brief communication

Use-dependent block of single sodium channels by lidocaine in guinea pig ventricular myocytes

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ABSTRACT Single sodium channel openings have been recorded from cell-attached patches of isolated guinea pig ventricular myocytes. A paired pulse protocol was used to test the hypothesis that channel openings are required for lidocaine block. While the averaged ensemble current during the test pulse was much reduced, there was no correlation between the appearance of channel openings during the conditioning pulse and the subsequent test pulse. Analysis of single channel records demonstrated that the unit conductance of open channels was not changed by lidocaine. The block of ensemble I_{Na} was explained by roughly equal reductions in number of open channel events, and in the average duration of opening for each event. These results suggest that lidocaine binding to Na^+ channels is dependent upon voltage, but may occur before channel opening. A lidocaine-modified channel can still open, but will be less likely to remain open than a drug-free channel. These results are consistent with block of a pre-open state of the channel.

INTRODUCTION

Sodium channel block represents an important mode of action for both antiarrhythmic drugs and local anesthetics. This mode of drug action has been particularly well studied in nerve preparations, and two major hypotheses, the modulated receptor hypothesis and the guarded receptor hypothesis, have been put forth to explain it (Hondeghem and Katzung, 1977; Hille, 1977; Starmer et., 1984). In experiments that involve single cardiac myocytes, lidocaine has received the most attention thus far. Several important features of drug action have been reported, including use-dependent block of channels, and ability of the drug to bind to channels that have entered the inactivated state (Bean et al., 1983; Nilius et al., 1987; Hondeghem and Katzung, 1984; Reuter et al., 1985). However, progress has been hindered by the difficulty of controlling membrane potential in entire cells, especially under physiologic conditions. Furthermore, it is difficult to deduce the state-dependence of drug action from experiments in which large numbers of channels contribute simultaneously to the recording. To avoid these difficulties, we have studied the effects of lidocaine on single sodium channel currents in isolated guinea pig ventricular myocytes. We have utilized a double pulse protocol, which permits the use-dependence of specific parameters of channel behavior to be tested directly. We find that neither opening nor inactivation is mandatory for the blocking effect on single channels to develop. Instead, we find a use-dependent reduction of channel open time, which can be explained by binding of the drug to a pre-open state of the channel.

METHODS

Experiments were performed on guinea pig ventricular myocytes isolated using a modification of the method described by Mitra and Morad (1985). After pentobarbitol anesthesia, heparinization, and cervical dislocation, hearts were removed rapidly from 300–500-g guinea pigs and perfused by a Langendorff apparatus. Initial perfusion was performed at 37°C and pH 7.2 with a Hepes-buffered saline (HBS) (135 mM NaCl, 4 mM KCl, 1.2 mM $MgCl_2$, 5 mM glucose, 10 mM Hepes) for 5 min, followed by perfusion with HBS containing type II collagenase 270 U/ml (Cooper Biochemical Corp., Freehold, NJ) and type XIV protease 1.27 U/ml (Sigma Chemical Co., St. Louis, MO) for 8 min, and a final washout with HBS containing 0.2 mM Ca^{2+} for 5 min. Myocytes were dispersed by gentle agitation of beakers containing minced tissue. Dispersed myocytes were washed and resuspended in HBS containing 1.8 mM Ca^{2+} and stored at room temperature for 2–18 h. All solutions were gassed with 100% oxygen during the isolation procedure.

Micropipettes for patch-clamp recording were made by the method of Hamill et al. (1981) and had tip resistances of 8–16 MΩ. Bath and pipette solutions were identical and contained 145 mM NaCl, 4 mM KCl, 2.0 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5 mM glucose, and 10 mM Hepes. pH was adjusted to 7.40 with NaOH. Lidocaine, 50 μM (Astra Scientific International, Inc., Santa Clara, CA), was added to the solutions in both the bath and pipette. All experiments were performed at room temperature (19–21°C). Single channel currents were recorded using a model 8900 patch clamp amplifier (Dagan Corp., Minneapolis, MN) with a 10 GΩ feedback resistance headstage. Signals were filtered at 2.5 kHz with a low-pass filter (model 901 F, Frequency Devices, Haverhill, MA), and digitized at 10 kHz using a Tecmar 12-bit A/D-D/A converter (Scientific Solutions, Cleveland, OH) and stored directly onto floppy disc with an IBM-PC/AT (IBM Instruments, Danbury, CT).

Single channel recordings were made in the cell-attached configuration and voltage protocols controlled by a computer program and D/A converter. Holding potential was set at 60 mV negative to resting cell membrane potential and depolarizing pulses of 80 mV were applied at a frequency of 0.5 Hz with conditioning pulses of varying duration.

Capacity and leak currents were subtracted digitally using pulses of opposite polarity. Ensemble currents were obtained by averaging the current at each time point during a series of consecutive sweeps with identical stimulus pulses. Single channel open times were determined using a semiautomatic event detection program with threshold set at half the amplitude of single channel currents. Patches with frequent multiple overlapping openings (i.e., >5% of open events) were not used for kinetic analysis of single channels. Patches with infrequent overlapping events were analyzed according to the method of Kunze et al. (1985). This method defines overlap as present when the signal level exceeds 1.5 times the mean amplitude of the single channel event. Pairs of overlapping events were assigned one long and one short open time rather than two more intermediate open times.

Channel opening behavior was also quantified by taking the integral of current during the first 10 ms of each pulse. This procedure allowed opening events to be compared or correlated during consecutive pulses, without requiring that all of the openings in a sweep be assigned unequivocally to the same physical channel.

A nonlinear, least squares fitting routine was used to fit exponential curves to open time histograms (Schreiner et al., 1985). Mean currents, open times, and open event frequencies were compared by paired *t*-tests and analysis of variance. Values of variables are stated as mean ± SEM.

RESULTS

The average resting potential, determined by patch rupture after gigaohm seal formation, in 12 cells under similar ionic conditions and temperature was −70.4 ± 0.9 mV. Records of Na^+ channel currents from a cell-attached patch in the presence of 50 μM lidocaine are shown in Fig. 1. During each sweep, the membrane potential was stepped from a holding potential 60 mV negative to resting cell potential (Rp, −60 mV) to 20 mV positive to resting potential (Rp, +20 mV) in two sequential pulses (V_c, conditioning pulse; V_t, test pulse) separated by 20 ms.

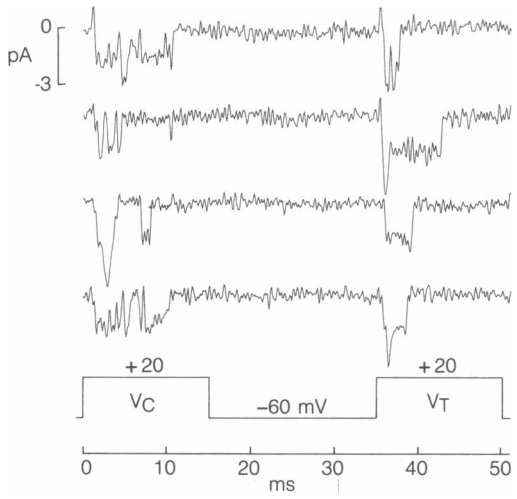

FIGURE 1 Single Na^+ channel current traces from a patch-clamped guinea pig ventricular myocyte in the cell-attached configuration. Upper traces show four records obtained during a two-pulse voltage protocol with 50 μM lidocaine present in the pipette and bath solutions. Bottom part of figure shows the voltage protocol. Cells selected for single channel analysis had few or no overlapping events.

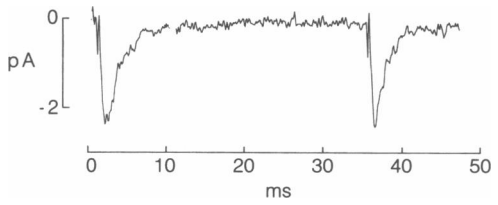

FIGURE 2 Summed and averaged current traces from 38 sweeps in a myocyte not exposed to lidocaine. The voltage protocol during each of the sweeps was identical to Fig. 1. There was full recovery of the peak sodium current in the absence of lidocaine. The number of open channel events was slightly smaller in V_t than in V_c (see Table 2).

Mean ensemble currents for each cell were constructed from 16 to 112 sweeps using this pulse protocol. In the absence of lidocaine, there was no reduction in peak Na^+ current during the test pulse (Fig. 2), and only a minor (5 ± 3%) reduction in mean ensemble current integrals (Table 1), relative to values measured during the conditioning pulse. Results from three cells that were exposed to 50 μM lidocaine are shown in Fig. 3 (right panels). Lidocaine caused a 32.4 ± 4.4% reduction of the mean ensemble current integrals in V_t relative to V_c (n = 10 cells). This reduction in current represented the degree of use-dependent Na^+ channel block after a recovery period of 20 ms.

The dependency of drug-channel binding on channel opening was investigated by a sweep-by-sweep correlation of the integral of current for V_t vs. V_c. If channel opening was required for drug binding to the Na^+ channel, a

TABLE 1 Opening events, and ensemble current block during the control (V_c) and test pulse (V_t) in five cells not treated with lidocaine

Mean open time		No. of openings		
V_c	V_t	V_c	V_t	% Block
0.570	0.600	540	506	0
1.070	1.240	105	92	14
0.710	0.700	137	128	7
0.730	0.820	282	277	0
0.970	1.030	126	88	5
0.810 ± 0.091	0.878 ± 0.115	238 ± 82	218 ± 80	5 ± 3
V_t/V_c = 1.07 ± 0.03		V_t/V_c = 0.89 ± 0.05		

Pulse protocols and data analysis are identical to Table 2, except that the data are from five cells not treated with lidocaine.

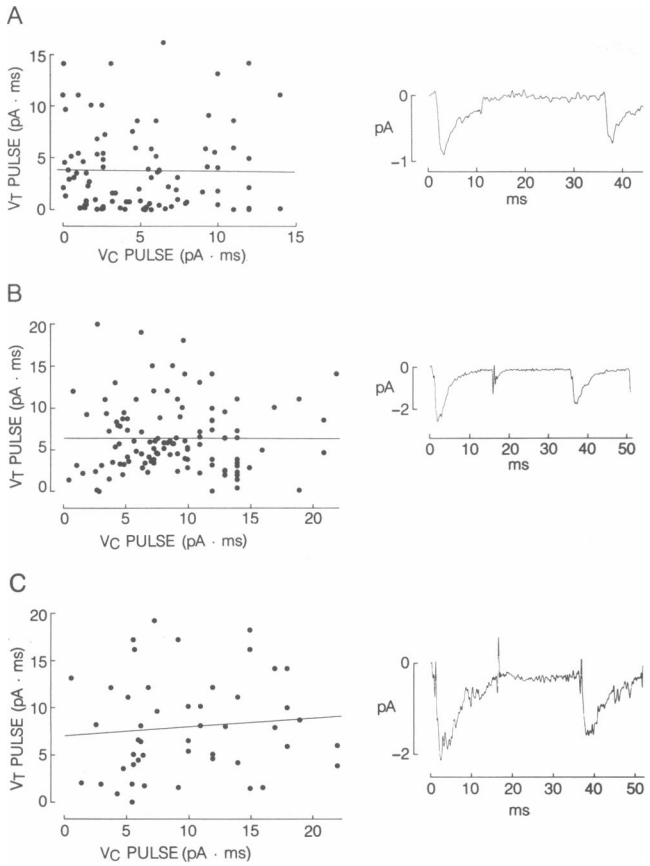

FIGURE 3 Use-dependence and channel state–independence of lidocaine block of Na^+ current. Right side of figure shows summed and averaged current tracings for three separate cells (*A*, *B*, and *C*). Each cell was exposed to 50 μM lidocaine, and was stimulated according to the voltage pulse protocol of Fig. 1. Left side shows corresponding scatter plots of integrated current from each single tracing. Integrated current during V_c was plotted on the abscissa against the current during V_t along the ordinate. Regression lines demonstrate lack of correlation of channel opening in V_t with V_c. Exclusive binding of lidocaine to open channels should produce such a correlation, even if multiple channels were present in the patch. In that case, the number of openings expected during V_t would vary inversely with the number of channels that had opened and potentially bound to the drug during V_c.

significant negative correlation of integrated V_t current with integrated V_c current would be expected. This type of analysis was performed in 10 cells, and no correlation of V_t with V_c was observed (Fig. 3, left panels). The average regression slope was 0.15 with an average correlation coefficient of 0.14. This result indicates that channel opening during V_c was not required for the channel to be blocked during V_t. It follows that whereas use-dependent binding of lidocaine to Na^+ channels is a consequence of membrane depolarization, it does not depend on whether the channel enters the open state.

The role of depolarization in the use-dependent action of lidocaine was examined using a pulse protocol in which the duration of the conditioning pulse varied, but the holding potential, pulse amplitudes, and interval between V_c and V_t remained constant. Integrated ensemble currents during V_t were measured, and plotted against the duration of V_c in Fig. 4 (top curves). The bottom curve of Fig. 4 shows the mean normalized V_t current integrals for 16 cells treated with lidocaine, expressed as a fraction of the corresponding current integrals for four cells not treated with lidocaine. These results demonstrate that lidocaine block continued to accumulate at V_c durations well beyond those required to inactivate most of the Na^+

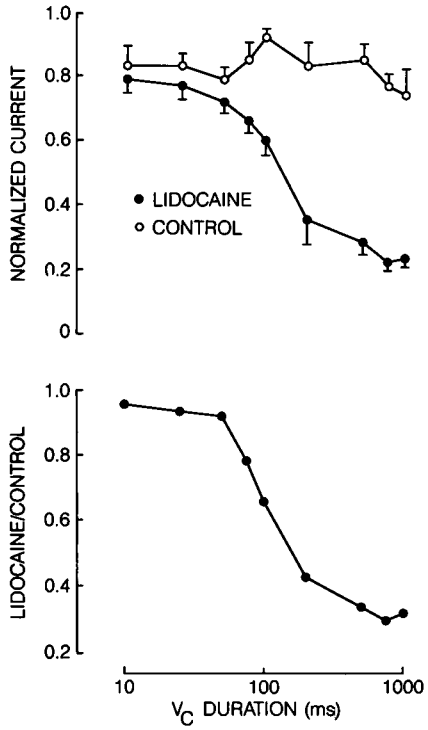

FIGURE 4 Effects of conditioning pulse duration on lidocaine block of Na^+ current during the test pulse. Conditioning pulses (V_c) of 10–1,000 ms duration depolarized the membrane to Rp +50 mV, followed by return to a holding potential of Rp −60 mV for 25 ms. Identical 25-ms test pulses (V_t) to Rp +20 mV were then given, and the resulting channel activity was measured as the integral of the averaged ensemble current during the first 10 ms of V_t. Integrated V_t current was expressed as a fraction of the integrated current when V_c was omitted, and this fraction was plotted along the ordinate (top two curves) with the duration of V_c pulse along the abscissa. Open circles show integrated V_t current in the absence of lidocaine (mean values from four cells). Closed circles show corresponding V_t integrals in the presence of 50 μM lidocaine (mean values from 16 cells). The bottom curve shows the mean normalized V_c current integral from the lidocaine-treated cells, expressed as a fraction of the normalized V_c current integral from the control cells at each point in time. Block of the Na^+ current by lidocaine continued to accumulate when V_c duration extended beyond the period when channels were open.

TABLE 2 Opening events, ensemble current block, and correlation of openings during the control (V_c) and test pulse (V_t) in seven cells treated with 50 μM lidocaine

Mean open time		No. of openings			
V_c	V_t	V_c	V_t	% Block	R
0.582	0.504	235	211	23	0.108
0.580	0.548	250	198	29	0.103
0.750	0.614	440	423	24	0.094
0.757	0.660	316	261	24	0.098
0.746	0.615	56	47	50	0.083
1.012	0.823	550	430	32	−0.005
1.269	1.005	39	21	58	0.369
0.814 ± 0.093	0.681 ± 0.066	269 ± 71	228 ± 61	34 ± 5	0.123 ± 0.043
$V_t/V_c = 0.85 \pm 0.02$*		$V_t/V_c = 0.81 \pm 0.05$‡			

*$P < 0.001$.
‡$P < 0.01$.
Openings during V_c and V_t have been analyzed for a series of sweeps in each of seven cells (16–112 sweeps/cell). The integrated current during each V_c pulse is correlated against current during the V_t pulse, to give correlation coefficients (R values), which are insignificant. Current is then integrated across sweeps to give the ensemble current integral, which is reduced during the V_t pulse by the percentage shown (% block). Mean open times are in milliseconds. Pulse protocol shown in Fig. 1.

channels. This finding confirms that accumulation of drug block during depolarization is not entirely dependent on channel opening, or on the availability of the channel to open.

The mechanism of use-dependent block of Na^+ currents by lidocaine was further investigated using single channel analysis in seven cells subjected to the two pulse protocol of Fig. 1 (Table 2). During the test pulse there were significantly fewer open channel events than during the conditioning pulse (15%, $P < 0.01$). There was also a 16% reduction ($P < 0.001$) in the mean open channel durations during V_t compared with V_c (0.81 ± 0.09 ms vs. 0.68 ± 0.07 ms; V_c vs. V_t, respectively). The reduction in open channel duration is seen clearly in the representative histograms obtained during the V_c pulse (A) and V_t pulse (B) in Fig. 5. The mean amplitudes of open channel unit conductance were not significantly different during the two pulses (−1.93 ± 0.04 pA and −1.88 ± 0.04 pA; for V_c and V_t, respectively).

The specific involvement of lidocaine in the use-dependent reduction of mean channel open time was confirmed in five cells that were stimulated with the same pulse protocol, but in the absence of drug (Table 1). This analysis showed a small (8%) reduction in the number of open-channel events during the test pulse, which occurred in every cell, but did not reach statistical significance. Mean channel open times were not significantly different for the two pulses (0.81 ± 0.09 ms vs. 0.88 ± 0.12 ms for V_c and V_t, respectively), and there was no trend toward shorter open times in V_t.

DISCUSSION

Local anesthetics are thought to bind preferentially to, and thereby block, open sodium channels. This concept was originally based on studies using very hydrophilic or quaternary drugs, often in cooled nerve preparations (Courtney, 1975; Strichartz, 1973; Yeh et al., 1986). It is not clear whether this binding of drug to open channels occurs generally, for example, in the action of antiarrhythmic drugs in cardiac muscle under physiological conditions. Our two-pulse protocol tests, directly, the importance of open channels in the use-dependent block by lidocaine of the sodium current in mammalian cardiac myocytes. The lack of a negative correlation between the integrated current in V_c compared with V_t (Fig. 3 and Table 2) suggests that use-dependent block of the sodium channel by lidocaine does not require channel opening. We also found that for durations of membrane depolarization greater than that required for channel opening, more lidocaine blocking action occurs (Fig. 4). The apparent plateau of block seen in this experiment probably reflects saturation of the drug receptors during the conditioning pulse, and is analogous to the behavior of the macroscopic sodium current described by Bean et al. (1983, their Fig. 10 B).

Mean open times are very sensitive to membrane holding potential and pulse potential (Grant et al., 1983; Kunze et al., 1985; Fozzard, 1985). In our experimental protocol the open times for V_c and V_t were measured sequentially in the same cell under the same experimental

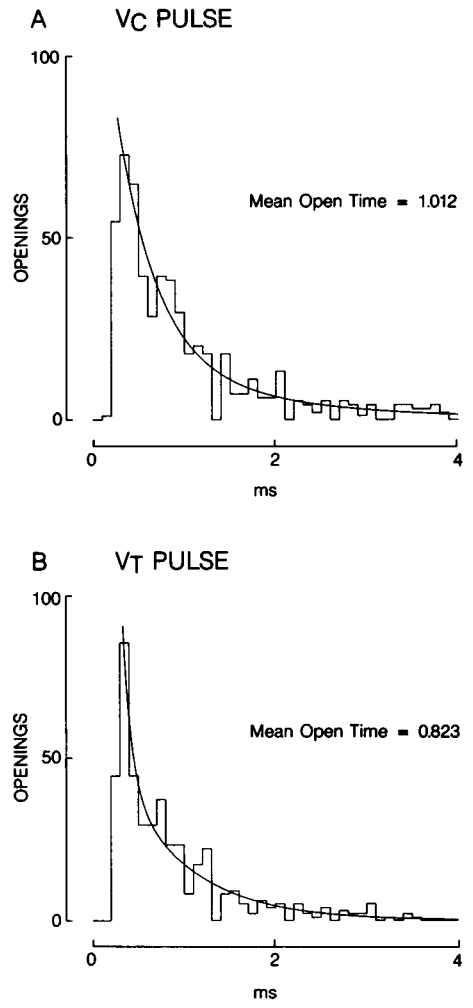

FIGURE 5 Open duration histograms of Na^+ channels from a single cell in the presence of 50 μM lidocaine. Two pulse voltage protocol as described in Fig. 1. (*A*) Events during the conditioning pulse best fit to a monoexponential, mean open duration of 1.012 ms. (*B*) Events from the test pulse, mean open duration of 0.823 ms.

conditions. Thus, cell-to-cell variation in resting membrane potential did not significantly affect our results or interpretation. Mean open times during V_t are consistently shorter than those during V_c. This finding would suggest that lidocaine-bound Na^+ channels can still open, but that rates of transition back to the drug-bound closed state (C*D), or into the drug-bound inactivated state (ID), are accelerated, giving a less stable drug-bound open state (OD):

	Closed	Pre-open	Open	Inactivated
Drug-free	C	C*	O	I
Drug-bound		C*D	OD	ID

Binding of drug to the channel during depolarization would be contingent upon transition from a baseline closed state (C), to a higher affinity pre-open state (C*; see Strichartz and Wang, 1986; Wang et al., 1987). Channels that have entered the pre-open state may also bind to the drug after progression to the open (O) or inactivated (I) states, but their probability of binding would not be increased by these transitions, compared to channels that waited in C* (result of Fig. 3).

In addition to decreased mean open durations in V_t there were significantly fewer openings. Thus, it appears that use-dependent binding of lidocaine also decreases the probability of channel opening. This result would most likely be due to accumulation of drug-bound channels in an absorbing inactivated state (ID), which is consistent with the result in Fig. 4. The average reduction in mean open times (16%), together with the average reduction in openings (15%), accounts quantitatively for the 34% reduction in average ensemble Na^+ current in V_c compared with V_t ($[1.00 - 0.16] \times [1.00 - 0.15] \doteq [1.00 - 0.34]$). From these results we can hypothesize that use-dependent binding of lidocaine to Na^+ channels produces a drug-channel complex that can open, but with altered kinetics compared to drug-free channels.

Results consistent with our formulation can be found in the previous work of others. For example, Nilius et al. (1987) have found that during application of single depolarizations, the mean channel open time in the presence of lidocaine is reduced compared with control values. However, the reduction in open time did not occur when membrane potential was strongly hyperpolarized before application of the depolarizing stimulus. This suggests that tonic channel block might be required for a reduction in open times to be observed. Tonic channel block (i.e., reduced availability of channels during unconditioned depolarizations) would then be one manifestation of drug-receptor occupancy, whereas reduction of mean open time would be another manifestation of this receptor occupancy. According to this view, lidocaine does not occlude the channel and does not necessarily prevent the channel from opening. Instead the drug can (*a*) make it less likely for channel opening to occur during depolarizations, and (*b*) make it less likely that a channel, once open, will remain open. Binding of the drug to the channel is an electrically silent event, and channel opening per se is not required for binding to occur.

We did not see consistent evidence for reductions of late opening events in either the single channel or ensemble current traces, as others have reported (Reuter et al., 1985; Bennett, 1987). For the brief depolarizations used in our experiments (Fig. 1), we found that roughly equal reductions in number of channel openings and in open times can account for the use-dependent block that we observe (Table 2).

In summary, we have used single channel recordings of cardiac sodium channels to test the hypothesis that channel opening is a necessary step in the blocking action of lidocaine. Our results show that channel opening is not required. We also find that lidocaine-modified channels can open, but for a shorter period of time. We infer the existence of a drug-bound pre-open state. The partial activation gating required to attain this state would contribute to the use-dependence and voltage-dependence of channel block. It is possible that transitions among the closed, pre-open, and drug-bound states could be further elucidated through the study of gating currents, which have recently been recorded in single cardiac cells (Hanck et al., 1987).

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