Adenylyl cyclase 2 (AC2) selectively couples to EP\textsubscript{2} receptors while adenylyl cyclase 3 (AC3) is not receptor regulated in airway smooth muscle

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List of non-standard abbreviations: AC, adenylyl cyclase; ßAR, beta-adrenergic receptor;
mBSMC, mouse bronchial smooth muscle cells; cAMP, cyclic 3’,5’ adenosine monophosphate;
PGE2, prostaglandin E2; EP2R, prostacyclin EP2 receptor; RT-PCR, reverse transcriptase
polymerase chain reaction; PKA, protein kinase A; PKC, protein kinase C, PDE,
phosphodiesterase; AKAP, A Kinase Anchoring Protein.
ABSTRACT

Adenyl cyclases (AC) are important regulators of airway smooth muscle function, as β-adrenergic receptor (βAR) agonists stimulate AC activity and cAMP production. We have previously shown in a number of cell types that AC6 selectively couples to βAR and that these proteins are co-expressed in lipid rafts. We overexpressed AC2, AC3 or AC6 in mouse bronchial smooth muscle cells (mBSMC) and HEK-293 cells using recombinant adenoviruses and assessed their localization and regulation by various G protein-coupled receptors (GPCR). AC3 and AC6 were expressed primarily in caveolin-rich fractions, whereas AC2 expression was excluded from these domains. AC6 expression enhanced cAMP production in response to isoproterenol but did not increase responses to butaprost, reflecting the colocalization of AC6 with β2AR but not EP2R in lipid raft fractions. AC2 expression enhanced butaprost-stimulated cAMP production but had no effect on the β2AR-mediated response. AC3 did not couple to any GPCR we tested. Forskolin-induced arborization of mBSMC was assessed as a functional readout of cAMP signaling. Arborization was enhanced by overexpression of AC6 and AC3, but AC2 had no effect. GPCR-stimulated arborization mirrored the selective coupling observed for cAMP production. With addition of the PDE4 inhibitor, rolipram, AC2 did accelerate forskolin-stimulated arborization. Thus, AC2 selectively couples to EP2R but signals from this complex are limited by PDE4 activity. AC3 does not appear to couple to GPCR in either mBSMC or HEK-293 cells, so likely exists in a distinct signaling domain.
INTRODUCTION

Smooth muscle tone is influenced by extracellular hormones and neurotransmitters, many of which activate G protein-coupled receptors (GPCR’s) that modulate the activity of effector enzymes and the level of intracellular second messengers. Intracellular calcium and cyclic AMP (cAMP), key second messengers of GPCR’s, exert opposite effects on smooth muscle contraction, with Ca$^{2+}$ causing contraction and cAMP inducing relaxation (Torphy et al., 1982; Billington and Penn, 2003). ß-adrenergic receptor (ßAR) agonists, which stimulate cAMP production via activation of G_s and adenylyl cyclase (AC) activity, induce relaxation of smooth muscle (Kume et al., 1994). However, several investigators have found that ßAR agonists induce relaxation of airway smooth muscle via both cAMP-dependent and -independent mechanisms, possibly indicating other roles for cAMP signaling (Torphy, 1994; Ostrom and Ehlert, 1998; Spicuzza et al., 2001). In addition, other hormones can also regulate cAMP production in smooth muscle. GPCR-mediated stimulation or inhibition of AC activity regulates formation of cAMP which, via its activation of PKA, initiates both rapid actions, such as regulation of ion channels and effects on carbohydrate, protein and lipid metabolism, as well as more delayed effects, such as changes in gene expression, cell growth and proliferation (Billington et al., 1999; Scott et al., 1999).

Nine different transmembrane AC isoforms exist, each with different amino acid sequence, tissue and chromosomal distribution, and regulation (Hurley, 1999; Hanoune and Defer, 2001). Differences in regulation include stimulation or inhibition by G$\beta$γ, Ca$^{2+}$ and various protein kinases. AC5 and AC6 represent a subfamily of AC’s related in structure and regulation. These isoforms are inhibited by PKA, Ca$^{2+}$, nitric oxide, Gi and G$\beta$γ (McVey et al., 1999; Hill et al., 2000; Hanoune and Defer, 2001). By contrast, AC3 can be either stimulated by Ca$^{2+}$/calmodulin or specifically inhibited by calmodulin kinase-II while AC2 is activated by G$\beta$γ (Wei et al., 1996; Hanoune and Defer, 2001). While these unique properties of AC isoforms have been appreciated for some time (based on reconstituted enzymatic assays), the effect these features have on cell physiology are poorly understood (Sadana and Dessauer, 2009). One likely reason
for this is the fact that most cells express at least three or four different AC isoforms, implying a high degree of duplicity (Ostrom and Insel, 2004). Also, since all AC’s are activated by the same G protein and there are no good isoform-specific activators, the means by which one can selectively activate an AC isoform has been lacking.

It is becoming a commonly accepted notion that various proteins involved in GPCR signal transduction are enriched and spatially organized within plasma membrane microdomains (Neubig, 1994; Steinberg and Brunton, 2001; Ostrom, 2002). Numerous studies have focused on caveolae and lipid rafts as membrane microdomains where receptors, G proteins, effector molecules and even second messengers are concentrated (Shaul and Anderson, 1998; Davare et al., 2001; Ostrom and Insel, 2004). Caveolae are cholesterol- and sphingolipid-enriched portions of the membrane that form distinct flask-like invaginations when the protein caveolin is expressed (Anderson, 1998). Lipid rafts are microdomains of the plasma membrane that are biochemically similar to caveolae but morphologically indistinguishable from the rest of the plasma membrane (Hooper, 1999; Simons and Toomre, 2000; Galbiati et al., 2001). Data from several cell types indicates that AC6 selectively couples to βAR and not prostanoid EP receptors due to colocalization of AC6 and βAR in lipid rafts (Ostrom et al., 2001; Liu et al., 2008; Bogard et al., 2011). Non-raft localized EP receptors do activate cAMP production, but it is unknown which AC isoform(s) they couple to in order to regulate cell function.

The goal of this study was to determine the receptor coupling and functional consequences of two other AC isoforms, AC2 and AC3, and to determine what AC’s EP receptors signal through. In mBSMC and HEK-293 cells, AC2 is excluded from lipid raft domains and is regulated primarily by non-raft localized EP2 receptors. AC3 is localized to lipid raft fractions, much like AC6, but did not couple efficiently to any receptor we examined. Regulation of a downstream response, arborization, was also different between AC isoforms, with AC3 and AC6 able to accelerate arborization of mBSMC but AC2 unable to due to phosphodiesterase (PDE) activity. These data support the idea that GPCR-AC signaling can be highly compartmentalized and underscore the importance of defining the signaling proteins in these complexes and their
functional end-points.

MATERIALS AND METHODS

Materials: Primary antibodies for caveolin-1 (monoclonal, 610057), caveolin-2 (monoclonal, 610684) and caveolin-3 (monoclonal, 610420) were obtained from BD Biosciences. Primary antibodies for AC5/6 (polyclonal, sc-25500), AC2 (polyclonal, sc-587), AC3 (polyclonal, sc-588), β2-adrenergic receptor (polyclonal, sc-569), EP2 receptor (polyclonal, sc-20675) were obtained from Santa Cruz Biotechnology. PDE4 primary antibody was obtained from FabGennix (polyclonal, PD4-101AP). Secondary antibodies were obtained from Santa Cruz Biotechnology. Beraprost and butaprost were obtained from Cayman Chemical. All other chemicals and reagents were obtained from Sigma Aldrich.

Isolation of mBSMC: Male FVB/N mice were euthanized by intraperitoneal injection of ketamine/xylazine followed by exsanguination. Lungs with major airways were removed and put into KREBS buffer. Lower trachea and first bronchial branches were carefully dissected free of fatty and connective tissues and were opened by cutting the cartilage rings opposite to the trachealis muscle. The airways were then incubated with collagenases type F (2 mg/ml) and type H (1 mg/ml), under gentle agitation, at 37°C for 10 min. Single cells were picked with a micropipet under a microscope then cultured in SmGM-2 medium (Lonza Biosciences). Cells were used the following day or were cultured 3-5 days when larger cell populations were required.

Reverse-transcriptase PCR (RT-PCR): AC isoform expression was assessed by RT-PCR using the primer pairs previously described (Bogard et al., 2011). Total RNA was extracted from mBSMC grown to 60-70% confluency using RNeasy RNA isolation kit (Qiagen). A DNase reaction was performed to eliminate DNA contaminants and the RNA was reverse transcribed using Superscript II (Invitrogen) and poly-dT primer. PCR reactions with each primer pair were performed on cDNA, genomic DNA (positive control) and minus RT (negative control). The thermal profile for the reactions was 50°C for 2 min and 95°C for 10 min, followed by 35 cycles.
of 95°C for 10 sec and 60 - 62°C for 1 min. PCR products were analyzed by agarose gel electrophoresis and visualized under UV light with ethidium bromide.

**Measurement of cAMP accumulation:** mBSMC were grown to 70% confluency then washed three times with serum- and NaHCO₃-free DMEM supplemented with 20 mM HEPES, pH 7.4 (DMEH). After equilibration at 37°C for 30 min, cells were incubated with the indicated drugs plus 0.2 mM isobutylmethylxanthine, a broadly specific PDE inhibitor, for 10 min. In some assays, isobutylmethylxanthine was omitted and incubations times were shortened to 5 min. Assay medium was aspirated and 200 µL 7.5% trichloroacetic acid was added to each well to terminate each reaction. cAMP content of the lysis buffer extract was quantified using the cAMP EIA Kit (Cayman Chemical) using the manufacturer’s acetylation protocol. Data were normalized to the amount of protein in each sample, which was measured using a dye-binding protein assay (Bio-Rad). HEK-293 cells were grown to 80% confluency then incubated 16 h with 1 µCi/well of [³H]adenine. Cells were washed three times with serum- and NaHCO₃-free DMEM supplemented with 20 mM HEPES, pH 7.4 (DMEH). After equilibration at 37°C for 30 min, cells were incubated with the indicated drugs plus 0.2 mM isobutylmethylxanthine, for 10 min. Assay medium was aspirated and 250 µL 7.5% trichloroacetic acid was added to each well to terminate each reaction. Approximately 1,000 cpm of [³²P]cyclic AMP is added to each sample as an internal standard, then [³H]cyclic AMP and [³H]ATP were separated using the chromatography method described by Salomon et al (Salomon et al., 1974). Loss of internal standard was used to correct each sample before expressing data as percent conversion of cAMP from ATP.

**Non-detergent isolation of caveolar and non-caveolar membranes:** Cells grown to 70-80% confluency were washed twice in cold PBS. 1.5 ml of 500 mM sodium carbonate, pH 11, was added and cells scraped off the plate. Cells were homogenized with 20 strokes in a tissue grinder then three 20 sec bursts with an ultrasonic cell disruptor on medium power. A 1 min rest period was included in between each ultrasonic burst. An equal volume of 90% sucrose in MBS (25 mM MES and 150 mM NaCl, pH 6.5) was added to the homogenate to make 45% sucrose
and loaded in an ultracentrifuge tube. Two discontinuous sucrose layers were formed on top of the sample by placing 2 ml of 35% sucrose in MBS with 250 mM sodium carbonate then adding 1 ml of 5% sucrose (also in MBS/Na$_2$CO$_3$). The gradient was centrifuged at 46,000 rpm on a SW55Ti rotor (Beckman Coulter, Fullerton, CA) for 16 h at 4°C. Fractions were collected from the top in 0.5 mL increments, yielding a total of 10 fractions. An equal volume of each individual fraction was then subjected to SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) before being transferred to a PVDF membrane (Millipore) via electroblotting. After blocking membranes in 20 mM phosphate buffered saline (PBS) with 3% nonfat dry milk, primary antibody (see Materials and Methods) was added for 12 h at 4°C with constant rocking. Bound primary antibodies were visualized using appropriate secondary antibody with conjugated horseradish peroxidase (Santa Cruz Biotechnology) and ECL reagent (Pierce). Some primary antibodies recognized multiple non-specific protein species. In these cases, appropriately sized immunoreactive bands were identified based upon expected molecular weight of the protein of interest and only these bands are shown.

**Immunofluorescent confocal microscopy:** mBSMC were fixed in 3% paraformaldehyde/PBS at RT for 15 min and permeabilized with 0.1% Brij 98/PBS for 2 min. Cells were incubated with primary monoclonal antibody for 2-4 hr followed by a fluorochrome-conjugated secondary antibody at RT for 1 hr. Cells were imaged using a Bio-Rad 1024 confocal microscope (Bio-Rad, Hercules, CA). For confocal microscopic analysis, cell samples were visualized using single line excitation at 488 nm or 555 nm for GFP or Alexa 594, respectively, with appropriate emission filters. In each experiment, approximately 70 to 100 cells were analyzed for each treatment, and each experiment was performed at least three times.

**Arborization:** mBSMC were cultured in chamber slides and incubated for 24 hr with the indicated recombinant adenovirus. Titer of each virus was optimized to obtain similar increases in forskolin-stimulated cAMP production (Figure 4A). Cells were placed on a heated stage and visualized with a digital inverted microscope with a DIC objective. Images were captured prior to and each minute following addition of either forskolin (0.1 µM), isoproterenol (0.1 µM),
beraprost (0.3 µM) or butaprost (0.3 µM) for a total of 40 min. Pixel intensity of individual cells was calculated for each frame and the change in intensity was expressed as a percent of basal (prior to drug addition). 3-4 cells per experiment were analyzed then averaged to obtain a single rate of arborization for each condition in that experiment. The arborization rate was then expressed relative to the rate measured in control (LacZ) cells from the same experiment.

Data analysis and statistics: Data are presented as the mean ± S.E.M., and in some cases as representative images, of at least 3 separate experiments. Statistical comparisons (t-tests and one-way analysis of variance) and graphics were performed using Prism 5.0 (GraphPad Software).

RESULTS

In order to study the signaling by overexpressed AC isoforms one must first define the native expression of AC isoforms. We performed RT-PCR analysis with isoform-specific primers to detect mRNA for each of the nine mammalian, G protein-regulated AC isoforms in mBSMC. While RNA template (sample not treated with reverse transcriptase, noRT) yielded no PCR product for any of the primers, we did detect appropriately sized PCR products for ß-actin (positive control), AC1, AC3 and AC5 (Figure 1A). In some experiments we detected faint bands for AC4 but we were unable to sequence this small amount of PCR product. Sequencing of each of these PCR products confirmed the appropriate sequence. Thus, mBSMC express AC1, AC3 and AC5 mRNA and possibly low levels of AC4.

To determine the expression of receptors capable of stimulating AC activity in mBSMC, we measured cAMP accumulation in response to maximal or near-maximal concentrations of agonists selective for GPCR known to couple to G_s in other cells. Ten minutes after drug addition and in the presence of a phosphodiesterase inhibitor, IBMX, we detected increased cAMP levels in response to the AC activator forskolin (10 µM), isoproterenol (1 µM), prostaglandin E_2 (PGE_2, 1 µM), butaprost (1 µM), beraprost (1 µM) and adenosine-5’N-ethylcarboxamide (NECA, 1 µM). We did not detect increased cAMP levels after incubating
mBSMC with calcitonin gene-related peptide (CGRP, Figure 1B), pituitary adenylate cyclase activating peptide, 5-hydroxytryptamine or dopamine (data not shown). These data are consistent with the expression of ßAR, prostaglandin EP₂/4R, prostacyclin IPR and adenosine A₂a/2b receptors in mBSMC.

We next sought to understand the localization of AC isoforms and GPCR expressed in mBSMC by fractionating cells and isolating lipid raft fractions. Using a non-detergent method to fractionate cells, we detected expression of all three isoforms of caveolin in buoyant fractions from mBSMC (Figure 2). Immunoblot analysis from native mBSMC using isoform-specific antibodies detected only faint bands for AC3 and AC5/6 (the latter antibody is unable to differentiate between AC5 and AC6 isoforms) and no bands of appropriate size for other AC isoforms (Supplemental Data, Figure 1). We did not attempt to detect AC1 expression, as no suitable antibody is commercially available. We did detect native ß₂AR and EP₂R expression by immunoblot analysis, with ß₂AR expressed exclusively in buoyant, lipid raft fractions enriched in caveolins and EP₂R detected only in non-raft fractions (Figure 2). PDE4 exists in four different genes (PDE4A-D) each with splice variants that yield 15 different isoforms (Houslay and Adams, 2003). Some PDE4 isoforms do localize to lipid rafts (Abrahamsen et al., 2004). We detected multiple PDE4 isoforms (using a pan-PDE4 antibody) primarily in non-raft fractions, but immunoreactivity for one isoform was faintly detectable in lipid raft fractions.

mBSMC were then incubated with recombinant adenoviruses expressing either AC2, AC3 or AC6 and fractionated to ascertain the expression and localization of these AC isoforms. Incubating cells with an adenovirus expressing AC6 (AdV-AC6) increased AC5/6 immunoreactivity primarily in buoyant, lipid raft fractions (Figure 2). Incubating cells with AC2 or AC3 adenoviruses also led to detectible AC expression, with AC2 in non-raft fractions and AC3 in lipid raft fractions. Commercially available AC isoform antibodies yield multiple bands from mBSMC and many other cell types (Liu et al., 2008; Bogard et al., 2011). Genuine immunoreactive bands were confirmed by comparing blots from control and AC-overexpressing cells (Supplemental Data, Figure 1). Consistent with findings from other cell types, AC3 and
AC5/6 localize to lipid rafts while AC2 localized to non-raft membranes (Ostrom et al., 2000; Ostrom et al., 2002; Ostrom and Insel, 2004; Bogard et al., 2011).

Because cell fractionation studies can suffer from artifactual problems, we also assessed AC isoform localization using immunofluorescent confocal microscopy. mBSMC were cultured on coverslips, permeabilized and fixed, then incubated with antibodies for AC isoforms and caveolin-1. In native mBSMC we detected staining for both AC3 and AC5/6 and each of these proteins displayed approximately 20% overlap with caveolin-1 staining (Figure 3 and Table 1). We detected very low levels of staining for AC2. Based on immunoblot analyses, a significant amount of non-specific staining for natively expressed AC isoforms is expected with the antibodies available. This limits the usefulness of staining for native AC’s, particularly those expressed at lower levels. To partially overcome this limitation of these antibodies we assessed immunofluorescent microscopy in cells overexpressing each AC. In mBSMC incubated with AdV-AC2, we detected immunofluorescent staining that had less than 10% overlap with caveolin-1. By contrast, cells incubated with either AdV-AC3 or AdV-AC6 displayed specific staining for AC3 and AC5/6, respectively, that overlapped with caveolin-1 staining by approximately 30%. Thus, staining of intact cells supports the conclusion that mBSMC express AC3 and AC5/6 and that these isoforms localize to lipid-rafts. AC2 is not natively expressed in mBSMC, but when it is exogenously expressed it localizes in non-raft membranes.

One key question is if overexpression of different AC isoforms alters the responses to various Gs-coupled receptors. In order to directly compare the effects of expressing different AC isoforms, we varied the viral titer for each condition to attain equivalent levels of maximal (10 µM) forskolin-stimulated cAMP production. This is a sensitive measure that corresponds directly with the level of AC expression and circumvents the problem of using antibodies that are varied in their specificity and sensitivity (Gao et al., 1998). Using the viral titers that produced equivalent forskolin-stimulated cAMP levels, we observed that basal cAMP accumulation did not differ between control cells and cells overexpressing AC2 or AC6, but did increase slightly when AC3 was overexpressed (Figure 4A).
We then analyzed how receptor-specific agonist responses differed in cells overexpressing different AC isoforms. We used cAMP production as a measure of coupling efficiency between a receptor and an overexpressed AC isoform. cAMP accumulation responses were normalized to lacZ (control) cells to determine if adenoviral expression of a given AC isoform led to increased cAMP signaling by a given agonist. The agonist concentrations used stimulated approximately half-maximal cAMP production in concentration-response curves (performed in HEK-293 cells, data not shown). AC2 expression increased cAMP responses by 0.1 µM forskolin and 0.3 µM butaprost, the EP2 receptor-selective agonist but did not increase responses to 0.1 µM isoproterenol or 0.3 µM beraprost (Figure 4B). AC3 overexpression increased responses to forskolin but did not enhance response to any of the receptor agonists we used. AC6 expression led to increased responses to forskolin, isoproterenol and beraprost but did not alter butaprost responses. Thus, AC2 appears to only couple to EP2R while AC6 couples to only β2AR and IPR in mBSMC. AC3 does not appear to couple to any of the GPCR we activated. These data are consistent with the idea that GPCR’s couple only to co-localized AC isoforms. However, the fact that AC3 does not couple to lipid raft resident β2AR implies that intramolecular interactions, not just raft or non-raft membrane localization, may be critical.

Similar studies were conducted in HEK-293 cells to determine the generalizability of the observed specific GPCR-AC coupling. HEK-293 cells natively express AC2, AC4, AC5/6 and AC9 isoforms with their predicted lipid raft (AC5/6) and non raft (AC2, AC3 and AC9) localization (Ostrom and Insel, 2004). Maximal forskolin-stimulated cAMP production was equivalent in cells treated with each of the recombinant adenoviruses, indicating expression and activity of each AC isoform (Figure 4C). AC2 overexpression enhanced PGE2-stimulated cAMP production but had no effect on responses to isoproterenol. AC3 expression did not enhance cAMP production to either isoproterenol nor PGE2. AC6 overexpression selectively enhanced isoproterenol responses. Thus, the selective coupling of EP2/4 receptors to AC2 and the lack of GPCR coupling of AC3 appears to be a generalized phenomenon.
Our measures of bulk cellular cAMP levels do not necessarily reflect local, compartmentalized cAMP generation due to the standard practice of including a broad-spectrum PDE inhibitor. We re-examined cAMP production stimulated by forskolin but excluded the PDE inhibitor in order to determine if PDE activity might constrain cAMP diffusion in specific compartments. Without IBMX, the time to peak cAMP levels in forskolin-stimulated mBSMC was 5 min (data not shown). Therefore, we measured cAMP levels 5 min after drug addition and compared this response to mBSMC expressing each of the AC isoforms. Forskolin (10 µM) stimulated increases in cAMP production 2.3-fold over basal levels in control cells (Figure 4D). AC3 and AC6 expression increased this forskolin-stimulated response to 3-4 fold over basal, but AC2 expression had no effect on the forskolin response. Thus with the cell’s full complement of PDE activity, we could not observe increased bulk cytosolic cAMP levels emanating from AC2.

We hypothesized that cAMP signaling compartments exist that couple the GPCR-AC signalosomes defined above to distinct cellular functions. One response that can be assayed in single cells is arborization. Arborization is a cell shape change that is stimulated by cAMP signaling in smooth muscle cells (Gros et al., 2006). We measured the rate of arborization in individual cells by analyzing the pixel density change over time (40 min) under a digital microscope. The rate of arborization following drug addition in cells overexpressing either AC2, AC3 or AC6 was then compared to the rate measured in lacZ (control) mBSMC. Forskolin-stimulated arborization rate was increased by AC3 or AC6 expression but was unaltered in mBSMC expressing AC2 (Figure 5A). The inability of AC2 to increase arborization rate parallels it’s inability to increase bulk cytosolic cAMP levels in the absence of PDE inhibition (Figure 4). We also examined how receptor-specific agonists regulate arborization of mBSMC. Isoproterenol-stimulated arborization was accelerated by overexpression of AC6, but not AC2 or AC3. Butaprost, an EP2R agonist, stimulated a slow arborization response in mBSMC and none of the AC isoforms that we expressed accelerated this response. Beraprost, an IPR agonist, stimulated arborization, the rate of which was accelerated by AC6 expression but was
unaltered by expression of AC2 or AC3. Therefore, arborization in mBSMC can be mediated by signaling through AC3 and AC6, but not through AC2.

It has been recently demonstrated that PDE’s are critical enzymes in establishing and maintaining cAMP signaling compartments (Jin et al., 1998; Zaccolo and Pozzan, 2002). We hypothesized that PDE activity might restrict AC2-derived cAMP from diffusing from the site of generation, not allowing it to stimulate arborization. Concentrations as low as 2 µM IBMX accelerated arborization, making any further drug-induced response difficult to measure. Therefore, we treated cells with a PDE4-specific inhibitor, rolipram (1 µM) and measured arborization responses. In these conditions, forskolin-stimulated arborization rate was accelerated by AC2, AC3 and AC6 expression (Figure 5B). Isoproterenol-stimulated arborization remained unaffected by AC2 and AC3 in these conditions, indicating that PDE4 activity does not effect the selective GPCR-AC coupling we have observed. We could not observe butaprost-mediated arborization responses in the presence of rolipram, perhaps due to the small size of this response being overwhelmed by the PDE inhibitor (data not shown). Nonetheless, these data are consistent with the idea that a PDE, likely PDE4, spatially constrains signaling by cAMP emanating from AC2.

DISCUSSION

One goal of these studies was to determine the AC isoform expression profile of mouse airway smooth muscle in order to understand how this model might relate to humans. Our data show that mBSMC express mRNA for AC1, AC3 and AC5 along with low levels of AC4. We could only detect expression of AC3 and AC5/6 protein, although immunological approaches to define AC’s can be problematic (Bogard et al., 2011). This AC expression profile differs significantly from human airway smooth muscle cells, which express AC2, AC4 and AC6 (Bogard et al., 2011). AC2 and AC4 expression predominates in human airway smooth muscle, meaning these cells express significant non-raft localized AC’s. This contrasts with mouse, which expresses mostly lipid raft isoforms AC3 and AC5. Thus mice may not be a particularly
good model of GPCR-AC regulation of airway tone due to these key differences in AC isoform expression.

mBSMC, as with all other cells we have examined, express AC3 and AC6 in caveolin-rich lipid raft fractions. A significant proportion of β2AR appear to also reside in these domains in the basal state. AC2 expression appeared exclusively in non-raft fractions where we observed expression of EP2R. We have previously used the increased $E_{\text{max}}$ of an agonist at stimulating cAMP production as an index of GPCR-AC coupling (Ostrom et al., 2001; Liu et al., 2008; Bogard et al., 2011). These prior studies indicate that increased AC expression principally alters $E_{\text{max}}$ without effect on EC$_{50}$. We found that GPCR-stimulated responses in mBSMC were effected differently by expression of each AC isoform. βAR-mediated cAMP production was only enhanced by expression of AC6, not AC2 or AC3. This was somewhat surprising given the lipid raft colocalization between AC3 and β2AR and may reflect the fact that specific signaling complexes are formed within lipid rafts. Prostacyclin (IP) receptors displayed a similar coupling profile, raising the possibility that AC3 does not couple effectively to GPCR’s. EP2R, localized in non-raft fractions, coupled only to fellow non-raft resident, AC2. Similar studies in HEK-293 cells show that these effects can be generalized to other cell types. Therefore, lipid rafts define microdomains that may compartmentize cAMP signaling by GPCR, but other factors may help define how a receptor preferentially couples to a particular AC isoform.

Commercially available antibodies for AC isoforms, which we use in the present studies, do have significant limitations. In general, these antibodies cross-react with several other proteins yielding multiple immunoreactive bands (see Supplemental data). One must be able to separate these other proteins and validate genuine AC bands by expressing the AC of interest and comparing to immunoreactivity in control cells. As we show in Figure 3, immunofluorescent microscopy is difficult in cells not overexpressing AC due to high non-specific binding. Another issue is that certain AC isoform antibodies are more reliable than others. Therefore, one should not rely upon these tools for characterizing AC isoform expression in cells or tissues without significant corroborating evidence using other approaches (Erdorf and Seifert, 2011).
Previous studies of vascular smooth muscle cells show that PGE$_2$ signals primarily through AC3, a result that conflicts with our present findings (Wong et al., 2001). These investigators found PGE$_2$-mediated cAMP production and growth inhibition were reduced in used AC3 knockout mice. A few key experimental differences could explain these different outcomes. First, Wong et al. only used PGE$_2$ in their studies, not the selective EP$_2$R agonist butaprost we employed, making it likely that they activated multiple prostanoid receptors in their studies. Second, we examined airway smooth muscle and HEK-293 cells while Wong and co-workers investigated vascular smooth muscle, raising the possibility that these cell types differ in their formation of GPCR-AC complexes. Of course, we also used overexpression of AC3 instead of knockout to probe for receptor coupling. Native AC3 does couple to GPCR in certain cells, particularly in the olfactory bulb (Choi et al., 1992).

Pieri et al. described increased basal activity of AC2 compared to AC6 when expressed in Sf9 insect cells (Pieri et al., 1995). We did not observe increased levels of cAMP in mBSMC (mammalian) cells when either AC2 or AC6 was overexpressed (Figure 4). This difference in AC2 basal activity may be due to the cellular environment in which it is expressed, with distinct regulatory proteins and molecules in these cell types. For example, AC2 is stimulated by G$\beta\gamma$ signaling, which may be more tightly regulated in mBSMC than Sf9 cells. Furthermore, the studies by Pieroni were performed in isolated membranes with defined Mg$^{2+}$ concentrations where our studies use intact cells and the native cellular milieu. More studies are needed to fully understand the differences in AC isoform activity when expressed in different contexts.

Arborization of smooth muscle cells is a useful response to monitor since it is mediated by cAMP and can be readily detected in single cells (Gros et al., 2006). We found that arborization rate stimulated by forskolin (which activates all AC isoforms, although not necessarily to the same degree (Erdorf et al., 2011)) is enhanced by overexpression of AC3 or AC6 but not by AC2. These data imply that cAMP regulation of cytoskeletal reorganization is compartmentalized such that non-raft generated second messenger can't access the arborization machinery. Overexpression of AC6 enhances arborization in response to βAR or prostacyclin receptor
activation, but does not effect responses to EP₂R. Overexpression of AC3 did not alter responses to any of the GPCR agonists tested, despite enhancing forskolin-mediated arborization, reflecting it’s inability to couple to receptors. AC2 expression had no effect on arborization rate in any of our conditions.

With the hypothesis that PDE activity limits diffusion of cAMP from the site of generation, we repeated arborization studies in cells treated with a specific PDE4 inhibitor. In these conditions, AC2 did accelerate forskolin-mediated arborization but had no effect on βAR-mediated arborization. These findings are consistent with the idea that PDE4 limits access of AC2-derived cAMP to the arborization machinery, but has no role in determining the specific coupling between GPCR’s and a given AC isoform. This hypothesis is supported by the fact that we could not detect increased cAMP levels in the bulk cytosol of cells when AC2 was overexpressed and no PDE inhibitor was used. Further support comes from immunoblot analysis of all PDE4 isoforms showing the bulk of PDE4 in mBSMC resides in non-raft fractions (Figure 2). Thus, AC2 appears to exist in a compartment in which PDE expression and activity severely limits the diffusion and action of cAMP. A schematic diagram of our hypothesized arrangement of signaling in mBSMC is shown (Figure 6).

Previous work in vascular smooth muscle supports the notion that different AC isoforms can be coupled to specific cellular responses. Gros et. al. showed that AC1, but not AC6, could regulate ERK1/2 activity and cell proliferation while only AC6 could regulate VASP phosphorylation (Gros et al., 2006). Thus AC isoforms appear to be central players in organized signaling complexes, which include AKAP’s, PDE’s and other proteins, that can specifically regulate a subset of cellular responses (Efendiev and Dessauer, 2011; Ostrom et al., 2012). Specific AKAP’s can bring PDE’s to the sites of cAMP synthesis via distinct interactions with isoforms of AC, and this localized PKA activity can regulate PDE and AC activity and/or GPCR function in near membrane compartments (Rich et al., 2007; Dessauer, 2009). Yotiao is one such AKAP that is known to bind both PDE4 and AC2 (Piggott et al., 2008; Terrenoire et al., 2009). More studies are needed to assess the role of AC’s, and specific AKAP’s and PDE’s, in
the compartmentalized responses we observe. Because of long AC protein half-lives, we have not successfully knocked down AC isoform expression in mBSMC. This type of approach is needed to determine what AC’s mediate arborization, or other cellular responses, in native smooth muscle cells.

Given that the AC isoform expression profile largely determines the compartments in which cAMP is generated, and that mouse airway smooth muscle AC expression profile differs significantly from human, we suggest that mouse models of airway function regulated by GPCR-AC pathways may not accurately reflect responses in humans. Nonetheless, it is clear that airway smooth muscle signaling is highly compartmentalized at proximal steps (GPCR and AC) with lipid rafts/caveolae acting as sites for βAR-AC5/6 signalosomes and non-raft membranes serving as sites for EP<sub>2</sub>R-AC2 signalosomes. cAMP regulation of cytoskeletal reorganization, a more distal event, is also compartmentalized, with PDE, particularly PDE4, activity required for maintaining the observed compartmentation. Therapies that take advantage of these distinct signaling complexes in order to more specifically alter bronchodilation would represent exciting new treatment modalities.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Bogard, Adris and Ostrom.

Conducted experiments: Bogard, Adris and Ostrom.

Contributed new reagents or analytic tools:

Performed data analysis: Bogard and Ostrom.

Wrote or contributed to the writing of the manuscript: Bogard and Ostrom.

Other: Ostrom acquired funding for the research.
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FOOTNOTES

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FIGURES

**Figure 1.** A: AC isoform mRNA expression in mBSMC was measured by RT-PCR. AC isoform-specific primer pairs we used with 35 cycles of RT-PCR (see *Materials and Methods*). Representative image of 3 experiments is shown. Arrows indicate expected size of PCR product. Molecular weight markers (MW) are shown in lane 1, β-actin primers were used with no reverse transcriptase (noRT) or cDNA (β-actin) templates. B: cAMP accumulation in whole cells pretreated with IBMX was measured (see *Materials and Methods*) in response to various agonists known to stimulate AC activity in other cells. Data are presented as mean ± S.E.M. of n=3-4. * denotes p < 0.05, ** denote p > 0.01 by paired t-test as compared to basal (no drug).

**Figure 2.** Immunoblot analysis of fractions from lipid raft isolation from mBSMC. Cells were fractionated using a non-detergent method and separated by sucrose density centrifugation (see *Materials and Methods*). Gradients were collected in ten, 0.5 mL fractions and analyzed for appropriate separation of marker proteins (not shown). Fractions were separated by SDS-PAGE and analyzed by immunoblotting using the indicated primary antibody. In some studies, cells were incubated with recombinant adenoviruses (AdV) expressing AC2, AC3 or AC6 for 24 hr. Shown are regions of the gels at the approximate molecular weight of the expected immunoreactive band. Images shown are representative of 3-5 experiments.

**Figure 3.** Colocalization of AC isoforms and caveolin-1 in mBSMC. Cells were blocked with 1% BSA and then incubated with rabbit anti-AC2, AC3, or AC6, or mouse anti-caveolin-1 antibodies at 37°C for 1 h. After washing, the cells were incubated with Alexa 594-conjugated goat anti-mouse IgG and Alexa 488-conjugated goat-anti-rabbit IgG for 1 hr. In some studies, cells were incubated with recombinant adenoviruses (AdV) expressing AC2, AC3 or AC6 for 24 hr. Images shown are representative of 4-5 experiments. Degree of overlap calculated from the merged images is shown in Table 1.
Figure 4. cAMP accumulation in mBSMC or HEK-293 cells expressing AC2, AC3 or AC6. mBSMC (A, B and D) or HEK-293 cells (C) were incubated with recombinant adenoviruses expressing either lacZ (control), AC2, AC3 or AC6 then cAMP production was measured (see Materials and Methods). A: mBSMC were stimulated with a maximal concentration of forskolin (Fsk) in the presence of IBMX. * denotes p < 0.05, ** denotes p > 0.01 by paired t-test as compared to basal. # denotes p < 0.05 by paired t-test as compared to lacZ condition. B: mBSMC were treated with 0.1 µM Fsk, isoproterenol (Iso 0.1 µM), butaprost (0.3 µM) or beraprost (0.3 µM) in the presence of IBMX. Data are presented as the percent increase in cAMP levels when an AC was expressed over the lacZ condition, with the dashed line indicating no increase over lacZ. * denotes p < 0.05, ** denotes p > 0.01 by paired t-test as compared to lacZ. C: HEK-293 cells were treated with vehicle, 10 µM Fsk, 1 µM Iso, or 1 µM PGE₂. Data are presented as the fold increase in basal cAMP levels (vehicle-treated lacZ cells), with the dashed line indicating no increase over basal. * denotes p < 0.05, ** denotes p > 0.01 by paired t-test as compared to lacZ. D: mBSMC treated with a maximal concentration of forskolin (Fsk) or vehicle without the inclusion of a PDE inhibitor. * denotes p < 0.05, ** denotes p > 0.01 by paired t-test as compared to lacZ. # denotes p < 0.05 by paired t-test as compared to basal. All data are presented as mean ± S.E.M. of n=3-5.

Figure 5. Arborization of mBSMC following overexpression of either AC2, AC3 or AC6. Cells were incubated with the indicated drug and time-lapse photography was used to monitor cell shape changes for 40 min after addition of the indicated drug in the absence (panel A) or presence of the PDE4-specific inhibitor, rolipram (1 µM, panel B). The pixel density of 3-4 cells in each image was calculated and the rate of increase in pixel density over time was used to calculate arborization rate. The percent change in arborization rate versus lacZ cells was calculated and data is presented as the mean ± S.E.M. of 3 experiments (9-12 observations). * denotes p < 0.05, ** denotes p > 0.01 by paired t-test as compared to lacZ. Representative images of the cell shape change is shown at top.
Figure 6. Schematic diagram of GPCR/AC localization and signaling in mBSMC. β2AR and prostacyclin receptors (IPR) primarily signal via AC6 localized in lipid raft microdomains. AC3, when overexpressed, localizes in these same domains but does not appear to couple to any GPCR. Signals emanating from this domain mediate arborization of the cells. In non-raft membranes, prostanoid EP2 receptors localize with AC2, but cAMP signals from these locales do not appear to regulate the arborization response. PDE4 is expressed in this location and its activity appears responsible for limiting cAMP in this compartment, as treatment of cells with rolipram uncovers an EP2R-AC2 stimulation of arborization.
TABLE 1

Co-localization of native and overexpressed AC isoforms with caveolin-1 in mBSMC.

<table>
<thead>
<tr>
<th></th>
<th>AC2</th>
<th>AC3</th>
<th>AC6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control cells</strong></td>
<td>1.4 ± 0.8%</td>
<td>21.3 ± 5.1%</td>
<td>20.2 ± 2.3%</td>
</tr>
<tr>
<td><strong>Overexpression</strong></td>
<td>9.6 ± 1.2%</td>
<td>31.4 ± 6.8%</td>
<td>29.7 ± 1.8%</td>
</tr>
</tbody>
</table>
Figure 6

lipid raft

caveola

AC3

Iso

β2AR

G

Gs

AC5/6

Gs

IPR

Bera

Buta

EP2R

Gs

AC2

PDE4

Arborization

pDE4

IBMX or Rolipram

cAMP

cAMP

cAMP

non-raft

MB