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Short Term Desensitization of the A_1 Adenosine Receptors in DDT_1MF-2 Cells

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SUMMARY

Previous studies have indicated that desensitization of the A_1 adenosine receptor (A_1AR), unlike other adenosine receptor subtypes and G protein-coupled receptors, required prolonged exposure to agonists. We more closely studied this observation by focusing on changes in the A_1AR signal transduction pathway after short term agonist exposure (0.5–4 hr) in the hamster vas deferens smooth muscle cell line (DDT_1MF-2 cells). Incubation of these cells with 1 μM (*R*)-phenylisopropyladenosine [(*R*)-PIA] produced a time-dependent loss in binding of the agonist radioligand [^{125}I]N^6-2-(4-amino-3-iodophenyl)ethyladenosine but not of the antagonist radioligand [^{3}H]8-cyclopentyl-1,3-dipropylxanthine. This was accompanied by a reduction in the high affinity (G protein-coupled) state of this receptor from 63 $\pm$ 8% to 37 $\pm$ 12% after treatment for 4 hr. Moreover, cells treated with (*R*)-PIA demonstrated reduced agonist-stimulated GTPase activity and diminished inhibition of adenylyl cyclase activity but no change in expression of α_i and β subunits. The decreases in agonist binding in the desensitized cells were reversible after treatment of DDT_1MF-2 cell membranes with alkaline phosphatase or protein phosphatases 1 and 2A, suggesting a role of phosphorylation in the uncoupling and desensitization of the A_1AR. Incubation of cells with (*R*)-PIA led to rapid translocation of G protein-coupled receptor kinase (GRK) from the cytosol to the plasma membrane within 1 hr of exposure. In addition, purified preparations of the A_1AR that were phosphorylated with purified recombinant GRK-2 demonstrated enhanced affinity for arrestin over G_i/G_o. These results indicate rapid and functional desensitization of the A_1AR by brief exposure to agonist. The mechanism underlying this event seems to involve phosphorylation of the A_1AR, presumably by the GRK or GRKs.

Adenosine, a metabolite of ATP, produces profound effects on the central and peripheral nervous, cardiovascular, and immune systems. These effects of adenosine are mediated, in part, by activation of cell surface ARs. Four subtypes of ARs (A_1, A_{2a}, A_{2b}, A_3) have been identified (1). Activation of the A_1AR has generally been implicated in the inhibition of adenylate cyclase activity via a pertussis toxin-sensitive G protein. In some tissues, this receptor subtype also couples to inhibition of the voltage-sensitive Ca^{2+} channel and activation of the K^+ channel, the low-K_m cAMP phosphodiesterase guanylate cyclase, and phospholipase C (2).

It has generally been recognized that prolonged activation of the A_1AR triggers subsensitivity of the receptor to subsequent agonist challenge, a phenomenon termed desensitization. Examples of desensitization of the A_1AR were described *in vivo* in the rat adipocyte (3, 4), cultured rat adipocytes (5), cardiac myocytes (6, 7), cells transfected with the A_1AR (8), and the DDT_1MF-2 clonal cell line (9). It was generally believed that desensitization of the A_1AR differed from the A_{2a}AR because of its relatively slower rate (9). This slower rate of desensitization might be important *in vivo* during ischemia by helping to maintain adequate tissue perfusion in presence of elevated levels of adenosine.

Desensitization of the adipocyte A_1AR after prolonged *in vivo* agonist exposure could be explained in part by a decrease in receptor number, decrease in the expression of G_i protein α subunits, and uncoupling of these receptors from G protein (3). However, in the DDT_1MF-2 cell culture, exposure to (*R*)-PIA led to a decrease in the number of A_1AR without any significant loss in G protein expression. The difference in response observed could be due to differences in cell types or might be attributed to hormonal changes that accompany *in vivo* administration of (*R*)-PIA. Phosphorylation of A_1AR has been described in cells desensitized to (*R*)-PIA (9). More recent studies using purified preparations of A_1AR have implicated GRK-2 in phosphorylation and uncoupling of the A_1AR from G proteins (10). The cloned canine, rat, and bovine A_1ARs possess a potential phosphorylation site for GRK-2 in the third cytoplasmic loop, a serine moiety flanked

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ABBREVIATIONS: AR, adenosine receptor; APNEA, N^6-2-(4-amino-3-iodophenyl)ethyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; (*R*)-PIA, (*R*)-N^6-phenylisopropyladenosine; GRK, G protein-coupled receptor kinase.

on the amino-terminal side by glutamic acid (11, 12). Despite this evidence, Palmer *et al.* (8) were not able to demonstrate phosphorylation of the epitope-tagged A_1AR in Chinese hamster ovary cells after short term (1-hr) agonist exposure.

In the current study, we focused on three issues. First, we assessed whether agonist exposure leads to rapid uncoupling and desensitization of the A_1AR. Second, we determined the role of phosphorylation of the A_1AR in the process of uncoupling and desensitization. Third, we determined the potential involvement of GRKs in these processes.

Experimental Procedures

Materials. HEPES, Tris·HCl, soybean trypsin inhibitor, pepstatin, benzamidine, and adenosine were from Sigma Chemical (St. Louis, MO). Cell culture supplies were obtained from GIBCO BRL (Grand Island, NY). (*R*)-PIA and adenosine deaminase were from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Electrophoresis reagents were from BioRad Laboratories (Hercules, CA). [^{3}H]DPCPX (160 Ci/mmol), [α-^{32}P]ATP (30 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), [γ-^{32}P]GTP, and [^{125}I]Na were from DuPont-New England Nuclear (Boston, MA). Alkaline phosphatase and protein phosphatase types 1 and 2A were from Calbiochem (La Jolla, CA). Purified G proteins were from Dr. Pat Casey (Duke University Medical Center, Durham, NC). Purified rhodopsin and arrestin were from Dr. Paul Hargrave (University of Florida, Gainesville, FL). Antibodies against GRK-2 were from Santa Cruz Biochemicals (Santa Cruz, CA). G protein α and β subunit antibodies were kindly provided by Dr. Tom Gettys (Medical University of South Carolina, Charleston, SC). Recombinant GRK-2 was obtained from Dr. Jeff Benovic (Thomas Jefferson University, Philadelphia, PA). All other reagents were of the highest available grade and were purchased from standard sources.

Cell culture. DDT_1MF-2 cells were grown as monolayers in 75-cm^2 flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were grown at 37° in the presence of 5% CO_2/95% ambient air. The flasks were supplemented with fresh media 12 hr before agonist treatment, and adenosine deaminase (0.3 unit/ml) was added 1 hr before agonist treatment. For agonist exposure, (*R*)-PIA (1 μM) was added to the culture media for the indicated period of time. After agonist exposure, the flasks were placed on ice, and the same concentration of (*R*)-PIA was added to control flasks for 3 min. Cells were then washed three times (20 ml each) with Krebs' phosphate buffer (128 mM NaCl, 1.4 mM $MgCl_2$, 10 mM Na_2HPO_4, pH 7.4) and pelleted by centrifugation at 1000 × *g*.

Membrane preparation. The cells were lysed in buffer A (50 mM Tris·HCl buffer containing 10 mM $MgCl_2$, 1 mM EDTA), 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine, and 2 μg/ml pepstatin. Cells were then homogenized briefly with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at setting 7 for 25 sec and centrifuged at 1,000 × g for 5 min. The supernatant was then centrifuged at 40,000 × g for 10 min, and the resulting pellet was resuspended in buffer A containing protease inhibitors, as described above. Membranes were then incubated with adenosine deaminase (3 units/ml) at 37° for 15 min to degrade the endogenous adenosine before the radioligand binding assay was performed.

[^{3}H]DPCPX binding assay. Membranes (75–100 μg/assay tube) were incubated with increasing concentrations of [^{3}H]DPCPX (0.5–12 nM) in a total volume of 250 μl of buffer A at 37° for 1 hr. Theophylline (1 mM) was used to define nonspecific binding, which usually ranged from 10% to 40% of the total binding with increasing concentrations of the radioligand. After incubation, membranes were filtered through GF/B glass-fiber filters using a cell harvester (Brandel, Gaithersburg, MD) and washed with 9 ml of ice-cold buffer containing 0.01% CHAPS. Filters were allowed to extract overnight in toluene-based scintillation fluid before counting. For competition binding, increasing concentrations of (*R*)-PIA were used to inhibit the binding of [^{3}H]DPCPX (1.0 nM).

[^{125}I]APNEA binding assay. Radioligand binding experiments were performed similarly to [^{3}H]DPCPX binding, with concentrations of [^{125}I]APNEA for saturation binding ranging from 0.5 to 27 nM. To attain these high concentrations of the radioligand, the specific activity of the [^{125}I]APNEA was diluted 10-fold with [I]APNEA.

Gel electrophoresis. Electrophoresis was performed according to the method of Laemmli (13) using homogenous gels, with the stacking gel containing 3% acrylamide and the separating gel containing 12% acrylamide. Electrophoresis was performed at a constant current at 7.5 mA. Premixed SDS-PAGE standards were iodinated using the chloramine T method. These standards contain albumin (M_r 66,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 29,000), trypsinogen (M_r 24,000), and soybean trypsin inhibitor (M_r 20,000). After electrophoresis, gels were dried using a BioRad gel dryer and exposed to X-ray film [XA(R)-5] in a cassette containing Cronex Lightening Plus intensifying screens for 1–2 days at −80°.

Adenylyl cyclase assay. Membranes were prepared from both controls and cells treated with (*R*)-PIA (1 μM, 4 hr) and pretreated with adenosine deaminase. Twenty microliters of membrane preparations (~50 μg of protein) was incubated with 20 μl of a reaction mixture containing 0.14 mM ATP, 5 mM phosphocreatine, 1 μM cAMP, 30 units/ml creatine phosphokinase, 5 μM GTP, ~1.5 μCi of [α-^{32}P]ATP, and 10 μl of water, forskolin (10 μM), or (*R*)-PIA. Papaverine (0.1 mM) was included in all assay tubes to inhibit the low-K_m cAMP phosphodiesterase. Assay tubes were incubated at 30° for 15 min, and the reaction was terminated by addition of 1 ml of ice-cold stop solution containing [^{3}H]cAMP (~15,000 cpm), 0.3 mM cAMP, and 0.4 mM ATP. cAMP was isolated as previously described (14).

GTPase assay. GTPase activity stimulated by AR agonist was determined as previously described (10). Briefly, 50 μl of membranes (25 μg of protein) from control and (*R*)-PIA-treated cells were incubated with 20 μl of buffer or AR agonist and 50 μl of reaction mixture, which consisted of 20 mM Tris, pH 8.0, 10 mM $MgCl_2$, 2 mM EDTA, 1 mg/ml bovine serum albumin, 2 mM dithiothreitol, 2 mM APP(NH)p, and 0.4 μM [γ-^{32}P]GTP. Incubations were for 30 min at 30° and were terminated by the addition of 500 μl of 5% ice-cold charcoal mixture. The samples were then centrifuged for 3 min in a microfuge tube at 4°. The supernatants (450 μl) were placed in 16 × 150-mm glass tubes containing 1.6 ml of molybdate solution (52 mM sodium molybdate in 1 M HCl) and briefly vortexed. Two milliliters of benzene/isobutanol mixture (1:1) was then added to the tubes, and these were vortexed for 20 sec. The upper organic layers (1.4 ml) were removed, added to 15 ml of scintillation fluid, and counted with the use of a β-counter.

Determination of the activity of GRKs. DDT_1MF-2 cells were treated without or with (*R*)-PIA (1 μM) for 1 hr, washed thoroughly in phosphate-buffered saline, rapidly lysed, and gently homogenized in a hypotonic solution containing 10 mM Tris, pH 7.4, 5 mM EDTA, 2 μg/ml pepstatin, 5 μg/ml benzamidine, and 5 μg/ml soybean trypsin inhibitor. Membrane and cytosolic fractions were prepared by centrifugation at 40,000 × g for 15 min. Cytosolic and particulate fractions (10 μl) were incubated in a final volume of 50 μl for 1 hr at 30° in the presence of 20 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 100 μM [γ-^{32}P]ATP (1 cpm/fmol), and 10 pmol of rhodopsin. The reactions were terminated by the addition of SDS-PAGE buffer and electrophoresed on a 12% acrylamide gel. The dried gel was subjected to autoradiography, after which the rhodopsin bands were excised and the radioactivity was measured in a scintillation counter to determine the relative GRK activities in cytosol and membrane.

Western blotting experiments. DDT_1 MF-2 cell membranes were electrophoresed on a 12% acrylamide gel, and the proteins were transferred to nitrocellulose filters using a Nova Blot apparatus (Pharmacia, Piscataway, NJ). Filters were blocked with Blotto (130 mM NaCl, 2.7 mM KCl, 1.8 mM Na_2HPO_4, 1.5 mM KH_3PO_4, 0.1% NaN_3, and 5% low-fat skim milk) and then incubated at 4° overnight with a specific antibodies against G protein α and β subunits and

GRK-2. The blots were then washed five times (10 min each) with Blotto and incubated with ^{125}I-labeled goat-anti-rabbit IgG for 1 hr at room temperature. This was followed by five washes (10 min each) with Blotto containing 1% Triton X-100 before exposure to autoradiographic films.

Protein determination and data analysis. Saturation curves were analyzed using Prism (GraphPAD Software, San Diego, CA), and competition curves were analyzed by a computer-assisted curve-fitting program (15, 16) equipped with a statistical package. Other statistical analyses were performed using the Student's *t* test and analysis of variance. Error bars shown in the text and in the figures represent standard errors. Protein concentrations were determined according to the method of Bradford (17), using bovine serum albumin as standard.

Results

Short term agonist exposure uncouples A_1AR. Pretreatment of DDT_1MF-2 cells with (*R*)-PIA (1 μM) led to a time-dependent loss in the binding of the agonist [^{125}I]APNEA. The effect on binding was observed 30 min after (*R*)-PIA exposure and progressed to a 45% decrease by 4 hr (Fig. 1). Withdrawal of (*R*)-PIA from the culture media led to a time-dependent restoration of [^{125}I]APNEA binding to that observed in the control membranes. To further characterize the changes induced by (*R*)-PIA, full [^{125}I]APNEA saturation curves were performed. Cells exposed to (*R*)-PIA for 4 hr showed a significant reduction in maximum binding sites (B_{max}) for [^{125}I]APNEA from a control level of 342 ± 82 to 251 ± 67 fmol/mg of protein, whereas the equilibrium dissociation constant (K_d) were not statistically different (Fig. 2, *top*). K_d values were 7.8 ± 2.2 and 7.2 ± 2.2 nM for control and (*R*)-PIA-treated preparations, respectively.

To determine whether the decrease in [^{125}I]APNEA binding reflects a true loss in receptor number or uncoupling of the receptor from its G protein, quantification of the A_1AR with an antagonist radioligand was initiated. Unlike the agonist, the antagonist binding does not require receptor coupling to G protein. The A_1AR-selective antagonist radioligand [^{3}H]DPCPX was used for this purpose. Saturation binding assays using [^{3}H]DPCPX showed similar levels of A_1AR in the control and treated cells (Fig. 2, *bottom*). If anything, [^{3}H]DPCPX binding was higher in the treated group, with B_{max} values of 465 ± 92 and 569 ± 139 fmol/mg of protein in control and (*R*)-PIA-treated cells, respectively. This is likely the result of uncoupling of the A_1AR from its G proteins because it has been shown that guanine nucleotide-mediated uncoupling of this receptor subtype leads to increased antagonist binding (18–20). Furthermore, no significant change in K_d was observed. K_d values were 2.3 ± 0.5 and 3.4 ± 0.9 nM for control and (*R*)-PIA-treated preparations, respectively. Thus, the reduced agonist binding after (*R*)-PIA exposure likely reflects uncoupling of A_1AR from G_i proteins and not from reduction in the number of these receptors.

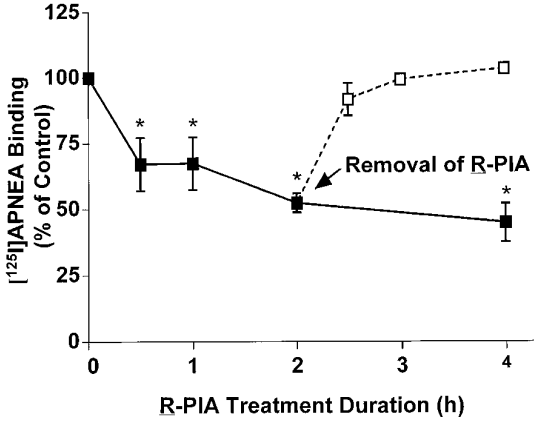

Fig. 1. Time course of the decrease in [^{125}I]APNEA binding sites initiated by agonist exposure. Membranes were prepared from controls and cells exposed to (*R*)-PIA (1 μM) for different time periods up to 4 hr. Membranes were incubated with 1 nM [^{125}I]APNEA (undiluted) in the absence (nonspecific binding) or presence (nonspecific binding) of 1 mM theophylline. Values are mean ± standard error of six independent experiments, each performed in triplicate. *, $p < 0.05$, statistically significant difference from control (Student's *t* test).

To exclude the possibility that the decrease in [^{125}I]APNEA binding was due to competition from residual (*R*)-PIA remaining on the membranes even after washing, several steps were taken. First, (*R*)-PIA (1 μM) was added to the media of control cells just before detachment of the cells. Under this condition, the difference between control and treated cells was maintained. Second, pretreatment of the cells with the agonist plus antagonist [either 8-(4-[(([2-amino-ethyl-amino]carbonyl)methyl)oxyl]phenyl)-1,3-dipropylxanthine or theophylline] blocked the effect of the (*R*)-PIA administered alone (data not shown).

The uncoupling of the A_1AR required the presence of both the agonist [(*R*)-PIA] and a physiological temperature (37°). For example, cells exposed to (*R*)-PIA (at 37°) together with theophylline (1 mM) led to a blockade of agonist-mediated uncoupling of the A_1AR. In addition, incubation of the cells with (*R*)-PIA at 4° abrogated uncoupling of the A_1AR (data not shown).

To further characterize the change of A_1AR after short term agonist exposure, agonist competition of [^{3}H]DPCPX binding was performed using (*R*)-PIA. In control membranes, (*R*)-PIA competition curves were more appropriately fitted to a two-state model with equilibrium dissociation constants of the high (K_H) and low (K_L) affinity states being 1.5 ± 0.4 and 392 ± 49 nM, respectively, and the percentage of A_1AR in the high affinity state (R_H) detected was 63 ± 8% (Table 1). Exposure to (*R*)-PIA for 4 hr resulted in a rightward shift in the competition curves (Fig. 3). The K_H value was unchanged at 3.4 ± 1.3 nM, whereas the R_H value was significantly decreased to 37 ± 12% ($p < 0.05$) after (*R*)-PIA exposure (Table 1). No significant change in K_L value was observed, with values in control and (*R*)-PIA-treated cells of 414 ± 38 and 392 ± 49 nM, respectively.

To test whether uncoupling results from inefficiency in A_1AR/G protein coupling, membranes from both control and (*R*)-PIA-treated cells were solubilized with CHAPS, as previously described (10), and then reconstituted with purified preparations of a mixture of bovine brain G_i/G_o (1.5 nM final concentration). As can be seen in Fig. 4, membranes obtained from cells exposed to (*R*)-PIA showed a decrease in [^{125}I]APNEA binding. The addition of G_i/G_o to these preparations reversed the binding toward control levels without affecting the level of binding in the membranes prepared from control cells. This finding suggests that agonist exposure renders the A_1AR in these cells less able to interact with their endogenous G proteins but that the addition of a stoichiometric excess of G proteins "forced" coupling of these receptors. No change in the expression of the G_i α or β subunits were

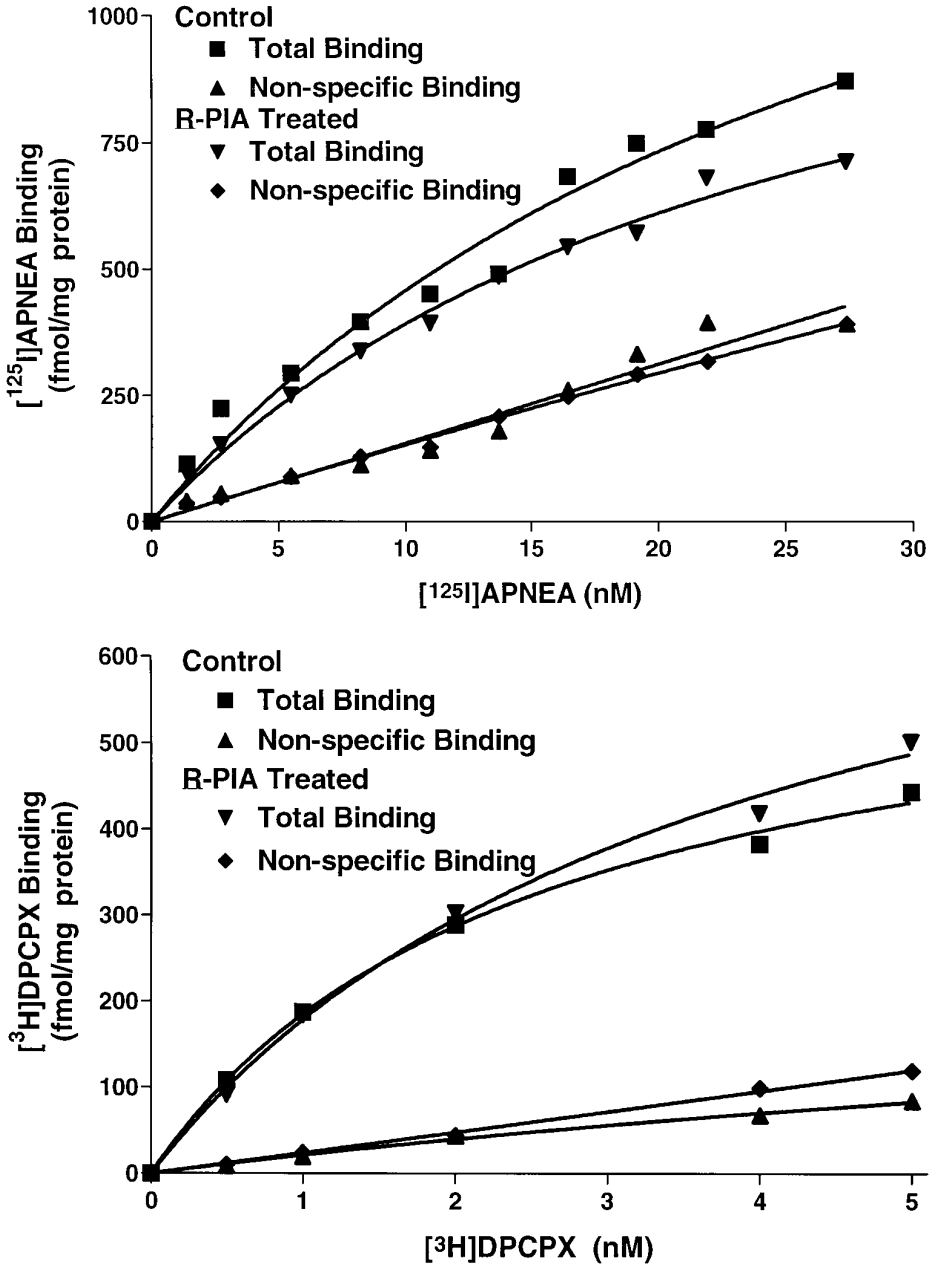

Fig. 2. Changes in A_1AR number after agonist treatment. *Top*, loss of A_1AR high affinity sites. DDT_1MF-2 cells were treated without or with (*R*)-PIA (1 μM, 4 hr) at 37°, and plasma membranes were prepared from each group by differential centrifugation. [^{125}I]APNEA was diluted 10-fold with [I]APNEA and used to determine the level of high affinity A_1AR binding sites. Nonspecific binding was defined using 1 mM theophylline. The curves were fitted by Prism (GraphPAD Software) using a one-site fit. Results are representative of four experiments with similar results. *Bottom*, lack of change in the number of the A_1AR sites after short term agonist exposure. Membranes were incubated with [^{3}H]DPCPX in the absence and presence of 1 mM theophylline. Experimental procedures were the same as described above. Results are representative of four similar experiments with similar results.

detected in membrane preparations obtained from untreated cells compared with cells previously exposed to (*R*)-PIA for 4 hr. Levels (mean ± standard error) of $G_{\alpha i1,2}$, $G_{\alpha i3}$, and G_β in the treated preparations were 102 ± 11%, 129 ± 19%, and 127 ± 11% of control (untreated), respectively.

Functional consequences of uncoupling of the A_1AR. To determine whether uncoupling of the A_1AR/G protein complex was manifested as a deficit in receptor signaling processes, agonist-stimulated GTPase activity was determined. In membrane preparations, (*R*)-PIA produced dose-dependent stimulation of GTPase activity that was reversed with theophylline (data not shown). Pretreatment of DDT_1MF-2 cells with (*R*)-PIA for 4 hr led to a diminution in this response compared with the control cells (Fig. 5).

Additional studies were performed to test whether agonist pretreatment also led to attenuation of (*R*)-PIA-mediated inhibition of adenylyl cyclase in membrane preparations. As seen in Fig. 6, (*R*)-PIA produced a dose-dependent inhibition of adenylyl cyclase, with peak effects observed at 1 μM concentration of the agonist. Membranes prepared from cells treated with (*R*)-PIA for 4 hr showed reduced agonist-mediated inhibition of adenylyl cyclase compared with control. This was manifested in a decrease in the maximum level of inhibition [at 1 μM (*R*)-PIA] compared with control. Stimulation of adenylyl cyclase activity in control cells observed between 1 and 10 μM (*R*)-PIA likely reflects activation of the A_2AR present in these cells.

Role of phosphorylation in A_1AR uncoupling in intact cells. Several G protein-coupled receptors are rapidly regulated as a consequence of phosphorylation by kinases such as protein kinase A, protein kinase C, and GRKs (21). Previously, we have shown that desensitization of the A_1AR after longer term exposure to (*R*)-PIA (24 hr) in DDT_1MF-2 cells led to increased phosphorylation of this receptor (9).

TABLE 1

Effect of *(R)*-PIA (1 μM, 4 hr) treatment on the competition of [^{3}H]DPCPX binding by the agonist *(R)*-PIA

[^{3}H]DPCPX was used as the antagonist radioligand at a concentration of 1 nM. Values are mean ± standard error.

Treatment	One site	Two sites			*n*
	K_i	K_H	K_L	R_H	
	nM	nM		%	
Control	75.7 ± 34.6	1.52 ± 0.42	392 ± 49	63 ± 8	3
(*R*)-PIA	220.2 ± 14.9[a]	3.37 ± 1.25	414 ± 38	37 ± 12[a]	3

[a] Statistically significantly different from control ($p < 0.05$, Student's *t* test).
K_H and K_L are the high and low dissociation constants, respectively, calculated assuming a two-state model.
Percentage of R_H is the percentage of total receptors in the high affinity state.
n, Number of individual experiments performed in control and treated cells.

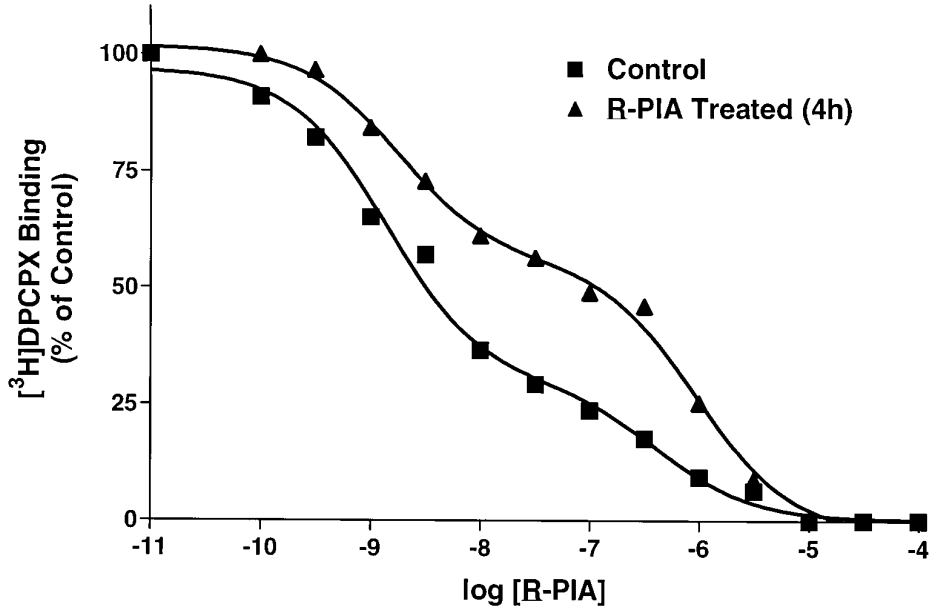

Fig. 3. Quantification of agonist high and low affinity sites by competition binding assays. Cells were treated without or with (*R*)-PIA (1 μM, 4 hr). Membranes were incubated with 1 nM [^{3}H]DPCPX and increasing concentrations of (*R*)-PIA, with 1 mM theophylline used to define nonspecific binding. The results are presented as percent of specific binding in the absence of competitor. Specific binding in the absence of competitor was 127 ± 21 and 130 ± 15 fmol/mg of protein for control and cells treated with (*R*)-PIA, respectively. Results are representative of three similar experiments, each performed in triplicate. The curves were analyzed by a curve-fitting program (Scatfit, Duke University Medical Center, Durham, NC) according to a two-state model (15, 16).

Furthermore, we also provided evidence that indicates that the A_1AR is a substrate of GRK (10). To investigate the possible role of receptor phosphorylation in A_1AR/G protein uncoupling, we used alkaline phosphatase to test whether dephosphorylation had any effect on agonist interaction with the A_1AR. Membranes from both control and (*R*)-PIA-treated (1 μM, 2 hr) cells were incubated with calf intestinal alkaline phosphatase at 30° for 1 hr, and the residual alkaline phosphatase was washed off by resuspension and centrifugation three times (20 ml each) with buffer A before binding assays were performed. The concentration range of alkaline phosphatase used in this study produced no significant inhibition of agonist binding to the A_1AR in control membranes when tested in competition binding assay. In contrast, alkaline phosphatase stimulated [^{125}I]APNEA binding when added to membrane preparations at both 25 and 200 units/ml. Alkaline phosphatase (25 and 200 units/ml) was effective in reversing the loss in agonist binding to A_1AR produced by agonist exposure (Fig. 7, *top*). On the other hand, the binding of the antagonist [^{3}H]DPCPX to A_1AR was unaffected after agonist treatment and by alkaline phosphatase treatment (Fig. 7, *top*). Similar patterns of reversal in [^{125}I]APNEA binding were observed after treatment of membranes with protein phosphatases types 1 and 2a (Fig. 7, *bottom*). Previous studies have shown that GRK-2 can phosphorylate a subset of G protein-coupled receptors that included the A_1AR (10). A consequence of agonist occupation of these receptors is translocation of GRK-2/3 from the cytosol to the membrane (22, 23), where it associates with the βγ subunits of G proteins. Thus, it was intriguing to test the possible translocation of the enzyme from cytosol to membrane in DDT_1MF-2 cells after agonist treatment. As shown in Fig. 8, activation of A_1AR by (*R*)-PIA induced translocation of the enzyme activity from cytosol to membrane, as detected by phosphorylation of rhodopsin, a substrate of GRKs (24). The GRK activity (detected by phosphorylation of the 36-kDa band) was barely detectable in the membrane in the absence of the agonist. However, a ~2–3-fold increase in enzyme activity was detected on exposure to (*R*)-PIA for 1 hr. On the contrary, the cytosol of the control cells showed higher GRK activity, which decreased by ~50% after agonist treatment for 1 hr. No 36-kDa band was observed when these experiments were performed in the absence of added rhodopsin (data not shown), suggesting that this band represents phosphorylated rhodopsin. The agonist-induced translocation of GRK from cytosol to membrane was also confirmed by Western blotting through the use of an antibody specific for the GRK-2 (Fig. 8). Quantification of the radioactivity of the specific bands indicated that the level of GRK-2 in the membrane increased by 394 ± 167%, 390 ± 134%, 492 ± 216%, 449 ± 216%, and 616 ± 327% of control after exposure to (*R*)-PIA for 15, 30, 45, 60, and 120 min, respectively (four experiments). These re-

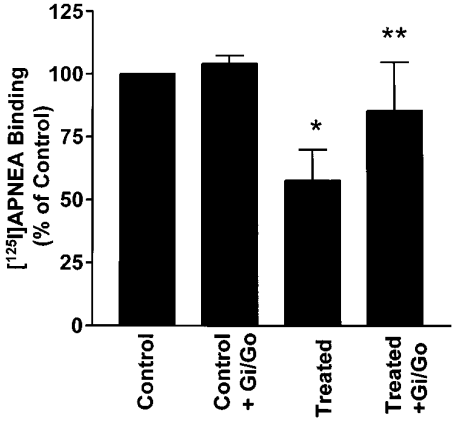

Fig. 4. Differential reconstitution of plasma membranes from control and desensitized cells with G_i/G_o mixtures. Plasma membranes from control DDT_1MF-2 cells and cells desensitized to (*R*)-PIA (1 μM) for 4 hr were solubilized in CHAPS, and equal amounts of solubilized preparations were reconstituted in a mixture of G_i/G_o (1.5 nM) on ice for 30 min. These preparations were then used for [^{125}I]APNEA binding. Results are mean ± standard error of four experiments, each performed in triplicate. *, $p < 0.05$, significant decrease in binding from control. **, $p < 0.05$, G_i/G_o promotes significant recovery in binding from the desensitized group.

sults suggest that activation of A_1AR leads to translocation of GRK to the membrane, where it becomes available for phosphorylation of the A_1AR.

Uncoupling of the A_1AR/G protein complex by arrestin. Purified A_1AR preparation (from bovine brain) was incubated with purified preparation of GRK-2 in the absence and presence of adenosine (10 μM). The adenosine was then degraded with exogenously added adenosine deaminase (3 units/ml). The resulting preparation was mixed with a purified preparation of bovine brain G_i/G_o (1 pmol) and increasing concentrations of arrestin (30–300 pmol). After a 15-min incubation on ice, the preparations were used for determination of [^{125}I]APNEA binding. Fig. 9 indicates a dose-dependent decrease in [^{125}I]APNEA binding by arrestin in preparations previously exposed to GRK-2 and adenosine. The concentration of arrestin producing 50% of maximum inhibition was ~90 pmol. Little inhibition of [^{125}I]APNEA binding was observed in the preparations exposed to GRK-2 alone. These results indicate increased affinity of arrestin for the GRK-2-phosphorylated A_1AR.

Discussion

This study provides novel information concerning regulation of the A_1AR after short term agonist exposure. Our data indicate rapid agonist-mediated uncoupling of the A_1AR from its associated G protein or proteins. This finding was not appreciated in an earlier study using DDT_1MF-2 cells because agonist binding experiments were not performed after these short periods of exposure. In fact, desensitization of the A_1AR was considered unique from that of the $A_{2a}AR$, which showed a more rapid rate (9). Uncoupling of the A_1AR in these cells was associated with other functional deficits as measured by GTPase activity and loss of inhibition of adenylyl cyclase. Additional experiments provide indirect evidence of phosphorylation of the A_1AR, which might trigger the process of uncoupling. Finally, this study provides some support for a role of GRK or GRKs in mediating desensitization of the A_1AR.

During the time frame of agonist exposure (0.5–4 hr), uncoupling of the A_1AR was not associated with receptor down-regulation in that no change in antagonist binding was detected in these cells. It is proposed that this uncoupling process represents the initial step in desensitization of the A_1AR and precedes receptor down-regulation. This latter phenomenon, which requires a more prolonged exposure to the receptor agonist, might result from internalization and/or sequestration of the receptor protein. The rapid recovery of A_1AR/G protein coupling after washout of the drug also points to some post-translational modification of the receptor protein (i.e., by phosphorylation). Desensitization of the A_1AR was not accompanied by changes in the concentrations of G proteins in the cell membrane, even though agonist-stimulated GTPase activity was decreased. This finding provides additional support for the contention that deficit at the

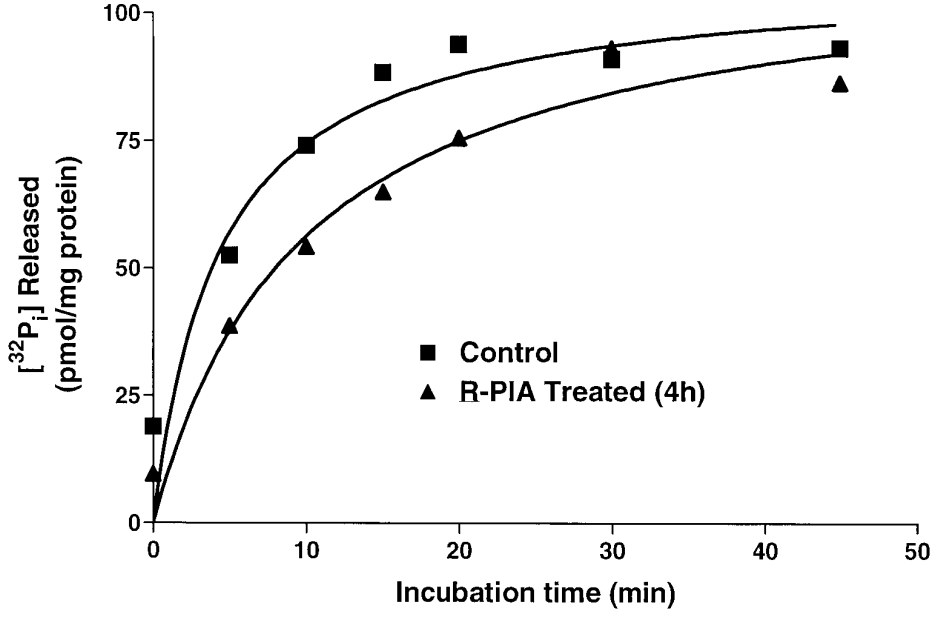

Fig. 5. A_1AR-stimulated GTPase activity in control and desensitized cells. Cells were treated without or with (*R*)-PIA (1 μM) for 4 hr. Membrane protein (20–50 μg/tube) were incubated with 1 μM [γ-^{32}P]GTP in a reaction mixture containing 1 μM (*R*)-PIA (described in Experimental Procedures) at 30° for the time periods as indicated. Nonspecific activity was determined by the addition of 100 μM GTP or guanosine-5′-*O*-(3-thio)triphosphate into the reaction mixture. Experiments were performed three times with similar results.

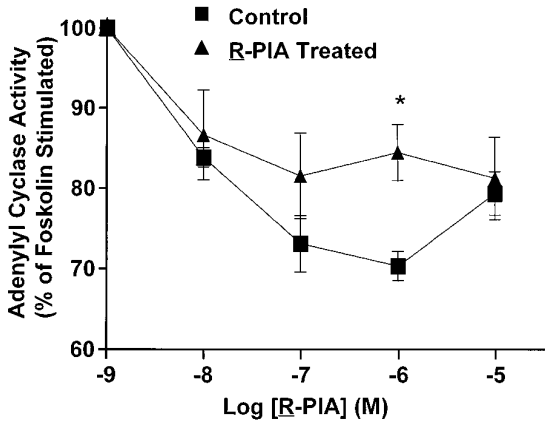

Fig. 6. Attenuation of (*R*)-PIA-mediated inhibition of adenylyl cyclase activity in DDT_1MF-2 cell membranes pretreated with agonist. Cells were treated without (control) or with (*R*)-PIA (1 μM, 4 hr). Adenylyl cyclase activity was determined by incubation of 20 μl of membranes (50 μg of protein) with 1.5 μCi of [α-^{32}P]ATP. Papaverine (0.1 mM) was included to inhibit the low-K_m cAMP phosphodiesterase. The reaction mixtures were incubated at 30° for 10 min and terminated by the addition of 1 ml of ice-cold stop solution. cAMP was isolated as described in Experimental Procedures. The fold stimulation by forskolin (10 μM) was 5.9 ± 0.8 and 4.8 ± 0.9 for control and membranes from (*R*)-PIA-treated cells, respectively. *, Statistically significant difference ($p < 0.05$) between control and (*R*)-PIA-treated groups (four experiments).

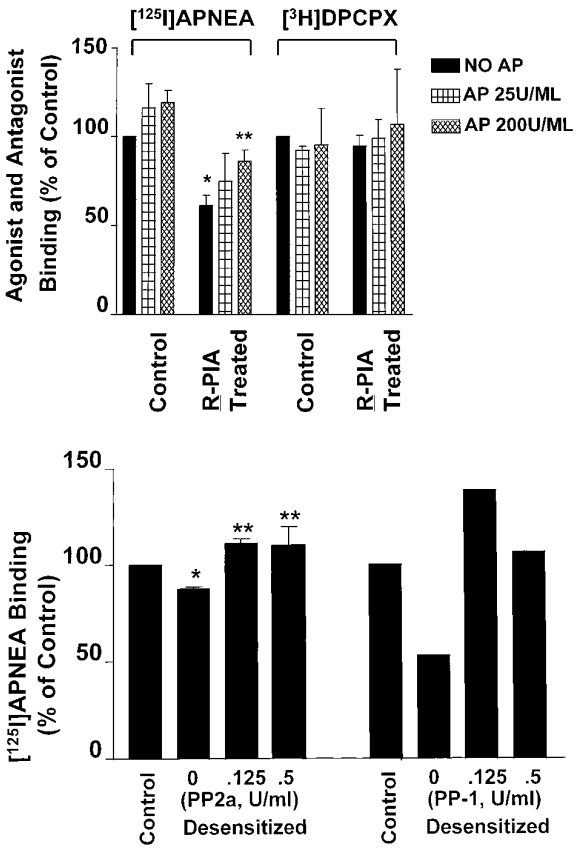

Fig. 7. Reversal of agonist-induced uncoupling of the A_1AR in DDT_1MF-2 cells by protein phosphatases. Membranes obtained from control DDT_1MF-2 cells and cells desensitized to (*R*)-PIA (1 μM) for 4 hr were incubated with (*top*) alkaline phosphatase (*AP*) (25 or 200 units/ml), and radioligand binding experiments were performed using both [^{125}I]APNEA and [^{3}H]DPCPX. *Bottom*, reversal of A_1AR uncoupling by protein phosphatases (*PP*) 1 and 2a. The different protein phosphatases were incubated with membrane preparations from control and cells desensitized to (*R*)-PIA after a 4-hr incubation with agonist. The binding of [^{125}I]APNEA was used to monitor A_1AR/G protein coupling. The concentrations of each phosphatase (in units/ml) is listed. Values are mean ± standard error of four independent experiments for protein phosphatase 2a and the mean of two experiments for protein phosphatase 1. *, Statistically significant reductions from control ($p < 0.05$). **, Significant reversal of uncoupling at both 0.125 and 0.5 unit/ml ($p < 0.05$).

level of the A_1AR protein itself is the primary reason for receptor desensitization.

Modification of G protein-coupled receptors by phosphorylation seems to be a primary mechanism for initiating desensitization (21). This study provides data supporting phosphorylation of the A_1AR in the process of uncoupling. The incubation of membrane preparations from desensitized cells with various phosphatases, including alkaline phosphatase, protein phosphatase 1, and protein phosphatase 2a, resulted in reversal of the A_1AR to its G protein-coupled state. A small but nonsignificant increase in coupling (as assessed by [^{125}I]APNEA binding) was observed in membranes from control cells. This suggests some uncoupling of the A_1AR in the absence of exogenous agonist and probably reflects the effect of endogenous adenosine, which was not completely eliminated by pretreatment with adenosine deaminase. A previous study, using rat adipocyte membranes, demonstrated increased sensitivity of the A_1AR to GTP after exposure to alkaline phosphatase (25). This study lends additional support that coupling of the A_1AR could be modulated by phosphorylation.

Previous work performed by this group has provided evidence for the involvement of GRKs, specifically β-adrenergic receptor kinase, in the phosphorylation of the A_1AR. However, the levels of phosphorylation detected in both intact cells and purified preparations of the A_1AR were low compared with other G protein-coupled receptors that are substrates of GRKs (10). Nevertheless, phosphorylation of the A_1AR by GRK-2 produced significant deficits in receptor function as assessed by receptor coupling, stimulated GTPase activity, and guanosine-5′-*O*-(3-thio)triphosphate binding. Although the reason for this discrepancy is not clear, it is possible that the purified A_1AR is partially phosphorylated under normal culture conditions and that the level of phosphorylation cannot be significantly increased under the assay conditions used.

Several pieces of evidence support a role of GRK-2 in the phosphorylation and uncoupling of the A_1AR. First, incubation of DDT_1MF-2 cells with (*R*)-PIA produced translocation of GRK-2 activity from the cytosol to the membrane, as assessed by the phosphorylation of rhodopsin. This coincided with an increase in GRK-2 immunoreactivity in the membrane fraction. Furthermore, the purified A_1AR that was incubated with agonist and purified GRK-2 in phosphorylation buffer demonstrated increased sensitivity to arrestin, as determined by a loss in agonist binding. This suggests modification of the A_1AR during the incubation period, presumably by phosphorylation, which increased its affinity for arrestin versus G_i. Phosphorylation of the βAR by GRK-2 has similarly been shown to increase its affinity for arrestin (26). In addition, pretreatment of DDT_1MF-2 cells with phorbol esters or dibutyryl cAMP to activate the protein kinase C and protein kinase A pathways, respectively, has no effect on the

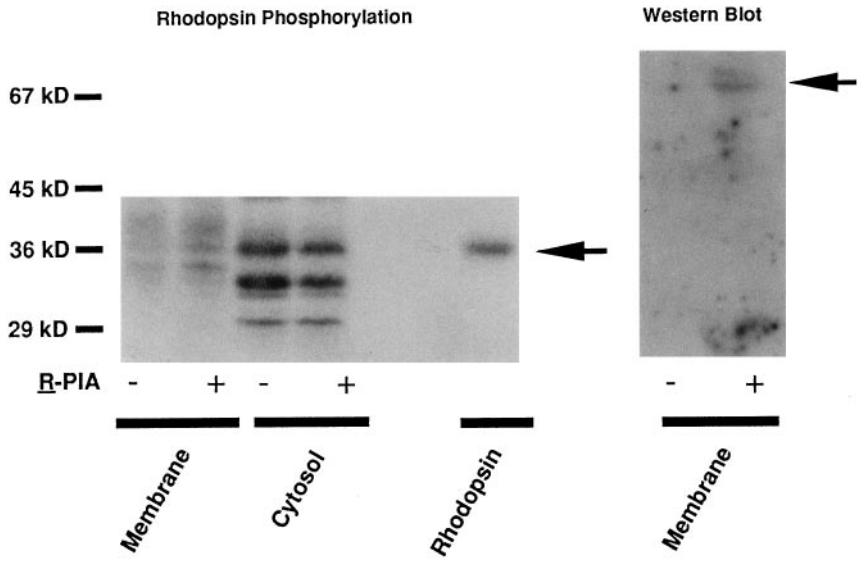

Fig. 8. (*R*)-PIA elicits translocation of GRK-2 activity from cytosol to membrane. DDT_1MF-2 cells were treated with (*R*)-PIA (1 μM) for 1 hr, and the activity of GRK-2 was determined in both membrane and cytosol. Phosphorylation activity was determined by incubating rhodopsin (10 pmol) with 100 μM of [γ-^{32}P]ATP (2000 cpm/pmol) and 10 μl of cytosolic or membrane protein at 30° for 1 hr. The samples were then solubilized in SDS-PAGE buffer, and the mixture was resolved on a 12% acrylamide gel and subjected to autoradiography. *Left arrow*, 36-kDa band is an identical preparation of rhodopsin that was phosphorylated by GRK-2. The translocation of GRK-2 was also confirmed by Western blots (*right arrow*).

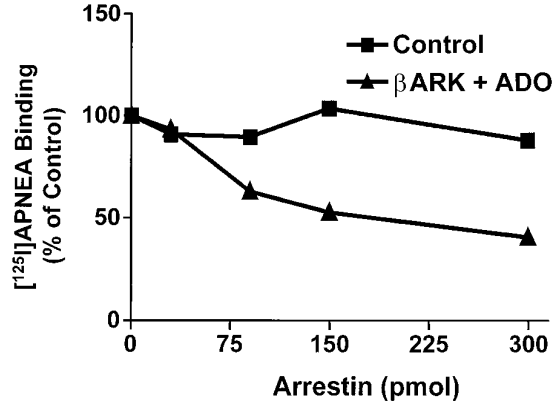

Fig. 9. Arrestin preferentially blocks reconstitution of the phosphorylated A_1AR with the G_i/G_o. Purified preparations of A_1AR obtained from bovine brain were incubated with purified GRK-2 [β-adrenergic receptor kinase 1 (*βARK*)] in the absence (control) and presence of 100 μM of adenosine (*ADO*) for 1 hr at 30°. After treatment, the preparations were incubated with adenosine deaminase (2 units/ml) for an additional 10 min. The mixtures were then reconstituted with a mixture of G_i/G_o (1 pmol) in presence of increasing concentrations of arrestin (0–300 pmol). Radioligand binding experiments using [^{125}I]APNEA were performed to quantify the extent of reconstitution of the A_1AR. Results are expressed as the mean of three individual experiments for each treatment group.

desensitization of the A_1AR induced by (*R*)-PIA.[1] This finding tends to rule out a role of either of these kinases in the process of A_1AR desensitization.

The targets of GRK-2 are serine and threonine residues, which are flanked by acidic residues on the amino-terminal side of the protein sequence. The A_1AR cloned from several species indicate two such putative GRK-2 phosphorylation sequences (Ser235, which is flanked by glutamic acid on the amino-terminal side, and Thr44, which is flanked by aspartic acid on the amino-terminal side). This seems to be a low number compared with other receptor substrates of GRK-2, such as the α_2-adrenergic receptor (27, 28), M_2 muscarinic acetylcholine receptor (29), β_2-adrenergic receptor (30), and the receptor for substance P (31). This might explain the poor A_1AR phosphorylation signals in intact cells (9) and purified receptor preparations (10). The first site (Ser235) is located on the third cytoplasmic loop of the deduced receptor structure. Because the third cytoplasmic loop seems to be intimately involved in G protein coupling (32), it is likely that phosphorylation of this serine would lead to disruption in receptor coupling.

This study presents the evidence supporting a role of GRK in the rapid desensitization of the A_1AR in an intact cell model. It is possible that this process promotes the subsequent steps in the desensitization process, such as receptor sequestration or internalization, which are generally associated with longer term agonist exposure. Desensitization of the A_1AR will tend to reduce the beneficial effects of adenosine *in vivo*, such as its ability to mediate cytoprotection during an ischemic episode. As such, inhibition of the function of GRK-2 might be clinically useful. In the β-adrenergic receptor system, inhibition of GRK-2 activity seems to prolong the beneficial action of β-agonists *in vivo* (33). A few other receptors that exhibit negative coupling to adenylyl cyclase have also been shown to be targets of GRK-2 (27–29).

In summary, the current data provide evidence of rapid inactivation of the A_1AR in DDT_1MF-2 cells after agonist exposure. Desensitization was associated with receptor phosphorylation and uncoupling of the A_1AR from G proteins. Furthermore, the data provide support for the involvement of GRK or GRKs in the phosphorylation and uncoupling of the A_1AR.

Acknowledgments

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