Na\textsuperscript{+} current contribution to the diastolic depolarization in newborn rabbit SA node cells

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Baruscotti, Mirko, Dario DiFrancesco, and Richard B. Robinson. Na\textsuperscript{+} current contribution to the diastolic depolarization in newborn rabbit SA node cells. Am J Physiol Heart Circ Physiol 279: H2303–H2309, 2000.—Isolated newborn, but not adult, rabbit sinoatrial node (SAN) cells exhibit spontaneous activity that (unlike adult) are highly sensitive to the Na\textsuperscript{+} current (I\textsubscript{Na}) blocker TTX. To investigate this TTX action on automaticity, cells were voltage clamped with ramp depolarizations mimicking the pacemaker phase of spontaneous cells (−60 to −20 mV, 35 mV/s). Ramps elicited a TTX-sensitive current in newborn (peak density 0.89 ± 0.14 pA/pF, n = 24) but not adult (n = 5) cells. When depolarizing ramps were preceded by steplike depolarizations to mimic action potentials, ramp current decreased 54.6 ± 8.0% (n = 3) but was not abolished. Additional experiments demonstrated that ramp current amplitude depended on the slope of the ramp and that TTX did not alter steady-state holding current at pacemaker potentials. This excluded a steady-state Na\textsuperscript{+} window component and suggested a kinetic basis, which was investigated by measuring TTX-sensitive I\textsubscript{Na} during long step depolarizations. I\textsubscript{Na} exhibited a slow but complete inactivation time course at pacemaker voltages (τ = 33.9 ± 3.9 ms at −50 mV), consistent with the rate-dependent ramp data. The data indicate that owing to slow inactivation of I\textsubscript{Na} at diastolic potentials, a small TTX-sensitive current flows during the diastolic depolarization in neonatal pacemaker myocytes.

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CARDIAC PACEMAKING is a basic physiological function performed by pacemaker myocytes located, in the mammalian heart, in the sinoatrial node (SAN) region. The generation of spontaneous activity requires a net inward current flowing at pacemaker potentials to drive the diastolic depolarization. The need for an inward component led DiFrancesco (9) to reinterpret the outward potassium current (I\textsubscript{Ko}) as a mixed Na\textsuperscript{+}-K\textsuperscript{+} current now known as I\textsubscript{f} or pacemaker current. Other inward currents have been postulated to contribute to the net influx, including the I\textsubscript{Ca} and T-type Ca\textsuperscript{2+} currents, a transient Na\textsuperscript{+} current (I\textsubscript{Na}), and a background I\textsubscript{Na} (12–14, 19, 22, 25, 34). All of these studies were carried out on cells isolated from adult animals; only a few studies considered development as a source of potential differences in the currents underlying such a delicate and essential event as pacemaking (31, 32).

Previous experiments from our laboratories focused on a novel TTX-sensitive I\textsubscript{Na} that is present in the SAN of newborn rabbits and slowly disappears during the first 40 days after birth (4). The effect of TTX on rate of contraction revealed an important difference between newborn and adult cells: although in adult cells superfusion with TTX did not alter significantly the frequency of spontaneous action potentials, in newborn myocytes a dramatic reduction was demonstrated. Any current variation that alters the balance of the net ionic flux flowing during the pacemaker phase would be expected to alter spontaneous activity. Thus the effect of TTX observed in newborn cells could be generated by a direct contribution of I\textsubscript{Na} during this pacemaker phase. Alternatively, the effect of TTX could reflect more complex events secondary to the block of a fast inward component during the action potential upstroke and subsequent alteration of the time course of repolarization. This paper addresses the issue of a direct contribution of this TTX-sensitive current to maintain diastolic depolarization by testing for the presence of steady-state and time-dependent components. The results rule out the presence of a steady-state I\textsubscript{Na} and demonstrate that slow inactivation of this newborn SAN-expressed Na\textsuperscript{+} channel contributes to its effect on automaticity.

METHODS

Protocols employed in experiments conducted in the United States were reviewed and approved by the Columbia University Institutional Animal Care and Use Committee. The same protocols conformed to the guidelines of the care and use of laboratory animals established by Italian (DL-116/1992) and European directives (86/609/CEE).

The experiments described in this paper were performed on single cells isolated from the SAN region of newborn (3–8 days old) rabbit hearts. Animals were first anesthetized by intramuscular injection of a mixture of 2.3 mg/kg xylazine.
and 30 mg/kg ketamine (Sigma Aldrich) and then euthanized by cervical dislocation and exsanguinations. Hearts were quickly removed and placed in prewarmed (37°C) Tyrode solution containing 500 units of heparin. The SAN tissue, delimited by the cristal terminalis and interatrial septum, was dissected free and cut into five or six strips on which the cell isolation procedure was performed. A two-step (enzymatic and mechanical) procedure was adopted to obtain single cells using the methods described previously by our group (4). During experiments cells were placed in a chamber on a modified stage of an inverted microscope and superfused via a fast perfusion device that permitted exchange of the ionic environment near the cell in 1–2 s. All experiments were carried out at room temperature (22−24°C).

The whole cell patch-clamp approach, both in rupture and perforated-patch configuration, was used to study the characteristics of the $I_{\text{Na}}$ expressed in newborn SAN cells. Mainly two types of recordings are presented in RESULTS: whole cell ramp depolarizations and whole cell step depolarizations. In all experiments the external solution was (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, 10 glucose, and 5 CsCl (to block K⁺), with pH 7.4. In a subset of experiments, this solution also contained (in mM) 1 BaCl₂, 2 MnCl₂, 0.01 nifedipine, and 0.1 NiCl₂ to block contaminating Ca²⁺ and K⁺ currents. The composition of the pipette solution depended on the type of recording configuration. In ramps and steady-state experiments, the perforated-patch (amphotericin B, 300–600 μM) configuration was mainly chosen to maintain the integrity of cytoplasmic constituents, and the pipette solution was as follows (in mM): 120 potassium aspartate, 10 KCl, 10 NaCl, 0.4 CaCl₂, 2 MgCl₂, 10 HEPES, and 1 EGTA, with pH 7.2. Ramp protocols were generated every 5 s, and the current traces were digitized at 3.3 kHz and filtered at 500 Hz. In whole cell step (and occasionally ramp and steady-state) voltage-clamp experiments, the ruptured-patch approach was chosen to minimize the effect of series resistance. In this case the pipette solution contained (in mM) 130 potassium aspartate, 10 NaCl, 2 CaCl₂ (pCa = 7), 2 MgCl₂, 10 HEPES, 5 EGTA, 2 ATP(Na₂), 0.1 GTP, and 5 creatine phosphate, with pH 7.2. Currents during voltage-clamp steps were digitally acquired at 50 kHz and filtered at 5 kHz; series resistance errors were minimized by series resistance compensation (AxoPatch 200B, Axon Instruments) and the level of compensation was always >85%. Only cells with adequate voltage control were used for the data presented in this paper; the quality of the clamp was ensured both by discarding cells where a sudden jump in the current amplitude was evident at voltages near the threshold and by verifying that the time to peak at depolarized potentials was not dependent upon current amplitude.

Experimental measurements of errors due to bath-to-liquid junction potentials yielded values of −7.8 ± 0.2 mV and −7.7 ± 0.2 mV, respectively, for the ruptured-patch and the perforated-patch conditions. The data presented below have not been corrected for these offsets to ease comparison with other papers. All the experiments were generated and analyzed using pCLAMP 6 and 7 software (Axon Instruments). Graphic display and editing were done using Origin 5.0 software (Microcal Software). All data are expressed as means ± SE.

RESULTS

The physiological relevance of a TTX-sensitive inward current in newborn SAN cells has been described previously by our group (4). The series of experiments presented in this section were directed at determining whether an influx of Na⁺ occurred via a TTX-sensitive channel during diastolic depolarization and, if so, at elucidating the underlying mechanism. We first considered the possibility of a steady-state window current in the diastolic potential range, because our earlier study (4) had raised the possibility of such a current. To investigate this, cells were tested for the presence of a TTX-sensitive component developing during a slow depolarization with a ramp protocol. A 2-s conditioning pulse at −60 mV followed by a 1.14-s ramp depolarization from −60 to −20 mV with a slope of 35 mV/s was generated every 5 s. The slope of the ramp was chosen to approximate the average diastolic depolarization slope measured in spontaneously active newborn SAN cells (4) so as to determine whether such a rate of voltage change resulted in the appearance of a TTX-sensitive $I_{\text{Na}}$. A conditioning step of 2 s at −60 mV was chosen to maximize the availability of channels at that voltage. The integrity of cytoplasmic constituents was preserved by using the perforated-patch technique described in METHODS. Figure 1A illustrates the effect of the ramp-clamp protocol on a representative single cell obtained from a newborn animal.

The trace recorded in control conditions revealed the presence of a clear inward-going deflection that was completely abolished by the addition of the $I_{\text{Na}}$-specific blocker TTX (3 μM). This TTX-sensitive current is referred to below as “ramp” current. In some experiments Ba²⁺ and Mn²⁺ were omitted from the external solution; because no evident difference in the properties of the ramp current was revealed, all of the data were pooled. A total of 30 cells were tested and the presence of a measurable TTX-sensitive current was observed in 80% of them. Peak current density was calculated only for those newborn cells exhibiting the ramp current and was 0.89 ± 0.14 pA/pF (n = 24). The voltage dependence of the peak spanned a very narrow range and the average current at which the peak current was measured in the same 24 cells was −49 ± 0.8 mV. For comparison the same approach was then performed on adult SAN cells, and, as apparent in the Fig. 1B example, no TTX-sensitive current was detected in the adult cell population (n = 5). Figure 1, C and D, shows the effect of TTX superfusion on the spontaneous activity of the same cells as Fig. 1, A and B. As previously reported (4), TTX slowed the spontaneous rate in the neonatal cell but not in the adult myocyte. In nine of the newborn cells investigated, the effect of TTX during spontaneous activity (current clamp) also was determined; TTX caused a 64.6 ± 12.1% decrease in the spontaneous rate (four cells, including that shown in Fig. 1, were arrested). In two of the five adult cells in which TTX was applied while recording ramp currents, the action of TTX on spontaneous activity also was measured and only minor rate reductions were observed (8.5% and 2.2%).

The experiments described in Fig. 1 suggested that a $I_{\text{Na}}$ could be elicited upon a relatively slow depolarization, but did not provide insight into possible mechanisms for the generation of this ramp current. Specifically, the results could reflect either a steady-state...
window or noninactivating) current or a slowly inactivating current. The first step in elucidating the mechanism was to test for the presence of a steady-state window component. To determine whether superfusion with 3 μM TTX could have any blocking effect on the steady-state current, cells were held at different potentials within the range of pacemaker depolarization, and TTX was superfused for about 10 s (Fig. 2A). In all cells tested in the −60- to −20-mV range (11 data points from a total of 6 cells), no detectable variation of the holding current level was observed upon drug perfusion. In the example indicated in Fig. 2A, holding currents at −50 and −40 mV are shown, and TTX had no effect. The presence of a TTX-sensitive current in this particular cell was confirmed by using a step depolarization from −65 mV to 0 mV. Figure 2B shows the TTX-sensitive current and the original records before digital subtraction (inset); records such as those shown in Fig. 2B were always obtained after observing the absence of steady-state currents.

The absence of a detectable steady-state, TTX-sensitive current raised questions concerning the basis for the previously observed ramp current. We reasoned that if the ramp current arose from a true steady-state window component, then it should be detectable even at slow ramp rates. In contrast, if it arose from a slowly inactivating current, then it would be expected to be rate dependent and to disappear or be reduced at slower rates. To investigate this, three different ramp rates were employed: standard (35 mV/s), fast (105 mV/s), and slow (11.7 mV/s). Fast and slow ramps are respectively three times faster and slower than the standard ramp. Representative recordings with this protocol are shown in Fig. 3. To better appreciate the time and voltage dependence of ramp current elicited by the three different protocols, traces shown in Fig. 3...
are plotted on both time (top axis) and voltage (bottom axis) scales. It is evident that the size of the elicited ramp current strictly depends on the speed at which the voltage variation is imposed on the cell membrane and is fully abolished at the slowest rate. Similar results were observed in five other cells.

In the previous experiments, the ramp depolarization always followed a 2-s conditioning step at −60 mV to maximize the steady-state Na\(^+\) channel availability at that voltage. A more physiological action-potential-like protocol was utilized to verify the presence of a TTX-sensitive current in a non-steady-state situation. Traces shown in Fig. 4 are representative of data obtained in five cells and qualitatively confirm the relevance of the current. In three of the cells the amplitude of the TTX-sensitive peak current obtained with the regular ramp preceded by 2 s at −60 mV was directly compared with the magnitude of the current generated by the action potential-like voltage protocol; quantitative analysis of this subset of cells yielded a reduction in peak amplitude of 54.6 ± 8.0% when the action potential-like voltage protocol was employed, but in every case a TTX-sensitive current was still evident.

Results presented in Fig. 4 and the previous figures argue against the presence of a “window” component and suggest that the mechanism generating the TTX-sensitive current during the diastolic depolarization depends on the gating properties of these channels. To investigate whether the kinetic properties of the channel were consistent with a contribution of I\textsubscript{Na} during slow diastolic depolarization, the inactivation time course of currents elicited during long (300 ms) depolarizing steps was analyzed. Typical TTX-sensitive, whole cell current traces obtained upon step depolarizations are shown in Fig. 5A, and in Fig. 5B the mean values of time constants obtained from several measurements are plotted for voltages ranging from −50 to −10 mV. It is noteworthy that at pacemaker potentials (i.e., −40 or −50 mV) the time-constant values are in the tens of milliseconds range, suggesting that opening of new channels during diastolic depolarization could provide a significant contribution of Na\(^+\) flux.

Data in Fig. 5B were satisfactorily fit by a first-order exponential function \[ \tau = \tau_0 \times \exp(-V/k) \], yielding the following values: \( \tau_0 = 0.15 \text{ ms} \), and \( k = 9.25 \text{ mV} \). The rather slow inactivation rate at negative voltages encompass-
ing the beginning of the diastolic depolarization is in agreement with the presence of ramp current elicited by the regular diastolic slope of 35 mV/s.

**DISCUSSION**

The contribution of a TTX-sensitive $I_{Na}$ to pacemaker activity of newborn rabbit SAN cells was investigated in a previous paper (4), and the results of those experiments suggested a functional participation of this current in the generation of the nodal action potential. Data presented in this paper indicate that a small inward flux of Na$^+$ is transiently present during the diastolic depolarization and that this results from unusually slow inactivation kinetics of the nodal $I_{Na}$ at potentials in the diastolic range.

The role of a Na$^+$ influx in the original Hodgkin-Huxley (15, 16) model was to generate the upstroke phase of the nerve action potential. For many years the physiological importance of $I_{Na}$ was in general strictly connected to this upstroke generation; only in the last decade or so have several authors shown that due to subtle differences in the inactivation properties, fast-activating $I_{Na}$ could be extremely important also in other phases of the action potential. In ventricular myocytes, Kunze et al. (23) proposed a contribution of slowly inactivating $I_{Na}$ to the plateau phase, and in mammalian central neurons, Crill’s group (2, 3, 8) has shown that a persistent TTX-sensitive $I_{Na}$ (<1% of the peak current) has a functional significance. The presence of a $I_{Na}$ in adult SAN cells and its contribution to the generation of spontaneous activity has long been debated and was previously addressed (4). In this respect Honjo and coauthors (17) found no evidence of TTX-sensitive current in small nodal cells, although a TTX-sensitive component was present in larger cells. In addition, Kodama et al. (22) reported no effect of TTX on spontaneous activity recorded from central nodal cells, although an evident decrease in the upstroke velocity was evident in cells isolated from the periphery. It is therefore possible that in adult animals the physiological contribution of $I_{Na}$ is limited to secondary pacemaking cells. Although it is difficult to compare physiological mechanisms in animals as distantly related as mammals and amphibians, it is worth noting the functional relevance of a TTX-sensitive persistent $I_{Na}$ in cells isolated from the pacemaker region of the toad (20). Although this current shows a similar high sensitivity to TTX as the neonatal mammalian SAN current, its contribution to pacemaking results from incomplete inactivation, rather than the slow (but complete) inactivation reported here.

Experiments like the one shown in Fig. 1 were utilized to determine whether during a ramp depolarization with a rate of voltage change value similar to the slope of phase 4 depolarization measured in newborn rabbits (Table 1 in Baruscotti et al. (4)), a TTX-sensitive component could be identified. Adult cells did not reveal any $I_{Na}$, although in the majority of newborn cells a TTX-sensitive current was evoked. The negative threshold for current activation and the voltage at which the current peaked suggested that a TTX-sensitive component could be flowing in the pacemaker.
range of potentials during spontaneous activity. The net amount of current necessary to maintain the diastolic depolarization is on the order of a few picocamperees (10); thus even a small current density, such as the one shown in Figs. 1A and 4, can play a substantial role. Indeed the paired measurement of ramp currents and TTX effect on rate in the same cell (Fig. 1, A and C) strongly supports the importance of an inward TTX-sensitive contribution during the pacemaker phase. Furthermore, given the voltage dependence of the ramp, it is likely that this current exerts its major contribution during middiastole.

A characterization of the mechanism underlying the presence of the ramp current was then undertaken. The possibility of a current generated by the overlap of activation and inactivation curves was excluded by experiments like the one shown in Fig. 2. Although our previous data (4) suggested the possibility of a window component, the present results (Figs. 2 and 3) clearly demonstrate that there is no steady-state component of $I_{Na}$ in the diastolic potential range. This apparent inconsistency is not surprising, and in fact Muramatsu et al. (25) found similar results. The discrepancy may arise from the different conditions of the two sets of recordings; the activation and inactivation curves were obtained in an external low-Na$^+$ (50 mM), Ca$^{2+}$-free solution, and the steady-state measurements were done in a more physiological Tyrode solution.

Rather, the extent of contribution of $I_{Na}$ during diastole clearly depends on the rate of depolarization (Fig. 3), suggesting that the time course of inactivation relative to activation is the relevant determinant of ramp current. Because the presence or absence of the current was strictly kinetically dependent, it was important to verify that the Na$^+$ channel availability preceding the onset of the pacemaker depolarization would also apply under conditions similar to spontaneous activity. Data shown in Fig. 4 confirm that under conditions mimicking physiological activity the channel availability at the onset of the pacemaker depolarization is not a limiting factor that prevents the functional presence of the current. Nevertheless, a reduction of 54.6 ± 8.0% with respect to the current elicited from a state of maximal availability (at −60 mV) confirms that the amount of TTX-sensitive inward current flowing during the pacemaker potentials also depends on the dynamic availability of the channels. This observation allows speculation concerning a possible protective effect of the $I_{Na}$ of muscarinic activation, where a more hyperpolarized maximum diastolic potential will permit a faster and larger recovery of Na$^+$ channels that in turn will help to depolarize the cell and protect against excess vagal tone and a resultant sinus bradycardia.

Given the slow kinetics of the ramp current, it was important to verify that currents elicited during a voltage step in the diastolic potential range also had a slowly inactivating phase. The analysis of current decay was performed on TTX-sensitive currents to allow precise definition of the zero-current level and voltage steps of long duration to accurately determine the slow time constants. As a result, the values obtained (Fig. 5) are greater than those previously reported (4). The reported fitting functions found in the literature for $I_{Na}$ inactivation are mainly restricted to single or double exponentials (7, 11, 23, 27, 29, 30); unless a specific kinetic model is tested or assumed, the fitting functions are chosen when they can describe the time-dependent behavior of the current. Our data were well fit by single exponential functions and although more complex algorithms could fit the data as well, we adopted the simplest fitting procedure without any model implication. The data presented in Fig. 5 show that the slow kinetics of current inactivation are compatible with the presence of a ramp current during slow depolarizations. Slow inactivation appears mainly at voltages within the pacemaker range of potentials, where the time constants of inactivation are on the order of tens of milliseconds and where even small contributions of current could play an important role in modifying electrical activity.

Although pharmacological and in situ hybridization experiments indicate that the $I_{Na}$ in the newborn nodal cells is generated by a neuronal type I-like channel isoform (5), the slow inactivation observed in the present experiments (Fig. 5) suggests that the kinetic properties of this channel resemble those of the cardiac isoform. Indeed, inactivation processes could be generated by a variety of single-channel behaviors (1, 18, 21, 23), including multiple reopenings of the same channels, as found in cardiac isoforms (6, 26, 28, 33). The mechanism underlying the macroscopic slow inactivation of the $I_{Na}$ present in neonatal SAN myocytes remains to be determined. In this respect, the only published study to date on expressed neuronal type I channels (30) demonstrated that the rate of inactivation strongly depends on the coexpression of subsidiary subunits ($\beta_1$ and $\beta_2$). It is therefore possible that the presence or absence of one or both of these $\beta$-subunits contributes to the physiological role of this channel in the newborn SAN.

In conclusion, the experiments presented in this paper indicate a direct contribution of the SAN TTX-sensitive $I_{Na}$ to the electrical events occurring during diastole in newborn pacemaker cells. The presence of an active $I_{Na}$ during this phase of the action potential is not dependent on a steady-state contribution, but on a slow inactivation in the pacemaker range of potentials. The observation that newborn rabbits have a higher intrinsic heart rate than adults (24, 31) is thus consistent with our previous finding that expression of the $I_{Na}$ decreases after birth, and disappears after 40 days of age (4). At birth the high density of functional Na$^+$ channels (4) determines an important inward current flow that decreases with postnatal age, thereby generating a shallower phase 4 depolarization. However, this current clearly also contributes to the action potential upstroke in the newborn SAN (4), and the subsequent alteration in action potential repolarization (overshoot, maximum diastolic potential) is likely to have secondary effects on other currents (e.g., $I_{K1}$) that also may affect diastolic depolarization. Thus the
**I_{Na}** present in the newborn SAN likely has both direct and indirect effects on diastolic depolarization and spontaneous rate.

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