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

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Krogh-Madsen, Trine, Peter Schaffer, Anne D. Skriver, Louise Kold Taylor, Brigitte Pelzmann, Bernd Koidl, and Michael R. Guevara. An ionic model for rhythmic activity in small clusters of embryonic chick ventricular cells. Am J Physiol Heart Circ Physiol 289: H398–H413, 2005. First published February 11, 2005; doi:10.1152/ajpheart.00683.2004.—We recorded transmembrane potential in 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 effects of the blockers D-600, diltiazem, almokalant, and Ba2+. Electrical activity in small clusters is very different from that in reaggregates of several hundred embryonic chick ventricular cells, e.g., TTX-sensitive fast upstrokes in reaggregates vs. TTX-insensitive slow upstrokes in small clusters (maximum upstroke velocity ~100 V/s vs. ~10 V/s). On the basis of our voltage- and current-clamp results and data from the literature, we formulated a Hodgkin-Huxley-type ionic model for the electrical activity in these small clusters. The model contains a Ca2+ current (I_{Ca}), three K+ currents (I_{Ks}, I_{Kc}, and I_{Kr}), a background current, and a seal-leak current. I_{Ca} generates the slow upstroke, whereas I_{Kc}, I_{Ks}, and I_{Kr} contribute to repolarization. All the currents contribute to spontaneous diastolic depolarization, e.g., removal of the seal-leak current increases the interbeat interval from 392 to 535 ms. The model replicates the spontaneous activity in the clusters as well as the experimental results of application of blockers. Bifurcation analysis and simulations with the model predict that annihilation and single-pulse triggering should occur with partial block of I_{Ca}. 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 initiation and termination of reentrant rhythms; our model allows investigation of these topics through numerical simulation.

pacemaker; seal-leak current; rapid delayed rectifier potassium current block; slow inward calcium current block; bifurcation analysis

SPONTANEOUS ACTIVITY based on generation of the pacemaker potential (spontaneous phase 4, or diastolic, depolarization) is not normally found in adult ventricular muscle 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 in small clusters of cells isolated from the embryonic chick ventricle (10, 17, 26, 49, 51, 78, 95), in the embryonic mouse ventricle (117), and in the neonatal rat ventricle (86).

After a couple of days in culture, the electrical activity in an isolated embryonic chick ventricular cell, in a small cluster of a few such cells, or in a sparse monolayer is very different from that in situ or in a reaggregate of hundreds or thousands of cells isolated from the ventricle. For example, when trypsin-dispersed ventricular cells from 7-day embryonic chick hearts are used, the upstroke velocity is much lower in single cells, in small clusters of cells, and in sparse monolayers (17, 49, 51, 95) than in reaggregates (14, 16, 19) or in the intact ventricle (19, 97, 98, 118). Spontaneous beating can be abolished by addition of tetrodotoxin (TTX), a blocker of the fast inward Na+ current (I_{Na}), to the medium bathing reaggregates 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 addition of either of the Ca2+ channel blockers D-600 or diltiazem (49, 51).

We carried out an experimental study to characterize the electrical properties of spontaneously beating clusters of cells isolated from the 7-day embryonic chick ventricle and then assembled a mathematical model of this activity. The goal is to use this model to investigate phenomena seen in experiments, such as irregularity of beating in small clusters (10), 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 spontaneous initiation and termination of spiral-wave reentrant motions in monolayers (6). Although ionic models of reaggregates of embryonic chick atrial cells have been described (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 by means of techniques previously described (50) with modifications (79). The hearts of 7-day embryos were removed, and the ventricles were chopped off, minced, and transferred to flasks containing 0.25% trypsin (bovine pancreas; Boehringer Mannheim, Deisenhofen, Germany) in a nominally Ca2+- and Mg2+-free Hanks’ balanced salt solution (HBSS; in mM: 137 NaCl, 5.4 KCl, 0.34 Na2HPO4, 0.44 KH2PO4, 4.2 NaHCO3, and 5 glucose, pH 7.4). The flasks were placed in a shaker bath at 37°C for 7 min. The resulting cell suspension was gently agitated with a pipette and filtered through a 100-μm mesh. HBSS, supplemented with fetal calf serum (5% final...
concentration), was added to stop trypsin activity. The cell suspension was centrifuged at ~100 g for 5 min at 4°C, the supernatant was discarded, and the cell pellet was 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 density of 5 × 10^5 cells/ml.

The cell suspension was separated into aliquots on nonadhesive plastic culture dishes that were incubated at 37°C in a water-saturated atmosphere of 95% air-5% CO2. 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 coverslips. Coverslips with attached flexiPERM rings were placed in cell culture dishes (Greiner) 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 the cells were plated, on clusters containing two to four cells. Recordings were made in the whole cell mode from clusters that were spontaneously beating before they were patched. 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 (\(I_{\text{seal}}\); 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 coverslip 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 (in mM: 137 NaCl, 5.4 KCl, 1.8 CaCl2, 1.1 MgCl2, 2.2 NaHCO3, 0.4 NaH2PO4, 10 Na-HEPES, and 5.6 glucose, with 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 (\(V\)) was recorded using the whole cell recording mode of the patch-clamp technique. Patch pipettes (~2 MΩ resistance) were pulled from glass capillary tubes and filled with pipette solution (in mM: 110 KCl, 4.3 K2-ATP, 2 MgCl2, 1 CaCl2, 11 EGTA, and 10 K-HEPES, with pH adjusted to 7.4 with KOH) with estimated free Ca^{2+} concentration ([Ca^{2+}]) < 10^{-8} M. Electrode potentials were zeroed before seal formation. After the patch was broken, the transmembrane potential was recorded with an amplifier (model EPC-7, List, Darmstadt, Germany). The membrane capacitance was measured by integration of the capacitive transient in response to a voltage-clamp step from ~50 to ~60 mV. After capacity compensation, we compensated for series resistance 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 >50% of series resistance. For generation of voltage-clamp protocols and for recording voltage and current, a personal computer equipped with pCLAMP version 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 and the overshoot potential (OS) 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 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 between two consecutive samples on the upstroke (1-ms sampling interval).

Simulation Methods

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

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

where \(C_m\) is membrane capacitance and \(I_i\) represents the individual ionic currents described below. (A 4th-order Runge-Kutta integration scheme produced virtually superimposable results.) With our choice of time step (\(\Delta t = 0.1\) ms), the change in voltage from time \(t\) to \(t + \Delta t\) was kept to <1 mV. The value of each activation or inactivation variable (\(\xi\)) at time \(t + \Delta t\) was obtained from its value at time \(t\) using the analytic formula

\[
\xi(t + \Delta t) = \xi(t) - \left(\frac{\xi(t) - \xi_0(t)}{\tau}\right) e^{-\Delta t/\tau}
\]

where \(\xi(t)\) is the steady-state or asymptotic value of \(\xi\) at \(V(t)\) and \(\tau\) is the time constant of \(\xi\) at \(V(t)\). L’Hôpital’s rule was used to calculate \(\alpha_m\) 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 (supplemental data for this article may be found at http://ajpheart.physiology.org/cgi/content/full/00683–4.2004.DC1).

Formulation of the Model

Our model consists of six currents: a slow inward Ca^{2+} current (\(I_{\text{CaL}}\)), a slow delayed K\(^+\) current (\(I_{\text{Ks}}\)), a rapid delayed rectifier K\(^+\) current (\(I_{\text{Kr}}\)), an inward rectifier K\(^+\) current (\(I_{\text{Ki}}\)), a linear time-independent background current (\(I_{\text{b}}\)), and a linear nonspecific leak current (\(I_{\text{seal}}\)) generated by the leakage of ions through the gigahm seal of the recording pipette. 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: 1) 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 2) degeneracy, with nonuniformity of equilibrium solutions such as steady states and limit cycles (23, 24, 30, 37, 102).

Drift has been managed in several ways: 1) by finely adjusting parameters to achieve flux balance across the membrane (21, 2) by adding an electroneutral Na$^+$ current of a precise size to produce stability of concentrations (74), 3) by monitoring the stimulus current in a paced quiescent cell (37, 45), and 4) by ensuring that certain ionic concentrations remain fixed (3, 55, 103, 121). It is not clear whether strategies 1 and 2 are robust, because a change in some parameter in the model might require further fine adjustment of the stabilizing parameters. (This is reminiscent of a neutrally stable equilibrium.)

Strategy 3 is, of course, of no use in an unpaced pacemaker cell. Strategy 4 defeats, at least in part, the initial intent in formulating the model as a second-generation model; e.g., when all the internal and external iconic concentrations are held constant, the Na$^+$--K$^+$ pump current ($I_{Na,K}$) and the Na$^+$--Ca$^{2+}$ exchange current ($I_{Na,ca}$) are effectiveness 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. In second-generation models of several different types of cardiac cells, 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 by means of continuation techniques (103; see also Refs. 55 and 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 as a result of Ca$^{2+}$ buffering with EGTA and dialysis of the cell contents of a very small cell volume against the much larger pipette volume (55, 121). Making concentrations fixed then also removes 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 use a first-generation model, as have some authors of other quite recent studies (3).

Capacitance. Unless stated otherwise, ventricular and atrial cells will refer to embryonic chick ventricular and atrial cells, respectively, and $n$-day will refer to a cell isolated from the embryo after $n$ days of incubation. Because the capacitance of a single 7-day ventricular cell in our laboratory is 8--9 pF, we set the capacitance in our three-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) and 4--7 pF (44)]. We model the cluster, which is a mutually synchronized population oscillator, as an isopotential preparation (27).

$I_{Ca}$, $I_{Ca,L}$ has been described in 7-day reaggregates of ventricular cells (72), in small clusters of ventricular cells (28, 79), in single ventricular cells (13, 28, 42, 44), and at the single-channel level (43, 68). In one laboratory, L-type $I_{Ca}$ ($I_{Ca,L}$) was found in 11 of 12 6-day ventricular cells, and T-type $I_{Ca}$ ($I_{Ca,T}$) was found in the remaining cell (13). In another laboratory, only $I_{Ca,T}$ was found in 7-day ventricular cells (68). In a third study, both currents were found, but in only 45% of 7-day ventricular cells was $I_{Ca}$ clearly separable into $I_{Ca,L}$ and $I_{Ca,T}$ on the basis of the voltage threshold for activation (44). In these cells, $I_{Ca,T}$ is half-inactivated at $-49$ mV, whereas $I_{Ca,L}$ is half-inactivated at $-27.5$ mV. In the 55% of cells with nonseparable $I_{Ca}$ half-inactivation occurs at $-42.8$ mV.

We have chosen to use a nonseparable description of $I_{Ca}$ in our model

$$I_{Ca} = g_{Ca}d(V - E_{Ca})$$

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 on the basis of our own voltage-clamp results (79). The equations governing $d$ and $f$ are given in the appendix.

The steady-state inactivation ($f_s$) curve of the nonseparable 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. Because the steady-state activation ($d_s$) curve of the nonseparable current was not reported, we assume that it lies between the $I_{Ca,L}$ and $I_{Ca,T}$ activation curves of Fig. 10 of Ref. 44 and that it has an intermediate slope factor. We have chosen the values of parameters so that the $d_s$ curve is closer to the $I_{Ca,L}$ than to the $I_{Ca,T}$ activation curve, in order that the peak-current current-voltage ($I$-$V$) curve has a maximum at $-60$ mV, which is close to the experimental value for the nonseparable current (44). Figure 1A shows the steady-state $d_s$ and $f_s$ curves.

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

We have set $g_{Ca}$ so that $V_{max}$ is 9.5 V/s in the model, which is close to our mean experimental result (8.5 V/s). The peak-current $I$-$V$ curve then has a maximum value of $-30$ pA/pF at $-40$ 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, scaled for differences in capacitance and...
temperature (Q10 = 3 for peak current amplitude for guinea pig ventricular cells (7)). Figure 1D shows that the current traces from a voltage-clamp protocol in the model are similar in shape to those previously reported from our laboratory at 37°C (79; see also Fig. 7B of Ref. 44, scaled for temperature and capacitance).

Because internal Ca2+ is buffered by our pipette solution, we do not include Ca2+-dependent inactivation of Ik, (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 reaggregates (72).

Delayed K+ currents. The delayed K+ currents Ik and Ik, have been described in reaggregates of ventricular cells (13, 14, 88) and atrial cells (9, 88, 89), in small clusters of ventricular cells (79), and in single ventricular (8) and atrial (12) cells. These currents have also been observed at the single-channel level in ventricular cells (79; see also Fig. 1C of Ref. 8).

Two components of the delayed current, initially termed Ix1 and Ix2, have been seen in atrial reaggregates (89). These two currents correspond to those more recently termed Ik and Ik, respectively, in isolated adult mammalian ventricular cells. Ix2, or Ik, has been described in 7-day ventricular reaggregates (14, 15), in single ventricular cells (8), and in small clusters of such cells (79). However, although Ix1 is robust in experiments carried out using sharp microelectrodes on atrial (89) and ventricular (13) reaggregates, 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 reaggregates and small clusters of atrial cells can be accounted for by the absence of Ix1 in the latter (see Fig. 9 of Ref. 12 and Fig. 17 of Ref. 13). In contrast to the above-mentioned reports, in our 7-day ventricular clusters, the envelope-of-tails test shows two components (79), and application of the specific Ik blocker almokalant (113) removes the more rapidly deactivating component of the tail current. In addition, almokalant produces changes in the action potential consistent with Ik block (see below). At the single-channel level, a K+ channel that activates over a voltage range similar to that over which Ik activates has been described (8). We thus incorporate the Ik and Ik components into our model.

For Ik, we use the formulation

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

where \( n \) is the activation variable. We set the reversal potential (Eks) equal to −75 mV, the value found in our cells (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 37°C, external [K+] of 5.4 mM, and pipette [K+] of 129 mM. This relatively depolarized value of Eks has been found in many studies on Ik, and has been attributed to a slight permeability of the channel to Na+ (65, 121). In obtaining the formulas for the rate constants \( \alpha_n \) and \( \beta_n \) (see APPENDIX), we have first divided the original equations (52) by a factor of 3, to obtain a \( \tau_n \) curve consistent with the experimental values in single ventricular cells at room temperature (see Fig. 2C of Ref. 8), and then multiplied \( \alpha_n \) and \( \beta_n \) by a factor of 2, in correspondence with the reported Q10 (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 I-V curve (Fig. 2C), similar to that seen experimentally (see Fig. 1C of Ref. 8), scaled for capacitance and temperature (Q10 = 2) (111). The steady-state activation curve (Fig. 2A) and the voltage-clamp currents (Fig. 2D) are similar to those previously reported from our laboratory at 37°C (79), as well as those reported elsewhere (8), when compensated for temperature.

For Ik, we use the formulation

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

where \( z(V) \) is the activation variable, and we introduce \( z(V) \) to provide inward rectification so that the fully activated I-V relation (Fig. 3C) is similar in shape to that seen in atrial reaggregates (see Fig. 8C of Ref. 8).

Fig. 2. Characteristics of slow delayed K+ current (Ik) in the model. A: steady-state activation \((n_s)\) curve. B: time constant of activation \((\tau_n)\). C: fully activated \((art)\) Ik. D: simulated current during voltage-clamp steps from a holding potential of −60 mV to −20, 0, and +20 mV (protocol of Ref. 8).
Our form of \( z(V) \) (see APPENDIX; Fig. 3A) gives more current at depolarized voltages than the fit originally used in Fig. 8C of Ref. 89, which corresponds to the fact that we observe a maintained alkamolant-sensitive current at positive voltages (see RESULTS). The function \( z(V) \) represents the very rapid inactivation described for the human ether-a-go-go-related gene (HERG) subunit of \( I_{Ks} \) (92, 93) and for \( I_{K1} \) in single SA node cells (54, 76). The reversal potential \( E_{K1} \) was set to \(-81\) mV on the basis of observations in our laboratory. (This closeness of \( E_{K1} \) to the Nernst potential of \(-85\) mV for \( K^+ \) is characteristic of \( I_{K1} \) in many other cardiac preparations.) Our setting of the maximal conductance \( g_{K1} \) yields a maximum value of the fully activated current (Fig. 3C) comparable to that seen in atrial reaggregates (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 Fig. 5, A and B, of Ref. 89 (the original equations in Table I do not give the fitted curves shown in Fig. 5, A and B). With these modifications, our \( s_{K1} \) and \( g_{K1} \) curves (Fig. 3, A and B) are very close to the data in Fig. 5, A and B, of Ref. 89. A voltage-clamp protocol (Fig. 3D) gives currents similar to those in Fig. 4 of Ref. 89. We use only one time constant of activation; in SA node cells, two time constants of activation of \( I_{K1} \) have been described (76).

\( I_{K1} \), I-V curves of reaggregates of 7-day ventricular cells (14, 15), in small clusters of 7-day ventricular cells (79), and in single 7-day ventricular cells (4) show marked inward rectification at very negative potentials because of the presence of \( I_{K1} \). 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)

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

where we set \( E_{K1} = -81 \) mV, which is, as commonly observed, slightly depolarized to the calculated Nernst potential for \( K^+ (-85 \) mV). Although a time-independent description of \( I_{K1} \) (i.e., \( I_{K1} = I_{K1s} \)) 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 (unpublished observations). However, the time constant of this current is so small \( (\tau_{K1} < 0.2 \) ms over the operative range of voltage; Fig. 4B) that the current is virtually identical in the time-dependent and time-independent descriptions. The maximal conductance \( g_{K1} \) is reduced from the guinea pig value to reflect the smaller \( I_{K1s} \) earlier in development (15, 41).

The steady-state I-V curve for the total current (see curve in Fig. 7A) is then very flat between \(-70 \) and \(-30 \) mV, which agrees with our experimental results (see symbols in Fig. 7A). The \( I_{K1} \), I-V curve (see Fig. 7C) is the main contributor to the positive slope of the total-current I-V curve at very hyperpolarized potentials (see Fig. 7A) and is similar to the Ba\(^{2+}\)-sensitive current at hyperpolarized potentials (4, 79).

\( I_n \). In addition to \( I_{K1} \), which is outward at potentials depolarized to \(-81 \) mV, there is inward background current in 7-day ventricular reaggregates (14). This component has been modeled as a \( Na^+ \) current

\[
I_n = g_n(V - E_n)
\]

where \( E_n = 40 \) mV and \( g_n \) is obtained from Fig. 11 of Ref. 14, scaled for capacitance. Figure 7D gives the I-V relation for this linear current. \( I_{seal} \). It has been pointed out that \( I_{seal} \) flowing through the pipette gigahm 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 nonspecific \( I_{seal} \) in our model

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

with \( g_{seal} \) corresponding to a nominal seal-leak resistance of 5 G\( \Omega \) (20) and \( E_{seal} = 0 \) mV. Figure 7D gives the I-V relation of \( I_{seal} \).

**Currents Not Included in the Ionic Model**

\( I_{Na} \). There is voltage-clamp evidence for the existence of \( I_{Na} \) in reaggregates 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 of 7-day ventricular clusters in our laboratory show a fast inward current 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), suggesting that \( I_{Na} \) might not contribute appreciably to the upstroke phase, especially because Ca\(^{2+}\) channel blockers abolish spontaneous activity (see Fig. 9, A and B). The MDP (\(-60 \) mV in the clusters and \(-67 \) mV in the model) is sufficiently depolarized to essentially render \( I_{Na} \) fully inactivated, because the foot of the \( b_{Na} \) steady-state inactivation curve lies at about \(-50 \) to \(-60 \) mV in 7-day ventricular reaggregates (22) and 7-day ventricular cells (29, 82). Indeed, addition of \( I_{Na} \) to our model, on the basis of the conductance and the activation and inactivation curves from single 7-day ventricular cells (29) and the time constants from 11-day reaggregates (22), slightly increases \( V_{max} \) from 9.5 to 10.2 V/s. In contrast, reaggregates of trypsin-dissociated 7-day ventricular cells have a TTX-sensitive upstroke velocity of 120 V/s in 1.3 mM K\(^+\) and 91 V/s in 4.5 mM K\(^+\), presumably due to the more hyperpolarized MDP of about \(-90 \) 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 h 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 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 h, 64% at 24 h, and 100% at 48 h (57). In contrast, reaggregates of trypsin-dissociated 7-day ventricular cells that are cultured for 24–72 h 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 postnatum, 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, modeling work has suggested a role for a persistent component of a mutated \( I_{Na} \) in the generation of diastolic depolarization in long Q-T (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 Na\(^+\) channels occasionally (1 of 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). Because these long openings do not persist into diastole (see Fig. 1 of Ref. 64), they would not contribute to diastolic depolarization. Incorporation of our standard Hodgkin-Huxley-type \( I_{Na} \) into the
model, as described above, results in a slight 3-ms increase in APD$_{50}$ and a slight 5-ms increase in APD$_{100}$.

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

**Transient outward current.** Although the size of the transient outward current ($I_{to}$) increases with development, the number of isolated ventricular cells possessing $I_{to}$ is extremely low: 7 of 300 cells at 3 days, 5 of $\sim$200 cells at 10 days, and 5 of $\sim$100 cells at 17 days (84). At the single-channel level, an early outward channel appeared in only 1 of 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 reaggregates (9). Moreover, in our own voltage-clamp experiments, we have also found no evidence of $I_{to}$ in clusters. In the SA node, the $I_{to}$ density is smaller in cells with a smaller capacitance (58, 109). For these reasons, we do not include $I_{to}$ in our model.

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

$I_{NaCa}$ and $I_{NaCaB}$. Currents provided by ion pumps and exchangers, e.g., $I_{NaK}$, $I_{NaCa}$, and the Ca$^{2+}$ pump, also contribute to $V$. We employ a first-generation model, which does not have an Na$^{+}$-$K^{+}$ pump, an Na$^{+}$-$Ca$^{2+}$ exchanger, a Ca$^{2+}$ pump, internal Ca$^{2+}$ dynamics, and variable ionic concentrations. However, $I_{NaK}$ 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, because this would result in a second-generation model.

In our laboratory, 10 $\mu$M ouabain has been used to block $I_{NaK}$ in 7- to 10-day ventricular cells or in small clusters of such cells (48). After 1 min of superfusion, the IBIs first decreases, due to an increase in DDR, OS and MDP gradually fall, APD rises, and IBIs increase, 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 artifact due to dialysis and current rundown was minimal.) A similar result is seen in 11-day cells cultured as a confluent layer or polystrand, except the initial effect is seen immediately, presumably because of the use of a perfusion system with rapid perfusate changeover (half-time of $\sim$5 s), 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 reaggregates (100). In another report on 11-day reaggregates 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 (from $-60$ to +20 mV) (see Fig. 1 of Ref. 99). This value scales to $\sim$0.5 pA/pF at an internal [Na$^{+}$] 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 IBIs from 392 to 337 ms and depolarizing the MDP from $-67$ to $-61$ mV; both effects are seen immediately upon block of $I_{NaK}$ in an experiment (see Fig. 18 of Ref. 39). Because the electrogenic component of $I_{NaK}$ is removed within a few seconds of the start of block (Fig. 1 of Ref. 100), some secondary change must be responsible for the cessation of activity that occurs some minutes later (see Fig. 1 of Ref. 39). The most likely candidates are the rises in internal [Na$^{+}$] and [Ca$^{2+}$] after $I_{NaK}$ 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 reaggregates (100) and 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 although $I_{NaCa}$ 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 Ca$^{2+}$ pump in embryonic chick ventricular cells.

Given the above problems, as well as other problems described 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 $I_{K1}$, whereas the time course of $I_{Ca}$ in our model very closely resembles the action potential clamp record (i.e., sum of $I_{Ca}$ and $I_{NaCa}$, and any Ca$^{2+}$-activated currents) obtained in the SA node when Ca$^{2+}$ entry is blocked (120).

**Other currents.** Other currents, such as $I_{La}$ [a sustained inward current, carried by Na$^{+}$, insensitive to TTX, and sensitive to Ca$^{2+}$ channel blockers (71, but see Ref. 107)] and $I_{K(Ca)}$ [a Ca$^{2+}$-activated K$^{+}$ current, for which evidence is found only in the perforated-patch configuration (120)], exist in the SA node. Because there are no reports of these currents in ventricular cells, we do not include them in our model.

**RESULTS**

**Spontaneous Activity**

Action potentials recorded from 17 small clusters clearly show considerable cluster-to-cluster variability (Fig. 5) (17). Figure 6A shows a recording of $V$ obtained from one small
cluster (cluster 7 in Fig. 5), whereas Fig. 6B shows the phase-plane trajectory, in which the rate of change of $V$ ($V_t$) is plotted vs. $V$. For this cluster, the mean values of the parameters, averaged over 100 cycles of activity, were as follows: IBI = 458 ms, MDP = −57 mV, APA = 89 mV, $V_{max}$ = 7.5 V/s, DDR = 85 mV/s, APD$_{50}$ = 124 ms, and APD$_{100}$ = 224 ms. The action potential parameters (means ± SD) of the 17 clusters are given in Table 1. Because of beat-to-beat variability, the action potential parameters for each cluster were averaged over ∼100 beats before the population average was taken. Figure 6, C and D, gives the voltage-time series and the phase-plane trajectory for the ionic model, and Table 1 gives the action potential parameters in the model, which are quite close to the mean experimental values.

**Steady-State I-V Relations**

The curve in Fig. 7A gives the steady-state I-V relation for the total current in the model. This curve corresponds closely to the mean I-V data points obtained from five 7-day ventricular clusters in our laboratory (Fig. 7A, symbols); see also Ref. 4. The steady-state I-V relations of the individual currents in the model are shown in Fig. 7, B–D.

**Currents Underlying the Action Potential**

We previously mentioned that the spontaneous activity in the model (Fig. 6, C and D) has action potential parameters that compare well with the mean experimental values (Table 1). Figure 8 shows $V$ during approximately one cycle of spontaneous activity in the ionic model (A) and the various currents (B-D) and the various activation and inactivation variables (E and F). The upstroke phase is clearly generated by $I_{Ca}$ (Fig. 8C), which rapidly activates (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 Fig. 2B) contributes increasingly to repolarization (Fig. 8C); $I_{Ks}$ is small (Fig. 8C), despite rapid activation (s in Fig. 8F, see Fig. 3B) because of its strong inward rectification (Fig. 3C); there is also a smaller contribution from $I_{seal}$, which is outward but becomes less outward 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 Fig. 7C). There is also a secondary increase of $I_{Ca}$ (Fig. 8C), which serves to maintain the plateau phase of the action potential, despite decreased activation and increased inactivation of $I_{Ca}$ (d and f in Fig. 8E); this 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 po-

![Image](H404.png)

**Table 1. Action potential parameters: experiment vs. model**

<table>
<thead>
<tr>
<th></th>
<th>IBI, ms</th>
<th>$V_{max}$, V/s</th>
<th>MDP, mV</th>
<th>APA, mV</th>
<th>DDR, mV/s</th>
<th>APD$_{50}$, ms</th>
<th>APD$_{100}$, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment</strong></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>
</tr>
<tr>
<td><strong>Model</strong></td>
<td>392</td>
<td>9.5</td>
<td>−67</td>
<td>102</td>
<td>90</td>
<td>108</td>
<td>181</td>
</tr>
</tbody>
</table>

IBI, interbeat interval; $V_{max}$, maximum rate of rise of upstroke; MDP, maximum diastolic potential; APA, action potential amplitude; DDR, diastolic depolarization rate; APD$_{50}$ and APD$_{100}$, action potential duration at 50 and 100% repolarization. For each cluster, mean values of action potential parameters were computed for a train of ∼100 action potentials. Experimental values are means ± SD of these mean values for 17 clusters.
Depolarization but gradually become less inward (Fig. 8).

Although $I_{\text{Ca}}$ 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). $I_{\text{Kr}}$ and $I_{\text{seal}}$ are also inward throughout phase 4 depolarization but gradually become less inward (Fig. 8D).

Currents Underlying the Pacemaker Potential

Because DDR in a three-cell cluster is $\sim 100$ mV/s (Table 1), the net current during diastolic depolarization is tiny ($\sim 2.6$ pA); it is not even appreciable on the scale of Fig. 8B. $I_{\text{Ca}}$ 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). $I_{\text{Kr}}$ and $I_{\text{seal}}$ are also inward throughout phase 4 depolarization but gradually become less inward (Fig. 8D).

Although $I_{\text{Ks}}$, $I_{\text{Kr}}$, and $I_{\text{K1}}$ are outward during phase 4 depolarization, $I_{\text{K1}}$ becomes much less outward (Fig. 8D), $I_{\text{Ks}}$ gradually becomes slightly less outward (not visible on the scale of Fig. 8C) but does not contribute much current, and $I_{\text{Kr}}$ contributes increasingly less outward current as a result of slow deactivation (Fig. 8, C and F).

$I_{\text{Kr}}$ deactivates slowly during diastolic depolarization (Fig. 8, C and F), because the time constant for activation ($\tau_a$) is several hundred milliseconds over the pacemaker range of potentials (Fig. 3B). Hence, $I_{\text{Kr}}$ is not fully deactivated by the beginning of the upstroke of the action potential. However, the increase in voltage during the upstroke rapidly abolishes $I_{\text{Kr}}$ (Fig. 8C) because of its profound inward rectification (Figs. 3C and 8F). As the membrane then repolarizes, fast recovery from the inactivation of $I_{\text{Kr}}$ is responsible for its rectification (Fig. 8D).

The time course of $I_{\text{Kr}}$, during spontaneous activity is very different from that seen in an atrial reaggregate model (see Fig. 15 of Ref. 54), where $I_{\text{Kr}}$ deactivates much more rapidly because of its shorter time constant at the more hyperpolarized MDP of the reaggregate model: about $-90$ mV (89) vs. $-67$ mV (present study). However, action potential clamp studies of rabbit SA cells, which are more depolarized than the chick atrial reaggregate, show a time course of $I_{\text{Kr}}$ very similar to that in our model (see Fig. 1C of Ref. 76), as do SA node models that incorporate a sharply rectifying $I_{\text{Kr}}$ component (see Figs. 6, D and E, of Ref. 54).

Effect of Ca$^{2+}$ Channel Blockers on Spontaneous Activity

Application of D-600, a Ca$^{2+}$ channel blocker, on 7- to 10-day cells and small clusters in our laboratory results in the abolition of spontaneous activity (Fig. 9A), with mean resting potential of $-36.2$ mV ($n = 14$) (49). We observed similar results with another Ca$^{2+}$ channel blocker, diltiazem (51). 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 $I_{\text{Ca}}$ in the model, starting at 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 $\sim 120$ s when $\sim 90\%$ of $I_{\text{Ca}}$ is blocked. Because D-600 blocks $I_{\text{Ca,L}}$, and not the nonseparable $I_{\text{Ca,S}}$ close to $I_{\text{Ca,L}}$, the modeling intervention is similar to the experimental intervention of applying an $I_{\text{Ca,L}}$ blocker. In the experiment and the model, the loss of OS initially proceeds at a slow rate (from just after arrow 1 to just after arrow 3 in Fig. 9C); then the rate of loss accelerates just before spontaneous activity is 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 the experiment and the model, the phase of the 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$\text{100}$ increases (Fig. 9, B and D). Effects in many ways opposite to those described above are seen in our laboratory with administration of a Ca$^{2+}$ channel agonist (BAY K 8644): there are increases in MDP, APD$\text{100}$ increases (Fig. 9B), with mean resting potential of $-36.2$ mV ($n = 14$) (49). We observed similar results with another Ca$^{2+}$ channel blocker, diltiazem (51). 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.

Figure 9E gives the bifurcation diagram for $I_{\text{Ca}}$ block, computed using XPPAUT (25). The bifurcation parameter is $g_{\text{Ca}}$, and the bifurcation variable is $V$. The periodic activity of

Fig. 8. Spontaneous activity in the model. A: transmembrane potential. B: total current. C and D: individual currents. Peak value of $I_{\text{Ca}}$ is $-262$ pA (off-scale deflection in C). E and F: activation and inactivation variables in the model. Also shown is the function $z(V)$, describing rectification of $I_{\text{Kr}}$.  

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The resting membrane potential corresponding to this stable steady state produced in the subcritical Hopf bifurcation. If the control value of 30 nS (see Figs. 4 and 6 of Ref. 49). During ongoing superfusion of these cells with D-600, the number of nondriven action potentials in any one trial gradually decreases from tens of action potentials, then the membrane does not produce triggered action potentials, and finally the membrane becomes excitable (see Fig. 4 of Ref. 49).

A response similar to that shown in Fig. 9, A–D, is seen with \( I_{Ca,L} \) block in the SA node in experiments (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

The model corresponds to the existence of a stable limit cycle in the six-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 maximum value of the \( V \) coordinate of the limit cycle (i.e., the OS) and its minimum value (i.e., overshoot potential (OS)) and minimum (i.e., maximal diastolic potential (MDP)) values of \( V \) are plotted: •, stable; ◦, unstable; HB, Hopf bifurcation.

The coexistence of a saddle-node bifurcation in Fig. 9E is consistent with three prior observations from our laboratory: 1) During washout of D-600, transient flurries of action potentials occur spontaneously before spontaneous activity is permanently reestablished. 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. 2C of Ref. 49). 2) 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 (see 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” behavior is consistent with the existence of a saddle-node bifurcation of limit cycles and can be seen in simulations with the model. 3) 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 (see Figs. 4 and 6 of Ref. 49). During ongoing superfusion of these cells with D-600, the number of nondriven action potentials in any one trial gradually decreases from tens of action potentials, then the membrane does not produce triggered action potentials, and finally the membrane becomes excitable (see Fig. 4 of Ref. 49).

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A response similar to that shown in Fig. 9, A–D, is seen with \( I_{Ca,L} \) block in the SA node in experiments (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

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

In the model, 100% block of $I_{Kr}$ (Fig. 10B shows 50%, as well as 100%, block of $I_{Kr}$) results in a marked depolarization of MDP, a slight slowing of the terminal rate of repolarization, a small increase in APD100, a decrease in APD50, 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 $I_{Kr}$ to remain considerably more outward during the entire pacemaker potential and even during the early part of the action potential (Fig. 10C shows $I_{Kr}$ time course during 100% $I_{Kr}$ block), which is the major cause of the decrease in APD50 in Fig. 10B. This secondary increase in $I_{Kr}$ 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 $I_{Kr}$, this indirect effect of $I_{Kr}$ block on $I_{Kr}$ would not occur, and one would expect a prolongation of APD, as observed in SA node cells in which $I_{Kr}$ was not found (106). [In later studies, however, $I_{Kr}$ was clearly found in SA node cells (59, 110).] The fall in $V_{max}$ and OS is due to a decrease in $I_{Ca}$, with a fall in peak value from 263 to 207 pA during 100% $I_{Kr}$ block as a result of greater inactivation of $I_{Ca}$ during diastolic depolarization. Similarly, in experiments on the SA node, effects on action potential parameters due to selective block of $I_{Kr}$ with the compound E-4031 have been found to be due to “a combination of direct and indirect effects on various ionic currents” (106).

The shortening of APD50 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 the rundown of $I_{Kr}$ that occurs during the 10-min period between rupture of the patch (to enter whole cell recording mode) and initiation of the recording of the effect of almokalant (79). Figure 10D shows the combined effect in the model of 50% block of $I_{Kr}$ (to simulate almokalant) and 20% block of $I_{Kr}$ (to simulate the degree of rundown seen experimentally (79)): APD50 is no longer decreased.

To avoid the above-mentioned artifact 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. Although three clusters stopped beating on exposure to almokalant (and the effect was reversible on washout of almokalant from the bath), another four clusters did not stop beating: IBI was increased in three of four clusters, and there was no change in the remaining cluster. In response to 0.1 mM E-4031, another specific $I_{Kr}$ blocker, half of the single SA node cells stopped beating in one study (106), whereas none stopped in another study (76). When the concentration was raised to 1.0 mM, at which 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 mM E-4031, activity was abolished in all balls; with 0.1 mM E-4031, activity was sometimes extinguished in balls from the central area, but not in those from more peripheral areas (46). The fact that 0.1 mM E-4031 abolishes spontaneous activity in smaller, but not in larger, single SA node cells agrees with this observation (59), provided that small cells do indeed stem from the central area of the node and larger cells stem 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” (see Fig. 13 of Ref. 54).

Effect of Ba2+ on Spontaneous Activity

In 7-day ventricular cells, a low concentration of Ba2+ (0.2 mM) blocks/$I_{K1}$ without greatly affecting $I_{Kr}$ and $I_{Kr}$ (8). Figure 11A shows the effect of 0.1 mM Ba2+ in an experiment, and Fig. 11B shows the effect of blocking $I_{K1}$ completely in the model. In the experiment and the model, there is a depolarization of MDP, a decrease in $V_{max}$, a loss of OS, and a decrease in IBI. With the average drift of action potential parameters in control recordings after 10 min (the time after which the effects due to Ba2+ were measured) taken into account, MDP depolarizes by 5%, $V_{max}$ decreases by 13%, APA decreases by 7%, and IBI decreases by 9% ($n = 4$) (79), whereas in the model, MDP depolarizes by 15%, $V_{max}$ decreases by 28%, APA decreases by 13%, and IBI decreases by 11%. However, the increases in APD and DDR in the experiments (12% increase in APD50 and 28% increase in APD90) are not replicated in the model. Because we have observed neither an increase in the peak $I_{Ca}$ nor slowed inactivation kinetics of $I_{Ca}$ with elevated Ba2+ (79), we attribute the increase in APD in the experiments...
at least partially to rundown of \( I_{Ks} \), as we observed with almokalant (see Effect of Almokalant on Spontaneous Activity).

Increasing \( Ba^{2+} \) 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 \( I-V \) relation when 0.5 mM \( Ba^{2+} \) is applied to small clusters. Figure 11D shows the effect on the steady-state \( I-V \) relation in the model of 100% block of \( I_{K1} \), 33% block of \( I_{Ks} \), and 33% block 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 \( Ba^{2+} \) in Fig. 11C). The steady-state \( I-V \) curve is no longer N-shaped. The effect on spontaneous activity in the experiment and the model is a more marked change from control in the action potential parameters than at the lower \( Ba^{2+} \) concentration (Fig. 11, A and B). Specifically, in the experiment, again with the average drift over a period of 10 min in action potential parameters in control recordings taken into account, the MDP depolarizes by 11%, \( V_{max} \) decreases by 49%, APA decreases by 15%, and IBI decreases by 34% (\( n = 8 \)) (79), whereas in the model, MDP depolarizes by 27%, \( V_{max} \) decreases by 39%, APA decreases by 23%, and IBI decreases by 20%.

At an even higher concentration (1 mM), \( Ba^{2+} \) 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 \( Ba^{2+} \). Depolarization of MDP and cessation of spontaneous activity have previously been described in 6- to 7-day ventricular cells exposed to 5–10 mM \( Ba^{2+} \) (96). Cessation of spontaneous activity is also seen in the model if, in addition to complete block of \( I_{K1} \), \( I_{Ks} \), 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), in contrast to the result of \( I_{Ca} \) block (Fig. 9). Blocking the other background current in the model (\( I_{Ca} \)) results in the cessation of spontaneous activity via an infinite-period bifurcation.

**Effect of \( I_{seal} \) on Spontaneous Activity**

It is possible that \( I_{seal} \) is essential for the generation of spontaneous activity in our model. However, when \( I_{seal} \) is removed, spontaneous beating continues in the model, although 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 they are subjected to patching; i.e., the depolarizing \( I_{seal} \) does not induce spontaneous beating in our clusters. However, patching onto a single cell can change the IBI or even abolish preexisting spontaneous activity (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 \( G\Omega \) in our model (20). This is a worst-case scenario, because 5 \( G\Omega \) is toward 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 \( G\Omega \), which is toward the higher end of that used in our laboratory (79), IBI increases from 392 to 475 ms. Thus differences in seal-leak resistance might account for part of the prepara-

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**Fig. 11. Effect of \( Ba^{2+} \) (experiment and model). A: effect of 0.1 mM \( Ba^{2+} \) on spontaneous activity in a cluster (solid line) vs. control (dashed line). Cluster is not among those (clusters 1–17) in Fig. 5. B: effect of 100% \( I_{K1} \) block (solid line) vs. control (dashed line) in the model. C: steady-state \( I-V \) curve with 0.5 mM \( Ba^{2+} \) (solid line) and in control (dashed line). Values are means of 9 clusters, none of which are shown in Fig. 5. D: steady-state \( I-V \) curve with 100% block of \( I_{K1} \) and 33% block of \( I_{Ks} \) and \( I_{Kr} \) (solid line) vs. control \( I-V \) (dashed line) in the model.**

**Fig. 12. Effect of removal of \( I_{seal} \) in the model.**

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**Effect of \( I_{seal} \) on Spontaneous Activity**

It is possible that \( I_{seal} \) is essential for the generation of spontaneous activity in our model. However, when \( I_{seal} \) is removed, spontaneous beating continues in the model, although 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 they are subjected to patching; i.e., the depolarizing \( I_{seal} \) does not induce spontaneous beating in our clusters. However, patching onto a single cell can change the IBI or even abolish preexisting spontaneous activity (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.

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tion-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, those that use 7-day ventricular cells at an external [K+] of 2.7–5.4 mM generally report that the cells have an MDP between −60 and −70 mV and a slow upstroke velocity of ~10 V/s. One systematic study, which used sharp microelectrodes, reported MDP of −68.4 ± 1.0 mV and OS of 22 ± 0.9 mV (n = 30) at an external [K+] of 4.2 mM (17). Our findings in small clusters (Table 1) are in agreement with the findings of these previous studies.

How does the electrical activity of small clusters compare with that of the in situ ventricular muscle? The APA and APD of our small clusters (and in the other reports on single cells, small clusters, and sparse monolayers mentioned 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_{max} of 70–94 V/s at 5–7 days (19, 98, 118) vs. 8.5 V/s (present study). The absence of diastolic depolarization in the in situ 7-day chick ventricle could be due to overdrive suppression, because, in the only 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 or the time spent in culture (104) could change the electrophysiological properties of cultured cells. Adding the inward background current is needed to generate a steady-state I-V curve that agrees with the experiment (Fig. 7A) and to allow the membrane to come to rest at about −40 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) reaggregates, 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-mentioned 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 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). I_{Na} has been reported during diastolic depolarization in spontaneously active single SA node cells (71, but see Ref. 107); intriguingly, I_{Na} is not present in quiescent SA node cells (71). We know of no reports of I_{Na} in embryonic chick cells.

I_{seal} injected through the gigahm seal-leak resistance is a source of artifact in our experimental recordings. This is true even if the perforated-patch, rather than the ruptured-patch, technique were to be used. In contrast to the case in relatively large adult mammalian ventricular cells with input resistances on the order of tens of megohms, I_{seal} has significant effects when smaller cells (e.g., SA node cells and embryonic cells) with 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 I_{seal} is causing depolarization to the point that I_{Na} and I_{Ca,T} although present, become inactivated and I_{f} becomes deactivated. However, in our model, subtraction of I_{seal} hyperpolarizes the MDP by only 6 mV (resp. 2 mV) while increasing the IBI from 392 to 533 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}, because when I_{Na} is added to the model (see METHODS), removal of the 5-GΩ seal-leak resistance results in a decrease in IBI from 392 to 533 ms and an increase in V_{max} from 10.2 to 10.9 V/s. Similarly, when I_{f} is added to...
the model (see METHODS), the extra hyperpolarization does not activate \( \ell_{I} \) significantly: in this case, removal of \( I_{\text{seal}} \) increases the IBI from 390 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) and 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 an artifact stemming from differences in dissociation conditions from one culture to another (and even from cluster to cluster within the same culture). \( I_{\text{seal}} \) 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). Although at first sight these cell-to-cell differences might appear to be so large as to be problematic for physiological 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 the experiment (Table 1). However, 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 16, the IBI is much smaller than in cluster 17, and the APD is much shorter than in cluster 2. In each of these cases, the model could presumably be modified to replicate the electrical activity 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 or increasing the conductance of \( I_{\text{Ca}} \) 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 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 the 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 various extents in different clusters. Because ~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 review see Ref. 80). It is quite conceivable that some such homeostatic process is occurring here, because, 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 [\( Ca^{2+} \)] that occurs should IBI be changed. Because data are not available for all the currents in 7-day embryonic chick ventricular cells, there are uncertainties in some of the currents: \( I_{\text{K1}} \) is taken from experiments on atrial reaggregates, and we use a nonseparable \( I_{\text{Ca}} \). Although 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 \( I_{\text{Na}} \), which occasionally results in a longer-lasting current (40, 64). As in all work on ionic models of spontaneously active cells, \( I_{\text{h}} \), 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 \( Ca^{2+} \) cycling contributes to generating spontaneous diastolic depolarization (56). Because 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 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 showed 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 (unpublished observations).

**APPENDIX**

**Model Equations**

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

**Membrane Potential**

\[
\frac{dV}{dt} = - (I_{\text{Ca}} + I_{\text{KI}} + I_{\text{K1}} + I_{\text{h}} + I_{\text{seal}})/C_m
\]

**Table 2. Model parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( g_{\text{Ca}} )</td>
<td>Maximal ( I_{\text{Ca}} ) conductance</td>
<td>30 nS</td>
</tr>
<tr>
<td>( g_{\text{Kr}} )</td>
<td>Maximal ( I_{\text{Kr}} ) conductance</td>
<td>7.8 nS</td>
</tr>
<tr>
<td>( g_{\text{K1}} )</td>
<td>Maximal ( I_{\text{K1}} ) conductance</td>
<td>6.0 nS</td>
</tr>
<tr>
<td>( g_{\text{h}} )</td>
<td>Maximal ( I_{\text{h}} ) conductance</td>
<td>3.6 nS</td>
</tr>
<tr>
<td>( g_{\text{seal}} )</td>
<td>Maximal ( I_{\text{seal}} ) conductance</td>
<td>0.2 nS</td>
</tr>
<tr>
<td>( E_{\text{Ca}} )</td>
<td>Reversal potential of ( I_{\text{Ca}} )</td>
<td>40 mV</td>
</tr>
<tr>
<td>( E_{\text{Kr}} )</td>
<td>Reversal potential of ( I_{\text{Kr}} )</td>
<td>(-75) mV</td>
</tr>
<tr>
<td>( E_{\text{K1}} )</td>
<td>Reversal potential of ( I_{\text{K1}} )</td>
<td>(-81) mV</td>
</tr>
<tr>
<td>( E_{\text{h}} )</td>
<td>Reversal potential of ( I_{\text{h}} )</td>
<td>40 mV</td>
</tr>
<tr>
<td>( E_{\text{seal}} )</td>
<td>Reversal potential of ( I_{\text{seal}} )</td>
<td>0 mV</td>
</tr>
<tr>
<td>( C_m )</td>
<td>Membrane capacitance</td>
<td>25.5 pF</td>
</tr>
</tbody>
</table>

\( I_{\text{Ca}}, Ca^{2+} \) current; \( I_{\text{KI}}, \) slow delayed \( K^+ \) current; \( I_{\text{K1}}, \) rapid delayed rectifier \( K^+ \) current; \( I_{\text{h}}, \) inward rectifier \( K^+ \) current; \( I_{\text{seal}}, \) seal-leak current.
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