Communication

The Role of Sequestration in G Protein-coupled Receptor Resensitization

REGULATION OF β2-ADRENERGIC RECEPTOR DEPHOSPHORYLATION BY VESICULAR ACIDIFICATION

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G protein-coupled receptor kinases phosphorylate the agonist-occupied conformation of G protein-coupled receptors in the plasma membrane, leading to their desensitization. Receptor resensitization requires receptor dephosphorylation, a process which is mediated by a plasma and vesicular membrane-associated form of PP-2A. We present evidence that, like receptor phosphorylation, receptor dephosphorylation is tightly regulated, requiring a specific receptor conformation induced by vesicular acidification. In vitro, spontaneous dephosphorylation of phosphorylated receptors is observed only at acidic pH. Furthermore, in intact cells upon agonist stimulation, phosphorylated receptors traffic from the plasma membrane to vesicles where they become physically associated with the phosphatase and dephosphorylated. Treatment of cells with NH4Cl, which disrupts the acidic pH found in endosomal vesicles, blocks association of the receptors with the phosphatase and blocks receptor dephosphorylation. These findings suggest that a conformational change in the receptor induced by acidification of the endosomal vesicles is the key determinant regulating receptor dephosphorylation and resensitization.

As with other members of the G protein-coupled receptor superfamily, the functional status of the β2AR is determined by its phosphorylation state (1). One group of kinases which phosphorylate the receptor in an agonist-dependent manner consists of members of the family of G protein-coupled receptor kinases (GRKs) (2, 3). Phosphorylation of the receptors and the subsequent binding of members of a family of cytosolic proteins (arrestins or β-arrestins) serves to uncouple the receptor from its cognate G protein (4, 5). This results in a decreased responsiveness of the signaling system to agonist, termed desensitization.

Upon removal of agonist, the attenuated responsiveness is reversed in a process known as resensitization, which involves dephosphorylation of the receptors (6). Much less is known about the resensitization and dephosphorylation processes. We have recently identified a membrane-associated phosphatase which dephosphorylates GRK phosphorylated G-protein-coupled receptors (7). This phosphatase, referred to as GRP (G protein-coupled receptor phosphatase), is a member of the PP-2A family of protein serine/threonine phosphatases. Surprisingly, under in vitro conditions, GRP-mediated dephosphorylation of phosphorylated receptors is entirely latent at neutral pH, with activity being observed only in the presence of activators of PP-2A such as protamine or freeze/thawing (7). This indicates that some form of activation must be necessary for receptor dephosphorylation to occur under in vivo conditions.

Over the same time frame as desensitization and resensitization occur, the β2AR becomes sequestered into vesicles (1). Although evidence indicates that sequestration is not required for desensitization (8–10), it appears to be required for resensitization. Agents which block sequestration, as well as sequestration-deficient mutants of the β2AR, do not resensitize (6, 10–12). These data, together with earlier findings which indicate that receptors in a vesicular fraction are in a less phosphorylated state than receptors in the plasma membrane (12), led to a model proposing that dephosphorylation of the receptor occurs upon its sequestration into a vesicle population (10). Consistent with these results, a recent paper by Pippig et al. (6) shows that treatment of cells with concanavalin A in addition to agonist prevents not only sequestration and resensitization but also dephosphorylation of the β2AR. Since sequestration of the receptors into internalized vesicles appears to be required for their dephosphorylation, and since these vesicles were previously identified as endosomes (13, 14), an acidified vesicle population (15), we tested the hypothesis that the uniquely low pH of the vesicles is the key regulator of GRP-mediated receptor dephosphorylation.

EXPERIMENTAL PROCEDURES

Materials

Okadaic acid was purchased from Calbiochem. The monoclonal antibodies 12CA5 and M2 were obtained from Berkeley Antibody Co. and Eastman Kodak Co., respectively. Antibody recognizing the catalytic subunit of PP-2A was obtained from Promega.

Methods

Phosphatase Assays—Dephosphorylation of GRK2 phosphorylated β2AR (phosphorylated to a stoichiometry of 2–4 mol of Pi/mol of receptor) (16), utilizing salt-washed bovine brain membranes as the source of GRP (7), was performed by incubating 10 μl of phosphorylated β2AR (100 nm) with 10 μl of GRP and 10 μl of 50 mM acetic acid-acetate buffer. The proportion of acetic acid relative to acetate in the 50 mM buffer was used to adjust the pH of the overall assay between 4.0 and 6.5. For assays performed at pH 7.0, 50 mM Tris-HCl (pH 7.0) substituted for the acetic acid-acetate buffer. Where indicated, 1 mg of protamine sulfate/ml or 5 μM okadaic acid was added to the reaction mixture. Incubations were performed for 60 min at 30 °C and were terminated by the addition of SDS sample buffer. The amount of labeled receptor was quantified by PhosphorImager analysis and the percentage of phosphate released was calculated relative to controls containing okadaic acid (7). Dephosphorylation of phosphorylated casein (17) or phosphorylase a (18) was performed by incubating the respective substrate (at
a concentration of 1 mg/ml with an aliquot of GRP for 15 min at 30 °C, at the indicated pH (7).

Sucrose Density Gradient Fractionation—HEK293 cells transfected with DNA encoding FLAG-tagged β2AR were incubated for 15 min either in medium alone, medium containing 10 μM isoproterenol, or medium containing both 20 mM NH4Cl and isoproterenol. Cells were placed on ice, washed, and incubated with phosphate-buffered saline containing 250 μg of concanavalin A/ml to minimize vesicularization of the plasma membrane (19) during Dounce homogenization (in 50 mM Tris-HCl (pH 8.0) and 5 mM EDTA (TE) containing 5% sucrose and protease inhibitors). Intact cells and nuclei were removed by centrifugation at 300,000 g for 5 min. Crude membranes were obtained by centrifuging the resulting supernatant at 300,000 g for 30 min. Pellets were resuspended in TE containing 5% sucrose, layered on top of a 5–50% continuous, nonlinear sucrose gradient (19) and centrifuged at 100,000 g for 100 min at 4°C. Gradient fractions were collected and assayed for 125I-labeled cyanopindolol binding to determine the localization of β2ARs. The sucrose density in each fraction was determined by refractometry.

Immunoprecipitation of FLAG-tagged β2AR—Fractions from the sucrose gradients containing receptor associated with either vesicles or plasma membrane were pooled, diluted with TE and centrifuged at 300,000 g for 30 min at 4 °C. Pellets were resuspended in buffer B (10 mM Hepes (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, and 1% CHAPS) (20), and receptor complexes were immunoprecipitated with 15 μg of monoclonal antibody M2 in the presence of protein G-coupled agarose beads. Immunoprecipitates were washed in buffer B and resuspended in SDS sample buffer. Samples were subjected to SDS-PAGE, transferred to nitrocellulose, probed with antibody against the PP-2A catalytic subunit, and visualized by incubation with a horseradish peroxidase-conjugated secondary antibody followed by processing of the blot as recommended by the manufacturer (ECL by Amersham Corp.).

Whole Cell Dephosphorylation of β2AR—HEK293 cells were transfected with DNA encoding HA-tagged β2AR. The intracellular ATP pool was 32P-labeled as described (21). Cells were incubated with 10 μM isoproterenol either alone or in the presence of 20 mM NH4Cl for 10 min at 37 °C. Cells were washed with phosphate-buffered saline and either harvested or incubated an additional 20 min with medium alone or medium containing 20 mM NH4Cl prior to harvesting. The β2AR was immunoprecipitated as described (21), and equivalent amounts of protein were subjected to SDS-PAGE. The amount of labeled receptor was quantified by PhosphoImager analysis with the percentage remaining on the receptor after the 20-min incubation calculated relative to the amount of label present after the initial agonist stimulation.

RESULTS AND DISCUSSION

The effect of acidic pH on the in vitro dephosphorylation of GRK2 phosphorylated β2AR catalyzed by GRP was examined (Fig. 1A). While no GRP-mediated dephosphorylation of the receptor was observed at pH 7.0, significant dephosphorylation occurred as the pH was decreased below 7.0, with maximal dephosphorylation occurring at pH 4.85. This pH-dependent dephosphorylation was completely inhibited by okadaic acid, an inhibitor of PP-2A (Fig. 1A). In contrast, when assayed in the presence of protamine, a PP-2A activator, significant dephosphorylation of the receptor occurred at pH 7.0, while less dephosphorylation occurred as the pH was lowered (Fig. 1A). These data indicate that acidification stimulates GRP-mediated β2AR dephosphorylation in vitro. Since under these conditions both the receptor and the phosphatase are exposed to the acidic pH, the in vitro assays do not reflect the cellular situation. In the cell, the sites that are phosphorylated, and which therefore must be dephosphorylated on the β2AR, lie on the cytoplasmic face of the plasma membrane (1). When sequestration of the β2AR into endosomes occurs, these phosphorylated sites presumably face the cytosol. Since it is the intravesicular side that undergoes acidification, the sites of phosphorylation as well as the catalytic site of the phosphatase would not be directly exposed to the decreased pH. This suggests a pH induced conformational change in the receptor which regulates sensitivity to phosphatase actions.

To examine the role receptor conformation plays in facilitating the pH-dependent dephosphorylation of the β2AR, the effect of acidic pH on the ability of GRP to dephosphorylate native phosphorylated β2AR versus heat-denatured phosphorylated β2AR was examined (Fig. 1B). The stimulatory effect of low pH on dephosphorylation was markedly blunted for the denatured receptor. When the effect of pH on dephosphorylation of nonreceptor substrates such as phosphorylase α and phosphorylated casein by the GRP was examined, more dephosphorylation was observed at pH 7.0 than 4.85 (all substrates were soluble over the pH range explored, Fig. 1B). These data indicate that the receptor conformation plays a critical role in determining the phosphorylation status of the receptor.

If vesicular acidification plays an important role in regulating β2AR conformation and GRP-mediated β2AR dephosphorylation in a cellular system, then phosphorylated β2AR and GRP should be associated within endosomes containing sequestered β2AR (13, 14). Continuous, nonlinear sucrose gradients were utilized to separate β2AR localized in the plasma membrane

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** A, dephosphorylation of the receptor is stimulated at acidic pH. Dephosphorylation of GRK2 phosphorylated β2AR by GRP was performed at the indicated pH. The incubation of receptor with GRP was performed in either buffer alone (top panel), buffer with 5 μM okadaic acid (middle panel), or buffer with 1 mg of protamine sulfate/ml (bottom panel). Samples were subjected to SDS-PAGE and autoradiograms of representative gels are shown. B, dephosphorylation observed at acidic pH is influenced by receptor conformation. GRK2-phosphorylated β2AR was heat-denatured by incubation at 70 °C for 30 min and subsequently cooled. Dephosphorylation of denatured or untreated (labeled as Native) phosphorylated receptors (left side) as well as phosphorylated casein or phosphorylase α (right side) by GRP was assayed at either pH 7.0 (open bars) or 4.85 (solid bars). A representative of three or more experiments is shown.
Acidification Regulates β2-Adrenergic Receptor Dephosphorylation

FIG. 2. A, sucrose density gradient fractionation resolves plasma membrane-associated β2AR from sequestered β2AR. HEK293 cells transfected with FLAG-tagged β2AR DNA were incubated either in medium alone (○) or medium containing 10 μM isoproterenol (□) for 15 min. Crude membranes were subjected to sucrose gradient fractionation. Fractions were assayed for [3H]labeled cyano-pindolol binding (○ and □) and sucrose density (— — —). A representative of five experiments is shown. B, agonist-dependent association of the GRP catalytic subunit with sequestered β2AR. Membranes from fractions corresponding to Peak 1 (vesicles, V) or Peak 2 (plasma membrane, M) from A were solubilized and the FLAG-tagged β2AR was immunoprecipitated. Immunoprecipitated protein complexes (left panel) as well as samples of the fractions taken prior to immunoprecipitation (right panel) were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with antibody against the PP-2A catalytic subunit. An immunoblot of a representative of three experiments is shown.

from that sequestered within vesicles (Fig. 2A). Peak 1, localized near the top of the gradient, represented receptors localized to vesicles since these fractions were composed of clathrin coated vesicles, as determined by electron microscopy and immunoblotting (data not shown). Peak 2 represented plasma membrane-derived material, as determined by the presence of adenylate cyclase activity (data not shown). Incubating the cells with a β2AR agonist (10 μM isoproterenol), conditions which promote GRK-mediated β2AR phosphorylation (1, 10, 12), increases the quantity of β2AR present in the vesicular pool (Peak 1) from 5–10% to about 30% of the total β2AR present (Fig. 2A, ○ versus □), consistent with the occurrence of sequestration.

The association of plasma membrane or vesicular localized β2AR with phosphatase was subsequently examined (Fig. 2B). FLAG-tagged β2AR was immunoprecipitated from Peak 1 and Peak 2, corresponding to β2AR localized to vesicles or plasma membrane, respectively. Phosphatase associated with the immunoprecipitated receptor was visualized by immunoblotting, utilizing antibody that recognizes the catalytic subunit of PP-2A, a component of GRP (7). In the absence of isoproterenol treatment, no phosphatase was found associated with the β2AR either in the plasma membrane or in the vesicles (Fig. 2B, left panel), despite its presence in both fractions (Fig. 2B, right panel). The PP-2A present in these fractions represented GRP, as defined by its ability to dephosphorylate GRK2 phosphorylated β2AR (data not shown). When the cells were incubated with isoproterenol to phosphate transfected β2AR prior to harvesting, phosphatase was found associated with the β2AR specifically in the vesicles but not in the plasma membrane (Fig. 2B, left panel). Together, these data emphasize that the vesicular environment is critical for the association of receptor and phosphatase.

To determine specifically whether a decrease in pH, as occurs in endosomal vesicles containing sequestered β2AR, is a critical regulator of receptor dephosphorylation, we examined the effects of incubation of HEK293 cells with NH4Cl on receptor dephosphorylation and on the association of receptor with phosphatase (Fig. 3). NH4Cl is a weak base used to raise the pH of acidic cellular compartments such as endosomes and lysosomes (22, 23). The effect of NH4Cl on dephosphorylation of the β2AR is shown in Fig. 3A. In the absence of agonist, little phosphatase of the receptor was observed. Agonist stimulated a 3.5-fold increase in β2AR phosphorylation, which was unaffected by the presence of NH4Cl (Fig. 3A, time 0). Dephosphorylation of 42% of the phosphorylated β2AR occurred when agonist was removed, and the cells were incubated an additional 20 min in medium alone, corresponding to the time during which receptor resensitization occurs (10). In contrast, only 8% of the phosphorylated β2AR was dephosphorylated when NH4Cl was included in this 20-min incubation (Fig. 3A, time 20). Agonist-induced sequestration of receptors was not altered by the addition of NH4Cl (data not shown). This excludes the possibility that an inhibition of receptor sequestration was responsible for the decreased dephosphorylation observed in the presence of NH4Cl. Thus NH4Cl, an agent which inhibits vesicular acidification, significantly impairs β2AR dephosphorylation in HEK293 cells. In A431 cells, a recent paper by Pippig et al. (6) showed that monensin, a carboxylic ionophore that has also been used to increase vesicular pH (22, 23), inhibited resensitization but did not affect dephosphorylation of the β2AR in A431 cells. We used the weak base NH4Cl rather than monensin to avoid the possibility that previously reported...
Acidification Regulates β2-Adrenergic Receptor Dephosphorylation

Fig. 4. Model depicting the role of sequestration in β2AR resensitization. Details of the model are outlined in the text. “A” represents agonist. “PP” represents the sites of GRK phosphorylation on the carboxyl terminus of the receptor (31).

Effects of monensin, such as effects on Golgi morphology and vesicular trafficking (24–26), might influence the dephosphorylation observed.

Fig. 3B (left panel) shows that NH4Cl inhibits receptor dephosphorylation by inhibiting the interaction of GRP with the receptor. While the catalytic subunit of PP-2A was found associated with vesicular (sequestered) β2AR following incubation of cells with agonist alone (Fig. 3B, left panel, −NH4Cl), almost no interaction of GRP with β2AR was detected when sequestered receptor from cells treated with agonist and NH4Cl was subjected to immunoprecipitation (Fig. 3B, left panel, +NH4Cl). Expression and localization of the phosphatase appeared to be unaffected by NH4Cl treatment (Fig. 3B, right panel). These studies indicate that acidification is an important cellular mechanism for regulating the association of phosphatase with phosphorylated receptor and thus dephosphorylation.

These data suggest the following model for β2AR resensitization (Fig. 4). Upon agonist binding, the receptor becomes desensitized by GRK-mediated phosphorylation and the subsequent binding of β-arrestin. Recent data indicate that β-arrestin, in addition to its role in desensitization, is also involved in receptor sequestration (27). The receptor becomes sequestered into endosomes (13, 14) (Fig. 2A) which become acidic (15). Acidification facilitates receptor and GRP association in the vesicles (Fig. 2B) and the subsequent dephosphorylation of the β2AR (Fig. 3). Since the sites of phosphorylation on the receptor are not directly exposed to the decrease in pH within the endosome, how might this acidification stimulate dephosphorylation? Residues that are exposed to this decrease in pH include those that face the extracellular space when the receptors are localized in the plasma membrane. Protonation of these residues appears to produce a conformational change in the receptor that allows the receptor to be dephosphorylated. Residues on this face include those involved in agonist binding. It has been established that ligands of certain transmembrane receptors are dissociated in endosomal compartments upon acidification (23, 28). Perhaps a similar event occurs for the β2AR, producing a conformational change that causes the GRP to associate with the receptor and facilitate its dephosphorylation. Indicative of just such a specific conformational change within native β2AR is the markedly diminished effect of low pH on dephosphorylation of the denatured receptor and its inhibitory effect on GRP-mediated dephosphorylation of nonreceptor substrates (Fig. 1B). A change in receptor conformation facilitating GRP-mediated dephosphorylation of the receptor is analogous to the conformational requirement for agonist-depen-dent GRK-mediated phosphorylation of the β2AR. Following dephosphorylation, receptor is recycled back to the plasma membrane where agonist binding can initiate a new round of signal transduction (Fig. 4). This model may be applicable to a larger population of the G protein-coupled receptor superfamily since the Neurokinin receptor was recently shown to require vesicular acidification for receptor resensitization (29, 30).

The identification of acidification as a novel mechanism regulating β2AR dephosphorylation explains the requirement of sequestration for β2AR resensitization (6, 8–10). Sequestration facilitates the localization of the β2AR to an acidified compartment, where a conformationally altered receptor associates with the GRP and is dephosphorylated. These findings underscore the importance of receptor conformation in the regulation of both receptor phosphorylation as well as dephosphorylation. Whereas binding of agonist produces a receptor conformation that is readily phosphorylated by GRKs, an acidic pH environment produces a receptor conformation capable of being dephosphorylated by GRP.

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