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$G\alpha_q$ potentiation of adenylate cyclase type 9 activity through a Ca^{2+}/calmodulin-dependent pathway

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Abstract

Adenylate cyclase (EC 4.6.1.1) type 9 (AC9) activity has been shown to be inhibited by PMA activation of novel protein kinase C (nPKC) isoforms. In the current study the effect on AC9 activity of activating PKC in physiological relevant manner was examined. Contrary to the anticipated inhibitory effect of activating PKCs through G_q-coupled receptors, activation of transiently expressed G_q-coupled serotonin 5-HT_{2A} or muscarinic M_5 receptors resulted in the potentiation of isoproterenol-stimulated cyclic AMP accumulation in HEK293 cells stably expressing AC9 (HEK-AC9). Consistent with G_q-mediated activation of PKC, the addition of the PKC inhibitor bisindolylmaleimide further potentiated isoproterenol-stimulated cyclic AMP accumulation. Expression of a constitutively active mutant of $G\alpha_q$ in HEK-AC9 cells also produced an enhancement in basal and isoproterenol-stimulated cyclic AMP accumulation. We also examined the role of $G\alpha_q$-mediated release of intracellular Ca^{2+} on the observed potentiation of AC9 activity, by depleting intracellular Ca^{2+} stores with thapsigargin. In Ca^{2+}-depleted HEK-AC9 cells, activation of transiently expressed M_5 receptors resulted in inhibition of isoproterenol-stimulated cyclic AMP accumulation that was blocked by bisindolylmaleimide, indicating that M_5 potentiation of AC9 activity requires Ca^{2+}. This prompted us to examine the effects of the calmodulin antagonist W7 and the Ca^{2+}/calmodulin-dependent kinase II (CaMK II) inhibitor KN-93. Pretreating cells with W7 and KN-93 significantly inhibited M_5-mediated potentiation of isoproterenol-stimulated cyclic AMP accumulation in HEK-AC9 cells, suggesting that $G\alpha_q$ potentiation of AC9 activity involves Ca^{2+}/calmodulin and CaMK II. This data provides evidence for Ca^{2+}-mediated potentiation of AC9 activity.

Keywords: Adenylate cyclase; Protein kinase C; Calmodulin; G alpha q; Learning and memory; Muscarinic receptor

1. Introduction

The cyclic AMP signaling pathway is present in nearly all cells and controls a number of cellular processes such as cell growth, differentiation, and neuronal plasticity. Adenylate cyclases (EC 4.6.1.1) produce the second messenger cyclic AMP in response to a wide range of signal transduction pathways. To date, nine membrane-bound members of the mammalian adenylate cyclase family (AC1–9) have been identified. Although all adenylate cyclase isoforms are activated by the GTP-bound α subunit of Gs ($G\alpha_s$), each isoform displays complex and distinctive regulatory features [1]. AC9, the most recent of the membrane-bound adenylate cyclases to be identified, is a unique member of the mammalian adenylate cyclase family that exhibits reduced sensitivity to forskolin stimulation and is the most divergent in sequence of all the isoforms. Northern blot, immunocytochemistry, in situ hybridization, and RNA protection analysis indicate that AC9 is expressed widely in the central nervous system as well as in other major organs [2–5]. Of particular interest is the relative abundant expression of AC9 message and protein in the hippocampus, a region important for learning and memory [3]. One of the well-characterized regulatory features of AC9 is inhibition of basal activity by Ca^{2+} in calcineurin-dependent manner [4,6]. Calcineurin is a protein phosphatase that has been shown to be colocalized with AC9 in postsynaptic membranes of some hippocampal and cortical neurons [5]. Recently, we have demonstrated that isoproterenol-stimulated AC9 activity is inhibited by $G_{i/o}$ proteins and phorbol-12-myristate-13-acetate (PMA) activation of novel protein kinase C (PKC) isoforms [7].

Physiologically relevant activation of PKC can occur through $G\alpha_q$-mediated signaling. Activation of G_q-coupled receptors leads to the $G\alpha_q$-mediated activation of phos-

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pholipase C and release of diacylglycerol and inositol triphosphate from the plasma membrane. Subsequently, this precipitates the release of Ca^{2+} from intracellular IP_3-sensitive stores and activation of PKC as well as other Ca^{2+}-dependent pathways. Activation of $G\alpha_q$ has been shown to modulate AC1 and AC2 activity through different mechanisms. Receptor-activated $G\alpha_q$ enhances forskolin- and $G\alpha_s$-stimulated AC1 activity in a Ca^{2+}/calmodulin-dependent manner, whereas $G\alpha_q$ potentiation of G protein $\beta\gamma$- and $G\alpha_s$-stimulated AC2 activity occurs through PKC [8–11]. Having previously demonstrated that PMA activation of PKC can inhibit isoproterenol-stimulated AC9 activity, we sought to explore whether we could mimic these effects by activating PKC in a more physiologically relevant manner. To achieve this, we used molecular and pharmacological techniques to explore the effects of activating G_q-coupled receptors on AC9 activity. We report that $G\alpha_q$ activation leads to bimodal regulation of isoproterenol-stimulated AC9. Activated $G\alpha_q$ potentiated isoproterenol-stimulated AC9 activity through a Ca^{2+}/calmodulin-dependent pathway, and, consistent with our previous observations, $G\alpha_q$-mediated activation of PKC inhibited isoproterenol-stimulated AC9 activity [7]. This data provides for a novel mode of AC9 regulation, which may represent a mechanism for modulating cyclic AMP signaling in brain regions expressing high levels of AC9 such as the hippocampus.

2. Materials and methods

2.1. Materials

[^{3}H]Cyclic AMP was purchased from Perkin-Elmer Life Science Products (Boston, MA). 2-[*N*-(2-Hydroxyethyl)]-*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine) (KN-93), phorbol-12-myristate-13-acetate (PMA), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), and A23187 (calcimycin) were purchased from Calbiochem (La Jolla, CA). (−)-Quinpirole, carbachol, serotonin, and isoproterenol were purchased from RBI/Sigma (Natick, MA). Fetal clone serum and bovine calf serum were purchased from Hyclone (Logan, UT). The cDNAs for the serotonin 5-HT_{2A} and muscarinic M_5 receptors were purchased from the Guthrie cDNA Resource Center (www.cdna.org; Sayre, PA). The human AC9 cDNA was obtained from Dr. Daniel Storm (University of Washington). All other reagents were purchased from Sigma (St. Louis, MO) unless indicated otherwise.

2.2. Cell culture and transient transfection

HEK293 cells expressing D_{2L}, AC1, AC2, or AC9 were derived and maintained as previously described [12]. For transient transfections cells were grown in 24-well plates until approximately 90% confluent. Cells were transfected using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to manufacture's instructions. A ratio of 2 μl LipofectAMINE 2000/1 μg cDNA construct was used for all transfections. Cells were assayed 24–48 h following transfection.

2.3. Cyclic AMP accumulation assay

Cells were seeded at densities between 100,000 and 150,000 cells/well in 24-well cluster plates and grown to confluence. The cells were preincubated for 10 min with 200 μl/well of assay buffer (Earle's balanced salt solution containing 0.02% ascorbic acid and 2% bovine calf serum). The cells were then placed on ice and the indicated drugs were added. The cells were then incubated in a 37 °C water bath for 15 min. Following the incubation, the stimulation media was decanted and the reaction was terminated with 200 μl/well of ice-cold 3% trichloroacetic acid. The 24-well cluster plates were stored at 4 °C for up to one week prior to analysis.

2.4. Capacitative calcium entry (CCE)

Depletion of intracellular calcium stores was performed as previously described [13] with minor modifications. In brief, cells were washed once and incubated for 10 min at 37 °C in Ca^{2+}-free Krebs buffer (120 mM NaCl, 4.75 mM KCl, 1.44 mM $MgSO_4$, 11 mM glucose, 25 mM HEPES, and 0.1% bovine serum albumin—adjusted to pH 7.4 with 2 M Tris) containing 100 nM thapsigargin. The buffer was decanted and cells were placed on ice. Activators of cyclic AMP accumulation (as indicated in figures) were added to the cells in Ca^{2+}-free or 4 mM Ca^{2+}-supplemented Krebs buffer. Ca^{2+}-free conditions were also supplemented with 100 μM EGTA. Cells were incubated at 37 °C for 15 min and the assay was terminated by the addition of ice-cold 3% trichloroacetic acid.

2.5. Quantification of cyclic AMP

Cyclic AMP accumulation was quantified using a competitive binding assay as previously described [14] with minor modifications. Duplicate samples of trichloroacetic acid cell extracts (15 μl) were added to reaction tubes. [^{3}H]Cyclic AMP (∼1 nM final concentration) and cyclic AMP binding protein (ca. 150 mg) were diluted in cyclic AMP assay buffer (100 mM Tris/HCl pH 7.4, 100 mM NaCl, 5 mM EDTA) and then added to each well for a total volume of 550 μl. The tubes were incubated on ice for 2 h and harvested by filtration (Packard Unifilter GF/C) using a 96-well Packard Filtermate Cell harvester (Meriden, CT). The filters were allowed to dry, and Microscint O scintillation fluid was added. Radioactivity on the filters was determined using a Packard TopCount scintillation/luminescence detector. Cyclic AMP concentrations in each

sample were estimated in duplicate from a standard curve ranging from 0.1 to 300 pmol of cyclic AMP per assay.

2.6. Data analysis

One-way ANOVA followed by Bonferroni's post hoc analysis was used for statistical comparison between multiple stimulation, transfection, and treatment conditions. Statistical analysis was performed using GraphPad Prism (San Diego, CA).

3. Results

3.1. G_q-signaling and cyclic AMP accumulation in HEK293 cells

Activation of receptors that couple to G_q have been shown to modulate cyclic AMP accumulation in wide range of cells. Before addressing G_q regulation of AC9, we initially characterized the effects of G_q signaling on cyclic AMP accumulation in wild-type HEK293 cells. Although HEK293 cells express mRNA for AC2, AC3, AC6, and AC7 [15], they are a useful cellular model for the study of adenylate cyclase regulation because they produce relatively modest levels of basal and isoproterenol-stimulated cyclic AMP accumulation. In fact, the low levels of endogenous adenylate cyclase activity have allowed us to identify conditions to study recombinant adenylate cyclases [12]. To investigate the effects of G_q signaling on endogenous adenylate cyclases expressed in wild-type HEK293 cells, we measured cyclic AMP accumulation in HEK293 cells transiently transfected with one of two G_q-coupled receptors: the serotonin 5-HT_{2A} (HEK-5-HT_{2A}) or the muscarinic M_5 receptor (HEK-M_5). In all transfection conditions, no significant changes were observed in basal cyclic AMP accumulation or in cyclic AMP accumulation under conditions in which endogenous G_s-coupled β-adrenergic receptors were activated with 1 μM isoproterenol (Table 1). We also explored the effects of adding G_q-coupled receptor agonists (10 μM serotonin to HEK-5-HT_{2A} cells or 10 μM carbachol to HEK-M_5 cells) on isoproterenol-stimulated cyclic AMP accumulation. Addition of the cognate receptor agonist to HEK-5-HT_{2A} or HEK-M_5 cells did not significantly alter isoproterenol-stimulated cyclic AMP levels when compared to isoproterenol alone (Table 1). These data provide evidence that 5-HT_{2A} or M_5 receptor activation of G_q does not modulate endogenous adenylate cyclases in wild-type HEK293 cells.

3.2. G_q potentiation of isoproterenol-stimulated AC9 activity

We have recently demonstrated that isoproterenol-stimulated AC9 activity is attenuated by PMA activation of PKC [7]. To examine the effects on AC9 of activating PKC through a more physiologically relevant pathway, we transiently expressed the 5-HT_{2A} or M_5 receptor in HEK293 cells stably expressing AC9 (HEK-AC9) or the PKC-stimulated adenylate cyclase isoform AC2 (HEK-AC2).

In vector-transfected HEK-AC9 cells the addition of isoproterenol resulted in robust cyclic AMP accumulation (Fig. 1A). Consistent with our previous observations [7], PKC activation with 100 nM PMA significantly attenuated isoproterenol-stimulated cyclic AMP accumulation in HEK-AC9 cells, and the inhibitory effects of PMA were blocked by the PKC inhibitor bisindolylmaleimide (Fig. 1A). Similarly, the addition of PMA significantly reduced isoproterenol-stimulated cyclic AMP accumulation in HEK-AC9 cells transiently expressing the 5-HT_{2A} receptor (AC9/5-HT_{2A} cells; Iso 453 ± 72 pmol/well, Iso + PMA 304 ± 42 pmol/well, $n = 7$) or the M_5 receptor (AC9/M_5 cells; Iso 445 ± 75 pmol/well, Iso + PMA 185 ± 45 pmol/well $n = 4$) demonstrating that the ability of PKC to inhibit AC9 activity remained intact in these cells. In contrast to the expected inhibitory effect of activating PKC through a G_q-coupled receptor, the addition of 10 μM serotonin to AC9/5-HT_{2A} cells or 10 μM carbachol to AC9/M_5 cells significantly potentiated isoproterenol-stimulated cyclic AMP accumulation (Fig. 1B and C). Serotonin alone had no significant effect on isoproterenol-stimulated cyclic AMP accumulation in vector-trans-

Table 1
G_q signaling and cyclic AMP accumulation in wild-type HEK293 cells

Stimulation condition	Transfections			
	Vector	5-HT_{2A}	M_5	$G\alpha_q$(Q209L)
Cyclic AMP accumulation (pmol/well)[a]				
Vehicle	1.88 ± 0.43	4.07 ± 2.13	1.55 ± 0.89	2.02 ± 0.11
Iso	3.57 ± 0.84	5.75 ± 2.72	2.73 ± 0.62	4.18 ± 2.03
Iso + 5-HT	4.32 ± 0.46	1.56 ± 0.62	–	–
Iso + Carb	–	–	2.10 ± 0.77	–

HEK293 cells were transiently transfected with vector, the 5-HT_{2A} receptor, the M_5 receptor, or $G\alpha_q$(Q209L). Cyclic AMP accumulation was stimulated with 1 μM isoproterenol (Iso) or isoproterenol in the presence of 10 μM serotonin (Iso + 5-HT) or 10 μM carbachol (Iso + Carb). Data shown are mean ± standard error of the mean of three to five independent experiments.

[a] No statistically significant changes were observed in cyclic AMP accumulation among the different transfection or stimulation conditions (one-way ANOVA with Bonferroni's post hoc test).

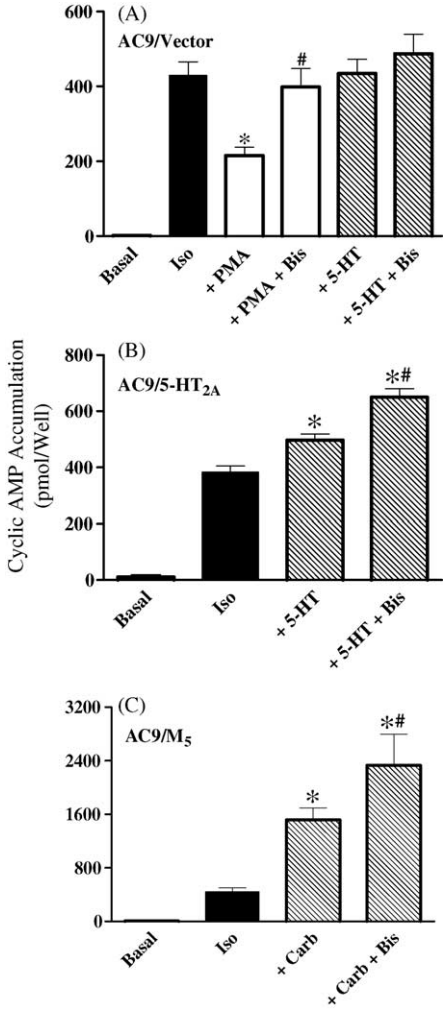

Fig. 1. G_q-coupled receptor potentiation of AC9 activity. HEK-AC9 cells were transiently transfected with (A) vector control, (B) the 5-HT_{2A} receptor, or (C) the M_5 receptor. Cyclic AMP accumulation was measured following incubation with vehicle (Basal), 1 μM isoproterenol (Iso), or isoproterenol in the presence of 100 nM PMA (+PMA), 10 μM serotonin (+5-HT), or 10 μM carbachol (+Carb). Where indicated cyclic AMP accumulation was stimulated in the presence of 1 μM bisindolylmaleimide (+Bis). Data shown are the mean ± standard error of the mean of three to four independent experiments. $^{*}p < 0.05$ compared to isoproterenol-stimulated cyclic AMP accumulation, $^{\#}p < 0.05$ compared to (A) isoproterenol plus PMA, (B) isoproterenol plus serotonin, and (C) isoproterenol plus carbachol (one-way ANOVA with Bonferroni's post hoc test).

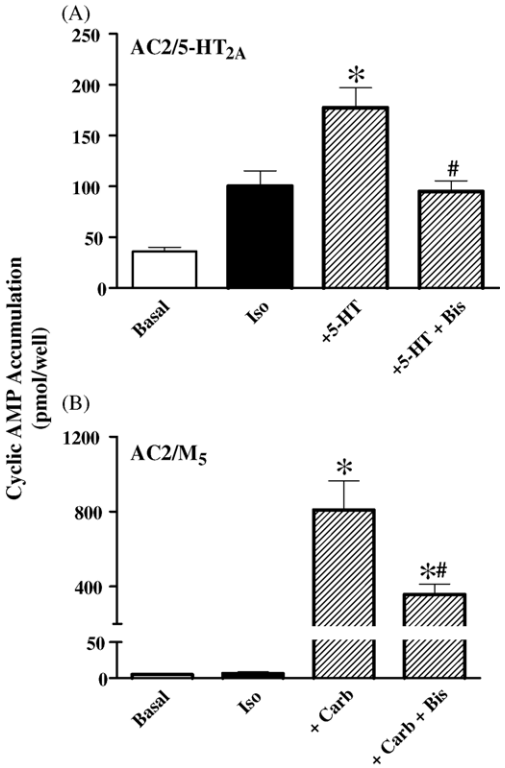

Fig. 2. G_q-coupled receptor potentiation of AC2 activity. HEK-AC2 cells were transiently transfected with (A) the 5-HT_{2A} receptor or (B) the M_5 receptor. Cyclic AMP accumulation was measured following incubation with vehicle (Basal), 1 μM isoproterenol (Iso), or isoproterenol in the presence of 10 μM serotonin (+5-HT) or 10 μM carbachol (+Carb). Where indicated cyclic AMP accumulation was measured in the presence of 1 μM bisindolylmaleimide (+Bis). Data shown are the mean ± standard error of the mean of three to four independent experiments. $^{*}p < 0.05$ compared to isoproterenol-stimulated cyclic AMP accumulation, $^{\#}p < 0.05$ compared to (A) isoproterenol plus serotonin, and (B) isoproterenol plus carbachol (one-way ANOVA with Bonferroni's post hoc test).

fected HEK-AC9 cells (Fig. 1A). To determine if the effects of 5-HT_{2A} or M_5 receptor stimulation involved PKC activation, we also measured the effect of 1 μM bisindolylmaleimide on serotonin or carbachol potentiation of isoproterenol-stimulated cyclic AMP accumulation in AC9/5-HT_{2A} or AC9/M_5 cells. The addition of bisindolylmaleimide significantly enhanced the ability of the G_q-coupled receptor agonists to potentiate isoproterenol-stimulated cyclic AMP accumulation in both AC9/5-HT_{2A} and AC9/M_5 cells (Fig. 1B and C). Together, these observations suggest that activation of G_q-coupled receptors can lead to both potentiation and inhibition of $G\alpha_s$-stimulated AC9 activity.

To establish that the G_q-mediated potentiation observed in HEK-AC9 cells was specific to the AC9 expressed in these cells, we also explored the effects of activating transiently expressed 5-HT_{2A} (AC2/5-HT_{2A}) or M_5 (AC2/M_5) receptors on isoproterenol-stimulated cyclic AMP accumulation in HEK-AC2 cells. The addition of serotonin to AC2/5-HT_{2A} cells or carbachol to AC2/M_5 cells significantly potentiated isoproterenol-stimulated cyclic AMP accumulation (Fig. 2A and B). Consistent with the well-established PKC-dependent G_q modulation of AC2, the G_q-mediated potentiation of isoproterenol-stimulated cyclic AMP accumulation observed in AC2/5-HT_{2A} and AC2/M_5 was attenuated by bisindolylmaleimide (Fig. 2A and B). The difference in the effect of bisindolylmaleimide on G_q-mediated modulation of cyclic AMP accumulation in HEK-AC2 (attenuation) versus HEK-AC9 (potentiation) cells confirms that the G_q-mediated potentiation observed in HEK-AC9 is specific to the AC9 expressed in these cells, and provides more evidence for the bimodal regulation of AC9 by G_q-signaling pathway.

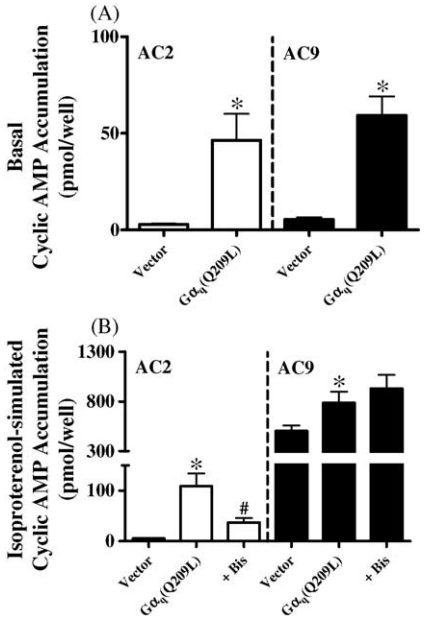

Fig. 3. Constitutively active $G\alpha_q$ potentiation of AC2 and AC9 activity. HEK-AC2 or HEK-AC9 cells were transiently transfected with vector or $G\alpha_q$(Q209L), and cyclic AMP accumulation was measured under (A) basal conditions or (B) in the presence of 1 μM isoproterenol. Where indicated cyclic AMP accumulation in $G\alpha_q$(Q209L) transfected cells was measured in the presence of 1 μM bisindolylmaleimide (+Bis). Data shown are the mean ± standard error of the mean of four independent experiments. $^{*}p < 0.05$ compared to vector-transfected cells, $^{\#}p < 0.05$ compared to isoproterenol alone (one-way ANOVA with Bonferroni's post hoc test).

A recognized characteristic of G protein-coupled receptors is their promiscuity in coupling to different G proteins. Thus, we wanted to determine if G_q activation was sufficient to potentiate isoproterenol-stimulated cyclic AMP accumulation. To circumvent receptor-mediated activation of G_q, we used a constitutively active mutant of $G\alpha_q$, $G\alpha_q$(Q209L). Transient transfection of $G\alpha_q$(Q209L) into HEK293 cells had no effect on basal or isoproterenol-stimulated cyclic AMP accumulation (Table 1), whereas in HEK-AC2 and HEK-AC9 cells $G\alpha_q$(Q209L) transfection produced significant increases in basal cyclic AMP accumulation (Fig. 3A). The ability of G_q signaling to enhance AC9 activity, in the absence of agents that activate the G_s pathway, was also confirmed by the observation that in AC9/M_5 cells the addition of carbachol alone significantly elevated cyclic AMP levels above basal (basal, 6.3 ± 2.0 pmol/well; carbachol, 22 ± 4 pmol/well $n = 3$). In HEK-AC2 cells transiently transfected with $G\alpha_q$(Q209L) the addition of 1 μM bisindolylmaleimide reduced basal cyclic AMP accumulation by 62 ± 9%, consistent with a PKC-dependent effect (data not shown). Furthermore, $G\alpha_q$(Q209L) synergistically enhanced isoproterenol-stimulated cyclic AMP accumulation in HEK-AC2 and HEK-AC9 cells (Fig. 3B). Bisindolylmaleimide significantly attenuated the isoproterenol/$G\alpha_q$(Q209L)-stimulated cyclic AMP accumulation in HEK-AC2 cells, but did not significantly alter cyclic AMP levels in HEK-AC9 cells (Fig. 3B). The results from $G\alpha_q$(Q209L) experiments are consistent with the 5-HT_{2A} or

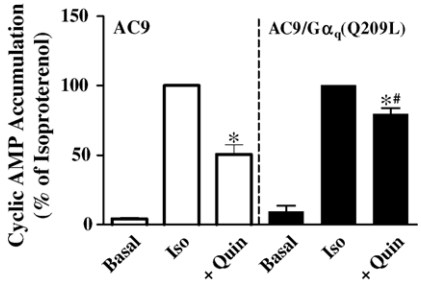

Fig. 4. D_{2L} receptor inhibition of $G\alpha_q$ potentiated AC9 activity. HEK-D_{2L} cells were transfected with AC9 or AC9 in combination with $G\alpha_q$(Q209L). Cyclic AMP accumulation was measured following incubation with vehicle (Basal) or 1 μM isoproterenol in the absence (Iso) or presence of 10 μM quinpirole (+Quin). Data are normalized to isoproterenol-stimulated cyclic AMP accumulation (100%) observed following transfection with AC9 (180 ± 35 pmol/well, $n = 3$) or AC9 with $G\alpha_q$(Q209L) (481 ± 96 pmol/well, $n = 3$). Data shown are the mean ± standard error of the mean of three independent experiments. $^{*}p < 0.05$ compared to isoproterenol-stimulated cyclic AMP accumulation, $^{\#}p > 0.05$ compared to quinpirole-mediated inhibition in AC9 transfected cells (one-way ANOVA with Bonferroni's post hoc test).

M_5 receptor agonist studies and further support the hypothesis that the enhancement of $G\alpha_s$-stimulated AC9 activity results from the ability of these receptors to couple to G_q-mediated signaling.

Recently, we have shown that activation of D_{2L} dopamine receptors attenuates isoproterenol-stimulated AC9 activity through a $G_{i/o}$-dependent pathway [7]. The present study examined the effect of G_q signaling on D_{2L}-mediated inhibition of isoproterenol-stimulated AC9 activity in HEK293 stably expressing the D_{2L} receptor (HEK-D_{2L}). Isoproterenol stimulation of transiently expressed AC9 or AC9 with $G\alpha_q$(Q209L) in HEK-D_{2L} cells produced robust cyclic AMP accumulation that was significantly attenuated by the addition of the D_2 agonist quinpirole (Fig. 4). However, the extent of D_{2L}-mediated inhibition of AC9 activity was reduced in cells co-expressing AC9 and $G\alpha_q$(Q209L), suggesting that the $G\alpha_q$-mediated potentiation of AC9 activity may attenuate $G_{i/o}$ inhibition of AC9 (Fig. 4).

Although the experiments with $G\alpha_q$(Q209L) suggest that the α subunit of G_q is sufficient for the potentiation of AC9 activity, we sought to examine directly the role of βγ subunits by sequestering these subunits with Gα transducin ($G\alpha_T$). A number of reports have demonstrated that expression of $G\alpha_T$ can attenuate βγ-mediated signaling without affecting signaling through Gα subunits [16–18]. Expression of $G\alpha_T$ in HEK-AC9 cells significantly enhanced isoproterenol-stimulated cyclic AMP accumulation consistent with the ability $G\alpha_T$ to attenuate βγ-mediated desensitization of endogenous β-adrenergic receptors in these cells (Fig. 5A) [19]. In contrast, $G\alpha_T$ expression had no effect on $G\alpha_q$(Q209L) potentiation of isoproterenol-stimulated cyclic AMP accumulation in HEK-AC9 cells (Fig. 5B). The lack of a $G\alpha_T$ effect on $G\alpha_q$(Q209L)-mediated potentiation of AC9 activity indicates that the observed potentiation occurs independent of

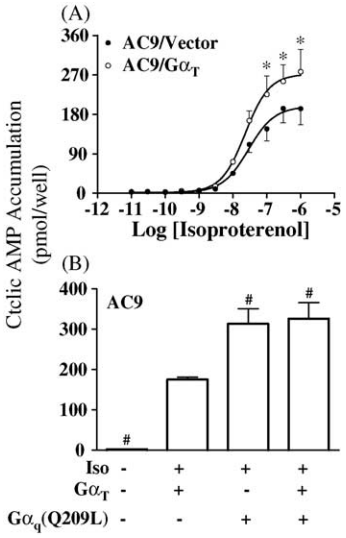

Fig. 5. The effect of $G\alpha_T$ on $G\alpha_q$ potentiated AC9 activity. (A) Isoproterenol-stimulated cyclic AMP accumulation in HEK-AC9 cells transiently transfected with vector or $G\alpha_T$. (B) Basal and isoproterenol-stimulated cyclic AMP accumulation were measured in HEK-AC9 cells transiently transfected with vector, $G\alpha_T$, or $G\alpha_T$ in combination with $G\alpha_q$(Q209L). Data shown are the mean ± standard error of the mean of four independent experiments. $^{*}p < 0.05$ compared to vector-transfected cells, $^{\#}p < 0.05$ compared to isoproterenol-stimulated cyclic AMP accumulation in $G\alpha_T$-transfected cells (one-way ANOVA with Bonferroni's post hoc test).

βγ signaling and substantiates the role of the α subunit of G_q in this pathway.

3.3. Ca^{2+}/calmodulin-dependent potentiation of AC9

In addition to activating PKC, $G\alpha_q$ activation of PLC also results in the release of intracellular Ca^{2+} from IP_3-sensitive stores. The role of Ca^{2+} in $G\alpha_q$-mediated potentiation of AC9 activity was examined by depleting intracellular Ca^{2+} stores with 1 μM thapsigargin (a potent inhibitor of the ATP-dependent Ca^{2+} pump of the endoplasmic reticulum) pretreatment and by removing extracellular Ca^{2+} [20]. In Ca^{2+}-depleted AC9/M_5 cells, activation of M_5 receptors with 10 μM carbachol resulted in the inhibition of isoproterenol-stimulated cyclic AMP accumulation, which could be blocked with the addition of 1 μM bisindolylmaleimide, indicating that the observed inhibition was PKC-mediated (Fig. 6). These results suggested that the $G\alpha_q$-mediated (using carbachol, see Fig. 1C) potentiation of AC9 activity was Ca^{2+}-dependent. Elevating intracellular Ca^{2+} levels with the Ca^{2+} ionophore A23187 has been reported to have no significant effect on isoproterenol-stimulated AC9 activity, whereas capacitative Ca^{2+} entry (CCE) has been shown to inhibit basal AC9 activity through the activation of the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin [2,4]. In order to explore potential mechanisms for the Ca^{2+}-dependent $G\alpha_q$ potentiation of AC9 activity in HEK-AC9 cells, the effects of A23187 and CCE on AC9 activity were characterized. Prior to those studies, however, control experiments examined the effects of A23187 and CCE on cyclic AMP accumulation in HEK293 cells expressing the Ca^{2+}/calmodulin activated adenylate cyclase AC1 (HEK-AC1). The addition of 3 μM A23187 to HEK-AC1 cells dramatically increased cyclic AMP levels (Fig. 7A). Similarly,

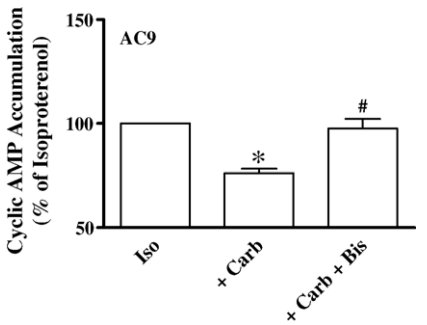

Fig. 6. $G\alpha_q$ modulation of AC9 activity in Ca^{2+}-depleted cells. To deplete intracellular Ca^{2+}, HEK-AC9 cells transiently-transfected with the M_5 receptor were pretreated for 10 min with 1 μM thapsigargin in Ca^{2+}-free Krebs buffer. Cyclic AMP accumulation was measured following incubation with 1 μM isoproterenol alone (Iso), isoproterenol in the presence of 10 μM carbachol (+Carb), or isoproterenol plus carbachol and 1 μM bisindolylmaleimide (+Carb + Bis). Data were normalized to isoproterenol-stimulated cyclic AMP accumulation (100%) and represent the mean ± standard error of the mean of three independent experiments. The average value for isoproterenol-stimulated cyclic AMP accumulation was 223 ± 49 pmol/well. $^{*}p < 0.05$ compared to isoproterenol-stimulated cyclic AMP accumulation, $^{\#}p < 0.05$ compared to isoproterenol plus carbachol-stimulated cyclic AMP accumulation (one-way ANOVA with Bonferroni's post hoc test).

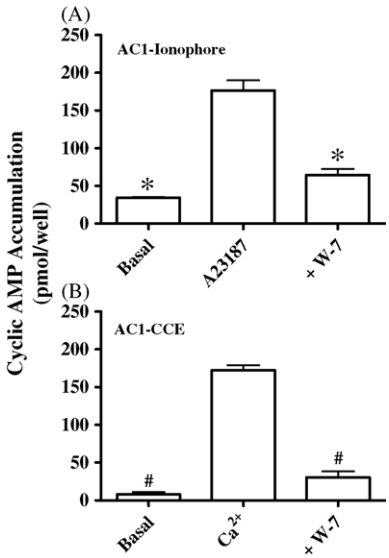

Fig. 7. Ca^{2+} stimulation of AC1 activity. (A) Cyclic AMP accumulation in HEK-AC1 cells was measured under basal conditions or in the presence of 3 μM A23187 or A23187 plus 100 μM W7 (+W7). (B) HEK-AC1 cells were depleted of intracellular Ca^{2+} as described in Section 2: basal cyclic AMP accumulation was measured in Ca^{2+}-free Krebs buffer and capacitative calcium entry (CCE)-stimulated cyclic AMP accumulation was measured by adding 4 mM $CaCl_2$ (Ca^{2+}) or $CaCl_2$ plus W7 (+W7). Data shown are the mean ± standard error of the mean of three independent experiments. $^{*}p < 0.05$ compared to A23187-stimulated cyclic AMP accumulation, $^{\#}p < 0.05$ compared to Ca^{2+}-stimulated (CCE) cyclic AMP accumulation (one-way ANOVA with Bonferroni's post hoc test).

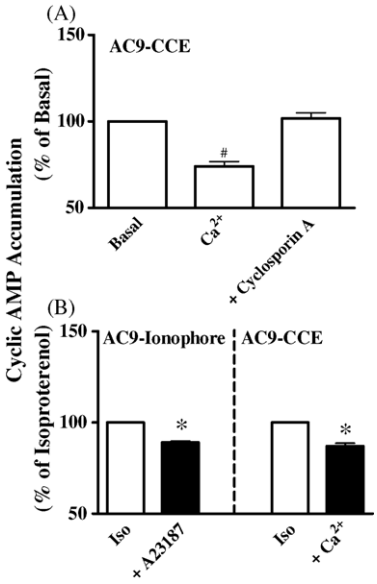

Fig. 8. Effects of Ca^{2+} on AC9 activity. HEK-AC9 cells were depleted of intracellular Ca^{2+} as described in Section 2: (A) basal cyclic AMP accumulation was measured in Ca^{2+}-free Krebs buffer (Basal) and the effects of capacitative calcium entry (CCE) on cyclic AMP accumulation were measured by adding 4 mM $CaCl_2$ alone ($+Ca^{2+}$) or $CaCl_2$ in the presence of 1 μM cyclosporin A. (B) Cyclic AMP accumulation in HEK-AC9 cells was measured in the presence of 1 μM isoproterenol (Iso) in the absence or presence of 3 μM A23187 (+A23187) or 4 mM $CaCl_2$ ($+Ca^{2+}$). Data were normalized to basal (A) or isoproterenol (B)-stimulated cyclic AMP accumulation (100%) under the experimental conditions described. Data shown are the mean ± standard error of the mean of three to four independent experiments. $^{\#}p < 0.05$ compared to basal cyclic AMP accumulation, $^{*}p < 0.05$ compared to isoproterenol-stimulated cyclic AMP accumulation (one-way ANOVA with Bonferroni's post hoc test).

increasing intracellular Ca^{2+} through CCE resulted in cyclic AMP accumulation in HEK-AC1 cells comparable to that produced by A23187 (Fig. 7B). CCE and A23187 stimulation of AC1 activity was blocked by pretreating cells with 100 μM of the calmodulin antagonist *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) (Fig. 7A and B). Consistent with previous observations [2,4], basal AC9 activity was significantly reduced by CCE (Fig. 8A). Inhibition of basal AC9 activity by CCE was also prevented by 1 μM cyclosporin A, consistent with studies reporting inhibition of AC9 by calcineurin (Fig. 8A). In contrast to the stimulatory effects in HEK-AC1 cells, CCE and A23187 produced a small (approximately 10%), but significant decrease in isoproterenol-stimulated cyclic AMP accumulation in HEK-AC9 cells (Fig. 8B). Combined, these observations provide evidence that the $G\alpha_q$ potentiation of AC9 activity is independent of Ca^{2+} entry and may require the release of Ca^{2+} from intracellular stores.

Ca^{2+} can modulate the activity of numerous proteins, including AC1, AC3, and AC8, by activating the protein calmodulin [21]. Therefore, the ability of the calmodulin antagonist W-7 to attenuate $G\alpha_q$ potentiation of AC9 activity was examined. Pretreatment for 10 min with 100 μM W-7 attenuated completely carbachol-induced

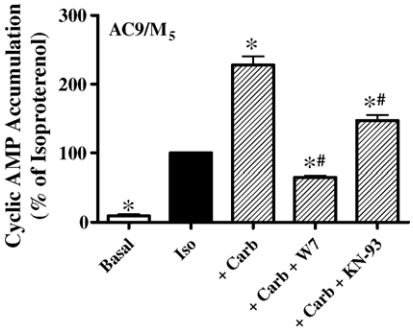

Fig. 9. Role of calmodulin and CaMK II in potentiation of AC9 activity. HEK-AC9 cells were transiently transfected with the M_5 receptor. Cyclic AMP accumulation was measured following incubation with vehicle (Basal), 1 μM isoproterenol (Iso), isoproterenol in the presence of 10 μM carbachol (+Carb), isoproterenol in the presence of carbachol and 100 μM W7 (+Carb + W7), or isoproterenol in the presence of carbachol and 50 μM KN-93 (+Carb + KN-93). Data shown are the mean ± standard error of the mean of three to four independent experiments. $^{*}p < 0.05$ compared to isoproterenol-stimulated cyclic AMP accumulation, $^{\#}p < 0.05$ compared to isoproterenol and carbachol (one-way ANOVA with Bonferroni's post hoc test).

potentiation of isoproterenol-stimulated cyclic AMP accumulation in AC9/M_5 cells (Fig. 9). Because AC9 has previously been shown to be insensitive to direct modulation by Ca^{2+}/calmodulin in reconstitutions studies using membranes from AC9-infected Sf9 cells and purified calmodulin in the presence of Ca^{2+}, we explored the possibility that Ca^{2+}/calmodulin-dependent kinases may be involved in the potentiation of AC9 activity [22]. In AC9/M_5 cells, 10 min pretreatment with 50 μM KN-93, an inhibitor of Ca^{2+}/calmodulin-dependent kinase II (CaMK II), produced a significant reduction (>60%) in carbachol-induced potentiation of isoproterenol-stimulated cyclic AMP accumulation (Fig. 9). These findings suggest that the $G\alpha_q$ potentiation of AC9 activity described in the present study involves Ca^{2+}/calmodulin as well as CaMK II.

4. Discussion

In the course of determining if activation of PKC through G_q-coupled receptors could mimic previously reported PKC-mediated inhibitory effects of PMA on isoproterenol-stimulated AC9 activity [7], we uncovered a novel regulatory feature of AC9 (summarized in Fig. 10). The present study revealed that activation of the G_q-coupled serotonin 5-HT_{2A} or the muscarinic M_5 receptor led to a net increase in $G\alpha_s$-coupled receptor-stimulated AC9 activity that was comprised of both an inhibitory and stimulatory component. The recently described inhibitory component [7] was revealed only in the presence of the PKC inhibitor bisindolylmaleimide. Surprisingly, $G\alpha_q$-mediated signaling through these receptors or through a constitutively active $G\alpha_q$ mutant potentiated both basal and isoproterenol-stimulated AC9 activity in a Ca^{2+}-dependent manner. Using biochemical and pharmacological strategies

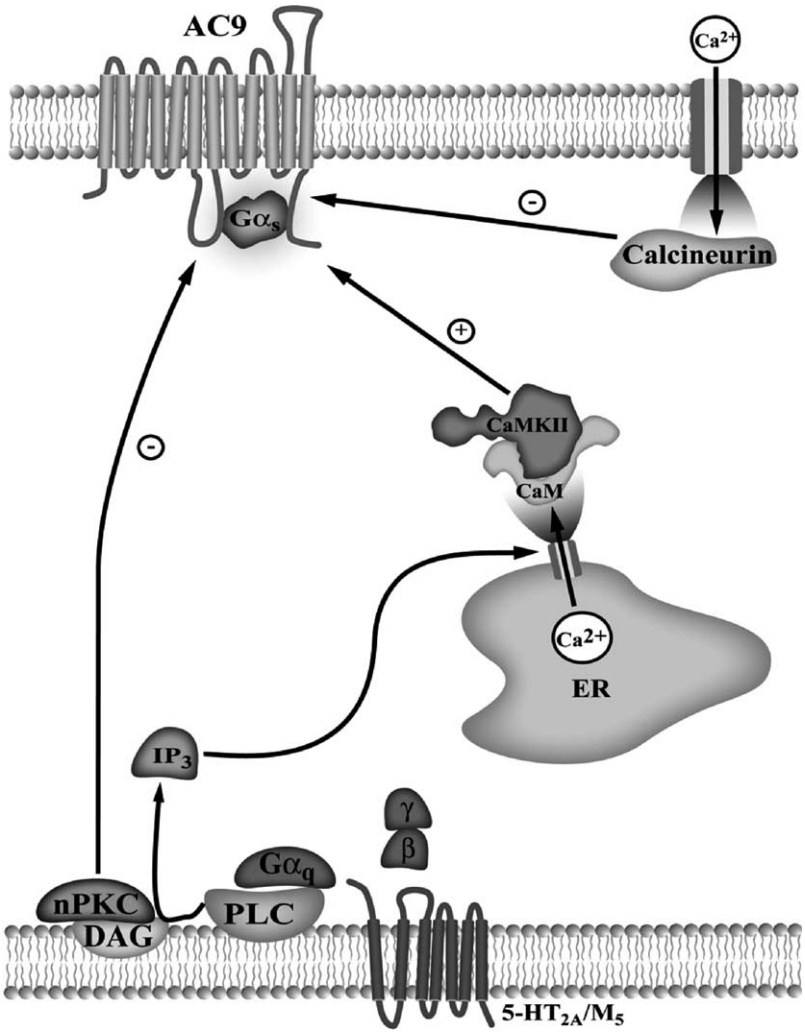

Fig. 10. Bimodal $G\alpha_q$ regulation of AC9 activity. The data presented in this study suggest that activation of G_q-coupled receptors modulates AC9 activity in two opposing ways. Activation of G_q-coupled receptors leads to the $G\alpha_q$-mediated activation of phospholipase C and release of diacylglycerol (DAG) and inositol triphosphate (IP_3) from the plasma membrane. Subsequently, DAG activates PKC leading to the inhibition of $G\alpha_s$-coupled receptor-stimulated AC9 activity, and IP_3 precipitates the release of Ca^{2+} from intracellular stores leading to CaM-kinase II-dependent potentiation of $G\alpha_s$-stimulated AC9 activity. The data also provides additional evidence for inhibition of basal and $G\alpha_s$-stimulated AC9 activity by capacitative Ca^{2+} entry.

the Ca^{2+}-dependent potentiation of AC9 appeared to involve Ca^{2+}/calmodulin and CaMK II. Although the stimulatory effect of calmodulin and CaMK II on AC9 may be direct or indirect, the present study provides evidence for a novel form of cyclic AMP regulation.

Regulation of adenylate cyclases by G_q proteins is thought to occur primarily through the down-stream effects of $G\alpha_q$ [23]. In HEK293 cells stably expressing the Ca^{2+}-stimulated adenylate cyclase isoform AC1, carbachol stimulation of endogenously expressed M_1-muscarinic like receptors has been shown to increase cyclic AMP levels and synergistically enhance $G\alpha_s$-coupled receptor activation of AC1 in a Ca^{2+}-dependent manner [8,9]. AC2, an adenylate cyclase that is directly stimulated by PKC and conditionally activated by βγ subunits in the presence of $G\alpha_s$, is also regulated by down-stream effects of $G\alpha_q$. $G\alpha_q$-mediated activation of PKC can augment $G\alpha_s$-stimulated AC2 activity and permits for βγ stimulation of AC2 in the absence of activated $G\alpha_s$ [10,11]. Another example of G_q modulation of adenylate cyclase activity has been observed in cardiac fibroblasts. Isoproterenol-stimulated cyclic AMP accumulation in cardiac fibroblasts is augmented by concomitantly activating endogenous G_q-coupled receptors [24]. The augmentation in cyclic AMP accumulation has been shown to be Ca^{2+}-dependent and inferred to be a consequence of $G\alpha_s$ and Ca^{2+}-calmodulin acting on the only calcium-stimulated adenylate cyclase detected in cardiac fibroblasts, AC3 [25]. Unlike $G\alpha_s$ and $G\alpha_{i/o}$, however, no evidence exists for direct modulation of adenylate cyclase activity by $G\alpha_q$ [23]. Consistent with the indirect mechanisms for $G\alpha_q$ regulation of adenylate cyclase, the results of the present study demonstrate that $G\alpha_q$ mediates inhibitory and stimulatory effects on AC9 through the down-stream effects of PLC activation.

Studies examining Ca^{2+} regulation of AC9 have focused primarily on inhibition by the protein phosphatase calcineurin. Inhibition of AC9 by Ca^{2+} has been demonstrated

to occur over the same concentrations that stimulate AC1 and, with the use of pharmacological agents, to be calcineurin-dependent [4,6,26]. The studies that examined calcineurin regulation of AC9 have focused entirely on basal cyclic AMP levels and relied on capacitive calcium entry (CCE) as a method of increasing intracellular Ca^{2+} [27]. CCE is considered to be the main mechanism for Ca^{2+} entry into non-excitable cells. Mechanistically, CCE requires release of Ca^{2+} from intracellular stores, which in turn, through an unknown mechanism, facilitates Ca^{2+} entry into the cell from extracellular sources through plasma membrane calcium channels [27]. The effects of calcineurin on $G\alpha_s$-stimulated AC9 activity has not been examined previously. However, $G\alpha_s$-coupled receptor-stimulated AC9 activity has been reported to be insensitive to Ca^{2+} ionophore-mediated Ca^{2+} entry, although some reports have demonstrated that this form of Ca^{2+}entry is insufficient to modulate the activity of other Ca^{2+}-sensitive adenylate cyclase isoforms [2,13,28]. Therefore, it has not been clear whether $G\alpha_s$-stimulated AC9 activity is modulated by Ca^{2+} or if Ca^{2+} regulation of AC9 is dependent on the source of Ca^{2+}, extracellular sources versus intracellular stores. In the present study, the effect of increasing cytosolic Ca^{2+} by different mechanisms was examined. The Ca^{2+} ionophore A23187 and CCE produced a small decrease in isoproterenol-stimulated cyclic AMP accumulation in HEK-AC9 cells. CCE also produced a significant decrease in basal AC9 activity through a calcineurin-dependent pathway, consistent with previous reports [4]. Since Ca^{2+} entry from an extracellular source by CCE or A23187 produced inhibition of isoproterenol-stimulated AC9, it is likely that the observed $G\alpha_q$-mediated potentiation of AC9 activity demonstrated in this study involves IP_3-dependent release of Ca^{2+} from intracellular stores, which is consistent with the primarily cytosolic localization of CaMK II in HEK293 cells [29]. An intriguing aspect of Ca^{2+} regulation of AC9 is that calcineurin inhibits AC9 activity and that CaMK II potentiates AC9 activity, suggesting that calcineurin and CaMK II act on AC9 with opposing effects. This may present a feedback mechanism for Ca^{2+} regulation of AC9. Release of intracellular Ca^{2+} would initially activate CaMK II and consequently potentiate AC9 activity, while also facilitating the entry of Ca^{2+} from extracellular sources through CCE negating the stimulatory effects of CaMK II through the actions of calcineurin. Interestingly, release of intracellular Ca^{2+} has been shown to potentiate CCE through CaMK II [30]. That CCE produces only modest inhibition of isoproterenol-stimulated cyclic AMP accumulation may reflect the ability of calcineurin to inhibit the potentiating effect of CaMK II, but not $G\alpha_s$-stimulated AC9 activity.

Perhaps the most exciting implication of the ability of calmodulin and CaMK II to potentiate AC9 activity is the possibility that this mechanism may play a role in hippocampal neuronal plasticity. The hippocampus is a region important for certain forms of learning and memory. A widely excepted model for the cellular changes associated with learning and memory is long-term potentiation (LTP) of glutamate-activated postsynaptic neurons in the hippocampus [31]. LTP is established following high frequency stimulation of hippocampal neurons, which facilitates calcium influx through *N*-methyl-D-aspartate (NMDA) receptors resulting in Ca^{2+}/calmodulin-dependent increases in cyclic AMP [32,33]. Ca^{2+}/calmodulin-stimulated cyclic AMP accumulation is important for the establishment of LTP, as well as memory formation [34,35]. The most convincing evidence for the role of cyclic AMP in learning and memory comes from genetically altered mice that lack AC1 and AC8, the two primary Ca^{2+}-stimulated adenylate cyclase isoforms expressed in neuronal tissue [36]. These knockout mice are incapable of establishing late phase LTP and show deficits in memory formation.

In addition to the ionotropic NMDA and AMPA receptors, the hippocampus also expresses $G_{q/11}$-coupled metabotropic glutamate receptors ($mGluR_1$ and $mGluR_5$) [37]. Mice lacking $mGluR_5$ exhibit reduced NMDA receptor-dependent LTP and deficiencies in hippocampal-dependent spatial memory [38]. Consistent with this, a recent report has provided evidence that signaling through $G\alpha_{q/11}$ sets the threshold for the formation of LTP [39]. Cyclic AMP has also been shown to be important in gating the early phase of LTP presumably through the actions of PKA [34]. In the context of the present study, it is possible to hypothesize that $G\alpha_{q/11}$-mediated signaling in the hippocampus can activate CaMK II, through the release of Ca^{2+} from intracellular stores and other mechanisms, which would in turn activate AC9 and increase cyclic AMP levels generating a priming effect and lowering the threshold for LTP induction. Although cyclic AMP is critically important for establishing LTP and memory formation, it may be that fine-tuning of the cyclic AMP pathway is essential for memory formation. This may explain the colocalization of AC9 and calcineurin in the postsynaptic densities of the hippocampus, since this would allow for tight regulation of cyclic AMP production [5]. The proposed role of AC9, and Ca^{2+}/calmodulin regulation of AC9, in LTP and hippocampal-dependent learning and memory will need be explored in AC9 knockout mice.

Acknowledgements

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