Agonist-dependent Phosphorylation and Desensitization of the Rat A3 Adenosine Receptor

EVIDENCE FOR A G-PROTEIN-COUPLED RECEPTOR KINASE-MEDIATED MECHANISM*

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A3 adenosine receptor (A3AR) activation contributes to both the cardioprotective and antihypertensive effects of adenosine. To date, no studies have examined the mechanisms by which this receptor undergoes rapid homologous desensitization. Therefore, a functional hemagglutinin epitope-tagged A3AR has been stably expressed in Chinese hamster ovary cells, and its regulation by the AR agonist 5'-N-ethylcarboxamidoadenosine (NECA) has been studied. Cellular exposure to NECA induces rapid (t1/2 = ~1 min) A3AR phosphorylation on serine and threonine residues. This is associated with a functional desensitization and a 30–40% reduction in the number of high affinity agonist binding sites as determined by radioligand binding assays. Activation of second messenger-regulated kinases could not mimic the effect of NECA, suggesting a role for G-protein-coupled receptor kinases (GRKs). In vitro phosphorylation assays demonstrate that phosphorylation of agonist-occupied A3ARs is enhanced by GRK2 and that cellular pretreatment with NECA dramatically inhibits subsequent GRK2-mediated phosphorylation in vitro. Therefore, the A3AR is phosphorylated in situ by a kinase similar or identical to GRK2, and this may be involved in rapid functional desensitization of the A3AR.

The multiple physiological effects of adenosine are mediated by the activation of cell surface adenosine receptors (ARs).1 Biochemical and molecular cloning studies have demonstrated the existence of four AR subtypes designated A1, A2a, A2b, and A3 (1). A3AR cDNA clones were initially isolated from rat testis and brain libraries (2, 3), but subsequently isolated cDNAs from sheep and human sources, which encode proteins with a 70% identity to the rat protein, have also been designated as A3Rs (4, 5). Characterization of the pharmacological properties of the recombinant rat A3AR led to the realization that the A3AR is the “atypical” AR expressed in a rat mast cell-derived tumor cell line RBL-2H3 (6, 7).

Despite its relatively recent discovery, A3AR activation has already been implicated in contributing to several important physiological effects of adenosine, including vasodilation, bronchoconstriction, and cardioprotection (8). Moreover, evidence has been presented to suggest that these effects are initiated by A3AR activation of mast cells, thereby leading to the release of allergic mediators that are directly responsible for the observed phenomena (8). Therefore, an understanding of how A3AR signaling is regulated would be a significant advance toward controlling these events.

The initial characterization of the A3AR expressed in RBL-2H3 cells demonstrated that agonist-stimulated calcium mobilization is subject to a rapid, homologous desensitization (6, 7). However, the molecular events responsible for this effect are currently unknown. Rapid termination of signaling by G-protein-coupled receptors is typically initiated by receptor phosphorylation events catalyzed by either second messenger-activated kinases or G-protein-coupled receptor kinases (GRKs) (9). The latter constitute a growing family of proteins that specifically phosphorylate agonist-occupied receptors. To date, six such kinases, termed GRKs 1 through 6, have been cloned from mammalian sources but, with the exception of GRK1 (rhodopsin kinase), the spectrum of receptor substrates for each GRK in vivo remains unknown (10).

To examine a role of receptor phosphorylation in regulating A3AR signaling, a functional hemagglutinin epitope-tagged rat A3AR has been expressed in Chinese hamster ovary cells. We report the first visualization of a recombinant A3AR and the first detailed characterization of adenosine receptor phosphorylation both in situ and in vitro. Moreover, evidence is presented that supports a role for a specific GRK isoform in mediating A3AR phosphorylation and desensitization.

**EXPERIMENTAL PROCEDURES**

Materials—125I-AB-MECA was synthesized and purified by high performance liquid chromatography as described previously (11). 125I-MECA (12) was the generous gift of Dr. Kenneth Jacobson (National Institutes of Health, Bethesda, MD). Cell culture supplies were from Life Technologies Inc. Radionucleides were from DuPont NEN. Horseradish peroxidase-conjugated streptavidin and biotin LC-hydrazide were from Pierce. Cellulose-coated plastic-backed chromatography plates were from Eastman Kodak Co. Monodonal antibody 12CA5 was from Berkeley Antibody Company. Sources of other materials have been described elsewhere (11, 13).

Receptor cDNA Constructs and Expression—The six amino acid influenza hemagglutinin (HA) epitope, DVPDYA, recognized by monoclonal antibody 12CA5 (14) was inserted at both the amino and carboxyl termini of the rat A3AR by insertion of polymerase chain reaction products generated using the previously described pCMV5/rat A3AR

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1 The abbreviations used are: AR, adenosine receptor; NECA, 5'-N-ethylcarboxamidoadenosine; GRK, G-protein-coupled receptor kinase; AB-MECA, 4-aminobenzyl-5'-N-methylcarboxamidoadenosine; IB-MECA, N9-(3-iodobenzyl)adenosine-5'-N-methyluronamide; HA, hemagglutinin epitope; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; LC, long chain alkyl spacer group.
A3 Adenosine Receptor Phosphorylation

Cellswerescrapedinto1mllysisbuffer(50mM Hepes,pH7.5,5mM EDTA,2.5mM MgCl2)supplementedwith1 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 NECA. 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 mM sodium phosphate, pH 7.5, and 10 mM EDTA). Membranes were pelleted by centrifugation (14,000 × g for 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 polyvinylidene difluoride membrane. After overnight autoradiography, the region of the membrane corresponding to the phosphorylated HA-A3AR was excised, hydrated, and hydrolyzed at 110°C in 200 μl of 5.7 mM 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 (20). Standards were visualized by ninhydrin staining, and 32P-labeled amino acids were visualized by autoradiography.

RESULTS

Functional Expression of HA-A3AR in CHO Cells—Saturation binding analysis of transfected cell membranes demonstrated that the expressed receptor bound the high affinity A3AR agonist radioligand 125I-AB-MECA with Kd and Bmax values of 2.04 ± 0.38 nM and 0.89 ± 0.38 pmol/mg, respectively (four experiments) (Fig. 1A). The Kd value observed is similar to that exhibited by the untagged rat A3AR after expression in CHO cells and by the native rat A3AR in RBL-2H3 cells (11). Adenylyl cyclase assays demonstrated that the A3AR agonist 18-β-glycerol inhibited 5 μM forskolin-stimulated adenylyl cyclase activity in a dose-dependent manner (IC50 = 48.3 ± 11.3 μM; three experiments) producing a maximal inhibition of 75 ± 5% (three experiments) (Fig. 1B). Therefore, the addition of HA-epitope sequences to the A3AR fails to diminish its ability to bind agonist radioligand with high affinity or interact productively with Gβ-proteins to inhibit adenylyl cyclase.

To identify the HA-A3AR protein, we utilized the presence of three predicted sites for N-linked glycosylation within the A3AR sequence (3). Cell surface carbohydrate residues were covalently labeled with biotin by sequential treatment of cell monolayers with periodate and biotin-LC-hydrazide. After immunoprecipitation with 12CA5 as described above, SDS-PAGE resolved proteins were transferred to a polyvinylidene difluoride membrane and nonspecific protein binding sites blocked by a 60-min incubation in blocking buffer (5% [w/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 solution containing 0.1% (v/v) Triton. After three washes in PBS, reactive proteins were visualized by an enhanced chemiluminescence protocol in accordance with the manufacturer’s instructions (Renasant, DuPont NEN).

GRK2 Purification—Recombinant bovine GRK2 was purified from S9 fractions by previously published procedures (48 h after infection with the baculovirus construct pSMV5HA-A3AR). Using light-activated rhodopsin as a substrate, the specific activity of the purified enzyme was approximately 1 μmol/min/mg protein.

In Vitro Assay of Receptor Kinase Activity—After treatment of HA-A3AR cells in T-75 flasks with or without agonist, incubations were terminated by placing the cells on ice, rapidly washing three times with ice-cold PBS, and scraping the cells into lysis buffer (10 mM Hepes, pH 7.5, 2 mM EDTA, 0.25 mM NaCl, 10 μg/ml each of soybean trypsin inhibitor and leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride). After Dounce homogenization on ice (20 strokes), membranes were

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10 μM NECA for 10 min. This protein migrated exactly with the HA-A₃AR as identified by biotin labeling (Fig. 1C) and was not immunoprecipitated from nontransfected CHO cells (Fig. 2A). Therefore, the HA-A₃AR protein is rapidly phosphorylated upon cellular exposure to agonist. Phosphoamino acid analysis revealed that phosphorylation was predominantly on threonine residues with some phosphoserine also being detected but no phosphotyrosine (Fig. 2B). In an attempt to identify which kinases may be responsible, transfected CHO cells were exposed to activators of several second messenger-regulated ki-
nases. However, activation of protein kinase C by phorbol 12-myristate 13-acetate, Ca²⁺-calmodulin kinases by the calcium ionophore A23187, or cyclic nucleotide-dependent kinases by forskolin and 8-bromo-cyclic GMP all failed to stimulate HA-A3AR phosphorylation under conditions in which NECA was effective (Fig. 2C).

Characterization of NECA-stimulated HA-A3AR Phosphorylation — The ability of NECA to stimulate HA-A3AR phosphorylation was dose-dependent (Fig. 3A). Modelling of data pooled from three experiments produced an EC₅₀ of 0.16 ± 0.04 μM (Fig. 3A). Time course experiments revealed that HA-A3AR phosphorylation was extremely rapid. Phosphorylation was detectable at the first time point examined (15 s) and was half-maximal by 60 s (Fig. 3B). The response was maximal by 4 min and was sustained for at least 20 min in the presence of agonist (Fig. 3B).

Effect of NECA Pretreatment on HA-A3AR Function — To determine whether HA-A3AR phosphorylation was associated with changes in receptor function, transfected cells were treated with 10 μM NECA for 10 min prior to membrane preparation for radioligand binding. These experiments demon-

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**Fig. 2.** Agonist-dependent phosphorylation of HA-A3AR. A, nontransfected and HA-A3AR-expressing CHO cells were metabolically labeled with [³²P]orthophosphate, exposed to 10 μM NECA or vehicle for 10 min at 37 °C, and then lysed for solubilization and immunoprecipitation with 12CA5. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography as described under “Experimental Procedures.” B, transfected cells were treated with agonist and immunoprecipitated with 12CA5 as described for A. Following SDS-PAGE, proteins were transferred to a polyvinyldene difluoride membrane, and the region corresponding to the phosphorylated HA-A3AR was excised for phosphoamino acid analysis as described under “Experimental Procedures.” The migration of ninhydrin-stained phosphoamino acid standards in this TLC buffer system is indicated. C, HA-A3AR phosphorylation in response to various stimuli. After labeling with [³²P]orthophosphoric acid, transfected cells were incubated for 10 min at 37 °C in the absence of any ligand (None), 10 μM NECA, 100 nM phorbol 12-myristate 13-acetate (PMA), 10 μM calcium ionophore A23187 in the presence of 1.8 mM calcium chloride (A23187), 100 μM forskolin (Forskolin), and 100 μM 8-bromo-cyclic GMP (8BrcGMP). Membranes were then prepared for solubilization and immunoprecipitation with 12CA5 as described under “Experimental Procedures.”
Strated that agonist pretreatment resulted in a 34 ± 10% reduction in the B max for 125I-AB-MECA binding (p < 0.05, four experiments) compared with untreated controls without significantly changing the K d (control, 2.04 ± 0.38 nM; versus treated, 2.54 ± 0.10 nM, four experiments) (Fig. 4A). This reduction in agonist binding sites was associated with a desensitization of HA-A 3 AR function, as manifested by an 8-fold increase in the IC 50 value for IB-MECA-mediated inhibition of forskolin-stimulated adenylyl cyclase activity (Fig. 4B, Table I). Therefore, exposure of transfected cells to agonist under conditions that promote receptor phosphorylation results in a loss of high affinity agonist binding sites and a functional desensitization.

**Fig. 4. Effects of agonist treatment on HA-A 3 AR function.** A, transfected cells were incubated with 1 unit/ml adenosine deaminase in the absence (○) or presence (●) of 10 μM NECA for 10 min at 37 °C. Membranes were then prepared for radioligand binding with increasing concentrations of 125I-AB-MECA as described under "Experimental Procedures." Scatchard transformations are shown of the binding data from one of four such experiments. B, transfected cells were treated as described for A, and membranes were prepared for assay of adenylyl cyclase activity in the presence of 5 μM forskolin and increasing concentrations of IB-MECA as described under "Experimental Procedures." Composite data from multiple experiments are given in Table I.

**TABLE I.**

| Basal activity | Stimulation at 5 μM forskolin | Maximal inhibition | IC 50 |
|----------------|-------------------------------|------------------|------|
| pmol/min/mg    | -fold                         | %                | NM   |
| Control        | 3.86 ± 1.20                   | 8.77 ± 0.50      | 75 ± 5 | 48.3 ± 11.5 |
| Treated        | 4.40 ± 0.53                   | 8.41 ± 0.84      | 77 ± 10 | 390 ± 25 a |

a Indicates p < 0.05.

The data are presented as the means ± S.E. for three experiments.

**DISCUSSION.**

Despite the growing appreciation of the contribution of A 3 AR activation toward mediating some of the physiological effects of adenosine, only limited information is available on how A 3 AR function is regulated. We have recently demonstrated that chronic exposure of A 3 AR-expressing CHO cells to the agonist NECA induces a functional desensitization that is associated with the specific down-regulation of G α -3 and G-protein β-subunits (13). However, the time-course of G-protein down-regulation (t 1/2 = -6 h) would suggest that although this event may be an important adaptive mechanism to prolonged agonist exposure, it cannot account for the rapid functional desensitization observed for the native rat A 3 AR in response to acute agonist treatment (6, 7). By examining the effects of agonist...
exposure in CHO cells expressing a recombinant epitope-tagged A3AR, the current study now provides a testable working model with which to explain the phenomenon of rapid A3AR desensitization. The validity of the model system we have chosen is proven by two observations. Firstly, both native and recombinant epitope-tagged A3ARs undergo a rapid functional desensitization (Fig. 4B and Refs. 6 and 7). Secondly, in membranes from transfected CHO cells (Fig. 4A) and RBL-2H3 cells,2 this desensitization is associated with a 30–40% reduction in the number of agonist binding sites recognized by 125I-AB-MECA in radioligand binding assays. Therefore, it is likely that similar adaptive processes operate to diminish A3AR signaling in CHO and RBL-2H3 cells.

Several lines of evidence support a role for G-protein-coupled receptor kinase involvement in A3AR desensitization. Firstly, the A3AR is rapidly phosphorylated in an agonist-dependent manner, and this cannot be mimicked by simple activation of second messenger-regulated kinases alone. Moreover, the EC50 value for NECA-stimulated A3AR phosphorylation (0.15 μM) is essentially the same as its Kᵢ value for displacing 125I-AB-MECA binding from the A3AR (11). Such a correlation between extents of receptor occupancy and phosphorylation coupled with the lack of any effect of second messenger-regulated kinases strongly suggest that one or more GRK isoforms is responsible for A3AR phosphorylation, because these kinases specifically phosphorylate agonist-occupied receptors (9, 10).

A specific role for GRK2 or a related kinase is suggested from the in vitro phosphorylation experiments, which demonstrated that GRK2 was capable of enhancing the agonist-dependent A3AR phosphorylation observed in isolated membranes. Although it is theoretically possible that GRK2 mediates its stimulatory effect on A3AR phosphorylation in vitro indirectly via interaction with nonreceptor proteins present in the membrane preparation, this is unlikely considering the high degree of substrate specificity that GRKs exhibit toward G-protein-coupled receptors (17–19, 22, 23). Therefore, it is most likely that the agonist-occupied A3AR is directly phosphorylated by GRK2 in vitro. More importantly, pretreatment of transfected cells with agonist reduced the subsequent level of GRK2-stimulated, agonist-dependent A3AR phosphorylation observed in vitro.

The simplest explanation for this phenomenon would be that agonist pretreatment induces A3AR phosphorylation in situ on some of these residues by GRK2 or a very similar kinase such that they are not available for subsequent phosphorylation in vitro. However, interpretation of these results is complicated by the observation that agonist pretreatment in situ results in an agonist-independent component of endogenous kinase- and GRK2-mediated receptor phosphorylation in vitro. Because this effect is reversible in a time-dependent manner with agonist washout, it cannot be simply due to the carry-over of residual NECA from the cellular treatment. Because agonist pretreatment would result in A3AR activation and a resulting dissociation of Gₛ-proteins in the proximity of the receptor, it is possible that localized release of βγ-subunits near the receptor may be sufficient to translocate sufficient GRK2 such that in the absence of agonist some receptor phosphorylation can occur. The ability of βγ-subunits alone to stimulate low level GRK2-mediated phosphorylation of antagonist-occupied β₂-adrenergic receptors in vitro has been previously described (24).

Alternatively, it is possible that agonist-dependent phosphorylation of the receptor on specific residues in situ primes the receptor for subsequent agonist-independent phosphorylation in vitro. Such a sequential model of protein phosphorylation, whereby phosphorylation at specific residues is required in order to observe phosphorylation at other sites, has been well described for the kinases that control the enzymes involved in

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2 T. M. Palmer, unpublished data.
glycogen metabolism (25). Moreover, recent experiments using synthetic peptide substrates (22), as well as a glutathione S-transferase fusion protein containing the COOH-terminal sequence of the fMet-Leu-Phe receptor (26), have indicated that GRK2 may utilize such a sequential phosphorylation mechanism.

Cellular pretreatment with agonist does not completely abolish subsequent A3AR phosphorylation in vitro. Therefore, it seems likely that the A3AR may contain multiple sites for GRK phosphorylation with only a subset of these being utilized in CHO cells in situ such that incubation with GRK2 in vitro results in the phosphorylation of some of the remaining sites. This is not an unexpected finding because studies of GRK-mediated phosphorylation of rhodopsin and the β2-adrenergic receptor have demonstrated that although high phosphorylation stoichiometries are reported in vitro, the stoichiometries exhibited by receptors in situ are much lower (27, 28).

Although A3AR phosphorylation is associated with the onset of functional desensitization, we cannot directly determine whether the phosphorylated receptor is impaired in its ability to signal downstream. This will require the purification of the receptor and its reconstitution with inhibitory G-proteins in order to directly measure the receptor’s signaling capacity in the absence of any other components. It is possible that additional factors, such as arrestins, are required to elicit a profound desensitization in response to GRK phosphorylation, as has been shown for rhodopsin and the human β2-adrenergic receptor (29).

Unfortunately, we were unable to determine the stoichiometry of A3AR phosphorylation. This will be possible if either an A3AR antagonist radioligand is developed or purified preparations of HA-A3AR become available to allow us to accurately measure receptor content in 12CA5 immunoprecipitates. However, a knowledge of the A3AR sequence (3) and the substrate has been shown for rhodopsin and the human A3AR antagonist radioligand is developed or purified preparation of the manuscript.

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