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Receptor Number and Caveolar Co-localization Determine Receptor Coupling Efficiency to Adenylyl Cyclase*

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Recent evidence suggests that many signaling molecules localize in microdomains of the plasma membrane, particularly caveolae. In this study, overexpression of adenylyl cyclase was used as a functional probe of G protein-coupled receptor (GPCR) compartmentation. We found that three endogenous receptors in neonatal rat cardiomyocytes couple with different levels of efficiency to the activation of adenylyl cyclase type 6 (AC6), which localizes to caveolin-rich membrane fractions. Overexpression of AC6 enhanced the maximal cAMP response to β_1-adrenergic receptor (β_1AR)-selective activation 3.7-fold, to β_2AR-selective activation only 1.6-fold and to prostaglandin E_2 (PGE_2) not at all. Therefore, the rank order of efficacy in coupling to AC6 is β_1AR > β_2AR > prostaglandin E_2 receptor (EP_2R). β_2AR coupling efficiency was greater when we overexpressed the receptor or blocked its desensitization by expressing βARKct, an inhibitor of G protein-coupled receptor kinase activation, but was not significantly greater when cells were treated with pertussis toxin. Assessment of receptor and AC expression indicated co-localization of AC5/6, β_1AR, and β_2AR, but not EP_2R, in caveolin-rich membranes and caveolin-3 immunoprecipitates, likely explaining the observed activation of AC6 by βAR subtypes but lack thereof by PGE_2. When cardiomyocytes were stimulated with a βAR agonist, β_2AR were no longer found in caveolin-3 immunoprecipitates; an effect that was blocked by expression of βARKct. Thus, agonist-induced translocation of β_2AR out of caveolae causes a sequestration of receptor from effector and likely contributes to the lower efficacy of β_2AR coupling to AC6 as compared with β_1AR, which do not similarly translocate. Therefore, spatial co-localization is a key determinant of efficiency of coupling by particular extracellular signals to activation of GPCR-linked effectors.

Regulation of cells by G protein-coupled receptors (GPCRs)[1]

[1] The abbreviations used are: GPCR, G protein-coupled receptor;

involves the sequential flow of information from the receptors via heterotrimeric G proteins to effector molecules. Among the GPCRs, probably the most well studied are the β-adrenergic receptors (βAR), which are widely expressed in cells throughout the body, including cardiac myocytes. Cardiac βAR, which are activated by neuronally released and circulating catecholamines, change several aspects of cardiac function, including increases in the rate and force of contraction and the rate of relaxation. βAR exerts the majority of its effects by coupling to the heterotrimeric G protein, G_s, and the stimulation of adenylyl cyclase (AC) activity. Activity of AC produces the second messenger cAMP, which, via activation of protein kinase A, alters intracellular Ca^{2+} dynamics and contractile function by phosphorylating calcium channels, troponin I, and phospholamban (1, 2). The β_1AR is the predominant subtype in cardiac myocytes, outnumbering β_2AR by ∼4:1 (3, 4). However, interest has grown recently in the possibility that these receptor subtypes are not redundant, but differentially regulate cardiac function. The distinct physiological actions of β_1AR and β_2AR may represent coupling to different signaling pathways (5–8) and/or different spatial localization within the heart or within single cells (9, 10).

Expression of both βAR and AC in distinct caveolar microdomains of the plasma membrane has been recently demonstrated in both non-cardiac and cardiac cells (10–14). Caveolae, detectable as plasma membrane invaginations, are enriched in the protein caveolin and in sphingolipid and cholesterol, thereby representing a distinct protein and lipid environment, which appears to attract and retain a subset of plasma membrane proteins (15, 16). Compartmentation of βAR and AC (and portions of cellular G_s) challenges the concept that components of GPCR signal transduction are randomly distributed protein entities that interact via diffusion over long distances in the plasma membrane. Instead, the sparsely expressed signaling proteins may be restricted to such plasmalemmal microdomains to facilitate rapid and specific signal transduction (17, 18) and regulation by co-localized molecules (19).

We previously reported that overexpression of AC6 in neonatal rat cardiac myocytes selectively enhances responses to βAR activation but not to activation of prostanoid, adenosine, glucagon, or histamine receptors and found that AC6 and β_1AR are co-localized in caveolar membranes of cardiomyocytes (13). The goal of the present study was to test the role of receptor-

β-AR, β-adrenergic receptor; AC, adenylyl cyclase; Iso, isoproterenol; PGE_2, prostaglandin E_2, EP_2R, prostanoid EP_2 receptor; GRK, G protein receptor kinase; βARK, β-adrenergic receptor kinase; βARKct, C-terminal peptide of βARK; MOI, multiplicity of infection; MES, 4-morpholineethanesulfonic acid; PKA, cAMP-dependent kinase; PKC, protein kinase C; PBS, phosphate-buffered saline.

effector co-localization in caveolae as a contributor to the ability of different GPCRs to regulate second messenger generation. We used AC6 overexpression as a read-out of receptor coupling by comparing the degree to which it enhanced maximal cAMP response to receptor activation. We show here that β_1AR couple more efficaciously to AC6 as compared with β_2AR, but that increasing total expression of β_2AR or blocking their desensitization and translocation out of caveolae enhances the coupling of this receptor to AC6. Taken together with the fact that prostanoid receptors, which do not couple to AC6, were not found in caveolae, we conclude that GPCR ability to couple to the stimulation of AC6 depends upon the co-localization of sufficient active receptors in caveolin-rich membrane microdomains, the predominant subcellular site of AC6 expression.

<div align="center">EXPERIMENTAL PROCEDURES</div>

Materials—Adenovirus-expressing murine AC6 was generated as described previously (20). βARKct adenovirus was a gift from Robert Lefkowitz (Duke University). Primary antibodies for β_1AR, β_2AR, and AC5/6 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibody for caveolin-3 was obtained from BD PharMingen. Radiolabeled chemicals were obtained from PerkinElmer Life Sciences. All other chemicals and reagents were obtained from Sigma Chemical.

Measurement of Adenylyl Cyclase Activity—Neonatal cardiac myocytes were prepared and maintained as described previously (13, 20). Cells were infected 2 days after plating with indicated adenoviral construct(s) for 20 h (10–100 MOI/cell). Control cells were treated with an identical adenoviral construct containing the *lacZ* gene. After infection, cells were washed extensively and allowed to equilibrate for 24 h. Membranes were prepared by rinsing cells twice in ice-cold PBS then scraping cells into hypotonic homogenizing buffer (30 mM NaHEPES, 5 mM MgCl$_2$, 1 mM EGTA, 2 mM dithiothreitol, pH 7.5) and homogenizing with 20 strokes in a Dounce homogenizer. Homogenate was spun at $300 \times g$ for 5 min at 4 °C. Supernatant was then transferred to a clean centrifuge tube and spun at $5,000 \times g$ for 10 min. The pellet was suspended in membrane buffer (30 mM NaHEPES, 5 mM MgCl$_2$, 2 mM dithiothreitol, pH 7.5) to attain ~1 mg/ml total protein concentration. In other studies, AC activity was measured in caveolin-3 immunoprecipitates. 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 dithiothreitol, 0.5% Igepal CA-630, plus mammalian protease inhibitor mixture). Homogenate was precleared with 50 μl of protein A-agarose for 30 min at 4 °C then incubated with caveolin-3 monoclonal antibody (BD PharMingen) for 1 h. Antibody complexes were then precipitated following incubation with protein A-agarose and resuspended in membrane buffer. The assay was conducted by adding 30 μl of sample (membranes or immunoprecipitate) into tubes containing assay buffer (30 mM NaHEPES, 100 mM NaCl, 1 mM EGTA, 10 mM MgCl$_2$, 1 mM isobutylmethylxanthine, 1 mM ATP, 10 mM phosphocreatine, 5 μM GTP, 60 units/ml creatine phosphokinase, and 0.1% bovine serum albumin, pH 7.5.) and drugs of interest. The mixture was incubated for 15 min at 30 °C, and reactions were stopped by boiling for 5 min. cAMP content of each tube was assayed for cAMP content by radioimmunoassay as described previously (13). Total protein concentration was determined using a dye-binding protein assay (Bio-Rad).

Measurement of cAMP Accumulation—Neonatal cardiac myocytes were prepared and treated as described above and were washed three times with serum-free and NaHCO$_3$-free Dulbecco's modified Eagle's medium supplemented with 20 mM HEPES, pH 7.4 (DMEH). Cells were equilibrated for 30 min and then assayed for cAMP accumulation by incubation with drugs of interest in the presence of 0.2 mM isobutylmethylxanthine for 10 min. When antagonists were used, these agents were preequilibrated with cells for 10 min before addition of agonists. Assay medium was aspirated and 250 μl of ice-cold trichloroacetic acid (7.5% w/v) was immediately added to each well to terminate reactions. TCA extracts were assayed for cAMP content by radioimmunoassay.

Membrane Fractionation—Neonatal rat cardiac myocytes were fractionated using a detergent-free method adapted from Song *et al.* (21). Approximately 30 million cells were washed twice in ice-cold PBS and scraped off the plates in 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 25 mM MES, 150 mM NaCl, pH 6.5 (MBS) and loaded into an ultra-

centrifuge 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$_2$CO$_3$). The gradient was centrifuged at 39,000 rpm on a SW41Ti rotor (Beckman Instruments) for 16–20 h at 4 °C. The upper light scattering band at the 5–35% sucrose interface was collected as the caveolin-rich fraction and the lower band (45% sucrose layer) was collected as the non-caveolin fraction. In some studies, cell were exposed to isoproterenol (Iso) (1 μM) for 10 min before being washed in cold PBS and scraped.

Immunoprecipitation—Immunoprecipitations were performed using a protein A-agarose method. Briefly, 10-cm plates of neonatal rat cardiac myocytes 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 mixture) and homogenized on ice with 10 strokes in a Dounce homogenizer. Samples were precleared with protein A-agarose (Roche Molecular Biochemicals) then incubated with primary antibody for 1–3 h on a rocking platform at 4 °C. Antibody conjugates were precipitated by incubating with protein A-agarose overnight on a rocking platform at 4 °C and centrifuging at $13,000 \times g$ for 5 min. Protein A-agarose pellets were then washed once in lysis buffer followed by washes in wash buffer 2 (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2% Igepal CA-630) then wash buffer 3 (10 mM Tris-HCl, pH 7.5, 0.2% Igepal CA-630). Pellets were suspended in sample buffer containing 20% β-mercaptoethanol and heated at 70 °C for 10 min. Proteins in the immunoprecipitates were analyzed by immunoblot analysis.

Immunoblot Analysis—Proteins in individual fractions, whole cell lysates, or immunoprecipitations were separated by SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide precast gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane by electroblotting. In some experiments, protein from samples were precipitated with trichloroacetic acid before electrophoresis by incubating with 8% trichloroacetic acid for 30 min on ice, then centrifuging at $3000 \times g$ for 15 min. Trichloroacetic acid was aspirated, and pellets were washed with ethyl ether and dissolved in $4\times$ sample buffer for SDS-polyacrylamide gel electrophoresis. Membranes were blocked in 20 mM PBS with 3% nonfat dry milk and incubated with primary antibody overnight at 4 °C. Bound primary antibodies were visualized using appropriate secondary antibody with conjugated horseradish peroxidase (Santa Cruz Biotechnology) and ECL reagent (Amersham Pharmacia Biotech). All bands shown migrated at the appropriate size, as determined by comparison to molecular weight standards (Santa Cruz Biotechnology). The amount of protein per fraction was determined using a dye-binding protein assay (Bio-Rad).

<div align="center">RESULTS</div>

Coupling of Endogenous GPCRs to Activation of AC6—Previous work demonstrates that overexpression of AC6 using an adenovirus expression system in neonatal rat cardiac myocytes selectively enhances βAR responses compared with several GPCR that are less efficaciously coupled to G$_s$ and the stimulation of cAMP accumulation (13). To rule out the possibility that factors requiring the intact cell (*e.g.*, intracellular Ca^{2+}, cytoskeletal proteins, etc.) might contribute to this selective effect, we assayed AC activity in membranes from neonatal rat cardiac myocytes. Membranes from control cells or from cells overexpressing AC6 were assayed using maximal concentrations of Iso (1 μM), forskolin (10 μM), or PGE$_2$ (10 μM). Iso and forskolin responses were substantially greater in membranes from AC6-overexpressing cells (Fig. 1*A*). In contrast, PGE$_2$ (10 μM) response, which stimulated AC activity 2.0-fold over basal levels, was not different in membranes from control or AC6-overexpressing cells. Therefore, AC6 overexpression selectively enhances βAR-mediated activation of AC activity but not response to PGE$_2$.

Coupling Efficiency of βAR Subtypes to Activation of AC6—βAR-mediated responses in cardiac myocytes derive from activation of two different receptors, β_1AR and β_2AR. We thus designed experiments to determine the effects of AC6 overexpression on responses to the two subtypes of βAR (and by inference, receptor coupling to AC6) by using subtype-selective antagonists and measuring responses to various concentration of Iso. Response to Iso yielded an EC$_{50}$ of 74 ± 1.3 nM and a maximum of 56 ± 4 pmol of cAMP/mg protein in control cells

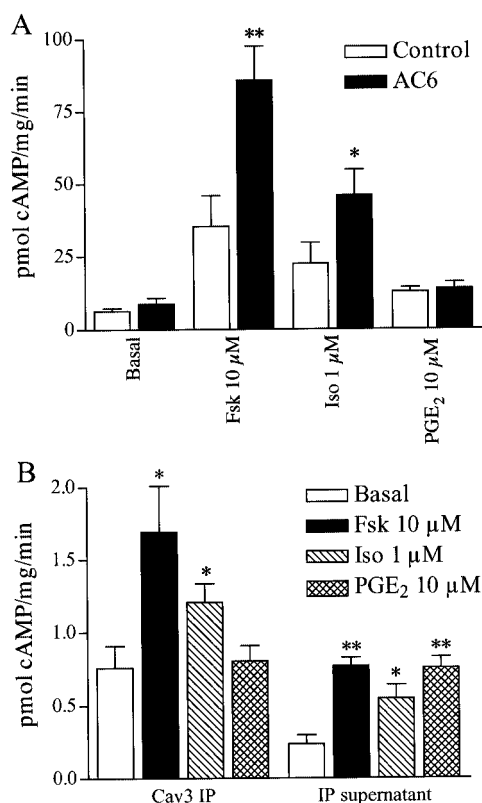FIG. 1. **βAR-mediated adenylyl cyclase activity is selectively enhanced by AC6 overexpression relative to PGE_2 response in cardiac myocyte membranes and caveolin-3 immunoprecipitates.** *A,* adenylyl cyclase activity in membranes from myocytes expressing either lacZ (*open bars*) or AC6 (*closed bars*). Membranes were prepared from cultured neonatal rat cardiomyocytes as described under "Experimental Procedures." Denotes significantly different from control: *, $p \geq 0.05$ and **, $p \geq 0.01$ by paired Student's t test. *B,* adenylyl cyclase activity in caveolin-3 immunoprecipitates and the IP supernatants from cardiomyocytes-overexpressing AC6 (as described under "Experimental Procedures"). Data are expressed as the mean ± S.E. of 3–5 experiments. Denotes significantly different from basal: *, $p \geq 0.05$ and **, $p \geq 0.01$ by paired Student's t test.

FIG. 2. **Cardiac myocyte β_1AR-mediated increase in cAMP accumulation is more prominently enhanced by overexpression of AC6 as compared with β_2AR response.** cAMP accumulation in response to various concentrations of Iso in intact myocytes expressing either LacZ (*squares*) or AC6 (*circles*) in the absence (*A*) or presence of either 0.1 μM ICI-118,551, a β_2-adrenergic selective antagonist (*B*), or 0.1 μM CGP 207124, a β_1-adrenergic-selective antagonist (*C*). Data are expressed as the mean ± S.E. of 4–5 experiments. Observed K_i of CGP-20712A was 10 nM in LacZ and AC6 cells; observed K_i of ICI-118,551 was 13 nM in LacZ and AC6 cells (K_d for CGP-20712A at β_1AR is 5 nM and K_d for ICI 118,551 at β_2AR is 3.1 nM).

and an EC_{50} of 110 ± 1.3 nM and a maximum of 181 ± 15 pmol of cAMP in cells overexpressing AC6 (Fig. 2*A* and Table I), consistent with previous studies that increasing expression of AC6 enhances maximal response without decreasing EC_{50} (13, 20). However, the impact of AC overexpression was different between β_1AR and β_2AR: the maximal response to Iso in the presence of ICI-118,551 (β_1AR preferential activation) was enhanced 3.7-fold in AC6-overexpressing cells as compared with control (Fig. 2*B*). In contrast, the maximal response to Iso in the presence of CGP-20712A (β_2AR preferential activation) was enhanced only 1.6-fold (Fig. 2*C*). Consistent with their known actions as competitive antagonists, both ICI-118,551 and CGP-20712A increased EC_{50} values for Iso in control and AC6-overexpressing cells (Table I). Therefore, the data show that AC6 overexpression enhances β_1AR-mediated response more than β_2AR-mediated response, and for both receptors maximal response was enhanced.

Recent reports indicate that β_2AR may couple to both G_s and G_i (7, 22). To investigate whether coupling of this receptor to G_i might limit its efficacy in stimulating AC, we measured cAMP responses to a maximal concentration of Iso in the absence or presence of either ICI-118,551 (0.1 μM) or CGP-20712A (0.1 μM) in cells treated with pertussis toxin (100 ng/ml for 18 h). Pertussis toxin treatment had no effect on the level of enhancement by AC6 overexpression to Iso (6.0-fold, Fig. 3) or on β_1AR selective response (*i.e.* Iso + ICI 118,551, 4.0-fold, data not
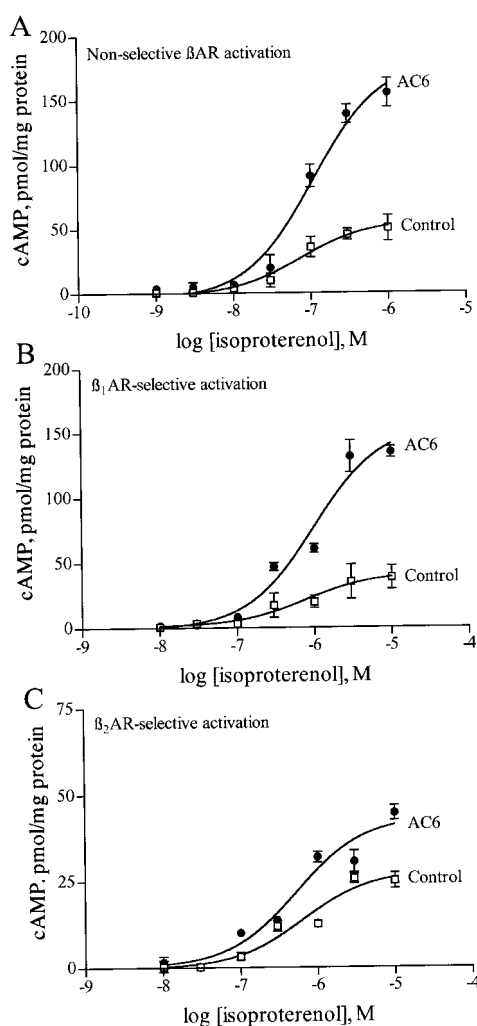
shown). However in PTX-treated cells, AC6 overexpression had a somewhat greater effect on the β_2AR-selective response (Iso + CGP-20712A) than in untreated cells (5.2-fold). This effect did not reach levels of statistical significance ($p = 0.077$ by paired Student's t test) and did not raise the response to levels attained by β_1AR activation. Because neither basal levels of cAMP nor overall response to Iso were enhanced by treatment of cells with pertussis toxin, G_i would not appear to exert a substantial role on cardiomyocyte cAMP generation in response to βAR activation.

Effect of Increased Expression or Blocked Desensitization on β_2AR Coupling to AC6—Because the total βAR population in cardiac myocytes is comprised of ~75% β_1AR and 25% β_2AR (3), we sought to determine whether the lack of effect of AC6 overexpression on β_2AR responses might be attributable to lower expression of this receptor subtype. We used an adenovirus to express an epitope-tagged β_2AR in cardiomyocytes such that the resultant level of β_2AR expression was 218-fold higher than endogenous levels (data not shown). Measuring cAMP production under conditions that preferentially activate

TABLE I
Effect of AC and/or receptor expression on the potency and efficacy of isoproterenol in stimulating cAMP production

EC_{50} and maximum values (pmol of cAMP/mg of protein) of Iso stimulation of cAMP accumulation in the presence of $\beta_1 AR$- and/or $\beta_2 AR$-selective antagonists CGP-20712A (0.1 μM) and ICI-118,551 (0.1 μM) from control and AC6-overexpressing cardiomyocytes. Values are also shown in cardiomyocytes expressing either $\beta_2 AR$ or $\beta ARKct$ in addition to LacZ or AC6.

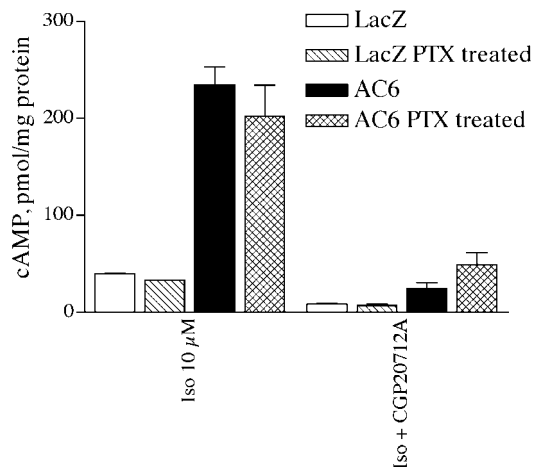|	LacZ (control)		AC6	
	EC_{50}	Maximum	EC_{50}	Maximum
	nM	*pmol*	*nM*	*pmol*
Iso	74.0	56.0	110	181
Iso + CGP-20712A	650	27.7	540	43.5
Iso + ICI-118,551	780	41.6	1000	156
Iso + CGP-20712A and ICI-118,551	16380	11.5	12210	26.2
$\beta_2 AR$ overexpression Iso + CGP-20712A	170	80.3	340	270
$\beta ARKct$ expression Iso + CGP-20712A	56	42.4	68	149

FIG. 3. **Coupling of neonatal cardiac myocyte $\beta_2 AR$ to AC6 to pertussis toxin-sensitive G proteins.** cAMP accumulation was measured in response to Iso alone or in the presence of a $\beta_2 AR$ subtype-specific antagonist in LacZ- (*open bars*) or AC6-overexpressing cells (*solid bars*) or cells treated with pertussis toxin (100 ng/ml for 18 h; LacZ, *hatched bars*; AC6, *cross-hatched bars*). Data are expressed as the mean ± S.E. of three experiments. Pertussis toxin treatment did not significantly increase the $\beta_2 AR$-selective response in AC6 overexpressing cells ($p = 0.077$ by paired Student's t test).

$\beta_2 AR$ in $\beta_2 AR$-overexpressing cells, isoproterenol showed a severalfold shift to the left (*i.e.* increase in potency) and increase in maximal response as compared with control cells (Fig. 4*A* and Table I). Overexpressing AC6 in addition to $\beta_2 AR$ resulted in a maximal response that was much greater as compared with cells overexpressing only $\beta_2 AR$. Therefore, overexpression of $\beta_2 AR$ enhances its ability to couple to cardiomyocyte AC6 and to stimulate cAMP formation via AC6.

It has been recently reported that $\beta_2 AR$, but not $\beta_1 AR$, translocate out of caveolin-rich membrane fractions from neonatal rat cardiac myocytes upon agonist stimulation (10). We hypothesized that this translocation might be associated with βARK (GRK_2)-mediated desensitization of $\beta_2 AR$ and thereby contribute to the lower ability of this receptor to couple to the stimulation of AC6. To test this idea, we used an adenovirus to express the C-terminal peptide of βARK ($\beta ARKct$), which blocks activation of endogenous βARK by sequestering $G_{\beta\gamma}$ subunits (23), thereby blunting desensitization of βAR (and other GPCRs) and increasing βAR-mediated signaling (24). We found that activation of $\beta_2 AR$ in cardiomyocytes engineered to express $\beta ARKct$ yielded about a 12-fold decrease in EC_{50} and a 1.5-fold increase in maximal cAMP as compared with control (Fig. 4*B* and Table I). In this setting of increased functional $\beta_2 AR$, overexpression of AC6 increased the maximal response to Iso 3.5-fold higher than in cells that only expressed $\beta ARKct$. These data imply that blockade of βAR desensitization facilitates greater coupling of $\beta_2 AR$ to AC6. In contrast, $\beta ARKct$

expression was not able to enhance coupling of EP receptors to overexpressed AC6, implying that the effect of $\beta ARKct$ was specific to βAR receptors, and not to G_s or AC activation (Fig. 4*C*). $\beta ARKct$ also did not enhance forskolin-stimulated cAMP formation in either control of AC6-overexpressing cardiomyocytes (Fig. 4*C*).

Determination of Receptor and AC6 Co-localization—Immunoblot analysis of caveolin-rich membrane fractions isolated from cardiomyocytes using a detergent-free method has indicated that native AC5/6 is predominantly expressed in caveolin-rich (cav) fractions (10, 13). Overexpression of AC6 substantially increases AC5/6 immunoreactivity in this same fraction (Fig. 5*A* and Ref. 13). In addition, native $\beta_1 AR$ and adenovirally expressed FLAG-$\beta_2 AR$ were predominantly (but not exclusively) detected in cav fractions while EP_2 receptor immunoreactivity was detectable only in non-cav fractions (Fig. 5*A*). These data provide an explanation for the pattern of receptor coupling to overexpressed AC6 (Fig. 1*A*): the absence of an effect on EP_2 receptor responses following AC6 overexpression correlates with the lack of co-localization of this receptor and AC6.

As an alternative approach, we performed immunoprecipitations and analyzed the proteins that co-precipitated using immunoblotting and by measuring AC activity. AC activity measured in caveolin-3 immunoprecipitates (designated as IP) was stimulated by forskolin and isoproterenol, but not by PGE_2 (Fig. 1*B*, *IP*). In contrast, AC activity in the IP supernatant (all cellular material not bound by caveolin-3 antibody) could be stimulated by PGE_2. Basal AC activity was 3.2-fold higher in the caveolin-3 IP than in the IP supernatants. Immunoblot analysis of the caveolin-3 IP demonstrated the presence of $\beta_1 AR$, AC5/6, and expressed FLAG-$\beta_2 AR$ (Fig. 5*B*) but not $EP_2 R$ (data not shown). Conversely, caveolin-3 immunoreactivity was detected in both $\beta_1 AR$ and AC5/6 IP from native cells as well as in the FLAG IP from cells expressing FLAG-$\beta_2 AR$. Further IP studies indicated that $\beta_1 AR$ and AC5/6 co-immunoprecipitated but that $\beta_2 AR$ did not immunoprecipitate with either $\beta_1 AR$ or AC5/6 (data not shown). Therefore, $\beta_1 AR$ and AC5/6 and caveolin-3, and to a lesser extent $\beta_2 AR$, form signaling complexes that exclude $EP_2 R$.

Rybin *et al.* (10) reported that agonist exposure causes a translocation of $\beta_2 AR$, but not $\beta_1 AR$, out of caveolin-rich fractions from cardiomyocytes (9). We exposed cells expressing FLAG-$\beta_2 AR$ to Iso (1 μM) for 10 min before performing immunoprecipitations and found that FLAG-$\beta_2 AR$ immunoreactivity was reduced in caveolin-3 IP. When these studies were repeated in cardiomyocytes also expressing $\beta ARKct$, overall FLAG-βAR immunoreactivity in caveolin-3 IP was increased and Iso exposure no longer diminished FLAG-$\beta_2 AR$ co-precipitation with caveolin-3 (Fig. 5*B*). Neither Iso stimulation nor $\beta ARKct$ expression altered the co-precipitation of $\beta_1 AR$ or AC5/6. Therefore, whether assessed by either cell fractionation
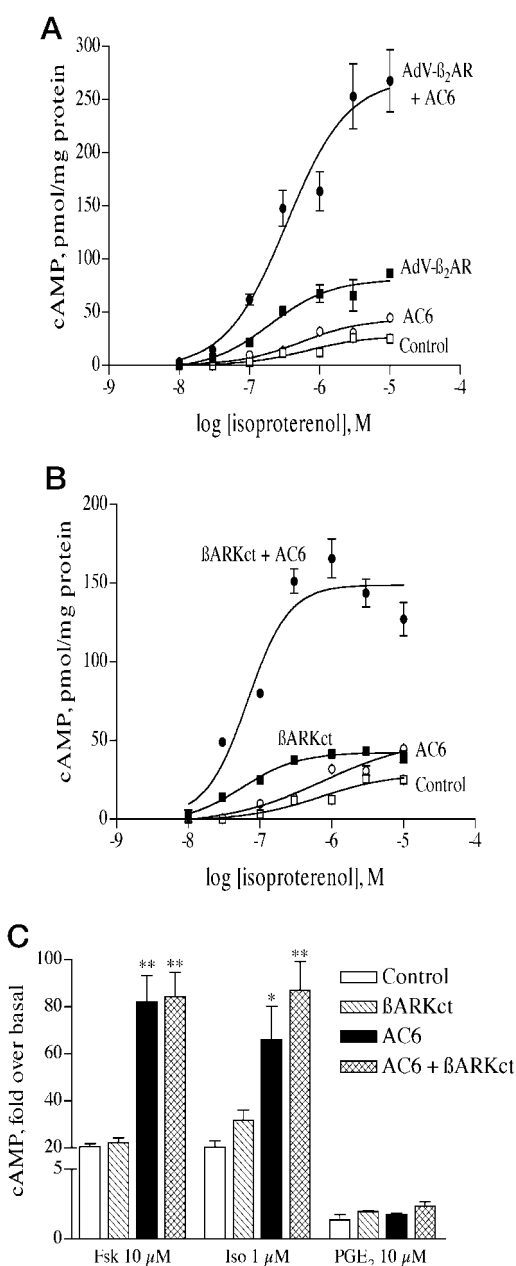
FIG. 4. **Increased expression of β_2AR or inhibition of GRK in cardiac myocytes uncovers an enhancement of β_2AR responses by AC6 overexpression.** cAMP accumulation in response to various concentrations of Iso in the presence of a β_1AR antagonist (0.1 μM CGP 207124) in intact myocytes expressing either LacZ (*squares*) or AC6 (*circles*). *A,* cells overexpressing β_2AR (*closed squares*) in addition to either LacZ or AC6 overexpression alone are shown for comparison (*open symbols*, data from Fig. 2). *B,* cells in which GRK activation was blocked by expressing the carboxyl-terminal peptide of βARK ($\beta ARKct$, *closed squares*). Responses from LacZ and AC6 overexpression alone are shown for comparison (*open symbols*, data from Fig. 2). *C,* cAMP accumulation responses to forskolin, Iso, and PGE$_2$ in control cells (*open bars*) or cells expressing βARKct (*hatched bars*), AC6 (*closed bars*), or βARKct and AC6 (*cross-hatched bars*). Data are expressed as the mean $\pm$ S.E. of 4–5 experiments. Denotes significantly different from control: *, $p \geq 0.05$ and **, $p \geq 0.01$ by paired Student's t test.
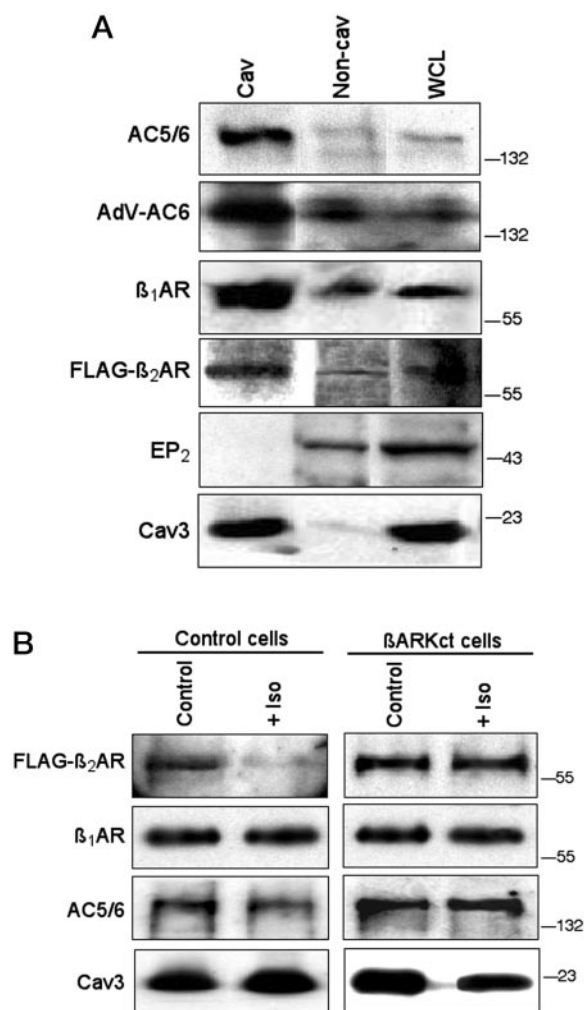
FIG. 5. **βARKct expression blocks agonist-induced transloca-tion of β_2AR out of cardiac myocyte caveolin-rich fractions.** *A,* expression of caveolin-3, AC5/6, β_1AR, FLAG-β_2AR, and EP$_2$R was assessed by immunoblot analysis of caveolin-rich and caveolin-poor membrane fractions and whole cell lysates from cardiomyocytes. Fractions from cells overexpressing AC6 (AdV-AC6) were also analyzed for AC5/6 immunoreactivity. *B,* caveolin-3 immunoprecipitates (*IPs*) from control cells or cells expressing βARKct that were exposed to either vehicle or Iso (1 μM for 10 min, *Iso*) were analyzed by immunoblotting. Immunoreactivity for β_2AR was lost in caveolin-3 immunoprecipitates from Iso-treated cells unless βARKct was expressed. β_1AR and AC5/6 immunoreactivity was unchanged by these treatments. Images are representative of at least three experiments.

effect likely resulting from an increased number of functional receptors in the compartment enriched in AC6.

DISCUSSION

It is well established that cells achieve their differentiated state and fulfill specialized cellular functions by expressing only a certain subset of genes. For example, the complement of cell-surface receptors that a given cell expresses determines the extracellular signals to which that cell will respond. However, because receptors may share common signal transduction mechanisms, spatial targeting to particular domains of receptors with similar signaling characteristics provides a means of achieving specificity in signaling. Such observations contradict the classical paradigm that receptors and other integral membrane proteins are randomly distributed in the plasma membrane. The present work demonstrates that caveolin-rich domains, plasma membrane microdomains that are enriched in many signaling molecules, can co-localize receptors and effectors to couple only certain receptors to a given effector. Data

(9) or caveolin-3 immunoprecipitation (Fig. 5*B*), β_2AR translocate out of caveolae upon agonist exposure. This translocation appears dependent upon GRK and presumably occurs as requisite for β_2AR internalization via clathrin-coated pits (25, 26). We also show that βARKct expression blocks this β_2AR translocation and enhances β_2AR coupling to AC (Fig. 4), the latter

from previous work by our laboratory and others suggest that such caveolin-rich domains represent morphologic caveolae (13, 27). Thus spatial, as well as temporal, regulation of signaling can be a critical determinant for individualizing cellular response to extracellular hormones and neurotransmitters (18).

Consistent with previous findings, we find that cardiomyocytes compartmentalize a significant proportion of βAR-AC signaling in buoyant, caveolin-rich membranes (10, 11, 13). We show that another type of receptor capable of stimulating AC, the EP_2 receptor, is excluded from caveolin-rich fractions and is unable to couple to overexpressed AC6. Therefore, localization in this subcellular compartment is a determinant for efficacy of GPCR coupling to an effector molecule. β_1AR, expressed at larger numbers and apparently more permanently residing in caveolae, couple with high efficacy to AC6. β_2AR, expressed at lower levels as compared with β_1AR, also reside in caveolae but translocate out of this microdomain upon agonist activation (10). This translocation occurs within the time frame of cAMP accumulation assays and appears to contribute to the decreased ability of β_2AR to maximally stimulate AC6. These data for β_1AR, β_2AR and EP_2R provide direct demonstration of the functional significance of co-localization of GPCR signaling molecules in caveolin-rich domains and do so using endogenous receptors in primary cultures of a physiologically important cell type.

Cardiomyocyte caveolae may serve as specialized regions that integrate multiple signaling pathways with other signaling events and concentrate critical signaling proteins involved in creating temporal and spatial patterns of cell regulation. For example, caveolae have been implicated as sites of Ca^{2+} sparks (28) in cardiomyocytes and the location of capacitive Ca^{2+} entry in other cells (19). If Ca^{2+} entry is localized in caveolae, expression of AC6 (a Ca^{2+}-inhibitable isoform) in this same microdomain should result in cAMP levels that are highly sensitive to Ca^{2+} entry from outside the cell. In fact, this is the case in C6 glioma cells, which express AC6 and require intact caveolae for capacitive Ca^{2+} entry to inhibit AC activity (19). Such co-localization in cardiomyocytes might contribute to the changes in cAMP levels observed in heart during the cardiac cycle (29) and might contribute to regulation of cAMP levels by nitric oxide (30). Changing the localization of AC6 expression would be predicted to result in a different pattern of regulation of cAMP levels. Consistent with this hypothesis, AC activity is increased when cardiomyocyte caveolae are disrupted with cyclodextrin, a moiety that removes cholesterol from the plasma membrane and presumably dissociates sites of Ca^{2+} entry from endogenous AC (10).

A further prediction that would derive from our findings is that overexpression of different isoforms of AC would result in different patterns of regulation and have different physiological consequences. Indeed, overexpression of AC isoforms other that AC6 has been reported to produce quite different effects on cellular and cardiac physiology. Overexpression of AC8, which is poorly activated by GPCRs, leads to a Ca^{2+}-dependent regulation of the enzyme without enhancement of GPCR responses (31). Transgenic overexpression of AC5 results in increases in basal AC activity and little enhancement, on a percent basis, of βAR-mediated responses (32). These data contrast with data presented here and with data from transgenic overexpression of AC6 (33). AC5 and AC6 are similar in their regulation by Ca^{2+} and PKA, but AC5 is stimulated while AC6 is inhibited by PKC. This difference in regulation may be quite relevant given that PKC translocates to caveolae upon activation (27). However, most previous work has not involved assessment of co-localization of AC and receptors that regulate

its activity. The present work illustrates the importance of understanding the subcellular localization of both AC and receptor expression.

There are certain caveats of the present findings. One is that the co-localization of proteins relies upon a biochemical isolation of buoyant membrane fractions. These fractions may not necessarily be pure morphologic caveolae. Although our efforts to perform electron microscopic studies confirm a caveolae-like structure (13), such fractions may also represent different microdomains of the cell, such as lipid rafts (34). While the immunoprecipitation studies in this report were designed to mitigate this shortcoming, definitive proof of co-localization of receptors and AC will likely require additional approaches, such as morphologic studies at the electron microscopic level. Unfortunately, antibodies presently available are of limited usefulness in these types of studies.[2] Also, it is conceivable that localization patterns of components in the neonatal cardiomyocyte may not be identical with those in adult cardiomyocytes. Direct studies to test this will need to be undertaken. In addition, it is important to consider that βARKct blocks GRK activation by sequestering $G_{\beta\gamma}$ subunits (23). Thus, any changes in cAMP formation resulting from βARKct expression may be attributable, in part, to the termination of signaling by $G_{\beta\gamma}$ and mechanisms other than blockade of desensitization.

In conclusion, these studies of GPCR-AC signal transduction in cardiomyocytes demonstrate that compartmentation of receptors and AC isoforms can determine the efficacy of receptor coupling in that different GPCR show different patterns of cAMP generation, which correlate with co-localization in caveolin-rich domains. The co-localization of GPCR-signaling components in such microdomains has important implications for the regulation of cellular responses to extracellular hormones and neurotransmitters. Most previous work that has examined mechanisms of signal transduction has involved isolation, purification, cloning, and reconstitution of purified components. The studies here emphasize the potentially critical role of spatial organization, at a subcellular level, of key signaling molecules in defining the ability of cells to respond to extracellular stimuli. It will be of interest to learn how such spatial organizational changes as a consequence of development, physiologic state, and in disease settings. The recognition of signaling microdomains in caveolin-rich regions of cardiomyocytes and other cells may provide a unifying hypothesis to help account for the rapidity, high fidelity, and specificity of GPCR-mediated signal transduction.

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Receptor Number and Caveolar Co-localization Determine Receptor Coupling Efficiency to Adenylyl Cyclase

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