An ionic model for rhythmic activity in small clusters of embryonic chick ventricular cells

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ABSTRACT

We recorded the transmembrane potential in the whole-cell recording mode from small clusters (2-4 cells) of spontaneously beating 7-day embryonic chick ventricular cells after 1-3 days in culture, and investigated the effects of adding the blockers D-600, diltiazem, almokalant, and Ba++. Electrical activity in small clusters is very different from that in re-aggregates made up of several hundred embryonic chick ventricular cells: e.g. re-aggregates have tetrodotoxin-sensitive fast upstrokes, while small clusters have tetrodotoxin-insensitive slow upstrokes (maximum upstroke velocity ~100 V s$^{-1}$ vs. ~10 V s$^{-1}$). Based on our voltage- and current-clamp results and on data from the literature, we formulated a Hodgkin-Huxley type of ionic model for the electrical activity in these small clusters. The model contains a calcium current ($I_{Ca}$), three potassium currents ($I_{Ks}$, $I_{Kr}$, and $I_{Kl}$), a background current ($I_b$), and a seal-leak current ($I_{seal}$). $I_{Ca}$ generates the slow upstroke, while $I_{Ks}$, $I_{Kr}$, and $I_{Kl}$ contribute to repolarization. All of the currents contribute to spontaneous diastolic depolarization: e.g. removal of $I_{seal}$ increases the interbeat interval from 392 ms to 535 ms. The model replicates the spontaneous activity in the clusters, as well as the experimental results of applying blockers. Bifurcation analysis and simulations with the model predict that annihilation and single-pulse triggering should occur with partial $I_{Ca}$ block. Embryonic chick ventricular cells have been used as an experimental model to investigate various aspects of spontaneous beating of cardiac cells, e.g. mutual synchronization, regularity of beating, and spontaneous start-up and stopping of reentrant rhythms; our model now opens the possibility of investigating these topics through numerical simulation.

Index keywords/phrases: Pacemaker, seal-leak current, $I_{Kr}$ block, $I_{Ca}$ block, bifurcation analysis
INTRODUCTION

Spontaneous activity based on generation of the pacemaker potential (spontaneous phase-4 or diastolic depolarization) is not normally found in adult ventricular muscle \textit{in situ}; nor is it normally found in single cells freshly isolated from adult ventricular muscle. In contrast, early enough during development, ventricular muscle (or areas of the heart destined to eventually become ventricular muscle) can beat spontaneously (1; 97). Spontaneous electrical activity can also be seen in single cells and small clusters of cells isolated from the embryonic chick ventricle (10; 17; 26; 49; 51; 78; 95), the embryonic mouse ventricle (117), and the neonatal rat ventricle (86).

After a couple of days in culture, the electrical activity in an isolated embryonic chick ventricular cell, a small cluster of a few such cells, or a sparse monolayer is very different from that seen \textit{in situ} or in a re-aggregate made up of hundreds or thousands of cells isolated from the ventricle. For example, when trypsin-dispersed ventricular cells from 7-day-old embryonic chick hearts are used, the upstroke velocity is much lower in single cells, small clusters of cells, and sparse monolayers (17; 49; 51; 95) than in re-aggregates (14; 16; 19) or in the intact ventricle (19; 97; 98; 118). Spontaneous beating can be abolished by adding tetrodotoxin (TTX), a blocker of the fast inward sodium current, to the medium bathing re-aggregates made up of trypsin-dispersed 7-day ventricular cells (16; 70), but spontaneous activity continues in single cells and monolayers (57; 70; 81; 95). However, spontaneous activity in some TTX-insensitive preparations can be abolished by adding either of the Ca$^{++}$-channel blockers D-600 or diltiazem (49; 51).

We decided to carry out an experimental study to characterize the electrical properties of spontaneously beating clusters of cells isolated from the 7-day embryonic chick ventricle, and to
then assemble a mathematical model of this activity. The goal is to use this model to investigate phenomena seen in experiment, such as the irregularity of beating in small clusters (10), the mutual synchronization of pairs of cells (18), phase-resetting and phase-locking (9; 11; 52; 53), current-pulse-induced annihilation of spontaneous activity (90; 94), and the spontaneous start-up and stopping of spiral-wave reentrant motions in monolayers (6). While ionic models of re-aggregates of embryonic chick atrial cells exist (9; 13; 52; 89; 90), we are not aware of any models of small clusters of isolated embryonic chick ventricular cells.
METHODS

Cell Isolation

Ventricular myocytes were isolated from embryonic chick hearts using techniques previously described (Ref. 50, with modifications described in Ref. 79). The hearts of 7-day-old embryos were removed, and the ventricles chopped off, minced, and transferred to flasks containing 0.25% trypsin (bovine pancreas, Boehringer Mannheim, Deisenhofen, Germany) in a nominally Ca\(^{++}\) and Mg\(^{++}\)-free Hank’s balanced salt solution (HBSS) of composition (mM): NaCl 137, KCl 5.4, Na\(_2\)HPO\(_4\) 0.34, KH\(_2\)PO\(_4\) 0.44, NaHCO\(_3\) 4.2, glucose 5 (pH = 7.4). The flasks were placed into a shaker bath at 37 °C for 7 minutes. The resulting cell suspension was gently agitated with a pipette and filtered through a 100 µm mesh. HBSS, supplemented with fetal calf serum (final concentration 5%), was then added to stop trypsin activity. The cell suspension was centrifuged at ~100 g for 5 min at 4 °C, the supernatant discarded, and the cell pellet resuspended in fresh trypsin-free HBSS. The centrifugation and resuspension processes were then repeated. The solution was centrifuged for a third time and resuspended in cell culture medium (M199 (Sigma) supplemented with 4% fetal calf serum, 2% horse serum, and 0.7 mM glutamine; pH = 7.4) to yield a cell density of 5 \times 10^5\text{ cells ml}^{-1}.

The cell suspension was then aliquoted out into non-adhesive plastic culture dishes which were incubated at 37 °C in a water-saturated atmosphere of 95% air and 5% CO\(_2\). In order to obtain small clusters of cells, 0.6 ml aliquots of cell suspension were removed after 0.5-2 days and placed into the lumen of flexiPERM silicone rings (Heraeus, Hanau, Germany), which were attached to microscope-slide cover slips. Cover slips with attached flexiPERM rings were placed in cell culture dishes (Greiner, Austria) and stored in the incubator. This procedure allowed the myocytes to adhere to the glass surface where they could divide and form small clusters of cells.
However, we can offer no guarantee that a particular cluster is made up entirely of cells that divided in culture.

Experiments were performed 2-36 h after plating, on clusters containing 2 to 4 cells. Recordings were made in the whole-cell mode from clusters that were seen to be spontaneously beating before patching. We use clusters of a few cells, rather than single cells, because we were unable to obtain recordings of spontaneous activity from single cells (it is also easier to successfully impale a cell in a cluster rather than a single cell if a conventional sharp microelectrode is used (17;26)). In addition, the effect of the seal-leak current (see below) on spontaneous activity is expected to be considerably smaller for a cluster than for a single cell (but see Ref. 78). For electrophysiological recording, the cover glass with attached myocytes was used to form the bottom of the experimental chamber, which was placed on the stage of an inverted microscope (Zeiss, Axiovert). The experimental chamber was perfused with extracellular solution (composition in mM: NaCl 137, KCl 5.4, CaCl2 1.8, MgCl2 1.1, NaHCO3 2.2, NaH2PO4 0.4, Na-HEPES 10, and glucose 5.6, pH adjusted to 7.4 with NaOH) at 36-37°C with a flow rate of 1.5 ml/min.

Electrophysiological Recording

The transmembrane potential was recorded using the whole-cell recording mode of the patch-clamp technique. Patch pipettes (resistance ~2 MΩ) were pulled from glass capillary tubes, and filled with pipette solution (composition in mM: KCl 110, K2-ATP 4.3, MgCl2 2, CaCl2 1, EGTA 11, K-HEPES 10, adjusted with KOH to a pH of 7.4; estimated free [Ca^{2+}] < 10^{-8} M). Electrode potentials were zeroed before seal formation. After breaking the patch, the transmembrane potential was recorded with a List EPC-7 amplifier (List, Darmstadt Germany). The membrane
capacitance was measured by integrating the capacitive transient in response to a voltage-clamp step from –50 to –60 mV. Following capacity compensation, series resistance compensation was carried out by turning up the series-resistance compensation control (which controls the amount of positive feedback) to just below the value where ringing in the current monitor signal would occur. Usually, compensation could be made for more than 50% of series resistance. For generation of voltage-clamp protocols and for recording voltage and current, a personal computer equipped with pCLAMP vers. 5.5.7 software (Axon) and a DigiData 1200 interface (Axon) were used. Signals were also digitized at 44 kHz, pulse-code modulated, and stored on video-cassette tape for offline analysis. The signal was played back, sampled at 1 kHz (Axotape, Axon Instruments), and stored on a disk-file for computerized analysis.

Action Potential Parameters

Several parameters are measured to characterize spontaneous electrical activity. Interbeat interval (IBI) is the time between consecutive crossings of 0 mV on the upstroke of the action potential. The maximal diastolic potential (MDP) is the most negative voltage recorded during an action potential, while the overshoot potential (OS) is the most positive. The action potential amplitude (APA) is the difference between MDP and OS. Action potential duration (APD) is the time from the crossing of 0 mV on the upstroke to the time of either 50% repolarization (APD_{50}) or 100% repolarization (APD_{100}). The diastolic depolarization rate (DDR) is the slope of the chord joining the point where (MDP + 1 mV) is crossed and the point 70 ms later (108; 114). The maximum rate of rise of the upstroke ($V_{\text{max}}$) is computed using the greatest voltage difference occurring between two consecutive samples on the upstroke (sampling interval = 1 ms).
Simulation Methods

Numerical integration of the Hodgkin-Huxley-type ionic model was carried out using a forward Euler scheme, with the transmembrane potential \( V \) at time \( t + \Delta t \) being calculated from

\[
V(t + \Delta t) = V(t) - (\Delta t / C_m) \sum I_i(t),
\]

where \( C_m \) is the membrane capacitance and the \( I_i \) are the individual ionic currents described below (using a fourth-order Runge-Kutta integration scheme produced virtually superimposable results). With our choice of time step \( (\Delta t = 0.1 \text{ ms}) \), the change in voltage from time \( t \) to time \( t + \Delta t \) was kept to less than 1 mV. The value of each activation or inactivation variable \( \xi_i \) at time \( t + \Delta t \) was obtained from its value at time \( t \) using the analytic formula

\[
\xi_i(t + \Delta t) = \xi_i(\infty) - \left[ \xi_i(\infty) - \xi_i(t) \right] e^{-\Delta t / \tau_i},
\]

where \( \xi_i(\infty) \) is the steady-state or asymptotic value of \( \xi_i \) at \( V(t) \) and \( \tau_i \) is the time-constant of \( \xi_i \) at \( V(t) \). L'Hôpital's rule was used to calculate the value of \( \alpha_n \) when \( V \) came to within \( \pm 0.1 \) mV of the value producing an indeterminate form. The numerical integration routine was written in C and all variables were double precision (~16 significant decimal places).

Bifurcation analysis was carried out using AUTO, as incorporated in XPPAUT (25). The model equations file for use with XPPAUT is available as Supplemental Material.

Formulation of the Model

Our model consists of six currents: a slow inward \( \text{Ca}^{++} \) current \( (I_{Ca}) \), a slow delayed \( K^+ \) current \( (I_{Ks}) \), a rapid delayed rectifier \( K^+ \) current \( (I_{Kr}) \), an inward rectifier \( K^+ \) current \( (I_{K1}) \), a linear time-independent background current \( (I_b) \), and a linear non-specific seal-leak current generated by the leakage of ions through the gigaohm-seal of the recording pipette \( (I_{seal}) \). We now give the
rationale for including each of these currents and for the particular formulation that we employ for each of these currents, as well as reasons for using a “first-generation” rather than a “second-generation” model.

**Difficulties with “second-generation” models.** In the earlier Hodgkin-Huxley-type ionic models of cardiac cells, all the concentrations of the various ionic species were held fixed, so that no provision had to be made for pumps and exchangers to regulate these concentrations. We refer to models that incorporate both of these refinements as “second-generation” models, in contrast to the earlier “first-generation” models. We formulate our model below as a more primitive first-generation model because there are two major problems with the more physiologically realistic second-generation models: (i) drift, with very slow long-term trends in some of the variables, particularly some ionic concentrations (3; 21; 23; 24; 37; 45; 103; 116), and (ii) degeneracy, with non-uniqueness of equilibrium solutions such as steady-states and limit cycles (23; 24; 30; 37; 102).

Drift has been dealt with in several ways: (i) finely adjusting parameters to achieve flux-balance across the membrane (21); (ii) adding an electroneutral Na\(^+\) current of a precise size to produce stability of concentrations (74); (iii) keeping track of the stimulus current in a paced quiescent cell (37; 45); and (iv) making certain ionic concentrations fixed (3; 55; 103; 121). It is not clear whether the first two strategies are robust, since making a change to some parameter in the model might require further fine adjustment of the stabilizing parameters (this is reminiscent of a neutrally stable equilibrium). The third strategy is of course of no use in an unpaced pacemaker cell. The fourth strategy defeats, at least in part, the initial intent in formulating the model as a second-generation model: e.g. when internal and external Na\(^+\), K\(^+\), and Ca\(^{++}\)
concentrations are held constant, the sodium-potassium pump current and the sodium-calcium exchange current are then effectively background currents, and one is left essentially with a first-generation model, in which activity-dependent effects due to changes in certain ionic concentrations are not manifest.

The other major problem noted with second-generation models is degeneracy. It was found in second-generation models of several different types of cardiac cells that the system of differential equations could be rewritten as a system of \(N-1\) equations in \(N\) unknowns (30; 102). The Jacobian is then singular and there is a continuum of equilibrium points (rather than isolated equilibrium point(s)), so that, e.g., the resting potential of a quiescent system depends on the initial conditions (30; 102). A similar finding of degeneracy holds for the limit cycle that corresponds to spontaneous activity (24; 30). It has been suggested that the original \(N\)-variable fully-differential model should be recast as a differential-algebraic system, with the equation controlling voltage being algebraic and the remaining \(N-1\) equations being differential (23; 24; 30; 37; 45; 102). In one report in a sinoatrial (SA) node model in which the differential-algebraic formulation was used, it was stated that there was no long-term drift (23). In earlier work in which drift was abolished by making some ionic concentrations fixed, this also had the unintended benefit of removing the degeneracy, thus allowing the bifurcation analysis of isolated equilibria using continuation techniques (Ref. 103; see also Refs. 55, 121).

Finally, in situations such as ours in which cells are studied using patch micropipettes, a more realistic model of the experimental situation is one in which internal concentrations are kept fixed, due to \(\text{Ca}^{++}\)-buffering with EGTA and dialysis of the cell contents of a very small cell-volume against the much larger pipette volume (Ref. 55; see also Ref. 121). Making concentrations fixed then also has the side-benefit of removing degeneracy and drift. Given all
the above uncertainties and complications, and given that very little information is available
about the control of intracellular ionic concentrations in our cells, we decided to stick with a
first-generation model, as have some authors of other quite recent studies (e.g. Ref. 3).

_Capacitance._ In what follows, unless stated otherwise, the term ventricular (resp. atrial) cell will
refer to an embryonic chick ventricular (resp. atrial) cell, and the term n-day will refer to a cell
isolated from the embryo following n days of incubation. Since the capacitance of a single 7-day
ventricular cell in our lab is 8-9 pF, we set the capacitance in our 3-cell model cluster to 25.5 pF.
Our value of the single-cell capacitance agrees with that reported in several whole-cell voltage-
clamp studies employing single 7-day ventricular cells (e.g. 5-10 pF (29); 4-7 pF (44)). We
model the cluster, which is a mutually synchronized population oscillator, as an isopotential
preparation (27).

_Ca\textsuperscript{++} current (I_{Ca})._ The slow inward calcium current has been described in 7-day re-aggregates
of ventricular cells (72), small clusters of ventricular cells (28; 79), single ventricular cells (13;
28; 42; 44), and at the single-channel level (43; 68). In one lab, $I_{Ca,L}$ was found in 11 out of 12 6-
day ventricular cells studied, with $I_{Ca,T}$ being found in the one remaining cell (13). In another
lab, only $I_{Ca,L}$ channels were found in 7-day ventricular cells (68). In a third study, both currents
were found, but only 45% of 7-day ventricular cells had an $I_{Ca}$ that was clearly separable into
$I_{Ca,L}$ and $I_{Ca,T}$ components based on the voltage threshold for activation (44). In these cells, $I_{Ca,T}$
is half-inactivated at $-49$ mV, while $I_{Ca,L}$ is half-inactivated at $-27.5$ mV. In the 55% of cells
having a non-separable $I_{Ca}$, half-inactivation occurs at $-42.8$ mV.

We have chosen to use a non-separable description of $I_{Ca}$ in our model:
$I_{Ca} = g_{Ca} df(V - E_{Ca})$, 

where $g_{Ca}$ is the maximal conductance, $d$ is the activation variable, $f$ is the inactivation variable, and $E_{Ca}$ is the reversal potential, which we set to 40 mV based on our own voltage-clamp results (79). The equations governing $d$ and $f$ are given in the Appendix.

The steady-state inactivation curve ($f_x$) of the non-separable current in 7-day ventricular cells, which lies between the inactivation curves for $I_{Ca,L}$ and $I_{Ca,T}$, is taken from Fig. 9 of Ref. 44. Since the steady-state activation curve ($d_x$) of the non-separable current was not reported, we take it to lie between the $I_{Ca,L}$ and $I_{Ca,T}$ activation curves of Fig. 10 of Ref. 44, and to have an intermediate slope factor. We have chosen the values of parameters so that the $d_x$ curve lies closer to that of $I_{Ca,L}$ than to $I_{Ca,T}$, in order that the peak-current current-voltage (IV) curve has a maximum at about 0 mV, a voltage close to the experimental value for the non-separable current (44). Figure 1A shows the steady-state activation and inactivation curves.

There are no systematic reports of the time-constants of activation ($\tau_a$) and inactivation ($\tau_f$) of the non-separable current. We therefore take the expressions unchanged from Ref. 66, which describes $I_{Ca,L}$ in a 37°C guinea-pig model. The $\tau_a$ curve (Fig. 1B) has a typical bell-shape, while the $\tau_f$ curve (Fig. 1B) has the U-shape that is seen in mammalian cells and in 7-day ventricular re-aggregates (72).

We have set the maximal conductance ($g_{Ca}$) so that $\dot{V}_{\text{max}}^i$ is 9.5 V s$^{-1}$ in the model, which is close to our mean experimental result (8.5 V s$^{-1}$). The peak-current IV curve then has a maximum value of $\sim$30 pA/pF at about 0 mV (Fig. 1C), which is within the range seen in our cells (79) (see also Fig. 4C of Ref. 44 and Fig. 1 of Ref. 13, scaling for differences in capacitance and temperature ($Q_{10} = 3$ for peak current amplitude for guinea-pig ventricular cells (7))).
1D shows that the current traces from a voltage-clamp protocol in the model are similar, both in magnitude and in time-course, to the corresponding experimental traces in Ref. 79 (see also Fig. 7B of Ref. 44, scaled for temperature and capacitance).

Since internal Ca\(^{++}\) is buffered by our pipette solution, we do not include Ca\(^{++}\)-dependent inactivation of \(I_{Ca}\) (13) in our description; instead, we use only voltage-dependent inactivation to reproduce the time-course of the inactivation process that is seen experimentally (44). Nor do we include the two time-constants of inactivation seen in 7-day ventricular re-aggregates (72).

**Delayed K\(^{+}\) currents (\(I_{Ks}\) and \(I_{Kr}\)).** Delayed K\(^{+}\) currents have been described in re-aggregates of ventricular cells (13; 14; 88) and atrial cells (9; 88; 89), in small clusters of ventricular cells (79), as well as in single ventricular (8) and atrial (12) cells. These currents have also been observed at the single-channel level in ventricular cells (8; 67).

Two components of the delayed current, initially termed \(I_{s1}\) and \(I_{s2}\), have been seen in atrial re-aggregates (89). These two currents correspond to those more recently termed \(I_{Kr}\) and \(I_{Ks}\), respectively, in isolated adult mammalian ventricular cells. The \(I_{s2}\) or \(I_{Ks}\) current has been described in 7-day ventricular re-aggregates (14; 15), in single ventricular cells (8), and in small clusters of such cells (79). However, while \(I_{s1}\) is robust in experiments carried out using sharp microelectrodes on atrial (89) and ventricular (13) re-aggregates, it has not been seen in whole-cell clamp experiments carried out using patch pipettes on isolated 7- to 10-day ventricular cells (8) or on single 6- to 11-day atrial cells or small clusters of such cells (12). This might be due to an intrinsic absence of the current (e.g. there is good evidence for cell-cell contact-dependent regulation of expression of two different K\(^{+}\)-channels in cultured adult rat ventricular cells (35)) or to rapid washout of this current in the whole-cell ruptured-patch recording mode. Indeed, it
has been suggested that the main difference in the action potentials of atrial re-aggregates and small clusters of atrial cells can be accounted for by the absence of $I_{Ks1}$ in the latter (Fig. 9 of Ref. 12; Fig. 17 of Ref. 13). In contrast to the above reports, in our 7-day ventricular clusters the envelope-of-tails test shows the presence of two components (79), and application of the specific $I_{Kr}$ blocker almokalant (113) removes the more rapidly deactivating component of the tail current. In addition, almokalant produces changes in the action potential consistent with $I_{Kr}$ block, as we describe below. At the single-channel level, a $K^+$-channel that activates over a voltage range similar to that over which $I_{Kr}$ activates has been described (8). We thus incorporate both the $I_{Ks}$ and $I_{Kr}$ components into our model.

For $I_{Ks}$, we use the formulation previously used in an atrial re-aggregate model (52):

$$I_{Ks} = g_{Ks} n(V - E_{Ks}),$$

where $n$ is the activation variable. We set $E_{Ks} = -75$ mV, the reversal potential found in our cells (Ref. 79; see also Fig. 1C of Ref. 8). This value is depolarized to the equilibrium potential for $K^+$ of $-85$ mV, computed from the Nernst equation at a temperature of 37 °C, an external $[K^+]$ of 5.4 mM, and a pipette $[K^+]$ of 129 mM. This relatively depolarized value of $E_{Ks}$ has been found in many studies on $I_{Ks}$, and has been attributed to a slight permeability of the channel to $Na^+$ (e.g., Refs. 65, 121). In obtaining the formulae for the rate-constants $\alpha_n$ and $\beta_n$ (see Appendix), we have first divided the original equations (52) by a factor of 3, in order to obtain a $n$-curve consistent with the experimental values in single ventricular cells at room temperature (Fig. 2C of Ref. 8), and then multiplied $\alpha_n$ and $\beta_n$ by a factor of two, in correspondence with the reported $Q_{10}$ (111), to obtain values appropriate for our experimental temperature of 36-37 °C (Fig. 2B). The maximal conductance, $g_{Ks}$, was set to give a fully-activated IV curve (Fig. 2C) similar to that seen experimentally (Fig. 1C of Ref. 8), scaled for capacitance and temperature.
The steady-state activation curve (Fig. 2A) and the voltage-clamp currents (Fig. 2D) are similar to those previously reported from our lab at 37°C (79), as well as to those of Ref. 8 (compensated for temperature).

For $I_{Kr}$, we use the formulation:

$$I_{Kr} = g_{Kr} s z(V)(V - E_{Kr}),$$

where $s$ is the activation variable, and we introduce $z(V)$ to provide inward rectification, so that the fully-activated IV relationship (Fig. 3C) is similar in shape to that seen in atrial re-aggregates (Fig. 8C in Ref. 89). Using our form of $z(V)$ (see Appendix and Fig. 3A) gives more current at depolarized voltages than the fit originally used in that figure, which corresponds to the fact that we observe a maintained almokalant-sensitive current at positive voltages (see Results below). The function $z(V)$ represents the very rapid inactivation described for the HERG subunit of the $I_{Kr}$ channel (see, e.g., Refs. 92, 93) and for $I_{Kr}$ in single SA node cells (54; 76). The reversal potential, $E_{Kr}$, was set to –81 mV, based on observations in our lab (this closeness of $E_{Kr}$ to the Nernst potential of –85 mV for $K^+$ is characteristic of $I_{Kr}$ in many other cardiac preparations). Our setting of the maximal conductance $g_{Kr}$ yields a maximum value of the fully activated current (Fig. 3C) comparable to that seen in atrial re-aggregates (89).

The gating variable $s$ is governed by equations (see Appendix) slightly modified from those in Table I of Ref. 89, so as to fit more closely the data points in their Figs. 5A and 5B (the original equations in their Table I do not give the fitted curves shown in those two figures). With these modifications, our $s_{\infty}$ and $z_{1}$ curves (Figs. 3A and 3B) are very close to the data in Figs. 5A and 5B of Ref. 89. A voltage-clamp protocol (Fig. 3D) gives currents similar to those of Fig. 4 of Ref. 89. We use only one time-constant of activation; in SA node cells, two time constants of activation of $I_{Kr}$ have been described (76).
**Inward rectifier K⁺ current (I_{K1}).** Re-aggregates of 7-day ventricular cells (14; 15), small clusters of 7-day ventricular cells (79), and single 7-day ventricular cells (4) have IV curves that show marked inward rectification at very negative potentials, due to the presence of the inward rectifier K⁺ current $I_{K1}$. There is also evidence for this current at the single-channel level in 7-day ventricular cells (67). We have thus included an $I_{K1}$ component in our model (Fig. 4), taking the formulation from a guinea-pig ventricular cell model (65):

$$I_{K1} = g_{K1} K1(V - E_{K1}),$$

where we set $E_{K1} = -81$ mV, which is, as commonly observed, slightly depolarized to the calculated Nernst potential for K⁺ (−85 mV). While a time-independent description of $I_{K1}$ (i.e. $K1=K1_{\infty}$) was originally used (65), we employ the time-dependent description to allow us to later formulate a model with stochastic gating kinetics to investigate beat-to-beat fluctuations in IBI (Krogh-Madsen T, Schaffer P, Skriver AD, Taylor LK, Koidl B, and Guevara MR, in preparation). However, the time constant of this current is so small ($\tau_{K1} < 0.2$ ms over the operative range of voltage — see Fig. 4B) that the current is virtually identical in the time-dependent and the time-independent descriptions. The maximal conductance $g_{K1}$ is reduced from the guinea-pig value to reflect the smaller size of $I_{K1}$ earlier in development (15; 41). The steady-state IV curve for the total current (curve in Fig. 7A) is then very flat between −70 mV and −30 mV, which agrees with our experimental results (asterisk symbols in Fig. 7A). The inwardly rectifying $I_{K1}$ IV curve (Fig. 7C) is the main contributor to the positive slope of the total-current IV curve at very hyperpolarized potentials (Fig. 7A), and is similar to the Ba⁺⁺-sensitive current at hyperpolarized potentials (4; 79).
**Background current** ($I_b$). In addition to $I_{K1}$, which is outward at potentials depolarized to −81 mV, there is inward background current present in 7-day ventricular re-aggregates (14). This component has been modeled as a sodium current:

$$I_b = g_b (V - E_b)$$

where $E_b = 40$ mV, and $g_b$ is obtained from Fig. 11 of Ref. 14, scaled for capacitance. Figure 7D gives the IV relationship for this linear current.

**Seal-leak current** ($I_{seal}$). It has been pointed out that the seal-leak current flowing through the pipette gigaohm-seal can be appreciable with respect to the total current flowing during diastolic depolarization for a very small cell with a high input resistance (20). We therefore add a non-specific seal-leak current, $I_{seal}$, in our model:

$$I_{seal} = g_{seal} (V - E_{seal})$$

with $g_{seal}$ corresponding to a nominal seal-leak resistance of 5 GΩ (20) and $E_{seal} = 0$ mV. Figure 7D gives the IV relationship of $I_{seal}$.

**Currents not included in the Ionic Model**

**Fast inward Na⁺ current** ($I_{Na}$). There is voltage-clamp evidence for the existence of $I_{Na}$ in re-aggregates of 7- to 11-day ventricular cells (22; 72), in single 2- to 18-day ventricular cells (29; 40; 82; 83; 112), and at the single-channel level in 7-day ventricular cells (64; 112). Voltage-clamp studies on 7-day ventricular clusters in our lab show that there is indeed a fast inward current present upon a depolarizing clamp-step from potentials more hyperpolarized than about −60 mV. However, our clusters have a very low upstroke velocity (8.5 V s⁻¹), suggesting that $I_{Na}$ might not contribute appreciably to the upstroke phase, especially since Ca++-channel blockers
abolish spontaneous activity (Fig. 9 A,B). The MDP (−60 mV in the clusters, −67 mV in the model) is sufficiently depolarized to essentially render $I_{Na}$ fully inactivated, since the foot of the $I_{Na}$ steady-state inactivation curve lies at about −50 to −60 mV in 7-day ventricular re-aggregates (22) and in 7-day ventricular cells (29; 82). Indeed, adding $I_{Na}$ to our model, based on the conductance and the activation and inactivation curves from single 7-day ventricular cells (29) and the time constants from 11-day re-aggregates (22), produces a slight increase in $V_{\text{max}}$ from 9.5 V s$^{-1}$ to 10.2 V s$^{-1}$. In contrast, re-aggregates made of trypsin-dissociated 7-day ventricular cells have a TTX-sensitive upstroke velocity of 120 V s$^{-1}$ in 1.3 mM [K$^+$] and 91 V s$^{-1}$ in 4.5 mM [K$^+$], presumably due to the more hyperpolarized MDP of about −90 mV and −76 mV respectively (16; 19).

$I_{Na}$ can also be involved in generating the pacemaker potential. There is indeed evidence that $I_{Na}$ is necessary in some isolated embryonic cells for the generation of spontaneous activity. After 24 hours in culture, 37% of single cells dissociated using trypsin from 7-day hearts (whole hearts, atria, or ventricles) stop beating after the addition of $10^{-5}$ g ml$^{-1}$ TTX (70), showing that $I_{Na}$ is crucial in generating spontaneous activity in these cells. However, the percentage of TTX-insensitive cells increases with time spent in culture: 43% at 4 hours, 64% at 24 hours, and 100% at 48 hours (57). In contrast, re-aggregates made of trypsin-dissociated 7-day ventricular cells and cultured for 24-72 hours stop beating when exposed to TTX (16; 70). This difference in the response to TTX almost certainly indicates the importance of cell-to-cell interactions (19; 69).

In newborn rabbit SA node, a TTX-sensitive current, which gradually disappears within the first 30 days post-natum, has been implicated in the generation of diastolic depolarization (2). This contribution is not due to the $I_{Na}$ window-current, but rather is a consequence of relatively slow inactivation of $I_{Na}$ in the pacemaker range of potentials. Recently, modelling work has suggested
a role for a persistent component of a mutated $I_{Na}$ channel in the generation of diastolic depolarization in LQT3 syndrome (105).

A third role for $I_{Na}$ is maintenance of the plateau of the action potential, e.g. via a window-current contribution. Application of TTX results in a shortening of the APD in some 7-day ventricular cells before they stop beating (64). Single $I_{Na}$ channels occasionally (on 1/100 beats) stay open throughout the action potential plateau (64), and burst for $> 150$ ms in 16% of trials during a long voltage-clamp step (40). Since these long-openings do not persist into diastole (Fig. 1 of Ref. 64), they would not contribute to diastolic depolarization. Incorporating our standard Hodgkin-Huxley type of $I_{Na}$ into the model, as described above, results in APD$_{50}$ increasing by only 3 ms and APD$_{100}$ increasing by only 5 ms.

**Pacemaker current ($I_f$).** The pacemaker current has been reported in ventricular re-aggregates (4; 14; 15; 87; 88), as well as in single atrial and ventricular cells and small clusters of such cells (4; 5; 85). The mid-point of the activation range of $I_f$ is $\sim 30$ mV more negative in single ventricular cells and in small clusters than in re-aggregates, with the foot of the activation curve of this hyperpolarization-activated current lying at $-70$ mV in the single cells and small clusters (5). In our clusters, we find $I_f$ activated at potentials negative to $-70$ mV (79). Based on the conductance, reversal potential, kinetics, and activation curve described in Ref. 5, we find that adding $I_f$ to the model causes only a very slight decrease in IBI from 392 ms to 390 ms. The MDP in our cells is hence too depolarized for $I_f$ to activate and contribute significantly to pacemaking activity, and so we do not include it in our model.

**Transient outward current ($I_{to}$).** While the size of the transient outward current increases with development, the number of isolated ventricular cells possessing $I_{to}$ is extremely low: 7 of $\sim 300$
cells at 3 days, 5 of ~200 cells at 10 days, and 5 of ~100 cells at 17 days (84). At the single-channel level, an early outward channel appeared in only 1/80 patches from 7-day ventricular cells (67). \(I_{to}\) was not seen in single atrial cells (12) nor was it “clearly observed” in 7- to 12-day atrial re-aggregates (9). Moreover, in our own voltage-clamp experiments we have also not found any evidence of \(I_{to}\) in the clusters. In the SA node, the \(I_{to}\) current density is smaller in cells with a smaller capacitance (58) (but see Ref. 109). Given the above facts, we do not include \(I_{to}\) in our model.

**Cl\(^-\) current** (\(I_{Cl}\)). A time-independent Cl\(^-\)-sensitive current has been described in isolated 11-day-old ventricular cells (63). We do not include \(I_{CI}\) explicitly in the model, but consider it to be lumped in as a component of the background current, \(I_b\).

**\(Na^+\)-\(K^+\) pump current** (\(I_{NaK}\)) and **\(Na^+\)-\(Ca^{++}\) exchanger current** (\(I_{NaCa}\)). Currents provided by ion pumps and exchangers — e.g. the sodium-potassium pump current, the sodium-calcium exchanger current, and the calcium pump — also contribute to the transmembrane potential. We employ a first-generation model, which does not have a \(Na^+\)-\(K^+\) pump, a \(Na^+\)-\(Ca^{++}\) exchanger, a \(Ca^{++}\) pump, internal \(Ca^{++}\) dynamics, and variable ionic concentrations. However, both \(I_{NaK}\) and \(I_{NaCa}\) are present in 7-day ventricular cells. Although these currents are included in several recent ionic models of cardiac tissue, we do not include them in our model, since this would result in a second-generation model.

In our lab, 10 \(\mu\)M ouabain has been used to block \(I_{NaK}\) in 7- to 10-day ventricular cells or small clusters of such cells (48). After one minute of superfusion, the IBI first decreases, due to an increase in DDR, followed by a gradual fall in OS and MDP, a rise in APD, and an increase in
IBI, so that within a few minutes spontaneous activity ceases, with the membrane coming to rest at about –30 mV (sharp microelectrodes were used in these experiments, so that artefact due to dialysis and current rundown is minimal). A similar result is seen in 11-day cells cultured as a confluent layer or polystrand, except that the initial effect is seen immediately, presumably due to the use of a perfusion system with rapid perfusate change-over (half-time of ~5 seconds), with the membrane coming to rest at about –40 mV (Fig. 1 of Ref. 39). It has been estimated that $I_{NaK}$ contributes 0.35 pA/pF at –70 mV in spontaneously beating 11-day re-aggregates (100). In another report on 11-day re-aggregates at an internal $[Na^+]$ of 41 mM, the ouabain-sensitive current amounts to 1.7 pA/pF and is independent of voltage over the operating range effective in our clusters (–60 to +20 mV) (Fig. 1 of Ref. 99). This value scales down to ~0.5 pA/pF at an internal $[Na^+]$ of 10 mM (Fig. 7 of Ref. 99), which agrees with SA node modeling work (54).

In our model, we can thus mimic the effect of blocking the electrogenic component of $I_{NaK}$ by adding a constant depolarizing current of 10.2 pA (i.e. 0.4 pA/pF). This has the effect of decreasing IBI from 392 ms to 337 ms, and depolarizing the MDP from –67 mV to –61 mV, both of which effects are seen immediately upon block of $I_{NaK}$ in experiment (Fig. 1B of Ref. 39). The fact that the electrogenic component of $I_{NaK}$ is removed within a few seconds of the start of block (Fig. 1 of Ref. 100) implies that some secondary change must be responsible for the cessation of activity that occurs some minutes later (Fig. 1 of Ref. 39). The most likely candidates are the rises in internal $[Na^+]$ and $[Ca^{++}]$ that are seen following $I_{NaK}$ block, which occur with a time-course on the order of minutes (38; 39).

There is clear evidence for $I_{NaCa}$ in 11-day ventricular re-aggregates (100) and in 11-day cells cultured as a confluent layer or polystrand (38). The maximum amplitude of $I_{NaCa}$ in embryonic chick cells is about the same as in guinea-pig ventricular cells (62). However,
because of the difficulties inherent in interpreting experiments attempting to characterize $I_{NaCa}$, many of its fundamental properties (e.g. stoichiometry) remain uncertain in embryonic chick ventricular cells (62). The extent to which $I_{NaCa}$ is involved in generating diastolic depolarization in SA node cells is controversial (56) and is very different in different SA node models, to the extent that while $I_{NaCa}$ is inward in most models, it is outward in at least one (see Fig. 7 of Ref. 54). In addition, in a model of spontaneous activity induced by suppression of $I_{K1}$ in guinea-pig ventricular cells, $I_{NaCa}$ is inward during the pacemaker potential (91). We are not aware of any studies of the calcium pump in embryonic chick ventricular cells.

Given the above problems, as well as other problems detailed earlier involving degeneracy and drift in models where pumps and exchangers have been added, we have chosen not to include these currents in our model. Rather, $I_{NaK}$ can be thought of as being incorporated into the background current, while the time-course of $I_{Ca}$ in our model resembles very much the action-potential-clamp record (i.e. sum of $I_{Ca}$ and $I_{NaCa}$ and any Ca++-activated currents) obtained in the SA node when calcium entry is blocked (120).

Other currents. Other currents, such as $I_{st}$ (a sustained inward current, carried by Na+, insensitive to TTX, but sensitive to Ca++-channel blockers (Ref. 107, but see Ref. 71)) and $I_{K(Ca)}$ (a Ca++-activated K+ current, for which evidence is only found in the perforated-patch configuration (120)) exist in the SA node. Since there are no reports of these currents in ventricular cells, we do not include them in our model.
RESULTS

Spontaneous Activity

Figure 5 shows action potentials recorded from 17 small clusters. It is clear that there is considerable cluster-to-cluster variability (see also Ref. 17). Figure 6A shows a recording of the transmembrane voltage ($V$) obtained from one small cluster (no. 7 in Fig. 5), while Fig. 6B shows the phase-plane trajectory, in which the rate of change of $V$ ($\dot{V}$) is plotted vs. $V$. For this particular cluster, the mean values of the parameters, averaged over 100 cycles of activity, were: $\text{IBI} = 458 \text{ ms}$, $\text{MDP} = -57 \text{ mV}$, $\text{APA} = 89 \text{ mV}$, $\dot{V}_{\text{max}} = 7.5 \text{ V s}^{-1}$, $\text{DDR} = 85 \text{ mV s}^{-1}$, $\text{APD}_{50} = 124 \text{ ms}$, and $\text{APD}_{100} = 224 \text{ ms}$. The action potential parameters (mean ± standard deviation) of the 17 clusters are given in Table 1 (top row). Due to beat-to-beat variability, the action potential parameters for each cluster were averaged over ~100 beats before the population average was taken. Figures 6C,D give the voltage time-series and the phase-plane trajectory for the ionic model, while Table 1 (bottom row) gives the action potential parameters in the model, which are quite close to the mean experimental values (top row).

Steady-State IVs

The curve in Fig. 7A gives the steady-state IV relationship for the total current in the model. This curve corresponds closely to the mean IV data points obtained from five 7-day ventricular clusters in our lab (asterisk symbols in Fig. 7A; see also Ref. 4). Figures 7B-D give the steady-state IVs of the individual currents in the model.

Currents Underlying the Action Potential

We have previously mentioned that the spontaneous activity in the model (Figs. 6C,D) has action
potential parameters that compare well with the mean experimental values (Table 1). Figure 8A shows the transmembrane potential during approximately one cycle of spontaneous activity in the ionic model, while Figs. 8B-D show the various currents, and Figs. 8E,F show the various activation and inactivation variables. The upstroke phase is clearly generated by $I_{Ca}$ (Fig. 8C), which rapidly activates (activation variable $d$ in Fig. 8E; see also Fig. 1B). During the first third of the action potential, the slow activation of $I_{Ks}$ ($n$ in Fig. 8F; see also Fig. 2B) contributes increasingly to repolarization (Fig. 8C); $I_{Kr}$ is small (Fig. 8C) despite rapid activation ($s$ in Fig. 8F; see also Fig. 3B) because of its strong inward rectification ($z$ in Fig. 8F; see also Fig. 3C); there is also a smaller contribution from $I_{seal}$, which is outward, but which becomes less so with time (Fig. 8D); $I_b$ is inward, and gradually becomes more inward with time (Fig. 8D); $I_{K1}$ plays no role here (Fig. 8D) because of its strong inward rectification ($K1$ in Fig. 8E; see also Fig. 7C). There is also a secondary increase of $I_{Ca}$ (Fig. 8C), which serves to maintain the plateau phase of the action potential; this occurs despite decreased activation and increased inactivation of $I_{Ca}$ ($d$ and $f$ in Fig. 8E) and is due to an increase in driving force. The overall shape of the waveform of $I_{Ca}$ during the action potential resembles that seen in action-potential-clamp studies on SA node cells (120) and in some models of such cells (see, e.g., Fig. 6 of Ref. 54). During the middle part of repolarization, $I_{Ca}$, following its secondary peak (Fig. 8C), falls due to both inactivation and deactivation ($f$ and $d$ in Fig. 8E), which would per se promote repolarization. Due to a decrease in driving force, $I_{Ks}$ decreases (Fig. 8C) and later starts to deactivate ($n$ in Fig. 8F). There is also a fall in the outward $I_{seal}$, which eventually becomes an inward current (Fig. 8D). During the final stage of repolarization, there are now contributions from $I_{Kr}$ (Fig. 8C) and $I_{K1}$ (Fig. 8D), which are no longer completely rectified ($z$ and $K1$ in Figs. 8F,E).
**Currents Underlying the Pacemaker Potential**

Since the DDR in a 3-cell cluster is about 100 mV s\(^{-1}\) (Table 1), the net current during diastolic depolarization is tiny (~2.6 pA), not even being appreciable on the scale of Fig. 8B. The current \(I_{Ca}\) is inward and gradually becomes more inward throughout phase-4 depolarization (Fig. 8C), which agrees with the results from ruptured-patch action-potential-clamp studies on single SA node cells (120). The currents \(I_b\) and \(I_{seal}\) are also inward throughout phase-4 depolarization, but gradually become less so (Fig. 8D). While \(I_{Ks}\), \(I_{Kr}\), and \(I_{K1}\) are outward during phase-4 depolarization, \(I_{K1}\) becomes much less outward (Fig. 8D), \(I_{Ks}\) gradually becomes slightly less outward (not visible on scale of Fig. 8C) but does not contribute much current, and \(I_{Kr}\) contributes increasingly less outward current due to slow deactivation (Figs. 8C,F).

\(I_{Kr}\) deactivates slowly during diastolic depolarization (Figs. 8C,F), since the time constant for activation, \(\tau_a\), is several hundred milliseconds over the pacemaker range of potentials (Fig. 3B). Hence, \(I_{Kr}\) is not fully deactivated by the time that the upstroke of the action potential starts. However, the increase in voltage during the upstroke rapidly abolishes \(I_{Kr}\) (Fig. 8C) due to its profound inward rectification (Figs. 3C, 8F). As the membrane then repolarizes there is fast recovery from the inactivation of \(I_{Kr}\) that is responsible for its rectification (Fig. 8F). The time-course of \(I_{Kr}\) during spontaneous activity is very different here from that seen in an atrial re-aggregate model (Fig. 15 of Ref. 89), where \(I_{Kr}\) deactivates much more rapidly due to its shorter time-constant at the more hyperpolarized MDP of the re-aggregate model (about −90 mV there vs. −67 mV here). However, action-potential clamp studies of rabbit SA cells, which are more depolarized than the chick atrial re-aggregate, show a time course of \(I_{Kr}\) very similar to that in our model (see Fig. 1C of Ref. 76), as do SA node models that incorporate a sharply rectifying \(I_{Kr}\) component (see Figs. 6D,E of Ref. 54).
Effect of Ca\textsuperscript{++}-Channel Blockers on Spontaneous Activity

Application of D-600, a Ca\textsuperscript{++}-channel blocker, on 7- to 10-day cells and small clusters in our lab results in the abolition of spontaneous activity (Fig. 9A), with the mean resting potential being \(-36.2\) mV (n=14) \((49)\). We have seen similar results with another Ca\textsuperscript{++}-channel blocker, diltiazem \((51)\). Note that in Fig. 9A, a sharp microelectrode is used, so that the cessation of spontaneous activity is not due to dialysis of the pipette contents against the intracellular medium leading to effects such as current rundown.

Gradually increasing block of $I_{Ca}$ in the model, starting at $t = 45$ s in Fig. 9C, gives a time-course of the voltage that is similar to the experimentally observed effect of D-600, with the membrane eventually coming to rest at \(-37\) mV at $t \approx 120$ s when \(~90\%\) of $I_{Ca}$ is blocked. Since D-600 blocks $I_{Ca,L}$ and because our non-separable $I_{Ca}$ is close to $I_{Ca,L}$, the modeling intervention is similar to the experimental one of applying a $I_{Ca,L}$ blocker. In both experiment and model, the loss of overshoot (OS) initially proceeds at a slow rate (from just after arrow 1 up to just after arrow 3 in Fig. 9C), but then the rate of loss speeds up just prior to spontaneous activity being extinguished (i.e. just after arrow 3 in Fig. 9C). The MDP initially drifts slowly positive and then suddenly depolarizes much more quickly (starting at arrow 2 in Fig. 9C) before spontaneous activity ceases. In both experiment and model, the phase of more rapid loss of MDP precedes the phase of more rapid loss of OS. The upstroke velocity gradually decreases throughout the course of the block, and APD\textsubscript{100} increases (experiment: Fig. 9B; model: Fig. 9D).

Effects in many ways opposite to those described above are seen in our lab with administration of a Ca\textsuperscript{++}-channel agonist (BAY-K-8644): there are increases in $V_{\text{max}}$, OS, DDR, and APD, as well as a hyperpolarization of MDP and the threshold or take-off potential, and a fall in IBI \((28)\); these changes are also seen in the model.
Figure 9E gives the bifurcation diagram for $I_{Ca}$ block, computed using XPPAUT (25). The bifurcation parameter is $g_{Ca}$, and the bifurcation variable is the transmembrane voltage ($V$). The periodic activity of the model corresponds to the existence of a stable limit-cycle in the 6-dimensional phase-space of the system. As $g_{Ca}$ is reduced from its nominal value of 30 nS, the limit cycle decreases in size, so that the APA falls: — the top set of filled circles in Fig. 9E gives the maximum value of the $V$-coordinate of the limit-cycle (i.e. the OS), while the bottom set gives its minimum value (i.e. the MDP). The dashed line in Fig. 9E indicates the locus of the $V$-coordinate of the unstable steady-state that is also present in the phase-space of the system (this corresponds to the zero-current crossing of the total current IV-curve in Fig. 7A). At $g_{Ca} = 4.2$ nS, there is a subcritical Hopf bifurcation (“HB” in Fig. 9E), producing an unstable limit-cycle oscillation (open circles), which grows in amplitude as $g_{Ca}$ is reduced further, until the stable and unstable limit cycles collide at $g_{Ca} = 3.7$ nS in a reverse saddle-node bifurcation of limit cycles (31).

When $g_{Ca}$ is reduced relatively slowly (“quasi-statically”) from its control value of 30 nS (Fig. 9C), this corresponds to moving from right to left along the stable limit-cycle branch of the bifurcation diagram in Fig. 9E. Eventually, at $g_{Ca} \approx 3.7$ nS, the state-point will leave the stable periodic branch, and head towards the stable steady-state produced in the subcritical Hopf bifurcation (solid line in Fig. 9E indicates the resting membrane potential (RMP) corresponding to this stable steady-state). This agrees with the simulation of Fig. 9C, where spontaneous activity is abolished with ~90% block of $I_{Ca}$. This “falling-off” is responsible for the rapid phase of decline in the OS seen after arrow 3 just before spontaneous activity is abolished in Fig. 9C (this is also seen in experiment – Fig. 9A). In the model (Fig. 9C), the more rapid phase of decline of MDP starts earlier, before the falling-off (just after arrow 2), at $g_{Ca} \approx 6$ nS, which
agrees with the change in the slope of MDP in the bifurcation diagram (Fig. 9E) (this pattern is also seen in the experiment – Fig. 9A). When a trace such as that shown in Fig. 9C, with distinct phases of change of MDP and OS, is seen experimentally, one should begin to think that abolition of spontaneous activity might involve a subcritical, rather than a supercritical, Hopf bifurcation.

The coexistence of a stable limit cycle and a stable steady-state for $3.7 \text{ nS} < g_{\text{Ca}} < 4.2 \text{ nS}$ in Fig. 9E implies that over this range one should be able to trigger activity from the resting state by injecting a stimulus, and that, once so triggered, one should be able to annihilate this activity by injection of a single well-timed stimulus (31). We have indeed observed both single-pulse triggering and annihilation in the model at $g_{\text{Ca}} = 3.9 \text{ nS}$. Annihilation has been seen in isolated ventricular cells (94) and in re-aggregates of atrial cells exposed to TTX (90). We do not know whether the bistable range in Fig. 9E would be wide enough in experiment to allow single-pulse triggering and annihilation to be seen in these clusters, since this would necessitate adjusting the D-600 concentration to a value within a rather narrow range, which will be different from cluster to cluster and which will be unknown a priori. However, there is other experimental evidence to support the scenario of Fig. 9E, which we now outline.

The existence of a saddle-node bifurcation in Fig. 9E is consistent with three prior observations from our lab: (i) During washout of D-600, transient flurries of action potentials occur spontaneously before spontaneous activity is permanently re-established. The amplitude of the first action potential in each flurry is relatively large, with the amplitude of the following action potentials gradually declining during the course of each episode of transient triggered activity (see Fig. 2(c) of Ref. 49). (ii) Once beating has stopped under the influence of diltiazem, injection of a hyperpolarizing bias current can provoke an episode of transient triggered activity,
with the first action potential being an anodal-break response (Fig. 2 of Ref. 51). As time proceeds and the degree of block continues to increase during quiescence, the number of action potentials in an episode decreases. This “critical slowing down” behaviour is consistent with the existence of a saddle-node bifurcation of limit cycles and can be seen in simulations with the model. (iii) In some cells that are initially found to be not spontaneously active, injection of a single hyperpolarizing current pulse again elicits a flurry of triggered action potentials, with the action potential amplitude gradually decreasing during each flurry (Figs. 4,6 of Ref. 49). During ongoing superfusion with D-600 of these cells, the number of non-driven action potentials in any one trial gradually decreases from tens of action potentials, then the membrane does not produce triggered action potentials, and then finally the membrane becomes inexcitable (Fig. 4 of Ref. 49).

A response similar to that shown in Figs. 9A-D is seen with $I_{Ca,L}$ block in the SA node, both in experiment (see references in Ref. 31) and in several ionic models (31; 54; 55). However, in some of these SA node models, a supercritical Hopf bifurcation occurs so that annihilation and single-pulse triggering cannot occur.

Effect of Almokalant on Spontaneous Activity

When 1 or 2 mM almokalant, a specific blocker of $I_{Kr}$ (113), is added to the bath, there is a slowing of the terminal rate of repolarization, a small depolarization of the MDP, and a slight loss of OS (Fig. 10A).

When 100% of $I_{Kr}$ is blocked in the model (Fig. 10B shows 50%, as well as 100% block of $I_{Ks}$) there is a marked depolarization of MDP, a slight slowing of the terminal rate of repolarization, a small increase in $APD_{100}$, a decrease in $APD_{50}$, a decrease in IBI, a fall in $V_{max}$,
and a decrease in OS. The slowing of the terminal rate of repolarization and the depolarization of the MDP are due to the absence of the $I_{Kr}$ contribution normally present (see $I_{Kr}$ trace in Fig. 8C during control activity). The resultant relative depolarization then causes $I_{Ks}$ to remain considerably more outward during the entire pacemaker potential and even during the early part of the action potential (Fig. 10C shows $I_{Ks}$ time-course during 100% $I_{Kr}$ block), which is the major cause of the decrease in APD$_{50}$ in Fig. 10B. This secondary increase in $I_{Ks}$ also accounts for the relatively small effect of the loss of $I_{Kr}$ on the rate of terminal repolarization in Fig. 10B. In cells that do not have $I_{Ks}$, this indirect effect of $I_{Kr}$ block on $I_{Ks}$ would not occur, and one would expect a prolongation of APD. This is exactly what is seen in SA node cells that were found to not possess $I_{Ks}$ (106) (but in later studies, $I_{Ks}$ was clearly found in SA node cells (59; 110)). The fall in $V_{\text{max}}$ and OS are due to a decrease in $I_{Ca}$ (peak value falls from 263 pA to 207 pA during 100% $I_{Kr}$ block), as a result of greater inactivation of $I_{Ca}$ during diastolic depolarization. It has been noted previously in experiments done on the SA node that effects on action potential parameters due to selective block of $I_{Kr}$ with the compound E-4031 are due to “a combination of direct and indirect effects on various ionic currents” (106).

The shortening of APD$_{50}$ seen in the model with $I_{Kr}$ block (Fig. 10B) does not occur in the experiment (Fig. 10A). One reason that might account for this discrepancy is that during the 10-minute period between rupturing the patch (to enter whole-cell recording mode) and the start of the recording of the effect of almokalant, there is rundown of $I_{Ks}$ (79). Figure 10D shows the combined effect in the model of 50% block of $I_{Kr}$ (to simulate almokalant) and 20% block of $I_{Ks}$ (to simulate the degree of rundown seen experimentally (79)): APD$_{50}$ is no longer decreased.

To avoid the above artefact due to dialysis of cytoplasm against the pipette contents and rundown of currents, we also used visual monitoring of mechanical beating to assess the effect of
almokalant. While three clusters stopped beating upon exposure to almokalant (and the effect was reversible upon washout of almokalant from the bath), another four clusters did not stop beating (3/4 had an increased IBI, and the remaining one had no change in IBI). In response to 0.1 µM E-4031, another specific $I_{Kr}$ blocker, half of the single SA node cells stopped beating in one study (106), while none stopped in another study (76). When the concentration was raised to 1.0 µM, at which concentration there are still negligible effects on other currents, all cells ceased spontaneous activity in both studies. A similar response was found in small balls of tissue isolated from the SA node: with 1.0 µM E-4031, activity was abolished in all balls, while with 0.1 µM E-4031 activity was sometimes extinguished in balls taken from the central area, but not in those from more peripheral areas (46). The fact that 0.1 µM E-4031 abolishes spontaneous activity in smaller single SA node cells, but not in larger ones, agrees with this observation (59), provided that small cells do indeed stem from the central area of the node and larger ones from the periphery.

Complete block of $I_{Kr}$ does not abolish spontaneous activity in the model (Fig. 10B). In contrast, cessation of spontaneous activity does occur with complete $I_{Kr}$ block in some SA node models, sometimes via a supercritical Hopf bifurcation, sometimes via what is probably a subcritical Hopf bifurcation, and sometimes through an intermediary phase of “irregular dynamics” (Fig. 13 of Ref. 54).

**Effect of Ba$$^{++}$$ on Spontaneous Activity**

In 7-day ventricular cells, a low concentration of Ba$$^{++}$$ (0.2 mM) blocks $I_{K1}$ without greatly affecting $I_{Ks}$ and $I_{Kr}$ (8). Figure 11A shows the effect of adding 0.1 mM Ba$$^{++}$$ in an experiment, while Fig. 11B shows the effect of blocking $I_{K1}$ completely in the model. In both experiment and
model, there is a depolarization of MDP, a decrease in $\dot{V}_{\text{max}}$, a loss of OS, and a decrease in IBI. Taking into account the average drift of action potential parameters seen in control recordings after 10 mins (the time after which the effects due to Ba$^{++}$ were measured), MDP depolarizes by 5%, $\dot{V}_{\text{max}}$ decreases by 13%, APA decreases by 7%, and IBI decreases by 9% ($n=4$) (79), whereas in the model MDP depolarizes by 15%, $\dot{V}_{\text{max}}$ decreases by 28%, APA decreases by 13%, and IBI decreases by 11%. However, the increases in APD and DDR seen in the experiments (APD$_{50}$ increases by 12%, APD$_{90}$ increases by 28%) are not replicated in the model. Since we have observed neither an increase in the peak $I_{Ca}$ nor slowed inactivation kinetics of $I_{Ca}$ with elevated Ba$^{++}$ concentrations (77), we attribute the increase in APD seen in the experiments at least partially to rundown of $I_{Kr}$, as with our almokalant results presented earlier.

Increasing the concentration of Ba$^{++}$ to 0.5-1.0 mM results in partial block of $I_{Ks}$ (8; 79) and $I_{Kr}$ (79), in addition to complete block of $I_{K1}$. Figure 11C shows the mean effect ($n=9$) on the steady-state IV when 0.5 mM Ba$^{++}$ is applied to small clusters. Figure 11D shows the effect on the steady-state IV in the model of blocking 100% of $I_{K1}$, 33% of $I_{Ks}$, and 33% of $I_{Kr}$ (the degree of block of $I_{Ks}$ and $I_{Kr}$ in the model was chosen to make the blocked current comparable with the combined effects of rundown and Ba$^{++}$ in Fig. 11C). The steady-state IV curve is no longer N-shaped. The effect on spontaneous activity in both experiment and model is a more marked change from control in the action potential parameters than at the lower concentration of Ba$^{++}$ (Figs. 11A,B). Specifically, in experiment, again taking into account the average drift seen over a period of 10 minutes in action potential parameters in control recordings, the MDP depolarizes by 11%, $\dot{V}_{\text{max}}$ decreases by 49%, APA decreases by 15%, and IBI decreases by 34% ($n=8$) (79), while in the model the MDP depolarizes by 27%, $\dot{V}_{\text{max}}$ decreases by 39%, APA decreases by 23%, and IBI decreases by 20%.
At an even higher concentration (1 mM), Ba\(^{++}\) abolishes spontaneous activity in the clusters, with the membrane coming to rest at about \(-35\) mV (79). The effect is reversible, in that spontaneous activity resumes with washout of Ba\(^{++}\). Depolarization of MDP and cessation of spontaneous activity has previously been described in 6- to 7-day ventricular cells exposed to 5-10 mM Ba\(^{++}\) (96). Cessation of spontaneous activity is also seen in the model if, in addition to complete block of \(I_{K1}\), both \(I_{Ks}\) and \(I_{Kr}\) are blocked by 68\%, with the membrane coming to rest at \(-17\) mV. In this case, the activity is abolished through a supercritical Hopf bifurcation. This modeling result agrees with there being only one distinct phase of change of MDP and OS in the experiment (79), unlike the result of \(I_{Ca}\) block (Fig. 9). Blocking the other background current in the model (\(I_b\)) results in the cessation of spontaneous activity via an infinite-period bifurcation.

**Effect of Seal-Leak Current on Spontaneous Activity**

The possibility exists that the inward seal-leak current is essential for the generation of spontaneous activity in our model. However, when \(I_{seal}\) is removed, spontaneous beating still continues in the model, albeit at a considerably increased IBI of 535 ms and a more hyperpolarized MDP of \(-72\) mV (Fig. 12). This result agrees with the fact that we record from clusters that are seen to be beating before patching; i.e. the depolarizing seal-leak current does not induce spontaneous beating in our clusters. However, patching onto a single cell can change the IBI or even abolish pre-existing spontaneous activity (see also Ref. 112). Indeed, if our model (with the nominal seal-leak resistance of 5 G\(\Omega\)) is reformulated for a single cell (i.e. capacitance = 8.5 pF), the MDP depolarizes to \(-51\) mV and the IBI decreases to 293 ms; with a seal-leak resistance of 3 G\(\Omega\) spontaneous activity is abolished in the single-cell model.
We have used a nominal seal-leak resistance of 5 GΩ in our model (20). This is in fact a worst-case scenario, since this value is towards the lower end of the range of values reported in experimental work on chick cells (42; 63; 79). When the seal-leak resistance in the model is increased to 20 GΩ, which is in fact towards the higher end of what is used in our lab (79), the IBI increases from 392 to 475 ms. Thus, differences in seal-leak resistance might account for part of the preparation-to-preparation differences in IBI and DDR seen experimentally (Fig. 5).
DISCUSSION

Spontaneous Activity in Isolated Chick Ventricular Cells

Electrical recordings of spontaneous activity in single embryonic chick ventricular cells, in small clusters of such cells, and in sparse monolayers have been made using conventional sharp microelectrodes (17; 26; 48; 49; 51; 78; 95) or patch pipettes in the whole-cell recording mode (64; 67; 112). Among these studies, the ones that use 7-day ventricular cells at an external \([K^+]\) of 2.7-5.4 mM generally report that the cells have an MDP lying between −60 and −70 mV and a slow upstroke velocity of \(\approx 10\) V s\(^{-1}\). One systematic study, which used sharp microelectrodes, reported MDP = −68.4 ± 1.0 mV and OS = 22 ± 0.9 mV (n=30) at an external \([K^+]\) of 4.2 mM (17). Our findings in small clusters (Table 1, top row) are in agreement with the findings of these prior studies.

How does the electrical activity of small clusters compare with that of the in situ ventricular muscle? The APA and APD in our small clusters (and in the other reports on single cells, small clusters, and sparse monolayers mentioned just above) are quite similar to those of in situ ventricular muscle (98; 101; 118). In contrast, in situ ventricular muscle, with one exception (97), has not been reported to show spontaneous phase-4 depolarization (61; 101; 118) and has a much larger \(V_{\text{max}}\) \((70-94\) V s\(^{-1}\) at 5- to 7-days (19; 98; 118) vs. 8.5 V s\(^{-1}\) here). The absence of diastolic depolarization in the in situ 7-day chick ventricle could be due to overdrive suppression since, in the one report in which pacemaker potentials were seen in intact 7-day ventricles, such potentials were seen in only 20-40\% of intact ventricles, but in 100\% of cut 7-day ventricular fragments (97). Alternatively, the dissociation procedure itself or the time spent in culture (104) could change the electrophysiological properties of cultured cells. Adding the \(I_{Na}\) known to be present in the small clusters to the model (see Methods) and increasing \(I_{K1}\) to the point where
spontaneous activity stops with a resting membrane potential of $-73 \text{ mV}$, gives a $\dot{V}_{\text{max}}$ of an
induced action potential of $54 \text{ V s}^{-1}$, suggesting that the low value of $\dot{V}_{\text{max}}$ observed in the
clusters is indeed due to virtually complete inactivation of $I_{\text{Na}}$.

The action potential parameters of 7-day embryonic chick ventricular cells are very close
to those of isolated SA node cells (compare data in Table 1 with the data from the many
experimental studies summarized in Table 1 of Ref. 54). This is not too surprising, given that a
similar mix of currents seems to be present in the central part of the SA node and in our cells.
For example, in the smallest SA node cells (presumably from the central part of the node), which
have a capacitance about the same as one of our 3-cell clusters (i.e. $\sim 20 \text{ pF}$), currents such as
$I_{\text{Ca,L}}, I_{Kr}, I_{Ks}$ and background currents are present, but other currents, such as $I_{\text{Na}}, I_{f}$, and $I_{\text{Ca,T}}$,
either are absent or the membrane is too depolarized to allow activation (e.g. $I_{f}$) or to allow
removal of inactivation (e.g. $I_{\text{Na}}$) (36). Indeed, a minimal SA node model with only $I_{\text{Ca}}, I_{K}$, and a
background current produces very respectable-looking spontaneous activity (33).

**Currents Underlying Diastolic Depolarization in the Model**

To produce a DDR of $\sim 100 \text{ mV s}^{-1}$ (Table 1) in a 3-cell cluster with a capacitance of $25.5 \text{ pF}$
requires that the net current flowing during spontaneous diastolic depolarization be $2.55 \text{ pA}$. In
our model, this net current is obtained from the algebraic sum of several much larger inward and
outward currents (Fig. 8). In models of SA node cells, these individual currents contribute to
varying extents to produce this tiny net current (Fig. 4 of Ref. 115; Figs. 6 and 7 of Ref. 54). At
present, we have no way of knowing exactly which currents are flowing, and in what amounts,
during diastolic depolarization in a given cell; to make matters worse, this mix of currents almost
certainly changes from cell to cell, especially in cells from structures as inhomogeneous as the SA node (47).

The current for which we have the least direct experimental evidence in our model is $I_b$ (14). We have added this current to the model because we know that an inward background current is needed to generate a steady-state IV curve that agrees with experiment (Fig. 7A) and to allow the membrane to come to rest at about $-40 \, \text{mV}$ when $I_{Ca}$ is blocked (Fig. 9). Background currents have also been described in SA node cells (34; 106). One or more inward background currents are incorporated into all models of atrial (9; 52; 88-90) and ventricular (11; 88) re-aggregates, as well as into all models of SA node cells (see Fig. 4 of Ref. 115 and Fig. 7 of Ref. 54). As in many of the above models, the inward background current is necessary for spontaneous activity in our model, in that deletion of the inward background current results in the cessation of spontaneous activity.

Very early on during development (3 days), the steady-state inactivation curve of $I_{Na}$ is shifted in the depolarizing direction (82); simulations suggest that the window component of $I_{Na}$ might then contribute to diastolic depolarization (83). A sustained inward Na$^+$ current ($I_{st}$) has been reported during diastolic depolarization in spontaneously active single SA node cells (Ref. 71, but see Ref. 107); intriguingly, $I_{st}$ is not present in quiescent SA node cells (71). We know of no reports of this current in embryonic chick cells.

The seal-leak current injected through the gigaohm seal-leak resistance is a source of artefact in our experimental recordings. This is true even if the perforated-patch, rather than the ruptured-patch, technique were to be used. Unlike the case in relatively large adult mammalian ventricular cells having input resistances on the order of tens of MΩ, this current has significant effects when smaller cells (e.g. SA node cells, embryonic cells) having membrane resistances on
the order of 1 GΩ at −70 mV (as do our clusters; see also Ref. 29) are studied (20). It is thus conceivable that the seal-leak current is causing depolarization to the point that $I_{Na}$ and $I_{Ca,T}$, although present, become inactivated, and $I_f$ deactivated. However, in our model subtraction of the seal-leak current hyperpolarizes the MDP by only 6 mV (resp. 2 mV) while increasing the IBI from 392 ms to 535 ms (resp. 472 ms) when the seal-leak resistance is 5 GΩ (resp. 20 GΩ). This extra hyperpolarization is not sufficient to activate significant amounts of $I_{Na}$, since when $I_{Na}$ is added to the model (see Methods), removal of the 5 GΩ seal-leak resistance results in IBI decreasing from 392 ms to 533 ms, and $V_{max}$ increasing from 10.2 V s$^{-1}$ to 10.9 V s$^{-1}$. Likewise, when $I_f$ is added to the model (see Methods) the extra hyperpolarization does not activate $I_f$ significantly: in this case removal of the seal-leak current increases the IBI from 390 ms to 520 ms.

**Spontaneous Activity: Cell-to-Cell Variability and Activity-Dependent Conductances**

There is considerable variability in electrical parameters from cluster to cluster (Fig. 5). The extent to which this is a result of true inhomogeneity within the cellular population (e.g., the regional heterogeneities in the early embryonic chick heart (1; 101); the apex-to-base and endocardial-to-epicardial ventricular gradients known to be present in adult muscle (77)) is not clear. There is almost certainly artefact stemming from differences in dissociation conditions from one culture to another (and even from cluster to cluster within the same culture). The seal-leak current is also different from cluster to cluster. Population inhomogeneity exists in ventricular cells isolated from the adult rat heart (77) and the adult guinea-pig heart (119), as well as in cells isolated from the rabbit SA node (75). While at first sight these cell-to-cell differences might appear to be so large as to be problematic for physiologic function,
experimental and modeling work indicates that cell-to-cell differences in APD would be largely smoothed out in situ by gap-junctional coupling (119); a similar conclusion holds for IBI (108).

The action potential parameters in the model are very close to the mean values obtained in experiment (Table 1). But, because of the inhomogeneity of the cellular population, a particular action potential parameter in a given cluster can be quite different from that in the model. For example, in Fig. 5 the MDP in the model is more depolarized than in cluster no. 16, the IBI is much smaller than in cluster no. 17, and the APD is much shorter than in cluster no. 2. In each of these cases the model could presumably be modified to replicate the electrical activity seen in the particular cluster by changing particular currents (e.g. increasing the conductance of one or more of the K+-currents to produce a more hyperpolarized MDP, increasing the conductance of $I_{Ca}$ to extend the APD). However, this is an exercise in “theorizing in a vacuum”, given that one does not know (and cannot know, with the currently available technology) the exact parameters describing all the ionic currents in a given cell.

The wide variety of shapes of the action potentials seen in experiment and the corresponding cell-to-cell variability in the individual currents must mean that the currents influencing APD and DDR (and hence IBI) are mixed to varying extents in different clusters. Given the fact that ~40% of our clusters do not beat (50), it is perhaps not surprising that there is a great deal of cluster-to-cluster variability in IBI and in the waveform of the action potential. It has been suggested that activity-dependent conductances might provide a negative-feedback mechanism by which spontaneous activity can be regulated (for a review see Ref. 80). It is quite conceivable that some such homeostatic process is going on here, since, e.g., it is known that messenger-RNA expression and protein level of one K+-channel are decreased as a result of KCl-induced depolarization in pituitary cells (60). It is also possible that the individual currents,
through their voltage-dependence, negatively feed back on each other to regulate spontaneous activity (73).

Limitations of the Model

Our first-generation model contains no pumps or exchangers and has fixed ionic concentrations. It thus cannot be used to realistically model phenomena such as the rise in internal [Na\(^+\)] seen when \(I_{\text{NaK}}\) is blocked, or the change in internal [Ca\(^{++}\)] that occurs should IBI be changed. Because data is not available for all the currents in 7-day embryonic chick ventricular cells, there are uncertainties in some of the currents: e.g. \(I_{\text{Kr}}\) is taken from experiments on atrial re-aggregates, and we use a non-separable \(I_{\text{Ca}}\). While we have used the classic Hodgkin-Huxley description for the gating of all the currents in our model, there are channels that do not always behave in this way (e.g. the “bursting” mode of \(I_{\text{Na}}\) that occasionally results in a longer-lasting current (40; 64)). As in all work on ionic models of spontaneously active cells, the background current, which in our case is a composite current (including, e.g., \(I_{\text{NaK}}\)), is titrated to produce a reasonable IBI. There is evidence from recent work on the SA node that internal Ca\(^{++}\)-cycling contributes to generating spontaneous diastolic depolarization (e.g. Ref. 56). Since there is no firm experimental evidence for this mechanism in embryonic chick ventricular cells, we do not entertain this possibility in our model.

The deterministic Hodgkin-Huxley type of model we present here shows no beat-to-beat fluctuation in action potential parameters. In contrast, all of our experimental recordings show considerable beat-to-beat fluctuations in the various action potential parameters (e.g. IBI). Previous modeling studies of rabbit SA node cells have shown that the beat-to-beat fluctuations observed experimentally can be accounted for by the stochastic opening and closing of
membrane channels (32; 114). Development of a stochastic single-channel version of the
deterministic model we have described above allows investigation of this hypothesis (Krogh-
APPENDIX: Model Equations

Some model parameters (maximal conductances, reversal potentials, and capacitance) are given in Table 2.

Membrane Potential

\[
dV/dt = -(I_{ca} + I_{Ks} + I_{Kr} + I_{K1} + I_{b} + I_{seal})/C_m
\]

Slow Inward Ca\(^{++}\) Current

\[
I_{ca} = g_{ca}d(V - E_{ca})
\]

\[
\frac{dd}{dt} = \frac{1}{\tau_d}(d_\infty -d)
\]

\[
\frac{df}{dt} = \frac{1}{\tau_f}(f_\infty - f)
\]

\[
f_\infty = \frac{1}{1+\exp\left\{(V + 42.8)/8.4\right\}}
\]

\[
d_\infty = \frac{1}{1+\exp\left\{-(V + 10.0)/6.2\right\}}
\]

\[
\tau_d = 0.001\times\left[1-\exp\left\{-(V+10)/6.24\right\}\right]\times\left[1+\exp\left\{-0.035(V+10)\right\}\right]
\]

\[
\tau_f = 0.001\left[0.0197\exp\left\{-(0.0337(V+10))^2\right\}+0.02\right]
\]

Slow Delayed K\(^+\) Current

\[
I_{Ks} = g_{Ks}n(V - E_{Ks})
\]

\[
\frac{dn}{dt} = \alpha_n(1-n) - \beta_n n
\]

\[
\alpha_n = (2/3)\times0.08(V - 15)/\left[1-\exp\left\{-0.08(V-15)\right\}\right]
\]

\[
\beta_n = (2/3)\times0.156\exp\left\{-0.055(V-15)\right\}
\]
Rapid Delayed Rectifier $K^+$ Current

\[ I_{Kr} = g_{Kr}z(V)(V - E_{Kr}) \]

\[ z(V) = 1/(1 + \exp\{(V + 100)/25\}) \]

\[ \frac{ds}{dt} = \alpha_s(1 - s) - \beta_s s \]

\[ \alpha_s = 23.0\exp\{0.13(V + 9.0)\} \]

\[ \beta_s = 0.036\exp\{-0.09(V + 9.0)\} \]

Inward Rectifier $K^+$ Current

\[ I_{K1} = g_{K1}K1(V - E_{K1}) \]

\[ \frac{dK1}{dt} = \alpha_{K1}(1 - K1) - \beta_{K1}K1 \]

\[ \alpha_{K1} = 1000 \times 1.02/[1 + \exp\{0.2385(V - E_{K1} - 59.215)\}] \]

\[ \beta_{K1} = 1000 \times [0.49124\exp\{0.08032(V - E_{K1} + 5.476)\} + \exp\{0.06175(V - E_{K1} - 594.31)\}] / [1 + \exp\{-0.5143(V - E_{K1} + 4.753)\}] \]

Background Current

\[ I_b = g_b(V - E_b) \]

Seal-Leak Current

\[ I_{seal} = g_{seal}(V - E_{seal}) \]

Voltages are in mV, currents in pA, conductances in nS, capacitance in nF, and time in s. The initial conditions are \(V = -66.5526\) mV, \(d = 1.0932 \times 10^{-4}\), \(f = 0.6309\), \(n = 0.0764\), \(s = 0.8019\), \(K1 = 0.2953\).
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REFERENCES


FIGURE LEGENDS

Fig. 1  Characteristics of $I_{Ca}$ in the model.

A: Steady-state activation ($d_{x}$) and inactivation ($f_{x}$) curves.
B: Time constants of activation (τ_a) and inactivation (τ_f).
C: Peak $I_{Ca}$ in voltage-clamp steps from a holding potential of –80 mV to various test potentials (protocol of Ref. 44).
D: Simulated current during voltage-clamp steps from a holding potential of –80 mV to the indicated test potentials (protocol of Ref. 44).

Fig. 2 Characteristics of $I_{Ks}$ in the model.

A: Steady-state activation ($n_{x}$) curve.
B: Time constant of activation (τ_n).
C: Fully-activated $I_{Ks}$.
D: Simulated current during voltage-clamp steps from a holding potential of –60 mV to the indicated test potentials (protocol of Ref. 8).

Fig. 3 Characteristics of $I_{Kr}$ in the model.

A: Steady-state activation ($s_{x}$) curve and rectification variable (z).
B: Time constant of activation (τ_s).
C: Fully activated $I_{Kr}$: notice the pronounced rectification.
D: Simulated current during voltage-clamp steps from a holding potential of –26 mV to the indicated test potentials (protocol of Ref. 89).
Fig. 4  Characteristics of $I_{K1}$ in the model.

A: Steady-state activation ($K_{1\infty}$) curve.

B: Time constant of activation ($\tau_{K1}$).

Fig. 5  Transmembrane potential recorded during spontaneous activity from all 17 clusters studied. Ticmarks indicate 0 mV.

Fig. 6  A: Transmembrane potential ($V$) recorded during spontaneous activity from one cell in a cluster (cluster no. 7 in Fig. 5).

B: Phase-plane trajectory of 8 cycles in the cluster ($dV/dt$ or $\dot{V}$ plotted vs. $V$).

C: Transmembrane potential during spontaneous activity in model.

D: Phase-plane trajectory in the model ($dV/dt$ or $\dot{V}$ plotted vs. $V$).

Initial conditions (see Appendix) closely approximate a point on the limit cycle corresponding to spontaneous activity. In C and D a transient of 2 s was removed.

Fig. 7  Steady-state current-voltage (IV) curves.

A: Steady-state IV relationship of total current in the experiments (*) (n=5) and model (curve).

B-D: IV curves of individual currents in the model.
Fig. 8  Spontaneous activity in the model.
A: Transmembrane potential.

B: Total current

C,D: Individual currents. Peak value of $I_{Ca}$ is $-262$ pA (off-scale deflection in C).

E,F: Activation and inactivation variables in the model. Also shown is the function $z(V)$ describing rectification of $I_{Kr}$.

Fig. 9  Block of $I_{Ca}$ (experiment and model).
A,B: Block of $I_{Ca}$ with D-600 in experiments (reproduced from Ref. 49 with permission). This preparation is thus not one of 17 shown in Fig. 5; sharp microelectrode used.

C,D: Block of $I_{Ca}$ in the model. The maximal conductance of $I_{Ca}$ ($g_{Ca}$) was decreased linearly starting at $t = 45$ s at a rate of $0.36$ nS s$^{-1}$. At $t \approx 120$ s, when spontaneous activity stops, about 90% of $g_{Ca}$ is blocked.

E: Bifurcation diagram with $g_{Ca}$ as the bifurcation parameter. For a steady state, the bifurcation variable is the voltage-coordinate ($V$) of that point (solid line: stable (RMP = resting membrane potential); dashed line: unstable); for a limit cycle, the maximum (i.e. OS) and minimum (i.e. MDP) values of $V$ are plotted (filled circles: stable; unfilled circles: unstable). HB = Hopf bifurcation.
Fig. 10  Effect of almokalant (experiment and model).

A: Effect of almokalant on cluster no. 9 in Fig. 5 (solid line) vs. control (dashed line).

B: Effect of block of 50% (solid line) and 100% (dash-dotted line) of $I_{Kr}$ in the model.

C: $I_{Ks}$ during 100% block of $I_{Kr}$ in the model (solid line).

D: Effect in model of 50% block of $I_{Kr}$ and 20% block of $I_{Ks}$ (solid line; latter to simulate rundown).

Fig. 11  Effect of Ba$^{++}$ (experiment and model).

A: Effect of 0.1 mM Ba$^{++}$ on spontaneous activity in a cluster (solid line) vs. control (dashed line). This cluster is not one of the 17 shown in Fig. 5.

B: Effect in model of 100% $I_{K1}$ block (solid line) vs. control (dashed line).

C: Steady-state IV curve with 0.5 mM Ba$^{++}$ (closed symbols) and in control (open symbols). Mean values of nine clusters, none of which are shown in Fig. 5.

D: Steady-state IV curve with block of 100% of $I_{K1}$ and 33% of $I_{Ks}$ and $I_{Kr}$ (solid line) vs. control IV (dashed line) in the model.

Fig. 12  Effect of removal of the seal-leak current ($I_{seal}$) in the model.

Dashed line: standard model with $I_{seal}$. Solid line: $I_{seal}$ removed from the model.
TABLE 1

Action potential parameters: experiment vs. model

<table>
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<tr>
<th></th>
<th>IBI (ms)</th>
<th>$V_{\text{max}}$ (V s$^{-1}$)</th>
<th>MDP (mV)</th>
<th>APA (mV)</th>
<th>DDR (mV s$^{-1}$)</th>
<th>APD$_{50}$ (ms)</th>
<th>APD$_{100}$ (ms)</th>
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<tr>
<td>Experiment (mean ± s.d.)</td>
<td>389 ± 78</td>
<td>8.5 ± 4.6</td>
<td>−60 ± 4</td>
<td>93 ± 7</td>
<td>110 ± 30</td>
<td>108 ± 24</td>
<td>176 ± 39</td>
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<td>Model</td>
<td>392</td>
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<td>−67</td>
<td>102</td>
<td>90</td>
<td>108</td>
<td>181</td>
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For each cluster, the mean values of action potential parameters were computed for a train of ~100 action potentials. The values presented are mean ± s.d. of these mean values for 17 clusters.
<table>
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<tr>
<th>Parameter</th>
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<th>Value</th>
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</table>
$I_{Ca}$
$I_{Ks}$

**Figure 2**

A

$\eta_s$ vs. Voltage (mV)

B

$\tau_n$ vs. Voltage (mV)

C

Fully act. $I_{Ks}$ (pA) vs. Voltage (mV)

D

$I_{Ks}$ (pA) vs. Time (s)
FIGURE 3

$I_{Kr}$

A

B

C

D

Voltage (mV)

Voltage (mV)

Voltage (mV)

Time (s)
FIGURE 4

$I_{K1}$

A

B

$K_{1\infty}$

$\tau_{K1}$

Voltage (mV)

Voltage (mV)
FIGURE 8

A

\[ \text{Voltage (mV)} \]

\[ \begin{array}{c}
0 & 100 & 200 & 300 & 400 & 500 \\
0 & 10 & 20 & 30 & 40 & 50 \\
\end{array} \]

\[ \text{Time (ms)} \]

B

\[ \text{Current (pA)} \]

\[ \begin{array}{c}
0 & 100 & 200 & 300 & 400 & 500 \\
-250 & -150 & -50 & 50 & 150 & 250 \\
\end{array} \]

\[ \text{Time (ms)} \]

C

\[ \text{Current (pA)} \]

\[ \begin{array}{c}
0 & 100 & 200 & 300 & 400 & 500 \\
-80 & -40 & 0 & 40 & 80 \\
\end{array} \]

\[ \text{Time (ms)} \]

D

\[ \text{Current (pA)} \]

\[ \begin{array}{c}
0 & 100 & 200 & 300 & 400 & 500 \\
-30 & -15 & 0 & 15 & 30 \\
\end{array} \]

\[ \text{Time (ms)} \]

E

\[ \text{Gating variables} \]

\[ \begin{array}{c}
0 & 1 & 2 & 3 & 4 & 5 \\
0 & 1 & 2 & 3 & 4 & 5 \\
\end{array} \]

\[ \text{Time (ms)} \]

F

\[ \text{Gating variables} \]

\[ \begin{array}{c}
0 & 1 & 2 & 3 & 4 & 5 \\
0 & 1 & 2 & 3 & 4 & 5 \\
\end{array} \]

\[ \text{Time (ms)} \]
FIGURE 12

[Graph showing voltage over time for control and no seal-leak conditions]