Expression and Function of a Biological Pacemaker in Canine Heart

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Background—We hypothesized that localized overexpression of the hyperpolarization-activated, cyclic nucleotide-gated (HCN2) pacemaker current isoform in canine left atrium (LA) would constitute a novel biological pacemaker.

Methods and Results—Adenoviral constructs of mouse HCN2 and green fluorescent protein (GFP) or GFP alone were injected into LA, terminal studies performed 3 to 4 days later, hearts removed, and myocytes examined for native and expressed pacemaker current (Iₚ). Spontaneous LA rhythms occurred after vagal stimulation–induced sinus arrest in 4 of 4 HCN2+GFP dogs and 0 of 3 GFP dogs (P<0.05). Native Iₚ in nonexpressed atrial myocytes was 7±4 pA at −130 mV (n=5), whereas HCN2+GFP LA had expressed pacemaker current (Iₚ_HC2) of 3823±713 pA at −125 mV (n=10) and 768±365 pA at −85 mV.

Conclusions—HCN2 overexpression provides an Iₚ-based pacemaker sufficient to drive the heart when injected into a localized region of atrium, offering a promising gene therapy for pacemaker disease. (Circulation. 2003;107:1106-1109.)

Key Words: arrhythmia ■ pacemakers ■ electrophysiology

Implantable electronic devices have represented state-of-the-art therapy for high degrees of heart block since the 1960s. Such devices save lives, and refinements in design have made them far more palatable to patients than they had been originally. Nonetheless, the ideal pacemaker, in terms of both physiological function of the heart and adaptability to the human body, would be biological.¹⁻⁻³ The search for such a pacemaker has centered on 3 gene therapy strategies: (1) ventricular myocardial cells⁴; and (3) adenoviral transfer of that the balance of inward currents suffices to depolarize ventricular myocardial cells⁵; and (3) adenoviral transfer of α (hyperpolarization-activated cyclic nucleotide–gated [HCN2])⁶ and/or β (minK-related peptide 1 [MiRP1])⁷ subunits of the endogenous human pacemaker current to induce autonomically responsive pacemaker function in ventricular myocytes. The first two approaches have seen proof of concept demonstrated in animal models.¹⁻⁻³ The third approach might be less problematic and proarrhythmic in that it incorporates the endogenous pacemaker channel gene, which selectively activates only during diastole. The present study provides proof of concept that HCN2 overexpression locally in left atrium (LA) induces both current and in situ pacemaker function.

Methods

Protocols were approved by the Columbia University Animal Care and Use Committee.

Viral/Genetic Preparation

We prepared an adenoviral construct of mouse HCN2 (mHCN2, GenBank: AJ225122) driven by the cytomegalovirus promoter, as previously described.¹ The construct AdHCN2 was purified through plaque assay, amplified to a large stock, and titrated after CsCl banding. The same procedure was used to construct an adenoviral vector of enhanced green fluorescent protein (AdGFP), the sequence of which was taken from its original vector pIRE2-EGFP (Clontech) at BamHI and NotI sites and subcloned into the shuttle vector pDC516. The final titer for AdHCN2 was 3.4×10¹⁰ ffu/mL and AdGFP 1.4×10¹⁰ ffu/mL. In each experiment, 2 to 3×10¹⁰ ffu of each virus was injected.

Intact Animal Studies

Under sterile conditions and after sodium thiopental induction (17 mg/kg IV) and inhalational isoflurane (1.5% to 2.5%) anesthesia, 23- to 27-kg male or female mongrel dogs were subjected to a pericardectomy. We injected AdGFP+AdHCN2 or AdGFP alone subepicardially in 0.6 mL of solution into the root of the LA appendage and sewed a reference electrode to the right atrium.

Animals recovered for 3 to 4 days and then were anesthetized. Both cervical vagal trunks were isolated and the chest opened. During continuous ECG monitoring, graded right and/or left vagal stimulation was performed via bipolar platinum iridium electrodes⁸ to suppress sinus rhythm such that escape pacemaker function might
Dissociation of Myocytes and Studies of HCN Current

Atrial myocytes were dissociated by modifying a previously published procedure.8 Excised atrial tissue was cut into strips, triturated in collagenase-protease solution, and exposed to 2 to 4 digestion cycles in enzyme. Enzyme concentrations were reduced after the initial cycle.

Isolated cells were transferred to a stage-mounted chamber of an inverted epifluorescence microscope to identify green fluorescent protein (GFP)-expressing cells. To measure pacemaker currents, cells were superfused with 35°C Tyrode solution containing (in mmol/L): aspartic acid, 130; KOH, 146; NaCl, 140; NaOH, 2.3; MgCl₂, 1; KCl, 10; CaCl₂, 1.0; MnCl₂, 2; BaCl₂, 4; HEPES, 5; and 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; and HEPES-KOH, 10 (pH 7.2). To record pacemaker current, cells were held at −50 or −55 mV and stepped to −65 to −145 mV for 6 seconds, followed by an 8-second step to −125 mV to measure tail current.

Statistical Analysis

Fisher’s exact test or Student’s t test was used as appropriate. Data are expressed as mean±SEM. *P<0.05 was considered significant.

Results

Intact Animal Studies

Three animals received GFP alone. None showed spontaneous atrial rhythms during vagal stimulation, whereas all four dogs receiving GFP + HCN2 showed spontaneous rhythms during vagal stimulation (P<0.05 versus GFP alone). Moreover, mapping with a hand-held electrode demonstrated early LA activation above the injection site (representative experiment, Figure 1). Increased vagal stimulation terminated atrial activity (data not shown).

Ion Channels

There was no measurable native pacemaker current (Iₚ) in 5 nonexpressing cells from injected animals, but we identified an Iₚ of 7±4 pA at −130 mV in 4 of 5 cells from control animals studied with cAMP in the pipette.9 Membrane capacitance was 97.4±29.6 pF and 90.9±12.9 pF, respectively (P>0.05). In contrast, 10 atrial cells receiving GFP+HCN2 manifested expressed pacemaker current (I_{HCN}) 500-fold greater, 3823 ±173 pA (45.3±12.5 pA/pF) at −125 mV and 768±365 pA (8.6±3.8 pA/pF) at −85 mV (representative experiments, Figure 2, A and B). Membrane capacitance here was 105±15.2 pF, not different from the others (P>0.05). The I_{HCN} activation threshold was −75 mV. The activation-voltage relation generated by the HCN2 tail current at −125 mV is shown in Figure 2C. Mean voltage of half-maximum activation was −95.2±0.4 mV, and slope factor was 7.9±0.4 mV.

Discussion

Complete heart block and sinus node dysfunction are major indications for pacemaker implantation.10 Although such electronic devices have had excellent success and minimal morbidity, it would be optimal to offer a therapy incorporating the same plasticity as the normal sinus node and atrioventricular conducting system. Several approaches to provide biological pacemaker function have been tested. After injection of plasmids to overexpress human β₂-adrenergic receptors in porcine right atrium, heart rates were 50% faster than those of controls.11 Although clearly demonstrating the value of gene transfer to modify pacemaker function, this study focused on modulating native pacemaker cells rather than the pacemaker itself.

A dominant negative strategy to reduce I_{K1}, which normally maintains ventricular myocytes at negative membrane potentials, induced spontaneous impulse initiation in guinea pig heart.5 This provided proof of concept for biological pacemaking, although the site of pacemaker initiation was not identified in vivo, and there was no attempt to achieve localized expression of pacemaking. There is little likelihood that the source of inward current providing pacemaker func-

Figure 1. ECG leads I, II, and RA, and LA (overlying injection site) electrograms during normal sinus rhythm in an anesthetized dog previously injected with HCN2+GFP into LA (initial 2 beats). Vagal stimulation induces asystole. An idioventricular escape beat and 2 ectopic P waves (arrows) follow. Note that the LA electrogram precedes the RA. Horizontal axis indicates milliseconds before (negative values) and after (positive values) onset of vagal stimulation (0).
The present approach to biological pacemaking, using the molecular correlate of \( I_f \), recruits the pacemaker current endogenous to the heart. The unique voltage dependence of the pacemaker channel means that additional current will flow during diastole and not during the action potential. We anticipated that impulses might be initiated in atrium, given its low \( I_{K1} \) and relatively positive activation of native pacemaker current.\(^{12,13} \) Similarly, we expect success on administration of this construct in Purkinje fibers, inasmuch as these, too, have low \( I_{K1} \) and relatively positive native \( I_f \).\(^{6} \) In many ways the proximal bundle-branch system would be an optimal site for administering pacemaker constructs because it provides organized propagation to the ventricles. This possibility is presently being tested.

There are still many obstacles to overcome before such pacemaker constructs become feasible for clinical testing. Our current practice is to induce transient expression, the duration of which is only as long as virus and resulting protein construct survive in the host. Needed to ensure long-term function of such constructs are identification and utilization of an appropriate delivery system in which the construct is effectively immortalized. Although other candidates for these functions are currently available, uncertainties remain with regard to safety (eg, retrovirus) and permanence of expression (eg, adeno-associated virus). Regulating the level of expression to achieve optimal pacemaker rate is also critical.

Autonomic responsiveness of this pacemaker is another issue. We have demonstrated that HCN2 overexpressed in cultured myocytes responds to alterations in intracellular cAMP\(^{3} \) as well as to the \( \beta \)-adrenergic and muscarinic agonists that physiologically regulate cellular cAMP (data not shown). This suggests that HCN-based biological pacemakers would be subject to modulation by autonomic neurohumors. Moreover, as stated with regard to Figure 1, the LA pacemaker site was suppressed by vagal stimulation, which suggests this limb of autonomic responsiveness is intact.

In summary, we have developed a biological pacemaker using an HCN family isoform that is responsible for pacemaker current in mammalian heart. Although significant issues require solution before testing such constructs in human heart, there are now enough data to suggest that application of such a pacemaker can be effectively explored in animal models. Additional questions to be answered relate to ideal sites for implantation (atrium, Purkinje system), the extent to which diseased sinus nodes can be returned to normal function, and the ideal constructs to be used. Among the candidates are not only HCN2, but also the accessory subunit, MiRP1, which coexpresses in cell membranes with HCN2 and increases pacemaker current.\(^{6} \) Also to be considered are various mutations to HCN channels, whose properties of activation might render them more favorable to initiating impulses in the physiological milieu.

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