Serine 232 of the α_{2A}-Adrenergic Receptor Is a Protein Kinase C-Sensitive Effector Coupling Switch†

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ABSTRACT: α_2-adrenergic receptors (α_2AR) couple to multiple effectors including adenylyl cyclase and phospholipase C. We hypothesized that signaling selectivity to these effectors is dynamically directed by kinase-sensitive domains within the third intracellular loop of the receptor. Substitution of Ala for Ser232, which is in the N-terminal region of this loop in the α_{2A}AR, resulted in a receptor that was markedly uncoupled (∼82% impairment) from stimulation of inositol phosphate accumulation while the capacity to inhibit adenylyl cyclase remained relatively intact. In S232A α_{2A}AR transfected cell membranes, agonist-promoted [^{35}S]GTPγS binding was reduced by ∼50%. Coexpression of modified G proteins rendered insensitive to pertussis toxin revealed that the S232A receptor was uncoupled from both G_i and G_o. S232 is a potential PKC phosphorylation site, and whole cell phosphorylation studies showed that the mutant had depressed phosphorylation compared to wild type (1.3- vs 2.1-fold/basal). Consistent with S232 directing coupling to phospholipase C, PMA exposure resulted in ∼67% desensitization of agonist-promoted inositol phosphate accumulation without significantly affecting inhibition of adenylyl cyclase. The dominant effect of mutation or phosphorylation at this site on inositol phosphate as compared to cAMP signaling was found to most likely be due to the low efficiency of signal transduction via phospholipase C vs adenylyl cyclase. Taken together, these results indicate that S232 acts as a selective, PKC-sensitive, modulator of effector coupling of the α_{2A}AR to inositol phosphate stimulation. This represents one mechanism by which cells route stimuli directed to multifunctional receptors to selected effectors so as to attain finely targeted signaling.

G protein coupled receptors represent the largest superfamily of signaling molecules in the genome. Stabilization of receptors in the active conformation by agonists results in their binding and activation of heterotrimeric G proteins, which consist of α and $\beta\gamma$ subunits. Early paradigms proposed that a specific α subunit transduced the receptor's signal to effectors and that each receptor or family of receptors coupled to one signaling pathway. Over the past decade it has become clear that some receptors have the capacity to couple to multiple effectors. In some cases the mechanisms of these multifunctional properties have been identified and include receptors signaling via several distinct G_α subunits (*1*, *2*) and/or the $\beta\gamma$ subunits (*3*, *4*), activation of several effectors by the same G protein (*5*−*7*), and direct (non G protein) interactions between receptors and effectors (*8*). The α_2-adrenergic receptors (α_2AR),[1] which include the α_{2A}, α_{2B}, and α_{2C} subtypes, are among the most versatile receptors, displaying a wide range of signaling via many effectors. Activation of α_2AR have been reported to decrease adenylyl cyclase activity, increase adenylyl cyclase activity, activate inward rectifying K^+ currents, inhibit Ca^{2+} currents, increase inositol phosphate accumulation, increase intracellular Ca^{2+} concentrations, and activate MAP kinase (*1*, *3*, *4*, *9*). Although cell-type specific expression of particular effectors may limit the spectrum of α_2AR-mediated responses, mechanisms that regulate the specificity of α_2AR coupling remain to be determined.

In the current work we hypothesized that protein kinase C (PKC) phosphorylation sites within G protein coupling regions of the α_{2A}AR play a role in such signal directivity. Like other G protein coupled receptors, the amino-terminal and carboxy-terminal portions of the third intracellular loop have been identified as critical for G protein binding, activation, or specificity (*10*, *11*). The third intracellular loop of the human α_{2A}AR consists of ∼158 amino acids. Within the first 16 residues there are two potential PKC phosphorylation sites at residues 227 and 232. In the C-terminal region of the third intracellular loop, within the last 16 residues there are also two potential sites, at residues 360 and 373. The latter has been shown to be highly sensitive to mutagenesis, in that certain substitutions result in constitutive activation (*12*), analogous to the α_1AR (*13*) and β_2AR (*14*). In our initial studies, we substituted Ala for Ser or Thr at

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[1] Abbreviations: α_2AR, α_2-adrenergic receptor; β_2AR, β_2-adrenergic receptor; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; MAP kinase, mitogen-activated protein kinase; SDS, sodium dodecyl sulfate.

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positions 227, 232, and 360 and expressed the mutated receptors in CHO cells. One mutation, S232A, markedly reduced agonist-promoted inositol phosphate accumulation but had relatively preserved inhibition of adenylyl cyclase. In this report we delineate the basis of this signaling divergence and show that S232 is regulated by PKC phosphorylation, which serves to selectively desensitize receptor signaling via phospholipase C.

MATERIALS AND METHODS

DNA Constructs and Mutagenesis. A polymerase chain reaction (PCR) based strategy was used to substitute the Ser residue at amino acid position 232 of the third intracellular loop of the human α_{2A}AR to Ala. The receptor was epitope tagged at the amino terminus with the influenza hemaglutinin nonopeptide as described (*15*) and ultimately subcloned into the vector pBC12BI. Pertussis toxin-insensitive mutants of rat $G_{\alpha o}$ and $G_{\alpha i2}$ were obtained from Dr. R. Taussig, University of Michigan, and contained Cys to Ser mutations in their respective ADP-ribosylation sites (*16*). These constructs were subcloned into pCDNA1.1 Amp. The integrity of all constructs was verified by sequencing.

Transfection and Cell Culture. For stable expression of the wild-type and mutant α_{2A}AR, CHO cells in monolayers at ~30% confluence were cotransfected using a calcium precipitation method (*17*). Cells were transfected with 3 μg of pSV_2neo, which provides for G-418 resistance, and 20 μg of the wild-type or mutant α_{2A}AR constructs. Cells were selected in 1000 μg/mL G-418, and clones were screened for expression of the wild-type or mutant α_{2A}AR using a [^{3}H]yohimbine binding assay as described below. CHO cells were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 80 μg/mL G-418 (to maintain selection pressure) at 37 °C in a 5% CO_2 atmosphere. CHO cells stably expressing the wild-type and mutant α_{2A}AR in monolayers at ~90% confluence were utilized. Multiple clones expressing each receptor were studied. For transient expression of pertussis toxin-insensitive $G_{\alpha o}$ and $G_{\alpha i2}$, CHO cells stably expressing the wild-type or mutant α_{2A}AR in monolayers at 60−90% confluence were transfected with the indicated vectors using GenePORTER (Gene Therapy Systems, San Diego, CA). Assays were carried out 48 h after transfection.

Inositol Phosphate Turnover. Inositol phosphate accumulations in whole cells were determined as described previously (*18*). Briefly, nearly confluent CHO cells stably expressing the wild-type and mutant α_{2A}AR were incubated with [^{3}H]myoinositol (5 μCi/mL) in media without fetal calf serum for 16−18 h at 37 °C in a 5% CO_2 atmosphere. Cells were then washed and incubated with warm PBS for 30 min, followed by incubation with 20 mM LiCl in PBS for 30 min at 37 °C in a 5% CO_2 atmosphere. Cells were then treated with vehicle alone (basal), 10 μM epinephrine, or 5 units/mL thrombin for 5 min; for desensitization conditions, cells were first treated with 0.1 μM PMA for 2 min and then 10 μM epinephrine for 5 min. The reactions were terminated by aspiration of the media and addition of 0.4 M perchloric acid for 10 min at 25 °C. After neutralization of the extracts with KOH and $KHCO_3$, they were loaded on Dowex AG1-X8 resin formate columns, and total inositol phosphates were eluted with a solution containing 0.1 M formic acid and 1 M formate.

Radioligand Binding Assays. Expression levels of the wild-type and mutant α_{2A}AR were determined by radioligand binding with [^{3}H]yohimbine. CHO cells expressing the wild-type or mutant α_{2A}AR in monolayers were washed three times with cold PBS, and membranes were prepared by hypotonic lysis in cold buffer (5 mM Tris, 2 mM EDTA, pH 7.4) and scraping with a rubber policeman, followed by centrifugation at 42000*g* for 10 min at 4 °C. The crude membrane pellets were then resuspended in a buffer which contained 75 mM Tris, pH 7.4, 12.5 mM $MgCl_2$, and 2 mM EDTA. Saturation and competition binding assays were carried out as described previously (*18*). Reactions were terminated by dilution with the cold 10 mM Tris, pH 7.4, followed by vacuum filtration over glass fiber filters.

Adenylyl Cyclase and cAMP Assays. Membrane adenylyl cyclase activities were determined in the presence of buffer alone, 5.0 μM forskolin, or 5.0 μM forskolin with the indicated concentrations of agonist using methods similar to those previously described (*19*). Briefly, membranes were resuspended in a buffer that provided for a final assay concentration of 1.6 mM $MgCl_2$, 0.8 mM EDTA, and 40 mM HEPES, pH 7.4. Membranes (15−25 μg) were incubated in the above buffer with 2.7 mM phosphoenolpuruvate, 0.6 μM GTP, 0.1 mM cAMP, 0.12 mM ATP, 50 μg/mL myokinase, 0.05 mM ascorbic acid, and 2 μCi of [α-^{32}P]-ATP for 30 min at 37 °C. Reactions were stopped by the addition of 1.0 mL of a cold solution containing ~100000 dpm of [^{3}H]cAMP and excess ATP and cAMP. [^{32}P]cAMP was isolated by chromatography over disposable alumina columns with [^{3}H]cAMP used to quantitate individual column recovery. Results are expressed as the percent inhibition of forskolin-stimulated activity. For whole cell cAMP accumulation experiments, cells were incubated with [^{3}H]adenine (10 μCi/mL) and 0.1 mM isobutylmethylxanthine (IBMX) in media without fetal calf serum for 2 h at 37 °C in a 5% CO_2 atmosphere. After a single wash, cells were incubated with media alone, media with 30 μM forskolin, or media with 30 μM forskolin and various concentrations of epinephrine at 37 °C for 2 min. The reactions were stopped by adding an equal volume of perchloric acid (5% w/v) plus 0.2 mM cAMP for 10 min at 25 °C. After neutralization of the extracts with $^1/_{10}$ volume of 4.2 N KOH, 20000 cpm of [^{14}C]cAMP was added to each tube. Labeled cAMP was isolated by sequential chromatography over Dowex AG 50W-X4 and alumina columns, with [^{14}C]cAMP used to quantitate individual column recovery. Results were calculated as percent inhibition of forskolin-stimulated conversion of ATP to cAMP.

[^{35}S]GTPγS Binding Assays. G protein coupling to α_{2A}-AR was measured by ^{35}S-radiolabeled guanosine 5′-*O*-(3-thiotriphosphate) ([^{35}S]GTPγS) binding in CHO cell membranes as described (*20*). Briefly, membranes were resuspended in a buffer providing a final concentration of 36 mM Tris, pH 7.4, 6 mM $MgCl_2$, 100 mM NaCl, and 1 mM EDTA and incubated with the indicated concentrations of agonist and 1 mM dithiothertol, 1 μM GDP, and 2 nM [^{35}S]GTPγS in a 100 μL reaction volume for 15 min at 25 °C. Nonspecific binding was defined in the presence of 100 μM GTPγS. Incubations were stopped by dilution with cold 10 mM Tris, pH 7.4, followed by vacuum filtration over glass fiber filters.

Intact Cell Receptor Phosphorylation. Whole cell phosphorylation studies were carried out in a manner similar to

that described previously (*15*). For these studies, COS-7 cells were transiently transfected in order to achieve expression levels of 10−15 pmol/mg of α_2AR. Briefly, cells expressing the wild-type or the S232A α_{2A}AR were incubated with 0.5 mCi/mL [^{32}P]orthophosphate in six-well plates for 1 h at 37 °C in 5% CO_2. Cells were then incubated with vehicle or 0.1 μM PMA for 10 min, washed twice with cold PBS, and scraped in a solubilization buffer consisting of 150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, pH 8.0, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM NaF, and 10 mM sodium pyrophosphate, with the following protease inhibitors: 0.1 mM PMSF, 1 mM benzamidine, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 5 μg/mL aprotinin, and 1 μg/mL pepstatin A. Particulates were solubilized at 4 °C for 1 h on a rotating wheel, and nonsolubilized material was removed by centrifugation at 40000*g* for 15 min at 4 °C. The supernatant was incubated with a 1:200 dilution of a monoclonal antibody to the HA-epitope (12CA5, Boehringer Mannheim, Indianapolis, IN) and protein A/Sepharose CL-4B beads (Amersham Pharmacia, Chicago, IL) with 2% (w/v) BSA for 3 h at 4 °C. The beads were washed three times in the above solubilization buffer, and the immune complexes were dissociated by incubation of the beads in SDS sample buffer at 37 °C for 1 h. Proteins were fractionated on 12.5% SDS−polyacrylamide gels and detection and quantitation of radioactive bands carried out on the dried gels using a Molecular Dynamics PhosphorImager.

Data Analysis. Data are represented as the mean ± SE. Comparisons were by a two-way *t*-test, with significance considered when $p < 0.05$. Curve fitting was carried out using GraphPad Prism Software (GraphPad, San Diego, CA).

RESULTS

To investigate the relevance of potential PKC phosphorylation sites in a G protein coupling domain of the third intracellular loop of the α_{2A}AR, Ser232 was mutated to Ala, and wild-type and S232A receptors were expressed in CHO cells. Lines with equivalent expression levels, typically ∼1000 fmol/mg (see figure legends) were utilized. The mutated receptor displayed no alterations in [^{3}H]yohimbine binding affinity compared to wild type ($K_d = 2.4 \pm 0.08$ vs 2.0 ± 0.19 nM, $n = 3$), nor was the affinity for the agonist epinephrine altered by the mutation ($K_i = 266 \pm 93$ vs 242 ± 77 nM, $n = 4$). However, initial functional studies showed a marked decrease in maximal agonist-promoted inositol phosphate accumulation in cells expressing S232A compared to wild-type α_{2A}AR (Figure 1). Wild-type α_{2A}AR-stimulated inositol phosphate accumulation was 44 ± 2.5% over basal compared to 8.1 ± 2.2% for the mutant, amounting to an ∼81.6% impairment. The response to thrombin was similar between the two lines (see legend to Figure 1). In contrast to this inositol phosphate-coupling defect, the coupling of the mutated receptor to inhibition of adenylyl cyclase was nearly intact (∼26.6% impairment; Figure 2). Taken together, these studies point toward the mutant having a somewhat selective loss of coupling to phospholipase C compared to adenylyl cyclase.

To assess the receptor−G protein coupling defect further, [^{35}S]GTPγS binding studies were carried out in membranes from CHO cells expressing equivalent levels of the receptors.

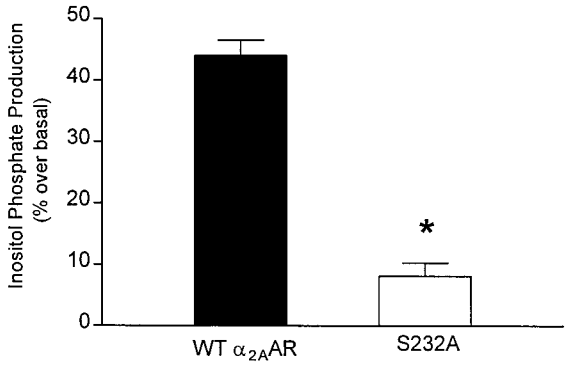

FIGURE 1: The S232A α_{2A}AR demonstrates impaired agonist-promoted inositol phosphate production. CHO cells stably expressing WT or S232A α_{2A}ARs were preincubated with [^{3}H]inositol and treated with vehicle, 10 μM epinephrine, or 5 units/mL thrombin for 5 min. The receptor expression levels were 1287 ± 131 fmol/mg of protein for WT vs 1410 ± 166 fmol/mg of protein for S232A. Epinephrine-stimulated inositol phosphate production was 8.1 ± 2.2% over basal in S232A compared with 44.0 ± 2.5% over basal in WT, amounting to an ∼82% impairment of S232A signaling. In contrast, thrombin-stimulated inositol phosphate production over basal was 262.3 ± 9.6% in WT and 219.0 ± 8.7% in S232A (*, $p < 0.001$, $n = 4$).

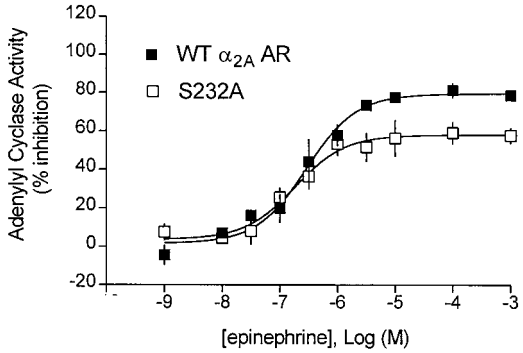

FIGURE 2: Agonist-promoted inhibition of adenylyl cyclase is relatively preserved with the S232A receptor. Shown are the results from studies with CHO clonal lines expressing WT or S232A α_{2A}-AR at matched levels (WT, 1524 ± 56 fmol/mg of protein; S232A, 1566 ± 245 fmol/mg of protein). Maximal inhibition of adenylyl cyclase activity of S232A was impaired ∼26 ± 6% compared to WT α_{2A}AR ($p < 0.01$, $n = 4$). There were no differences in the EC_{50} for epinephrine for the WT or S232A receptor (173 ± 48 vs 201 ± 52 nM).

As shown in Figure 3, S232A displayed an ∼50% decrease in agonist-promoted binding. To delineate whether this coupling defect was specific for $G_{\alpha o}$ vs $G_{\alpha i}$, cells expressing each receptor were transiently transfected with pertussis toxin-insensitive $G_{\alpha o}$ or $G_{\alpha i2}$ constructs and then treated with pertussis toxin to ablate all other G_o/G_i coupling. Membranes were prepared and [^{35}S]GTPγS binding studies carried out. In preliminary studies, Western blots were used to optimize the amount of cDNA constructs utilized in the transfection to achieve maximal expression (∼10-fold over levels of the native G protein) of the mutated $G_{\alpha o}$ or $G_{\alpha i}$ proteins. Importantly, this expression was always equivalent between the cells expressing the wild-type or the S232A α_{2A}ARs (data not shown). For wild-type α_{2A}AR, expression of pertussis toxin-insensitive $G_{\alpha o}$ or $G_{\alpha i2}$ under these conditions increased epinephrine-stimulated [^{35}S]GTPγS binding to ∼50% and 30%, respectively, of that observed in the absence of pertussis toxin treatment and G protein transfection. (Even with maximal expression of the modified protein, full rescue to

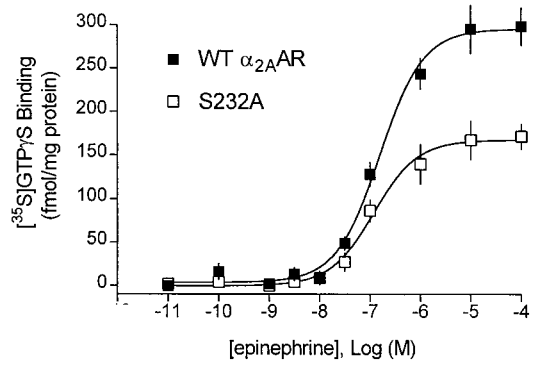

FIGURE 3: S232A has decreased agonist-promoted [^{35}S]GTPγS binding. Membranes of CHO cells expressing matching levels of WT or S232A α_{2A}AR were incubated with [^{35}S]GTPγS and indicated concentrations of epinephrine to induce the exchange of bound GDP for [^{35}S]GTPγS. Maximal levels of binding between S232A receptor and G proteins were ~50% lower than the WT α_{2A}AR ($p < 0.01$, $n = 4$). The EC_{50} values were not different between WT and S232A (153 ± 33 vs 132 ± 20 nM).

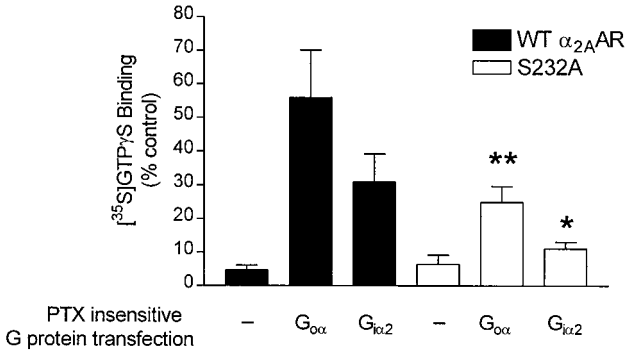

FIGURE 4: S232A is defective in coupling to $G_{\alpha o}$ and $G_{\alpha i2}$. CHO cells stably expressing WT or S232A α_{2A}AR were transiently transfected with pertussis toxin-insensitive $G_{\alpha o}$ or $G_{\alpha i2}$ proteins. Cells were treated with vehicle or 10 μg/mL pertussis toxin at 37 °C and 5% CO_2 for 4 h. [^{35}S]GTPγS binding studies were carried out as described in Materials and Methods. Data are expressed as the percent of the response from receptor-expressing cells that were neither transfected with the G protein constructs nor treated with pertussis toxin (control cells). Cotransfection of pertussis toxin-insensitive $G_{\alpha o}$ increased [^{35}S]GTPγS binding activities to 55.9 ± 14.3% of control in wild type but to only 24.8 ± 4.7% of control in the S232A. Cotransfection of pertussis toxin-insensitive $G_{\alpha i2}$ restored [^{35}S]GTPγS binding activities to 31.0 ± 8.23% in wild type and 11.1 ± 2.0% in S232A (**, $p < 0.01$, and *, $p < 0.05$, compared to wild type under the same conditions, $n = 4$).

wild-type receptor [^{35}S]GTPγS binding in the absence of toxin was not obtained, so the ~50% and ~30% levels were considered the maximal achievable.) With the S232A mutant, expression of either modified $G_{\alpha o}$ or $G_{\alpha i2}$ proteins failed to increase [^{35}S]GTPγS binding to the same extent observed with wild-type α_{2A}AR (Figure 4). These data indicate that S232A has both G_o and G_i coupling defects.

Given the substantial alteration of inositol phosphate signaling which occurred when the potential PKC site (VPPS*RR) at position 232 was altered, we next assessed whether PMA-promoted phosphorylation of the receptor was altered when Ala was substituted at this position. We have previously shown that PMA-promoted phosphorylation of wild-type α_{2A}AR is blocked by PKC inhibitors (*17*). In the current work, cells expressing wild-type and the S232A α_{2A}-AR were incubated with [^{32}P]orthophosphate and exposed to 0.1 μM PMA for 10 min, and receptors were immunoprecipitated using an antibody to the HA tag and resolved by electrophoresis. The results from a typical autoradiograph are shown in Figure 5A, and a summary of four such experiments is shown in Figure 5B. PMA-promoted phosphorylation of the S232A receptor over background was markedly depressed compared to wild type. While the latter showed a 2.1 ± 0.16-fold increase, the mutant displayed 1.3 ± 0.11-fold increase ($p < 0.05$, wild type vs mutant). The marked reduction in PMA-promoted α_{2A}AR phosphorylation by the S232A mutation strongly suggests that S232 is in fact phosphorylated by PKC. Since we have shown that when this residue is altered via mutagenesis that inositol phosphate signaling is selectively attenuated, we hypothesized that modification of the serine residue by PKC phosphorylation would impair α_{2A}AR signaling to inositol phosphate production but have little effect on adenylyl cyclase inhibition. As shown in Figure 6, this indeed turned out to be the case. Brief exposure of wild-type α_{2A}AR cells to PMA resulted in a 67% desensitization of agonist-promoted inositol phosphate stimulation (41 ± 4.3% vs 15 ± 2.7%, $p < 0.01$, Figure 6A). In contrast, agonist-promoted inhibition of adenylyl cyclase trended toward being slightly affected by PMA, but the maximal inhibition was not statistically different between control (57 ± 3.3%) and PMA treatment (49 ± 2.4%, Figure 6B).

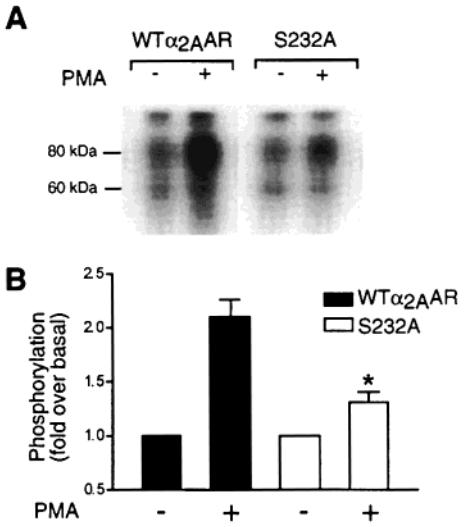

FIGURE 5: Mutation of S232 of the α_{2A}AR alters PKC phosphorylation of the receptor. CHO cells were preincubated with [^{32}P]-orthophosphate and exposed to 0.1 μM PMA for 10 min, and the α_{2A}AR was purified as described in Materials and Methods. (A) Representative autoradigraph. (B) Mean results from four independent experiments (*, $p < 0.05$, $n = 4$).

Since the selective inositol phosphate uncoupling engendered by the mutation (or phosphorylation) of S232 does not appear to be due to differential coupling to G_o or G_i (see Figure 4), we wondered whether the inositol phosphate pathway was more susceptible to these modifications due to a lower signal transduction efficiency compared to inhibition of adenylyl cyclase. To address this, whole cell assays of epinephrine-stimulated inositol phosphate accumulation and epinephrine-promoted inhibition of forskolin stimulated cAMP levels were carried out in parallel. As shown in Figure 7, there is indeed a marked rightward shift of the dose response curve for inositol phosphate accumulation compared to cAMP inhibition (EC_{50} = 11.5 ± 0.7 μM vs 40.1 ± 10.3 nM, respectively). Taken together, the data indicate that

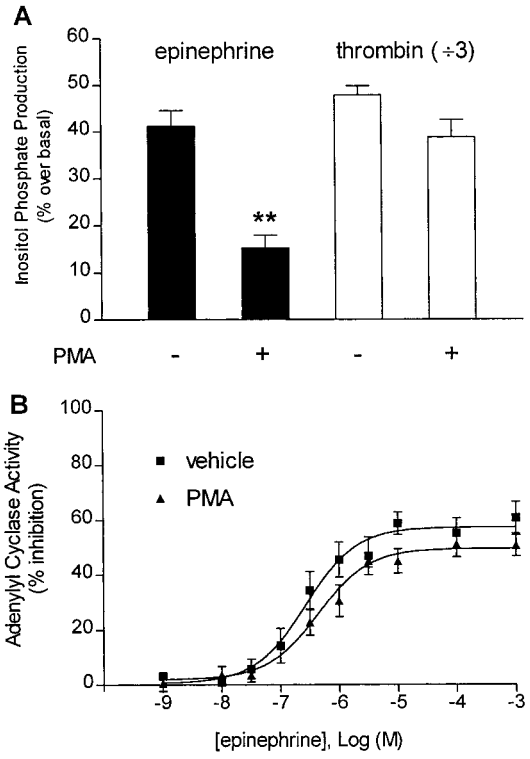

FIGURE 6: Effects of PKC activation on α_{2A}AR coupling to inositol phosphate and adenylyl cyclase. (A) CHO cells stably expressing wild-type α_{2A}AR were preincubated with [^{3}H]inositol and treated with vehicle or 0.1 μM PMA for 2 min and then vehicle (basal) or 10 μM epinephrine for 5 min at 37 °C. Without PMA treatment, there was a 41 ± 3.4% increase of inositol phosphate production by epinephrine; with PMA treatment, the production was only 15.3 ± 2.7% by epinephrine (*, $p < 0.01$), amounting to 67 ± 7% desensitization. Receptor expression was 1462 ± 149 fmol/mg. Results are from 11 experiments. (B) CHO cells stably expressing wild-type α_{2A}AR were treated with vehicle or 0.1 μM PMA for 5 min and adenylyl cyclase activities in membranes measured in the presence of forskolin and epinephrine. The maximal inhibitions were 57.4 ± 3.3% in the control group and 49.6 ± 2.4% with PMA treatment, which were not statistically different. Nor were the EC_{50} values different with PMA exposure (310 ± 71 vs 424 ± 153 nM, $n = 4$). The receptor expression level was 1520 ± 215 fmol/mg. Results are from four experiments.

PKC-mediated phosphorylation at S232 affects α_{2A}AR coupling to G_i/G_o that is manifested as a selective impairment of inositol phosphate stimulation as compared to cAMP inhibition because the former signaling is a low-efficiency process.

Our interpretation of the coupling efficiency of the α_{2A}AR is similar to that of Limbird and colleagues with the D79N α_{2A}AR (*5*, *6*, *21*). This receptor, which has a conserved Asp residue mutated to Asn in the second transmembrane spanning domain, has wild-type coupling to inhibition of adenylyl cyclase but fails to activate K^+ channels. Using several approaches, these investigators have also concluded that this loss of one effector pathway over another with the D79N receptor is due to the low coupling efficiency of native α_{2A}AR to activation of K^+ channels compared to inhibition of adenylyl cyclase. Since the D79 residue is not known to be polymorphic in nature, or to undergo posttranslational modification, it does not represent a physiologically regulated switch residue. Nevertheless, data from the D79N mutant (as well as our S232A mutant) are consistent with the concept that lower efficiency pathways of α_{2A}AR signaling are more dramatically affected compared to higher efficiency pathways during conditions of moderate G protein uncoupling.

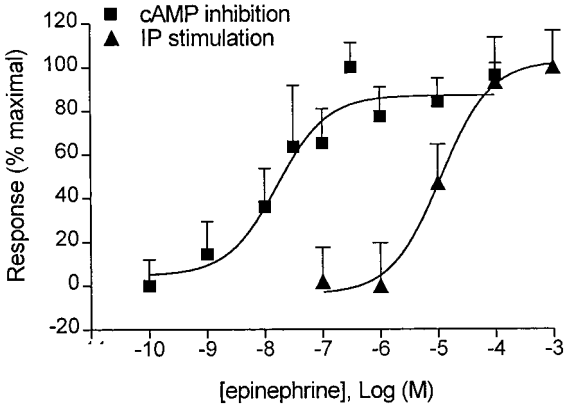

FIGURE 7: Efficiency of α_{2A}AR signaling to inositol phosphate accumulation and cAMP inhibition. Epinephrine-mediated inhibition of forskolin-stimulated whole cell cAMP levels and stimulation of inositol phosphate levels were measured in intact cells. The *y* axis represents the two responses (% inhibition of cAMP or % stimulation of inositol phosphates) which are normalized to 0−100% for comparison. The EC_{50} for whole cell cAMP inhibition was 40.1 ± 10.3 nM vs 11.5 ± 0.7 μM for inositol phosphate stimulation ($n = 4$ experiments).

DISCUSSION

Coupling of a single receptor to multiple effectors has been well documented for a number of G protein coupled receptors. The basis for such diversity of signaling has been found to be due to several mechanisms. In some cases, the receptor couples to several different G proteins, whose α subunits subsequently engage different effectors (*1*, *2*). Multifunctional signaling can also be due to free $\beta\gamma$ interactions with effectors. $\beta\gamma$ subunits activate certain isoforms of adenylyl cyclase (*22*), stimulate MAP kinase activation (*4*), and activate phospholipase C β isoforms (*3*). Agonist-promoted inositol phosphate accumulation and subsequent increases in intracellular calcium via α_{2A}AR are due to G_i/G_o $\beta\gamma$ activation of phospholipase Cβ (*3*). Receptor colocalization with effectors in microdomains within the cell also can dictate which signals are elicited by receptor activation (*23*, *24*). There also is precedent for postreceptor modifications, such as receptor phosphorylation, acting to direct signaling to a given pathway (*25*). The best example may be the β_2AR, which is traditionally considered a G_s coupled receptor. However, when phosphorylated by PKA at a site in the third intracellular loop, the β_2AR also couples to G_i. Subsequent downstream consequences include activation of MAP kinase by G_i $\beta\gamma$ and inhibition of adenylyl cyclase by $G_{\alpha i}$ (*25*, *26*). Indeed, we have recently shown that receptor−G_i coupling is a mechanism by which β_2AR undergoes agonist-promoted functional desensitization of adenylyl cyclase signaling (*26*).

In our current work we found that the Ser at position 232 of the α_{2A}AR, which is within the amino-terminal portion of the third intracellular loop, is critical for coupling to stimulation of inositol phosphate but appears to play a smaller role in coupling to the inhibition of adenylyl cyclase. Since this was considered a potential PKC phosphorylation site, we hypothesized that this residue may act as a physiologically relevant switch, preferentially dampening inositol phosphate

Table 1: Potential PKC Phosphorylation Sites in the Third Intracellular Loops of G_i-Coupled Receptors[a]

Gene Name	Third Intracellular Loop
ADORA1	E V F Y L I R K Q L N K K V Ⓢ A S S G D P Q K Y Y G K E L K I A K Ⓢ
ADRA2A	R I Y Q I A K R R Ⓣ R V P P [Ⓢ] R R . . A A K A **S** R W R G R Q N R E K R F Ⓣ F
ADRA2B	R I Y L I A K R Ⓢ N R R G P R A K . . G G Q W W R R R A Q L Ⓣ R E K R F Ⓣ F
ADRA2C	R I Y R V A K L R Ⓣ R Ⓣ L Ⓢ E K . . R Ⓢ Ⓢ V C R R K V A Q A R E K E R F Ⓣ F
CCR1	K I L L R R P N E K K Ⓢ K A V R
CHRM2	H I Ⓢ R A Ⓢ K Ⓢ R I K K D K . . K M Ⓣ K Q P A K K K P P P Ⓢ R E K K V Ⓣ R Ⓣ
CX3CR1	Q T L F Ⓢ C K N H K K A K A I
EDG1	R I Y Ⓢ L V R Ⓣ R Ⓢ R R L T F R K N I Ⓢ K A Ⓢ R Ⓢ Ⓢ E N V A L L K
EDG6	I F R L V Q A Ⓢ G Q K A P R P A A R R K A R R L L K Ⓣ V
FPRL1	K I H K K G M I K Ⓢ Ⓢ R P L R V
FPRL2	K I H R N H M I K Ⓢ Ⓢ R P L R V
GPR10	V R V Ⓢ V K L R N R V V P G C V T Q S Q A D W D R A R R R R Ⓣ F
GPR8	D L L R R L R A V R L R Ⓢ G A K A
GRM3	K Ⓣ R K C P E N F N E A K
HTR1A	I F R A A R F R I R K Ⓣ V K K V E K . . N A E A K R K M A L A R E R K Ⓣ V K
IL8RB	G F Ⓣ L R Ⓣ L F K A H M G Q K H R A M R
LTB4R	Y S D I G R R L Q A R R F R R Ⓢ R R Ⓣ G R
NPY1R	K I Y I R L K R R N N M M D K M R D N K Y R Ⓢ Ⓢ E Ⓣ K R I N I
NPY2R	R I W Ⓢ K L K Ⓢ H V S P G A A N D H Y H Q R R Q K Ⓣ Ⓣ K
SSTR3	K V R Ⓢ A G R R V W A P Ⓢ C Q R R R R Ⓢ E R R V Ⓣ R

[a] Due to the diversity of loop sizes, each sequence was originated in the left-hand column at the beginning of the predicted amino terminus of the third intracellular loop. The S232 residue is outlined. A (..) indicates a deletion of the midportion of long loops. Open letters are potential PKC phosphorylation sites. The gene names are from the GenBank listing.

signaling. Since both inositol phosphate and cAMP responsiveness are ablated by pertussis toxin, signaling to both pathways is via G_i/G_o. Using transfections of pertussis toxin-insensitive $G_{\alpha o}$ and $G_{\alpha i}$ constructs, we found that the S232A mutant had depressed coupling to both G proteins. In other studies we confirmed in CHO cells that inositol phosphate stimulation could occur via released $\beta\gamma$ from G_i or G_o, while as expected inhibition of adenylyl cyclase was mediated via $G_{\alpha i}$ (data not shown). So while it was attractive to consider that perhaps G_o coupling alone was affected by the S232A mutation, this did not appear to be the case. We found that the inositol phosphate stimulation is of relatively poor coupling efficiency compared to inhibition of cAMP. Indeed, the EC_{50}s for the two processes differ by ~200-fold, so a moderate decrease in G_o/G_i coupling would be expected to have a greater impact on inositol phosphate stimulation, with little or no effect on the more efficient adenylyl cyclase coupling pathway.

To ascertain whether S232 is indeed a PKC phosphorylation site, intact cell phosphorylation studies were carried out with wild-type α_{2A}AR and the S232A mutant. The latter showed attenuated PMA-promoted phosphorylation, indicating that this Ser is indeed a PKC phosphorylation site. Given the results of the aforementioned functional studies, we predicted that PMA-promoted desensitization of α_{2A}AR-mediated inhibition of adenylyl cyclase would be negligible, while desensitization of receptor mediation stimulation of inositol phosphate would be substantial. Such studies confirmed these concepts, with brief PMA exposure resulting in marked inositol phosphate desensitization, while adenylyl cyclase inhibition underwent virtually no desensitization. It should be noted that most studies with G protein coupled receptors that examine the role of kinase-mediated desensitization find that mutation of the putative phosphoacceptor site has no effect on functional coupling (*27*). And, in fact, overall agonist-promoted signaling in live cells over time is often enhanced, due to a loss of the influence of the regulatory kinase (*28*, *29*). In contrast, here we have identified a site of the α_{2A}AR that plays a role in G protein coupling, and that mutation or phosphorylation by PKC acts to diminish such coupling sufficiently to virtually ablate signaling to phospholipase C but not to adenylyl cyclase. Of note, it is recognized that this specific Ser residue may not necessarily represent the critical contact point between receptor and G protein but may well establish a conformation of the loop which provides for productive interactions at several residues. It is also possible, but we feel unlikely, that the disruption of this conformation by mutagenesis actually alters PKC phosphorylation at a site other than Ser232. Finally, it should be noted that we have previously shown that PMA-promoted phosphorylation of α_{2A}AR is sensitive to PKC inhibitors (*17*) and in the current work have shown that removal of a classic PKC consensus sequence depresses PMA-promoted phosphorylation. Nevertheless, PKC may be modulating activities of other kinases, such that coupling or phosphorylation of the α_{2A}AR is affected by additional complex mechanisms other than exclusively by direct receptor phosphorylation by PKC.

This mechanism thus represents one way by which cells sort incoming stimuli at multifunctional receptors to selected effectors so as to attain finely targeted/regulated signaling. Since PKC sites are not uncommon within coupling domains of G protein coupled receptors, this mechanism may also be present in other receptors as well. For example, both of the other two α_2AR subtypes (α_{2B} and α_{2C}) have potential PKC sites in their third intracellular loops in analogous positions

of S232 of the α_{2A}AR (Table 1). Indeed, a random sampling of 27 G_i-coupled receptor sequences reveals that ~75% have PKC consensus sites within their third intracellular loops (Table 1). It is not uncommon, though, for studies of PKC effects on receptor function to be focused on a single signaling pathway, so whether this mechanism of preferential modulation of a specific pathway represents a general paradigm will require additional studies with multiple other receptors.

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