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Ionic currents that generate the spontaneous diastolic depolarization in individual cardiac pacemaker cells

(Ca^{2+} current/inward rectification/time- and voltage-dependent K^+ current)

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ABSTRACT **An enzymatic dispersion procedure has been developed to obtain viable, spontaneously active single myocytes from cardiac pacemaker tissue: the bullfrog (*Rana catesbeiana*) sinus venosus. Recordings of time- and voltage-dependent Ca^{2+} and K^+ currents have been made by using a single suction-microelectrode technique. The results show that two time- and voltage-dependent currents interact to modulate the slope of the pacemaker potential. These are: (*i*) the decay of a delayed rectifier K^+ current and (*ii*) the activation of a Ca^{2+} current. In addition, the data strongly suggest that cardiac pacemaker tissue does not have an inwardly rectifying background K^+ current.**

The initiation of the heartbeat in vertebrates is known to be of myogenic origin. An essential component of this electrophysiological response is the pacemaker potential, a slow spontaneous depolarization that precedes and triggers the action potential. Quantitative analysis of the mechanisms of cardiac pacing requires very rapid and precise measurements of transmembrane ionic current(s). Although the voltage-clamp technique was first applied to amphibian and mammalian cardiac pacemaker tissue nearly 10 years ago, none of the presently available data provide an unambiguous answer to the question: Which ionic current(s) generate(s) the cardiac pacemaker potential? This may arise from technical difficulties intrinsic in the application of the voltage-clamp technique to multicellular syncytial preparations and/or from the anatomical and electrophysiological heterogeneity of the mammalian sinoatrial node (1). Thus, although the work of Irisawa and colleagues and that of Brown and colleagues have provided useful descriptions of time- and voltage-dependent ionic currents in small strips of rabbit pacemaker tissue and have demonstrated selective effects of various transmitters and pharmacological agents (2–4), no quantitative account of the mechanism of pacing has been published.

In the present series of experiments, we have used an enzymatic dispersion procedure that yields viable, spontaneously discharging single cells from the sinus venosus of the bullfrog, *Rana catesbeiana*. A whole-cell voltage-clamp technique (5–7) has been adapted for these myocytes. Our results consistently show that the interaction of two time- and voltage-dependent ionic currents, I_K and I_{Ca}, superimposed upon a time-independent background inward current, generate the cardiac pacemaker potential in this preparation.

MATERIALS AND METHODS

The enzymatic dispersion procedure, the electrophysiological recording method, and the voltage-clamp apparatus and technique were very similar to those previously used in studies of isolated atrial cells in this laboratory (5, 6). Only recent improvements or novel aspects of these methods will be described here.

The sinus venosus region of the excised bullfrog heart was removed, adhering cardiovascular smooth muscle and connective tissue were dissected away, and the entire sinus venosus was incubated sequentially in enzyme solutions of the following compositions: (*i*) collagenase (300 units/mg) and trypsin (2300 units/mg) in nominally Ca^{2+}-free Ringer's solution for 45 min; (*ii*) elastase (120 units/mg) in Ca^{2+}-free Ringer's solution for 30 min; and finally (*iii*) collagenase (100 units/mg) in Ca^{2+}-free Ringer's solution for 20 min. After the completion of these enzymatic incubations, the remaining tissue fragments were dispersed by aspiration with a blunted, fire-polished Pasteur pipette (tip diameter, approximately 2 mm). The resulting cell suspension was transferred to the recording chamber, and superfusion with normal Ringer's solution was begun. Microelectrodes were fabricated as described previously from glass shanks, having a star-shaped lumen (Radnoti Glass Technology, Monrovia, CA). When back-filled with 1.0 M potassium gluconate/60 mM KCl, these electrodes had dc resistances of 3–5 MΩ and tip potentials of approximately −3 mV.

Fig. 1*A* shows the typical morphology of an isolated cardiac pacemaker cell from the sinus venosus of *R. catesbeiana*. Suction-microelectrode impalements in these cells consistently yield the regular spontaneous electrical activity shown in Fig. 1*B*. Note that the maximum diastolic potential is approximately −75 mV and that the action potential overshoots to approximately +40 mV. The development of the pacemaker potential and the initiation of the action potential in these pacemaker cells are insensitive to high doses of tetrodotoxin (3 μM) but are depressed by inorganic ($CdCl_2$, 100 μM; $LaCl_3$, 50 μM) or organic (D-600, 1 μM; nitrendipine, 0.1 μM) Ca^{2+} channel blockers.

RESULTS

The first series of experiments attempted to identify and characterize the slow outward current(s) in individual sinus venosus pacemaker cells. The time- and voltage-dependent onset and decay of this current is shown in the superimposed records of Fig. 2*A*. Individual records were obtained by applying a paired pulse protocol from a holding potential of −75 mV. P_I was not varied; it depolarized the cell to +30 mV for 5 sec and elicited a reproducible outward current, which turned on with a somewhat sigmoid time course. P_{II} was systematically changed before each clamp cycle so that the

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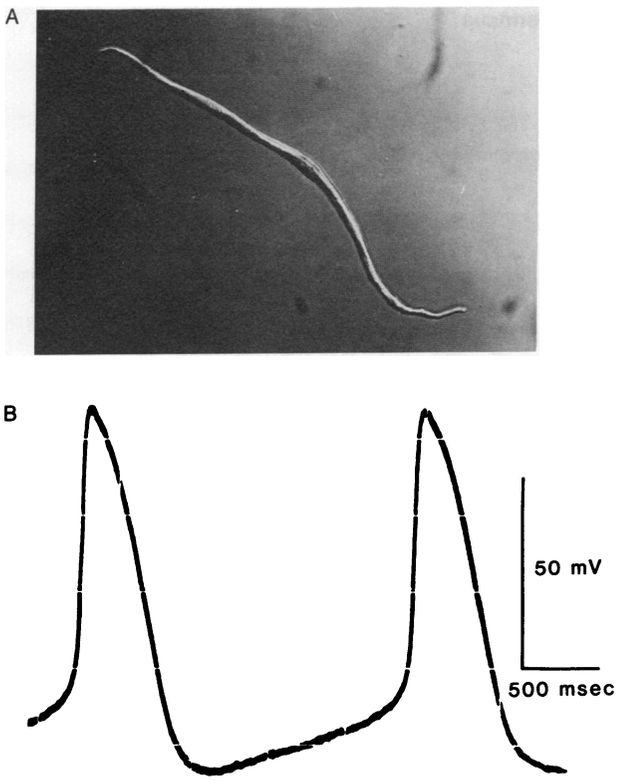

FIG. 1. Intracellular suction microelectrode recording of spontaneous pacemaker activity in an isolated myocyte from the bullfrog (*R. catesbeiana*) sinus venosus. (*A*) A typical cell, viewed with differential interference contrast (Nomarski) optics at ×400. The maximum diameter of this cell is 6 μm and its length is approximately 220 μm. Transmission electron microscopy of these cells has shown that they are individual myocytes and that they lack a transverse tubule system and have only very limited sarcoplasmic reticulum. Both of these findings are typical of the atria and ventricles of the frog heart (8). (*B*) Normal spontaneous pacemaker activity recorded in a 3 μM tetrodotoxin-containing Ringer's solution: 90 mM NaCl/20 mM $NaHCO_3$/2.5 mM KCl/5 mM $MgCl_2$/2.5 mM $CaCl_2$/10 mM glucose, pH 7.4. Note that the heart rate and the action potential parameters are very similar to those previously recorded from the intact sinus venosus or from trabeculae dissected from the sinus venosus (9).

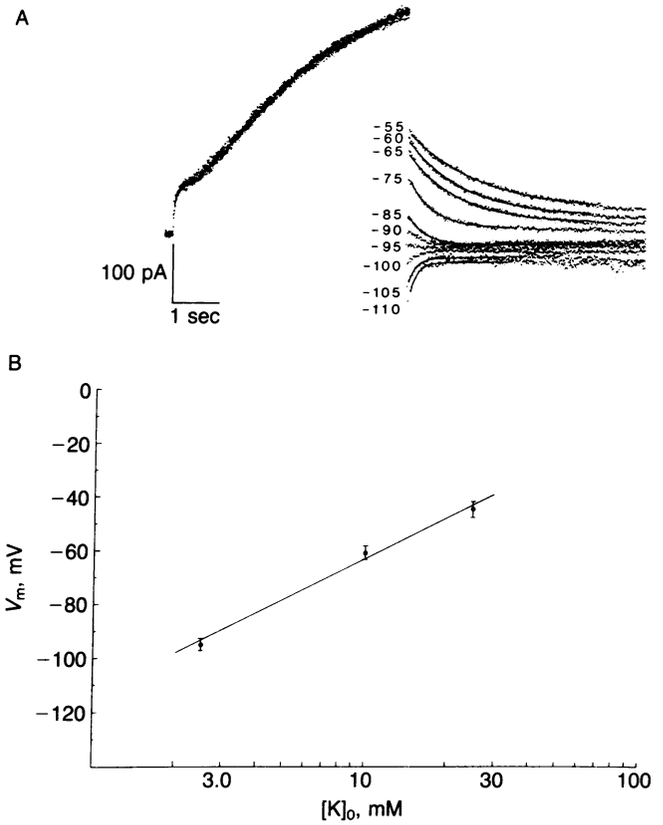

FIG. 2. Kinetics and selectivity of the time- and voltage-dependent outward K^+ current in an isolated cardiac pacemaker cell from the bullfrog sinus venosus. (*A*) The 10 superimposed records show a measurement of the reversal potential, E_{rev}, of this current in normal Ringer's solution (2.5 mM $[K^+]_o$). A double-pulse protocol was used; P_I depolarized the cell from the holding potential, −75 mV, to +30 mV for 5 sec; P_{II} lasted 5 sec and was varied from −55 to −110 mV in 5-mV steps. Note that the reversal potential is between −95 and −100 mV, very near E_K. The kinetics of decay of each current tail were determined by fitting it to a function consisting of a sum of exponentials. By using the criteria developed by Provencher (10), a single exponential function (denoted as the solid black curve) was consistently shown (in 80–85% of all trials) to give the best fit. (*B*) Change in E_{rev}, as a function of $[K^+]_o$. Error bars denote the mean ± SEM ($n = 4$). The best-fitting straight line has a slope of 51 mV per 10-fold change in $[K^+]_o$, showing that this time- and voltage-dependent outward current is carried mainly by K^+. V_m, membrane voltage.

decay of the slow outward current could be recorded at a variety of transmembrane potentials. Each of the superimposed decay "tails" was fitted by a single exponential function. These results illustrate three important points: (*i*) a single Hodgkin–Huxley conductance mechanism may be used to describe the kinetics of this current change; (*ii*) the reversal potential is near −95 mV, as predicted from K^+-selective microelectrode data (11); and (*iii*) the rate of decay of this current is strongly voltage-dependent in the pacemaker range of potentials (−80 to −55 mV).

Fig. 2*B* provides conclusive evidence that the ion-transfer mechanism of this time- and voltage-dependent current is highly selective for K^+. Reversal potential measurements identical to that in Fig. 2*A* were repeated four times in each of three different extracellular K^+ concentrations, $[K^+]_o$. The data, expressed as mean (±SEM) were best-fitted with a straight line having a slope of 51 mV per 10-fold change in $[K^+]_o$. Hence, this current was designated I_K.

Is this time- and voltage-dependent K^+ current importantly involved in generating the pacemaker depolarization in these spontaneously firing single myocytes? A qualitative but quite conclusive answer can be obtained by noting that the "average" transmembrane potential during the pacemaker depolarization is between −70 and −60 mV (see Fig. 1*B*) and that this "average" is maintained for 1–1.5 sec. The data in Fig. 2*A* show that at −65 mV the K^+ current decays with a time constant of approximately 1 sec. Thus, the decay of this K^+ current definitely is a significant factor in controlling the rate of diastolic depolarization. Somewhat similar findings have been reported from two-microelectrode voltage-clamp studies in a variety of multicellular cardiac pacemaker tissues (12–14).

Further inspection of the current tails in Fig. 2*A* yields another important result: P_{II} voltage-clamp steps negative to the maximum diastolic potential activate only a very small steady inward current—i.e., there appears to be no inwardly rectifying background K^+ current, I_{K_1}. Additional evidence for this is shown in Fig. 3. Here, the response to hyperpolarizing clamps steps to −110, −120, and −130 mV are compared in atrial cells (Fig. 3*A*) and sinus venosus pacemaker cells (Fig. 3*B*). The atrial myocytes have been shown (6) to exhibit a substantial I_{K_1}. In contrast (Fig. 3*B*), there is no detectable I_{K_1} in single cells from sinus pacemaker tissue. In the absence of an inwardly rectifying background K^+ current, the data in Fig. 2*A* also provide an estimate of the size of the steady inward current which "holds" the sinus venosus cell

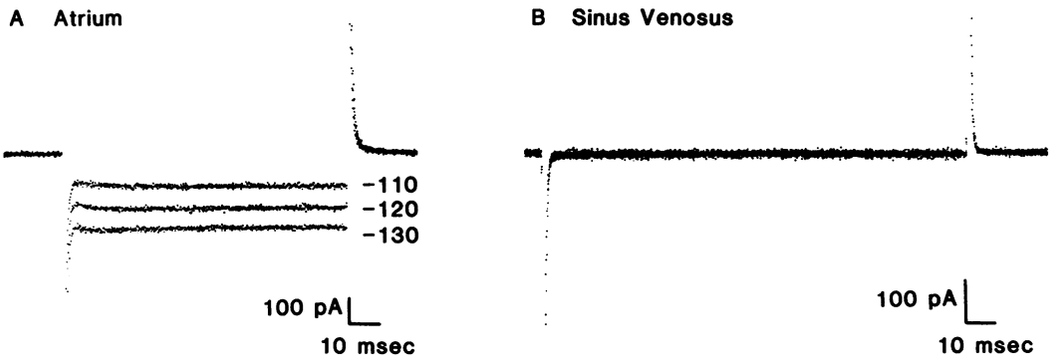

FIG. 3. Superimposed records of I_{K_1}, the inwardly rectifying background K^+ current in bullfrog cardiac cells from the atrium (*A*) and the sinus venosus (*B*). In both experiments the cell was held at −70 mV, and hyperpolarizing pulses were applied to −100, −120, and −130 mV. In atrial cells a conventional I_{K_1} is present; in contrast, in sinus venosus pacemaker cells, I_{K_1} is absent.

membrane potential positive to E_K: this is approximately 20 pA. Although the ion(s) that carry this inward current has not been identified conclusively, Na^+ is the most likely candidate (cf. ref. 4).

Is the time- and voltage-dependent K^+ current the only significant current change underlying the diastolic depolarization or pacemaker potential? In previous experiments using the double sucrose-gap technique to voltage-clamp trabeculae from bullfrog sinus tissue, we (9) attempted to answer this question by holding the membrane potential at the maximum diastolic potential and then applying brief, small (5–10 mV) depolarizations. Our results suggested that a tetrodotoxin-insensitive inward current was activated during the last one-third to one-half of the pacemaker depolarization. We now have extended these findings by making quantitative measurements of the activation of the tetrodotoxin-resistant inward current, I_{Ca}, in single sinus venosus myocytes. Fig. 4 shows typical records of the activation and inactivation of this transient inward current in response to brief (200 msec) depolarizing voltage-clamp steps. In this experiment the pacemaker cell was held at −65 mV and clamped to potentials varying from −60 to +60 mV. The nine I_{Ca} records are superimposed (Fig. 4*A*); peak inward or minimum outward currents are plotted as a *I–V* curve in Fig. 5 (solid line). With regard to the mechanism of pacing, the most important aspect of this data is its pattern of activation in the range of potentials corresponding to the pacemaker potential. Fig. 4*B* illustrates high-gain records of I_{Ca} activation at three potentials in the pacemaker range: −60, −55, and −50 mV. These data show that I_{Ca} is first activated between −60 and −55 mV. Reference to Fig. 1*B* confirms that these potentials are in the pacemaker range: I_{Ca} is activated in the last one-third of the normal potential "swing" of the diastolic depolarization or pacemaker potential. It may appear that this very small amount of inward current is completely insignificant. However, calculation of the amount of net current needed to produce the pacemaker potential, provides the basis for considering any 2- to 5-pA current change to be functionally important. Our measurements show that (*i*) the total cell capacitance is approximately 80 pF and (*ii*) the maximum rate of depolarization during development of the pacemaker depolarization is 0.02–0.05 V/sec. In a uniformly polarized cell (i.e., one in which the regenerative response is a "membrane" as opposed to a "conducted" action potential), the required net inward current is given by:

$$i_{ion} = i_{cap} = -C_m \frac{dV}{dt},$$

where i_{cap} is the capacitative current, C_m is the membrane capacitance, V is voltage, and t is time. Substitution of the values of C_m and dV/dt shows that the net current necessary for generating the pacemaker depolarization in this single-cell preparation is indeed very small: $2–4 \times 10^{-12}$ A.

A second very important finding in these experiments (Fig. 5) is illustrated by the data obtained in response to hyperpolarizing clamp steps. In the range of potentials −80 to −120 mV, cardiac muscle normally exhibits marked inward-going rectification. In contrast, these isolated pacemaker cells exhibited a nearly linear *I–V* relationship with a very large input resistance, R_{in}: 1–2 GΩ. As shown in Fig. 3, this is very different than in the immediately adjacent right atrium in which prominent inward-going rectification is consistently observed. In addition, it is worth noting that in this preparation these hyperpolarizing clamp steps do not

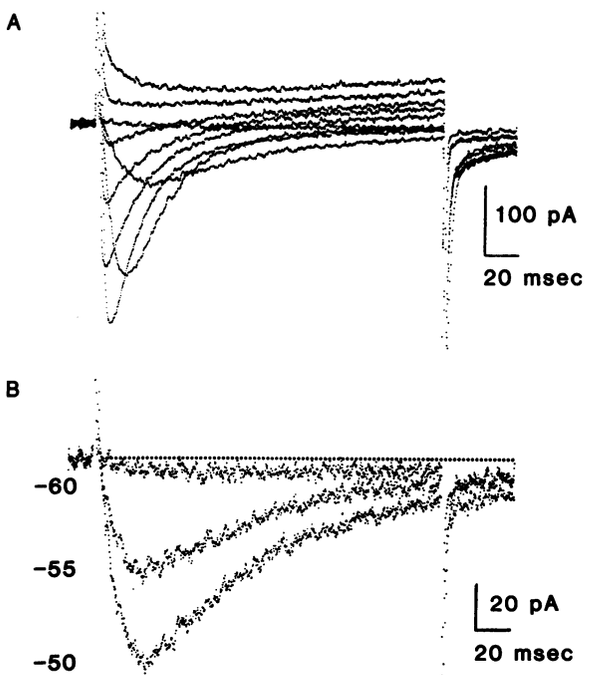

FIG. 4. Tetrodotoxin-resistant Ca^{2+} current in an isolated pacemaker cell from bullfrog sinus venosus. (*A*) Superimposed family of currents elicited by holding the cell at −65 mV and applying 200-msec clamp steps from −60 to +60 mV. The corresponding *I–V* curve, obtained from the peak inward or minimum outward current at each potential, is shown in Fig. 5. Although these data were obtained in Ringer's solution containing 3 μM tetrodotoxin, additional experiments (not shown) done in the absence of TTX gave very similar results, suggesting that cardiac pacemaker cells from the bullfrog lack a conventional I_{Na}. (*B*) Activation of I_{Ca} at potentials corresponding to the last one-third (most depolarized) of the pacemaker potential. Note that at potentials positive to −60 mV a significant net inward current can be recorded.

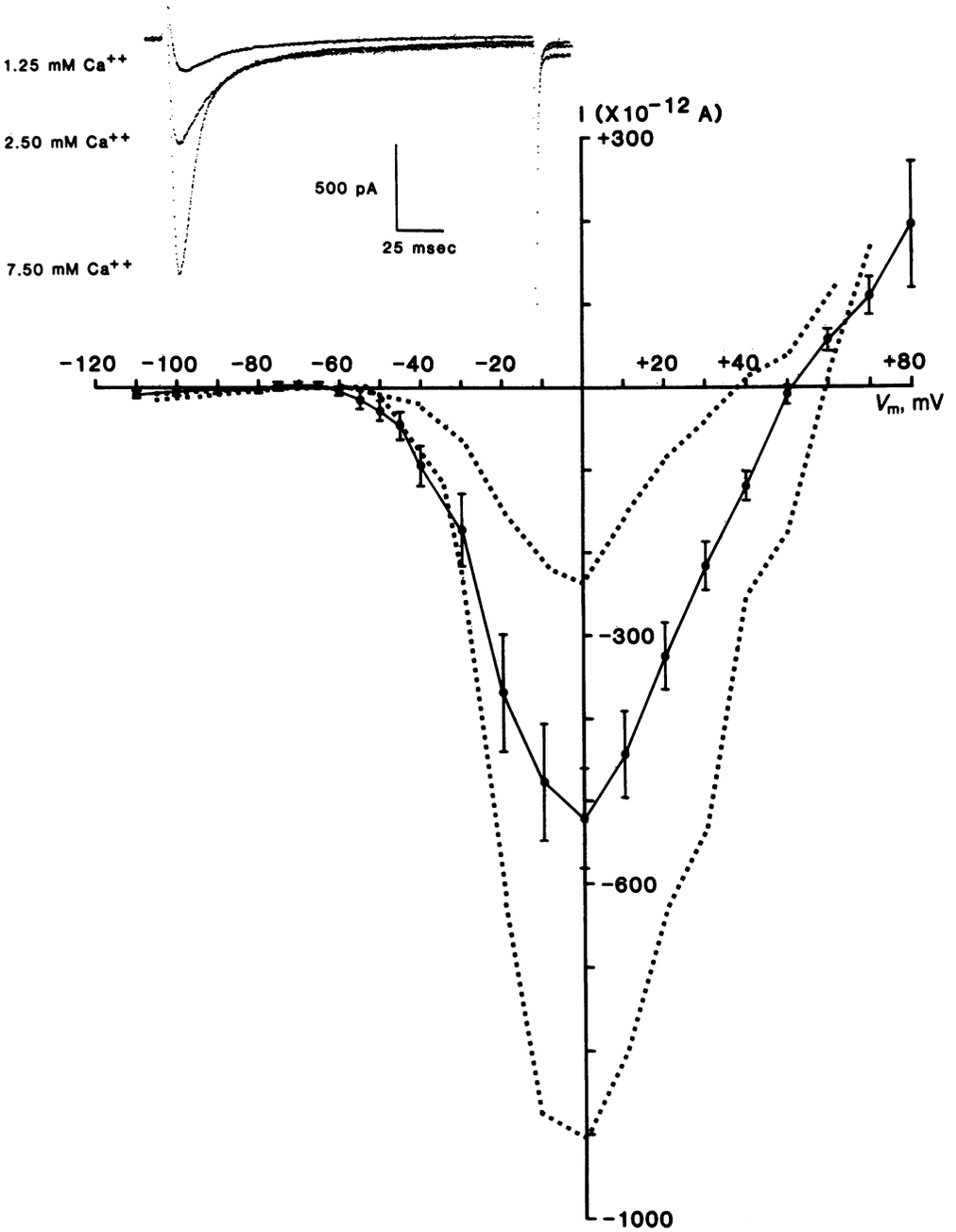

FIG. 5. Dependence of the tetrodotoxin-resistant transient inward current, I_{Ca}, on $[Ca^{2+}]_o$. Control data obtained in 2.5 mM $[Ca^{2+}]_o$ Ringer's solution are illustrated by the *I–V* solid curve, which was constructed using data from 18 different cells, each held at −80 mV and clamped in the range −110 to +80 mV in 10-mV steps. The *I–V* curve was plotted as peak inward or minimum outward currents, denoted as mean ± SEM. The dotted *I–V* curves were obtained in 1.25 and 7.5 mM $[Ca^{2+}]_o$, respectively. The inset shows that the current at 0 mV is strongly modulated by changes in $[Ca^{2+}]_o$; and the superimposed *I–V* curves further illustrate the voltage-dependence of this effect. In these experiments when $[Ca^{2+}]_o$ was changed, the total divalent cation concentration in the Ringer's solution was held constant by addition or withdrawal of $MgCl_2$. Note that in these pacemaker cells, hyperpolarizing voltage clamp steps do not activate any measureable inwardly rectifying background K^+ current.

elicit any measurable slow time- and voltage-dependent inward current i_h or i_f (cf. ref. 15).

Fig. 5 *Inset* provides further information regarding the selectivity of this tetrodotoxin-resistant inward current, I_{Ca}. The three superimposed records, each obtained in response to a 200-msec depolarization to 0 mV from −70 mV show that changes in $[Ca^{2+}]_o$ result in marked changes in this transient inward current. The superimposed *I–V* curves illustrate the voltage-dependence changes in I_{Ca} when $[Ca^{2+}]_o$ is altered. The apparent reversal potentials were approximately +35, +50, and +58 mV in 1.25, 2.5, and 7.5 mM $[Ca^{2+}]_o$, respectively—yielding a dependence of this reversal potential on $[Ca^{2+}]_o$ of 27 mV per 10-fold change in $[Ca^{2+}]_o$.

DISCUSSION

Our results show that it is possible to isolate electrophysiologically viable cardiac pacemaker cells from the bullfrog sinus venosus by using a modification of a relatively simple enzymatic dispersion technique. These individual myocytes exhibit a prominent time- and voltage-dependent outward current, which is carried mainly by K^+ ions. This current, I_K, is activated during the action potential and then decays in the range of potentials in which the pacemaker potential develops; the kinetics of its decay show that it is a major controlling factor of the initial slope of the diastolic depolarization. In addition, a Ca^{2+} current is activated later in the pacemaker

depolarization. This current is an important determinant of heart rate; moreover, it is a very likely target for modulation of the autonomic transmitters (16). The resulting working hypothesis for the mechanism of pacing based on these single-cell measurements is very similar to that previously proposed by Brown *et al.* (9). These new, quantitative measurements, however, provide more direct evidence for the relative roles of I_{Ca} and I_K and the steady-inward background current in cardiac pacemaking.

Finally, it is interesting that these cardiac pacemaker cells do not have an inwardly rectifying background K^+ current. Recently Noma *et al.* (17) have found that mammalian cardiac pacemaker cells also lack I_{K_1}, and Giles and van Ginneken (18) have shown that in rabbit subsidiary pacemaker tissue, the crista terminalis, the I_{K_1} current is exceedingly small in comparison to atrium or ventricle. This may explain the well-known lack of sensitivity of cardiac pacemaker cells to physiological changes in $[K^+]_o$.

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