Adenoviral Gene Transfer of the Human V2 Vasopressin Receptor Improves Contractile Force of Rat Cardiomyocytes

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Background—In congestive heart failure, high systemic levels of the hormone arginine vasopressin (AVP) result in vasoconstriction and reduced cardiac contractility. These effects are mediated by the V1 vasopressin receptor (V1R) coupled to phospholipase Cβ-isofoms. The V2 vasopressin receptor (V2R), which promotes activation of the Gs/adenylyl cyclase system, is physiologically expressed in the kidney but not in the myocardium. Expression of a recombinant V2R (rV2R) in the myocardium could result in a positive inotropic effect via the endogenous high concentrations of AVP in heart failure.

Methods and Results—A recombinant adenovirus encoding the human V2R (Ad-V2R) was tested for its ability to modulate the cardiac Gs/adenylyl cyclase system and to potentiate contractile force in rat ventricular cardiomyocytes and in H9c2 cardiomyoblasts. Ad-V2R infection resulted in a virus concentration-dependent expression of the transgene and led to a marked increase in cAMP formation in rV2R-expressing cardiomyocytes after exposure to AVP. Single-cell shortening measurements showed a significant agonist-induced contraction amplitude enhancement, which was blocked by the V2R antagonist, SR 121463A. Pretreatment of Ad-V2R-infected cardiomyocytes with AVP led to desensitization of the rV2R after short-term agonist exposure but did not lead to further loss of receptor function or density after long-term agonist incubation, thus demonstrating resistance of the rV2R to downregulation.

Conclusions—Adenoviral gene transfer of the V2R in cardiomyocytes can modulate the endogenous adenylyl cyclase-signal transduction cascade and can potentiate contraction amplitude in cardiomyocytes. Heterologous expression of cAMP-forming receptors in the myocardium could lead to novel strategies in congestive heart failure by bypassing the desensitized β-adrenergic receptor signaling. (Circulation. 1999;99:925-933.)

Key Words: receptors ■ myocardial contraction ■ heart failure

Many neurotransmitters, hormones, and autocrine and paracrine factors exert their physiological functions by binding to heptahelial receptors which activate or inhibit specific signal transduction pathways that couple with different classes of heterotrimeric G proteins.1,2 Vasopressin receptors, belonging to the superfamily of heptahelial receptors, exist as 4 subtypes: the V1A vasopressin receptor (V1AR), which is mainly expressed in the endothelium, smooth muscle cells, and the myocardium; the V1BR and the probably identical V3R subtype, which is expressed in the hypophysis; and the V2 vasopressin receptor (V2R), which is exclusively expressed in the renal collecting tubule cells. Stimulation of V2R results in the activation of the Gs/adenylyl cyclase system and promotes the reabsorption of fluid via insertion of water pores into the luminal membrane.3,4

Congestive heart failure is a syndrome that confers significant morbidity and mortality despite recent advances in clinical therapy.5 Numerous compensation mechanisms occur in this syndrome, including an increased activity of the sympathoadrenergic system and of the renin-angiotensin-aldosteron complex and an increased release of several peptide hormones, such as arginine vasopressin (AVP) and atrial natriuretic peptide. The elevated concentrations of catecholamines and AVP in the systemic circulation of patients with congestive heart failure correlate with their prognosis.6,7 Despite high levels of catecholamines, the failing myocardium is not able to build up a sufficient blood supply for the organism. In the failing heart, the β-adrenergic signaling cascade is functionally inactivated by a selective downregulation of β1-adrenergic receptor (AR)8 and an upregulation of the β-AR kinase, an enzyme that specifically phosphorylates and uncouples the activated β-AR.9

The present study investigates the feasibility of overexpressing a heterologous cAMP-coupling receptor in cardio-
myocytes which could potentially bypass the defective β-adrenergic signal transduction observed in heart failure. We chose as model systems, adult ventricular cardiomyocytes and cardiomyoblasts. H9c2 cardiomyoblasts were used because they possess β-adrenergic signal transduction characteristics similar to the human myocardium and they can be cultured in a standardized manner, thereby facilitating the comparability of large-scale experiments. Isolated rat adult ventricular cardiomyocytes retain most contractile properties of the intact adult heart.

Recently, replication-deficient recombinant adeno viral vectors have been used for efficient gene transfer into the myocardium and into isolated cardiac myocytes. Adeno viral vectors have important characteristics that make them well suited for myocardial gene transfer. The efficiency and expression levels of adeno virus-mediated gene transfer in vitro and in vivo are substantially better than those seen with other types of gene-delivery systems, such as liposomal transfection or direct plasmid DNA injection.

The aim of this study was to explore whether an adeno virally mediated transfer of the human V2R would lead to a sufficient recruitment of the endogenous GS/ adenylyl cyclase system to potentiate contraction amplitude in cardiomyocytes.

**Methods**

**Construction and Purification of Recombinant Adenovirus**

Generation of a recombinant (E1 deficient) adenovirus (Serotype 5) carrying the human V2R has been described recently. Briefly, the coding sequence of the hemagglutinin-tagged wild-type V2R, including the nontissue-specific cytomegalovirus promoter and SV40 polyadenylation signal, was taken from the hemagglutinin-V2-pcd-FS vector and inserted into the polylinker sequence of the pmCD-PS vector. The resulting plasmid was then cotransfected with the pJM17 plasmid into subconfluent HEK 293 cells by using a modified calcium phosphate coprecipitation method. After plaque isolation, recombinant virus, referred to as Ad-V2R, was amplified, and individual virus stocks were analyzed by polymerase chain reaction and restriction analysis.

After isolation, Ad-V2R were prepared at large scale as described previously. Adenoviral titers were determined using plaque titration on HEK 293 cells.

**Cell Culture and Adenoviral Infection of H9c2 Cardiomyoblasts**

H9c2 cardiomyoblasts (ATCC CRL 1446, cardiac myoblasts from rat) were cultured in monolayers in DMEM, 10% fetal bovine serum, 2 mmol/L glutamine, penicillin (100 IU/mL), streptomycin (100 μg/mL), and gentamicin (100 μg/mL) in 7% CO2 in a humidified incubator at 37°C.

The cells were used for the individual experiments 36 to 48 hours after adenoviral infection (performed as described).

**Preparation and Culture of Adult Ventricular Cardiomyocytes**

Single calcium-tolerant ventricular myocytes were isolated from 12- to 16-week-old male Wistar rats and cultured in M199 medium (supplemented with vitamins, nonessential amino acids, 25 mmol/L HEPEES, 10 μg/mL insulin, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 100 μg/mL gentamicin), on laminin-precoated dishes (5- to 10 μg/cm2 at a density of 104 cells/cm2), in a humidified atmosphere (5% CO2) at 37°C. The infection of the ventricular cardiomyocytes with the adenoviruses was performed 6 to 8 hours after plating in M199 culture medium.

**Confocal Laser Scanning Microscopy**

Immunofluorescence microscopy of Ad-V2R-infected cardiomyoblasts was performed as described previously.

**Contraction Experiments**

Measurement of contraction amplitude of Ad-V2R- or Ad-β galactosidase (Gal)-infected rat cardiomyocytes was performed with an electro-optical monitoring system. The contraction amplitude of the cardiomyocytes was recorded because excellent correlation between this parameter and contraction velocity was reported for an identical preparation of adult cardiomyocytes. The experiments were performed on ventricular cardiomyocytes in a single-cell investigation system (Scientific Instruments), in a temperature-controlled cuvette (37°C) at a constant medium flow of 0.5 mL/min and at a constant electrical field. As medium, a 1.8-mmol/L Ca2+-Tyrode’s-solution was used. The cardiomyocytes were paced by an external stimulation of 50 V and an 800-ms pulse duration to achieve a contraction frequency of ~70 minutes. After the contraction amplitude reached stability, the experiments were started by applying increasing concentrations of AVP or isoproterenol. The AVP-treated cells were finally superfused with isoproterenol (0.1 μmol/L) to control comparability.

**Determination of Intracellular cAMP Concentrations and Sarcolemmal Adenyl Cyclase Activity**

These assays were performed in ventricular cardiomyocytes or H9c2 cardiomyoblasts plated at the density of 1×105 cells/cm2, following the protocols previously described.

**Radioligand Binding**

Cells were harvested 48 hours after adenoviral infection. Membranes were prepared and radioligand binding was performed as described. The protein content of each sample was determined by the method of Bradford.

**β-Galactosidase Expression**

To estimate infection efficiencies, cells were infected with varying concentrations of a recombinant adenovirus carrying the lacZ gene under the expression control of a Rous sarcoma virus promoter. After adenoviral infection, the cells were fixed in 0.05% glutaraldehyde in PBS for 10 minutes at 4°C and then stained with 3 mmol/L K4Fe(CN)6, 3 mmol/L K3Fe(CN)6, 1 mmol/L MgCl2, 15 mmol/L NaCl, and 1 mg/mL X-Gal-(5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) in 44 mmol/L HEPES for 1 hour at 37°C.

**Receptor Desensitization and Downregulation Protocol**

Desensitization of β-adrenergic or V2R was induced by incubating the cells for 15 minutes at 37°C in PBS containing 1 μmol/L of isoproterenol or 10 nmol/L of vasopressin and 1 μmol/L of IBMX. For receptor downregulation, the cells were treated for 20 hours with the indicated agonists. The incubation was stopped by washing the cells 3 times in PBS. Afterward, the intracellular cAMP content or the density of rV2R or β-AR in the cell membranes was determined.

**Data Analysis**

To compare the statistical significance of the differences between the means of 2 independent groups, the Student t test with 2-tailed distribution was used. The data presented in Figures 2, 3 and 5 were analyzed by 1-way ANOVA followed by Scheffé’s analysis.

**Results**

**Infection With Ad-βGal and Ad-V2R**

Adenoviral constructs for the human V2R (Ad-V2R) and LacZ (Ad-βGal) were used in all experiments. Robust expression of the 2 adenoviral transgenes was demonstrated after infection of both cardiomyoblasts and isolated adult cardiomyocytes. Figure
1A shows that after infection with the adenovirus for β-Gal (moi: 100 pfu/cell) and subsequent staining with X-Gal, almost all H9c2 cardiomyoblasts showed a positive nuclear staining, whereas Ad-V2R-infected cardiomyoblasts remained colorless under these conditions (Figure 1A).

Expression of the adenoviral transgene for the V2R was demonstrated by confocal fluorescence microscopy using a polyclonal antibody raised against a C-terminal peptide of the receptor. As shown in Figure 1B, ≈80% of the Ad-V2R-infected H9c2 cardiomyoblasts showed an intense, intracellular staining of the rV2R transgene after permeabilization.

Radioligand binding with 3H-AVP documented marked expression of rV2R after infection with Ad-V2R. A concentration-dependent effect was observed with increasing titers of the adenoviral construct for the V2R which resulted in a B_max value for 3H-AVP-binding of 998 ± 119 fmol/mg protein at moi of 100 pfu/cell (Figure 2A). The decrease in rV2R density at higher adenoviral titers is probably a result of viral toxicity. Noninfected control cardiomyoblast membranes showed specific 3H-AVP binding with a B_max-value of 270 ± 87 fmol/mg protein resulting from endogenously expressed V1AR. Infection with either Ad-βGal or Ad-V2R did not significantly alter β-AR density in the membranes of H9c2 cardiomyoblasts (81 ± 18 fmol/mg protein). The K_d value for 3H-AVP binding was 0.16 ± 0.09 nmol/L.

To differentiate the receptor subtype populations, the selective V2R antagonist SR 121463A, which binds to V2R (in comparison with V1R) with a selectivity of 2400:1, was used. Figure 2B shows that in cardiomyoblasts infected with either Ad-βGal or Ad-V2R (moi: 100 pfu/cell), a native population of V1AR existed with a B_max-value of ≈270 fmol/mg protein. In Ad-V2R-infected cardiomyoblasts, the total amount of binding sites for 3H-AVP increased to 880 ± 110 fmol/mg protein. By application of the selective V2R antagonist SR 121463A, this increase in 3H-AVP binding was almost completely blocked (Figure 2B). These data demonstrate the dominant expression of the rV2 receptor in comparison with the endogenous V1A population in Ad-V2R-infected cells.

Also, in adult ventricular cardiomyocytes, we detected a 3-fold increase in 3H-AVP binding after infection with Ad-V2R (B_max value of 155 ± 18 fmol/mg protein versus 48 ± 9 fmol/mg protein in Ad-βGal-infected control cardiomyocytes [n=4];
moi: 100 pfu/cell). The basal and heterologous expression levels of V1R and V2R were each 6-fold lower in cardiomyocytes versus H9c2 cardiomyoblasts.

To examine the time dependence and the optimum adenoviral titer for rV2R expression in cardiomyoblasts, receptor-dependent cAMP formation was determined in intact cardiomyoblasts infected with increasing titers of Ad-V2R. A concentration-dependent effect was seen with a maximum 4-fold increase at an moi of 100 pfu Ad-V2R/cell, corresponding to ^3H-AVP binding experiments in Figure 2A. Intracellular cAMP formation peaked at 48 to 72 hours after infection at optimal adenoviral titer.

Intracellular cAMP Formation and Sarcolemmal Adenylyl Cyclase Activity After Infection With Ad-V2R
In Ad-βGal-infected or noninfected adult rat ventricular cardiomyocytes, intracellular cAMP formation did not change after addition of increasing concentrations of AVP. After V2R transgene expression, receptor-stimulated intracellular cAMP production reached a maximum increase of 4-fold with an EC50 value of ≈0.5 nmol/L in ventricular cardiomyocytes (Figure 5A).

Sarcolemmal adenylyl cyclase activity was measured in H9c2 cardiomyoblasts. In membrane fractions of Ad-V2R-infected cardiomyoblasts, AVP-stimulated membrane adenylyl cyclase activity increased to values 35-fold above basal. The EC50 value for the AVP-stimulated adenylyl cyclase activity was calculated to be 0.16 nmol/L. In comparison, a maximum 7-fold increase in cyclase activity was observed after stimulation of endogenous β-ARs with up to 10 μmol/L of isoproterenol. The half-maximal concentration for isoproterenol-stimulated cAMP formation was observed at 20 nmol/L (data not shown).

Homologous and Heterologous Receptor Desensitization and Downregulation of rV2R in Comparison to Native β-AR
Receptor regulation of the heterologous protein was studied (1) after short-term (15 minutes) agonist exposure corre-
Radioligand binding experiments with $^3$H-AVP and $^3$H-CGP 12177 were performed on crude membrane extracts from Ad-V2R-infected cardiomyoblasts (A) and ventricular cardiomyocytes (B). Cells were infected with Ad-V2R at a moi of 80 pfu/cell and $\beta$-AR- and V2R-densities were determined after agonist exposure of isoproterenol (solid bars) and AVP (shaded bars) for 0 and 15 minutes and 20 hours. The data represent the mean±SEM of 3 independent experiments, each performed in triplicate. *$P<0.05$; **$P<0.0005$.

Figure 3. Studies of ligand-induced receptor downregulation. Radioligand binding experiments with $^3$H-AVP and $^3$H-CGP 12177 were performed on crude membrane extracts from Ad-V2R-infected cardiomyoblasts (A) and ventricular cardiomyocytes (B). Cells were infected with Ad-V2R at a moi of 80 pfu/cell and $\beta$-AR- and V2R-densities were determined after agonist exposure of isoproterenol (solid bars) and AVP (shaded bars) for 0 and 15 minutes and 20 hours. The data represent the mean±SEM of 3 independent experiments, each performed in triplicate. *$P<0.05$; **$P<0.0005$.

Figure 4A and 4B show the functional effect of homologous desensitization on cAMP formation in Ad-V2R-infected cardiomyoblasts. Control concentration-response curves of the Ad-V2R-infected cardiomyoblasts showed EC$_{50}$ values of $\approx$0.15 and 12 nmol/L for AVP and isoproterenol, respectively. The homologous receptor desensitization resulted in a reduction of maximal cAMP stimulation of 18% for the rV2R and of 42% for the $\beta$-AR. Additionally, the agonist concentration needed for half-maximal receptor stimulation shifted to higher EC$_{50}$ values (from 0.15 to 1.2 nmol/L for AVP and from 15 to 50 nmol/L for isoproterenol).

Figure 4C and 4D demonstrate the effect of long-term agonist incubation for a time which is commonly sufficient to induce complete homologous receptor downregulation. In Ad-V2R-infected cells, maximal receptor-dependent cAMP production via rV2R was only reduced by $\approx$12%, whereas cAMP formation after stimulation of endogenous $\beta$-AR was almost blunted (78% reduction).

Figure 5A shows the effect of Ad-V2R infection on AVP-induced cAMP generation in adult ventricular myocytes. Also, in these cells, the effect of short-term and long-term agonist incubation was tested (Figure 5B). It can be seen that in cardiomyocytes, 15 minutes of agonist exposure decreased AVP-dependent cAMP formation by $\approx$20%, whereas no further decrease after long-term incubation with AVP occurred (Figure 5B). This is in contrast to the marked loss of adenyl cyclase $\beta$-adrenergic stimulation observed in rat ventricular cardiomyocytes after long-term incubation with isoproterenol.

**Physiological Effect of rV2R Expression on Contraction Amplitude in Rat Ventricular Cardiomyocytes**

To study the effect of heterologous expression of the rV2R on contractile responsiveness of ventricular cardiomyocytes, myocyte shortening was measured after infection with an moi of 100 pfu/cell of Ad-V2R. Cell culture conditions were chosen to maintain stable contraction characteristics of cardiomyocytes. Rat cardiomyocytes were electrically stimulated at a rate of $\approx$70 contractions per minute (Figure 6A and 6B). The baseline contraction amplitude was in the range of 3.4 to 3.8 $\mu$m and was not significantly altered by adenoviral infection. Baseline and isoproterenol-dependent contraction amplitude did not differ significantly between freshly isolated cardiomyocytes and isolated cardiomyocytes after 48 to 72 hours of culture (data not shown), which corresponded well to data from identical cell preparations. Superfusion of Ad-V2R-infected cardiomyocytes with AVP led to a significant increase in the contraction amplitude to the same extent as could be reached by $\beta$-adrenergic stimulation (Figure 6A). Control cells infected with Ad-$\beta$Gal did not show any contractile response to stimulation with AVP at the submillimolar concentrations used (Figure 6B). The effect of AVP in Ad-V2R-infected cells was concentration-dependent and reached a maximum 3-fold increase in contraction amplitude. The observed EC$_{50}$ was in the subnanomolar range, well below concentrations needed for stimulation of the natively present V1AR. Addition of the V2 antagonist, SR 121463A, inhibited the AVP-induced increase in contraction amplitude by 65% (inset of Figure 6A).
Discussion

In the present study, we demonstrate that a heterologous receptor is capable of modulating cardiac adenylyl cyclase-signal transduction and is able to improve contractile function of cardiomyocytes.

Adenoviral Gene Transfer to Cardiomyocytes

Adenoviral infection of cultured cardiomyoblasts or isolated cardiomyocytes resulted in an almost 100% transduction efficiency as assessed by X-Gal staining. Also, we have demonstrated in immunofluorescence studies and radioligand binding that infection with Ad-V2R led to a robust transgene expression.

Adenoviral infection did not enhance cAMP formation, per se, because there was no difference in the amount of AVP-stimulated cAMP formation between uninfected myocytes and those infected with high titers of Ad-βGal. In immunoblotting experiments, Ad-V2R infection did not significantly affect levels of G protein α-subunits of the Gs- and Gi-family, as assessed by others.23 In conclusion,
adenovirus-mediated gene transfer proved to be an efficient and feasible gene delivery system to cardiac cells with our vectors in vitro.

Overexpression of the rV2R and Functional Implications in Cardiomyocytes

After adenoviral infection, rV2R signaling was studied by measuring the accumulation of intracellular cAMP in intact myocytes or by studying the activation of adenyl cyclase in sarcoplasmic membranes. AVP-stimulated adenyl cyclase activity increased to a maximum of 35-fold over basal in infected cardiomyoblasts. In comparison, a maximum 7-fold increase could be reached by stimulation of the native β-AR population. The receptor ratio between the rV2R and the native β-AR was ≈12:1 in both cardiomyoblasts and ventricular cardiomyocytes, although absolute expression levels of the receptor molecules were quite different in the 2 cell populations. Lower expression of rV2R in adult ventricular cardiomyocytes explains why, in these cells, AVP-dependent cAMP production did not reach the same level as in cardiomyoblasts. In both cell types, however, rV2R-dependent cAMP formation exceeded β-AR-dependent increases in cAMP.

Noninfected cardiomyoblast membranes showed a basal 3H-AVP binding, which was caused by the expression of native V1AR. Because no specific V1R antagonists exist, we differentiated both receptor subtypes in rV2R-expressing cells by addition of the selective V2R antagonist SR 121463A\textsuperscript{21} (Figure 2B). Taken together, Ad-V2R infection induced a genetic receptor subtype shift in both types of cardiomyocytes, overexpressing rV2R versus native V1R by a factor of 3:1.

The modulation of the endogenous cardiac signal transduction by the renal receptor led to an improvement of contraction amplitude in rat ventricular cardiomyocytes. Ad-V2R-infected cardiomyocytes showed a dose-dependent increase in contraction amplitude to a maximum of 300% after stimulation with AVP. The half-maximal concentration for the AVP-stimulated cAMP response was ≈0.16 nmol/L, which corresponds to the peak AVP concentrations in vivo in the circulation of patients with congestive heart failure.\textsuperscript{7} Expression of the rV2R in the myocardium of these patients might allow to use the high systemic levels of AVP, which are involved in reducing ventricular contractility via V1R, for a beneficial, positive inotropic effect mediated by rV2R. The present study represents the first report about the functional modulation of the cardiac contractile apparatus by overexpression of a heterologous, positive inotropic receptor. It complements the attempts of earlier studies to improve cardiac contraction cycle by overexpressing calcium-regulating proteins.\textsuperscript{24}

In the failing myocardium, numerous alterations of the β-adrenergic signaling pathway occur. One approach that might be used to enhance cardiac function is to “resensitize” the β-adrenergic signaling cascade. Actually, this mechanism might explain at least part of the positive effect of some β-blockers, such as metoprolol, in the treatment of heart failure; these agents increased LV function and β-AR-induced contractility, and most probably, cardiac cAMP levels.\textsuperscript{25} A molecular tool to simulate this receptor resensitization might be the overexpression of a βARK-1 inhibitor protein (“βARKmini”), consisting of the carboxyl terminus.
of βARK-1. Transgenic mice with overexpressed βARK-mini in their hearts showed increased cardiac contractility and increased cAMP levels for the duration of their lives, without any signs of myocardial damage. βARK-mini transgenic mice also showed stable LV function after aortic banding, in contrast to the marked loss of LV contractility in their aortic-banded wild-type littermates. In contrast, other transgenic mice with specific molecular interventions in cardiac G protein-coupled signal transduction showed quite distinct characteristics: (1) overexpression of β2-AR led to mild cardiac fibrosis and even cardiomyopathy. Taken together, specific molecular interventions in the G protein-coupled receptor system, leading to increased cAMP levels, might produce beneficial effects in the long run in the treatment of patients with heart failure, despite the failure of cAMP-raising pharmacological agents, such as phosphodiesterase III inhibitors. Despite the disappointing results with these cAMP-raising drugs, a long-term beneficial effect of an intermittent stimulation of cardiomyocytic cAMP levels by a specific molecular mechanism might still be possible and should be further investigated.

Our approach was to investigate whether an ectopic expression of the V2R could be a sufficient alternative method to bypass the deficient β-adrenergic signaling pathway in cardiomyocytes. Because AVP levels in patients with heart failure vary with hemodynamic changes, cAMP would only be intermittently stimulated via rV2R when hemodynamic conditions are especially bad. rV2Rs were subject to endogenous mechanisms which promote homologous desensitization after short-term agonist exposure because we consistently observed an 20% loss of receptor function in both types of cardiomyocytes after short-term exposure to AVP. In contrast, the adenovirally introduced V2R was not submitted to receptor downregulation because we detected stable protein levels and no further reduction of cAMP responsiveness after prolonged agonist exposure in neither cardiomyoblasts nor adult cardiomyocytes. The reasons for this lack of downregulation cannot be determined, but it seems probable that the cytomegalovirus promoter used to drive receptor expression is not subject to endogenous mechanisms leading to receptor downregulation in cardiomyocytes. It remains to be seen whether this phenomenon also holds true for recombinant expression of other proteins.

Limitations of the Study
It remains to be determined whether a similar biological effect can be observed in failing myocardium. Any approach to the treatment of heart failure will have to be verified by investigating a hemodynamic and survival benefit in animal models of congestive heart failure. Moreover, the capacity of enhanced cAMP levels to improve systolic dysfunction, despite alterations downstream of membranous cAMP formation, needs to be evaluated in the failing heart.

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