (Iso) for 5 min at 37 °C. ERK activation was assayed as described under "Experimental Procedures." Expression of MAGI-3 was found to dramatically inhibit $\beta_1$-AR-mediated ERK activation (A) but have little effect on ERK activation by the V477A mutant receptor (B). C: quantification of the effect of MAGI-3 on $\beta_1$-AR- and V477A-mediated ERK activation. The amount of phospho-ERK immunoactivity observed following isoproterenol stimulation was expressed as a fold of the phospho-ERK immunoactivity in the absence of stimulation. D: time course of the effect of MAGI-3 on $\beta_1$-AR-mediated ERK activation. The results from cells transfected with $\beta_1$-AR alone are indicated by the squares and solid line, whereas the results from cells co-transfected with $\beta_1$-AR/ MAGI-3 are indicated by the triangles and dashed line. For both C and D, the data and error bars represent means ± S.E. for three independent experiments. The asterisks indicate a significant decrease (p < 0.05) relative to phospho-ERK immunoactivity in the absence of transfected MAGI-3. WT, wild-type.

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from that observed for $\beta_1$-AR-CT. As shown in Fig. 6A, $\beta_1$-AR-CT bound strongly to NHERF-1 PDZ1, NHERF-2 PDZ1, NHERF-2 PDZ2, and PDZK1 PDZ2 but bound only weakly or not at all to other PDZ domains on the array. To study the importance of the terminal amino acid in mediating this difference in PDZ binding specificity between $\beta_1$-AR-CT and $\beta_2$-AR-CT, the aforementioned $\beta_1$-AR-CT point mutant with the terminal Val mutated to Leu (ESKL) was utilized to mimic the terminal position of $\beta_2$-AR. Strikingly, this mutant did not detectably bind to any of the PDZ domains that bound well to the wild-type $\beta_1$-AR-CT. Instead, the ESKL mutant bound strongly to NHERF-1 PDZ1, NHERF-2 PDZ2, PDZK1 PDZ2, and also bound weakly to MAGI-3 PDZ5 (Fig. 6B), whereas mutation of the terminal Val residue to Ala (ESKA) resulted in a fusion protein that did not bind strongly to any of the PDZ domains on the array (data not shown). These data emphasize the specificity of binding between PDZ domains and their targets, as a single point mutation to the $\beta_1$-AR-CT resulted in a switch of its PDZ domain binding specificity to a profile much more comparable with that of the $\beta_2$-AR-CT.
The association of the two receptors with unique sets of PDZ scaffolds is likely a key factor that underlies the distinct cellular actions of $\beta_1$AR and $\beta_2$AR. Differential association with PDZ scaffolds represents both a potential molecular explanation for differences between $\beta_1$AR subtypes and also a probable mechanism underlying cell-specific differences in $\beta_1$AR signaling and trafficking. Like most GPCRs, $\beta_1$AR exhibits marked variation in its behavior in distinct cell types, with substantial differences in the rate and extent of agonist-promoted internalization being especially notable (12–15). Most PDZ scaffolds are not expressed uniformly across all tissues but instead tend to exhibit profound differences in expression levels between different tissues and cell types (1). Thus, interactions of GPCRs such as $\beta_1$AR with PDZ scaffolds that exhibit distinctive patterns of expression across different tissues may account for many examples of cell-type specific regulation of GPCR signaling and trafficking.

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