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Subtype-Specific Kinetics of Inhibitory Adenosine Receptor Internalization Are Determined by Sensitivity to Phosphorylation by G Protein-Coupled Receptor Kinases

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ABSTRACT

Despite coupling to the same class of inhibitory G proteins and binding the same physiological ligand, the human A_1 and rat A_3 adenosine receptors (ARs) desensitize at different rates in response to sustained agonist exposure. This is due to the ability of the A_3AR, but not the A_1AR, to serve as a substrate for rapid phosphorylation and desensitization by members of the G protein-coupled receptor kinase (GRK) family. The aim of this study was to investigate whether these differences were also manifested in their abilities to undergo agonist-dependent receptor internalization. For the first time, we report that A_3ARs internalize profoundly in response to short-term exposure to agonist but not activators of second messenger-regulated kinases. The A_3AR-selective antagonist MRS1523 blocked both A_3AR phosphorylation and internalization. Moreover, in contrast to the A_1AR, which internalized quite slowly ($t_{1/2}$ = 90 min), A_3ARs internalized rapidly ($t_{1/2}$ = 10 min) over a time frame that followed the onset of receptor phosphorylation. A nonphosphorylated A_3AR mutant failed to internalize over a 60-min time course, suggesting that receptor phosphorylation was essential for rapid A_3AR internalization to occur. In addition, fusion onto the A_1AR of the A_3AR C-terminal domain containing the sites for phosphorylation by GRKs conferred rapid agonist-induced internalization kinetics ($t_{1/2}$ = 10 min) on the resulting chimeric AR. In conclusion, these data suggest that GRK-stimulated phosphorylation of threonine residues within the C-terminal domain of the A_3AR is obligatory to observe rapid agonist-mediated internalization of the receptor.

Desensitization has been defined traditionally as the process whereby a guanine nucleotide-binding regulatory protein (G protein)-coupled receptor (GPCR)-initiated response plateaus and then diminishes despite the sustained presence of agonist. However, several recent studies have suggested that the molecular processes that desensitize signaling pathways at the plasma membrane can simultaneously initiate alternate pathways after receptor clustering and internalization (Lefkowitz, 1998). From work performed predominantly on the β_2-adrenergic receptor, it has been suggested that agonist-stimulated phosphorylation of the receptor protein by GPCR kinases (GRKs) stimulates the binding of arrestin proteins (Krupnick and Benovic, 1998; Pitcher et al., 1998). This serves at least three functions: 1) uncoupling of plasma membrane-located receptors from heterotrimeric G proteins, thereby leading to a functional desensitization of G protein-linked signaling; 2) clustering of phosphorylated receptors into clathrin-coated pits; and 3) recruitment and activation of src family tyrosine kinases, which ultimately result in the activation of the mitogen-activated protein kinase (MAPK) signaling cascade (Luttrell et al., 1997, 1999). Although evidence supporting this model of β_2-adrenergic receptor stimulation of MAPK is growing, it is unlikely that it is applicable to all other GPCRs. For example, m_3 muscarinic acetylcholine and κ-opioid receptor stimulation of MAPK occur independently of receptor internalization (Budd et al., 1999; Li et al., 1999). Also, several GPCRs, including the A_1 adenosine receptor (AR), transiently activate MAPK activity over time courses that temporally precede the onset of receptor internalization (Ciruela et al., 1997; Dickenson et al., 1998). Thus, to manipulate GPCR signaling, it is essential that we understand the role of receptor phosphorylation and/or internalization in initiating or terminating specific signaling cascades.

Although we have demonstrated that the rat A_3AR is phos-

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ABBREVIATIONS: G protein, guanine nucleotide-binding regulatory protein; GPCR, G protein-coupled receptor; AR, adenosine receptor; GRK, G protein-coupled receptor kinase; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; LC, long alkyl spacer chain; (*R*)-PIA, (*R*)-N^6-(phenylisopropyl)adenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; WT, wild type; MAPK, mitogen-activated protein kinase.

phorylated rapidly by one or more GRKs in response to short-term agonist exposure (Palmer et al., 1995, 1996), it is not known whether the receptor internalizes. In this study, we used the human A_1 and a panel of rat A_3AR mutants to examine whether differential sensitivity to phosphorylation regulates inhibitory AR internalization in stably transfected Chinese hamster ovary (CHO) cells. Our results demonstrate that agonist-occupied A_1 and A_3ARs internalize over markedly different time courses. In addition, using both loss-of-function and gain-of-function mutagenesis approaches, we demonstrate that the distinct internalization kinetics displayed by the A_1ARs and A_3ARs are determined by their markedly different sensitivities to agonist-stimulated phosphorylation in situ by GRKs.

Experimental Procedures

Materials and Cell Lines. Biotin-long alkyl spacer chain (LC)-hydrazide and horseradish peroxidase-conjugated streptavidin were obtained from Pierce-Wariner. The A_3AR-selective antagonist MRS1523 [5-propyl 2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate; Li et al., 1998] was the generous gift of Dr. Ken Jacobson (National Institutes of Health, Bethesda, MD). The sources of all other materials and the generation of CHO cell lines stably expressing the indicated AR cDNAs have been documented previously (Palmer et al., 1995, 1996; Palmer and Stiles, 2000).

Receptor Phosphorylation. Receptor-expressing CHO cells were plated onto 6-well dishes at a density of approximately 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 in the same medium supplemented with 1 U/ml adenosine deaminase and 0.2 mCi/ml [^{32}P]orthophosphate. After incubation with 5′-*N*-ethylcarboxamidoadenosine (NECA), (*R*)-*N*^{6}-(phenylisopropyl)adenosine [(*R*)-PIA], or MRS1523 for the times indicated in the figure legends, reactions were terminated by placing the cells in ice and washing three times with ice-cold PBS. All subsequent procedures were performed at 4°C unless stated otherwise. Cells were solubilized by the addition of 0.5 ml of immunoprecipitation buffer (50 mM sodium HEPES, pH 7.5, 5 mM EDTA, 10 mM sodium phosphate, 10 mM sodium fluoride, 0.1 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml pepstatin A, and 10 μg/ml concentration each of soybean trypsin inhibitor and benzamidine). After a 60-min incubation on a rotating wheel, insoluble material was removed by centrifugation (14,000*g* for 15 min). Extracts were then equalized by protein assay and precleared of nonspecific binding proteins by incubation with protein A-Sepharose in the presence of 0.2% (w/v) IgG-free BSA. Receptors were then immunoprecipitated from precleared supernatants by incubation for 2 h with protein A-Sepharose and 1 μg of 12CA5. Immune complexes were isolated by centrifugation, washed twice with immunoprecipitation buffer supplemented with 0.2 M ammonium sulfate and once with immunoprecipitation buffer alone, and eluted from the protein A-Sepharose by the addition of electrophoresis sample buffer and incubation at 37°C for 1 h. Analysis was by SDS-polyacrylamide gel electrophoresis (PAGE) using 10% (w/v) polyacrylamide resolving gels and autoradiography.

Receptor Internalization Assay. AR-expressing CHO cell lines were plated onto 6-well dishes at a density of 1×10^6 cells/well. The next day, the cells were washed, and 0.75 ml/well normal medium was applied. Incubations were initiated by the addition of adenosine deaminase with vehicle, (*R*)-PIA, or MRS1523 for the times indicated in the figure legends. Reactions were terminated by placing the cells on ice and washing monolayers three times with ice-cold PBS. All subsequent procedures were performed at 4°C unless stated otherwise. The alcohol groups on cell-surface glycoproteins were oxidized to aldehydes by a 30-min incubation with 10 mM sodium periodate in PBS. After the removal of periodate and washing with PBS, monolayers were washed twice with 0.1 M sodium acetate, pH 5.5, and incubated for 30 min in the same buffer supplemented with 1 mM biotin-LC-hydrazide. This reacts with the newly formed alcohol groups, thereby labeling all cell-surface glycoproteins with biotin. Labeling was terminated by removal of the biotin-LC-hydrazide solution and washing monolayers three times with PBS. Cells were then solubilized for receptor immunoprecipitation with 12CA5 as described earlier. After fractionation of immunoprecipitated receptors by SDS-PAGE, proteins were transferred to a nitrocellulose membrane. Nonspecific protein-binding sites were blocked by incubation in Blotto [5% (w/v) skimmed milk in PBS supplemented with 0.2% (v/v) Triton X-100]. Cell surface biotin-labeled receptors were then identified by incubation of the membrane with 1 μg/ml horseradish peroxidase-conjugated streptavidin for 60 min at room temperature. After three washes with Blotto and two washes with PBS, reactive proteins were visualized by enhanced chemiluminescence (Renaissance; New England Nuclear Research Products, Boston, MA). Agonist-induced loss of cell-surface receptor was quantified by densitometric scanning of blots. Data are presented as mean ± S.E. for the number of experiments indicated. Statistical significance was determined by two-tailed Student's *t* tests with significance assessed at $P < .05$.

Results

Agonist Stimulation of A_3AR Internalization. To determine whether agonist treatment could induce internalization of the A_3AR, we used the presence of multiple consensus sites for *N*-linked glycosylation in the N-terminal domain and second extracellular loop. This allowed us to label cell-surface A_3AR glycoproteins with a membrane-impermeable derivative of biotin (Palmer et al., 1995) and assay the ability of specific drugs to induce a loss in cell-surface receptor levels. The treatment of wild-type (WT) A_3AR-expressing cells with either a 1 μM concentration of the AR agonist (*R*)-PIA or a 10 μM concentration of the agonist NECA for 30 min induced a 56 ± 18% ($P < .05$ versus vehicle-treated controls, $n = 3$) reduction in the levels of cell-surface A_3AR (Fig. 1, A and B). This effect could not be mimicked by several activators of second messenger-regulated protein kinases, including the phorbol ester phorbol-12-myristate-13-acetate, the calcium ionophore A23187, and 8-bromo-cGMP, which activates cGMP-dependent protein kinase (Fig. 1A).

Interestingly, elevation of intracellular cAMP levels with the diterpene forskolin resulted in both a small reduction in cell-surface A_3AR levels and a reduced mobility of the A_3AR protein on SDS-PAGE (Fig. 1A). This effect was not unique to forskolin as similar results were observed with the nonhydrolyzable membrane-permeable cAMP analog 8-bromo-cAMP (data not shown). Although phosphorylation is known to reduce the electrophoretic mobility of a variety of GPCRs (Ali et al., 1993), we were unable to detect any increase in A_3AR phosphorylation in response to forskolin or 8-bromo-cAMP treatment, consistent with our previous observations (Palmer et al., 1995). We are currently investigating this issue but want to note here that elevation of cAMP levels is unlikely to be responsible for the effects of agonist on A_3AR internalization for three reasons. First, multiple studies have shown that activation of the A_3AR in CHO cells reduces intracellular cAMP levels (Zhou et al., 1992; Linden et al., 1993; Palmer et al., 1995). Second, unlike the effect of forskolin/8-bromo-cAMP treatment, agonist exposure does not

appreciably alter the mobility of the A_3AR protein on SDS-PAGE. Finally, agonist treatment induces a much greater internalization of cell-surface A_3ARs than that observed after forskolin treatment.

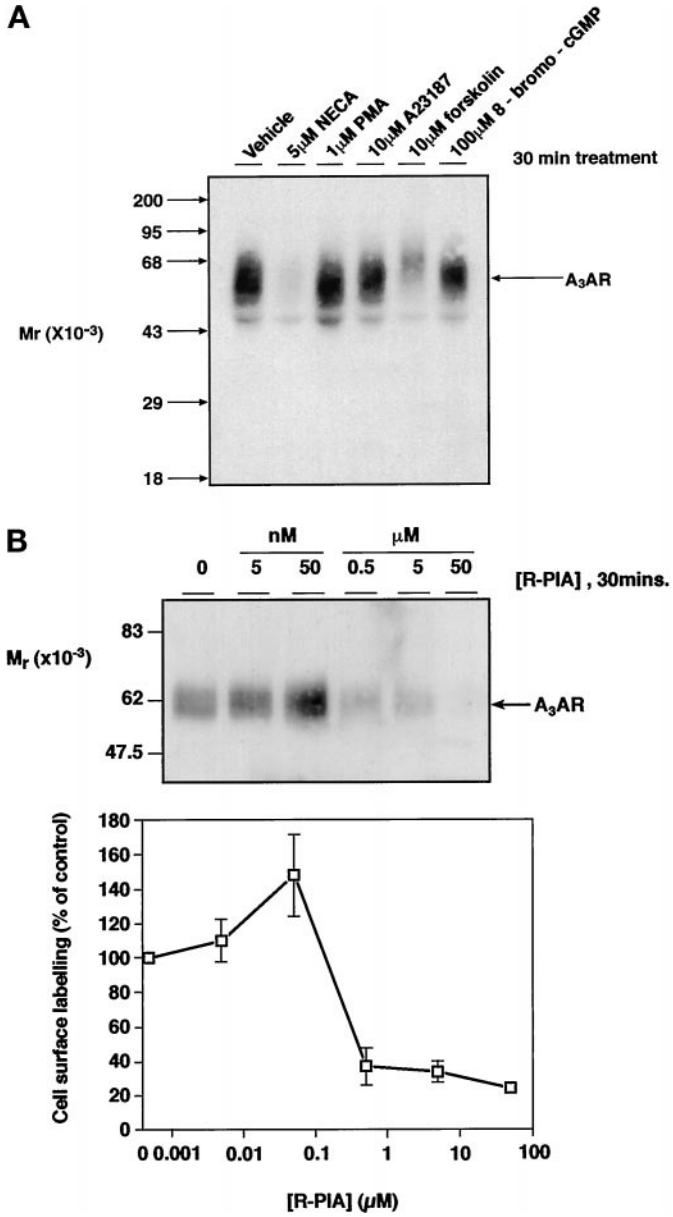

Fig. 1. Effects of exposure to agonist and activators of second messenger-regulated kinases on levels of cell-surface A_3ARs. A, CHO cells stably expressing a hemagglutinin epitope-tagged WT A_3AR were exposed for 30 min at 37°C to either vehicle, 5 μM concentration of the AR agonist NECA, 1 μM concentration of phorbol-12-myristate-13-acetate (PMA), 10 μM concentration of the calcium ionophore A23187 in the presence of 1.8 mM calcium chloride, 10 μM forskolin, or 100 μM 8-bromo-cGMP. Cell-surface glycoproteins were then labeled with biotin before receptor immunoprecipitation with 12CA5. After fractionation of immunoprecipitates by SDS-PAGE, biotin-labeled cell-surface A_3ARs were visualized by transfer to nitrocellulose and probing with horseradish peroxidase-streptavidin as described in *Experimental Procedures*. B, WT A_3AR-expressing CHO cells were incubated with the indicated concentrations of (*R*)-PIA for 30 min at 37°C. Cell-surface glycoproteins were then labeled with biotin before receptor immunoprecipitation with 12CA5 and visualization as described in *Experimental Procedures*. Blots were quantified by densitometric scanning. Values represent mean ± S.E. for three experiments, with the levels of cell-surface A_3AR observed in the absence of agonist set at 100%.

Analysis of the effects of increasing (*R*)-PIA concentrations on cell-surface A_3AR levels produced a biphasic response curve (Fig. 1B). (*R*)-PIA-mediated loss of cell-surface A_3ARs occurred with an EC_{50} value of approximately 0.2 μM (Fig.

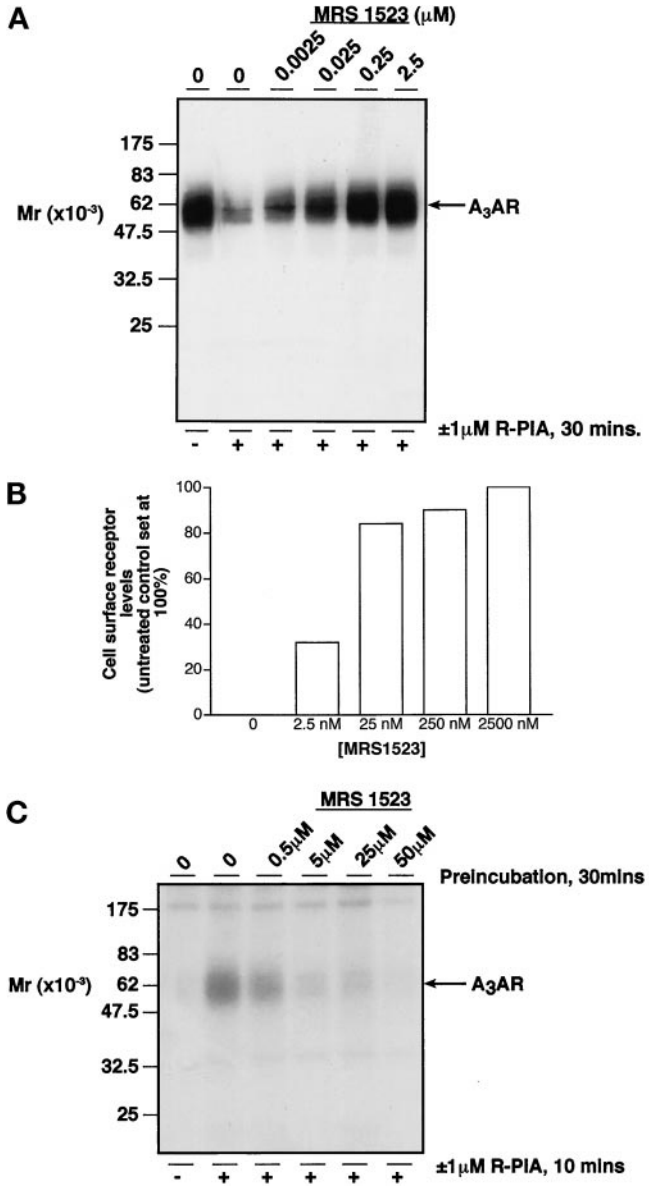

Fig. 2. Effect of the A_3AR-selective antagonist MRS1523 on agonist-stimulated phosphorylation and internalization of the WT A_3AR. A, WT A_3AR-expressing CHO cells were incubated with the indicated concentrations of MRS1523 for 30 min at 37°C before the addition of (*R*)-PIA to a final concentration of 1 μM for an additional 30 min. Cell-surface glycoproteins were then labeled with biotin before receptor immunoprecipitation with 12CA5 and visualization as described in *Experimental Procedures*. B, quantitative analysis of the results in A. The levels of cell-surface receptor in untreated control cells were set at 100%, whereas those in cells treated only with agonist were set at 0%. The levels of cell-surface receptor observed for the other conditions were then normalized with respect to these limits. These data are representative of three experiments that produced similar results. C, ^{32}P-prelabeled WT A_3AR-expressing CHO cells were incubated with the indicated concentrations of the A_3AR-selective antagonist MRS1523 for 30 min at 37°C before the addition of (*R*)-PIA to a final concentration of 1 μM for an additional 10 min. Reactions were then terminated and receptors were immunoprecipitated with 12CA5 as described in *Experimental Procedures*. After fractionation of immunoprecipitated proteins by SDS-PAGE, phosphorylated A_3ARs were visualized by autoradiography of the dried gel. This is one of three experiments that produced identical results.

1B). This is almost identical with the K_i value for (*R*)-PIA-mediated displacement of agonist radioligand binding from the rat A_3AR (Zhou et al., 1992) and suggests that the extent to which (*R*)-PIA mediates A_3AR internalization is linked closely to receptor occupancy. However, low nanomolar concentrations of (*R*)-PIA (5–50 nM) consistently produced a small yet significant increase in the levels of cell-surface A_3AR (Fig. 1B).

Antagonist Blockade of A_3AR Phosphorylation and Internalization. To determine whether A_3AR internalization was agonist-specific, we assessed the effect of preincubating transfected CHO cells with the A_3AR-selective antagonist MRS1523 (Li et al., 1998) before measuring (*R*)-PIA-mediated A_3AR internalization. Although MRS1523 alone had no effect on levels of cell-surface A_3ARs, it was able to inhibit (*R*)-PIA-mediated receptor internalization in a concentration-dependent manner (Fig. 2A). Quantitative analysis of these experiments produced an IC_{50} value for MRS1523 between 2.5 and 25 nM (Fig. 2B). The reported K_i value for this antagonist at the rat A_3AR is 130 nM (Li et al., 1998). A similar inhibitory effect of MRS1523 on (*R*)-PIA-mediated A_3AR phosphorylation was also observed (Fig. 2B). Therefore, A_3AR phosphorylation and internalization are agonist-mediated processes that can be blocked by the A_3AR-selective antagonist MRS1523.

Time Courses of (*R*)-PIA-Mediated Internalization of WT A_1 and A_3ARs. Previous studies have demonstrated that the A_1AR does not serve as a good substrate for agonist-mediated phosphorylation by GRKs either in situ or in vitro (Palmer et al., 1996). Given that the A_3AR is, by contrast, an excellent substrate for GRK phosphorylation, it was possible that these receptors might also exhibit differences in their abilities to undergo agonist-mediated internalization. To test this hypothesis, we assessed the time courses for (*R*)-PIA-mediated internalization of WT A_1 and A_3ARs stably expressed in CHO cells (Fig. 3, A and B). Although treatment with 1 μM (*R*)-PIA produced a slow reduction in cell-surface A_1AR levels ($t_{1/2}$ = 90 min; Fig. 3A), the same agonist reduced A_3AR levels at a much faster rate ($t_{1/2}$ = 10 min; Fig. 3B). Moreover, the time course of A_3AR internalization followed that of receptor phosphorylation ($t_{1/2}$ = 1 min; Fig. 3C). Therefore, the A_1 and A_3AR are distinguishable not only by their differing sensitivities to phosphorylation by GRKs but also by the markedly different rates at which they undergo agonist-dependent internalization.

Effects on A_3AR Internalization of Mutating GRK Phosphorylation Sites. Because the A_3AR internalized considerably faster than the nonphosphorylated A_1AR, it was possible that phosphorylation of the A_3AR by GRKs was the trigger that initiated the more rapid internalization process. To test this theory, we used a mutant A_3AR rendered resistant to GRK phosphorylation by the mutation to alanine of three threonine residues (Thr^{307}, Thr^{318}, and Thr^{319}) present in the C-terminal domain (Palmer and Stiles, 2000). In con-

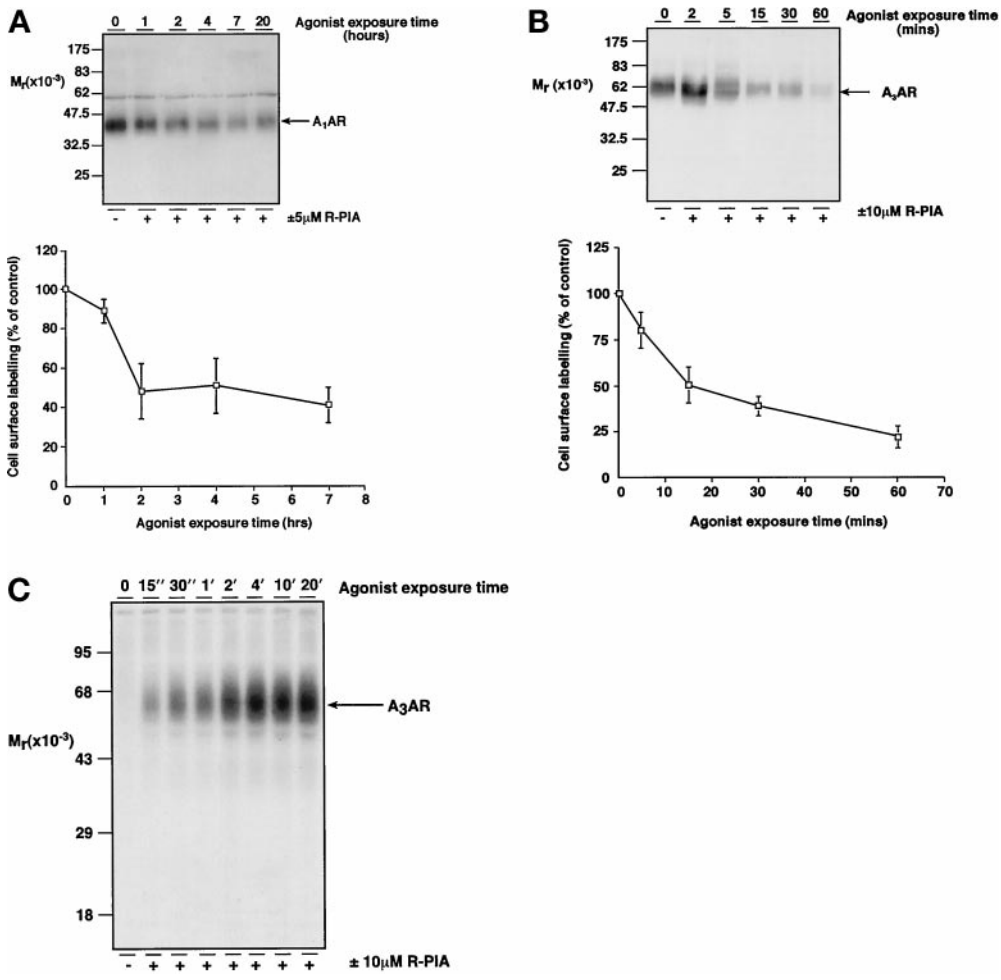

Fig. 3. Time courses of (*R*)-PIA-mediated internalization of the A_1 and A_3ARs. A_1AR (A)- and A_3AR (B)-expressing CHO cells were incubated with 1 μM (*R*)-PIA for the indicated times at 37°C. Cell-surface glycoproteins were then labeled with biotin before receptor immunoprecipitation with 12CA5 and visualization as described in *Experimental Procedures*. Blots were quantified by densitometric scanning. Values represent mean ± S.E. for three experiments, with the levels of cell-surface A_3AR observed in the absence of agonist set at 100%. C, ^{32}P-prelabeled WT A_3AR-expressing CHO cells were incubated with 10 μM (*R*)-PIA for the indicated times. Reactions were then terminated and receptors were immunoprecipitated with 12CA5 as described in *Experimental Procedures*. After fractionation of immunoprecipitated proteins by SDS-PAGE, phosphorylated A_3ARs were visualized by autoradiography of the dried gel. This is one of multiple experiments.

trast to the rapid and profound agonist-induced loss of WT A_3AR from the cell surface, a small loss of mutant receptor was only detectable after a 60-min agonist exposure (Fig. 4, A and B). Thus, disruption of the sites of GRK phosphorylation dramatically impairs the ability of the A_3AR to undergo rapid agonist-mediated internalization.

Agonist-Dependent Internalization of a Chimeric A_1-A_3AR (A_1CT3AR). Even though mutation of the GRK phosphorylation sites within the A_3AR C-terminal domain abolished receptor internalization, we could not eliminate the possibility that this had arisen from a nonspecific disruption of receptor structure within this region. Thus, to determine whether the addition of the C-terminal regulatory domain of the A_3AR could confer rapid internalization kinetics on a slowly internalizing receptor, we used a chimeric A_1-A_3AR, termed A_1CT3AR, which we have described previously (Palmer et al., 1996). This chimeric receptor comprises amino acids 1 to 310 of the human A_1AR (encompassing all of the receptor up to and including its predicted palmitoylation site) to which the C-terminal 14 amino acids of the rat A_3AR have been fused. Thus, although this receptor behaves pharmacologically like the A_1AR, it is rapidly phosphorylated and desensitized in response to acute agonist exposure in a manner similar to the WT A_3AR but unlike the WT A_1AR (Palmer et al., 1996). Time course experiments revealed that agonist stimulation of chimeric receptor phosphorylation occurred with a $t_{1/2}$ of approximately 1 min (Fig. 5A). Analysis of the (*R*)-PIA-mediated loss of A_1CT3AR from the cell-surface revealed that the chimeric receptor internalized over a time frame that followed that of receptor phosphorylation and was similar to that of the WT A_3AR ($t_{1/2}$ = 10 min; Fig. 5B). Thus, the GRK phosphorylated C-terminal domain of the A_3AR is able to confer the property of rapid agonist-induced internalization on a predominantly A_1AR-containing chimeric AR.

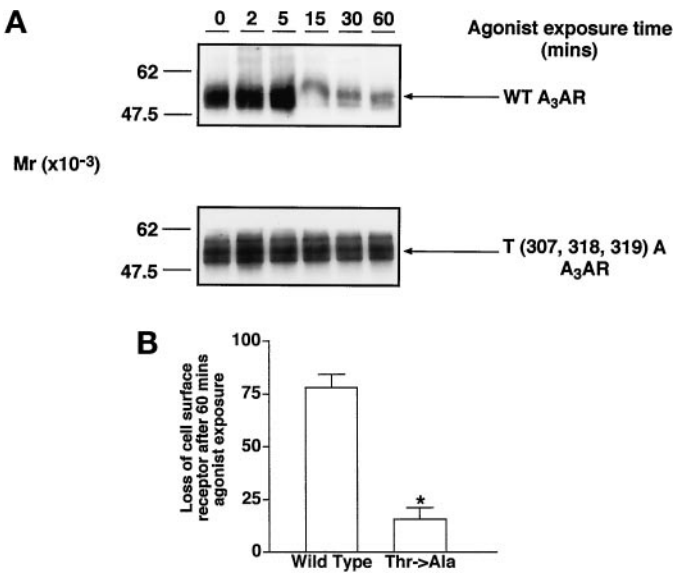

Fig. 4. Comparison of the time courses of agonist-stimulated internalization of WT and phosphorylation-resistant $Thr^{307,318,319}$ → Ala-mutated A_3ARs. A, CHO cells stably expressing either the WT A_3AR (top) or a phosphorylation-resistant $Thr^{307,318,319}$ → Ala mutant A_3AR (bottom) were incubated with 5 μM (*R*)-PIA at 37°C for the indicated times. Cell-surface glycoproteins were then labeled with biotin before receptor immunoprecipitation with 12CA5 and visualization as described in *Experimental Procedures*. This is one of three experiments that produced essentially identical results. Quantitative analysis of the internalization observed for each receptor after a 60-min agonist exposure is presented in B. *Statistically significant difference ($P < .05$) from the WT A_3AR.

Discussion

The A_1 and A_3ARs represent attractive targets for therapeutics aimed at combating asthma, ischemic heart disease, and stroke (Von Lubitz et al., 1994; Kohno et al., 1996; Auchampach and Bolli, 1999). Although several G_i-coupled signaling cascades have been shown to be activated by these receptors, the roles of receptor phosphorylation and internalization in regulating these pathways are unknown. As a first step toward addressing how such events may regulate distinct A_1 and A_3AR-activated signaling cascades, we have used a panel of mutant and chimeric ARs to examine the relationship between inhibitory AR phosphorylation and internalization.

For the first time, we demonstrated that the AR agonists (*R*)-PIA and NECA induced a rapid ($t_{1/2}$ = 10 min) concentration-dependent loss of A_3ARs from the cell surface as measured using a sequential cell-surface biotin labeling-immunoprecipitation assay (Palmer et al., 1996). In contrast, exposure of the A_1AR to the same agonists resulted in a much slower rate of receptor internalization ($t_{1/2}$ = 90 min). A similar slow rate of agonist-mediated internalization has also been observed for A_1ARs expressed endogenously in a DDT_1 MF-2 hamster smooth muscle cell line (Ciruela et al., 1997), suggesting that this is a characteristic feature of the A_1AR and not simply a reflection of the CHO host cell line we have used to express the recombinant receptor. Interestingly, the onset of agonist-mediated A_1AR internalization in CHO cells correlates temporally with the onset of reduced A_1AR/G_i coupling as detectable by copurification of receptor/G protein complexes and agonist radioligand binding (Gao et al., 1999). Therefore, sequestration of agonist-bound A_1ARs away from plasma membrane-associated G_i proteins represents one potential mechanism by which A_1AR desensitization could occur in this system, although this will ultimately need to be tested by comparing the subcellular distribution and colocalization patterns of G_i proteins and A_1ARs before and after agonist treatment.

Both loss-of-function and gain-of-function experimental approaches suggested strongly that A_3AR internalization is critically dependent on phosphorylation of three threonine residues (Thr^{307}, Thr^{318}, and Thr^{319}) within the C-terminal domain by one or more GRKs (Palmer et al., 1996; Palmer and Stiles, 2000). Other studies have demonstrated that discrete regions within the C-terminal tails of several GPCRs are involved in controlling internalization, including the β-isoform of the thromboxane A_2 receptor (Parent et al., 1999), the thrombin receptor (Shapiro et al., 1998), and the choriogonadotropin receptor (Rodriguez et al., 1992). In each of these instances, the C-terminal domains contain sites for phosphorylation by GRKs and second messenger-regulated protein kinases. However, for receptors like the β_2-adrenergic receptor (Gabilondo et al., 1997), additional structural elements within the C-terminal tail, such as dileucine repeats that bind AP1 and AP2 adaptor proteins associated with clathrin-coated pits, seem to be critical for agonist-induced internalization to be fully manifested on receptor phosphorylation. In the case of the A_3AR, no such motifs are present within the receptor's small cytoplasmic domains, so

it is likely that phosphorylation of Thr^{307}, Thr^{318}, and Thr^{319} is the predominant, if not the only, factor controlling the rapid internalization of this receptor. Based on observations made with other GPCRs (Cao et al., 1998; Li et al., 1999; McConalogue et al., 1999), it is likely that phosphorylation of the A_3AR C-terminal domain triggers the binding of arrestin proteins, which then target the phosphorylated receptors for internalization. We are currently performing experiments to test the validity of this hypothesis. By extension, our observations also suggest that the slow rate of A_1AR internalization reflects the absence of C-terminal GRK phosphorylation sites and the resultant inability of the agonist-occupied receptor to serve as a GRK substrate in this system (Palmer et al., 1996; Gao et al., 1999).

The markedly different behavior of the A_1AR compared with the A_3AR represents a prime example of how pharmacologically related receptors can be regulated differentially in response to agonist challenge. Given that GPCR internalization is now thought to be regulated predominantly by receptor phosphorylation, our results raise important questions about the molecular mechanisms controlling the slow agonist-mediated internalization of the A_1AR. Although internalization of other GPCRs, such as the secretin receptor (Holtmann et al., 1996), has also been shown to occur independently of phosphorylation, these receptors still internalize within a few minutes of agonist exposure. Interestingly, agonist-mediated redistribution of cell-surface A_1ARs into punctate plasma membrane clusters on the surface of DDT_1 MF-2 cells is observed within 5 min of agonist addition, suggesting that there is a considerable delay between receptor aggregation and internalization (Ciruela et al., 1997). The reasons for this delay are not immediately obvious, but it is possible that additional proteins must be recruited to sites of A_1AR clustering for internalization to occur. Related to this point, it is still unknown whether A_1AR and A_3AR internalization pathways occur via clathrin-coated pits or whether alternative trafficking pathways, such as those involving caveolin, are involved.

Finally, the combined results of both the current study and our previous investigations of inhibitory AR phosphorylation (Palmer et al., 1996; Palmer and Stiles, 2000) raise the intriguing question of why two inhibitory ARs have evolved that are regulated by markedly divergent mechanisms after agonist binding. Given the recently appreciated role of GPCR phosphorylation and internalization processes in initiating specific signaling pathways (Della Rocca et al., 1999; Luttrell et al., 1999), an exciting possibility is that subtype-specific regulation of the A_1 and A_3ARs may allow each receptor to activate distinct subsets of the increasing array of signaling pathways now known to be initiated by GPCRs (Gutkind,

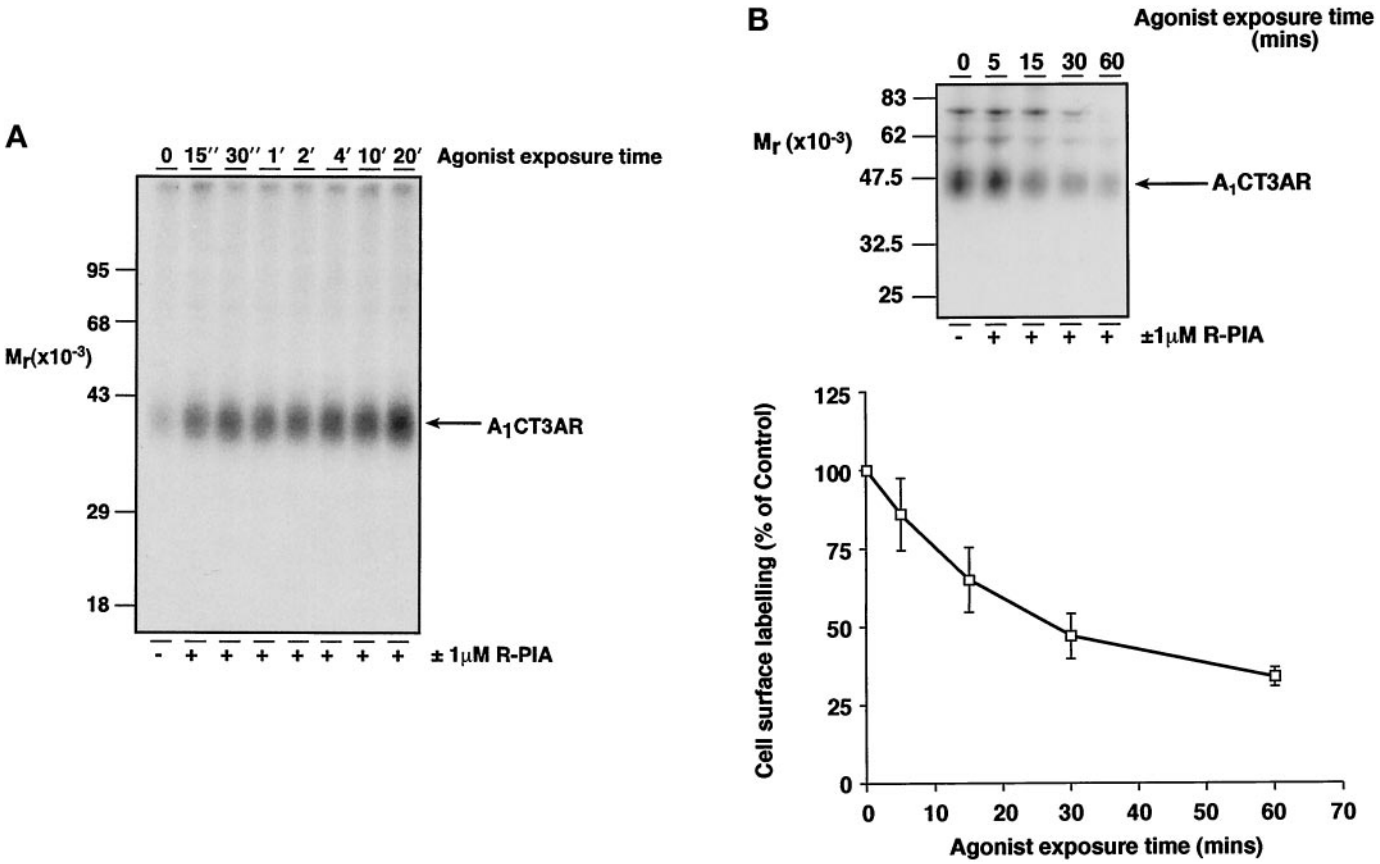

Fig. 5. Comparison of the time courses of agonist-stimulated phosphorylation and internalization of a chimeric A_1CT3AR. A, ^{32}P-prelabeled CHO cells stably expressing the chimeric A_1CT3AR were incubated with 5 μM (*R*)-PIA for the indicated times at 37°C before termination of the reactions and receptor immunoprecipitation with 12CA5 as described in *Experimental Procedures*. After fractionation of immunoprecipitated proteins by SDS-PAGE, phosphorylated A_1CT3ARs were visualized by autoradiography of the dried gel. This is one of three experiments that produced identical results. B, A_1CT3AR-expressing CHO cells were incubated with 5 μM (*R*)-PIA for the indicated times at 37°C. Cell-surface glycoproteins were then labeled with biotin before receptor immunoprecipitation with 12CA5 and visualization as described in *Experimental Procedures*. Blots were quantified by densitometric scanning. Values represent mean ± S.E. for three experiments, with the levels of cell-surface A_1CT3AR observed in the absence of agonist set at 100%.

1998). In this respect, the mutant and chimeric A_1ARs and A_3ARs generated from our analysis of receptor regulation will be invaluable tools for elucidating the molecular mechanisms via which these receptors initiate therapeutically important protective pathways in the myocardium and central nervous system (Von Lubitz et al., 1994; Auchampach and Bolli, 1999).

References

Ali H, Richardson RM, Tomhave ED, Didsbury JR and Snyderman R (1993) Differences in phosphorylation of formylpeptide and C5a chemoattractant receptors correlate with differences in desensitization. *J Biol Chem* **268:**24247–24254.

Auchampach JA and Bolli R (1999) Adenosine receptor subtypes in the heart: therapeutic opportunities and challenges. *Am J Physiol* **276:**H1113–H1116.

Budd DC, Rae A and Tobin AB (1999) Activation of the mitogen-activated protein kinase pathway by a $G_{q/11}$-coupled muscarinic receptor is independent of receptor internalization. *J Biol Chem* **274:**12355–12360.

Cao TT, Mays RW and von Zastrow M (1998) Regulated endocytosis of G-protein-coupled receptors by a biochemically and functionally distinct subpopulation of clathrin-coated pits. *J Biol Chem* **273:**24592–24602.

Ciruela F, Saura C, Canela EI, Mallol J, Lluis C and Franco R (1997) Ligand-induced phosphorylation, clustering and desensitization of A_1 adenosine receptors. *Mol Pharmacol* **52:**788–797.

Dickenson JM, Blank JL and Hill SJ (1998) Human adenosine A_1 receptor and P_{2Y2}-purinoceptor-mediated activation of the mitogen-activated protein kinase cascade in transfected CHO cells. *Br J Pharmacol* **124:**1491–1499.

Gabilondo AM, Hegler J, Krasel C, Boivin-Jahns V, Hein L and Lohse MJ (1997) A dileucine motif in the C terminus of the β_2-adrenergic receptor is involved in receptor internalization. *Proc Natl Acad Sci USA* **94:**12285–12290.

Gao Z, Robeva AA and Linden J (1999) Purification of A_1 adenosine receptor-G-protein complexes: Effects of receptor down-regulation and phosphorylation on coupling. *Biochem J* **338:**729–736.

Gutkind JS (1998) The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. *J Biol Chem* **273:**1839–1842.

Holtmann MH, Roettger BF, Pinon DI and Miller LJ (1996) Role of receptor phosphorylation in desensitization and internalization of the secretin receptor. *J Biol Chem* **271:**23566–23571.

Kohno Y, Ji X-d, Mawhorter SD, Koshiba M and Jacobson KA (1996) Activation of A_3 adenosine receptors on human eosinophils elevates intracellular calcium. *Blood* **9:**3569–3574.

Krupnick JG and Benovic JL (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol* **38:**289–319.

Lefkowitz RJ (1998) G protein-coupled receptors. III: New roles for receptor kinases and β-arrestins in receptor signalling and desensitization. *J Biol Chem* **273:** 18677–18680.

Li A-H, Moro S, Melman N, Ji X-d and Jacobson KA (1998) Structure-activity relationships and molecular modeling of 3,5-diacyl-2,4-dialkylpyridine derivatives as selective A_3 adenosine receptor antagonists. *J Med Chem* **41:**3186–3201.

Li J-G, Luo L-Y, Krupnick JG, Benovic JL and Liu-Chen L-Y (1999) U50,488H-induced internalization of the human κ opioid receptor involves a β-arrestin- and dynamin-dependent mechanism: κ Receptor internalization is not required for mitogen-activated protein kinase activation. *J Biol Chem* **274:**12087–12094.

Linden J, Taylor HE, Robeva AS, Tucker AL, Stehle JH, Rivkees SA, Fink JS and Reppert SM (1993) Molecular cloning of a sheep A_3 adenosine receptor with widespread tissue distribution. *Mol Pharmacol* **44:**524–532.

Luttrell LM, Daaka Y, Della Rocca GJ and Lefkowitz RJ (1997) G protein-coupled receptors mediate two functionally distinct pathways of tyrosine phosphorylation in rat 1a fibroblasts: Shc phosphorylation and receptor endocytosis correlate with activation of Erk kinases. *J Biol Chem* **272:**31648–31656.

Luttrell LM, Ferguson SSG, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F-T, Kawakatsu H, Owada K, Luttrell DK, Caron MG and Lefkowitz RJ (1999) β-Arrestin-dependent formation of β_2-adrenergic receptor-src protein kinase complexes. *Science (Wash DC)* **283:**655–666.

McConalogue K, Dery O, Lovett M, Wong H, Walsh JH, Grady EF and Bunnett NW (1999) Substance P-induced trafficking of β-arrestins: The role of β-arrestins in endocytosis of the neurokinin-1 receptor. *J Biol Chem* **274:**16257–16268.

Palmer TM, Benovic JL and Stiles GL (1995) Agonist-dependent phosphorylation and desensitization of the rat A_3 adenosine receptor: Evidence for a G-protein-coupled receptor kinase-mediated mechanism. *J Biol Chem* **270:**29607–29613.

Palmer TM, Benovic JL and Stiles GL (1996) Molecular basis for subtype-specific desensitisation of inhibitory adenosine receptors: Analysis of a chimeric A_1-A_3 adenosine receptor. *J Biol Chem* **271:**15272–15278.

Palmer TM and Stiles GL (2000) Identification of threonine residues controlling the agonist-dependent phosphorylation and desensitization of the rat A_3, adenosine receptor. *Mol Pharmacol* **57:**539–545.

Parent J-L, Labrecque P, Orsini MJ and Benovic JL (1999) Internalization of the TXA_2 receptor α and β isoforms: Role of the differentially spliced COOH terminus in agonist-promoted receptor internalization. *J Biol Chem* **274:**8941–8948.

Pitcher JA, Freedman NJ and Lefkowitz RJ (1998) G protein-coupled receptor kinases. *Annu Rev Biochem* **67:**653–692.

Rodriguez MC, Xie Y-B, Wang H, Collison K and Segaloff DL (1992) Effects of truncations of the cytoplasmic tail of the luteinizing hormone/chorionic gonadotropin receptor on receptor-mediated hormone internalization. *Mol Endocrinol* **6:**327–336.

Shapiro MJ and Coughlin SR (1998) Separate signals for agonist-independent and agonist-triggered trafficking of protease-activated receptor 1. *J Biol Chem* **273:** 29009–29014.

Von Lubitz DKJE, Lin RC-S, Popik P, Carter MF and Jacobson KA (1994) Adenosine A_3 receptor stimulation and cerebral ischaemia. *Eur J Pharmacol* **263:**59–67.

Zhou Q-Y, Li C, Olah ME, Johnson RA, Stiles GL and Civelli O (1992) Molecular cloning and characterisation of an adenosine receptor: The A_3 adenosine receptor. *Proc Natl Acad Sci USA* **89:**7432–7436.

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