Gene Transfer of a Synthetic Pacemaker Channel Into the Heart

A Novel Strategy for Biological Pacing

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Background—One key element of natural pacemakers is the pacemaker current encoded by the hyperpolarization-activated nucleotide-gated channel (HCN) gene family. Although HCN gene transfer has been used to engineer biological pacemakers, this strategy may be confounded by unpredictable consequences of heteromultimerization with endogenous HCN family members and limited flexibility with regard to frequency tuning of the engineered pacemaker.

Methods and Results—To circumvent these limitations, we converted a depolarization-activated potassium-selective channel, Kv1.4, into a hyperpolarization-activated nonselective channel by site-directed mutagenesis (R447N, L448A, and R453I in S4 and G528S in the pore). Gene transfer into ventricular myocardium demonstrated the ability of this construct to induce pacemaker activity with spontaneous action potential oscillations in adult ventricular myocytes and idioventricular rhythms by in vivo electrocardiography.

Conclusions—Given the sparse expression of Kv1 family channels in the human ventricle, gene transfer of a synthetic pacemaker channel based on the Kv1 family has novel therapeutic potential as a biological alternative to electronic pacemakers. (Circulation. 2006;114:1682-1686.)

Key Words: gene therapy • ion channels • pacing

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In the sinoatrial node, pacemaker activity is generated by a balance of depolarizing and repolarizing currents whose gating and permeation properties, in ensemble, create a stable oscillator.1 Hyperpolarization-activated nucleotide-gated channel (HCN) family genes figure prominently in physiological automaticity, and transfer of such genes into quiescent heart tissue has been explored as 1 way of creating a biopacemaker.2–4 However, use of HCN genes may be confounded by unpredictable consequences of heteromultimerization with multiple endogenous HCN family members in the target cell.5,6 Because HCN is expressed in ventricular myocytes and may contribute to arrhythmogenesis,7,8 HCN gene transfer in vivo may have unpredictable consequences. Moreover, the use of wild-type channels offers little flexibility with regard to frequency tuning of the engineered pacemaker. To avoid these limitations with HCN gene transfer in biopacemakers, we created a synthetic pacemaker channel (SPC). By targeted mutagenesis involving <2% of the sequence, we converted the human Kv1.4 depolarization-activated potassium-selective channel into a hyperpolarization-activated nonselective channel suitable for biopacing applications.

Methods

Plasmid Construction, Adenovirus Preparation, and Mutation

Human Kv1.4 cDNA was subcloned from XL-4 (Origene Technologies, Inc, Rockville, Md) to pTracerCMV2 (Invitrogen, Carlsbad, Calif) by EcoRI and NotI sites. The bicistronic adenovirus shuttle vector pAdCIG was used for generation of adeno/SPC-IRES green fluorescent protein (GFP). Adenovirus was produced as previously described.9 Oligonucleotide mutagenesis was performed with a site-direct mutagenesis kit (Stratagene, La Jolla, Calif).

Transient Transfections

Twenty-four hours before transfection, HEK293 cells (ATCC, American Type Culture Collection, Manassas, Va) were seeded at a density of 2.0×10^5 per 35 mm. Cells were transfected with 2 μg per well plasmid DNA with Lipofectamine 2000 (Invitrogen. After 4 hours, transfection media were replaced with normal growth media.

Electrophysiology

Experiments were performed with the use of the whole-cell patch-clamp technique10 at 37°C with an Axopatch 200B amplifier (Axon Instruments Inc, Foster City, Calif) while sampling at 10 kHz for voltage-clamp or 2 kHz for current-clamp recordings filtered at 2 kHz. Pipettes had tip resistances of 2 to 4 mol/LΩ when filled with the internal recording solution. Because we had demonstrated that

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adenovirus infection itself did not modify the electrophysiology of guinea pig myocytes,11 control patch-clamp experiments was performed on uninfected (nongreen) left ventricular myocytes isolated from SPC adenovirus (AdSPC)–injected guinea pig.

Cells were superfused with a physiological saline (Tyrode’s) solution containing 135 mmol/L NaCl, 5 mmol/L KCl, 1.8 mmol/L CaCl2, 10 mmol/L glucose, 1 mmol/L MgCl2, and 10 mmol/L HEPES; pH was adjusted to 7.4 with NaOH. The pipette solution was composed of 130 mmol/L K-glutamate, 10 mmol/L KCl, 5 mmol/L Na-HEPES, 2 mmol/L ethyleneglycoltetraacetic acid, 5 mmol/L Mg-adenosine triphosphate, and 1 mmol/L MgCl2; pH was adjusted to 7.3 with KOH. Action potential (AP) oscillations were initiated by brief depolarizing current pulses (2 ms, 300 to 700 pA, 110% threshold) at 0.33 Hz at 32°C. When we measured SPC current in adult myocytes, 5 μmol/L BaCl2 was added in bath solution. Data are mean ± SEM.

Animal Procedure and Myocyte Isolation
Adenoviruses were injected into the left ventricular free wall of guinea pigs. Adult female guinea pigs (weight: 250 to 300 g; Hilltop Lab Animals, Inc, Scottsdale, Pa) were anesthetized with 3% to 5% as judged by epifluorescence imaging, was 3% to 5% as judged by visual assessments when cells were dispersed into the electrophysiology recording chamber. The work presented was performed in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and was performed in accordance with the guidelines of the Animal Care and Use Committee of Johns Hopkins University.

Electrocardiograms
Surface ECGs (MP100; BIOPAC Systems, Inc, Goleta Calif) were recorded 72 hours after adenoviral injection as previously described.12 Guinea pigs were lightly sedated with isoflurane, and needle electrodes were placed under the skin. Electrode positions were optimized to obtain maximal-amplitude recordings. ECGs were simultaneously recorded from standard limb leads I, II, and III. To detect ventricular beats effectively, we used methacholine (0.1 to 0.5 mg/g; Sigma Chemical Co, St. Louis, Mo) by intraperitoneal injection to induce bradycardia. We confirmed the origin of ventricular beats by mapping the left ventricular free wall with a hand-held electrode.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results
Creation of SPC
In the Kv1.4 backbone, we introduced 3 point mutations (R447N, L448A, and R453I) in the S4 segment and a single mutation (G528S) in the pore (Figure 1).14,15 When expressed in HEK293 cells, wild-type human Kv1.4 showed the expected depolarization-activated outward current (Figure 2A). The S4 triple mutant changed the gating to hyperpolarization-activated from depolarization-activated (Figure 2B, panel a; current record in high-potassium external solution) but did not alter the K+-selective reversal potential. As shown in Figure 2B, panel b, the reversal potentials of this mutant in normal Tyrode’s or high potassium external solution were −79.1 ± 2.0 or 0.8 ± 0.5 mV, respectively (n=5). To create a repolarizing current at negative potentials, we further mutated the pore (G528S) so as to render the channel nonsel ective for Na+ versus K+. The pore mutant alone expressed depolarization-activated outward current with little inward current (Figure 2C). Combining the S4 and pore mutations resulted in SPC that showed hyperpolarization-activated inward current under physiological conditions (Figure 2D). The mean current density of SPC at −130 mV was −30.3 ± 4.4 pA/pF (n=10). Tail current analysis revealed a reversal potential of −10.5 ± 1.7 mV (n=5) and no significant deactivation in a physiological voltage range (Figure 2E). We detected little depolarization-activated current in SPC by another protocol (data not shown). With the use of normal Tyrode’s external solutions, the sodium-to-potassium permeability ratio (PNa/PK) was calculated to be 0.66 by the Goldman-Hodgkin-Katz formula. CsCl (2 mmol/L) did not affect SPC current (data not shown), whereas it completely blocked currents encoded by the HCN gene family.16

No Heteromultimerization of SPC With HCN Gene Family
Wild-type Kv1.4 has been reported previously not to multimerize with the HCN gene family.17 Before in vivo use of SPC, we verified that SPC was unable to multimerize with HCN1 by cotransfection into human embryonic kidney cells and by analyzing reversal potentials. Wild-type HCN1 (Figure 3A, panel a, left), when expressed alone, had a reversal potential of −36.1 ± 4.0 mV, whereas HCN cotransfected with SPC exhibited a reversal potential of −22.0 ± 8.0 mV (n=5 for each; tail currents not shown). Superfusion with 2 mmol/L CsCl to block HCN1 homomultimers left behind a current that reversed at −11.1 ± 2.3 mV, which is indistinguishable from the reversal potential of SPC alone (Figure 3A, panel c, right). The clean pharmacological separation suggests the absence of any functional SPC-HCN heteromultimers. We also excluded the possibility that SPC expression might affect native sodium, potassium, or calcium currents in adult guinea pig myocytes (data not shown).

Pacemaker Abilities of SPC In Vivo
Next, to test its pacemaker ability in the adult ventricle, we made bicistronic (GFP-tagged) AdSPC and injected it into guinea pig heart. Seventy-two hours after virus injection,
isolated ventricular myocytes transduced with AdSPC were examined by whole-cell voltage clamp. There was little measurable pacemaker current in control cells from injected animals (data not shown). In contrast, we detected hyperpolarization-activated inward current in AdSPC-transduced myocytes (Figure 3B, panel a). Mean current densities at −80 or −160 mV equalled −7.2±1.3 or −59.7±5.5 pA/pF, respectively (n=5 each; Figure 3B, panel b). We also examined APs in control (n=13) and SPC-transduced cells (n=14). Control cells never exhibited spontaneous AP oscillations, whereas half of SPC-transduced cells (7 of 14) showed spontaneous AP oscillations. In the experiment

Figure 2. A, Current records of human wild-type Kv1.4. B, panel a, Currents of S4 triple mutations in high-potassium external solution (140 mmol/L); panel b, I-V relationship of instantaneous currents at various test potentials (20 to −140 mV) measured 4 to 8 ms after a 1.7-ms prepulse to −120 mV. HiK indicates high-potassium external solution; NT, normal Tyrode’s external solution. I-V relationship was fitted by linear regression (R2=0.985 for NT, R2=0.979 for HiK). C, Currents through the pore mutant. Wild-type Kv1.4 showed typical transient outward current on depolarization. In pore mutant, current magnitude was reduced compared with wild-type, and the reversal potential was changed to −10 mV from −80 mV. D, Currents through a channel carrying S4 triple and pore mutations (SPC) and I-V relationship from mean current density. By combining S4 and pore mutations, we created SPC showing hyperpolarization-activated measurable inward current in physiological voltage range. E, Tail currents of SPC and I-V relationship of instantaneous currents at various test potential (20 to −100 mV) between 4 and 8 ms after 1.7-ms prepulse to −120 mV. I-V relationship was fitted by linear regression (R2=0.981). Reversal potential in normal Tyrode’s external solution was −10.5±1.7 mV. There was little significant deactivation in a physiological voltage range. We recorded all currents in normal Tyrode’s as an external solution. For S4 triple mutant, we also used high-potassium external solution to highlight hyperpolarized-activated inward current.

Figure 3. A, Currents and I-V relationship of instantaneous currents at various test potentials (20 to −140 mV) measured 4 to 8 ms after 1.7 ms prepulse to −120 mV. A, panel a, Wild-type mouse HCN1. Reversal potential was −36.1±1.4 mV. I-V relationship was fitted by second-order polynomial function. A, panel b, Cotransfection of wild-type mHCN1 and SPC. I-V relationship was fitted by second-order polynomial function. A, panel c, Cotransfection after addition of 2 mmol/L CsCl in the external solution of A, panel b. I-V relationship was fitted by linear regression (R2=0.998). B, panel a, Currents in AdSPC-transduced myocyte with the use of normal Tyrode’s without potassium in the external solution in order to suppress IK1. We did not use barium to prevent IK1 because barium affected SPC current (data not shown). B, panel b, I-V relationship from mean current density of B, panel a. In this condition, mean current density was −7.2±1.3 pA/pF at −80 mV. C, Spontaneous AP oscillation after triggered AP by brief depolarizing current pulses in AdSPC-transduced myocyte.
shown here, we could detect fast spontaneous AP oscillations (mean rate >200 bpm; Figure 3C), with maximal diastolic potential and phase-4 slope of 53.6±2.5 mV and 10.4 mV/s, respectively. There was no significant difference in evoked AP durations (306.2±12.5 ms in control versus 303.2±10.9 ms in AdSPC-transduced cells). Given these results, we concluded that SPC can induce pacemaker activity in guinea pig myocytes.

When the mechanism of spontaneous AP oscillation is considered, the combination of a positive shift of resting membrane potential and generation of hyperpolarization-activated inward current is key. As membrane potential shifts positively, membrane resistance becomes lower, such that even small currents can produce relatively large changes of membrane potential. Neonatal myocytes exhibit spontaneous AP oscillations partially because their resting membrane potential is depolarized relative to that of adult myocytes, in addition to native $I_f$. In our case, a small current could be produced by SPC at the level of maximal diastolic potential (from −55 to −40 mV). Furthermore, transduction of SPC whose reversal potential is −10 mV shifted the membrane potential positively. Taken together, SPC transduction resulted in a positive shift of membrane potential in adult myocytes, in which even small hyperpolarization-activated inward current produced by SPC could contribute to spontaneous AP oscillation.

To confirm the ability of SPC to induce pacemaker activity in vivo, ECGs were performed 72 hours after AdSPC injection. During ECG recording, methacholine (0.1 to 0.5 mg/g) was administered by intraperitoneal injection to induce bradycardia. Control animals (GFP adenovirus; n=6) showed no ectopic ventricular beats, whereas frequent monomorphic idioventricular beats could be detected in animals injected with AdSPC (n=6). In representative experiments (Figure 4), ECG with pace mapping demonstrated idioventricular rhythms (150 bpm) originating from the injection site (left ventricular free wall). These results demonstrated directly that SPC worked as a pacemaker in vivo.

**Discussion**

The number of SPC-transduced cells that are minimally necessary for pacemaker activity is an important and as-yet-unanswered issue. SPC was delivered by focal intramuscular injection to the apex of the heart. The 2% to 3% of transduction rate thus reflects the very limited site of virus injection, thereby creating a concentrated number of transduced cells in a small area (yielding a small percentage of transduced cells among all ventricular cells). Because induced ventricular pacemakers do not enjoy the impedance mismatch of the sinoatrial node, it is likely that more ventricular cells will have to be “pacemakers” for the biopacemaker to function in vivo (relative to the number of cells in the sinoatrial node).

**Flexibility for Frequency Tuning of SPC**

Unlike previous studies with adenoviral HCN2 delivered into other regions of the heart,2,3 we induced biopacemaker activity with SPC in ventricular myocardium. An alternative approach has been to use mesenchymal stem cells as a platform for gene delivery to the ventricle.4 Such cells do not fully differentiate into heart cells (although they can differentiate into bone, cartilage, or adipose tissue18), and their persistence over time has not been demonstrated. Direct gene transfer of SPC avoids many of these potential complications and uncertainties (while admittedly introducing others). Another potential advantage of SPC is its flexibility for frequency tuning of synthetic pacemaker strategy. We investigated 3 sets of S4 mutations and 5 different pore mutations, yielding a total of possible 15 combinations of S4 and pore mutations. Some of these other mutants also expressed hyperpolarization-activated inward current in physiological conditions. For example, combining the S4 triple mutation...
with another pore mutation (V525S, VGYG→SGYG) displayed a current density of −6.1 pA/pF at −100 mV with a reversal potential of −25 mV in human embryonic kidney cells (Figure I in the online-only Data Supplement). When expressed in vivo, this V525S pore mutant combined with the S4 mutations also showed slow idioventricular rhythms (55 bpm) for short periods (Figure II in the online-only Data Supplement). These results indicate that specific mutations could favor specific heart rates that can be achieved in vivo by combining the S4 mutations with different pore mutants. Thus, by combining various S4 mutations with pore mutations, we can prepare a broad range of candidates for synthetic pacemakers and choose the one best suited to accomplish a therapeutic goal, namely, pacing at any given desired basal heart rate.

In summary, by selective mutagenesis of S4 and the pore in the human Kv1.4 channel, we succeeded in creating a novel pacemaker channel. This channel showed hyperpolarization-activated inward currents with steady activation under physiological conditions. Gene transfer of SPC induced pacemaker activity in guinea pig adult ventricular myocardium and produced idioventricular rhythms on ECG. Given the sparse expression of Kv1 family channels in the human ventricle and the capability of tuning the frequency of oscillation to any given desired rate range, SPCs based on the Kv1 family have the potential to be novel therapeutic tools for the creation of biopacemakers.

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Disclosures
Excigen, Inc has licensed intellectual property related to biological pacemakers from Johns Hopkins University. Dr Marbán is a founder of, stockholder of, and consultant to Excigen. No research funding was provided by Excigen. The other authors report no conflicts.

References

CLINICAL PERSPECTIVE
Electronic pacemakers are effective but have limitations, including the risks of hardware implantation, limited battery life, and insufficient rate response to physiological stimuli. Engineered biological pacemakers promise to overcome these limitations. With the use of simple gene delivery methods, heart cells that are normally not pacemakers can be converted into automatically firing cells. This form of gene therapy is focal and reversible by conventional electrophysiological approaches. In the present study, we show that a pacemaker channel gene can be engineered from scratch to create a “synthetic pacemaker channel.” The use of this man-made gene enables the selective modulation of pacemaker properties, such as tailoring the rate to meet an individual patient’s needs. Important issues, including duration of transgene expression and long-term safety of the gene therapy approach, need to be addressed before clinical studies can be initiated.
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