Ca\(^{2+}\)-Stimulated Adenylyl Cyclase AC1 Generates Efficient Biological Pacing as Single Gene Therapy and in Combination With HCN2

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**Background**—Biological pacing performed solely via HCN2 gene transfer in vivo results in relatively slow idioventricular rates and only moderate autonomic responsiveness. We induced biological pacing using the Ca\(^{2+}\)-stimulated adenylyl cyclase AC1 gene expressed alone or in combination with HCN2 and compared outcomes with those with single-gene HCN2 transfer.

**Methods and Results**—We implanted adenoviral HCN2, AC1, or HCN2/AC1 constructs into the left bundle branches of atrioventricular-blocked dogs. During steady-state gene expression (days 5–7), differences between AC1, HCN2/AC1, and HCN2 alone were evident in basal beating rate, escape time, and dependence on electronic backup pacing. In HCN2, AC1, and HCN2/AC1, these parameters were as follows: basal beating rate: 50±1.5, 60±5.0, and 129±28.9 bpm (P<0.05 for HCN2/AC1 versus HCN2 or AC1 alone), respectively; escape time: 2.4±0.2, 1.3±0.2, and 1.1±0.4 seconds (P<0.05 for AC1 and HCN2/AC1 versus HCN2); and percent electronic beats: 34±8%, 2±1%, and 6±2% (P<0.05 for AC1 and HCN2/AC1 versus HCN2). Instantaneous (SD1) and long-term (SD2) heart rate variability and circadian rhythm analyzed via 24-hour Holter recordings showed a shift toward greater sensitivity to parasympathetic modulation in animals injected with AC1 and a high degree of sympathetic modulation in animals injected with HCN2/AC1.

**Conclusion**—AC1 or HCN2/AC1 overexpression in left bundle branches provides highly efficient biological pacing and greater sensitivity to autonomic modulation than HCN2 alone. (Circulation. 2012;126:528-536.)

**Key Words:** gene therapy ■ heart rate ■ pacemaker, artificial

**Clinical Perspective on p 536**

Gene therapy–based strategies reported include engaging the β-adrenergic signaling cascade via overexpression of the β₂-adrenergic receptor\(^{10,11}\) or its downstream target AC6,\(^{12}\) dominant-negative knockdown of inward rectifier channels to eliminate the I\(_{K1}\) contribution to resting membrane potential,\(^{13}\) or overexpressing ion channels to generate inward current such as I\(_{f}\) encoded by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels\(^{14–18}\) or mutated potassium channels.\(^{19}\) Each strategy has inherent advantages and shortcomings, although the HCN-based approach appears currently favored for several reasons. First, HCN channels generate de novo pacemaker function in various tissues and large-animal models, whereas β₂-adrenergic receptor or AC6 generates pacemaker activity only in response to catecholamines.\(^{10–12}\) Second, HCN channels are activated on hyperpolarization and remain open during diastole, thereby avoiding the prolongation of repolarization that complicates the dominant-negative I\(_{K1}\) strategy.\(^{20}\) Third, HCN channels respond to autonomic modulation via the cAMP binding

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**Arrhythmia/Electrophysiology**
domain located in the carboxy terminus. This permits direct modulation of biological pacemaker function by cholinergic and adrenergic stimuli, a property not incorporated into potassium channel–based strategies.

Although HCN2-based biological pacemakers respond to catecholamine administration, physical activity, and emotional arousal, their basal rates are sufficiently low that electronic backup pacing of 30% to 40% of beats is required. Introducing HCN mutants has provided some improvement in the biological pacemaker profile. For example, the HCN2 mutant E324A showed improved sensitivity to catecholamine stimulation compared with wild-type HCN2, and the HCN1 deletion within the S3–S4 linker (235–7EYV) induced spontaneous activity in cultured guinea pig myocytes, whereas wild-type HCN1-overexpressing myocytes remained silent.

Finally, an HCN212 chimera incorporating the N and C termini of HCN2 and the transmembrane region of HCN1 increased beating rates in vivo but also induced ventricular tachycardias exceeding 200 bpm. Furthermore, considerable dependence on electronic backup pacing persisted with these constructs, with pauses >2 seconds in response to overdrive pacing. Hence, optimization of gene-based pacemakers is desirable. Although “optimization” has not been specifically defined in the literature, we have suggested that an optimally firing biological pacemaker implanted in an adult human likely would have basal rates in the 60- to 90-bpm range and peak catecholamine- or exercise-stimulated rates of ~130 to 160 bpm.

One approach to optimization might be offered by the Ca2+-stimulated adenylyl cyclase gene AC1, which is abundant in the sinoatrial node and shows enhanced activity in response to Ca2+. The latter property likely contributes to the elevated baseline cAMP levels of sinoatrial node cells, which importantly affect not only I1 but the entire spectrum of pacemaker mechanisms in sinoatrial node cells. In a study of the mechanism of Ca2+-dependent β-adrenergic modulation of HCN2, AC1 overexpression in neonatal myocytes increased baseline cAMP levels, positively shifted the activation, V1/2 of overexpressed HCN2, and increased spontaneous beating. As expected, comparable overexpression of the working myocardial isoform AC6 did not modify these parameters.

The outcomes of these in vitro studies led us to hypothesize that AC1 overexpression in the left bundle branch will generate highly efficient biological pacemaker activity, both alone and in combination with overexpressed HCN2. We tested this in a previously reported canine model of complete heart block.

**Methods**

Experiments were performed under protocols approved by the Columbia University Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85–23, revised 1996).

**Adenoviral Constructs**

Adenoviral constructs of green fluorescent protein, mouse HCN2, and FLAG-tagged AC1 (a generous gift of D. Ross Feldman, University of West Ontario, London, ON, Canada), all driven by the cytomegalovirus promoter, were prepared as described previously. Consistency with earlier studies, we prepared 3×10^10 fluorescent focus-forming units of 1 adenovirus and combined this suspension with an equal amount of another adenovirus in a total volume of 700 μL to obtain the following groups: an HCN2 group (n=12) made up of 7 previously reported HCN2/green fluorescent protein–treated animals and an HCN2/green fluorescent protein–treated group made up of 5 animals. The other groups received AC1/green fluorescent protein (designated AC1; n=5) or HCN2/AC1 (n=7).

**Intact Canine Studies**

Adult mongrel dogs (Chestnut Ridge Kennels, Chippensig, PA) weighing 22 to 25 kg were anesthetized with propofol (6 mg/kg IV) and inhalational isoflurane (1.5%–2.5%). An electronic pacemaker (Guidant, Discovery II, Flexextend lead, Guidant Corp, Indianapolis, IN) was implanted and set at VVI 35 bpm. Using a steerable catheter, we injected adenoviral vectors into 3 left bundle-branch sites as previously described. We paced each injection site transiently via the catheter electrode to facilitate pace mapping of the origins of idioventricular rhythms electrocardiographically during follow-up. Complete atrioventricular block was induced via radiofrequency ablation. ECGs, 24-hour Holter monitoring, pacemaker log record review, and overdrive pacing at either 80 bpm or 5% faster than intrinsic rates were performed daily. ECG intervals were calculated from baseline ECGs recorded on day 5 to 7. To correct the QT intervals, we used both the Bazett formula and the 1-parameter logarithmic formula of Matsunaga et al: Q(Tc) = log600×QT/log RR. For each dog, the percent of electronically induced beats was calculated daily. Biological pacemaker function on the first day after construct injection is usually minimal and is typically confounded by ectopic activity resulting from the injection trauma; therefore, we excluded data from day 1 from our analysis.

In a subset of the HCN2/AC1-injected animals (n=4) that exhibited rapid idioventricular rhythms during resting ECG recordings, we turned off the electronic pacemaker, infused ivabradine (1 mg/kg diluted in 30 mL saline intravenously) over 5 minutes, and monitored the ECG continuously for 30 minutes to evaluate the effect of treatment. Continuous 24-hour monitoring was performed thereafter. Ivabradine was kindly provided by Servier Laboratories (Courbevoie, France).

We performed 24-hour monitoring using a Holter ECG (Rozinn [Scotcare], Glendale, NY). We defined basal beating rate as the rate that was recorded daily with the animal resting quietly on the examination table. We calculated maximal beating rates daily from 30-second strips of a stable rhythm at maximal rate. Pace mapping was performed on the basis of 6-lead ECG recordings obtained at the time of implantation during pacing from the injection site. During subsequent daily 6-lead ECG testing, we identified rhythms of comparable morphology and QRS axis and recorded them simultaneously with our 3-lead Holter system. In this way, we could identify and determine the characteristics of both matching and nonmatching rhythms on 6-lead ECG and on Holter (Figures I and II in the online-only Data Supplement).

We performed detailed heart rate variability (HRV) analysis on Holter recordings registered during steady-state gene expression (days 5–7; 1 day per animal). In this analysis, we calculated the percentage of matching beats, the percentage of nonmatching beats, the percentage of paced beats, and the 24-hour average beating rate of the matching beats. Furthermore, we classified all pace-mapped beats as “normal” to calculate the standard deviation of their instantaneous RR-interval variability (SD1) and the standard deviation of long-term continuous RR-interval variability (SD2). To analyze circadian modulation, we compared the rate of pace-mapped beats and dependence on electronic backup pacing during sleep (2–4 AM) versus during feeding and physical activity (8–10 AM). Investigators involved in the Holter analysis were not blinded to study group.

To evaluate β-adrenergic responsiveness at the termination of the study (day 7–8), we infused epinephrine (1.0, 1.5, and 2.0 μg·kg^-1·min^-1 IV) for 10 minutes as previously reported and recorded the rate response of the pace-mapped rhythm.

**Immunohistochemistry**

HCN2 and AC1 overexpression was validated by immunohistochemistry (Figure 1). The threshold for HCN2 detection was set above the level required to detect endogenous HCN2 so that we could detect HCN2 only in animals that had received HCN2
adenovirus. Similarly, FLAG staining was positive only in animals that received AC1 adenovirus.

Tissue blocks (left bundle branch and surrounding endocardium) were snap-frozen in liquid nitrogen, and 5-µm serial sections were cut with a cryostat (Microm HM505E) and air dried. Sections were washed in PBS, pretreated for 10 minutes with 0.2% triton X, blocked for 20 minutes with 10% goat serum, and incubated overnight at 4°C with anti-FLAG antibody (1:200; Sigma-Aldrich, St. Louis, MO) and anti-HCN2 antibody (1:200; Alomone, Jerusalem, Israel). Antibody bound to target antigen was detected by incubating sections for 2 hours with goat anti-mouse IgG labeled with Cy3 (red fluorescence for AC1) and goat anti-rabbit IgG labeled with Alexa 488 (green fluorescence for HCN2). Images were recorded with a Nikon E800 fluorescence microscope.

Statistical Analysis

Data are presented as mean±SEM. Statistical significance was examined by *t* test or by ANOVA followed by the Bonferroni post hoc test. In the following data sets, we did not detect a normal gaussian distribution: percent matching rhythm, percent nonmatching rhythm, percent paced (Holter), morning rate of matching, percent paced (morning), and maximal duration of electronic pacing. In these cases, we examined statistical significance by Wilcoxon matched-pairs signed-rank test and Kruskal-Wallis 1-way ANOVA followed by the Dunn multiple comparison test. *P*<0.05 was considered significant.

The authors had full access to and take full responsibility for the integrity of the data.

Results

Basal Function

Basal beating rates did not differ significantly in the AC1 and HCN2 groups, but that for the HCN2/AC1 group was more rapid than desirable and significantly faster than that for the HCN2 group (Figure 2A). Analysis of the ECG intervals indicated significantly shorter cycle lengths in HCN2/AC1-injected animals compared with those injected with HCN2 alone. Furthermore, in HCN2/AC1-injected animals with cycle lengths <600 milliseconds, QT and QTc-log were significantly shortened (*P*<0.05 versus HCN2) with the Matsunaga et al. formula, whereas QTc by the Bazett formula was significantly prolonged (*P*<0.05 versus HCN2; Figure 2B). The reemergence of pacemaker activity after overdrive pacing was significantly more rapid in the AC1 and HCN2/AC1 groups than in the group with HCN2 alone (Figure 3). The differences in basal rate and escape time had a sizeable impact on the dependence on electronic backup pacing, which was significantly reduced in the AC1 and HCN2/AC1 groups compared with the HCN2 alone group (Figure 4A).

Maximal Beating Rates and Response to Ivabradine

Using-24 hour Holter recordings, we investigated the maximal rates achieved in the 3 groups. Rates were significantly more rapid in HCN2/AC1-injected animals than in the other groups throughout the study (Figure 4B). The maximal rates attained in the HCN2/AC1 group, sometimes exceeding 250 bpm, we treated four of these animals with the *I* 

A 24-Hour Analysis of Pace-Mapped Rhythms and Autonomic Modulation

The percentage of matching pace-mapped beats (Figures I and II in the online-only Data Supplement) was significantly higher in the AC1 and HCN2/AC1 groups (>95% and >75%, respectively) compared with HCN2 alone (~40%; *P*<0.05 versus the other groups; Figure 6A, left). This increase was accompanied by a significant reduction in the percentage of electronically paced beats in AC1- versus HCN2-injected animals (*P*<0.05; Figure 6A, right). The 24-hour average rate of pace-mapped rhythms is summarized in Figure 6B, showing a significant increase in rate in the HCN2/AC1 group compared with the other groups (*P*<0.05), which correlated with the day 5 to 7 averages of basal and maximal beating rates reported above. The greater dependence on electronic backup pacing in the HCN2/AC1 versus AC1 groups derived from the finding that HCN2/AC1-induced rhythms sometimes overdrive-suppressed themselves, a process that did not occur in animals injected with AC1 (Sample tracings of overdrive suppression are shown in Figure III in the online-only Data Supplement). The maximal durations of episodes of electronic pacing are summarized in Figure 6C. The maximal duration of electronic pacing was significantly shorter in AC1-injected animals than in HCN2-injected animals.

To test whether the changes in beating rate and dependence on backup electronic pacing occurred in accordance with what would be expected on the basis of a normal circadian rhythm, we compared these parameters during 2 hours of sleep (2–4 AM) and 2 hours of feeding and activity (8–10 AM). Slower matching rhythms and higher percentages of electronic backup pacing, which was significantly reduced in the AC1 and HCN2/AC1 groups compared with the HCN2 alone group (Figure 4A).

![Immunohistochemical staining for HCN2 and AC1. Positive HCN2 staining (green) and AC1 staining (red; FLAG antibody) were detected in the injected left bundle branches from animals that received the corresponding adenovirus. Nuclei were stained blue with DAPI. Bars represent 50 µm.](http://circ.ahajournals.org/content/110/24/600/F1.large.jpg)
ically paced beats occurred in the HCN2-injected animals, primarily during the night (P<0.05 versus AC1 and HCN2/AC1; Figure 6D). In addition, beating rates increased in all groups from the resting state during the night to the active state in the morning (P<0.05). In the HCN2 and HCN2/AC1 groups, the percentage of electronically paced beats was also lower in the morning than in the night (P<0.05).

Poincaré plots of pace-mapped rhythms showed differences in HRV among the 3 groups (Figure 7A). Significant modulation of heart rates occurred in HCN2-injected animals (Figure 7A, left). AC1-injected animals showed more pronounced modulation that occurred over a wider range of heart rates than HCN2-injected animals (Figure 7A, center). HCN2/AC1-injected animals showed pronounced modulation that occurred primarily during intermediate to very rapid heart rates (Figure 7A, right).

Quantitative analysis of SD1, SD2, and SD1/SD2 revealed that the level of sympathetic modulation expressed by long-term variation of heart rates (SD2) was comparable between HCN2- and AC1-injected animals and was maximal in animals injected with HCN2/AC1 (P<0.05 versus HCN2 and AC1; Figure 7B, middle). Parasympathetic modulation, as expressed by short-term variation of heart rates (SD1), was comparable between HCN2 and HCN2/AC1 and was maximal in animals injected with AC1 (P<0.05 versus HCN2; Figure 7B, left). Furthermore, the significantly increased ratio of SD1/SD2 in the AC1-injected animals supports the notion of greater sensitivity to parasympathetic modulation in this group than in the other groups (P<0.05 versus HCN2 and HCN2/AC1; Figure 7B, right).

On the final day of the study, we tested the response to epinephrine. Figure 8A illustrates the individual rate responses during 10 minutes of infusion with 1.0 g/kg min 1 epinephrine. After the 10-minute exposure to epinephrine, all groups exhibited a significant increase in rate compared with

**Figure 2.** Basal beating rates in HCN2/AC1-injected animals are faster than in the other groups. A, Summary data pooled for days 2 to 4 and 5 to 7. B, Summary data on ECG intervals pooled for days 5 to 7. HCN2, n=12; AC1, n=5; HCN2/AC1, n=7. CL indicates cycle length. +P<0.05 vs HCN2 alone.

**Figure 3.** Reemergence of pacemaker activity after overdrive suppression in AC1- and HCN2/AC1-injected animals is faster than with HCN2. A through C, Representative examples of overdrive suppression experiments in HCN2-, AC1-, and HCN2/AC1-injected animals, respectively. D, Summary data pooled for days 2 to 4 and 5 to 7. HCN2, n=12; AC1, n=5; HCN2/AC1, n=7. +P<0.05 vs HCN2 alone.
and beating rates in the HCN2/AC1 group were significantly faster than in the HCN2 group (P < 0.05; Figure 8B). Animals that responded with an increase in rate 50% received higher doses of epinephrine up to 2.0 g/kg min in the HCN2 group, this did not result in a more extensive effect; in the other groups, the numbers were too low to draw further conclusions in relation to the higher doses.

Discussion

This study demonstrates significant changes in biological pacemaker function achieved with AC1 or HCN2/AC1 gene transfer. It is noteworthy that biologically induced rhythms at physiological beating rates based on overexpression of AC1 alone were generated for >95% of beats. Additionally, AC1 gene transfer resulted in robust sensitivity to sympathetic and parasympathetic modulation to a greater degree than has been reported for any single gene–based pacemaker strategy. Sensitivity to sympathetic modulation was further enhanced when HCN2 and AC1 were injected together, but this combination also resulted in an excessive increase in basal beating rate.

Biological Pacemaker Function in Relation to Other Approaches

In comparing the approaches tested, we used the following criteria as optimal biological pacing outcomes: basal beating rates of 60 to 90 bpm, escape times closely matching the basal cycle length (ie, if the basal rate is 60 bpm, escape times of ≈1 second would be optimal and represent a situation in which no beat is missed), an autonomic response resulting in rate increases to 130 to 160 bpm, and low to absent dependence on electronic backup pacing. Among the 3 groups studied, overall outcomes in the AC1 group were superior to those in the HCN2 and HCN2/AC1 groups. For AC1 alone on days 5 to 7, beating rates were about 60 bpm (Figure 2), escape times were <1.5 seconds (Figure 3), and the dependence on electronic backup pacing was reduced to <2% (Figure 4A). These outcomes compare favorably with those reported previously for gene- and cell-based pacemakers. Biological pacemakers based on HCN2 or the mutant HCN2-E324A have consistently exhibited episodes of excess bradycardia requiring significant (≈35%) dependence on electronic backup pacing. A truncated HCN1 construct administered to the left atrium shifted the kinetics of activation positively and reduced dependence on electronic backup pacing to only ≈15%. This construct was used to provide atrial pacing in a porcine model of sinoatrial node dysfunction, which is a different setting than atrioventricular block and demand ventricular pacing. Finally, preliminary experiments have shown highly efficient biological pacemaker function based on combined gene transfer of HCN2 and the skeletal muscle sodium channel (SkM1). Potential advantages of the AC1-based approach may include the smaller gene size of AC1 compared with SkM1, facilitating packaging into smaller
delivery vehicles such as adeno-associated virus, and the lesser complexity of a single gene–based approach.

Rates generated in the HCN2/AC1 group were excessive, but the outcomes provide insight at several levels. First, they show that when coexpressed, AC1 and HCN2 can function synergistically to increase basal beating rates (Figure 2) and sensitivity to sympathetic modulation (Figures 7 and 8). Second, although rhythms with the AC1/HCN2 combination were excessively rapid (Figure 4B), pacemaker activity remained stable, as indicated by the infrequent need for electronic backup pacing (Figure 4A), high number of beats that pace mapped to the injection site (Figure 6A), and intact autonomic modulation (Figure 6–8). Third, the results support the earlier finding that if excessive rate accelerations occur as a result of HCN-associated pacing, then effective treatment is provided by I_{f} blockade (Figure 5).24 Finally, the outcomes in the HCN2, AC1, and HCN2/AC1 groups suggest that a biological pacemaker profile superior to the approaches using either HCN2 or AC1 may be generated if gene expression is sufficiently down-titrated in the combination strategy.

Mechanisms Underlying AC1-Based Biological Pacemaker Function

In vitro studies have confirmed some of the mechanisms contributing to AC1-induced pacemaker function.30 They demonstrated that in AC1-overexpressing neonatal myocytes, cAMP was increased in basal conditions and that the increase in cAMP affected downstream targets of cAMP. The outcome was the positive activation shift of coexpressed HCN2 channels, rendering more channels available for membrane depolarization. Additionally, in the setting of overexpressed HCN2-R/E, a mutant channel that is insensitive to modulation by cAMP, the kinetics of I_{f} was, as expected, unmodified by overexpression of AC1. Yet, neonatal myocytes that coexpressed HCN2-R/E and AC1 had significantly faster beating rates than cells that overexpressed HCN2-R/E alone, further supporting the notion that AC1 can enhance pacemaker mechanisms other than I_{f}.30 Therefore, it is likely that the pacemaker function generated by overexpressing AC1 results from direct effects of cAMP elevation on targets that are sensitive to cyclic nucleotides such as HCN channels and from indirect effects on calcium-handling proteins that may be enhanced via protein kinase A–mediated phosphorylation. Examples of phosphorylation targets that may importantly affect pacemaker function include the L-type calcium channels, phospholamban, ryanodine receptors, and K channels.29 Our finding that pacemaker activity in HCN2/AC1 in vivo was not silenced completely on administration of ivabradine was consistent with the above-mentioned preliminary data suggesting that AC1 stimulates pacemaker mechanisms other than I_{f} (Figure 5). This observation was in contrast to our previous investigation with the chimera HCN212, in which a similar ivabradine protocol completely suppressed HCN212-based and endogenous idioventricular pacemaker activity,24 whereas sinoatrial node pacemaker activity remained unaffected. In summary, in vitro and in vivo data are consistent with the enhancement of I_{f} and other likely calcium-based pacemaker mechanisms by AC1. With regard to the debate over calcium clock versus HCN roles in pacemaker func-

**Figure 6.** A, Summary data of percentage of matching beats (corresponding to pace mapping), nonmatching, and electronically paced beats. HCN2, n=12; AC1, n=5; HCN2/AC1, n=7. **B,** The 24-hour average rate of matching beats. **C,** Summary data of day/night modulation of pace-mapped beating rates (left) and dependence on electronic backup pacing (right). **D,** Summary data on the maximal duration of electronic pacing. **B** through **D,** HCN2, n=11; AC1, n=5; HCN2/AC1, n=7. *P<0.05 vs HCN2 alone; †P<0.05 vs HCN2 or AC1 alone; ‡P<0.05 for morning vs night.
tion, our work supports the likelihood that a combination of calcium-based and $I_f$-based mechanisms is responsible.

**Autonomic Modulation of HCN2-, AC1-, and HCN2/AC1-Based Pacemakers**

Direct sensitivity to autonomic modulation is a potential key advantage of biological over electronic pacemakers. With this in mind, we concluded that HCN2- and AC1-based biological pacemakers incorporate a similar degree of sensitivity to sympathetic modulation based on SD2 (Figure 7B, middle), night versus morning rhythms (Figure 6B, left), and response to catecholamine infusion (Figure 8). Furthermore, the higher SD2 in HCN2/AC1 versus HCN2 or AC1 alone (Figure 7B, middle) and increased beating rates in the morning (Figure 6B, left) or after catecholamine administration (Figure 8) indicate that the response to sympathetic modulation may be further enhanced when HCN2 and AC1 are combined.

Also of interest is the high degree of HRV in the AC1 group observed in the fan-like pattern in the Poincaré plot (Figure 7A, middle), which closely resembles the pattern of sinus rhythm and is indicative of vagosympathetic modulation. Heightened sensitivity to parasympathetic stimulation was confirmed by the significant increase in SD1 and SD1/SD2 (Figure 7B). Given the strong correlation between higher HRV values and improved cardiovascular health and the association of severe phenotypes of diabetic neuropathy, myocardial infarction, and heart failure with reduced HRV and poor prognosis, the increased HRV in the AC1 group might suggest potential benefit in the modulation of cardiovascular chronotropy. This would be consistent with the association of increased HRV with interventions that improve prognosis such as disease-altering pharmacotherapy and physical exercise.

**QT Interval Measurements**

The QT and QTc intervals (Figure 2B) were not affected by AC1 alone, whereas for the HCN2/AC1 intervention, at cycle lengths <600 milliseconds, a significant shortening of QT and QTc-log was seen with the Matsunaga et al formula, and QTc with the Bazett formula was significantly prolonged. It should be noted that the Bazett formula overestimates the actual values at short RR intervals while underestimating them at long RR intervals in humans (see Reference 32 for review) and dogs. Without entering into the remaining controversies on which QTc correction to use in which experimental setting, we note the following. First, the Matsunaga et al formula has been found to be preferable to the Bazett formula for testing QTc prolongation in beagle dogs and likely other breeds. Second, despite the first point, the QTc prolongation with the Bazett formula in the combined AC1/HCN2 biological pacemaker setting (at cycle lengths <600 milliseconds) should call for vigilance in the evaluation of the occurrence of proarrhythmia either spontaneously or induced via electrophysiological testing. Third, the occurrence of QTc prolongation with overexpressed HCN2/AC1 at low cycle lengths, but not with HCN2/AC1 at higher cycle lengths or with sole overexpression of AC1 or HCN2, points out the complexity of events that may occur when various gene therapies are administered singly versus together in settings in which their impact on a physiological process is being assessed.
and its demonstration is crucial. There is also a need for caution related to the overexpression of AC1, either alone or in combination with HCN2. In settings of myocardial infarction or heart failure, elevating cAMP is known to have arrhythmogenic consequences. Safety testing for proarrhythmia during ischemia or cardiac hypertrophy will be useful in the further exploration of these biological pacemaker strategies.

Conclusions

AC1 generates efficient biological pacemaker function either alone or when coexpressed with HCN2. In addition, the baseline function and autonomic responsiveness generated by AC1 are superior to those of HCN2 alone. Finally, our in vivo and previous in vitro studies suggest that AC1 enhances $I_f$-dependent and $I_r$-independent pacemaker mechanisms, which may explain why pacemaker activity is more robust than in the setting of HCN2 overexpression alone.

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Disclosures

None.

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**SUPPLEMENTAL MATERIAL**

Supplementary Figure 1 illustrates the process of identifying matching and non-matching rhythms in an HCN2-injected animal. All rhythms were recorded simultaneously on both 6 lead ECG and Holter before and after episodes of electronic overdrive suppression pacing. Pace-mapping in the Holter analysis was performed after first determining that a matching rhythm was/was not present on 6 lead ECG. When matching QRS complexes were present the Holter ECG was used to determine rate and rhythm for beats with those complexes as well as to identify rhythms for non-matching beats. Supplementary Figure 2 provides examples of rhythms that pace-mapped to the injection site and rhythms with distinct morphology that were non-matching. Supplementary Figure 3 shows overdrive suppression occurring in an animal injected with HNC2/AC1. Note the rapid rhythm (rate = 230 bpm) on the left which ceases abruptly and is succeeded by the first electronically paced beat after a pause of 1.76 seconds.

**Legends**

**Supplementary Figure 1.** 6 Lead ECG (upper panels) and concurrent Holter (lower panels) during matching (day 7) and non-matching (day 1) escape beats observed after overdrive suppression electronic pacing (EP) in an HCN2-injected animal. Rhythms were classified matching when the QRS morphology closely resembled the QRS morphology observed during electronic pacing from the injection site (EP during implant at day 0; right panel).

**Supplementary Figure 2.** Holter ECG tracings of matching and non-matching rhythms. Typical example tracings of three Holter leads are shown for animals injected with HCN2 (A), AC1 (B), and HCN2/AC1 (C). Left panels show tracings that pace-mapped to the injection site which were therefore designated as “matching” rhythms. Right panels show tracings of rhythms that did not originate from the injection site and were therefore designated as “non-matching” rhythms.

**Supplementary Figure 3.** Intrinsic overdrive suppression of rapid pacemaker activity. Holter tracing from an HCN2/AC1-injected animal. A rapid rhythm that overdrive-suppressed itself resulted in abrupt termination of biological pacing followed by onset of electronic pacing.
Supplementary Figure 1.
Supplementary Figure 2.

**A**

**HCN2**

Matching rhythm | Non-Matching rhythm

**B**

**AC1**

Matching rhythm | Non-Matching rhythm

**C**

**HCN2/AC1**

Matching rhythm | Non-Matching rhythm

500 msec
Supplementary Figure 3.

![Supplementary Figure 3](image.png)