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Cardiac-Directed Expression of Adenylyl Cyclase VI Facilitates Atrioventricular Nodal Conduction

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OBJECTIVES The purpose of this study was to test the hypothesis that cardiac-directed expression of adenylyl cyclase VI (AC_{VI}) facilitates atrioventricular (AV) nodal conduction.

BACKGROUND Cardiac-directed expression of AC_{VI}, unlike other strategies to increase cyclic adenosine monophosphate generation, reduces mortality in murine cardiomyopathy. Recent reports suggest that AC_{VI} expression may also protect against lethal bradycardia.

METHODS We performed immunofluorescence staining for AC_{VI} in the AV node of transgenic mice. We then performed electrophysiologic studies (EPSs) using a 1.7-F octapolar catheter at the AV junction in 11 transgenic AC_{VI} mice and 14 control mice.

RESULTS Immunofluorescence staining revealed increased AC_{VI} expression in the AV node of transgenic mice versus controls. During EPS, AV intervals approximated PR intervals (R^2 = 0.99) and related linearly to atrial-to-His intervals (R^2 = 0.98; both $p < 0.0001$). Thus, we studied AV intervals to avoid electrocardiogram pacing artifacts and inconsistent inscription of His bundle electrograms. At baseline, AC_{VI} mice had shorter AV intervals (47 ± 9 ms) than controls (57 ± 11 ms; $p = 0.02$), despite similar sinus rates. In pacing, AV intervals were shorter in AC_{VI} mice than controls for a wide cycle-length range ($p < 0.01$). The AC_{VI} mice also had shorter AV Wenckebach cycle lengths (AC_{VI}: 114 ± 12 ms; control: 131 ± 28 ms; $p = 0.05$) and ventriculo-atrial effective refractory periods (AC_{VI}: 97 ± 21 ms; control: 127 ± 15 ms; $p = 0.05$). We observed no differences between groups in sinus node function, and ventricular arrhythmias were not inducible.

CONCLUSIONS Cardiac-directed expression of AC_{VI} facilitates AV nodal conduction without altering sinus node function. These results suggest the need to define a role for AC_{VI} gene transfer in treating diseases of AV conduction. (J Am Coll Cardiol 2006;48:559–65) © 2006 by the American College of Cardiology Foundation

Congestive heart failure (CHF) affects more than 5 million people in the U.S. and is a significant cause of morbidity and mortality. Clinically, inhibition of the renin-angiotensin-aldosterone system (1) or antagonism of beta-adrenergic receptors (βARs) (2,3) reduces the mortality and morbidity of CHF. Conversely, pharmacological agents that increase intracellular levels of cyclic adenosine monophosphate (cAMP) typically shorten life (4), despite favorable effects on left ventricular function and symptoms. These observations are mirrored in animal studies, in which cardiac-directed βAR and $G_{\alpha s}$ expression initially increase cardiac contractile function but are ultimately associated with adverse outcomes (5–7).

Cardiac-directed expression of adenylyl cyclase type VI (AC_{VI}), in contrast, is emerging as a useful strategy to increase contractile function while avoiding deleterious effects usually associated with agents that increase intracellular cAMP (8–10). Adenylyl cyclase type VI, a major cardiac isoform of AC, increases cAMP generation after βAR stimulation without increasing basal cAMP. In murine cardiomyopathy (10) and pacing-induced porcine heart failure (11), increased cardiac AC_{VI} improves cardiac function (9–11) and reduces mortality (10). Clinical trials to study the benefits of AC_{VI} gene transfer in patients with severe CHF will commence shortly.

However, the electrophysiologic effects of increased cardiac AC_{VI} expression are unknown. In particular, although AC_{VI} is expressed in the sinus node (12), its expression and functional importance in the atrioventricular (AV) node are unclear. Studies from our laboratory show that AC_{VI} mice are protected against AV nodal block after coronary artery ligation (13), although the mechanism is unknown. In addition, while AC_{VI} expression enhances sinus node chronotropy (8), it is unclear to what extent cardiac AC_{VI} expression, like other adrenergic interventions, pre-disposes to atrial or ventricular arrhythmias.

In the present study, we confirmed the expression of AC_{VI} in the AV node of transgenic mice, and then tested the hypothesis that cardiac-directed AC_{VI} expression facilitates AV nodal conduction. Furthermore, we performed detailed invasive electrophysiologic studies (EPSs) to determine whether increased cardiac AC_{VI} expression increases susceptibility to atrial or ventricular arrhythmias.

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Abbreviations and Acronyms

AC_{VI}	= adenylyl cyclase type VI
AH	= atrial-to-His
ANOVA	= analysis of variance
AV	= atrioventricular
AVERP	= atrioventricular nodal effective refractory period
βAR	= beta-adrenergic receptor
cAMP	= cyclic adenosine monophosphate
CHF	= congestive heart failure
CL	= cycle length
CSNRT	= corrected sinus node recovery time
ECG	= electrocardiogram
EPS	= electrophysiologic study
ERP	= effective refractory period
MHC	= major histocompatibility complex
SNRT	= sinus node recovery time
VA	= ventriculo-atrial

METHODS

Animals. The study protocol was approved by the Animal Care Program of the San Diego Veterans Affairs Medical Center, and we maintained humane treatment of all study subjects. Transgenic mice with cardiac-directed AC_{VI} expression (n = 20; mean age 11 months) were generated using the major histocompatibility complex (MHC) promoter as previously described (8). Transgene-negative siblings (n = 5) and wild-type mice of the same strain (C57BL/6; n = 14) were used as controls (mean age 8 months). We could not obtain data on 14 of these 39 mice: 11 died during surgical preparation before EPS (7 AC_{VI}, 4 control), and we could not obtain suitable catheter position in 3 (2 AC_{VI}, 1 control). This report is based on data from the remaining 25 mice.

Surgery. Mice were anesthetized with intraperitoneal injections of ketamine (100 mg/kg) and xylazine (5 mg/kg) (14) and received 100% oxygen (1 l/min) via nose cone. Mice were placed on their dorsa on a heating pad maintained at 37°C, and surface electrocardiogram (ECG) leads were recorded from electrodes on each limb; ECG and respiratory rate were monitored throughout the procedure.

Transvenous electrode placement. A 10- to 15-mm longitudinal paratracheal incision was made at the level of the larynx. Blunt dissection aided by an operating microscope was used to expose the right external jugular vein. Microscissors were used to create a venotomy, and a 1.7-F octapolar pace/sense catheter (Numed Inc., Hopkinton, New York) was carefully advanced to the AV junction such that right atrial and ventricular electrograms were observed on proximal and distal bipoles, respectively. Figure 1 shows the surgical field, external jugular vein, and catheter. Electrogram recordings were optimized by adjusting the catheter position and signal gain, and then the catheter was sutured in place.

Programmed stimulation. Intracardiac electrograms and the surface ECG were filtered between 5 and 500 Hz and 30 and 100 Hz, respectively, and analyzed at a scale of 200 mm/s from our physiologic recorder (Labsystem Duo, Bard, Massachusetts). Atrial and ventricular pacing were performed using 1-ms pulse widths at twice diastolic threshold, and each maneuver was performed twice.

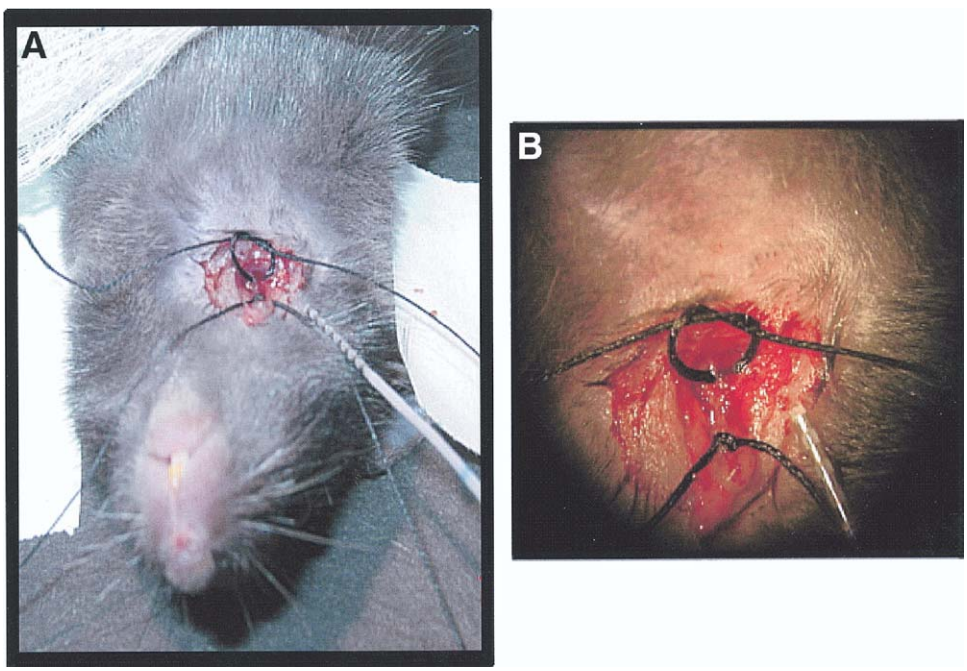

Figure 1. Surgical field and electrophysiology catheter. **(A)** View of right external jugular cutdown and 1.7-F electrophysiology catheter in an anesthetized mouse. **(B)** Close-up of venous cutdown with proximal and distal sutures. The venotomy is made between these sutures, and the catheter is advanced to the atrioventricular junction and then sutured in place.

Sinus node recovery time (SNRT) was measured as the return atrial cycle length (CL) after 30 s of overdrive atrial pacing at CL 150 ms, or 10 to 20 ms less than sinus CL, whichever was shorter. Corrected sinus node recovery time (CSNRT) was obtained by subtracting the pre-pacing sinus CL (mean of 5 cycles) from SNRT. Atrioventricular intervals closely approximated PR intervals at a variety of CLs (linear regression: slope = 0.99, intercept = 0.50 ms; R^2 = 0.99, $p < 0.0001$). Thus, we studied AV intervals, because ECG P waves were often obscured by artifacts during atrial pacing. Since the inscription of His bundle electrograms was inconsistent during stimulation, as is often seen in mice (14,15), AV intervals were also used as surrogates for atrial-to-His (AH) intervals. We validated this surrogate (14,16) via the linear relationship of AV to AH intervals at all CLs when both were measurable (R^2 = 0.98; slope = 0.90; $p < 0.0001$).

Incremental atrial pacing was performed to determine the AV interval–CL relationship, AV Wenckebach, and 2:1 AV block CLs. Atrioventricular nodal effective refractory period (AVERP) and atrial effective refractory period were determined using 15-beat atrial drive-train stimuli (S1) at CL 150 ms followed by progressively earlier extrastimuli (S2). Induction of atrial arrhythmias was attempted using double and triple extrastimuli, coupled progressively earlier until loss of capture, and then burst atrial pacing.

Programmed ventricular stimulation commenced with burst pacing to determine ventriculo-atrial (VA) conduction, Wenckebach, and 2:1 block CL. Effective refractory period was determined for ventricular and VA conduction (when present) using 15 beats of drive train (S1) stimulation at CL 150 ms and progressively earlier S2. Ventricular arrhythmia induction was attempted with up to 6 extrastimuli (S2 to S7), each coupled more prematurely until loss of capture, followed by burst ventricular pacing to CL 50 ms. Induced ventricular arrhythmias were defined as 3 or more repetitive ventricular responses.

Finally, intraobserver and interobserver reproducibility were assessed. For PR, AV, and AH intervals, Pearson coefficients for interobserver measurements (AS, SMN) were r = 0.98, 0.97, and 0.98, respectively ($p < 0.0001$ for each), with no difference between means. For intraobserver PR, AV, and AH interval measurements, Pearson coefficients were 0.99, 0.98, and 0.98, respectively ($p < 0.0001$ for each), with no difference between means.

At the conclusion of the experiment, mice were killed using an overdose of ketamine and xylazine.

AC_{VI} expression in AV node. Fresh frozen sections (10 μm) of hearts obtained from mice with cardiac-directed AC_{VI} expression and transgene-negative siblings were fixed in 100% methanol for 10 min at −20°C. Fixed sections were blocked in 1% bovine serum albumin for 20 min and incubated with Cx45 mouse monoclonal primary antibodies (Chemicon Inc., Temecula, California) to stain AV nodal tissue, and AC_V/AC_{VI} rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, California) at 1:100 dilution for 24 h at 4°C. After primary incubation, sections were washed in TBS-Tween (0.1%, 3X, 5 min) and incubated with FITC and rhodamine-conjugated secondary antibodies (Molecular Probes, Inc., Eugene, Oregon) for 1 h. The sections were washed again in TBS-Tween (0.1%, 6X, 5 min) to remove non-specific binding and incubated with DAPI (1:5000) to stain nuclei. Stained sections were mounted with gelvatol on glass coverslips and allowed to dry for 24 h before imaging. Images were captured with a DeltaVision deconvolution epifluorescence microscope system (Applied Precision, Inc., Issaquah, Washington). Ten optical sections spaced by 0.2 μm were taken at 10× or 60× magnification. Exposure times were set such that the camera response was in the linear range for each fluorophore and standardized for all images.

Statistical analysis. Two-tailed unequal variance *t* tests were used to compare data between AC_{VI} and control groups, and repeated measures analysis of variance (ANOVA) was used to detect group differences in AV conduction at various CLs. Pearson coefficient was used to assess interobserver and intraobserver variability in AH, AV, and PR intervals, with *t* tests to assess differences between the means. Chi-square analysis was used to compare the presence or absence of retrograde VA conduction between groups. The null hypothesis was rejected when $p = 0.05$.

RESULTS

Control mice. In this study, we used 2 groups of control mice: 5 transgene-negative siblings, and 9 wild-type mice of the same strain. Statistical testing between these 2 groups of control mice revealed no differences in any measure of electrophysiologic function. Therefore, these groups were combined into a single group (n = 14) and compared to the AC_{VI} transgene-positive animals (n = 9).

AV and VA conduction. At baseline, the AV interval was shorter in AC_{VI} mice (AC_{VI}: 47 ± 9 ms; control: 57 ± 11 ms; $p = 0.02$), despite similar baseline sinus CL (Table 1). Although His bundle electrograms were obtained in many mice, they were visible throughout the entire protocol in only a subset (for example, Fig. 2). However, when intracardiac AV and AH intervals were both observed, they correlated linearly ($p < 0.0001$), excluding significant independent effects from His-ventricular (HV) interval changes in either group.

On atrial pacing (Table 1), AV intervals were shorter in AC_{VI} than in control mice ($p < 0.01$, ANOVA) (Fig. 3). The AV Wenckebach CL (Fig. 4) was shorter in AC_{VI} mice (AC_{VI}: 114 ± 12 ms; control: 131 ± 28 ms; $p = 0.05$), as was the longest AV interval before the non-conducted P-wave (AC_{VI}: 96 ± 10 ms; control: 109 ± 20 ms; $p < 0.05$). The AV 2:1 block CL (AC_{VI}: 96 ± 11 ms; control: 113 ± 25 ms; $p = 0.07$) and AVERP (AC_{VI}: 81 ± 26 ms; control: 98 ± 32 ms; $p = 0.17$) trended to be shorter in AC_{VI} mice.

Table 1. Electrophysiologic Measurements

	AC_{VI} (n = 11)	Control (n = 14)	p Value
Baseline measurements			
Sinus CL (ms)	235 ± 77	208 ± 70	0.38
Range in sinus CL (ms)	183 ± 120	166 ± 78	0.69
Baseline AV interval (ms)	47 ± 9	57 ± 11	0.02
Atrial pacing			
SNRT (ms)	383 ± 159	420 ± 162	0.57
(C)SNRT (ms)	163 ± 153	218 ± 132	0.35
A ERP (ms)	64 ± 20	70 ± 15	0.51
AV Wenckebach CL (ms)	114 ± 12	131 ± 28	0.05
AV 2:1 CL (ms)	96 ± 11	113 ± 25	0.07
Longest AV interval (ms)	96 ± 10	109 ± 20	0.05
AV ERP (ms)	81 ± 26	98 ± 32	0.17
Ventricular pacing			
VA Wenckebach CL (ms)	136 ± 21	166 ± 27	0.07
VA ERP (ms)	97 ± 21	127 ± 15	0.05
V ERP (ms)	70 ± 29	70 ± 10	>0.9

A = atrium; AC_{VI} = adenylyl cyclase type VI; AV = atrioventricular; CL = cycle length; (C)SNRT = (corrected) sinus node recovery time; ERP = effective refractory period; V = ventricular; VA = ventriculo-atrial.

On ventricular pacing, AC_{VI} mice trended toward exhibiting VA conduction more often than controls (p = 0.08). In mice showing VA conduction, VA effective refractory period (ERP) was shorter in AC_{VI} mice (AC_{VI}: 97 ± 21 ms; control: 127 ± 15 ms; p = 0.05), and VA Wenckebach CL trended to be shorter (AC_{VI}: 136 ± 21 ms; control: 166 ± 27 ms; p = 0.07).

Other measurements. No group differences were seen in SNRT (AC_{VI}: 383 ± 159 ms; control: 420 ± 162 ms; p = 0.57) or CSNRT (AC_{VI}: 163 ± 153 ms; control: 218 ± 132 ms; p = 0.35), nor in atrial or ventricular ERP.

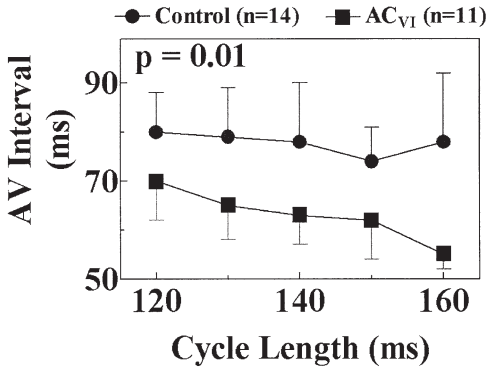

Figure 3. Atrioventricular (AV) conduction and cycle length. Atrioventricular intervals were shorter in adenylyl cyclase type VI (AC_{VI}) mice than in controls for a range of cycle lengths. The p value from analysis of variance; mean values ± 1 SEM are shown.

Arrhythmias. Two mice, 1 control mouse (age 4 months), and 1 AC_{VI} mouse (age 5.5 months) exhibited spontaneous non-sustained (<30 s) accelerated junctional rhythm (Fig. 5A). One control mouse developed a single episode of atrial tachycardia of CL 36 ms and duration 600 ms (Fig. 5B), likely induced by atrial pacing within the vulnerable period of the preceding P-wave (labeled), with accelerated and variable ventricular rate (4:1 and 5:1 AV conduction). Termination was either spontaneous or related to the next atrial pacing stimulus. The tachycardia mechanism could not be elaborated on further because this finding was not reproduced. Ventricular tachycardia (≥3 beats) could not be induced in any animal despite aggressive stimulation.

AC_{VI} expression in AV node. To determine whether increased AV conduction was a direct consequence of increased

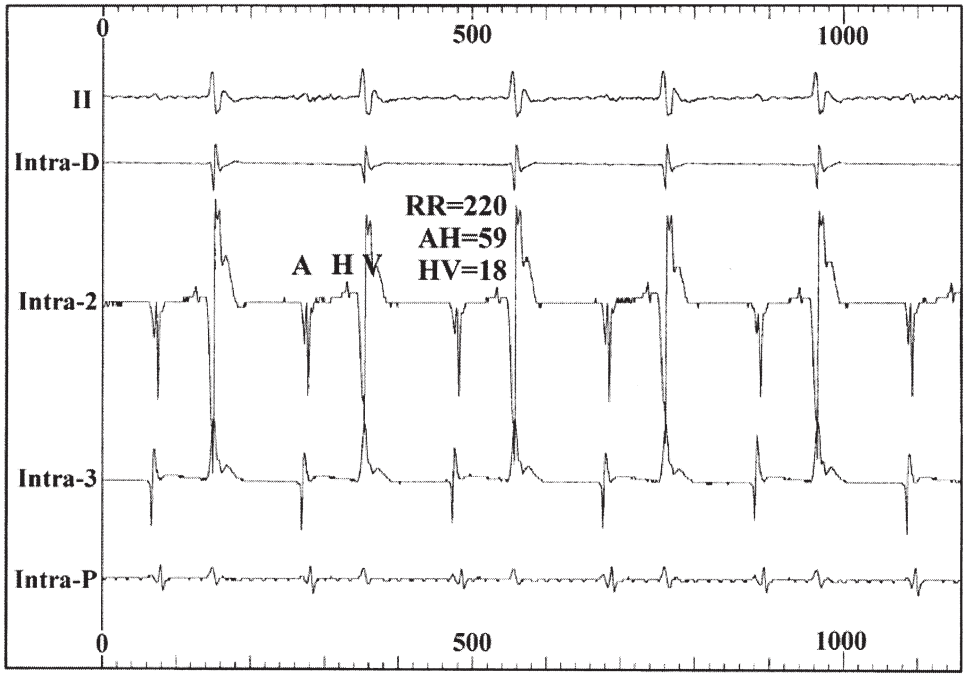

Figure 2. Electrocardiogram and intracardiac electrograms during sinus rhythm, showing atrial, His, and ventricular electrograms from an octapolar catheter at the atrioventricular junction. Bipoles are labeled Intra-P (proximal, atrial), Intra-3 (atrioventricular junction), Intra-2 (His position), and Intra-D (distal, ventricular), respectively. Scale markings are in ms and intervals are marked. AH = atrial-to-His; HV = His-to-ventricular.

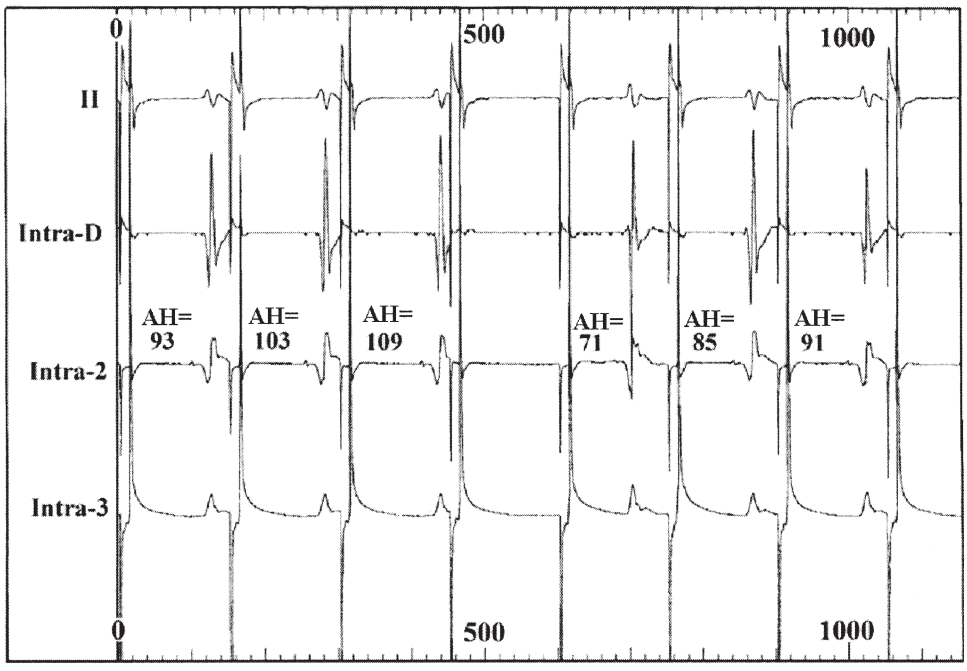

Figure 4. Atrioventricular nodal Wenckebach during atrial pacing at cycle length 120 ms, showing atrial-to-His (AH) Wenckebach (intervals marked). Bipoles and timescale are labeled as in Figure 2.

AC_{VI} expression in AV nodal tissue, rather than an indirect (e.g., reflex-mediated) effect, we used immunodetection with AV node-specific and AC-specific antibodies. Increased expression of AC_{VI}in the AV node was shown by immunofluorescence staining in regions that also demonstrated connexin 45 staining (denoting AV node). The AC_{VI} expression was increased in the AV node and myocardium of transgene-positive versus -negative mice (Fig. 6C).

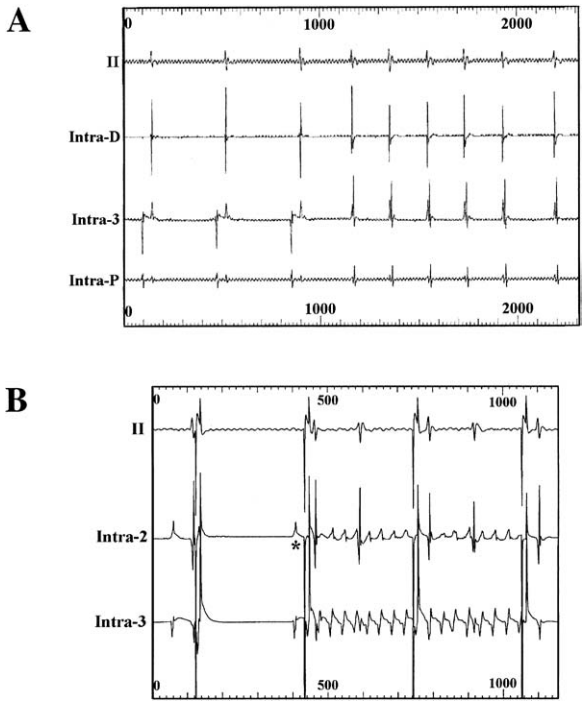

Figure 5. (A) Transient accelerated junctional rhythm. Episodes were of short duration (lasting seconds), self-limited, and seen in 1 animal from each group. **(B)** Transient atrial tachycardia. This rhythm was observed in a control-group mouse (cycle length 36 ms), likely induced by atrial pacing within the vulnerable period of the preceding native P-wave (labeled *). Note accelerated and variable ventricular rate during tachycardia. This finding was not reproduced and was seen only in this mouse. Bipoles and timescale are labeled as in Figure 2.

DISCUSSION

This is the first study to demonstrate that AV nodal conduction is facilitated by cardiac-directed expression of AC_{VI} via the αMHC promoter, and the first to provide immunohistochemical confirmation of AV nodal expression of AC_{VI}. At EPS, AV nodal conduction was facilitated in transgenic mice at baseline, during constant-rate pacing, and after premature extrastimuli. In contrast, AC_{VI} expression conferred no clear phenotype in sinus nodal function, nor in atrial or ventricular refractory periods, despite known expression in these tissues. Cardiac-directed AC_{VI} expression has already been shown to increase inotropic response to catecholamines in normal (8) and cardiomyopathic (9) mice, and in normal (17) and cardiomyopathic pigs (11). The current study suggests the intriguing possibility that cardiac-directed AC_{VI} expression may have a role in treating disorders of AV conduction or attenuating the negative dromotropic effects of βAR antagonists in individuals with heart failure.

Atrioventricular nodal function. Not only did AC_{VI} mice exhibit increased antegrade AV conduction (shorter AV intervals, AV Wenckebach, and 2:1 block CL), but retrograde VA conduction was correspondingly enhanced. These effects are consistent with the expected effects of increased βAR signaling on the AV node, and they suggest that AC_{VI}

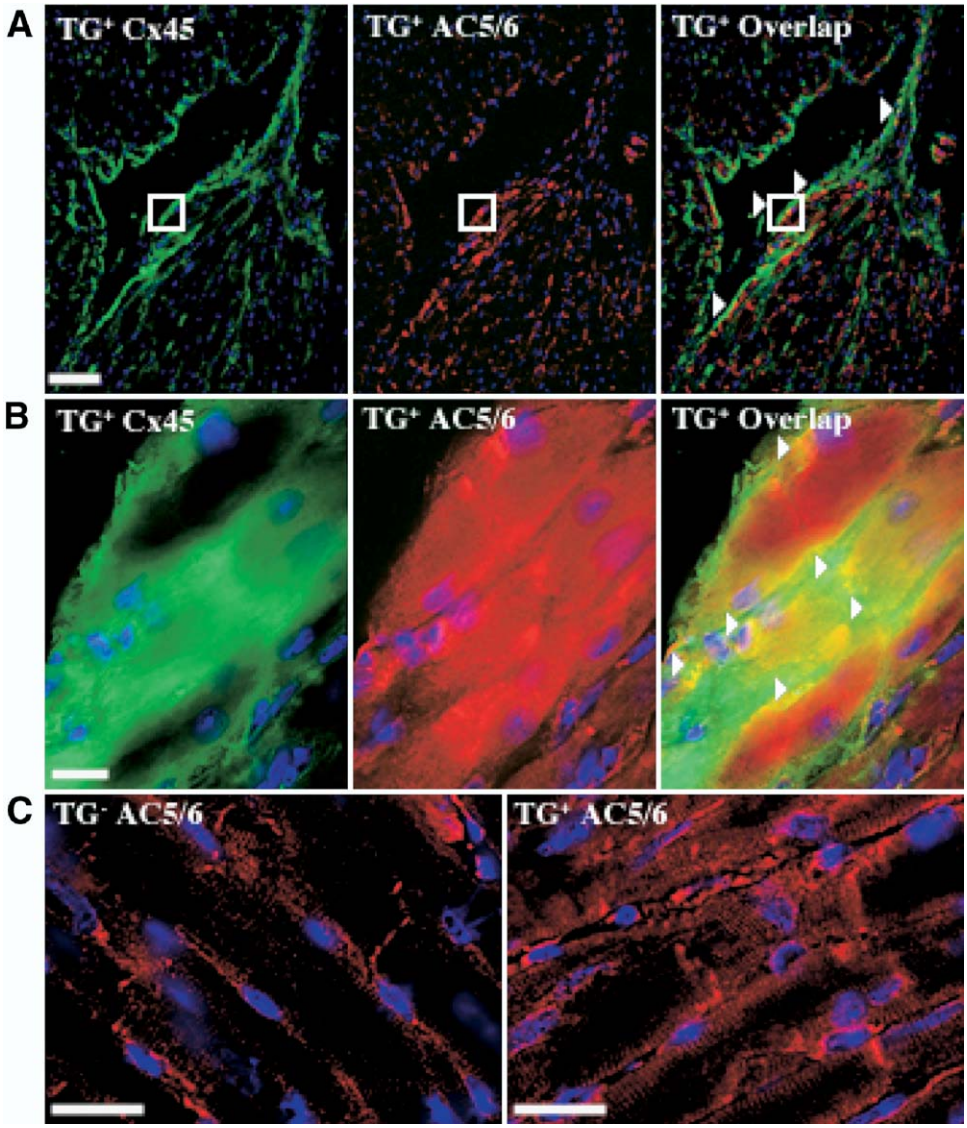

Figure 6. Expression of AC_{VI} in AV node. **(A)** 10× images of AV node region in heart from a mouse with cardiac-directed expression of AC_{VI}. **Green** indicates staining for Cx45, a marker for AV nodal tissue; **red** indicates $AC_{V/VI}$ protein; overlap image **(right)** indicates co-expression of Cx45 and $AC_{V/VI}$. **(B)** 60× images of area contained in **white box** in **(A)**. There is considerable co-localization of Cx45 AV nodal stain with $AC_{V/VI}$. **(C)** 60× image of AC_{VI} and transgene-negative sibling mouse (control); heart sections exposed to $AC_{V/VI}$ antibodies show increased AC_{VI} expression compared to heart sections from transgene-negative sibling. **White bar** lengths are **(A)** 100 μm; **(B)** 20 μm; **(C)** 20 μm. Abbreviations as in Figures 2 and 3.

expression facilitates βAR signaling at baseline as well as during stimulation.

Sinus nodal function. This study confirms previous results that AC_{VI} expression does not alter baseline sinus CL or heart rate variability (18) (Table 1).

The apparent selectivity of the AC_{VI} phenotype for the AV node rather than the sinoatrial node is intriguing. We have previously shown widespread AC_{VI} expression in cardiac tissues of transgenic mice, including the sinoatrial node (12), and the present study is the first to confirm expression in the AV node. First, phenotypic differences may reflect the influences of autonomic innervation in each region, in that baseline vagal tone may influence the sinus node (and attenuate its AC_{VI} phenotype in transgene-positive mice) to a greater extent than the AV node. Notably, we recently demonstrated, using telemetry and pharmacological interventions, that sinus rate variability (and hence autonomic tone per se [19]) is unaltered in AC_{VI} versus control animals (18). Second, apparent selectivity may be a consequence of differing effects of anesthesia on the sinus versus AV nodes. Finally, SNRT and CSNRT may provide only limited information on the sinus nodal phenotype of AC_{VI} expression, and alternative indices including chronotropic reserve may be required to fully characterize sinus nodal function.

ATRIAL AND VENTRICULAR ARRHYTHMIAS. Although cardiac-directed AC_{VI} expression increases βAR responsiveness (1,12,18), our results suggest that it is not proarrhythmic. Despite aggressive atrial stimulation, we ob-

served only 2 cases of self-limited accelerated junctional rhythm, once in a control animal and once in an AC_{VI} mouse. The 1 transient (<1 s) episode of atrial tachycardia was observed in a control animal.

Similarly, AC_{VI} expression did not increase susceptibility to ventricular tachycardia or ventricular fibrillation in these mice, unlike traditional sympathomimetic interventions (20). This was true despite very aggressive ventricular stimulation protocols that have been shown to induce ventricular arrhythmias in mice with structural heart disease (15,21). We also found no differences in ventricular refractory periods in AC_{VI} versus control mice. Indirectly, these results also support our prior studies that cardiac-directed AC_{VI} expression is not associated with abnormalities in cardiac structure (8).

Clinical implications. These results suggest a mechanism by which cardiac-directed AC_{VI} expression may prevent morbidity or mortality from AV block, with or without co-treatment with βAR antagonists, in patients with heart failure. Indeed, in a study of mice with implanted telemetry devices, we found that AC_{VI} mice were protected against bradycardic mortality after ligation of the left coronary artery when compared to control mice (13).

Study limitations. Difficulties in consistently recording His-bundle electrograms in mice for prolonged periods have been noted in epicardial (14) as well as endocardial (15) recordings. However, our use of AV intervals as previously reported surrogates for the AH interval (15,16,21) was supported by a strong linear correlation between AH and AV intervals. We used endocardial recordings to reduce potential autonomic (and thus electrophysiologic) changes from the surgery and pain involved with epicardial electrodes.

Theoretically, an important limitation of attempted arrhythmia induction is that these protocols are honed for humans (20), with unproven efficacy in mice. Nonetheless, such protocols have been used to induce arrhythmias in mice with various structural heart diseases (15,21). Finally, although differences in sedation between mice are a potential confounding factor, differences should apply equally between groups, and our protocols were also similar to previously described murine sedation schemes (16).

Finally, the use of transgenic mice limits extrapolation of our results to AC_{VI} gene therapy for AV nodal disease in CHF. In addition, further studies should explore how AV nodal facilitation would interact with the high sympathetic tone of CHF, or whether shortening of anterograde block CL would elevate ventricular rate in CHF-associated atrial fibrillation.

Conclusions. Electrophysiologic testing showed that cardiac-directed expression of AC_{VI} enhances anterograde and retrograde conduction through the AV node at baseline and during pacing, without affecting basal sinus rate or sinus node function, or inducing pro-arrhythmia. Immunofluorescence confirmed that cardiac-directed expression of AC_{VI} involved the AV node. We speculate that gene transfer of AC_{VI} to the AV node may be useful to ameliorate the negative dromotropic effects of βAR antagonists in patients with heart failure. This presupposes that the levels of expression obtained by gene transfer would be adequate to have such an effect and would require additional studies.

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