Late Sodium Current and Its Contribution to Action Potential Configuration in Guinea Pig Ventricular Myocytes

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We used the patch clamp technique to study the nature of the late sodium current in guinea pig ventricular myocytes. In a cell attached mode of single channel recording at room temperature (22-24°C), two kinds of late (100 msec or more after beginning of the depolarizing pulse) sodium channel activities were recognized. One is isolated brief openings appearing once for about 120 depolarizations per channel (background type), while the other type is sustained openings with rapid interruptions (burst type) that occurred only once for 2,700 depolarizations per channel. The time constant obtained from the open time histogram of the burst type (1.05 msec) was about five times longer than that of background type (0.18 msec, measured at the potential 10 mV above the threshold). Magnitude of the late sodium current flowing through the entire surface of a myocyte was estimated with tetrodotoxin (60 μM), a specific inhibitor of sodium channels, in whole-cell clamp experiments. The steady tetrodotoxin-sensitive current of 12 to 50 pA was registered at −40 mV (26±14 pA, mean±SD, n=5), in good agreement with the late sodium current calculated from the single channel recording. Tetrodotoxin produced small (−10%) but significant decreases in the action potential duration. These results suggest the presence of a small but significant late sodium current with slow inactivation kinetics and that this current probably plays a significant role in maintaining the action potential plateau and the duration in guinea pig ventricular myocytes. (Circulation Research 1989;64:389–397)

There are reports of a slow or late sodium current that flows for a period sufficient to contribute to the action potential plateau of cardiac muscles.1,2 In voltage clamp experiments on sheep Purkinje fibers, Attwell et al1 found a tetrodotoxin (TTX)-sensitive inward current and explained that this was a steady-state "window current" that flows at certain potentials where the steady-state activation and inactivation parameters are not zero. Shortening of the action potential duration (APD) induced by TTX or some local anesthetics has been attributed in part to a block of such a sodium current by these drugs.1,2

In the rabbit3-4 and canine Purkinje fibers,5 it was shown that the TTX-sensitive current decays with time and flows over much wider potential ranges than expected from an overlap of m and h curves.1 Thus, the current should be regarded as the slowly inactivating or late sodium current (see review by Fozzard et al9). More precise information on the late sodium current was obtained from single channel recordings in ventricular myocytes7,8 and from whole-cell clamp experiments in rat cardiac neocytes.9 Patlak and Ortiz10 studied the late sodium current by using frog skeletal muscle preparations. They noted two types of late sodium channel activities: isolated brief unitary openings (background type) and activities lasting for several hundred milliseconds (burst type). However, precise information on kinetics of such a late sodium current in cardiac cells has not been obtained.

In the heart, the late sodium current would have a special meaning from the clinical point of view: 1) the current may affect the APD, thereby altering the effective refractory period; 2) class I antiarrhythmic drugs may also alter the effective refractory periods; 3) in depolarized heart muscles, as is the case in ischemia, the current may contribute to development of the ectopic pacemaker.

Using the patch clamp technique and guinea pig ventricular myocytes, we 1) examined characteristics of single sodium channel activities, which would contribute to the late sodium current; 2) measured the amplitude and kinetics of the late sodium current; and 3) quantitatively analyzed the contribution...
of late sodium current to the action potential configuration. The latter two were conducted based on the assumption that TTX is a specific inhibitor of the sodium channel.

Materials and Methods

Cell Isolation

Single ventricular myocytes were prepared according to Taniguchi et al.11 In brief, the hearts from guinea pigs were mounted on a Langendorff's perfusion system. Initially, the hearts were perfused with normal Tyrode's solution to remove the blood, and then the perfusate was changed to calcium-free Tyrode's solution containing 0.4 to 1.0 mg/ml collagenase (Type I, Sigma Chemical, St. Louis, Missouri). After 5 to 10 minutes of perfusion, the hearts were immersed in a "storage solution" with the following millimolar composition: KCl 25, glutamic acid 70, taurine 10, oxalic acid 10, KH2PO4 5, HEPES 5, glucose 11, and EGTA 0.5 (pH was adjusted to 7.4 by adding KOH, resulting in a final potassium concentration of about 136 mM). The cells were dispersed on the test chamber mounted on the X-Y stage of an inverted microscope (TMD, Nikon, Tokyo) and were perfused with modified Tyrode's solution of the following millimolar composition: NaCl 137, KCl 5.4, CaCl2 1.8, MgCl2 0.5, NaHCO3 3.0, NaH2PO4 0.16, glucose 5.5, and HEPES 5 mM (pH was adjusted to 7.4 by adding NaOH). Only rod-shaped cells with clear edges were used. The experiments were done at room temperature (22-24°C).

Single Channel Recording of Sodium Current

Glass pipettes were prepared from a Pyrex glass tube, and the single channel current was recorded, as described by Hamill et al.12 The shank of the electrodes was coated with silicon rubber (KE106, Shin-Etsu Chemical, Tokyo) to reduce background noise.13 The pipettes were filled with calcium-free Tyrode's solution prepared by simply removing CaCl2 from components of the Tyrode's solution mentioned above. The pipette resistance ranged from 7 to 10 MΩ. The tip of the pipette was gently placed on the cell surface with the aid of a mechanical micromanipulator (Leitz, FRG), and a small negative pressure (<50 cm H2O) was applied to the pipette. The single sodium channel current was recorded with a patch clamp amplifier (EPC-7, List Electronics, FRG). The capacitive and leakage currents in the single channel recording were subtracted by addition of an averaged current obtained from 30 to 50 pulses with the same amplitude of an opposite polarity.

The surface area of a typical ventricular myocyte was estimated with a digitizer (KD4030, Graphtec, Tokyo) coupled with a computer (PC9801, NEC, Tokyo). First, using a digitizer, we measured the projected area and the circumferential length of the cell on the magnified photograph. The cell thickness was measured as the focal distance between the top and bottom surface of the cell viewed microscopically. The total cell surface area was then calculated as follows:

\[ \text{cell surface area} = (\text{projected cell membrane area}) \times 2 + (\text{circumferential length}) \times (\text{cell thickness}) \]

It was assumed that the cell surface was flat and composed of two equal areas of the membrane intervened by a constant thickness.

Whole-Cell Recording of TTX-Sensitive Current

To record whole-cell currents, glass pipettes with a large (>3 μm) tip diameter were used. The pipettes were filled with solution that contained (mM) KCl 140, MgCl2 2, ATP-Na2 5, EGTA 11, CaCl2 1, and HEPES 10 (pH 7.2, potassium-containing pipette solution). The late sodium current was recorded as a TTX (Sankyo Pharmaceutical, Tokyo)-sensitive inward current. In some experiments, the TTX-sensitive current was measured under conditions in which the outward potassium current and calcium inward current were suppressed. For this purpose, the pipettes were filled with solution that contained (mM) CsCl 140, MgCl2 2, ATP-Na2 2, EGTA 5, and HEPES 5 (pH 7.4), and the dihydropyridine calcium-antagonist nicardipine was added to the external solution at a concentration of 0.2 μM. Because satisfactory voltage control was not always achieved during the very early phase of the sodium current, we did not analyze the initial TTX-sensitive sodium current flowing within 50 msec from the beginning of the pulse.

Action potentials were elicited by intracellular injection of the depolarizing current with a supra-threshold intensity and at a rate of 0.2 Hz. For this purpose, the potassium-containing pipettes were used. Because the action potential configuration of the ventricular myocyte sometimes fluctuates from beat to beat, we averaged the action potentials elicited in 30 seconds (six action potentials). Therefore, the APDs described hereafter refer to the APDs averaged from six consecutive action potentials. The significance of TTX-induced change in the APD was assessed with paired Student's t test. A value of \( p<0.05 \) was considered significant.

Data Sampling and Processing

The current and potential signals were stored on magnetic tapes using a PCM data recording system (RP-880, NF Corp, Tokyo), replayed and processed by computers. In single channel analysis, the current signals were low pass filtered (cut-off frequency was 2.9 KHz) and digitized with a sampling interval of 100–120 μsec over 4,096 points and processed by a mini-computer (7T18 NEC San-ei Instruments Ltd, Tokyo). In the whole-cell voltage clamp and current clamp experiments, the current signals were sampled at 2 msec.
Results

Single sodium channel currents were recorded from guinea pig ventricular myocytes in a “cell attached” mode. The resting membrane potential (VR) measured under these conditions was -77±4 mV (mean±SD, n=9). The cell surface area measured from 47 cells obtained from two hearts was 4,530±1,248 µm² (mean±SD).

Two Types of Late Sodium Channel Activities

Figure 1 shows examples of the single channel current. The patch membrane was held at VR-50 mV, and depolarizing pulses to VR+10 mV were applied at a rate of 1.0 Hz. Duration of the pulses was 500 msec. In this figure, we show one type of late sodium channel activity, which resembled that identified in skeletal muscle as the “background type.” In this figure, we picked up the traces with channel openings after 100 msec from the beginning of the pulse. At the initial part of each trace, there are conspicuous activities of the sodium channel that may contribute to the rapid upstroke of the action potential. In the majority of cases, the overlap of more than two channel events occurred at the very early phase of depolarization.

In most of the current traces, no channel activities were observed after 50 msec from the beginning of the 500-msec pulse. However, in 600 successive current traces, 25 traces (=4%) yielded channel activities even after 100 msec from the beginning of the pulse. This type of late sodium channel activity was most often sporadic with brief openings and was isolated from other channel events.

We found that hyperpolarization before applying step depolarization was not necessary to induce this type of sodium channel activity. In the experiment shown in Figure 2, after confirming the threshold potential of sodium channel opening to be VR+10 mV, using step depolarization from the holding potential of VR-50 mV, we held the membrane potential at VR+20 (Figure 2B) or VR+30 mV (Figure 2A) for several minutes. Nevertheless, inward channel activities were sometimes apparent. This finding suggests that depolarization from the holding membrane potential more negative than the threshold is...
Figure 2. The "background" type channel activity induced by steady state depolarization. Each trace is a segment of long current traces with apparent channel activity. The voltage of patch membrane was kept at $V_R + 30$ mV in (A) and $V_R + 20$ mV in (B) for over 3 minutes. Sampling rate was 100 μsec/point. Different preparation from Figure 1.

not a prerequisite for induction of the background type of sodium channel activity.

Figure 3 shows another type of sodium channel activity with an extraordinarily long life. Since this type of channel activity was similar to channel events noted in skeletal muscle, we refer to this type of sodium channel activity as "burst type" after the terminology used by Patlak and Ortiz. The bursting was ignited from the beginning of the pulse and continued for up to an entire period of depolarization (500 msec). This type of channel activity occurred on only rare occasions, that is, in about seven of 3,840 depolarizations in this particular patch. As reported by others, this type of channel activity did not seem to occur in a random manner. The number placed on the left of each current trace in Figure 3 indicates the sequential number from the start of recording. It should be noted that six out of seven bursts occurred in pairs. Though the probability of this occurrence was low, it may carry an enormous amount of charges once activated, as compared with the background type. The number of the sodium channel in the particular patch was estimated to be five, from the maximum overlap of channel events during the initial phase of depolarizations. The averaged number of sodium channels thus measured was $4.5 \pm 2.2$ in 15 patches examined.

The averaged unit current of the burst type was identical to that of the background type (2.01 pA at $V_R + 10$ mV). The unit current amplitude of the initial openings had also the same unit current value. In addition, there was no appreciable difference in the single channel conductance (e.g., the single channel conductance of initial sodium channel activities was 24.4 pS) while those of the background and burst type channel activity were 24.8 and 26.0 pS, respectively. The latter value (26 pS) is, however, somewhat unreliable because it was calculated from the data at only two potential levels ($V_R + 10$ and $V_R + 20$ mV).

As there was no significant difference in single channel conductance, we postulate that the two types of late sodium channel activity were produced by the same set of sodium channels that contributed to activities at the very initial phase of depolarization. If each sodium channel related to the late sodium current functioned independently, then the probability of occurrence of these two types of channel activity can be estimated. For the background type, it was once per 120 depolarizations and was only once per 2,700 depolarizations for the burst type.

Comparison of Open Time Distribution Among Three Kinds of Sodium Channel Activities

We then compared the open time distribution of three types of sodium channel activities to determine possible kinetic differences. The sodium channel activities provoked immediately after the depolarization.
FIGURE 3. Examples of "burst" type sodium channel activity. The same patch as shown in Figure 1. Only seven of 3,840 traces revealed sustained repetitive channel activities, which lasted for almost the entire duration of depolarization. Six out of seven bursts occurred in pairs. The number to the left of each trace indicates the sequential number from the start of recording.

Depolarization overlapped in most cases, thereby making the measurement of open time difficult. Therefore, an analysis was made using only the current traces where there was no overlapping. The open time histograms of three types are compared in Figure 4. Each open time histogram was able to be fitted by a single exponential component. It is evident that the open time histogram of the burst type differs from the other two types. The time constants of the initial and background type sodium channel activity showed a close resemblance, that is, 0.22 and 0.18 msec, respectively. The time constant of the burst type (1.05 msec) was about five times longer than those of the other two types.

Amplitude of Late Sodium Current Measured From an Ensemble Averaged Current in Single Channel Recordings

As shown in Figure 5, we obtained the ensemble current from 3,840 consecutive current traces. The
FIGURE 5. A late sodium current reconstituted from 3,840 successive current traces in single channel recording. The holding potential was $V_H - 50 \text{ mV}$ and the test potential, $V_T + 10 \text{ mV}$. Depolarizing voltage steps with the duration of 500 msec were applied at a rate of 1 Hz. Start and the end of the pulse are shown by filled triangles.

The holding potential was $V_H - 50 \text{ mV}$ and step pulses with a duration of 500 msec were applied to $V_T + 10 \text{ mV}$, at a rate of 1.0 Hz. In this patch, $V_T + 10 \text{ mV}$ corresponded to 10 mV positive potential from the sodium channel threshold. Each current trace was idealized as follows: The threshold level for determination of the open event was set at the amplitude of a half unit current from the baseline. The channel was considered to be in the open state when a given point in a current trace was below the threshold. In Figure 5, the steady inward current with an amplitude of 6 to 7 pA is clearly visible even after 500 msec from the beginning of depolarization.

Amplitude of TTX-Sensitive Current in Whole-Cell Clamp Experiments

In the following experiments, we assessed the amplitude of the late sodium current as a TTX-sensitive current, using a tight-seal whole-cell voltage clamp configuration.

A typical example of the effect of TTX on the whole-cell current is shown in Figure 6A-a. Depolarizing voltage steps to $-40 \text{ mV}$ were applied from a holding potential of $-80 \text{ mV}$. Following equilibrium periods (5 to 10 minutes) after rupture of the patch membrane, TTX at a concentration of 60 $\mu$M was added to the perfusing solution. The currents obtained before (Figure 6A-a, unfilled circle) and after application of TTX (Figure 6A-a, filled circle) were superimposed. The application of TTX produced an outward shift of the current during depolarization. The current shown in Figure 6A-b was obtained by subtracting the current in the presence of TTX from that in the absence of TTX. This TTX-sensitive current was virtually constant throughout the depolarization and had an amplitude of about 20 pA at 200 msec from the beginning of the pulse. From five trials on three cells, the TTX-sensitive current was measured at 12 to 50 pA ($26 \pm 14 \text{ pA}$).

In such experiments conducted under conditions of intact calcium and potassium channels, the possibility that currents other than sodium current were modified by TTX was not ruled out. We then brought in another series of experiments. To reduce the outward potassium current, the pipette was filled with cesium (140 mM)-containing solution. We added 0.2 $\mu$M of nicardipine, an organic calcium channel antagonist, to the extracellular solution. This concentration was sufficient to block the calcium current in the guinea pig ventricular myocytes (authors' unpublished observation). Figure 6B shows the result from one such experiment and illustrates the TTX-sensitive current obtained by subtraction of the current in the presence of TTX from the current obtained before application of...
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**FIGURE 6.** The tetrodotoxin (TTX)-sensitive current in whole cell clamp experiments. A-a: Effect of TTX (60 μM) on whole cell currents. The pipette solution contained 140 mM KCl and no channel blockers other than TTX were present. The current indicated by an open circle was before and that indicated by a filled circle was after application of TTX. TTX produced an outward shift of the current. A-b: The TTX-sensitive current obtained by subtracting the current in the presence of TTX from that of the control. B: A TTX-sensitive current obtained in the experiment in which potassium and calcium currents were blocked by internally applied cesium ions and external nicardipine, respectively. Different preparation from A.

TTX. From three experiments, the amplitude of the TTX-sensitive current was 31±11 pA at −50 mV.

**Effects of TTX on Action Potential Configurations**

In the single channel as well as whole-cell clamp experiments, there seemed to be a slowly inactivating component of the sodium current in ventricular myocytes. Whether this late sodium current plays a role in maintaining the plateau of ventricular action potentials was thus given attention.

**FIGURE 7.** The effect of tetrodotoxin (TTX) on the action potential configuration of a single ventricular cell. The cell was stimulated at 0.2 Hz by an intracellular injection of suprathreshold current. The temperature was 22° C. Each trace shows the averaged action potential from six successive records. A: control. B: 4 minutes after application of 60 μM TTX. C: second control after washout of TTX for 5 minutes. D: The time course of the change in action potential duration (APD) measured at 95% repolarization levels. TTX induced slight but consistent shortening in action potential duration.

It is evident that TTX produced a reversible shortening of APD (by about 15%) in this particular cell.

We summarized the effect of TTX on the APD obtained from 10 trials conducted on seven cells. After a 5-minute application, TTX significantly (p<0.01) shortened the APD from 510±204 msec to 484±208 msec (mean±SD). After a 5-minute washout of TTX, the APD recovered to 512±193 msec, with a statistical significance (p<0.05).

**Discussion**

Using the patch clamp technique and ventricular myocytes isolated from guinea pig, we obtained evidence for two types of late (more than 100 msec from the beginning of depolarization) sodium channel activity. One is isolated, brief openings appearing once for about 120 depolarizations per channel (background type) and the other is sustained openings with rapid interruptions occurring only once for 2,700 depolarizations per channel (burst type). To our knowledge, this is the first report confirming the existence of "background type" sodium channel activity in cardiac cells. The burst type activity was noted in the rat and rabbit ventricular myocytes.7,8

We interpret the TTX-induced shortening of duration of action potential to be a blockade of these late sodium channel openings by TTX. There seems to have been no report, at least on cardiac ventricular myocytes, in which the contribution of the late sodium current to duration of the action potential was studied, quantitatively.

**Late Sodium Current in Single Channel Recording**

The nature of single sodium channel current which may contribute to the late sodium current in
Late Sodium Current as a Contributor to TTX-Sensitive Current and Action Potential Duration

The validity of the use of different current before and after application of TTX as a measure of late sodium current (Figures 6 and 7) is based on the assumption that TTX selectively blocks the late sodium current and has negligible effects on other current systems. There are circumstantial findings to support this assumption. 1) With TTX (60 μM) in the pipette, all sodium channel activity was abolished (n=2). 2) In the case of rabbit Purkinje fibers, significant amounts of TTX-sensitive late current are present and this current is apparently responsible for the change in action potential configuration induced by TTX or by some local anesthetics. 3) TTX had negligible effects on other current systems such as delayed rectifier potassium current or calcium current. Therefore, we consider that the TTX-sensitive current (26±14 pA) in whole cell clamp experiments (cf., Figure 6A) is a reliable index of the late sodium current. The TTX-sensitive current measured in the presence of internal cesium for blocking channels and external nicardipine for blocking calcium channels, did not significantly differ from the value obtained in the absence of such blockers.

The ensemble current obtained by summing 3,840 single channel current traces revealed a fairly constant magnitude (6–7 pA) of inward current after 100 msec from the beginning of the depolarizing pulse (Figure 5). As this particular patch contained five sodium channels, the reconstituted late sodium current may be tantamount to the current size induced by a single depolarization of about 19,200 (=3,840 x 5) channels. In other words, synchronized opening of 19,200 sodium channels yielded a current of 6–7 pA. Therefore, if the total number of sodium channels on a cell surface is known, one can estimate the amplitude of the late sodium current flowing through a single ventricular myocyte.

We estimated the total number of sodium channels in a cell, in two ways: 1) As described in the “Results,” the cell surface area of a single ventricular cell was 4,530±1,248 μm² (mean±SD, n=47). This value is of the same order of magnitude as estimated for rat ventricular myocytes (4,000 to 8,000 μm²). The average number of sodium channels in a cell was about 20,400 (=4.530 μm² x 4.5/1 μm²). 2) In whole-cell clamp experiments, the peak amplitude of the sodium current was about 100 nA at room temperature. As estimated from the single channel current measurement in our study, the unit channel current amplitude was about 1.5 pA at the potential during which the ensemble averaged current reached its peak (Vh=40 mV). Assuming that the open probability of the sodium channel at the peak of the sodium current is unity, we estimated the number of sodium channels per cell to be 67,000 (=100 nA/ 1.5 pA). Thus, the number of sodium channels in one entire cell surface would be close to 20,400 or 67,000. For measurement of the surface area, there would be a considerable underestimation of the cell surface area due to membrane infoldings and the T-system, and hence in the total number of sodium channels in case of the first method. Therefore, in our calculation, we assumed the total number of sodium channels on an entire cell surface to be 67,000.

Since simultaneous opening of 19,200 sodium channels yielded 6–7 pA of late sodium current, 67,000 channels should produce 21 to 24 pA at about –50 mV (10 mV positive to the threshold). These values are comparable to those obtained in whole-cell clamp experiments in which the TTX-sensitive current of 26 to 31 pA was registered (Figures 6A and 6B).

Consistent shortening in the APD after application of TTX (Figure 7) would be secondary to a blockade of the TTX-sensitive inward sodium current with the very slow inactivation kinetics we evidenced. Similar effects of TTX on the action potential configuration have been noted in Purkinje fibers.

Although amplitude of the late sodium current in a ventricular cell is small, the current significantly contributed to the action potential duration by as much as 10%, at room temperature. Local anesthetics such as mexiletine (30 μM) produced a more pronounced shortening (by 38%) of the APD,
in the same cell preparations. We attribute such a marked shortening effect of this drug on the APD to a significant blockade of the calcium current, aside from its authentic blocking action on the sodium current.

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References

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