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Temperature Dependence of Pyrethroid Modification of Single Sodium Channels in Rat Hippocampal Neurons

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Abstract. Pyrethroid modulation of sodium channels is unique in the sense that it is highly dependent on temperature, the potency being augmented by lowering the temperature. To elucidate the mechanisms underlying the negative temperature dependence of pyrethroid action, single sodium channel currents were recorded from cultured rat hippocampal neurons using the inside-out configuration of patch-clamp technique, and the effects of the pyrethroid tetramethrin were compared at 22 and 12°C. Tetramethrin-modified sodium channels opened with short closures and/or transitions to subconductance levels at 22 and 12°C. The time constants of the burst length histograms for tetramethrin-modified channels upon depolarization to −60 mV were 7.69 and 14.46 msec at 22 and 12°C, respectively (Q_{10} = 0.53). Tetramethrin at 10 μM modified 17 and 23% of channels at 22 and 12°C, respectively, indicating that the sensitivity of the sodium channel of rat hippocampal neurons to tetramethrin was almost the same as that of tetrodotoxin-sensitive sodium channels of rat dorsal root ganglion neurons and rat cerebellar Purkinje neurons. The time constants for burst length in tetramethrin-modified sodium channels upon repolarization to −100 mV from −30 mV were 8.26 and 68.80 msec at 22 and 12°C (Q_{10} = 0.12), respectively. The prolongation of tetramethrin-modified whole-cell sodium tail currents upon repolarization at lower temperature was ascribed to a prolongation of opening of each channel. Simple state models were introduced to interpret behaviors of tetramethrin-modified sodium channels. The Q_{10} values for transition rate constants upon repolarization were extremely large, indicating that temperature had a profound effect on tetramethrin-modified sodium channels.

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Key words: Sodium channel — Temperature — Hippocampal neuron — Pyrethroid — Tetramethrin — Single channel

Introduction

Pyrethroids are synthetic derivatives of pyrethrins, which are toxic components contained in the flower of *Chrysanthemum cinerariaefolium.* The pyrethroids are used widely as near-ideal insecticides due to their high insecticidal potencies, low mammalian toxicities and biodegradability. Pyrethroids may be classified into two types based on the structure and the symptoms of poisoning. Type I pyrethroids lack the α-cyano group and produce T syndrome characterized by aggressive sparring, elevated startle response, whole body tremor, and prostration in the rat, whereas type II pyrethroids which contain the α-cyano group produce CS syndrome characterized by burrowing behavior, coarse tremors, clonic seizures, sinuous writhing, and profuse salivation with lacrimation (also referred to as choreoathetosis/salivation) (Verschoyle & Aldridge, 1980; Casida et al., 1983). A considerable degree of the selectivity of toxicity among various insect species and mammals exists, and low mammalian toxicity of pyrethroids as compared with insect toxicity was ascribed at least in part to the differences in enzymatic degradation (Jao & Casida, 1974*a*,*b;* Miyamoto, 1976; Lawrence & Casida, 1982; Casida et al., 1983). However, our recent study has clearly indicated that the difference in sodium channel sensitivity to pyrethroids plays a major part in selective toxicity between insects and mammals (Song & Narahashi, 1996*a*).

While type I pyrethroids generally produce repetitive discharges in the nervous system as a result of an increase in depolarizing after-potential, type II pyrethroids cause in most cases membrane depolarization

without eliciting repetitive after-discharges and evoke sensory discharges (Narahashi, 1992). However, these two types of changes in nerve function are ascribed to the modulation of sodium channels (Ruigt, 1984; Narahashi, 1989, 1992, 1996; Vijverberg & van den Bercken, 1990; Narahashi et al., 1995; Soderlund, 1995).

Pyrethroids stabilize sodium channel gating particles (Salgado & Narahashi, 1993), resulting in slowing of the movements of both the activation and inactivation gates (Chinn & Narahashi, 1986), and shift the voltage dependence of the gates in the hyperpolarizing direction (Tatebayashi & Narahashi, 1994). These changes cause a prolonged flow of sodium current into a cell, leading to sustained membrane depolarization. Several neurotoxin binding sites on the sodium channel have been identified on the basis of electrophysiological, biochemical and molecular biological studies (Catterall, 1992). However, pyrethroids do not act at any of the sites previously defined for other sodium channel toxins and seem to have a specific binding site (Trainer et al., 1997).

It is well known that pyrethroids have more potent effects on sodium channels at lower temperatures than at higher temperatures (Wang, Narahashi & Scuka, 1972; Vijverberg et al., 1983). This negative temperature dependence is an important property that contributes significantly to the selective toxicity in mammals and insects (Song & Narahashi, 1996*a*). Although there are several studies that examined the negative temperature dependence of pyrethroid action either in current clamp or in whole-cell voltage clamp conditions, only one study dealt with this issue at the single-channel level (Chinn & Narahashi, 1989). Pyrethroid-induced slowing of the activation and inactivation gates of sodium channels (Chinn & Narahashi, 1986; Salgado & Narahashi, 1993) was more pronounced at low temperatures than at high temperatures (Chinn & Narahashi, 1989).

The present study was aimed at pursuing the mechanism of action of tetramethrin at the single-channel level as a function of temperature. A variety of parameters of tetramethrin-modified single sodium channels were compared at 22 and 12°C. Simple state models were proposed in order to explain the negative temperature dependence of tetramethrin action.

Materials and Methods

Choice of Pyrethroid and Neuron Preparation

Tetramethrin, a type I pyrethroid, was chosen as a test compound for several reasons: (i) A large amount of information has been accumulated regarding its mechanism of action on sodium channels in both mammals and invertebrates (e.g., Lund & Narahashi, 1981*a,b,* 1982, 1983; Yamamoto, Quandt & Narahashi, 1983; Tatebayashi & Narahashi, 1994; Song & Narahashi, 1996*a,b*). (ii) Tetramethrin has proven potent in modifying the sodium channel activity in mammalian neurons despite its relatively low mammalian toxicity which is due likely to be ascribed to high metabolic degradation (Jao & Casida, 1974*a,b;* Miyamoto, 1976; Lawrence & Casida, 1982; Casida et al., 1983). (iii) A purified (+)-*trans*-isomer of tetramethrin was available to us. In light of the lack of information about the exact sites of action of pyrethroids in the mammalian brain (Casida et al., 1983), hippocampal neurons were chosen as the material, since they are among the most thoroughly examined neurons in the brain.

Dissociation and Culture of Neurons

Hippocampal neurons were prepared from 17-day-old embryos of a Sprague-Dawley pregnant rat under methoxyflurane anesthesia. After an adequate level of anesthesia was reached as indicated by lack of responses to pinching the hind legs, a transverse incision was made in the lower abdomen of the pregnant rat and the fetuses were withdrawn from the abdomen. The heads of the fetuses were immediately cut off, and the hippocampi were dissected and collected in a calcium- and magnesium-free phosphate-buffered saline solution (PBS). The mother rat was euthanized immediately thereafter by decapitation. Neurons were dissociated by repeated passage through a flame-narrowed Pasteur pipette and diluted with Neurobasal Medium supplemented with B-27 and 2 mM L-glutamic acid (GIBCO/Life Technologies, Grand Island, NY). The final suspension was plated onto 12 mm poly-L-lysine-coated coverslips at a density of 200,00/well. Cells were maintained in humidified atmosphere of 90 air and 10% CO_2 at 37°C, and were used within 3 weeks of culture. Neurons having triangular cell bodies were selected for experiments.

Electrical Recording

Single-channel currents were recorded using the inside-out configuration of the patch-clamp technique (Hamill et al., 1981). Patch pipettes fabricated from borosilicate glass capillary tubes (1.5 mm inner diameter) had resistances of 5–10 MΩ when filled with pipette solution.

The pipette solution contained (in mM): NaCl, 250; CsCl, 5; $CaCl_2$, 1.8; $MgCl_2$, 1; and Hepes, 5. It should be noted that the pipette sodium concentration was doubled to 250 mM to obtain larger single sodium channel currents (Motomura et al., 1995). The pH was adjusted to 7.4 with NaOH. Normal Krebs solution contained (mM): NaCl, 120; KCl, 5; $CaCl_2$, 1.8; $MgCl_2$, 1; Hepes, 5; and glucose, 25. The pH was adjusted to 7.4 wit NaOH. After making gigaohm seal in the Krebs solution, it was switched to the test bath solution, which contained (in mM): NaF, 1.4; CsF, 145; sucrose, 200; Hepes, 5; and EGTA, 5. The pH was adjusted to 7.2 with CsOH. After the solution exchange, a membrane patch was excised.

Single-channel currents passing through the pipette were recorded by a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA). The currents were filtered at 2–10 kHz with a four-pole Bessel filter, digitalized at 10–50 kHz (at least 5 times larger than the frequency of filtering) through an analog-to-digital converter (Digidata 1200, Axon Instruments), and stored on hard disk for later analysis. The baseline of currents at the holding potential was continuously recorded with a pen recorder. Programmed sequences of voltage pulses (by the software pClamp6, Axon Instruments) were applied to the preparation from the computer using a digital-to-analog converter (Digidata 1200). Voltage steps were applied after an initial stabilization period of about 15 min. Temperature of the solution was controlled by the temperature controller (TC-10, Dagan Corporation, Minneapolis, MN). All experiments were performed at 12 ± 1 or 22 ± 1°C. Results are expressed as means ± SEM, and *n* represents the number of patches unless otherwise stated.

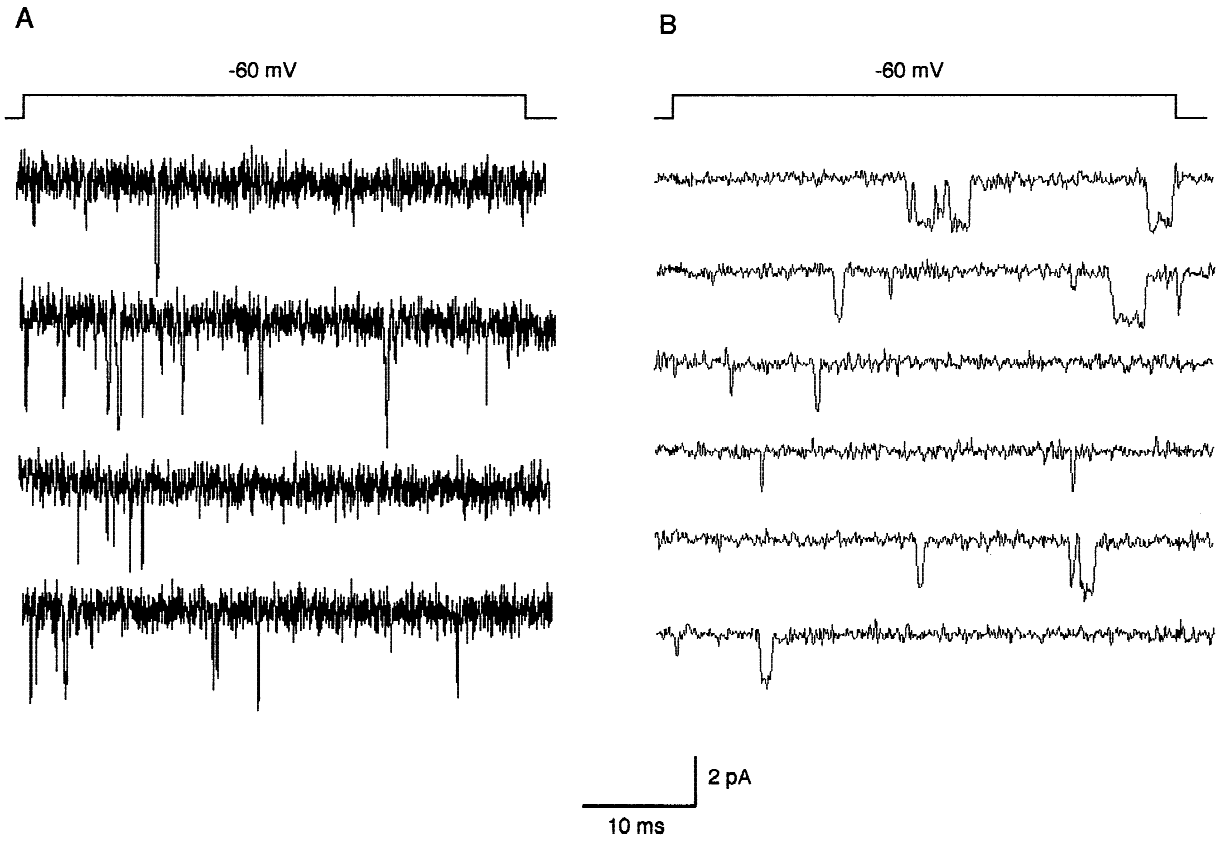

Fig. 1. Single sodium channel currents recorded from cultured rat hippocampal neurons at 22 and 12°C. Single-channel currents were evoked by a test pulse (V_t) to −60 mV from a holding potential (V_h) of −100 mV. The duration of V_t was 45 msec, and the interpulse interval was 5 sec. Final cutoff frequency of *filtering* was 5 and 2 kHz in *A* and *B*, respectively; *A* at 22°C; *B* at 12°C. *A* and *B* were derived from separate patch membranes. In this and subsequent figures, downward and upward deflections represent inward and outward currents, respectively.

Analysis of Single-Channel Currents

pClamp6 and TAC 3.0 (Bruxton Corporation, Seattle, WA) softwares were used for single-channel current analysis. A Gaussian filtration was applied if necessary. Capacitive and leakage currents were eliminated by subtraction of an averaged null trace from a trace with channel openings. Each corrected trace was carefully checked by eye and used for further analysis. Openings and closings of the channel were detected using the half-amplitude threshold analysis (Colquhoun & Sigworth, 1995). When the effective cutoff frequency was 2 and 5 kHz, the rise times of the signal (T_r) were approximately 180 and 70 μsec, respectively, and an opening must have a duration of at least $2 \times T_r$ for its amplitude to be measured reliably. Therefore, only events with well-established open levels having durations longer than $2 \times T_r$ were used for amplitude histogram analysis. Openings or closings having a duration longer than T_r were selected for dwell histogram analysis.

Chemicals

The insecticidally active (+)-*trans*-isomer of tetramethrin was provided by Sumitomo Chemical (Takarazuka, Japan). Stock solutions of tetramethrin were made in dimethylsulfoxide (DMSO) at a concentration of 10 mM. Final concentrations of tetramethrin and DMSO were, respectively, 10 μM and ≤0.1% v/v. Drug effect was compared with control experiments which were performed in the presence of DMSO (0.1% v/v). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Results

Single Sodium Channel Openings on Depolarization at 22 and 12°C

Normal single sodium channel currents were recorded using the inside-out configuration at 22 and 12°C. Figure 1*A* shows representative traces recorded at 22°C with the final cutoff filtering of 5 kHz. The membrane was depolarized to −60 mV from a holding potential of −100 mV for a duration of 45 msec. Very brief openings were observed in response to depolarizations. Figure 1*B* shows traces obtained at 12°C with the final cutoff filtration of 2 kHz using the same protocol as described above. Many channel openings were seen, and current amplitudes were smaller and open durations were longer than those at 22°C. In the presence of 200 nM tetrodotoxin in the pipette, these channel openings were not observed ($n = 4$).

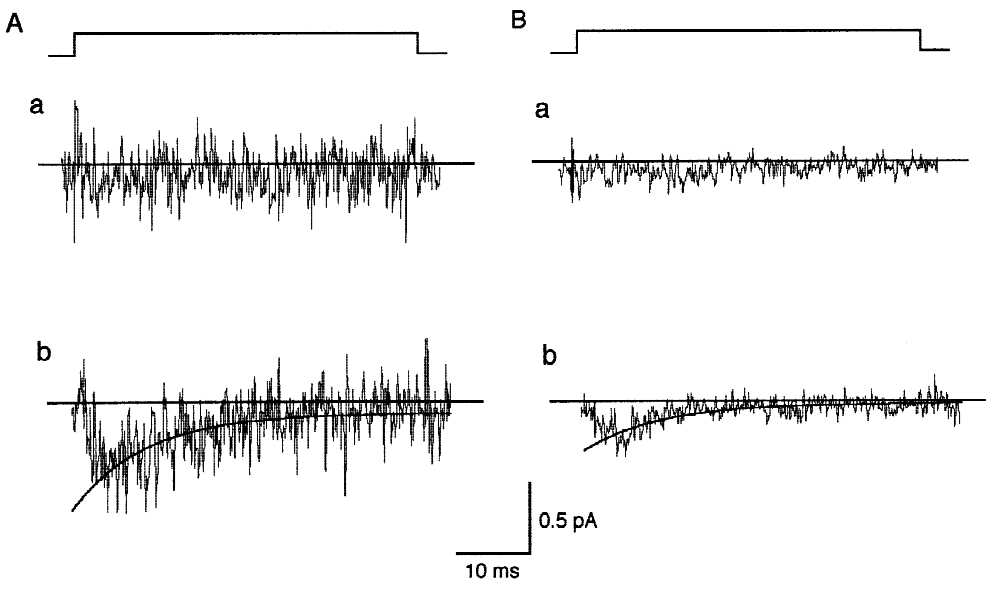

Fig. 2. Averaged currents of normal channels at different temperatures and voltages. Fifty consecutive single-channel traces recorded in different conditions were averaged; *A* at 22°C, a: −60 mV b: −50 mV; *B* at 12°C, a: −60 mV b: −50 mV. *A* and *B* were from separate patch membranes.

In order to mimic whole-cell sodium currents, consecutive single-channel traces were summed and divided by the number of summed traces to calculate averaged currents. Figure 2 represents averaged control currents at −50 mV (*a*) and −50 mV (*b*) at 22°C (*A*) and 12°C (*B*). Averaged currents were derived from two different patches. At −60 mV, averaged currents did not indicate apparent rising and decay phases, but at −50 mV their phases were obvious at two temperatures. The channel opening pattern and tetrodotoxin sensitivity indicated that this single-channel activity was derived from tetrodotoxin-sensitive sodium channels.

Tetramethrin-Modified Single-Channel Currents on Depolarization at 22 and 12°C

Tetramethrin was included in the internal solution at a concentration of 10 μM. The patch membranes were depolarized for 100 msec to −60 mV from a holding potential of −100 mV. Figure 3*A* shows traces acquired at 22°C. Two types of openings were observed here; one was a very brief opening which may have represented the activity of normal channels, the other was a much longer opening which may have represented the activity of tetramethrin-modified channels. Furthermore, even after the membrane was repolarized to −100 mV, channel openings were occasionally observed (*see* the top trace of Fig. 3*A*). Traces recorded at 12°C (Fig. 3*B*) showed a similar tendency to those in Fig. 3*A*, although their open durations were much longer than those at 22°C and their amplitudes were smaller than those at 22°C.

Single-Channel Currents upon Repolarization During Tetramethrin Application at 22 and 12°C

Single-channel currents upon repolarization were obtained with the identical protocol at two temperatures; membrane patches were depolarized to −30 mV for 5 msec, and subsequently repolarized to −100 mV. Figure 4*A* and *B* shows traces obtained at 22 and at 12°C, respectively. There was a striking difference between them. Channel openings upon repolarization at 22°C were observed only for tens of milliseconds, whereas channels opened for hundreds of milliseconds at 12°C. Openings contained many brief closures and/or transitions to subconductance levels both at 22 and 12°C. These deflections to baseline seemed to be more prominent and frequent at 12 than at 22°C, possibly due to the limitation of filtering.

According to the previous whole-cell experiments, pyrethroid-modified sodium tail currents often have their own rising phase and subsequent decay phase. The rising phase of tail current was diminished by the treatment with a blocker of sodium channel inactivation such as chloramine T (Song & Narahashi, 1996*a*). Therefore, this hooked tail current has been interpreted as being due to reopening of the inactivation gate and prolonged opening of the activation gate during repolarization. With the protocol described above, most openings upon repolarization were a continuation of openings during depolarization. Nonetheless, we could also observe channel openings which were regarded as latent openings of channels only when either larger or longer depolarizing pulses were applied. Figure 5*A* shows typical traces in which larger depolarizations (to 0 mV, 5 msec) some-

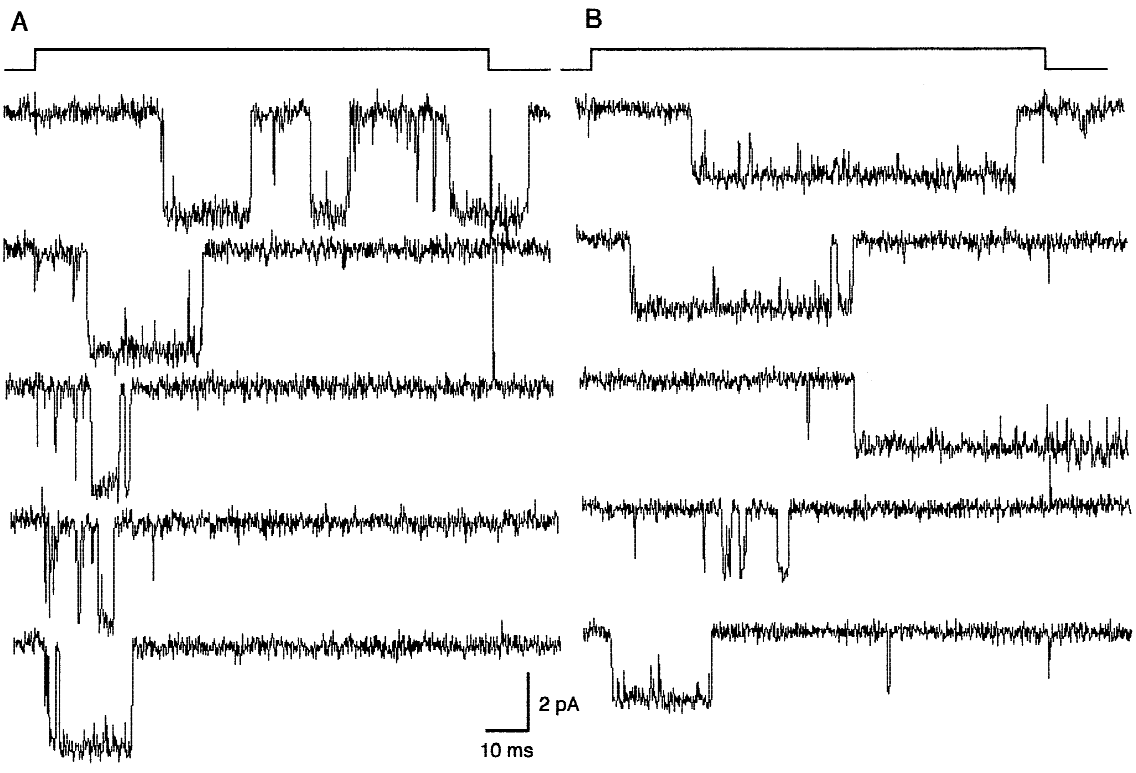

Fig. 3. Single sodium channel currents during 10 μM tetramethrin application. Single-channel currents were evoked by a V_t to −60 mV from V_h of −100 mV during 10 μM tetramethrin application. The duration of V_t was 100 msec; *A* at 22°C; *B* at 12°C. *A* and *B* were from separate patch membranes. The final cutoff frequency of filtering was 2 kHz.

times caused latent openings upon repolarization at 22°C although many channel openings upon repolarization in response to this pulse protocol were a continuation of openings during depolarization (top trace). Figure 5*B* shows latent openings upon repolarization when longer (50 msec) depolarizations to −60 mV were applied at 12°C; each opening upon repolarization occurred with an apparent delay from the moment of repolarization. These single-channel observations were in keeping with the interpretation derived from whole-cell currents.

Averaged Currents During Tetramethrin Application at 22 and 12°C

Figure 6*A* and *B* represents averaged current traces compiled from single-channel currents recorded at 22 and 12°C, respectively, in the presence of 10 μM tetramethrin in the bath solution. The voltage protocol was identical to that of Fig. 4*A* and *B*. The lower traces are the expansion of the upper ones. A few tetramethrin-modified channels were contained in these patches. Solid curves indicate fitting by single exponential functions. The decay time constants for the averaged currents were 10.5 ± 1.6 msec (the number of averaged traces = 11) and 84.4 ± 9.9 msec (n = 13) at 22 and at 12°C, respectively. The difference between the two temperatures was approximately 8 times, which is consistent with the previous whole-cell data showing that the decay time constant increased steeply with a decrease in temperature (*see* Fig. 7 in Song & Narahashi, 1996*a*). Averaged currents compiled from the present data did not show an apparent "hooked" tail current observed with dorsal root ganglion (Tatebayashi & Narahashi, 1994) and cerebellar Purkinje neurones (Song & Narahashi, 1996*a*). This indicates that latent openings upon repolarization were rare in response to the voltage step protocol shown in Fig. 6 (*see also* Fig. 4). Figure 6*C* shows a representative averaged current at 12°C recorded without tetramethrin exposure. The decay time constant for these normal averaged currents was 0.40 ± 0.05 msec (n = 6). Therefore, the decay time constant for tetramethrin-modified currents upon repolarization was approximately 200 times longer than that for normal sodium current at 12°C.

Amplitude Histograms for Unmodified and Tetramethrin-Modified Sodium Channel Currents

Amplitude histograms for normal channel currents at 22 and 12°C are shown in Fig. 7*A* and *B*, respectively, and

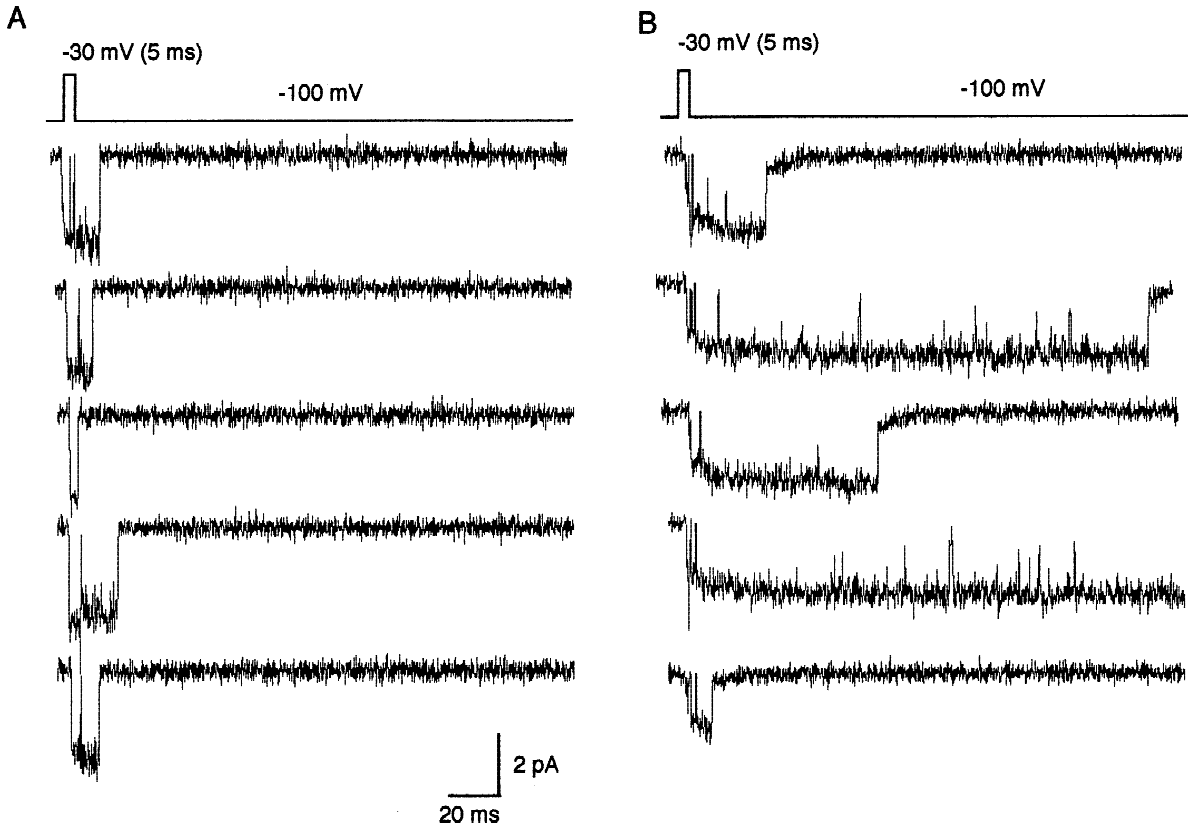

Fig. 4. Tetramethrin induces single-channel current openings upon repolarization. Membrane patches were depolarized to −30 mV for 5 msec, and subsequently repolarized to −100 mV; *A* at 22°C; *B* at 12°C. *A* and *B* were from separate patch membranes. Tetramethrin concentration was 10 μM. The final cutoff frequency of filtering was 2 kHz.

those for tetramethrin-modified channel currents at 22 and 12°C are shown in Fig. 7*C* and *D*, respectively. All opening events here were derived from traces during depolarization to −60 mV. Events having longer durations than 2 × rise time (T_r) (i.e., 0.35 and 0.15 msec at 2 and 5 kHz filtering, respectively, *see* Materials and Methods) and reaching their full amplitudes were selected for histograms.

The distribution of normal channel current amplitudes was well fitted by a Gaussian distribution with a mean of 3.00 pA and 2.19 pA at 22 and 12°C, respectively. The variance of histograms was large, possibly because histograms might include subconductance level openings (Benndorf, 1994). Another possible reason for the large variance of histograms might be due to the limitation of filtering, although the theoretically reliable criteria described above were used.

The amplitude histograms for tetramethrin-modified sodium channel currents were compiled only from events that had longer durations than 1 msec at 22°C and 2 msec at 12°C in order to eliminate normal channel openings (*see* burst length histograms, Fig. 9*A* and *B*). Events reaching their full amplitudes were selected. The mean amplitude of tetramethrin-modified sodium channel currents at −60 mV was 3.46 pA at 22°C and 2.12 pA at 12°C. Tetramethrin did not much change the amplitude of sodium channel current at 12°C, whereas the amplitude of the tetramethrin-modified channel current at 22°C was larger than that of the normal sodium channel current.

Current-Voltage Relationships for Unmodified and Tetramethrin-Modified Sodium Channels

The mean amplitude of single-channel currents at each potential level was plotted as a function of voltage. Fig. 8*A* and *B* shows current-voltage relationships for normal channels and tetramethrin-modified channels, respectively. The curves in these figures are those predicted by the Goldman-Hodgkin-Katz current equation (Hodgkin & Katz, 1949). The current-voltage curve for normal channels at 22°C was derived from only two data points because it was difficult to accumulate enough reliable events due to brief and early openings at more depolarized potentials (Fig. 8*A*). The conductance of normal channels obtained by linear regressions was 29.8 and 20.0 pS at 22 and 12°C, respectively (Q_{10} = 1.49). The amplitude of single-channel currents for tetramethrin-modified channels increased at potential from 0 to −60

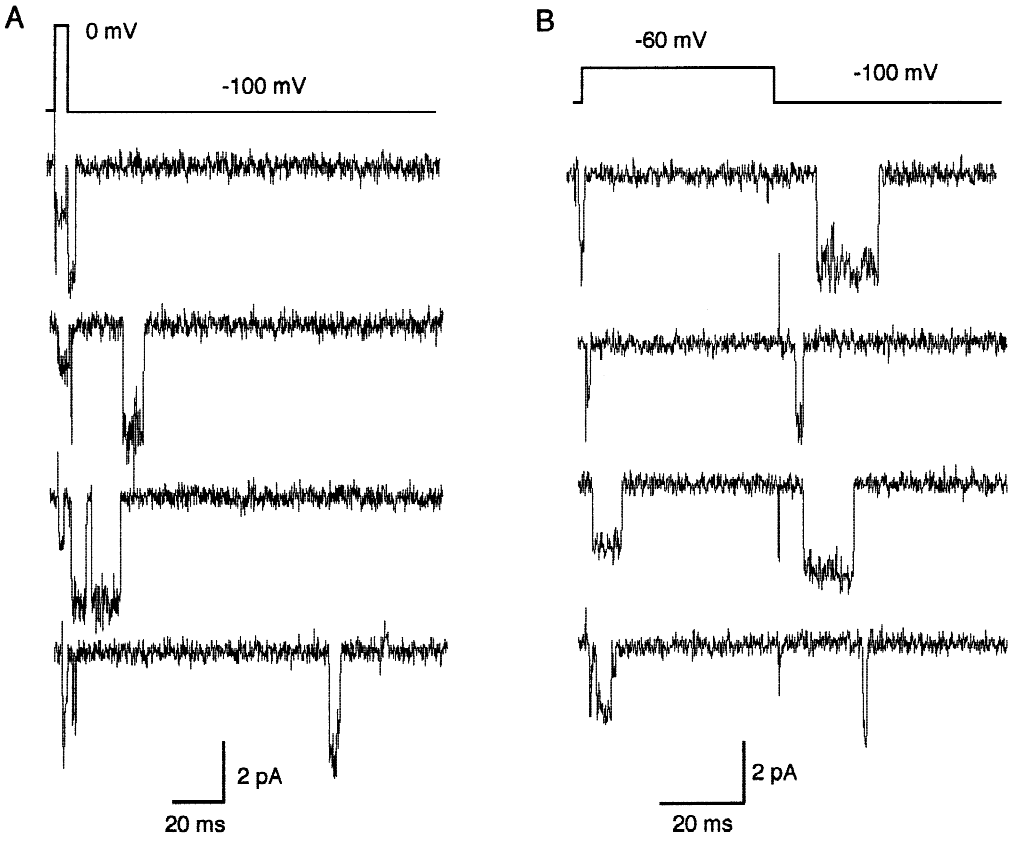

Fig. 5. Latent openings of tetramethrin-modified single sodium channel upon repolarization. (*A*) Membrane patches were depolarized to 0 mV for 5 msec, and subsequently repolarized to −100 mV at 22°C. (*B*) Membrane patches were depolarized to −60 mV for 45 msec, and subsequently repolarized to −100 mV at 12°C. The final cutoff frequency of filtering was 2 kHz.

mV, as expected from an increase in driving force. However, hyperpolarizations to the level more negative than −60 mV did not increase unit amplitude as expected from the theoretical curve. This rectification observed at hyperpolarized potentials was mainly due to voltage-dependent divalent cation block as reported previously (Yamamoto, Yeh & Narahashi, 1984). The conductance of tetramethrin-modified channels calculated from the slope between −60 and 0 mV was 30.9 and 18.5 pS at 22°C and 12°C, respectively (Q_{10} = 1.67). The Q_{10} values of normal channels (1.49) and tetramethrin-modified channels (1.67) were smaller than the Q_{10} values of 2.11 and 2.15 for normal and deltamethrin-modified sodium channels, respectively, in mouse neuroblastoma cells (Chinn & Narahashi, 1989), but larger than the Q_{10} of 1.3 for simple aqueous diffusion (Hille, 1992). It was concluded that the Q_{10} value for single-channel conductance was not much affected by tetramethrin modification.

Analysis of Open Time During Depolarization

Open time histograms for normal sodium channels at 22 and 12°C are shown in Figs. 9*A* and *B*, respectively. Events having longer durations than T_r (i.e., 0.17 and 0.07 msec at 2 and 5 kHz filtration, respectively) were selected. All opening events were less than 1 msec at 22°C or 2 msec at 12°C. The time constants calculated by fitting with a single exponential function were 0.089 msec and 0.31 msec at 22 and 12°C, respectively, although the reliability of their values were somewhat limited because of the limitation of filtering.

To assemble appropriate burst length histograms from tetramethrin-modified single-channel traces was not so easy. All closed time histograms should be compiled at first from a patch containing only one channel in order to determine the upper limit of brief close duration in a burst (Colquhoun & Sigworth, 1995). However, the patch membrane having only one channel rarely showed long openings after tetramethrin application and the accumulation of tetramethrin-modified channel opening events was difficult in the present study. This could be due to the relatively low sensitivity of tetrodotoxin-sensitive sodium channels to 10 μM tetramethrin (Tatebayashi & Narahashi, 1994; Song & Narahashi, 1996*a*). Therefore, most patches having a tetramethrin-modified channel contained at least another unmodified channel, causing difficulties in compiling reliable closed time histograms.

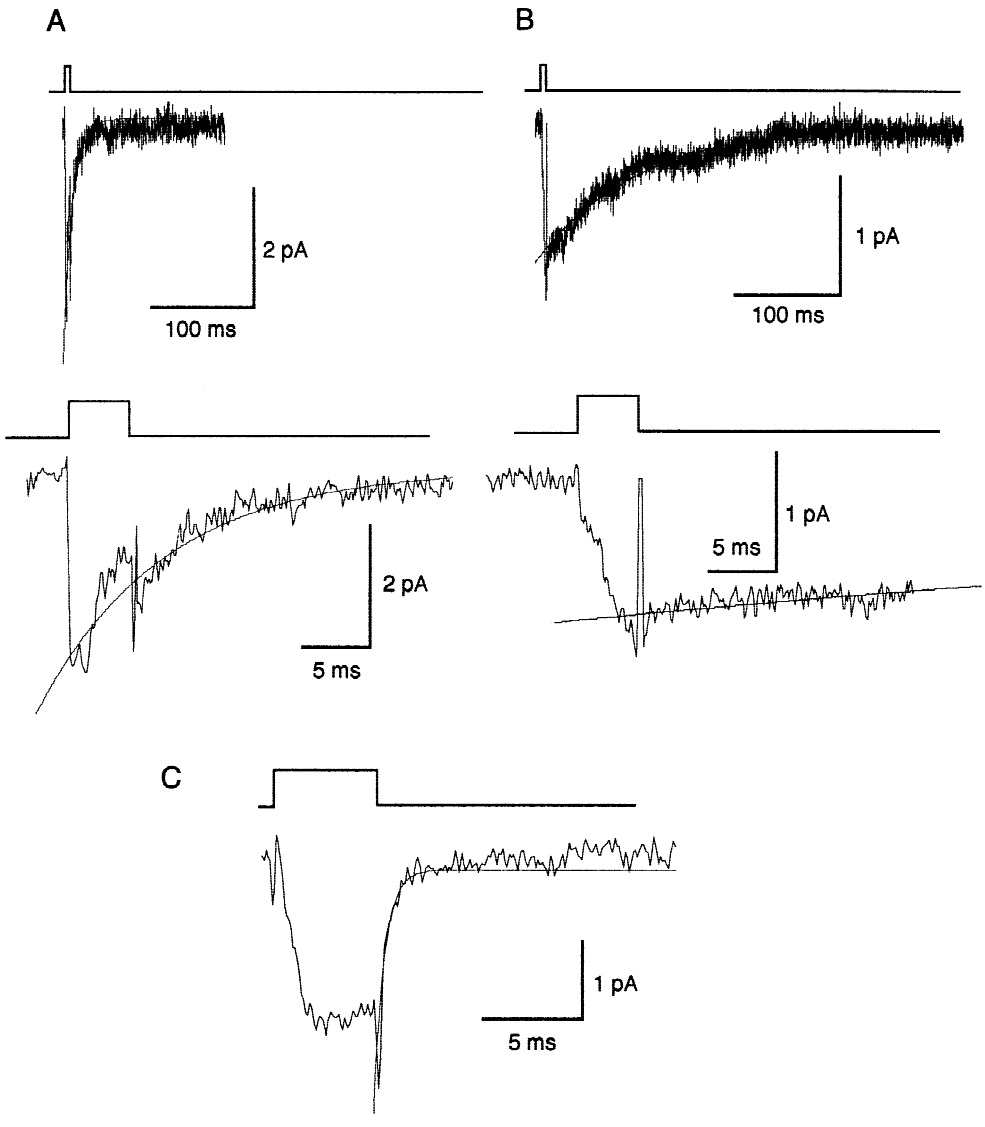

Fig. 6. Comparison of the decay phase of averaged currents upon repolarization. Membrane patches were depolarized to −30 mV for 5 msec, and subsequently repolarized to −100 mV. Fifty consecutive traces were averaged. *A* and *B*, during 10 μM tetramethrin application at 22°C (*A*) and 12°C (*B*). Lower trace in *A* or *B* was the expansion of the upper trace. *A* and *B* were from separate patch membranes. (*C*) The averaged current of normal channels at 12°C. The membrane patch contained multiple channels. Curves superimposed on the averaged currents are single exponential fits to the decay phase of currents upon repolarization.

Figure 9*C* and *D* show the open time histograms for tetramethrin-modified sodium channels at 22 and 12°C, respectively, analyzed using the 50% amplitude threshold method. Note that logarithmic scale is used for the abscissa. As was shown in Fig. 3, the histograms here should contain both unmodified and modified channel opening events. The open time histogram at 22°C has two apparent peaks, whereas the histogram at 12°C shows only one peak. This might be due to the existence of many brief deflections to baseline which were detectable by the 50% threshold method (*see* Fig. 3).

We defined here that a close or deflection to baseline shorter than 1 msec which is located between two opening events should be regarded as an intraburst closure or transition to subconductance levels, and that at least one opening in a burst should have a longer duration than 1 msec at 22 or 2 msec at 12°C. This complicated definition was designed to eliminate misidentification that repetitive normal sodium channel openings might be regarded as a burst.

Burst length histograms shown in Fig. 9*E* (22°C) and *F* (12°C) were analyzed by using this criterion. Both histograms had two peaks which were well fitted by two exponential functions. The time constants for two components were 0.20 and 7.69 msec at 22°C, and 0.76 and 14.46 msec at 12°C. The fast component should corre-

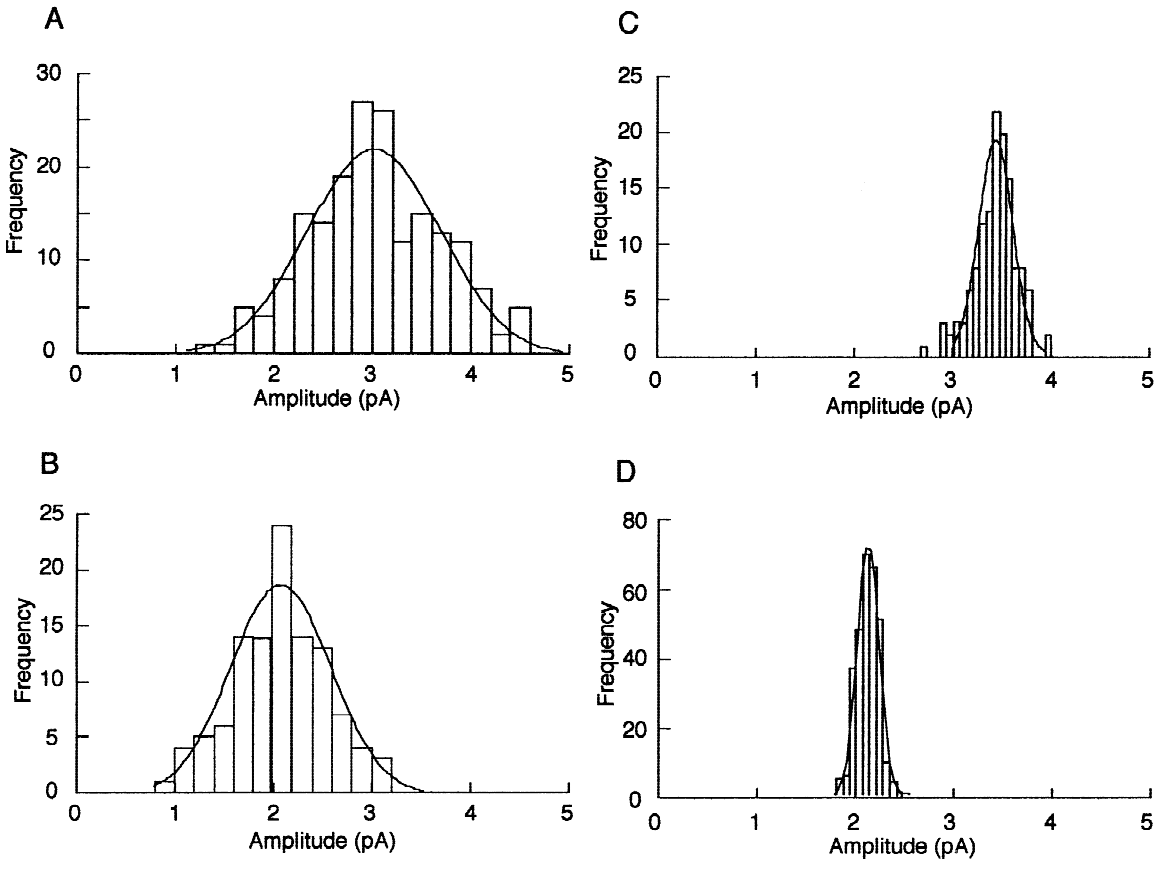

Fig. 7. Amplitude histograms of normal (*A*, *B*) and tetramethrin-modified (*C*, *D*) sodium channel currents. (*A* and *B*) Histograms of unmodified channel openings at 22°C (*A*) and 12°C (*B*). Bin size was 0.2 pA in both *A* and *B*. (*C* and *D*) Histograms of tetramethrin-modified channel currents at 22°C (*C*) and 12°C (*D*). Bin size was 0.067 pA in both *C* and *D*. Curves superimposed on the histograms are single Gaussian fits to the data points. All histograms were derived from traces evoked by a V_t to −60 mV.

spond to unmodified channel openings, and the slow component should correspond to tetramethrin-modified channel openings.

Reconstructed Currents from Tetramethrin-Modified Openings

Since tetramethrin-modified whole-cell currents usually consist of modified and unmodified channel openings, any comparison of kinetic properties between unmodified and modified currents is difficult. One convenient assumption that has been used for the analysis of whole-cell currents is that peak currents in the presence of pyrethroid derive mainly from unmodified channel openings and steady-state current at the end of the depolarization is due mainly to modified channel openings. Thus, we reconstructed tetramethrin-modified whole-cell currents from tetramethrin-modified single-channel opening events. Figure 10 shows reconstructed current traces compiled only from tetramethrin-modified opening events at −60 mV. Openings with duration longer than 1 msec at 22°C or 2 msec at 12°C were selected as tetramethrin-modified channel openings and used for reconstruction. Current traces were reconstructed by using their own values of latencies and burst lengths. Here we assumed that each event had the same unit amplitude. Although brief events were not included in reconstructed currents, its influence should be negligible since their open times were much shorter than those of tetramethrin-modified events. Interestingly, the reconstructed currents had both the rising and decay phases that normal averaged currents did not show at −60 mV (Fig. 2*A* and *B*). It should be noted that tetramethrin-modified reconstructed currents had an apparent decay phase during depolarization, suggesting that the inactivation process take place even at a depolarization to −60 mV. The decay time constants obtained by fitting with a single exponential function were 21.2 and 29.5 msec at 22 and 12°C, respectively. Thus, it was suggested that the decay phase of reconstructed currents at −60 mV were not so much affected by lowering the temperature.

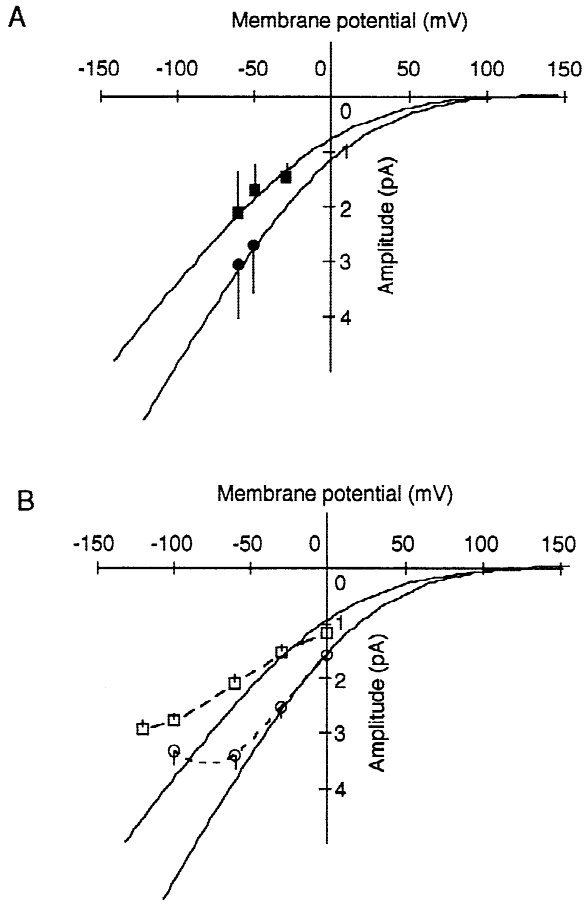

Fig. 8. Current-voltage relationships for normal (*A*) and tetramethrin-modified (*B*) channels. (*A*) Normal sodium channel. Filled circles, at 22°C; filled squares, at 12°C. (*B*) Tetramethrin-modified channel. Open circles, at 22°C; open squares, at 12°C. The solid curves are theoretical curves calculated from the following equation:

$$I = AE[[Na^+]_i - [Na^+]_o \exp(BE)/[1\text{-}\exp(BE)],$$

where I is the current amplitude, A is a constant, E is the voltage, B is $-F/RT$, and $[Na^+]_i$ and $[Na^+]_o$ are inside and outside Na^+ concentrations, respectively. The two sets of plots were derived from separate patch membranes. Standard deviations of each data point are shown with data points.

Analysis of Currents Upon Repolarization

Single-channel currents upon repolarization were regarded as bursts with brief closures and transitions to subconductance levels. Burst length histograms were compiled from data at 22°C (Fig. 11*A*) and at 12°C (Fig. 11*B*) in the presence of tetramethrin. The histogram at 22°C showed one peak which was fitted by a single exponential function with a time constant (τ) of 8.26 msec. The histogram at 12°C had two apparent peaks; the faster component represents normal channel openings upon repolarization (τ = 0.22 msec), which could not be detected at 22°C, while the slower components represent tetramethrin-modified channel openings (τ = 68.80 msec).

Figure 11*C* and *D* shows closed time histograms during bursts at 22 and 12°C, respectively. The time constants obtained by single exponential fittings were 0.075 and 0.29 msec at 22 and 12°C, respectively, although the reliability of their values were somewhat limited because of the limitation of filtering.

Voltage Dependence of Burst Lengths of Tetramethrin-Modified Channels

Figure 12 shows the voltage dependence of burst length of tetramethrin-modified single-channel currents at two temperatures. Opening events derived from different patches were accumulated and burst length histograms were compiled at each potential. Time constants were estimated by fitting with two exponential functions. Although the fast component that corresponds to unmodified channel openings at less negative potentials were not so accurate because of the short and early openings during depolarization, the slow component that corresponds to modified channel openings were relatively reliable. The ordinate represents a reciprocal of time constant at each potential. Thus, burst length decreased with larger depolarizations at both temperatures. The result is compatible with the previous whole-cell data showing that the difference between control and tetramethrin is much less prominent at less negative potentials (Tatebayashi & Narahashi, 1994; Song & Narahashi, 1996*a*). The interpretation of this tendency is not so easy since burst durations at each potential are associated with multiple and different processes: the gating reactions during a repolarization may be a combination of deactivation and the recovery from inactivation and activation. Gating at potentials positive to −60 mV may be associated with activation, deactivation, and onset of inactivation.

Discussion

After tetramethrin application, sodium channels showed two patterns of openings; one was brief openings similar to normal sodium channel openings and the other was longer ones (Fig. 3). At least two possible interpretations of these two patterns of openings are conceivable. First, brief and longer openings are deemed to represent unmodified and tetramethrin-modified channel openings, respectively (Song & Narahashi, 1996*b*). Second, both opening patterns are of tetramethrin-modified channels but with different modes. Our present data showed that there was the fast component in the burst length histogram at 12°C during repolarization (Fig. 11*B*). In addi-

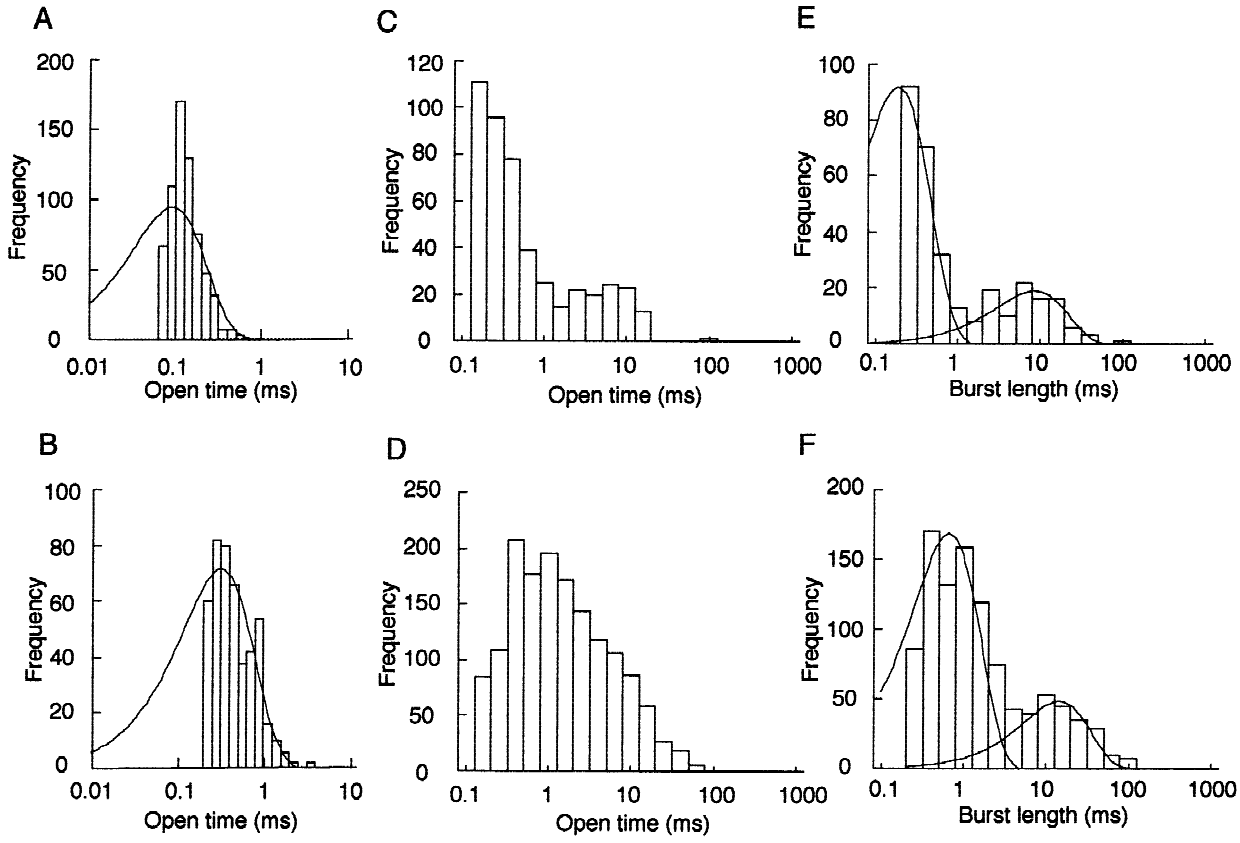

Fig. 9. Open time histograms for channel openings before (*A, B*) and during tetramethrin application (*C, D, E* and *F*). All histograms were derived from traces evoked by V_t to −60 mV. Unitary currents shorter than 0.07 msec (*A*) and 0.17 msec (*B, C, D, E* and *F*) duration were excluded from the histogram to avoid truncation errors introduced by the limited frequency response of the recording system. (*A* and *B*) Open time histograms for normal sodium channel openings at 22°C (*A*) and 12°C (*B*). (*C* and *D)* Open time histograms for channel openings during 10 μM tetramethrin application at 22°C (*C*) and 12°C (*D*). (*E* and *F*) Burst length histograms for channel openings during tetramethrin application at 22°C (*E*) and 12°C (*F*). Curves superimposed on the histograms are double exponential fits to the data points.

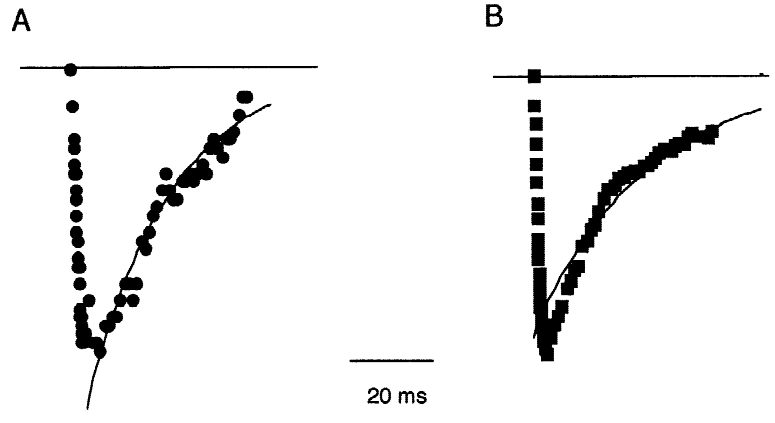

Fig. 10. Reconstructed currents derived from tetramethrin-modified opening events. Openings longer than 1 msec at 22°C or 2 msec at 12°C on depolarization to −60 mV were selected. The values of their own latencies and open durations were used for current reconstruction; (*A*) at 22°C; (*B*) at 12°C. The abscissa is standardized current amplitude. Curves superimposed are single exponential fits to the decay phase of currents.

tion, this component was very similar to the normal channel openings at 12°C upon repolarization (Fig. 6*C*). These observations suggest that the normal unmodified channels are contained in the membrane patch after tetramethrin application. Although we cannot exclude the possibility that brief openings may be due to the tetramethrin molecule being dissociated from its binding site earlier resulting in short openings, we take the first interpretation that brief and longer openings represent unmodified and tetramethrin-modified channel openings,

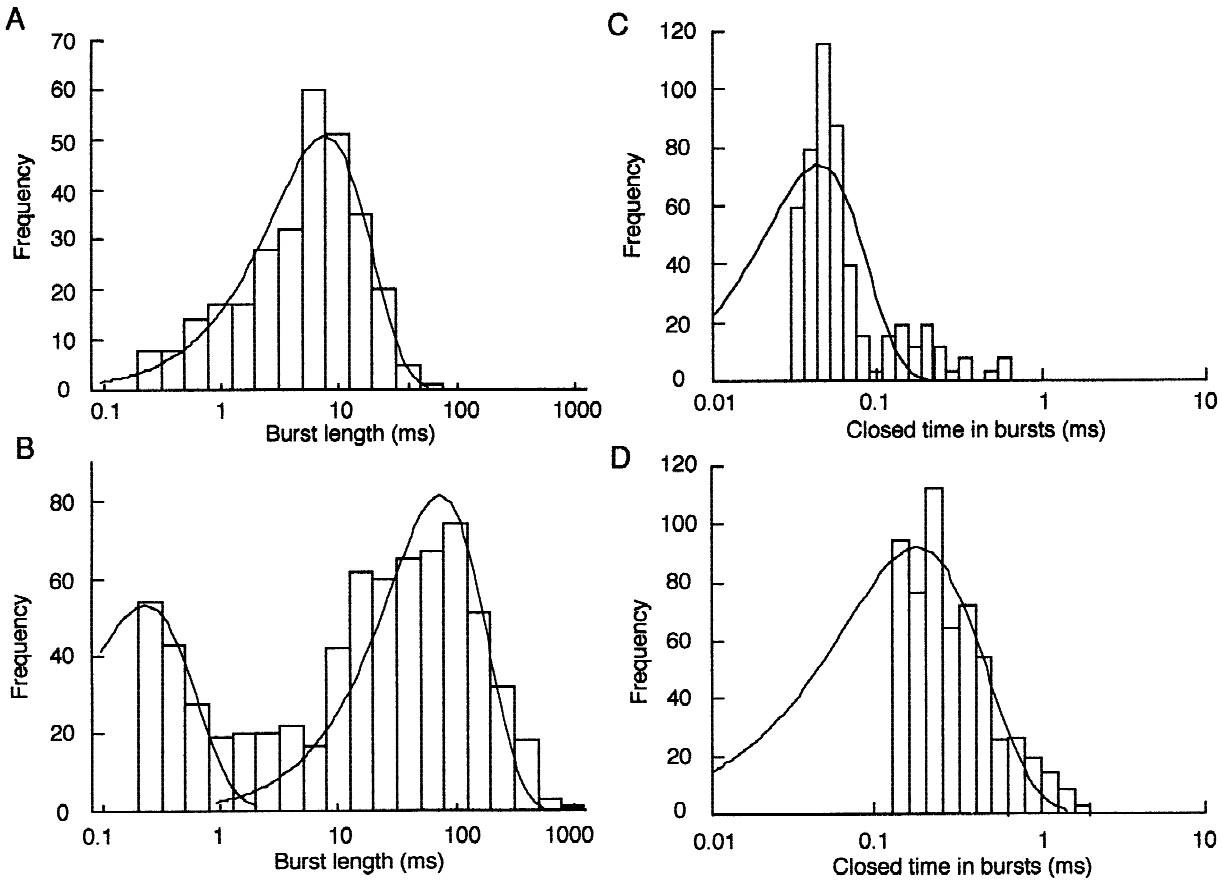

Fig. 11. Burst length histograms and closed time histograms in bursts upon repolarization in tetramethrin. (*A* and *B*) Burst length histograms upon repolarization at 22°C (*A*) and 12°C (*B*). (*C* and *D*) Closed time histograms in bursts upon repolarization at 22°C (*C*) and 12°C (*D*). Only histogram C was compiled from traces whose final cutoff frequency was 5 kHz. Others were derived from traces filtrated at 2 kHz. All histograms were derived from traces upon repolarization to −100 mV from −30 mV.

respectively, as a more likely possibility throughout this paper.

It should be noted that the neurons investigated were assumed to express a single population of sodium channels in the present study. Although inactivation-resistant persistent sodium currents in hippocampal neurons have been reported (e.g., French et al., 1990; Hammarström & Gage, 1998), their population is much smaller than that of large transient ordinary sodium currents (1.5 and 0.7–1% in French et al. (1990) and Hammarström & Gage (1998), respectively). Besides, the single-channel data derived from 36 patches were compiled in the present study. The heterogeneity of sodium channels could be negligible, although we cannot completely exclude the possible contaminations of other sodium channel populations.

The tetramethrin-modified sodium channels opened forming bursts with brief closures and/or transitions to subconductance levels during depolarization (Fig. 3) and upon repolarization (Fig. 4). This feature was less prominent at 22 than at 12°C partly because of the limitation of filtering. Tetramethrin at 10 μM modified 17% of channels at 22°C (Fig. 9*E* and Table 1), with an assumption that the open probability for modified channels was the same as that for unmodified channels. It should also be noted that the value of 17% could be overestimated since unmodified channel openings at 22°C were too short to be detected and counted compared to those at 12°C. The percentage of tetrodotoxin-sensitive sodium channels modified by the same concentration of tetramethrin was 12% in rat dorsal root ganglion neurons (Tatebayashi & Narahashi, 1994) and 23% in rat cerebellar Purkinje neurons (Song & Narahashi, 1996*a*). Moreover, the characteristic prolongation of the pyrethroid-modified sodium tail current which was more prominent at lower temperatures (Song & Narahashi, 1996*a*) seems to be similar to the present result based on averaged single-channel current traces (Fig. 6). Thus, hippocampal neurons of rats are endowed with sodium channels, which exhibit approximately the same tetramethrin sen-

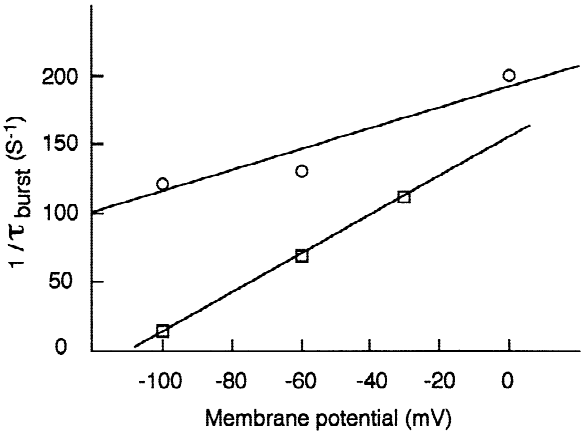

Fig. 12. Voltage dependence of burst length of tetramethrin-modified channels. Ordinate indicates membrane potentials, while abscissa indicates a reciprocal of time constant of burst length at each potential. Open squares; at 22°C, open circles; at 12°C. Lines show the results of linear regression on data points.

sitivity as that of tetrodotoxin-sensitive sodium channels of rat dorsal root ganglion neurons and cerebellar Purkinje neurons.

Openings of sodium channels during depolarization to −60 mV were greatly prolonged by tetramethrin modification both at 22 and 12°C (Fig. 9*A*, *B*, *E* and *F*). A previous single-channel experiment showed that the open time constant became approximately four times longer after modification with tetramethrin, although it was performed with a different preparation and with different experimental conditions (Song & Narahashi, 1996*b*). The present results indicate that tetramethrin prolongs sodium channel open time 60–90 times both at 22 and 12°C.

It has been shown that the tail current of the pyrethroid-modified sodium channel is greatly prolonged at lower temperatures in frog nodes of Ranvier (Vijverberg et al., 1983) and in rat cerebellar Purkinje neurons (Song & Narahashi, 1996*a*). Three possibilities are considered in order to explain the prolongation of whole-cell tetramethrin-modified sodium currents by lowering the temperature. First, a remarkable increase in percentages or opening frequency of modified channels occurs at a low temperature. If the total number of modified channel openings increases drastically, it may cause the prolongation of the current. Second, the frequency of reopenings during the late portion of repolarization is increased at lower temperature. Third, the open time of each channel is prolonged at low temperature.

Percentages of modified channels at different temperatures are important factors in the negative temperature dependence of pyrethroid action. To estimate percentages of modified channels during repolarization is difficult since there could be multiple modified channels. Instead, the percentages of modified channel openings to total openings during depolarization were calculated with an assumption that percentages of modified channels were constant during depolarization and repolarization. The proportion of tetramethrin-modified channel openings among all openings during depolarization, estimated by a_{fast} and a_{slow} values (*a:* ratio of each component to total event number) in burst length histograms (Fig. 9*E* and *F*), were not so different between 22 and 12°C ($Q_{10} = 0.74$ in Table 1). In order to estimate the contribution of tetramethrin-modified single-channel openings to whole-cell currents, $a_{fast}\,\tau_{fast}$ and $a_{slow}\,\tau_{slow}$ were calculated and compared between the two temperatures. The ratio of $a_{slow}\,\tau_{slow}$ to $(a_{fast}\,\tau_{fast} + a_{slow}\,\tau_{slow})$ was almost the same and a remarkable difference that

Table 1. Comparison of various single sodium channel parameters between two temperatures

	22°C	12°C	Q_{10}	Source (Figure)
Normal channel				
Current amplitude (−60 mV)	3.0 pA	2.19 pA	1.30	1, 7*A*, *B*
Conductance	29.8 pS	20.0 pS	1.49	8*A*
Open time constant (−60 mV)	0.089 msec	0.31 msec	0.29	9*A*, *B*
Tetramethrin modified channel				
⟨Depolarization⟩				
Current amplitude (−60 mV)	3.46 pA	2.12 pA	1.63	3, 7*C*, *D*
Conductance	30.9 pS	18.5 pS	1.67	8*B*
Burst length (−60 mV) τ_{fast}	0.20 msec	0.76 msec	0.24	9*E*, *F*
τ_{slow}	7.69 msec	14.46 msec	0.53	9*E*, *F*
$a_{slow}/(a_{fast} + a_{slow})$	0.17	0.23	0.74	9*E*, *F*
$a_{slow}\,\tau_{slow}/(a_{fast}\,\tau_{fast} + a_{slow}\,\tau_{slow})$	0.89	0.84	1.06	9*E*, *F*
⟨Repolarization (−100 mV)⟩				
Burst length τ_{fast}		0.22 msec		4, 11*A*, *B*
τ_{slow}	8.26 msec	68.80 msec	0.12	4, 11*A*, *B*
Brief closures in burst	0.075 msec	0.29 msec	0.26	11*C*, *D*
Decay time constant of averaged current	10.5 msec	84.4 msec	0.12	6

would cause the marked prolongation of tail current at low temperature was not found (Table 1). These observations are compatible with the previous whole-cell data (Song & Narahashi, 1996*a*). Thus, it was concluded that the prolongation of tetramethrin-modified whole-cell currents at low temperature was not due either to an increase in percentages of modified channels or to an increase in open frequency of the modified channels. In addition, a second possibility that calls for the increase in the frequency of reopenings during the late portion of repolarization by lowering the temperature was not likely since latent openings during the late portion of repolarization were very rare (Figs. 4 and 6).

Comparison of burst length at two temperatures showed striking differences. Tetramethrin-modified single-channel currents, especially upon repolarization, indicated a remarkable difference between two temperatures; the time constant for burst length at 12°C upon repolarization was eight times as long as that at 22°C (Figs. 8*A* and *B*, $Q_{10} = 0.12$ in Table 1), whereas the time constant for burst length at 12°C during depolarization was only twice as long as that at 22°C ($Q_{10} = 0.53$ in Table 1). Thus, the prolonged flow of sodium whole-cell current at lower temperatures was attributed mainly to a prolongation of each opening.

Prolonged openings during repolarization contained brief closures and/or transitions to subconductance levels (Fig. 4) and the time constant of the closed time in bursts histogram at 12°C was over three times longer than that at 22°C (Figs. 11*C* and *D*, Table 1). It was possible that these brief transitions to baseline could be a similar phenomenon at different temperatures. To quantify and interpret these changes in channel behavior, simple state models are introduced. Four different states are assumed; openings to full conductance, openings to subconductances, brief closures and long closures.

$$\begin{array}{l}\text{open (subconductance)}\\ \quad\updownarrow\\ \text{open} \leftrightarrows \text{close (short closure)} \leftrightarrows \text{close (long closure)}\end{array}$$

The state dependency of channel modification by pyrethroids has been extensively investigated with various preparations by using electrophysiological techniques. There are at least two possibilities of pyrethroid modification of sodium channels; the pyrethroid molecule binds to the sodium channels either in the closed state or in the open state (Narahashi, 1996). However, whether or not the pyrethroid molecule leaves its receptor site before the channel can close is still unknown. To simplify the model, we assume here that the tetramethrin-modified channel has its own kinetic pathway and that entering closed states does not necessarily imply the dissociation of the tetramethrin molecule from the channel binding site. During a burst, we can see many deflections to the baseline. The existence of subconductance states in sodium channels has been demonstrated by many investigators (*see* Patlak, 1991), including pyrethroid-modified sodium channels (Chinn & Narahashi, 1989). Some deflections should be regarded as short closures, others as transitions to subconductance levels. However, since the objective distinction between them is difficult, the interpretation of brief transitions to baseline was important to quantify our data.

We applied two other different state models (models 2 and 3) derived from the original state model (model 1) in order to interpret channel behaviors upon repolarization; the brief transitions to baseline were taken into consideration in model 2, but not in model 3. These two models were used for analysis separately and the results were compared.

$$\text{open} \underset{\alpha}{\overset{\beta}{\leftrightarrows}} \text{close1 (short closure)} \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrows}} \text{close2}$$

First, we assume that transitions to subconductance levels are neglected and that most deflections are regarded as short closures. This assumption changes the model 1 to the model 2, which is a simple linear model having one open state and two closed states. The closed state 1 represents very brief closures in bursts, while the closed state 2 exhibits longer closures that is regarded as an absorbing state in this model. The two closed states can communicate with each other, but an open channel can enter the closed state 2 only through the closed state 1.

Rate constants between two states can be defined as described above and can be estimated from experimental values (time constants for burst length and brief closure, and numbers of brief closures in bursts), using the method originally developed for the analysis of single-channel currents activated by acetylcholine. In this estimation, missed events due to the limitation of filtering should be corrected (Colquhoun & Sakmann, 1985; Colquhoun & Hawks, 1995; Colquhoun & Sigworth, 1995). Although the model should incorporate binding and unbinding of tetramethrin to the channel, we mainly used the model 2 in the present study in order to evaluate brief transitions in bursts at both temperatures.

$$\text{open} \underset{\alpha'}{\overset{\beta'}{\leftrightarrows}} \text{close}$$

Alternatively, we can assume that both transitions to subconductance levels and short closures can be neglected since an objective distinction between them is difficult. This assumption changes the model 1 to another simple model, having only one open state and one closed state (model 3). A burst opening including many short closings and transitions to subconductance levels is regarded as a single opening. Rate constants between the

Table 2. Comparison of calculated rate constants according to state models

Parameter	22°C	12°C	Q_{10}
⟨*Model 2*⟩			
Mean number of brief closures per burst (corrected value)	1.815	2.164	0.84
Calculated open time	2.89 msec	21.55 msec	0.13
Rate constant			
α	350 sec^{-1}	46 sec^{-1}	7.5
β	8,600 sec^{-1}	680 sec^{-1}	15.0
k_{-1}	4,740 sec^{-1}	320 sec^{-1}	12.6
⟨*Model 3*⟩			
α'	120 sec^{-1}	15 sec^{-1}	8.0

two states are defined as described above. Thus, the rate constant, α', can be calculated as a reciprocal of the time constant.

Table 2 shows comparisons of calculated parameters at 22 and 12°C, and Q_{10} values between them according to two-state models (models 2 and 3). Although the reliability of rate constants in the analysis of model 2 is somewhat limited because of very short closed time constants in bursts, the mean number of brief closures per burst at 22°C was similar to that at 12°C in model 2 (Table 2). The calculated open time at 12°C was 8 times larger than that at 22°C, similar to the time constant of burst length at 12°C which was 8 times larger than that at 22°C during repolarization (Table 1). In addition, every rate constant decreases with a decrease in temperature and each Q_{10} value is at least 7.5 between the two temperatures. Chinn and Narahashi (1986) suggested that pyrethroid stabilizes a variety of channel states by reducing the transition rates between them. The large Q_{10} value of every rate constant in the present study may mean that this stabilization effect by pyrethroid becomes more prominent by lowering the temperature. Either result based on two different models (models 2 and 3) suggests that open time of channels is prolonged approximately 8 times by lowering the temperature.

McLarnon & Wang (1991) examined temperature dependence of drug block of a calcium-dependent potassium channel and reported that the rate constant which was not related to drug blocking step exhibited a considerably low dependence on temperature ($Q_{10} = 1.5$) compared with the blocking step (e.g., $Q_{10} = 2.7$). Correa, Bezanilla & Latorre (1992) who investigated the properties of batrachotoxin-modified sodium channels reported that Q_{10} values for rate constants were around 4 to 5. The Q_{10} values for rate constants in Table 2, together with these previous reports, seem to be extremely large, which may reflect unique effects of pyrethroid at low temperatures.

Compared with the apparent change in the deactivation process by lowering the temperature, the accurate evaluation of the temperature dependence of the activation process by tetramethrin was difficult in our present study because of the shift of the conductance-voltage curves during tetramethrin modification (Tatebayashi & Narahashi, 1994; Song & Narahashi, 1996*a*). The time to peak of reconstructed currents in Fig. 10 shows that the delay of the activation process by tetramethrin is not so apparent and not so enhanced by lowering the temperature. However, further investigations are required since a small shift of the conductance-voltage curve may cause large change in the time to peak of reconstructed currents. The whole-cell current analysis of the type II pyrethroid deltamethrin revealed an apparent delay for the time to peak of deltamethrin-modified current at each depolarized potential (Tabarean & Narahashi, 1998). This suggests that pyrethroids have effects on the activation process.

Temperature dependence of inactivation process is another interesting point. The reconstructed current traces having apparent decay phases (Fig. 10) and strong voltage dependence of burst length (Fig. 12) suggest that the inactivation process is not negligible at a membrane potential of −60 mV. However, the observation that the decay phase of reconstructed currents was not so much affected by lowering the temperature (Fig. 10) indicates that a similar temperature-independent process might be involved in the closing of modified channels at −60 mV at the two temperatures. Further investigation will be required to evaluate the involvement of inactivation process in modified channels as a function of temperature.

Although we could not evaluate the temperature effect on the recovery from inactivation based on the present single-channel data, it has been shown in other preparations that the difference in Q_{10} values between the deactivation and the recovery from inactivation process was quite different. For instance, in rat cerebellar Purkinje neurons (Song & Narahashi, 1996*a*), whole-cell tail current comparisons revealed a large difference; the Q_{10} value for deactivation calculated from the time constants of decay phase of tail currents between 20 and 25°C was 37.75, whereas the Q_{10} for recovery from inactivation, estimated by the time constants for rising phase of tail currents, was 2.25. It is suggested that the recovery from inactivation process is less affected than the deactivation process by lowering the temperature.

In summary, we presented here comparisons of various parameters of tetramethrin-modified sodium channels based on single-channel data. The tetramethrin-modified sodium channel opens longer than the normal sodium channel, with brief closures and/or transitions to subconductance levels in bursts both at 22 and 12°C. The prolongation of tetramethrin-modified whole-cell current at low temperature upon repolarization is not due to an increase in the percentage of modified channels, an increase in opening frequency of the modified channel,

or an increase in the frequency of reopenings during the late portion of repolarization. It is due to the prolongation of each channel opening in a burst pattern. Furthermore, simple state models introduced to interpret the tetramethrin-modified channel behaviors reveal that the Q_{10} values for rate constants upon repolarization are extremely large.

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