Inhibition of inactivation of single sodium channels by a site-directed antibody

PETER VASSILEV, TODD SCHEUER, AND WILLIAM A. CATTERALL
Department of Pharmacology, SJ-30, University of Washington, School of Medicine, Seattle, WA 98195

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ABSTRACT The effects of site-directed antibodies on single sodium channel currents in excised membrane patches from rat brain neurons have been examined. Of six antibodies directed against different intracellular domains of the sodium channel α subunit, only an antibody directed against a highly conserved intracellular segment between homologous transmembrane domains III and IV induced late single channel openings and prolonged single channel open times during depolarizing test pulses, resulting in nearly complete inhibition of sodium channel inactivation. The antibody effect was not observed if the membrane patches were depolarized to inactivate sodium channels before exposure to the antibody, indicating that the intracellular sequence recognized by the antibody is rendered inaccessible by inactivation. The results show that a conformational change involving the intracellular segment between domains III and IV of the α subunit of the sodium channel molecule is required for fast sodium channel inactivation and suggest that this segment may be the fast inactivation gate of the sodium channel.

Voltage-gated sodium channels mediate a rapidly activated inward movement of sodium ions that underlies the rising phase of the action potential in excitable membranes. Upon depolarization, sodium channels activate and then inactivate within a few milliseconds. The sodium channel purified from rat brain consists of three glycoprotein subunits: α (260 kDa), β1 (36 kDa), and β2 (33 kDa) (1). The primary structures of three rat brain α-subunit subtypes, Rα, NHII, and NHIII, have been determined by cDNA cloning and sequencing (2, 3). Messenger RNA encoding the NHII or NHIII α subunits is sufficient to direct the synthesis of functional sodium channels in Xenopus oocytes or mammalian somatic cells (4–8). A major focus of current research is to determine which parts of the α subunit are responsible for the known functions of sodium channels. In a previous study, we found that an anti-peptide antibody directed against a highly conserved intracellular segment between homologous domains III and IV of the α subunit slowed inactivation of macroscopic sodium currents (9). The onset of this effect was substantially slowed at depolarized holding potentials at which sodium channels were inactivated. It was concluded that the peptide segment between domains III and IV of the α subunit is directly involved in rapid sodium channel inactivation during large depolarizations. To further elucidate the mechanism of action of this antibody, we have studied its effects on single channels in inside-out membrane patches excised from rat brain neurons in cell culture.

EXPERIMENTAL PROCEDURES

Preparation of Cells. Dissociated cell cultures. Minced forebrain tissue from 20-day rat embryos was dissociated by incubation in Ca2+- and Mg2+-free phosphate-buffered saline solution for 30 min at 37°C in the presence of trypsin at 2 mg/ml. After centrifugation for 10 min at 600 x g, the cells were resuspended in growth medium consisting of 85% Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (vol/vol) heat-inactivated horse serum, 5% (vol/vol) newborn calf serum, and 10 μg of penicillin and 20 μg of streptomycin per ml. The cells were washed twice by centrifugation and resuspension, filtered through a sterile Nitex filter, and plated on 35-mm Falcon Petri dishes coated with collagen and poly(t-lysine) from a solution of 0.5% of each. The cell cultures were incubated at 37°C in 7.5% CO2/92.5% air for 2–10 days before electrophysiological studies.

Explant cultures. In some experiments, cells were prepared as explant cultures without protease treatment. Forebrain tissue was minced and approximately six 1- to 2-mm3 blocks of tissue were placed in uncoated Petri dishes filled with growth medium. After 4–8 days, cells with neuronal morphology grew out of the adherent explants. These cells were used for electrophysiological studies.

Antibodies. Antisera were raised against synthetic peptides whose amino acid sequence corresponded to that of distinct 15- to 20-residue segments of the α subunit, and the antibodies were purified by adsorption to immobilized sodium channel α subunits as described (10, 11). The antibodies are directed against the following peptide segments of the α subunit: SP16, residues 31–47; SP11, residues 467–486; SP20, residues 1106–1126; SP19, residues 1491–1508; SP27, residues 1321–1337; and SP12, residues 1987–2005 (2). Affinity-purified antibodies were dialyzed against intracellular solution (see below). They were used at concentrations that completely immunoprecipitate 0.5 nM sodium channels, as determined in previous studies (10, 11).

Patch-Clamp Experiments. Single sodium channels were studied by using the excised patch-clamp method (12). To minimize the quantities of affinity-purified antibodies used, we developed a hanging-drop configuration of the patch-clamp technique to be described in detail elsewhere (P.V., unpublished data). Briefly, membrane patches were formed on the tip of a pipette filled with extracellular solution and excised into a bath filled with the same solution. The pipette tip was lifted out of the bath and introduced immediately into a small drop hanging at the tip of an agar-bridge capillary. The drop and the capillary contained intracellular solution at 21–23°C and were connected to a chamber containing an Ag/AgCl reference electrode. Decreases in drop diameter due to evaporation were monitored and compensated by addition of fresh intracellular solution. The voltage dependence of activation and inactivation of Na currents shifted in the negative direction during the first few min of recording and then remained stable for up to 2 hr. After stabilization, control recordings were taken. The antibodies dissolved in small volumes (1–3 μl) of intracellular solution were then added to the hanging drop. The onset of the antibody effect usually occurred in several minutes dependent upon the...
holding voltage, and recordings were continued for 20–120 min. The intracellular solution was 105 mM CsF/40 mM CsCl/10 mM NaF/5 mM EGTA/5 mM Heps, adjusted to pH 7.2 with CsOH; the extracellular solution was 150 mM NaCl/1.5 mM CaCl2/1.0 mM MgCl2/5.0 mM glucose/5.0 mM Heps, adjusted to pH 7.4 with NaOH.

Data Acquisition and Analysis. Membrane currents in excised patches were measured by using Ag/AgCl electrodes connected to a List L/M-EPCL-7 patch-clamp amplifier and filtered at 3 kHz (3 decibels) prior to digitization. Pulses were generated, and filtered currents were sampled on line at 20–50 μs per point with programs based on the Fastlab system (Indec Systems, Sunnyvale, CA). Partial compensation of leakage and capacity current was obtained by using the internal amplifier circuitry. Further digital compensation was obtained by subtraction from the experimental records of exponentials fitted to blank traces or to scaled records from hyperpolarizing pulses (13).

RESULTS AND DISCUSSION

Specificity of the Antibody Effect. The functional effects of six anti-peptide antibodies on single sodium channels were examined (Fig. 1). These antibodies were directed against segments of all five major predicted intracellular domains of the α subunit as well as the short predicted intracellular segment between transmembrane segments S4 and S5 of homologous domain IV. In all control traces, sodium channel openings are clustered near the onset of the depolarizing pulses (Fig. 1A). Openings after the first few milliseconds of the pulses are rare events. In contrast, single channel openings of identical current amplitude continue throughout the 25-ms depolarizations in the presence of the antibody to SP19 (AbSP19; Fig. 1B, SP19). In other experiments, sodium channels continue to open and close throughout 250-ms depolarizing pulses in the presence of AbSP19 (not shown). These results indicate that AbSP19 completely blocks the process of rapid sodium channel inactivation. As a result, ensemble average currents are dramatically prolonged (Fig. 1C, SP19).

None of the other five antibodies tested caused repetitive openings of single sodium channels throughout the depolarizing pulses (Fig. 1B), and none had a marked effect on the time course of the ensemble average (Fig. 1C). The striking effect of AbSP19 on single sodium channel currents and the lack of effects of other antibodies that bind to different intracellular segments provide substantial evidence for our previous conclusion (9) that the peptide segment between domains III and IV of α subunit is specifically involved in sodium channel inactivation.

The number of channels recorded varied from patch to patch. Typically, 1–5 functional channels were observed, and multiple superimposed openings clustered at the onset of the depolarizing pulses were recorded (Fig. 1). AbSP19 dramatically increased the probability of channel opening late in the depolarizing pulses regardless of the number of channels recorded in the patch. Since the number of late openings was always much greater than the apparent number of functional channels, we assume that these late openings represent reopenings of individual channels rather than openings of channels for the first time after a long latency.

Voltage Dependence of AbSP19 Action. The onset of the effect of AbSP19 in muscle cells under whole-cell voltage

![Fig. 1](image-url)  

**Fig. 1.** Effects of site-directed antibodies on single sodium channel currents. Depolarizing test pulses to −20 mV for 25 ms were applied from a holding potential of −140 mV. Pulses were delivered at a frequency of 1 Hz. Two representative scaled current traces for each membrane patch under control conditions (A), four representative scaled current traces in the presence of antibody (B), and superimposed average (AV.) currents from 20 consecutive sweeps under control conditions and in the presence of antibody (C) are shown for each of the tested antibodies. The ensemble average currents in the presence of each of the antibodies were scaled and superimposed on the corresponding control currents to compare the time courses of inactivation in C. (Bars = 1 pA.)

![Fig. 2](image-url)  

**Fig. 2.** Dependence of the rate of action of AbSP19 on holding potential. Sodium currents were stimulated by 25-ms test pulses to −10 mV. (A Left) Control records were taken during a 15-min period at a holding potential of −140 mV. Five representative single channel traces and an average of 20 consecutive sweeps are presented. (A Middle) The holding potential was changed to −70 mV and then to −80 mV. AbSP19 was added to the cytoplasmic side of the membrane patch as indicated by the arrow, and the holding potential was maintained for an additional 6.5 min at −80 mV. Five single channel current traces and an average of 20 consecutive sweeps recorded between 6 and 6.5 min after adding AbSP19 are shown. (A Right) The holding potential then was changed to −140 mV. After 50 s, five single channel traces and an average of 20 consecutive traces were recorded. (B Left) In a different membrane patch, the membrane potential was held at −140 mV for 5 min in the absence of AbSP19 and five single channel traces and an average of 20 consecutive sweeps were recorded. (B Right) AbSP19 was added as indicated by the arrow, and 90 s later five single channel traces and an average of 20 consecutive traces were recorded.
clamp was more rapid at negative holding potentials (9). The excised patch preparation allows examination of this effect with better time resolution because barriers to diffusion of the antibody to its site of action are reduced. Fig. 2 illustrates the dependence of the onset of the effect of Absp19 on the holding potential. At −140 mV, single channel traces and ensemble average currents show channel openings clustered at the beginning of 25-ms depolarizing pulses (Fig. 2A Left). After further control recordings at a holding potential of −80 mV (not shown), Absp19 was added to the intracellular side of the membrane patch. No alterations in sodium channel inactivation were observed during a period of 6 min after adding the antibody while holding the membrane potential at −70 or −80 mV (Fig. 2A Center). Returning the holding potential to −140 mV caused modification of the inactivation kinetics within 50 sec, as evidenced by the long, late channel openings throughout the depolarizing pulses (Fig. 2A Right). After the antibody effect has occurred at −140 mV, it is not reversed by depolarization to −80 mV.

In a similar experiment, several records were taken under control conditions at −140 mV (Fig. 2B Left). Within 20 s after addition of Absp19, the appearance of late prolonged channel openings indicated inhibition of inactivation (Fig. 2B Right). Thus, the onset of the effect of Absp19 depends strongly on the holding voltage. At −140 mV, where sodium channels are not inactivated, Absp19 acts rapidly to inhibit the inactivation process; at −70 or −80 mV, where sodium channels are substantially inactivated (see below), Absp19 does not alter channel inactivation within several minutes. These results indicate that the effect of Absp19 on sodium channel inactivation is all-or-none when analyzed at the single channel level. In contrast, the effect of the antibody increases slowly to a maximum at a voltage-dependent rate when analyzed in whole-cell voltage clamp (9). The progressive action of the antibody observed in whole-cell experiments must represent the time-dependent diffusion and binding of the antibody to sodium channels rather than a time-dependent, progressive modification of channel properties.

Effects of Absp19 on Single Sodium Channel Currents at Different Test Potentials. In our previous whole-cell voltage-clamp study, it was found that the antibody-induced slowing of inactivation was more prominent during depolarizations to more positive test potentials than −30 mV. Fig. 3 shows single channel traces and ensemble average currents recorded at seven different test potentials under control conditions (Fig. 3A) and in the presence of Absp19 (Fig. 3B). Under control conditions, channel openings cluster at the onset of depolarization at all membrane potentials, although apparent channel reopenings are typically observed at test potentials of −50 mV and −60 mV. In the presence of Absp19, both the frequency of channel opening and the probability of one or more channels being in the open state late in the depolarizing test pulses are significantly increased at all test potentials (Fig. 3B). However, the effect is much more striking at more positive test potentials. For example, in test pulses to −60 mV, channel openings are observed late in the depolarizations in the absence of antibody, but their frequency and duration are increased in the presence of antibody. In contrast, in test pulses to −10 mV, single sodium channels activate and inactivate completely within 2 ms under control conditions but continue to open throughout the 25-ms depolarizations in the presence of the antibody.

Inspection of the single-channel current recordings made in the presence and absence of Absp19 (Fig. 3) suggests that Absp19 causes individual channel opening events to be prolonged. This effect is most striking at pulse potentials more positive than −30 mV, where openings longer than 10 ms in duration are frequently observed in the presence of Absp19 but never in the control. To make a quantitative assessment of the effect of Absp19 on the mean open times of the sodium channels, we analyzed the open time durations during test pulses to −20 mV, approximately the peak of the I–V curve. Fig. 4A and B show frequency histograms of apparent mean open times obtained from single-channel opening events recorded under control conditions (335 events, Fig. 4A) and in the presence of Absp19 (333 events, Fig. 4B). The data was fit well by a single exponential decay with a half-time of 0.69 ms in the control and by a sum of two exponentials with half-times of 0.73 ms (46% of openings) and 5.62 ms (54% of openings) in the presence of Absp19. We assume that the single channel openings, having an unchanged mean open time in the presence of Absp19, are due to unmodified sodium channels, while those with prolonged open times are antibody-modified. Thus, these results indicate that binding of Absp19 induces approximately an 8-fold increase in the mean open time at −20 mV. The effect of Absp19 on mean open time is even greater at more positive membrane potentials, since mean open times are prolonged even more markedly at more positive potentials in the presence of antibody but change relatively little at more positive membrane potentials in control (Fig. 3; refs. 14 and 15).

Fig. 3. Effect of Absp19 on single Na channel currents at different test potentials. Sodium currents were elicited from a constant holding potential of −140 mV by 25-ms pulses to different test voltages as indicated. Three representative single channel sweeps and one ensemble average (Av.) current trace shows for each test potential (T.P.) under control conditions (A) and in the presence of Absp19 (B). Arrows above the top current traces indicate the onset of the depolarizing pulses. In the traces showing the average currents in B, the zero current levels have been marked with straight lines.
contrast, following a 250-ms prepulse to −60 mV (Fig. 4C) or a 100-ms prepulse to −30 mV (Fig. 4D), the early peak in the ensemble current is inactivated, but the sustained current is unaffected by the prepulse. Similar results are observed whether or not channel openings occur during the prepulse. Evidently, antibody-modified channels not only reopen repetitively during test pulses but also do not inactivate during 100-ms or 250-ms prepulses to less-depolarized potentials. Because the peak in ensemble average currents at the beginning of test pulses in the presence of Absp19 has a similar time course to control sodium currents and inactivates during depolarizing prepulses, we tentatively assign this current component to unmodified sodium channels.

The amplitudes of the ensemble-average currents were measured at the peak of sodium current and 10 ms after the beginning of the test pulse and were plotted as a function of the applied test potential (Fig. 4E). No change in the voltage dependence of activation of single sodium channels by Absp19 was observed when peak currents were measured. The potential at which the maximum-sustained sodium currents were recorded appeared to be 5–10 mV more positive than that required for maximum peak currents in some recordings (Fig. 4E), but this was not a statistically significant effect.

To determine the effect of Absp19 on slow inactivation of single sodium channels, the holding potential was varied, and the amplitudes of ensemble average currents during 25-ms test pulses to −10 mV were measured at the peak of sodium current and 10 ms after the beginning of the test pulse (Fig. 4F). The voltage dependence of slow inactivation of both the early and sustained sodium current components was unaffected by Absp19. Thus, Absp19 completely inhibits fast sodium channel inactivation measured during a test pulse or during 100- or 250-ms prepulses but does not markedly alter the voltage dependence of slow sodium channel inactivation.

**Mechanism of Inhibition of Sodium Channel Inactivation by Absp19.** In previous whole-cell voltage-clamp studies (9), we found that Absp19 caused progressive slowing of the rate of inactivation but did not remove it completely. The rate of action of the antibody was decreased by a factor of 3 by prior inactivation of the sodium channels by prolonged depolarization. We concluded that Absp19 bound preferentially to the “not inactivated” conformation of a portion of the inactivation gate formed by the intracellular segment between domains III and IV of the sodium channel α subunit and inhibited its function in inactivating the channel (9). The present single-channel recording results extend and further clarify the mechanisms underlying these conclusions and allow us to propose a specific working model for Absp19 action.

A successful model for the action of Absp19 must provide a mechanistic basis for understanding the following effects: (i) Absp19 inhibits fast inactivation of individual sodium channels completely but does not substantially alter slow inactivation; (ii) sodium channels modified by Absp19 have prolonged mean open times and greatly enhanced reopening probability but are otherwise functionally intact; (iii) reopening probability and mean open times of modified sodium channels are greatly increased at more positive test pulse potentials; and (iv) Absp19 binds much more rapidly to individual sodium channels that are not inactivated.

We have utilized the following minimal gating model (15–17) to explain our results with Absp19:

\[
\begin{align*}
I_s & \quad \text{slow} \\
C_s & \quad C_2 \quad C_1 \quad O \\
C_1 & \quad I_f
\end{align*}
\]
This model assumes multiple closed states, two inactivated states, and a single open state. Voltage-dependent activation of sodium channels during depolarization from a negative holding potential involves transitions from $C_0$ to $O$. In our use of this model, we consider that fast inactivation during strong depolarizations represents primarily the transition from $O$ to $I_f$, whereas at smaller depolarizations (e.g., to $-60$ mV or $-50$ mV), the decay of sodium current represents transitions to both $C_1$ and $I_f$. Inactivation of sodium channels measured after prepulses of 20-200-ms duration to membrane potentials in the range of $-90$ to $-60$ mV, where few channels reach the open state, results primarily from transitions from $C_1$ to $I_f$. Slow inactivation on the time scale of seconds to minutes results from transitions to a different inactivated state, $I_s$. In the context of this model, $Absp_{19}$ is proposed to block specifically the two pathways leading to the fast inactivated state, $I_f$. Block of transitions to this single conformational state provides a qualitative explanation of all four of the antibody effects that we have observed.

If reaction pathway $O \rightarrow I_f$ represents the only route for rapid transition from the open state to an inactivated state, $Absp_{19}$ would be expected to block fast inactivation essentially completely as observed in our experiments. Single channel conductance and voltage dependence of activation should be relatively unaffected. If inactivation measured in experiments with 100- to 250-msec prepulses to potentials more negative than $-50$ mV primarily represents transitions to the rapidly inactivated state by another pathway ($C_1 \rightarrow I_s$), this process should also be inhibited by $Absp_{19}$, as we have observed. Since slow inactivation involves a conformational transition to a different state, $I_s$, it is not affected by $Absp_{19}$. The lack of effect of $Absp_{19}$ on slow inactivation indicates that sodium channels enter $I_s$ directly from one or more closed states rather than from $I_f$.

Block of fast inactivation by $Absp_{19}$ results in steady-state channel activity by causing individual sodium channels to open upon depolarization and then to fluctuate between the open state and final closed states ($C_1, C_2$, etc.). This fluctuation is observed in our recordings as steady-state reopening of individual channels. The probability of reopening at steady state represents a balance of transitions among states $C_2$, $C_1$, and $O$.

The dependence of the effects of $Absp_{19}$ on test-pulse potential is also accommodated by this model. In the control, for small depolarizations, a substantial fraction of channel openings are terminated by the return to $C_1$. For these depolarizations, $Absp_{19}$ has minor effects as observed in our records at $-60$ mV and $-50$ mV. For strong depolarizations (more positive than $-30$ mV), most openings are terminated by transitions to $I_f$. Block of this transition by $Absp_{19}$ unMASKS the high intrinsic probability of the open state characteristic of the voltage-dependent activation process at these depolarized potentials. This causes the long open times with occasional flickers to the closed state that are observed in our records at positive test potentials. Thus, block of conformational transition to a single state by $Absp_{19}$ is sufficient to induce all of the major changes of behavior of single sodium channels recorded in our experiments.

The voltage dependence of the binding of $Absp_{19}$ may also follow directly from its inhibition of transition to this single conformational state. Our results show that $Absp_{19}$ binds rapidly to its recognition site on the sodium channel in its resting conformation and prevents fast inactivation but does not bind to this site when the sodium channel is inactivated. We conclude that the intracellular peptide segment between domains III and IV, which binds $Absp_{19}$, undergoes a conformational change during the process of inactivation making it inaccessible to $Absp_{19}$. The antibody inhibits inactivation by binding with high affinity to its recognition site in the "not inactivated" conformation and preventing the transition to the rapidly inactivated state. The peptide segment recognized by $Absp_{19}$ is required for fast inactivation and is directly involved in that process. We propose that it may act as an "inactivation gate" by folding into the transmembrane pore of the sodium channel and occluding it in the inactivated state of the channel. This conformational transition is detected in our experiments as a loss of accessibility to $Absp_{19}$.

Inactivation of sodium channels is also inhibited by intracellular perfusion with proteases and amino-acid-specific reagents (reviewed in ref. 17). The action of proteases is slowed by depolarization, suggesting that their site of action is also made inaccessible by inactivation (18). Since the peptide segment recognized by $Absp_{19}$ contains multiple pairs of basic amino acid residues that are favorable targets for proteolytic cleavage, proteases may inhibit inactivation by cleaving the $\alpha$ subunit at the site of action of $Absp_{19}$.

Note Added in Proof. Stühmer et al. (19) report that mutations that introduce cuts in the intracellular segment between domains III and IV block sodium channel inactivation in support of the conclusion (ref. 19 and this report) that this channel segment is required for inactivation.

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