A Key Role for Protein Kinase A in Homologous Desensitization of the $\beta_2$-Adrenergic Receptor Pathway in S49 Lymphoma Cells*

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We have used a $[^3H]$forskolin binding assay to assess $G_\alpha$-adenyl cyclase interactions in intact wild-type (WT) and kin$^{-}$ S49 cells under conditions that desensitize the $\beta_2$-adrenergic receptor ($\beta_2$-AR) system. This assay provides a measurement of $G_\alpha$-adenyl cyclase interaction that does not rely on the determination of second messenger accumulation or enzyme activity in broken cells. Kin$^{-}$ S49 cells lack protein kinase A (PKA) activity and provide a unique system in which to study the relative importance of this enzyme in $\beta_2$-AR desensitization. Although both WT and kin$^{-}$ S49 cells display similar kinetics of cAMP accumulation and agonist-induced cell-surface $\beta_2$-AR loss, we found that these cell types exhibited very different extents of desensitization of forskolin binding following agonist treatment. Specifically, 10 $\mu$m isoproterenol (37 °C, 30 min) induced the loss of 70% of $[^3H]$forskolin binding sites in WT cells but only 30% in kin$^{-}$ cells. This loss of sites in WT cells displayed a $t_1/2$ of $\sim$ 7 min, was agonist concentration-dependent (EC50 $\sim$ 60 nm), was not mimicked by 8-Br-cAMP, and could be blocked by the PKA inhibitor, H89. The difference between WT and kin$^{-}$ cells in agonist-induced desensitization of the $\beta_2$-AR pathway was also noted in studies of cAMP accumulation in cells. In addition, preincubation of intact cells with isoproterenol did not inhibit guanine nucleotide-dependent $[^3H]$forskolin binding in permeabilized cells. Overall, data obtained from $[^3H]$forskolin binding assays demonstrate the involvement of PKA in the agonist-dependent uncoupling of $\beta_2$-AR and $G_\alpha$; thus we conclude that PKA plays an important role in the homologous desensitization of the $\beta_2$-AR-$G_\alpha$-adenyl cyclase pathway in intact cells.

Desensitization of receptor-mediated cellular responses is a complex process which can involve multiple pathways including: (a) receptor uncoupling from G protein, (b) sequestration of receptors away from the plasma membrane, and (c) down-regulation of signaling proteins (1–4). It has been demonstrated that uncoupling of receptor and G protein occurs rapidly (within 10 min) in response to receptor activation and appears to involve receptor phosphorylation (5). In the case of the $\beta_2$-adrenergic receptor ($\beta_2$-AR)$^3$ system, agonist-occupied receptors activate the stimulatory G protein, $G_s$, which in turn promotes the production of cAMP by adenyl cyclase. Increased cAMP levels activate the cAMP-dependent protein kinase (PKA) resulting in the phosphorylation of several proteins, including the $\beta_2$-AR and other receptors. In addition, agonist-occupied $\beta_2$-AR are phosphorylated by one or more isoforms of the $\beta$-adrenergic receptor kinase ($\beta$-ARK, also termed G protein receptor kinase; GRK) resulting in the uncoupling of receptor and $G_s$ (6–10). Therefore, both PKA and $\beta$-ARK can contribute to desensitization of the $\beta_2$-AR system.

In contrast to the effect of $\beta$-ARK, PKA-mediated $\beta_2$-AR phosphorylation is most often associated with heterologous desensitization (that which is agonist nonspecific and therefore not dependent on $\beta_2$-AR occupancy), but is generally thought to be of little importance in homologous desensitization (agonist-specific and dependent on $\beta_2$-AR occupancy) (11–14). Therefore, while the $\beta_2$-AR can be phosphorylated by both PKA and $\beta$-ARK, most recent work has focused on the importance of $\beta$-ARK and related proteins (reviewed in Refs. 6 and 15–17) as mediators of homologous desensitization.

The kin$^{-}$ S49 murine lymphoma cell lacks PKA activity and thus provides a unique system in which to study the relative importance of PKA in mediating various cellular responses. Importantly, both WT and kin$^{-}$ S49 cells express only the $\beta_2$-AR isoform and display similar profiles of $\beta$-ARK agonist-induced desensitization of cAMP production and cell-surface $\beta_2$-AR loss (14, 18). The ability of the cell-permeable cAMP analog, 8-Br-cAMP, to mimic the effect of epinephrine in decreasing $\beta$-AR agonist-induced adenyl cyclase activity in membranes prepared from treated cells suggests that the effect of PKA activation is not dependent on the presence of agonist (11). These results suggest that PKA is not involved in homologous desensitization of the $\beta_2$-AR.

In the current studies, we have used a $[^3H]$forskolin binding assay to define more precisely the role of PKA in desensitizing the $\beta_2$-AR system in intact S49 cells. Since high-affinity forskolin interaction with adenyl cyclase requires the activation of $G_\alpha$, the amount of $[^3H]$forskolin associated with cells can be used as an index of $G_\alpha$-adenyl cyclase interaction (19–21). This assay, therefore, both provides a direct means of assessing hormone-stimulated $G_\alpha$-adenyl cyclase interaction in intact cells that is independent of second messenger accumulation and maintains components of the intracellular milieu important for regulating adenyl cyclase activity and that may be altered in membrane preparations. Using forskolin binding assays, we compared $G_\alpha$-adenyl cyclase interactions in WT and kin$^{-}$ S49 cells under conditions that desensitize the $\beta_2$-AR system. We reasoned that a decrease in the $\beta_2$-AR-stimulated $[^3H]$forskolin binding would reflect the functional desensitization.

The abbreviations used are: $\beta_2$-AR, $\beta_2$-adrenergic receptor; WT, wild-type S49 cells; PKA, protein kinase A; $\beta$-ARK, $\beta$-adrenergic receptor kinase; GRK, G protein-coupled receptor kinase; cAMP, 3',5'-cyclic adenosine monophosphate; PGE$_1$, prostaglandin E$_1$; PDE, phosphodiesterase; 8-Br-cAMP, 8-bromo-cAMP; GTP-$\gamma$S, guanosine 5'-3-O-(thio)triphosphate.
tion of the β2-AR pathway and that a difference in the extent of desensitization between WT and kin- cells would demonstrate the importance of PKA in this process. Using this [3H]forskolin binding assay, we found that, in contrast to previous studies (11–14), PKA plays an important role in homologous desensitization of the β2-AR.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Permeabilization—**Wild-type S49 lymphoma cells and kin- S49 clonal variants were used in these studies, the kin- variant lacks cAMP-dependent protein kinase activity (22, 23). All cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium containing 10% horse serum as described previously (24). Cells were maintained in logarithmic growth conditions (0.3–1.2 × 10⁶ cells/ml). Cell number and viability were determined using a Coulter ZBI cell counter/Channelizer. Prior to permeabilization, cells (1 × 10⁶/ml) were centrifuged (200 × g, 5 min, 4 °C) and suspended at 2 × 10⁶/ml in a “reverse” buffer (100 mM KCl, 20 mM NaCl, 1 mM Na₂HPO₄, 20 mM Hepes, 1 mM MgSO₄, pH 7.4). Saponin (10 μg/ml in Me₂SO) was added to the cell suspension at a final concentration of 30 μg/ml and cells incubated at 37 °C for 10 min. The cells were then centrifuged and suspended in reverse buffer. Using these conditions, ≥97% of cells were permeabilized as assessed by uptake of trypan blue.

[3H]Forskolin Binding—[3H]Forskolin binding was determined as described previously (19, 21). Briefly, intact S49 cells were incubated in Dulbecco’s modified Eagle’s medium buffered with 20 mM Hepes, pH 7.4 (DMEH) and permeabilized cells were incubated in reverse buffer. Following pretreatment (as indicated in figure legends), cells (5 × 10⁵) were incubated with 40 nM [3H]forskolin in the absence (total binding) or presence (nonspecific binding) of 10 μM unlabeled forskolin for 25 °C for 10 min. The cells were rapidly filtered over Whatman GF/C filters with 5 ml of ice-cold wash buffer (50 mM Tris-HCl, 10 mM MgCl₂, pH 7.4) and washed with an additional 20 ml of cold buffer. [3H]Forskolin remaining associated with cells was determined by scintillation counting of the filters.

CAMP Assays—Cells (1 × 10⁶) were incubated in the presence or absence of isoproterenol (1 μM) or forskolin (1 μM) for 30 min at 37 °C. Following this treatment, isobutylmethylxanthine (200 μM, final concentration, to inhibit phosphodiesterase activity) together with either buffer, isoproterenol (1 μM) or forskolin (1 μM), were added and incubations continued for an additional 7 min. Reactions were terminated by the addition of trichloroacetic acid (7.5% w/v final). The amount of cAMP in samples was quantitated by radioimmunoassay following the acetylation of aliquots according to manufacturer’s protocol (Calbiochem, La Jolla, CA).

Materials—Reagents were purchased from the following sources: [3H]forskolin from NEN DePont; forskolin and anti-CAMP antibody were from Calbiochem; saponin, isoproterenol, 8-Br-cAMP, and isobutylmethylxanthine were from Sigma.

**RESULTS**

In response to isoproterenol, WT and kin- S49 cells display a similar amount of [3H]forskolin binding (19, and data not shown). This increase is dependent upon β-AR-mediated activation of the stimulatory G protein, Gsα (19, 25). However, the preincubation of cells with isoproterenol (10 μM) for various times (5–60 min) WT and kin- cells differ in the extent to which the agonist promotes an increase in forskolin binding sites (Fig. 1). Specifically, whereas only 28 ± 3% of control isoproterenol-stimulated [3H]forskolin binding remained following a 60-min preincubation of WT S49 cells with agonist, kin- S49 cells retained 64 ± 7% of these sites (p < 0.05 versus WT). In each case, the extent of desensitization of forskolin binding reached maximum by about 30 min of incubation with agonist and displayed a t₁/₂ ∼ 7 min. When WT cells were treated with the PKA inhibitor H89 (3 μM) the extent of isoproterenol-induced desensitization of [3H]forskolin binding was similar to that observed for kin- cells (Table I). Importantly, this concentration of H89 had no effect either on the activity of recombinant GRK2 (β-ARK₂, data not shown) or on the extent of desensitization observed in kin- cells (data not shown).

Since PKA activation is dependent on cAMP production, we compared the concentration dependence for isoproterenol-induced desensitization of forskolin binding with that previously described for isoproterenol-stimulated cAMP production (25, 26). Importantly, the ability of isoproterenol to induce desensitization of [3H]forskolin binding (EC₅₀ = 56.5 ± 2.1 μM) parallels its ability to stimulate cAMP production (cf. Fig. 2 and Refs. 25 and 26). Together with data presented in Fig. 1, these results indicate that the different extents of isoproterenol-induced desensitization of [3H]forskolin binding observed in WT and kin- cells results from cAMP-dependent activation of PKA. Furthermore, PKA activity is responsible for about 50% (% desensitization in WT − % desensitization in kin-) of desensitization in WT × 100%; Fig. 1) of the total desensitization of [3H]forskolin binding observed in intact WT S49 cells; the desensitization occurring in kin- cells is presumably due to the activity of β-ARK and related proteins (5).

To determine if results from isoproterenol-induced desensi-
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Desensitization of [3H]forskolin binding assays correlate with an effect on second messenger production, we compared cAMP accumulation in WT and kin- cells preincubated with either isoproterenol or forskolin (Fig. 3). Following 30 min of treatment with isoproterenol (1 μM), WT cells displayed a much greater decrease in cAMP production following the addition of forskolin (1 μM) compared to kin- cells (40 versus 70% of control cAMP production remaining for WT and kin-, respectively; p = 0.03). Treatment of cells with forskolin (1 μM) did not inhibit cAMP production following the addition of isoproterenol (1 μM), indicating that the desensitization of cAMP accumulation depends on the presence of isoproterenol in the preincubation. These results are consistent with results obtained from [3H]forskolin binding assays in that WT cells display a greater extent of agonist-dependent desensitization relative to kin- cells. Thus, we conclude that β2-AR-induced desensitization of both forskolin binding and cAMP production is dependent on the activity of PKA.

While the above data demonstrate the importance of PKA in β2-AR-induced desensitization of Gαs-adenyl cyclase interaction, they do not indicate the site(s) or mechanism (heterologous versus homologous) by which this effect occurs. The β2-AR contains two consensus PKA phosphorylation sequences and thus could represent one site for PKA-mediated desensitization (27). In addition, it has been suggested that certain isoforms of adenyl cyclase are subject to direct regulation by PKA (28). This is particularly important in that one such isoform, Type VI, is expressed in S49 cells (29). To test whether PKA-mediated desensitization of the β2-AR pathway in WT S49 cells occurs via homologous or heterologous pathways, we tested (a) the ability of guanine nucleotide to stimulate [3H]forskolin binding in saponin-permeabilized cells following isoproterenol pretreatment, and (b) the ability of agonists that increase cAMP, and hence activate PKA, to mimic the effect of isoproterenol preincubation on [3H]forskolin binding in intact WT cells.

To determine if the effect of isoproterenol preincubation on subsequent [3H]forskolin binding results from alterations in the ability of Gox to bind guanine nucleotides or interact with adenyl cyclase, we examined GTPγS-stimulated [3H]forskolin binding in saponin-permeabilized WT S49 cells. As shown in Fig. 4, isoproterenol (10 μM) did not increase [3H]forskolin binding in permeabilized cells. However, [3H]forskolin binding in permeabilized cells was increased by the addition of GTPγS (30 μM) either alone or with isoproterenol. While isoproterenol did not affect the total amount of [3H]forskolin bound in the presence of GTPγS, the agonist decreased the time required to achieve the full extent of forskolin binding (data not shown).
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The amount of guanine nucleotide-stimulated [3H]forskolin binding in permeabilized cells was similar to that observed in intact cells incubated in the presence of isoproterenol. Incubation of cells with isoproterenol (10 M, 60 min) prior to permeabilization did not affect the amount of [3H]forskolin bound in the presence of GTPγS following permeabilization. Since isoproterenol affected the rate of [3H]forskolin binding to permeabilized cells, the concentration of isoproterenol (10 M) used in the preincubation of intact cells was maintained throughout permeabilization and subsequent incubation with [3H]forskolin in order to avoid dissociation of the $\beta_2$-AR agonist from receptor. These results indicate that the ability of Ga$_s$ to bind guanine nucleotide and to interact with adenyl cyclase is not impaired by isoproterenol preincubation. Therefore, PKA-mediated desensitization of [3H]forskolin binding observed in WT cells appears not to result from PKA-mediated effect on Ga$_s$ or adenyl cyclase but rather from a functional uncoupling of the $\beta_2$-AR and G$_s$.

Since PKA-mediated phosphorylation of receptors has been associated with heterologous desensitization (11–14), we reasoned that increasing intracellular cAMP levels (thereby activating PKA) with cell-permeable cAMP analogs and phosphodiesterase inhibitors should mimic the effect of isoproterenol preincubation on [3H]forskolin binding. As shown in Table I, preincubation of WT S49 cells with 8-Br-cAMP (1 mM) and phosphodiesterase inhibitors (isobutylmethylxanthine (100 M) + RO201724 (100 M)) for 60 min did not alter the amount of [3H]forskolin bound following the addition of isoproterenol. These results agree with those obtained from cAMP assays showing that preincubation of WT cells with forskolin does not induce desensitization of the $\beta_2$-AR response (see Fig. 3). Heterologous desensitization of $\beta_2$-AR-stimulated [3H]forskolin binding and cAMP production following treatment with agents which increase intracellular cAMP levels was not observed under these conditions. Heterologous desensitization in intact cells is often manifest by an increase in the concentration of hormone required to elicit a response without changes in the maximal effect of the agonist (11). For this reason, the conditions used in these experiments examining maximal $\beta_2$-AR-stimulated Ga$_s$-AC interaction and cAMP production presumably would not detect heterologous desensitization. Overall, we conclude that increasing cAMP does not mimic the isoproterenol-induced desensitization of the $\beta_2$-AR system in WT S49 cells, suggesting that PKA is involved in agonist-dependent (i.e. homologous) $\beta_2$-AR desensitization.

To examine further the role of PKA in homologous receptor desensitization, we compared the ability of prostaglandin E, another receptor agonist that increases cAMP in S49 cells, with the ability of isoproterenol to stimulate [3H]forskolin binding in cells pretreated with either prostaglandin E, or isoproterenol. As shown in Fig. 5, isoproterenol-stimulated forskolin binding was inhibited by about 70% following pretreatment of cells with isoproterenol (10 M, 60 min; * p < 0.05 versus control). Similarly, preincubation of cells with PGE$_1$ (30 M, 60 min) inhibited subsequent PGE$_1$-stimulated [3H]forskolin binding by ~75% (*, p < 0.05 versus control). In contrast, isoproterenol stimulation of [3H]forskolin binding was only slightly (~25%; p > 0.05) inhibited by pretreatment of cells with PGE$_1$. Thus, the desensitization of [3H]forskolin binding following either isoproterenol or PGE$_1$ pretreatment is specific for the $\beta_2$-AR and prostaglandin receptor, respectively. Together with data presented above, our results demonstrate that in WT S49 cells PKA serves an important role in homologous receptor desensitization.

**DISCUSSION**

Desensitization of receptor-mediated cellular responses is a complex process that can involve multiple pathways. For the $\beta_2$-AR, phosphorylation by both PKA and $\beta$-ARK can uncouple receptor and G protein (7, 10, 30, 31). It has been suggested that the ability of PKA to desensitize the $\beta_2$-AR seemingly does not depend on receptor occupancy by agonist and therefore mediates heterologous desensitization (7, 11, 13, 32). In contrast, phosphorylation of $\beta_2$-AR by $\beta$-ARK depends on receptor occupancy and occurs only in the presence of $\beta_2$-AR agonists (5–7, 9). Thus, in contrast to PKA, $\beta$-ARK-mediated phosphorylation is generally thought to be critical for homologous desensitization of the $\beta_2$-AR.

We examined activation and subsequent desensitization of the $\beta_2$-AR-G$_s$-adenyl cyclase system in intact cells using an approach different than previous studies (i.e. adenyl cyclase enzyme assays in membranes). This is of particular physiological importance since adenyl cyclase activity is regulated by a complex network of interacting pathways (for review, see Refs. 33–37). For example, adenyl cyclase isoforms present in S49 cells, Type VI and Type VII (29, 38), are regulated by Ca$^{2+}$, PKA, PKC, G$_{o-s}$, and G$_{o-i}$ (28, 38, 39 and references therein). Furthermore, the ability to detect receptor desensitization using adenyl cyclase assays in membranes is influenced by Mg$^{2+}$ concentration (31, 40, 41). Thus, multiple modulators of adenyl cyclase activity can be affected during the preparation and treatment of membranes.

Assessment of forskolin binding provides a means to examine the role of PKA in $\beta_2$-AR-mediated desensitization that circumvents the problems associated with determining adenyl cyclase activity in membranes. The interaction of forskolin with adenyl cyclase is enhanced by the activation of G$_s$ (19, 37, 42, 43). Therefore, the amount of [3H]forskolin associated with cells following agonist treatment can be used as an index of G$_s$-adenyl cyclase interaction (19–21). Using this assay, we show here that WT and kin$^-$/S49 cells display very different
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extents of receptor-mediated desensitization. This is, whereas about 70% of isoproterenol-stimulated[^3H]forskolin binding sites desensitize in WT cells, only about 30% undergo desensitization in the absence of PKA (i.e., in kin^- cells) or in the presence of a PKA inhibitor. This desensitization is detectable on receptor occupancy, as evidenced by the inability of PGE1, and other agents that raise cAMP to mimic the effect of isoproterenol. Moreover, treatment of intact WT cells with isoproterenol did not effect the amount of guanine nucleotide-dependent forskolin binding following permeabilization of cells. In agreement with[^3H]forskolin binding results, WT cells exhibited a greater extent of agonist-dependent desensitization of cAMP production than kin^- cells. Overall, these results argue that PKA is important for homologous (i.e., agonist-specific) desensitization of the β2-AR pathway in WT S49 cells.

Several lines of evidence in both WT and kin^- S49 cells suggest that PKA-mediated phosphorylation of β2-AR is important for heterologous β2-AR desensitization and that β-ARK mediates homologous desensitization. For example, membranes prepared from WT cells treated with agents that increase cAMP levels (and hence the activity of PKA) display decreased epinephrine-stimulated adenyl cyclase activity relative to membranes from untreated cells (11). Likewise, treatment of WT S49 cell membranes with PKA in vitro inhibited epinephrine-stimulated adenyl cyclase activity (32). However, there was no additional effect of PKA treatment on adenyl cyclase activity in membranes prepared from WT cells previously treated with β-AR agonist. These results suggest that activation of PKA either by β-AR agonists or by heterologous pathways has a similar effect on β2-AR desensitization and that this desensitization can occur independent of receptor occupancy. In contrast, treatment of WT or kin^- cells with isoproterenol reduced to the same extent both receptor affinity for agonist and isoproterenol-stimulated adenyl cyclase activity in membranes prepared from treated cells (5, 14, 44). These effects were dependent on the presence of β-AR agonist. Since β2-AR-stimulated adenyl cyclase activity was inhibited equally in both WT and kin^- cells, it was concluded that homologous desensitization was mediated by a receptor-specific kinase and not by PKA. Taken together, these results support the conclusion that PKA and β-ARK mediate heterologous and homologous β2-AR desensitization respectively.

Previous results, however, have not conclusively demonstrated an important role for PKA in homologous β2-AR desensitization. For example, while Shih and Malbon show that isoproterenol treatment of several cell lines decreased the subsequent cAMP response elicited following agonist rechallenge and that the extent of inhibition was substantially reversed in cells treated with PKA antisense cDNA, the requirement for β2-AR occupancy on this desensitization was not investigated (45). In other studies, Liggett et al. demonstrated that cells expressing the wild-type β2-AR displayed a rapid decrease in the rate of cAMP accumulation after 2 min of agonist stimulation, whereas the increased rate of cAMP production induced by agonist was prolonged in cells expressing β2-AR mutant constructs lacking consensus sites for PKA phosphorylation. Different studies with β2-AR constructs have suggested that mutation of either the putative PKA or β-ARK consensus phosphorylation sites can reduce β-AR agonist-mediated desensitization of adenyl cyclase activity (47). In contrast, other studies have shown that deletion of putative PKA phosphorylation sites in the β2-AR inhibited heterologous but not homologous receptor desensitization (13). These seemingly contradictory observations can be reconciled by noting that these mutated β2-AR receptors contain multiple mutations in different domains of the receptor which could indirectly affect receptor function and desensitization (48). Moreover, the extent of receptor expression can substantially alter β-AR desensitization (49). We believe our data obtained using[^3H]forskolin binding assays are the first to demonstrate a role for PKA in homologous β2-AR desensitization in a native cell.

Overall, the data presented here indicate that assessment of[^3H]forskolin binding provides a useful measure to examine desensitization of β2-AR-induced responses in S49 cells. Results from forskolin binding studies indicate that β-AR-induced PKA activation promotes an uncoupling of the β2-AR receptor and Gs, thereby reducing the responsiveness of this system. The inability of cAMP analogs and forskolin to mimic this effect and the ability of guanine nucleotide to stimulate[^3H]forskolin binding in permeabilized cells pretreated with isoproterenol further suggest that PKA is important for homologous desensitization of the β2-AR-Gs-adenylyl cyclase system. Taken together with previous results, our data are consistent with a model whereby the concerted phosphorylation of receptor by both PKA and one or more forms of GRK is important for the homologous desensitization of the β2-AR system observed in intact cells.

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