LOW CONDUCTANCE SODIUM CHANNELS IN CANINE CARDIAC PURKINJE CELLS

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ABSTRACT Low conductance sodium (Na) channels have been observed in nerve, skeletal muscle, and cardiac cells. In cardiac tissues the higher amplitude, more commonly observed Na channel was first investigated in detail by Cachelin et al. (Cachelin, A. B., J. E. de Peyer, S. Kokubun, and H. Reuter, 1983, *J. Physiol. (Lond.)*, 340:389–402). They also reported low amplitude Na channel events. We have studied this low conductance Na channel in single canine cardiac Purkinje cells using cell-attached patches. Patch pipette solutions contained either 140 or 280 mM NaCl, and cells were bathed in a solution of 150 mM KCl to bring their resting potential close to zero. In 140 mM Na^+, during steps to −50 mV, the lower and higher openings had amplitudes of 0.57 ± 0.2 and 1.2 ± 0.2 pA (means ± SD of Gaussian fits). In 280 mM Na^+ at −50 mV, amplitudes were 0.72 ± 0.2 and 1.55 ± 0.2 pA. Over a substantial voltage range, the lower events had amplitudes of about one-third that of the higher events. The frequency of the low conductance openings varied in different patches from zero to 22% of total openings. Histograms of open durations and latencies at several voltages suggested no difference in kinetics between the two channel events. The behavior of the low conductance channels was more consistent with a second population of channels rather than a second open state.

INTRODUCTION

Diversity of sodium (Na) channels has been clearly demonstrated using both electrophysiological and biochemical techniques. The Na channel from *Electrophorus electricus* is reported to consist of only one large polypeptide (Noda et al., 1984; Agnew et al., 1978), whereas those from rat skeletal muscle (Barchi, 1983) and rat brain (Hartshorne and Catterall, 1984) have, in addition, two smaller subunits. Isolation of mRNA that codes for rat brain Na channels has shown two and possibly three different sequences for the large subunit (Noda et al., 1986). There are differences between Na channels in various tissues in their susceptibility to block by tetrodotoxin (TTX) (Lombet et al., 1982; Sherman et al., 1983) and by divalent cations such as Cd^{2+} and Zn^{2+} (Frelin et al., 1986). Electrophysiological evidence has been presented for a second population of Na channels in squid giant axons that activate in the threshold region and that show slow inactivation kinetics (Gilly and Armstrong, 1984). In frog node of Ranvier two Na currents were shown to be separable on the basis of their TTX and niflumic acid sensitivities and by their different rates of inactivation (Benoit et al., 1985).

Single channel studies have demonstrated two types of Na channel activity with different single channel conductances. In non-cardiac tissues low amplitude single channel Na currents have been observed in mouse neuroblastoma cells (Nagy et al., 1983), in developing rat skeletal muscle (Weiss and Horn, 1986), and in cultured embryonic *Xenopus* myocytes (DeCino and Kidokoro, 1985). In cardiac tissue several single channel studies have reported the occurrence of low amplitude Na channel openings in addition to Na channels with the more commonly observed single channel conductance of ~12 pS. Cachelin et al. (1983) found low amplitude openings in two of 20 patches from neonatal rat cardiac cells. However, the small size and rarity of these currents precluded detailed study. Kunze et al. (1985) also saw low amplitude openings but did not study them further.

Low amplitude openings in cardiac cells, if not artifactual, might represent a subconductance state of the Na channel, a biochemically modified Na channel, or an entirely different channel population. A subconductance state has been reported for the acetylcholine-activated channel (Hamill and Sakmann, 1981; Auerbach and Sachs, 1984). Two open states have been proposed for the Na channel (Chandler and Meves, 1970; Armstrong and Bezanilla, 1977; Sigworth, 1981; Patlak and Horn, 1982). However, in those studies the second open state was suggested to account for observed kinetic properties and not because of evidence of a second conductance level. In skeletal muscle a second population of Na channels is likely to exist because the proportion of events with the two conductances changes with development (Weiss and Horn, 1986).

We have observed low amplitude openings in several cardiac Purkinje cell patches. Here we report the charac-

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teristics of these low amplitude openings in five patches. They appear to be Na channel events with a single channel slope conductance that is about one-third of the more commonly seen Na channel events at 10°–12°C. Latencies (waiting times) and open duration distributions of the two types of Na channel events were similar. Other than the lower single channel conductance, the only difference found for the low amplitude events was that they may open less frequently than the higher amplitude events at more depolarized potentials. In patches where low amplitude events were observed, their frequency was at most one-fifth that of the more frequently observed larger amplitude events. The available evidence favors the conclusion that the low amplitude Na channel openings are from a separate population of Na channels, although a single population with two open states and conductances cannot be totally excluded. For convenience, the larger amplitude events will be referred to below as "common" events or as the "common" channel. Some of these data have been reported in abstract form (Scanley and Fozzard, 1987).

METHODS

The method for preparing single canine cardiac Purkinje cells has been described in detail elsewhere (Sheets et al., 1983). Briefly, canine cardiac Purkinje fibers were cut into short segments, placed in Eagle's minimal essential medium modified to contain 0.1 mM free Ca^{2+} (by addition of EGTA), 5.6 mM Mg^{2+}, 5.0 mM Hepes, 1 mg/ml albumin, and 5 mg/ml type I collagenase (pH 6.2) (Worthington Biochemical Corp., Freehold, NJ) at 37°C, gassed with 100% O_2, and gently agitated in a water bath shaker for 3–4 h. After 15 min in 130 mM K-glutamate, 5.7 mM Mg^{2+}, 0.1 mM EGTA, 5.0 mM glucose, and 5.0 mM Hepes (pH 6.2), the cells were mechanically dispersed and maintained in Eagle's minimal essential medium with 1.0 mg/ml albumin and 5.0 mM Hepes (pH 7.2) at room temperature. They were studied within 12 h of isolation.

Patch pipettes were made according to the method of Hamill et al. (1981). Patches 10/10A and 10/10B had pipette solutions containing 280 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes buffer at pH 7.4. Pipette solutions for patches 10/3A and 10/3B had 140 mM NaCl instead of 280 mM NaCl; 10/3B had no added Ca^{2+}. Freshly prepared single canine Purkinje cells were placed in a bath solution containing 150 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes (pH 7.4), and 10 mM glucose at a temperature of 10°–12°C. All studies were on cell-attached patches containing four or fewer channels and in which seal resistances were stable.

Single channel recordings were made with a Dagan 8900 patch clamp/whole cell clamp (Dagan Corp., Minneapolis, MN). The head stage was constructed according to a custom design (J. Rae and R. Levis, Rush Medical College, Chicago, IL). The signal was filtered at 2 kHz with an 8-pole Bessel filter (Frequency Devices, Inc., Haverhill, MA) and then digitized at 10 kHz. Voltage steps and data acquisition were controlled by protocols written in this lab for an IBM-PC (IBM Instruments, Inc., Danbury, CT) and a Tekmar 12-bit TM-100 digital-to-analog and analog-to-digital convertor (Tekmar Co., Cincinnati, OH). Data were stored directly onto floppy disk. A capacity compensation circuit was used to minimize the capacity transient, and further correction was performed during analysis by the subtraction of averaged sweeps without openings. Patches were depolarized repetitively at 1 Hz to step potentials lasting 45 ms, from a holding potential that was usually −120 to −140 mV (assuming a resting membrane potential of 0 mV in 150 mM KCl).

Average single channel currents were derived from histograms of the set of all sampled current values that exceeded threshold (Fig. 2). Threshold was set for the common events at half of their amplitude. For each low amplitude event threshold was set with a cursor by eye. The baseline noise is thus suppressed in these amplitude distributions, but the open channel noise is a factor in determining the shape of the distribution. In plots of the distribution of all sampled points the baseline noise is centered at zero and in most patches it had a standard deviation of 0.12 pA. The open durations were measured as the time above threshold. For overlapping openings the earlier closure was matched with the earlier opening. This occurred in only a few percent of the openings, but it would bias slightly toward short open times. Mean channel open durations were obtained from single exponential fits to histograms of open durations. The first 0.5 ms was discarded for these fits because the frequency response of the recording system prevented accurate measurement of very short openings. Gaussian curves were fit using a modified Gauss–Newton method to determine the means and standard deviations of the amplitudes. Exponential curves were fit as a sum of exponentials by a Fourier method (Provencher, 1976). The number of common channels in each patch was determined by a maximum likelihood method (Patlak and Horn, 1982), but this did not differ from the maximal number of simultaneous openings seen during the recordings.

RESULTS

Fig. 1 shows records of low and common amplitude openings in 140 Na^+ (*A*) and 280 Na^+ (*B*) both at steps to −50 mV. At this potential the amplitudes are clearly separable. We have observed these low amplitude openings in seven of seven patches recorded using this headstage. In earlier experiments using another headstage and with filtering at 1 kHz the low amplitude events were not apparent in three out of seven patches. One of these patches contained only a single Na channel of the common amplitude type and in that patch no low amplitude events

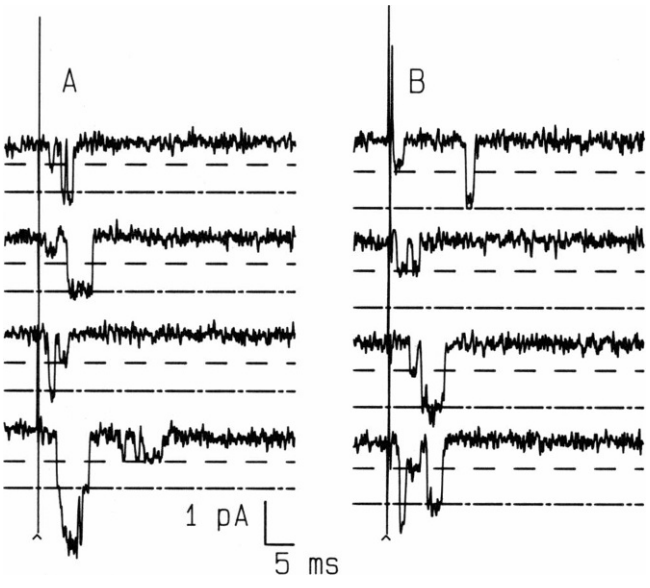

FIGURE 1 Patch clamp recordings showing Na channel openings of two different amplitudes. Double-dashed lines are drawn at the level of the amplitude of the higher amplitude events, dashed lines at the level of the lower amplitude events. Step potential −50 mV; arrows at bottom show when the depolarizing step occurred. The sweeps shown are nonconsecutive. (*A*) Patch 10/3A, pipette [Na] = 140 mM. (*B*) Patch 10/10B, pipette [Na] = 280 mM.

were seen. The low amplitude events may have been present in those patches but obscured by the higher noise level and lower filter frequency.

Single Channel Conductance

Amplitude histograms at −50 mV, for the two patches shown in Fig. 1, are illustrated in Fig. 2. Gaussian fits are superimposed on the distributions. In 140 Na^+ openings had amplitudes of 0.57 ± 0.2 and 1.2 ± 0.2 pA (mean ± SD). In 280 Na^+ the amplitudes were 0.72 ± 0.2 and 1.55 ± 0.2. i/V plots for the low and common events in one patch with 280 Na^+ and two patches with 140 Na^+ in the pipette are shown in Fig. 3.

The observed low amplitude currents were inward (with only Na^+ as an available charge carrier in that direction), their amplitude declined with depolarization, and the currents were increased by using a higher pipette Na^+ concentration (see below). While these observations do not ensure that the two types of Na channel openings have the same selectivity, they do indicate that under physiological conditions the low amplitude events are primarily transporting Na^+.

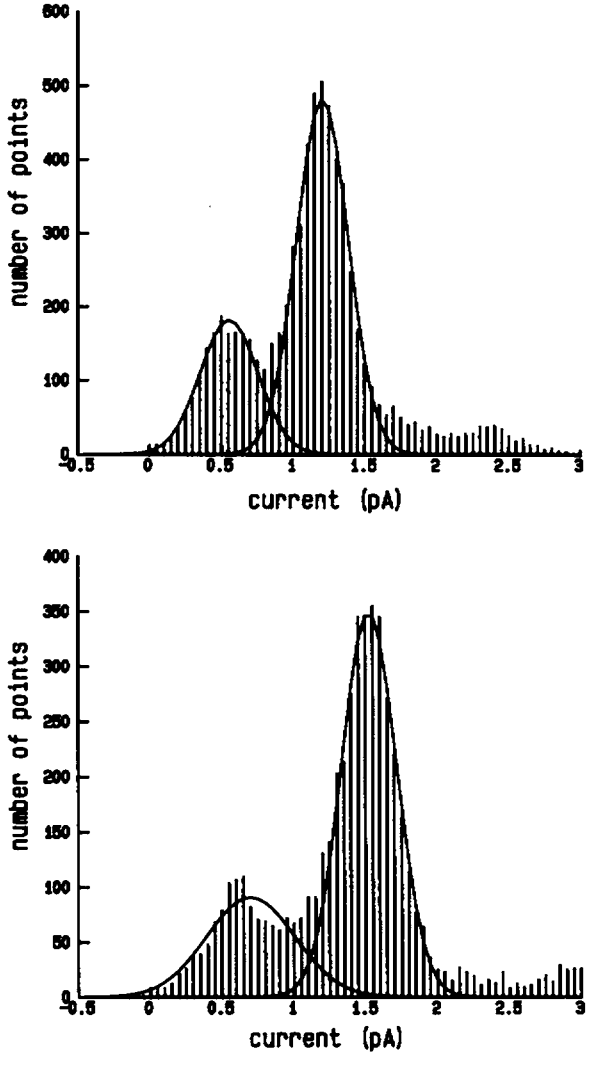

FIGURE 2 Amplitude histograms of all sample points that were taken within an event, suppressing baseline data (see text for description). Same patches and voltage as the records in Fig. 1. Curves superimposed on the histograms are double Gaussian fits. (*Top*) Patch 10/3A, −50 mV, Gaussian fits gave values of 0.57 ± 0.2 and 1.2 ± 0.2 pA (mean ± SD). (*Bottom*) Patch 10/10B, −50 mV, means were 0.72 ± 0.2 and 1.55 ± 0.2.

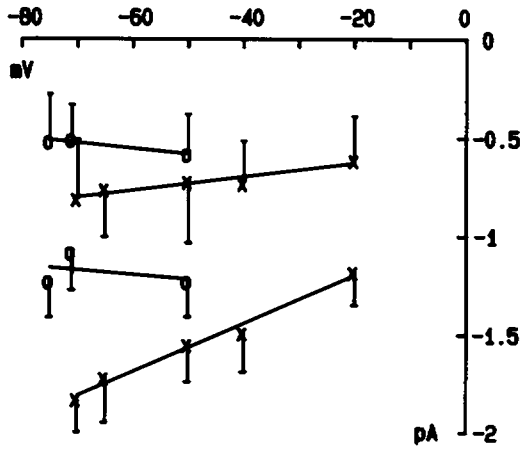

FIGURE 3 Current–voltage relation for low and common events. Patch 10/10B with a pipette [Na] of 280 mM (×). Patches 10/3A at −75 mV and −50 mV and 4/11E at −71 mV (o) in 140 mM [Na]. Brackets indicate SD. Lines drawn were best linear least squares fits. In 280 Na^+ the slopes were 3.5 and 12.3 pS for the low and common openings, respectively. In 140 Na^+ the slopes were −2.8 and −2.3 pS. This was probably because the effect of Ca^{2+} block on the single channel Na current was more pronounced at the lower Na^+ concentration.

Kinetics

A comparison of the distributions of the times to all openings (all latencies) at −65 and −50 mV for the low and common events is shown in Fig. 4. The times to all openings was used, rather than times to first openings, to avoid the effect that channel number has on the latter. While we can determine the number of common channels present in the patch, we have no way to determine the number of putative low conductance channels. This all latency distribution for common events resembles the time course of current owing to the common channel activity, and it may be compared with the low conductance event distribution. Open duration histograms for the two types of events, also at −65 and −50 mV in the same patch, are shown in Fig. 5. Because very brief common amplitude events fall below the threshold set for their detection, some of these events may be included in the open duration histogram for the low amplitude events, skewing it towards shorter durations. While there are not enough low events to make fits to the distributions, the kinetics of the low conductance events appear to be similar to those of the common Na channel events. This implies that the gating properties of the low conductance events may be the same as those of the common events.

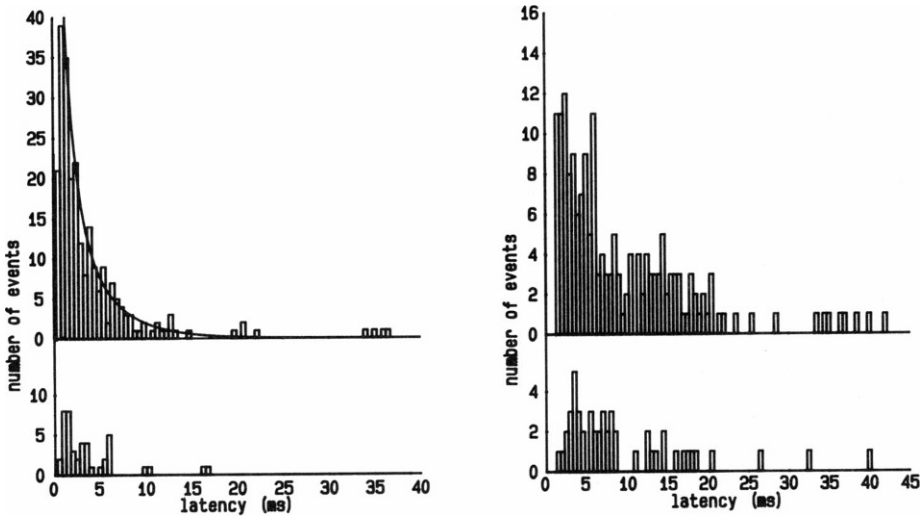

FIGURE 4 Latencies to all openings. Patch 10/10B. (*Left*) Step potential = −50 mV, upper histogram includes all common openings, lower histogram includes all low openings, the scale is the same for upper and lower histograms. The double exponential fit has τ_1 = 1.2 and τ_2 = 4.1 ms. (*Right*) Step potential = −65 mV, format as in the left panel. The fit is not shown, as the distribution of latencies is more dispersed at this potential.

The voltage dependence of the frequency of low events was compared with that of the common openings by determining the ratio of the number of low to high openings at several voltages in four patches (see Table I). No clear difference was evident. In patches 10/10B, 10/3B, and 10/10A the fraction of low events appeared to decrease with depolarization. However, in patch 10/3A the reverse was true. The frequency of low events never exceeded 22% of the total activity in these patches, which contained two to four common channels. At potentials more positive to −30 mV there were few sweeps without common channel activity and all of the events occurred within the first few milliseconds of depolarization. Therefore, at very positive potentials it became difficult to assess the occurrence of low events. If low openings occurred they would usually have coincided with common channels and only when simultaneous events had well separated opening and closing times could the low events be clearly distinguished.

Two Substates or Two Populations

To address the question of whether the low events were a separate population of channels or a substate of the common channel, we tested the independence of occurrence of the two types of events. The probability that a sweep would have a common event (No. of sweeps with common events/total No. of sweeps) and the probability that a sweep would have a low event (No. of sweeps with low events/total No. of sweeps) were multiplied to derive the predicted number of sweeps with both low and common events. This was compared with the actual number of sweeps with both types of events (see Table II). The predicted values coincided very closely with the observed occurrence of sweeps with common and low openings. For each recording, the data were analyzed as a fourfold table, and the weighted Z value was calculated to be 0.301. The weighting was according to the estimated variance (Canner, 1983; Bliss, 1967). This Z value fails to support the

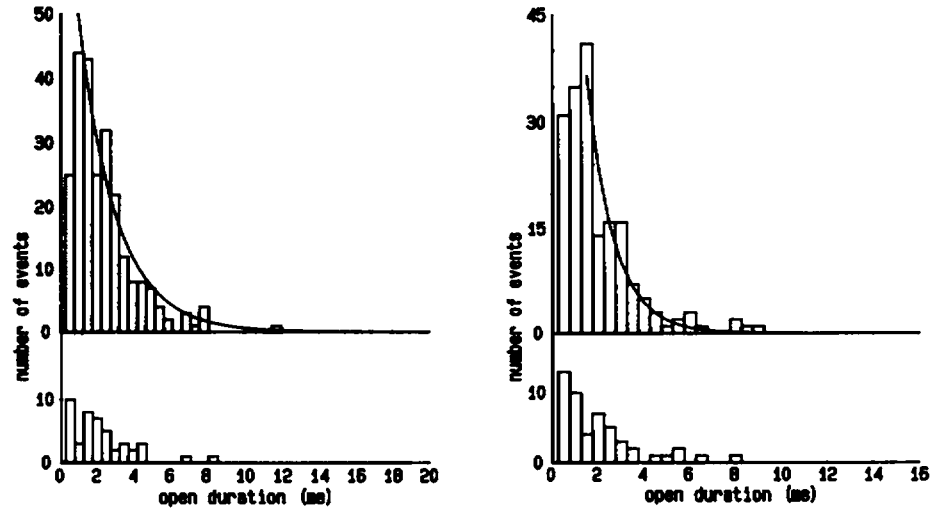

FIGURE 5 Open durations. Patch 10/10B, format as in Fig. 4. (*Left*) Step potential = −50 mV, single exponential fit to common amplitude events gave τ = 2.1 ms. (*Right*) Step potential = −65 mV, τ = 1.2 ms.

dependence of low amplitude events on the presence or absence of common events and is consistent with the idea of independence. Runs analysis for two patches with the longest recording periods showed no phasic changes in the ratio of common to low amplitude openings over 10-min periods. Interestingly, in patch 10/10A at −60 mV of 180 sweeps in which there were 119 openings only three low openings were seen and these three openings all occurred in one sweep. It therefore seems likely that the low amplitude channel may reopen before inactivating, as occurs with the common channels (Kunze et al., 1985; Horn and Vandenberg, 1984).

The low conductance level could be assumed to be a substate if frequent transitions between conductance levels without closings were found. There were two transitions of this kind observed in 72 low events from patch 10/3A at −50 mV and no transitions of this kind observed in 45 low events from patch 10/10B at −50 mV. The rarity of these transitions suggests that instead of being state transitions they represent closed durations that were too brief to be resolved.

TABLE I
VOLTAGE DEPENDENCE OF LOW EVENTS

Patch	No. of channels	Step potential	No. of events (low/total)	Low events
		mV		%
10/10B	4	−70	22:105	21
		−65	50:229	22
		−50	45:286	16
		−40	22:112	19
10/3A	4	−75	6:91	7
		−50	72:404	22
10/3B	2	−70	30:155	19
		−40	0:161	0
10/10A	4	−70	3:21	14
		−60	3:119	2.5
		−40	1:224	0.4

DISCUSSION

Possible Artifactual Origin of the Low Conductance Events

It has been suggested that channels under the rim of the patch pipette can exhibit a lower current amplitude (Neher et al., 1978; Hamill et al., 1981). The observation that the amplitude distributions show two clearly separable peaks and not one skewed distribution argues against this sort of artifact. Furthermore, their amplitude and kinetic properties were reproducible from patch to patch, and other investigators have observed similar events.

Two Populations vs. Two Open States

Either two channel populations or two different states of a channel could explain the lower amplitude events. The behavior of our low conductance events was not typical of better-studied channels having more than one open state. If the low events were a substate of the common channel with a constant relative probability of entering either the lower or higher amplitude state, then there should be a constant ratio of low to common events from patch to patch, independent of the number of channels in the patch. In our studies the ratio of low to common events was different in different patches. Additionally, the idea of two open states, each accessible from the same closed state, is unlikely because in each patch the probability of observing the low conductance events was not dependent on the presence of the common events. If the low and common events were alternate states of the same channel the occurrence of one type of event should influence the probability of the occurrence of the other.

Because of our need to filter the single channel recordings at 2 kHz, a rapidly blocked and unblocked channel opening would be recorded as a steady reduction in current amplitude. One possible explanation for the low conductance events is that the channel is engaged in such flickering, analogous to the Ca^{2+}-induced reduction in single channel current (Yamamoto et al., 1985; Sheets et al., 1987). However, with a uniformly Ca^{2+}-sensitive popula-

TABLE II
PREDICTED AND EXPERIMENTAL OCCURRENCE OF SWEEPS WITH BOTH TYPES OF EVENTS

Patch	Step potential	No. of events					Fraction of sweeps with both low and common events			
		Low	High	Both	None	Total	Predicted	Experimental	χ^2	Var
	mV									
10/10B	−70	20	51	8	57	120	0.071	0.066	0.008	29.7
	−65	32	98	19	62	173	0.105	0.110	0.025	43.0
	−50	36	124	25	33	168	0.158	0.148	0.180	38.9
10/3A	−50	55	188	43	36	236	0.186	0.182	0.028	53.1
10/3B	−70	24	70	9	153	238	0.030	0.038	0.180	57.8

tion of channels, Ca^{2+} block causes all channels in the patch to be affected rather than only one of several. The low amplitude events observed in patch 10/3B, which had a nominally Ca^{2+} free pipette solution, were not proportionately larger than those in patch 10/3A, which had 1.8 mM Ca^{2+}. Thus, the low amplitude events probably do not represent a population of channels with a very high Ca^{2+} sensitivity.

Comparison to Other Low Conductance Sodium Channel Openings

Nagy et al. (1983), in recordings from mouse neuroblastoma cells, demonstrated a distribution of single Na channel current amplitudes that was best fit by the sum of two Gaussian distributions. The mean amplitude of the smaller current was 80% of its higher counterpart. Their open time histograms were best fitted by the sum of two exponentials, which could be explained by different mean open times for the two amplitude types. They favored the conclusion that the low events represented a substate of the channel because in their experiments the proportion of low events was always less than that of high events. Kunze et al. (1985), studying ventricular myocyte Na channels, fit a double Gaussian to their amplitude distributions in which the lower peak was 60% that of the higher. Cachelin et al. (1983) also estimated that the low events that they observed had amplitudes 60% that of the more common, higher events.

Weiss and Horn (1986) have studied the characteristics of two populations of Na channels in developing skeletal muscle. Their two populations showed very different TTX sensitivity and developmental distribution. The developmentally earlier channel type was less sensitive to TTX and had a current amplitude that was 70% of the TTX-sensitive channel. They suggested that the low amplitude channel was activated at more negative voltages. The low amplitude and TTX resistance of the Na channels that they reported resembled that of the tetramethyloxonium (TMO) modified brain Na channels in artificial bilayers (Worley et al., 1986). The TMO modified channels had low amplitudes and decreased sensitivity to saxitoxin and to block by Ca^{2+}. However, Frelin et al. (1986) reported that they found TTX resistance paralleled sensitivity to block by Cd^{2+} and Zn^{2+}. Whereas TTX sensitivity was not studied in the patch clamp experiments reported here, whole cell TTX studies on these cardiac Purkinje cells have shown that the Na currents require micromolar concentrations of TTX to achieve complete block (Hanck, D. A. and M. F. Sheets, unpublished communication).

The low events observed here have amplitudes of about one-third that of the common channel. Relative to the common events they are thus smaller than the events described in previous patch clamp studies. The TMO modified channels, however, had amplitudes 36% of the unmodified channels, which is closer to the values that we found.

Physiological Role for the Low Conductance Events

Single channel recordings give little insight into the possible cellular role of low conductance channels. Cardiac Purkinje cells do have a "pacemaker channel," which is monovalent cation selective, and admits inward Na current at negative potentials (DiFrancesco, 1981). However, the voltage dependence of these low conductance events is quite different from the pacemaker current. The kinetics of the low amplitude events is different from that of "threshold" Na channels which inactivate slowly (Gilly and Armstrong, 1984). The low conductance events would contribute little to the peak Na current, because of their relative rarity and their lower conductance. For example, if the frequency of low events is 20% that for common events, and with only 40% of the current carrying capacity, their contribution would be $<10\%$. If they were to be activated in a more negative range, then they could play a role in threshold events. However, at present no voltage range was found at which they constituted more than 22% of all events.

An interesting question is whether or not the low events represent openings of biochemically modified common channels and that this is a means by which cells could modulate their excitability. The Na channel can exist in phosphorylated or dephosphorylated forms (Costa and Cattarall, 1984). We found no evidence in these studies that the low amplitude channel varied in its activity during the recording period, but that does not exclude metabolic control on a longer time scale.

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