0026-895X/02/6204-983–992$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 62:983–992, 2002

Vol. 62, No. 4
1708/1016459
Printed in U.S.A.

Localization of Adenylyl Cyclase Isoforms and G Protein-Coupled Receptors in Vascular Smooth Muscle Cells: Expression in Caveolin-Rich and Noncaveolin Domains

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Received March 1, 2002; accepted July 24, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

A number of different agonists activate G protein-coupled receptors to stimulate adenylyl cyclase (AC), increase cAMP formation, and promote relaxation in vascular smooth muscle. To more fully understand this stimulation of AC, we assessed the expression, regulation, and compartmentation of AC isoforms in rat aortic smooth muscle cells (RASMC). Reverse transcription-polymerase chain reaction detected expression of AC3, AC5, and AC6 mRNA, whereas immunoblot analysis indicated expression of AC3 and AC5/6 protein primarily in caveolin-rich membrane (cav) fractions relative to noncaveolin (noncav) fractions. β_1-Adrenergic receptors (AR), β_2AR, and G_s were detected in both cav and noncav fractions, whereas the prostanoid receptors EP_2R and EP_4R were excluded from cav fractions. We used an adenoviral construct to increase AC6 expression. Overexpressed AC6 localized only in noncav fractions. Two-fold overexpression of AC6 caused enhancement of forskolin-, isoproterenol- and prostaglandin E_2-stimulated cAMP formation but no changes in basal levels of cAMP. At higher levels of AC6 overexpression, basal and adenosine receptor-stimulated cAMP levels were increased. Stimulation of cAMP levels by agents that increase Ca^{2+} in native cells was consistent with the expression of AC3, but overexpression of AC6, which is inhibited by Ca^{2+}, blunted the Ca^{2+}-stimulable cAMP response. These data indicate that: 1) RASMC express multiple AC isoforms that localize in both caveolin-rich and noncaveolin domains, 2) expression of AC6 in non–caveolin-rich membranes can increase basal levels of cAMP and response to several stimulatory agonists, and 3) Ca^{2+}-mediated regulation of cAMP formation depends upon expression of different AC isoforms in RASMC. Compartmentation of GPCRs and AC is different in cardiomyocytes than in RASMC, indicating that targeting of these components to caveolin-rich membranes can be cell-specific. Moreover, our results imply that the colocalization of GPCRs and the AC isoforms they activate need not occur in caveolin-rich fractions.

Multiple G protein-coupled receptors (GPCR) expressed in vascular smooth muscle, including β-adrenergic receptors (βAR), can reduce vascular tone. These GPCRs exert their effects by coupling to the heterotrimeric G protein G_s and stimulating adenylyl cyclase (AC) activity. Activity of AC in smooth muscle produces the second messenger cAMP, which, via activation of protein kinase A, alters intracellular Ca^{2+} dynamics and contractile function by phosphorylating calcium channels, Ca^{2+}-ATPases, and myosin light-chain kinase (Nishikawa et al., 1984; Lincoln and Cornwell, 1991; Somlyo and Somlyo, 1994). In addition, βAR agonists seem to induce relaxation via a cAMP- and PKA-independent mechanism in smooth muscle from some tissues (Kume et al., 1994; Ostrom and Ehlert, 1998; Spicuzza et al., 2001). Hypertension and aging both seem to be associated with compromised βAR (and perhaps other GPCR)-mediated signaling in vascular smooth muscle (Feldman, 1987; Werstiuk and Lee, 2000). This decrease in responsiveness to catecholamines is probably attributable to several factors, including a decrease in βAR expression, increased G_i protein and increased G protein receptor kinase 2 expression (Anand-Srivastava, 1992; Brodde and Michel, 1992; Gros et al., 2000). Recent data document the fact that increasing cellular expression of AC has the potential to improve and

This work was supported by National Institutes of Health grants HL63885 and HL53773.
R.S.O. and X.L. contributed equally to this work.

ABBREVIATIONS: GPCR, G protein-coupled receptor; AR, adrenergic computer; AC, adenylyl cyclase; RASMC, rat aortic smooth muscle cells; CaMK, calmodulin-dependent kinase II; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; MES, 2-[*N*-morpholino]ethanesulfonic acid; MBS, MES-buffered saline; DTT, dithiothreitol; NB, nuclei buffer; WCL, whole-cell lysates; CB, cacodylate buffer; HPMA, hydroxypropyl methacrylate; Ro20-1724, 4-(3-butoxy-4-methoxybenzyl)2-imidazolidinone; EP_xR, prostanoid EP receptor (x = 2 or 4); NECA, 5′-*N*-ethylcarboxamidoadenosine; PGE, prostaglandin E; ICI-118,551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3[(1-methylethyl)amino]-2-butanol; CGP-20712A, [2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluormethyl-2-imidazolyl)-phenoxy]-2-propanolmethanesulfonate.

restore βAR function in cardiovascular disease (Roth et al., 1999; Feldman, 2002).

It has recently been appreciated that GPCRs and their associated signaling components are not randomly dispersed throughout the plasmalemma. In several cultured cell models and cardiac muscle cells, expression of both βARs and AC is enriched in distinct caveolin- and sphingolipid/cholesterol-rich microdomains of the plasma membrane, i.e., caveolae (Schwencke et al., 1999a,b; Ostrom et al., 2000b, 2001; Rybin et al., 2000). In these microdomains, one observes interaction with (or retention of) proteins that have particular post-translational modifications, such as palmitoylation (Shaul et al., 1996). In addition, caveolin in caveolae contains a binding ("scaffolding") domain that interacts with certain signaling molecules, thereby facilitating localization of such signaling molecules in caveolae. Compartmentation of signaling molecules challenges the concept that components of GPCR signal transduction are randomly distributed and have extensive mobility in the plasma membrane. Instead, these sparsely expressed proteins seem to be restricted to plasmalemmal microdomains, probably facilitating rapid and specific signal transduction (Anderson, 1998; Okamoto et al., 1998; Ostrom et al., 2000a).

In the present study, we tested the hypothesis that expression and localization of GPCRs and isoforms of AC might be critical determinants of how vascular smooth muscle cells respond to extracellular signals. We thus assessed GPCR-AC compartmentation in rat aortic smooth muscle cells (RASMC). Analysis of the expression of GPCR and AC isoforms in caveolin-rich membrane fractions from these cells shows that these proteins seem not to be as highly compartmentalized in these microdomains as such fractions from cardiomyocytes. Overexpression of different levels of a particular isoform of AC, AC6, altered both the regulation of basal levels of cAMP production and the coupling of GPCRs to cAMP generation. Furthermore, we observe that regulation of cAMP levels by intracellular Ca^{2+} concentrations in native RASMC is consistent with expression of a Ca^{2+}-stimulable isoform of AC. We propose that GPCRs and postreceptor signaling components show differential, cell-specific patterns of subcellular compartmentation, implying that the determinants of such localization cannot be exclusively dependent upon primary structure of the interacting proteins but instead probably involves other, as-yet-unknown factors. Cellular compartmentation of AC isoforms and components that regulate their activity thus seem to be a means by which differentiated cells "tailor" their responses to extracellular and intracellular signals.

Materials and Methods

Materials and Cell Culture. Primary antibodies for caveolin isoforms and calmodulin-dependent kinase II (CaMK-II) were obtained from BD PharMingen (San Diego, CA). Trp1 antibody was obtained from Sigma (St. Louis, MO). EP_2R and EP_4R primary antibodies were a generous gift from John W. Regan (University of Arizona, Tucson, AZ). All other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The AC5/6 antibody from this commercial source does not distinguish between AC5 and AC6. Radiolabeled chemicals were obtained from PerkinElmer Life Sciences. Other chemicals and reagents were obtained from Sigma. RASMC were obtained from Dr. Wolfgang Dillmann (University of California San Diego, La Jolla, CA). These cells were isolated from thoracic aortas of 8- to 12-week-old male Sprague-Dawley rats (Chamley-Campbell et al., 1979; Seasholtz et al., 1999). RASMC were maintained in high-glucose DMEM with 16% fetal bovine serum and penicillin/streptomycin and kept in a 37°C incubator with 10% CO_2 and were used between passages 5 and 12 for all experiments.

β-Galactosidase Staining. Cells were incubated in growth medium for 18 h with either vehicle or lacZ-expressing adenovirus (1000–3000 viral particles/cell) then washed and equilibrated for 24 h. RASMC were then washed twice with PBS and fixative solution (2% formaldehyde, 0.05% glutaraldehyde in PBS) for 5 min at room temperature. Fixative solution was then aspirated, cells were washed 3 times with PBS and incubated with 5-bromo-4-chloro-3-indolyl-β-D-galactoside solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside in PBS) at 37°C for 3 to 6 h before visualization and documentation with a light microscope and mounted 35-mm camera.

Reverse Transcriptase PCR. Total RNA was extracted from RASMC grown to 80 to 90% confluence on 10-cm plates using TRIzol reagent (Invitrogen, Carlsbad, CA). A DNase reaction was performed to eliminate DNA contaminants and the RNA was reverse transcribed using Superscript II (Invitrogen) and poly(T) priming. Primer pairs for each known isoform of AC were designed to unique sequences in the highly homologous C1a or C2a regions of AC. PCR reactions with each primer pair were performed on cDNA template, genomic DNA (positive control) and minus RT (negative control) template. Primer sequences used are shown in Table 1. PCR products were analyzed by agarose gel electrophoresis and visualized under UV light with ethidium bromide.

Membrane Fractionation. Cells were fractionated using a detergent-free method adapted from Song et al. (1996) as described previously (Ostrom et al., 2000b). Two 15-cm plates containing 70 to 80% confluent RASMC were washed twice in ice-cold PBS and scraped into a total of 2 ml of 500 mM sodium carbonate, pH 11. Cells were homogenized with a tissue grinder with three 10-s bursts and then a sonicator with three 20-s bursts. The homogenate was brought to 45% sucrose by addition of an equal volume of 90% sucrose in MBS (25 mM MES and 150 mM NaCl, pH 6.5) and loaded in an ultracentrifuge tube. A discontinuous sucrose gradient was layered on top of the sample by placing 4 ml of 35% sucrose prepared in MBS with 250 mM sodium carbonate then 4 ml of 5% sucrose (also in MBS/Na_2CO_3). The gradient was centrifuged at 39,000 rpm on a SW41Ti rotor (Beckman Coulter, Fullerton, CA) for 16 to 18 h at 4°C. The top 2.0 ml of the gradient were discarded and the faint light-scattering band was collected from the 5 to 35% sucrose interface (caveolin-enriched membranes). The bottom 4 ml of the gradient (45% sucrose) was collected as noncaveolar membranes.

Purification of Nuclei. Approximately 30 million cells were washed twice with ice-cold PBS, then incubated in 5 ml of DMEM

TABLE 1
Primer sequences used

AC1	5′-ACC AGC CAA GAG GAT GAA GTT-3′
	5′-ATA CCA GCA GCA GCA GGA CAG-3′
AC2	5′-CAT GTT TGC CTC CAT TCC-3′
	5′-GCA TCC AGT TTT CCC ACC-3′
AC3	5′-ACC AAG ATC AAA ACC ATC G-3′
	5′-GAA AAA GGT CAG AAG CTC C-3′
AC4	5′-ACC AAG GCT ACA CTC AAC TAC-3′
	5′-GGT TCA TCT TGG CGA TCA-3′
AC5	5′-TCT CAT CCC TCT CAC ACC-3′
	5′-CAT ACA GCA CAC TCA CTC C-3′
AC6	5′-ATG ACT CTA GCA AAG AGA ACC-3′
	5′-AAC AGA TGA AGC AGA AAA CC-3′
AC7	5′-GCT CCT ACT GAA GCC CAA GTT C-3′
	5′-AAT CAC TCC AGC AAT CAC AGG C-3′
AC8	5′-TGT ACT AAC CAA ACT CAC C-3′
	5′-GTG TAA AAA GCA CGT AGC-3′
AC9	5′-CAC CGC AAA ATA CTT AGA TGA CG-3′
	5′-CCT TCT CCT GCA AGA TCT CAC AC-3′

plus 10 μM cytochalasin B for 40 min at 37°C. The cells were washed twice with ice-cold PBS and scraped into 2 ml of PBS. The cells were centrifuged at 800*g* for 5 min to collect cells, and the supernatant was discarded. The pellet was resuspended in 2 ml of NB buffer (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, and protease inhibitor mix, pH 7.5) and centrifuged at 800*g* for 5 min. The supernatant was removed, and the pellet was resuspended in 1 ml of NB buffer containing 10 μM cytochalasin B and incubated on ice for 20 min. The cells were transferred to a chilled glass homogenizer and disrupted with 20 gentle strokes. The homogenate was transferred to a microcentrifuge tube, and 200 μl of NB buffer containing 40% sucrose was layered underneath the homogenate, then was centrifuged at 800*g* for 15min. The pellet was resuspended in 200 μl of PBS and analyzed by immunoblotting.

Measurement of Adenylyl Cyclase Activity. AC activity was measured in caveolin-3 immunoprecipitates as described previously (Ostrom et al., 2001). Briefly, cells were scraped and homogenized in a modified Lysis buffer with a lower concentration of detergent (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, and 0.5% Igepal CA-630, plus mammalian protease inhibitor cocktail), incubated with caveolin-3 monoclonal antibody (BD PharMingen) for 1 h, precipitated by incubation with protein A-agarose then resuspended in membrane buffer (30 mM Na-HEPES, 5 mM $MgCl_2$, 2 mM DTT, pH 7.5). Sample (30 μl; immunoprecipitate or supernatant) was added into tubes containing 30 mM Na-HEPES, 100 mM NaCl, 1 mM EGTA, 10 mM $MgCl_2$, 1 mM isobutylmethylxanthine, 1 mM ATP, 10 mM phosphocreatine, 5 μM GTP, 60 U/ml creatine phosphokinase, and 0.1% bovine serum albumin, pH 7.5, and drugs of interest. Tubes were incubated for 15 min at 30°C and reaction was stopped by boiling for 5 min. cAMP content of each tube was assayed for cAMP content by radioimmunoassay as described previously (Ostrom et al., 2000b). Total protein concentration was determined using a dye-binding protein assay (Bio-Rad, Hercules, CA).

Immunoprecipitation. Immunoprecipitations were performed as described previously (Ostrom et al., 2001). Briefly, 15-cm plates of RASMC were washed twice with cold PBS, scraped in 1 ml of Lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, plus mammalian protease inhibitor cocktail) and homogenized in a Dounce homogenizer. Samples were incubated with primary antibody for 1 to 3 h then precipitated by incubating with protein A-agarose overnight. Pellets were washed once in lysis buffer followed by washes in wash buffers 2 (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.2% Igepal CA-630) and 3 (10 mM Tris-HCl, pH 7.5, and 0.2% Igepal CA-630). Immunoprecipitated proteins were analyzed by immunoblot analysis.

Immunoblot Analysis. Individual fractions and whole cell lysates (WCL) were separated by SDS-polyacrylamide gel electrophoresis (Nu-PAGE, Invitrogen). Equal volumes of each fraction and a half-volume of WCL were loaded, resulting in approximately 6-fold lower amount of protein loaded in the cav fraction lanes. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) by electroblotting. Membranes were blocked in 20 mM phosphate-buffered saline (PBS) with 3% nonfat dry milk and incubated with primary antibody (see *Materials and Methods*) overnight at 4°C. Bound primary antibodies were visualized using appropriate secondary antibody with conjugated horseradish peroxidase (Santa Cruz Biotechnology) and enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ). Most primary antibodies recognized multiple nonspecific species. Only the band representing the appropriately sized molecule is shown. The amount of protein per fraction was determined using a dye-binding protein assay (Bio-Rad). In some cases, membranes were stripped using Re-Blot reagent (Chemicon International, Temecula, CA) and reprobed with another primary antibody.

Transmission Electron Microscopy. RASMCs were washed in PBS and fixed for 2 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (CB), then washed three times with CB and fixed with 1% osmium tetroxide (OsO_4) in CB. Cells were washed briefly in CB and treated with 0.5% fresh tannic acid in CB. Dishes were additionally washed with CB followed by two washes with ddH_20. EM blocks were stained with 2% uranyl acetate in 10% ethanol for 1 h at room temperature. The sections were then dehydrated through a standard series of 5 min each in 50%, 75%, 2× 95%, and 2× 100% ethanol/HPMA (1:1) then at 100% HPMA/LX112 (1:1) (Ladd Research Industries, Burlington, VT) followed by 1× at 100% LX112 for 10 min each. The dishes were then embedded in 100% fresh LX112, polymerized overnight at 60°C, sectioned, then stained with uranyl acetate and lead nitrate.

Measurement of cAMP Accumulation. RASMC were incubated with adenovirus for 24 h and then were washed extensively and allowed to equilibrate in maintenance media for 24 h. Cells were washed three times with serum- and $NaHCO_3$-free DMEM supplemented with 20 mM HEPES, pH 7.4, and equilibrated for 30 min. Assay for cAMP accumulation was performed by incubation with drugs of interest and 0.2 mM isobutylmethylxanthine, a cyclic nucleotide phosphodiesterase inhibitor, for 10 min. In some experiments, an alternative phosphodiesterase inhibitor, 10 μM Ro20-1724, was used instead of isobutylmethylxanthine. Basal and agonist-stimulated cAMP levels were similar in RASMC incubated with isobutylmethylxanthine and Ro20-1724. To terminate reactions, assay medium was aspirated, and 250 μl of ice-cold trichloroacetic acid (7.5%, w/v) was immediately added to each well. cAMP content in trichloroacetic acid extracts was determined by radioimmunoassay, as described previously (Ostrom et al., 2000b). Production of cAMP was normalized to the amount of protein per sample as determined using a dye-binding protein assay (Bio-Rad).

Results

AC Isoform Expression and Colocalization with GPCR. A dearth of information exists regarding the expression and localization of AC isoforms in vascular smooth muscle cells. Therefore, we first performed RT-PCR analysis with isoform-specific primers for all nine transmembrane isoforms of AC, detecting only AC3, AC5, and AC6 mRNA in RASMC (data not shown). Recent work has described that caveolae or buoyant membrane fractions expressing caveolins, are enriched in various signaling proteins, including AC, arguing for the existence of distinct signaling microdomains in the plasmalemma (Anderson, 1998; Okamoto et al., 1998; Schwencke et al., 1999a,b; Ostrom et al., 2000a,b; Rybin et al., 2000; Steinberg and Brunton, 2001). Lipid raft domains, which are enriched in cholesterol and sphingolipids but lack caveolins, also contain signaling proteins (Oh and Schnitzer, 2001). To ascertain whether compartmentation of receptors, AC, and other key signaling components play a role in the regulation of cAMP production in RASMC, we used immunoblot analysis to assess expression of AC isoforms, β-AR, $G_{\alpha s}$, and EP receptor isoforms in caveolin-rich and noncaveolin membrane fractions. We isolated caveolin-rich microdomains by fractionating RASMC in detergent-free conditions [i.e., by using sonication in sodium carbonate (see *Materials and Methods*)], and isolated buoyant membranes via ultracentrifugation on a discontinuous sucrose gradient. Immunoblot analyses were performed on these buoyant fractions (cav) as well as on nonbuoyant fractions (noncav) and whole-cell lysate (WCL) from the same cellular preparations. Cav fractions from these preparations contained 64.4 ± 3.6 μg protein, whereas the noncav fraction contained 760.8 ± 34.8 μg of protein. Approximately 6% of the total cellular protein was recovered in the buoyant cav fraction. Equal volumes of each fraction were loaded in adjacent lanes for separation of pro-

teins by SDS-PAGE and immunoblotting. Because the cav fraction volume totals 2 ml and the noncav fraction totals 4 ml, immunoreactivity for a protein equally distributed between the cav and noncav fractions will seem 2-fold more intense in the cav lane.

Consistent with previous results (Ishizaka et al., 1998), immunoreactivity was detected for all three caveolin isoforms in cav fractions and WCL from RASMC (Fig. 1). Immunoreactivity for β-adaptin, a marker of clathrin-coated pits, and calnexin, a marker of endoplasmic reticulum, was detected only in noncav and WCL. Immunoblot analysis using antibodies specific for each of the nine isoforms of AC detected only AC3 and AC5/6 (this latter antibody does not distinguish between AC5 and AC6) in RASMC. Immunoreactivity for both AC3 and AC5/6 was detectable primarily, but not exclusively, in cav fractions in control RASMC (Figs. 1 and 6b). The antibody for β_1AR detected bands that migrate at 96, 70, and 53 kDa primarily in noncav fractions (Fig. 1); previous data indicate that these species all represent β_1AR (Rybin et al., 2000). β_2AR and two prostanoid receptors, EP_2R and EP_4R, were detected solely in noncav fractions (Fig. 1). An antibody directed toward the C-terminal of $G_{\alpha s}$ primarily detected a 44-kDa band, corresponding to the short form of this G protein, in both cav and noncav fractions. Only a faint 52-kDa band, corresponding to the long form of $G_{\alpha s}$, could be detected. Therefore, several key GPCRs coupled to the stimulation of AC activity, β_1AR , β_2AR, EP_2R, and EP_4R, seem not to be highly enriched in caveolin-rich membrane microdomains of RASMC.

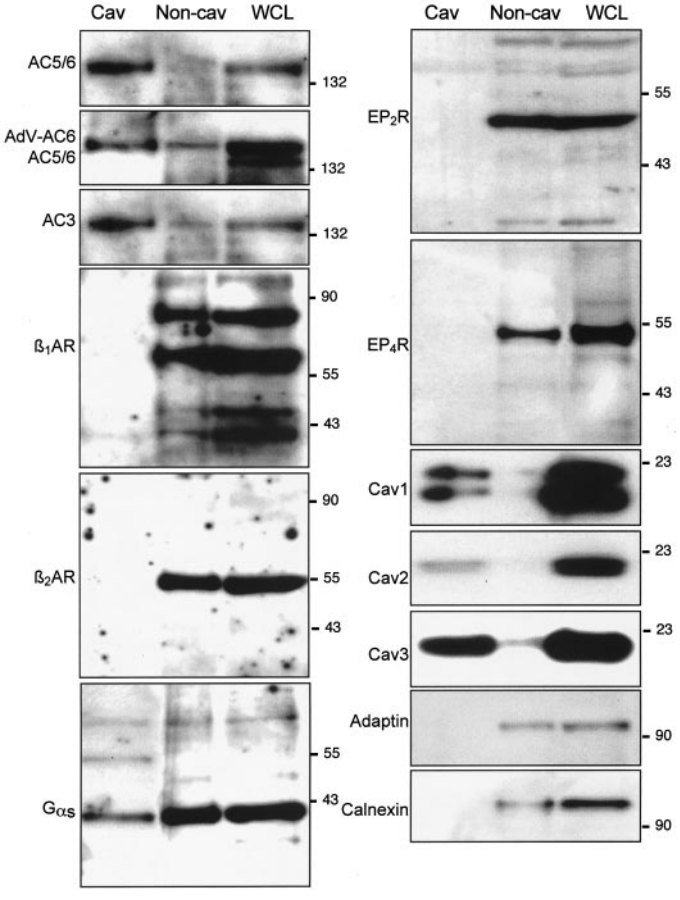

Fig. 1. Expression and localization of AC isoforms, β-adrenergic, and prostanoid receptors in RASMC caveolin-rich fractions. Expression of caveolin subtypes, AC isoforms, βAR, and EP receptors was assessed by immunoblot analysis of caveolin-rich (cav) and noncaveolin rich (noncav) membrane fractions and WCL from RASMC. Each fraction was separated by SDS-PAGE, transferred to membrane, and probed with antibodies specific for AC1, AC2, AC4, AC7, AC8, and AC9 (not shown) and AC3, AC5/6, β_1AR, β_2AR, EP_2, EP_4, $G_{\alpha s}$, caveolin 1, 2, and 3, β-adaptin, and calnexin. Fractions from cells overexpressing AC6 (AdV-AC6, low titer) were also analyzed for AC5/6 immunoreactivity.

Noncav fractions in the above studies contain cellular protein not associated with buoyant, caveolin-rich membranes. Because several GPCR seemed not to localize in cav fractions, we tested for the possible localization in other membranes, in particular intracellular membranes associated with the nucleus, because these could be isolated to relative purity. The bulk of βAR and EPR subtypes were detected by immunoblot analysis in non-nuclear membranes from RASMC (data not shown). These data suggest that intracellular membranes or, more probably, caveolin-poor plasma membrane regions express these GPCRs in RASMC.

As a complementary approach, we investigated the colocalization of components by performing immunoprecipitation followed by immunoblot analysis. Caveolin-3 immunoprecipitates (Cav3 IP) from RASMC contained AC3, AC5/6, and caveolin-3 immunoreactivity, but not immunoreactivity for β_1AR or β_2AR (Fig. 2A). AC3 immunoprecipitates (AC3 IP) from control RASMC also contained immunoreactivity for AC3, AC5/6, and caveolin-3 but, in addition, displayed immunoreactivity for β_1AR and β_2AR. By contrast, AC5/6 immunoprecipitates (AC5/6 IP; Fig. 2A) from RASMC overexpressing AC6 (low-titer adenoviral incubation, see below) contained immunoreactivity for β_1AR, β_2AR, and AC5/6 but not caveolin-3. Taken together with the results shown in Fig. 1, these data indicate that βAR and native AC are not completely colocalized in control RASMC but can colocalize in noncaveolin domains in RASMC that overexpress AC6.

As an independent measure of the localization of AC in caveolin-domains, we performed caveolin-3 immunoprecipitations using reduced detergent (0.5% Nonidet P-40) to isolate detergent-insoluble, caveolin-rich membranes and assayed for AC activity in these membranes and in supernatant fractions. Immunoblot analysis of the resulting pellet and supernatant indicated that 85% of the total caveolin-3 was precipitated, along with 70% of caveolin-1, 93% of AC3, and 12.5% of AC5/6. βAR immunoreactivity was difficult to detect in either the pellet or supernatant. Forskolin-stimulated AC activity was detectable in both cav and supernatant fractions; overexpression of AC6 increased AC activity only in the supernatant fraction (Fig. 2b). These results are consistent with a predominant localization of overexpressed AC6 in noncaveolin-rich membrane domains.

Because primary cells in culture can rapidly lose their differentiated phenotype, we examined RASMC morphology using transmission electron microscopy. Ultrastructural examination of passage 5 RASMC indicated that these cells possess both morphologic caveolae (light vesicular structures, Fig. 3, top) and smooth muscle contractile filaments (Fig. 3, bottom, arrow). These studies indicate that cultured RASMC possess both caveolae and morphologic features consistent with a contractile phenotype (Thyberg, 2000).

AC6 Overexpression Enhances Basal and GPCR-Stimulated cAMP Formation. Multiple GPCRs that regulate cAMP formation are expressed in vascular smooth muscle (Feldman and Gros, 1998). To determine the coupling of

various GPCRs to a particular isoform of AC in RASMC, we overexpressed AC6 using adenoviral gene delivery and then measured the ability of various agonists to stimulate the formation of cAMP. We first quantified the efficiency of gene delivery by adenovirus in RASMC by exposing cells to various titers of AdV-lacZ for 24 h, then fixed and stained cells for β-galactosidase activity. Sixty percent of RASMC exposed to approximately 2000 viral particles/cell AdV-lacZ stained for β-galactosidase activity, indicating that these cells are moderately susceptible to gene delivery with adenovirus (Fig. 4A).

RASMC responded to isoproterenol, forskolin, PGE_2, and an adenosine receptor agonist (NECA) with increases in cAMP formation (Fig. 4B). The responses to these agonists were similar in control and AdV-lacZ treated RASMC (data not shown). Isoproterenol-, forskolin- and PGE_2-mediated cAMP formation, but neither basal cAMP levels nor NECA-stimulated responses were enhanced in RASMC that overexpressed AC6 (Fig. 4B, Table 2). Based on previous data that indicate the amount of increased response to forskolin is proportional to the level of AC6 overexpression (Gao et al., 1998), we calculate that these AC6-overexpressing RASMC expressed approximately 2-fold more AC than did control cells. In RASMC treated with a higher titer of AC6 adenovirus, the forskolin response was enhanced 2.7-fold, consistent with the idea that these RASMC expressed approximately 40% more AC than with the lower titer of adenovirus. In these studies, basal cAMP levels and NECA responses were

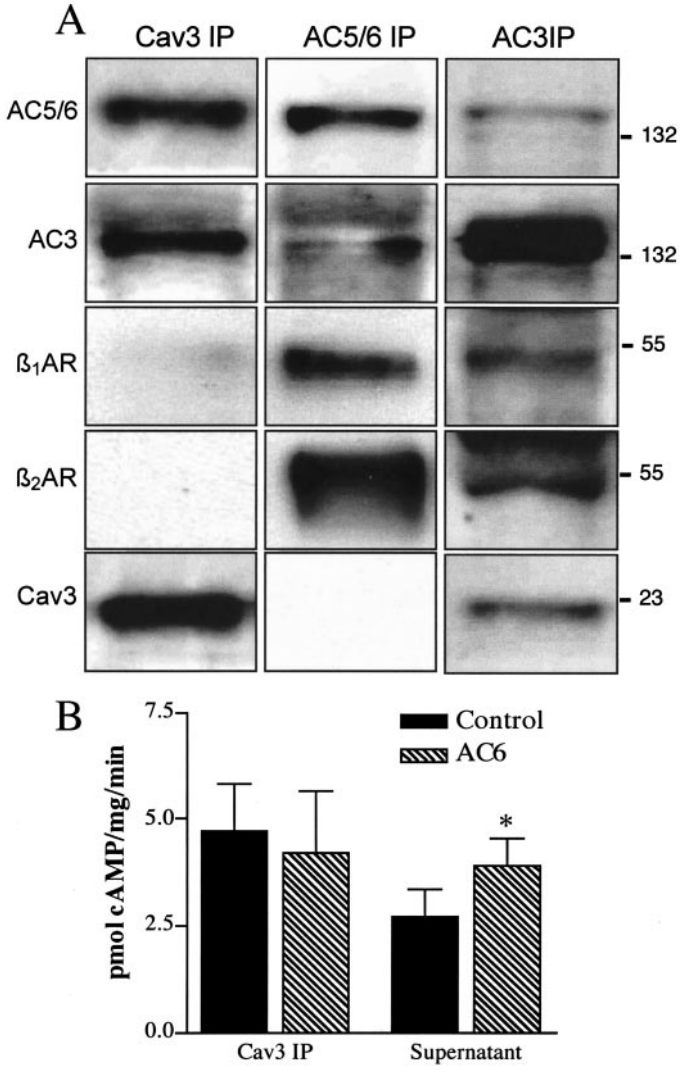

Fig. 2. Detection of AC isoforms and β-adrenergic receptors in immunoprecipitates from RASMC. A, immunoblot analysis of caveolin-3 and AC3 immunoprecipitates from control RASMC and AC5/6 immunoprecipitates from AC6 overexpressing RASMC (low titer). Representative image from two to three experiments is shown. B, forskolin (10 μM)-stimulated adenylyl cyclase activity was measured in modified caveolin-3 immunoprecipitates (Cav3 IP) and the IP supernatant from control RASMC and RASMC overexpressing AC6 (high titer). Data are expressed as fold over basal and each bar represents average ± S.E.M. of three experiments. *, $p < 0.05$ compared with control.

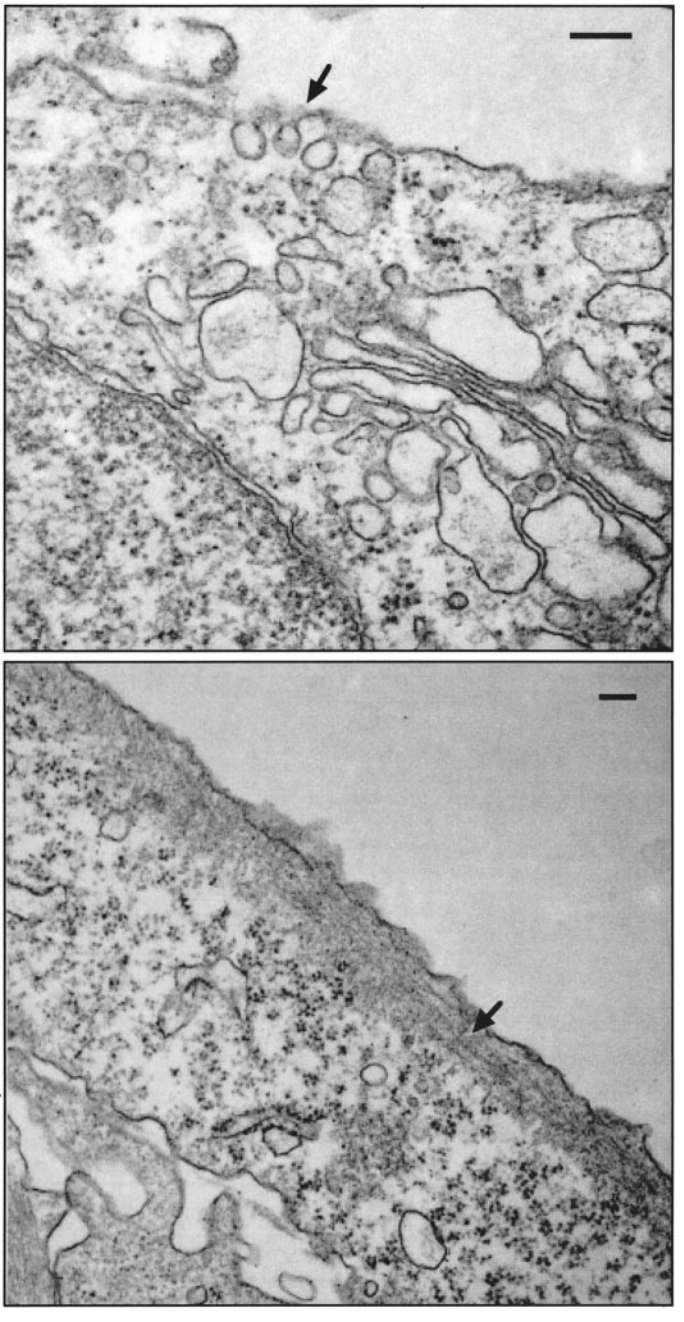

Fig. 3. Ultrastructural analysis of RASMC morphology. Transmission electron microscopy of low passage RASMC shows clusters of caveolae (top, caveolae indicated by arrow) and morphology indicative of contractile phenotype (bottom, arrow indicates smooth muscle contractile filaments). Scale bar, 0.1 μm.

about 2-fold higher (Fig. 4C, Table 2). Therefore, a low level of AC6 overexpression selectively enhanced responses elicited by isoproterenol and PGE_2, but higher levels of AC6 overexpression were able to enhance basal cAMP levels and activation of AC by adenosine receptors. These data are in accordance with the finding that AC6 overexpression localized primarily in noncav fractions, the same fraction in which βAR and EPR were detected (Fig. 1).

Overexpression of AC6 increased levels of cAMP produced in response to multiple concentrations of isoproterenol without an apparent increase in potency of the agonist (Fig. 5A). Response of RASMC to βAR agonists represents the contribution of two βAR subtypes, β_1AR and β_2AR. To determine the effect of AC6 overexpression on the responses mediated

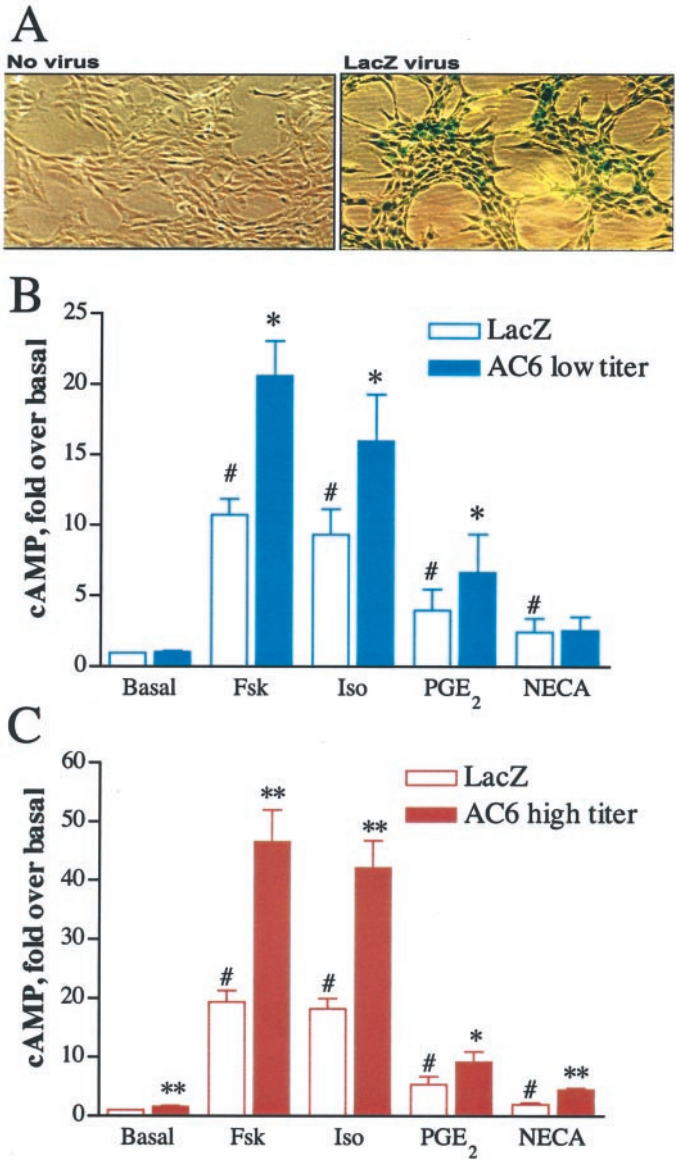

Fig. 4. Level of overexpression of AC6 in RASMC alters basal and hormone-stimulated cAMP levels. A, β-galactosidase staining of control RASMC (no virus) or RASMC incubated with a high titer of adenoviral lacZ (LacZ virus). Approximately 60% of cells stained positively for β-galactosidase activity. B, cAMP accumulation stimulated by forskolin (10 μM), isoproterenol (1 μM), PGE_2 (10 μM), or NECA (10 μM) was measured in RASMC incubated with either adenoviral lacZ (control, open bars) or a low titer of adenoviral AC6 (closed bars). C: In separate experiments cAMP accumulation stimulated by these same agents was measured in RASMC incubated with either adenoviral lacZ (control, open bars) or a high titer of adenoviral AC6 (closed bars). Each bar or point represents average ± s.e.m. of 6 experiments. *, $p < 0.05$; **, $p < 0.01$ compared with lacZ; #, $p < 0.05$ compared with basal by paired Student's t test.

TABLE 2

Enhancement of cAMP production elicited by agonists selective for various GPCRs in RASMC overexpressing different amounts of AC6

Increase in the response elicited by various agonists in cells exposed to approximately 100 v.p./cell (low titer) or 10,000 v.p./cell (high titer) of AC6 adenovirus. Fold increases are calculated by comparing the response in AC6 overexpressing RASMC to uninfected cells (mean ± S.E. of six experiments).

	AdV-AC6	
	Low Titer	High Titer
	-fold over control	
Basal	1.07 ± 0.09	1.65 ± 0.13*
Forskolin, 10 μM	1.91 ± 0.07*	2.70 ± 0.17**
Isoproterenol, 1 μM	1.64 ± 0.15*	2.30 ± 0.05**
PGE_2, 10 μM	1.61 ± 0.11*	1.84 ± 0.27*
NECA, 10 μM	1.07 ± 0.04	2.34 ± 0.21**

* $P < 0.05$ by paired Student's t test.
** $P < 0.01$ by paired Student's t test.

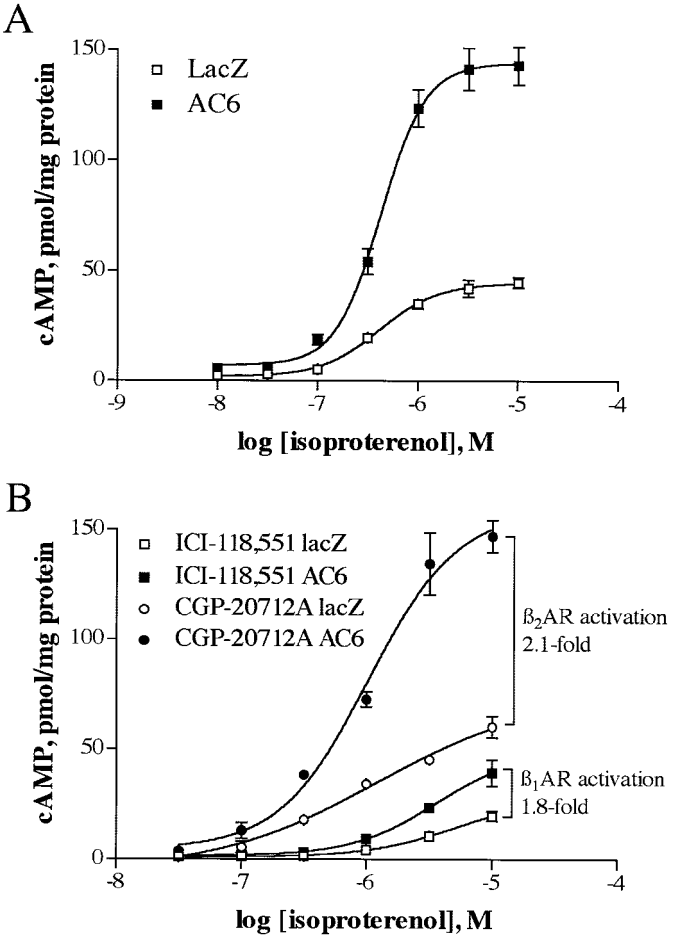

Fig. 5. AC6 overexpression enhances both β_1AR and β_2AR coupling efficacy. A, cAMP accumulation stimulated by various concentrations of isoproterenol in RASMC incubated with either adenoviral lacZ (control, open) or high-titer adenoviral AC6 (closed). B, cAMP accumulation stimulated by isoproterenol was measured in RASMC incubated with either adenoviral lacZ (open symbols) or high-titer adenoviral AC6 (closed symbols) using conditions to preferentially activate either β_1AR (inclusion of 0.1 μM ICI 118,551, a β_2AR-selective antagonist) or β_2AR (inclusion of 0.1 μM CGP-20712A, a β_1AR-selective antagonist). Each point represents average ± S.E.M. of three to six experiments.

by the two major βAR subtypes in these cells, we used low titer AC6 adenovirus and measured cAMP production stimulated by various concentrations of isoproterenol in the presence of either a β_1AR or β_2AR-selective antagonist, thus "isolating" responses to each of the receptor subtypes. Isoproterenol-stimulated cAMP production in the presence of ICI-118,551 (0.1 μM, a β_2AR-selective antagonist) displayed an EC_{50} of 5.0 ± 2.09 μM and a maximum of 28.0 ± 10.8 pmol of cAMP/mg of protein (Fig. 5B). This same response in AC6 overexpressing RASMC was similar in potency (EC_{50} = 4.0 ± 1.66 μM) but had a 1.8-fold higher maximal response (49.5 ± 12.9 pmol/mg of protein). Isoproterenol-stimulated cAMP production in the presence of CGP-20712A (0.1 μM, a β_1AR-selective antagonist) displayed an EC_{50} of 1.3 ± 1.66 μM and a maximum of 49.5 pmol cAMP/mg of protein. This response in AC6 overexpressing cells was similar in potency (EC_{50} = 1.0 ± 1.23 μM) but had a 2.1-fold higher maximal response (160 ± 13.3 pmol/mg of protein). These data indicate that β_1AR- and β_2AR-mediated responses, and by inference, coupling of the two βAR subtypes to the additional AC6, are similar in RASMC. Moreover, as indicated above, RASMC in which we expressed AC6 using an adenoviral AC6 construct showed increased AC5/6 immunoreactivity primarily in noncav fractions and whole-cell lysate (Figs. 1 and 2A). Expression levels and localization of β_1AR, β_2AR, EP_2R, EP_4R, $G_{\alpha s}$, and AC3 were not altered in RASMC overexpressing AC6 (data not shown).

Calcium-Stimulated cAMP Formation via AC3. Contractile tone in smooth muscle is regulated by intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), which activate the contractile machinery, and cAMP, which inhibits contraction via PKA-dependent phosphorylation of myosin light-chain kinase and activation of Ca^{2+} uptake via sarcoplasmic Ca^{2+}-ATPases (Nishikawa et al., 1984; Lincoln and Cornwell, 1991). Cross talk between increased $[Ca^{2+}]_i$ and cellular generation of cAMP probably depends upon the isoforms of AC expressed (Hanoune and Defer, 2001). Some isoforms (AC1, AC3, and AC8) are stimulated by Ca^{2+}, whereas others (AC5 and AC6) are inhibited by Ca^{2+} (Bakalyar and Reed, 1990; Tang and Gilman, 1992; Hanoune and Defer, 2001) However, AC3 can also be inhibited by increases in $[Ca^{2+}]_i$ via activation of CaMK-II (Wei et al., 1996). Therefore, we measured the effects of agents that can increase $[Ca^{2+}]_i$ on cAMP production in RASMC to determine the interaction between these two critical messengers. The Ca^{2+} ionophore, ionomycin, increased basal cAMP accumulation but did not increase or decrease either isoproterenol or PGE_2-stimulated cAMP levels (Fig. 6A).

AC3-dependent inhibition of cAMP levels by Ca^{2+}-elevating agents has been noted in murine aortic smooth muscle cells; this inhibition is thought to be mediated by phosphorylation of AC3 by CaMK-II (Wong et al., 2001). We hypothesized that the Ca^{2+}-stimulated cAMP formation that we observed in RASMC (Fig. 6A) might be caused by the lack of colocalization of Ca^{2+} entry channels and/or CaMK-II with a Ca^{2+}-stimulable AC isoform. Therefore, we assessed expression of Trp1 [a capacitative Ca^{2+} entry channel (Brough et al., 2001)] and CaMK-II in cav and noncav fractions. We found that both Trp1 and CaMK-II (generally considered a cytosolic protein) are predominantly localized in noncav fractions, whereas AC3 predominantly localizes in caveolin-rich membranes (Figs. 1 and 6B). We conclude that the lack of

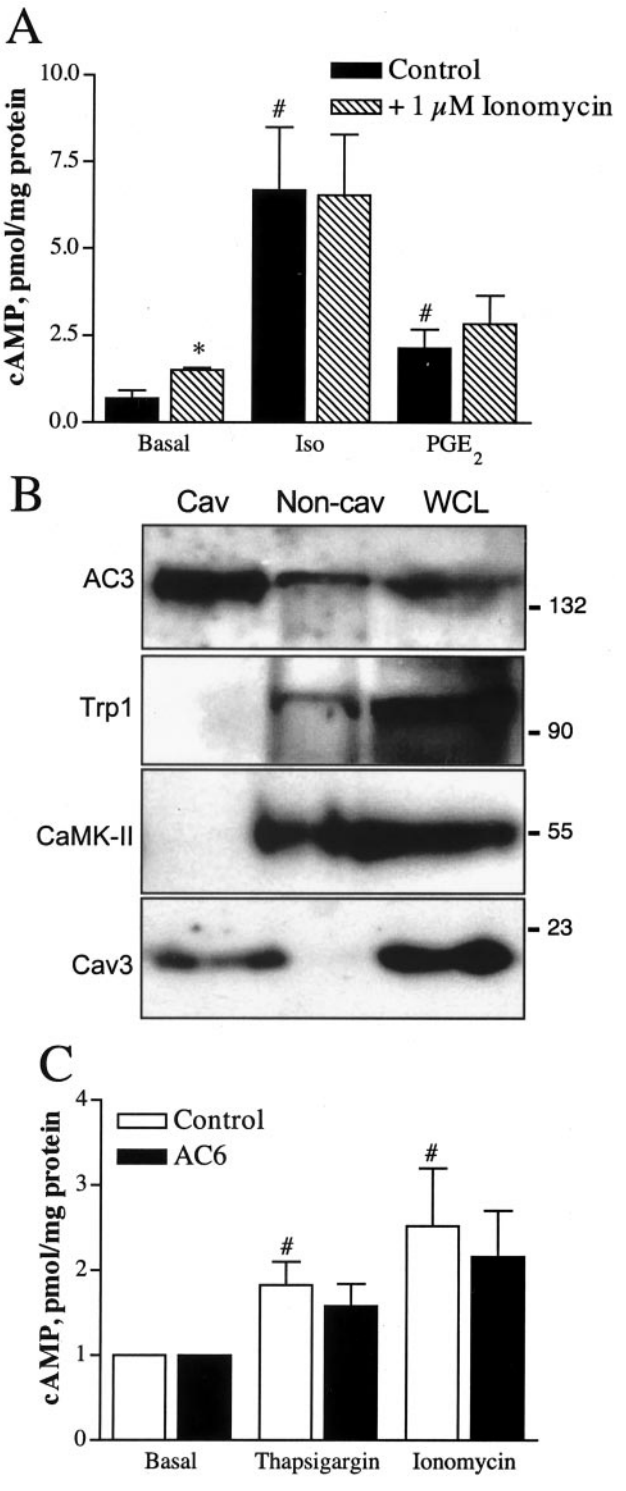

Fig. 6. Regulation of cAMP production is stimulated by increases in intracellular Ca^{2+} because the Ca^{2+}-stimulable isoform AC3 does not highly colocalize with the capacitive Ca^{2+} entry channel, Trp1. A, basal cAMP accumulation and that stimulated by isoproterenol (1 μM) or PGE_2 (10 μM) in the presence and absence of ionomycin (1 μM). B, expression and localization of AC3, Trp1, and CamK-II were assessed by immunoblot analysis of caveolin-rich (cav) and noncaveolin rich (noncav) membrane fractions and WCL from RASMC (see *Materials and Methods*). Representative image from at least three experiments is shown. C, cAMP accumulation stimulated by thapsigargin (10 μM) or ionomycin (10 μM) in cells overexpressing AC6 (incubated with low titer adenoviral AC6, solid bars) compared with control cells (□). Each bar represents average ± S.E.M. of five experiments done in triplicate. #, $p < 0.05$ compared with basal; *, $p < 0.05$ compared with control by paired Student's t test.

Ca^{2+}-inhibitable AC activity in RASMC is probably attributable to two factors: 1) the limited colocalization of the site of Ca^{2+} entry, the kinase responsible for mediating the Ca^{2+}-dependent phosphorylation and the Ca^{2+}-stimulable AC isoform, and 2) the less prominent role of Ca^{2+}-inhibitable isoforms (e.g., AC5 and AC6) compared with that of AC3 in native RASMC.

Because exogenous AC expression alters the proportion among AC isoforms in RASMC, we tested whether regulation of cAMP production by signals other than GPCRs was altered after AC6 overexpression. In control RASMC, thapsigargin (1 μM), an inhibitor of sarcoplasmic Ca^{2+}-ATPase, and ionomycin stimulated cAMP accumulation 4.1- and 2.9-fold over basal levels, respectively (Fig. 6C). In RASMC that overexpressed AC6, the stimulation of cAMP levels by thapsigargin or ionomycin was not enhanced. Therefore, cAMP levels in control RASMC are regulated in a Ca^{2+}-stimulable fashion, consistent with a prominent expression of AC3; overexpression of AC6, an isoform inhibited by Ca^{2+}, does not increase Ca^{2+}-stimulable cAMP production, a finding in contrast with what is observed for several GPCRs (Figs. 4 and 5). Taken together with other data shown above, the results for response to thapsigargin and ionomycin imply that overexpressed AC6 retains its regulatory properties in RASMC and that this occurs in noncav domains.

Discussion

There has been growing interest in caveolin-rich domains (e.g., caveolae), as plasma membrane microdomains that seem to attract and retain certain signaling molecules (Okamoto et al., 1998; Ostrom et al., 2000a; Simons and Toomre, 2000; Galbiati et al., 2001). Spatial organization of multiple component signal transduction cascades provides a means to generate signals with high fidelity and efficiency. Therefore, the goal of the present study was to characterize the compartmentation of GPCR-AC signaling components in primary cultures of vascular smooth muscle cells, RASMC.

Our results show that in RASMC, AC3 and AC5/6 are predominant AC isoforms preferentially expressed in caveolin-rich fractions, whereas several GPCRs, including β_1AR, β_2AR, EP_2R, and EP_4R, are substantially localized in noncav fractions. Increased expression of AC6 localized primarily to noncav fractions and enhanced cAMP formation in response to agonists for several GPCRs. Low levels of AC6 overexpression did not enhance basal levels of cAMP or responses to another GPCR that increases cellular cAMP production, the adenosine A_{2b} receptor [the subtype expressed in RASMC (Dubey et al., 2000; Peyot et al., 2000)]. In contrast, higher levels of AC6 overexpression enhanced basal and A_{2b} receptor responses, thereby implying differences in receptor coupling and regulation of AC6 that depend upon amount of the effector enzyme. These results contrast with those from studies of cardiomyocytes, where up to 10-fold increases in AC6 expression do not increase basal levels of cAMP production (Gao et al., 1998; Ostrom et al., 2000b). Overexpressed AC6 localizes similarly to endogenous AC6 in cardiomyocytes while in RASMC overexpressed AC6 does not (Ostrom et al., 2001; Ostrom et al., 2000b).

It is curious that endogenous AC5/6 localizes in caveolin-rich fractions while overexpressed AC6 protein does not. The reason for this is unknown, but there are several possible explanations. One is that RASMC predominantly express AC5 protein, which localizes differently than AC6. Because no antibody is currently available to distinguish AC5 and AC6 protein, this hypothesis cannot be tested. RT-PCR studies showed expression of both AC5 and AC6 mRNA in RASMC. A second possibility relates to mechanisms that govern AC localization to caveolin-rich fractions; such mechanisms remain poorly defined. AC6 localizes to caveolin-rich fractions in native RASMC, but these cells may lack the capacity to appropriately localize additional AC6 protein. These results thus highlight a potential pitfall of studies using exogenously expressed proteins to assess localization of native proteins.

The present findings contrast with previous work on cardiomyocytes in another way. Cardiomyocyte AC5/6 and βAR are both predominantly localized in cav fractions (Ostrom et al., 2001; Ostrom et al., 2000b; Rybin et al., 2000). Thus, comparison of the two cell types suggests that both βAR and AC isoforms can partition into buoyant, caveolin-rich membrane fractions in a cell-specific manner (Table 3). Although different patterns of protein expression in various cell types are responsible for their differentiated state and specialized cellular functions, we speculate that the localization of AC and the components that influence AC activity in different cells might help "tailor" the ability of cells to respond to extracellular and intracellular signals by defining a precise environment in which the second messenger will be generated. The precise nature and action of factors that serve as determinants of such cell-specific localization remain to be identified. Because high-level AC6 overexpression in cardiomyocytes localizes in caveolin-rich fractions (Ostrom et al., 2000b; 2001; Rybin et al., 2000), cardiomyocytes and RASMC must differ in their capacity to appropriately localize overexpressed AC6.

This spatial organization of signaling is likely to be an important factor in vascular smooth muscle cell regulation, in particular with respect to regulation of cAMP formation, which leads to decreased contractile tone, decreased vascular resistance and decreased blood pressure (Werstiuk and Lee, 2000). In addition, the AC isoforms expressed by vascular smooth muscle are probably key determinants of the regulation of cAMP production because different isoforms can be regulated in an opposite manner by various cellular signals such as Ca^{2+} and $G_{\beta\gamma}$ (Hanoune and Defer, 2001). Therefore, the identity of the AC isoform expressed, combined with the colocalization of the isoform with the source of other signals

TABLE 3
Differential localization of components involved in GPCR-AC signaling in cardiomyocytes and RASMC
Cardiomyocyte data is compiled from published reports (Rybin et al., 2000; Ostrom et al., 2001). RASMC data is summarized from the present work.

	Cardiomyocytes		RASMC	
	Cav	Noncav	Cav	Noncav
β_1-AR	+			+
β_2-AR	+			+
EP_2R		+		+
EP_4R		+		+
$G_{\alpha s}$	+	+	+	+
AC5/6	+		+	
AdV-AC6	+			+
AC3	N.D.	N.D.	+	

N.D., not detected.

that regulate its activity, dictates both basal cAMP levels and the hormonal signals that stimulate or inhibit AC activity (Fagan et al., 2000; Smith et al., 2002). The importance of caveolae in smooth muscle regulation has also been suggested by evidence that GPCRs that link to other signaling pathways are enriched in caveolae (Ishizaka et al., 1998; Taggart, 2001; Ushio-Fukai et al., 2001).

AC3 is generally regarded as a Ca^{2+}-stimulable isoform; however, increases in $[Ca^{2+}]_i$ can inhibit AC3 activity due to phosphorylation by CamK-II (Wei et al., 1996). RASMC exhibit Ca^{2+}-stimulable AC activity, as evidenced in the present studies (Fig. 6) and by the findings of Zhang et al. (1997), who reported that vasopressin enhances βAR-stimulated cAMP formation in a Ca^{2+}-dependent manner in RASMC. Lack of Ca^{2+}-mediated inhibition of cAMP in the present studies may be due to the poor colocalization of AC3 with the Ca^{2+} entry channel (Trp1) and CamK-II, which are probably required for the regulation of AC activity. Without sufficient colocalization of Trp1 and CamK-II, the direct stimulatory effect of Ca^{2+} may predominate and cause activation of AC3 (Fig. 6).

PGE_2, a major product of arachidonic acid metabolism by cyclooxygenases, potently activates the EP subfamily of prostanoid receptors. Of the four receptors in the EP class, EP_2 and EP_4 characteristically couple to $G_{\alpha s}$ and the stimulation of cAMP production and are expressed in smooth muscle from many vascular beds (Breyer et al., 2001). Wong et al. (2001) recently reported that PGE_2 activation of both cAMP generation and concomitant inhibition of vascular smooth muscle cell proliferation were diminished in AC3 knockout animals, implying that receptors activated by PGE_2 could only couple to AC3. In the present studies, we find that receptors on which PGE_2 acts can couple to AC6 when this AC isoform is overexpressed in RASMC. Our results suggest that EP_2R and EP_4R may not couple efficiently to AC3 in RASMC because of their lack of colocalization (Fig. 4). However, inconsistencies between our results in RASMC and those of Wong et al. (who studied human fetal and adult mouse aortic smooth muscle) may relate to cell-specific or species differences in expression and localization of these various signaling components.

One key caveat of the present work is that the colocalization of proteins that we studied relies upon isolation of buoyant membrane fractions. The membrane fractions isolated in this way are not exclusively morphologic caveolae, and may include additional microdomains of the cell (e.g., lipid rafts) (Simons and Toomre, 2000; Galbiati et al., 2001; Oh and Schnitzer, 2001). Definitive proof of colocalization of receptors and AC will probably require additional approaches, such as studies at the electron microscopic level, similar to those in Fig. 3, c and d. Unfortunately, antibodies presently available for GPCR and AC are of limited usefulness for these types of studies (data not shown). These studies only examined the localization of GPCR signaling components in quiescent cells. It will be important to understand the location of these GPCR and other proteins after activation by agonists.

In conclusion, these studies of GPCR-AC signal transduction in RASMC provide new information regarding subcellular localization and compartmentation of these signaling components that regulate smooth muscle function. The colocalization of GPCR signaling components in plasmalemmal microdomains has important implications for the regulation of cellular, in particular smooth muscle cell, responses to extracellular hormones and neurotransmitters. Most previous work that has examined mechanisms of signal transduction has emphasized isolation, purification, cloning, and reconstitution of purified components. The studies described here emphasize the potentially critical role of spatial organization of key signaling molecules in helping to define the manner by which vascular smooth muscle cells respond to extracellular and intracellular stimuli. Moreover, the findings indicate cell-specific differences in the identity of signaling molecules in membrane microdomains, in particular caveolin-rich domains.

Acknowledgments

We are grateful to Dr. John W. Regan (University of Arizona) for providing EP_2R and EP_4R antibodies and to Dr. Marilyn G. Farquhar and Ingrid Neisman for assistance with electron microscopy. We also express our gratitude to Dr. Wolfgang Dillmann (University of California San Diego) for providing cells and Linda Pan and Miki Hase for assistance with cell culture. We wish to also acknowledge Ryan M. Drenan for his help with RT-PCR.

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