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TWO TYPES OF VOLTAGE DEPENDENT Na CHANNELS SUGGESTED BY DIFFERENTIAL SENSITIVITY OF SINGLE CHANNELS TO TETRODOTOXIN

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Tetrodotoxin (TTX) can reduce the upstroke velocity and decrease the duration of the plateau phase of myocardial action potentials. In fact, the plateau duration of canine Purkinje fibers is more sensitive to TTX than is the upstroke (Coraboeuf et al., 1979), suggesting that cardiac Na current has either a slowly inactivating component or a steady-state noninactivating voltage-dependent Na-leak component, and that the sensitivity to TTX of the channels involved in upstroke production may be different from that of the channels conducting current during the plateau phase (termed "window current"). We examined these hypotheses with single-channel Na currents elicited during voltage clamp by isolated cultured chick ventricular myocytes. We asked: (*a*) During sustained depolarization to potentials in the range of the action potential plateau voltage, do Na channels open and conduct TTX-sensitive current? If so, then (*b*) do the channels that carry current during sustained depolarization eventually fail to open (i.e., slowly inactivate), or does their probability of opening remain >0 despite sustained depolarization to voltages at which "window current" is elicited? (*c*) Are there any electrophysiologically definable differences between the channels that open early on during voltage clamp to the "window" level and those that open 20 or more ms later? And finally, (*d*) is the sensitivity to TTX of the early opening channels different from that of the later-opening channels?

METHODS

Voltage-dependent single Na-channels having short opening latencies (<20 ms) after the onset of depolarizing voltage-clamp steps were compared with those opening with long latencies (20–160 ms). We used outside-out membrane patches isolated from cultured cardiac ventricular myocytes obtained from 11-d old chick embryos. Myocytes were dispersed from the ventricles of 6–10 embryos by gentle agitation in a 0.25% trypsin solution, as described by Lehmkuhl and Sperelakis (1963). Harvested myocytes suspended in culture medium were transferred to 2-cm glass coverslips, placed in covered culture dishes; for 2–5 d at 37°C; then placed in a shallow chamber mounted on the moveable stage of an inverted microscope and perfused with a conventional HEPES-buffered physiological extracellular solution maintained at constant temperature (± 0.3°C) between 13° and 14° C. The composition of the extracellular solution was NaCl 135 mM, KCl 4 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1.2 mM, HEPES 20 mM, Glucose 11 mM; the pH was adjusted to 7.30 ± 0.05.

Elementary single-channel TTX-sensitive Na currents were recorded during voltage clamp using the gigaohm-seal technique of Hamill et al. (1981). Heat-polished machine-pulled glass pipettes having inside diameters of ~0.3 μm and DC resistances of 5–20 MΩ when filled with "internal solution" were used. The composition of the internal solution was K^+ 100 mM, Na^+ 1.0 mM, HEPES 20, EGTA 20, glutamate 54, sucrose ~100 (used to adjust osmolarity to 340 mosmoles) and pH was adjusted with KOH to 7.2 ± 0.05.

Isolated outside-out membrane patches were obtained by a technique similar to that used by Horn and Patlak, 1980. After good seal (2–20 GΩ)

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was obtained, the membrane under the pipette was ruptured by light suction. Isolation was achieved by carefully pulling the pipette away from the cells. Leakage and capacitative currents evoked by voltage-clamp pulses were subtracted by electronic compensation and by conventional computer-averaging and subtraction techniques. The signals produced by application of patch-clamping currents through the pipette were filtered at 1.4 KHz and sent directly to the computer for processing and storage. Single-channel open time, current amplitude, and opening latency were measured and correlated with membrane voltage before, after, and during exposure of patches to TTX in concentrations ranging from one to 1,000 nM.

RESULTS

When patch potential was held equal or negative to −70 mV, depolarizing voltage pulses positive to −40 mV evoked inward-directed single-channel elemetary currents similar to those recorded from other excitable cells said to have voltage-gated Na channels (Sigworth and Neher, 1980; Rudy, 1981; Quandt and Narahashi, 1982). Fig. 1 shows traces having at least one channel opening; the bottom left trace shows two coincident channel openings.

The number of channel openings and their opening frequency was influenced by both holding and pulse potentials. Channel openings were never seen during depolarizing pulses when holding potential was positive to −50 mV, and opening events were more numerous as the holding potential became more negative. In most preparations, holding negative to −80 mV and pulsing positive to −30 mV produced a larger-than-elementary inward current during the first 5 ms of the pulse that appeared to be composed of at least 4–6 essentially coincident single-channel openings. It was not possible to estimate accurately the number of openings under these conditions. However, by holding at −70 to −60 mV it was possible to obtain an easily counted small number of discrete opening events even during pulses to 0 or +10 mV. When the holding potential was −70 mV, increasing pulse amplitude increased the number of opening events registered as discrete inward-directed elementary currents.

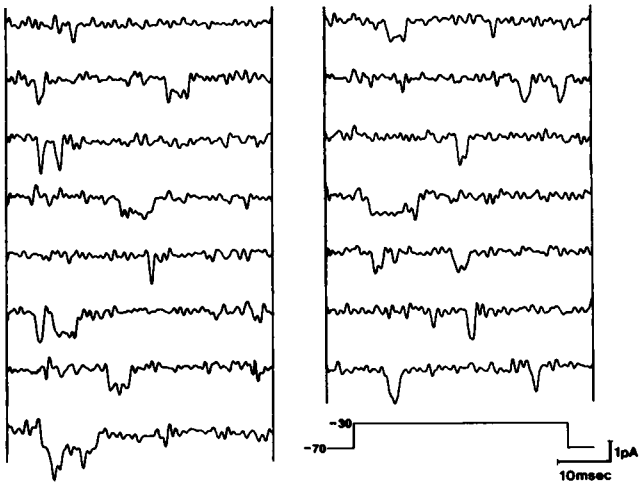

FIGURE 1 15 selected samples of single-channel TTX-sensitive currents recorded from a representative outside-out isolated membrane patch. Voltage clamps from −70 to −30 mV for 40 ms were applied at 0.25 Hz. Capacitative current was subtracted. Inward current registered as downward deflection. Temperature was 13.3°C.

Fig. 1 also reveals that opening events occurred more often during the first third of the pulse period than during the second two-thirds of the period. However, openings occurred even quite late in the course of the 40-ms pulses (see right side, second from top panel, for instance), and that late events occurred independent of any early events. Late openings (events occurring 20 ms or more after the onset of a depolarizing voltage pulse) occurred only during depolarization to voltages between −40 and −10 mV, with the greatest number and frequency occurring at ~−30 mV (provided holding potential was negative to −70 mV). In contrast, openings occurring during the first 20 ms of a depolarizing pulse were seen at all voltages between −40 and at least +30 mV, with the greatest frequency occurring at or positive to 0 mV (provided holding potential was −60 mV or greater). As opposed to when the holding potential was negative to −70 mV and patches were pulsed to −30 mV, when holding potential was −30 mV, no channel openings with either short or long opening latencies were seen.

To be certain that the channels with opening latencies longer than 20 ms were in fact Na channels and not another type of inward-current channel, isolated patches were exposed to 30, 100, and 300 nM TTX. Exposure of all isolated membrane patches to 300 nM TTX abolished virtually all inward-directed elementary currents evoked by pulses to −30 mV from −80 mV, confirming that the observed opening events, whatever their opening latency, had been produced by TTX-sensitive Na channels.

An unexpected finding was made on outside-out patches exposed to 10–30 nM TTX. The number of openings having short latencies seemed to be reduced more than those having long latencies. We therefore asked, can differences between channels opening with short latencies and those opening with long latencies account for this, or does TTX change the electrophysiological character of the unblocked channels? Outside-out patches were depolarized to −30 mV to evoke opening events with both short (<20 ms) and long (>20 ms) opening latencies. Holding potential was carefully selected so that a burst or uncountable number of openings with short latencies would not occur, yet enough openings with long latencies would be produced to permit meaningful analysis. Fig. 2 shows the results of analysis of one such experiment. Under TTX-free conditions at 13–14°, the single-channel conductance calculated from the currents obtained during depolarizations to −40 and −20 mV was 8.5 pS. Poisson analysis of the frequency of open times (i.e., length of time channels remained in the conducting state following the opening event) indicates that open times were exponentially distributed and had a mean channel-closing rate of 605 s^{-1} (n = 10). The observed mean open time was 2.36 ± 0.28 ms (n = 10). It should be understood that frequency response of our

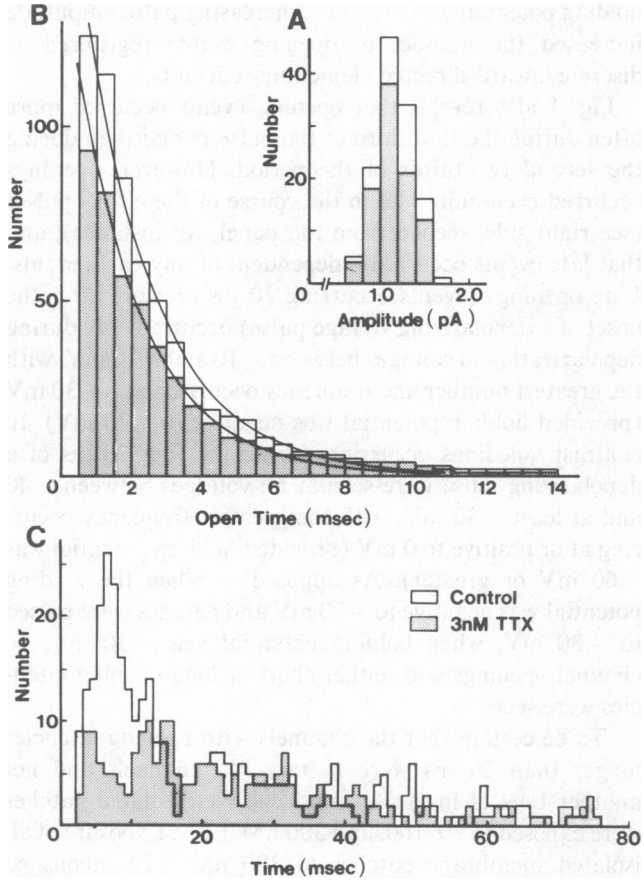

FIGURE 2 Analysis of single-channel TTX-sensitive current data evoked by 100 successive voltage clamps from −80 to −30 mV for 80 ms at 0.25 Hz before and during 5 min exposure to 3 nM TTX (Temperature was 13.0°C). For all panels: unfilled bars, control; filled bars; during exposure to 3 nM TTX. *A*, frequency distribution of single-channel current amplitudes. *B*, frequency distribution of single-channel open times. The upper (control) and lower (3-nM TTX) curves are exponentials best fitting the data (Control: $N = 191.3 \exp(-0.49t)$; TTX: $N = 149.3 \exp(-0.52t)$ where N is the number of events and t is the open time in ms). *C*, frequency distribution of channel-opening latencies taken as time between pulse onset and the opening event. Histogram developed from same population of events used to develop panel *B* histogram. No overlapping or simultaneous events were observed. Assuming a single channel was in the patch, the maximal opening probability during control was 0.26 with a 7–8-ms latency.

voltage clamping circuit precluded resolution of openings lasting <0.8 ms, so channels open for <0.8 ms will be indistinguishable from noise. The average channel latency was ~12 ms. Exposure to 3-nM TTX reduced the number of opening events to ~3/4 of control but altered neither the mean current amplitude (Fig. 2 *A*) nor the closing rate (Fig. 2 *B*) of the unblocked channels, indicating that neither mean channel conductance nor activation kinetics was affected by 3-nM TTX. At 13°C the number of channels with opening latencies roughly between 2 and 15 ms was reduced to about half of control, whereas the number of channels with latencies between 15 and ~40 ms was only nominally affected, and the average latency was prolonged by ~10 ms. Results obtained from three other isolated outside-out patches exposed to either 3- or 10-nM TTX were qualitatively similar in that the percentage reduction in the number of events with latencies <20 ms was always greater than that with latencies >20 ms; yet, neither the amplitude of the single-channel current nor the closing time histogram of the unblocked channels (normalized to the number of channels with open times between 1 and 2 ms for each experimental condition) was altered by TTX. After exposure to 3- or 10-nM TTX, the seal in three of the four patches was still well-maintained, so these patches were next exposed to 300-nM TTX. No openings were observed during TTX exposure, yet opening events reappeared during TTX wash out. Therefore, all the channels were confirmed to be Na channels.

DISCUSSION

At the membrane voltages and for the durations associated with the chick action potential plateau (−30 mV and 80 ms), Na channels have a finite opening probability. However, sustained depolarization to voltages positive to −40 mV seems to eliminate all opening events. Na channels opening with latencies >20 ms could underlie the component of Na conductance that contributes to TTX-sensitive plateau or window current. The "disappearance" of channels with long latencies during sustained depolarization suggests that the Na component of the window current is a slowly inactivating rather than a noninactivating voltage-dependent Na current. Considering that experiments were done at 13–14°C, the values obtained for single-channel Na conductance, open time, and opening latency are also quite similar to those described for Na channels in other tissues from other species (Neher, 1981; Rudy, 1981).

That channels with short opening latencies were rather more sensitive to blocking by TTX than were those with longer opening latencies is very provocative. There are at least two possible explanations. Despite the fact that (*a*) in Purkinje fibers Na channel block by TTX is enhanced by depolarization (Baer et al., 1976; Cohen et al., 1981), the observation could reflect a voltage dependence that enhances TTX binding to well-polarized channels; TTX could be leaving blocked channels during the depolarizing pulse, so that channels initially blocked at the pulse onset become conducting as the depolarized period lengthens. One would thus predict more events with very long latencies than events with only moderately long latencies. Fig. 2 *C* does not support this prediction.

The finding also could reflect (*b*) a greater affinity of TTX for binding sites associated with or in short latency channels than in long; this affinity would cause TTX to bind preferentially to channels with short latencies. The molecular structure and/or charge-binding sites of short latency channels may be slightly different from those of longer latency channels; each could represent one subset of Na channels having its own voltage-dependent probability

of opening; the sensitivity of each subset to specific cardioactive drugs may be somewhat different and permit a semi-selective block of one or the other subset of Na channels. Because we have employed membrane taken from embryonic myocardium, questions involving the maturation and development of the Na channel are cogent.

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CONDUCTION, BLOCKADE AND GATING IN A Ca^{2+}-ACTIVATED K^+ CHANNEL INCORPORATED INTO PLANAR LIPID BILAYERS

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Ca^{2+}-activated K^+ channels of large unitary conductance have been identified in several different types of cells (Schwartz and Passow, 1983; Latorre and Miller, 1983). To study this channel in a simplified, cell-free system and as a first step towards reconstitution, we have incorporated it into planar lipid bilayer membranes (Latorre et al., 1982; Vergara and Latorre, 1983; Moczydlowski and Latorre, 1983). We found that the Ca^{2+}-activated K^+ channel incorporated into artificial membranes has about the same conductance and gating kinetic characteristics as those found in cultured rat muscle by means of the patch-clamp technique (e.g., Barret et al., 1982; Methfessel and Boheim, 1982). Here, we report some of the conduction, blockade, and gating properties of this channel.

CONDUCTANCE-ACTIVITY RELATIONSHIP

Fig. 1 shows g vs. a_K data for bilayers of pure phosphatidylethanolamine (PE) in the absence of Tris.[1] The *inset* of Fig. 1 is a Scatchard or Eadie-Hofstee plot of the same data. It is apparent that the conductance-activity relationship deviates markedly from a Langmuir isotherm. The latter would be expected for a single-ion channel with fixed energy barriers (Lauger, 1973). In the *inset*, we have drawn two straight lines to show limiting behavior in the low and high activity range that we define for the sake of discussion, as high- and low-affinity "sites." The high-affinity site is roughly characterized by a maximal conductance of 220 pS and an apparent dissociation constant of 3 mM; the respective parameters for the low-affinity site are

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[1]The presence of mM amounts of Tris appears to depress the unit conductance of the channel at low K^+, resulting in nearly perfect Michaelis-Menten behavior as shown previously (Latorre and Miller, 1983).