Reciprocal interaction between $I_{K1}$ and $I_f$ in biological pacemakers: A simulation study

Running Title: Role of $I_{K1}$ and $I_f$ in bio-pacemaking

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Abstract

Pacemaking dysfunction (PD) may result in heart rhythm disorders, syncope or even death. Current treatment of PD using implanted electronic pacemaker has some limitations, such as finite battery life and the risk of repeated surgery. As such, the biological pacemaker has been proposed as a potential alternative to the electronic pacemaker for PD treatment. Experimentally it has been shown that bio-engineered pacemaker cells can be generated from non-rhythmic ventricular myocytes (VMs) by knocking down genes related to the inward rectifier potassium channel current (I_{K1}) or by overexpressing hyperpolarization-activated cyclic nucleotide gated channel genes responsible for the “funny” current (I_{f}). Such approaches can turn the VM cells into rhythmic pacemaker cells. However, it is unclear if a bio-engineered pacemaker based on the modification of I_{K1}- and I_{f}-related channels simultaneously would enhance the ability and stability of bio-engineered pacemaking action potentials (APs). This study aimed to investigate by a computational approach the combined effects of modifying I_{K1} and I_{f} density on the initiation of pacemaking activity in human ventricular cell models. First, the possible mechanism(s) responsible for VMs to generate spontaneous pacemaking APs by changing the density of I_{K1} and I_{f} were investigated. Then the integral action of targeting both I_{K1} and I_{f} simultaneously on the pacemaking APs was analysed. Our results showed a reciprocal interaction between I_{K1} and I_{f} on generating stable and robust pacemaking APs in VMs. In addition, we thoroughly investigated the dynamical behaviours of automatic rhythms in VMs in the I_{K1} and I_{f} parameter space, providing optimal parameter ranges for a robust pacemaker cell. In conclusion, to the best of our knowledge, this study provides a novel theoretical basis for generating stable and robust pacemaker cells from non-pacemaking VMs, which may be helpful in designing engineered biological pacemakers for application purposes.

Keywords: bio-pacemaker, sinoatrial node, funny current (I_{f}), inward rectifier potassium channel current (I_{K1}), reciprocal interaction, stable pacemaking
Author Summary

Pacemaking dysfunction has become one of the most serious cardiac diseases, which may result in arrhythmia and even death. The treatment of pacemaking dysfunction by electronic pacemaker has saved millions of people in the past fifty years. But not every patient can benefit from it because of possible limitations, such as surgical implication and lack of response to autonomic stimulus. The development of bio-pacemaker based on gene engineering technology provides a promising alternative to electronic pacemaker by manipulating the gene expression of cardiac cells. However, it is still unclear how a stable and robust bio-pacemaker can be generated. The present study aims to elucidate possible mechanisms responsible for a bio-engineered pacemaker by using a computational electrophysiological model of pacemaking cells based on modifying ion channel properties of $I_{K_1}$ and incorporating $I_f$ in a human ventricular cell model, mimicking experimental approaches of gene engineering. Using the model, possible pacemaking mechanisms in non-pacemaking cells, as well as factors responsible for generating robust and stable biological pacemaker, were investigated. It was shown that the reciprocal interaction between reduction of $I_{K_1}$ and incorporation of $I_f$ played an important role for producing robust and stable pacemaking. This study provides a novel insight into understanding of the initiation of pacemaking behaviours in non-rhythmic cardiac myocytes, providing a theoretical basis for experimental designing of biological pacemakers.

Introduction

Currently, the electronic pacemaker implantation is the only non-pharmacological therapy for some patients with pacemaking dysfunctions (PD), such as sick sinus syndrome and atrioventricular heart-block, but it has some possible limitations (1). Implantation of pacemaker device may have complications for patients, especially for aged ones because of their infirm health (2). Pediatric patients can receive electronic pacemakers; however, the device has to be replaced as they grow and repeated surgeries are needed (3). Electronic devices can be subject to electromagnetic interference (4), which causes inconvenience to the patients. A further issue is that classical electronic pacemakers are insensitive not only to hormone stimulation (5) but also to autonomic
emotion responsiveness (4), although there are some attempts to make them respond to autonomic nervous control (6). In addition, the long-term use of electronic pacemakers has been reported to increase the risk of heart failure (7). Appropriately designed biological pacemakers (bio-pacemakers) have the potential to overcome some of the limitations of electrical device use (8). For example, engineered bio-pacemakers could potentially involve only minor surgical trauma for implantation as well as opportune chronotropic responses to creature emotion (9). In previous experimental studies, it has been shown that a bio-pacemaker can be engineered via adenoviral gene transduction (10-12) or lentiviral vector (13, 14) techniques, by which non-pacemaking cardiac myocytes (CMs) can be transformed to rhythmic pacemaker-like cells.

The native cardiac primary pacemaker, sinoatrial node (SAN), is a special region comprised of cells with distinct electrophysiological properties to cells in the working myocardium. Such intrinsic and special electrophysiological properties of SAN cells are mainly manifested by their small if not absent inward rectifier potassium channel current ($I_{K1}$) (15), but a large “funny” current ($I_f$) (16) that is almost absent in atrial and ventricular cells. In addition to absence of $I_{K1}$ and presence of $I_f$, T-type $Ca^{2+}$ channel current ($I_{CaT}$) (17) and sustained inward current ($I_{st}$) (18) also contribute to spontaneous pacemaking activity in SAN cells. Such unique electrophysiological properties of SAN cells form a theoretical basis to engineer non-pacemaking CMs into spontaneous pacemaker cells. These non-pacemaker cells include native CMs, such as ventricular (11, 19-21), atrial (22) or bundle branch myocytes (23). They can also be stem cells, such as embryonic stem cells (24-26), bone marrow stem cells (13, 27, 28), adipose-derived stem cells (29-31), or induced pluripotent stem cell (14, 32, 33).

With gene therapy, these cells are manipulated to provoke automaticity. In previous studies, knocking off the $Kir2.1$ gene to reduce the expression of $I_{K1}$ promoted spontaneous rhythms in newborn murine ventricular myocytes (VMs) (19); by reprogramming the $Kir2.1$ gene in guinea-pigs, VMs also produced pacemaker activity when $I_{K1}$ was suppressed by more than 80% (11, 20). As $I_f$ plays an important role in the native SAN cell pacemaking, a parallel gene therapy manipulation to create engineered bio-pacemaker has been carried out by expressing the $HCN$ gene family in non-rhythmic cardiac myocytes (34). It has
been shown that expressing HCN2 produced escape beats in canine CMs (22, 23) and initiated spontaneous beats in neonatal rat VMs (21). HCN expression in stem cells-induced-CMs also enhanced their pacemaking rate (13, 27, 28, 35, 36). Overexpressing HCN4 can also induce spontaneous pacemaking activity in mouse embryonic stem cells (37). However, acute expression of the HCN gene might have a side effect on the normal cardiac pacemaking activity (38-40) and the overexpression of the HCN gene in VMs can cause ectopic pacemaker automaticity and even arrhythmicity (41).

It has been suggested that a combined manipulation of I_{K1} and I_f may be a better alternative for creating a bio-pacemaker (42). It has been demonstrated that the expression of transcriptional regulator TBX18, which influenced both I_f and I_{K1} expression, generated appropriate autonomic responses in non-pacemaking CMs (10, 31, 43). In addition, reprogramming TBX18 in porcine VMs did not show the increase of arrhythmia risk (12), indicating the probable superiority of manipulating I_{K1} and I_f jointly for generating a bio-pacemaker. Furthermore, Chen et al. (44) attempted to explore the generation of oscillation by manipulating the expressing level of Kir2.1 and the HCN genes and suggested that a dynamic balance between Kir2.1 and HCN was essential to initiate oscillation in HEK293 cells. In the absence of I_{K1}, spontaneous rhythmic oscillations might be inhibited due to an insufficiently repolarized membrane potential to activate I_f. However, HEK293 cells lack all the other ionic channels present in native SAN myocytes.

Computational modelling offers a means to investigate different approaches to generating stable pacemaking activity. It has been used to study possible roles of down-regulating I_{K1} in VMs (45-47) and combined action of overexpression of I_f with down-regulation of I_{K1} in inducing spontaneous pacemaking activity in SAN (42). Bifurcation analysis has also been used to explore the effect of changes in some individual ion channel current on the pacemaking activities of SAN cells (48) and genesis of automaticity in VMs (46, 49), showing the role of I_{K1}, I_s, I_{ad} and Na^+/Ca^{2+} exchange current (I_{NaCa}) in modulating the initiation and stability of pacemaking activity (49). But this approach was applied to simplified CMs model (46) and interplay between more than one ion channel currents on modulating bio-pacemaker APs has not been comprehensively investigated yet.
In this study, we constructed a bio-pacemaker model based on a human non-rhythmic VMs model (50) by manipulating $I_{K1}$ and incorporating $I_f$ (51) into the model. The aim of this study was to investigate (i) possible mechanism(s) underlying the pacemaking activity of the VMs in the $I_{K1}$ and $I_f$ parameter space; and (ii) the reciprocal interaction of reduced $I_{K1}$ and increased $I_f$ in generating stable pacemaking action potentials (APs) in them. In addition, possible factors responsible for impaired pacemaking activity due to inappropriate ratio between $I_{K1}$ and $I_f$ were also investigated. This study provides insightful understandings to generating stable and robust engineered bio-pacemaker.

Results

Initiation of transient spontaneous depolarization

In the basal VM cell model with the suppression of $I_{K1}$ by 70% (the density of $I_{K1}$ at -80 mV was 0.297 pA/pF), incorporating of $I_f$ (with a current density of -0.63 pA/pF at -80 mV in the I-V curve of $I_f$ (S1B Fig)) was unable to depolarize the membrane potential and lead to spontaneous pacemaking activity because the excessive outward current of $I_{K1}$ counteracted the inward depolarizing current. This state can be described by State-1 as shown in Eq. 8. However, when the density of $I_f$ was increased to -1.89 pA/pF, spontaneous depolarization was provoked at the beginning of the transition period, however, the automaticity self-terminated after $1.63 \times 10^5$ ms (Fig 1 A), showing a State-2 behaviour as described in Eq. 9.

We analysed possible ion channel mechanisms responsible for unstable and self-terminating pacemaking APs with the current densities of ($I_{K1}$, $I_f$) at (0.297pA/pF, -1.89 pA/pF) (‘CASE 1’). Results in Fig 1 showed that during the time course of the spontaneous pacemaking, there were changes of intracellular ionic concentrations and the MDP. Through the Na⁺ permeability of $I_f$, there was extra Na⁺ flowing into the cytoplasm during each of the APs, leading the intracellular Na⁺ concentration ([Na⁺]$_i$) to increase from 7.67 to 11.8 mM (Fig 1 B). The increased [Na⁺], augmented the feedback mechanism of Na⁺/K⁺ pumping activity, by which the Na⁺/K⁺ pump current ($I_{NaK}$) increased gradually with time (Fig 1 E). In addition, there was also an accumulation of the intracellular Ca²⁺ concentration ([Ca²⁺]$_i$, Fig 1 C) during the time course of spontaneous pacemaking APs. Such an
accumulation of \([\text{Ca}^{2+}]_i\) was due to the fact that the automaticity in VMs shortened the DI between two successive APs, leaving insufficient time for \(\text{Ca}^{2+}\) in the cytoplasm to be extruded to restore to its initial value after each cycle of excitation. This consequently led to overload in \([\text{Ca}^{2+}]_i\), which suppressed the extent of the activation degree of the L-type calcium current \(I_{\text{CaL}}\) (Fig 1 F), especially during the phase 0 of the pacemaking action potential. Furthermore, the overloaded \([\text{Ca}^{2+}]_i\) increased the \(I_{\text{NaCa}}\) (S2 Fig) gradually with time, resulting in an elevated MDP (Fig 1 H) that inhibited the activation degree of the fast sodium channel current \(I_{\text{Na}}\) (Fig 1 D). All of these factors worked together, inhibiting the membrane potential to reach the take-off potential, leading to self-terminated automaticity at 1.63×10^5 ms (Fig 1 G).

![Fig 1. Transient spontaneous pacemaking behaviour.](image)

(A-F) Membrane potential (V), intracellular Na\(^+\) concentration ([Na\(^+\)]\(i\)), intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)), fast sodium current \(I_{\text{Na}}\), L-type calcium channel current \(I_{\text{CaL}}\) and Na\(^+\)/K\(^+\) pumping current \(I_{\text{NaK}}\) with the current densities of \(I_{\text{K1}}, I_f\) at (0.297pA/pF, -1.89 pA/pF) during the entire simulating period of 800 s. (G) Expanded plots of (A-F) for the time course of maximum diastolic potential (MDP).
pacemaking self-termination (1.6×10^5 ms to 1.64×10^5 ms). H: Maximum diastolic potential of spontaneous pacemaking behaviour of (A).

It was also possible to generate automaticity in the model by fixing the current density of I_f at a low value, but with a further reduction in I_{K1} density. S3 Fig shows the results when I_f was held at -0.63 pA/pF, the current density of I_{K1} was reduced to 0.178 pA/pF. In this case, pacemaking activity appeared in the model, but the automaticity was unstable and self-terminated due to similar mechanisms as shown in Fig 1 for the increased-I_f situation.

**Bursting pacemaking behaviour**

Fig 2 shows the intermittent bursting behaviour, which is generated with a different combination of I_{K1} and I_f current densities in the model. In the figure, the current densities of (I_{K1}, I_f) were held at (0.297 pA/pF, -2.52 pA/pF) (defined as ‘CASE 2’). Such kind of pacemaking state can be classified as State-3 (Eq. 10).

![Fig 2. Bursting pacemaking behaviour.](image-url)
(A-F) Membrane potential ($V$), intracellular $Na^+$ concentration ([Na$^+$]), intracellular $Ca^{2+}$ concentration ([Ca$^{2+}$]), fast sodium channel current ($I_{Na}$), $Na^+$/K$^+$ pumping current ($I_{NaK}$) and L-type calcium channel current ($I_{CaL}$) with the current densities of ($I_{K1}$, $I_f$) at (0.297 pA/pF, -2.52 pA/pF) during the entire simulating period of 800 s. (G) Expanded plots of (A-F) for the time course of pacemaking resumption (3.83 $\times$ 10$^5$ ms to 3.85 $\times$ 10$^5$ ms).

H: Maximum diastolic potential of automatic pacemaking activity when $I_f$ is -2.52 and -1.89 pA/pF (solid and dotted line respectively).

In CASE 2, the spontaneous oscillation was unstable, characterized by self-termination and then resumption after a quiescent period (Fig 2 A). Similar to CASE 1, the self-termination was accompanied by the overload of [Na$^+$] (Fig 2 B), the accumulation of [Ca$^{2+}$] (Fig 2 C), which caused the reduction of $I_{Na}$ (Fig 2 D), the increase of $I_{NaK}$ (Fig 2 E) and the decrease of $I_{CaL}$ (Fig 2 G).

This suggested that the underlying mechanisms responsible for the self-termination of the pacemaking APs were similar to those of CASE 1.

It is of interest to analyse the mechanism(s) for the resumption of the pacemaking APs after a long pause. It was shown that, during the time course of the quiescent interval (from 2.36 $\times$ 10$^5$ ms to 3.84 $\times$ 10$^5$ ms in Fig 2 A-F), the intracellular $Na^+$ (Fig 2 B) continued to be extruded out of the cell by $I_{NaK}$ (Fig 2 E), and the intracellular $Ca^{2+}$ (Fig 2 C) extruded by the $I_{NaCa}$ (S4A Fig). As such, the [Na$^+$], (Fig 2 B) and [Ca$^{2+}$], (Fig 2 C) gradually decreased over time. A decrease in [Na$^+$] led to a gradually reduced $I_{NaK}$ over the time course of quiescence (Fig 2 E), which decreased its suppressive effect on depolarization. During the time period of quiescence, $I_{CaL}$ kept at a small magnitude (Fig 2 F). Via $I_{NaCa}$ (S4 Fig), the intracellular $Ca^{2+}$ was kept to be extruded out of the cell, leading to a decreased [Ca$^{2+}$]. Consequently, a decrease in [Ca$^{2+}$], resulted in a reduced calcium-dependent inactivation of $I_{CaL}$, leading to an increased $I_{CaL}$ (Fig 2 G), which facilitated the action potential generation. Moreover, as compared with CASE 1, the increase in $I_f$ also helped to produce a more depolarized MDP (Fig 2 H), allowing the membrane potential more easily to reach the take-off potential for initiation of the upstroke. All of these actions worked in combination to produce a full course of action potential with a sufficient amplitude that activated sufficient outward currents to repolarize the cell membrane to a range that...
activated \( I_f \) and \( I_{Na} \), facilitating the resumption of the spontaneous pacemaking activity at \( 3.847 \times 10^5 \) ms (Fig 2 G). This process of self-termination and resumption repeated alternately, which constituted bursting behaviour.

**Persistent pacemaking activity**

A further increase in \( I_f \) ((\( I_{K1}, I_f \)) at (0.297 pA/pF, -3.15 pA/pF)) produced a series of persistent spontaneous APs. Results are shown in Fig 3 (grey lines) for APs (Fig 3 A), together with a phase portrait of membrane potential (V) and total membrane channel current (\( I_{total} \)) (Fig 3 B), \( I_{K1} \) (Fig 3 C), and \( I_{CaL} \) (Fig 3 D). Although the spontaneous APs were sustained during the entire simulation period of 800 s, there were some incomplete depolarizations observed periodically (Fig 3 A, grey line) which can be classified as State-4 according to Eq. 11. This pacemaking situation was termed as ‘CASE 3’.

**Fig 3. Persistent pacemaking activity.**

The current densities of (\( I_{K1}, I_f \)) of stable pacemaking activity and periodically incomplete pacemaking activity are at (0.248 pA/pF, -3.15 pA/pF) (black lines) and (0.297 pA/pF, -3.15 pA/pF) (grey lines) respectively. (A-D) The membrane potential (V), phase portraits of membrane potential against the total membrane channel current (\( I_{total} \)), inward rectifier potassium channel
current ($I_{K1}$) and L-type calcium channel current ($I_{CaL}$) with simulating time course from $7.6 \times 10^5$ to $7.63 \times 10^5$ ms. (Inset)

Expanded plot for phase diagram during $V$ from -75 to -45 mV and $I_{total}$ from -0.1 to 0 pA/pF.

When the density of $I_{K1}$ was further reduced to 0.248 pA/pF (Fig 3 C, black line), a stable pacemaking activity was established (Fig 3A, black line), with an average CL of 895 ms and MDP of -72.63 mV. This kind of pacemaking state can be described as State-5 by Eq. 12. In this condition, the pacemaking activity was robust and the pacing CL was close to that of the native human SAN cells (approximately 800 - 1000 ms (52)). We termed stable pacemaking activity with ($I_{K1}$, $I_f$) at (0.248 pA/pF, -3.15 pA/pF) as ‘CASE 4’.

In order to understand potential mechanism(s) underlying the genesis of incomplete pacemaking potentials in CASE 3, phase portraits of membrane potential against $I_{total}$ for incomplete (grey line) and complete (black line) depolarization APs were plotted and superimposed for comparison, as shown in Fig 3 B, with a highlight of phase portraits during the diastolic pacemaking potential range from -75 mV to about -45 mV being shown in the inset. In the case of incomplete depolarization (CASE 3), there was a greater $I_{K1}$ (Fig 3 C) that counteracted the inward depolarizing current, leading to a smaller $I_{total}$ during the diastolic depolarization phase (see the grey line in Fig 3 B and the inset). Consequently the membrane potential failed to reach the take-off potential for the activation of $I_{CaL}$ (Fig 3 D, grey line), leading to an incomplete course of action potential (Fig 3 A, grey line). In CASE 4, with a reduced $I_{K1}$ (Fig 3 C, black line), there was a greater $I_{total}$ during the diastolic depolarization phase (Fig 3 B and the inset, black line), which drove the membrane potential to reach the take-off potential for the activation of $I_{CaL}$ (Fig 3 D, black line), leading to the upstroke of the action potential.

The frequency for the appearance of the incomplete AP was dependent on the density of $I_{K1}$. Incomplete depolarization occurred less frequently, with progressively smaller $I_{K1}$. By way of illustration, the incomplete depolarization appeared once every three cycles with the $I_{K1}$ density at 0.297 pA/pF in CASE 3 (S5A Fig), but this became once every five cycles when $I_{K1}$ density was reduced to 0.277 pA/pF $I_{K1}$ (S5B Fig). This suggested that a large residual of $I_{K1}$ in the VM model might result in the failure of
**Dynamic analysis in $I_{K1}$ and $I_f$ parameter space**

Dynamic pacemaker AP behaviours were dependent on the balance of $I_{K1}$ and $I_f$ interactions. Further simulations were conducted in the $I_{K1}$ and $I_f$ parameter space to characterize this dependence. Results are shown in Fig 4. With differing combinations of $I_{K1}$ and $I_f$ density, five different regions for distinctive pacemaking dynamics could be discerned, including stable pacemaking activity (blue area, features described by Eq. 12), intermittence of failed depolarization (yellow area, features described by Eq. 11), bursting pacemaking behaviour (orange area, features described by Eq. 10), transient pacemaking activity (green area, features described by Eq. 9), and no automaticity (grey area, features described by Eq. 8). In each category, representative membrane potentials are illustrated at the bottom panel of the Fig 4.

![Fig 4](image-url)
bursting pacemaking behaviour. Green: transient spontaneous pacemaking behaviour. Gray: no spontaneous pacemaking behaviour. In each category, the typical pacemaking action potentials are illustrated at the bottom panel.

With a fixed $I_f$ density, alterations to $I_{K1}$ could produce different types of pacemaking activities and this also applied when $I_{K1}$ was fixed whilst $I_f$ was changed. To illustrate, when $I_f$ density was fixed at a density between -0.63 and -2.52 pA/pF, with a 60 – 80% block of $I_{K1}$ (i.e., $I_{K1}$ density at -80 mV was in the range of 0.198-0.396 pA/pF), pacemaking activity was generated but with self-termination (Fig 4, green area). Then, a further reduction in $I_{K1}$ or a slight increase in $I_f$ induced bursting pacemaking behaviour, as shown by the orange area in Fig 4, which was between the boundaries marking the persistent automaticity and transient pacemaking activity regions. A further increase in $I_f$ or suppression in $I_{K1}$ could produce persistent automaticity (Fig 4, yellow and blue area). But when $I_{K1}$ was greater than about 0.248 pA/pF, incomplete depolarization appeared periodically (Fig 4, yellow area). Finally, a stable and spontaneous pacemaking activity could be generated when $I_{K1}$ was decreased to less than 0.248 pA/pF at -80 mV with $I_f$ included (Fig 4, blue area).

**Reciprocal role of $I_f$ and $I_{K1}$ in generating pacemaking APs**

Further analysis was conducted to investigate the reciprocal role of reduced $I_{K1}$ and increased $I_f$ in generating pacemaking APs. By sufficiently reducing $I_{K1}$ to a density of 0.05 pA/pF alone, the model was able to generate spontaneous APs with a CL of 1011 ms. In this case, the incorporation of $I_f$ with a small density helped to boost the pacemaking activity and increase the pacemaking frequency. It was shown that with the incorporation of $I_f$ at a density of 0.63 pA/pF, the CL was reduced by 233 ms, changing from 1011 ms to 778 ms (Fig 5 A). Compared with the case of $I_f$ absence, incorporation of $I_f$ - even with a small density (-0.63 pA/pF) - helped to depolarize cell membrane potential during the early DI phase (Fig 5 A and C). Moreover, the incorporation of $I_f$ led to an accumulation of $[Na^+]_i$ (Fig 5 B) then increased amplitude of $I_{NaCa}$ (Fig 5 D), which also contributed to the depolarization of membrane potential. As a result, the incorporation of $I_f$ facilitated genesis of spontaneous APs and shortened the DI significantly, thus decreased the CL.
Fig 5. Role of $I_f$ in pacemaking ability.

(A-D) The membrane potential ($V$), intracellular $\text{Na}^+$ concentration ([Na$^+$]), hyperpolarization-activated channel current ($I_h$), $\text{Na}^+$/Ca$^{2+}$ exchange current ($I_{\text{NaCa}}$) with simulating time course from $4 \times 10^5$ to $4.03 \times 10^5$ ms when the current densities of ($I_{K1}$, $I_f$) are at (0.05 pA/pF, 0 pA/pF) and (0.05 pA/pF, -0.63 pA/pF) (dotted and solid line respectively). (Inset A-B) Expanded plots of [Na$^+$]$_i$ traces for the time course marked by the horizontal brackets with asterisks in (B).

With a fixed $I_{K1}$ density of 0.05 pA/pF, the relationship between the computed CL of pacemaking APs and $I_f$ density was found to be nonlinear as shown in Figure 6 A. In a range from -0.126 to -2.52 pA/pF, an increase in $I_f$ density produced a marked decrease in the CL (Fig 6 A), which was associated with an increase in the rate of membrane depolarization during the DI (diastolic depolarizing rate) (S6B Fig). In this range, an increase in $I_f$ caused an elevated MDP (Fig 6 B), as well as an accumulation of [Na$^+$]$_i$ (Fig 6 C), which enhanced $I_{\text{NaCa}}$ (Fig 6 D). All of these contributed to the acceleration of the pacemaking activity. However, when $I_f$ density was over -2.52 pA/pF, there was a less dramatic decrease in CL with an increase of $I_f$ (Fig 6 A). This was
attributable to a reduced $I_{Na}$ (Fig 6 F) as a consequence of gradual elevation of the MDP (Fig 6 B). Another factor was that the maximum density of $I_{f}$ was not increased linearly (Fig 6 E) because of elevated MDP.

**Fig 6. Effect of $I_{f}$ density on the pacemaking cycle length under fixed $I_{K1}$ density.**

$I_{K1}$ density is fixed at 0.05 pA/pF. (A-B) Change of cycle length and maximum diastolic potential (MDP) with the increase of $I_{f}$ from 0 to -6.3 pA/pF. (C-F) Change of maximum intracellular $Na^{+}$ concentration ([$Na^{+}$]), maximum $Na^{+}$/Ca$^{2+}$ exchange current (INaCa), maximum funny current ($I_{f}$) and maximum fast sodium current ($I_{Na}$) during a pacemaking period with the increase of $I_{f}$.

Depending on the $I_{K1}$ density, the relationship between CL and $I_{f}$ density could also be biphasic as shown in Fig 7. With a small $I_{K1}$ density (0.05 pA/pF), the measured CL decreased monotonically with the increase in $I_{f}$ density (Fig 7 A, dotted line). However, with a large $I_{K1}$ (0.198 pA/pF), the measured CL first decreased with an increased $I_{f}$ density, but then increased with it.
when $I_f$ density was greater than -3.15 pA/pF, implicating a slowdown in the pacemaking activity with the increase of $I_f$ (Fig 7 A, solid line). Such a slowdown of pacemaking APs with an increased $I_f$ was mainly due to the prolonged DI (S7 Fig). This was attributable to the delicate balance between the total integral of three dominant inward currents ($I_{Na}$, $I_{NaCa}$, and $I_f$) ($I_{in}$ as defined in Eq. 14) and the total integral of four dominant outward currents ($I_{K1}$, $I_{NaK}$, $I_{Kr}$ and $I_{Ks}$) ($I_{out}$ as defined in Eq. 15) during the DI phase (Fig 7 B and C). At a low $I_{K1}$ density of 0.05 pA/pF, with the increase in $I_f$ density, there was a monotonic increase in both of $I_{in}$ and $I_{out}$, and the balance of them resulted in a monotonic decrease of CL (Fig 7, dotted lines). However, at a large $I_{K1}$ density (e.g. 0.198 pA/pF), an increase of $I_f$ was associated with a monotonic increase in $I_{out}$ (Fig 7 C, black line), but had a different impact on $I_{in}$, which reached at a flat magnitude after $I_f$ density was greater than -3.15 pA/pF (Fig 7 B, black line).

Consequentially, at large $I_f$, a greater $I_{out}$ out balanced the $I_{in}$ leading to a prolonged DI that increased the CL.

**Fig 7. Effect of $I_f$ density on the pacemaking cycle length under different $I_{K1}$ density.**

$I_{K1}$ density is 0.198 (solid line) and 0.05 pA/pF (dotted line) at -80 mV. (A) Change of cycle length with the increase of $I_f$ from 0 to -6.3 pA/pF. (B) Change of the total integral of main inward currents (Integral $I_{in}$) during diastolic interval phase. The inward currents include fast sodium current ($I_{Na}$), Na$^+$/Ca$^{2+}$ exchange current ($I_{NaCa}$) and funny current ($I_f$). (C) Change of the total integral of main outward currents (Integral $I_{out}$) during diastolic interval phase. The outward currents include inward rectifier potassium channel current ($I_{K1}$), Na$^+$/K$^+$ pumping current ($I_{NaK}$), rapid delayed rectifier potassium channel current ($I_{Kr}$) and slow delayed rectifier potassium channel current ($I_{Ks}$).
Pacemaking cycle length in $I_{K_1}$ and $I_f$ parameter space

A systematic analysis of the relationship between the calculated CL in the $I_{K_1}$ and $I_f$ density parameter space is presented in Fig 8.

In the figure, the measured CL was coloured from 650 ms in dark red to 1000 ms in yellow. In this study, we regarded persistent pacemaking action potential with CL 1000 ms or less as ‘valid pacemaking activity’, therefore, only the CLs of the valid pacemaking potentials are shown in Fig 8.

**Fig 8. Measured cycle length in the $I_{K_1}$ and $I_f$ density parameter space.**

The density of $I_{K_1}$ is from 0 to 0.396 pA/pF and the density of $I_f$ is from 0 to -6.3 pA/pF at -80 mV. The measured CL is coloured from 650 ms in dark red to 1000 ms in yellow. White means that pacemaking cycle length is ‘invalid’ (more than 1000 ms) or membrane potential is not persistent during the whole simulating time course.

It was shown that a sufficient depression in $I_{K_1}$ (up to 75%; $I_{K_1}$ density < 0.248 pA/pF) was required to produce a stable pacemaking action potential with a ‘valid’ pacemaking frequency. With the increase of the $I_{K_1}$ inhibition level, the CL became shortened at all $I_f$ densities considered. Also, the more that $I_{K_1}$ was inhibited, the less $I_f$ was needed to provoke ‘valid’ spontaneous pacemaking activity. By contrast, the effect of $I_f$ on the CL presented two phases, which was dependent on the $I_{K_1}$ density. When
$I_{K1}$ was less than 0.198 pA/pF, the pacemaking ability became robust with the increase in $I_f$ density. However, when $I_{K1}$ was increased from 0.198 to 0.248 pA/pF, an increase in $I_f$ actually slowed the pacemaking activity, leading to an increased CL.

Discussion

Summary of major findings

In this study, we construct a virtual bio-engineered pacemaking cell model based on a human VM model by a combination of reduction of $I_{K1}$ and incorporation of $I_f$. Using the developed bio-pacemaker model, we investigate the combined actions of different $I_{K1}$ and $I_f$ permutations on the dynamical behaviours of membrane potential, ionic channel currents and intracellular ionic concentrations. It is shown that robust and stable pacemaking activity can be established by balancing the actions of reduced $I_{K1}$ and increased $I_f$, though the effect of each manipulation on pacemaking activity is different. While the action of a reduced $I_{K1}$ on the pacemaking activity is consistent, that of an increased $I_f$ is biphasic. Whilst the incorporation of $I_f$ at an appropriate level promotes pacemaking activity, excessive $I_f$ might result in abnormal pacemaking activity accompanied by abnormal intracellular ionic concentrations, which could be proarrhythmic. As a result, the reciprocal interaction between $I_{K1}$ and $I_f$ is crucial for producing stable spontaneous pacemaking activity in VMs. The results of this study may be useful for optimizing the future design of engineered bio-pacemakers.

Role of $I_{K1}$ suppression on pacemaking activity

The suppression of $I_{K1}$ plays an important role in the generation of pacemaking activities in the VM cell model. Our simulation results have shown that a significant $I_{K1}$ suppression (at least 60%) with the incorporation of $I_f$ is required to provoke auto-rhythmicity in the model. With a modest suppression (i.e. 60-75% suppression of $I_{K1}$), only unstable spontaneous APs can be produced. When $I_{K1}$ is further suppressed by 75% - 100%, persistent, steady pacemaking behaviour can be initiated in our model with appropriate incorporation of $I_f$ (Fig 4). These simulation results are in consistence with those of experimental findings, where it has been found that $I_{K1}$ could be suppressed by 50 – 90 % by knocking out the Kir2.1 gene, and more than 80% inhibition of $I_{K1}$
was required to produce a pacemaking phenomenon in guinea-pig’s ventricular cavity (11, 20). It is also in agreement with
previous bifurcation analyses in showing that it required $I_{K1}$ to be reduced to at least 15% of the control value to transform a VM
cell model to be auto-rhythmic (46), and a complete block of $I_{K1}$ produced a spontaneous pacemaking activity with a CL of 795
ms (46), close to 833 ms when $I_{K1}$ was totally suppressed in the present study. We have also found a monotonic relationship
between the measured CL and the degree of $I_{K1}$ suppression. It suggests that when $I_{K1}$ is inhibited enough to induce automaticity,
the more the $I_{K1}$ is blocked, the faster the pacemaking activity is with all $I_f$ densities considered (Fig 8). Similar results have also
been observed in another ventricular cell model developed for human VMs (53) based on modifications of the model of O’Hara
and Rudy (54) (S1 Text and S8 Fig, solid and dotted lines).

Though our simulation results suggest an important role of sufficient suppression of $I_{K1}$ for generating persistent and stable
pacemaking APs, it is noteworthy that the deficiency of $I_{K1}$ has been reported to be lethal for adult rodents (55); and loss function
of $Kir2$ gene may prolong QT intervals as well as cause Andersen’s syndrome (56). Consequently, suppression of $I_{K1}$ from VM
for generating a biological pacemaker may only be suitable when applied to highly localized, designated ‘pacemaker’ regions.

**Role of $I_f$ in pacemaking activity**

$I_f$ has been shown to play an important role in generating pacemaking APs in both native (13, 21, 27, 28, 35, 36) and engineered
pacemakers (42). Experimentally it has been shown that high expression of $HCN2$ can initiate spontaneous beats in neonatal rat
VMs (21, 35) and improve spontaneous beats in rabbit CMs (13). $HCN4$ incorporation by the expression of $TBX18$ can also
initiate spontaneous pacemaking activity in both rodent VMs (10) and porcine VMs (12).

In the present study, we have also highlighted the role of $I_f$ in generating pacemaking activity. Our simulation results have shown
that with different $I_f$ densities, the pacemaking activity may present different behaviours, including transient automaticity with
self-termination, bursting behaviour, and persistent pacemaking (Fig 4). The incorporation of low amplitude of $I_f$ can help to boost
the pacemaking activity in the VM-based pacemaker model induced by $I_{K1}$-inhibition (Fig 5). It helped to promote pacemaking
activity, via its action of depolarization during the diastolic depolarization phase as well as its action on the intracellular ion concentrations. It has been shown that the inclusion of $I_f$ in the VM cell model causes the accumulation of $[\text{Na}^+]_i$, which enhances $\text{Na}^+/\text{Ca}^{2+}$ exchange, thus promoting membrane potential depolarization especially during the early stage of DI (Fig 5). Such a promoting action of $I_f$ in bio-pacemaking was also shown in another ventricular cell model as shown in S1 Text (S8 Fig, solid and dashed lines).

An increase in $I_f$ density can enhance the automaticity in most cases. However, the effect of $I_f$ on the pacemaking activity was observed to be biphasic. In our simulation, increasing $I_f$ from a small initial density was associated with an increased pacemaking rate manifested by a decreased CL. But when it was increased to be over -2.52 pA/pF, excessive $I_f$ resulted in an elevated MDP (Fig 6 B), which caused a reduced activation of $I_f$ and $I_{Na}$ (Fig 6 E and F), leading to a slowdown of the ability of pacemaking activity. The negative effect of excessive $I_f$ on pacemaking APs was also observed in another ventricular pacemaker model (53) (S1 Text). A further increase in $I_f$ density even terminated pacemaking activity (S9 Fig). This mechanism is verified by the fact that in the bio-pacemaker induced by HCN2 expression (57), co-expression of the skeletal muscle sodium channel 1 ($\text{SkM1}$), in order to hyperpolarize the action potential threshold, helped to counterbalance the negative effect of $I_f$ overexpression, producing an accelerated depolarization phase. Furthermore, when $I_{K_1}$ was at a high value (e.g. density at 0.198 pA/pF), acute $I_f$ even lengthened pacemaking period (Fig 7 A, black line). This simulation result is in agreement with a previous biological experimental study that observed a negative action of acute HCN gene expression in cardiac automaticity (41).

**Reciprocal interaction between $I_{K_1}$ and $I_f$**

Our study demonstrated that the reciprocal interaction between $I_{K_1}$ and $I_f$ plays a crucial role in creating stable and persistent pacemaking. The present study has shown that only an optimal combination of $I_{K_1}$ and $I_f$ can initiate stable pacemaking activity (Fig 4). In the presence of $I_f$, the greater the degree of $I_{K_1}$ suppression, the smaller was the $I_f$ density required for the generation of spontaneous oscillation (Fig 4). And modulation of the two currents simultaneously helps to create a physiologically-like
pacemaker that is better than that produced by manipulating $I_{K1}$ or $I_f$ alone (Fig 8). Such observation of reciprocal interaction between $I_{K1}$ and $I_f$ in pacemaking is consistent with previous experimental observations. Previous studies have shown that although suppressing $I_{K1}$ (11, 20), or incorporating sufficient $I_f$ (21) alone was able to initiate pacemaking activity in VM cells, a pacemaker constructed by $TBX18$ showed greater stability, due to its combined actions of $I_{K1}$ reduction and $I_f$ increase (10).

Another experiment in porcine VMs (12) also indicated that $TBX18$ expression did not increase the risk of arrhythmia, which means that a mixed-current approach is probably a superior means of producing a bio-pacemaker. Experiments in a $Kir2.1/HCN2$ HEK293 cell (44) and $Kir2.1/HCN4$ (42) showed that $I_{K1}$ may actually recruit more $I_f$ by activating current at more negative membrane voltages, because $I_{K1}$ was the only negative current in these experiments. Our simulation, however, did not yield such a result because the interaction of other positive currents (such as $I_{NaK}$, $I_{Kr}$ and $I_{Ks}$) contributed to the hyperpolarization of membrane potential and helped the activation of $I_f$.

In addition, simulation results showed that $I_{K1}$ expression level may influence the $I_f$’s effect on the pacemaking activity. Excessive $I_{K1}$ hindered $I_f$’s ability to modulate pacemaking activity (Fig 7A and Fig 8). An experiment showed a coincident result that the expression of $HCN2$ in adult rat VMs could not cause spontaneous beats due to the high expression of $I_{K1}$ (21), but in neonatal rats, the $I_{K1}$ was less so that expressing $HCN2$ could provoke automaticity.

**Limitations**

The limitations of human VMs model we used in this study has been described elsewhere (58). In this study, the $I_f$ formulation of human SAN (51) was incorporated into the original VMs model. The properties of $I_f$, including the conductance of $I_f$, the half-maximal activation voltage ($V_{1/2}$) and time constants of the activation, may present species-dependence. In this study, we only consider the conductance of $I_f$ but have not discussed other properties of $I_f$.

In addition, in this study, we only investigated the pacemaking action potential at the single-cell level, without considering the intercellular electrical coupling between pacemaker cells as presented in the SAN tissue. Mathematical analysis showed that the
incorporation of I_{st} and I_{CaT} into VMs may promote the pacemaking ability of ventricular pacemaker in the coupled-cell model (49). However, up to now, there is no experimental study conducted yet to produce pacemaker cells from VMs by modifying the expression of I_{st} and I_{CaT}, thus we did not discuss them in the present study. It is necessary to highlight these limitations, they nevertheless do not affect our conclusions on the underlying pacemaking mechanisms of engineered bio-pacemaker cells, especially regarding the reciprocal interaction of I_{K1} and I_{f} for a robust bio-pacemaker in modified VMs.

**Methods**

**Single bio-pacemaker cell model**

Previous experimental studies (10, 11, 19-21) implemented the suppression of Kir2.1, the incorporation of HCN channels and the expression of TBX18 to induce pacemaking in VMs. In this study, we used a mathematical model of human VMs (50) as the basal model to investigate possible pacemaking mechanisms in VM-transformed pacemaking cells. In brief, the basal VM cell model can be described by the following ordinary differential equation:

$$\frac{dV}{dt} = -\frac{I_{\text{ion}}}{C_m}$$

(1)

where V is the voltage across cell membrane surfaces, t is time, I_{\text{ion}} is the sum of all transmembrane ionic currents, and C_m is cell capacitance.

The I_{\text{ion}} in the original ventricular model is described by the following equation:

$$I_{\text{ion}} = I_{Na} + I_{K1} + I_{to} + I_{Kr} + I_{Ks} + I_{CaL} + I_{NaCa} + I_{NaK} + I_{pCa} + I_{pK} + I_{bCa} + I_{bNa}$$

(2)

where I_{Na} is fast sodium channel current, I_{K1} is inward rectifier potassium channel current, I_{to} is transient outward current, I_{Kr} is rapid delayed rectifier potassium channel current, I_{Ks} is slow delayed rectifier potassium channel current, I_{NaCa} is Na^+/Ca^{2+} exchange current, I_{NaK} is Na^+/K^+ pump current, I_{pCa} and I_{pK} are plateau Ca^{2+} and K^+ currents, and I_{bCa} and I_{bNa} are background Ca^{2+} and Na^+ currents. The formulations and their parameters for the ionic channels of human VM cells were listed in Ref. (50, 58).
To mimic the reduction of Kir2.1 expression (11, 19, 20) or suppressing I\textsubscript{K1} by expressing TBX18 (10), in simulations, I\textsubscript{K1} was decreased by modulating its macroscopic channel conductance (G\textsubscript{K1}). To mimic the incorporation of I\textsubscript{f} in VMs experimentally (21), we modified the basal model of Eq. 2 by incorporating human SAN I\textsubscript{f} formulation (51). In simulations, I\textsubscript{f} was modulated by changing its channel conductance (G\textsubscript{f}).

As a result, the I\textsubscript{ion} for the bio-pacemaker model can be described as:

\[
I_{\text{ion}} = I_{\text{Na}} + I_{\text{K1}} + I_{\text{to}} + I_{\text{Kn}} + I_{\text{CaL}} + I_{\text{NaCa}} + I_{\text{NaK}} + I_{\text{pCa}} + I_{\text{pK}} + I_{\text{bCa}} + I_{\text{bNa}} + I_{\text{f}}
\]

where I\textsubscript{K1} could be expressed by

\[
I_{\text{K1}} = S_{K1} G_{K1} \sqrt{\frac{K_o}{5.4}} x_{K1=\infty} (V_m - E_K)
\]

where G\textsubscript{K1} is the conductance of I\textsubscript{K1}, \(x_{K1=\infty}\) is a time-independent inward rectification factor, K\textsubscript{o} is extracellular K\textsuperscript{+} concentration and E\textsubscript{K} is the equilibrium potentials of K\textsuperscript{+}. S\textsubscript{K1} is a scaling factor used to simulate the change of I\textsubscript{K1} expression level.

I\textsubscript{f} has two ionic channels and could permeate Na\textsuperscript{+} and K\textsuperscript{+} respectively. I\textsubscript{f} could be described by

\[
I_f = I_{f,\text{Na}} + I_{f,K}
\]

\[
I_{f,\text{Na}} = S_f G_{f,\text{Na}} y (V - E_{\text{Na}})
\]

\[
I_{f,K} = S_f G_{f,K} y (V - E_K)
\]

where G\textsubscript{f,Na} and G\textsubscript{f,K} are maximal I\textsubscript{f,Na} and I\textsubscript{f,K} channel conductance, y is a time-independent inward rectification factor that is a function of voltage, E\textsubscript{Na}, E\textsubscript{K} are equilibrium potentials of Na\textsuperscript{+} and K\textsuperscript{+} channels respectively, and S\textsubscript{f} is a scaling factor used to simulate the change of I\textsubscript{f} expression level.

Formulations of other channel currents for the VM cell model are the same as those in the original model in Ref. (50).
Evaluating criterion of the pacemaking stability and ability

To analyse the effect of $I_{K1}$ and $I_f$ on pacemaking activity, we simulated the membrane potential under different current densities of $I_{K1}$ and $I_f$, with $I_{K1}$ being reduced systematically by from 60% to 100% (i.e., $I_{K1}$ density at -80 mV changed from 0.396 to 0 pA/pF while the $I_{K1}$ density in the original basal model is 0.99 pA/pF at -80 mV in I-V curve). The representative I-V relation curve under different inhibition of $I_{K1}$ is shown in S1A Fig. $I_f$ density was increased by from 0 to 10 folds with a basal value of -0.63 pA/pF at -80 mV in I-V curve (i.e., $I_f$ density changed from 0 to -6.3 pA/pF at -80 mV). The representative I-V relation curve under different incorporation of $I_f$ is shown in S1B Fig.

Two characteristics were used to quantify the state of membrane potentials generated by the ventricular pacemaker model: the continuity and validity of spontaneous APs. The continuity was used to quantify whether or not the automaticity of membrane potential could sustain with time; whilst the validity was used to characterize whether every automatic wave was biologically-valid or not. As such, we defined the following:

- $W$: a valid wave. An action potential whose wave trough was less than -20 mV and wave crest was more than 20 mV could be considered as a valid wave.
- $\alpha W$, $\alpha<1$: an incomplete wave.
- $R$: a resting period lasting 1000 ms.
- $W^n$: the concatenation of $n$ $W$'s.
- $(W R)$: the concatenation of $W$ and $R$.

As such, none pacemaking behaviour during the entire simulation period could be described as State-1:

$$R^n, n \in \mathbb{N}^+$$ (8)
Transient spontaneous pacemaking behaviour could be described as State-2:

\((W^m, R^n), m, n \in \mathbb{N}^+\) \hspace{1cm} (9)

Bursting pacemaking behaviour could be described as State-3:

\((W^{mi}, R^{ni})^M, i \in [1,M], M, m_i, n_i \in \mathbb{N}^+\) \hspace{1cm} (10)

Persistent pacemaking activity with periodically incomplete depolarization could be described as State-4:

\((W^m, \alpha W)^M, \alpha<1, m, M \in \mathbb{N}^+\) \hspace{1cm} (11)

Stable pacemaking activity could be described as State-5:

\(W^m, m \in \mathbb{N}^+\) \hspace{1cm} (12)

With regard to the pacemaking ability, when the pacemaking behaviour was stable, the cycle length (CL) under varied \(I_{k1}\) and \(I_f\) was calculated. The CL was defined as the averaged wavelength of pacemaking activity over a period of simulation of over \(4 \times 10^5\) ms, ensuring the accuracy of the computed CL. As the basal model was for VMs, a long simulation period was necessitated to achieve a completely stable pacemaking status and minimize the effect of the transition period.

**Characteristics of pacemaking during diastolic interval**

The length of the diastolic interval (DI) is an important measure to characterize the pacemaking ability. In this study, we defined that DI as the time interval between the time of maximum diastolic potential (MDP) (S6A Fig, \(t_1\)) and the time when the membrane potential reaches at -55 mV (i.e., around the activation potential of the \(I_{CaL}\)) (S6A Fig, \(t_2\)). The diastolic upstroke velocity during DI was defined as the change rate of the membrane potential, taking the following formulation:

\[
\text{diastolic upstroke velocity} = \frac{\text{MDP} - (-55)}{t_2 - t_1}
\] \hspace{1cm} (13)

The unit of diastolic upstroke velocity was V/s.
The main inward currents which helped to depolarize membrane potential during DI are $I_{Na}$, $I_{NaCa}$ and $I_f$. Their contribution can be described by an average integral during DI:

$$I_{in} = \frac{\int_{t_1}^{t_2} (I_{Na} + I_{NaCa} + I_f) \, dt}{t_2 - t_1}$$

(14)

Similarly, the main outward currents which held membrane potential at diastolic potential during DI are $I_{K1}$, $I_{NaK}$, $I_{Kr}$ and $I_{Ks}$, the integral of which can be described as:

$$I_{out} = \frac{\int_{t_1}^{t_2} (I_{K1} + I_{NaK}) \, dt}{t_2 - t_1}$$

(15)

**Supporting Information**

**S1 Fig.** The I-V curve of $I_{K1}$ and $I_f$ with different expression level.

$S_{K1}$ and $S_f$ are defined as scaling factors used to simulate the change of $I_{K1}$ and $I_f$ expression level. (A) The I-V curve of $I_{K1}$ with $S_{K1}$ of 1, 0.4, 0.1 that gives $I_{K1}$ densities in the I-V curve at -80 mV 0.99, 0.396 and 0.099 pA/pF respectively. (B) The I-V curve of $I_f$ with $S_f$ of 1, 5, 10 that gives $I_f$ densities in the I-V curve at -80 mV -0.63, -3.15 and -6.3 pA/pF respectively.

**S2 Fig.** $Na^+/Ca^{2+}$ exchange current ($I_{NaCa}$) of a transient pacemaking behaviour.

(A) $I_{NaCa}$ during the entire simulating period of 800 s with the current densities of ($I_{K1}$, $I_f$) at (0.297pA/pF, -1.89 pA/pF). (B) Expanded plots of $I_{NaCa}$ traces for the time course from $1.6 \times 10^5$ to $1.64 \times 10^5$ ms marked by the horizontal brackets with asterisks in (A).

**S3 Fig.** Transient spontaneous pacemaking behaviour.

Membrane potential (V) during the entire simulation period of 400 s with the current densities of ($I_{K1}$, $I_f$) at (0.178 pA/pF, -0.63 pA/pF).

**S4 Fig.** $Na^+/Ca^{2+}$ exchange current ($I_{NaCa}$) of a bursting pacemaking behaviour.
(A) $I_{\text{NaCa}}$ with the current densities of ($I_{K1}$, $I_f$) at (0.297 pA/pF, -2.52 pA/pF) during the entire simulating period of 800 s. (B) Expanded plots of $I_{\text{NaCa}}$ traces for the time course from $3.83 \times 10^5$ ms to $3.85 \times 10^5$ ms marked by the horizontal brackets with asterisks in (A).

**S5 Fig. Partial failure of spontaneous action potentials with different densities of $I_{K1}$.**

(A) Membrane potential ($V$) with the current densities of ($I_{K1}$, $I_f$) at (0.297 pA/pF, -3.15 pA/pF) during simulating time course from $3.6 \times 10^5$ to $3.7 \times 10^5$ ms. (B) Membrane potential ($V$) with the current densities of ($I_{K1}$, $I_f$) at (0.277 pA/pF, -3.15 pA/pF) during simulating time course from $3.6 \times 10^5$ to $3.7 \times 10^5$ ms.

**S6 Fig. Change of diastolic depolarising rate with the increase of $I_f$ density.**

(A) Definition of diastolic depolarising rate. MDP: maximum diastolic potential; $t_1$: the time when membrane potential is MDP; $t_2$: the time when potential arrives -55 mV (i.e., around the activation potential of the $I_{\text{CaL}}$). (B) Change of diastolic depolarising rate with the increase of $I_f$ density from 0 to -6.3 pA/pF when $I_{K1}$ density at -80 mV is at 0.05 pA/pF.

**S7 Fig. Prolonged cycle length under greater $I_f$ density.**

Membrane potential ($V$) with the current densities of ($I_{K1}$, $I_f$) at (0.05 pA/pF, -3.78 pA/pF) and (0.05 pA/pF, -5.04 pA/pF) (solid and dotted line respectively).

**S8 Fig. Model-dependence test – stable pacemaking behaviour.**

Membrane potential ($V$) with the current densities of ($I_{K1}$, $I_f$) at (0.198 pA/pF, -0.63 pA/pF), (0.178 pA/pF, -0.63 pA/pF) and (0.198 pA/pF, -0.756 pA/pF) (solid, dotted and dashed line respectively) based on a human ventricular myocytes model.

**S9 Fig. Model-dependence test – failed pacemaking activity.**

Membrane potential ($V$) with the current densities of ($I_{K1}$, $I_f$) at (0.198 pA/pF, -1.26 pA/pF) based on a human ventricular myocytes model.

**S1 Text. Model-dependence test.**
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References


