Stimulation of A_{2A} Adenosine Receptor Phosphorylation by Protein Kinase C Activation: Evidence for Regulation by Multiple Protein Kinase C Isoforms†

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ABSTRACT: Activation of the A_{2A} adenosine receptor (A_{2A}AR) contributes to the neuromodulatory and neuroprotective effects of adenosine in the central nervous system. Here we demonstrate that, in rat C6 glioma cells stably expressing an epitope-tagged canine A_{2A}AR, receptor phosphorylation on serine and threonine residues can be increased by pretreatment with either the synthetic protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) or endothelin 1, which increases PKC activity via binding to endogenous endothelin$_A$ receptors. Under conditions in which PMA was maximally effective, activation of other second messenger-regulated kinases was without effect. While basal and PMA-stimulated phosphorylation were unaffected by the A_{2A}AR-selective antagonist ZM241385, they were both blocked by GF109203X (a selective inhibitor of conventional and novel PKC isoforms) and rottlerin (a PKCδ-selective inhibitor) but not Go6976 (selective for conventional PKC isoforms). However, coexpression of the A_{2A}AR with each of the α, βI, and βII isoforms of PKC increased basal and PMA-stimulated phosphorylation. Mutation of the three consensus PKC phosphorylation sites within the receptor (Thr298, Ser320, and Ser335) to Ala failed to inhibit either basal or PMA-stimulated phosphorylation. In addition, phosphorylation of the receptor was not associated with detectable changes in either its signaling capacity or cell surface expression. These observations suggest that multiple PKC isoforms can stimulate A_{2A}AR phosphorylation via activation of one or more downstream kinases which then phosphorylate the receptor directly. In addition, it is likely that phosphorylation controls interactions with regulatory proteins distinct from those involved in the classical cAMP signaling pathway utilized by this receptor.

Adenosine plays a central role in the maintenance of cardiovascular and central nervous system homeostasis (*1*). During the hypoxic conditions associated with the onset of ischemia, extracellular adenosine levels in heart and brain can rise dramatically (*2*, *3*). The ability of target cells to adapt and respond to these rapid changes in extracellular adenosine levels is determined by their complement of adenosine receptors (ARs).[1] To date, four subtypes of ARs have been identified by pharmacological and molecular cloning studies, with each exhibiting the seven transmembrane-spanning segment topography characteristic of almost all G protein-coupled receptors (GPCRs) (*4*, *5*). While the A_1 and A_3ARs initiate their downstream effects by coupling to G proteins of the G_i/G_o family, the A_{2A} and A_{2B}ARs can activate G_s to stimulate adenylyl cyclase activity and elevate intracellular cAMP levels (*4*, *5*).

Although the neuromodulatory, antihypertensive, and antiinflammatory effects of A_{2A}AR activation have been well-documented, other physiological consequences of receptor activation are now being examined for their potential therapeutic utility. These include the ability of the A_{2A}AR to antagonize D_2 dopamine receptor signaling in striatopallidal neurones, which suggests that A_{2A}AR antagonism may be a novel therapeutic strategy to enhance dopaminergic signaling in Parkinson's disease (*1*, *2*, *6*). Also, agonist occupation of the A_{2A}ARs present on endothelial cells can not only reduce hypoxia-induced cell death but also increase mitogenesis, a phenomenon that may be important in regulating the increased blood vessel growth observed in chronically hypoxic solid tumors (*7*, *8*).

Despite the myriad of physiological effects ascribed to the A_{2A}AR, relatively little is known about receptor regulation at the molecular level. We have recently determined that a

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[1] Abbreviations: AR, adenosine receptor; GPCR, G protein-coupled receptor; PKC, protein kinase C; ZM241385, 4-(2-[7-amino-2-{2-furyl}-{1,2,4}triazolo{2,3-*a*}{1,3,5}triazin-5-yl-amino]ethyl)phenol; PMA, phorbol 12-myristate 13-acetate; GF109203X, bisindolylmaleimide 1; CHO, Chinese hamster ovary; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IgG, immunoglobulin G; PBS, phosphate-buffered saline; Ro201724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazoline; CGS21680, 2-(*p*-carboxyethyl)phenylamino-5′-*N*-ethylcarboxamidoadenosine; Go6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole; LC, long alkyl chain.

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small region of the A_{2A}AR carboxyl-terminal domain, proximal to the seventh transmembrane-spanning region, is important in conferring the ability to desensitize rapidly in response to agonist exposure (*9*). However, the ability of other signal transduction pathways to regulate A_{2A}AR function or expression has not been examined in any detail. This represents a potentially important area of investigation since, in vivo, cells will receive information from a complex extracellular milieu containing an ever-changing compliment of activators of multiple signal transduction pathways. The ability of parallel signaling pathways to cross-regulate each other in a specific manner provides one level at which this vast array of information can be integrated to produce an appropriate cellular response. This has been demonstrated most elegantly for the various chemoattractant receptors, which can regulate each other's phosphorylation status and function in a receptor-specific manner (*10*).

In a previous study on the molecular mechanisms of agonist regulation of A_{2A}AR function, we noted that the A_{2A}AR was phosphorylated under basal conditions (*9*). Further examination of this phenomenon has revealed a previously unappreciated regulation of A_{2A}AR phosphorylation by both conventional and novel protein kinase C (PKC) enzymes. This may have implications for the potential cross-regulation of the multiple cAMP-independent signaling pathways recently shown to be activated by the A_{2A}AR in specific cell types.

EXPERIMENTAL PROCEDURES

Materials. ZM241385 was the generous gift of Dr. Simon Poucher (Zeneca Pharmaceuticals, Macclesfield, U.K.). ^{125}I-ZM241385 was synthesized and purified as we have previously described (*11*). Other radiochemicals were from Dupont−NEN. Polyclonal antibodies against the A_{2A}AR were from Affinity Bioreagents Inc. (Golden, CO). Antibodies versus PKC isoforms were the generous gifts of Professor Yusuf Hannun (MUSC, Charleston, SC) and their characterization has been described elsewhere (*12*). Protein A−Sepharose 4FF and 8-bromo-cGMP were from Sigma. PMA, A23187, forskolin, GF109203X, Go6976, and rottlerin were from Calbiochem. CGS21680 was from Research Biochemicals International. Sources of other materials have been described elsewhere (*13*−*15*).

cDNA Constructs and Expression. The canine A_{2A}AR was subcloned as a *Hin*dIII/*Sma*I fragment from pBC12BI (*9*) into similarly digested pCMV5 expression vector and hemagglutinin epitope-tagged on its amino and carboxyl termini by a polymerase chain reaction protocol analogous to that previously described for the A_1 and A_3 ARs (*14*, *15*). Preliminary experiments demonstrated that the addition of epitope tags to both ends of the receptor did not alter its ability to bind agonist and antagonist ligands or stimulate adenylyl cyclase activity in transiently transfected Chinese hamster ovary (CHO) cells compared with the untagged receptor (data not shown). Phosphorylation site-mutated A_{2A}ARs were generated by a two-step polymerase chain reaction-based protocol with pCMV5/epitope-tagged A_{2A}AR cDNA as the template (*9*). Rat PKCα, PKCβI, and PKCβII (*16*) subcloned into a pBΔG expression vector were provided generously by Professor Yusuf Hannun.

Rat C6 glioma cells stably expressing the epitope-tagged A_{2A}AR were generated by cotransfecting cells with the pCMV5/epitope-tagged A_{2A}AR cDNA and pSVNeo in a 20:1 ratio by a standard calcium phosphate/glycerol shock procedure as previously described (*13*−*15*). After selection in the presence of the neomycin analogue G418, resistant clones were isolated, expanded, and screened for receptor expression by radioligand binding with ^{125}I-ZM241385. Cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% (w/v) fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were used between passages 2 and 15. For phosphorylation experiments, C6 cells were split into six-well dishes at a density of (1.5−5) $\times$ 10^6 cells/well and cultured overnight. COS-P cells were transiently transfected by a standard DEAE-dextran protocol (*17*, *18*) with pCMV5/epitope-tagged A_{2A}AR (15 μg/100 mm dish) together with either empty pBΔG vector or pBΔG containing PKCα, PKCβI, or PKCβII (10 μg/dish). Approximately 24 h posttransfection, cells were split into six-well dishes at a density of (1.5−5) $\times$ 10^6 cells/well. Phosphorylation experiments were performed 48−72 h posttransfection. For CHO cell phosphorylations, near-confluent 100 mm dishes were transfected with 20 μg of pCMV5/epitope-tagged A_{2A}AR per dish and split into six-well dishes 24 h later as described for COS cells. To examine regulation of adenylyl cyclase activity, 25 μg of pCMV5/epitope-tagged A_{2A}AR was used to transfect a near-confluent 150 mm dish of CHO cells. Twenty-four hours later, the cells were split into three 100 mm dishes and assays were performed 48−72 h posttransfection. COS-P and CHO cells were propagated as described previously (*11*, *17*).

Intact Cell Receptor Phosphorylation. Transfected cells in six-well dishes were washed twice with phosphate-free Dulbecco's modified Eagle's medium and incubated for 90 min at 37 °C in the same medium supplemented with 0.2 mCi/mL [^{32}P]orthophosphate and 1 unit/mL adenosine deaminase. After incubation with the stimulants indicated in the figure legends, reactions were terminated by placing the cell monolayers on ice and washing them twice with ice-cold phosphate-buffered saline (PBS). All subsequent procedures were carried out at 4 °C unless indicated otherwise. Cells were solubilized by scraping into 0.75 mL of immunoprecipitation buffer [50 mM Na Hepes, pH 7.5, 5 mM EDTA, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.15 M sodium chloride, 10 mM sodium fluoride, 10 mM sodium phosphate, 0.1 mM phenylmethanesulfonyl fluoride, 10 μg/mL each soybean trypsin inhibitor and leupeptin, and 1 μg/mL pepstatin A) and incubation on a rotating wheel for 1 h. Lysates were clarified by centrifugation (14000*g*, 15 min) and supernatants were assayed for protein content. Equivalent amounts of soluble protein from each sample (typically 0.23−0.26 mg/sample for C6 and CHO cells and 0.28−0.35 mg/sample for COS-P cells) were then brought up to a volume of 0.65 mL with immunoprecipitation buffer and precleared of nonspecific binding proteins by a 1 h incubation with protein A−Sepharose in the presence of 0.2% (w/v) IgG-free bovine serum albumin. Receptors were immunoprecipitated from the precleared supernatants by incubation with protein A−Sepharose and 12 μL of 12CA5 ascitic fluid for 2 h on a rotating wheel. Immune complexes were isolated by brief centrifugation, washed twice with immunoprecipitation buffer supplemented with 0.2 M ammonium sulfate and once with immunopre-

cipitation buffer alone, and eluted from the beads by the addition of 60 μL of Laemmli sample buffer and incubation at room temperature for 45 min. Analysis was by SDS−PAGE and autoradiography for between 16 and 40 h at −80 °C as previously described (*14*, *15*). Quantitation was by either densitometric scanning of autoradiographs or phosphorimaging.

For COS-P cells expressing different combinations of receptor and PKC cDNAs, it was important to equalize the amount of receptor loaded for SDS−PAGE from each transfected cell population. Therefore, receptor levels for each transfection were determined by radioligand binding with a saturating concentration (approximately 8 nM) of ^{125}I-ZM241385. The amount of receptor in each transfected cell population (picomoles per milligram of protein) multiplied by the protein content of the solubilized fraction from the equivalently transfected sample used for the immunoprecipitation (milligrams of protein per sample) produces a value for the level of receptors in each sample (picomoles per sample). For SDS−PAGE analysis, the receptor content of each sample was normalized to that of the sample with the least receptor, and loading volumes were equalized by the addition of Laemmli sample buffer.

Phosphoamino Acid Analysis. Following SDS−PAGE, ^{32}P-labeled immunoprecipitated receptors were transferred to a poly(vinylidene difluoride) membrane. Following overnight autoradiography, the region of the membrane corresponding to the phosphorylated A_{2A}AR was excised, hydrated, and hydrolyzed in 0.2 mL of 5.7 M hydrochloric acid for 90 min at 110 °C. 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−ammonium hydroxide (5:3 v/v) buffer system as described previously (*14*, *15*). Standards were visualized by ninhydrin staining and ^{32}P-labeled phosphoamino acids were visualized by autoradiography.

Immunoblotting. For immunoblotting of C6 cell membranes, crude cell membranes were prepared from confluent 35 mm wells from a six-well dish and solubilized by sonication in 100 μL of Laemmli sample buffer. After equalization by protein assay, 20 μg aliquots of solubilized samples were resolved by discontinuous SDS−PAGE on 10% (w/v) polyacrylamide mini resolving gels and transferred to nitrocellulose membranes. Membranes were blocked by a 1 h incubation in blocking buffer [5% (w/v) skimmed milk in PBS with 0.2% (v/v) Triton X-100] and incubated overnight at 4 °C with either 12CA5 (1:500 dilution of ascitic fluid) or anti-A_{2A}AR antibody (0.5 μg/mL epitope affinity-purified IgG) diluted in fresh blocking buffer. After extensive washing with three changes of blocking buffer, membranes were incubated at room temperature for 1 h with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG in a high detergent skimmed milk solution. After further washing with blocking buffer and PBS, immunoreactive proteins were visualized by an enhanced chemiluminescence protocol.

For detection of PKC isoforms, confluent COS-P cells in 35 mm wells in six-well dishes were harvested by scraping into 0.2 mL of lysis buffer (10 mM Tris-HCl, pH 7.5 at 4 °C, 5 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride and 1 μg/mL pepstatin A), transferred to microcentrifuge tubes, and lysed by vigorous vortexing. A cytosolic fraction was isolated by centrifugation (14000*g*, 15 min) and 10 μg aliquots were used for discontinuous SDS−PAGE on 8% (w/v) polyacrylamide mini resolving gels. Immunoblotting was performed as described above with antibodies specific for PKCα (1:10 000 dilution), PKCβI (1:1000 dilution), and PKCβII (1:1000 dilution).

Cell-Surface Labeling with Biotin-LC Hydrazide. This was performed exactly as we have described previously on confluent monolayers of transfected C6 cells (*14*, *15*).

Radioligand Binding and Adenylyl Cyclase Assays. Radioligand binding assays with ^{125}I-ZM241385 were performed exactly as we have described previously (*11*). Adenylyl cyclase assays were performed and analyzed exactly as we have described previously except that 20 μM Ro201724 was used as a phosphodiesterase inhibitor instead of papaverine (*9*, *13*−*15*). To account for interexperimental variations in adenylyl cyclase assays, the maximal stimulation elicited by CGS21680 in membranes from control cells was set at 100% and the basal value was set at 0%. The stimulation achieved by the various concentrations of CGS21680 in membranes from control and PMA-treated cells were then normalized with respect to these limits. We have previously used this method to assess A_{2A}AR desensitization (*9*).

Data Analysis. Data are presented as means ± standard deviations for the number of experiments indicated. Statistical significance was determined by two-tailed Student *t*-tests with significance assessed at $p < 0.05$.

RESULTS

Expression of an Epitope-Tagged A_{2A}AR in C6 Glioma Cells. Saturation binding analysis of transfected C6 cells membranes with the A_{2A}AR-selective antagonist ^{125}I-ZM241385 revealed that the epitope-tagged A_{2A}AR bound this radioligand with K_d values of 1.10 ± 0.29 nM and B_{max} values that ranged between 2.57 and 6.02 pmol/mg of membrane protein over five experiments (Figure 1A). No specific binding was detectable in membranes from nontransfected C6 cells. To identify the expressed A_{2A}AR protein, membranes from transfected C6 cells were used for immunoblotting experiments (Figure 1B). Both anti-HA antibody 12CA5 and an anti-A_{2A}AR polyclonal antibody recognized a 48−52 kDa immunoreactive band specifically in membranes from transfected C6 cells. The lack of any A_{2A}AR signal in membranes from nontransfected cells (Figure 1B) confirmed the lack of an endogenous A_{2A}AR in this system.

Phorbol Ester-Stimulated Phosphorylation of the A_{2A}AR. The A_{2A}AR contains multiple potential phosphorylation sites within its long C-terminal domain, including three consensus sites for phosphorylation by PKC family members (*19*). To examine the possibility that these sites are utilized, A_{2A}AR phosphorylation was assessed after exposure of transfected cells to 1 μM synthetic PKC activator phorbol 12-myristate 13-acetate (PMA). Figure 2A demonstrates that the A_{2A}AR existed as a phosphoprotein under basal conditions and that the addition of PMA for 10 min increased phosphorylation dramatically (over 15 experiments, 1 μM PMA increased A_{2A}AR phosphorylation by between 4.9- and 12.4-fold after

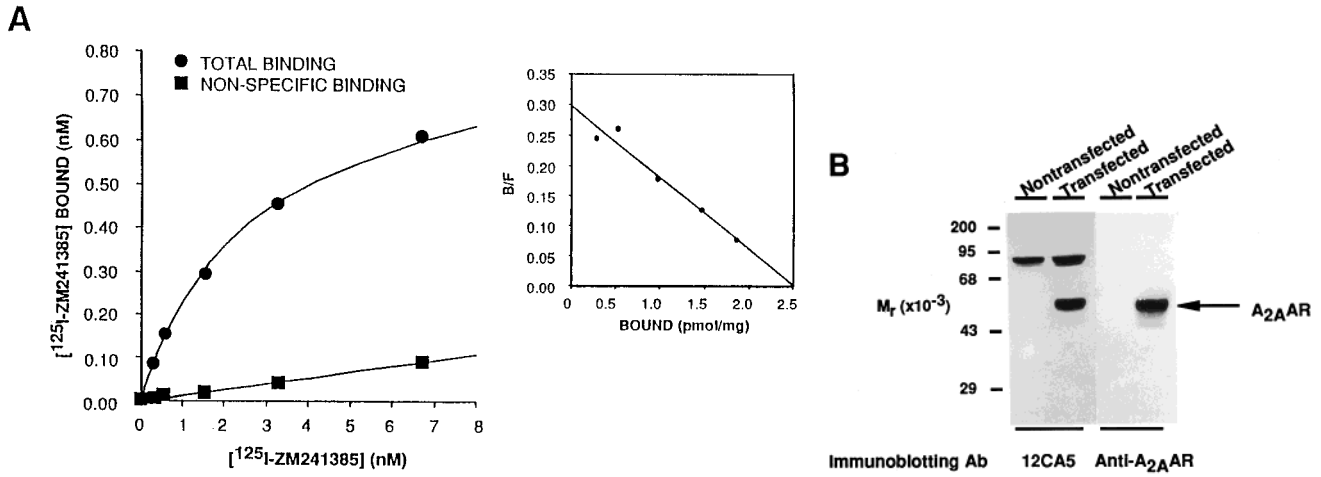

FIGURE 1: Stable expression of an epitope-tagged A_{2A}AR in C6 glioma cells. (A) Representative saturation isotherm for the binding of ^{125}I-ZM241385 to membranes for transfected C6 cells. (Inset) Scatchard analysis of the specific binding data from this experiment. This is one of five experiments, data from which are given in the Results section. (B) Aliquots (20 μg) of membranes from nontransfected and transfected C6 cells were solubilized in Laemmli sample buffer and analyzed by SDS−PAGE and immunoblotting with either anti-epitope tag monoclonal antibody 12CA5 or a polyclonal anti-A_{2A}AR antibody as indicated. This is one of multiple experiments performed for both antibodies.

10 min). The ability of PMA to increase A_{2A}AR phosphorylation was unique among regulators of second messenger-activated kinases, since activation of either Ca^{2+}−calmodulin-activated kinases or the cyclic nucleotide-activated kinases failed to stimulate A_{2A}AR phosphorylation under conditions in which PMA was maximally effective (Figure 2B). Phosphoamino acid analysis revealed that the A_{2A}AR was phosphorylated on serine and threonine residues in the basal state and that exposure to PMA increased incorporation into both of these amino acids (Figure 2C).

Sensitivity of A_{2A}AR Phosphorylation to ZM241385 and GF109203X. We have demonstrated previously that agonist occupation of the A_{2A}AR stimulates receptor phosphorylation in CHO cells (*9*). Therefore, it was possible that basal phosphorylation reflected a low-level activation of the A_{2A}AR by ambient levels of adenosine. Also, since PKC is known to activate ecto-5′-nucleotidase and stimulate cellular adenosine release (*20*), it was possible that enhanced phosphorylation reflected increases in both the release of adenosine and the subsequent agonist-stimulated phosphorylation of the A_{2A}AR. It was also important to determine whether the effects of PMA on A_{2A}AR phosphorylation were due exclusively to its ability to activate PKC or whether other non-PKC high-affinity phorbol ester receptors, such as n-chimaerin (*21*), were involved. To address these issues, the effects on A_{2A}AR phosphorylation of the A_{2A}AR-selective antagonist ZM241385 were compared with those of GF109203X, a potent and selective inhibitor of conventional and novel PKC enzymes (*22*). Under conditions in which PMA produced a 8−10-fold increase in A_{2A}AR phosphorylation, preincubation with ZM241385 was without effect at a concentration at which it completely blocks agonist binding to this receptor (*23*). In contrast, GF109203X inhibited basal and PMA-stimulated phosphorylation by between 75% and 95% over three experiments (Figure 3A,B). Moreover, the effect of GF109203X was not attributable to any dramatic changes in expression of the A_{2A}AR over the time course of these experiments (Figure 3C). Dose−response experiments revealed that basal and PMA-stimulated phosphorylation were similarly sensitive to increasing concentrations of GF109203X (Figure 3D). While significant inhibition of phosphorylation was observed at 0.5 μM, >75% inhibition of phosphorylation required preincubation with 5 μM GF109203X (Figure 3D). Therefore, basal and PMA-stimulated A_{2A}AR phosphorylation are similarly sensitive to inhibition of PKC enzymes, and PMA stimulation of A_{2A}AR phosphorylation did not reflect either a PKC-mediated increase in extracellular adenosine levels or a PKC-independent effect of PMA on A_{2A}AR regulation.

Sensitivity of A_{2A}AR Phosphorylation to Go6976 and Rottlerin. To more accurately identify the PKC isoform responsible for A_{2A}AR phosphorylation in C6 cells, the effects of rottlerin and Go6976 on basal and PMA-stimulated receptor phosphorylation were compared. Go6976 inhibits calcium-dependent PKC isoforms with IC_{50} values in the nanomolar range (*24*). Rottlerin selectively inhibits the calcium-independent PKCδ isoform with an IC_{50} of approximately 5 μM; 10−30-fold higher concentrations are required to inhibit conventional PKC isoforms and the other calcium-independent isoforms (*25*). Under conditions in which GF109203X was maximally effective, 1 μM Go6976 had no significant effect on basal or PMA-stimulated A_{2A}AR phosphorylation (Figure 4). However, in the absence of PMA, 10 μM rottlerin significantly inhibited A_{2A}AR phosphorylation to the same degree as GF109203X (Figure 4). While 10 μM rottlerin was also able to inhibit PMA-stimulated A_{2A}AR phosphorylation, the inhibition was not as marked as that produced by GF109203X (Figure 4). These data suggest strongly that while PKCδ is the isoform that predominantly regulates A_{2A}AR phosphorylation in the basal state, additional calcium-independent GF109203X-sensitive PKCs also contribute to the phosphorylation observed in response to PMA.

Concentration and Time Dependences of PMA-Stimulated A_{2A}AR Phosphorylation. The ability of PMA to increase A_{2A}AR was concentration-dependent. Curve-fitting of data pooled from three experiments produced an EC_{50} value for PMA of 6.94 ± 0.63 nM (Figure 5A). Time-course experiments revealed that stimulation of phosphorylation was readily detectable after only 15 s of exposure to PMA (Figure 5B). Half-maximal phosphorylation was achieved by 60 s,

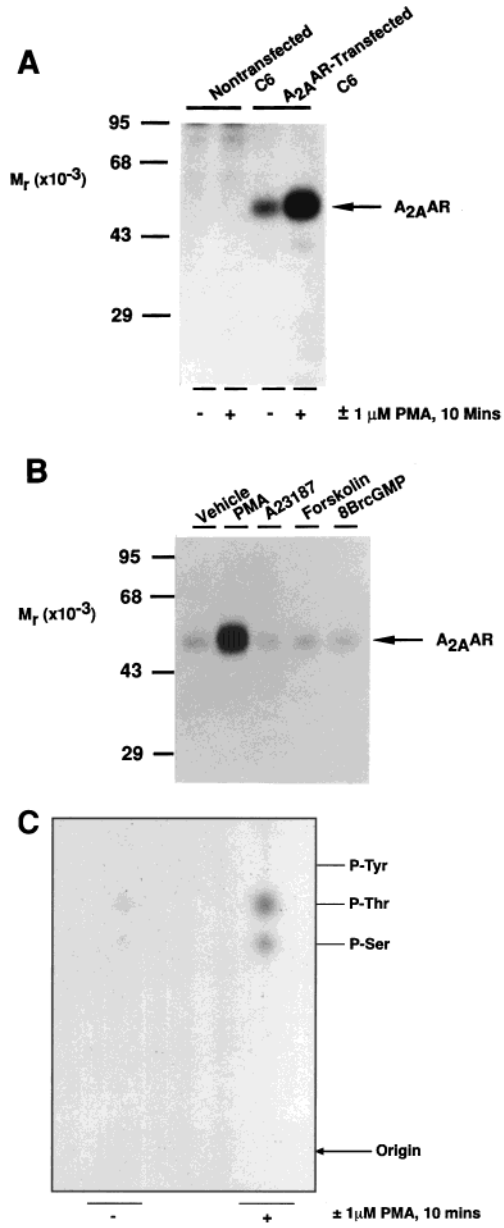

FIGURE 2: PMA stimulation of A_{2A}AR phosphorylation. (A) Nontransfected and transfected C6 cells were metabolically labeled with [^{32}P]orthophosphate, exposed to 1 μM PMA for 10 min at 37 °C as indicated, solubilized for immunoprecipitation with 12CA5, and analyzed as described under Experimental Procedures. (B) ^{32}P-Labeled transfected C6 cells were incubated for 10 min at 37 °C in the absence of any ligand (vehicle, lane 1) or in the presence of 1 μM PMA (PMA, lane 2) 10 μM calcium ionophore A23187 and 1.8 mM calcium chloride (A23187, lane 3) 100 μM forskolin (forskolin, lane 4), or 100 μM 8-bromo-cyclic GMP (8BrcGMP, lane 5) as indicated. Cells were then solubilized for analysis of A_{2A}AR phosphorylation by immunoprecipitation with 12CA5 as described under Experimental Procedures. (C) Transfected C6 cells were treated with or without 1 μM PMA for 10 min as described for panel A. Following SDS−PAGE, proteins were transferred to a poly(vinylidene difluoride) membrane prior to excision of phosphorylated A_{2A}ARs for phosphoamino acid analysis as described under Experimental Procedures. The migration of ninhydrin-stained phosphoamino acid standards relative to the origin is indicated.

and maximal phosphorylation reached after 10 min (Figure 5B).

Regulation of A_{2A}AR Phosphorylation by Endothelin 1. To determine whether A_{2A}AR phosphorylation could be stimulated upon agonist binding to an endogenous PKC-activating receptor, we took advantage of the presence of functional endothelin$_A$ receptors in C6 cells. Exposure of C6 cells to endothelin 1 results in a rapid and sustained increase in PKC activity due to the seqential activation of phospholipases C and D (*26*). Exposure of A_{2A}AR-expressing C6 cells to a maximally effective concentration of endothelin 1 resulted in an increase in A_{2A}AR phosphorylation comparable to that observed with a saturating concentration of PMA (Figure 6). In addition, GF109203X inhibited both PMA- and endothelin 1-stimulated A_{2A}AR phosphorylation events to similar extents (Figure 6), suggesting that they both induce receptor phosphorylation by activating PKC.

Effects of Conventional PKC Isoform Overexpression on A_{2A}AR Phosphorylation. While PKCδ predominantly regulates A_{2A}AR phosphorylation in C6 cells (Figure 4), it is possible that other isoforms may also regulate receptor phosphorylation in different cell types. To test this possibility, receptor phosphorylation was assessed in COS-P cells cotransfected with an A_{2A}AR expression vector and expression constructs encoding PKCα, PKCβI, and PKCβII. In cells expressing the A_{2A}AR alone, the addition of PMA stimulated receptor phosphorylation by between 1.5- and 2-fold (Figure 7A,B). The predominant effect of overexpressing each of the α, βI, and βII isoforms of PKC was to elevate basal levels of receptor phosphorylation such that addition of 1 μM PMA resulted in little further increase (Figure 7A,B). Immunoblotting of cytosolic extracts prepared from each of the transfected cell populations confirmed the transfection-dependent overexpression of each PKC isoform (Figure 7C).

Effect of Mutation of Consensus PKC Sites on A_{2A}AR Phosphorylation. On the basis of synthetic peptide (*19*) and oriented peptide library studies (*27*), the canine A_{2A}AR contains three consensus sites for PKC phosphorylation within the receptor's long cytoplasmic C-terminal domain: Thr 298, Ser 320, and Ser 335. Simultaneous mutation of each residue to a nonphosphorylatable alanine produced a receptor that bound agonist and antagonist ligands with similar affinities to those of the wild-type A_{2A}AR (data not shown). Surprisingly, upon expression in COS-P cells, the mutant A_{2A}AR displayed levels of basal and PMA-stimulated phosphorylation identical to those of the wild-type receptor (Figure 8). Therefore, despite being regulated by PKC-stimulated phosphorylation, the A_{2A}AR is not phosphorylated on its consensus PKC phosphorylation sites.

Effects of PMA Treatment on A_{2A}AR Function. While many G protein-coupled receptors are sequestered from the cell surface upon binding agonist, some receptors are also internalized in response to phosphorylation by PKC (*28*). However, treatment of transfected C6 cells with 1 μM PMA over a 30 min time course failed to alter significantly the levels of A_{2A}ARs present on the cell surface, as determined by a sequential cell surface biotin labeling−receptor immunoprecipitation protocol (Figure 9A). To assess any potential changes in adenylyl cyclase regulation, adenylyl cyclase assays were performed on membranes from A_{2A}AR-transfected CHO cells, in which PMA exposure stimulated A_{2A}AR phosphorylation above basal to an extent similar to that observed in C6 cells (Figure 9B, inset). However, consistent with our previous report (*29*), PMA exposure failed to alter the stimulation of adenylyl cyclase activity observed over a range of concentrations of CGS21680, an A_{2A}AR-selective

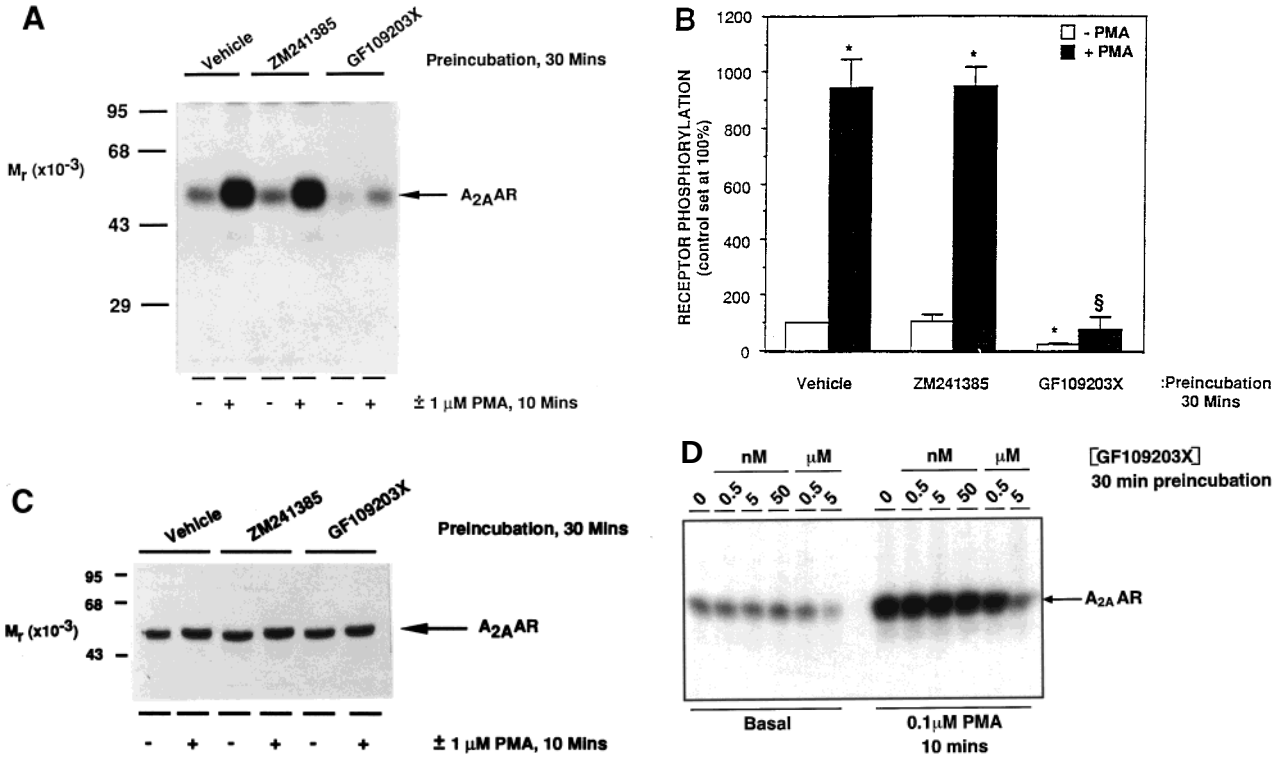

FIGURE 3: Effects of ZM241385 and GF109203X on A_{2A}AR phosphorylation. (A) ^{32}P-Labeled transfected C6 cells were incubated for 30 min in the absence (lanes 1 and 2) or presence of 1 μM ZM241385 (lanes 3 and 4) or 2.5 μM GF109203X (lanes 5 and 6) prior to stimulation with or without 1 μM PMA for 10 min at 37 °C as indicated. Cells were then solubilized for analysis of A_{2A}AR phosphorylation by immunoprecipitation with 12CA5 as described under Experimental Procedures. Panel B shows a quantitative analysis of data obtained from three such experiments. The data are normalized to the basal phosphorylation observed in the absence of any inhibitor. The average stimulation of A_{2A}AR phosphorylation in these experiments was (9.5 ± 1.0)-fold. An asterisk indicates a significant difference ($p < 0.05$) from the phosphorylation observed in the absence of PMA and inhibitors. § indicates a significant difference ($p < 0.05$) from the phosphorylation observed in the presence of PMA alone. (C) Aliquots (20 μg) of solubilized membranes from transfected C6 cells treated as indicated were analyzed by SDS−PAGE and immunoblotting with 0.5 μg/mL anti-A_{2A}AR antibody and visualized by enhanced chemiluminescence as described under Experimental Procedures. This is one of three experiments that produced similar data. (D) ^{32}P-Labeled transfected C6 cells were incubated for 30 min in the absence (0) or presence of increasing concentrations of GF109203X prior to stimulation with or without 0.1 μM PMA for 10 min at 37 °C as indicated. Cells were then solubilized for analysis of A_{2A}AR phosphorylation by immunoprecipitation with 12CA5 as described under Experimental Procedures. This is one of three experiments that produced similar results.

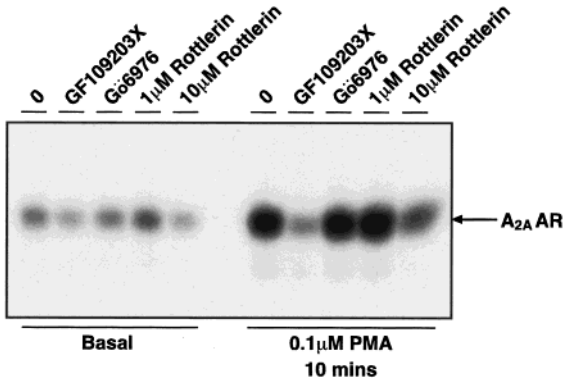

FIGURE 4: Effects of Go6976 and rottlerin on A_{2A}AR phosphorylation. (A) ^{32}P-Labeled transfected C6 cells were preincubated for 30 min at 37 °C with either vehicle, 5 μM GF109203X, 1 μM Go6976, or the indicated concentrations of rottlerin. Cells were then incubated in the absence or presence of 0.1 μM PMA for 10 min as indicated prior to solubilization for receptor immunoprecipitation with 12CA5 as described under Experimental Procedures. This is one of three experiments in which the average *x*-fold stimulation of A_{2A}AR phosphorylation above basal produced by 0.1 μM PMA was 7.8 ± 2.0.

agonist (Figure 9B). Also, agonist competition experiments versus ^{125}I-ZM241385 binding performed on transfected C6 cell membranes failed to reveal any changes in the number or affinity of agonist binding sites or increased sensitivity to uncoupling by guanine nucleotides in response to pretreatment with PMA (data not shown). Therefore, it appears that A_{2A}AR phosphorylation in response to PKC activation does not regulate either the subcellular distribution of the A_{2A}AR or its ability to stimulate G proteins to activate adenylyl cyclase.

DISCUSSION

In this study, we have demonstrated that, in C6 glioma cells, phosphorylation of the A_{2A}AR on serine and threonine residues can be rapidly enhanced upon activation of PKC by either phorbol ester or agonist occupation of endothelin$_A$ receptors. By using a panel of isoform-selective PKC inhibitors, we have also determined that the calcium-independent PKCδ isoform is partly responsible for this phenomenon. However, coexpression of the A_{2A}AR with each of the calcium-dependent PKCα, PKCβI, and PKCβII isoforms is also capable of sustaining A_{2A}AR phosphorylation in an intact cell environment. This is an important observation because the complement of PKC isoforms expressed in a given cell varies in a tissue-dependent fashion. Therefore, the fact that multiple PKC isoforms, including two that are expressed ubiquitously (PKCα and PKCδ), can sustain A_{2A}-

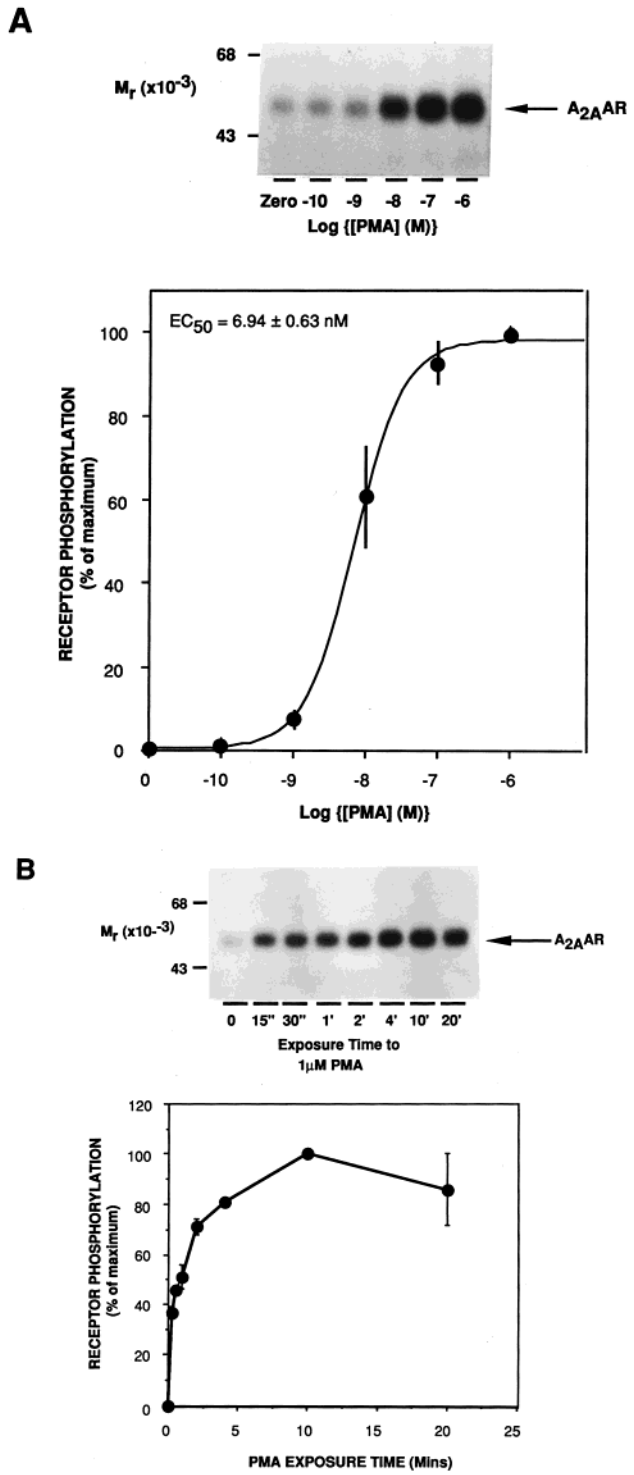

FIGURE 5: Concentration and time dependences of PMA-stimulated A_{2A}AR phosphorylation. (A) ^{32}P-Labeled transfected C6 cells were incubated for 10 min at 37 °C with the indicated concentrations of PMA. Cells were then solubilized for analysis of A_{2A}AR phosphorylation by immunoprecipitation with 12CA5 as described under Experimental Procedures. Quantitative analysis of receptor phosphorylation is from data pooled from three experiments in which the average maximal stimulation of phosphorylation induced by 1 μM PMA was (11.2 ± 2.5)-fold above basal. (B) ^{32}P-Labeled transfected C6 cells were incubated for the indicated times at 37 °C with 1 μM PMA. Cells were then solubilized for analysis of A_{2A}AR phosphorylation by immunoprecipitation with 12CA5 as described under Experimental Procedures. Quantitative analysis of receptor phosphorylation is from data pooled from three experiments in which the average maximal stimulation of phosphorylation induced by 1 μM PMA was (7.7 ± 0.9)-fold above basal.

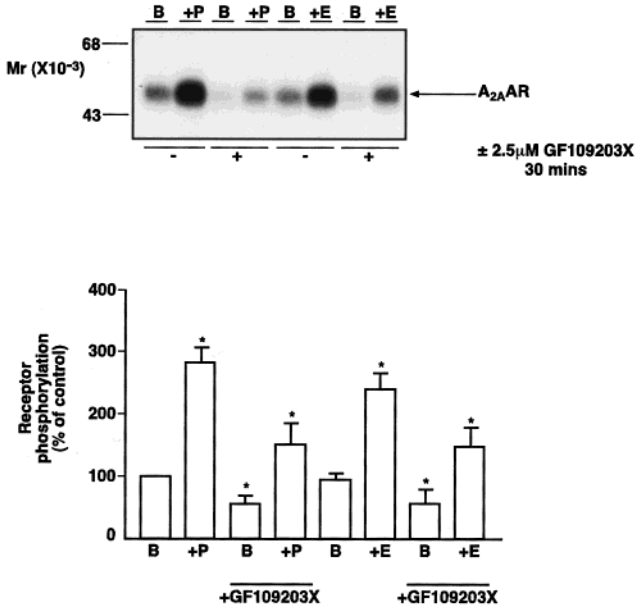

FIGURE 6: Comparison of PMA- and endothelin 1-stimulated A_{2A}-AR phosphorylation in C6 cells. ^{32}P-Labeled transfected C6 cells were incubated for 30 min in the absence or presence of 2.5 μM GF109203X prior to stimulation without (B) or with 1 μM PMA (+P) or 100 nM endothelin 1 (+E) for 10 min at 37 °C as indicated. Cells were then solubilized for analysis of A_{2A}AR phosphorylation by immunoprecipitation with 12CA5 as described under Experimental Procedures. The bar graph shows a quantitative analysis of data obtained from three such experiments. The data are normalized to the basal phosphorylation observed in the absence of any additions. The average stimulation of A_{2A}AR phosphorylation by endothelin 1 in these experiments was (2.3 ± 0.3)-fold. An asterisk indicates a significant difference ($p < 0.05$) from the phosphorylation observed without any additions.

AR phosphorylation suggests that this phenomenon is likely to be displayed by multiple A_{2A}AR-expressing cell types upon agonist occupation of receptors that can increase PKC activity. Indeed, in the current study, each of the three cell lines used to express the A_{2A}AR heterologously (C6 glioma, COS-P, and CHO cells) exhibited enhanced A_{2A}AR phosphorylation upon activation of PKC.

To our knowledge, the current study represents the first attempt to assess the ability of multiple PKC isoforms to phosphorylate a GPCR in intact cells. However, given that there are at least 12 distinct PKC isoforms, as well as the postulated existence of other less extensively characterized lipid-regulated enzymes, more studies will be necessary to assess the capacity of these enzymes to phosphorylate the A_{2A}AR in situ.

Two important observations argue that PKC-stimulated phosphorylation of the A_{2A}AR represents a physiologically relevant mechanism by which this important receptor is regulated. First, A_{2A}AR phosphorylation is sensitive to low nanomolar concentrations of PMA and occurs rapidly (readily detectable 15 s after PMA exposure). Similar rapid time courses and low nanomolar sensitivities to PMA-stimulated phosphorylation have been observed for other proteins that are known to be phosphorylated by multiple PKC isoforms in vitro and in situ: these include myristoylated alanine-rich C-kinase substrate (MARCKS) and the multidrug resistance protein (*30*−*33*). In addition, the low nanomolar sensitivity to PMA of A_{2A}AR phosphorylation compares favorably with the estimates of phorbol ester affinities for

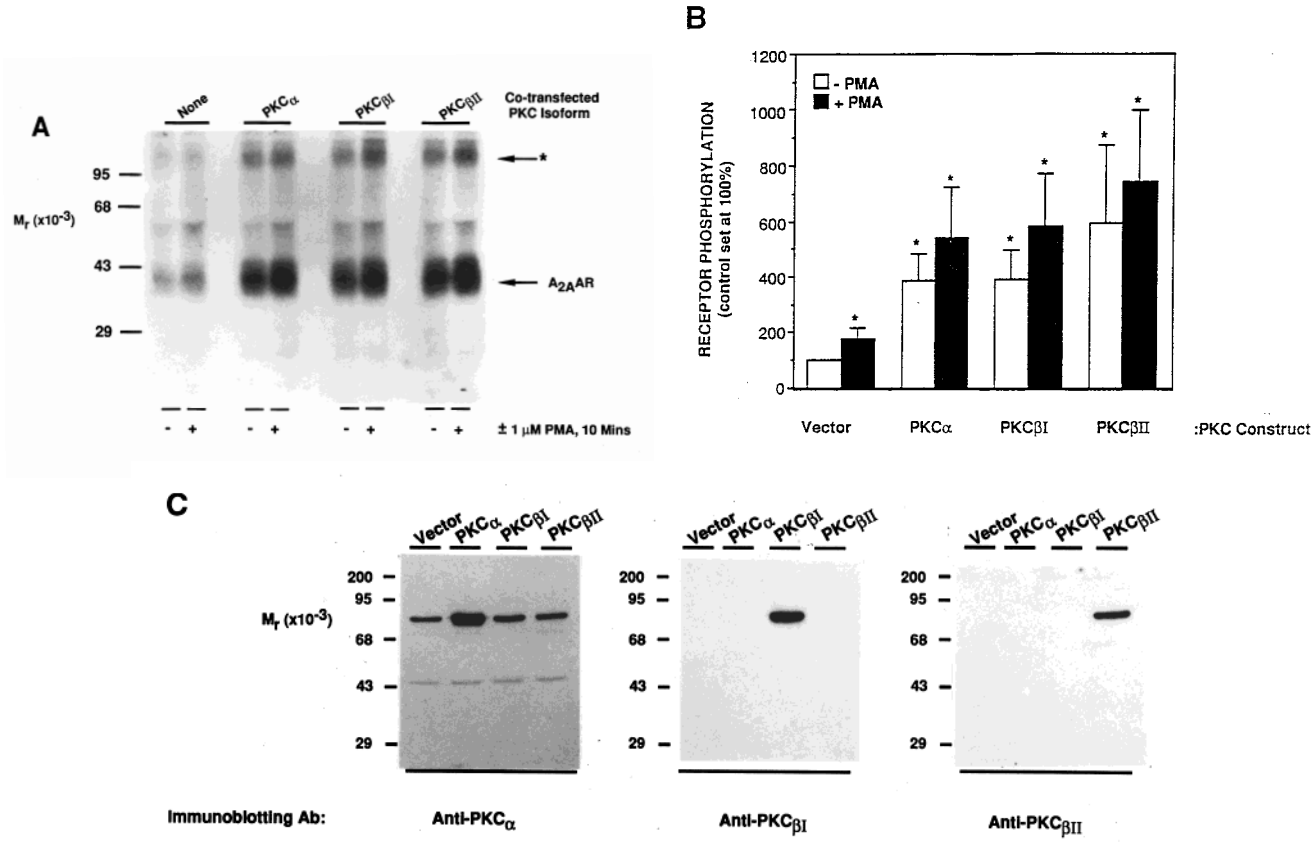

FIGURE 7: Effects of PKC isoform overexpression on PMA-stimulated A_{2A}AR phosphorylation. (A) COS-P cells were cotransfected with pCMV5/epitope-tagged A_{2A}AR and either pBΔG (none), pBΔG/PKCα (PKC$_\alpha$), pBΔG/PKCβI (PKC$_{\beta I}$), or pBΔG/PKCβII (PKC$_{\beta II}$). ^{32}P-Labeled cells were then incubated in the absence or presence of 1 μM PMA for 10 min at 37 °C prior to cell solubilization and analysis of A_{2A}AR phosphorylation by immunoprecipitation with 12CA5 as described under Experimental Procedures. The asterisk indicates a transfection-dependent band whose appearance was variable between experiments and may represent a receptor aggregate. Immunoblotting of transfected COS-P membranes with an anti-A_{2A}AR antibody demonstrated that the 40−43 kDa protein and the presumed aggregate were the predominant transfection-dependent A_{2A}AR species observed in this system (data not shown). Panel B shows a quantitative analysis of data obtained from three phosphorylation experiments. In these experiments, the data were normalized to the basal phosphorylation levels observed in cells transfected with pCMV5/epitope-tagged A_{2A}AR and empty pBΔG vector. In this panel, an asterisk indicates a significant ($p < 0.05$) increase in phosphorylation over control conditions. The levels of A_{2A}AR expression in these experiments, as determined by radioligand binding with a saturating concentration of ^{125}I-ZM241385, were between 10 and 15 pmol/mg of membrane protein and were not consistently different between each group of transfected cells. (C) Cytosolic extracts (10 μg portions) prepared from COS-P cells transfected with pCMV5/epitope-tagged A_{2A}AR and pBΔG either without (vector) or with the indicated PKC isoform cDNA were analyzed by SDS−PAGE and immunoblotting with the indicated antibodies as described under Experimental Procedures. This is one of three immunoblots performed with each antibody.

purified PKC isoforms obtained from ^{3}H-phorbol dibutyrate binding assays (*34*, *35*). Second, A_{2A}AR phosphorylation is enhanced by activation of endogenously expressed endothelin$_A$ receptors, which elevate PKC activity by increasing diacylglycerol formation from phospholipase C- and D-mediated hydrolysis of membrane lipids (*26*).

While we are unable to determine unequivocally whether the A_{2A}AR is phosphorylated directly by PKC or indirectly via activation of intermediate kinases, some of our observations are consistent with the latter possibility. The most important of these is that mutation to alanine of the three consensus PKC phosphorylation sites within the A_{2A}AR has no effect on either basal or PMA-stimulated receptor phosphorylation. Second, attempts to assess the ability of A_{2A}ARs immunoprecipitated from C6 cells to act as substrates for purified recombinant PKC isoforms in in vitro immune complex kinase assays have been unsuccessful (T. M. Palmer, unpublished observations). While it is possible that detergent solubilization of the A_{2A}AR alters receptor conformation such that the kinases may not be able to either recognize or have access to the phosphorylation sites utilized in situ, this could also be explained if A_{2A}AR phosphorylation occurs via an indirect mechanism in intact cells. We are currently attempting to identify the amino acids phosphorylated in response to PKC activation and use this information to isolate candidate receptor kinases. Excluding Thr298, Ser320, and Ser335, the 121-residue cytoplasmic tail of the receptor also contains two threonines and seven serines, making it a prime candidate for the locus of receptor phosphorylation.

The specific ability of PMA, but not other activators of second messenger-regulated kinases, to stimulate A_{2A}AR phosphorylation might suggest that this phenomenon has a important role in regulating A_{2A}AR signaling. Typically, PKC-stimulated phosphorylation of GPCRs is associated with the onset of heterologous desensitization (*36*−*41*). In the cases of the glucagon-like peptide 1 and chorionic gonadotropin receptors, PMA-responsive phosphorylation sites have been mapped to their C-terminal domains (*36*, *37*). By contrast, we were unable to observe any PMA-induced uncoupling of the receptor from G protein regulation or diminution of adenylyl cyclase stimulation. Mutagenesis of

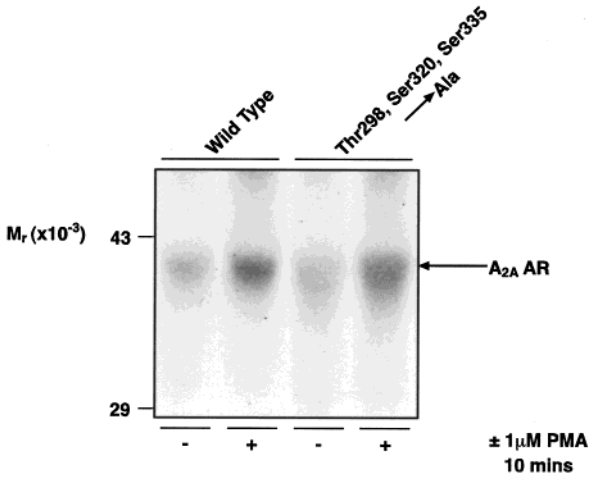

FIGURE 8: Effect of mutating consensus PKC phosphorylation sites to Ala on A_{2A}AR phosphorylation. COS-P cells were transfected (15 μg/100 mm dish) with either a pCMV5/epitope-tagged WT A_{2A}-AR cDNA or a mutant A_{2A}AR in which each of Thr298, Ser320, and Ser335 had been mutated simultaneously to Ala. Forty-eight hours posttransfection, ^{32}P-labeled transfected cells in six-well dishes were incubated in the absence or presence of 1 μM PMA for 10 min at 37 °C prior to cell solubilization and analysis of A_{2A}-AR phosphorylation by immunoprecipitation with 12CA5 as described under Experimental Procedures. This is one of three experiments in which the average *x*-fold increases in A_{2A}AR phosphorylation over basal produced by 1 μM PMA were 2.2 ± 0.3 (WT) and 2.5 ± 0.5 (mutant).

the A_{2A}AR has demonstrated that the C-terminal 95 amino acids do not influence the ability of the receptor to either bind ligand with the appropriate pharmacology, stimulate adenylyl cyclase via G_s, or desensitize in response to agonist exposure (*9*, *42*, *43*). Therefore, it is tempting to speculate that the C-terminal tail of the A_{2A}AR has a unique role independent from the adenylyl cyclase signaling pathway and that the function of the C-terminal tail may be regulated by PKC-stimulated phosphorylation. Several recent studies have demonstrated that specific physiologically important A_{2A}-AR-mediated signaling events occur independently of cAMP generation, although the molecular components of these signaling pathways have yet to be elucidated. These include the p21^{ras}-dependent A_{2A}AR-mediated stimulation of extracellular signal-regulated kinase (ERK/MAP kinase) activity in endothelial cells (*8*), activation of L-type calcium channels in ventricular myocytes (*44*), and antagonism of D_2 dopamine receptor signaling in the caudate putamen (*6*). In this regard, evidence is now accumulating that GPCRs can initiate signaling pathways utilizing molecular mechanisms that do not involve interactions with heterotrimeric G proteins. For example, the extreme C-terminal domains of the human β_2-adrenergic and P2Y1 purinergic receptors each contain a tetrapeptide motif that directs receptor binding to the PDZ domain of the Na^+/H^+ exchanger regulatory factors NHERF and NHERF-2 (*45*). It has also been reported that the C-terminal tail of the mGluR5 metabotropic glutamate receptor binds calcium−calmodulin and that this interaction is regulated by PKC-mediated phosphorylation (*46*). It has also recently been demonstrated that upon binding to agonist-occupied phosphorylated GPCRs, arrestin proteins not only terminate heterotrimeric G protein-mediated signaling but also initiate the MAP kinase signaling cascade by binding and activating src family tyrosine kinases (*47*). In all these cases, the C-terminal tail of the appropriate receptor acts as a scaffold upon which signaling complexes can be assembled. Should the C-terminal domain of the A_{2A}AR function in a similar way, identification of proteins that interact with the A_{2A}AR in a manner which is regulated by PKC-stimulated phosphorylation will be essential in order to understand how this receptor initiates multiple signaling pathways upon agonist binding.

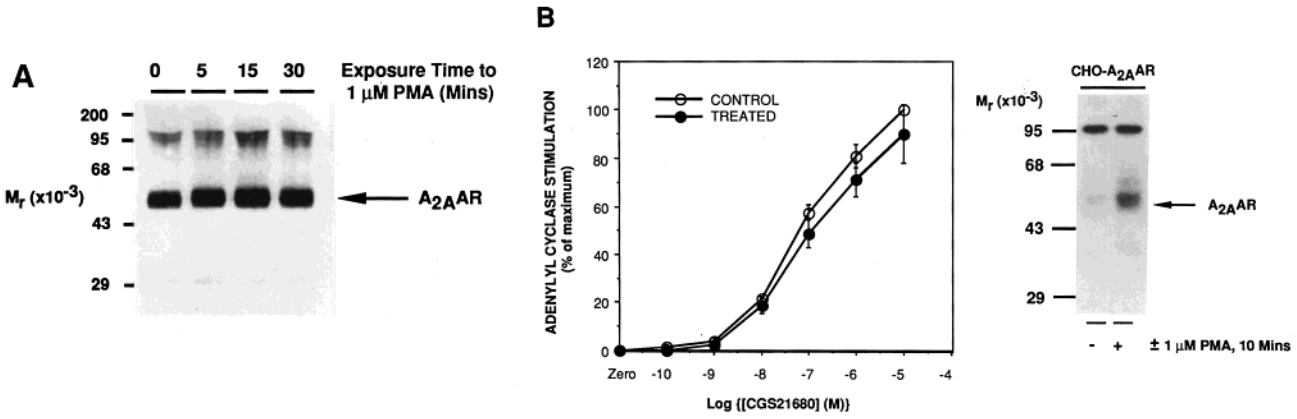

FIGURE 9: Effect of PMA treatment on A_{2A}AR internalization and adenylyl cyclase activation. (A) Transfected C6 cells were incubated in the absence (0) or presence of 1 μM PMA for the indicated times at 37 °C prior to labeling of cell-surface glycoproteins by sequential periodate/biotin-LC hydrazide treatment. Cells were then solubilized for immunoprecipitation of A_{2A}ARs with 12CA5 as described under Experimental Procedures. After SDS−PAGE, resolved proteins were transferred to a nitrocellulose membrane and probed with horseradish peroxidase-conjugated streptavidin to visualize the levels of biotin-labeled A_{2A}ARs in each condition. This is one of three experiments. (B) CHO cells transiently transfected with pCMV5/epitope-tagged A_{2A}AR were serum-starved for 90 min prior to incubation without (○) or with (●) 1 μM PMA for 10 min at 37 °C. Membranes were then prepared for assay of adenylyl cyclase activity in the absence or presence of increasing concentrations of CGS21680. Average basal activities were (in picomoles per minute per milligram of membrane protein) 2.30 ± 0.38 and 2.71 ± 0.19 for vehicle- and PMA-treated samples, respectively. The average stimulation of adenylyl cyclase activity above basal elicited by 10 μM CGS21680 in membranes from vehicle-treated cells was (6.29 ± 1.60)-fold. The data are pooled from three separate experiments and normalized as described under Experimental Procedures. (Inset) pCMV5/epitope-tagged A_{2A}AR-transfected CHO cells were metabolically labeled with [^{32}P]orthophosphate, exposed to 1 μM PMA for 10 min at 37 °C as indicated, and then solubilized for analysis of A_{2A}AR phosphorylation by immunoprecipitation with 12CA5 as described under Experimental Procedures. This is one of four experiments in which the average increase in A_{2A}AR phosphorylation induced by 1 μM PMA was (3.8 ± 0.7)-fold over basal.

In conclusion, we have determined that the A_{2A}AR exists as a phosphoprotein under basal conditions and that either biochemical activation of PKCδ or overexpression of conventional PKC isoforms increases receptor phosphorylation. However, mutation of consensus PKC phosphorylation sites fails to inhibit PKC-stimulated phosphorylation, suggesting that phosphorylation occurs via activation of an intermediate kinase/kinases, which then interact with and phosphorylate the receptor directly. In addition, elevated receptor phosphorylation does not alter the subcellular distribution of the A_{2A}AR or impair its ability to activate G_s and stimulate adenylyl cyclase activity. Given that the A_{2A}AR C-terminal domain contains multiple potential phosphorylation sites and that no function has yet been assigned to this region of the A_{2A}AR, it is likely that phosphorylation within this domain regulates previously unappreciated interactions of the A_{2A}AR with regulatory proteins. Moreover, it is possible that these proteins may be involved in controlling cAMP-independent signaling events shown recently to be initiated upon A_{2A}AR activation in cardiac myocytes, endothelial cells, and striatopallidal neurones (*6*, *8*, *44*). Experiments to test these hypotheses are currently underway.

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