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Protein Kinase C and Epidermal Growth Factor Stimulation of Raf1 Potentiates Adenylyl Cyclase Type 6 Activation in Intact Cells

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ABSTRACT

Adenylyl cyclase type 6 (AC6) activity is inhibited by protein kinase C (PKC) in vitro; however, in intact cells, PKC activation does not inhibit the activity of transiently expressed AC6. To investigate the effects of PKC activation on AC6 activity in intact cells, we constructed human embryonic kidney (HEK) 293 cells that stably express wild-type AC6 (AC6-WT) or an AC6 mutant lacking a PKC and cyclic AMP-dependent protein kinase (PKA) phosphorylation site, Ser674 (AC6-S674A). In contrast to in vitro observations, we observed a PKC-mediated enhancement of forskolin- and isoproterenol-stimulated cyclic AMP accumulation in HEK-AC6 cells. Phorbol 12-myristate 13-acetate also potentiated cyclic AMP accumulation in cells expressing endogenous AC6, including Chinese hamster ovary cells and differentiated Cath.a differentiated cells. In HEK-AC6-S674A cells, the potentiation of AC6 stimulation was significantly greater than in cells expressing AC6-WT. The positive effect of PKC activation on AC6 activity seemed to involve Raf1 kinase because the Raf1 inhibitor 3-(3,5-dibromo-4-hydroxybenzylidene-5-iodo-1,3-dihydro-indol-2-one (GW5074) inhibited the PKC potentiation of AC6 activity. Furthermore, the forskolin-stimulated activity of a recombinant AC6 in which the putative Raf1 regulatory sites have been eliminated was not potentiated by activation of PKC. The ability of Raf1 to regulate AC6 may involve a direct interaction because AC6 and a constitutively active Raf1 construct were coimmunoprecipitated. In addition, we report that epidermal growth factor receptor activation also enhances AC6 signaling in a Raf1-dependent manner. These data suggest that Raf1 potentiates drug-stimulated cyclic AMP accumulation in cells expressing AC6 after activation of multiple signaling pathways.

There are nine known membrane-bound isoforms of adenylyl cyclase (AC1–9), and each isoform is differentially regulated by calcium, $G\alpha_{i/o}$, $G\alpha_s$, $G\beta\gamma$, and serine/threonine kinases such as PKA and PKC (Defer et al., 2000). Investigations into the regulation of adenylyl cyclase by protein kinases are often performed in vitro using overexpressed adenylyl cyclase isoforms in cell membranes incubated in the presence of purified kinases. These studies are ideal for determining the direct effects of kinase phosphorylation on adenylyl cyclase activity. However, examining the regulatory properties of adenylyl cyclase in intact cells may offer additional insight into the ability of adenylyl cyclases to act as coincidence detectors for multiple intracellular signaling pathways.

AC6 and the closely related AC5 isoform share high amino acid sequence identity. AC5 and AC6 are both highly expressed in brain and cardiomyocytes (Chern, 2000; Defer et al., 2000). AC6 is abundantly expressed in several other tissues, including kidney, liver, and lung (Chern, 2000; Defer et al., 2000). Both isoforms are stimulated by $G\alpha_s$ and are inhibited by $G\alpha_i$ and submillimolar levels of calcium (Chabardes et al., 1999; Defer et al., 2000). PKC increases AC5 activity (Kawabe et al., 1994, 1996); however, PKC phosphorylation inhibits AC6 activation in insect or mammalian cell membranes (Lai et al., 1997, 1999; Lin et al., 2002; Wu et al., 2001). Although PMA activation of PKC is reported to have no significant effect on drug-stimulated AC6 activity in transiently transfected HEK293 cells (Jacobowitz et al., 1993; Yoshimura and Cooper, 1993), a recent study suggested that PKC may be involved in δ-opioid receptor-induced heterolo-

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ABBREVIATIONS: AC, adenylyl cyclase; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; HEK, human embryonic kidney; CHO, Chinese hamster ovary; EGF, epidermal growth factor; ERK, extracellular-regulated kinase; PVDF, polyvinylidene difluoride; WT, wild type; CAD, Cath.a differentiated; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; IBMX, isobutylmethylxanthine; GW5074, 3-(3,5-dibromo-4-hydroxybenzylidene-5-iodo-1,3-dihydro-indol-2-one; Ro 20-1724, 4-[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone; PD98059, 2′-amino-3′-methoxyflavone.

gous sensitization of endogenous AC6 in CHO cells (Varga et al., 1998, 2003). The same CHO cell model has also been used to demonstrate that the Raf1 inhibitor GW5074 partially attenuated heterologous sensitization (Varga et al., 2002). Furthermore, both phosphorylation of AC6 and forskolin stimulation of AC6 are enhanced by orthovanadate-mediated inhibition of tyrosine phosphatase activity in HEK293 cells, and these effects were abolished by cotransfection of a dominant-negative Raf1 construct (Tan et al., 2001). A recent study has also provided evidence that Raf1 is capable of physically and functionally interacting with several recombinant adenylyl cyclases, including AC6 (Ding et al., 2004). The identification of PKC and Raf1 as possible positive regulators of AC6 coupled with studies demonstrating that PMA activation of PKC can activate Raf1 in intact cells (Ueda et al., 1996) suggests the hypothesis that activation of PKC leads to a Raf1-dependent enhancement of AC6 signaling.

To address directly this hypothesis and to examine the regulation of AC6 by protein kinases in intact cells, we used HEK293 cells expressing wild-type and genetically engineered AC6, as well as CAD and CHO cells that endogenously express AC6. In contrast to data obtained from in vitro observations, we report a PKC-dependent enhancement of both forskolin- and isoproterenol-stimulated cyclic AMP accumulation in HEK-AC6 cells. Likewise, EGF receptor activation enhanced drug-stimulated cyclic AMP accumulation in HEK-AC6 cells. Biochemical, immunological, and genetic studies revealed that the potentiation of drug-stimulated AC6 activity by PKC or EGF receptor activation involves Raf1 kinase and serine residues in the fourth intracellular loop of AC6.

Materials and Methods

Materials. [^{3}H]Cyclic AMP (32 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Most drugs and the FLAG antibody were purchased Sigma-Aldrich (St. Louis, MO). H89 was purchased from Calbiochem (San Diego, CA). ERK1/2, phospho-ERK1/2, and MYC antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Western blotting gels and membranes were purchased from Bio-Rad (Hercules, CA).

Cell Culture and Transfection. The AC6-S674A mutation was made by site-directed mutagenesis of FLAG-AC6 cDNA using the following primers: forward 5′-CTTGAGAAGAAGTATGCACG-GAAAGTAGATCCTCGC-3′, reverse 5′-GCGAGGATCTACTTTC-CGTGCATACTTCTTCTCAAG-3′. The AC6-SIC4A construct was made using primers described in Tan et al. (2001) using the primers for the "A" mutant with minor modifications. The vector for all AC6 constructs is pcDNA3 and the vector for MYC-Raf1 and MYC-Raf1-CAAX is pMT. The Raf4N construct is in pCGN vector. Stable cell lines were constructed by transfection of AC6-WT or AC6-S674A into HEK293 cells and selection using G418. G418-resistant colonies were screened and selected for expression of adenylyl cyclase by immunoblotting and by assaying isoproterenol- and forskolin-stimulated cyclic AMP accumulation. Cells stably expressing adenylyl cyclases were maintained in Dulbecco's modified Eagle's medium containing 5% Fetalclone1 serum and 5% bovine calf serum with penicillin (100 units/ml), streptomycin (100 μg/ml), and G418 (300 μg/ml). Cells were grown in a humidified incubator in the presence of 5% CO_2 at 37°C. For transient transfections, constructs were transfected into HEK293 cells using LipofectAMINE 2000 according to manufacturer's instructions (Invitrogen, Carlsbad, CA).

Cyclic AMP Accumulation Assay. Cells were seeded in 48-well cluster plates at a density of between 100,000 and 150,000 cells/well. For the CAD cell differentiation experiments, the cells were washed in serum-free media at 60% confluence and incubated in serum-free media for 48 to 72 h before use. For pretreatments, cells were incubated with drugs at 37°C in a humidified incubator in the presence of 5% CO_2 for 2 to 18 h. After pretreatment or for short-term experiments, the cells were washed once for 10 min with 200 μl of assay buffer (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) was 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 as described previously (Watts and Neve, 1996).

Western Blotting. For adenylyl cyclase and Raf1 immunoblots, the cells were maintained in six-well plates. The medium was aspirated, and cells were put on ice. Lysis buffer (1 mM HEPES, 2 mM EDTA, 1 mM dithiothreitol, 0.15 mM phenylmethylsulfonyl fluoride) was added to each well for 10 min. Lysates were scraped into centrifuge tubes, homogenized briefly, and centrifuged at 30,000*g* for 20 min. The supernatant was removed and the pellet was resuspended in resuspension buffer (15 mM HEPES, 1 mM dithiothreitol, and 0.15 mM phenylmethylsulfonyl fluoride, pH 7.5). Protein content was quantified using the bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). Equal amounts of total protein were resolved by SDS-PAGE using 7.5% acrylamide gels, and resolved samples were electrotransferred to a PVDF membrane. Raf1 and AC6 expression levels were determined using a primary antibody directed against the MYC epitope (1:500) or the FLAG epitope (3 μg/ml), respectively. Immunoreactivity was detected by enhanced chemifluorescence according to manufacturer's instructions (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). For ERK1/2 immunoblots, cells were seeded in six-well cluster plates. Cells were incubated with drugs for 5 or 15 min as indicated, washed twice in ice-cold phosphate-buffered saline, and lysed in Laemmli buffer for 10 min. Lysates were scraped into clear polystyrene tubes, sonicated, and incubated on ice for 30 min. The resolved samples were electrotransferred to a PVDF membrane and an anti-phospho-ERK1/2 primary antibody (1:1000) was used to detect ERK1/2 phosphorylation. Equal loading was confirmed by stripping the immunoblot and probing for total ERK1/2 levels using and anti-ERK1/2 antibody (1:1000).

Immunoprecipitation. HEK-AC6 cells were transfected with vector control or MYC-Raf1-CAAX in 10-cm plates. After 48 h, cells were washed in ice-cold phosphate buffer. Lysis buffer (50 mM Tris, pH 7.5, 120 mM NaCl, 1.5% Nonidet P-40, 5.8 trypsin inhibitory units of aprotinin, and 1 mM Na_3VO_4) was added, and cells were incubated on ice for 30 min. Samples were centrifuged at 50,000*g* for 1 h to remove cellular debris, and the supernatant was retained. Equal volumes of samples were incubated with antibodies directed against the FLAG or MYC epitopes as well as a rabbit anti-mouse bridging antibody (Pierce Chemical) for 4 h. Protein A-Sepharose beads (Amersham Biosciences UK, Ltd.) were subsequently added and incubated with samples for another 2 h. The beads were washed two times each with wash buffer (200 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1.5% Nonidet P-40, 5.8 trypsin inhibitory units of aprotinin, and 1 mM Na_3VO_4) or 1 M NaCl. Loading buffer was added after the last wash, and samples were boiled for 5 min and loaded onto gels as described above. The resolved samples were electroblotted onto nitrocellulose, and the membranes were probed with the anti-MYC antibody and detected by enhanced chemifluorescence as described above.

Results

Stable Expression of AC6-WT and AC6-S674A in HEK293 Cells. To examine the regulation of AC6 by PKC in an intact cell system, we stably transfected HEK293 cells with a wild-type AC6 (AC6-WT) or an AC6 mutant in which

the PKC/PKA serine phosphorylation site had been mutated to an alanine (AC6-S674A). Clonal cell lines were initially selected based on cyclic AMP accumulation responses to 1 μM forskolin (Fig. 1A). Subsequent selection for the clones used in the present studies was based on similar levels of AC6 expression as determined by immunodetection of the FLAG epitope on the N terminus of AC6 (Fig. 1B). Functional studies with the clones used for the present studies revealed that 1 μM forskolin-stimulated cyclic AMP accumulation values were elevated approximately 18-fold in cells expressing AC6-WT and 8-fold in cells expressing AC6-S674A compared with HEK-WT cells.

Effect of PKC Activation on AC6 Activity in Intact Cells. To determine the effects of PKC activation on AC6 activity in our stably transfected HEK-AC6 cells, we activated PKC by incubating the cells with the phorbol ester PMA. PMA (100 nM) had no effect on basal cyclic AMP accumulation levels in HEK-AC6 cells (basal, 2.9 ± 0.3 pmol/well; 100 nM PMA, 3.1 ± 0.2 pmol/well; $n = 4$). However, PMA markedly enhanced forskolin-stimulated cyclic AMP accumulation in HEK-AC6 cells (Fig. 2A). The effect of PMA was not restricted to forskolin because PMA also potentiated isoproterenol-stimulated cyclic AMP accumulation (Fig. 2A). The ability of PMA to potentiate AC6 activity was also observed in additional clonal HEK cell lines stably expressing AC6 (data not shown) as well as after transient transfection of AC6 (Fig. 6).

The ability of PMA to modulate drug-stimulated cyclic AMP of endogenous AC6 was initially explored in a novel neuronal cell model, Cath.a differentiated (CAD) cells (Johnston et al., 2002, 2004). In their undifferentiated form, CAD cells express robust levels of both AC6 and AC9, and PMA does not potentiate significantly forskolin or $G\alpha_s$-stimulated cyclic AMP accumulation in these cells (Johnston et al., 2004; functional data not shown). However, upon removal of serum from the growth medium, the CAD cells differentiate, develop neuronal-like processes, lose AC9 expression almost completely, and retain robust AC6 expression (Johnston et al., 2004). In these differentiated CAD cells, PMA potentiated both forskolin and adenosine 2A receptor-stimulated cyclic AMP accumulation (Fig. 2B). Subsequent studies with wild-type and PKA-deficient CHO cells that express endogenous AC6 also examined the effect of PMA on forskolin-stimulated cyclic AMP accumulation (Singh et al., 1985; Ventura and Sibley, 2000). Similar to the results in HEK-AC6 and differentiated CAD cells, PMA potentiated forskolin-stimulated cyclic AMP accumulation in wild-type and mutant CHO cells (Table 1).

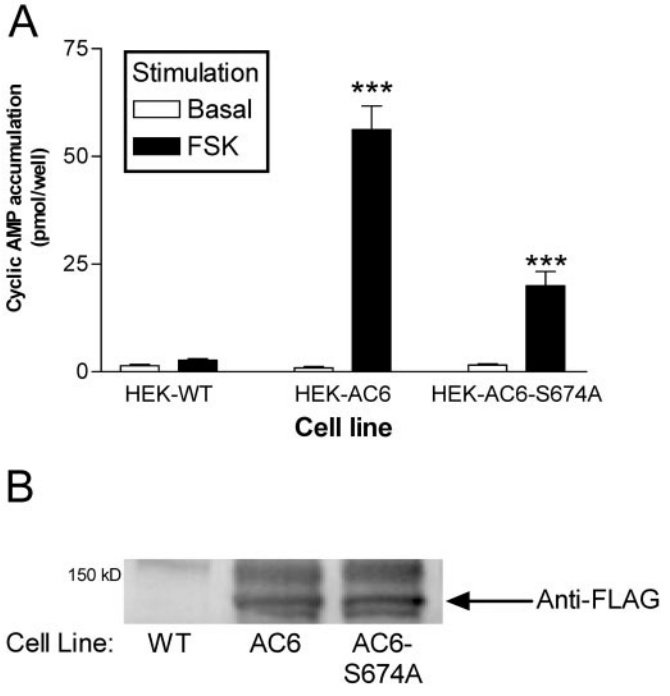

Fig. 1. Expression and function of AC6 and AC6-S674A in stably transfected HEK293 cells. A, HEK-WT, HEK-AC6, and HEK-AC6-S674A cells were incubated for 15 min with vehicle (basal) or 1 μM forskolin (FSK). Data shown are mean ± standard error of the mean of four to five experiments. ***, $p < 0.001$ compared with forskolin-stimulated values in HEK-WT, (one-way analysis of variance with Bonferroni's post test). B, HEK-WT, HEK-AC6, and HEK-AC6-S674A cells were lysed, and lysates were prepared as described under *Materials and Methods*. Equal amounts of protein from each sample were separated on polyacrylamide gels, transferred to PVDF membrane, and probed with the anti-FLAG antibody. Immunoblot is representative of four independent experiments.

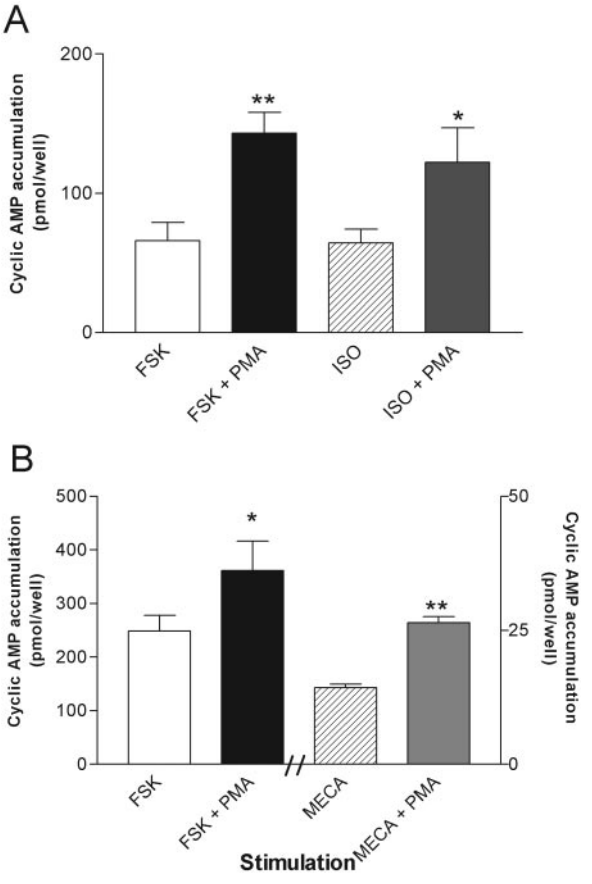

Fig. 2. Effect of PMA on drug-stimulated cyclic AMP accumulation in cells expressing recombinant or endogenous AC6. A, HEK-AC6 cells were incubated for 15 min with 1 μM forskolin (FSK), FSK + 100 nM PMA (FSK + PMA), 10 μM isoproterenol (ISO), or isoproterenol + PMA (ISO + PMA). Data shown are mean ± standard error of the mean of six to eight experiments. **, $p < 0.01$ compared with forskolin alone; *, $p < 0.05$ compared with isoproterenol alone (one-way ANOVA with Bonferroni's post test). B, differentiated CAD cells were incubated in serum-free media for 48 to 72 h to induce differentiation. After serum-free media incubation, the CAD cells were incubated for 15 min with 1 μM FSK, FSK + 100 nM PMA (FSK + PMA), 100 nM 5′-*N*-methylcarboxamidoadenosine (MECA) or MECA + PMA (MECA + PMA). All experiments were performed in the presence of 100 μM Ro 20-1724. *, $p < 0.05$ compared with forskolin alone; **, $p < 0.01$ compared with MECA alone (Student's paired t test).

We also examined the effect of PMA in HEK-AC6-S674A cells because Ser674 is a site for negative regulation by PKC (Lin et al., 2002). These studies revealed that the magnitude of potentiation by PMA of forskolin-stimulated cyclic AMP accumulation on in HEK-AC6-S674A cells was increased significantly compared with cells expressing AC6-WT (2.9 ± 0.4-fold in HEK-AC6 cells; 5.8 ± 0.5-fold in HEK-AC6-S674A cells; $p < 0.01$; $n = 6$). The difference in the potentiation of adenylyl cyclase activation between cells expressing AC6-WT and cells expressing AC6-S674A suggests a role for Ser674 in the negative regulation of AC6 by PKC in intact cells (Lin et al., 2002). We exploited previous findings with AC6-S674A to explore further the negative portion of this bidirectional modulation of AC6. Ser674 is also a site of negative regulation for PKA in vitro (Chen et al., 1997); therefore, we examined the effects of PKA modulators on AC6 or AC6-S674A in intact cells in the absence of the confounding positive effects of PKC activators. To increase endogenous cyclic AMP levels and activate PKA, we pretreated cells with forskolin for 0 to 4 h. Forskolin pretreatment reduced the subsequent responsiveness of AC6-WT as early as 1 h with a near-maximal effect by 3 h (Fig. 3). In contrast to HEK-AC6 cells, no decrease in cyclic AMP accumulation was observed in cells expressing AC6-S674A under any conditions tested after pretreatment with forskolin (Fig. 3). Additional studies revealed that pretreatment with the cyclic AMP analog 8-(4-chlorophenylthio) adenosine 3′:5′-cyclic monophosphate also decreased subsequent forskolin-stimulated cyclic AMP accumulation in AC6-WT cells to 70 ± 4% of control values ($p < 0.05$; $n = 4$). In contrast, pretreatment with the PKA inhibitor H89 increased subsequent AC6 responses to 140 ± 6% of control values ($p < 0.05$; $n = 5$). Cyclic AMP accumulation in HEK-AC6-S674A cells was not altered by 8-(4-chlorophenylthio) adenosine 3′:5′-cyclic monophosphate (100 ± 1% of control) or H89 (101 ± 1% of control) pretreatment. These data demonstrate that Ser674 is a site for negative regulation of AC6 in intact cells as well as in vitro (Chen et al., 1997; Lin et al., 2002).

TABLE 1

PMA potentiates cyclic AMP accumulation in CHO cells

WT (10001) and PKA-deficient (10260) CHO cells were incubated for 15 min with 30 μM forskolin (FSK) in the absence or presence of 100 nM PMA. Data shown are mean ± S.E.M. of four to five experiments.

Cell Line	Cyclic AMP Accumulation	
	− PMA	+ PMA
	pmol/well	
CHO 10001 (WT)	16.0 ± 6.7	39.8 ± 11[a]
CHO 10260 (PKA-deficient)	15.1 ± 6.2	36.3 ± 16[a]

[a] $p < 0.05$ compared with forskolin alone for each cell line (Student's paired t test).

The Effects of PMA on AC6 Activation Are Mediated by PKC. To determine whether the effect of PMA on AC6 stimulation was caused by activation of PKC, we examined the effect with the PKC inhibitor bisindolylmaleimide on cyclic AMP accumulation. The addition of bisindolylmaleimide blocked the PMA potentiation of forskolin-stimulated cyclic AMP accumulation in HEK-AC6 cells (Fig. 4A). To further confirm that the effect of PMA on forskolin-stimulated cyclic AMP accumulation in HEK-AC6 cells was caused by PKC activation, we took advantage of the observation that prolonged activation of PKC by phorbol esters down-regulates PKC protein levels and activity (Rodriguez-Pena and Rozengurt, 1984; Stabel et al., 1987). To down-regulate PKC, HEK-AC6 and HEK-AC6-S674A cells were pretreated for 18 h with 1 μM PMA. Pretreatment with PMA abolished the ability of subsequent PMA treatment to potentiate forskolin-stimulated cyclic AMP accumulation in HEK-AC6 cells (Fig. 4B) as well as in HEK-AC6-S674A cells (data not shown). However, after PMA pretreatment, the ability of forskolin alone to stimulate cyclic AMP accumulation in HEK-AC6 cells was markedly reduced from 80.5 ± 6.0 pmol/well in vehicle-treated cells to 26.3 ± 1.0 pmol/well in PMA-treated cells ($p < 0.01$; $n = 5$) despite similar AC6 expression levels after the PMA pretreatment (Fig. 4B, inset). In contrast, PMA pretreatment enhanced subsequent forskolin-stimulated cyclic AMP accumulation in HEK-AC6-S674A cells from 29.8 ± 0.7 to 68.9 ± 1.0 pmol/well ($p < 0.001$; $n = 5$). The results of these studies provide support for the argument that the effects of PMA on AC6 activity involve PKC and further suggest a role of Ser674 in the PKC regulation of AC6 (Lin et al., 2002). To determine whether the prolonged (18-h) pretreatment with PMA altered phosphodiesterase activity, we examined the effects on PKC down-regulation on drug-stimulated cyclic AMP accumulation in the absence or presence of the phosphodiesterase inhibitor IBMX. The data presented in Table 2 demonstrate that PKC down-regulation attenuated the short-term effects of PMA on forskolin-stimulated cyclic AMP in both the absence and presence of IBMX. Likewise, PKC down-regulation markedly reduced the response of AC6 to forskolin under both stimulation conditions, suggesting that effects of prolonged (18-h) pretreatment with

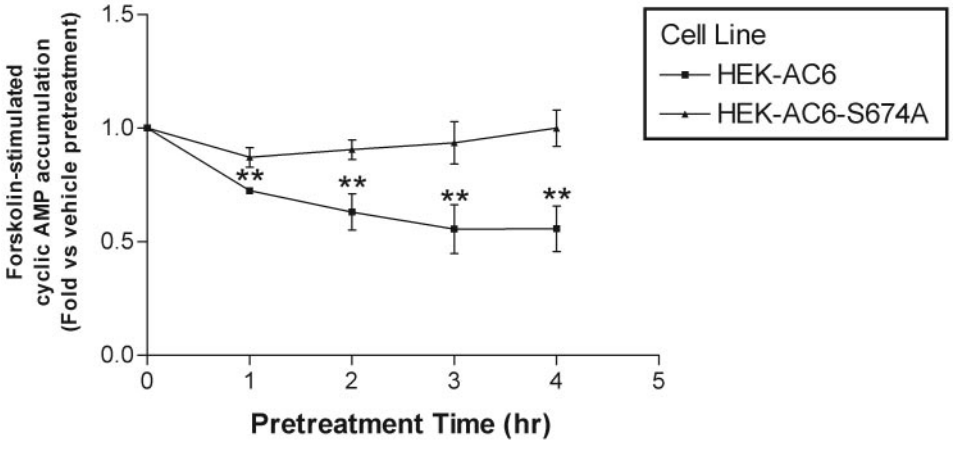

Fig. 3. Regulation of AC6 at Ser674 by PKA. HEK-AC6 and HEK-AC6-S674A cells were pretreated for 0 to 4 h with 1 μM forskolin (FSK). After pretreatment, cells were washed and incubated with 1 μM forskolin for 15 min. Data shown are mean ± standard error of the mean of four experiments. Data are normalized to forskolin-stimulated cyclic AMP values after vehicle pretreatment for each cell line and were HEK-AC6, 38.5 ± 3.7 pmol/well, and HEK-AC6-S674A, 64.3 ± 21. **, $p < 0.01$ compared with vehicle-pretreated cells (one-way analysis of variance with Dunnett's post test).

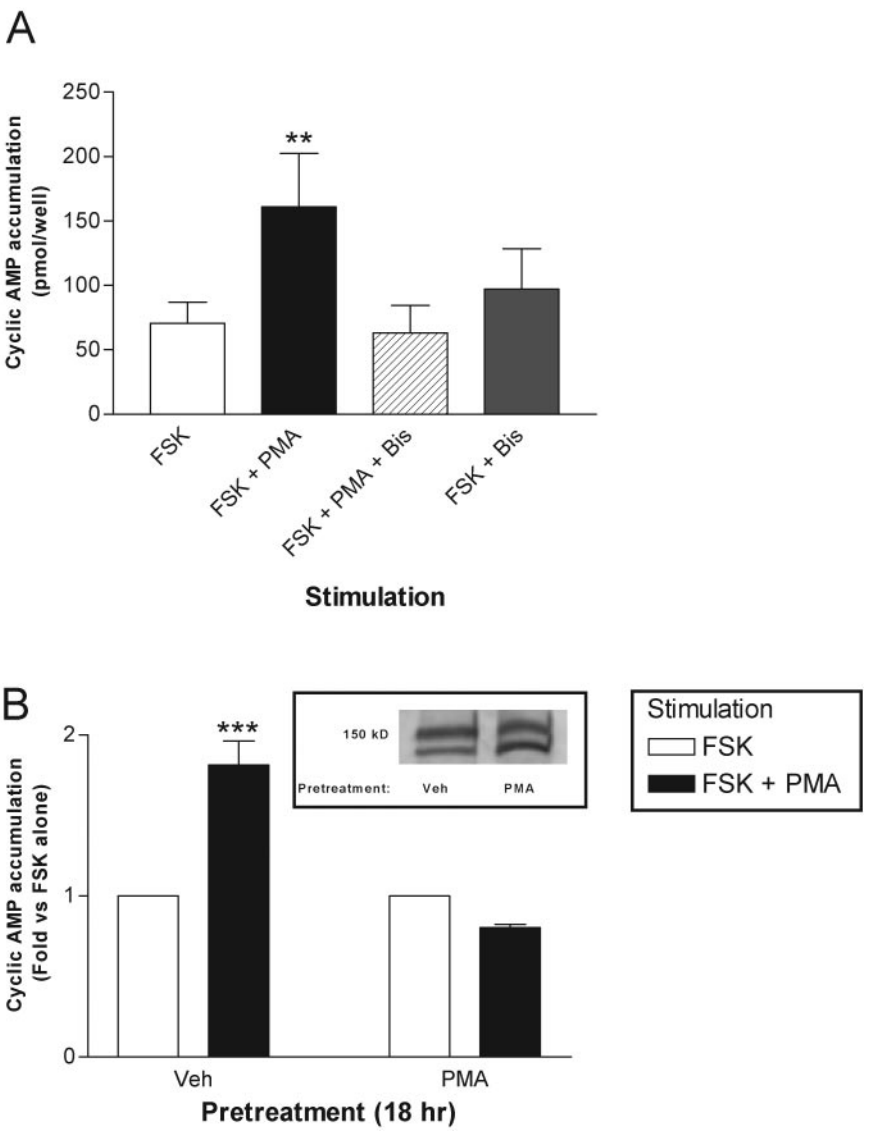

Fig. 4. Effect of bisindolylmaleimide and PKC down-regulation on PMA potentiation of drug-stimulated cyclic AMP accumulation. A, HEK-AC6 cells were incubated for 15 min with 1 μM forskolin (FSK), forskolin + 100 nM PMA (FSK + PMA), forskolin + PMA + 1 μM bisindolylmaleimide (FSK + PMA + Bis), or forskolin + bisindolylmaleimide (FSK + Bis). Data shown are mean ± standard error of the mean of six experiments. **, $p < 0.01$ compared with forskolin-stimulated values (one-way analysis of variance with Dunnett's post test). B, HEK-AC6 cells were pretreated for 18 h with vehicle (Veh) or 1 μM PMA (PMA). After pretreatment, cells were washed and incubated with 1 μM forskolin in the absence (FSK) or presence of 100 nM PMA (FSK + PMA) for 15 min. Data shown are mean ± standard error of the mean of five experiments. Data are normalized to forskolin alone after each pretreatment. ***, $p < 0.001$ compared with forskolin alone in vehicle-pretreated cells (Student's paired t test). Inset, cell membrane preparations were prepared from HEK-AC6 cells after 18-h pretreatment with vehicle (Veh) or 1 μM PMA (PMA). Membranes were probed with the anti-FLAG antibody. Immunoblot is representative of four independent experiments.

TABLE 2
Effect of IBMX on cyclic AMP accumulation after PKC down-regulation
HEK-AC6 cells were pretreated overnight (18 h) with vehicle or 1 μM PMA. After pretreatment, cells were washed and incubated for 15 min with 1 μM forskolin (FSK) or 1 μM forskolin and 100 nM PMA (FSK + PMA) in the absence or presence of 500 μM IBMX. Data shown are mean ± S.E.M. of eight experiments.

Pretreatment (18 h)	− IBMX		+ IBMX	
	FSK	FSK + PMA	FSK	FSK + PMA
	pmol/well			
Vehicle	50 ± 16	132 ± 28[a]	127 ± 17	207 ± 31[a]
PMA	7.6 ± 3.4	6.8 ± 2.8	40 ± 8.2	36 ± 6.9

[a] $p < 0.01$ compared with forskolin alone under matched condition (Student's paired t test).

PMA were not the result of enhanced phosphodiesterase activity.

The Role of Raf1 in the Potentiation of AC6 Stimulation by PKC. Because PKC directly inhibits AC6 in vitro (Lin et al., 2002), we examined the possibility that the positive effects of PMA and PKC on AC6 activity were caused by the activation of other intracellular proteins or pathways. PMA has been shown to lead to an activation of Raf1 in intact cells (Ueda et al., 1996), and Raf1 has recently been identified as a kinase that may be involved in an enhancement of forskolin-stimulated AC6 activity (Tan et al., 2001; Varga et al., 2002). Initial studies examined the effect of the Raf1 inhibitor GW5074 on the ability of PMA to potentiate forskolin-stimulated cyclic AMP accumulation in HEK-AC6 cells. Coincubation with GW5074 inhibited the potentiation of forskolin-stimulated AC6 activity by PMA in HEK-AC6 cells (Fig. 5A). This inhibitory effect of GW5074 was restricted to PMA potentiation of forskolin-stimulated cyclic AMP accumulation because GW5074 did not alter the response of forskolin alone in HEK-AC6 cells (Fig. 5A). Likewise, the addition of GW5074 failed to alter $G\alpha_s$-coupled receptor (using isoproterenol to activate the endogenous β-adrenergic receptor) stimulation of cyclic AMP accumulation in HEK-AC6 cells (isoproterenol alone, 10.6 ± 2.1 pmol/well above basal versus isoproterenol + GW5074, 14.7 ± 1.6 pmol/well above basal; $n = 5$). To examine further any nonspecific effects of GW5074 on adenylyl cyclase or PKC activity, we took advantage of the regulatory properties of another adenylyl cyclase isoform, AC2. AC2 is stimulated upon direct phosphorylation by PKC, and PKC activation synergizes with forskolin to

increase cyclic AMP accumulation in cells expressing AC2 (Jacobowitz et al., 1993; Yoshimura and Cooper, 1993; Jacobowitz and Iyengar, 1994; Bol et al., 1997). The present studies demonstrate that GW5074 did not seem to inhibit directly PKC activity because the ability of PMA to stimulate cyclic AMP production in HEK-AC2 cells was not reduced by coincubation with GW5074 (Table 3). Subsequent experiments revealed that GW5074 failed to alter forskolin-stimulated cyclic AMP accumulation as well as the synergistic cyclic AMP response to the combination of forskolin and PMA in HEK-AC2 cells (Fig. 5A). Together, these studies provide evidence that the effects of GW5074 are selective to PMA-mediated enhancement of AC6 activity and do not involve nonspecific effects on PKC, $G\alpha_s$, or adenylyl cyclase. The specificity and functional effects of GW5074 were further explored by examining the ability of GW5074 to block PMA-induced ERK1/2 phosphorylation in HEK-AC6 and HEK-AC2 cells. PMA induced a marked increase in ERK1/2 phosphorylation in both HEK-AC6 and HEK-AC2, whereas GW5074 alone seemed to reduce slightly basal ERK1/2 phosphorylation (Fig. 5B). Furthermore, these studies revealed that coincubation with GW5074 blocked completely PMA-stimulated ERK1/2 phosphorylation in both HEK-AC6 and HEK-AC2 cells (Fig. 5B). Our data suggest that PMA activation of PKC leads to a potentiation of forskolin-stimulated cyclic AMP accumulation that is dependent on Raf1 activation in HEK-AC6 cells. In contrast, PMA stimulation alone or PMA and forskolin-stimulated cyclic AMP accumulation in HEK-AC2 cells are independent of Raf1.

EGF Receptor Enhancement of AC6 Activity Is Raf1-Dependent. To examine the ability of receptors upstream of Raf1 to modulate AC6 activity, we incubated HEK-AC6 cells with forskolin in the absence or presence of EGF to activate endogenous EGF receptors. Similar to PMA, EGF did not alter cyclic AMP accumulation alone at concentrations up to 1 μg/ml (data not shown). However, EGF treatment robustly potentiated forskolin-stimulated cyclic AMP (Fig. 6A). Coincubation with the Raf1 inhibitor GW5074 completely blocked EGF potentiation of cyclic AMP accumulation in HEK-AC6 cells. In contrast, coincubation with the mitogen-activated protein kinase kinase inhibitor PD98059 did not attenuate the effects of EGF on cyclic AMP accumulation (Fig. 6B). Neither GW5074 nor PD98059 altered cyclic AMP accumulation by forskolin alone (data not shown). These data suggest that in addition to activation of PKC by PMA, activation of endogenous EGF receptors in HEK-AC6 cells can also potentiate drug-stimulated cyclic AMP responses in a Raf1-dependent manner.

Dominant-Negative Raf1 Blocks EGF, but Not PMA-Induced Potentiation of AC6. To further investigate the role of Raf1 in mediating the PMA- and EGF-induced potentiation of drug-stimulated cyclic AMP accumulation in HEK-AC6 cells, we transfected the cells with a dominant-negative Raf1 construct (Raf4N) consisting of two tandem Ras-binding domains and cysteine-rich domains. Expression of the Raf4N construct blocked the ability of EGF to potentiate forskolin-stimulated cyclic AMP accumulation (Fig. 7A). In contrast, Raf4N failed to block the more robust PMA potentiation of AC6 activation (Fig. 7B). This was despite the fact that the dominant-negative Raf1 attenuated both EGF- and PMA-induced phosphorylation of ERK (Fig. 7, A and B, inset). Because Raf4N interferes with Raf1 binding to Ras (Schaap et al., 1993; Brtva et al., 1995), these data may suggest that although PMA-induced ERK phosphorylation is Ras-dependent, PMA-mediated potentiation of AC6 activation is Ras-independent.

Putative Sites Involved in the Enhancement of AC6 Activity by PKC and Raf1. In addition to implicating Raf1 as a positive regulator of AC6, Tan et al. (2001) also identified two series of serine to alanine mutations that abolished both the phosphorylation and increased activation of AC6 by vanadate treatment. We constructed one of those mutants in

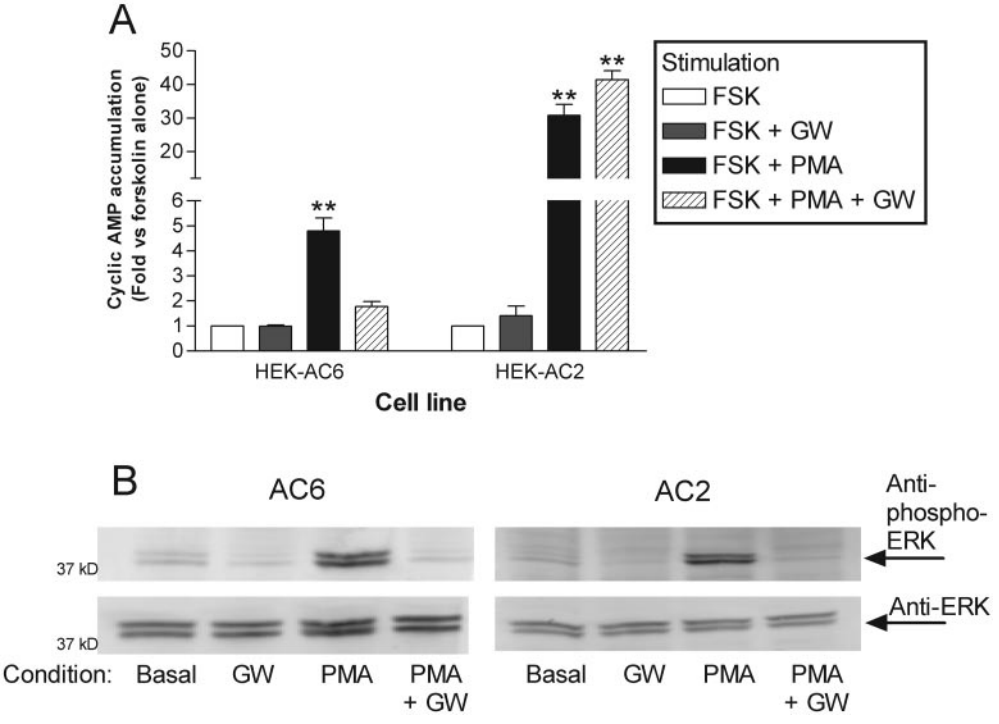

Fig. 5. Effect of Raf1 inhibition on adenylyl cyclase activation and ERK1/2 phosphorylation. A, HEK-AC6 and HEK-AC2 cells were incubated for 15 min with 1 μM forskolin (FSK), forskolin + 100 μM GW5074 (FSK + GW), forskolin + 100 nM PMA (FSK + PMA), or forskolin + PMA + GW5074 (FSK + PMA + GW). Data are normalized to cyclic AMP accumulation with forskolin alone for each cell line that were 12.1 ± 3.2 pmol/well in AC2 cells and 12.5 ± 1.3 pmol/well in AC6 cells. Data shown are mean ± standard error of the mean of four to five experiments. **, $p < 0.01$ compared with forskolin alone in each cell line (one-way analysis of variance with Dunnett's post test for each cell line). B, HEK-AC6 and HEK-AC2 cells were incubated with vehicle (basal), 100 μM GW5074 (GW), 100 nM PMA, or PMA + GW5074 for 15 min. Cells were lysed, and equal volumes were separated on polyacrylamide gels, transferred to PVDF membrane, and probed with the anti-phospho-ERK1/2 antibody. Membranes were subsequently stripped and reprobed with the anti-ERK1/2 antibody to confirm equal protein loading. Immunoblots shown are representative of three to four independent experiments.

TABLE 3
Effect of PKC and Raf1 inhibitors on AC2 activation by PMA
HEK-AC2 cells were incubated for 15 min with vehicle (basal) or 100 nM PMA (PMA) in the absence (control) or presence of 1 μM bisindolylmaleimide or 100 μM GW5074. Data shown are mean ± standard error of the mean of four experiments.

	Cyclic AMP Accumulation in HEK-AC2 cells		
	Control	+ Bisindolylmaleimide	+ GW5074
		pmol/well	
Basal	3.4 ± 1.3	4.5 ± 7.8	4.9 ± 1.5
+ PMA	41.8 ± 6.8	6.2 ± 1.6[a]	64.7 ± 7.6[b]

[a] $p < 0.001$ compared with PMA alone.
[b] $p < 0.01$ compared with PMA alone (one-way analysis of variance with Bonferroni's post test).

which Ser744, Ser746, Ser750, and Ser754 in the fourth intracellular loop were mutated to alanines (AC6-SIC4A). We transiently transfected AC6-WT or AC6-SIC4A into HEK293 cells to examine the effect of PMA on forskolin-stimulated cyclic AMP accumulation. Similar to the results of our studies using stably transfected HEK293 cells, we found that PMA potentiated forskolin-stimulated cyclic AMP accumulation in cells transiently transfected with AC6-WT (ca. 2.7-fold). In contrast, PMA had no significant effect in cells transiently transfected with vector alone or with AC6-SIC4A (Fig. 8A). The inability of PMA to potentiate forskolin-stimulated cyclic AMP accumulation in cells transfected with AC6-SIC4A did not seem to reflect reduced expression of the AC6-SIC4A mutant (Fig. 8B). These data suggest that the potentiation of AC6 stimulation by PMA activation of PKC requires at least one of the four serine residues in the intracellular 4 loop of AC6.

AC6 Interacts with Constitutively Active Raf1 in Intact Cells. Raf1 has recently been shown to physically interact with AC6 in enriched membrane preparations (Ding et al., 2004). To investigate the physical relationship between Raf1 and AC6 in our intact cell studies, we transiently transfected HEK-AC6 cells with vector control or a MYC-tagged Raf1-construct that contains a CAAX box on the C terminus that increases membrane localization and activity (Leevers et al., 1994; Stokoe et al., 1994). After transfection, lysates were immunoprecipitated with an anti-FLAG antibody to pull down the FLAG epitope-tagged AC6 in the HEK-AC6 cells. In cells transfected with MYC-Raf1-CAAX, the FLAG antibody was able to precipitate MYC immunoreactivity at 75 kDa, corresponding to the expected molecular mass of Raf1 (Fig. 9). Furthermore, the 75-kDa band corresponded to immunoreactivity precipitated with an anti-MYC antibody (MYC-Raf1-CAAX), as well as a Western blot sample prepared from cells transfected with MYC-Raf1-CAAX (Fig. 9). These data suggest that the ability of Raf1 to modulate AC6 activity may involve a direct protein-protein interaction.

Discussion

In the present study, we propose a model for AC6 regulation by PKC and EGF receptor activation (Fig. 10). Activation of PKC or EGFR leads to an increase in the ability of drugs to stimulate AC6. The observed increase in forskolin-stimulated cyclic AMP accumulation in HEK-AC6 cells by PKC activation is attenuated by the Raf1 inhibitor GW5074 and involves one or more serine residues in the fourth intracellular loop of AC6. Furthermore, EGF receptor activation also enhanced AC6 activation in a Raf1-dependent manner.

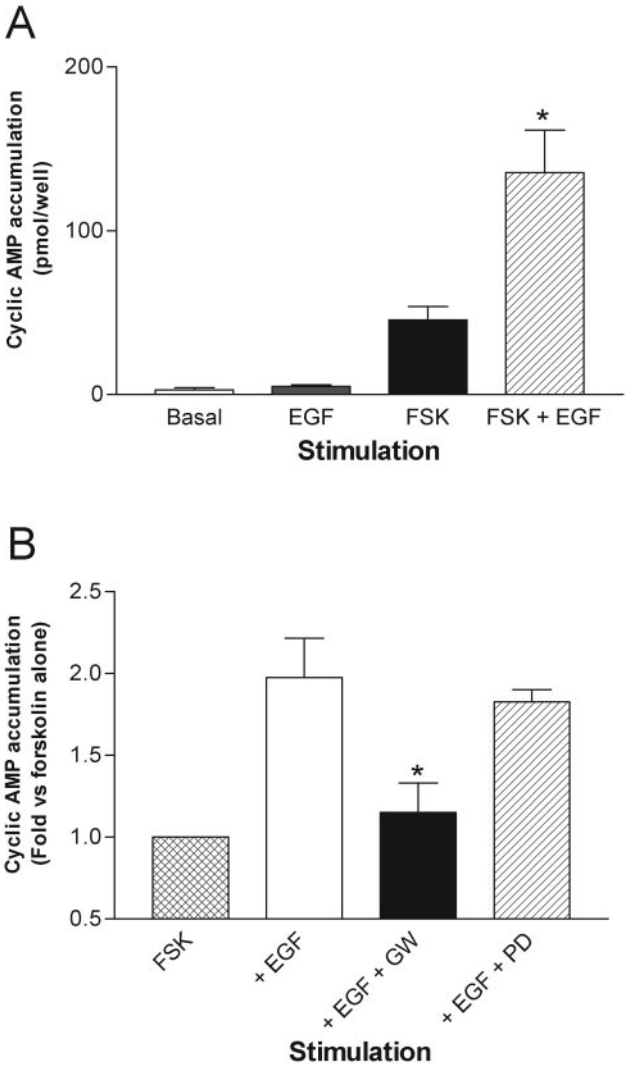

Fig. 6. Modulation of AC6 activity by EGF receptor activation. A, HEK-AC6 cells were incubated with vehicle (basal), 10 ng/ml EGF, 1 μM forskolin (FSK), or EGF + forskolin (FSK + EGF) for 15 min. Data shown are mean ± standard error of the mean of four experiments. *, $p < 0.05$ compared with forskolin alone (one-way analysis of variance with Bonferroni's post test). B, HEK-AC6 cells were treated with 300 nM forskolin (FSK), forskolin + 100 ng/ml EGF (+ EGF), FSK+ EGF + 100 μM GW5074 (+ EGF + GW), or FSK + EGF + PD89059 10 μM (+ EGF + PD). Data shown are mean ± standard error of the mean of three to five experiments. Data are normalized to cyclic AMP accumulation in response to forskolin alone that was 20.5 ± 7.8 pmol/well. *, $p < 0.05$ compared with FSK + EGF (one-way analysis of variance with Bonferroni's post test).

Previous intact cell studies initially identified PKC as a kinase involved in the desensitization of the cyclic AMP response by adenosine in PC12 cells; however, subsequent experiments directly examining the effect of PKC on AC6 activity were performed in vitro (Lai et al., 1997, 1999; Wu et al., 2001; Lin et al., 2002). PKC phosphorylation inhibits the ability of AC6 to be activated when expressed in insect or mammalian cell membranes by phosphorylating Ser10, Ser568, Ser674, and Thr931 (Lin et al., 2002). Mutation of Ser674 to alanine partially attenuates the ability of PKC to inhibit AC6 activity in vitro; however, additional mutations at either Ser10 or Ser568 are required to abolish the inhibition by PKC (Lin et al., 2002). In the present study, we

examined the regulation of AC6 by PKC in intact, stably transfected HEK293 cells as well as CHO and differentiated CAD cells that express endogenous AC6 using the phorbol ester PMA to activate endogenously expressed PKC isoforms. PMA treatment of HEK-AC6 cells enhanced forskolin- and isoproterenol-stimulated cyclic AMP accumulation, and this effect was blocked by down-regulation of PKC or the PKC inhibitor bisindolylmaleimide. Despite the enhancement of AC6 signaling by PMA, our data may provide indirect evidence that PKC activation leads to an inhibitory regulation in intact cells as it does in vitro. In particular, the potentiation of forskolin-stimulated cyclic AMP accumulation by PMA in HEK-AC6-S674A cells was greater than the potentiation observed in HEK-AC6 cells. This observation suggests that PMA activation in HEK-AC6 cells might result in two regulatory events. Additional studies using PKA activators in HEK-AC6 and HEK-AC6-S674A cells revealed that Ser674 is a site for negative modulation of AC6 in intact cells

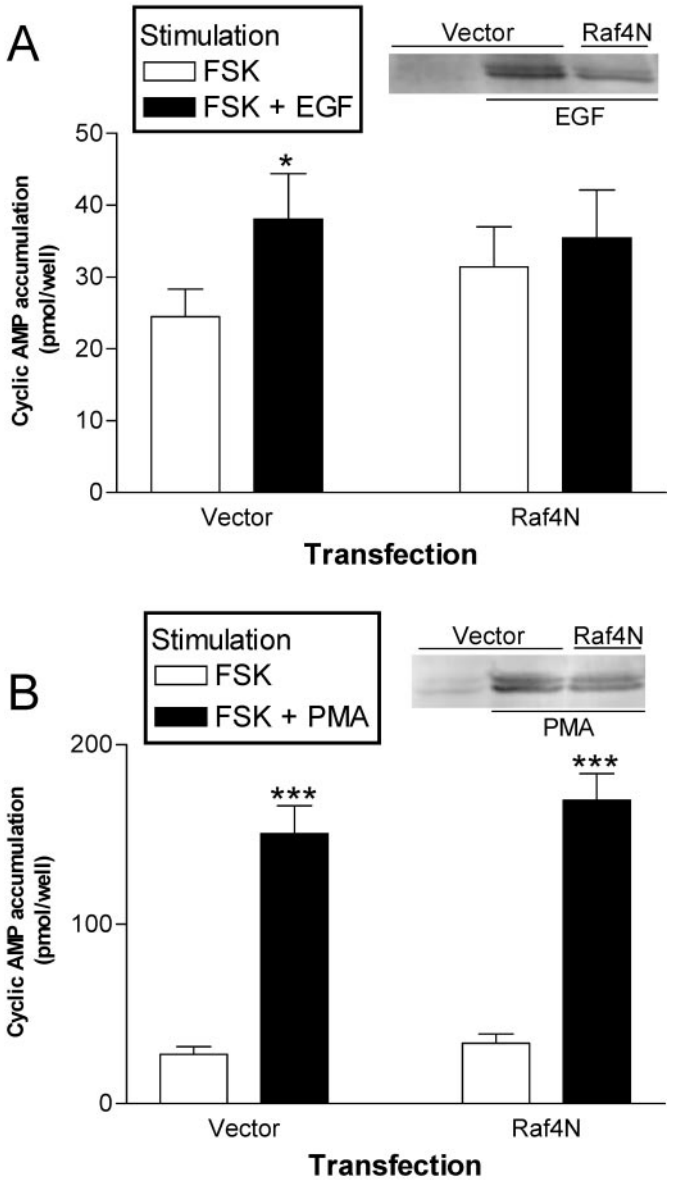

Fig. 7. Effect of dominant-negative Raf1 on PMA- and EGF-induced ERK phosphorylation and AC6 potentiation. HEK-AC6 cells were transfected as described under *Materials and Methods* section with vector control or Raf4N. At 24 to 48 h after transfection, cells were incubated with 1 μM forskolin in the absence (FSK) or presence of 10 ng/ml nM EGF (FSK + EGF) (A) or 100 nM PMA (FSK + PMA) (B). Data shown are mean ± standard error of the mean of five to seven experiments. *, $p < 0.05$ compared with forskolin alone in vector transfected cells (Student's paired t test); ***, $p < 0.001$ compared with forskolin alone in vector and Raf4N transfected cells (Student's paired t test). Insets, phospho-ERK activity in HEK-AC6 cells transfected with vector or Raf4N after incubation in the absence (vehicle) or presence of (A) 10 ng/ml EGF for 5 min or (B) 100 nM PMA for 15 min. Immunoblot shown is representative of two independent experiments.

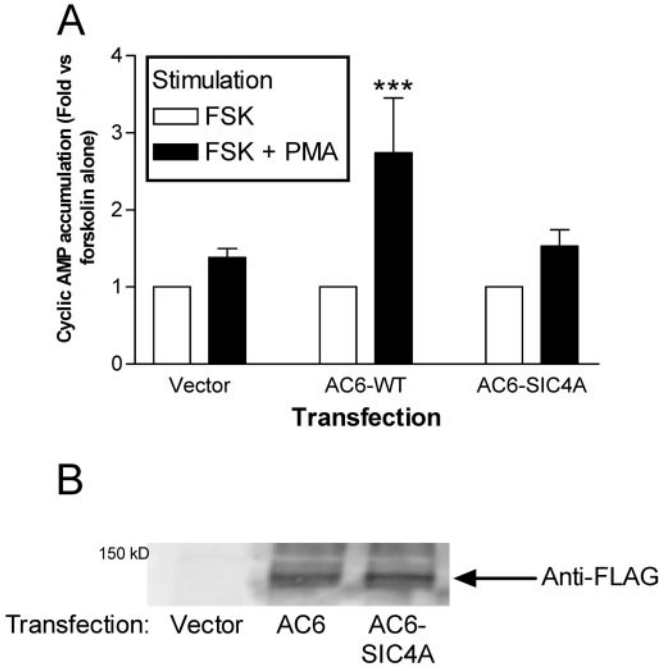

Fig. 8. Effect of PMA on AC6 lacking putative Raf1 phosphorylation sites. A, HEK293 cells were transfected as described under *Materials and Methods* with vector control, AC6-WT, or AC6-SIC4A. At 48 h after transfection, cells were treated with 3 μM forskolin in the absence (FSK) or presence of 100 nM PMA (FSK + PMA). Data shown are mean ± standard error of the mean of four experiments. Data are normalized to forskolin alone for each transfection. ***, $p < 0.001$ compared with forskolin alone (one-way analysis of variance with Bonferroni's post test). Forskolin-stimulated cyclic AMP accumulation values were 5.5 ± 1.1, 42.9 ± 9.3, and 11.4 ± 2.9 for cells transfected with empty vector, AC6-WT, and AC6-SIC4A, respectively. B, at 48 h after transfection, cells were lysed and lysates were centrifuged as described above. Equal amounts of protein from each sample separated on polyacrylamide gels, transferred to PVDF membrane, and probed with the anti-FLAG antibody. Immunoblot is representative of at least three independent experiments.

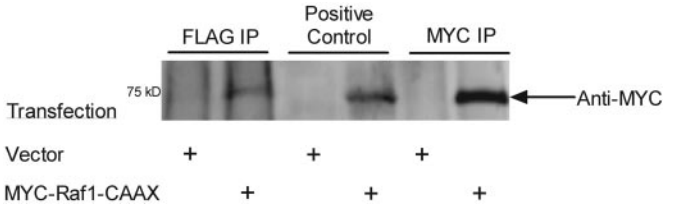

Fig. 9. Coimmunoprecipitation of AC6 and Raf1-CAAX. HEK-AC6 cells were transfected with Vector or MYC-Raf1-CAAX as described under *Materials and Methods*. At 48 h after transfection, cells were lysed and lysates were incubated with protein A-Sepharose beads in the presence or absence of anti-FLAG or anti-MYC antibodies and a linker antibody. After extensive washing, samples were boiled in sample buffer, separated on polyacrylamide gels, transferred, and immunoblotted with anti-MYC antibodies. Positive control samples are from Western blot samples of cells transfected with vector control or MYC-Raf1-CAAX. Immunoblot is representative of four independent experiments.

and provided indirect support for the bidirectional regulation of AC6 by PKC.

Because PKC directly inhibits AC6 in vitro (Lin et al., 2002), we examined other pathways that may be responsible for the PKC enhancement of AC6 signaling in intact cells. Recent studies have suggested that a downstream effector of the tyrosine kinase receptor may increase adenylyl cyclase activity. In particular, the tyrosine phosphatase inhibitor sodium orthovanadate enhanced forskolin-stimulated cyclic AMP accumulation in permeabilized HEK293 cells transfected with AC6 (Tan et al., 2001). The sodium orthovanadate-mediated increase in AC6 activity was blocked by cotransfection of a dominant-negative Raf1 construct (NΔRaf) and was abolished in cells expressing a Raf1-insensitive AC6 construct, AC6-SIC4A (Tan et al., 2001). To circumvent the potential nonspecific effects associated with orthovanadate treatment, we used the classical EGF receptor pathway to activate Raf1 as well as PMA-induced activation of Raf1 via PKC (Ueda et al., 1996; Prenzel et al., 2001). The Raf1 inhibitor GW5074 in HEK-AC6 cells attenuated both PKC and EGF receptor-mediated enhancement of AC6. The dominant-negative Raf1 construct Raf4N blocked EGF-, but not PMA-induced potentiation of AC6 activation. The lack of a Raf4N blockade on PMA potentiation may be caused by the relatively robust effect of PMA on AC6 stimulation or additional mechanisms for PKC-mediated enhancement of AC6 signaling that do not require Ras-Raf1 interactions, but that are attenuated by the Raf1 inhibitor GW5074. Furthermore, the PKC-mediated enhancement of cyclic AMP signaling was absent in cells transiently expressing AC6-SIC4A. This AC6 mutant contains the Ser750Ala mutation that was recently shown to be important for Raf1-stimulated AC6 phosphorylation and activation (Ding et al., 2004). The functional effects of Raf1 on AC6 activity seemed to involve physical interactions because AC6 and a constitutively active Raf1 were coimmunoprecipitated from HEK-AC6 cells. In addition, Raf1 has recently been demonstrated to interact with AC6 both physically and functionally in enriched membrane preparations (Ding et al., 2004). Together, the present findings and the recently published studies from Feldman and colleagues (Ding et al., 2004) provide strong evidence for a physical association of Raf1 with AC6. Moreover, these observations highlight the potential for tyrosine kinase receptor activation to modulate cyclic AMP signaling of individual adenylyl cyclase isoforms in several cellular models.

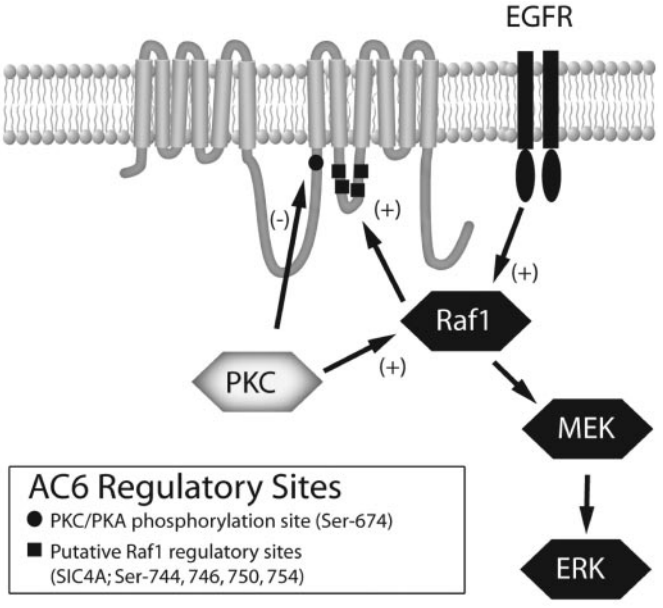

Fig. 10. Model for AC6 regulation by PKC and Raf1 in intact cells. PKC (and PKA) negatively regulate AC6 at Ser674. PMA activation of PKC leads to a Raf1-dependent increase in drug-stimulated cyclic AMP accumulation that involves one or more serine residues in the intracellular loop 4 of AC6. EGF receptor activation also enhances AC6 activity in a Raf1-dependent manner.

Previous studies of the closely related AC5 isoform have suggested a role for EGF receptor regulation of cyclic AMP accumulation in intact cells. In particular, EGF treatment of perfused rat hearts or isolated cardiomyocytes robustly increased cyclic AMP accumulation in a GTP-dependent manner (Nair et al., 1989, 1990; Yu et al., 1992). EGF receptor activation was shown to enhance AC5 activity via a phosphorylation of $G\alpha_s$ on one or more tyrosine residues (Nair et al., 1993; Poppleton et al., 1996). In HEK293 cells, EGF alone increased cyclic AMP accumulation in cells expressing AC5, but not AC6 (Chen et al., 1995). Here, we confirm that EGF alone does not effect cyclic AMP accumulation in HEK-AC6 cells, however, in the presence of forskolin, EGF enhanced cyclic AMP accumulation in a Raf1-dependent manner.

Studies linking tyrosine kinases to AC6 regulation may be relevant to identifying the mechanism(s) of heterologous sensitization of AC6. Heterologous sensitization of adenylyl cyclase occurs after the long-term activation of $G\alpha_{i/o}$-coupled receptors, and results in a subsequent increase in adenylyl cyclase activity (Watts, 2002). Heterologous sensitization after prolonged δ-opioid receptor activation in CHO cells correlates with an increase in phosphorylation of the predominant endogenous adenylyl cyclase isoform AC6 (Varga et al., 1998, 1999). The Raf1 inhibitor GW5074 or a combination of tyrosine kinase and PKC inhibitors have recently been demonstrated to markedly attenuate the magnitude of heterologous sensitization in CHO cells after prolonged δ-opioid receptor activation (Varga et al., 2002, 2003). Furthermore, it is well established that G protein-coupled receptor-mediated release of $G\beta\gamma$ leads to Raf1 activation (Schwindinger and Robishaw, 2001; Luttrell, 2002). That sequestration of $G\beta\gamma$ subunits prevents heterologous sensitization of AC6 and the closely related AC5 isoform may suggest a potential relationship where $G\beta\gamma$ induces an alteration in Raf1 signaling (Avidor-Reiss et al., 1996; Thomas and Hoffman, 1996; Rhee et al., 2000; Rubenzik et al., 2001). However, additional studies linking $G\beta\gamma$ dimers to modulate Raf1 activity in the context of heterologous sensitization and regulation of AC6 are necessary.

Although PKC phosphorylates and inhibits AC6 in vitro, the present study suggests that activation of PKC can also positively regulate AC6 in intact cells. A series of biochemical, genetic, and physical studies provided evidence that the positive effects of PKC activation on AC6 activity involve Raf1. We also demonstrated that EGF receptors conditionally enhanced forskolin-stimulated AC6 activity. These findings highlight the importance of cross-talk between multiple intracellular signaling pathways in intact cells to modulate cyclic AMP signaling and suggest that in addition to regulation by $G\alpha_s$- and $G\alpha_{i/o}$-coupled receptors, receptor tyrosine kinases are also important modulators of cyclic AMP signaling in cells expressing AC6.

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