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Biological Pacemaker Implanted in Canine Left Bundle Branch Provides Ventricular Escape Rhythms That Have Physiologically Acceptable Rates

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Background—We hypothesized that administration of the HCN2 gene to the left bundle-branch (LBB) system of intact dogs would provide pacemaker function in the physiological range of heart rates.

Methods and Results—An adenoviral construct incorporating HCN2 and green fluorescent protein (GFP) as a marker was injected via catheter under fluoroscopic control into the posterior division of the LBB. Controls were injected with an adenoviral construct of GFP alone or saline. Animals were monitored electrocardiographically for up to 7 days after surgery, at which time they were anesthetized and subjected to vagal stimulation to permit emergence of escape pacemakers. Hearts were then removed and injection sites visually identified and removed for microelectrode study of action potentials, patch clamp studies of pacemaker current, and/or immunohistochemical studies of HCN2. For 48 hours postoperatively, 7 of 7 animals subjected to 24-hour ECG monitoring showed multiple ventricular premature depolarizations and/or ventricular tachycardia attributable to injection-induced injury. Thereafter, sinus rhythm prevailed. During vagal stimulation, HCN2-injected dogs showed rhythms originating from the left ventricle, the rate of which was significantly more rapid than in the controls. Excised posterior divisions of the LBB from HCN2-injected animals manifested automatic rates significantly greater than the controls. Isolated tissues showed immunohistochemical and biophysical evidence of overexpressed HCN2.

Conclusions—A gene-therapy approach for induction of biological pacemaker activity within the LBB system provides ventricular escape rhythms that have physiologically acceptable rates. Long-term stability and feasibility of the approach remain to be tested. (*Circulation*. 2004;109:506-512.)

Key Words: arrhythmia ■ electrophysiology ■ gene therapy ■ pacemakers

Electrical pacemakers remain the state of the art for treating a variety of arrhythmias and conduction disturbances. However, molecular approaches to the development of a biological pacemaker have become conceptually attractive alternatives. Proof of concept for gene therapy to construct biological pacemakers has been provided as follows. First, overexpression of β_2 -adrenergic receptors was found to increase atrial rate in porcine hearts.^{1,2} Subsequently, replacement of 3 amino acid residues in the pore structure of Kir2.1 to create a dominant negative construct resulted in idioventricular pacemaker function in guinea pig ventricle.³ Most recently, overexpression of HCN2, an isoform of the gene encoding the wild-type pacemaker current I_h , in a region of canine left atrium provided pacemaker function when sinus rhythm was suppressed.⁴ In the latter 2 sets of experiments, an adenovirus was used as the vector for gene delivery.

Whereas all these approaches induced pacemaker function, none provided an analog to electrical pacemakers that might be considered physiologically sound. By this we mean that for any biological cardiac pacemaker to be considered a potential medical therapy, it should (1) provide a dominant focus of impulse initiation proximal enough in the conducting system to ensure physiological activation of the ventricles and (2) compete favorably with electrical pacemakers in duration and stability of effect. In the present study, we focused on the first requirement: we implanted an adenoviral construct into the left bundle-branch (LBB) system and determined whether the rhythm that developed during transient atrioventricular block was stable and organized. Moreover, we hypothesized that rhythms occurring in this setting would result from I_{HCN2} induction of automaticity in the region of the LBB injected with the construct. Testing the second requirement was not part of the present study, because it awaits a long-term trial.

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Methods

All studies were performed with protocols approved by the Columbia University Institutional Animal Care and Use Committee.

Viral/Genetic Preparation

An adenoviral construct of mouse HCN2 (mHCN2, GenBank AJ225122) driven by the cytomegalovirus promoter was prepared as described previously.⁵ The AdHCN2 construct was purified through plaque assay, amplified to a large stock, and harvested and titrated after CsCl banding. The identical procedure was used to construct an adenoviral vector of enhanced green fluorescent protein; AdGFP), the sequence for which was taken from its original vector pIRES2-EGFP (Clontech, Palo Alto, Calif) at *Bam*HI and *Nor*I sites and subcloned into the shuttle vector pDC516. The final titers were AdHCN2 3.4×10^{11} fluorescent focus units (ffu)/mL and AdGFP 1.4×10^{12} ffu/mL. When both AdHCN2 and AdGFP were injected, 2 to 3×10^{10} ffu of each virus was injected per experiment; when AdGFP was injected alone as a control, 4 to 6×10^{10} ffu was injected per experiment to achieve an equivalent viral load.

Intact Animal Studies

Thirteen male or female mongrel dogs aged 2 to 4 years and weighing 23 to 27 kg each were anesthetized with sodium thiopental induction (17 mg/kg IV) followed by inhalational isoflurane (1.5% to 2.5%). A custom-modified bipolar 8F steerable catheter (Guidant Corporation) was used for subendocardial delivery of the adenoviral constructs or saline. A 29-gauge needle, which could be advanced and retracted by 3 mm, was incorporated into the core lumen of the catheter. The catheter filled with sterile saline was introduced into the left ventricle (LV) under fluoroscopic control via an 8F arterial introducer sheath through the right carotid artery. After a stable bundle-branch potential electrogram recording had been ensured, the needle was advanced, and 0.1 to 0.2 mL of 3:1 saline-diluted contrast material (50% Hypaque, Nicomed) was injected to ensure that the needle tip was in the LV wall and not the LV cavity. An adenoviral construct (AdGFP+AdHCN2 or AdGFP alone) or normal saline solution (total, 0.6 mL) was then injected at 3 sites identified by electrogram as being in the LBB. After each injection, the needle was retracted and the catheter relocated before another injection was made. The 3 injection sites were within 4 mm of each other. During surgery, ECG and electrograms were monitored with Ponemah software (Gould Instrument Systems).

Animals were then allowed to recover for 4 to 7 days, during which time ECGs were recorded daily. In addition, continuous 24-hour recordings were made for 7 of the dogs with a Holter cassette recorder, and these were analyzed on a Holter for Windows System (Rozinn Electronics, Inc). Results were reviewed by 2 to 3 independent readers.

On the day of terminal study, animals were anesthetized as above, and both cervical vagal trunks were isolated. A quadripolar endocardial pacing catheter (EP Technologies, Inc) was placed into the right ventricle (RV) for electrogram recording and pace mapping. During continuous ECG monitoring (IOX acquisition system, EMKA Technologies), graded right and left vagal stimulation was performed (20 to 40 pulses/second, pulse duration 2 to 4 ms [Grass S88 stimulator, Grass Instruments Co]) via bipolar platinum iridium electrodes.⁶ This was done to suppress sinus rhythm and/or induce atrioventricular block such that escape pacemaker function might occur. Then a short period of RV pacing was performed at 150 bpm to demonstrate the configuration of rhythms that had an RV origin. LV and RV escape rhythms were identified on the basis of QRS complex morphology on ECG and temporal relationships between ECGs and onset of electrograms.

The chest was then opened, and the heart was removed and immersed in cold Tyrode's solution equilibrated with 95% O₂-5% CO₂ containing (in mmol/L) NaCl 131, NaHCO₃ 18, KCl 4, CaCl₂ 2.7, MgCl₂ 0.5, NaH₂PO₄ 1.8, and dextrose 5.5. The LV was opened, and the region of LBB that had been injected (the posterior division) was visually identified, photographed, and excised ($\approx 2.0 \times 1.5 \times 0.1$

cm), as were comparable regions from AdGFP- or saline-injected or control dogs. These were used for microelectrode study or study of ion channels or were frozen for immunohistochemical study as described below.

Microelectrode Studies

Preparations were placed in a 4-mL chamber perfused with Tyrode's solution (37°C, pH 7.3 to 7.4) at a rate of 12 mL/min and were permitted to beat spontaneously. The bath was connected to ground via a 3 mol/L KCl/Ag/AgCl junction. Preparations were impaled with 3 mol/L KCl-filled glass capillary microelectrodes that had tip resistances of 10 to 20 M Ω coupled by an Ag/AgCl junction to an amplifier with high input impedance and input capacity neutralization. Transmembrane action potential signals were digitized and stored on a personal computer for subsequent analysis as described previously.⁷

Dissociation of Myocytes and Studies of HCN Current

Purkinje myocytes were dissociated by modification of a previously published procedure.⁸ Excised bundle-branch regions were placed in standard Hank's balanced salt solution (Gibco, Invitrogen Corporation) nominally free of calcium and containing 1000 to 1500 U/mL collagenase (type II, Worthington), 1% BSA (Sigma), and 5 mmol/L HEPES-NaOH buffer (pH 6.7). Petri dishes with tissue were placed in a gyrator shaker and agitated (2 to 3 cycles per second) for 30 to 40 minutes at 37°C. Tissue samples were then washed twice in a high K⁺-saline solution (in mmol/L: potassium glutamate 160, HEPES-KOH buffer 5, MgSO₄ 5.4; pH 6.7), and individual cells were dispersed by gentle hand pipetting.

Isolated cells were transferred to a stage-mounted chamber of an inverted epifluorescence microscope to identify GFP-expressing cells. To measure pacemaker currents, cells were superfused with 35°C Tyrode solution containing (in mmol/L) NaCl 140, NaOH 2.3, MgCl₂ 1, KCl 5.4, CaCl₂ 1.0, MnCl₂ 2, BaCl₂ 4, HEPES 5, glucose 10 (pH 7.4). Pipette solution contained (in mmol/L) aspartic acid 130, KOH 146, NaCl 10, CaCl₂ 2, EGTA-KOH 5, Mg-ATP 2, HEPES-KOH 10 (pH 7.2). To record pacemaker current, cells were held at -55 mV and stepped to -125 mV for 6 seconds, followed by an 8-second step to -115 mV to measure tail current. A 0.5-second step to 5 mV at the end of each episode ensured full current deactivation.

Immunohistochemical Studies

Freshly dissected tissue was immersed in Tyrode's solution for 2 to 3 hours, pH 7.4 at 4°C. The tissue was rinsed in 30% sucrose in 0.1 mol/L phosphate buffer and then stored overnight in fresh sucrose buffer at -20°C. Cryostat sections were cut at 14 μ m, mounted on slides, and stored at 4°C until they were stained. Antibodies to HCN2 from Alomone Laboratories (AN-01) were used. Sections were hydrated in 0.1 mol/L phosphate buffer and then exposed to normal goat serum diluted 1:75 for 1 hour. The goat serum was drained off, and the primary antisera, diluted 1:200 with the phosphate buffer, were applied to the sections overnight at room temperature in a humidified chamber. Control slides were always prepared in parallel and consisted of sections exposed overnight to normal goat serum instead of the primary antibody. Slides were then washed 3 times in phosphate buffer and exposed to FITC-coupled goat anti-rabbit secondary antisera for 1 hour in the dark and then mounted on coverslips with Vectashield mounting medium (Vector Laboratories). Sections were examined via confocal microscopy.

Statistical Analysis

Measurements of cycle lengths of cardiac rhythms and of isolated LBB were made from at least 5 beats. Fisher's exact test, Student's *t* test, or 1-way ANOVA were used as appropriate. Data are expressed as mean \pm SEM. *P* < 0.05 was considered significant.

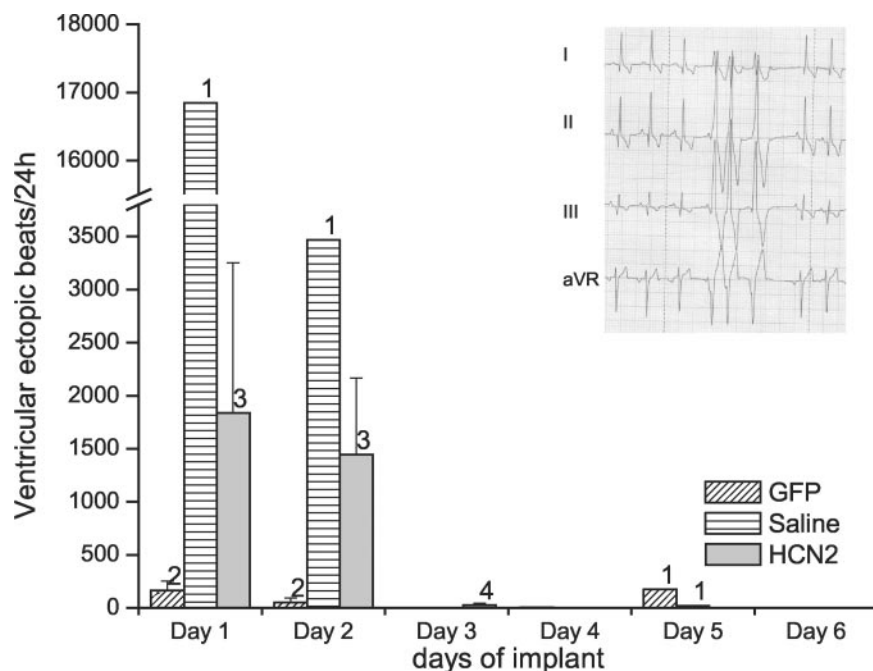


Figure 1. Results of 24-hour ECG recordings from 7 dogs (long-term monitoring was not performed in this fashion in remaining 6 dogs). All ventricular ectopic activity (premature depolarizations plus ventricular tachycardia) is displayed, as is ECG of 1 dog manifesting ventricular tachycardia (inset). During first 48 hours, there were large numbers of ventricular ectopic impulses, which thereafter were minimal. Numbers above each column refer to number of animals in each group.

Results

Intact Animal Studies

We studied 7 animals injected with HCN2+AdGFP, 3 injected with AdGFP alone, and 3 injected with saline. Seven of these animals underwent 24-hour ECG monitoring throughout their course of study. Figure 1 demonstrates that for 2 days after the injection procedure, there was increased irritability of the ventricles in all 7, seen as polymorphic premature ventricular depolarizations and/or ventricular tachycardia regardless of whether the animals were saline injected, AdGFP injected, or AdGFP+AdHCN2 injected. For this reason, no studies were done of pacemaker function until days 4 to 7.

Figure 2 demonstrates representative vagal stimulation experiments showing records from 1 dog injected with AdGFP alone (Figure 2A) and 1 injected with AdGFP+AdHCN2 (Figure 2B). Before vagal stimulation, both dogs had comparable sinus rates. After vagal stimulation, the rate of the AdGFP+AdHCN2-injected heart was more than 2 times faster than that of the heart injected with AdGFP alone. Moreover, the RV electrogram recordings (insets in Figure 2) show that in the AdGFP-injected animal, electrogram initiation was early in the QRS complex, whereas in the AdHCN2-injected animal, RV electrogram initiation occurred later.

Summary data for the vagal stimulation experiments were as follows (data from saline-injected and AdGFP-injected dogs did not differ from one another and were analyzed together as "controls"). Baseline sinus cycle length did not differ between control and AdHCN2-injected groups (484 ± 46 and 506 ± 11 ms, respectively; $P > 0.05$). During vagal stimulation, RV escape rhythms occurred in 4 of 6 controls and 3 of 7 HCN2 dogs ($P > 0.05$), whereas LV escape rhythms occurred in 4 of 6 controls and 6 of 7 HCN2-injected dogs ($P > 0.05$). Although idioventricular escape of RV or LV

origin occurred in all animals, the rate generated was significantly more rapid (nearly 60 bpm) in the hearts of animals that had received an LBB injection of AdHCN2 than was seen for LV rhythms of control animals and for RV rhythms of control or AdHCN2-injected animals (Table).

QRS duration during sinus rhythm was 59 ± 2.6 ms and did not differ among the animals regardless of treatment given ($P > 0.05$). During idioventricular rhythm, QRS duration was prolonged significantly in all groups ($P < 0.05$ by comparison with sinus rhythm) but was still within a range consistent with origin in the proximal ventricular conducting system (Table). Despite the differences in rate, no one group of idioventricular rhythms manifested QRS durations different from the others (Table). However, for the RV and LV origin control groups, there was greater variability in QRS duration.

Figure 3 shows the appearance of the endocardial surface of 1 heart at the time of its excision immediately after the vagal stimulation experiment. Note the hematoma formation at the injection site. This finding was consistent among animals regardless of the substance injected.

Isolated Tissue and Disaggregated Purkinje Myocyte Studies

Figure 4 shows the results of 16 experiments on bundle branches from animals injected with AdHCN2+AdGFP, AdGFP alone, or saline and from the same site in 5 control dogs that had not been injected at all. Note that rate was most rapid ($P < 0.05$) in the AdHCN2-injected regions. Although maximum diastolic potential tended to be lower in the AdHCN2-injected dogs, this result was not significant.

We have previously demonstrated that overexpression of HCN2 in rat myocytes in culture and canine atrium in vivo results in an I_{HCN2} more than 1000-fold greater than for wild-type I_f .^{4,5} Our intent here was not to repeat our earlier studies but simply to test qualitatively whether the procedures used to alter cardiac rhythm and Purkinje fiber electrophys-

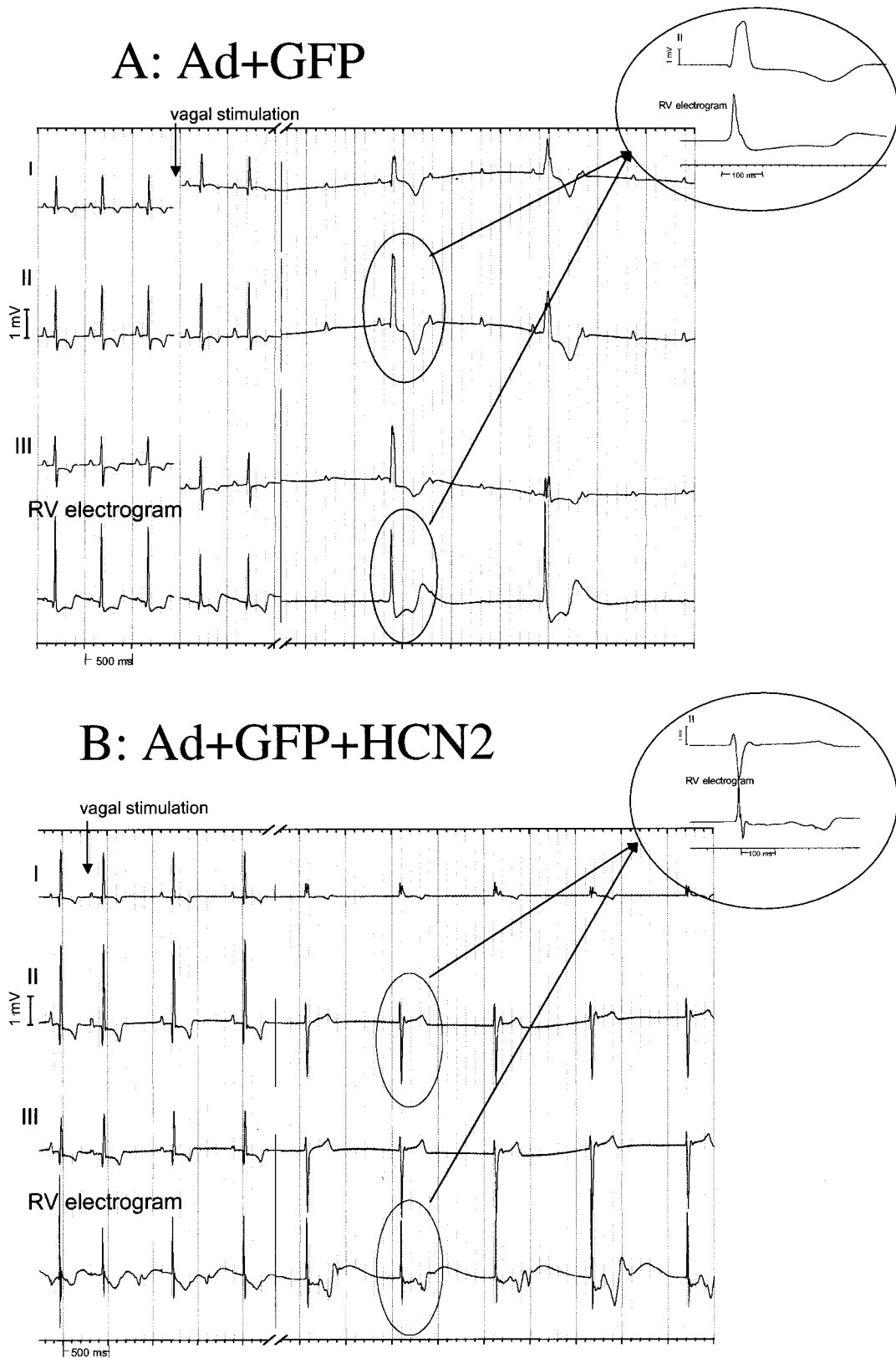


Figure 2. Representative experiments with AdGFP injection (A) and AdGFP+AdHCN2 injection (B). Note that at outset (left side of each panel), sinus rhythm of comparable rate occurs in both animals. In both dogs, primary effect of vagal stimulation was to induce AV block. This was followed by slow idioventricular rhythm in AdGFP-injected dog (A; interval between left and right traces lasted 22 seconds, near outset of which vagal stimulation was initiated). In AdHCN2-injected dog (B), vagal stimulation (arrow) was followed by far more rapid idioventricular rhythm. Interval between left and right traces was of 5-second duration. Insets are magnifications of lead II and RV electrogram impulses indicated in basic traces. See text for discussion.

Cycle Lengths and QRS Durations of Idioventricular Rhythms

	LV Origin		RV Origin	
	Cycle Length, ms	QRS, ms	Cycle Length, ms	QRS, ms
AdHCN2 injection	1091 ± 21*	80 ± 7	1637 ± 102	77 ± 4
Controls	1500 ± 34	87 ± 10	2025 ± 286	91 ± 9

* $P < 0.05$ for cycle length compared with all other groups, which do not differ from one another ($P > 0.05$). $n = 6$ dogs for HCN2, LV origin; 1 dog HCN2, RV origin; 2 dogs Control, LV origin; 4 dogs Control, RV origin. Mean ± SE was determined from all beats recorded from each animal.

iological properties, detailed above, were associated with the anticipated overexpression of I_{HCN2} assayed biophysically or histochemically. Figure 5 demonstrates the magnitude of I_{HCN2} in 1 of 2 Purkinje myocytes from an animal injected with AdHCN2+AdGFP and of I_f in 1 of 6 control Purkinje myocytes. Note the markedly greater current in the myocyte in which HCN2 is overexpressed, approximating that previously reported by us in atrium.⁴ Moreover, in the cells with overexpressed I_{HCN2} , this time-dependent current was observed on the first hyperpolarizing step to -65 mV, which suggests that I_{HCN2} can activate at all diastolic membrane potentials.

Figure 6 provides histochemical evidence of the presence of HCN2 by staining of tissue with antibodies to HCN2 using a fluorescently tagged secondary antibody (FITC). Figure 6A is an LBB region that was injected with the HCN2 vector, and Figure 6B is an LBB region that was not injected. Note that the staining in Figure 6B is less than in Figure 6A, consistent with a low level of endogenous HCN2.

Discussion

Proof in concept has previously been provided regarding the possibility of creating biological pacemakers.¹⁻⁴ However, none of the studies referred to, including our own of HCN2 injection into the canine left atrium,⁴ tested whether a

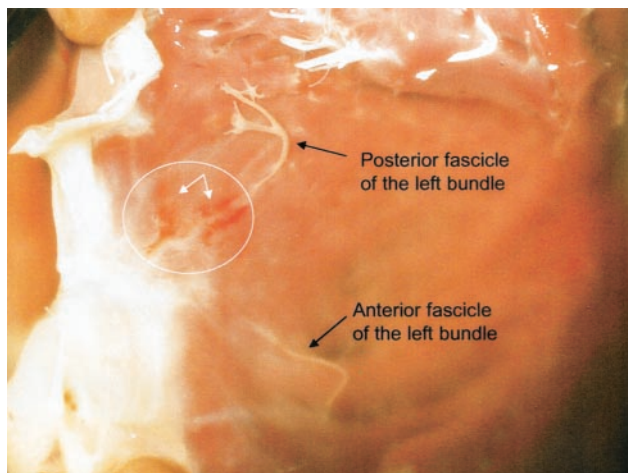


Figure 3. LV endocardial surface from 1 dog injected with AdGFP+AdHCN2. Cut mitral valve is on left, and both anterior and posterior Purkinje fascicles have been cut. Note hematoma formation at site of injection (circle and arrows).

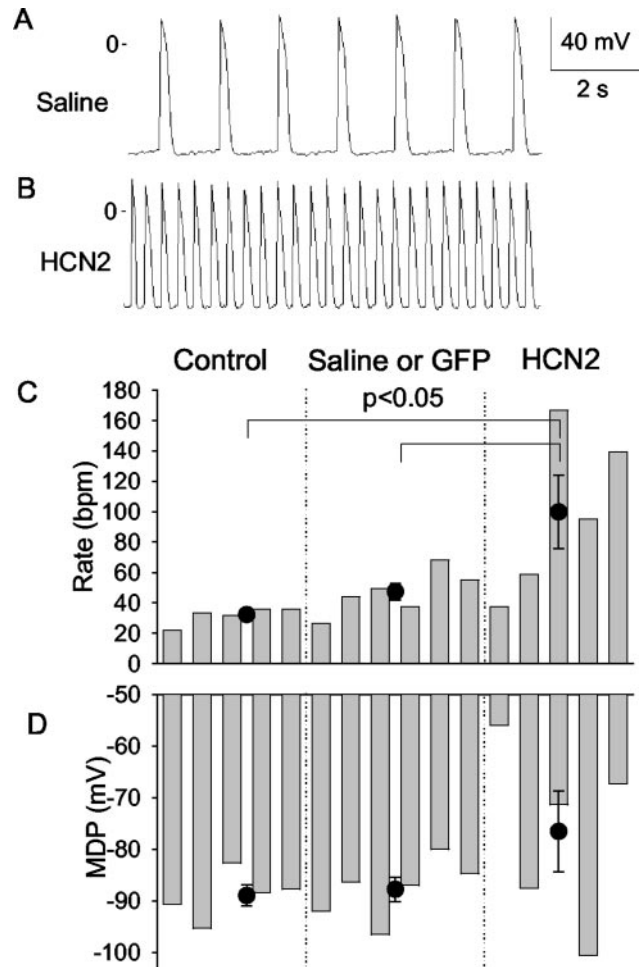


Figure 4. Automatic rate and maximum diastolic potential (MDP) in LBB preparations. A and B, Representative recordings from 1 saline- and 1 AdHCN2-injected dog. C and D, Rate (C) and MDP (D) for individual dogs, with black circles indicating mean ± SEM for each group.

biological pacemaker could be engineered into the ventricular conducting system. Moreover, none of the earlier studies demonstrated that an adenoviral construct of a pacemaker gene inserted into the proximal specialized conducting system of the ventricle could create a functioning biological pacemaker capable of driving the ventricle in a demand mode when the sinus node signal fails. This is most clearly demonstrated in Figure 2, in which the biological pacemaker activates the ventricle rapidly after vagal stimulation-induced atrioventricular block, clearly providing an escape function. This potential for the biological pacemaker to function adequately was further demonstrated in 3 ways. First, in 5 isolated LBB preparations, we studied the rate achieved, which was significantly greater than in LBB injected with GFP constructs or saline or in uninjected controls (Figure 4). Second, we disaggregated injected LBB from 2 dogs and found a marked increase in pacemaker current (Figure 5). Although we did not conduct a complete biophysical analysis of the expressed current in the isolated Purkinje myocytes, the recordings from the 2 cells studied demonstrated that current was expressed at high density and was active at physiological

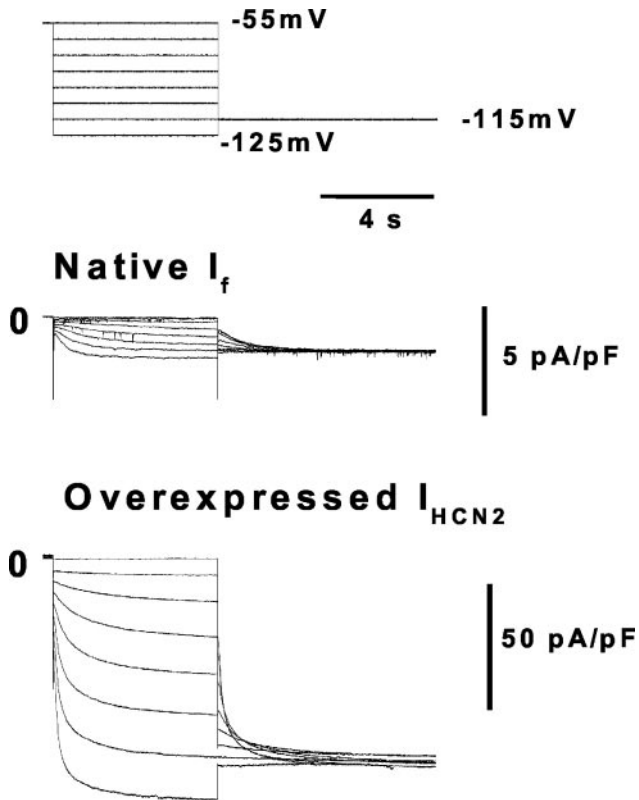


Figure 5. Patch clamp recording of native I_f (control dog, middle panel) and overexpressed I_{HCN2} (lower panel) in Purkinje cells. Note different scales in both panels and markedly greater current magnitude in presence of overexpressed HCN2. Upper panel is experimental protocol. In each panel, records have been cropped before final deactivation step to 5 mV.

potentials. Finally, the presence of a pacemaker channel was verified by immunohistochemical evidence of the HCN2 channel (Figure 6).

In considering the potential stability of a biological pacemaker, not only is the mean rate achieved in vivo important, but so is the variability of rate. As demonstrated in the Table, HCN2 injection resulted in the most rapid rate achieved (mean ≈ 60 bpm) and the smallest variance. Variance was also low with LV pacemaker origin and no HCN2 injection. In contrast, the RV pacemaker sites manifested a far greater variance in idioventricular rate (and multiple QRS configu-

rations, which suggests a lesser stability of pacemaker function or origin in the RV conducting system). It would be interesting to learn whether an RV injection of HCN2 would not only increase rate but (as we presume) also decrease variability.

There are several caveats with regard to the results of the present study. One relates to the injury created by the injection of the constructs, as is clearly seen in the hematoma formation (Figure 3). Hematoma formation occurred regardless of whether we injected AdHCN2+AdGFP, AdGFP alone, or saline. In other words, it was the result of trauma from the needle itself. It is likely that this injury contributed to the tachycardias that occurred in the first 24 to 48 hours after cardiac catheterization and that were universal across the 3 groups of animals. Moreover, the fact that both in vivo and in vitro accelerated pacemaker function was seen only in the animals and isolated LBBs that received HCN2, despite the fact that all 3 groups underwent injury, suggests that the expression of pacemaker function was not the result of injury.

That escape pacemaker function was observed in all animals should not be a source of confusion, because escape pacemaker function is a consistent property of the canine and the human ventricular conducting systems. Of particular interest here is that although all 3 interventions were performed at the same LV sites, when only saline or AdGFP was given, most escape rhythms (4 of 6) appeared to be of RV origin and regardless of site were slow in rate, whereas injection of AdHCN2 was associated with a left-sided pacemaker (6 of 7) and a narrow QRS complex, with rates of nearly 60 bpm.

In summary, we have demonstrated that implantation of a pacemaker gene into the LV conducting system results in escape pacemaker function in the canine heart. Given the early arrhythmias and hematoma formation, the method of administration does require refinement. Moreover, an understanding of whether and to what extent extracardiac gene expression occurs must be obtained. We also have no understanding of how long and how stably the next generation of pacemaker genes will operate; this information is a requisite before competition with electrical therapy can be attempted. Finally, future use of constructs that are not dependent on viral transfer should confer significant advantage over the present approach.

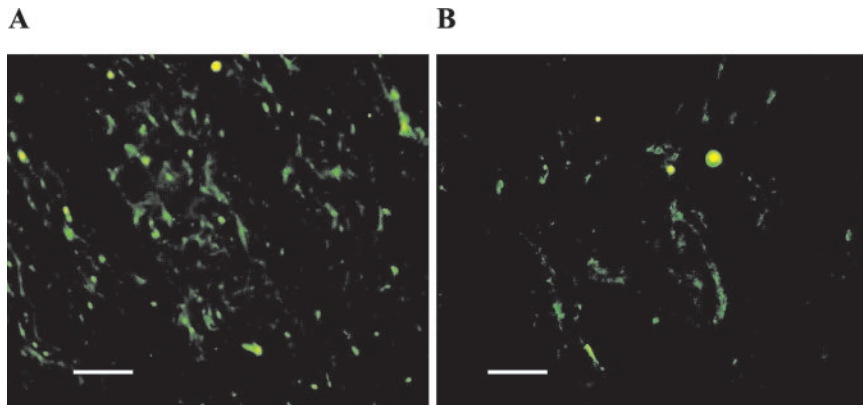


Figure 6. A, HCN2 injection site in canine LBB was stained with HCN2 antibody and FITC secondary antibody to acquire image shown. B, Uninjected LBB, stained as in A. Calibration bars=25 μm .

Acknowledgments

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