Identification of an A_{2a} Adenosine Receptor Domain Specifically Responsible for Mediating Short-Term Desensitization†

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ABSTRACT: In an attempt to delineate the structural requirements necessary for agonist-induced desensitization, we have stably expressed wild-type and mutant A_{2a} adenosine receptors (A_{2a}ARs) in Chinese hamster ovary cells and examined the effects of agonist pretreatment on adenylyl cyclase activity in subsequently isolated membranes. Deletion of 95 amino acids from the carboxyl-terminus of the A_{2a}AR, thereby removing 10 potential phosphorylation sites, failed to alter radioligand binding, adenylyl cyclase activation, or functional desensitization parameters as compared with the wild-type receptor. However, simultaneous mutation of Thr 298 and Ser 305 to Ala residues attenuated the desensitization observed in response to short-term (30 min) agonist treatment while not blocking the ability to desensitize after long-term (24 h) agonist exposure. Individual mutation of these residues revealed that mutation of Thr 298 alone was sufficient to diminish both short-term desensitization and agonist-stimulated receptor phosphorylation. These data suggest that while the majority of the A_{2a}AR carboxyl-terminus is dispensable with regard to observing functional desensitization, the integrity of Thr 298 is essential for observing agonist-stimulated receptor phosphorylation and short-term desensitization but not long-term desensitization of A_{2a}AR function.

Desensitization, or refractoriness, is defined as the ability of a receptor-mediated response to plateau and then diminish despite the continual presence of agonist. While this phenomenon has been described for responses elicited by many G-protein-coupled receptors, the molecular events leading to desensitization have been delineated for only a few well-characterized examples, namely, the β_2-adrenergic receptor and rhodopsin (Lohse *et al*., 1992). However, a number of studies on the regulation of recently cloned receptors after expression in various systems have suggested that several features of the basic mechanisms proposed for the β_2-adrenergic receptor and rhodopsin systems may be generally applicable. A good example of this is the A_{2a} adenosine receptor (A_{2a}AR),[1] the rapid homologous functional desensitization of which has been described in several systems (Newman & Levitzki, 1983; Anand-Srivastava *et al*., 1989; Ramkumar *et al*., 1991). Recently, we generated cell lines stably expressing an A_{2a}AR cDNA and characterized the various adaptive mechanisms induced upon agonist exposure; this study revealed that desensitization is a complex phenomenon involving multiple, temporally distinct components (Palmer *et al*., 1994). Short-term agonist exposure induced a rapid reduction in A_{2a}AR function which was associated with a reduced coupling efficiency between the A_{2a}AR and G_s, and with an agonist-stimulated phosphorylation of the receptor protein. No change in the functioning of either G_s or the catalytic unit of adenylyl cyclase could be detected, consistent with a modification of the receptor−G-protein interaction being solely responsible for the observed effect. Neither desensitization nor phosphorylation could be mimicked by elevation of intracellular cAMP levels with forskolin, suggesting involvement of a G-protein-coupled receptor kinase (GRK) (Palmer *et al*., 1994). Receptor sequestration also occurred over the same time span, but while blockade of this process did not affect the desensitization observed, the rapid recovery normally observed after agonist removal was attenuated. Longer agonist treatment times (several hours) caused a down-regulation in total receptor number and up-regulation of inhibitory G-protein α-subunits (Palmer *et al*., 1994).

The aim of the current study was to determine whether the different desensitization mechanisms induced after short- and long-term agonist treatments were mediated by structurally distinct regions of the receptor protein. This was achieved by generating stable cell lines expressing wild-type and mutant A_{2a}ARs and comparing their desensitization patterns. Specifically, mutations were generated within the long carboxyl-terminal tail of the A_{2a}AR, whose many serine and threonine residues suggest a regulatory function for this domain. By this approach, we have identified a small region of the receptor whose integrity is essential for observing short-term desensitization and agonist-stimulated phosphorylation but which is not required to observe desensitization in response to long-term agonist treatment.

EXPERIMENTAL PROCEDURES

Receptor cDNA Constructs and Expression. The pBC/A_{2a}AR construct we previously described (Palmer *et al*., 1994) was used as the template for all subsequent manipulations. Generation of point mutants was achieved using an oligonucleotide-directed polymerase chain reaction (PCR) method previously described (Olah *et al*., 1992). The cDNA encoding a truncated A_{2a}AR was generated using the same

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[1] Abbreviations: AR, adenosine receptor; GRK, G-protein-coupled receptor kinase; CHO, Chinese hamster ovary; PAPA-APEC, 2-[4-[2-[2-[[(4-aminophenyl)methylcarbonyl]amino]ethylaminocarbonyl]-ethyl]phenyl]ethylamino-5′-*N*-ethylcarboxamidoadenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine.

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protocol, converting the triplet encoding Gly 317 to an amber stop codon (GGT to TGA). All mutations were verified by dideoxynucleotide sequencing.

Introduction at the amino terminus of the influenza virus hemagglutinin HA1 epitope (YPYDVPDYA) recognized by monoclonal antibody 12CA5 was also achieved using PCR. The resulting plasmid was termed pBC/WT. After verification of introduction of the epitope by restriction digestion and dideoxynucleotide sequencing, a *Hin*d III/*Pvu*II cassette from pBC/WT was used for subcloning to similarly epitope-tag each of the mutant constructs described. Transient transfections of COS7 cells with each receptor cDNA were performed to ensure that each A_{2a}AR mutant was capable of expression to a similar extent as the wild-type receptor, as determined by radioligand binding (data not shown). Generation of cell lines stably expressing receptor cDNAs was achieved by co-transfecting CHO cells with 30 μg of receptor cDNA and 1.5 μg of pSV_2Neo as previously described (Olah *et al*., 1992). After selection in G418, stable transfectants were picked, expanded, and screened for receptor expression by radioligand binding using a 1 nM concentration of the A_{2a}AR-selective agonist radioligand ^{125}I-PAPA-APEC (Barrington *et al*., 1989). A DEAE-dextran protocol was employed for the transient transfection of COS7 and CHO cells (Cullen, 1987). We have previously shown CHO cells to be devoid of functional A_{2a}- and A_{2b}ARs (Palmer *et al*., 1994).

Identification of Receptors by Photoaffinity Labeling. ^{125}I-PAPA-APEC and ^{125}I-azidoPAPA-APEC were synthesized and purified by reverse phase high-performance liquid chromatography as described previously (Nanoff *et al*., 1991). Recombinant A_{2a}ARs in membranes from transfected CHO cells were photoaffinity labeled using 1 nM ^{125}I-azidoPAPA-APEC as previously described (Palmer *et al*., 1994). Nonspecific incorporation was assessed by the inclusion of 10 μM NECA in the labeling reaction.

Radioligand Binding. Saturation binding analyses were performed and analyzed as previously described using ^{125}I-PAPA-APEC (Palmer *et al*., 1994; Nanoff *et al*., 1991; DeLean *et al*., 1982).

Desensitization Conditions and Adenylyl Cyclase Assay. These were performed as we have previously described (Palmer *et al*., 1994) except that the final concentration of $MgCl_2$ in the adenylyl cyclase assay was reduced to 1 mM. To account for interexperimental variation, the maximal stimulation elicited by NECA in control membranes was set at 100% and the basal value set at 0%; the stimulations elicited by the various doses of NECA in control and treated membranes were then normalized with respect to these limits and the resulting curves plotted for analysis (DeLean *et al*., 1982).

Receptor Phosphorylation. Approximately 48 h after transfection, CHO cells were plated into six-well dishes at a density of approximately 1.5×10^6 cells/well and cultured overnight in regular media. The next day, cells were washed twice with phosphate-free Dulbecco's-modified Eagle's media and labeled for 60 min in the same media supplemented with 1 unit/mL adenosine deaminase and 200 μCi/mL [^{32}P]orthophosphate. After cells were exposed to vehicle or agonist at 37 °C for 30 min, reactions were stopped by chilling the cells on ice and washing the monolayers twice with ice-cold phosphate-buffered saline. Cells were then solubilized for 1 h at 4 °C with 750 μL of immunoprecipitation buffer (50 mM Hepes, pH 7.5, 5 mM EDTA, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.1 mM phenylmethanesulfonyl fluoride, and 10 μg/mL each of soybean trypsin inhibitor, leupeptin, and pepstatin A). Lysates were clarified by centrifugation (14000*g*, 15 min), and after equalization by protein assay, supernatants were precleared with protein A−Sepharose for 1 h at 4 °C. Receptors were immunoprecipitated from the precleared supernatants by incubation with 12CA5 and protein A−Sepharose for 2 h at 4 °C. Immune complexes were isolated by brief centrifugation, washed twice with immunoprecipitation buffer supplemented with 0.2 M ammonium sulfate and once with immunoprecipitation buffer alone, and proteins were eluted from the beads by the addition of electrophoresis sample buffer. Analysis was by SDS−PAGE and autoradiography, with quantitation by densitometric scanning.

RESULTS

Expression of A_{2a}ARs. The carboxyl-terminal domains of each of the A_{2a}ARs thus far cloned, while slightly divergent in strict amino acid identity, contain multiple serine and threonine residues suggestive of a role for this domain in receptor regulation (Figure 1A). Specifically, it has been proposed that this region may be a site for receptor phosphorylation events responsible for mediating desensitization (Stiles, 1992). Several lines of evidence now support the idea of a covalent modification being involved in A_{2a}AR desensitization. Firstly, both the onset of desensitization and recovery from the desensitized state after short-term agonist exposure are very rapid, consistent with a dynamically regulated modification of the receptor being responsible. Secondly, inhibition of receptor sequestration fails to inhibit desensitization, suggesting that receptor internalization is not responsible. Finally, we have observed an agonist-stimulated phosphorylation of A_{2a}ARs in stably transfected CHO cells within the same time span in which functional desensitization occurs (Palmer *et al*., 1994).

To examine in detail whether the A_{2a}AR carboxyl-terminal domain was responsible for mediating receptor desensitization, two A_{2a}AR cDNAs were generated which were mutated in this domain (Figure 1A). An A_{2a}AR truncated after Ala 316 (termed Tr316) resulted in the removal of 10 of the 12 putative phosphorylation sites from the receptor while leaving intact a highly basic stretch of amino acids which, by comparison with studies on other G-protein-coupled receptors, may be important in ensuring efficient receptor/G-protein coupling (Figure 1A; Okamoto *et al*., 1991). Consistent with the removal of 95 amino acids from the receptor protein, the stably expressed Tr316 A_{2a}AR migrated faster on SDS−PAGE compared with the wild-type receptor after visualization by photoaffinity labeling (Figure 1B). A second mutant was generated in which only the two putative phosphorylation sites closest to the seventh transmembrane domain (Thr 298 and Ser 305) were disrupted by mutation to Ala residues (Figure 1A). CHO cell lines stably expressing these cDNAs were generated and clones expressing similar levels of receptor studied further (Table 1). In line with observations made on a similar A_{2a}AR mutant (Pierson *et al*., 1994), removal of the carboxyl-terminal domain after Ala 316 had no effect on the ability of the receptor to bind agonist with high affinity or to stimulate adenylyl cyclase

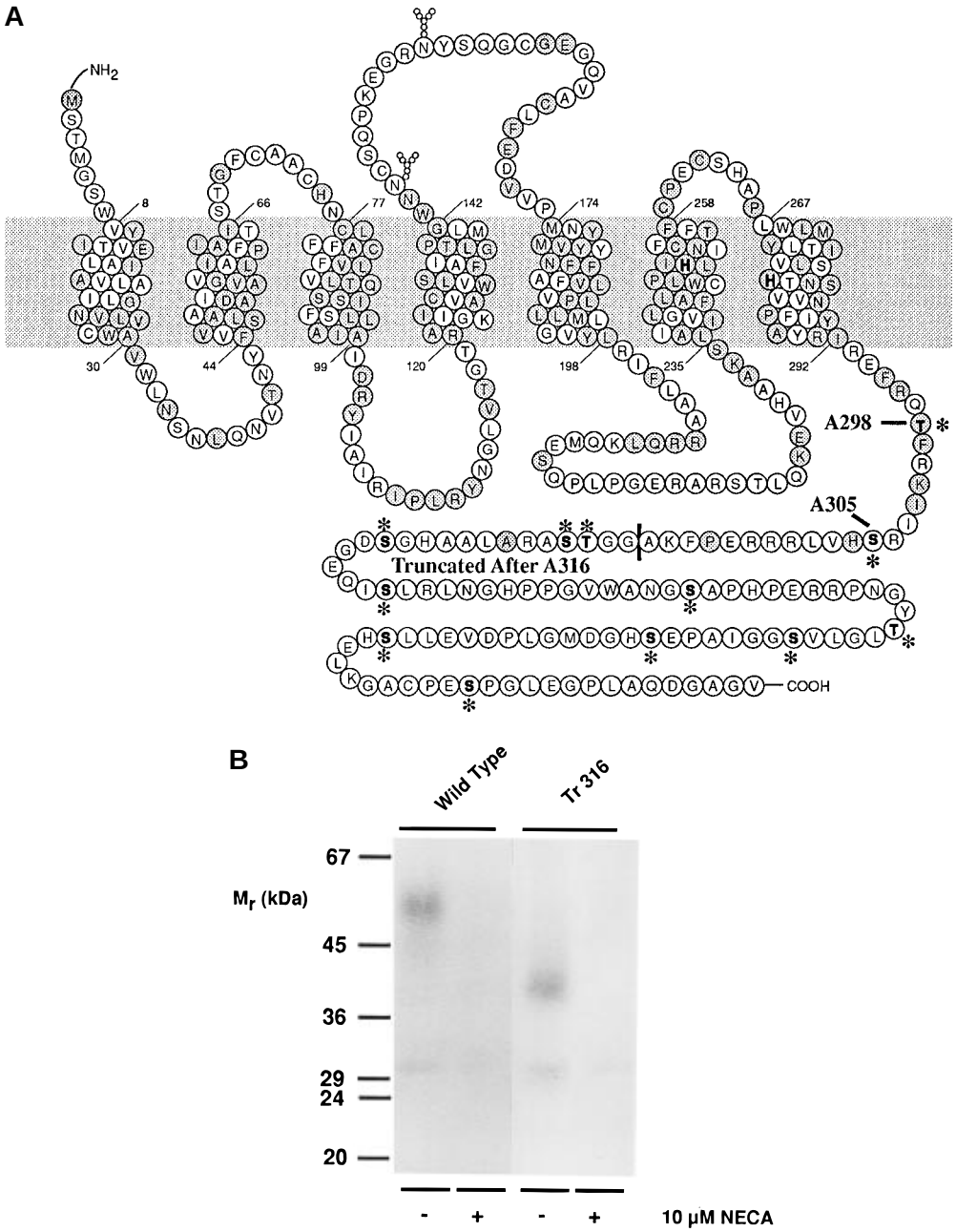

FIGURE 1: Mutational analysis of A_{2a}AR desensitization. (A) Presumed membrane-spanning topography of the A_{2a}AR. The sites at which mutations were introduced are indicated. Shaded residues represent amino acids conserved between the canine A_1 and A_{2a}ARs. The serine and threonine residues marked by asterisks represent potential phosphorylation sites. The two predicted sites for N-linked glycosylation on the second extracellular loop are indicated, although only one appears to be utilized (Palmer *et al*., 1992). Each of the constructs used also expressed the hemagglutinin epitope recognized by monoclonal antibody 12CA5 at their amino termini. (B) Identification of recombinant A_{2a}ARs by photoaffinity labeling. Membranes from CHO cells stably expressing wild-type and Tr316 truncated A_{2a}ARs were photoaffinity labeled using ^{125}I-azidoPAPA-APEC in the presence or absence of 10 μM NECA as described in the Experimental Procedures. After SDS−PAGE, A_{2a}ARs were visualized by autoradiography.

activity (Table 1). By contrast, mutation of Thr 298 and Ser 305 to Ala elevated the EC_{50} for NECA stimulation of adenylyl cyclase activity approximately 5-fold, suggesting that this portion of the carboxyl-terminal domain may be important in ensuring efficient receptor/G-protein coupling. However, both the maximal stimulation of adenylyl cyclase achieved and the K_d for ^{125}I-PAPA-APEC obtained in radioligand binding experiments were comparable to those of the wild-type receptor, indicating that receptor/G_s coupling was not severely diminished (Table 1).

Desensitization in Response to Short-Term Agonist Exposure. To determine the effect of the various mutations on short-term desensitization, stably transfected cells were incubated with or without 10 μM NECA for 30 min prior to assay of adenylyl cyclase activity (Figure 2, Table 2). Consistent with our previous study (Palmer *et al*., 1994), cells expressing wild-type receptor underwent a profound functional desensitization as manifested by a reduction in the ability of NECA to maximally stimulate adenylyl cyclase in membranes from NECA-treated cells compared with untreated controls (Figure 2A, Table 2). Surprisingly, the Tr316 A_{2a}AR mutant underwent a functional desensitization comparable to that exhibited by the wild-type receptor despite the removal of several putative phosphorylation sites (Figure 2B, Table 2). Other experiments demonstrated that both the rate of onset of desensitization and the dose-dependence of

Table 1: Characterization of CHO Cell Lines Stably Expressing Wild-Type and Mutant A_{2a}ARs[a]

	radioligand binding		adenylyl cyclase stimulation		
cell line	B_{max} range (pmol/mg)	K_d (nM)	basal [pmol/(min/mg)]	EC_{50} (μM)	FS
wild type	0.26−0.30	1.8 ± 0.3	3.2 ± 1.0	0.3 ± 0.1	19.7 ± 0.5
Tr316	0.21−0.26	2.5 ± 0.1	4.9 ± 1.1	0.4 ± 0.3	15.0 ± 5.4
$T^{298}A,S^{305}A$	0.40−0.44	3.6 ± 1.0	2.4 ± 0.4	1.3 ± 0.3	19.1 ± 2.4
$T^{298}A$	0.20−0.39	1.5 ± 0.2	2.6 ± 0.6	3.5 ± 1.8	16.2 ± 2.6
$S^{305}A$	0.25−0.32	1.7 ± 0.3	3.1 ± 0.7	4.3 ± 1.5	13.5 ± 2.2

[a] Membranes from CHO cells stably expressing the indicated A_{2a}AR cDNA were prepared for assay of adenylyl cyclase activity, ^{125}I-PAPA-APEC saturation binding experiments, and analysis as outlined in the Experimental Procedures. Data are presented for between 3 and 10 experiments and, where indicated, given as mean ± SE. FS. = fold stimulation above basal activity.

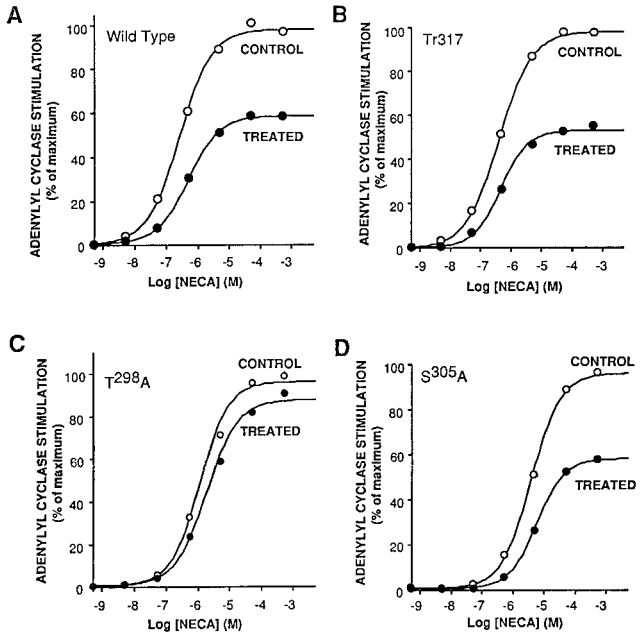

FIGURE 2: Short-term desensitization of wild-type and mutant A_{2a}ARs. CHO cells stably expressing either (A) wild-type, (B) Tr316, (C) $T^{298}A$, or (D) $S^{305}A$ A_{2a}AR cDNAs were treated with or without 10 μM NECA for 30 min prior to membrane preparation, assay of adenylyl cyclase activity, and analysis as described in the Experimental Procedures. Each curve represents the mean of 3 or 4 experiments. Composite data are given in Table 2.

desensitization to increasing NECA concentrations were similarly indistinguishable between the wild-type and Tr316 A_{2a}ARs (data not shown). In contrast, cells expressing the $T^{298}A,S^{305}A$ double mutant exhibited a severely attenuated functional desensitization compared with the wild-type A_{2a}AR (Table 2). Moreover, either increasing the agonist incubation time to 60 min or increasing the concentration of NECA employed from 10 to 50 μM (to account for this mutant's elevated EC_{50} for adenylyl cyclase activation) failed to unmask any significant desensitization response (data not shown). Therefore, the presence of Thr 298 and Ser 305 is essential to observe a desensitization in response to short-term agonist exposure.

To ascertain whether a single amino acid was responsible for this effect, A_{2a}AR cDNAs containing either $T^{298}A$ or $S^{305}A$ point mutations were generated and stably expressed in CHO cells (Table 1). The clonal cell lines chosen displayed parameters simlar to those of the $T^{298}A,S^{305}A$ double mutant, *i.e.*, an elevated EC_{50} but similar maximal

Table 2: Short-Term Desensitization of Wild-Type and Mutant A_{2a}ARs[a]

	% of control stimulation	EC_{50} (μM)	
cell line	at 50 μM NECA	control	treated
wild type	60 ± 9 (8)[b]	0.3 ± 0.1	0.5 ± 0.1 (4)
Tr316	58 ± 6 (8)[b]	0.2 ± 0.1	0.3 ± 0.1 (3)
$T^{298}A,S^{305}A$	95 ± 6 (6)	1.3 ± 0.3	2.9 ± 0.6 (3)
$T^{298}A$	90 ± 8 (4)	1.8 ± 0.3	2.1 ± 0.4 (4)
$S^{305}A$	58 ± 9 (4)[b]	3.7 ± 1.1	5.8 ± 1.6 (4)

[a] CHO cells stably expressing the indicated A_{2a}AR cDNA were treated for 30 min with or without 10 μM NECA prior to membrane preparation for assay of adenylyl cyclase activity and analysis as described in the Experimental Procedures. The % stimulation values are presented as mean ± SE for the number of experiments indicated in parentheses, whereas the EC_{50} values are derived from curve-fitting analysis of mean values from the number of desensitization experiments indicated. [b] Statistically significant difference from untreated controls ($p < 0.05$).

stimulation of adenylyl cyclase with similar K_d and B_{max} values for radioligand binding (Table 1). The desensitization pattern of the $T^{298}A$ mutant was virtually identical to that of the $T^{298}A,S^{305}A$ double mutant, with very little functional desensitization observed after 30 min agonist exposure (Figure 2C, Table 2). However, the $S^{305}A$ mutant A_{2a}AR exhibited a profound functional desensitization similar in magnitude to that of the wild-type and Tr316 A_{2a}ARs (Figure 2D, Table 2). This demonstrates that the reduced coupling efficiency exhibited by the $T^{298}A,S^{305}A$ double mutant is not responsible for its lack of desensitization since the $S^{305}A$ mutant exhibits a similar reduced coupling yet desensitizes similarly to the wild-type A_{2a}AR, and that the presence of Thr 298 is essential to observe a short-term desensitization response.

Effect of Thr 298 Mutation on Receptor Phosphorylation. To determine the effects of mutating Thr 298 on receptor phosphorylation, CHO cells were transiently transfected with wild-type or $T^{298}A$ A_{2a}AR cDNAs for ^{32}P-labeling and receptor immunoprecipitation with 12CA5. Transiently transfected cells were used for these experiments since this procedure results in receptor expression which is routinely some 10-fold higher (typically 2−3 pmol of receptor/mg) than that exhibited by the stable cell lines. In the absence of agonist, 12CA5 specifically immunoprecipitates a phosphoprotein of the appropriate size from wild-type and $T^{298}A$ A_{2a}AR-transfected cells but not nontransfected CHO cells (Figure 3). However, under conditions where the addition of agonist increased the labeling of the wild-type A_{2a}AR by some 41 ± 10% over basal (three experiments), no significant increase in the labeling of the $T^{298}A$ A_{2a}AR was observed (6 ± 12% decrease vs vehicle-treated controls, three experiments, $p > 0.05$) (Figure 3).

Desensitization in Response to Long-Term Agonist Exposure. We have previously demonstrated that the desensitization mechanisms induced upon short- and long-term agonist exposure are distinct, resulting in different rates of recovery after agonist removal depending on the duration of agonist pretreatment (Palmer *et al.*, 1994). Therefore, to determine whether A_{2a}ARs mutated at Thr 298 are completely defective in mediating desensitization, the various receptor-expressing cell lines were treated with or without 10 μM NECA for 24 h prior to assay of adenylyl cyclase activity (Figure 4A). Each receptor exhibited a functional desensitization after 24 h agonist treatment including the $T^{298}A$ and $T^{298}A,S^{305}A$

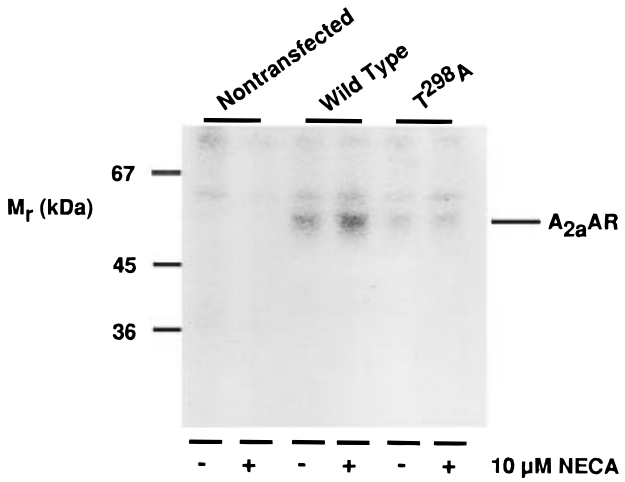

FIGURE 3: Phosphorylation of wild-type and $T^{298}A$ A_{2a}ARs. CHO cells transiently transfected with the indicated cDNAs were labeled with [^{32}P]orthophosphate prior to incubation with vehicle or 10 μM NECA for 30 min at 37 °C. After washing, cell monolayers were solubilized for immunoprecipitation with 12CA5 and protein A−Sepharose, with analysis by SDS−PAGE and autoradiography.

mutants which failed to desensitize after short-term agonist exposure. Moreover, the $T^{298}A$ mutant recovered slowly from the desensitized state after agonist removal in a manner indistinguishable from the recovery of the wild-type receptor (Figure 4B). Therefore, the attenuated desensitization pattern of A_{2a}ARs mutated at Thr 298 appeared to be restricted to short-term agonist treatment times.

DISCUSSION

The A_{2a}AR mediates several important physiological effects of adenosine, including inhibition of platelet aggregation and onset of vasodilation (Olsson & Pearson, 1990). However, exploitation of the potent antihypertensive effects of this receptor remains an unexplored area for therapeutic intervention. While adenosine itself would not prove useful for these purposes, due to its short biological half-life, several metabolically stable A_{2a}AR-selective agonists have been developed in recent years (Barrington *et al*., 1989; Jarvis *et al*., 1989). Nevertheless, the beneficial effects of these compounds would diminish over time due to the phenomenon of desensitization; indeed, desensitization of A_{2a}AR-mediated antihypertensive effects have been noted in several studies (Makujina & Mustafa, 1993; Hussain & Mustafa, 1993). Therefore, an understanding of the various desensitization mechanisms induced upon agonist exposure is an important first step in manipulating adaptive processes such that cells may remain responsive to agonist, thereby increasing any therapeutic effectiveness. This can only be achieved when the molecular determinants responsible for mediating the observed refractoriness have been delineated.

While homologous desensitization of A_{2a}AR-mediated cAMP production has been documented in several systems (Newman & Levitzki, 1983; Anand-Srivastava *et al*., 1989; Ramkumar *et al*., 1991), the various adaptive mechanisms responsible for this effect have been determined only recently (Palmer *et al*., 1994). Short-term exposure to NECA, but not forskolin or phorbol ester, induces a rapid diminution of A_{2a}AR function which is readily reversible upon removal of agonist. This phase of desensitization is associated with the phosphorylation of the receptor protein and a reduced

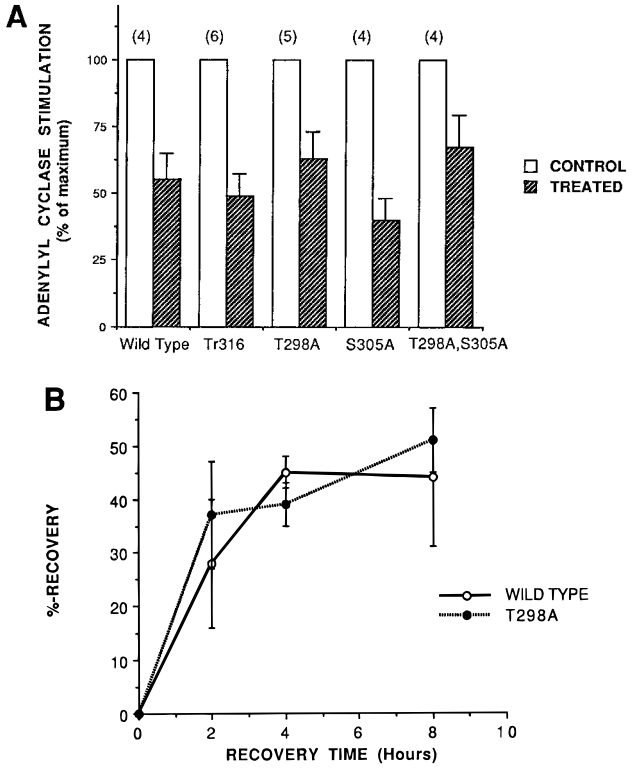

FIGURE 4: A_{2a}AR desensitization after long-term agonist exposure. (A) CHO cells stably expressing the indicated A_{2a}AR cDNAs were treated with or without 10 μM NECA for 24 h prior to membrane preparation, assay of adenylyl cyclase activity, and analysis as described in the Experimental Procedures. Data are presented as means ±SE for the number of experiments indicated in parentheses. (B) CHO cells stably expressing either the wild-type or $T^{298}A$ mutant A_{2a}ARs were treated with or without 10 μM NECA for 24 h. After treatment with agonist, monolayers were washed twice with 20 mL of prewarmed PBS and the cells incubated in agonist-free media for the indicated times prior to membrane preparation and assay of adenylyl cyclase activity in the presence or absence of 50 μM NECA as described in the Experimental Procedures. The % recovery at the appropriate time points was calculated as 100 × [fold stimulation (recovering cells) − fold stimulation (treated cells)/ fold stimulation (control cells) − fold stimulation (treated cells)]. Data are presented as means ± SE for 3 experiments. The average desensitization induced by agonist treatment in these experiments was to 54 ± 14% (wild type) and 71 ± 4% ($T^{298}A$ mutant) of the control stimulation at 50 μM NECA.

coupling efficiency between the receptor and G_s. Longer treatment times lead to receptor down-regulation, and although the extent of desensitization observed is similar to that seen at early time points, recovery from long-term treatment requires several hours. Overall, it seems that the importance of the different adaptive processes varies depending on the length of exposure to agonist (Palmer *et al*., 1994).

In this study, we have been able to demonstrate that the abilities to desensitize in response to short-term agonist exposure and exhibit an agonist-stimulated phosphorylation appear to depend on the presence of the Thr residue at position 298. These observations are consistent with a model whereby phosphorylation at Thr 298 is responsible for the reduced ability of the A_{2a}AR to stimulate adenylyl cyclase activity. It is also possible that mutation of Thr 298 induces a nonspecific conformational change in the receptor such that the ability of an agonist-occupied receptor to be recognized by the cell's desensitization "machinery" is attenuated. However, the ability of mutant A_{2a}ARs to display functional parameters comparable to those of wild-type

receptors when expressed in CHO cells argues against any nonspecific conformational effects (Table 1).

One difference from our previous report (Palmer *et al*., 1994) is our observation in this study of a basal phosphorylation state of the A_{2a}AR (Figure 3). This discrepancy most likely results from the different antibodies used to immunoprecipitate the A_{2a}AR. The lack of a basal phosphorylation in our previous study (Palmer *et al*., 1994) is most probably related to the somewhat poorer immunoprecipitating capacity of the polyclonal antibody used previously compared with the 12CA5 monoclonal antibody used here (Figure 3). Preliminary experiments utilizing photoaffinity-labeled A_{2a}ARs suggest that immunoprecipitations with 12CA5 produce an approximately 5-fold greater recovery of A_{2a}ARs in immunoprecipitates than our polyclonal antibody under the same conditions (data not shown). However, the observation of an agonist-stimulated phosphorylation of the A_{2a}AR above basal levels (Figure 3) is consistent both with our previous observations and with a potential role of receptor phosphorylation in mediating rapid A_{2a}AR desensitization (Palmer *et al*., 1994).

Thr 298 is located in a region of the A_{2a}AR that is relatively enriched in basic amino acids and that would be expected to be positively charged at physiological pH (Figure 1A). Similar stretches of basic residues have been shown to mediate receptor/G-protein coupling for some receptor subtypes (Okamoto *et al*., 1991). If Thr 298 was the site of a phosphorylation event, introduction of such a negatively charged group would be expected to affect the charge distribution in this region, which in turn could affect interaction with G_s. A role for this domain in receptor/G-protein coupling is suggested by the elevated EC_{50} value for NECA-mediated stimulation of adenylyl cyclase activity observed for the $T^{298}A$ and $S^{305}A$ point mutants as well as the $T^{298}A,S^{305}A$ double mutant (Table 1). Clearly, this coupling defect does not ultimately affect the abilities of the mutant A_{2a}ARs to maximally stimulate adenylyl cyclase activity and, more importantly, is not responsible for the inability of each of the Thr 298 mutants to undergo desensitization since the $S^{305}A$ mutant A_{2a}AR desensitizes in a manner similar to the wild-type receptor (Figure 2D, Table 2).

Our inability to detect any gross changes in agonist radioligand binding parameters despite the 5-fold increases in EC_{50} values observed for adenylyl cyclase stimulation in the $S^{305}A$ and $T^{298}A$ mutants may be due to any of several reasons. One of these may relate to the fact that agonist radioligand binding experiments are a measure of receptor/G-protein contact and do not provide any indication of the ability of the agonist-occupied receptor to induce the correct conformational change in the G-protein necessary to induce release of GDP. Therefore, a situation is possible whereby mutant receptors can interact with G_s (measurable as normal radioligand binding) but inefficiently induce the change in conformation of G_s required for GDP release and subsequent effector activation (manifested as an elevated EC_{50} for adenylyl cyclase activation).

The most surprising finding in the present study was the apparent functional redundancy of the carboxyl-terminal 95 amino acids of the A_{2a}AR. Removal of this region had no effect on the ability of the receptor either to couple to G_s or to undergo desensitization; the latter effect is particularly surprising since this region contains several putative phosphorylation sites (Figure 1A). Moreover, in many instances these sites are flanked on their amino-terminal side by acidic amino acids, which peptide phosphorylation studies have shown to be an important structural determinant in determining susceptibility to phosphorylation by GRK2 (Onorato *et al*., 1991). While the highly basic nature of the sequence surrounding Thr 298, and the functional redundancy of most of the C-terminal domain, may argue against a role for GRK2, it remains possible that GRKs may phosphorylate nonacidic motifs under certain conditions, as has recently been described for GRK2- and GRK5-mediated phosphorylation of the β_2-adrenergic receptor *in vitro* (Fredericks *et al*., 1996). To our knowledge, only three other G-protein-coupled receptors thus far studied have Ser/Thr-rich C-terminal domains which are not involved in agonist-induced desensitization. It has been reported that removal of potential phosphorylation sites from the tails of each of the $5\text{-}HT_2$, angiotensin II_{1A}, or endothelin A receptors fails to diminish the rate or extent of receptor desensitization as determined by measuring either agonist-stimulated inositol phosphate accumulation or mobilization of intracellular calcium (Vouret-Craviari *et al*., 1995; Thomas *et al*., 1995; Cyr *et al*., 1993). As far as we are aware, the A_{2a}AR is the first example of a mammalian G_s-coupled receptor which still desensitizes after similar carboxyl-terminal truncation.

As demonstrated for other G-protein-coupled receptors, the structural requirements necessary for observing short-term desensitization of the A_{2a}AR appear to be distinct from those mediating long-term adaptation (Lohse *et al*., 1992). Each of the receptors studied here exhibited a functional desensitization in response to long-term agonist treatment including the Thr 298 mutants which exhibited attenuated short-term desensitization. Therefore, mechanisms of short- and long-term adaptation are separable temporally, mechanistically, and with respect to the structural requirements of the A_{2a}AR which mediate them. As for other receptors, the exact sequences responsible for mediating long-term adaptation are unclear.

In conclusion, we have identified a small region of the A_{2a}AR carboxyl-terminal tail whose integrity is essential for observation of rapid, but not long-term, agonist-mediated desensitization, with the presence of Thr 298 being particularly important. Taken together with our previous work (Palmer *et al*., 1994), this suggests that rapid desensitization may be mediated by phosphorylation of the A_{2a}AR at this position, by kinases unknown, leading to reduced $A_{2a}AR/G_s$ coupling and adenylyl cyclase stimulation. We have also demonstrated that the presence of a large number of putative phosphorylation sites does not guarantee that they will all be required to induce functional desensitization *in vivo*. Indeed, the lack of a requirement for the majority of the A_{2a}AR carboxyl-terminal tail in mediating desensitization would suggest that this domain has a previously unappreciated role which can only be determined when the nature of the protein(s) with which it interacts is revealed.

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