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Angiotensin II Enhances Adenylyl Cyclase Signaling via Ca^{2+}/Calmodulin

G_q-G_s CROSS-TALK REGULATES COLLAGEN PRODUCTION IN CARDIAC FIBROBLASTS*

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Cardiac fibroblasts regulate formation of extracellular matrix in the heart, playing key roles in cardiac remodeling and hypertrophy. In this study, we sought to characterize cross-talk between G_q and G_s signaling pathways and its impact on modulating collagen synthesis by cardiac fibroblasts. Angiotensin II (ANG II) activates cell proliferation and collagen synthesis but also potentiates cyclic AMP (cAMP) production stimulated by β-adrenergic receptors (β-AR). The potentiation of β-AR-stimulated cAMP production by ANG II is reduced by phospholipase C inhibition and enhanced by overexpression of G_q. Ionomycin and thapsigargin increased intracellular Ca^{2+} levels and potentiated isoproterenol- and forskolin-stimulated cAMP production, whereas chelation of Ca^{2+} with 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid/AM inhibited such potentiation. Inhibitors of tyrosine kinases, protein kinase C, or Gβγ did not alter this cross-talk. Immunoblot analyses showed prominent expression of adenylyl cyclase 3 (AC3), a Ca^{2+}-activated isoform, along with AC2, AC4, AC5, AC6, and AC7. Of those isoforms, only AC3 and AC5/6 proteins were detected in caveolin-rich fractions. Overexpression of AC6 increased βAR-stimulated cAMP accumulation but did not alter the size of the ANG II potentiation, suggesting that the cross-talk is AC isoform-specific. Isoproterenol-mediated inhibition of serum-stimulated collagen synthesis increased from 31 to 48% in the presence of ANG II, indicating that βAR-regulated collagen synthesis increased in the presence of ANG II. These data indicate that ANG II potentiates cAMP formation via Ca^{2+}-dependent activation of AC activity, which in turn attenuates collagen synthesis and demonstrates one functional consequence of cross-talk between G_q and G_s signaling pathways in cardiac fibroblasts.

Cardiac hypertrophy is associated with increased cardiac mass, a gradual decline in contractile function and eventual heart failure. The remodeling associated with these changes involves an altered balance of synthesis and degradation of extracellular matrix (ECM)[1] by cardiac fibroblasts and can lead to abnormal accumulation of ECM in the interstitial space (*i.e.* fibrosis) (1, 2). Several G protein-coupled receptors (GPCR) that signal through G_q have been implicated in the pathogenesis of cardiac hypertrophy and failure (3, 4). Conversely, GPCRs that signal via G_s may inhibit collagen deposition by cardiac fibroblasts (5).

Two major pro-fibrotic signals in the heart are cytokines, such as transforming growth factor-β, and the peptide hormone ANG II, both of which increase collagen synthesis by cardiac fibroblasts (6, 7). ANG II also inhibits matrix metalloproteinase expression by cardiac fibroblasts, thereby attenuating degradation of ECM proteins (8). An anti-fibrotic role for cAMP is supported by evidence that adenosine and prostacyclin inhibit cardiac fibroblast proliferation and collagen synthesis through activation of A_{2b} and prostanoid receptors, respectively, which each couple via G_s, to enhanced cAMP production (9, 10). β-Adrenergic receptors have been shown to stimulate fibroblast proliferation via epidermal growth factor receptor transactivation (11) but their role in regulating collagen synthesis in cardiac fibroblasts is poorly documented.

Adenylyl cyclase (AC) catalyzes the synthesis of cAMP and its expression limits the ability of a cardiac cell to maximally produce this second messenger (12, 13). Nine different transmembrane AC isoforms exist, each with different amino acid sequence, tissue and chromosomal distribution, and regulation (14). Differences in regulation include stimulation or inhibition by Gβγ, Ca^{2+}, and various protein kinases. AC5 and AC6, which are the predominant isoforms expressed in cardiac myocytes (15), represent a subfamily of ACs that are related in structure and regulation. These isoforms are inhibited by protein kinase A, Ca^{2+}, G_i, Gβγ, and nitric oxide (14, 16, 17). By contrast, AC1, AC3, and AC8 are stimulated by Ca^{2+}/calmodulin (yet AC3 can also be inhibited by calmodulin kinase-II) (14, 18, 19). AC2 and AC4 are activated by Gβγ and AC2 and AC7 can be activated by phosphorylation by protein kinase A and/or PKC (14). Thus, the AC isoform expression in a cell determines the interaction between cAMP production and other signal transduction cascades.

Meszaros *et al.* (20) have recently described a signaling "cross-talk" between two key GPCR signal transduction pathways in cardiac fibroblasts. Isoproterenol (ISO), a β-adrenergic

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[1] The abbreviations used are: ECM, extracellular matrix; GPCR, G protein-coupled receptors; ANG II, angiotensin II; AC, adenylyl cyclase; PKC, protein kinase C; ISO, isoproterenol; βAR, β-adrenergic receptors; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; RT, reverse transcriptase; PLC, phospholipase C.

receptor (βAR) agonist, activates G_s and stimulates cAMP production 10-fold over basal levels. ANG II activates G_q-coupled angiotensin receptors that, by themselves, do not alter cAMP production in cardiac fibroblasts, but in combination with ISO potentiate the βAR response, resulting in a 2-fold potentiation of ISO-stimulated cAMP production. The goal of the present study was to identify the molecular mechanism and physiological consequence of the cross-talk between these two signaling pathways in cardiac fibroblasts. The current data indicate that G_q-mediated elevation of intracellular Ca^{2+} induced by ANG II potentiates G_s-stimulated cAMP formation, probably via stimulation of AC3. Moreover, we show that the potentiation is functionally relevant: combined treatment of cardiac fibroblasts with ANG II and ISO inhibits ANG II-promoted collagen synthesis more so than ISO alone. Thus, we identify an endogenous signaling pathway by which intracellular Ca^{2+} enhances cAMP production and limits collagen synthesis, and perhaps fibrosis, in the heart.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were obtained from Invitrogen. Primary antibodies to AC isoforms and all secondary antibodies were obtained from Santa Cruz Biotechnology. Antibodies to caveolin were from BD Pharmingen. ANG II, UTP, Fura-2/AM, forskolin, and BAPTA/AM were obtained from Calbiochem. All other drugs and reagents were of reagent grade and obtained from Sigma.

Preparation and Culture of Adult Rat Cardiac Fibroblasts—Cardiac fibroblasts were prepared from adult male 250–300-g Sprague-Dawley rat hearts. Following rapid excision of the hearts, the ventricles were isolated, minced, pooled, and placed in a collagenase/pancreatin digestion solution. After sequential digestions, the fibroblasts were pelleted and resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin, streptomycin, fungizone, and 10% fetal bovine serum (FBS, Gemini Bio-Products). After a 30-min period of attachment to uncoated culture plates, cells that were weakly attached or unattached were rinsed free and discarded. After 2–3 days, confluent cultures were amplified by trypsinization and seeding onto new dishes. For signaling assays, only early passage (≤3) cells grown to 80–90% confluency were used. The purity of these cultures was greater than 95% cardiac fibroblasts as determined by positive staining for vimentin and negative staining for smooth muscle actin and von Willebrand factor, as previously described (21).

Adenoviral Gene Transfer to Cardiac Fibroblasts—Wild-type G_q was cloned into the PACCMVpLpA shuttle vector for production of adenovirus, as previously described (3). We found that a titer of 200 virus particles per cell was optimal for G_q expression without cytotoxicity, as determined by trypan blue exclusion and morphological examination with phase-contrast light microscopy. We used 1000 viral particles per cell of an adenovirus construct to maximally overexpress the murine adenylyl cyclase type 6 (AC6) gene, produced as described previously (22). Cardiac fibroblasts were infected with the appropriate virus and incubated at 37 °C 18–24 h in serum-free DMEM prior to the assays. Preliminary experiments using LacZ expression and β-galactosidase staining indicated that the efficiency of the adenoviral construct to increase gene expression was 40% (data not shown).

Quantitation of cAMP Production—Cardiac fibroblasts cultured on 24-well plates were washed three times with 37 °C serum-free and $NaHCO_3$-free DMEM supplemented with 20 mM HEPES, pH 7.4. Cells were equilibrated for 30 min, then assayed for cAMP accumulation by incubation with drugs of interest in the presence of 0.2 mM isobutylmethylxanthine for 10 min. When antagonists or inhibitors were used, these agents were equilibrated with cells for 15 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. Trichloroacetic acid extracts were assayed for cAMP content by either Direct ELISA kit (Assay Designs) or radioimmunoassay as described previously (22). Data were corrected for the amount of total acid-precipitable protein per well. In other studies, AC activity was measured in cardiac fibroblast membranes as previously described (23). Briefly, membranes were prepared by rinsing cells twice in ice-cold phosphate-buffered saline then scraping cells into hypotonic homogenizing buffer (30 mM Na-HEPES, 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 × *g* for 5 min at 4 °C. Supernatant was then transferred to a clean centrifuge tube and spun at 5,000 × *g* for 10 min. Pellet was suspended in membrane buffer (30 mM Na-HEPES, 5 mM $MgCl_2$, 2 mM dithiothreitol, pH 7.5) to attain ~1 mg/ml total protein concentration. Assay was conducted by adding 30 μl of membranes into tubes containing assay buffer (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 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.

Intracellular Ca^{2+} Measurements—Fibroblasts (0.25×10^5 cells) were plated on 22-mm glass coverslips. The cells were washed once in phosphate-buffered saline and incubated in 1 ml of DMEM containing 1 μM Fura-2/AM at 37 °C for 30 min. Cells were then washed once with DMEM and placed in a 37 °C chamber containing 1.5 ml of HEPES-buffered saline (HBS: 130 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, 1.0 mM $CaCl_2$, 25 mM HEPES, pH 7.4), such that groups of 5–8 cells could be viewed using an inverted Olympus IX-70 microscope. Spectrofluorometric measurements were collected using Delta Scan System spectofluorometer (Photon Technology), where the field was excited at 380 and 340 nm and the emission ratio was collected at 511 nm. Agonists were administered from ×1000 stocks to maintain a constant volume of 1.5 ml.

Reverse Transcriptase-PCR to Identify AC Isoforms—Total RNA was extracted from cardiac fibroblasts grown to 80–90% confluency on 15-cm plates using Trizol reagent (Invitrogen). A DNase reaction was performed to eliminate DNA contaminants and the RNA was reverse transcribed using Superscript II (Invitrogen) and poly-T priming. Individual isoform-selective primer pairs were used to amplify each isoform of AC. Primers were based on rat or murine sequences as previously described (24). PCR reactions with each primer pair were performed on cDNA template, genomic DNA (positive control), and minus RT (negative control) template using 35 cycles and an annealing temperature of 56 °C. Sequence analysis was used to confirm the identity of all PCR products.

Purification of Caveolin-enriched Membrane Fractions—Cardiac fibroblasts were fractionated using a detergent-free method adapted from Song *et al.* (25), and described previously (22). The faint light-scattering band resulting from sucrose density centrifugation was collected from the 5–35% sucrose interface. The bottom 4 ml of the gradient (45% sucrose) was collected as non-caveolar membranes.

Immunoblot Analysis—Individual fractions or whole cell lysates were separated by SDS-polyacrylamide gel electrophoresis (Nu-PAGE, Invitrogen). Equal volumes of fractions were loaded so that each lane represents similar proportions from the cells, resulting in ~10-fold lower amounts of protein loaded in the caveolin-enriched membrane fraction lanes. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) by electroblotting and probed with primary antibody (see "Materials"). Bound primary antibodies were visualized by chemiluminescence. Images of immunoblot analyses and RT-PCR are representative of at least 3 separate experiments. In some experiments, the optical density of bands was calculated using a digital imaging system and LabWorks software (UVP Bioimaging Systems) and reported as arbitrary optical density units.

Assay of Collagenase-sensitive [^{3}H]Proline Incorporation—[^{3}H]Proline incorporation by cardiac fibroblasts was measured according to modified methods of Guarda *et al.* (26). Briefly, cells were transferred to 12-well plates then were serum starved in 0% FBS for 24 h. Proline incorporation was then assayed by adding 1 μCi/well of [^{3}H]proline (PerkinElmer Life Sciences) along with, where indicated, drugs of interest and 2.5% FBS for 24 h. Cells were removed by trypsinization and protein precipitated overnight with 20% trichloroacetic acid. Protein was pelleted by centrifugation and washed 3 times with 1.0 ml of 5% trichloroacetic acid + 0.01% proline. Pellets were dissolved with 0.2 M NaOH and the solutions titrated to neutral pH with 0.2 M HCl. Collagenase (2 mg/ml: Worthington Biochemical Corp.) in Tris/$CaCl_2$/*N*-ethymaleimide buffer was added to each tube and samples were allowed to incubate for 1 h at 37 °C. Samples were then placed on ice and proteins were precipitated with 10% trichloroacetic acid for 1 h. Samples were centrifuged at 14,000 rpm for 10 min and the collagenase-sensitive [^{3}H]proline in the supernatant was determined by liquid scintillation counting.

Analysis of Data—Statistical comparisons (*t* tests and one-way analysis of variance) and graphics were performed using Graph Pad Prism 3.0 (GraphPad Software).

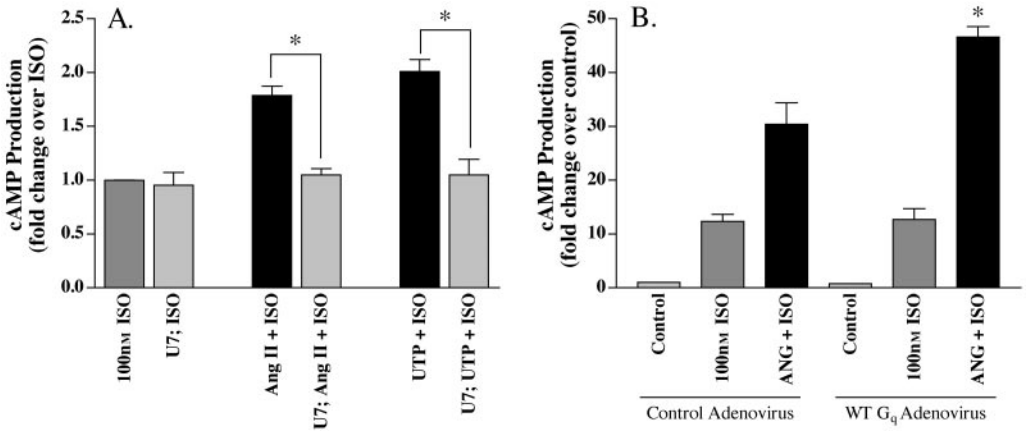

FIG. 1. **G_q-G_s cross-talk is dependent upon G_q and PLC activation.** *A*, cardiac fibroblasts were treated with PLC inhibitor U73122 (5 μM) for 5 min. Cells were co-stimulated with ANG II + ISO as well as with UTP + ISO. Cell lysates were extracted with 0.1 M HCl and cAMP was determined by an enzyme-linked immunosorbent assay. Data are mean ± S.E. expressed as fold-change over ISO. *, statistically significant differences ($p < 0.01$) by one-way analysis of variance. *B*, cardiac fibroblasts were infected with adenoviruses containing the *lacZ* gene (control) and the wild-type α subunit of G_q (10 virus particles/cell) for 18–24 h prior to 15-min reactions and cAMP determinations. Data are mean ± S.E. expressed as fold-change over control. *, statistically significant differences *versus* control virus ANG II + ISO ($p < 0.01$) by Student's *t* test.

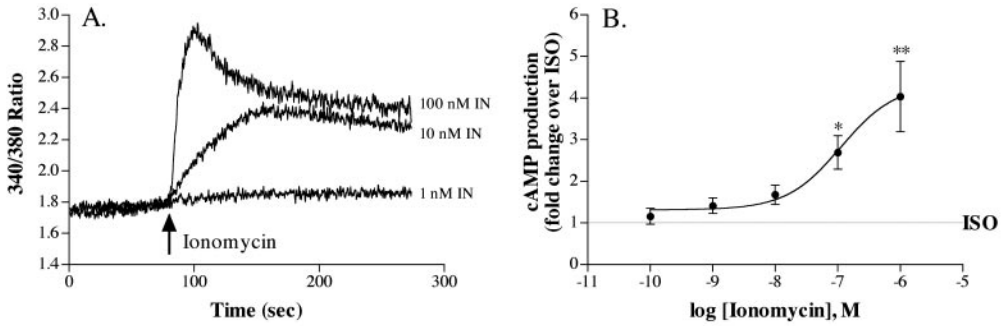

FIG. 2. **Ionomycin-induced intracellular Ca^{2+} transients enhance cAMP production.** *A*, cardiac fibroblasts were loaded with Fura-2/AM (1 μM) and treated with the calcium ionophore ionomycin (*IN*) at varying concentrations. As anticipated, IN elevated cytosolic Ca^{2+} in a dose-dependent manner. The fluorescence ratios are representative data of 3 similar experiments. *B*, cells were co-treated with ISO + IN in the presence of 0.1 mM isobutylmethylxanthine for 15 min at the indicated doses prior to cAMP measurements. Data are mean ± S.E. expressed as fold-change over ISO. Statistically significant differences were determined *versus* ISO alone by Student's *t* test (*, $p < 0.05$; **, $p < 0.01$).

RESULTS

Cross-talk between ANG II and βAR Signaling Pathways Is Mediated by Phospholipase C and G_q—Meszaros *et al.* (20) have described that ANG II, perhaps acting via G_q, potentiates βAR and G_s-mediated cAMP production in cardiac fibroblasts. In the first series of experiments we assessed whether cross-talk between G_q and G_s was dependent upon G_q-promoted phospholipase C (PLC) activity (promoted by G_q activation). We incubated cardiac fibroblasts with the specific PLC inhibitor, U73122, prior to hormonal stimulation with ANG II (100 nM) or UTP (30 μM) in combination with ISO (1 μM, Fig. 1). UTP provides a second class of agonist to assess cross-talk because it is an efficacious activator of the G_q/PLC/inositol trisphosphate pathway in these cells (20). Neither ANG II nor UTP alone caused altered cAMP accumulation. At the concentration used in these experiments, ISO routinely produces a 10–12-fold increase in cAMP levels in cardiac fibroblasts (20). ANG II and UTP potentiated the response to ISO by 1.8 ± 0.1 and 2.0 ± 0.1-fold (relative to ISO alone), respectively. Whereas U73122 alone did not affect basal cAMP accumulation or the ISO response, this inhibitor (at a concentration that reduced stimulated phosphoinositide hydrolysis 75–80%) completely eliminated the potentiation of cAMP accumulation by ANG II and UTP. These data suggest that G_q-linked activation of PLC is required for the effect of G_q potentiation of G_s-AC activity.

To assess whether activation of G_q might mediate the effects of ANG II on βAR signaling, we used adenoviral-mediated gene transfer of G_q to increase its expression in cardiac fibroblasts (Fig. 1*B*). Increased expression of G_q enhanced inositol phosphate accumulation by both ANG II (1.6-fold increase over control) and UTP (2.6-fold increase over control) and significantly enhanced the ANG II potentiation of ISO-stimulated cAMP (3.9 ± 0.5-fold over ISO alone) compared with control cells incubated with the null (PACCMVpLpA) virus (2.5 ± 0.3-fold over ISO alone). Thus, results in Fig. 1 are consistent with the conclusion that activation of GPCRs that activate G_q and PLC enhance βAR/G_s-mediated cAMP formation.

To determine whether signaling by Gβγ subunits generated by activation of G_q mediates the observed cross-talk, we used an adenovirus to express the C-terminal peptide of GRK_2 (βARK_1). This peptide (βARKct) binds to free Gβγ subunits, and inhibits both Gβγ signaling and GRK_2 activation (27). We exposed cardiac fibroblasts to either βARKct or null virus and measured cAMP accumulation stimulated by ISO with and without ANG II. Expression of βARKct did not attenuate ANG II-induced potentiation of ISO-stimulated cAMP accumulation in cardiac fibroblasts (null virus: ISO 10.8 ± 0.2-fold over basal, ANG II + ISO 19.4 ± 2.2; βARKct virus: ISO 9.2 ± 0.5, ANG II + ISO 20.8 ± 0.6) but did induce a 4-fold increase in ISO potency, consistent with its action blocking GRK_2 activation (27).

Another possible pathway by which ANG II could effect cAMP production is via receptor tyrosine kinase signaling. However, cross-talk was not inhibited in cells treated with

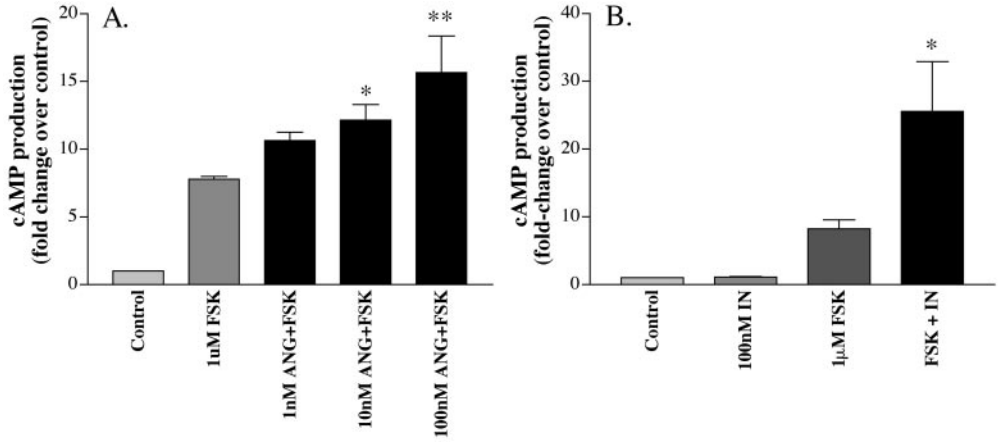

FIG. 3. **ANG II and ionomycin enhance forskolin-stimulated cAMP production.** *A*, the cells were given varying concentrations of ANG II in combination with 1 μM forskolin (*FSK*) for 15 min prior to cAMP determinations. Data are mean ± S.E. expressed as fold-change over control. Statistically significant differences were determined *versus* FSK alone by one-way analysis of variance (*, $p < 0.05$; **, $p < 0.001$). *B*, cardiac fibroblasts were co-treated with ionomycin (*IN*) + 1 μM FSK for 15 min and cAMP was determined. Data are mean ± S.E. expressed as fold-change over control. *, statistically significant differences *versus* FSK alone ($p < 0.05$) by Student's *t* test.

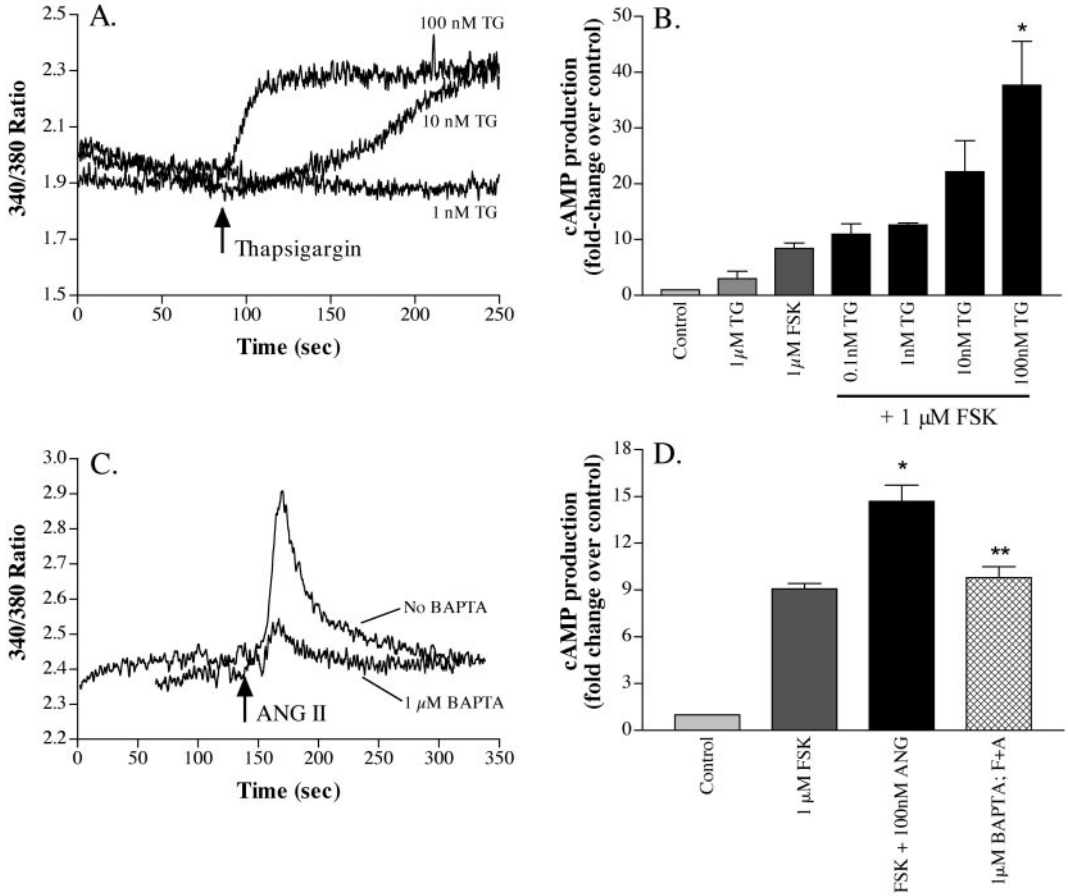

FIG. 4. **Intracellular Ca^{2+} transients enhance cAMP production and are blocked by Ca^{2+} chelation.** *A*, cardiac fibroblasts were loaded with 1 μM Fura-2/AM and the 340/380 fluorescence ratios were monitored. The cells were given varying concentrations of thapsigargin (*TG*), which produced dose-dependent increases in intracellular Ca^{2+}. Data are representative of 3 similar experiments. *B*, cells were co-treated with similar doses of TG (as in *A*) along with 1 μM FSK. A dose-dependent increase in cAMP levels was observed with increasing concentrations of TG. Data are mean ± S.E. expressed as fold-change over control. *, statistically significant differences *versus* FSK alone ($p < 0.01$) by Student's *t* test. *C*, cells were incubated in the absence and presence 1 μM BAPTA/AM for 30 min prior to monitoring intracellular Ca^{2+} levels in the presence of 100 μM ANG II. BAPTA/AM effectively buffers intracellular Ca^{2+} levels when stimulated with 100 nM ANG II. Data are representative of three similar experiments. *D*, cardiac fibroblasts were treated with 1 μM BAPTA/AM for 30 min, and then with the indicated hormone treatments for 15 min. BAPTA/AM effectively blocked the FSK/ANG II potentiation of cAMP. Data are mean ± S.E. expressed as fold-change over control. *, statistically significant differences *versus* FSK alone ($p < 0.01$) by Student's *t* test. **, statistically significant differences *versus* FSK + ANG II by Student's *t* test ($p < 0.001$).

AG1478 (10 μM), a specific tyrosine kinase inhibitor (ANG II + ISO, 2.0 ± 0.1-fold over ISO alone; ANG II + ISO + AG1478 2.0 ± 0.1) or with PP2 (1 μM), a selective Src inhibitor (data not shown). These findings and those of Fig. 1 indicate that ANG II enhances ISO-mediated cAMP production via the action of G_q and PLC rather than G$\beta\gamma$ or tyrosine kinase-associated signal-

ing, an important distinction given that ANG II activates both G protein and tyrosine kinase pathways in cardiac fibroblasts (28, 29).

Requirement for Elevation of Intracellular Ca^{2+} but Not PKC—Because G_q activation by ANG II elevates intracellular Ca^{2+} levels and activates PKC in cardiac fibroblasts (29), we examined the role of both responses as potential mechanisms by which ANG II influences cross-talk with G_s. We utilized the Ca^{2+} ionophore, ionomycin, to elevate intracellular Ca^{2+} levels in a concentration-dependent manner (Fig. 2*A*) that synergistically enhanced ISO-induced cAMP production (Fig. 2*B*) by 2.7 ± 0.4 to 4.0 ± 0.9-fold compared with ISO alone (indicated by the *dashed line*) using 10 and 100 nM ionomycin, respectively. Ionomycin alone did not alter basal cAMP production. Conversely, pharmacological inhibition of PKC by GF109203X (10 μM) failed to inhibit the ANG II-induced potentiation of the ISO response (ANG II + ISO 2.0 ± 0.1-fold over ISO alone, plus GF109203X 1.9 ± 0.1) but did reduce UTP-stimulated phospho-extracellular signal regulated kinase immunoreactivity (basal, 36 ± 6.1 OD; 100 μM UTP, 1037 ± 41; UTP + GF109203X, 180 ± 14.4). Similar negative results were seen with the PKC inhibitors calphostin C and staurosporine (data not shown). Thus, G_q/G_s cross-talk appears to be mediated by the effect of ANG II to elevate intracellular Ca^{2+} and not activation of PKC.

ANG II and Ionomycin Potentiate Forskolin-induced cAMP Production—To test whether the synergistic effects of ANG II on ISO-stimulated cAMP formation might occur via an effect distal to βAR, we utilized forskolin, which directly activates AC (albeit with some dependence upon G_s). Forskolin (1 μM) alone stimulated cAMP production by 8–10-fold over basal levels, a level of response similar to that seen with 0.1–1 μM ISO. ANG II potentiated forskolin-induced cAMP levels (over forskolin alone) in a concentration-dependent manner in the range tested (1–100 nM), reaching statistical significance at 10 and 100 nM (1.6 and 2-fold over forskolin alone, respectively, Fig. 3*A*). Likewise, ionomycin (100 nM) significantly potentiated forskolin-induced cAMP levels (from 8.3 ± 1.3-fold alone to 25.5 ± 7.4-fold in combination, relative to controls, Fig. 3*B*). These results indicate that the effect of ANG II is distal to the receptor and that ionomycin can mimic this effect by elevating intracellular Ca^{2+}.

Effects of Ca^{2+} Store Release and Ca^{2+} Buffering on G_q-G_s Cross-talk—We next sought to determine whether G_q-G_s cross-talk might occur via G_q-promoted Ca^{2+} release from intracellular stores and if we could inhibit the cross-talk by buffering intracellular Ca^{2+}. Thapsigargin, which inhibits the sarcoendoplasmic reticular Ca^{2+}-ATPase pump, elevated intracellular Ca^{2+} levels in a concentration-dependent manner (Fig. 4*A*). Thapsigargin at a concentration of 100 nM was maximally efficacious in stimulating rapid Ca^{2+} release; this concentration also synergistically enhanced forskolin-induced cAMP production (Fig. 4*B*) with slight but statistically insignificant effects on basal cAMP production. We obtained similar results when these experiments were performed in the absence of extracellular Ca^{2+} (data not shown), indicating that Ca^{2+} release from internal stores was sufficient to observe the enhancement in cAMP formation. These results, together with those from the studies with ionomycin, indicate that potentiation of the cAMP signal can occur from elevation of intracellular Ca^{2+}, either by storage release or influx promoted by a Ca^{2+} ionophore. Conversely, preincubation with 1 μM BAPTA/AM for 30 min prior to agonist stimulation prevented ANG II-induced Ca^{2+} transients (Fig. 4*C*) and blocked the synergistic effects of ANG II on forskolin-mediated cAMP production (Fig. 4*D*). Thus, elevation of intracellular Ca^{2+} is both necessary and sufficient for potentiation of cAMP production by ANG II. The

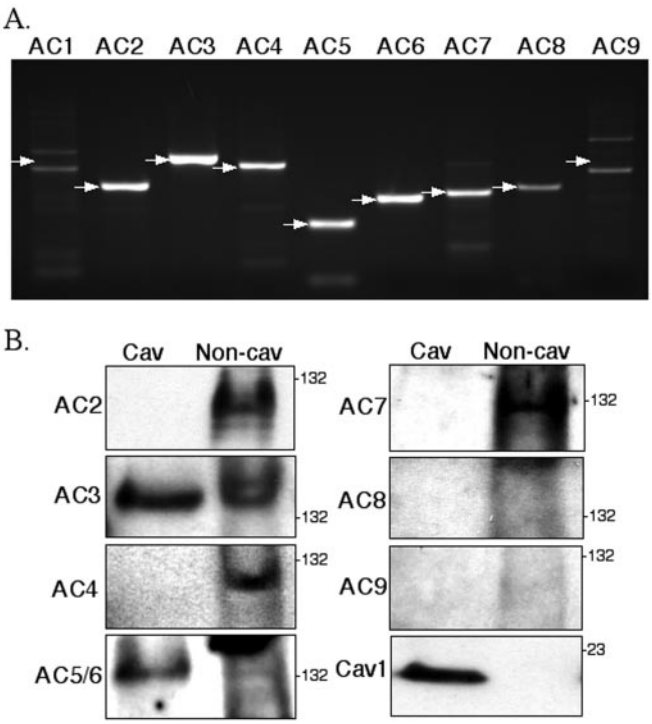

FIG. 5. **Cardiac fibroblasts express multiple AC isoforms with different subcellular distributions.** *A*, RT-PCR analysis was performed using AC isoform-specific primer pairs (see "Experimental Procedures") and cDNA, mRNA (no reverse transcriptase negative control, not shown), or genomic DNA (positive control, not shown). *Arrows* indicate the size of the expected PCR product from each primer pair. Image is representative of three experiments. *B*, expression of AC isoforms was assessed by immunoblot analysis of caveolin-rich (*cav*) and non-caveolin rich (*non-cav*) membrane fractions from cardiac fibroblasts (see "Experimental Procedures"). Each fraction was separated by SDS-PAGE, transferred to membrane, and probed with antibodies specific for AC1, AC2, AC3, AC4, AC5/6, AC7, AC8, AC9, and caveolin 1. Nonspecific immunoreactive bands are partially evident at the *top* of images for AC5/6 and AC8. Each image is representative of at least three experiments.

results seen with forskolin indicate that Ca^{2+} works at the level of G_s/AC rather than βAR.

AC Isoform Expression in Cardiac Fibroblasts—Because the identity of AC isoforms expressed in cardiac fibroblasts could result in specific signaling characteristics, we sought to define the AC isoforms expressed in these cells and in particular to assess isoforms that are regulated by Ca^{2+}. RT-PCR analysis using isoform-specific primers revealed that cardiac fibroblasts express mRNA for AC2, AC3, AC4, AC5, AC6, AC7, and AC8 (Fig. 5*A*). Each of the primer pairs amplified appropriate genomic DNA sequence but did not yield PCR products when RNA (no reverse transcriptase) was used as template (data not shown). PCR reactions using primers for AC1 and AC9 yielded products that were not of the expected size or sequence. We also conducted immunoblot analysis to detect expression of AC proteins. Because AC immunoreactivity in cardiac myocytes is enriched in buoyant, caveolin-rich fractions in a manner that improves immunological detection (22, 30), we fractionated cardiac fibroblasts to isolate caveolin-rich fractions and performed immunoblot analyses. As shown in Fig. 5*B*, caveolin-1 immunoreactivity was detected in buoyant fractions (caveolin-rich fraction) and was excluded from non-buoyant fractions. Immunoreactivity for AC3 and AC5/6 (the antibody used does not distinguish between AC5 and AC6) was detected primarily in caveolin-enriched membrane fractions, whereas immunoreactivity of AC2, AC4, and AC7 was detected only in non-caveolin-enriched membrane fractions (Fig. 5*B*). No immunoreac-

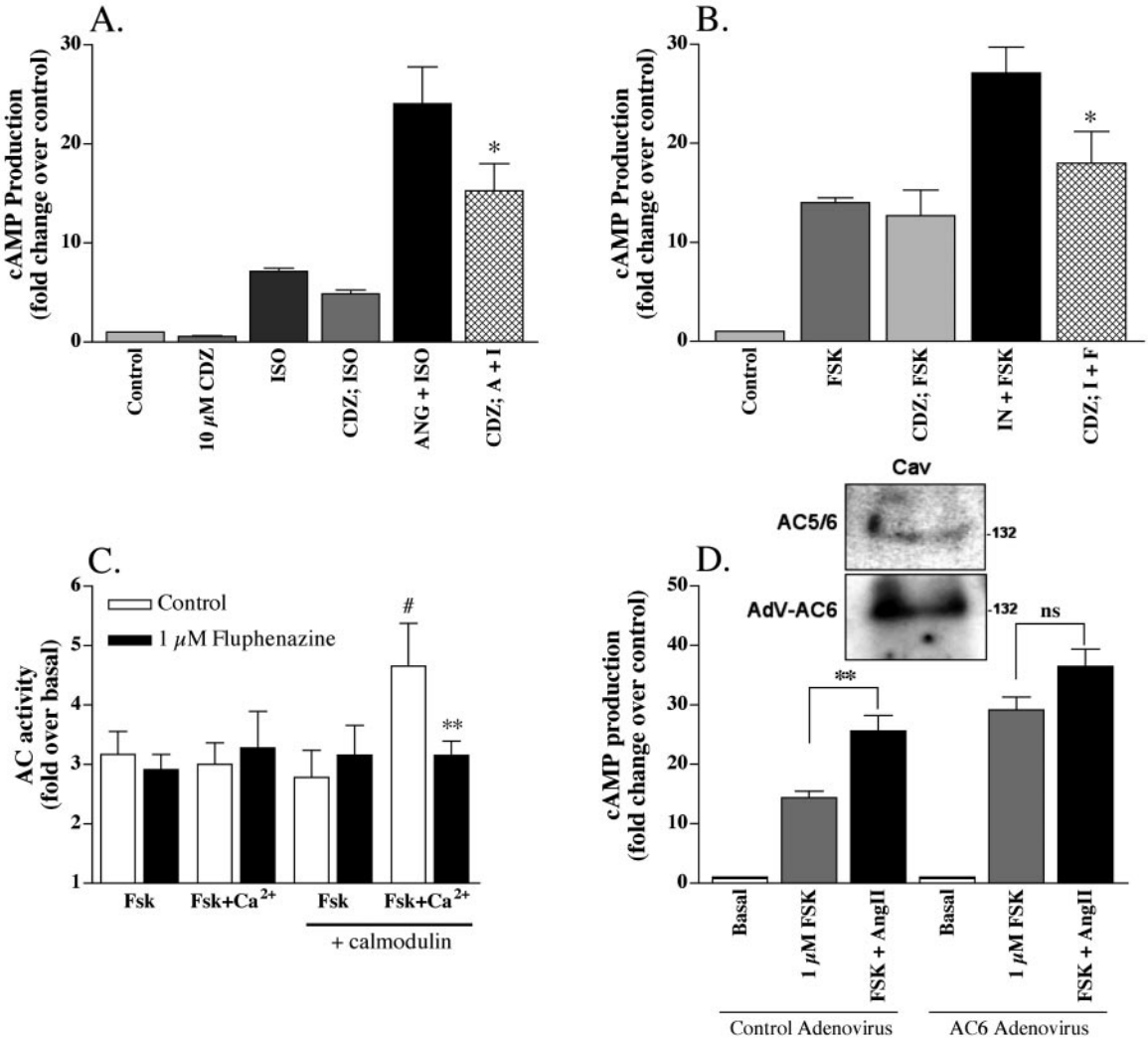

FIG. 6. **Calmidazolium and overexpression of AC6 inhibit cross-talk.** *A,* cardiac fibroblasts were pretreated with the calmodulin inhibitor calmidazolium (*CDZ*) for 30 min prior to reactions. The potentiation effect produced by the combination of ANG II and ISO was significantly blocked in the presence of CDZ, but did not have a significant effect on ISO alone. Data are mean ± S.E. expressed as fold-change over control. *, statistically significant differences *versus* ANG II + ISO (*, $p < 0.05$) by Student's *t* test. *B*, cells were pretreated with CDZ for 30 min prior to reactions. CDZ significantly inhibited cAMP potentiation induced by IN and FSK, but did not have a significant effect on FSK alone. Data are mean ± S.E. expressed as fold-change over control. *, statistically significant differences *versus* IN + FSK (*, $p < 0.05$) by Student's *t* test. *C,* AC activity was measured in cardiac fibroblast membranes with or without calmodulin (0.1 μM) and with or without fluphenazine (1 μM). Data are expressed as -fold over basal AC activity. #, statistically significant difference ($p < 0.05$) *versus* Fsk alone; **, statistically significant difference ($p < 0.01$) *versus* control by Student's *t* test. *D*, production of cAMP in response to forskolin or forskolin plus ANG II was measured in control and AC6-overexpressing cardiac fibroblasts. ANG II potentiation of FSK was significant ($p < 0.01$) by Student's *t* test) in control cells but not significant ($p = 0.14$) in AC6 overexpressing cells. Immunoblot analysis indicated an appreciable increase in AC5/6 protein in caveolin-enriched membrane (*Cav*) fractions of cardiac fibroblasts incubated with AC6 adenovirus (*inset*).

tivity was detected for AC8 or AC9 (Fig. 5*B*). Thus, cardiac fibroblasts express numerous AC isoforms but the isoforms appear to be differentially localized in caveolin-rich membrane microdomains.

AC Isoform Specificity of G_q-G_s Cross-talk—We hypothesized that expression of a Ca^{2+}-calmodulin-stimulable isoform of AC was essential for the cross-talk we observe. Thus, we pretreated cardiac fibroblasts with a specific calmodulin inhibitor (10 μM calmidazolium) and found that it significantly attenuated cross-talk induced by ANG II plus ISO or by ionomycin plus forskolin (Fig. 6, *A* and *B*, respectively). We also measured Ca^{2+} and forskolin-stimulated AC activity in membrane preparations from cardiac fibroblasts with and without exogenously added calmodulin; 3 μM free Ca^{2+} in the presence of forskolin (10 μM) did not stimulate AC activity unless calmodulin (0.1 μM) was added to the reaction (Fig. 6*C*). This Ca^{2+}-stimulable AC activity was inhibited by the inclusion of a calmodulin inhibitor (1 μM fluphenazine, which did not alter basal or forskolin-stimulated AC activity). These results indicate that Ca^{2+}-calmodulin activation of AC is a plausible mechanism for the effect of ANG II and ionomycin to enhance cAMP production by βAR.

We next probed whether it was possible to attenuate cross-talk between ANG II and cAMP production by altering the balance of Ca^{2+}-stimulated *versus* Ca^{2+}-insensitive AC isoforms by using an adenovirus to overexpress an isoform of AC that is not Ca^{2+}-stimulable, AC6 (14). Incubation of cardiac fibroblasts with the AC6 adenovirus for 18 h resulted in a 2-fold increase in maximal forskolin-stimulated cAMP production (Fig. 6*D*). Based on evidence that the extent of AC overexpression is proportional to the increase in maximal forskolin response (13), the conditions used here approximately doubled the total cellular content of AC and generated cardiac fibroblasts expressing predominantly AC6. Immunoblot analyses indicated an appreciable increase in detectible AC5/6 protein in caveolin-enriched membrane fractions (Fig. 6*D*, *inset*). ANG II

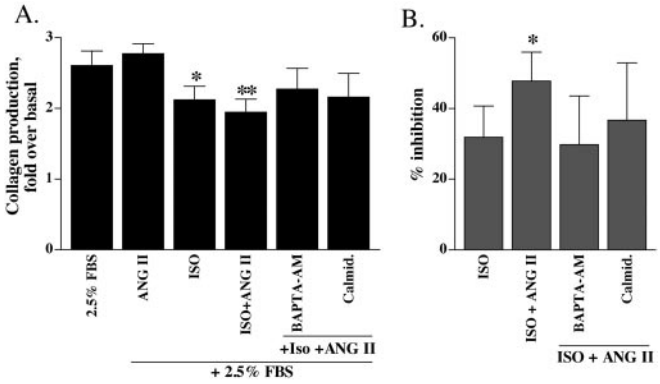

FIG. 7. **G_q-G_s cross-talk impacts cardiac fibroblast collagen synthesis.** *A*, collagen synthesis, measured by collagenase-sensitive [^{3}H]proline incorporation as described under "Experimental Procedures," was stimulated by adding 2.5% FBS to serum-starved cells in the absence and presence of ISO (100 μM), ANG II (10 nM), or ISO plus ANG II for 24 h. The Ca^{2+} chelator BAPTA/AM (0.1 μM) and the calmodulin inhibitor calmidazolium (0.1 μM) were also added in the presence of ISO and ANG II. *B*, data for ISO, ISO plus ANGII, and BAPTA/AM and calmidizolium are presented expressed as the percent inhibition of the FBS-stimulated collagen synthesis. *, statistically different $p < 0.05$; **, $p < 0.01$ by Student's *t* test as compared with 2.5% FBS or FBS + ANG II stimulated, respectively.

potentiation of forskolin-stimulated cAMP was unchanged by AC6 overexpression in terms of absolute amount of cAMP stimulated over forskolin alone (14.2 ± 1.4 pmol of cAMP/mg of protein in control cells *versus* 17.7 ± 8.8 in AC6 cells), but was significantly inhibited in terms of the fractional effect ($p = 0.14$, Fig. 6*D*). Thus, the potentiative effect of ANG II appeared not to extend to the overexpressed AC6. The inability of ANG II to potentiate forskolin-stimulated cAMP production to the same degree in AC6-overexpressing cells was not related to a limited capacity of cardiac fibroblasts to synthesize cAMP in this latter condition, as combined treatment of the cells with ISO and forskolin-stimulated cAMP levels to 27.6 ± 3.48 pmol/mg protein, a level nearly 3-fold higher than the combination of forskolin and ANG II. Thus, cross-talk is decreased in cells manipulated to express increased levels of AC6, an AC isoform not activated by Ca^{2+}. Taken together, the data in Figs. 1–6 implicate the central role of both Ca^{2+} influx and a Ca^{2+}-calmodulin-stimulable AC isoform in mediating the observed G_q-G_s cross-talk.

Role of Cross-talk on Collagen Synthesis—To investigate the functional relevance of the cross-talk between ANG II signaling and the cAMP pathway, we assessed the synthesis of collagen by cardiac fibroblasts. Increased cellular cAMP levels can inhibit cardiac fibroblast-mediated production of collagen (31). Thus, we tested whether simultaneous incubation with cAMP-elevating agents and ANG II would inhibit collagen synthesis to a greater extent than incubation with cAMP-elevating agents alone. FBS-stimulated collagenase-sensitive [^{3}H]proline incorporation was measured in cardiac fibroblasts as an index of collagen formation in the absence and presence of ANG II and in the absence and presence of ISO. 2.5% FBS stimulated collagen synthesis 2.6 ± 0.2-fold over basal levels. ANG II alone increased collagen synthesis (data not shown) but in the presence of 2.5% FBS induced no significant increase over FBS alone (Fig. 7*A*). ISO (100 μM) inhibited FBS-stimulated collagen synthesis (31 ± 9% inhibition, Fig. 7, *A* and *B*). However, in the presence of ANG II, ISO inhibited proline incorporation 48 ± 8%, an effect that was significantly different ($p < 0.01$) from serum-stimulated levels (Fig. 7, *A* and *B*). Moreover, the ANG II enhancement of the ISO effect was reduced to non-significant levels by chelation of Ca^{2+} (with 0.1 μM BAPTA/AM) or inhibition of calmodulin (with 0.1 μM calmidazolium). Thus, the data support the conclusion that the enhanced βAR-mediated cAMP production that ANG II promotes via elevation of Ca^{2+} and signaling via calmodulin can blunt collagen synthesis. Taken together, the data suggest that coincidental G_s and G_q signals interact to modulate cardiac fibroblast signaling and function, and thereby promote a G_s-mediated inhibition of collagen synthesis.

DISCUSSION

The focus of the present work was to define the mechanism of G_q-G_s cross-talk, which we previously described in adult rat cardiac fibroblasts (20). The striking potentiation of βAR signaling in the presence of ANG II might occur via several different mechanisms. ANG II activates AT_1 receptors in cardiac fibroblasts, thereby activating both G_q-coupled and tyrosine kinase signaling pathways (28, 29). Several pieces of evidence indicate that this cross-talk is mediated by activation of G_q: 1) at least 2 other G_q-coupled receptors (P2Y and bradykinin) are capable of inducing similar potentiation (Fig. 1*A* and Ref. 20); 2) blockade of PLC eliminates potentiation of ISO response by either ANG II or UTP (Fig. 1A); 3) overexpression of G_q enhances cross-talk (Fig. 1*B*); 4) chelation of a key second messenger of the G_q pathway, intracellular Ca^{2+}, results in attenuation of cross-talk; and 5) pharmacological agents that directly increase cytosolic Ca^{2+} concentrations (ionomycin, thapsigargin) also potentiate cAMP accumulation (Figs. 2 and 3). These data, along with evidence that exclusion of extracellular Ca^{2+} does not effect cross-talk (data not shown), indicate that ANG II-induced potentiation of βAR signaling is triggered by G_q activation and Ca^{2+} release from internal stores and that Ca^{2+} influx is not required for enhanced cAMP production. Consistent with this conclusion, pharmacological inhibition of the other major pathway activated by G_q, PKC, had no effect on cross-talk.

Our studies with forskolin allowed us to test the target at which Ca^{2+} acts to increase βAR signaling. As a direct activator of AC, albeit requiring G_s activation for full effect (32), forskolin-stimulated cAMP formation was also activated by coincident signals that elevate intracellular Ca^{2+} (*e.g.* ANG II, ionomycin, or thapsigargin, Figs. 3 and 4). These results led us to focus on the role of AC in G_q-G_s cross-talk. We were surprised to discover that cardiac fibroblasts express mRNA for seven of the nine transmembrane AC isoforms (Fig. 5). This expression of multiple isoforms contrasts with results from cardiac myocytes, which appear to express predominantly 2 isoforms, AC5 and AC6 (15). Of the isoforms expressed in cardiac fibroblasts, the protein for AC3 appears to be predominantly expressed, particularly in caveolin-rich fractions (Fig. 5*B*).

Given its level of expression and action as a Ca^{2+}-stimulable isoform, AC3 is likely critical for the cross-talk that we observe in cardiac fibroblasts. The other AC isoforms capable of being activated by Ca^{2+}, AC1 and AC8, were not detected by immunoblot analysis (although AC8 mRNA was detected in PCR studies). Previous data using heterologously expressed protein indicate that AC3 stimulation by Ca^{2+} is dependent upon coincidental activation of AC by either G_s or forskolin (18), results consistent with what we observe in cardiac fibroblasts. An important role for a Ca^{2+}-stimulable AC isoform in the observed cross-talk is inferred by data from vascular smooth muscle (33) and directly supported by our studies overexpressing AC6. By increasing AC6 expression, we altered the balance of AC isoform expression in favor of an isoform not stimulated by Ca^{2+} and found that the G_q-G_s cross-talk was fractionally reduced. Taken together, these data provide a clear-cut example of AC isoform expression imparting distinct signaling characteristics and regulation of cell function.

The membrane microdomains in which expression of AC isoforms occurs can influence cell responses (22, 34). Caveolae are a subset of lipid rafts, plasma membrane microdomains enriched in sphingolipid and cholesterol, and are the site of enrichment of many membrane-associated signaling molecules (35). We found that among numerous AC isoforms expressed in cardiac fibroblasts, only AC3 and AC5/6 localize in buoyant, caveolin-rich domains. These data are the first of which we are aware that show that native AC isoforms localize differently with respect to caveolae/lipid rafts. Smith *et al.* (36) recently reported that AC7 and AC8 heterologously expressed in HEK-293 cells localized differently with respect to caveolin-rich domains (AC8 in caveolar fractions, AC7 in non-caveolar fractions). The reason for the difference between the data from Smith *et al.* (36) and the present studies (we observe AC8 in non-caveolar fractions, Fig. 5) is unclear, but localization of GPCR and AC isoforms in caveolar fractions can be highly cell-specific and can differ between exogenously and endogenously expressed protein (24). More work is needed to understand the functional importance, if any, of the observed pattern of AC isoform expression in different plasma membrane microdomains of these cells.

Our studies of collagen synthesis (Fig. 7) suggest the physiological importance of G_q-G_s cross-talk. Agents that elevate cellular cAMP attenuate collagen synthesis by cardiac fibroblasts (5). In our studies, ISO alone (in the absence of a phosphodiesterase inhibitor) could significantly inhibit collagen synthesis, and the addition of ANG II, which produced a 2-fold increase in ISO-stimulated cAMP accumulation, led to a greater reduction in collagen synthesis. These results appear to conflict with observations that βAR antagonists can reduce fibrosis in heart failure (37, 38). However, the beneficial effects of βAR blockade are likely dominated by improvements in cardiac contractility (which would lead to decreased levels of renin-angiotensin and possibly other hormones) and myocyte survival, which in turn influence ECM formation and turnover as well as fibroblast proliferation. Our findings are consistent with data on certain other G_s-linked agonist action on cardiac fibroblasts (31, 39) and with a previous report that the βAR antagonist, propranolol, inhibits collagen deposition in pulmonary fibroblasts, cells not subjected to the mechanical forces of a contractile organ (40). In addition, our studies examine fibroblasts over a short period (24 h) and in isolation, removed from circulating catecholamines and the mechanical forces of cardiac contraction.

In conclusion, the current results demonstrate that ANG II enhances βAR signaling via activation of G_q and elevated intracellular Ca^{2+}. We propose that a Ca^{2+}-stimulable isoform of AC, likely AC3, is the regulatory site of this Ca^{2+}/calmodulin-dependent potentiation and acts as an integrator of these signaling pathways. Moreover, cross-talk between the G_q and G_s pathways (*i.e.* enhanced cAMP production) appears to have functional consequences for regulation of the ECM in myocardium, acting to limit collagen synthesis.

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Additions and Corrections

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Helical structure of the needle of the type III secretion system of *Shigella flexneri*.

Frank S. Cordes, Kaoru Komoriya, Eric Larquet, Shixin Yang, Edward H. Egelman, Ariel Blocker, and Susan M. Lea

Page 17107, Acknowledgments: The name of our collaborator was incorrectly listed. The correct name is David DeRosier.

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Angiotensin II enhances adenylyl cyclase signaling via Ca^{2+}/calmodulin. G_q-G_s cross-talk regulates collagen production in cardiac fibroblasts.

Rennolds S. Ostrom, Jennifer E. Naugle, Miki Hase, Caroline Gregorian, James S. Swaney, Paul A. Insel, Laurence L. Brunton, and J. Gary Meszaros

Page 24465: In Fig. 5 of this paper, we analyzed the localization of natively expressed isoforms of adenylyl cyclase (AC) in buoyant, caveolin-rich fractions from rat cardiac fibroblasts isolated on a discontinuous sucrose gradient. Fig. 5 shows and under "Results" we state that immunoreactivity to AC8 and AC9 was not detected. However, under "Discussion," we mistakenly stated that we observed AC8 immunoreactivity in non-caveolin-rich fractions. While a minority of our studies revealed a small amount of AC8 immunoreactivity in non-caveolin-rich fractions, our results were neither definitive nor consistent enough for us to conclude that AC8 is expressed in non-caveolin-rich fractions. Therefore, we conclude that AC8 protein is not detectable in cardiac fibroblasts by the methods that we have employed.

Angiotensin II Enhances Adenylyl Cyclase Signaling via Ca^{2+}/Calmodulin: Gq-Gs CROSS-TALK REGULATES COLLAGEN PRODUCTION IN CARDIAC FIBROBLASTS

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