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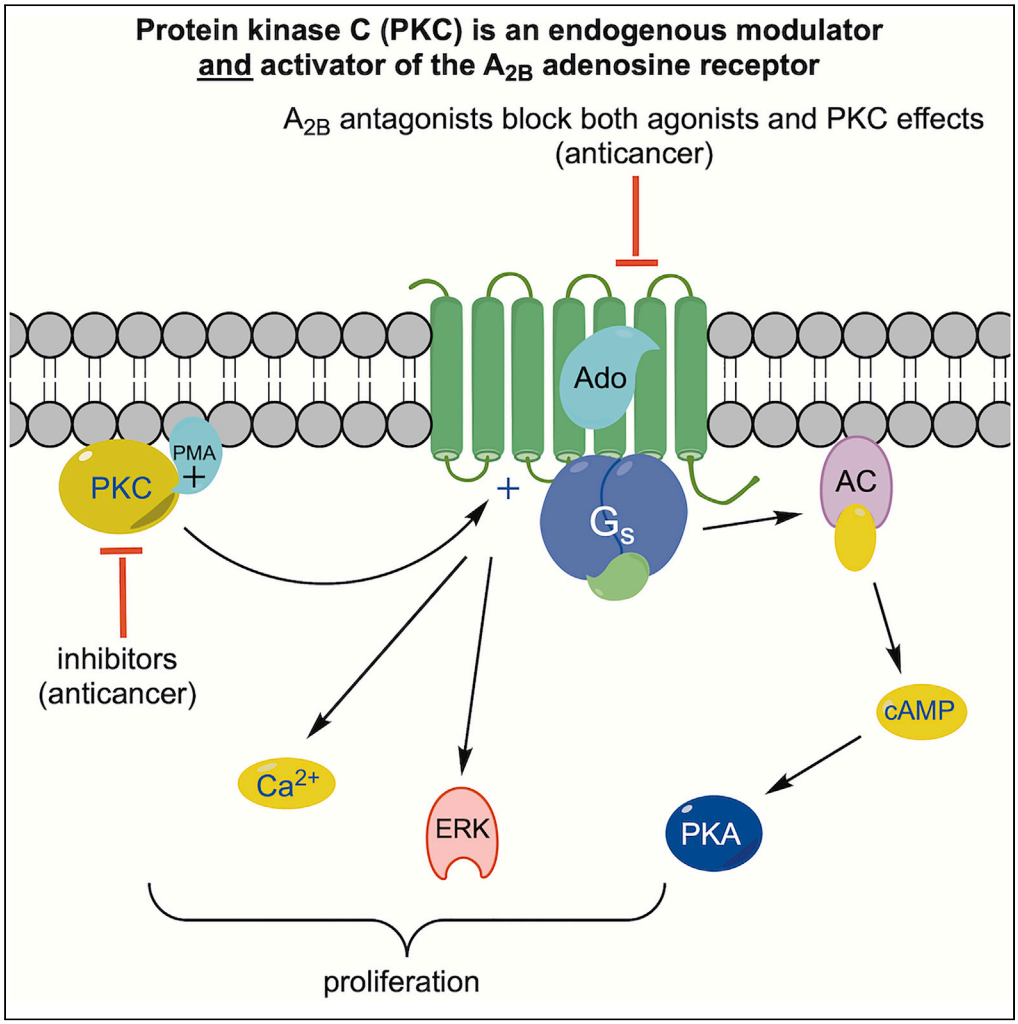

Zhan-Guo Gao, Ian M. Levitan, Asuka Inoue, Qiang Wei, Kenneth A. Jacobson

zg21o@nih.gov (Z.-G.G.)
kennethj@niddk.nih.gov (K.A.J.)

Highlights

PMA activated PKC to enhance A_{2B}AR-mediated, but not β_2-mediated, cAMP accumulation

PKC also activated A_{2B}AR to raise cAMP, especially in A_{2B}-overexpressing cells

Gαi isoforms and PKCγ isoform were involved in A_{2B}AR enhancement and activation

Findings are relevant to common A_{2B}AR and PKC function: cardioprotection and cancer

Gao et al., iScience 26, 107178
July 21, 2023
https://doi.org/10.1016/j.isci.2023.107178

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A_{2B} adenosine receptor activation and modulation by protein kinase C

Zhan-Guo Gao,[1,*] Ian M. Levitan,[1] Asuka Inoue,[2] Qiang Wei,[1] and Kenneth A. Jacobson[1,3,*]

SUMMARY

Protein kinase C (PKC) isoforms regulate many important signaling pathways. Here, we report that PKC activation by phorbol 12-myristate 13-acetate (PMA) enhanced A_{2B} adenosine receptor (AR)-mediated, but not β_2-adrenergic receptor-mediated, cAMP accumulation, in H9C2 cardiomyocyte-like and HEK293 cells. In addition to enhancement, PKC (PMA-treatment) also activated A_{2B}AR with low E_{max} (H9C2 and NIH3T3 cells endogenously expressing A_{2B}AR), or with high E_{max} (A_{2B}AR-overexpressing HEK293 cells) to induce cAMP accumulation. A_{2B}AR activation induced by PKC was inhibited by A_{2B}AR and PKC inhibitors but enhanced by A_{2B}AR overexpression. Gαi isoforms and PKCγ isoform were found to be involved in both enhancement of A_{2B}AR function and A_{2B}AR activation. Thus, we establish PKC as an endogenous modulator and activator of A_{2B}AR, involving $G_{i\alpha}$ and PKCγ. Depending on signaling pathway, PKC could activate and enhance, or alternatively inhibit A_{2B}AR activity. These findings are relevant to common functions of A_{2B}AR and PKC, e.g. cardioprotection and cancer progression/treatment.

INTRODUCTION

Protein kinase C (PKC) is an important modulator of many cell surface receptors, including G protein-coupled receptors (GPCRs) and ligand-gated ion channels (LGICs).[1–4] Various PKC isoforms are heavily involved in tumorigenesis, diabetes, cardiac ischemia, cell growth, and apoptosis, angiogenesis, cell proliferation and differentiation, membrane conductance and transport, potentiation and desensitization of receptor systems, smooth muscle contraction, etc.[1–5] PKC has both positive and negative signaling effects depending on the nature of the target proteins.[1] Although its modulatory mechanisms are diverse, it is generally accepted that PKC typically phosphorylates serine or threonine residues in basic sequences but displays significantly less specificity than protein kinase A (PKA).[6] Most recent work has been focusing on PKC-induced receptor modulation and desensitization.[2,7–10] However, to our knowledge, PKC has not been reported as a GPCR activator, although it has been reported to increase or decrease the function of many GPCR agonists.[11–13] There is no clear connection between PKC-induced receptor desensitization and a direct role of PKC in GPCR activation.

Both PKC and A_{2B}AR are involved in cancer progression[14,15] as well as cardiac preconditioning.[16,17] Epperson et al.[18] detected all four AR subtypes in rat ventricular cardiac fibroblasts, with the A_{2B}AR being the highest expressed subtype. Eckle et al.[17] examined the effects of ischemic preconditioning in mouse hearts with knockout of either A_1, A_{2A}, A_{2B}, or A_3 ARs, and found that ischemic preconditioning reduced infarction in all but the A_{2B}-KO mice. Thus, only the A_{2B}AR was an absolute requirement among the AR subtypes. The treatment with an A_{2B}AR-selective nonnucleoside agonist BAY60-6583 (BAY) protected wild-type, but not A_{2B}-knockout hearts.[17] Philipp et al.[16] showed that the pan-PKC activator (and diacylglycerol (DAG) mimetic) phorbol 12-myristate 13-acetate (PMA)-induced preconditioning protects rabbit hearts via PKC-A_{2B}AR signaling. The pan-PKC inhibitor chelerythrine decreased the protective effect of PMA but not pan-AR agonist NECA. However, the A_{2B}AR antagonist MRS1754 blocked preconditioning induced by either NECA or PMA.[17] Thus, it was suggested that PKC increased A_{2B}AR sensitivity to protect in cardiac ischemia.

Although both the A_{2B}AR and PKC isoforms are important targets for cardioprotection and cancer progression and treatment[14,15,19] and that PKC has been suggested to increase A_{2B}AR sensitivity,[17] the mechanisms of PKC-induced A_{2B}AR modulation have not been well-understood. For example, the role of PKC

[1]Molecular Recognition Section, Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA

[2]Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Miyagi 980-8578, Japan

[3]Lead contact

*Correspondence: zg21o@nih.gov (Z.-G.G.), kennethj@niddk.nih.gov (K.A.J.)

https://doi.org/10.1016/j.isci.2023.107178

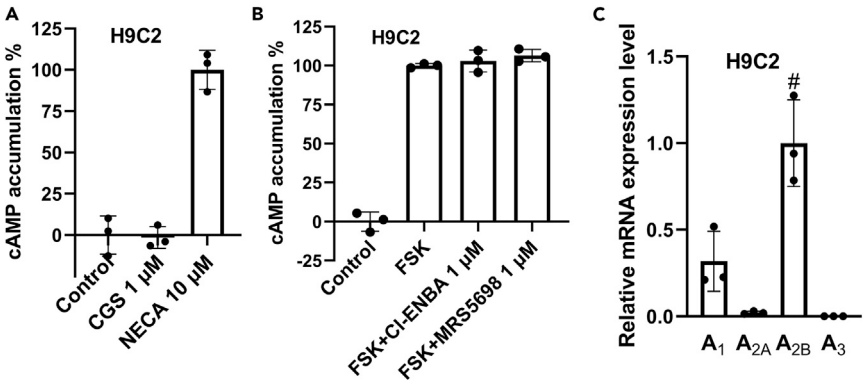

Figure 1. Characterization of four AR subtypes endogenously expressed in H9C2 cells

(A) A_{2A}AR and A_{2B}AR-mediated-cAMP accumulation.

Cells were treated with agonists CGS21680 (CGS) or NECA for 20 min. (B) A_1AR- and A_3AR-mediated inhibition of the adenylyl cyclase activator forskolin (FSK)-stimulated cAMP accumulation. Cells were treated with A_1AR agonist Cl-ENBA and A_3AR agonist MRS5698 for 20 min and then incubated with forskolin (10 μM) for 10 min. cAMP stimulation by forskolin was set as 100% (mean value; error bars represent SEM from three individual experiments). (C) Real-time PCR detection of the expression of AR subtypes. #Significantly different from A_1, A_{2A} and A_3 ($p < 0.05$, one-way ANOVA, Tukey's post-hoc test). Data shown are from three independent experiments.

in the modulation cAMP production in cardiomyocytes induced by the A_{2B}AR has not been examined, although the A_{2B}AR is G_s-coupled. The potential for A_{2B}AR activation by its plausible PKC-catalyzed phosphorylation has not been reported. Thus, in the present study we initially explored the potential modulation of the A_{2B}AR by PKC using the H9C2 rat cardiomyoblast cell line endogenously expressing the A_{2B}AR. The rationale for choosing H9C2 cells was primarily based on the observation that both the PKC activator PMA and A_{2B}AR activation protect rabbit heart, and both can be blocked by an A_{2B}AR antagonist MRS1754.[16] H9C2 cells showed almost identical pharmacologically induced hypertrophic responses to those observed in primary rat cardiomyocytes.[20] H9C2 cells have been previously used for the study of many GPCRs including the A_{2B}AR and β_2 adrenergic receptors.[21,22]

Modulation of three different signaling levels by PMA-induced PKC activation was evaluated in this manuscript: A_{2B}AR, adenylyl cyclase and G_s protein. In addition to H9C2, we also examined the role of PKC in the commonly used HEK293 cell line and in HEK293-A_{2B} cells.[23] We demonstrated that PKC could mimic an A_{2B}AR agonist effect and act as an enhancer, or alternatively an inhibitor depending on the signaling pathway examined. We also explored the mechanisms related to the actions of PKC and found that G_i proteins and PKCγ are critically involved in both the functional enhancement and activation of the A_{2B}AR.

RESULTS

Detection and characterization of AR subtypes in H9C2 cells

The relative expression levels and pharmacological profiles of the four AR subtypes in H9C2 cells have not been systematically compared. Here we first examined the role of A_1, A_{2A}, A_{2B} and A_3 AR subtypes in cAMP accumulation. Figure 1A shows that the nonselective A_{2B}AR agonist NECA but not A_{2A}AR selective agonist CGS21680 (CGS) induced cAMP accumulation, suggesting A_{2A}AR expression is probably very low in H9C2 cells in comparison to that of the A_{2B}AR. Figure 1B shows that neither A_1AR-selective agonist Cl-ENBA (1 μM) nor A_3AR-selective agonist MRS5698 (1 μM) inhibited cAMP accumulation stimulated by the adenylyl cyclase activator forskolin, which may indicate that A_1 and A_3AR expression levels in H9C2 cells are probably not high enough to significantly inhibit forskolin-stimulated cAMP accumulation, despite that the A_1AR has been reported to be involved in preconditioning.[24] To further prove whether the A_1AR is involved in cAMP production or not, a range of concentrations of Cl-ENBA were shown to be unable to inhibit forskolin's effect (Figure S1). In addition to cAMP accumulation, NECA-increased ERK1/2 activity in H9C2 cells was also shown to be A_{2B}AR-mediated and pertussis toxin (PTX)-sensitive, suggesting a G_i-mediated effect (Figure S2).

Real-time PCR data (Figure 1C) showed that the A_{2B}AR expression was the highest among the four ARs in H9C2 cells. A_1AR expression was about 30% of the level of A_{2B}AR expression. A_{2A}AR expression was over

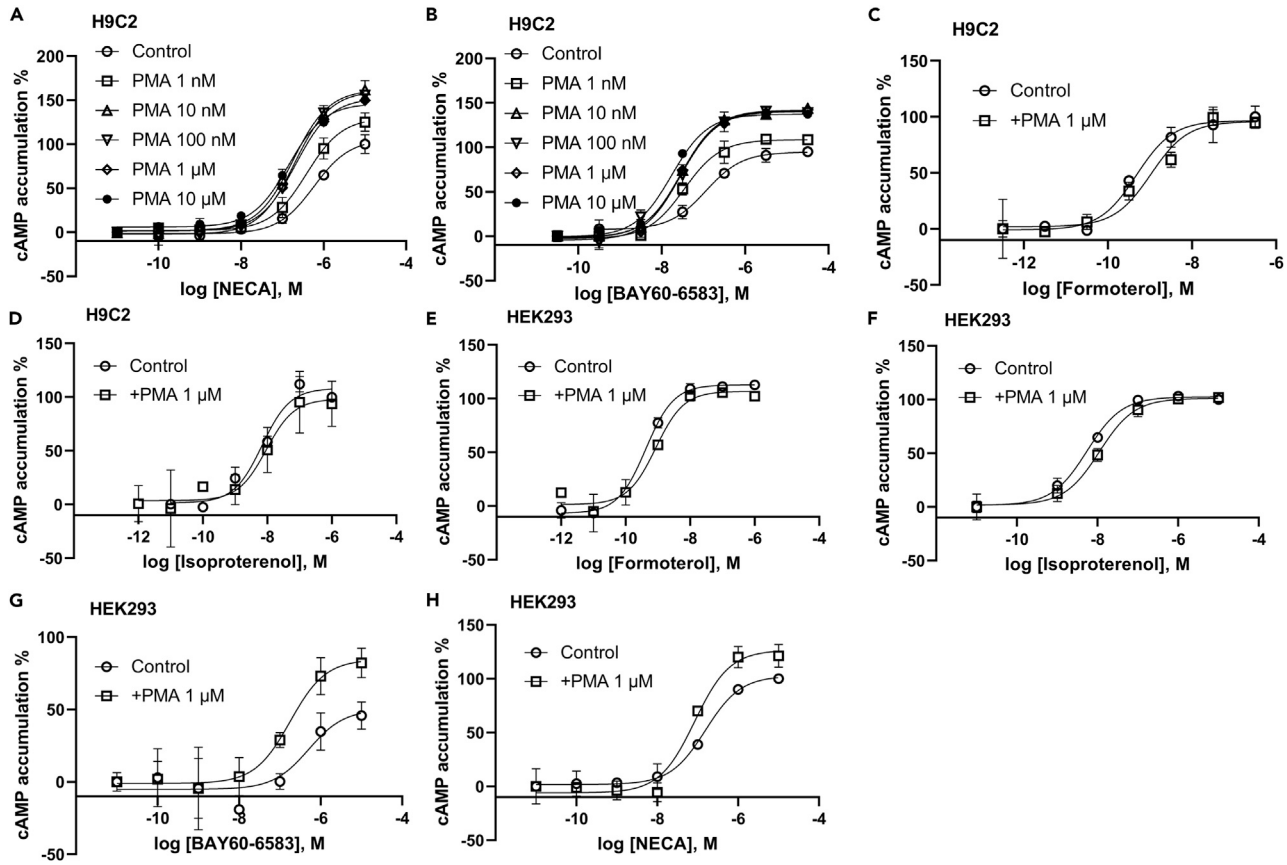

Figure 2. PMA pretreatment enhanced A_{2B}AR-mediated but diminished β_2 receptor-mediated cAMP accumulation

H9C2 or HEK293 cells were grown in 96-well plates in DMEM supplemented with 10% fetal bovine serum, 100 Units/ml penicillin, 100 μg/mL streptomycin, and 2 μmol/mL glutamine. After overnight growth, cells were treated with assay buffer containing rolipram (10 μM), and adenosine deaminase (3 units/ml) for 30 min. Cells were then treated with various concentrations of PMA for 40 min before the addition of agonists and incubated for another 20 min. Plots are combined data from two to four independent experiments performed in duplicate or triplicate. cAMP accumulation induced by 10 μM NECA was expressed as 100%, and basal cAMP levels were expressed as 0%. (A–D) H9C2 cells; (E–H) HEK293 cells. NECA and BAY60-6583 are full and partial agonists of the A_{2B} adenosine receptor, respectively. Isoproterenol and formoterol are nonselective and selective β2-adrenergic agonists, respectively.

10-fold lower than the A_{2B}AR, while A_3AR expression was negligible. Thus, the real-time PCR data are in line with the functional data that the A_{2B}AR is the highest expressed among the four AR subtypes (Figures 1A and 10C). The dominant A_{2B}AR endogenous expression in HEK293 cells has been reported previously.[23]

Pretreatment with PMA enhanced A_{2B}AR agonist-induced cAMP accumulation in H9C2 cells

As mentioned in the Introduction, the PKC activator PMA induces protection in ischemic rabbit hearts, which is blocked by PKC inhibitor chelerythrine and by selective A_{2B}AR antagonist MRS1754, thus suggesting a mechanism related to both PKC and A_{2B}AR signaling.[16] However, the role of PKC in A_{2B}-mediated cAMP accumulation in cardiomyocytes has not been defined yet.

In the present study, we first tested whether A_{2B}AR agonists NECA and BAY induce cAMP accumulation in the rat embryonic cardiomyocyte-like H9C2 cell line. We then examined if PMA pretreatment could affect A_{2B}AR-mediated cAMP accumulation in H9C2 cells. Figures 2A and 2B show that both BAY and NECA induced robust cAMP accumulation, and PMA pretreatment concentration-dependently enhanced agonist-induced cAMP accumulation. At a concentration of 1 nM, PMA produced a substantial enhancement (25%), reaching a maximum effect at about 10 nM. PMA pretreatment did not enhance β_2 agonist (isoproterenol or formoterol)-induced cAMP accumulation in H9C2 cells or HEK293 cells (Figures 2C–2F). On the contrary, PMA treatment decreased the potency of both formoterol and isoproterenol (EC_{50} values of Control vs. "PMA 1 μM" group). In H9C2 cells, the EC_{50}s in the absence and presence of PMA

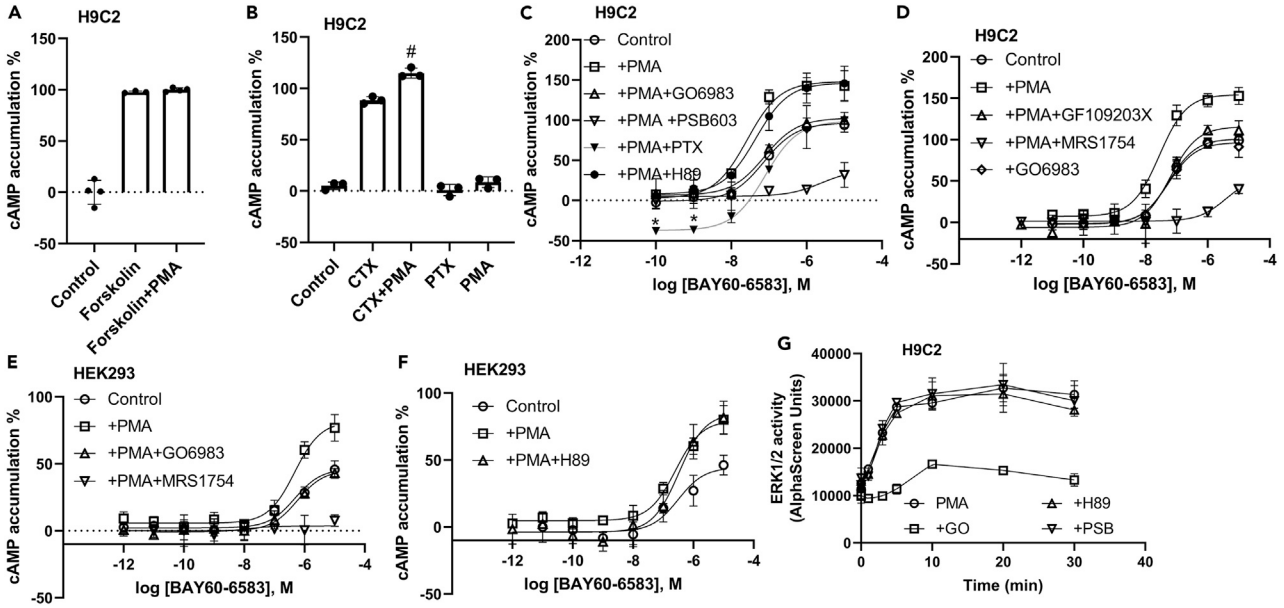

Figure 3. Probing the mechanisms of enhancement of cAMP accumulation by PMA treatment

(A and B) Effect of PMA pretreatment on forskolin (A) and CTX (B)-induced cAMP accumulation in H9C2 cells. Cells were pretreated with PMA (1 μM) for 40 min followed by addition of forskolin (10 μM) and incubate for 20 min or CTX (0.5 μg/mL) for 60 min. PTX (200 ng/mL) was incubated with cells overnight. The bar graph is shown in mean ± S.E.M. of at least three individual experiments performed in triplicate. #$p < 0.05$, significantly different from CTX group (Student's *t* test). CTX, cholera toxin. PTX, pertussis toxin. The mean value of maximal cAMP accumulation induced by 10 μM of forskolin was set as 100%. (C–F) Effects of kinase inhibitors and A_{2B}AR antagonists on enhancement of cAMP accumulation induced by PMA pretreatment. The maximum activation level of by NECA (10 μM) was set as 100%. The background of each individual cell type was set as 0%. Cells were incubated with PTX (200 ng/mL) overnight and with antagonists or inhibitors for 20 min before addition of PMA (1 μM) and incubated for 40 min, followed by addition of the A_{2B} agonist BAY and incubation continued for additional 20 min. PTX, pertussis toxin. The concentration of PSB603, MRS1754, GF109203X or GO6983 used was 1 μM; 10 μM H89 was used. (G) Time-course of PMA (1 μM)-induced increase of ERK1/2 activity. GO6983, H89, or PSB603 (1 μM) was added 20 min before PMA addition. Graphs shown are from three individual experiments performed in triplicate. *$p < 0.05$ (significantly different for the corresponding values in "+PMA" group, One-way ANOVA, Tukey's post hoc test).

were 0.47 and 1.05 nM for formoterol, and 4.92 and 9.63 nM for isoproterenol, respectively. In HEK293 cells, the EC_{50}s in the absence and presence of PMA were 0.42 and 0.86 nM for formoterol, and 5.46 and 11.1 nM for isoproterenol, respectively. Thus, the PKC enhancement was not general for G_s-coupled receptors. The effect of PKC-induced enhancement of the A_{2B}AR function is not unique to H9C2 cells, as similar enhancement was demonstrated in HEK293 cells (Figures 2G–2H). It is interesting to note that the pattern of enhancement of A_{2B}AR agonist E_{max} in naive H9C2 and naive HEK293 cells is, in this respect, similar to the A_3AR allosteric enhancer LUF6000, which also increases agonist E_{max}.[25]

Mechanisms of PKC enhanced cAMP accumulation induced by A_{2B}AR agonist: Involvement of PKC, G_s and G_i

A PKC-induced increase or decrease of receptor function has previously been suggested to be via receptors, G proteins, and adenylyl cyclase.[2,7,11,12,26–28] Here, we first tested if PMA treatment directly affects adenylyl cyclase activity in H9C2 cells. Figure 3A shows that PMA pretreatment did not inhibit or enhance the forskolin-stimulated cAMP accumulation in H9C2 cells, suggesting the action of PKC is probably at the receptor or G protein level, but not at the level of adenylyl cyclase. Figure 3B shows that PMA pretreatment modestly enhanced cholera toxin (CTX)-induced cAMP accumulation in H9C2 cells. The percentages of cAMP accumulation in the absence and presence of PMA pretreatment were 88.6 ± 2.0% and 112 ± 3% (cAMP level induced by 10 μM forskolin was set as 100%), respectively ($p < 0.05$, Student's *t* test), suggesting that enhancement of G_s function could at least be partially involved in the enhancement of cAMP accumulation in H9C2 cells.

Figure 3C shows that the enhancement of A_{2B}AR agonist BAY-induced cAMP accumulation by PMA pretreatment was blocked by a broad-spectrum PKC inhibitor GO6983, confirming a PKC-mediated effect by PMA. The selective A_{2B}AR antagonist PSB603 at a concentration of 1 μM inhibited both BAY-induced

and PMA-enhanced cAMP accumulation. However, the PKA inhibitor H89 did not inhibit this effect in H9C2 cells (Figure 3C). The G_i inactivator PTX eliminated the enhancing effect of PMA pretreatment suggesting that G_i protein is also involved in PKC's enhancing effect on A_{2B}-mediated cAMP accumulation (Figure 3C). Of interest, PTX also lowered the basal cAMP level in H9C2 cells in the presence of PMA (Figure 3C), although PTX alone had no effect on the basal cAMP level in H9C2 cells (Figure 3B). Nevertheless, the results suggest that both G_s and G_i, but not adenylyl cyclase, are involved in PKC-induced enhancement of A_{2B}AR-mediated cAMP accumulation in H9C2 cells. Figure 3D shows that another PKC inhibitor GF109203X also inhibited PKC-enhancement, and another A_{2B}AR selective antagonist MRS1754 inhibited both BAY-induced and PKC-enhanced cAMP accumulation. GO6983 did not affect BAY-induced cAMP accumulation (Figure 3D). The effects of PKC inhibitor GO6983 and A_{2B}AR antagonist MRS1754 in HEK293 cells were similar in H9C2 cells (Figure 3E). As expected, the effect of PMA pretreatment in HEK293 cells was also unaffected by PKA inhibitor H89 (Figure 3F). In HEK293 cells, PKC (PMA treatment) also had a large effect on the A_{2B}AR agonist BAY, a small effect on the G_s activator CTX, but did not affect adenylyl cyclase activator forskolin-stimulated cAMP accumulation, which is similar to that in H9C2 cells (Figure S3). We then examined PMA effects on downstream mediators of the A_{2B}AR.

PMA-increased ERK1/2 activity and effects of PMA pretreatment on A_{2B}-mediated ERK1/2 activity in H9C2 and HEK293 cells

Unlike in cAMP accumulation, the A_{2B}AR antagonists did not affect PMA-increased ERK1/2 activity in either H9C2 cells (Figures 3G, and S4) or HEK293 cells (Figure S5), although A_{2B}AR agonist-increased ERK1/2 activity was inhibited by MRS1754. Of interest, PMA treatment modulated A_{2B}AR-mediated ERK1/2 activity, which was inhibited by A_{2B}AR antagonists. Figure S4A illustrated the transient nature of NECA-induced ERK1/2 activity. Figure 3G shows that PMA pretreatment induced robust (>300% above baseline) and sustained ERK1/2 phosphorylation lasting for over 30 min, which was diminished by the PKC inhibitor GO6983, but not affected by PKA inhibitor H89 or A_{2B}AR antagonist PSB603. PMA pretreatment induced ERK1/2 phosphorylation with an EC_{50} of 46.8 $\pm$ 13.2 nM (Figure S4B), which was weaker than its enhancing effect on A_{2B}AR-mediated cAMP accumulation (about 1 nM). The lack of an effect of PSB603 on PMA-mediated ERK1/2 activity may suggest that PMA pretreatment-mediated ERK1/2 activity is probably not related its enhancing effect on A_{2B}-mediated cAMP accumulation, which was diminished by PSB603.

Effects of PMA-mediated PKC activation on A_{2B}AR-induced intracellular calcium mobilization

As shown in Figures 2 and 3, PMA-pretreatment enhanced A_{2B}-mediated cAMP accumulation in H9C2 cells. We next explored how PMA treatment affects A_{2B}AR's effect on intracellular calcium mobilization in H9C2 cells. However, as shown in Figure 4A, NECA did not induce calcium mobilization. As a control, P2Y receptor agonist UTP induced a robust calcium response which was completely inhibited by pretreatment of $G_{q/11}$ inhibitor YM254890 (YM) or the PKC activator PMA (Figure 4B). The effect of PMA pretreatment to inhibit UTP was completely reversed by pretreatment with GO6983, suggesting a PKC-mediated effect of PMA.

Because A_{2B}AR activation did not lead to robust calcium mobilization in H9C2 cells as demonstrated in the present study or in naive HEK293 cells demonstrated previously,[23] we then examined the potential effect of PMA pretreatment in HEK293-A_{2B} cells, which was shown earlier to produce a larger calcium response on A_{2B}AR activation. Figure 4C shows that NECA-induced intracellular calcium mobilization was inhibited by PMA pretreatment or PSB603. The inhibitory effect of PMA pretreatment was completely blocked by pretreatment with GO6983. Thus, PMA pretreatment evidently had opposite effects on cAMP and Ca^{2+} mobilization. In contrast to NECA, PMA itself did not induce calcium mobilization in HEK293-A_{2B} cells (Figure 4D).

PMA pretreatment enhanced A_{2B}AR agonist binding

Johnson et al.[12] and Plouffe et al.[13] found that the third intracellular loop (IL3) of the β_2-adrenergic and D_1 dopamine receptors, is required for the PMA-induced increase in epinephrine and dopamine function, respectively. The role of potential A_{2B}AR modification by PKC on agonist binding has not been previously reported. Thus, to examine if PMA pretreatment could affect A_{2B}AR radioligand binding, we first prepared membranes from H9C2 cells. Figure 5A shows that agonist radioligand [^{3}H]NECA (25 nM) binding could not be detected, although the specific binding of antagonist [^{3}H]DPCPX (10 nM) could be detected (Figure 5A). However, in membranes from HEK293-A_{2B} cells, the specific binding of both antagonist [^{3}H]DPCPX (Figure 5B) and agonist [^{3}H]NECA (Figure S6) was detectable. Therefore, we treated HEK293-A_{2B} cells with

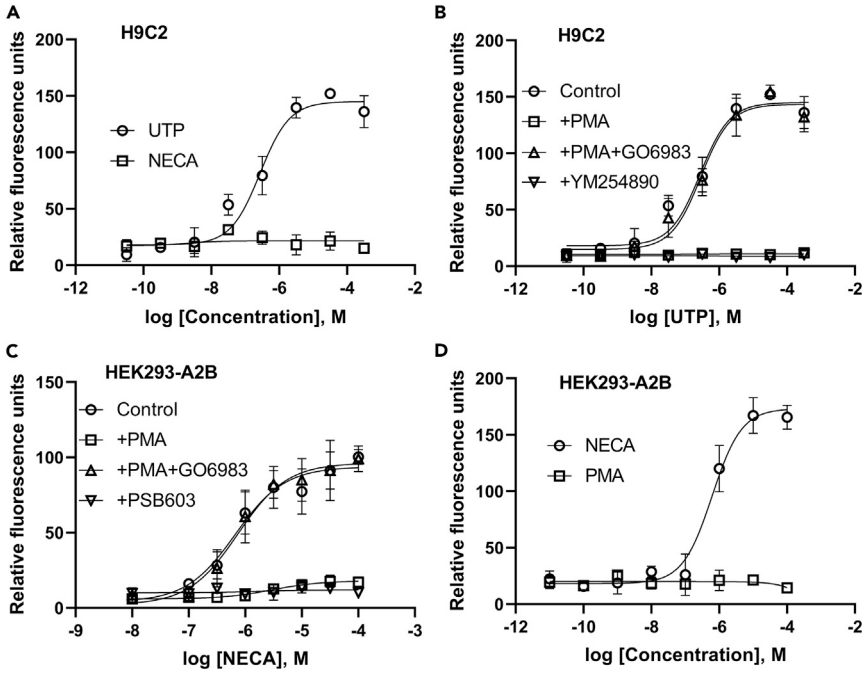

Figure 4. Intracellular calcium mobilization in H9C2 cells and HEK293-A_{2B} cells

(A and B) H9C2. (C,D). HEK293-A_{2B}. (B,C) Cells were incubated with 1 μM PSB603, PMA, or YM254890 for 20 min. For the PMA+GO6983 group, GO6983 (1 μM) was added 20 min followed by addition of PMA (1 μM) for 20 min, and intracellular calcium mobilization was measured after the addition of agonists using FLIPR Tetra as described in Methods section. D. Comparison of NECA- and PKC-induced calcium mobilization. Error bars represent SEM from at least three independent experiments performed in triplicate.

PMA. PMA treatment did not affect antagonist [^{3}H]DPCPX binding (Figure 5B) but significantly increased agonist binding (Figure S6). Therefore, we tested the effect of PKC on [^{3}H]NECA saturation binding (Figure 5C). Figure 5C shows that PMA pretreatment caused a modest, but significant increase of agonist binding affinity, and this effect could be eliminated by the pretreatment with the broad-spectrum PKC inhibitor GO6983 (1 μM). The K_d value of [^{3}H]NECA in the control group was calculated to be 74.9 ± 6.3 nM, which is significantly lower affinity compared to the PMA-treated group 34.2 ± 3.0 nM ($p < 0.05$), but not different from the group with both PMA pretreatment and GO6983 (EC_{50} = 82.8 ± 20.9 nM) ($p > 0.05$; one-way ANOVA, Tukey's post-hoc test). It is of note that increased receptor-G protein coupling is well known to increase agonist affinity.[12,13] PMA alone did not displace specific binding of [^{3}H]NECA (25 nM) to membranes from HEK293-A_{2B} cells (1.6 ± 1.2% vs. MRS1754 (100%) at a 1 μM concentration.

Enhancing effect of PMA pretreatment on A_{2B}AR-mediated cAMP accumulation in HEK293 cells overexpressing the A_{2B}ARs and the identification of PKC (PMA treatment) as an A_{2B}AR activator

Since it has now been demonstrated that PMA treatment enhanced A_{2B}AR-mediated cAMP accumulation in both H9C2 and HEK293 cells (Figure 2), but inhibited A_{2B}-mediated calcium mobilization in HEK293-A_{2B} cells (Figure 4C), we next examined how PMA pretreatment affects cAMP accumulation in HEK293-A_{2B} cells. However, to our surprise, Figure 6 shows that, unlike in naive HEK293 cells (Figure 2), PMA-pretreatment raised the basal cAMP level but not the E_{max} of NECA- or BAY-induced cAMP accumulation in HEK293-A_{2B} cells, which suggests that PKC could actually behave as an A_{2B}AR activator, in addition to its agonist E_{max}-enhancing effect. A similar pattern of enhancement (increase of basal cAMP level) by PMA treatment was also observed in CHO cells overexpressing the A_{2B}AR (Figure S7). This type of enhancement is in a way similar to that of the A_1AR allosteric enhancer MIPS521, which could also be considered a partial agonist.[29] It appears that the partial agonist-like property of PKC is more pronounced when the receptor is highly expressed, consistent with the Operational model of receptor activation.[30] Figure 6C shows the concentration-dependence of cAMP accumulation in HEK293-A_{2B} cells by PMA pretreatment in comparison to the known full A_{2B}AR agonist NECA. The EC_{50}s of NECA and PMA treatment

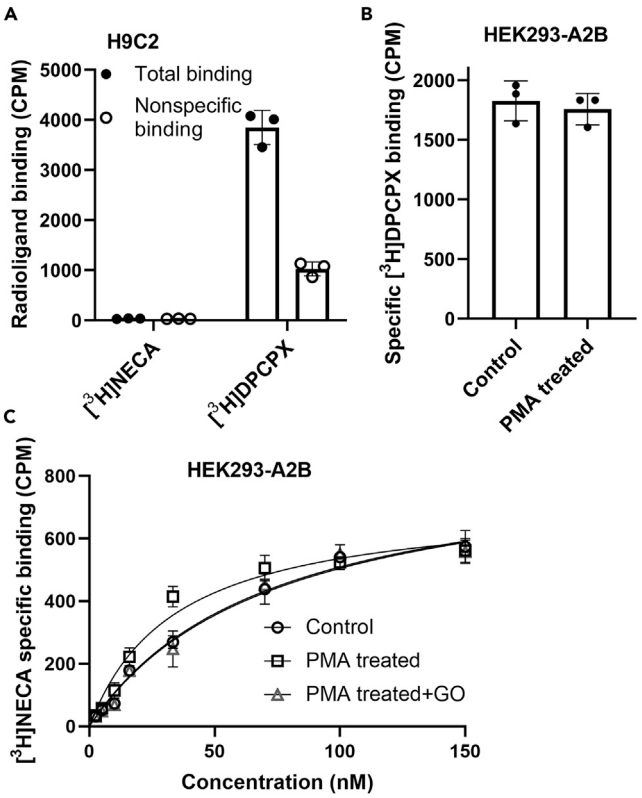

Figure 5. Effects of PKC phosphorylation on A_{2B}AR binding

(A) Binding of A_{2B}AR agonist ([^{3}H]NECA, 25 nM) and antagonist [^{3}H]DPCPX (10 nM) to membrane preparations from H9C2 cells endogenously expressing the rat A_{2B}AR. (B and C) HEK293 cells expressing both the endogenous and recombinant human A_{2B}ARs. Cells were treated with PMA (1 μM) for 40 min before they were collected for membrane preparations. The PKC inhibitor GO (GO6983) was added 20 min before the addition of PMA. Error bars represent SEM from at least three independent experiments.

(to be consistent with the Figure 6A, both were incubated for 60 min with cells) were 4.80 and 56.4 nM, respectively. The activation E_{max} of PMA was calculated to be 84.7% (the E_{max} of NECA was set as 100%). Here, we need to clarify again that PKC (rather than PMA itself) is the A_{2B}AR activator. The PKC inhibitor GO6983 blocked PKC- but not NECA-induced cAMP accumulation (Figure 6C). Figure 6D shows that the A_{2B}AR antagonist MRS1754 concentration-dependently inhibited PKC (100 nM PMA)-induced cAMP accumulation corresponding to an IC_{50} of 513 ± 207 nM, confirming that PKC-induced cAMP accumulation is via the A_{2B}AR. Figure S8 shows that MRS1754 and PSB603 have different patterns in inhibiting BAY- and PKC-induced cAMP accumulation. Schild analysis of MRS1754 antagonism against BAY (Figure S8) yielded a K_B value of 3.3 nM (Figure S9), which is over 100-fold more potent than its IC_{50} value in inhibiting the effect of PKC (Figure 6D), suggesting that MRS1754 inhibits the effects of PKC and BAY via different mechanisms.

In naive H9C2 cells endogenously expressing the A_{2B}AR, PMA-induced cAMP accumulation with lower E_{max}, about 20% of NECA, the effect of which was inhibited by A_{2B}AR antagonists PSB603 and MRS1754 (Figure S10). In naive HEK293 cells (also endogenously expressing the A_{2B}AR[23]), the maximal stimulation by PMA treatment was less than 10% of the E_{max} of NECA (Figure S10D), which is presumably because of a lower A_{2B}AR expression level.

Because many cancer cells endogenously express the A_{2B}AR at high levels,[31] we further tested the potential activation by PKC (PMA treatment) of the A_{2B}AR at two other previously characterized cell lines human PC-3 cells[32] and mouse NIH3T3 cells.[33] Figures 7A and 7B show that PMA treatment induced cAMP accumulation in NIH3T3 cells (34.9 ± 7.7% versus NECA = 100%), but did not in PC-3 cells. Because the cell lines used are of different species, they are not strictly comparable in terms of the A_{2B}AR expression levels.

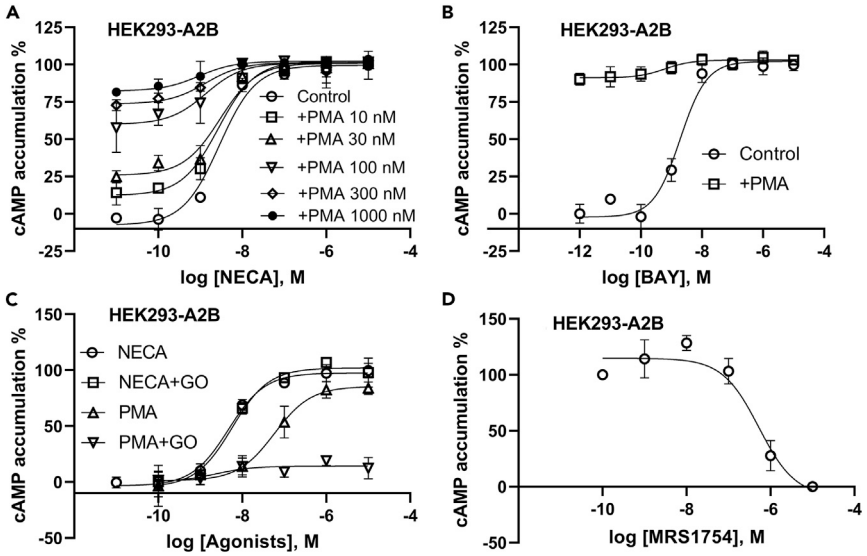

Figure 6. Effects of PMA pretreatment on enhancement of cAMP accumulation in HEK293 cells overexpressing the A_{2B}ARs and the identification of PKC as A_{2B}AR activator

(A and B) Cells were pretreated with PMA (1 μM or as indicated) for 40 min before the addition of agonists and incubated for another 20 min. The maximum activation level of cAMP by 10 μM NECA was set as 100%, while the basal cAMP level was set as 0%. (A) Enhancement of NECA-induced cAMP accumulation by increasing concentrations of PMA present in the preincubation. (B) Enhancement of BAY-induced cAMP accumulation in HEK293-A_{2B} cells by PMA (1 μM) treatment. (C) cAMP accumulation induced by treatment with various concentrations of PMA and NECA. GO6983 (1 μM) was added 20 min before NECA and PMA were added and incubated for a total of 60 min.

(D) Antagonism of PKC (treated with 100 nM of PMA)-induced cAMP accumulation by different concentrations of the A_{2B}AR antagonist MRS1754. Error bars represent SEM from three independent experiments performed in duplicate or triplicate.

However, in comparison to the E_{max} of partial agonist BAY, it appears that BAY has a higher E_{max} at NIH3T3 than at PC-3 cells, which may indicate that A_{2B}AR is probably expressed in NIH3T3 cells at a higher level that in PC-3 cells. We cannot exclude the possibility of cell-type dependent effect of PKC-induced A_{2B}AR activation.

To further examine if the A_{2B}AR expression level affects the capability of PKC to activate the A_{2B}AR, the recombinant rat and human A_{2B}ARs were expressed in H9C2 and HEK293 cells, respectively (0.3 μg/well of 6-well plate). Figure 7C shows that rat A_{2B}AR expression in H9C2 cells elevated the maximal cAMP level by PKC (PMA treatment) from 20.0 ± 0.36% to 65.4 ± 8.0% (cAMP level induced by 10 μM NECA was set as 100%). Figure 7D shows that recombinant human A_{2B}AR expression elevated the maximal agonist effect in HEK293 cells from 7.36 ± 1.08% to 35.5 ± 5.0%. Thus, PKC-induced cAMP accumulation can be enhanced by A_{2B}AR overexpression and can be inhibited by A_{2B}AR antagonists as well, both of which support a role of the A_{2B}AR. To further prove a specific role of the A_{2B}AR, the cAMP effect of PMA was shown to be weaker at the A_{2A}AR, a close A_{2B}AR homolog, overexpressed in HEK293 cells (Figures 6C and S11).

Because a G_q-couped receptor is known to activate PKC, we next examined whether the activation of the endogenous $P2Y_1$ receptor by selective agonist MRS2365 can modulate the A_{2B}AR function. However, Figure S12 shows that pretreatment with 1 μM MRS2365 did not show any effect on A_{2B}AR-mdiated cAMP accumulation, suggesting a different mechanism from direct PKC activation.

A_{2B}AR enhancement by PMA pretreatment in G_i-null HEK293 cells

Because a role of G_i protein in the enhancement of cAMP accumulation induced by PMA pretreatment in H9C2 cells has been suggested (Figure 3C), we further explored its role using the recently available G protein-null HEK293 cells.[23,34] Figures 8A and 8B show that both NECA and BAY concentration-dependently induced cAMP accumulation in HEK293 cells or in G_q- and G_i-null, but not G_s-null HEK293 cells. PMA pretreatment only induced a very small extent of cAMP accumulation in naive HEK293 cells, and we were not

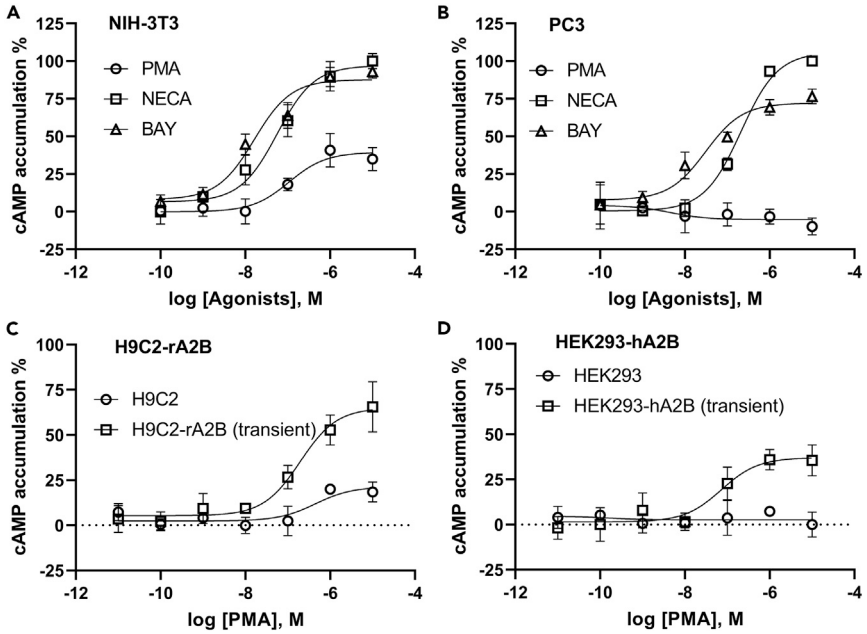

Figure 7. A_{2B}AR expression level contributes to PKC-induced A_{2B}AR activation

(A and B) NIH3T3 cells and PC-3 cells endogenously expressing the A_{2B}ARs.

(C and D) Expression of the recombinant rat A_{2B}ARs and human A_{2B}ARs in H9C2 cells and HEK293 cells, respectively. After overnight growth in 96-well plates, cells were treated with assay buffer containing rolipram (10 μM), and adenosine deaminase (3 units/ml) for 30 min. Cells were then treated with PMA or agonists for 20 min. The mean value of A_{2B}AR agonist NECA (10 μM)-induced cAMP accumulation from three individual experiments was set as 100%. Error bars represent SEM from three independent experiments. BAY, BAY60-6583. (C and D) Cells were transfected using Lipofectamine 2000 as instructed by the manual.

able to discern a difference of PMA-induced cAMP accumulation between wild-type and G_i-null HEK293 cells (Figure 8C). Of interest, although PMA pretreatment was still able to enhance the function of BAY in G_i-null HEK293 cells (Figure 8D), it was diminished (95% in wild-type vs. 37% in G_i-null HEK293 cells). Thus, a role of G_i protein in PKC-induced enhancement is also suggested in HEK293 cells. However, cells with higher A_{2B}AR expression are needed to clearly observe the role of G_i in A_{2B}AR activation induced by PMA-pretreatment. In addition to PMA, pretreatment with another structurally distinct PKC activator TPPB, a 3-oxo-1,4-benzodiazocine derivative, also enhanced the function of BAY (Figure S13).

PKC-induced enhancement and activation at G_i-, G_s- and G_q-null HEK293 cells expressing the recombinant human A_{2B}AR

The basal A_{2B}AR activity enhancement by PMA pretreatment was demonstrated in HEK293 and G_q-null HEK293-A_{2B}, but not in G_i-null HEK293-A_{2B} cells (Figure S14). To study the roles of G_i and G_q in PKC-induced activation we used an intermediate concentration of A_{2B}AR plasmids, i.e. 0.3 μg/well for transfection. Figure 9 shows cAMP accumulation in A_{2B}AR-transfected HEK293 cells induced by treatment with two PKC activators, PMA (Figure 9A) and TPPB (Figure 9B), and two A_{2B}AR agonists, BAY (Figure 9C) and NECA (Figure 9D). Figure 9A shows that G_q-knockout did not substantially affect cAMP accumulation induced by PMA-treatment, but activation was diminished by G_i-knockout. The E_{max} of PMA pretreatment in G_i-null HEK293-A_{2B} cells was 19.3 ± 3.1% in comparison to the E_{max} of PMA pretreatment in HEK293-A_{2B} cells of 49.3 ± 4.6%, which is significantly different ($p < 0.05$, Student's *t* test). Figure 9B shows that cAMP accumulation induced by another PKC activator, TPPB, was also diminished by G_i knockout. However, only G_s, but not G_i or G_q knockout affected cAMP accumulation induced by the A_{2B}AR agonists BAY and NECA (Figures 9C and 9D).

Characterization of PKC isoforms involved in enhancing A_{2B}AR-mediated cAMP accumulation in naive HEK293 cells and A_{2B}AR activation in HEK293-A_{2B} cells

As shown in Figure 3, both (at 1 μM) non-selective PKC inhibitor GO6983 and classical isoform (PKCα, PKCβI, PKCβII, and PKCγ) inhibitor GF109203X (Figures 3C and 3D) inhibited PKC-enhanced cAMP

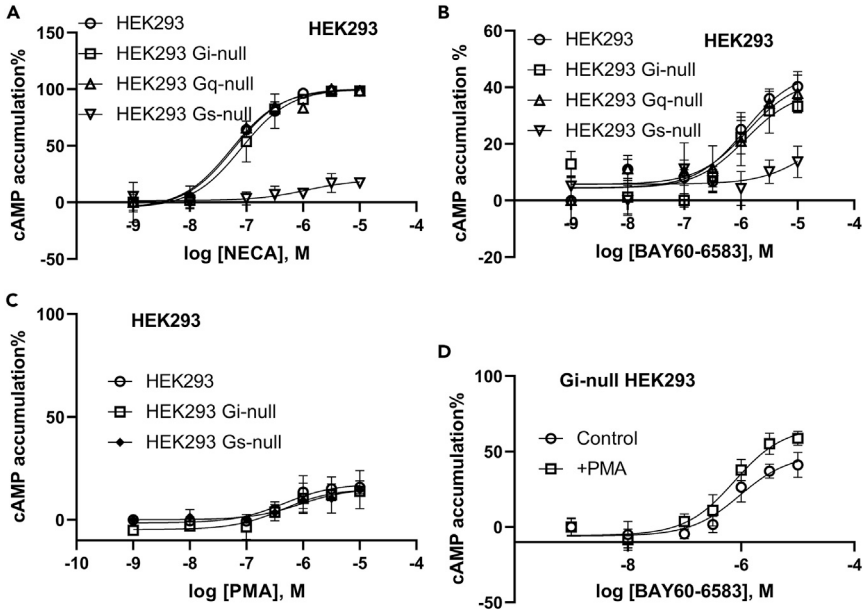

Figure 8. Enhancement of A_{2B}AR function by PKC activation in G_i-null HEK293 cells

(A,B and C) Activation by NECA, BAY, and PMA, respectively; the incubation time for NECA and BAY and PMA is 20 min. (D) Enhancement of BAY-induced cAMP accumulation by pretreatment with PMA (1 μM) in G_i-null HEK293 cells. Data shown are mean ± SEM, n = 3–5. After overnight growth in 96-well plates, cells were treated with assay buffer containing rolipram (10 μM), and adenosine deaminase (3 units/ml) for 30 min. (D) Cells were then treated with PMA 40 min before addition of BAY and incubate for another 20 min. The mean value of cAMP accumulation induced by the adenylyl cyclase activator forskolin (10 μM) from three individual experiments was set as 100%. Error bars represent SEM from three independent experiments. BAY, BAY60-6583.

accumulation induced by BAY in H9C2 cells. Here, Figure 10A shows that in naive HEK293 cells GO6983, RO320432 (non-selective) and GF109203X (all at 1 μM) also blocked PKC-induced enhancement. The substantial inhibition (74%) by GF109203X of PKC-induced enhancement indicates that one or more classical PKC isoforms plays a major role. Nevertheless, one or more undefined novel or atypical PKC isoforms may also play a minor role, based on the 26% remaining enhancement in HEK293 cells in the presence of 1 μM GF109203X. VTX-27 (IC_{50} = 0.08 and 16 nM for PKCθ and PKCδ, respectively) and the PKCζ inhibitor ZIP (10 μM) also did not significantly inhibit. Thus, the involvement of PKCδ, PKCζ and PKCθ is unlikely. Therefore, one of more classical PKC isoforms evidently play a major role in the enhancement, although another undefined non-classical isoform may also play a minor role based on the less-than-complete inhibition by 1 μM GF109203X. In contrast to the PKC inhibitors, the A_{2A}/A_{2B} antagonist AB928 (1 μM), which is currently in clinical trials for several types of cancers[15] (clinicaltrials.gov, accessed Dec. 13, 2022), inhibited both BAY-induced cAMP accumulation and PMA-induced enhancement.

The possible involvement of isoforms was further narrowed using additional inhibitors. Figure 10B shows that a lower concentration of GO6976 (30 nM; IC_{50} for PKCα and PKCβI are 2.3 nM and 6.2 nM, respectively) that is sufficient to block both PKCα and PKCβI did not significantly inhibit PMA-induced enhancement, thus suggesting that either or both PKCβII and PKCγ, but not PKCα or PKCβI played a major role. It is noted that at a higher concentration, GO6976 (1 μM) also produced a modest but significant inhibition (Figure 10B). It is not clear which isoform is involved in this minor inhibition. LY333531 (IC_{50} = 5 nM at both PKCβ1 and PKCβ2) and enzastaurin (IC_{50} = 6 nM at PKCβ) did not inhibit PMA-induced enhancement at a 30 nM concentration (Figure 10B), which may suggest that neither PKCβI nor PKCβII isoform plays a significant role. Therefore, PKCγ is the only possibility to be a major player, although this still needs to be confirmed with a PKCγ specific inhibitor or other approaches such as PKCγ siRNA. Nevertheless, to further prove the role of PKCγ, a higher concentration (1 μM) of enzastaurin (IC_{50} = 83 nM at PKCγ) and LY333531 (IC_{50} = 300 nM at PKCγ) was shown to produce a significant inhibition, albeit incomplete (Figure 10B). Thus, the results suggest that PKCγ plays a main role in PKC-induced enhancement in naive HEK293 cells,

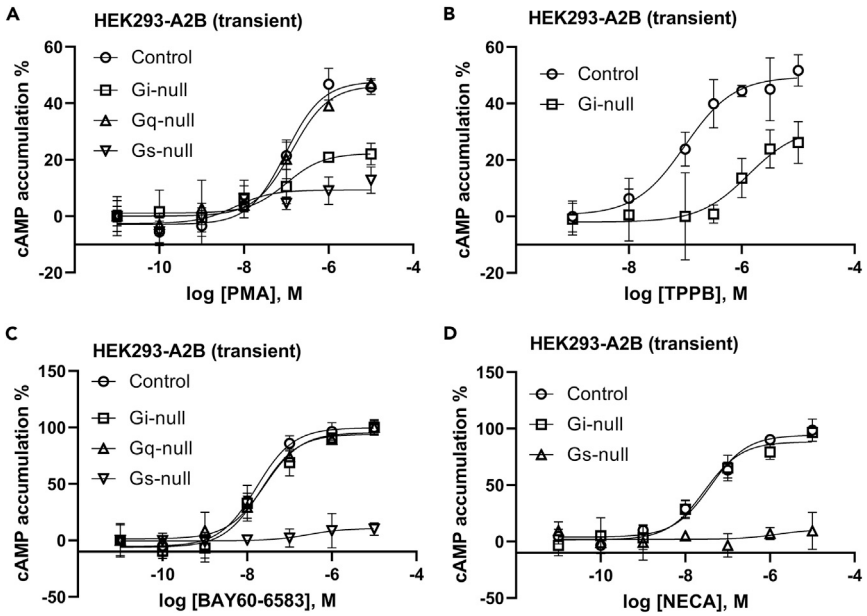

Figure 9. Activation of the A_{2B}ARs expressed in G_i-, G_s- and G_q-null HEK293 cells

(A) PMA. (B) TPPB. (C) BAY (BAY60-6583). (D) NECA. Cells were treated with agonists for 20 min to stimulate cAMP production. The maximum activation level of at the HEK293-A_{2B} by the adenylyl cyclase activator forskolin (10 μM) was set as 100%. The background of each individual cell type was set as 0%. Data shown are from three individual experiments performed in triplicate. Error bars represent SEM from three independent experiments. Cells were transfected with A_{2B}AR plasmids (0.3 μg/well of using 6-well plate) using Lipofectamine 2000 as instructed by the manual and described in the Methods section.

although another undefined non-classical isoform may also play a minor role based on the less-than-complete inhibition by 1 μM GF109203X (Figure 10A).

In addition to PKC-induced enhancement in naive HEK293 cells, we also probed the possible PKC isoforms involved in PKC-induced A_{2B}AR activation in HEK293-A_{2B} cells (A_{2B}AR overexpression). Figure 10C shows that both GO6983 and GF109203X significantly inhibited PKC-induced A_{2B}AR activation, which suggests that classical PKC isoforms are involved. The lack of inhibition by 30 nM GO6976 (inhibitor of PKCα (2.3 nM) and PKCβ1 (6.2 nM)) and by 30 nM of LY333531, and significant inhibition by 1 μM LY333531 (PKCγ IC_{50} = 0.3 μM) may suggest that PKCγ is involved in the A_{2B}AR activation by PKC.

DISCUSSION

In the present study, we demonstrated that PKC activation can lead to A_{2B}AR activation (Figures 6 and 7) and enhancement (Figure 2) or inhibition (Figure 4) of A_{2B}AR function as well. It is interesting that G_i proteins are involved in both the A_{2B}AR activation and enhancement by PKC, although the A_{2B}AR is generally G_s-coupled. The activation is more readily observed in cells with high A_{2B}AR expression than in cells with low A_{2B}AR-expression (Figures 6 and 7), which may diminish the clinical importance of this finding. However, it should be noted that this phenomenon has not been reported, and this kind of activation of a GPCR has not been elucidated mechanistically, which could be an important future direction. Of particular pathophysiological relevance, the specific interaction of PKC with the A_{2B}AR has already been demonstrated in rabbit cardioprotection.[16]

Concerning the antagonism of BAY- and PKC-mediated signaling events, it is noted that both the patterns and potencies of A_{2B}AR antagonists were different. In cAMP accumulation, A_{2B}AR antagonists (PSB603 and MRS1754) were surmountable and insurmountable against BAY- and PKC-mediated cAMP accumulation, respectively. The results that PSB603 and MRS1754 decrease the basal receptor activity may suggest that they are inverse agonists in nature. A_{2B}AR antagonists were able to inhibit PKC-mediated cAMP accumulation but not ERK1/2 activity, suggesting that PKC-mediated cAMP accumulation and ERK1/2 activity rely

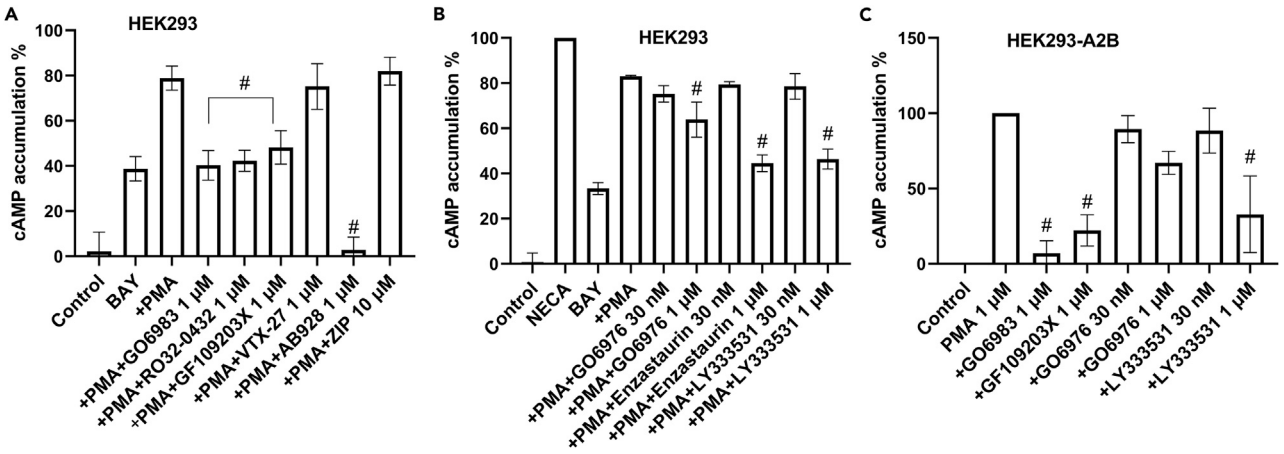

Figure 10. Effects of inhibitors of various PKC isoforms on enhancement of A_{2B}AR-mediated cAMP accumulation in naive HEK293 cells and A_{2B}AR activation by PMA pretreatment in HEK293 A_{2B}AR cells

(Panel A and B): The order of addition of compounds: first, cells were incubated with inhibitors (Panel A: all 1 μM, except ZIP 10 μM; Panel B: GO6976, enzastaurin and LY333531, each at 1 μM and 30 nM) for 20 min, followed by PMA (1 μM) 40 min, and BAY (10 μM) was added last, and incubation was continued for 20 min. In B, the concentration of full agonist NECA for comparison was 10 μM. (In Panel C): inhibitors were added 20 min before PMA and incubated for another 20 min. The maximal stimulation by 1 μM PMA was set as 100%. Results shown are from at least three independent experiments performed in triplicate. $^{\#}p < 0.01$ (compared with "PMA" or "+PMA" groups; one-way ANOVA with Tukey's post hoc test).

on different mechanisms. PKC-mediated inhibition of A_{2B}AR-induced calcium mobilization is consistent with its role in A_{2B}AR desensitization.

Considering that both PKC and the A_{2B}AR are distributed throughout the body and are involved in many common functions, such as cancer and cardioprotection, it is important to understand the mechanisms of interactions between these two important proteins. For some indications, boosting the A_{2B}AR signal would be beneficial,[16,17,19] and others would need A_{2B}AR suppression.[15] A_{2B}AR antagonists, including AB928, are in clinical trials for cancer treatment.[15] The data that AB928 can block the cAMP accumulation induced by both the A_{2B}AR and PKC may support its potential future use in cancer immunotherapy.

PKC isoenzymes belong to a family of serine/threonine kinases.[4,9] The modulatory role of PKC in phosphorylation of receptors, G proteins, and adenylyl cyclases has been suggested.[35–37] However, none of those earlier studies indicated that PKC could activate a GPCR by phosphorylation, although phosphorylation is required for the allosteric activation of guanylyl cyclase A.[38] Katada et al.[39] stated that "a subunit of the inhibitory guanine-nucleotide-binding regulatory component of adenylate cyclase may be a physiological substrate for protein kinase C." Jakobs et al.[40] also wrote that "protein kinase C activated by the phorbol ester interferes with the platelet adenylate cyclase system, leading to a specific alteration of the G_i-protein-mediated signal transduction to the adenylate cyclase". In human neuroblastoma SK-N-MC cells, PMA pretreatment increased isoproterenol-stimulated cAMP levels, and it also enhanced cAMP accumulation by dopamine, CTX, and forskolin. The authors suggested that PKCα is involved in the direct potentiation of adenylyl cyclase activity, and G_i is unlikely to be involved.[41]

The present study showed that G_i proteins are involved in the A_{2B}AR-mediated enhancement of cAMP accumulation in both H9C2 and HEK293 cells. Furthermore, G_i is also involved in PKC-induced A_{2B}AR activation in HEK293 cells overexpressing the A_{2B}AR as demonstrated using G_i-null HEK293 cells. It will be important to pinpoint the potential phosphorylation site(s) on the A_{2B}AR and to characterize the isoforms of G_i proteins and PKCs involved. Also, considering $G_{\alpha i}$ is required for functional β-arrestins to participate in certain signaling events,[34] the involvement of β-arrestins in A_{2B}AR modulation by PKC also needs to be examined. Few studies have addressed the specific G_i protein isoforms involved in PKC modulation. Nevertheless, PKC-induced attenuation of PGE2-induced cAMP accumulation in Jurkat cells has been suggested via G_{i2} and G_{i3}.[26] The α-subunit of $G_{i2\alpha}$ in the membrane fractions from UMR-106 osteosarcoma cells was proposed as a PKC phosphorylation substrate.[27] It remains to be examined which G_i isoform(s) are involved in the A_{2B}AR modulation by PKC.

The role of PKC in β-adrenergic receptor phosphorylation has been studied.[2,12,28] For example, PKC was reported to partially reduce isoproterenol-induced cAMP accumulation in HEK293 cells, with a larger effect on the β_1 than the β_2 receptor.[28] However, PMA pretreatment increased isoproterenol-stimulated cAMP levels in human SK-N-MC cells. Thus, the effects of PKC can be different depending on cell type. Furthermore, both PKA and PKC facilitate β_2 receptor phosphorylation and signaling.[42] PKA, PKC and GRK induced β_2 receptor desensitization via different mechanisms.[2] PKC activation induced by PMA pretreatment reduced β_2-dependent increase in Na-K ATPase activity and sodium transport.[35] PMA acting at PKC is reported to have opposite effects on isoproterenol-induced cAMP accumulation in astrocytes and microglia.[43] The inhibitory effect of PMA pretreatment on isoproterenol-induced cAMP accumulation in rat prostatic epithelium could be prevented by incubation of cells with PTX.[36,37] Teitelbaum showed that PMA-induced inhibition of arginine vasopressin-stimulated cAMP accumulation in cultured rat inner medullary collecting tubes was eliminated by PTX pretreatment.[37] Koch et al.[27] showed that PKC activation by PMA in UMR-106 cells had no effect on basal cAMP accumulation but enhanced parathyroid hormone (PTH)-stimulated cAMP accumulation. PTX-treatment also enhanced PTH-mediated cAMP production. By studying PKC-induced inhibition of catecholamine-stimulated cyclase, Johnson et al.[12] suggested that potential receptor phosphorylation and a possible direct effect on G_i rather than on adenylyl cyclases might explain the PMA effect. The findings from the present study that PKC-enhanced agonist [^{3}H]NECA binding and cAMP accumulation is impaired in G_i-null cells are in line with Johnson et al.[12]

Although the A_{2B}AR is a G_s-coupled receptor, its G_i coupling has been demonstrated in various cell types.[23,38] In addition to interactions with G_i, PKC, and TNF-α, the A_{2B}AR interacts with other proteins such as actinin-1 and netrin-1.[44,45] It is not entirely clear whether these molecules behave similarly to PKC or not. The role of TNF-α in modulating the function of endogenously expressed A_{2B}AR in astrocytes suggested that chronic PMA treatment (>24 h) markedly reduced agonist-dependent receptor phosphorylation on threonine residues and attenuated agonist-mediated A_{2B}AR desensitization.[46] In addition to the A_{2B}AR activation and regulation by PKC from the present study, several other GPCRs have recently been demonstrated to use large proteins as agonists or modulators. For example, a transmembrane protein CD69 has been shown to act as a sphingosine-1-phosphate receptor agonist.[47] Receptor activity-modifying proteins were shown to behave as negative allosteric modulators of the glucagon receptor.[48] Sadler et al.[49] suggested that IL3 could autoregulate β_3 adrenergic receptor activity and act as an allosteric site for the receptor. It remains to be examined if IL3 phosphorylation by PKC is the mechanism for A_{2B}AR activation or not.

Concerning PKC isoforms in signaling, PKCζ has been reported as a switch of signal transduction of TNF-α in U937 cells.[50] The PKC isoforms involved in the modulation of receptor function, especially cAMP accumulation, have not been extensively explored. PKCα but not PKCζ has been implicated in both enhancement of cAMP levels and desensitization in SK-N-MC cells.[41] Using constitutively activated PKC expression constructs transfected into HEK293 cells also expressing the β_2 and β_1 receptors, Guimond et al.[28] demonstrated that different PKCs decreased the maximal isoproterenol-induced cAMP accumulation-mediated by β_2 receptors with a rank order of PKCα ≥ PKCζ>PKCε>PKCβII. The modulation of β_1 by PKC isoforms had a different rank order: PKCβII>PKCα>PKCε>PKCζ. Thus, it seems PKC-mediated effects could be positive or negative, PKC isoform-dependent, receptor- and cell-dependent. Nevertheless, in the present study, we were able to observe PKC (PMA treatment)-induced cAMP accumulation in H9C2 and NIH3T3 cells via endogenously expressed A_{2B}ARs in addition to HEK293 cells overexpressing the recombinant A_{2B}ARs (Figures 6 and 7). It remains to be seen how PKC modulates A_{2B}ARs in other cell types, especially in many other types of cancer cells that endogenously express the receptor at high levels.[31]

In summary, we demonstrated that PKC activation by two structurally distinct PKC activators induced A_{2B}AR activation, and furthermore PKC could enhance or inhibit A_{2B}AR activity, depending on the signaling pathway. G_i protein isoforms are involved in both the A_{2B}AR activation and enhancement by PKC. PMA pretreatment also enhanced A_{2B}AR agonist radioligand binding. Considering that PKC positively modulates A_{2B}AR to induce cardioprotection,[16] and the A_{2B}AR is one of a few GPCRs that is highly expressed in various cancer cells,[31] it will be important to further explore the mechanistic basis of this interaction and its relevance for cancer, ischemia and other conditions. Remaining questions include identifying potential A_{2B}AR phosphorylation sites, possible involvement of β-arrestins, and possible dependence on particular isoforms of $G_{i\alpha}$ and PKC, in addition to PKCγ. Although robust activation was only observed in

cells with higher A_{2B}AR-expression, which may argue against the importance of this finding, it should be noted that neither this phenomenon nor the mechanisms related to this kind of activation of a GPCR have been documented, which could be an important direction for future clinically directed research.

Limitations of the study

The present study does not exclude the possibility of cell type-dependence of A_{2B}AR activation by PKC, although several types of cells were used and the A_{2B}AR expression level has been demonstrated to play an important role. There are no selective inhibitors for several PKC isoforms; therefore, the conclusion related to PKC isoforms involved depends on the reported selectivity profile of currently available PKC inhibitors. The phosphorylation sites on the A_{2B}AR by PKC have not been located, although it is assumed that the effect of PKC is via phosphorylation. It has not been examined whether one or more isoforms of Gαi are involved in the effect of PKC.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107178.

ACKNOWLEDGMENTS

Support from the NIDDK Intramural Research Program (ZIADK031117) is acknowledged. We thank Jürgen Wess and Liu Liu (NIDDK) for helpful discussions.

AUTHORSHIP CONTRIBUTIONS

Z-G.G.: Conceptualization, Pharmacological experiments, and Writing.

I.M.L.: Pharmacological experiments.

A.I.: Provided G protein-knockout cells.

Q.W.: Pharmacological experiments.

K.A.J.: Conceptualization and Writing.

DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

INCLUSION AND DIVERSITY

We support inclusive, diverse and equitable conduct of research.

Received: February 23, 2023
Revised: May 25, 2023
Accepted: June 15, 2023
Published: June 19, 2023

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals		
isoproterenol	Tocris	Cat. No. 1747/100
formoterol	Tocris	Cat. No. 1448/10
GO6983	Tocris	Cat. No. 2285/1
GO6976	Tocris	Cat. No. 2253/500U
RO32-0432	Tocris	Cat. No. 1587/1
GF109203X	Tocris	Cat. No. 0741/1
H89	Tocris	Cat. No. 2910/1
PSB603	Tocris	Cat. No. 3198/10
ESI-09	Tocris	Cat. No. 4773/10
TPPB	MedChemExpress	HY-12359
NECA	Sigma	CAS No.: Cat. No. 35920-39-9
MRS5698	Sigma	Cat. No. 5428/1
MRS2365	Sigma	Cat. No. 2157/1
CGS21680	Sigma	Cat. No. 1063/10
cholera toxin	Sigma	CAS No.: 9012-63-9
Pertussis toxin	Sigma	CAS No.: 70323-44-3
YM254890	MedChemExpress	HY-111557
enzastaurin	MedChemExpress	HY-10342
LY333531	MedChemExpress	HY-10195
VTX-27	MedChemExpress	HY-112782
AB928	MedChemExpress	HY-129393
BAY60-6583 (LUF6210)	Leiden University	https://www.universiteitleiden.nl/en
MRS1754	Sigma	M6316
ZIP	MedChemExpress	HY-P1284
PMA	Sigma	79346
Hydrofluor	National Diagnostics	Catalog Number: LS-111
Biological samples		
Cell membrane preparations	NIH	N/A
Experimental models: Cell lines		
H9C2	ATCC	CRL-1446
HEK293	ATCC	CRL-1573
NIH-3T3	ATCC	CRL-1658
PC-3	ATCC	CRL-1435
HEK293-Gi knockout	Tohoku University, Sendai, Miyagi 980–8578, Japan	http://www.pharm.tohoku.ac.jp/~seika/seika-e.html
HEK293-Gs knockout	Tohoku University, Sendai, Miyagi 980–8578, Japan	http://www.pharm.tohoku.ac.jp/~seika/seika-e.html
HEK293-Gq knockout	Tohoku University, Sendai, Miyagi 980–8578, Japan	http://www.pharm.tohoku.ac.jp/~seika/seika-e.html

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
HEK293-A2A cells	NIH	N/A
HEK293-A2B cells	NIH	N/A
CHO K1 cells	ATCC	CCL-61
CHO-A2B cells	NIH	N/A
Reagents and assay kits		
AlphaScreen cAMP kit, SureFire®p-ERK1/2 (Thr202/Tyr204) Assay Kit	PerkinElmer	ALSU-PERK-A500
AlphaScreen cAMP Detection Kit	PerkinElmer	6760635D
Calcium 6 assay kit	Molecular Devices	R8191
[^{3}H]NECA	PerkinElmer	NET811250UC
[^{3}H]DPCPX	PerkinElmer	NET974250UC
RNeasy®Mini Kit	Qiagen, Valencia, CA	Cat. No./ID:74004
Superscript III First Strand Synthesis Supermix® kit	Invitrogen, Carlsbad, CA	18080400
FAM-labeled MGB Taqman®probes	Applied Biosystems, Foster City, CA	4316034
Lipofectamine 2000	Thermo Fisher Scientific, Waltham, MA	11668019
Recombinant DNA		
A2BAR- PCDNA 3.1	https://www.cdna.org/	#ADRA2B0000
A2AAR- PCDNA 3.1	https://www.cdna.org/	Catalog Number: #ADRA2ATN00
Software		
Prism 9.1.0 software	GraphPad Software, San Diego, CA	N/A
Other		
All compounds were dissolved in DMSO except MRS2365, CTX and PTX were dissolved in water	N/A	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, K. A. Jacobson, kennethJ@niddk.nih.gov.

Materials availability

This study has generated constructs and cell lines. These materials can only be accessed through proper material transfer agreement following the guidelines of the National Institutes of Health.

Data and code availability

- This paper does not report original code.
- Any additional information required about data reported here is available.
- The lead contact may be contacted to obtain additional information.

MATERIALS AND METHODS

Materials

Isoproterenol, formoterol, GO6983, GO6976, RO32–0432, GF109203X, H89, PSB603, ESI-09, TPPB, NECA (adenosine-5′-*N*-ethyluronamide), MRS5698 and MRS2365 ([[(1R,2R,3S,4R,5S)-4-[6-amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid monoester trisodium salt) were purchased from Tocris (Ellisville, MO). CGS21680, carbachol, cholera toxin (CTX) and PTX were

from Sigma (St. Louis, MO). YM254890, enzastaurin, LY333531, VTX-27 and AB928 were purchased from MedChemExpress (Monmouth Junction, NJ, USA). BAY60-6583 (LUF6210, termed hereafter 'BAY') was synthesized at Leiden/Amsterdam Center for Drug Research and was provided by Ad IJzerman (Leiden, The Netherlands). All compounds were dissolved in DMSO except that CTX and PTX were in water, and proper controls were included in all experiments. AlphaScreen cAMP kit, SureFire *p*-ERK1/2 (Thr202/Tyr204) Assay Kit and AlphaScreen SureFire *p*-Akt 1/2/3 (*p*-Ser473) Assay Kit were purchased from PerkinElmer (Waltham, MA). G_s-null and $G_{q/11}$-null HEK293 cells were generated at Tohoku University, Sendai, Japan. HEK293 human embryonic kidney, PC-3 human prostate cancer, NIH-3T3 mouse fibroblast, and H9C2 rat cardiomyoblast cells were from ATCC (Manassas, VA); all other reagents were from standard commercial sources and of analytical grade.

Detection of gene expression by quantitative real-time PCR

Total mRNA was extracted from H9C2 cells was described in the protocol for the RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription was performed using Superscript III First Strand Synthesis Supermix kit (Invitrogen, Carlsbad, CA). The cDNA was amplified by PCR with gene-specific FAM-labeled MGB Taqman probes (Applied Biosystems, Foster City, CA) in 96-well plates using a 7300 Real-Time PCR System (Applied Biosystems) and default thermocycler program. The gene expression levels were calculated by the $2^{-\Delta\Delta CT}$method using GAPDH as the endogenous control.

INTRACELLULAR CALCIUM MOBILIZATION ASSAY

Cells were grown overnight in 100 μL of medium in 96-well plates (black, clear bottom) at 37°C and with 5% CO_2 until they reached 90% confluency at a density of 30,000 cells per well. Cells were washed with Hank's Buffer containing 20 mM HEPES (pH 7.4; without calcium and magnesium). The Calcium 6 assay kit was used as instructed by mixing Hank's Buffer containing 20 mM HEPES without calcium and magnesium. Probenecid (final concentration of 2.5 mM) was added to the loading dye to increase dye retention. Cells were incubated with 100 μL dye/probenecid for 60 min at room temperature. Cells were pretreated with inhibitors or antagonists for 20 min before the addition of agonists. For the "PMA+G6983" group, GO6983 was added 20 min followed by addition of PMA (1 μM) for 20 min. All reagents used were prepared using Hank's Buffer containing 20 mM HEPES without calcium. Samples were run in duplicate or triplicate using an FLIPR TETRA(Molecular Devices, Sunnyvale, CA) at room temperature. Cell fluorescence (excitation at 485 nm; emission at 525 nm) was monitored following exposure to agonists. Increases in intracellular calcium concentrations are reported as the maximum fluorescence value after exposure minus the basal fluorescence value.

Radioligand binding assays

H9C2 cells endogenously expressing the A_{2B}AR or HEK293 cells stably expressing the recombinant A_{2B}AR (HEK293-A_{2B}) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 Units/ml penicillin, 100 μg/mL streptomycin, and 2 μmol/mL glutamine. To prepare membranes, cells were detached from culture plates by scraping into cold PBS and centrifuged at 250 *g* for 5 min. The pellets were resuspended in ice-cold PBS buffer (pH 7.4) and then homogenized for 30 s. After homogenization and suspension, cell homogenates were centrifuged at 1000 *g* for 10 min, and the pellet was discarded. The suspension was then re-centrifuged at 20,000 *g* for 60 min at 4°C. The pellets were resuspended in buffer containing 3 Units/ml adenosine deaminase and incubated at 37°C for 30 min. The aliquots of membrane preparations were stored at −80°C until the binding experiments. To determine the total and nonspecific binding, [^{3}H]NECA (20 nM, 26 Ci/mmol) or [^{3}H]DPCPX (10 nM, 160 Ci/mmol) (both from PerkinElmer) were incubated membrane preparations from H9C2 cells (40 μg/tube) or HEK293 cells (20 μg/tube) for 60 min in a total assay volume of 200 μL. For saturation binding assays, membrane preparations (20 μg proteins/tube) were incubated at 25°C for 60 min with a range of concentrations of [^{3}H]NECA in a total assay volume of 200 μL of 50 mM Tris HCl, pH 7.4, containing 10 mM $MgCl_2$. Nonspecific binding was determined using 100 μM of xanthine amine congener (XAC, prepared at NIDDK). The reaction was terminated by filtration with GF/B filters using a Brandel 24-channel harvester. Filters were placed in scintillation vials containing 5 mL of Hydrofluor scintillation buffer and counted using a PerkinElmer Tricarb 2810TR liquid scintillation counter.

cAMP accumulation assay

H9C2, PC-3 cells, NIH-3T3 or HEK293 cells were grown in 96-well plates in DMEM supplemented with 10% fetal bovine serum, 100 Units/ml penicillin, 100 μg/mL streptomycin, and 2 μmol/mL glutamine. After overnight growth, cells were treated with assay buffer containing rolipram (10 μM), and adenosine deaminase (3 units/ml) for 30 min (for A_1, A_{2A} and A_3 AR assays, selective antagonist PSB603 (1 μM) was included to block the endogenous A_{2B}AR) followed by addition of agonists and incubated for 20 min. For A_1 and A_3 ARs, after incubation with agonists for 20 min, the adenylyl cyclase activator forskolin (10 μM) was added and the cells incubated for an additional 15 min. For the cAMP assay in the presence of PMA, cells were pretreated with PMA (1 μM) for 40 min before the addition of agonists and incubated for another 20 min. Antagonists or inhibitors were added 20 min, and PTX (200 ng/mL) was incubated with cells for overnight before addition of PMA. The reaction was terminated upon supernatant removal and the addition of 100 μL Tween 20 (0.3%). Intracellular cAMP levels were measured with an ALPHAScreen cAMP assay kit as instructed by the manufacturer (PerkinElmer). The characterization of HEK293 cells stably expressing the A_{2B}ARs has been reported earlier.[23] For transient A_{2B}AR expression, HEK293 cells or G protein-null HEK293 cells were transfected in 6-well plates using the plasmid amounts listed in individual experiments. Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) was used for the transfection of A_{2B}AR plasmids (PCDNA 3.1) according to the manufacturer manual. Cells were split into 96-well plates 24 h after transfection, and cAMP assays were performed after 24 h.

ERK1/2 activity assay

The method used was essentially as previously described.[23] H9C2 cells or HEK293 cells (30,000 cells/100 μL) were seeded in a 96-well plate in complete growth medium. After cell attachment, the medium was removed, and cells were serum starved overnight in 90 μL of serum-free medium. For A_{2B}AR agonist-induced ERK1/2 stimulation, cells were stimulated with agonist for 5 min. For PMA-PKC–mediated ERK1/2 stimulation, cells were pretreated with PMA for 10 min or longer. A_{2B}AR antagonists or PKC inhibitors, GO6983, H89, or PSB603, were preincubated with cells for 20 min before addition of agonists, except that PTX (200 ng/mL) was incubated overnight. After agonist treatment, the medium was removed, and cells were lysed with 1× lysis buffer (20 μL, AlphaScreen SureFire *p*-ERK1/2 (Thr202/Tyr204) Assay Kit; PerkinElmer). Lysate (4 μL/well) was transferred to a 384-well ProxiPlate Plus (PerkinElmer). Acceptor beads were diluted 1:50 in a 1:5 mixture of activation buffer in reaction mix and added to the 384-well plate (5 μL/well). The plate was sealed and incubated for 2 h at room temperature. Donor beads (2 μL) diluted 1:20 in dilution buffer were added, and the plate was incubated for another 2 h at room temperature. The plate was measured using an EnVision multilabel reader using standard AlphaScreen settings.

Data and statistical analysis

Binding and functional parameters were calculated using Prism 9.10 software (GraphPad Software, San Diego, CA). Statistical significance of differences was assessed using Student's *t* test (between two conditions) or a One-Way Analysis of Variance (ANOVA) followed by Bonferroni's or Tukey's multiple comparison tests where appropriate among multiple conditions. Differences yielding $p < 0.05$ were considered as statistically different. If data from functional assays need to be normalized, the signal levels of cells alone were considered 0%, and stimulation by the full agonist NECA or the adenylyl cyclase activator forskolin or the PKC activator PMA was set as 100%.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

G_s-null and $G_{q/11}$-null HEK293 cells were generated at Tohoku University, Sendai, Japan. HEK293 human embryonic kidney, PC-3 human prostate cancer, NIH-3T3 mouse fibroblast, and H9C2 rat cardiomyoblast cells were from ATCC (Manassas, VA).

Cell lines and culture methods

H9C2, HEK293, NIH-3T3 and PC-3 cells were grown at 37°C in proper media based on supplier instructions, supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 1% L-glutamine, and 5% CO_2.

Transfection and generation of stable cell lines

For transient A_{2B}AR expression, HEK293 cells or G protein-null HEK293 cells were transfected in 6-well plates using the amount of plasmids listed in individual experiments. Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) was used for the transfection of A_{2B}AR plasmids (PCDNA 3.1) according to the

manufacturer manual. Stable cell lines stably expressing the receptors were selected after transfection in the presence of 800 μg/mL G418 for 4–8 weeks. The resultant monoclonal or multiclonal cells was maintained in the presence of 500 μg/mL G418.

Detection of gene expression by quantitative real-time PCR

Total mRNA was extracted from H9C2 cells was described in the protocol for the RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription was performed using Superscript III First Strand Synthesis Supermix kit (Invitrogen, Carlsbad, CA). The cDNA was amplified by PCR with gene-specific FAM-labeled MGB Taqman probes (Applied Biosystems, Foster City, CA) in 96-well plates using a 7300 Real-Time PCR System (Applied Biosystems) and default thermocycler program. The gene expression levels were calculated by the $2^{-\Delta\Delta CT}$method using GAPDH as the endogenous control.

Intracellular calcium mobilization assay

Cells were grown overnight in 100 μL of medium in 96-well plates (black, clear bottom) at 37°C and with 5% CO_2 until they reached 90% confluency at a density of 30,000 cells per well. Cells were washed with Hank's Buffer containing 20 mM HEPES (pH 7.4; without calcium and magnesium). The Calcium 6 assay kit was used as instructed by mixing Hank's Buffer containing 20 mM HEPES without calcium and magnesium. Probenecid (final concentration of 2.5 mM) was added to the loading dye to increase dye retention. Cells were incubated with 100 μL dye/probenecid for 60 min at room temperature. Cells were pretreated with inhibitors or antagonists for 20 min before the addition of agonists. For the "PMA+G6983" group, GO6983 was added 20 min followed by addition of PMA (1 μM) for 20 min. All reagents used were prepared using Hank's Buffer containing 20 mM HEPES without calcium. Samples were run in duplicate or triplicate using an FLIPR TETRA(Molecular Devices, Sunnyvale, CA) at room temperature. Cell fluorescence (excitation at 485 nm; emission at 525 nm) was monitored following exposure to agonists. Increases in intracellular calcium concentrations are reported as the maximum fluorescence value after exposure minus the basal fluorescence value.

Radioligand binding assays

H9C2 cells endogenously expressing the A_{2B}AR or HEK293 cells stably expressing the recombinant A_{2B}AR (HEK293-A_{2B}) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 Units/ml penicillin, 100 μg/mL streptomycin, and 2 μmol/mL glutamine. To prepare membranes, cells were detached from culture plates by scraping into cold PBS and centrifuged at 250 *g* for 5 min. The pellets were resuspended in ice-cold PBS buffer (pH 7.4) and then homogenized for 30 s. After homogenization and suspension, cell homogenates were centrifuged at 1000 *g* for 10 min, and the pellet was discarded. The suspension was then re-centrifuged at 20,000 *g* for 60 min at 4°C. The pellets were resuspended in buffer containing 3 Units/ml adenosine deaminase and incubated at 37°C for 30 min. The aliquots of membrane preparations were stored at −80°C until the binding experiments. To determine the total and nonspecific binding, [^{3}H]NECA (20 nM, 26 Ci/mmol) or [^{3}H]DPCPX (10 nM, 160 Ci/mmol) (both from PerkinElmer) were incubated membrane preparations from H9C2 cells (40 μg/tube) or HEK293 cells (20 μg/tube) for 60 min in a total assay volume of 200 μL. For saturation binding assays, membrane preparations (20 μg proteins/tube) were incubated at 25°C for 60 min with a range of concentrations of [^{3}H]NECA in a total assay volume of 200 μL of 50 mM Tris HCl, pH 7.4, containing 10 mM $MgCl_2$. Nonspecific binding was determined using 100 μM of xanthine amine congener (XAC, prepared at NIDDK). The reaction was terminated by filtration with GF/B filters using a Brandel 24-channel harvester. Filters were placed in scintillation vials containing 5 mL of Hydrofluor scintillation buffer and counted using a PerkinElmer Tricarb 2810TR liquid scintillation counter.

cAMP accumulation assay

H9C2, PC-3 cells, NIH-3T3 or HEK293 cells were grown in 96-well plates in DMEM supplemented with 10% fetal bovine serum, 100 Units/ml penicillin, 100 μg/mL streptomycin, and 2 μmol/mL glutamine. After overnight growth, cells were treated with assay buffer containing rolipram (10 μM), and adenosine deaminase (3 units/ml) for 30 min (for A_1, A_{2A} and A_3 AR assays, selective antagonist PSB603 (1 μM) was included to block the endogenous A_{2B}AR) followed by addition of agonists and incubated for 20 min. For A_1 and A_3 ARs, after incubation with agonists for 20 min, the adenylyl cyclase activator forskolin (10 μM) was added and the cells incubated for an additional 15 min. For the cAMP assay in the presence of PMA, cells were pretreated with PMA (1 μM) for 40 min before the addition of agonists and incubated for another

20 min. Antagonists or inhibitors were added 20 min, and PTX (200 ng/mL) was incubated with cells for overnight before addition of PMA. The reaction was terminated upon supernatant removal and the addition of 100 μL Tween 20 (0.3%). Intracellular cAMP levels were measured with an ALPHAScreen cAMP assay kit as instructed by the manufacturer (PerkinElmer). The characterization of HEK293 cells stably expressing the A_{2B}ARs has been reported earlier [24]. For transient A_{2B}AR expression, HEK293 cells or G protein-null HEK293 cells were transfected in 6-well plates using the plasmids amount listed in individual experiments. Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) was used for the transfection of A_{2B}AR plasmids (PCDNA 3.1) according to the manufacturer manual. Cells were split into 96-well plates 24 h after transfection, and cAMP assays were performed after 24 h.

ERK1/2 activity assay

The method used was essentially as previously described [24]. H9C2 cells or HEK293 cells (30,000 cells/100μL) were seeded in a 96-well plate in complete growth medium. After cell attachment, the medium was removed, and cells were serum starved overnight in 90 μL of serum-free medium. For A_{2B}AR agonist-induced ERK1/2 stimulation, cells were stimulated with agonist for 5 min. For PMA-PKC–mediated ERK1/2 stimulation, cells were pretreated with PMA for 10 min or longer. A_{2B}AR antagonists or PKC inhibitors, GO6983, H89, or PSB603, were preincubated with cells for 20 min before addition of agonists, except that PTX (200 ng/mL) was incubated overnight. After agonist treatment, the medium was removed, and cells were lysed with 1× lysis buffer (20 μL) [AlphaScreen SureFire *p*-ERK1/2 (Thr202/Tyr204) Assay Kit; PerkinElmer]. Lysate (4 μL/well) was transferred to a 384-well ProxiPlate Plus (PerkinElmer). Acceptor beads were diluted 1:50 in a 1:5 mixture of activation buffer in reaction mix and added to the 384-well plate (5 μL/well). The plate was sealed and incubated for 2 h at room temperature. Donor beads (2 μL) diluted 1:20 in dilution buffer were added, and the plate was incubated for another 2 h at room temperature. The plate was measured using an EnVision multilabel reader using standard AlphaScreen settings.

QUANTIFICATION AND STATISTICAL ANALYSIS

Binding and functional parameters were calculated using Prism 9.10 software (GraphPad Software, San Diego, CA). Statistical significance of differences was assessed using Student's *t* test (between two conditions) or a One-Way Analysis of Variance (ANOVA) followed by Bonferroni's or Tukey's multiple comparison tests where appropriate among multiple conditions. Differences yielding $p < 0.05$ were considered as statistically different. If data from functional assays need to be normalized, the signal levels of cells alone were considered 0%, and stimulation by the full agonist NECA or the adenylyl cyclase activator forskolin or the PKC activator PMA was set as 100%. All experiments were repeated at least three times, and results were presented as average SEM. Statistical significance was assessed using one-way analysis of variance (ANOVA) including a Tukey's test for multiple comparisons. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.