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$G\alpha_q$-coupled receptor signaling enhances adenylate cyclase type 6 activation

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Abstract

Calcium signaling robustly inhibits AC6 activity in membrane preparations and in intact cells via capacitative calcium entry (CCE). However, the release of intracellular calcium has not been demonstrated to robustly alter AC6 signaling and activation of $G\alpha_q$-coupled receptors in tissues that express AC6 enhances cyclic AMP accumulation. To specifically examine the ability of $G\alpha_q$-coupled receptors to modulate AC6 signaling in intact cells, we used stably transfected HEK-AC6 cells. We demonstrate that AC6 activation is potentiated by activation of endogenous muscarinic receptors expressed in HEK293 cells. Muscarinic receptor activation failed to potentiate the activation of the closely related AC5 isoform. Expression of recombinant $G\alpha_q$-coupled muscarinic or serotonin receptors, or constitutively active $G\alpha_q$, also potentiated drug-stimulated cyclic AMP accumulation in HEK-AC6 cells. Muscarinic receptor-mediated potentiation of AC6 activation was not due to activation of PKC or modulation of $G\alpha_{i/o}$-mediated inhibition of AC6. We demonstrate that calcium chelation or inhibition of calmodulin attenuates the effect of carbachol on AC6 activation. These data support the hypothesis that $G\alpha_q$-coupled receptor-mediated calcium signaling potentiates AC6 activation in intact cells.

Keywords: Adenylate cyclase; G protein; Calcium; PKC; Muscarinic receptor

1. Introduction

There are nine membrane-bound isoforms of adenylate cyclase (AC1-9). Each isoform is differentially regulated by calcium, G protein, and protein kinase signaling [1]. AC5 and AC6 are members of the calcium-inhibited adenylate cyclase family. Both isoforms are highly expressed in cardiac myocytes and striatal neurons [1,2] and AC6 is abundantly expressed in several other brain regions and other tissues including liver and lung [1]. All adenylate cyclase isoforms are inhibited by high concentrations of calcium (10–25 μM) [3] and the activity of AC5 and AC6 examined in membrane preparations from several tissues and cultured cell lines revealed inhibition by submicromolar concentrations of calcium (0.2–0.6 μM) [3,4]. The precise mechanism for direct inhibition of AC5 and AC6 by calcium is unclear; but it postulated to involve the Mg^{2+}-binding domain [5,6]. The inhibition of AC5 and AC6 by calcium in vitro is not altered by the presence of calmodulin [7].

Although calcium directly inhibits AC5 and AC6 in membranes, elevating calcium concentrations in intact cells differentially affects AC5 and AC6 signaling, depending on the source of calcium. Capacitative calcium entry (CCE) into cells from extracellular sources inhibits cyclic AMP signaling in cells that express endogenous AC6; however, the release of intracellular calcium has little or no effect on AC6 activity [8]. In cardiac fibroblasts and gastric smooth muscle cells, both of which demonstrate AC5/6 immunoreactivity, activation of $G\alpha_q$-coupled receptors enhances adenylate cyclase signaling [9–11]. $G\alpha_q$-mediated enhancement of drug-stimulated cyclic AMP production in those tissue types has been postulated to be dependent upon $G\beta\gamma$ signaling or the expression of additional calcium-stimulated adenylate cyclase isoforms [9,11].

To explore the effects of $G\alpha_q$-coupled receptor activation specifically on AC6 signaling, we used previously

Abbreviations: AC, adenylate cyclase; BAPTA/AM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester; CaMK, calmodulin-dependent kinase; CCE, capacitative calcium entry; HEK, human embryonic kidney; PKC, protein kinase C; W7, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester

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characterized HEK-AC6 cells [12]. HEK293 cells express low levels of AC2, AC3, AC6, and AC7 and have been used frequently to examine the regulation of specific isoforms of adenylate cyclase [13–15]. We examined the ability of endogenous Gα_q-coupled muscarinic receptors expressed in HEK-AC6 cells to modulate isoproterenol (ISO)- or forskolin (FSK)-stimulated cyclic AMP accumulation. We report that carbachol treatment of HEK-AC6 cells enhanced both isoproterenol- and forskolin-stimulated cyclic AMP accumulation. In contrast, carbachol had no effect on drug-stimulated cyclic AMP accumulation in HEK-AC5 cells. The effect of carbachol was not due to PKC or G$\alpha_{i/o}$. Calcium chelation with BAPTA/AM or incubation with the calmodulin antagonist W7 attenuated the effects of carbachol on AC6 activation. These data suggest that Gα_q signaling potentiates the activation of AC6 in a calcium–calmodulin-dependent manner.

2. Materials and methods

2.1. Materials

[^{3}H]Cyclic AMP (32 Ci/mmol) was purchased from NEN Life Science products (Boston, MA, USA). W7 and BAPTA/AM were purchased from Calbiochem (La Jolla, CA, USA). All other drugs were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell culture and transfection

The HEK-AC6 stable cell line was constructed by transfection of pcDNA3-FLAG-AC6 into HEK293 cells [12]. Cells stably expressing adenylate cyclase were maintained in DMEM containing 5% fetalclone1 serum and 5% bovine calf serum with penicillin (100 units/mL), streptomycin (100 μg/ml) and G418 (300 μg/ml) or hygromycin (150 μg/ml) for the HEK-AC6 or HEK-AC5 cells, respectively. Cells were grown in a humidified incubator in the presence of 5% CO_2 at 37 °C. Cells were transfected using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and were assayed 24–48 h post-transfection. For all transfections, 1 μg of plasmid was used per well in 24-well plates.

2.3. Cyclic AMP accumulation assay

Cells were seeded in 48-well cluster plates at a density of between 100,000 and 150,000 cells per well. The cells were washed once for 10 min in EBSS (15 mM HEPES-buffered Earle's balanced salt solution containing 0.02% ascorbic acid and 2% bovine calf serum). The wash buffer was removed, drug(s) were added on ice, and the cells were incubated for 15 min at 37 °C. The medium was removed and the cells were lysed with ice-cold 3% trichloroacetic acid. The 48-well plates were stored at 4 °C until quantification of cyclic AMP. Cyclic AMP accumulation was determined as described previously [12].

2.4. Capacitative calcium entry

Depletion of intracellular calcium stores was performed as previously described [16] 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% BSA-adjusted to pH 7.4 with 2 M Tris) containing 100 nM thapsigargin. The buffer was decanted and cells were placed on ice. Forskolin was added to the cells in 100 μM EGTA- or 4 mM Ca^{2+}-supplemented Krebs buffer. 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. Data analysis

One-way ANOVA followed by Bonferroni's *post hoc* analysis was used for statistical comparison between multiple stimulation, transfection, and treatment conditions. Student's *t*-test was used to compare two conditions. A *p*-value <0.05 is considered statistically significant. Statistical analysis was performed using GraphPad Prism (San Diego, CA).

3. Results

3.1. Effect of carbachol on cyclic AMP signaling in HEK293, HEK-AC5, and HEK-AC6 cells

To investigate the regulation of calcium-inhibited adenylate cyclase isoforms by Gα_q-coupled receptors, we examined the ability of endogenous Gα_q-coupled muscarinic receptors in HEK293 cells [17] to modulate AC5 and AC6 activation. In wild-type HEK293 cells, forskolin did not robustly stimulate cyclic AMP production and carbachol was without effect on forskolin stimulation (Fig. 1). In HEK293 cells that stably express AC5 (HEK-AC5), carbachol did not alter forskolin-stimulated cyclic AMP accumulation (Fig. 1). However, in HEK-AC6 cells, carbachol significantly potentiated forskolin-stimulated cyclic AMP accumulation and this potentiation was attenuated by the muscarinic receptor antagonist, atropine (Fig. 1). Due to the robust forskolin response in the HEK-AC5 cell line, the potentiation of carbachol may be more difficult to detect. However, the cyclic AMP response is not saturated in this cell line using a concentration of 100 nM forskolin [13].

Carbachol also potentiated isoproterenol-stimulated cyclic AMP accumulation in HEK-AC6 cells (Table 1). The effect of carbachol on AC6 activation was not due to changes in phosphodiesterase activity because carbachol

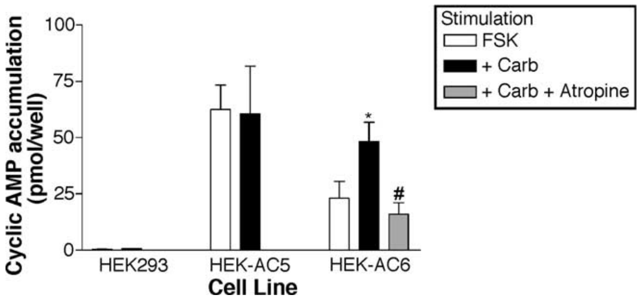

Fig. 1. Carbachol potentiates forskolin-stimulated cyclic AMP accumulation in HEK-AC6 cells. HEK293, HEK-AC5, and HEK-AC6 cells were incubated with 1 μM forskolin (FSK) (HEK293, HEK-AC6) or 100 nM forskolin (HEK-AC5) in the absence or presence of 50 μM carbachol (Carb) for 15 min. HEK-AC6 cells were also incubated with 1 μM atropine. Data shown are mean ± standard error of the mean of three to five experiments. $^{*}p < 0.05$ compared to forskolin-stimulated cyclic AMP values, $^{\#}p < 0.05$ compared to forskolin + carbachol-stimulated cyclic AMP values (one-way ANOVA with Bonferroni's post-test).

treatment also enhanced isoproterenol-stimulated cyclic AMP accumulation in the presence of 500 μM IBMX (Table 1). Carbachol did not significantly alter basal cyclic AMP levels in any of the cell lines tested.

3.2. Recombinant $G\alpha_q$-coupled receptors and constitutively active $G\alpha_q$ potentiate AC6 activation

To examine the effects of recombinant $G\alpha_q$-coupled receptors on drug-stimulated cyclic AMP accumulation, we transfected HEK-AC6 cells with the $G\alpha_q$-coupled muscarinic type 1 receptor (M_1) and the $G\alpha_q$-coupled serotonin (5HT) type 2A receptor. Transfection of the M_1 muscarinic receptor significantly enhanced forskolin-stimulated cyclic AMP accumulation in HEK-AC6 cells (Fig. 2A). Carbachol also potentiated forskolin-stimulated cyclic AMP accumulation in cells transfected with the M_1 receptor, however, the fold-potentiation of forskolin-stimulated cyclic AMP accumulation by carbachol was similar to that in vector-transfected cells (Fig. 2A). A

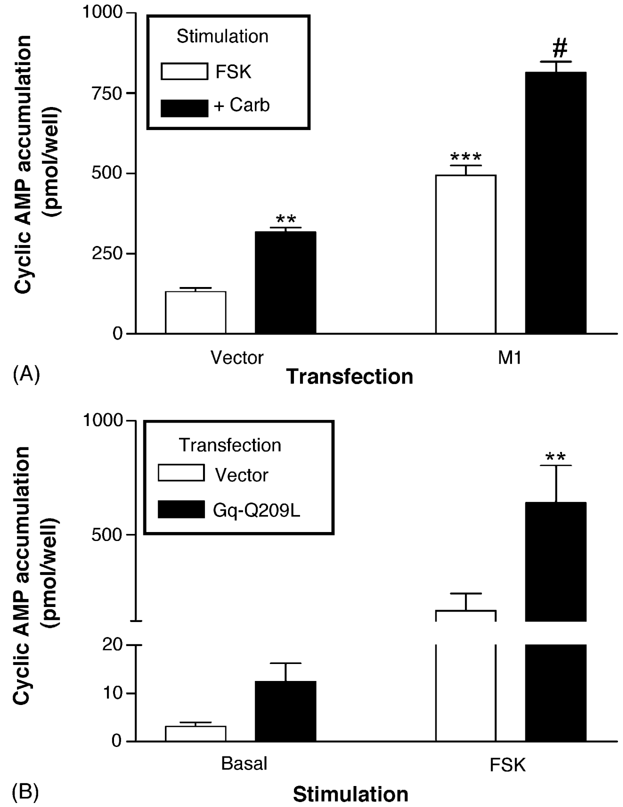

Fig. 2. Effect of transfection of $G\alpha_q$-coupled muscarinic receptors or constitutively active $G\alpha_q$ in HEK-AC6 cells. (A) HEK-AC6 cells were transiently transfected with pcDNA3 (Vector) or pcDNA3.1-M_1 (M1). After 24–48 h, cells were incubated with 1 μM forskolin (FSK) or forskolin + 50 μM carbachol (+Carb). Data shown are mean ± standard error of the mean of three experiments. $^{**}p < 0.01$ compared to forskolin-stimulated cyclic AMP values in vector-transfected cells, $^{***}p < 0.001$ compared to forskolin-stimulated cyclic AMP values in vector-transfected cells, $^{\#}p < 0.001$ compared to forskolin-stimulated cyclic AMP values in M_1-transfected cells (one-way ANOVA with Bonferroni's post-test). (B) HEK-AC6 cells were transiently transfected with vector or pcDNA3.1-$G\alpha_q$-Q209L. After 24–48 h, cells were incubated with vehicle (Basal) or 1 μM forskolin (FSK). Data shown are mean ± standard error of the mean of six experiments. $^{**}p < 0.01$ compared to forskolin-stimulated cyclic AMP values in vector-transfected cells (one-way ANOVA with Bonferroni's post-test).

Table 1
Carbachol potentiation of isoproterenol-stimulated cyclic AMP accumulation in HEK-AC6 cells does not involve alterations in phosphodiesterase activity

	Cyclic AMP accumulation (pmol/well) in HEK-AC6 cells			
	Basal	Carb	ISO	ISO + Carb
	1.8 ± 0.4	2.6 ± 0.4	13.3 ± 0.6[a]	33.6 ± 1.3[b]
+IBMX	7.8 ± 2.7	9.7 ± 2.7	80.3 ± 3.9[c]	115 ± 12.5[d]

HEK-AC6 cells were incubated with vehicle (Basal), 50 μM carbachol (Carb), 1 μM isoproterenol (ISO), or isoproterenol + carbachol in the absence or presence of 500 μM IBMX for 15 min. Data shown are mean ± standard error of the mean of seven experiments.

[a] $p < 0.001$ compared to basal cyclic AMP values.

[b] $p < 0.001$ compared to isoproterenol-stimulated cyclic AMP values.

[c] In the presence of IBMX, $p < 0.001$ compared to basal cyclic AMP values.

[d] $p < 0.01$ compared to isoproterenol-stimulated cyclic AMP values (one-way ANOVA with Bonferroni's post-test).

previous report in CHO cells transfected with the M_1 muscarinic receptor suggested that M_1 receptors could enhance cyclic AMP production in membranes directly via $G\alpha_s$ activation [18]. However, in contrast to the findings by Burford and Nahorski, carbachol alone did not enhance cyclic AMP accumulation in intact HEK-AC6 cells (see Table 1) nor in HEK-AC6 cells transiently transfected with the M_1 receptor (data not shown). In addition, transfection of the $G\alpha_q$-coupled $5HT_{2A}$ receptor also enhanced forskolin stimulated cyclic AMP accumulation 2.1 ± 0.3-fold compared to vector-transfected cells ($p < 0.05$, $n = 5$).

To directly examine $G\alpha_q$ signaling, we transfected HEK-AC6 cells with a constitutively active $G\alpha_q$ mutant (Q209L) that has diminished GTPase activity [19]. Transfection of $G\alpha_q$-Q209L also significantly enhanced forskolin-stimulated cyclic AMP accumulation (Fig. 2B) and appeared to enhance basal cyclic AMP levels, although this result was not statistically significant.

3.3. The carbachol-induced potentiation of AC6 activation is calcium-dependent

We have recently demonstrated that activation of PKC with phorbol esters potentiates AC6 activation in a Raf1-dependent manner [12]. Because $G\alpha_q$-coupled receptor activation can lead to an activation of PKC, we incubated the HEK-AC6 cells with carbachol in the absence or presence of PKC inhibitors. Inhibition of PKC failed to attenuate the enhancement of forskolin-stimulated cyclic AMP accumulation by carbachol in HEK-AC6 cells (Fig. 3). The concentrations of the PKC inhibitors used in Fig. 3 were sufficient to fully block the effects of PMA on AC6 or AC2, respectively, in recent publications from our laboratory [12,15]. Despite the lack of effect of the PKC inhibitors to attenuate carbachol-induced potentiation of AC6 activation, we investigated the possibility that Raf1 signaling was involved. Incubation of cells with the Raf inhibitor GW5074 did not significantly attenuate carbachol-induced potentiation of AC6 activation (data not shown).

There are five types of muscarinic receptors (M_1–M_5). M_1, M_3, and M_5 muscarinic receptors couple to $G\alpha_q$, whereas, the M_2 and M_4 subtypes couple to $G\alpha_{i/o}$ [20]. To confirm that the enhancement of drug-stimulated cyclic AMP accumulation by carbachol was not due to alterations in $G\alpha_{i/o}$ signaling, we pretreated the HEK-AC6 cells overnight with pertussis toxin (PTX) to inactivate $G\alpha_{i/o}$. Overnight pretreatment with pertussis toxin did not alter carbachol potentiation of forskolin-stimulated cyclic AMP accumulation in HEK-AC6 cells (Fig. 4A). The same

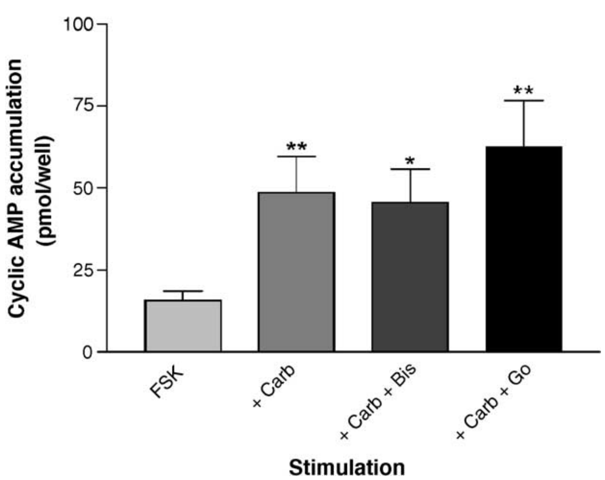

Fig. 3. Effect of PKC inhibitors on the carbachol potentiation of AC6 activation. HEK-AC6 cells were incubated with 1 μM forskolin (FSK) in the absence or presence of 50 μM carbachol (Carb), 1 μM bisindolylmaleimide (Bis), or 1 μM Gö6976 (Go). Data shown are mean ± standard error of the mean of four experiments. $^{*}p < 0.05$ compared to forskolin-stimulated cyclic AMP values, $^{**}p < 0.01$ compared to forskolin-stimulated cyclic AMP values (one-way ANOVA with Bonferroni's post-test).

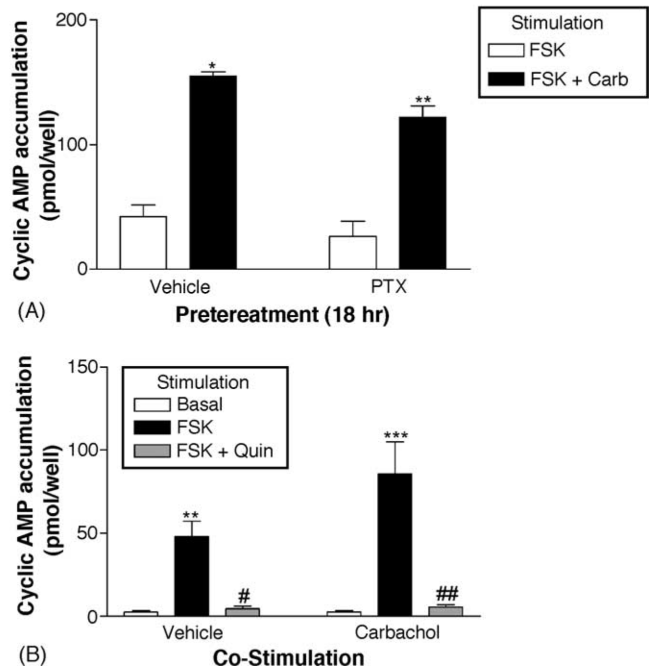

Fig. 4. Effect of carbachol on $G\alpha_{i/o}$ signaling. (A) HEK-AC6 cells were incubated overnight with vehicle or 25 ng/mL pertussis toxin (PTX). Cells were subsequently washed and incubated with 1 μM forskolin (FSK) or forskolin + 50 μM carbachol (FSK + Carb). Data shown are the mean ± standard error of three experiments. $^{*}p < 0.05$ compared to forskolin-stimulated cyclic AMP values in vehicle-pretreated cells, $^{**}p < 0.01$ compared to forskolin-stimulated cyclic AMP values after pertussis toxin pretreatment (Student's *t*-test). (B) HEK-AC6-D_{2L} cells were incubated with vehicle (Basal), 1 μM forskolin (FSK), or forskolin + 1 μM quinpirole (FSK + Quin) in the absence or presence of 50 μM carbachol. Data shown are mean ± standard error of the mean of four experiments. $^{**}p < 0.01$ compared to basal cyclic AMP values, $^{\#}p < 0.01$ compared to forskolin-stimulated cyclic AMP. In the presence of carbachol, $^{***}p < 0.001$ compared to basal cyclic AMP values, $^{\#\#}p < 0.01$ compared to forskolin-stimulated cyclic AMP values (one-way ANOVA with Bonferroni's post-test).

incubation period and concentration of pertussis toxin completely attenuated D_{2L} receptor inhibition of adenylate cyclase signaling in transiently-transfected HEK-AC9 cells [15]. Furthermore, in HEK-AC6 cells stably expressing D_{2L} dopamine receptors, carbachol enhanced forskolin-stimulated cyclic AMP accumulation but did not alter the ability of $G\alpha_{i/o}$ to inhibit cyclic AMP accumulation upon activation of the $G\alpha_{i/o}$-coupled D_{2L} dopamine receptor (Fig. 4B). These data suggest that the effect of carbachol on AC6 activation is not due to activation of, or interference with, $G\alpha_{i/o}$ signaling.

The effects of carbachol on AC6 activation did not appear to be due to the activation of PKC or Raf1, or interference with $G\alpha_{i/o}$ signaling. We, therefore, investigated the possibility that calcium signaling was mediating the carbachol potentiation of AC6 activation. In order to examine the role of calcium on the carbachol potentiation of AC6, we pre-incubated HEK-AC6 cells with vehicle or the cell-permeable calcium chelator, BAPTA/AM for 30 min. Pretreatment with BAPTA/AM did not alter basal cyclic AMP levels (3.0 ± 0.5 pmol/well versus 2.7 ± 0.5 pmol/well with or without BAPTA/AM pretreatment, $n = 5$). However, preincubation with BAPTA/AM decreased the ability of carbachol to potentiate isoproterenol-stimulated cyclic AMP accumulation (Fig. 5A). Incubation with the calmodulin antagonist, W7, also attenuated the effects of carbachol on isoproterenol-stimulated cyclic AMP accumulation (Fig. 5B). These data suggest that although AC6 is potently inhibited by calcium in vitro or by CCE [7,8], calcium signaling after muscarinic receptor activation enhances drug-stimulated cyclic AMP accumulation in cells that express AC6 cells in a calmodulin-dependent manner.

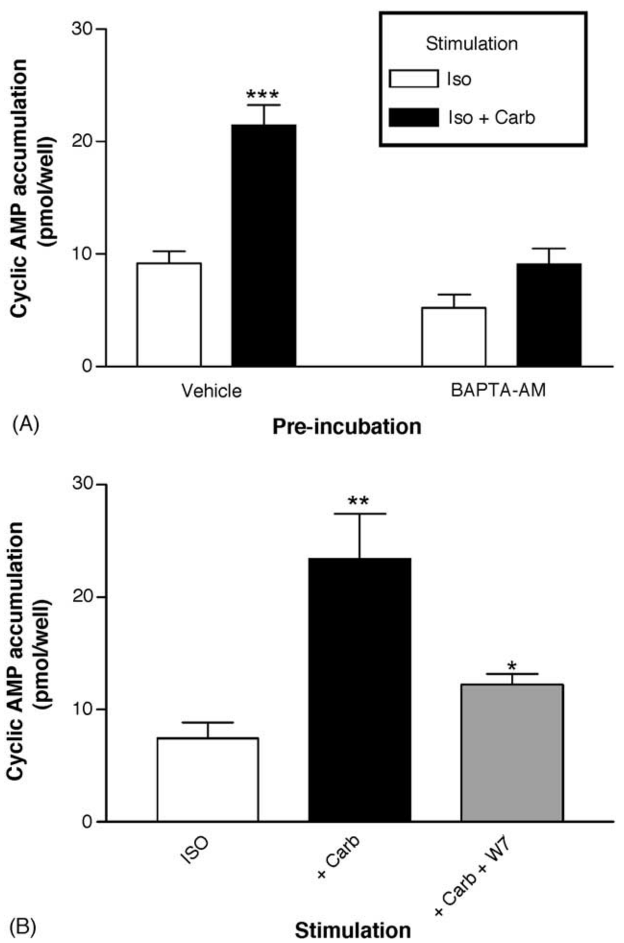

Fig. 5. The role of calcium signaling in the carbachol potentiation of AC6 activation. (A) HEK-AC6 cells were pre-incubated with 50 μM BAPTA/AM for 30 min. Following the pre-incubation, the cells were incubated for 15 min with 1 μM isoproterenol (ISO) in the absence or presence of 50 μM carbachol (ISO + Carb). Data shown are mean $\pm$ standard error of the mean of five experiments. $^{***}p < 0.001$ compared to isoproterenol-stimulated cyclic AMP values (Student's t-test). (B) HEK-AC6 cells were incubated for 15 min with 1 μM isoproterenol (ISO) in the absence or presence of 50 μM carbachol (+Carb) or 100 μM W7. Data shown are mean $\pm$ standard error of the mean of four experiments. $^{**}p < 0.01$ compared to isoproterenol-stimulated cyclic AMP values, $^{*}p < 0.05$ compared to isoproterenol + carbachol-stimulated cyclic AMP values (one-way ANOVA with Bonferroni's post-test).

3.4. Capacitative calcium entry inhibits AC5 and AC6

The unanticipated role of calcium in muscarinic receptor potentiation of AC6 signaling in intact cell experiments prompted additional studies to explore the role of the calcium source on AC6 activity. CCE has been demonstrated to inhibit cyclic AMP accumulation in cells that express endogenous or recombinant AC6 [8,21]. In HEK-AC6 cells, CCE robustly inhibited forskolin-stimulated cyclic AMP accumulation after depletion of intracellular calcium by thapsigargin (Fig. 6A). CCE also inhibited cyclic AMP accumulation in HEK-AC5 cells (Fig. 6B). Incubation with thapsigargin alone slightly decreased forskolin-stimulated cyclic AMP values in both HEK-AC6 and HEK-AC5 cells. These data are consistent with the ability of calcium to negatively regulate AC5 and AC6 following facilitated calcium entry from extracellular sources.

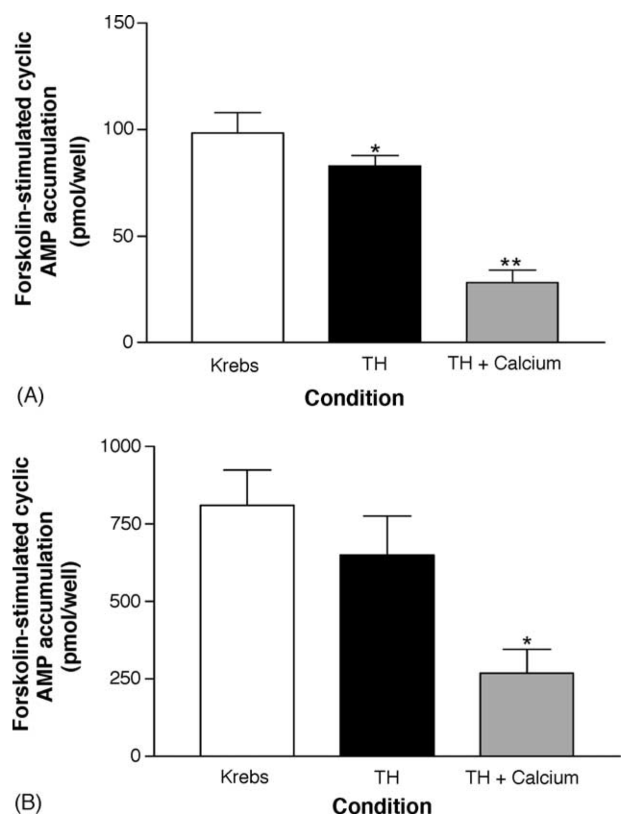

Fig. 6. CCE inhibits drug-stimulated cyclic AMP accumulation in both HEK-AC5 and HEK-AC6 cells. HEK-AC6 (A) or HEK-AC5 (B) cells were pre-incubated for 10 min in calcium-free Krebs buffer (Krebs) or 100 nM thapsigargin (TH). Cells were subsequently incubated with 1 μM (A) or 300 nM (B) forskolin in the absence or presence of 4 mM calcium (+calcium). Data shown are mean $\pm$ standard error of the mean of 4 experiments. (A) $^{*}p < 0.05$ compared to forskolin-stimulated cyclic AMP values after Krebs incubation, $^{**}p < 0.01$ compared to forskolin-stimulated cyclic AMP values after thapsigargin pre-incubation in the absence of calcium, (one-way ANOVA with Bonferroni's post-test). (B) $^{*}p < 0.05$ compared to forskolin-stimulated cyclic AMP values after thapsigargin pre-incubation in the absence of calcium, (one-way ANOVA with Bonferroni's post-test).

4. Discussion

In the present study, we propose a model for AC6 regulation by $G\alpha_q$-coupled receptors and calcium in intact cells (Fig. 7). This proposed model also provides for bi-directional regulation of AC6 activity by calcium. Using HEK293 cells, we examined the ability of endogenous and recombinant muscarinic receptors to regulate the calcium-inhibited adenylate cyclase isoforms. Muscarinic receptor activation in HEK-AC6 cells, but not HEK-AC5 cells, potentiated isoproterenol- and forskolin-stimulated cyclic AMP accumulation. In addition, transfection of $G\alpha_q$-coupled muscarinic or serotonin receptors, or constitutively active $G\alpha_q$, increase forskolin-stimulated cyclic AMP accumulation in HEK-AC6 cells.

$G\alpha_q$-coupled receptor activation has been reported to enhance cyclic AMP signaling in at least two cell types that demonstrate AC5/6 immunoreactivity [9,11]. Similar to the results presented here, $G\alpha_q$-coupled receptor activation in cardiac fibroblasts did not affect basal cyclic AMP but enhanced drug-stimulated cyclic AMP accumulation [9,10]. In cardiac fibroblasts, AC6 overexpression did not enhance but rather ''fractionally reduced'' the potentiation by $G\alpha_q$-coupled receptor activation. Cardiac fibroblasts also demonstrate robust AC3 expression, therefore, the enhancement of signaling observed by $G\alpha_q$-coupled receptors may be due to the expression of the calcium-stimulated AC3 isoform [9]. However, the data presented here suggest that $G\alpha_q$ signaling can enhance AC6 activation in intact cells. Further investigation of the adenylate cyclase isoform(s) responsible for the $G\alpha_q$-coupled receptor-mediated potentiation of cyclic AMP signaling in cardiac fibroblasts would be useful to characterize this signaling pathway.

In smooth muscle cells that demonstrate AC5/6 immunoreactivity, the M_3 muscarinic receptor enhances cyclic AMP accumulation [11]. Similar to the present study, PKC inhibitors failed to attenuate the effects of muscarinic receptor activation on cyclic AMP signaling [11]. Murthy and Makhlouf suggested that the M_3 muscarinic receptor-mediated enhancement of cyclic AMP signaling was dependent on $G\beta\gamma$ based on the observation that preincubation with anti-$G\beta$ antibodies in permeabilized cells blunted the stimulatory effect of carbachol [11]. However, in HEK-AC6 cells, transfection of $G\alpha_q$ potentiated drug-stimulated cyclic AMP accumulation, suggesting that $G\alpha_q$ signaling enhances AC6 activation. Chelation of calcium or antagonism of a calcium effector, calmodulin attenuated the effects of carbachol on AC6 activation, therefore, we suggest that $G\alpha_q$ signaling mediates the enhanced AC6 signaling in a calcium-dependent manner.

Although we demonstrate that the potentiation of AC6 by $G\alpha_q$ is calcium–calmodulin-dependent, the regulatory mechanisms downstream of calcium–calmodulin remain unknown. Calmodulin is a small, ubiquitously-expressed protein that can signal to several downstream effectors [22,23]. After binding to four calcium ions, calmodulin can activate myosins, calcium channels, GTPase activating proteins, and several others proteins [23]. Calcium–calmodulin can also activate several calcium–calmodulin-dependent kinases (CaMK) [22] that regulate adenylate cyclase signaling. Specifically, CaMKII and CaMKIV can phosphorylate and inhibit AC3 and AC1, respectively [24,25]. Although CaMK phosphorylation of AC1 and AC3 is inhibitory, recent studies with AC9 (Cumbay and Watts, in press) and the present study with AC6 suggest that a possible role of CaMK signaling should be further investigated as a mechanism for the calcium–calmodulin-mediated potentiation of adenylate cyclase activity.

The data presented here suggest that AC6 is regulated by calcium in a bi-directional manner. In vitro exposure to calcium ions or calcium influx from extracellular sources robustly inhibits AC6 [7,8], whereas, $G\alpha_q$-mediated calcium release potentiates AC6 signaling. Interestingly, non-selective increases in calcium using calcium ionophores or thapsigargin-mediated release of intracellular calcium, have little effect on AC6 signaling [8]. It is likely that the

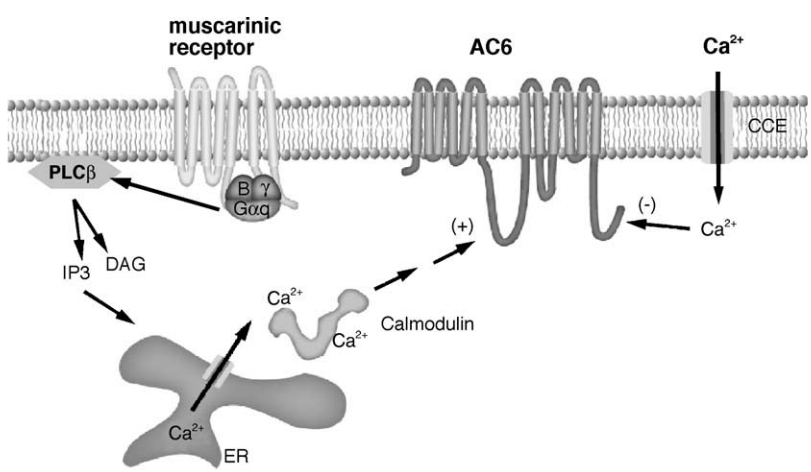

Fig. 7. In cells expressing AC6, CCE inhibits cyclic AMP accumulation. However, activation of $G\alpha_q$-coupled receptors leads to a calcium- and calmodulin-dependent enhancement of drug-stimulated cyclic AMP accumulation.

regulation of AC6 by extracellular calcium entry at the plasma membrane is due to a direct Ca^{2+} ion inhibition [26], whereas, calcium released from intracellular sources may not reach AC6 at the plasma membrane. Rather, calcium released from intracellular sources would rely on additional, calmodulin-dependent signaling pathways to modulate AC6 activation. It is also possible that carbachol is inducing an intracellular release of calcium as well as an influx of extracellular calcium [27]. CCE activation of AC8 and inhibition of AC6 is dependent upon intact membrane-microdomain structures [28,29], therefore, if the ability of carbachol to potentiate AC6 signaling is dependent upon extracellular calcium, the differential effects of CCE versus $G\alpha_q$-mediated extracellular calcium influx may be due to differentially localized pools of AC6 in intact cells.

Understanding the regulation of AC6 by $G\alpha_q$ signaling may lead to useful discoveries relevant to the development of cardiac hypertrophy. A current genetic model used to study cardiac hypertrophy and cardiac failure in mice involves the cardiac-directed over-expression of WT-$G\alpha_q$ [30]. These rats develop decreased ionotropic and chronotropic responsiveness to β-adrenergic receptor (βAR)-stimulated cyclic AMP signaling, decreased βAR-induced L-type channel calcium currents, elevated PKC activity, decreased AC5 expression, and increased $G\alpha_{i/o}$ expression [31]. Although transgenic over-expression of AC5 restores some signaling and functional deficits in the $G\alpha_q$-hypertrophic mice [32], cardiac-directed expression of AC6 rescues several functional deficits, survival rates, and hypertrophy [33]. In a cellular environment such as the hypertrophic cardiac myocytes, there is both an impairment of cyclic AMP signaling, and an excess of $G\alpha_q$ signaling. Therefore, the beneficial effects of AC6 over-expression in the $G\alpha_q$-hypertrophic mouse model may be due not only to enhancing cyclic AMP signaling by over-expression of an adenylate cyclase isoform, but by the over-expression of a specific cyclase (AC6) that is also potentiated by $G\alpha_q$ signaling.

Acknowledgements

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