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Sodium Currents in Toad Cardiac Pacemaker Cells

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Abstract. Cells in the pacemaker region of toad (*Bufo marinus*) sinus venosus had spontaneous rhythmic action potentials. The rate of firing of action potentials, the rate of diastolic depolarization and the maximum rate of rise of action potentials were reduced by TTX (10 nM to 1 μM). Currents were recorded with the whole cell, tight seal technique from cells enzymatically dissociated from this region. Cells studied were identified as pacemaker cells by their characteristic morphology, spontaneous rhythmic action potential activity that could be blocked by cobalt but not by TTX and lack of inward rectification. When calcium, potassium and nonselective cation currents (I_f) activated by hyperpolarization were blocked, depolarization was seen to generate transient and persistent inward currents. Both were sodium currents: they were abolished by tetrodotoxin (10 to 100 nM), their reversal potential was close to the sodium equilibrium potential and their amplitude and reversal potential were influenced as expected for sodium currents when extracellular sodium ions were replaced with choline ions. The transient sodium current was activated at potentials more positive than −40 mV while the persistent sodium current was obvious at more negative potentials. It was concluded that, in toad pacemaker cells, TTX-sensitive sodium currents contributing both to the upstroke of action potentials and to diastolic depolarization may play an important role in setting heart rate.

Key words: Pacemaker cells — Transient sodium currents — Persistent sodium currents

Introduction

The cells of most regions of the heart generate action potentials that depend on inward movement of sodium ions and can be blocked with TTX [13, 24]. Thus in ventricular, atrial and Purkinje cells in the heart, voltage-dependent sodium currents are activated by depolarization [14]. These currents show a rapid time-dependent inactivation during sustained depolarizations and are only fully reactivated upon returning to membrane potentials more negative than −100 mV [4, 5]. Characteristically, such sodium channels are blocked by tetrodotoxin, but only at relatively high concentrations [5, 9, 24, 28]. More recently, a "persistent" TTX-sensitive sodium current that has been recorded in voltage-clamped rat ventricular muscle has been shown to be activated at diastolic potentials [22, 29].

There are conflicting views, however, as to whether voltage-dependent sodium channels are present in cardiac pacemaker cells. As pacemaker cells continue to generate action potentials in the presence of TTX [23, 30], an inward TTX-sensitive sodium current is evidently not essential for action potential activity in these cells. Indeed, it has been proposed that there is no voltage-dependent sodium current in single cells isolated from regions of amphibian heart which contain pacemaker cells [8]. In mammalian pacemaker cells, sodium channels *are* thought to be present but inactivated in the range of membrane potentials that normally occur during pacemaker activity [23]. However, more recent studies have indicated that sodium channels are present in the majority of pacemaker cells isolated from the mammalian sinoatrial node and contribute to the upstroke of the action potential [10]. In the sinus venosus of the toad, the observation that TTX invariably reduces the rate of generation of pacemaker action potentials indicates that sodium channels are involved in normal activity [7, 12].

It is shown in this study that both transient and persistent, voltage-dependent, TTX-sensitive sodium currents are present in pacemaker cells in toad heart. The membrane potentials at which these currents are active suggest that they play a role in setting heart rate.

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Materials and Methods

Action potentials were recorded from preparations of sinus venosus of the toad, *Bufo marinus*. Toads were anaesthetised using an aqueous solution of 0.5% tricaine methanesulfonate. Preparations, consisting either of the sinus venosus alone or of the sinus venosus left in continuity with the two atria, were pinned out in a shallow recording chamber. The preparations were continuously perfused at a rate of 6 ml/min (bath volume 1 ml) with a physiological saline containing (mM): NaCl 115; KCl 3.2; $NaHCO_3$ 20; NaH_2PO_4 3.1; $CaCl_2$ 1.8; $MgCl_2$ 1.4; glucose 16.7; equilibrated with 95% oxygen/5% carbon dioxide. All experiments were carried out at room temperature (20–25°C). Action potentials were recorded from cells which lay in the exposed center of the dorsal wall of the sinus venosus, some 5 to 7 mm distant from the sinoatrial aperture, using conventional techniques with fine glass microelectrodes (resistances 80–150 MΩ when filled with 0.5 M KCl). Pacemaker cells were selected on the basis that the diastolic depolarization led smoothly into the upstroke of the pacemaker action potential. Recordings were taken from a number of muscle bundles until such recordings were obtained. All membrane potential records were low-pass filtered with a cutoff frequency of 1 kHz, digitized, and stored on disk for later analysis.

To record membrane currents, single cells were enzymatically dissociated from sinus venosus preparations. Toads were killed by decapitation and pithed. The heart was removed and the sinus venosus dissected free and placed into calcium-free Ringer solution bubbled with oxygen for five min. Intact sinus venosus tissue was then transferred into 25 μM calcium-Ringer solution containing 300 units/ml collagenase (Worthington) at 28°C for 60 min. The solution was bubbled with oxygen while being gently stirred. The tissue was transferred into fresh 25 μM calcium-Ringer solution containing 140 units/ml collagenase and 40 units/ml elastase (Sigma) for a further 50 min. The tissue was then placed in a calcium-free Ringer solution for one hour, before being triturated gently in 25 μM calcium-Ringer solution to dissociate the cells. The cells were allowed to settle onto glass coverslips and perfused with 200 μM calcium-Ringer's solution for 1 min, then perfused with 2 mM calcium-Ringer's solution. Action potentials or currents were recorded using the whole-cell patch clamp technique [17]. In order to record spontaneous action potentials in several experiments, nystatin (240 μg/ml) was added to the pipette solution to give a perforated patch with minimum disruption of the cell interior [18, 25].

Patch electrodes were made from borosilicate glass and had resistances of 5 to 10 MΩ. The intracellular (patch pipette) solution generally had the following composition (mM): CsF 40; CsCl 60; $MgCl_2$ 2; KEGTA 10; $CaCl_2$ 2; Na_2ATP 5; TES (N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid) 10; pH adjusted to 7.2 with KOH. The solution bathing the cells had the composition (mM): NaCl 100; KCl 2.5; CsCl 5; $CoCl_2$ 5; HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) 10; $CaCl_2$ 2, $MgCl_2$ 1, pH adjusted to 7.2 with NaOH. These solutions were designed to block calcium and potassium currents as well as I_f. Recordings were made at room temperature (20 to 22°C).

Results

Effect of Tetrodotoxin on the Frequency of Action Potentials in Sinus Venosus Preparations

Pacemaker cells in intact sinus venosus preparations were identified by the time course of their cyclical changes in membrane potential: the diastolic depolarization led smoothly into the upstroke of the action potential. Spontaneous action potentials in these cells had a frequency of 33 to 46 beats per min (38.7 ± 2.2; mean ± 1 SEM, 6 preparations). The maximum diastolic potential was in the range −63 to −69 mV (mean = −66.2 ± 1.2 mV). The peak amplitude of action potentials was in the range of 75 to 110 mV (98.0 ± 5.6 mV) and they had a maximum rate of rise of 3.2 to 11 V/s (7.5 ± 1.2 V/s).

The rate of generation of pacemaker action potentials is reduced in this preparation when TTX is added to the extracellular solution [7, 12]. This observation was briefly reconfirmed in this study. In each of three preparations, TTX (100 nM) reduced the rate of generation of pacemaker action potentials to about half the control rate. When lower concentrations of TTX were applied, it became apparent that even quite low concentrations of TTX slowed the rate. These effects are illustrated in Fig. 1. TTX at a concentration of 10 nM caused the rate of generation of pacemaker action potentials to fall from 43 beats/min to 34 beats/min (Fig. 1 *A* and *B*). In control solution (*A*), the frequency of action potentials was 43 min^{-1} and the diastolic potential immediately after an action potential was −68 mV. Since the peak diastolic potential led smoothly into the upstroke of an action potential and the maximum dV/dt was less than 10 V/s, the recording was identified as being made from a pacemaker cell. The middle set of traces (*B*) shows recordings from the same cell 10 min after adding TTX (10 nM) to the extracellular solution. The frequency of action potentials had fallen to 34 min^{-1}, the peak diastolic potential was −64 mV and the rate of rise of action potentials was reduced. The lower set of traces, *C*, shows recordings from the same cell 10 min after increasing the concentration of TTX from 10 nM to 100 nM. The frequency of action potentials was further reduced to 27 min^{-1}, the peak diastolic potential was −60 mV and the maximum rate of rise of pacemaker action potentials was reduced further. No further change was detected when the concentration of TTX was increased to 1 μM (*not shown*). By comparing records in *A*, *B* and *C*, it can be seen that the reduction in frequency in action potentials was associated with a slowing of diastolic depolarization. Similar observations were made in the other two preparations. These observations show that TTX-sensitive currents contribute both to the upstroke of action potentials and to diastolic depolarization in the sinus venosus. It was concluded that cells in the pacemaker region are able to generate TTX-sensitive currents which contribute to pacemaking activity.

Identification of Isolated Cells as Pacemaker Cells

Morphology

Experiments were carried out on cells isolated from preparations of sinus venosus which had been dissected

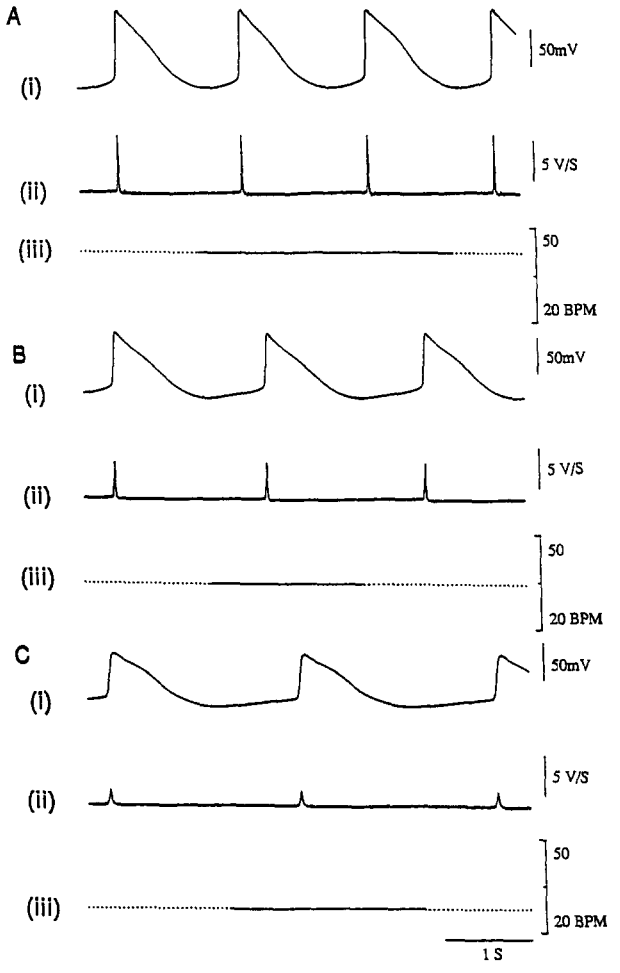

Fig. 1. Effects of TTX on action potentials recorded from a cell in toad sinus venosus. The three sets of traces in *A*, *B* and *C* represent the membrane potential (i), dV/dt (ii) and frequency of action potentials (iii). Records were obtained in control solution (*A*), in a solution containing 10 nM TTX (*B*) and in a solution containing 100 nM TTX (*C*). The time calibration bar refers to all records.

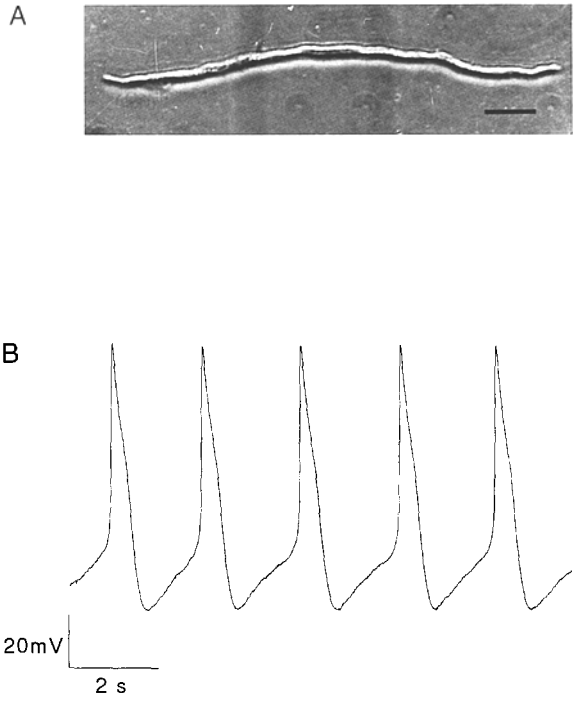

Fig. 2. Identification of pacemaker cells isolated from the sinus venosus. (*A*) an enzymically isolated single pacemaker cell from toad. The horizontal calibration bar denotes 20 μm. (*B*) spontaneous action potentials recorded from a single pacemaker cell using the nystatin perforated-patch technique. Maximum upstroke velocity was 4.1 V/s (bath temperature 18°C).

free of adjacent atrial muscle fibers. The dissection was performed under a microscope to ensure that only the central pacemaker region was used for cell dissociation. Cells dissociated from this region had diameters of 4 to 5 μm and lengths of some 100 to 150 μm. One of these cells is illustrated in Fig. 2*A*. Striations were less obvious in these cells than in atrial or ventricular cells prepared from the same animals. Pacemaker cells isolated from other amphibian hearts have been reported to have similar sizes and appearances to these cells [15].

Spontaneous Rhythmic Action Potentials

Many dissociated cells showed spontaneous contractile activity. Spontaneous action potentials could be recorded from these cells using the perforated patch technique (Fig. 2*B*). Action potentials persisted but at a lower frequency in the presence of TTX (10 nM) and disappeared when the extracellular solution contained 5 mM cobalt.

No Inward Rectifier

When electrophysiological recordings have been made from cardiac myocytes isolated from other amphibian and mammalian hearts, it has been found that cells isolated from either atria or ventricles always have an inwardly rectifying potassium current whereas pacemaker cells do not [15, 20, 21, 27]. As a check on the identity of the cells used in this study, some experiments were done with no caesium in solutions and 2.5 or 5 mM K^+ in the extracellular solution. Cells were held at 0 mV and their membrane potential stepped to potentials in a range from −10 to −150 mV. Under these conditions, cells isolated from the sinus venosus displayed no inwardly rectifying current whereas cells isolated from ventricles had a prominent inwardly rectifying current. This is illustrated in Fig. 3.

The small inward currents shown in the lower panel of Fig. 3*A* were generated by voltage steps to −30, −60, −90, −120 and −150 mV (upper panel, Fig. 3*A*) in a pace-

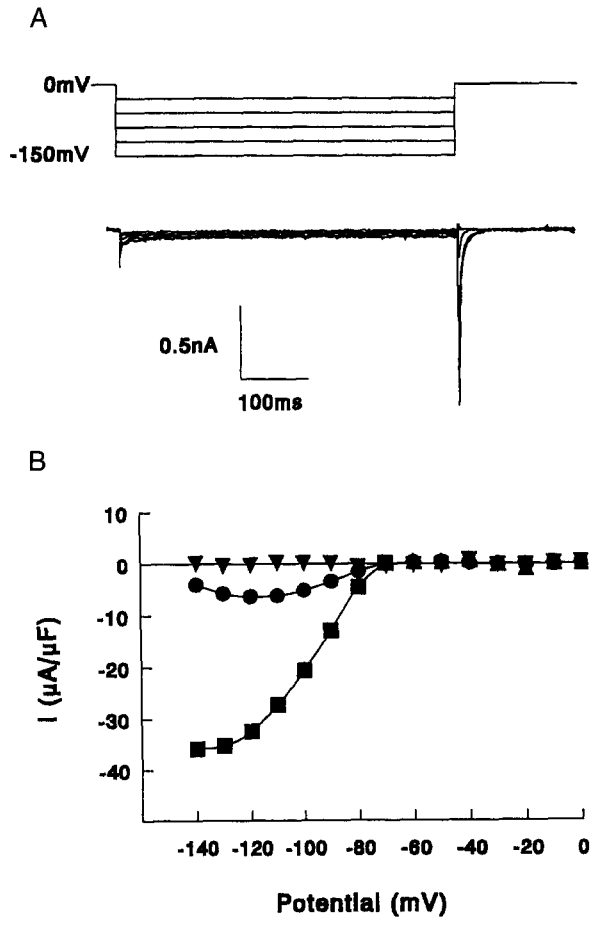

Fig. 3. Lack of inward rectification in pacemaker cells. (*A*) Currents generated by hyperpolarizing pulses in a pacemaker cell with 5 mM K^+ in the extracellular solution. The potential was held at 0 mV, stepped to −30, −60, −90, −120 and −150 mV for 500 msec and then returned to 0 mV (upper panel). The currents generated in a cell from the sinus venosus with this protocol are shown superimposed in the lower panel. Note that following pulses to potentials more negative than −30 mV, a large transient current was evoked when the membrane potential returned to 0 mV. (*B*) Comparison of currents produced with this protocol in a toad pacemaker (filled triangles), atrial (filled circles) and ventricular (filled squares) myocyte. The extracellular solution contained 2.5 mM K^+. Note that current is normalized to cell capacitance.

maker cell. There is essentially no inward current. In contrast, the same voltage protocol in atrial and ventricular myocytes generated significant inward currents (Fig. 3*B*). Current-voltage (*IV*) relationships for the inward currents (normalized to capacitance) produced by hyperpolarizing voltage pulses in a toad pacemaker (filled triangles), atrial (filled circles) and ventricular (filled squares) myocyte are shown for comparison in Fig. 3*B* which illustrates the lack of inward current in the pacemaker cells.

This lack of inward current was seen in all eight pacemaker cells examined in this way. It might have been expected that hyperpolarization would have activated a nonspecific cation current (I_f). However, pacemaker cells in amphibia appear to have very little I_f current because caesium, which blocks I_f, has little effect on pacemaking activity in intact toad [12] or other amphibian [15] preparations. In addition, the results shown here were obtained from cells which had been held in whole cell recording mode for at least 10 min. It has been reported that I_f is very labile and shows pronounced 'rundown' during prolonged recording periods [11]. The inward currents in the atrial and ventricular myocytes had the characteristics of the inwardly rectifying K^+ current [15, 19]: the voltage-dependence and magnitude of the current was influenced by extracellular K^+ and the current was abolished when barium ions were added to the external solution (*not shown*).

In summary, the cells isolated from sinus venosus preparations had an appearance typical of pacemaker cells, displayed spontaneous action potentials that were blocked by cobalt but not by TTX and lacked an inwardly rectifying potassium current. Thus, they fulfilled the criteria accepted for pacemaker cells [20, 31].

It can be seen in Fig. 3*A* that there was a prominent, rapid, transient, inward current when the potential was returned to 0 mV from potentials more negative than −30 mV. Atrial and ventricular cells, like those dissociated from sinus venosus, also generated transient inward currents under these conditions. It seemed likely that these transient inward currents were sodium currents that recovered from inactivation during the hyperpolarizing pulse and were then activated on return to 0 mV. The characteristics of the inward current after the hyperpolarizing pulse were explored using more conventional pulse protocols.

The Transient Inward Current in Pacemaker Cells is a Sodium Current

To prevent contamination of the inward currents with potassium currents, all subsequent experiments were carried out in solutions in which caesium ions were present in both the bathing solution and in the patch pipette (*see* Materials and Methods). Currents were recorded using the standard whole-cell tight seal technique [17] from 28 cells with an $[Na^+]_o$ of 104 mM: in 23 of them, the $[Na^+]_i$ was 10 mM and, in the other 5, $[Na^+]_i$ was 70 mM. In these cells, depolarization to potentials more positive than −40 mV evoked obvious transient inward currents. Currents generated by voltage pulses to potentials between −40 and +60 mV from a holding potential of −130 mV in one cell are shown in Fig. 4*A*. The currents reached a peak within 2 to 3 msec and rapidly inactivated over the following 10 to 20 msec.

The relationship between peak current and membrane potential is shown in Fig. 4*B*. It can be seen that the peak current reached a maximum at a potential of −10 to 0 mV and reversed at +54 mV. Data from eight pace-

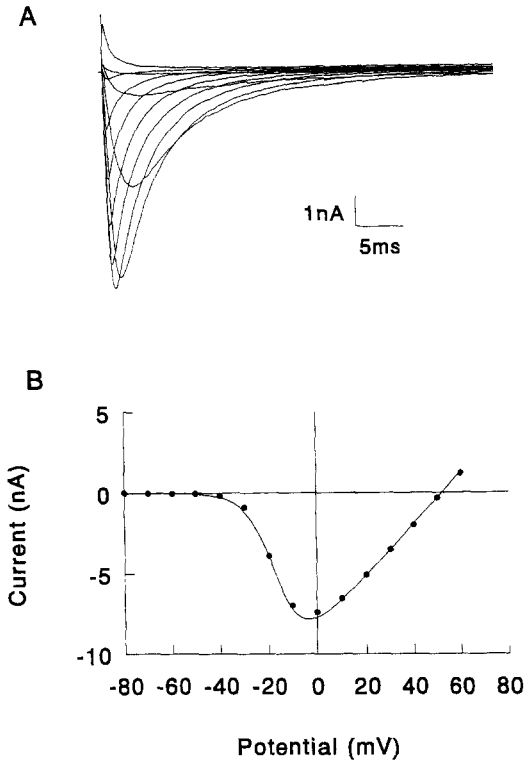

Fig. 4. Whole cell recording of sodium currents. (*A*) a family of currents evoked by depolarizing pulses to potentials between −40 and +60 mV from a holding potential of −130 mV. The external solution contained 104 mM sodium and the internal (pipette) solution contained 10 mM sodium. $CoCl_2$ and CsCl were also present in the external solution. (*B*) the current-voltage relationship for the currents shown above. Maximum peak current amplitude was −7.4 nA (evoked by a step to 0 mV), and the current reversed at about 54 mV. The line through the data points is $I = G_{max} \cdot (V - E_{Na})/(1 + \exp((V' - V)/k))$ with $E_{Na} = 54$ mV, $V' = -16$ mV and $k = 7$ mV.

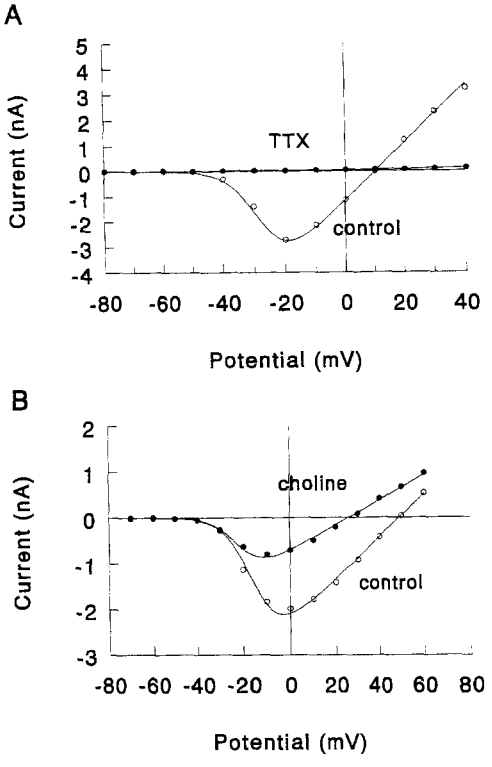

Fig. 5. Evidence that the transient current is a sodium current. (*A*) current-voltage curves recorded with an internal sodium concentration of 70 mM before (open circles) and after (filled circles) addition of TTX (1 μM) to the extracellular solution. The line through the open circles, fitted as in Fig. 4, gave a reversal potential of +10 mV ($V' = -27$ mV, $k = 5$ mV). (*B*) current-voltage curves recorded with an internal sodium concentration of 10 mM and an extracellular sodium concentration of either 104 mM (open circles) or 54 mM (filled circles, 50 mM choline chloride substituted for 50 mM sodium chloride). The reversal potentials were 48 mV (open circles) and 27 mV (filled circles).

maker cells gave an average reversal potential of +57 ± 1.2 mV, close to the calculated sodium equilibrium potential, E_{Na}, of +59 mV. In the five experiments carried out with an internal sodium concentration of 70 mM, the average reversal potential for the current was 13.1 ± 3.0 mV, also very close to the calculated E_{Na} of +10 mV. A current voltage curve for one of these cells can be seen in Fig. 5*A* (open circles). The inward current in this cell reversed at +10 mV. The shift in the current-voltage curve caused by changing the extracellular $[Na^+]$ from 104 to 54 mM in one of the experiments (choline substituted for Na^+) is illustrated in Fig. 5*B*. Currents were reduced in amplitude and the reversal potential shifted by about the 17 mV expected for a current carried predominantly by sodium ions.

Block of the Transient Current by TTX

The transient inward current was blocked by TTX (1 μM, filled circles, Fig. 5*A*). At lower TTX concentrations, the Na^+ current was partially blocked, as illustrated in Fig. 6*A*. Exposure of a cell to 10 nM TTX produced a significant reduction in sodium current. Increasing the TTX concentration to 100 nM blocked almost all of the current. The effect was not potential-dependent as can be seen in the current-voltage curves recorded from another cell (Fig. 6*B*) in control solution (open circles), in the presence of 10 nM TTX (filled triangles) and in the presence of 100 nM TTX (filled squares). The lines through the data points show the best fits of the Boltzmann equation $I = G_{max} \cdot (V - E_{Na})/(1 + \exp((V' - V)/k))$, where E_{Na} is the sodium equilibrium potential, I is the peak current, G_{max} is the maximum conductance, V is the membrane potential, V' is the potential at which I is half $G_{max} \cdot (V - E_{Na})$ and k is a slope factor. The fits to the three sets of data were obtained with an E_{Na} of +53 mV, V′ of −14.5 mV and k of 6 mV in each case. G_{max} values were 160, 45 and 5 nS for control solution, 10 nM and 100 nM TTX, respectively. Similar observations were made in another nine cells in addition to the two

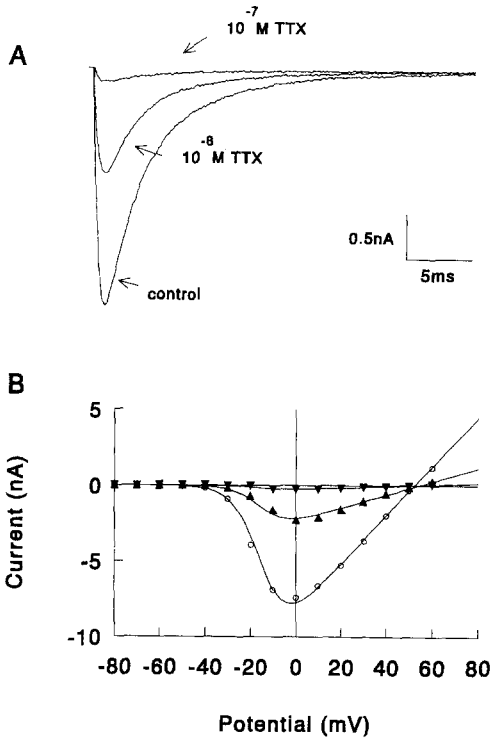

Fig. 6. Effect of TTX on the transient sodium current. (*A*) currents elicited by a voltage pulse from −130 to 0 mV in control solution and after exposure to 10^{-8} M and 10^{-7} M TTX. (*B*) current-voltage curves in control solution (open circles) and in the presence of 10^{-8} M TTX (filled triangles) and 10^{-7} M TTX (filled squares). The lines through the points in the curves each curve are best fits of the Boltzmann equation (see text) with E_{Na} = +53 mV, V' = −14.5 mV and k = 6 mV.

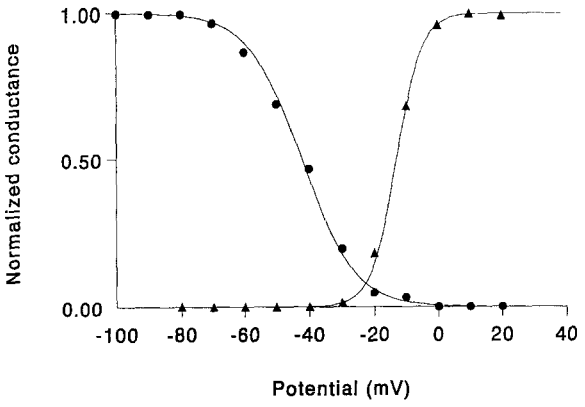

Fig. 7. Voltage dependence of activation and inactivation of the transient sodium current. Currents activated by positive voltage pulses from a holding potential of −130 mV were converted to conductance with an E_{Na} = 55 mV, normalized to the maximum conductance and plotted against pulse potential to obtain the activation curve (triangles). The line through the points is the best fit of the Boltzmann equation (*see text*) with V' = −13 mV and k = 5.5 mV. The inactivation curve (circles) was obtained by plotting the normalized peak amplitude of currents, evoked by a depolarizing step to 0 mV following a 100 msec conditioning prepulse to levels from −130 to +20 mV, against the prepulse potential. Peak current amplitude was normalized by dividing by the maximum peak current. The line through the circles is a best fit of the Boltzmann equation (*see text*) with E_{Na} = 55 mV, V' = −42 mV and k = 8 mV.

illustrated in Fig. 6. It is clear that the transient sodium current recorded in these isolated cells was sensitive to the same concentrations of TTX that changed the frequency and rate of rise of action potentials in the intact sinus venosus (*see* Fig. 1).

Voltage Dependence of the Transient Sodium Current

The observations above indicate that the transient inward currents recorded in single pacemaker cells were TTX-sensitive, voltage-dependent sodium currents. The functional role of this current in pacemaking activity depends, however, on whether the channels responsible can be activated over the range of potentials normally encountered in pacemaker cells. In normal pacemaker activity, the diastolic potential ranges from about −65 mV to about −50 mV, the threshold potential for action potentials [3, 7]. If sodium channels were completely inactivated in this potential range, they could not contribute to the action potential. With this in mind, activation and steady-state inactivation curves for the transient current were determined in eleven cells.

The fraction of channels activated at any potential was measured by stepping the membrane potential from a prepotential of −150 mV to potentials from −80 to +20 mV. The peak current was converted to conductance and normalized to the maximum conductance obtained at +20 mV. The voltage-dependence of inactivation was determined from the peak amplitude of the transient current, generated by a voltage pulse to 0 mV following a 300 msec prepulse to potentials from −130 to +20 mV, expressed as a fraction of the peak current obtained with the prepulse to −130 mV. Typical activation (triangles) and inactivation (circles) curves obtained in one cell are shown in Fig. 7. The inactivation and activation data were fitted with the Boltzmann equations $I = I_{max}/(1 + \exp((V - V')/k))$ and $G = G_{max}/(1 + \exp((V' - V)/k))$. V' values for activation and inactivation in Fig. 7 were −13 mV and −42 mV. Average V' values in eleven cells were −16 ± 1.9 mV for activation and −43 ± 2.1 mV for inactivation.

Similar experiments were done on single cells isolated from toad ventricles. These cells, like pacemaker cells, generated inward sodium currents when depolarized and the currents decreased in amplitude when TTX, 10 to 100 nM, was added to the extracellular solution. However, the half maximum values for activation and inactivation of the sodium currents in ventricular cells, measured under identical conditions, were more negative than in the pacemaker cells. The mean values of V′ for

activation and inactivation in 5 ventricular cells were −24 ± 3.6 mV and −55 ± 4.1 mV.

Persistent Sodium Currents in Sinus Venosus Cells

The TTX-sensitive persistent sodium current recorded in mammalian ventricular cells probably contributes to the inward current causing depolarization during diastole [22, 29]. The finding that TTX, as well as causing a fall in the rate of rise of pacemaker action potentials, also caused a fall in heart rate, suggested that a similar persistent sodium current might also be present in pacemaker cells and contribute to pacemaking activity. Experiments were done, therefore, to examine this possibility.

As the persistent current is very small and difficult to detect in the presence of other currents, the current was recorded by subtracting current obtained in the presence of TTX from that obtained in the same cell before exposure to TTX [22, 29]. In order to reduce the possibility of time-dependent changes in other currents, TTX was normally washed out and the currents recorded compared with those obtained before exposure to TTX: if any time-dependent changes were detected, the results were not used. In three experiments, the persistent inward current at negative potentials was abolished by substitution of choline for Na^+ in the extracellular solution providing further evidence that the inward current is a sodium current.

In 13 of 25 cells, subtraction of pre- and post-TTX records showed an obvious TTX-sensitive persistent current. In each case, washing the TTX from the bath caused a restoration of the persistent current: it is thus unlikely that the persistent current arose from changes in other currents not related to exposure to TTX. Currents recorded in one of these experiments are shown in Fig. 8. The two traces in Fig. 8*A* show currents evoked by a voltage step from −130 mV to +20 mV, before and after exposure of the cell to TTX (10 nM) for 3 min. It is clear that the TTX depressed both the transient current and a persistent current. Subtraction of currents recorded before and after exposure to the TTX shows the transient and persistent current blocked by TTX (Fig. 8*B*). The persistent current had an amplitude of about −100 pA (insert, Fig. 8*B*), about 4% of the size of the transient current initiated by the same voltage pulse.

The persistent current invariably appeared at more negative potentials than the transient current in the same cell. Fig. 9*A* shows TTX-sensitive currents evoked by depolarizing steps to potentials between −70 mV and −40 mV. At the more negative potentials, persistent inward currents could be seen in the absence of a transient current. When current was converted to conductance and plotted against voltage, the graph shown in Fig. 9*B* was obtained. In the thirteen cells, the average maximum

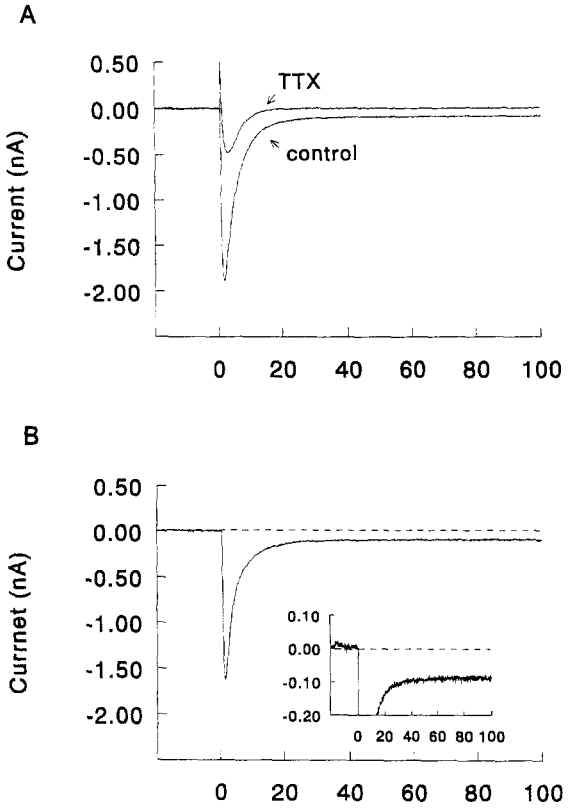

Fig. 8. TTX-sensitive persistent sodium current. (*A*) currents evoked by a voltage step to +20 mV from a prepulse potential of −130 mV. TTX (10^{-8} M) reduced the amplitude of the transient current by about 75% and also blocked a persistent inward current lasting more than 100 msec. (*B*) the TTX-sensitive current obtained by subtraction of the current recorded in the presence of TTX from the control current. Inset is the same trace at higher current gain.

persistent sodium conductance was 1.38 ± 0.25 nS. The persistent current in this cell was half activated at −39 mV compared with −24 mV for the transient current. In four cells in which it was possible to record the persistent current over a wide range of voltages, the Boltzmann fits gave an average potential for half maximum conductance of −30 ± 3.5 mV. Thus, as in mammalian ventricular myocytes [29], the persistent sodium current activates at more negative potentials than the transient sodium current.

It is possible that the persistent current in these experiments could have been generated by a window current [2]. Although it has been demonstrated [29] that the persistent sodium current in mammalian ventricular muscle is not due to such a "window" current, there *is* some overlap in activation and inactivation of the transient sodium current in pacemaker cells within a range from −40 mV to 0 mV (Fig. 7). If the Boltzmann fits are accurate descriptions of activation and inactivation of the transient current, a window current should not occur with a voltage step to +20 mV (Fig. 7) or to −50 mV (Fig. 9). However, to confirm that the persistent current recorded

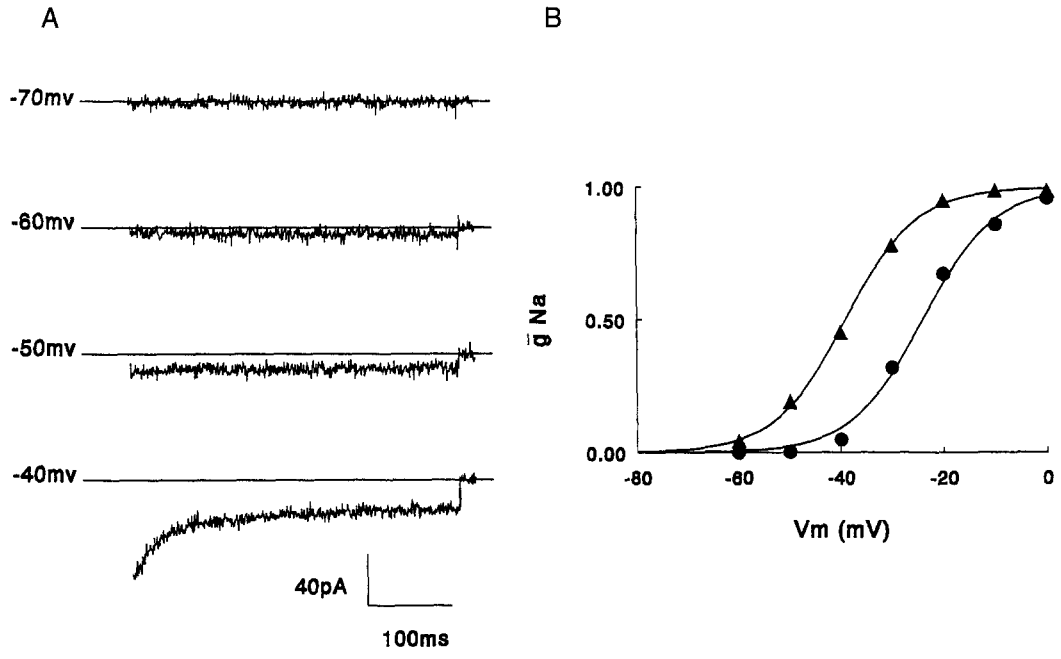

Fig. 9. Voltage-dependence of the persistent sodium current. (*A*) TTX-sensitive currents recorded during 400 msec-voltage steps to the potentials shown from a holding potential of −130 mV. The line shows the zero current level. (*B*) conductance at the peak of the transient current (filled circles) and at the end of the persistent current (filled triangles) recorded in a different myocyte are plotted against test potential. Lines are best fits of the Boltzmann equation (*see text*) to the data. The voltages for half maximal activation from the Boltzmann fits were −24 mV for the transient conductance and −39 mV for the persistent conductance.

was not due to overlap of the inactivation and activation curves, we also examined the current generated by a step to +60 mV which would be expected to produce rapid and complete inactivation of the transient current. In order to obtain a measurable current at this potential, the sodium concentration in the pipette was increased to 90 mM and the sodium concentration in the extracellular solution was reduced to 4 mM. Under these conditions, the sodium current is outward. Examples of currents produced by depolarizing a cell to +60 mV are shown in Fig. 10*A*. In the upper panel, there are three currents, all generated by a voltage step to +60 mV but following prepulses to −130 mV (largest transient current), −40 mV (small transient current) and 0 mV (no transient current). It is clear from the insert in the figure that the conditioning prepulses that inactivated the transient current had little effect on the persistent current. In other words, the transient current shows voltage-dependent inactivation whereas the persistent current does not. This is illustrated more clearly in Fig. 10*B* in which the voltage-dependences of inactivation of the transient and persistent currents recorded in one cell are compared. Similar curves were obtained in three other cells in which the effect of a wide range of conditioning prepulses was tested. This demonstration of a noninactivating current at very positive potentials makes it very unlikely that this current is a window current generated as a consequence of the properties of the transient current.

Discussion

TTX clearly reduced the rate of generation of action potentials in toad sinus venosus preparations. The more positive diastolic potentials in the presence of TTX are a secondary effect of blocking Na channels: the decrease in depolarization at the peak of the action potential in the presence of TTX leads to less delayed potassium conductance and hence a more positive diastolic potential [3, 7, 12].

We isolated pacemaker cells from sinus venosus preparations in order to record membrane currents. Cells were identified as pacemaker cells by their characteristic morphology, the presence of spontaneous rhythmic contractions and action potentials, and the absence of inward rectification [20]. Pacemaker cells identified in this way had a large transient and a small persistent inward TTX-sensitive sodium current when depolarized.

Thus, it would appear that pacemaker cells of the toad sinus venosus contain voltage-dependent sodium channels that influence the frequency of action potentials. At first sight, this observation may be surprising because action potentials are not blocked by TTX in pacemaker cells. However, action potentials in these cells are blocked by cobalt indicating that calcium current makes the major contribution to the upstroke of the action potential. Although pacemaker cells have been thought not to contain sodium channels [20], it has been

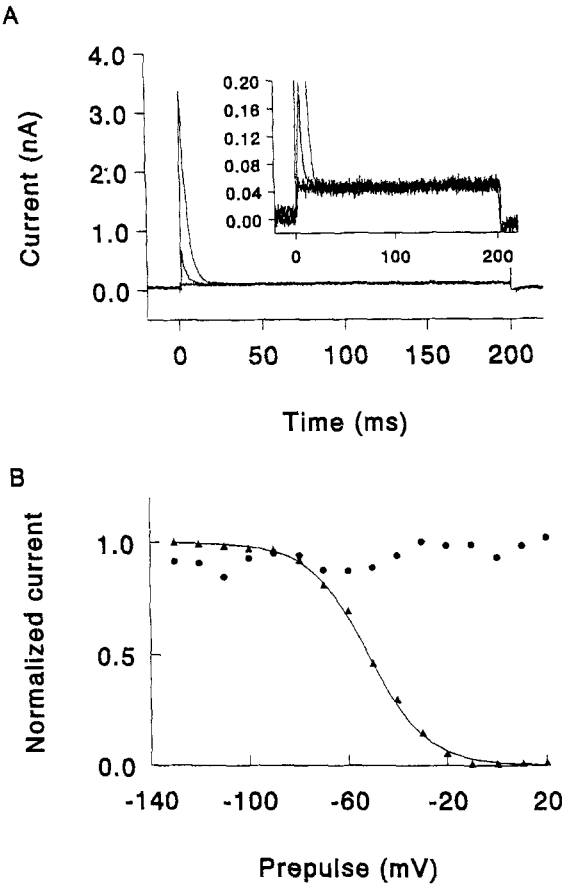

Fig. 10. The persistent current is not a "window" current. (*A*) currents were evoked by a voltage step to +60 mV following prepulse potentials of −130, −40 and 0 mV, first in control solution and then in the presence of 2×10^{-7} M TTX. The intracellular and extracellular Na^+ concentrations were 90 and 4 mM, respectively, so that outward currents were generated at these potentials. The currents shown are the TTX-sensitive currents obtained by subtraction of currents recorded before and after exposure to the TTX. As the prepulse potential became more positive, the transient current became smaller but the persistent current was unaffected. (*B*) Amplitudes (normalized to maximum amplitudes) of TTX-sensitive transient (triangles) and persistent currents (circles, measured 200 msec after the onset of depolarization) evoked by a voltage pulse to +60 mV are plotted against prepulse potential. The transient current shows inactivation whereas the persistent current does not.

well recognized that TTX can slow the heart rate in both amphibia [7, 12] and mammals [1], presumably by blocking sodium channels. Furthermore, sodium currents have been described previously in single isolated pacemaker cells not exposed to TTX [10]. The evidence, taken together, demonstrates that pacemaker cells do contain TTX-sensitive sodium channels.

The sodium currents recorded from pacemaker cells shared many of the characteristics of other TTX-sensitive sodium currents. However, activation and inactivation occurred at more positive potentials than normally found in other cardiac cells [24] and even in ventricular myocytes from the same animals recorded using identical procedures. The reason for this difference in pacemaker and ventricular cells is not clear.

It appears that toad heart muscle is more sensitive to TTX than mammalian heart muscle. Concentrations greater than 1 μM are normally required to completely block sodium currents in the mammalian heart [26] whereas lower concentrations of TTX are needed to block sodium currents in amphibian cardiac muscle [19]. This would explain the effects we obtained on toad heart rate with 10 nM TTX.

In addition to a transient TTX-sensitive sodium current, many pacemaker cells were found to contain a small TTX-sensitive persistent sodium current. This current is not the "background" sodium current that has been described in pacemaker cells [16] because it is voltage-dependent and blocked by TTX. A persistent TTX-sensitive sodium current has not been reported in pacemaker cells previously, probably because of its very small amplitude. It becomes obvious only when the "difference" current is revealed by subtraction of currents recorded with and without TTX. This current is active in the potential range more negative than the threshold for action potentials and would be expected to contribute to the pacemaker current. It is hardly surprising, therefore, that TTX slows the frequency of action potentials.

Our results show that pacemaker cells of the sinus venosus in cane toads generate TTX-sensitive transient and persistent sodium currents. These currents are activated over the range of membrane potentials normally seen in pacemaker cells. Although small, they would have a substantial influence on potential in these cells which have a high input impedance [8]. The slowing of heart rate caused by TTX can be explained by removal of the persistent current and consequent slowing of the rate of diastolic depolarization. Furthermore, since TTX is as effective as caesium in slowing pacemaking activity in these preparations [12], the persistent sodium current must make as significant a contribution to the pacemaking current as the cation channels activated by hyperpolarization [11]. Finally these experiments give further support to the idea that pacemaking activity results from the coordinated activity of a variety of ion channels [6].

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