Molecular Basis for Subtype-specific Desensitization of Inhibitory Adenosine Receptors

ANALYSIS OF A CHIMERIC A₁-A₃ ADENOSINE RECEPTOR*

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The differing effects of short-term agonist exposure on the two inhibitory adenosine receptor (AR) subtypes have been examined using Chinese hamster ovary cells stably expressing the hemagglutinin epitope-tagged human A₂AR and rat A₃AR. Under conditions in which exposure of transfected cells to 5 μM (-)-(R)-N⁶-(phenylisopropyl)adenosine resulted in the functional desensitization and phosphorylation of the A₁AR, neither property was exhibited by the A₃AR. However, a stably expressed chimeric A₁-A₃AR, termed A₁CT3AR, in which the C-terminal domain of the A₁AR distal to its predicted palmitoylation site was replaced by the corresponding region of the A₃AR, was able to undergo functional desensitization and agonist-stimulated phosphorylation in a manner similar to that exhibited by the A₁AR. Moreover, purified G-protein-coupled receptor kinases 2, 3, and 5 were each capable of enhancing the agonist-dependent phosphorylation of the A₁AR and A₁CT3AR in vitro. Taken together, these data demonstrate that the C-terminal domain of the A₁AR distal to its predicted palmitoylation site is responsible for this receptor's ability to undergo a rapid agonist-dependent desensitization and are consistent with a model in which phosphorylation of the A₁AR within this domain by one or more G-protein-coupled receptor kinases initiates the desensitization process.

The phenomenon whereby a biological response plateaus and then diminishes despite the continual presence of agonist is termed desensitization. At least three different processes may be involved in the desensitization of G-protein-coupled receptor function. The earliest detectable event, occurring within seconds of agonist exposure, is that of receptor phosphorylation by either second messenger-activated kinases or G-protein-coupled receptor kinases (GRKs). The latter represent a growing family of proteins that specifically phosphorylate agonist-occupied receptors (1). In the β₂-adrenergic receptor, m₃-muscarinic receptor, and rhodopsin systems, phosphorylation increases receptor affinity for arrestin molecules, which uncouple the receptors from their associated G-proteins (2, 3). Following receptor phosphorylation, many receptors undergo sequestration into a poorly defined intracellular compartment (4). While the molecular determinants controlling sequestration are defined for an increasing number of receptors, the function of this process remains unclear since, in many cases, inhibition of sequestration does not appear to impair desensitization (5, 6). Prolonged agonist exposure (several hours) can lead to the down-regulation of the receptor protein and/or its associated G-proteins (7, 8).

The many physiological effects of adenosine are mediated in part by its binding to multiple cell-surface adenosine receptor (AR) subtypes termed A₁, A₂₆, A₂₆, and A₃ (9). Given that both the A₁AR and A₃AR bind the same physiological ligand and activate Gᵢ proteins, the significance of the existence of multiple inhibitory ARs is unclear. Recent work on the regulation of multiple α₂-adrenergic (10, 11) and β-adrenergic (12, 13) receptors has demonstrated that subtype-specific adaptive desensitization mechanisms have evolved such that responses mediated by particular receptor subtypes may be turned off at distinct rates. Indeed, studies examining the agonist-induced regulation of the A₁AR and A₂₆AR have demonstrated that these receptors desensitize over quite distinct time courses (14–18). Specifically, A₁AR desensitization typically occurs over periods of several hours, or even days, and is temporally associated with receptor down-regulation either alone (14) or concomitantly with Gᵢ proteins (15, 16). In contrast, desensitization of the rat A₂₆AR expressed in the RBL-2H3 cell line undergoes a homologous functional desensitization that is apparent after only a few minutes of agonist exposure (17, 18). We have recently demonstrated that the rapid functional desensitization of a recombinant rat A₁AR expressed in CHO cells is associated with the agonist-dependent phosphorylation of the receptor by one or more GRKs (19).

To more accurately characterize subtype-specific differences in the short-term regulation of inhibitory ARs, we have characterized functional desensitization in CHO cell lines stably expressing hemagglutinin epitope-tagged human A₁AR (20) and rat A₁AR (21) cDNAs. In addition, we have exploited these differences to generate a chimeric A₁-A₃AR, which has revealed that the structural determinants conferring rapid A₁AR desensitization kinetics and sensitivity to GRK-mediated phosphorylation reside within a small region at its C terminus.
EXPERIMENTAL PROCEDURES

Materials—IB-MECA (22) was the generous gift of Dr. Kenneth Jacobson (National Institutes of Health, Bethesda, MD). Cell culture supplies were from Life Technologies, Inc. Radiochemicals were from DuPont NEN. Sources of other materials have been described elsewhere (19, 23).

Receptor cDNA Constructs and Expression—The CHO cell line stably expressing the hemagglutinin epitope-tagged rat A1AR has been previously characterized (19). The six-amino acid hemagglutinin epitope DVPDYA, recognized by monoclonal antibody 12CA5 (24), was inserted at both the amino and carboxyl termini of the human A3AR by insertion of polymerase chain reaction products generated using the previously described pCMV5/human A3AR cDNA as a template (20). Correct introduction of the epitope sequences was verified by dyeoxyxynucleotide sequencing. The chimeric A1-A3, termed A1CT3AR, was generated by a previously described three-step polymerase chain reaction protocol utilizing the epitope-tagged A1AR and A3AR cDNAs in pCMV5 as templates (23). The integrity of the chimeric insertion was confirmed by dyeoxyxynucleotide sequencing.

CHO cell lines stably expressing the epitope-tagged wild-type and chimeric ARs were generated by cotransfecting cells with the appropriate cDNA subconfluent in pCMV5 and pSV2 neo in a 2:1 ratio using a modified calcium phosphate precipitation/glycerol shock procedure previously described (25). After selection in G418, resistant colonies were isolated, expanded, and screened for receptor expression by radioligand binding. Cells were propagated in T-75 flasks with Ham's F-12 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin in a 37 °C humidified atmosphere containing 5% CO2.

Intact Cell Receptor Phosphorylation—Transfected CHO cells were plated into 6-well dishes at a density of ~1 × 10^6 cells/well and cultured overnight in regular medium. The next day, the cells were washed twice with phosphate-free Dulbecco's modified Eagle's medium and incubated for 90 min at 37 °C in the same medium supplemented with 1 unit/ml adenosine deaminase and 200 μg/ml [125I]protein A-Sepharose in a vial. After stimulation with the indicated agonists, reactions were terminated by placing the cells on ice and washing the monolayers twice with 3 ml of ice-cold phosphate-buffered saline. Cells were scraped into 1 ml of lysis buffer (50 mM Hepes, pH 7.5, 5 mM EDTA, 10 mM sodium fluoride, 10 mM sodium phosphate, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each soybean trypsin inhibitor, leupeptin, and pepstatin A), transferred to microcentrifuge tubes, and lysed by vigorous vortexing. Membranes were pelleted by centrifugation (14,000 × g, 10 min) and solubilized by resuspension in lysis buffer supplemented with 150 μl of 1% (v/v) SDS, followed by brief sonication and heating to 65 °C for 15 min. After chilling to 4 °C, 600 μl of a concentrated nonionic detergent mixture was added to the solubilized membranes such that the final mixture contained 1% (v/v) Triton X-100, 0.5% (v/v) sodium deoxycholate, 0.2% (w/v) SDS, and 150 μM sodium chloride in lysis buffer. Insoluble material was removed by centrifugation (14,000 × g, 10 min), and the supernatant was pre-cleared by incubation for 1 h at 4 °C with protein A-Sepharose in the presence of 0.2% (w/v) IgG-free bovine serum albumin. The pre-cleared supernatant was then incubated for 2 h at 4 °C with protein A-Sepharose and 7 μl of 12CA5 ascites. Immune complexes were isolated by centrifugation, washed twice with detergent buffer containing 0.2 M ammonium sulfate and once with detergent buffer alone, and eluted from the protein A-Sepharose by the addition of electrophoresis sample buffer and incubation at room temperature for 45 min. Analysis was by SDS-PAGE, using 10% (w/v) polyacrylamide resolving gels, and by autoradiography. Quantitation of phosphorylation was determined by either densitometry or excision from the dried gel and Cerenkov counting of bands of interest.

Cell-surface Labeling with Biotin-LC-Hydrazide—This was performed on cells in 6-well dishes basically as described by Lisanti et al. (26). Briefly, cell monolayers were washed with ice-cold PBS and then treated with 10 mM sodium periodate in PBS for 30 min at 4 °C in the dark. After removal of the periodate solution and further washing with PBS, the cells were incubated once with 20 μM sodium acetate, pH 7.4, then incubated for 30 min at 4 °C with 2 μg/ml biotin-LC-hydrazide in the same buffer; this procedure labels all cell-surface carbohydrate residues with biotin. Cells were then washed prior to membrane preparation and immunoprecipitation with 12CA5 as described above. After SDS-PAGE, resolved proteins were transferred to a PVDF membrane, and nonspecific binding sites were blocked by 0.5% (v/v) skimmed milk solution in PBS containing 0.2% (v/v) Triton X-100 and 0.02% (w/v) thimerosal. The membrane was then incubated for 60 min at room temperature with 1 μg/ml horseradish peroxidase-conjugated streptavidin in a high detergent/skimmed milk solution. After three washes in blocking buffer and two washes in PBS, reactive proteins were visualized by an enhanced chemiluminescence protocol in accordance with the manufacturer's instructions (Renaissance, DuPont NEN).

GRK Purification—Recombinant bovine GRK2, bovine GRK3, and human GRK5 were purified from Sf9 cells by previously published procedures 48 h after infection with the appropriate baculovirus construct (27, 28). Using light-activated rhodopsin as a substrate, the specific activity of each of the purified enzymes was ~1 μmol/min/mg of protein.

In Vitro Assay of Receptor Kinase Activity—Monolayers of transfected CHO cells were washed three times with ice-cold PBS and scraped into lysis buffer (10 mM Hepes, pH 7.5, 2 mM EDTA, 0.25 mM NaCl, and 0.1 mM phenylmethylsulfonyl fluoride containing 10 μg/ml each soybean trypsin inhibitor and leupeptin). After Dounce homogenization on ice (20 strokes), membranes were pelleted by centrifugation and resuspended in GRK assay buffer (25 mM Hepes, pH 7.5, 2.5 mM EDTA, and 7.5 mM MgCl2) supplemented with 1 unit/ml adenosine deaminase and protease inhibitors for immediate use. Assays consisted of 40 μl of membrane suspension, 40 μl of kinase mixture (GRK assay buffer supplemented with 0.25 mM ATP, 0.88 mM dithiothreitol, 0.15 μM okadaic acid, and 10 μCi of [γ-32P]ATP), 10 μl of vehicle or purified GRK, and 10 μl of vehicle or agonist. After incubation at 30 °C for 5 min, reactions were terminated by placing the tubes on ice and adding 0.5 ml of ice-cold stop solution (0.1% sodium phosphate, pH 7.5, and 10 mM EDTA). Membranes were pelleted by centrifugation (14,000 × g, 10 min), and the resulting pellets were solubilized in 1% (w/v) SDS prior to dilution in nonionic detergent buffer as described above. After centrifugation to remove insoluble material, detergent extracts were equalized by protein assay prior to immunoprecipitation with 12CA5 and analysis by SDS-PAGE and autoradiography as described above.

Phosphoamino Acid Analysis—Following SDS-PAGE, proteins were transferred to a PVDF membrane. After overnight autoradiography, the region of the membrane corresponding to the phosphorylated receptor was excised, hydrated, and hydrolyzed at 110 °C in 200 μl of 5.7 M HCl for 90 min. The resulting hydrolysate was lyophilized and resuspended in chromatography buffer supplemented with phosphoamino acid standards. After spotting onto cellulose-coated plates, samples were subjected to ascending chromatography in an isobutyric acid, 0.5 M ammonium hydroxide (5:3, v/v) buffer system (29). Standards were visualized by ninhydrin staining, and 32P-labeled amino acids were visualized by autoradiography.

Radioligand Binding and Adenylyl Cyclase Assays—Saturation binding experiments employing [3H]DPCPX were performed and analyzed as we have described previously (23). Adenylyl cyclase assays were performed exactly as described previously using IB-MECA or (R)-PIA (30). Dose-response curves were analyzed by a previously validated curve-fitting program (31).

RESULTS

Functional Receptor Expression—We have previously demonstrated that the rapid functional desensitization of the rat A1AR is associated with the agonist-dependent phosphorylation of the receptor in situ by a kinase of similar substrate specificity to GRK2 (19). It was also noted that the C-terminal domain of the A1AR distal to the predicted palmitoylation sites of the human A1AR, rat A1AR, and chimeric A1CT3AR are shown. Each receptor also expressed hemagglutinin epitope tags at its amino and carboxyl termini.

Inhibitory Adenosine Receptor Phosphorylation
fore, the chimeric A1-A3AR shown in Fig. 1 was constructed to determine whether this domain of the A3AR was sufficient to confer on the resulting predominantly A1-containing chimera the rapid regulatory properties of the A3AR.

Agonist-mediated regulation of the epitope-tagged A1AR, A3AR, and A1CT3AR was then studied after stable expression of each cDNA in CHO cells. We have previously demonstrated that the epitope-tagged rat A3AR functions comparably to the untagged rat A3AR in this system (19). The epitope-tagged A1AR bound the A1-selective antagonist radioligand [3H]DPCPX with a K_d of 3.21 ± 0.20 nM (five experiments), exhibiting B_max values ranging between 2.98 and 3.37 pmol/mg of protein (five experiments). The A1CT3AR chimera bound [3H]DPCPX with similarly high affinity (K_d = 2.94 ± 0.67 nM; four experiments) and was expressed at slightly higher levels than the A1AR (B_max = 4.70–7.60 pmol/mg of protein; four experiments). The K_d values for [3H]DPCPX of the epitope-tagged A1AR and A1CT3AR are similar to those reported by other investigators for the untagged recombinant human A1AR expressed in CHO cells (34) and the purified human brain A1AR (35).

In addition, both the A1AR and A1CT3AR were functional as determined by their ability to inhibit adenylyl cyclase activity in isolated membranes. In membranes from A1AR-expressing cells, (R)-PIA maximally inhibited 5 μM forskolin-stimulated adenylyl cyclase activity by 52 ± 6%, with an IC_{50} value of 18.8 ± 8.5 nM (three experiments). Under the same assay conditions, (R)-PIA maximally inhibited forskolin-stimulated adenylyl cyclase activity in membranes from A1CT3AR-expressing cells by 46 ± 6%, with an IC_{50} value of 5.5 ± 2.2 nM (three experiments). Therefore, the A1AR and A1CT3AR bind antagonist radioligand with similar affinity and can sustain comparable agonist-dependent inhibition of adenylyl cyclase.

Effects of (R)-PIA Pretreatment on Receptor Function—To study the capacity of the expressed inhibitory ARs to undergo a rapid functional desensitization, transfected CHO cells were exposed to vehicle or (R)-PIA prior to membrane preparation and assayed for agonist-mediated inhibition of forskolin-stimulated adenylyl cyclase activity (Fig. 2 and Table I). Consistent with our previous study (19), pretreatment of A3AR-expressing CHO cells with 5 μM (R)-PIA for 10 min resulted in a functional desensitization, as manifested by a 7-fold increase in the IC_{50} value for the agonist IB-MECA to inhibit forskolin-stimulated adenylyl cyclase activity, with no significant change in the maximal inhibition achieved (Fig. 2A and Table I). In contrast, similar agonist pretreatment had no effect on the ability of the A1AR to inhibit adenylyl cyclase activity under the same assay conditions (Fig. 2B and Table I). Moreover, increasing the agonist exposure time to 30 min failed to unmask any detectable A1AR desensitization (data not shown). However, exposure of A1CT3AR-expressing cells to (R)-PIA for 10 min resulted in a functional desensitization that was qualitatively similar to that observed for the A1AR, i.e. agonist pretreatment resulted in a 5-fold increase in the IC_{50} value for (R)-PIA-mediated inhibition without significantly changing the maximal inhibition observed (Fig. 2C and Table I). Therefore, while the A1AR and A1CT3AR are similar with respect to their abilities to inhibit adenylyl cyclase activity, A1CT3AR signaling undergoes an agonist-induced functional desensitization that is similar to that exhibited by the A1AR.

Agonist-stimulated Receptor Phosphorylation in Situ—Radioligand binding studies using [3H]DPCPX demonstrated that levels of the A1AR and A1CT3AR were essentially unaffected by prior agonist treatment (A1AR levels were reduced by 1 ± 13%, and A1CT3AR levels were decreased by 6 ± 11%; three experiments). No antagonist radioligand with high affinity for the

FIG. 2. Effects of short-term agonist exposure on inhibitory AR function in CHO cells stably expressing the rat A3AR (A), human A1AR (B), or chimeric A1CT3AR (C) were incubated with 1 unit/ml adenosine deaminase in the absence (CONTROL) or presence (TREATED) of 5 μM (R)-PIA for 10 min at 37°C. Membranes were then prepared for the assay of adenylyl cyclase activity in the presence of 5 μM forskolin and increasing concentrations of the indicated agonist as described under “Experimental Procedures.” Composite data from multiple experiments are given in Table I.
A3AR is currently available. However, comparative immunoblotting of membranes with 12CA5 revealed that no significant change in the level of A3ARs was induced by treatment with 5 μM (R)-PIA for 10 min (data not shown). Therefore, since the expression level of each of these receptor proteins in transfected cell membranes is unaffected by prior agonist exposure, a rapid loss of receptors from cell membranes cannot account for the agonist-induced functional desensitization observed for the A3AR and A1CT3AR.

To study receptor regulation in response to agonist exposure, we utilized the ability of monoclonal antibody 12CA5 to specifically recognize the hemagglutinin epitope tag sequences engineered into each receptor's coding sequence. To assess the capacity of 12CA5 to immunoprecipitate each of the expressed ARs, transfected cell monolayers were surface-labeled with biotin hydrazide and solubilized for receptor immunoprecipitation with 12CA5. After SDS-PAGE, resolved proteins were transferred to a PVDF membrane for probing with horseradish peroxidase-conjugated streptavidin as we have previously described (19). Fig. 3A demonstrates that 12CA5 could specifi-

**TABLE I**

|                          | A3AR       | A1AR       | A1CT3AR    |
|--------------------------|------------|------------|------------|
|                          | Control    | Treated    | Control    | Treated    | Control    | Treated    |
| Basal activity (pmol/min/mg) | 3.18 ± 0.33 | 3.24 ± 0.48 | 2.11 ± 0.29 | 2.11 ± 0.28 | 1.19 ± 0.49 | 1.23 ± 0.51 |
| -Fold stimulation at 5 μM forskolin | 8.50 ± 0.42 | 8.71 ± 0.55 | 10.09 ± 0.82 | 9.15 ± 0.71 | 10.30 ± 0.55 | 9.53 ± 1.58 |
| IC50 (nM)                | 100 ± 10a  | 760 ± 80ab | 18.8 ± 8.5  | 20.1 ± 10.2 | 5.5 ± 2.2  | 30.1 ± 15.7b |
| Maximal % inhibition     | 64.0 ± 11.0 | 59.1 ± 4.4  | 52.0 ± 5.6  | 63.0 ± 5.0  | 46.2 ± 6.3  | 52.3 ± 5.0   |

A1-MECA used as the agonist in A1AR adenyl cyclase assays.

b Significantly different from untreated control (p < 0.05).

**Fig. 3.** Effects of agonist pretreatment on inhibitory AR phosphorylation. A, CHO cells stably expressing the indicated ARs were treated with or without 5 μM (R)-PIA for 10 min at 37 °C as indicated prior to cell-surface biotin labeling, membrane preparation, and solubilization for immunoprecipitation with 12CA5. After SDS-PAGE, immunoprecipitated proteins were transferred to a PVDF membrane, probed with horseradish peroxidase-conjugated streptavidin, and visualized by enhanced chemiluminescence as described under “Experimental Procedures.” B, 32P-labeled CHO cells stably expressing the indicated ARs were treated with or without 5 μM (R)-PIA for 10 min at 37 °C as indicated prior to membrane preparation, solubilization, and immunoprecipitation with 12CA5. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. C, CHO cells stably expressing chimeric A1CT3ARs were treated with or without 5 μM (R)-PIA for 10 min at 37 °C as indicated prior to immunoprecipitation with 12CA5 as described above. Following SDS-PAGE, resolved proteins were transferred to a PVDF membrane for phosphoamino acid analysis as described under “Experimental Procedures.” The migrations of ninhydrin-stained standards in this thin-layer chromatography system are indicated. D, 32P-labeled A1CT3AR-expressing CHO cells were treated with 1 μM XAC or the appropriate vehicle as indicated prior to immunoprecipitation with 12CA5 and analysis by SDS-PAGE.
cally immunoprecipitate cell surface-labeled receptor proteins of the appropriate size from each of the transfected cell lines. Both the A2AR and A2CT3AR migrate as ~40-kDa glycoproteins, consistent with the approximate size of the purified human brain A2AR as identified by radioiodination (35). We have previously demonstrated that the epitope-tagged A2AR can be specifically immunoprecipitated from transfected CHO cells and that it migrates as a broad 50–70-kDa glycoprotein (Fig. 3A) (19). For the A2AR and A2AR, a 10-min agonist pre-treatment appeared to produce a small decrease in the levels of cell-surface receptor compared with vehicle-treated cells (over multiple experiments, the maximal reductions observed were 20% for the A2AR and 24% for the A2AR). No decrease was consistently observed for the A2CT3AR at this time point. Therefore, since total receptor levels were unaffected by agonist treatment, it seems that agonist pretreatment for 10 min can induce a small but detectable internalization of the A2AR and A2AR, but not the chimeric A2CT3AR.

To determine whether any of the inhibitory ARs were phosphorylated in response to agonist treatment, receptors were immunoprecipitated from 32P-labeled transfected cells following incubation with or without 5 μM (R)-PIA for 10 min. Consistent with our previous report (19), the A2AR became phosphorylated in an agonist-dependent manner (Fig. 3B). In contrast, no phosphorylation of the A2AR could be detected after treatment with or without agonist, despite our ability to immunoprecipitate this receptor under these conditions (Fig. 3, A and B). However, the A2CT3AR displayed a significant level of basal phosphorylation, and this was increased by some 3.3 ± 1.0-fold (three experiments) after incubation with agonist. Phosphoamino acid analysis revealed that in the basal state, the A2CT3AR was phosphorylated exclusively on threonine residues. Agonist treatment resulted in the appearance of phosphoserine and increased the levels of phosphothreonine residues. Agonist treatment of the A2CT3AR by GRK2 in situ was reflected in their sensitivity to phosphorylation by GRK2, in vitro phosphorylation experiments were performed using membranes from transfected CHO cells and purified GRK2. Under these conditions, no agonist-dependent phosphorylation of the A2AR by either endogenous membrane-associated receptor kinase activity or purified GRK2 was detectable (Fig. 4). However, under the same assay conditions, the A2CT3AR was phosphorylated in an agonist-dependent manner by an endogenous receptor kinase activity, and the level of agonist-dependent phosphorylation was increased by some 2.2 ± 0.6-fold (three experiments) in the presence of GRK2 (Fig. 4). Therefore, the distinct effects of agonist treatment on the phosphorylation of the A2AR and A2CT3AR in situ are associated with distinct sensitivities to phosphorylation by GRK2 in vitro.

Sensitivity of the A2AR and A2CT3AR to Phosphorylation by Multiple GRKs—Despite the fact that GRK2 is the most intensively studied GRK isoform with regard to its activity against a range of receptor substrates, it has become clear in the last few years that it is just one member in an expanding family of GRKs cloned to date (1). Six members of this family, termed GRK1–6, have been cloned from mammalian sources, but with the exception of GRK1 (rhodopsin kinase), the spectrum of substrates for each GRK in vivo remains unknown. One reason for this is that very few receptors have been tested for their ability to act as substrates for these kinases in vitro, and therefore, the structural determinants conferring receptor sensitivity to them are unclear.

In addition, it was shown that prior treatment of intact cells with agonist reduces the level of GRK2-stimulated agonist-dependent phosphorylation subsequently observed in vitro, suggesting that a GRK2-like kinase is responsible for the phosphorylation observed in the intact cell. To determine whether the distinct effects of agonist on A2AR and A2CT3AR phosphorylation in situ were reflected in their sensitivity to phosphorylation by GRK2, in vitro phosphorylation experiments were performed using membranes from transfected CHO cells and purified GRK2. Under these conditions, no agonist-dependent phosphorylation of the A2AR by either endogenous membrane-associated receptor kinase activity or purified GRK2 was detectable (Fig. 4). However, under the same assay conditions, the A2CT3AR was phosphorylated by an agonist-dependent manner by an endogenous receptor kinase activity, and the level of agonist-dependent phosphorylation was increased by some 2.2 ± 0.6-fold (three experiments) in the presence of GRK2 (Fig. 4). Therefore, the distinct effects of agonist treatment on the phosphorylation of the A2AR and A2CT3AR in situ are associated with distinct sensitivities to phosphorylation by GRK2 in vitro.

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Therefore, to determine whether the A2AR and A2CT3AR could be phosphorylated by multiple GRKs, in vitro phosphorylation experiments were performed using membranes from transfected CHO cells and each of purified GRK3 and GRK5, with GRK2 being used as a positive control (Fig. 5). Using membranes expressing the A2AR, it was found that GRK3 could stimulate agonist-dependent A2AR phosphorylation to a similar extent as GRK2, with which it exhibits an 85% identity at the amino acid level (Fig. 5, A and C) (36). It was also found that GRK5 could stimulate agonist-dependent A2AR phospho-

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M. E. Olah and G. L. Stiles, unpublished data.
tyrosine phosphorylation by multiple GRKs was slightly lower than that displayed by GRK5 (Fig. 5, B and C). Under similar assay conditions, no agonist-dependent phosphorylation of the A2AR could be produced by GRK2, GRK3, or GRK5 (data not shown). Therefore, it appears that the structural determinants conferring sensitivity to agonist-dependent phosphorylation by multiple GRKs in vitro reside in the C-terminal 14 amino acids of the A2AR.

DISCUSSION

Many studies have shown that the A1AR expressed natively in a variety of systems desensitizes over a time course of several hours or even days (14–16). For example, the rat adipocytes A1AR down-regulates and desensitizes after treatment of cultured cells with (R)-PIA for 4 days (15), while the hamster A1AR in the DDT1 MF-2 cell line undergoes functional desensitization with a t₁/₂ of ~6 h (14). In contrast, the native A3AR expressed in the rat mast cell-derived RBL-2H3 line undergoes a rapid functional desensitization detectable within a few minutes of agonist exposure (17, 18). By expressing the epitope-tagged human A3AR and rat A2AR in the same cell type under control of the same expression vector, we have shown that the differences observed in the short-term regulation of native receptor function are related to receptor structure and are not simply a reflection of the use of different cell lines to study these receptors. Specifically, we have demonstrated that under conditions in which the A2AR undergoes agonist-dependent phosphorylation and desensitization, the A3AR exhibits neither of these properties. Therefore, since it appeared that the A3AR lacked the molecular determinants necessary to desensitize in response to short-term agonist treatment, we reasoned that it would be a useful "acceptor" molecule into which candidate regulatory cytoplasmic domains from the A1AR could be introduced, resulting in the formation of chimeric A1-A3ARs. The extreme C-terminal 14-amino acid segment of the A3AR was introduced into the A1AR since we had previously demonstrated that in transfected CHO cells, the A1AR underwent a rapid desensitization that is associated with receptor phosphorylation by a GRK-related kinase (19). The A3AR contains multiple serine and threonine residues in the region of the C-terminal tail distal to the predicted palmitoylation site, and in several instances, these are flanked by acidic amino acids, which peptide phosphorylation studies have shown to be an important determinant for phosphorylation by GRK2 (32, 33). Moreover, the A3AR does not possess any potential phosphorylation sites within the same region, perhaps suggesting that this may be a site responsible for the subtype-specific adaptive responses to short-term agonist exposure.

Expression and functional analysis of the A1CT3AR chimera (Fig. 1) demonstrated that its behavior in response to short-term agonist exposure was qualitatively similar to that of the A3AR. Specifically, A1CT3AR function underwent a rapid functional desensitization in response to short-term agonist treatment, and this was associated with the increased phosphorylation of the chimera on serine and threonine residues. Neither of these properties was displayed by the A2AR under the same conditions. Taken together with our previous observation that a GRK-related kinase is responsible for agonist-dependent A1AR phosphorylation in situ (19), the current data strongly suggest that the agonist-stimulated phosphorylation of the C-terminal domain of the A1AR is responsible for initiating the events that lead to the rapid desensitization of receptor function.

One difference between the A2AR and A1CT3AR was the detection of a significant level of threonine phosphorylation of

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**Fig. 5.** Agonist-dependent phosphorylation of the A1AR and A1CT3AR by multiple GRKs in vitro. In vitro phosphorylations were performed as described under "Experimental Procedures" using membranes from CHO cells stably expressing either the A3AR (A) or A1CT3AR (B) incubated with or without 5 or 10 μM 5′-N-ethylcarboxamidoadenosine (NECA) in the presence or absence of a 50 nM concentration of the indicated GRK isoform at 30 °C for 5 min. Following the addition of stop solution, membranes were pelleted for solubilization and receptor immunoprecipitation with 12CA5. Analysis was by SDS-PAGE and autoradiography. C is a quantitative analysis of three such experiments performed for each receptor. For each receptor, phosphorylation is expressed relative to that observed in the presence of agonist, but in the absence of any added GRK (set at 100%).

GRK2, GRK3, and GRK5 being capable of stimulating agonist-dependent receptor phosphorylation in vitro, although the levels of phosphorylation produced by GRK2 and GRK3 were slightly higher than that displayed by GRK5 (Fig. 5, B and C).
the latter receptor in the absence of agonist (Fig. 3). It is unlikely that this is due to the presence of high amounts of adenosine in the medium since a high concentration of adenosine deaminase was included during cell incubation. Also, any effects of endogenous adenosine would be expected to similarly induce basal phosphorylation of the A2AR, which was not observed (Fig. 3). Instead, this phenomenon may relate to the observation that within a given population of A2ARs expressed, a certain percentage may be active in the absence of agonist. In a chimeric A2AT3AR, such low level activation would result in a basal phosphorylation since it is the activated form of the receptor, no matter what complement of GRK isoforms would be observed in many cell types natively expressing the receptor. Regardless of this issue, the basal phosphorylation state of the chimera does not noticeably alter the receptor-G-protein equilibrium as compared with the wild-type A1AR. Therefore, conditions producing the additional agonist-stimulated phosphorylation on serine and threonine residues are necessary to produce the observed functional desensitization of the A2AT3AR.

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