

Dynamic Complexes of β_2 -Adrenergic Receptors with Protein Kinases and Phosphatases and the Role of Gravin*

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Signals mediated by G-protein-linked receptors display agonist-induced attenuation and recovery involving both protein kinases and phosphatases. The role of protein kinases and phosphatases in agonist-induced attenuation and recovery of β -adrenergic receptors was explored by two complementary approaches, antisense RNA suppression and co-immunoprecipitation of target elements. Protein phosphatases 2A and 2B are associated with the unstimulated receptor, the latter displaying a transient decrease followed by a 2-fold increase in the levels of association at 30 min following challenge with agonist. Protein kinase A displays a robust, agonist-induced association with β -adrenergic receptors over the same period. Suppression of phosphatases 2A and 2B with antisense RNA or inhibition of their activity with calyculin A and FK506, respectively, blocks resensitization following agonist removal. Recycling of receptors to the plasma membrane following agonist-promoted sequestration is severely impaired by loss of either phosphatase 2B or protein kinase C. In addition, loss of protein kinase C diminishes association of phosphatase 2B with β -adrenergic receptors. Overlay assays performed with the RII subunit of protein kinase A and co-immunoprecipitations reveal proteins of the A kinase-anchoring proteins (AKAP) family, including AKAP250 also known as gravin, associated with the β -adrenergic receptor. Suppression of gravin expression disrupts recovery from agonist-induced desensitization, confirming the role of gravin in organization of G-protein-linked signaling complexes. The Ht31 peptide, which blocks AKAP protein-protein interactions, blocks association of β -adrenergic receptors with protein kinase A. These data are the first to reveal dynamic complexes of β -adrenergic receptors with protein kinases and phosphatases acting via an anchoring protein, gravin.

Chronic stimulation of G-protein-linked receptors (GPLRs)¹ provokes attenuation of the receptor-mediated signal, or desensitization (1). Protein phosphorylation is a critical element of

agonist-induced desensitization involving at least three prominent kinase activities (2), cyclic AMP-dependent protein kinase (protein kinase A), calcium and phospholipid-sensitive protein kinase (protein kinase C), and members of the G-protein-linked receptor kinase family, like the β -adrenergic receptor kinase. β_2 -Adrenergic receptors (β_2 AR) are substrates for protein kinase A, protein kinase C, G-protein-coupled receptor kinase, as well as growth factor receptors with intrinsic tyrosine kinase activity (3, 4). Study of the complex roles of protein kinases in the functional regulation of these multiply phosphorylated receptor substrates has been accelerated through the use of loss-of-function mutant cells in which target protein kinases have been suppressed by antisense oligodeoxynucleotides (2) and dominant negative mutant kinase (5), in addition to protein kinase inhibitors (6), receptor mutagenesis (7, 8), and reconstitution of purified elements *in vitro* (9).

Recently, we reported cell-type-specific roles of various protein kinases in agonist-induced desensitization using oligodeoxynucleotides to suppress these enzymes transiently (2). Suppression of protein kinase C, but not protein kinase A or β -adrenergic receptor kinase, amplified rather than attenuated agonist-induced desensitization in a variety of cell types. In the current work, we explore the role of protein kinases, phosphatases, and anchoring proteins in organizing associations with the β -adrenergic receptor. Protein kinase C is shown to be obligate for resensitization of GPLR, its action blocked by protein kinase C deficiency and by the protein kinase C inhibitor bisindolylmaleimide, and mimicked by FK506, a protein phosphatase 2B inhibitor. Protein kinase anchoring and scaffolding proteins have emerged as central elements in many aspects of cell signaling (10–13). The anchoring protein AKAP250, also known as gravin (14), is shown to associate with the β -adrenergic receptor and be required for recovery from agonist-induced desensitization to occur.

EXPERIMENTAL PROCEDURES

Cell Culture—Human epidermoid carcinoma cells (A431) were grown in Dulbecco's modified Eagles' medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT), penicillin (60 μ g/ml), and streptomycin (100 μ g/ml). A431 cells were transfected with the pLNCX plasmid using Lipofectin[®] (Life Technologies, Inc.) reagent according to the manufacturer's protocol. Stably transfected A431 cells expressing antisense RNA to protein kinase A and protein phosphatase 2A and 2B were screened for suppression of the target enzyme as outlined below. At least three separate clones of stable transfectants were selected and propagated for each antisense construct. The stable transfectant clones routinely were maintained in medium containing gentamycin (0.5 mg/ml; Life Technologies, Inc.).

Construction of the pLNCX Retroviral Vectors—The antisense sequences, 5'-TTGGCTTTGGCTAAGAATTCCTTCACGCTCTCC-3', 5'-TCGGCCAGCACCGCTC-CAGGTCCGCAT-3', and 5'-CTGCAGAA-GGTGGGCTGCTTGAAGAAGCG-3' derived from the complementary sequences of protein kinase A catalytic β -subunit (16), protein phosphatase 2A (17), and protein phosphatase 2B (18), respectively, were en-

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¹ The abbreviations used are: GPLR, G-protein-linked receptor; β_2 AR, β_2 -adrenergic receptor; ICYP, [¹²⁵I]iodocyanopindolol; PAGE, polyacrylamide gel electrophoresis; AKAP, A kinase-anchoring protein; EV, empty vector; CHO, Chinese hamster ovary; PKA, protein kinase A; PKC, protein kinase C; PP2A and PP2B, protein phosphatases 2A and 2B, respectively.

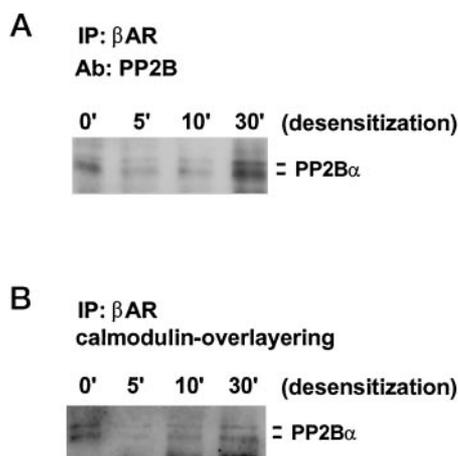


FIG. 1. Association of protein phosphatase 2B with β -adrenergic receptor: agonist stimulation of a dynamic association. Lysates were prepared from A431 cells treated with isoproterenol ($10 \mu\text{M}$) for periods up to 30 min. Cell lysates were incubated with antibodies to β -adrenergic receptors (CM-04), and then the immunocomplexes recovered by adsorption to protein A agarose beads. The immunocomplexes were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and stained either with antibody specific for the catalytic subunit of PP2B (panel A) or with calmodulin followed by an anti-calmodulin antibody (calmodulin-overlay assay, panel B). The immunocomplexes were made visible by the chemical luminescence method. The data presented are representative of at least four separate determinations performed with separate cell lysates.

gineered into the *HindIII/ClaI* sites of the pLNCX retroviral vector using standard recombinant DNA techniques (19). The pLNCX vector contains the gene to confer neomycin resistance under the control of the 5'- and 3'-long terminal repeats of the mouse Moloney virus, and the expression of the antisense RNA is under the control of the cytomegalovirus promoter. Antisense RNA to protein kinase A targets both α - and β -isoforms of the catalytic subunit of protein kinase A (16, 20).

Immunoblotting Analysis—Cells were harvested and homogenized in 10 mM Hepes buffer, pH 7.4, 2 mM MgCl_2 , 2 mM EDTA containing 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride. Nuclei were collected by low speed centrifugation. Fifty micrograms of post-nuclei fraction protein was subjected to 10% SDS-PAGE, and the separated proteins were transferred onto a nitrocellulose membrane. Expression of protein kinase C was probed with antibody against the α -isoform of protein kinase C (Life Technologies, Inc.).

Protein Kinase A and C Assays—Protein kinase A and C activities were assessed by using commercially available assay kits purchased from Life Technologies, Inc. The manufacturer's protocol was followed. Protein kinase A activity is defined as the amount of phosphate incorporated into a substrate peptide, Kemptide, in the presence of 10 μM cyclic AMP minus that incorporated in the presence of the protein kinase A inhibitor peptide (1 μM). The specific activity of protein kinase C is defined as the difference between the amount of phosphorylation of an acetylated peptide derived from myelin basic protein in the presence of 10 mM phorbol 12-myristate 13-acetate from the amount of phosphorylation occurring in the presence of 20 μM protein kinase C inhibitory peptide (protein kinase C α peptide 19–36).

Protein Phosphatase 2A and 2B Assays—Protein phosphatase 2A was isolated by immunoprecipitation with antibodies to the catalytic subunit of PP2A (Transduction Laboratories, Lexington, KY). The PP2A activity was measured using [^{32}P]phosphorylated glycogen phosphorylase A as a substrate and a commercially available kit (13188-016; Life Technologies, Inc.). PP2A-specific activity was defined as the activity sensitive to inhibition by 100 nM calyculin A or 1 nM okadaic acid. For protein phosphatase 2B, two complementary techniques were employed for assay: immunostaining with antibodies to the catalytic subunit of PP2B (C26920; Transduction Laboratories, Lexington, KY) and identification by the calmodulin-overlay assay. Calmodulin binding to the renatured SDS-PAGE gel (overlay) was detected using antibodies to calmodulin. The immunocomplexes then were made visible as described above. Both assays provide comparable results.

RII Overlay Assays of A Kinase-anchoring Proteins—The presence of AKAPs in A431 cells were detected by RII overlay assay as described (15). Briefly, fifty micrograms of cell lysate or membrane protein was separated by electrophoresis on an SDS-polyacrylamide gel and elec-

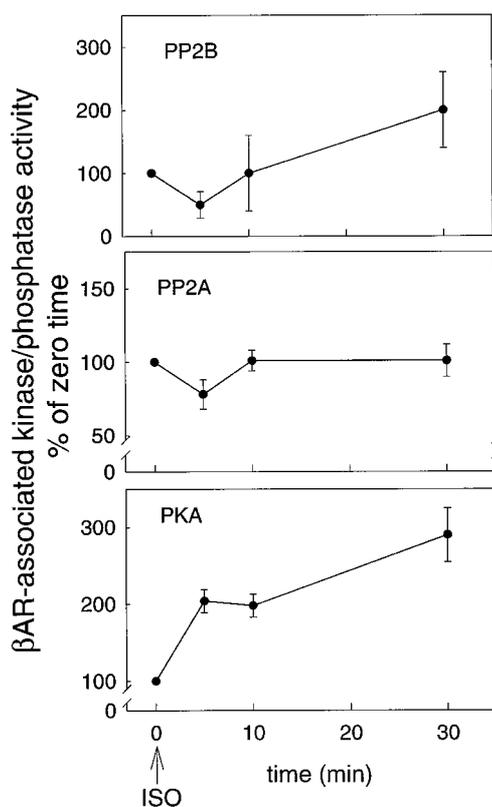


FIG. 2. Association of β -adrenergic receptors with protein phosphatase 2A, 2B, and protein kinase A is regulated by agonist stimulation. Lysates were prepared from A431 cells treated with isoproterenol ($10 \mu\text{M}$) for periods up to 30 min. Cell lysates were incubated with antibodies to β -adrenergic receptors (CM-04) and then the immunocomplexes recovered by adsorption to protein A agarose beads. The receptor immunocomplexes were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and stained with antibody specific for the catalytic subunit of PP2B, and the immunocomplexes were made visible by the chemical luminescence method. For PP2A, the collected immunocomplexes were resuspended in assay buffer and the liberation of [^{32}P]phosphorylase *a* substrate used to establish enzyme activity. The PP2A-specific activity was defined as that phosphatase activity sensitive to inhibition by 100 nM calyculin A or by 10 nM okadaic acid. For PKA, the immunocomplexes were resuspended in assay buffer in which the Kemptide was employed as substrate. PKA activity was defined as that activity stimulated in the presence of cyclic AMP and sensitive to inhibition with the protein kinase A inhibitory peptide. The results are displayed as the mean values \pm S.E. from at least three independent assays performed on as many separate cell preparations. The reproducibility of the immunoprecipitation of β -adrenergic receptors in these assays was probed independently by immunoblotting of the receptors in the immunoprecipitates, referenced against the samples from the time = 0 min (1.0) and the variance of the measurements (mean values \pm S.E., $n = 3$) were as follows: 5 min (1.02 ± 0.16), 10 min (1.10 ± 0.10), and 30 min (0.96 ± 0.1). Thus, the variance in immunoprecipitation, sample loading, and/or blotting of β -adrenergic receptors among the time points was in the aggregate $\leq 10\%$.

trotransferred to nitrocellulose. Filters were blocked with a Tris-buffered saline solution containing 10% heat-inactivated horse serum and incubated with RII α subunit for 2 h at room temperature. After washing, the presence of AKAPs was detected using a goat anti-RII α antibody, as described in immunoblotting analysis.

Suppression via Antisense Oligodeoxynucleotides—Antisense and control missense oligodeoxynucleotides with the same base composition, but in scrambled order, were synthesized and purified to cell culture-grade (Operon, Alameda, CA), as described (2). Before addition to cells, oligodeoxynucleotides were mixed at a ratio of 1:3 (w/w) with DOTAP (Boehringer Mannheim), a cationic diacylglycerol in liposomal form which serves as a delivery vehicle. A431 cells were treated with oligodeoxynucleotides (5 $\mu\text{g}/\text{ml}$) for 2 days prior to the analysis of the expression of the target molecule. Cells in which gravin was specifically suppressed by antisense oligodeoxynucleotides were then analyzed for

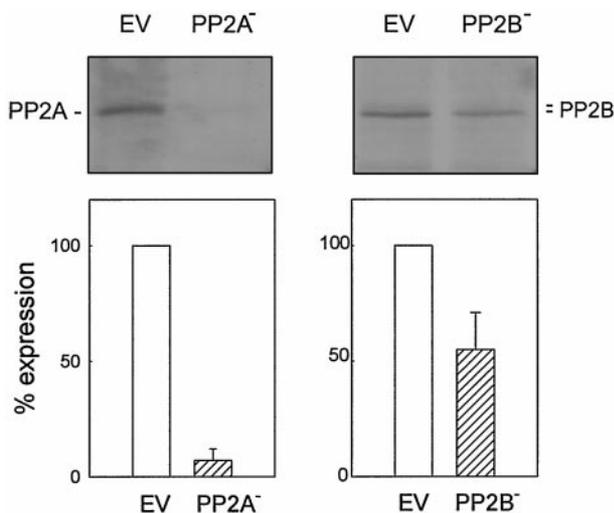


FIG. 3. Suppression of protein phosphatases 2A and 2B in A431 cells by antisense RNA. Stable transfectants harboring pLNCX vectors engineered to produce RNA antisense to catalytic subunits of either PP2A or PP2B were created and selected with gentamycin. Post-nuclei subcellular fractions were prepared from stable transfectants harboring either EV or an antisense region of PP2A (PP2A⁻) or of PP2B (PP2B⁻). Samples (35 μ g of protein/lane) of the fraction were subjected to SDS-PAGE, the resolved proteins transferred to nitrocellulose, and the blots probed with the appropriate antiserum to the catalytic subunit of either PP2A or PP2B. The immunocomplexes made visible on the blots (representative) were scanned. The results from three to five experiments quantified and are presented as mean \pm S.E. ($n = 4$).

agonist-induced (isoproterenol) desensitization and resensitization following a wash-out of agonist (2).

Immunoprecipitation—The association of β_2 -adrenergic receptor with other proteins was probed by use of immunoprecipitation. Cells were harvested, and cell membrane was prepared by homogenization. The membranes were solubilized in lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 6.0 μ M dithiothreitol, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, 100 μ g/ml bacitracin, 100 μ g/ml benzamide, 2 mM sodium orthovanadate, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 40 mM sodium pyrophosphate, 50 mM KH_2PO_4 , 10 mM sodium molybdate, and 20 mM Tris-HCl, pH 7.4). Immunoprecipitation was performed in the lysis buffer. The lysates were pre-cleared with protein A/G-agarose for 90 min and then subjected to immunoprecipitation for 2 h with antibodies specific either for the β -adrenergic receptor (CM4) or for AKAP250 gravin. The primary antibodies were linked covalently to a protein A/G-agarose matrix. The variance of immunoprecipitation, sample loading, and/or immunoblotting in these experiments in the aggregate was established at $\leq 10\%$.

Radioligand Binding Studies—The number of β_2 -adrenergic receptors was determined by radioligand binding. Intact A431 cell clones were incubated with 0.5 nM [¹²⁵I]iodocyanopindolol (ICYP; NEN Life Science Products) in the presence or absence of 10 μ M propranolol at 23 °C for 90 min. The incubation buffer contained 50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 , and 150 mM NaCl. The affinity constants for ICYP and isoproterenol (Sigma) binding were determined using crude membrane fractions (2). The dissociation constant for ICYP binding was calculated by Scatchard plot analysis with ICYP concentrations ranging from 0 to 1 nM (2).

Desensitization and Resensitization of $\beta_2\text{AR}$ —Two days prior to the analysis of agonist-induced desensitization, the A431 cells were seeded in 96-well microtiter plates at a density of 25,000–50,000 cells/well. Details of the desensitization protocol have been published elsewhere (2). For the actual assay, cells were washed and challenged with or without 1 μ M isoproterenol for 30 min at 37 °C. At the end of the first challenge, the cells were washed three times and then incubated in 20 mM Hepes buffer, pH 7.4, containing 0.1 mM Ro-20-1724 (Calbiochem, San Diego, CA) and 0.5 units/ml adenosine deaminase (Sigma) for 5 min prior to and again following a second challenge with 1 μ M agonist. For resensitization, the cells challenged with isoproterenol for 30 min prior were washed free of agonist and maintained in fresh buffer for an additional 30–60 min. Five minutes before the second challenge of the agonist, cells were incubated again in the presence of Ro-20-1724 and adenosine deaminase. The amount of cyclic AMP accumulated by cells in response to agonist was measured in the naive cells (activation), in

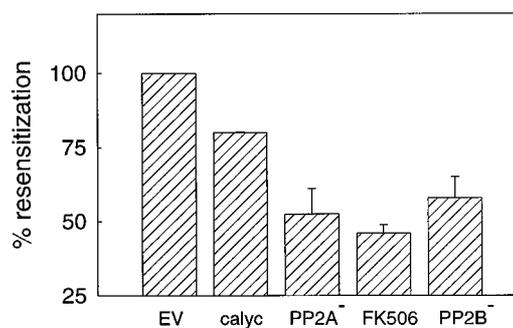


FIG. 4. Chemical inhibition of activity or suppression of the expression of protein phosphatases PP2A and PP2B by antisense RNA reduce recovery from agonist-induced desensitization. A431 clones stable transfected with EV or pLNCXAS engineered to suppress PP2A (PP2A⁻) or PP2B (PP2B⁻) were challenged with 10 μ M isoproterenol for 30 min (desensitization) and then washed free of agonist and allowed to resensitize for 60 min. For studies of the effects of protein phosphatase inhibitors, the EV clones were treated with either 10 nM calyculin to inhibit PP2A activity or 100 ng/ml FK506 to inhibit PP2B activity. Inhibitors were introduced 15 min prior to and then during the resensitization phase of the analysis. The results presented are the mean values \pm S.E. from four separate experiments.

the cells treated with agonist for 30 min prior to a second challenge with isoproterenol (desensitized), and in cells treated with agonist for 30 min prior to a second challenge with isoproterenol and washed free of agonist for 30–60 min (resensitized). The cyclic AMP accumulated by the cells was determined as described (2, 21). “0% desensitization” denotes a cyclic AMP response to a second challenge with agonist that is equivalent in magnitude to that obtained in naive cells in response to the first challenge. “100% desensitization” represents the maximal level of desensitization obtained by a 30-min pre-incubation of the cells with isoproterenol.

Effect of Protein Phosphatase Inhibitors on Receptor Resensitization—Cells were preincubated in the presence or absence of protein phosphatase inhibitors, such as FK506 (Fujisawa USA, Deerfield, IL) and calyculin A (Boehringer Mannheim) for 15 min prior to the addition of the agonist. Each inhibitor was included throughout the incubation for desensitization, washout, and resensitization, as described elsewhere (2).

Data Presentation and Analysis—Each of the experimental protocols were performed, as indicated, with three to five separate A431 clones. The values presented are means \pm S.E., and the autoradiograms are representative examples of data.

RESULTS AND DISCUSSION

Agonist-induced desensitization of G-protein-linked receptors, typified by studies of the β -adrenergic receptor, is sensitive to chemical inhibitors of protein phosphatases (2). Inhibition of PP2A and PP2B leads to enhanced desensitization, reflecting an attenuation of the recovery phase (2). Direct analysis of the association of β -adrenergic receptors with protein phosphatases 2A and 2B was probed by immunoprecipitating β -adrenergic receptors from detergent extracts from human epidermoid carcinoma A431 cells and exploring the nature of associated proteins either by immunoblotting or by direct assay of phosphatase activity of the immunoprecipitates (Fig. 1A). In the absence of exposure to the β -adrenergic agonist isoproterenol, immunoprecipitates of β -adrenergic receptors subjected to SDS-polyacrylamide gel electrophoresis and stained with antibodies to PP2B α reveal the presence of phosphatase PP2B in association with the β -adrenergic receptor (0-min desensitization time, Fig. 1A). A characteristic doublet band of PP2B with molecular mass values of 57 and 61 kDa are detected with antibodies to PP2B α , whereas staining either with a non-immune serum or with antibodies to unrelated antigens reveals no such bands (data not shown).

Challenging A431 cells with isoproterenol stimulates agonist-induced desensitization (2, 21, 22) and results in a decline of PP2B associated with the β -adrenergic receptors that was prominent within 5 min of the challenge (Fig. 1A). Within 10

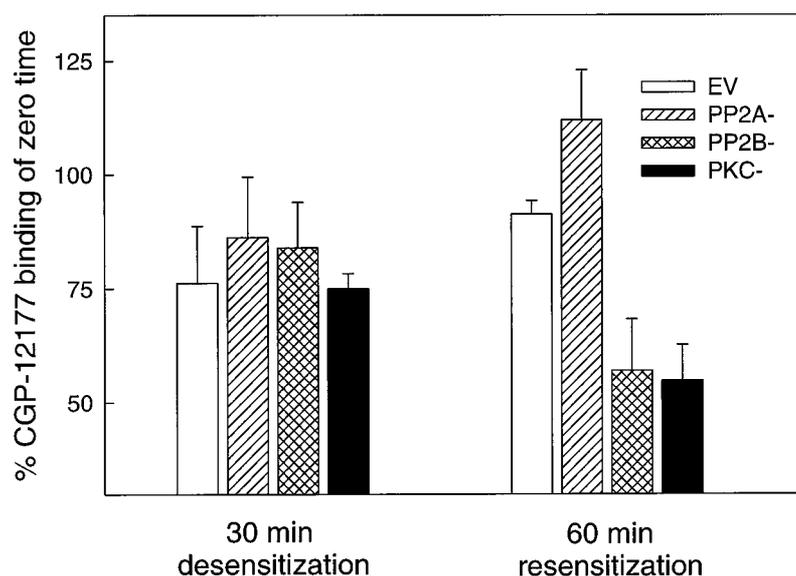


FIG. 5. Sequestration of β -adrenergic receptors in A431 cells subject to agonist-induced desensitization and then to resensitization: effects of deficiency in PP2A, PP2B, or protein kinase C. Stable transfectants of A431 cell clones harboring pLNCX engineered to suppress the expression of PP2A (PP2A⁻), PP2B (PP2B⁻) or protein kinase C (PKC⁻) were studied 30 min following a challenge with 10 μ M isoproterenol (desensitization) and again 60 min following a complete wash-out of agonist (resensitization). The data displayed for the PKC include both studies of the stably transfected clones in which protein kinase C is suppressed by antisense RNA (2) as well as data obtained from A431 cells treated with 300 nM bisindolylmaleimide, a selective inhibitor for protein kinase C. The results from either approach were identical. Receptor sequestration was assayed using the tritiated β -adrenergic ligand CGP-12177, which is hydrophilic and measures the availability of exofacial β -adrenergic receptors for binding to intact cells. The values presented are mean values \pm S.E. from at least three separate experiments.

min of agonist challenge, the amount of PP2B associated with β -adrenergic receptors began to return toward normal. By 30 min of challenge with isoproterenol, levels of PP2B associated with β -adrenergic receptors nearly doubled compared with that observed in the naive cells (Fig. 1A). Use of the calmodulin-overlay assay as an alternative to detect the 57- and 62-kDa forms of phosphatase PP2B confirms (i) the association of PP2B with β -adrenergic receptors in the naive cells, (ii) the decline in PP2B associated with the receptor upon early challenge with agonist, and (iii) the enhanced association of PP2B with the receptor at 30 min, a time when recovery from agonist-induced desensitization occurs (1, 2).

The relative levels of PP2A, PP2B, and protein kinase A (PKA) associated with β -adrenergic receptors was determined in A431 cells using this same strategy (Fig. 2). Summation of data from several independent assays confirms the results displayed in Fig. 1, *i.e.* PP2B associates with β -adrenergic receptors in the naive cells, is lost transiently early during agonist treatment, and associates in greater abundance as desensitization progresses to 30 min. Unlike the case for PP2B determinations, antibodies capable of detecting either PP2A or protein kinase A were found to be unsuitable for the purpose of detection of either protein in the immunoprecipitates. For measurement of PP2A associated with β -adrenergic receptors, PP2A activity was measured in immunoprecipitates of β -adrenergic receptors using [³²P]phosphorylated glycogen phosphorylase *a* as a substrate. PP2A activity was readily measurable in immunoprecipitates of β -adrenergic receptors from naive cells. Upon challenge with agonist, the amount of PP2A associated with the receptor declined by 20–25% at 5 min, and returned to control pre-challenge levels within the next 5 min, and remained rather constant over the next 20 min. Thus, both protein phosphatases PP2A and PP2B associate with the β -adrenergic receptors in naive cells and display a partial dissociation from the receptor early after challenge with an agonist. For PP2A the agonist-induced dissociation is transient and returns to normal, while PP2B actually displays increased association with the receptor as the time course continues to

the 30-min challenge with agonist. For protein kinase A, kinase activity is found in association with β -adrenergic receptors of naive A431 cells and increases approximately 3-fold over control levels at 30 min after challenge with isoproterenol (Fig. 2).

In an effort to explore further the role of PP2A and PP2B in agonist-induced desensitization and resensitization, stable transfectant clones of A431 cells were created that harbor pLNCX antisense RNA-expressing vectors (2). pLNCX antisense constructs, characterized previously (2, 23), were engineered to include 30-base pair sequences antisense to the catalytic subunit of either PP2A or PP2B. Each of these antisense expression vectors is driven by the cytomegalovirus promoter, harbors the neomycin resistance gene, and stably transfects clones of A431 cells, selected with the neomycin analog G418 (2, 25). Clones stably transfected with pLNCXASPP2A vector display an 85–95% suppression of PP2A levels (Fig. 3). Glycogen phosphorylase *a* is dephosphorylated by either protein phosphatase type 1 or PP2A. Using radiolabeled [³²P]phosphorylated glycogen phosphorylase *a* as a substrate, the sum of these two activities in whole-cell extracts was found to be reduced >75% in the clones stably transfected with the pLNCXASPP2A vector (data not shown). The suppression of PP2A expression (immunoblotting) by antisense RNA and corresponding loss of activity suggest that PP2A is largely responsible for the readout using phosphorylase *a* as the substrate for detection of PP2A in immunoprecipitates of β -adrenergic receptors from A431 cells (Fig. 3). Similar clones deficient in PP2B expression were sought using the pLNCXASPP2B form of the vector, with surprising results. Despite considerable experience with pLNCXAS constructs to suppress the expression of a variety of targets (2, 25, 26), we were unable to identify clones in which PP2B levels are suppressed in excess of 50–60% even after several independent rounds of transfection (Fig. 3). This observation leads us to speculate that suppression of PP2B beyond the 50–60% range may not be compatible with cell viability. Alternatively, some PP2B isoform not sensitive to the antisense RNA may be expressed in A431 cells. Notably, the growth rate of the stably transfected A431 cell clones with 50% the

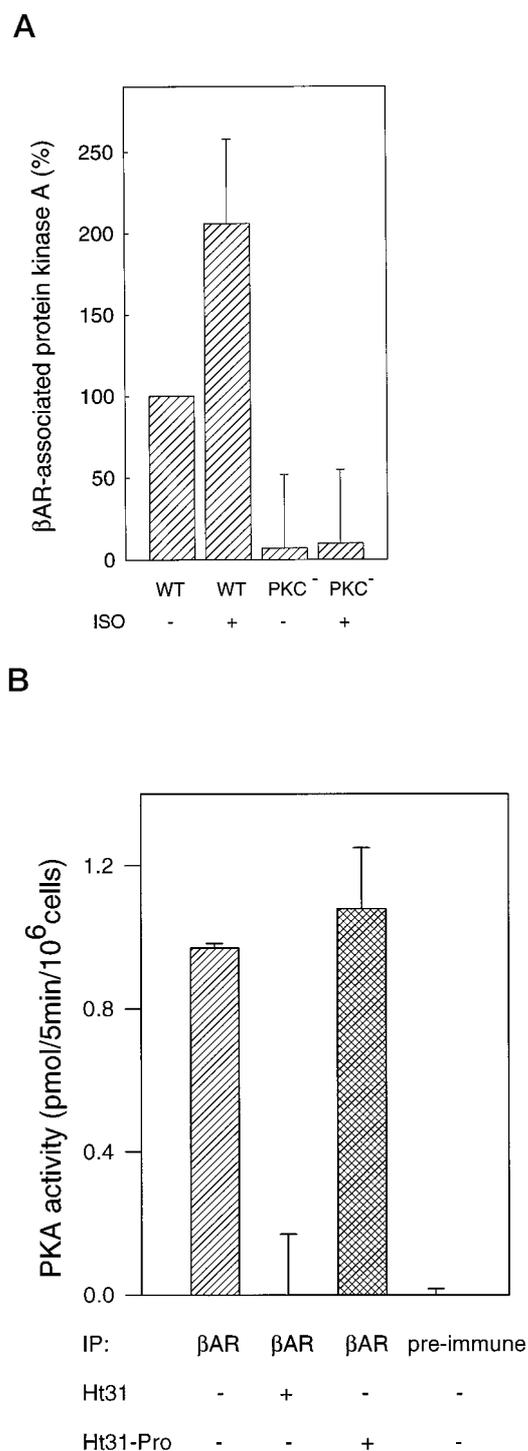


FIG. 6. Association of protein kinase A with β -adrenergic receptors in A431 cells is lost in clones made deficient in protein kinase C and by competition with the Ht31 peptide selective for interruption of AKAP protein-protein interactions. *Panel A*, lysates were prepared from A431 cells treated without (-) and with (+) the β -adrenergic agonist isoproterenol (10 μ M) for 30 min. Cell lysates were incubated with antibodies to β -adrenergic receptors (CM-04) and then the immunocomplexes recovered by adsorption to protein A-agarose beads. For PKA, the immunocomplexes were resuspended in assay buffer in which the Kemptide was employed as substrate. PKA activity was defined as that activity stimulated in the presence of cyclic AMP and sensitive to inhibition with the protein kinase A inhibitory peptide. The results are displayed as the mean values \pm S.E. from at least three independent assays performed on as many separate cell preparations. *Panel B*, the interaction between the β -adrenergic receptor and protein kinase A of A431 cell lysates were probed in the absence (-) and presence (+) of 1 μ M Ht31, a 24-amino acid, conserved amphipathic helix common to all AKAP family members.

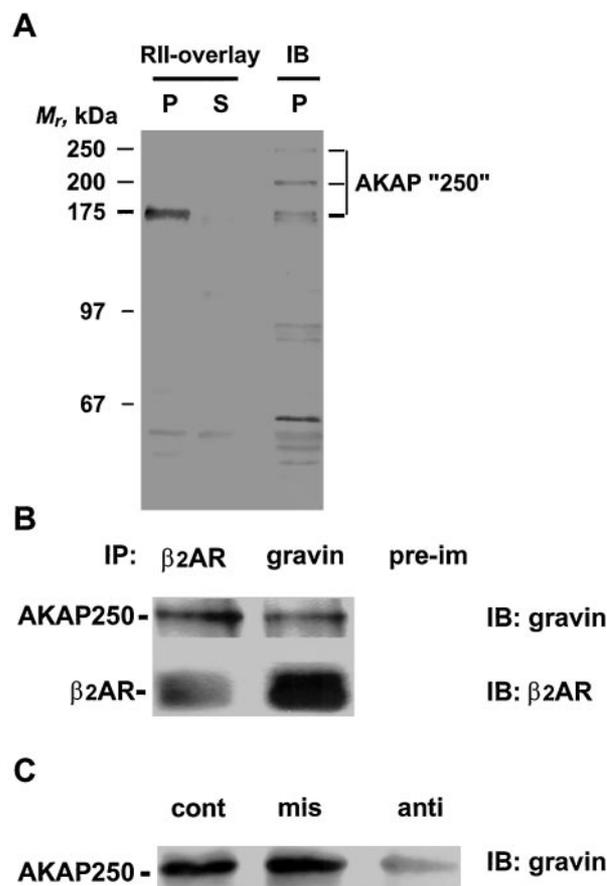


FIG. 7. The A kinase-anchoring protein AKAP250 (gravin) associates with the β -adrenergic receptors in A431 cells. *Panel A*, A431 cells were homogenized and a low speed, post-nuclei subcellular fraction (*pellet*) and supernatant (*super*) prepared. Samples (50 μ g of protein/lane) were subjected to SDS-polyacrylamide gel electrophoresis, the resolved proteins transferred to nitrocellulose, and the resultant blots stained with either radiolabeled RII subunit of PKA (*RII-overlay*) or antibodies against gravin (*IB*). Note that the gravin is found almost exclusively in the pellet, membrane-enriched fraction. The amount of the M_r 250,000 and 200,000 forms of gravin were variable, nicked to the M_r 175,000 form recognized prominently by both RII subunit and anti-gravin antibodies. *Panel B*, A431 cell lysates were incubated with antibodies to either β -adrenergic receptors (CM-04) or to AKAP250 (gravin) and then the immunocomplexes recovered by adsorption to protein A-agarose beads. The immunocomplexes were subjected to SDS-PAGE, the resolved proteins transferred to nitrocellulose, and the resultant blots stained either for AKAP250 or β -adrenergic receptors. Similar experiments were performed in CHO clones lacking β -adrenergic receptors (*CHO-K cells*) and stable transfectants of CHO cells that express β -adrenergic receptors under the cytomegalovirus promoter (*CHO-13d*). *Panel C*, A431 cells were treated with oligodeoxynucleotides antisense (*anti*) or missense (*mis*, same composition in scrambled order) to gravin or vehicle (*cont*) alone as described under "Experimental Procedures." The treated cells were harvested and a fraction of each group homogenized. Samples (50 μ g of protein/lane) were subjected to SDS-polyacrylamide gel electrophoresis, the resolved proteins transferred to nitrocellulose, and the resultant blots stained with antibody specific for the gravin. Note the suppression of gravin expression in the cells treated with oligodeoxynucleotides antisense, but not missense, to gravin. The results are representative of at least three separate experiments performed on separate occasions.

complement of PP2B was reduced by severalfold. Assay of PP2B activity in whole-cell extracts of the PP2B-deficient clones was performed, and the amount of PP2B activity was found to be similarly reduced to about 50–60% of the control levels (data not shown).

Agonist-induced desensitization was measured in the A431 clones stably transfected to suppress either PP2A or PP2B. The availability of chemical inhibitors for either PP2A or PP2B as

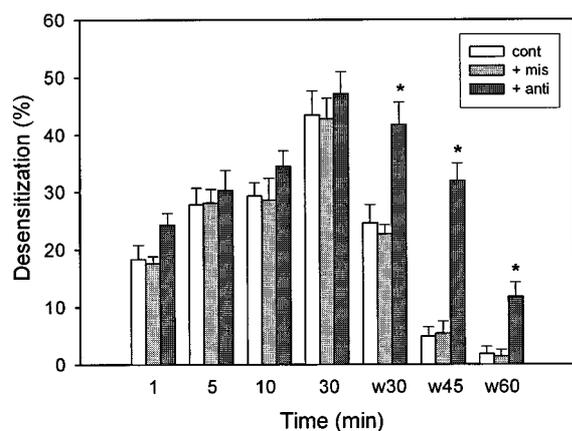


FIG. 8. **Suppression of AKAP250 gravin by antisense oligodeoxynucleotides disrupts recovery from agonist-induced desensitization for β -adrenergic receptors.** A431 cells were treated with oligodeoxynucleotides antisense (*anti*) or missense (*mis*, same composition in scrambled order) to gravin or vehicle (*cont*) alone as described under "Experimental Procedures." Treated cells were challenged with 10 μ M isoproterenol to induce desensitization, which was monitored at 1, 5, 10, and 30 min after challenge. A fractions of the desensitized cells were washed free of the agonist and the recovery from desensitization monitored at 30 (*w30*), 45 (*w45*), and 60 (*w60*) min after wash-out. The results displayed are mean values \pm S.E. ($n = 4$). Note that, in the cells made deficient in gravin (Fig. 7C), recovery from desensitization is markedly impaired.

well as clones in which levels of expression of either PP2A or PP2B are reduced provided two complementary strategies with which to study the functional significance of protein phosphatases PP2A and PP2B in agonist-induced desensitization. The amount of desensitization measured in either the clones expressing the empty vector or control A431 cells was set at 100% for these studies. A431 clones deficient in PP2A display a modest increase in the amount of agonist-induced desensitization in response to a challenge with 10 μ M isoproterenol, 137 \pm 09% of the control levels (mean \pm S.E.; $n = 4$). Treating cells with 10 nM calyculin A, a selective inhibitor for PP2A, yields agonist-induced desensitization, which is unaltered (105 \pm 12%, mean \pm S.E.; $n = 4$). The optimal concentration (100 nM) of calyculin A insuring maximal inhibition of PP2A activity could not be employed in the current studies. Calyculin A at 100 nM provoked cell death and detachment of the A431 cells. Thus, suppression of PP2A expression leads to a modest increase in the amount of desensitization, whereas partial inhibition of PP2A with 10 nM calyculin does not alter the extent of the desensitization.

A431 cells lacking >50% of the PP2B complement display normal levels of desensitization, 92 \pm 8% of control levels (mean \pm S.E.; $n = 4$). Treating cells with the PP2B inhibitor FK506 (100 ng/ml), in contrast, more than doubles (210 \pm 06%, mean \pm S.E.; $n = 4$) the amount of agonist-induced desensitization over that observed in the control, untreated A431 cells. The inability of the antisense RNA to suppress the expression of PP2B more than 50–60%, whereas the FK506 effectively blocks PP2B activity under the same conditions, provides an explanation for the apparent dichotomy in the inhibitor *versus* antisense data. In the desensitization paradigm employed for these studies, loss or inhibition of PP2A enhances the extent of desensitization only slightly, whereas inhibition of PP2B enhances markedly the extent of agonist-induced desensitization.

Earlier studies implicated PP2A in agonist-induced desensitization (1). Since protein phosphatases would likely have a more prominent role in re-sensitization rather than desensitization *per se*, the re-sensitization of the cyclic AMP response of A431 cells following a challenge with 1 μ M isoproterenol was

evaluated in cells for which levels of PP2A and PP2B were suppressed by either antisense RNA or through chemical inhibition of their activities (Fig. 4). A431 clones harboring the empty vector alone were employed as controls. Stable transfectant clones were treated with isoproterenol for 30 min to achieve full desensitization (1, 2). During the last 15 min of incubation with agonist, the A431 clones were challenged with either vehicle alone, calyculin A (10 nM), or FK506 (100 ng/ml). Cells were then washed free of agonist repeatedly with fresh buffer supplemented with or without one of the phosphatase inhibitors maintained at the same concentration. Resensitization was measured 60 min after washout of the agonist, a time at which full recovery from desensitization occurs.

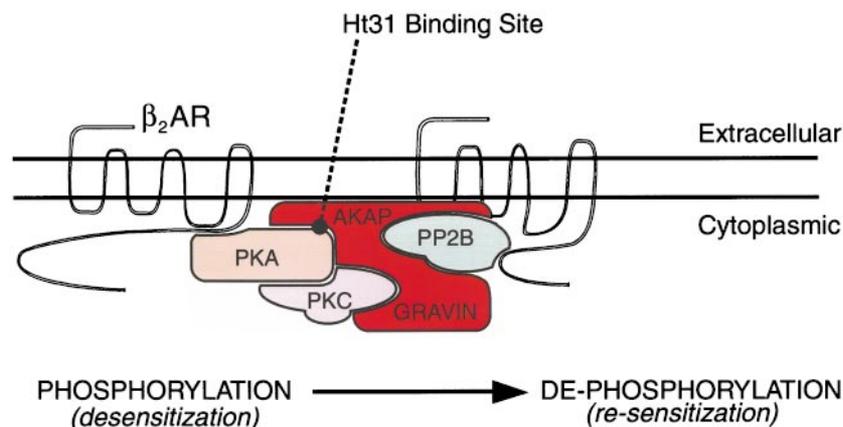
A431 stably transfected clones harboring the empty vector (EV) and naive, wild-type A431 cells displayed 100% resensitization by 60 min after wash-out of agonist (Fig. 4). For clones made deficient in PP2A with antisense RNA, the extent of resensitization was reduced to approximately half of that observed for A431 clones harboring the empty vector (Fig. 4). This sharp decline in resensitization in the PP2A-deficient cells provides an explanation for the apparent increase in desensitization, as measured on the "supply" side of the equation. Partial inhibition of PP2A with 10 nM calyculin A, much like PP2A-deficiency, reduced the extent of resensitization, but to a lesser extent (20–25%) than that provoked by suppression of PP2A (Fig. 3). The inability of calyculin A inhibition to mimic the full effects observed in PP2A-deficient cells likely reflects the suboptimal concentration of the PP2A inhibitor employed for these studies, as discussed above.

Suppression of PP2B activity markedly attenuated resensitization of the agonist response (Fig. 4). Resensitization in PP2B-deficient cells under these conditions is ~60% of the control level. Chemical inhibition of PP2B with FK506, like PP2B deficiency, attenuates resensitization, displaying levels of resensitization less than 50% of control. The enhanced agonist-induced desensitization observed in the presence of the FK506 likely reflects the reduction in resensitization accompanying inhibition of PP2B. The chemical inhibitor is more effective than antisense in both suppressing PP2B activity as well as attenuating resensitization. In view of the inability of the viable, antisense RNA-producing cells to suppress PP2B more than 50% of control values (Fig. 3), the more pronounced effect of the chemical inhibitor seems predictable (Fig. 4).

Receptor sequestration was assayed in order to define to what extent, if any, do PP2A and PP2B exert their influence on agonist-induced desensitization/resensitization through receptor sequestration. The hydrophilic β -adrenergic antagonist ligand CGP-12177 was employed to measure receptor sequestration (Fig. 5). Each of A431 clones under study display equivalent levels of β -adrenergic receptor expression, as measured by the binding of iodocyanopindolol (data not shown). In the absence of treatment with agonist, the amount of CGP-12177 binding measured in the intact cells is equivalent among all A431 clones (data not shown). To provoke sequestration, cells were challenged with 10 μ M isoproterenol for 30 min in the standard desensitization protocol and amount of CGP-12177 binding measured in the whole cells. Deficiency of either PP2A or PP2B had no significant effect on the extent to which CGP-12177 binding declined in response to agonist-induced desensitization. Each of the clones displayed a reduction in the amount of CGP-12177 bound ranging from 18% to 26%, typical for 30-min agonist-induced receptor sequestration in control A431 cells (1, 2).

Assay of CGP-12177 binding capacity at 60 min of resensitization following a wash-out of agonist revealed several features of protein phosphatase action. EV control clones treated with

FIG. 9. Schematic illustration of the signaling components targeted to β -adrenergic receptor complexes. See "Results and Discussion" for details.



agonist, washed free of agonist, and allowed to recover for 60 min displayed >95% of the CGP-12177 binding capacity of naive, untreated cells (Fig. 5). The suppression of PP2A by antisense RNA (Fig. 3) attenuated resensitization (Fig. 4), but did not alter recovery of CGP-12177 binding, which returned to ~110% of that observed in the naive, untreated cells. Partial inhibition of PP2A with 10 nM calyculin A yielded identical results, *i.e.* inhibition of PP2A failed to alter the ability of the cells to return to levels of CGP-12177 binding observed in naive, untreated cells (data not shown). Partial suppression of PP2B by antisense RNA (Fig. 3), in contrast, blocked the recovery from receptor sequestration (Fig. 5), as well as resensitization of the β -adrenergic receptor response (Fig. 4). Inhibition of PP2B with FK506 also blocked recovery of the agonist-induced decline in CGP-12177 binding by $60 \pm 11\%$ (Fig. 5). The results from assay of these two aspects of receptor function and cycling are in agreement and suggest that PP2B plays a key role in the recovery of β -adrenergic receptor from agonist-induced desensitization.

Previously, protein kinase C deficiency was shown to provoke enhanced agonist-induced desensitization (2), much like either PP2B deficiency or inhibition of PP2B with FK506 shown here (Figs. 4 and 5). CGP-12177 binding studies of protein kinase C-deficient, A431 stable transfectants reveal a similar pattern for protein kinase C deficiency as for either partial suppression of expression or chemical inhibition of PP2B (Fig. 5). A431 cells pre-treated for 15 min and thereafter in the presence of the protein kinase C inhibitor bisindolylmaleimide (300 nM, not shown) yielded identical data to those obtained with the protein kinase C-deficient cells (Fig. 5). PP2B associated with β -adrenergic receptors in the absence of agonist, dissociating during the early period of agonist-induced desensitization, but rebounding in association with the receptor as the desensitization continues (Figs. 1 and 2). Loss of either protein kinase C or PP2B shared many of the same features, leading to enhanced agonist-induced desensitization, to sustained loss of function following agonist removal ("resensitization"), and to an inability of the β -adrenergic receptors to recover from agonist-induced receptor sequestration.

For A431 cells, deficiency of protein kinase A (1, 2) attenuates whereas deficiency of protein kinase C potentiates agonist-induced desensitization (26). The effect of protein kinase C deficiency on the association of protein kinase A with the β -adrenergic receptors was explored. Whereas agonist treatment enhanced the amount of protein kinase A associated with the β -adrenergic receptor (Fig. 2), deficiency of protein kinase C disrupted the association of protein kinase A with the β -adrenergic receptors (Fig. 6A). Association of either PP2A or PP2B with β -adrenergic receptors, in contrast, was unaltered in the protein kinase C-deficient cells, being $105 \pm 12\%$ for PP2A and

$97 \pm 6\%$ for PP2B association (mean \pm S.E., $n = 4$), respectively. This interplay between protein kinase A and protein kinase C suggested the possible participation of either anchoring or scaffold proteins in agonist-induced desensitization (10–13).

Anchoring and scaffold proteins have been discovered that participate in cell signaling (10–13). The AKAPs represent a diverse family of proteins that assist in organizing signaling elements (10–16). The expression of AKAP proteins in A431 cells was probed using antibodies to two prominent members of the family, AKAP79 and AKAP250 (gravin). Antibodies to AKAP79 failed to identify any immunoreactive species with a mass of approximately 70–90 kDa (data not shown). Staining blots of cellular proteins of A431 cells with the RII α subunit of protein kinase A, the technique employed in the discovery of AKAPs (15), revealed one potential AKAP at M_r 175,000, which is a proteolytic product of a M_r 250,000 AKAP (Fig. 7). Subsequent staining of A431 cell proteins with antibodies to other AKAPs reveal the identity of the AKAP250 to be the protein gravin (13). As an additional test of a possible role of AKAPs in the organization of the β -adrenergic receptors and protein kinase A, the interaction between the β -adrenergic receptors and protein kinase A was probed with Ht31, a 24-amino acid, conserved amphipathic helix peptide common to all AKAP family members (12). Ht31, which blocks protein-protein interactions of AKAPs, effectively blocked the ability of the β -adrenergic receptors to bind protein kinase A (Fig. 6B). Ht31 blocked the association of protein kinase A with β -adrenergic receptors in naive cells (Fig. 6B), as well as in cells treated with β -adrenergic agonist (data not shown). The Ht31 peptide in which a prolyl residue has been inserted to disrupt the AKAP binding motif (Ht31-Pro) was without effect on PKA association with the β -adrenergic receptors, whereas the Ht31 peptide essentially abolished PKA association with the β -adrenergic receptors. Inclusion of either the active Ht31 or inactive Ht31-Pro peptide yielded a small (<10%) reduction in the PKA activity measurements.

To probe further the involvement of AKAP250 (gravin), immunoprecipitations of both β -adrenergic receptors as well as of gravin were performed and the immunoprecipitates subjected to SDS-polyacrylamide gel electrophoresis and staining with antibodies to either β -adrenergic receptors or gravin (Fig. 7, A–C). Subcellular fractionation of A431 cells followed by SDS-PAGE and staining with the RII subunit of protein kinase A (RII-overlay assay) revealed prominent staining of gravin in the subcellular cell membrane-enriched, "pellet" (P) fraction collected by low speed centrifugation (Fig. 7A). The supernatant (S) fraction, in contrast, was essentially devoid of immunoreactive gravin. Gravin (AKAP250) was rapidly nicked to a dominant M_r 175,000 species, which was stained prominently

by the RII subunit of PKA. The amount of the M_r 250,000 and 190,000 species of gravin was variably determined by some non-suppressible proteolytic activity in cell homogenates. In most preparations, the M_r 175,000 form of gravin was the major form and appears to be the limit cleavage product under these conditions. Immunoblotting of the pellet fraction with anti-gravin antibodies revealed gravin and its major proteolytic species. Thus, gravin is essentially confined to the membrane-enriched, pellet fraction, whether identified by either the RII overlay or immunoblotting with antibodies to gravin.

Staining of immunoprecipitates of β -adrenergic receptors of A431 cell extracts with antibodies to gravin revealed prominent staining of AKAP250, providing compelling evidence of gravin association with β -adrenergic receptors (Fig. 7B). Similarly, staining of immunoprecipitates of gravin revealed prominent staining of β -adrenergic receptor, found in association with gravin (Fig. 7B). Immunoprecipitations performed with antibodies to gravin and extracts from Chinese hamster ovary (CHO-K) clones that lack β -adrenergic receptors revealed no such staining or associations (data not shown).

The functional significance of gravin in agonist-induced desensitization/resensitization in A431 cells was explored. Antisense oligodeoxynucleotides were employed to suppress gravin production. Treatment with oligodeoxynucleotides antisense (*anti*), but not missense (*mis*), to gravin for 2–3 days suppresses the expression of gravin by >90% in A431 cells (Fig. 7C). Agonist-induced desensitization was examined in the cells treated with and without oligodeoxynucleotides antisense to gravin. The progress and extent of the agonist-induced desensitization in response to 1 μ M isoproterenol was equivalent in all of the cells, including those treated with oligodeoxynucleotides antisense to gravin (Fig. 8). To assess the role of gravin in resensitization, the cells were washed free of agonist and studied over the next 60 min (Fig. 8). Although altering neither the time course nor the extent of the desensitization, suppression of gravin provoked a dramatic loss of resensitization in A431 cells at 30, 45, and 60 min following the wash-out of agonist (Fig. 8). Thus, deficiency in gravin profoundly delayed the ability of the A431 cells to recover from agonist-induced desensitization.

Consistent with earlier observations (2), the association of protein kinase A with the β -adrenergic receptors was found to be dependent upon the expression of protein kinase C. Association of protein kinase A with β -adrenergic receptors was not detectable in A431 cells made deficient of protein kinase C. Similar studies in other systems have revealed a central role for protein kinase C in the association of AKAPs (13, 26). The AKAP gravin was shown to be associated with β -adrenergic receptors physically and functionally. Taken together, these data provide support for the formation of signaling complexes in which protein kinases and phosphatases that regulate a G-protein-linked receptor are maintained in proximity to the target protein (Fig. 9). Recent work in the mitogen-activated protein kinase cascade in yeast and mammalian systems implicates scaffold complexes in signaling (27–29). In neurons, AKAP proteins have been shown to coordinate three multifunctional enzymes: PKA, PKC, and PP2B (27). Herein we provide evidence for the existence of signaling complexes for a prominent member of the G-protein-linked receptor superfamily, the β -adrenergic receptor. According to this scheme, the β -adrenergic receptor is localized to the AKAP gravin, in association with PKA (sensitive to Ht31 inhibition) and PKC (PKA fails to associate in the PKC-deficient cells) as well as with PP2B. Thus, much like the organization in yeast and in neurons, G-protein-linked receptors are organized into complexes with

multifunctional enzymes.

The current work illuminates also several key features of agonist-induced desensitization, the resensitization that follows, and receptor sequestration. Protein phosphatase 2B plays an obligate role; partial deficiency of this protein phosphatase or inhibition of PP2B by FK506 disrupts all three parameters. Both protein kinase A and PP2B physically associate with β -adrenergic receptors, as analyzed through co-immunoprecipitation, and display a dynamic association. The ability of protein kinase C expression or deficiency to influence agonist-induced desensitization and protein kinase A association with β -adrenergic receptors provoked a search for possible AKAPs. Importantly, β -adrenergic receptors are shown to physically associate with AKAP250 (gravin). The suppression of gravin by antisense oligodeoxynucleotides is shown to disrupt resensitization. These results provide compelling evidence to support the notion that G-protein-linked receptors, such as the β -adrenergic receptor, that activate adenylyl cyclase participate in macromolecular complexes that are composed minimally of protein kinases and phosphatases in association with a prominent member of the AKAP family of anchor/scaffold proteins, gravin. These associations are functionally relevant, as disruption of specific interactions among these molecules influences agonist-induced desensitization, resensitization, and/or receptor sequestration.

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