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Research Article

Our laboratory has been testing the hypothesis that genetic modulation of the beta-adrenergic signaling cascade can enhance cardiac function. We have previously shown that transgenic mice with cardiac overexpression of either the human beta2-adrenergic receptor (beta2AR) or an inhibitor of the beta-adrenergic receptor kinase (betaARK), an enzyme that phosphorylates and uncouples agonist-bound receptors, have increased myocardial inotropy. We now have created recombinant adenoviruses encoding either the beta2AR (Adeno-beta2AR) or a peptide betaARK inhibitor (consisting of the carboxyl terminus of betaARK1, Adeno-betaARKct) and tested their ability to potentiate beta-adrenergic signaling in cultured adult rabbit ventricular myocytes. As assessed by radioligand binding, Adeno-beta2AR infection led to approximately 20-fold overexpression of beta-adrenergic receptors. Protein immunoblots demonstrated the presence of the Adeno-betaARKct transgene. Both transgenes significantly increased isoproterenol-stimulated cAMP as compared to myocytes infected with an adenovirus encoding beta-galactosidase (Adeno-betaGal) but did not affect the sarcolemmal adenylyl cyclase response to Forskolin or NaF. beta-Adrenergic agonist-induced desensitization was significantly inhibited in Adeno-betaARKct-infected myocytes (16+/-2%) as compared to Adeno-betaGal-infected myocytes (37+/-1%, $P < 0.001$). We conclude that recombinant adenoviral gene transfer of the beta2AR or an inhibitor of betaARK-mediated desensitization can potentiate beta-adrenergic signaling.

Potentiation of β-Adrenergic Signaling by Adenoviral-mediated Gene Transfer in Adult Rabbit Ventricular Myocytes

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Abstract

Our laboratory has been testing the hypothesis that genetic modulation of the β-adrenergic signaling cascade can enhance cardiac function. We have previously shown that transgenic mice with cardiac overexpression of either the human β_2-adrenergic receptor (β_2AR) or an inhibitor of the β-adrenergic receptor kinase (βARK), an enzyme that phosphorylates and uncouples agonist-bound receptors, have increased myocardial inotropy. We now have created recombinant adenoviruses encoding either the β_2AR (Adeno-β_2AR) or a peptide βARK inhibitor (consisting of the carboxyl terminus of βARK1, Adeno-βARKct) and tested their ability to potentiate β-adrenergic signaling in cultured adult rabbit ventricular myocytes. As assessed by radioligand binding, Adeno-β_2AR infection led to ~ 20-fold overexpression of β-adrenergic receptors. Protein immunoblots demonstrated the presence of the Adeno-βARKct transgene. Both transgenes significantly increased isoproterenol-stimulated cAMP as compared to myocytes infected with an adenovirus encoding β-galactosidase (Adeno-βGal) but did not affect the sarcolemmal adenylyl cyclase response to Forskolin or NaF. β-Adrenergic agonist-induced desensitization was significantly inhibited in Adeno-βARKct–infected myocytes (16±2%) as compared to Adeno-βGal-infected myocytes (37±1%, $P < 0.001$). We conclude that recombinant adenoviral gene transfer of the β_2AR or an inhibitor of βARK-mediated desensitization can potentiate β-adrenergic signaling. (*J. Clin. Invest.* 1997. 99:288–296.) Key words: gene therapy • β-adrenergic receptor • myocardium • cultured cells • congestive heart failure

Introduction

The activation of β-adrenergic receptors (β-ARs)[1] by catecholamines is a critical event in the regulation of cardiac function. Transduction of this signal is mediated by heterotrimeric guanine nucleotide binding (G) proteins and includes the generation of second messengers such as cAMP. In turn, cAMP-dependent protein kinase (PKA) is activated and phosphorylates proteins including L-type voltage-dependent calcium channels (1) and phospholamban (2), leading to enhanced cardiac chronotropy and inotropy. Though the β_1-AR is the predominant subtype present in the myocardium, ~ 20–30% of total cardiac β-ARs are β_2-ARs and they too contribute to the regulation of cardiac function (3), possibly through a distinct intracellular pathway (4, 5).

Congestive heart failure (CHF) is a clinical syndrome that confers significant morbidity and mortality despite recent advances in medical therapy (6). Numerous abnormalities in the β-AR signaling cascade occur in this syndrome including a selective downregulation of β_1ARs (7, 8) and an upregulation of the β-adrenergic receptor kinase (βARK), an enzyme that specifically phosphorylates and uncouples the activated β-AR (9, 10). Modulation of this pathway to improve cardiac inotropy is being explored by our laboratory as a novel therapeutic approach for CHF. Transgenic mice with selective cardiac overexpression of either the human β_2AR (11) or an inhibitor of βARK (12) have enhanced contractility. The current study was undertaken to explore whether recombinant adenoviral-mediated transfer of these transgenes would be able to potentiate β-adrenergic signaling in cultured adult rabbit ventricular myocytes.

Methods

Myocyte isolation and culture. All studies with rabbits were approved by the institutional review board at Duke University. Adult male New Zealand white rabbits were anesthetized, treated with heparin, and then intubated. The heart was excised and perfused by the Langendorff technique with Joklik's modified MEM containing hyaluronidase, collagenase, protease, and 12.5 μM $CaCl_2$ as previously described in references 13 and 14. When the heart turned soft, the ventricles were dissected free and agitated into a solution of Joklik's MEM with 10% fetal bovine serum. Cells were allowed to settle by gravity twice in 10 ml of culture medium (medium 199 with Earle's salts, 25 mM Hepes, and sodium bicarbonate without glutamine

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1. *Abbreviations used in this paper:* Adeno-β_2AR, Adenovirus encoding human β_2-adrenergic receptor; Adeno-βARKct, Adenovirus encoding βARK_{ct} transgene; Adeno-βGal, Adenovirus encoding a β-galactosidase transgene; βAR, β-adrenergic receptor; βARK, β-adrenergic receptor kinase; βARKct, carboxyl terminus fragment of bovine βARK1; CHF, congestive heart failure; CMV, cytomegalovirus; ISO, (−)-isoproterenol.

[Sigma Chemical Co., St. Louis, MO] containing 10% fetal bovine serum [GIBCO BRL, Gaithersburg, MD], 10 μM cytosine β-D-arabinofuranoside [ARA-C; Sigma Chemical Co.], 100 IU/ml penicillin, and 100 μg/ml streptomycin [Sigma Chemical Co.]). This procedure typically yielded 1–2 $\times$ 10^7 myocytes per rabbit heart, with 50–80% in a rod-shaped morphology. Myocytes were plated at a density of 1 $\times$ 10^5/35 mm well or 1 $\times$ 10^6 100 mm well on tissue culture plates that were precoated with 20 μg/ml of mouse laminin (GIBCO BRL) for 1 h. The myocytes were calcium tolerant as evidenced by their quiescent state throughout the experiments.

Adenoviral construction. Preparation of the β-galactosidase adenovirus (Adeno-βGal) (see Fig. 1) has been described in reference 15. The adenoviral backbone for Adeno-β_2AR and Adeno-βARKct was a replication-deficient "first-generation" adenovirus with deletions of the E1 and E3 genes. It contains the cytomegalovirus (CMV) promoter and bovine growth hormone polyadenylation (bGH) site separated by a polylinker containing a unique XbaI site. The Adeno-empty vector does not contain an insert in the polylinker region. 100 μg of Adeno-empty vector DNA was prepared by digestion of a large scale preparation of this virus (see below) with proteinase K (Sigma Chemical Co.) in the presence of 0.5% SDS at 55°C for 2 h, followed by phenol/chloroform (1:1 ratio) extraction and ethanol precipitation. This was then digested by XbaI overnight and the large fragment containing the bGH and adenovirus map units 9.3–100 was gel purified on a 0.6% agarose gel. This fragment served as the "right end" of both Adeno-β_2AR and Adeno-βARKct. The "left ends" of these viruses were constructed uniquely. For Adeno-β_2AR, plasmid pCMV4 (16) was digested by SpeI and XbaI. The resulting 740-bp band was subcloned into plasmid pACCMV (gift of Dr. Robert Gerard, University of Texas Southwestern Medical Center, Dallas, TX) creating plasmid pACCMV4. The human β_2AR (11) was PCR amplified using primers 5′-AATTGAATTCCCAGACTGCGCGCCATGG-3′ and 5′-ATATTCTAGATGCAGGTGGACTGCTACC-3′, digested with EcoRI and XbaI, and then subcloned into plasmid pACCMV4. This construct was then digested with PvuI and XbaI and the fragment (Adeno-β_2AR "left end") containing adenovirus map units 0.0–1.3, the CMV promoter, the AMV translational enhancer derived from plasmid pCMV4, and the human β_2AR was gel purified. For the "left end" of Adeno-βARKct, plasmid pACCMV4 was PCR amplified with primers 5′-AATGCCGGCGTTTAAACATCATCAATAATATACC-3′ and 5′-AATTCTAGATTAATTAAGCTAGCCTAGGATCCCCGGGTACCGAG-3′, digested with NgoMI and XbaI, and subcloned into plasmid pBluescript SK (Strategene, La Jolla, CA) creating plasmid pSKAC. The βARK1 carboxyl terminus fragment (βARKct; 17) was digested with EcoRI and XbaI and subcloned into pSKAC. The resulting construct was digested with PmeI and XbaI, and the fragment (Adeno-βARKct "left end") containing adenovirus map units 0.0–1.3, the CMV promoter, the AMV translational enhancer, and βARKct was gel purified. Approximately 100 ng of the appropriate gel-purified "left end" fragment of Adeno-β_2AR or Adeno-βARKct was then ligated to $\sim$ 1 μg of the "right end" fragment overnight at 16°C. The ligation mixture was transfected onto a single 60 mm dish of 293 human embryonal kidney cells (18) using lipofectamine (GIBCO BRL). The plate was allowed to lyse without an agar overlay. Individual viruses were then isolated by two consecutive rounds of plaque purification using an agar overlay.

After isolation, individual viruses were prepared at large scale by infecting 40 150 mm plates of EBNA transfected 293 cells (Invitrogen Corp., San Diego, CA) with the appropriate virus at a multiplicity of infection (moi) of three. 36–48 h after infection, when the majority of the cells were floating, the cells were harvested by gentle scraping and collected by a 5 min centrifugation at 1,000 *g* in a GSA rotor. The cell pellet was resuspended in 20 mM Tris HCl, pH 7.4, 2 mM EDTA, pH 8.0, and the cells were homogenized with 20 strokes in a Dounce homogenizer. DNase A was added to 100 μg/ml and the homogenate was incubated at room temperature for 5 min. The nuclei were removed by centrifugation at 2,500 *g* for 10 min. CsCl was added to the supernatant to 0.3 g/ml and the supernatant was then layered on top of a CsCl step gradient (1.3 and 1.4 g/ml prepared in virus storage buffer [VSB] 137 mM NaCl, 20 mM Tris HCl, pH 7.4, 5 mM KCl, and 1 mM $MgCl_2$) and centrifuged for 2 h at 32K rpm in a Sorvall Instruments Div. (Newton, CT) TH 64 rotor. The virus band that formed at the 1.3–1.4 g/ml interphase was removed with a 16 gauge needle. The virus preparation was layered on top of a 2 ml bed of Cl-6B (Pharmacia Fine Chemicals, Piscataway, NJ) prepared in a 3 ml syringe and centrifuged for 2 min at 1,000 rpm in a Beckman Instruments, Inc. (Fullerton, CA) tabletop centrifuge. This step was repeated once more and the virus concentration was adjusted to 1 $\times$ 10^{11} plaque forming units (pfu)/ml in VSB. Sucrose was added to 10% final and the virus preparation was stored in aliquots at −80°C. Each aliquot was used a maximum of two times and discarded. Adenoviral titers were determined using plaque titration on HEK 293 cells (17). Alternatively, plaque titers were estimated by determining the absorbance at 260 nm (pfu/ml = A_{260} $\times$ dilution $\times$ 10^{10}) of the final viral preparation.

Adenoviral infection. After myocytes became adherent to the tissue culture plates ($\sim$ 5 h after harvesting), they were infected with an appropriate titer of adenovirus in 1 ml medium 199. After 1 h, without aspirating the adenoviral-containing medium, culture medium was added back to the plates.

Cellular viability after adenoviral infection. 36 h after adenoviral infection, the medium was aspirated and the plates were washed twice with PBS. 1.5% glutaraldehyde in 50% PBS (19) was added to the plates as a fixative. The number of rod-shaped or round myocytes was counted at a magnification of 200. 20 fields per plate were counted. Only myocytes fully visible within the field were counted. Rod-shaped myocytes included those in which the length of the cell was at least two times its width with an overall linear morphology. Myocytes with mild end-plate changes were not excluded if the overall morphology met the above conditions. Only round myocytes that were at least one-half the size of the average round myocyte in a field were counted (to exclude cellular debris). To verify adenoviral infection in these experiments, transgene expression from concurrently infected plates was demonstrated as described below.

β-Galactosidase expression. 36 h after adenoviral infection, cells were fixed in 0.5% glutaraldehyde in 50% PBS for 5 min at room temperature, and then stained with 10 mM $K_4Fe(CN)_6$, 10 mm $K_3Fe(CN)_6$, 2 mM $MgCl_2$, and 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-d galactopyranoside) (20) in PBS for 30 min at 37°C. The staining solution was then aspirated and the cells were permanently fixed in 1.5% glutaraldehyde in 50% PBS.

Immunoblotting. 36 h after adenoviral infection, cells were harvested in lysis buffer (5 mM Tris-HCl, pH 7.4, and 5 mM EDTA) and Dounce homogenized with 10 strokes on ice. Samples were centrifuged 40,000 *g* to pellet membranes and supernatants were concentrated in a Centricon-10 (Amicon, Beverly, MA) at 5,000 *g* for 30 min at 4°C. The membrane fraction was resuspended in 75 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, and 2 mM EDTA. Protein concentration was determined by Bradford's method (21). For detection of the Adeno-βARKct transgene, 5 μg of the cytosolic extracts were electrophoresed on a 12% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. As described in reference 17, a rabbit polyclonal anti-serum that recognizes the carboxyl terminus of βARK was used as a primary antibody and chemiluminescent detection was achieved via an alkaline-phosphatase conjugated goat anti–rabbit IgG (Bio-Rad, Hercules, CA). For detection of membrane G protein levels, 50 μg of the membrane fraction were loaded on 4–20% SDS/polyacrylamide denaturing protein gels and electrophoresed, followed by electro-transfer to nitrocellulose membranes. Primary polyclonal antiserum used at 1:2,000 dilution was anti-Gsα (sc-262; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-$Gi\alpha_3$ (sc-823; Santa Cruz Biotechnology). The anti-$Gi\alpha_3$ does cross react with other Giα subtypes. Standard chemiluminescent detection was achieved with an ECL kit (Amersham Inc., Arlington Heights, IL).

Radioligand binding. Cells were harvested 36 h after adenoviral infection and lysed as above. Nuclei were pelleted at 500 *g.* Crude

membranes were prepared by centrifugation at 40,000 *g* and then resuspended in β-binding buffer (75 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, and 2 mM EDTA). β-AR density was determined by incubation of membranes with a saturating concentration (~ 300 pM) of [^{125}I] cyanopindolol (Dupont-NEN, Boston, MA) for 1 h at 37°C as described in reference 11 except that 10 μM alprenolol was used to determine nonspecific binding. Specific binding was normalized to membrane protein. To determine receptor affinity for CYP, saturation isotherms were carried out with increasing concentrations of [^{125}I] cyanopindolol (9–360 pM) in the absence and presence of 10 μM alprenolol.

Intracellular cAMP assay. Cells were labeled overnight in 1.5 μCi/ml [^{3}H]adenine (Dupont-NEN) in medium 199 and then preincubated in MEM (GIBCO BRL) containing 10 mM Hepes (GIBCO BRL) and 1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma Chemical Co.) for 30 min. Subsequently, the cells were stimulated with 10 μM (−)-isoproterenol (ISO) at 37°C in medium containing 100 μM ascorbic acid except in the ISO dose–response experiment where the ISO concentration used is explicitly stated. In some experiments either the selective β_2 antagonist, 100 nM ICI 118, 551 (Research Biochemicals International, Natick, MA) or the selective β_1 antagonist, 1 μM ICI 89.406 (Cambridge Research Biochemicals, Wilmington, DE) was included in the preincubation and stimulation steps. In another series of experiments, myocytes were handled as above except that they were stimulated for 5 min with 100 μM forskolin in the absence of ascorbic acid. The medium containing either ISO or forskolin was aspirated at the designated time point and 1 ml of ice-cold stop solution (2.5% perchloric acid, 100 μM cAMP, 10,000 cpm ^{14}C) was added to each well. cAMP was determined by anion exchange chromatography and a percent incorporation of the total ^{3}H uptake into [^{3}H]cAMP was calculated (22, 26). In some experiments, data were standardized such that the percent conversion in control myocytes (uninfected or Adeno-βGal–infected myocytes) was arbitrarily set at one, and all other values were described as a relative amount of cAMP to control.

Desensitization protocol. Myocytes were labeled as above except 10 μCi/ml of [^{3}H]adenine was used. All media were prewarmed to 37°C. For each condition described below, a percent conversion of total ^{3}H to [^{3}H]cAMP was determined (as in the intracellular cAMP assay). The myocytes were prestimulated either with MEM containing no agonist (naive cells) or 10 μM ISO (prestimulated cells). This medium was then aspirated at 10 min and each well was washed twice with MEM. Medium with no additives was then added to half of the naive cells (basal) and medium containing 10 μM ISO to the other half of the naive cells (naive/stimulated). After 3 min the second incubation was terminated by aspiration of medium and the addition of 1 ml of cold stop solution (see above). ISO-induced cAMP from naive cells was calculated as ([naive/stimulated] − [basal]). The prestimulated cells were handled in an analogous fashion; i.e., after prestimulation, half were incubated for 3 min with medium only (prestimulated only) and half were restimulated with 10 μM ISO (prestimulated/restimulated). The incubation was terminated with aspiration and addition of cold stop solution. ISO-induced cAMP from prestimulated cells was calculated as ([prestimulated/restimulated] − [prestimulated only]). Desensitization was defined as the percent loss of activity due to prestimulation; i.e.,

$$\text{Desensitization} = \left(1 - \frac{\text{ISO-induced cAMP from prestimulated cells}}{\text{ISO-induced cAMP from naive cells}}\right) \times 100\%$$

Sarcolemmal adenylyl cyclase activity. 36 h after adenoviral infection, membranes were prepared exactly as described above for radioligand binding, and protein concentration was measured. Adenylyl cyclase activity was determined by incubating 25 μg of protein for 10 min at 37°C with no agonist (basal) or in the presence of 100 μM ISO, 100 μM zinterol, 10 mM NaF, or 100 μM forkolin. [α-^{32}P]ATP was isolated and cAMP quantitated as we have described in reference 12. For a determination of EC_{50}, increasing concentrations (10^{-8} M up to 10^{-4} M) of ISO were incubated with sarcolemmal membranes as above.

Data analysis. Data represent the mean±SEM. The isoproterenol-cAMP dose–response curve was generated by GraphPad InPlot. The isoproterenol-adenylyl cyclase dose–response curve was generated by GraphPad Prism. β-Adrenergic receptor affinity for CYP was calculated using a nonlinear least squares fitting program (GraphPad Prism). To compare the statistical significance of the differences between the means of two independent groups, the Student's *t* test with a two-tailed distribution was used. Calculations were done on Microsoft EXCEL.

Results

Cellular viability. In 20 fields, the mean number of myocytes in the respective dishes was: uninfected 1,537±185; Adeno-βARKct 1,515±208; or Adeno-β_2AR infected 1,477±208 ($n = 3$ plates, for each condition). Adenoviral infection did not alter the percentage of rod-shaped myocytes (uninfected 65±1%; Adeno-βARKct 62±2%; Adeno-β_2AR 64±1%). This contrasts with a marked decrease in the percentage of rod-shaped myocytes after incubation with norepinephrine for 48 h as previously demonstrated by Mann et al. (23).

Expression of transgenes. The three adenoviral constructs depicted in Fig. 1 were used in all infections and subsequent experiments. Robust expression of each adenoviral transgene was demonstrated after myocyte infection. As shown in Fig. 2 *A*, 36 h after infection with Adeno-βGal at a moi of 100 and subsequent staining with X-gal, nearly all the myocytes stained positive. Uninfected myocytes or those infected with Adeno-β_2AR or Adeno-βARKct remained colorless under these conditions. Radioligand binding documented marked overexpression of β-ARs after infection with Adeno-β_2AR (Fig. 2 *B*). A dose-dependent effect is seen with increasing titers of Adeno-β_2AR that reaches a maximal receptor density (3,700±300

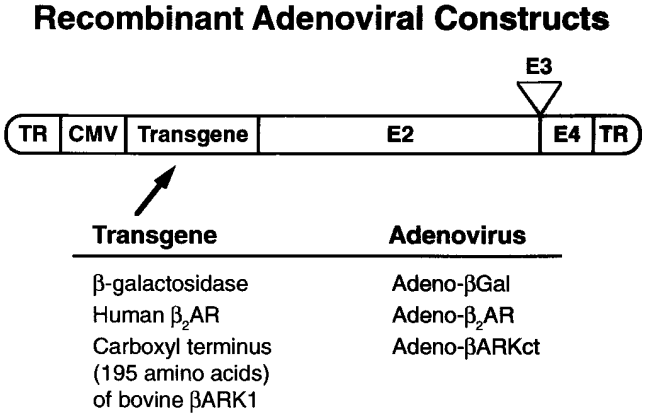

Figure 1. Diagram of recombinant adenoviral vectors. A first-generation adenoviral vector backbone (E1/E3 deletions) is ligated with the CMV promoter in front of the appropriate transgenes. Adeno-βGal contains the β-galactosidase marker gene, Adeno-β_2AR the human β_2AR, and Adeno-βARKct the 195–amino acid carboxyl terminus peptide fragment of bovine βARK1 described in reference 17. Adeno-empty vector (not shown) contains no transgene. TR is the terminal repeat of the adenoviral vector and E2/E3/E4 represent the respective adenoviral genes. Full details of the construction of these vectors is given in the Methods section.

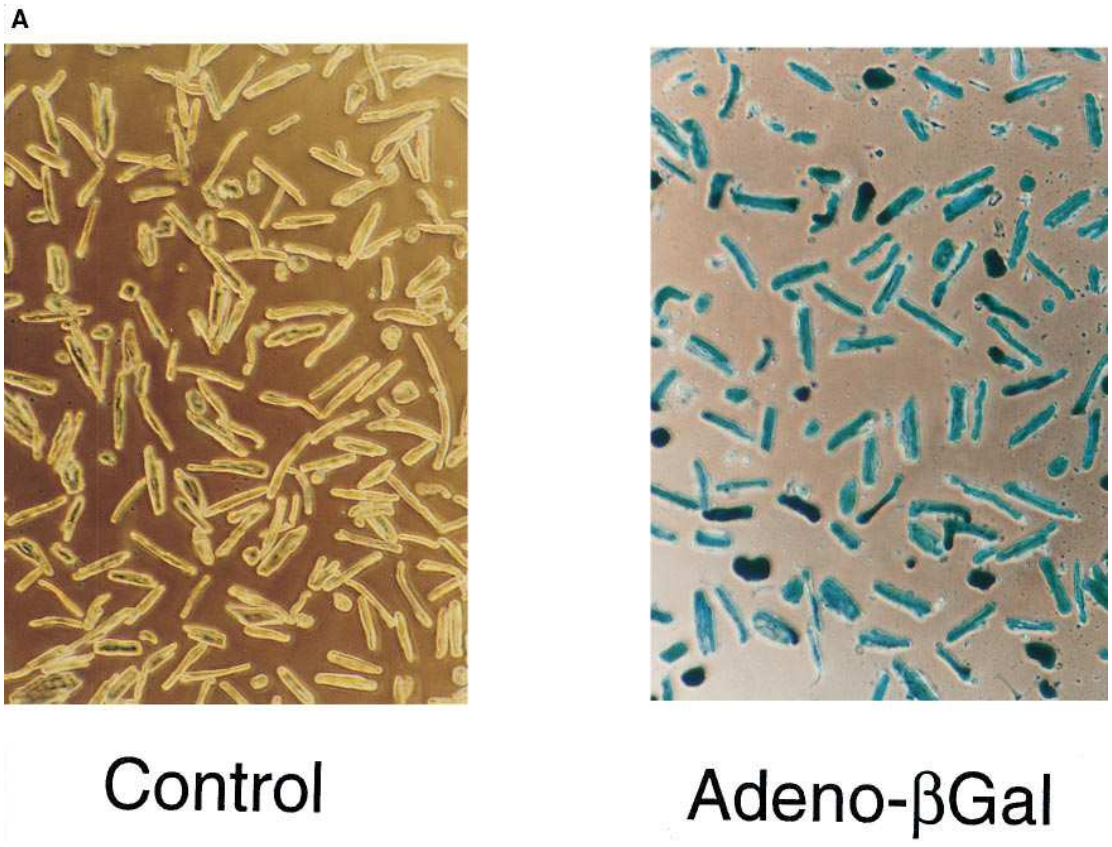

Figure 2. Expression of recombinant adenoviral transgenes in adult rabbit ventricular myocytes. Myocytes were infected at a MOI of 100 for 36 h. (*A*) As assessed by X-gal staining, Adeno-βGal transduces ~ 100% of myocytes (*right*) compared to uninfected (*Control*) myocytes (*left*) or those infected with Adeno-β_2AR or Adeno-βARKct. (*B*) Radioligand binding assays with [^{125}I] iodocyanopindolol performed on crude membranes from Adeno-β_2AR–infected myocytes demonstrate a viral titer-dependent expression of β-adrenergic receptors. The data represent the mean±SEM of four independent experiments, each performed in triplicate. (*C*) Protein immunoblots performed on cytosolic extracts from myocytes infected with the designated MOI of Adeno-βARKct. 5 μg extracts were subjected to SDS–PAGE, probed with a βARK antiserum that recognizes the carboxyl terminus of βARK1, and detected with an alkaline phosphatase-conjugated rabbit antiserum. The arrow on the left points to the expected size band of βARKct, the carboxyl terminus fragment of βARK1 described in reference 17. The arrows on the right depict the position of the molecular mass standards in kilodaltons.

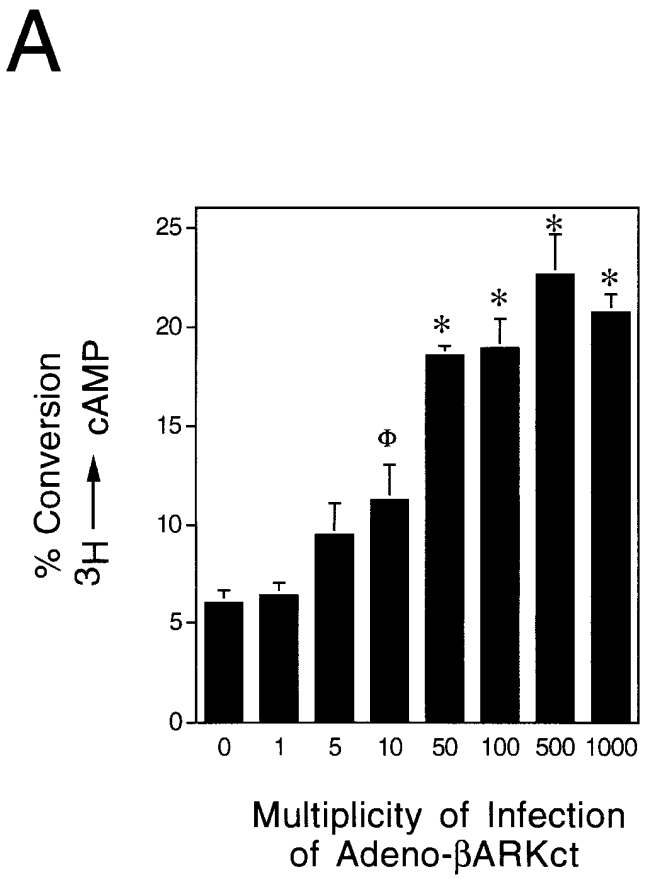

Figure 3. Isoproterenol-induced cAMP levels in Adeno-βARKct–infected myocytes. Experiments were performed 36 h after adenoviral infection. Myocytes are labeled overnight with 1.5 μCi/ml [^{3}H]adenine, and preincubated for 30 min with IBMX. The accumulation of intracellular cAMP following ISO stimulation at 37°C is expressed as a percent conversion from total ^{3}H uptake. Shown is the effect of increasing moi of Adeno-βARKct (*A*) or increasing concentration of ISO (*B*). In *A*, myocytes were stimulated with 10 μM ISO for 30 min. In *B*, myocytes were infected with a moi 100 of Adeno-βGal (○) or Adeno-βARKct (■) and stimulated with the designated concentration of ISO for 20 min. In *B*, the basal values were determined from myocytes labeled with 10 μCi/ml [^{3}H]adenine in preliminary experiments designed specifically to assess basal activity (n = 5). Infection with Adeno-βGal (moi 5,000) or Adeno-empty vector (moi 1,000) had no effect on cAMP accumulation after 15 min of ISO stimulation (data not shown). The data represent the mean±SEM of three (*A*) or four to five (*B*) independent experiments, each performed in triplicate. $^{\Phi}P < 0.05$, $^{*}P < 0.01$ vs. uninfected myocytes.

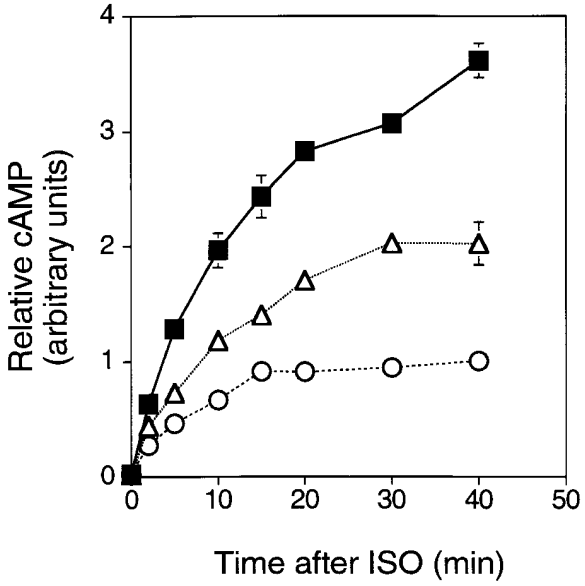

Figure 4. Time course of cAMP accumulation after exposure to 10 μM isoproterenol. Myocytes were infected with a moi 100 of Adeno-βGal (○), Adeno-β_2AR (△), or Adeno-βARKct (■) for 36 h and labeled overnight with 1.5 μCi/ml [^{3}H]adenine. Intracellular cAMP was assayed as in Fig. 3. The maximum percent conversion of total ^{3}H uptake to cAMP in Adeno-βGal–infected myocytes (5.7±1%) is arbitrarily set at one, and all other data are expressed as relative values.

fmol/mg membrane protein) with a moi of 100. In contrast, infection with either Adeno-βGal or Adeno-βARKct did not significantly alter β-AR density (uninfected 165±39; Adeno-βGal 131±23; Adeno-βARKct 134±30 fmol/mg membrane protein). There was no significant change in β-AR affinity for CYP (K_d, dissociation constant) (K_d) after infection with Adeno-β_2AR (26±8 pM) or Adeno-βARKct (33±9 pM) versus Adeno-βGal (31±10 pM). Protein immunoblotting with antiserum raised against the carboxyl terminus of βARK1 demonstrated expression of the novel, ~ 30-kD βARK peptide in myocytes infected with Adeno-βARKct (Fig. 2 *C*). Increasing titers of virus resulted in increased expression of protein with maximal expression at a moi of 100. At a moi of one, a faint band was seen in an overexposed blot (data not shown). As expected, cytosolic extracts from uninfected myocytes or those infected with Adeno-βGal or Adeno-β_2AR did not contain this band under any conditions. As assessed by protein immunoblots of myocyte membranes prepared 36 h after infection, transgene expression (β_2AR or βARKct) did not alter levels of Gs or Gi (data not shown).

Effect of transgenes on β-adrenergic signaling in intact myocytes. As shown in Fig. 3 *A*, we first assessed the effect of increasing titers of Adeno-βARKct on 10 μM ISO-stimulated (30 min) intracellular cAMP levels. A moi of 50 led to near maximal potentiation of response versus uninfected myocytes (19±1% vs. 6±1%, $P < 0.001$). This is concordant with the re-

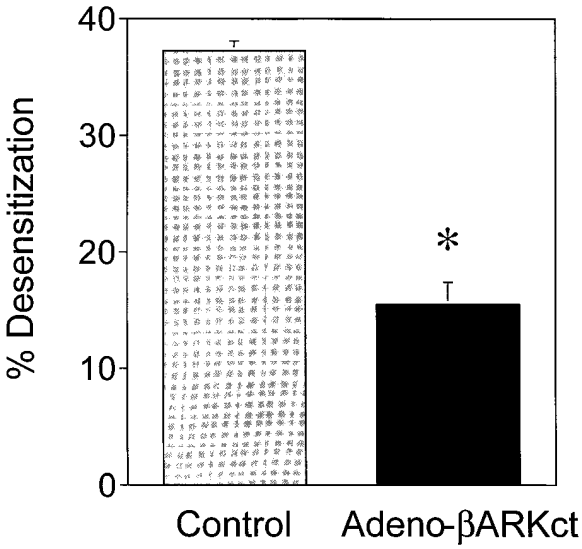

Figure 5. Desensitization in Adeno-βGal versus Adeno-βARKct–infected myocytes. ISO-induced cAMP accumulation was assayed as in Fig. 3 and was measured 36 h after infection with a MOI 100 of either Adeno-βGal (*Control*) or Adeno-βARKct. To quantify desensitization, a prestimulation–restimulation approach was used in which cells were initially treated with or without agonist (10 μM ISO) for 10 min (prestimulated myocytes or naive myocytes, respectively). The response to a subsequent 3 min stimulation with 10 μM ISO was compared. Desensitization, the percent loss of activity due to prestimulation (see Methods for calculations) was 37±1% in Adeno-βGal (*Control*) myocytes (*gray bar*) and 16±2% in Adeno-βARKct myocytes (*black bar*). Data are the mean±SEM of four independent experiments, each in triplicate. Desensitization in Adeno-βGal infected myocytes was not significantly different than in uninfected myocytes ($n = 2$, data not shown). *$P < 0.01$.

lationship of adenoviral titer and production of transgene protein (Fig. 2 *C*). In contrast, myocytes that are uninfected, Adeno-βGal infected (moi up to 5,000), or Adeno-empty vector infected (moi 1,000) accumulate a nearly identical amount of cAMP following 15 min of ISO stimulation (data not shown). An ISO dose–response curve done in myocytes infected with a moi 100 of either Adeno-βGal or Adeno-βARKct (Fig. 3 *B*) also shows potentiation of β-AR signaling by Adeno-βARKct. After 20 min of stimulation with increasing concentrations of ISO, an increased V_{max} was seen in Adeno-βARKct–infected myocytes (12±1%) as compared to Adeno-βGal–infected myocytes (5.4±0.5%, $P < 0.05$) as well as an approximately four fold leftward shift in the EC_{50} (100 nM Adeno-βGal vs. 28 nM Adeno-βARKct).

To further assess the effect of Adeno-βARKct or Adeno-β_2AR infection on the β-AR system, a time course of cAMP accumulation in response to ISO was conducted (Fig. 4). Basal cAMP level in Adeno-βGal–infected myocytes (0.1%) was unchanged by infection with Adeno-β_2AR (0.1±0.1%) or Adeno-βARKct (0.1%). However, ISO-stimulated cAMP accumulation is increased at 5, 15, and 30 min after ISO versus control in myocytes infected with Adeno-β_2AR (1.6±0.1-, 1.5±0.1-, and 2.2±0.1-fold) or Adeno-βARKct (2.8±0.4-, 2.7±0.1-, and 3.3±0.2-fold). In contrast, forskolin-stimulated cAMP accumulation is not significantly enhanced versus control (22% conversion to cAMP) after infection with Adeno-β_2AR (1.2±0.1-fold, $n = 5$) or Adeno-βARKct (1.3±0.2-fold, $n = 6$). The rate of accumulation of cAMP following ISO stimulation in Adeno-βGal myocytes rapidly diminishes such that from 15 to 30 min it has essentially plateaued (Fig. 4). In marked contrast, during this time interval (15–30 min) Adeno-βARKct–infected myocytes continue to accumulate significant rates of cAMP (0.05 relative cAMP U/min), comparable to the maximal rates seen in control myocytes (0.06 relative cAMP U/min during the interval 0–15 min). The prolongation of response to agonist after Adeno-βARKct infection is consistent with an acquired defect in desensitization.

Quantification of this desensitization defect is shown in Fig. 5. After prestimulation with 10 μM ISO for 10 min, desensitization is significantly inhibited in Adeno-βARKct–infected cells as compared to Adeno-βGal–infected cells ($P < 0.001$, $n = 4$). Nearly identical data were obtained following a 3 min prestimulation with 10 μM ISO (data not shown). Uninfected myocytes exhibited nearly the same level of desensitization (35%) as Adeno-βGal–infected cells ($n = 2$) after a 10 min prestimulation with 10 μM ISO.

Because the myocardium contains both β_1 and β_2 adrenergic receptors, we used selective antagonists to determine which receptor subtype would be affected by βARKct expression (Fig. 6). As previously shown in cultured myocytes (24), the β_1AR is the predominant mediator of ISO-induced cAMP ac-

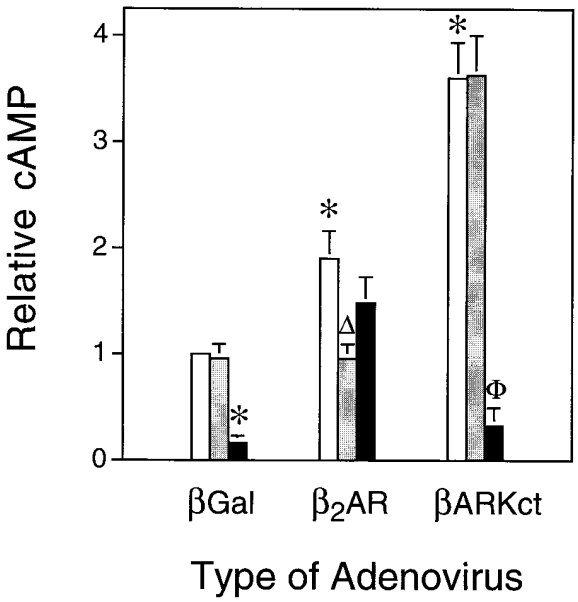

Figure 6. Effect of β_1 (ICI 89.406) or β_2 (ICI 118, 551) selective antagonists on ISO-induced cAMP levels. Myocytes were infected with a moi 100 of Adeno-βGal (*left*), Adeno-β_2AR (*middle*), or Adeno-βARKct (*right*) for 36 h. Intracellular cAMP was assayed after a 20 min stimulation with 10 μM ISO at 37°C in the absence of antagonists (*white bars*) and in the presence of ICI 118, 551, a selective β_2AR antagonist (*gray bars*) or ICI 89.406, a selective β_1AR antagonist (*black bars*). The percent conversion of total ^{3}H uptake to [^{3}H]cAMP after ISO stimulation in Adeno-βGAl–infected myocytes (5.4±1%) is arbitrarily set at one (*left*, *white bar*), and all other data are expressed as relative values. Data represent the mean±SEM of three (ICI 89.406), four (ICI 188,551), or five (no antagonist) independent experiments, each in triplicate. *$P < 0.05$ vs. Adeno-βGal no antagonist, $^{\Delta}P < 0.05$ vs. Adeno-β_2AR no antagonist, $^{\Phi}P < 0.01$ vs. Adeno-βARKct no antagonist.

Table I. Adenylyl Cyclase Activity in Sarcolemmal Membranes from Adenoviral-infected Adult Rabbit Ventricular Myocytes

	Basal	Isoproterenol (100 μM)	Zinterol (100 μM)	NaF (10 mM)	Forskolin (100 μM)
Adeno-βGal	36.9±3.1	63.4±4.8	48.0±4.0	120±9.5	223±13.6
Adeno-β_2AR	53.6±3.2*	104±6.7*	98.2±7.5*	122±8.2	202±16.2
Adeno-βARKct	41.3±4.4	73.8±8.8	58.4±8.0	121±11	205±13.9

Data represent pmol cAMP/mg protein/min±SEM, $n = 3-5$ cell isolations, assays done in duplicate. *$P < 0.005$ vs. Adeno-βGal.

cumulation. Thus, in Adeno-βGal–infected (control) myocytes, the response to ISO is only minimally affected by ICI 118,551, a β_2AR selective antagonist (*βGal*, *gray bar*) but is significantly inhibited by ICI 89.406, a β_1AR selective antagonist (*βGal*, *black bar*). After infection with Adeno-β_2AR, there is a 1.9±0.2-fold potentiation of ISO-induced cAMP levels (*β_2AR*, *white bar*). ICI 118, 551 reduces the accumulation of cAMP back to control levels, presumably by blocking exogenous β_2ARs (*β_2AR*, *gray bar*). This experiment demonstrates that 100 nM ICI 118,551 effectively blocks the β_2AR-mediated response. Since the same concentration of ICI 118,551 has little effect on diminishing the cAMP response in Adeno-βARKct–infected cells (*βARKct*, *gray bar*), this strongly suggests that the enhanced cAMP accumulation following βARKct expression is mediated via β_1ARs. Supporting this hypothesis is the effect of ICI 89.406, the β_1AR antagonist, which effectively attenuates this signal (*βARKct*, *black bar*).

Effect of transgenes on β-adrenergic signaling in myocyte sarcolemmal membranes. Adeno-β_2AR infection significantly increased both basal cyclase activity (53.6±3.2 pmol cAMP/mg/min) versus control (Adeno-βGal, 36.9±3.1 pmol/mg/min, $P < 0.005$) and ISO-stimulated cyclase (104±6.7 pmol cAMP/mg/min) versus control (63.4±4.8 pmol/mg/min, $P < 0.005$) (Table I). Consistent with this effect being mediated through the β_2AR, the stimulation of adenylyl cyclase by zinterol, a selective β_2AR agonist, is also significantly increased in Adeno-β_2AR–infected myocytes (98.2±7.5 pmol cAMP/mg/min) versus control (48±4 pmol/mg/min, $P < 0.005$). This potentiation of β-adrenergic signaling does not appear to be secondary to changes in either G proteins or adenylyl cyclase as the response to NaF or Forskolin is unchanged by Adeno-β_2AR infection (Table I). An ISO–adenylyl cyclase dose–response curve did not show significantly enhanced agonist potency (EC_{50}) after Adeno-β_2AR infection (48 nM) versus control (59 nM).

Similar to that demonstrated in transgenic mice (12), the Adeno-βARKct transgene does not enhance β-adrenergic agonist stimulation (either ISO or zinterol) of membrane adenylyl cyclase versus control. This result is expected since βARKct is a cytosolic peptide that is not present in the membrane fraction (12). Infection with Adeno-βARKct also does not affect the response of membrane adenylyl cyclase to NaF or forskolin as compared to control.

Discussion

A number of alterations in the myocardial β-adrenergic pathway occur in patients with congestive heart failure including downregulation of β_1ARs (8, 9, 25), uncoupling of the remaining β-ARs from adenylyl cyclase (7), and increased expression of βARK1 (9, 10), an enzyme that phosphorylates and uncouples only agonist-bound receptors (26) including the β_1AR (27, 28). Our laboratory has been studying the feasibility of reversing these alterations in the β-adrenergic cascade to restore cardiac inotropy to normal in patients with depressed systolic function. Transgenic mice that overexpress the human β_2AR under control of the cardiac specific α-myosin heavy chain promoter have a basal cardiac inotropy that rivals that achieved in wild-type mice after maximal ISO stimulation (11). Likewise, transgenic mice with cardiac overexpression of an inhibitor of βARK (βARKct) have increased basal contractility and an enhanced response to ISO (12).

Having established that cardiac function can be enhanced in transgenic mice by overexpression of either β_2ARs or an inhibitor of βARK, we are now attempting to use recombinant adenovirus encoding these transgenes to potentiate β-adrenergic signaling. This paper is the initial report of these efforts using a cell culture model consisting of adult rabbit ventricular myocytes. The relevance of a primary cardiomyocyte culture model is highlighted by the demonstration that isolated myocytes from human patients with CHF have demonstrable deficiencies in response to β-agonist stimulation (29). As others have shown (20, 30), we have found adenovirus to achieve ~100% transduction efficiency as assessed by X-gal staining (Fig. 2 *A*). We also have demonstrated robust transgene expression after infection with Adeno-β_2AR as assessed by radioligand binding (Fig. 2 *B*) and Adeno-βARKct by protein immunoblots (Fig. 2 *C*). As has been seen previously with chloramphenicol acetyltransferase or β-galactosidase (20, 30), expression of both the β_2AR and βARKct transgenes is dependent upon viral titer, and with our vector reaches a maximum at a moi between 50 and 100.

After adenoviral infection, β-adrenergic signaling was assessed by the accumulation of intracellular cAMP in the intact myocyte or by activation of adenylyl cyclase in sarcolemmal membranes. Infection with Adeno-β_2AR potentiated the β-adrenergic signal as assessed both by the intracellular cAMP response (Fig. 4) and by membrane adenylyl cyclase activity (Table I). Evidence that the potentiated signal following Adeno-β_2AR infection is mediated via β_2-adrenergic receptors includes the inhibition of the increased ISO-stimulated cAMP response by a selective β_2-adrenergic antagonist (Fig. 6) and the significantly increased stimulation of cyclase activity versus control by zinterol, a selective β_2-adrenergic agonist (Table I). While it might have been expected that the EC_{50} for isoproterenol stimulation of adenylyl cyclase would have been left shifted after β_2-adrenergic receptor overexpression, this was not observed. This most likely relates to the relatively modest overexpression of receptors in these experiments. In myocardial membranes from transgenic animals expressing very high concentrations of receptors (20–40 pmol/mg) (11), a 10-fold shift was observed. In animals expressing ~ 1.2 pmol/mg, about a fourfold shift was found (31). By contrast, in the isolated myocytes used for the experiments in which we determined EC_{50}'s for isoproterenol, only ~ 600 fmol/mg of β_2-adrenergic receptors were present.

Infection with Adeno-βARKct also potentiated β-adrenergic signaling in the intact myocyte, but its effect was mediated via the β_1-adrenergic receptor (Fig. 6). Notably, Adeno-βARKct infection does not affect β-adrenergic stimulation as assessed by membrane adenylyl cyclase activity (Table I).

These results are in agreement with previously published data from transgenic mice that overexpress the identical transgene and are to be expected because βARKct is a cytosolic peptide that is not present in the membrane fraction (12). Furthermore, these data strongly suggest that the potentiation of β_1-adrenergic signaling by Adeno-βARKct in the intact myocyte is not secondary to a change in expression of a membrane component of the β-adrenergic cascade (e.g., G proteins or adenylyl cyclase) in which case enhanced β-adrenergic stimulation of sarcolemmal adenylyl cyclase also should have been seen.

As predicted, the mechanism of potentiation of β_1AR signaling by Adeno-βARKct involves inhibition of receptor desensitization. βARKct is a 195–amino acid carboxyl terminus peptide fragment of bovine βARK1 that contains the $G_{\beta\gamma}$ binding domain. It presumably sequesters dissociated $G_{\beta\gamma}$, preventing it from recruiting βARK1 to the membrane, and thus inhibits βARK phosphorylation of its activated receptor substrate. These actions of the carboxyl terminal peptide of βARK1 have been well characterized previously in vitro (17, 32, 33). In this study, Adeno-βARKct inhibition of desensitization was documented in two ways. In a time course experiment of cAMP accumulation following ISO stimulation, the ability of myocytes infected with Adeno-βARKct to respond to ISO persisted far longer than in the control myocytes (Fig. 4). In a second set of experiments, using a prestimulation–restimulation approach to quantitate desensitization, Adeno-βARKct infection was again shown to significantly inhibit this process (Fig. 5). Because individual cell types have different mechanisms of desensitization (34), these experiments are informative by showing that in rabbit ventricular myocytes $G_{\beta\gamma}$ activation of βARK plays a central role in desensitization of the β_1AR.

Several other potential mechanisms for the enhanced β-adrenergic signal after infection with Adeno-β_2AR or Adeno-βARKct were excluded. Unlike a prior report based on neonatal myocytes (35), adenoviral infection did not enhance β-adrenergic signaling per se since there was no difference in the amount of ISO-stimulated cAMP between uninfected myocytes and those infected with high titers of Adeno-βGal or Adeno-empty vector. Adeno-β_2AR or Adeno-βARKct infection did not significantly affect levels of G proteins as determined by protein immunoblots and by the adenylyl cyclase response after NaF stimulation, nor did the transgenes significantly increase levels of adenylyl cyclase as assessed by its response to forskolin stimulation (Table I). As expected, Adeno-βARKct also did not increase expression of the β-adrenergic receptor.

In conclusion, recombinant adenoviral infection leading to overexpression of the β_2AR or an inhibitor of βARK-mediated desensitization can potentiate β-adrenergic signaling in cultured adult rabbit ventricular myocytes. We emphasize that large hurdles remain before genetic modulation of the β-adrenergic system can be considered a potential therapy for patients with systolic dysfunction. These hurdles include extending the limited duration of expression of recombinant adenoviral transgenes (36), obtaining global myocardial delivery of the adenovirus presumably via coronary artery injection (37), and demonstrating a hemodynamic and survival benefit in animal models of CHF.

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