

Dynamic Action Potential Clamp as a Powerful Tool in the Development of a Gene-Based Bio-Pacemaker

Arie O. Verkerk, Jan G. Zegers, Antoni C. G. van Ginneken, and Ronald Wilders, *Member, IEEE*

Abstract—The development of a genetically engineered ‘biological pacemaker’, or ‘bio-pacemaker’, is a rapidly emerging field of research. One of the approaches in this field is to turn intrinsically quiescent myocardial cells, i.e., atrial or ventricular cells, into pacemaker cells by making them express the cardiac hyperpolarization-activated ‘pacemaker current’ I_f (known in neurophysiology as I_h), which is encoded by the hyperpolarization-activated cyclic nucleotide-modulated (HCN) gene family. We carried out ‘dynamic action potential clamp’ (dAPC) experiments in which we record current from a HEK-293 cell transfected with HCN4, which is the dominant HCN isoform in the sinoatrial (SA) node. This HCN4-transfected HEK-293 cell is voltage-clamped by the action potential generated in a real-time simulation of a human atrial cell (Courtemanche-Ramirez-Nattel model). In a continuous feedback loop, this current is injected into the atrial cell, so that this cell effectively expresses an HCN4-based pacemaker current. With sufficiently high ‘expression levels’ of HCN4 current the atrial cell is turned into a pacemaker cell with an SA nodal like action potential. Lower expression levels are sufficient if the inward rectifier potassium current (I_{K1}), which is largely responsible for the stable resting potential of atrial cells, is ‘down-regulated’ by 50%, thus mimicking the gene therapy strategy to create a bio-pacemaker by down-regulation of I_{K1} and (over-)expression of I_f . Our dAPC experiments provide direct insights into the effects of introducing HCN4 current into an atrial cell, illustrating that dynamic action potential clamp can be a powerful tool in the process of developing a gene-based bio-pacemaker.

I. INTRODUCTION

WE recently developed the ‘dynamic action potential clamp’ (dAPC) technique, which was used to study the effects of ion channel mutations by effectively replacing a native ionic current of a cardiac myocyte with wild-type or mutant current expressed in cells of the HEK-293 human embryonic kidney cell line [1], [2]. Dynamic action potential clamp differs from traditional ‘dynamic clamp’ and ‘action potential clamp’ in that it combines current clamp, as used in dynamic clamp, and voltage clamp, as used in action potential clamp. Essentially, the traditional ‘action potential clamp’ is a particular refinement of the voltage clamp technique, in which a prerecorded action potential waveform is used as voltage clamp command potential instead of the

more common voltage clamp protocol with stepwise changes in command potential. This traditional action potential clamp may be used to study the behavior of ion channels expressed in cells of a cell line (by transfection with the ion channel gene cDNA), e.g., the HEK-293 cell line, that express little or no endogenous ion channels [3]. The current in response to a voltage clamp command potential with the shape of an action potential then reflects the behavior of the expressed ion channels during that action potential.

Traditionally, ‘dynamic clamp’ has been used to introduce artificial conductances into real excitable cells by injecting a real-time computed current into the current-clamped cell, thus simulating, e.g., synaptic input in an isolated neuron or the presence of an additional membrane ionic current in an isolated cardiac cell (see reviews by Goillard and Marder [4] and Wilders [5]). With ‘dynamic action potential clamp’, ion channels are again expressed in a cell line, e.g. in HEK-293 cells, and subjected to voltage clamp. However, the voltage clamp command potential is not a prerecorded action potential, but is the free-running membrane potential of a freshly isolated, patch-clamped cardiac myocyte (or cell model). The measured HEK cell current is injected into the cardiac myocyte in real time. Thus, there is continuous feedback between the action potential and the HEK cell current. The expressed ion channels are allowed to follow the natural time course of the cardiac action potential (through the voltage clamp), upon being simultaneously allowed to contribute current for the generation of this action potential as if they were incorporated into the membrane of the cardiac myocyte (through injection of the HEK cell current into the myocyte).

One approach in the field of creating a bio-pacemaker is the induction of pacemaking in intrinsically quiescent myocardial cells by modification of their expression pattern of membrane currents. This requires genetic modification at the site of interest, typically by gene transfer aimed at down-regulation of I_{K1} and/or up-regulation of I_f in atrial or ventricular myocardium (see editorial by de Bakker and Zaza [6] and primary references cited therein, and recent reviews by Marbán and Cho [7] and Rosen *et al.* [8]). In a series of dAPC experiments, we have tested whether expression of HCN current—HCN4 being the dominant HCN isoform in the sinoatrial (SA) node [9]—in a human atrial cell model [10], either per se or in combination with down-regulation of I_{K1} , induces SA nodal like pacemaker activity in this intrinsically quiescent cell type.

Manuscript received June 25, 2008.

A. O. Verkerk (e-mail: a.o.verkerk@amc.uva.nl), J. G. Zegers (e-mail: j.g.zegers@amc.uva.nl), A. C. G. van Ginneken (e-mail: a.c.vanginneken@amc.uva.nl), and R. Wilders (phone: +31-20-5665229; fax: +31-20-6976177; e-mail: r.wilders@amc.uva.nl) are with the Department of Physiology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands.

II. METHODS

A. Electrophysiology

HEK-293 cells were transfected with HCN4 cDNA using standard transfection procedures. Recordings were made in the whole-cell configuration of the patch-clamp technique at a temperature of 36°C and recording conditions were similar to those in recent studies on HCN based currents by Verkerk and coworkers [11], [12], except for the NaCl concentration in the patch pipette solution, which was raised to 10 mM. Furthermore, the delayed rectifier like endogenous current of HEK-293 cells [13], [14] was blocked by E-4031 (5 μ M). All potentials were corrected for liquid junction potential.

B. Dynamic Action Potential Clamp

Our dAPC setup was similar to that used in the studies by Berecki *et al.* [1], [2], using custom software running on a real-time Linux (RT-Linux) based platform [5]. The update rate of A/D input and D/A output values was 20 kHz. The corresponding time step of 50 μ s was used in the Euler-type integration scheme that we used to solve the differential equations of the mathematical models of a human atrial cell [10] or a rabbit SA nodal cell [15]. Our dAPC software is available for download from our institutional website (URL: <http://www.amc.nl/index.cfm?pid=4922>).

III. RESULTS

A. HCN4 Current in HEK-293 Cells

The voltage clamp experiment of Fig. 1 illustrates the presence of an I_f like inward current in HCN4-transfected HEK-293 cells. This current develops slowly (Fig. 1, bottom) upon a hyperpolarizing step to -100 mV from a holding potential of -30 mV (Fig. 1, top). The current reverses its sign upon stepping back from -100 mV to $+10$ mV, reflecting that the membrane potential of $+10$ mV is

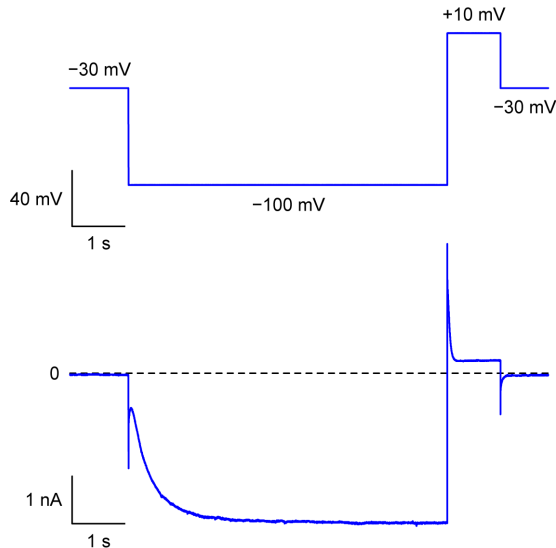


Fig. 1. Current recorded from a HCN4-transfected HEK-293 cell (bottom; zero-current level indicated by a dashed line) in response to a voltage clamp step protocol (top). Scale bars near traces.

positive to the HCN4 reversal potential. Also, there is fast deactivation of the HCN4 current at $+10$ mV. However, the current does not return to near-zero levels, but attains a steady value of 200–300 pA. This steady outward current may result from K_V channels that are not blocked by E-4031 [14], but may also reflect a fraction of HCN4 channels that remain open at positive potentials.

B. HCN4 Current in SA Nodal Pacemaker Cells

Next, we used the HCN4-transfected HEK-293 cell of Fig. 1 in combination with a comprehensive mathematical model of a rabbit SA nodal pacemaker cell [15] to test whether this HCN4 current could replace the I_f of the model.

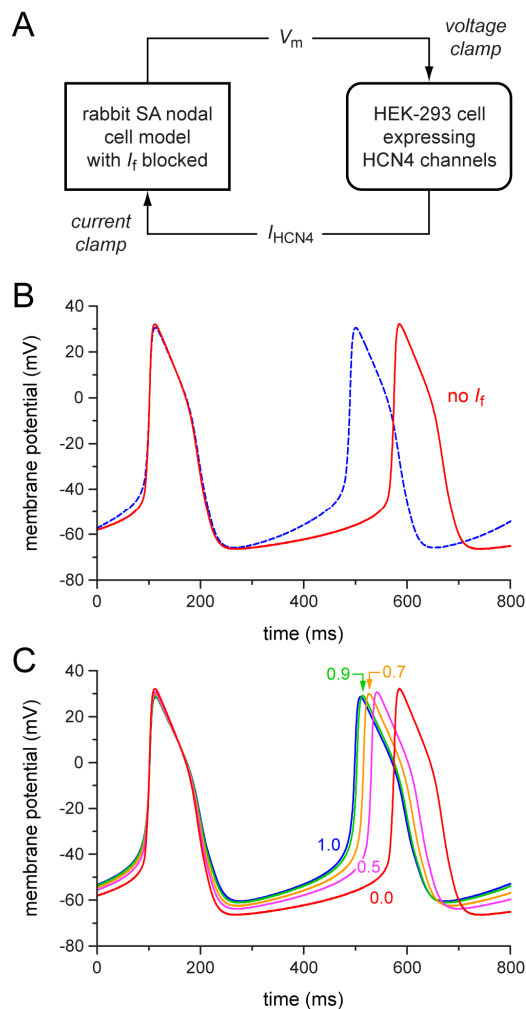


Fig. 2. Dynamic action potential clamp (dAPC) experiment with a real-time simulation of a sinoatrial (SA) nodal pacemaker cell and a HEK-293 cell expressing HCN4 channels. (A) Experimental configuration. An SA nodal pacemaker cell is simulated in real time using the Wilders *et al.* [15] model of a rabbit SA nodal myocyte. The HCN-encoded hyperpolarization-activated current I_f , also known as ‘pacemaker current’ or ‘funny current’, of the model cell is set to zero and replaced with HCN4 current recorded from the HEK-293 cell (I_{HCN4}). (B) Effect of blocking I_f on the action potential of the SA nodal model cell. (C) Effect of adding increasing amounts of HCN4 current to the SA nodal cell with its native I_f set to zero. A scaling factor of 0.0, 0.5, 0.7, 0.9, or 1.0%, as indicated by numbers near traces, was applied to the HCN4 current recorded from the HEK-293 cell.

In the model, as in other (rabbit) SA nodal cell models [16], the cycle length increases significantly upon blockade of I_f , mainly due to a decrease in the rate of diastolic depolarization (Fig. 2B). As diagrammed in Fig. 2A, we used the action potential of the model cell—with its I_f set to zero—to voltage-clamp the HEK-293 cell and fed the recorded HCN4 current back into the current-clamped model cell, thus establishing the dAPC configuration. Given the large current expressed in the HEK-293 cell (Fig. 1), we applied scaling factors of 0.0–1.0%, to this HCN4 current before adding it

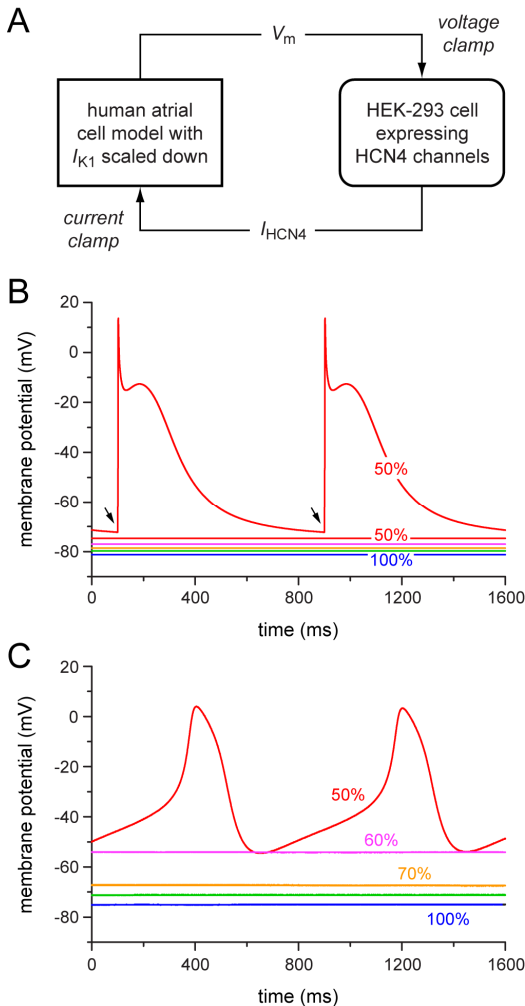


Fig. 3. Dynamic action potential clamp (dAPC) experiment with a real-time simulation of a human atrial cell and a HEK-293 cell expressing HCN4 channels. (A) Experimental configuration. A human atrial cell is simulated in real time using the Courtemanche *et al.* [10] model of a human atrial myocyte. The inward rectifier potassium current (I_{K1}) of the model cell is scaled down and HCN4 current recorded from the HEK-293 cell is added to the net membrane current of the model cell, thus mimicking the gene therapy strategy to create a bio-pacemaker by down-regulation of I_{K1} and (over-)expression of I_f . (B) Effect of down-scaling I_{K1} on the resting potential of the non-stimulated model cell. Traces obtained with 100, 80, 70, 60 or 50% of I_{K1} channels present, as indicated. Action potentials of the model cell with 50% I_{K1} obtained by stimulating the cell at a rate of 75 min⁻¹ (arrows). (C) Effect of adding HCN4 current to the non-stimulated atrial cell with 100, 80, 70, 60 or 50% of I_{K1} channels present, as indicated. A constant scaling factor of 1.0% was applied to the HCN4 current recorded from the HEK-293 cell.

to the model. With the scaling factor set to zero (Fig. 2C, red trace labeled ‘0.0’), the resulting action potential is identical to that of the model cell with its I_f set to zero (Fig. 2B, red solid line labeled ‘no I_f ’). With a scaling factor of 1.0% (Fig. 2C, blue trace labeled ‘1.0’), the cycle length shortens and becomes almost identical to that of the original model with its default I_f (Fig. 2B, blue dashed line). Intermediate shortening occurs with intermediate values for the scaling factor (Fig. 2C, traces labeled ‘0.5’, ‘0.7’ and ‘0.9’).

C. HCN4 Current in Atrial Cells

In the second type of dAPC experiment, we used the HCN4-transfected HEK-293 cell in combination with the Courtemanche-Ramirez-Nattel model of a human atrial cell [10] with its I_{K1} set to 50–100% of control (Fig. 3A). Thus, it could be tested whether the atrial cell developed SA nodal like pacemaker activity upon the inclusion of HCN4 current per se or upon the inclusion of HCN4 current in combination with down-scaling of I_{K1} . Figure 3B illustrates the effect of a reduction in I_{K1} on the resting potential of the human atrial cell model. The control value of the resting potential is -81 mV (Fig. 3B, blue trace labeled ‘100%’). It depolarizes with decreasing I_{K1} , up to -75 mV at 50% I_{K1} (Fig. 3B, horizontal red trace labeled ‘50%’), with intermediate values at 80, 70 and 60% I_{K1} . Figure 3B also shows action potentials of the model cell with 50% I_{K1} in response to short supra-threshold stimuli delivered at a rate of 75 min⁻¹ (arrows).

Figure 3C shows traces obtained with the same I_{K1} settings as in Fig. 3B, i.e., 100, 80, 70, 60 or 50% of control, but now with the inclusion of HCN4 current with a constant scaling factor of 1.0%, i.e., the highest value used in Fig. 2C. At 100% I_{K1} , the resting potential is now -75 mV, which is less negative than the corresponding resting potential value in Fig. 3B due to a steady HCN4 inward current at this potential. This resting potential again depolarizes with decreasing I_{K1} , up to -54 mV at 60% I_{K1} . In contrast with Fig. 3B, a further decrease in I_{K1} to 50% now leads to spontaneous pacemaker activity of the non-stimulated atrial cell with a cycle length of ≈ 800 ms (Fig. 3C, red trace).

The bio-pacemaker activity of Fig. 3C occurs in combination with a ‘down-regulated’ I_{K1} (50% of control). We also tested whether this bio-pacemaker activity could be achieved without down-scaling I_{K1} , using the dAPC configuration of Fig. 4A. We increased the scaling factor for the injected HCN4 current from 1.0%, as used in Fig. 3C, to 2.0 or 3.0%. With a scaling factor of 2.0%, the resting potential depolarizes from its value of -75 mV at 1.0% (Fig. 4B, blue trace labeled ‘1.0’) to -69 mV (Fig. 4B, orange trace labeled ‘2.0’). Bio-pacemaker activity develops if the scaling factor is further increased to 3.0% (Fig. 4B, red trace labeled ‘3.0’). However, the cycle length is considerably longer than in Fig. 3C (≈ 1450 vs. ≈ 800 ms). Also, the maximum diastolic potential is more negative (-66 vs. -54 mV).

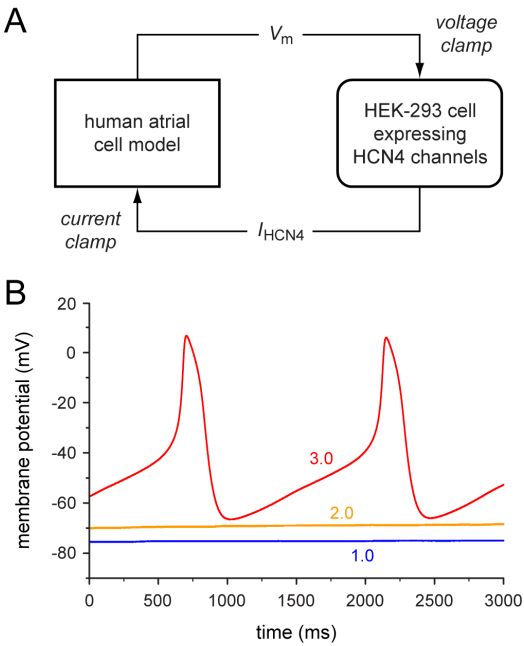


Fig. 4. Dynamic action potential clamp (dAPC) experiment with a real-time simulation of a human atrial cell and a HEK-293 cell expressing HCN4 channels. (A) Experimental configuration. A human atrial cell is simulated in real time using the Courtemanche *et al.* [10] model of a human atrial myocyte. HCN4 current recorded from the HEK-293 cell is added to the net membrane current of the model cell, thus mimicking the gene therapy strategy to create a bio-pacemaker by (over-)expression of I_f . (B) Effect of adding increasing amounts of HCN4 current to the non-stimulated atrial cell. The HCN4 current recorded from the HEK-293 cell was scaled by 1.0, 2.0, or 3.0%, as indicated.

IV. DISCUSSION

The experiment of Fig. 2 demonstrates that the HCN4 current can functionally, in terms of modulating pacemaker frequency, replace the native I_f of the model SA nodal cell. However, unlike I_f , the effect of increasing the HCN4 current is not limited to an increase in the rate of diastolic depolarization. This emphasizes that the kinetics of HCN4 need not be identical to those of native I_f channels [17] and that HCN4 channels should not simply be regarded as a replacement of I_f ‘pacemaker channels’ in gene therapy strategies. Ideally, the experiment of Fig. 2 should have been carried with a human SA nodal cell model instead a rabbit model, but such model is not available due to paucity of data from human SA nodal cells [11]. It should also be noted that we have not made attempts to assess the autonomic responsiveness of the dAPCed cell with the introduction of HCN4 current.

The experiments of Figs. 3 and 4 demonstrate that HCN4 channels can induce bio-pacemaker activity in an intrinsically quiescent atrial cell and that engineering of a single cell bio-pacemaker is facilitated by down-regulation of I_{K1} , but that such down-regulation may not be a prerequisite. These findings require confirmation in future dAPC experiments using freshly isolated human atrial myocytes.

V. CONCLUSION

Dynamic action potential clamp experiments provide direct insights into the effects of introducing a gene-based ‘pacemaker current’ into an intrinsically quiescent cardiac myocyte. We conclude that dynamic action potential clamp may prove a powerful tool in the process of developing a gene-based bio-pacemaker, particularly when investigating the effects of different (combinations of) candidate genes.

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