

Modified Kinetics and Selectivity of Sodium Channels in Frog Skeletal Muscle Fibers Treated with Aconitine

DONALD T. CAMPBELL

From the Department of Physiology, Yale University School of Medicine, New Haven, Connecticut 06510, and the Department of Physiology and Biophysics, University of Iowa School of Medicine, Iowa City, Iowa 52242

ABSTRACT The effect of the plant alkaloid aconitine on sodium channel kinetics, ionic selectivity, and blockage by protons and tetrodotoxin (TTX) has been studied in frog skeletal muscle. Treatment with 0.25 or 0.3 mM aconitine alters sodium channels so that the threshold of activation is shifted 40–50 mV in the hyperpolarized direction. In contrast to previous results in frog nerve, inactivation is complete for depolarizations beyond about -60 mV. After aconitine treatment, the steady state level of inactivation is shifted ~ 20 mV in the hyperpolarizing direction. Concomitant with changes in channel kinetics, the relative permeability of the sodium channel to NH_4 , K, and Cs is increased. This altered selectivity is not accompanied by altered block by protons or TTX. The results suggest that sites other than those involved in channel block by protons and TTX are important in determining sodium channel selectivity.

INTRODUCTION

This paper concerns the kinetic, ion-selective, and pharmacological properties of frog muscle sodium channels modified by aconitine, an alkaloid from the plant *Aconitum napellus*. In frog nerve it has been shown that aconitine alters sodium channel kinetics, eliminating inactivation and lowering the threshold for activation by ~ 50 mV (Schmidt and Schmitt, 1974; Mozhayeva et al., 1976). In addition, aconitine alters the selectivity of the sodium channel for a variety of cations (Mozhayeva et al., 1977), and it has been suggested that aconitine abolishes (Naumov et al., 1979a) or at least greatly reduces (Mozhayeva et al., 1982b) the voltage dependence that is normally observed in the reduction of peak sodium currents at low pH. These results were interpreted as supporting the notion that the site of block is the hypothetical constriction in the pore which has been called the “selectivity filter” (Hille, 1972, 1975d; Woodhull, 1973).

I report here a different kinetic effect of aconitine on sodium channels of frog muscle: as in nerve, the threshold of activation is shifted ~ 50 mV in the hyperpolarizing direction; however, inactivation, although slowed, is not eliminated in muscle. In addition, the question of whether aconitine modifies proton block of sodium channels has been re-examined in muscle using a

“tail” current protocol. In contrast to the previous reports in nerve, aconitine does not alter proton block of muscle sodium channels. An explanation is presented that resolves this difference between my results in muscle and the previous results, and bears on the question of the identity or separability of the proton blocking site and the site where ionic selectivity occurs.

METHODS

Pieces of single semitendinosus muscle fibers from *Rana pipiens* were studied under voltage-clamp conditions using the vaseline-gap technique (Hille and Campbell, 1976). This method uses vaseline seals to isolate an annular patch of membrane electrically, creating an artificial node. Voltage is measured and current is passed through separate cut ends of the fiber. In this method two areas of membrane are present that are not under strict voltage-clamp control: the transverse tubular system (T system) and the membrane under the vaseline seals. In some preparations, at relatively warm temperatures, small current bumps have been observed that are apparently caused by currents flowing in one of these poorly controlled regions (see Hille and Campbell, 1976, Fig. 11). Improved seal techniques (minimizing surface connective tissue, warming of the vaseline, and waiting 10–20 min for the seal to tighten) have resulted in the elimination of these anomalies from all but a small percentage of preparations. Although in theory it is predicted that the sodium channels in the T system should contribute similar anomalies to membrane currents measured in muscle, they are not evident in the majority of preparations. Preparations in which such anomalies were detected were discarded.

The improvements incorporated in the apparatus since the original description was published will be detailed in a subsequent paper. Briefly, in the present system, current is measured explicitly, with lower noise and higher resolution, using a current-to-voltage converter in the current-injecting pathway; separate voltage-measuring and current-passing electrodes in the nodal pool minimize the effects of series resistance; residual series resistance is compensated in the usual way except that the compensating signal is low-pass-filtered at 100 kHz to permit full compensation without instability (Sigworth, 1980). Compensation was generally set at 2–3 k Ω . The capacitance of the surface membrane was determined from the rapid phase of the current transient elicited by a 45-mV step from –135 mV, and the membrane area was calculated based on the assumption of 2.2 $\mu\text{F}/\text{cm}^2$ (Hille and Campbell, 1976).

Aconitine (Tridom-Fluka, NY) was dissolved in a small quantity of weak HCl solution and then diluted to a final concentration of 0.25 or 0.3 mM in Ringer at pH 7.4. During and after aconitine treatment the holding potential was set at –150 mV, which is sufficiently negative to prevent aconitine-modified channels from opening. In untreated fibers, a holding potential of –150 mV causes a 10–15% increase in peak current (presumably by removing long-term inactivation present at –90 mV; Brismar, 1977) and introduces a slight delay in the current transient (Hahin and Goldman, 1978). As reported previously in frog myelinated nerve (Mozhayeva et al., 1977), the rate of onset of the aconitine effect in muscle is stimulation dependent. The fiber was depolarized 10–20 times/s with a 6–10-ms step to a voltage slightly below E_{Na} . In 4–5 min, peak conductance was markedly decreased and inward currents could be evoked with depolarizations to –100 mV (~40–50 mV more negative than the threshold for opening normal sodium channels). After treatment, the aconitine Ringer was washed out of the chamber and subsequent experimental solutions contained no aconitine.

For selectivity experiments, external solutions contained 120 mM sodium or sodium

substitute, 2 mM Ca, and 4 mM HEPES-Tris buffer at pH 7.4. The anion was Cl except for tetramethylammonium (TMA)-Ringer, which contained 120 mM Br. These solutions are named by the predominant ion, e.g., Na Ringer. For the proton-blocking experiments, solutions contained either 110 NaCl or 110 NH₄Cl, 2 mM CaCl₂, 10 mM HEPES (pH 7.4), or MES (pH 5.0 and 6.0) adjusted to the appropriate pH with Tris. Fiber ends were bathed in an isotonic solution containing CsF with 5–10 mM NaF. The relative permeability of sodium channels to the substituted ion S, P_S/P_{Na} , was calculated from the expression:

$$P_S/P_{Na} = \exp(F\Delta E_{rev}/RT) \quad (1)$$

where $F/RT = 1/24$ mV at 5°C and ΔE_{rev} is the change in reversal potential of peak sodium channel currents measured upon replacing Na Ringer with the substituted Ringer (Hille, 1971).

Data was recorded on-line by a laboratory minicomputer and stored on floppy disks for later analysis. A digital stimulator (model PSG 1; Page Digital Electronics, Duarte, CA) received pulse sequence commands from the computer and generated the voltage-clamp command pulses and the digital sample trigger pulses. Approximate subtraction of linear leakage and capacity currents was accomplished with an analog electronic circuit. This subtracted current record was filtered at 10–40 kHz with an eight-pole Bessel filter and amplified 10–100 times before being digitized by a 12-bit A/D converter. During a single depolarizing step, 256 samples were taken at two different sample intervals. Six to eight control records were recorded for depolarizing steps to the holding potential from a prepulse hyperpolarization of 30–45 mV. These control currents were averaged and then appropriately scaled and subtracted from the test currents to remove the remaining linear leakage and capacity currents before displaying the records on the oscilloscope monitor for analysis.

Sodium current kinetics were described by fitting the expression

$$I = A[1 - \exp(-t/\tau_m)]^3 \exp(-t/\tau_h) \quad (2)$$

to individual current records using a least-squares algorithm. A is an amplitude factor, and τ_m and τ_h are time constants of activation and inactivation. This expression represents a simplification of the scheme originally used to describe sodium conductance kinetics in squid giant axon (Hodgkin and Huxley, 1952*b*) and assumes that the steady state level of activation is zero at the holding potential, a good assumption, and that steady state inactivation is complete at the test potential, which is approximately true for potentials more positive than –60 mV. At many voltages the rising phase of the ionic current is partially obscured by relatively large gating currents, which interfere with accurate determination of τ_m . Fitted values of inactivation time constant were confirmed by visually fitting a single exponential to the falling phase of the displayed current.

RESULTS

Three kinds of results will be presented: (a) the effect of aconitine on the size and kinetics of sodium currents; (b) the selectivity of aconitine-modified sodium channels; and (c) the block of aconitine-modified sodium channels by hydrogen ions and TTX.

Aconitine Decreases Peak Conductance

Fig. 1 shows sodium currents from a muscle fiber before and after a 5-min treatment with 0.3 mM aconitine. During the treatment, the fiber was

stimulated 10 times per second with a 7-ms depolarization to +50 mV. The resulting decrease of peak sodium current to ~20% of the pre-aconitine level was typical for this treatment protocol. Shorter-duration treatment, lower aconitine concentration, and fewer stimulating pulses all resulted in a smaller decrease in the peak sodium current. In some experiments the peak conductance increased slowly over time after washout of the aconitine containing Ringer, which suggested that the effect of aconitine may be partially reversible.

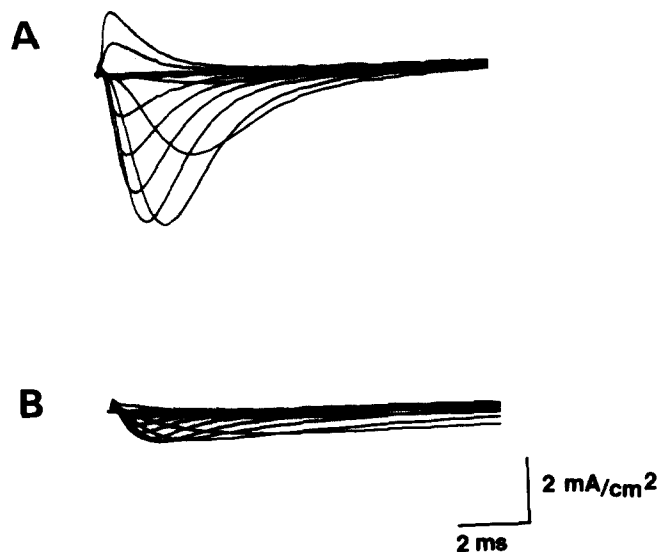


FIGURE 1. Sodium currents before and after treatment with 0.3 mM aconitine. A. Control currents before aconitine treatment. The holding potential was -90 mV, temperature 4.5°C . The test voltage steps, preceded by a 100-ms prepulse to -135 mV, are spaced 15 mV apart. The uppermost trace was elicited by a step to $+67.5$ mV. B. Currents from the same fiber recorded in normal Na Ringer after treating the fiber with 0.3 mM aconitine for 5 min. During aconitine treatment the fiber was stimulated at 10 Hz with 7-ms steps to $+50$ mV. During and after the aconitine treatment the holding potential was -150 mV. Voltage steps 15 mV apart, the top trace is for a step to $+67.5$ mV. Temperature 4.2°C , muscle 52.

Aconitine Modifies Sodium Channel Kinetics

The peak current-voltage relation in Fig. 2 illustrates that concomitant with the aconitine-induced decrease in peak sodium conductance is the appearance of a fraction of inward current turning on at potentials 40–50 mV hyperpolarized from the normal sodium current threshold. In Na Ringer, this shift in threshold is typically associated with the appearance of a new “bump” in the peak current-voltage relationship at about -50 mV, in addition to the maximum normally seen near 0 mV. The currents represented by the filled

circles in Fig. 2 were measured shortly after removing the aconitine-containing solution. In this example of strong aconitine effect, the peak current was nearly identical at all voltages between -52.5 and -7.5 mV. Later in the experiment, the peak near 0 mV had increased slightly, revealing the "two-bumped" shape characteristically seen in sodium Ringer (see Fig. 7 below). A simple interpretation of this result is that after aconitine treatment two populations of channels are present: one with normal voltage dependence and the other opening at unusually negative potentials. To estimate the fraction of channels not modified by aconitine, the current-voltage relation measured before aconitine treatment was scaled and subtracted from the post-aconitine

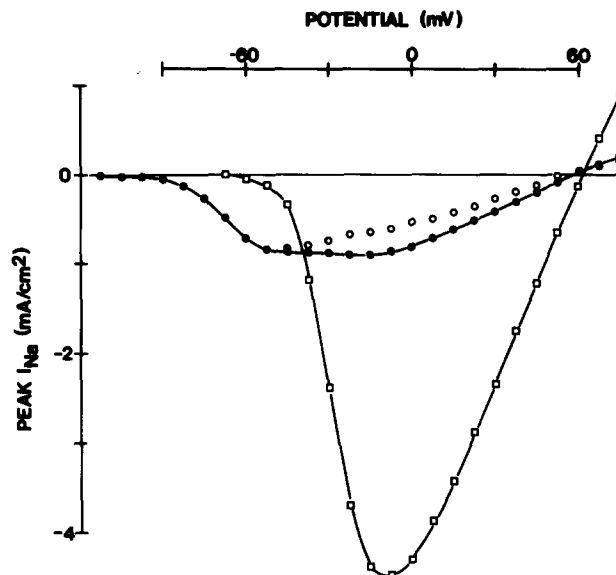


FIGURE 2. Peak sodium current-voltage relationship before and after aconitine. Peak currents from the experiment illustrated in Fig. 1, plotted as a function of voltage. Before aconitine (squares); after aconitine (filled circles). Open circles represent the post-aconitine peak currents minus 5.6% of the pre-aconitine peak currents measured at the same voltage.

current-voltage relationship. The open circles in Fig. 2 show the current-voltage relationship remaining after subtracting 5.6% of the pre-aconitine relationship. This derived curve has a single maximum near -50 mV. As will be described in more detail below, the kinetic changes induced by aconitine are accompanied by greatly increased permeability of sodium channels to Cs, NH_4 , and K, which are normally relatively impermeant. The current-voltage relations in these substituted Ringer solutions also exhibit a single maximum near -50 mV (see Fig. 7), lending support to the notion that the open circles of Fig. 2 represent the peak current-voltage relationship of the population of aconitine-modified channels. Thus, in this experiment it appears that $<6\%$ of

the channels remain unaffected by aconitine. Similar analysis of four other experiments resulted in estimates that 6–10% of the channels remain unmodified after the standard 4–6-min treatment with aconitine.

Aconitine Alters Sodium Channel Inactivation

Fig. 3 shows steady state inactivation determined with a two-pulse protocol: after aconitine, the voltage dependence of steady state inactivation is shifted ~ 20 mV in the hyperpolarized direction. Two changes were made in the protocol after aconitine treatment to reduce the contribution of currents through unmodified channels. First, the test potential was -30 mV (where fewer than half of the unmodified channels would be open at the peak of current). Second, NH_4 Ringer was used as the test solution because NH_4 permeates well through modified channels and poorly through normal sodium channels. Neither change by itself produces a detectable change in steady state inactivation determined in untreated fibers.

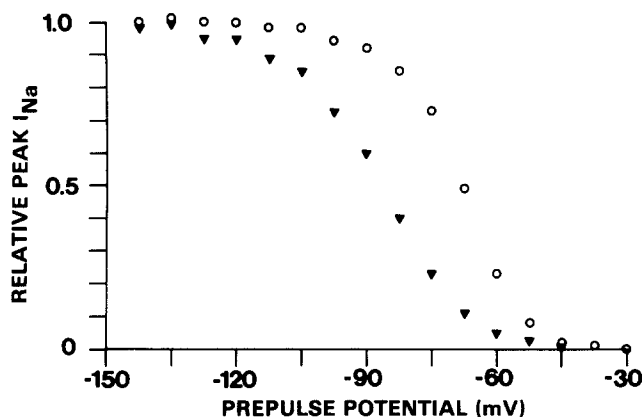


FIGURE 3. Steady state inactivation before and after 0.3 mM aconitine. Points represent relative peak currents elicited by test pulses to 0 mV (circles, Na Ringer, before aconitine) or -30 mV (triangles, NH_4 Ringer, after aconitine) plotted against the variable potential during 200-ms prepulses. The -30 -mV test pulse was used after aconitine treatment in order to minimize currents from unmodified channels. Muscle 47, temperature 6°C .

After aconitine treatment, inactivation is very slow and possibly incomplete for test pulses below -80 mV, slow but nearly complete for test pulses between -80 and 0 mV, and nearly normal for depolarizations more positive than 0 mV. This can be seen in Fig. 4, which shows individual current records from a single fiber before and after aconitine treatment. In Fig. 4A, the before- and after-current records, both elicited by a depolarization to -60 mV, are displayed at the same gain. Relatively large inward currents are present after aconitine treatment but not before. Although activation is reasonably rapid at this potential, inactivation is quite slow. In Figs. 4B–D, the current records

are scaled to permit comparison of the kinetics of the smaller post-aconitine currents with the kinetics of currents measured before treatment. For a step to -30 mV, activation is considerably faster and inactivation is considerably slower after aconitine treatment. As the test pulses become more positive, the kinetics of the post-aconitine sodium currents become more like the pre-aconitine currents. At $+30$ mV, the time course of inactivation is nearly identical in the two traces (the kinetics of activation in the post-aconitine trace are obscured by the early outward gating current, which is more visible in the post-aconitine record because it is displayed at 4.2 times the gain of the pre-aconitine control).

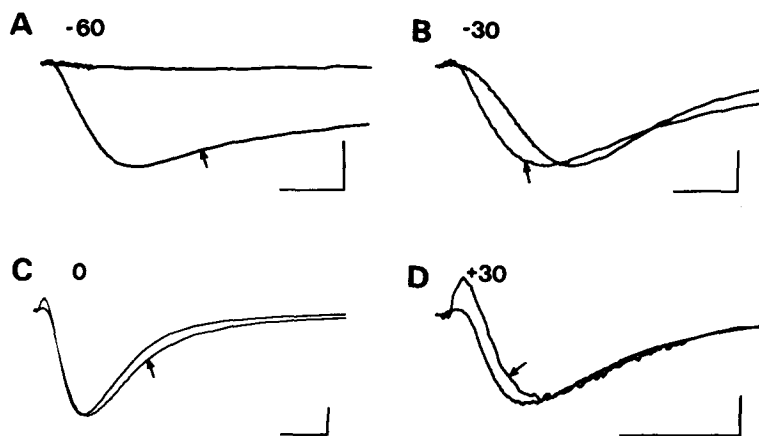


FIGURE 4. Currents recorded before and after treatment with 0.25 mM aconitine. A. Currents elicited by a step to -60 mV before and after (arrow) aconitine, displayed at the same gain (vertical bar represents 0.5 mA/cm²). B–D. Currents were scaled so that peaks are approximately the same size in order to compare the time courses. The vertical calibrations represent 1.0 mA/cm² in the pre-aconitine records. The post-aconitine records have been gained by 2.1, 3.1, and 4.2 in B, C, and D, respectively. In D, this additional gain reveals a prominent outward gating current at early times. Muscle 127, Na Ringer, temperature 4.7° C. Horizontal calibration bars represent 1 ms.

The slowing of inactivation after aconitine treatment is illustrated quantitatively in Fig. 5, in which the inactivation time constants determined from the experiment of Fig. 4 are plotted as a function of voltage. Previous work has shown that aconitine-modified channels in frog myelinated nerve inactivate very little if at all (Schmidt and Schmitt, 1974; Mozhayeva et al., 1976; Mozhayeva et al., 1980). To test whether this discrepancy was due to species differences or to differences between nerve and muscle, three experiments were performed on myelinated nerves from the same group of frogs used in a set of muscle experiments. In agreement with the earlier results, aconitine was found to eliminate inactivation in frog nerve.

Aconitine Modifies Sodium Channel Selectivity

The sodium channel of muscle (Campbell, 1976), like the sodium channel of nerve (Hille, 1971, 1972), is permeable to a variety of small metal and organic cations. After treatment with aconitine, the reversal potential in sodium Ringer is changed slightly if at all, but the relative permeability of the channel to NH_4 , K, and Cs is greatly increased. Fig. 6 shows sets of current traces in

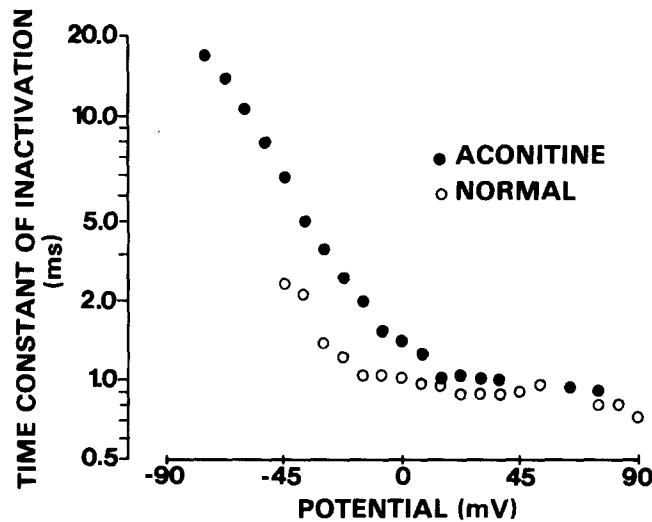


FIGURE 5. Inactivation of modified sodium channels. Time constants were determined by fitting a single exponential to the falling phase of sodium current. Muscle 127, temperature 4.7°C .

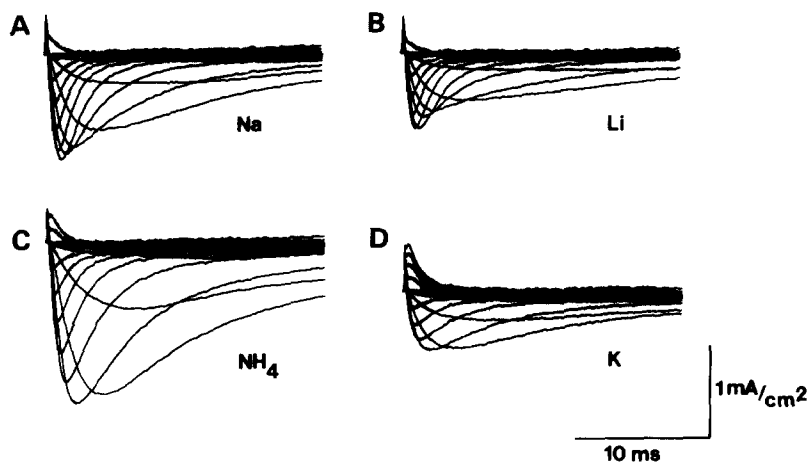


FIGURE 6. Currents from an aconitine-treated fiber bathed in sodium substitutes. All traces are displayed at the same gain. Traces are for voltage steps 15 mV apart; in all frames the top trace corresponds to a test pulse to $+67.5$ mV. Muscle 52, temperature 4.4 – 5.1°C .

sodium Ringer and in three substituted Ringer solutions. As is the case with normal sodium channels (Hille, 1972; Campbell, 1976), currents in Li Ringer are somewhat smaller than currents in Na Ringer, although the reversal potential is nearly identical in the two solutions. In contrast to normal sodium channels, in which NH_4 and K permeabilities are <0.1 of the Li permeability, the peak inward currents in K Ringer are nearly as large as the Li currents, and currents in NH_4 Ringer are twice as large as the Li currents.

Although the peak NH_4 and K currents are relatively large compared with currents in sodium Ringer, they reverse direction at membrane potentials 22 (NH₄) and 51 mV (K) more negative than do the sodium currents. From the changes in reversal potential measured in the experiment of Figs. 6 and 7, one can calculate the relative permeabilities for the sequence $\text{Li} > \text{Na} > \text{NH}_4 > \text{K} > \text{Cs}$

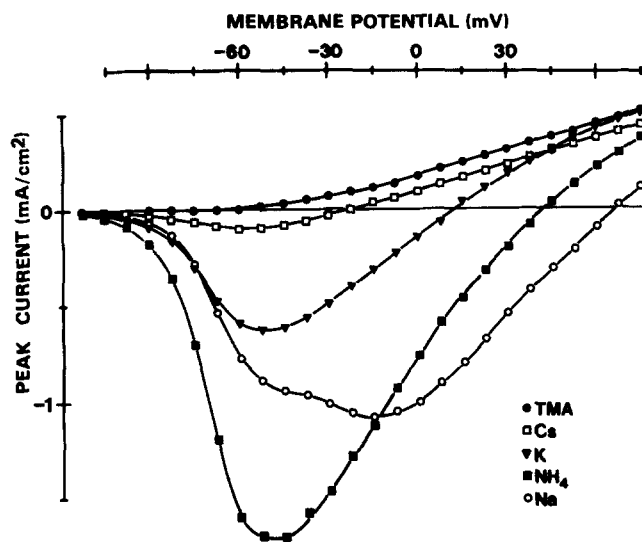


FIGURE 7. Peak current-voltage relations in substituted Ringer's after treatment with 0.3 mM aconitine. Same muscle as in Fig. 6.

of 1.16:1:0.4:0.11:0.03, whereas at -52.5 mV the relative magnitude of the current is $\text{NH}_4 > \text{Na} > \text{Li} > \text{K} > \text{Cs}$ in the ratio 1.91:1.0:0.74:0.72:0.1. One explanation for this discrepancy is that movement of sodium ions is far less independent after aconitine treatment. That is, sodium ions block the channels in addition to passing through them. Arguing against this interpretation is the observation (two experiments, not shown) that peak currents in $\frac{1}{2}$ Na Ringer are only slightly larger than predicted from the independence principle (Hodgkin and Huxley, 1952a). Another possible source of the discrepancy is that it arises from the presence of two populations of sodium channels in the preparation: the small fraction of normal channels and a majority of channels with kinetics and selectivity modified by aconitine. In this case, the reversal potential for total sodium channel current seen in NH_4 solution is actually the sum of an outward current through unmodified channels, which are relatively

impermeant to NH_4 , and an inward current through the aconitine-modified channels, which are highly permeant to NH_4 . In this manner, the residual, unmodified channels lower the apparent reversal potential and thereby cause an underestimation of the relative permeability of aconitine-modified channels to ions that are relatively impermeant to unmodified channels.

The notion that the reversal potential is determined by the sum of currents through two populations of channels with different kinetics and selectivity is supported by the kinetic differences seen in the two sets of traces shown in Fig. 8. In each frame six current traces are shown for voltage steps around the

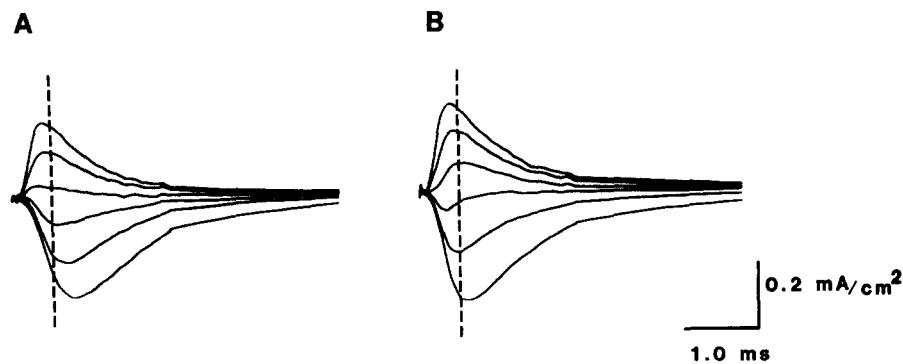


FIGURE 8. Currents in sodium and ammonium after aconitine treatment. Currents elicited by identical voltage steps are spaced 15 mV apart; the top trace is for a step to +90 mV, the bottom trace is for a step to +15 mV. The interrupted line is drawn 0.4 ms after the step to the test potential in order to facilitate comparison of kinetics in the two solutions. A. In Na Ringer, the time to peak of inward current traces is slower than in NH_4 Ringer. Instead of the orderly progression of increasingly shorter times to peak seen in normal fibers, the smallest outward current displays an especially early peak. B. In NH_4 Ringer, inward currents activate more quickly than in sodium Ringer. In contrast to the sodium currents in frame A, the smallest inward current displays the exceptionally early peak and unusual kinetics. This behavior is expected if two populations of channels are present, one more permeable to NH_4 than to sodium and with somewhat faster activation kinetics at these voltages, and the other more permeable to sodium than to NH_4 and with somewhat slower activation kinetics at these voltages. In addition to the standard electronic and digital subtraction of leakage and capacity currents, gating currents from the same fiber bathed in 100 nM TTX were scaled and subtracted from the records leaving only the nonlinear ionic currents shown. Muscle 179, temperature 5.5–6°C.

reversal potential in sodium Ringer (A) and in NH_4 Ringer (B). To facilitate comparison, the dashed lines are drawn 0.4 ms after the test step. Near the reversal potentials, which in this example of particularly strong aconitine effect are about +50 mV in both Na and NH_4 , discontinuities can be seen in the progression of the times to peak. A particularly early peak is seen just above the reversal potential in sodium Ringer and just below the reversal potential in NH_4 Ringer. This is the behavior expected if two populations of

channels with slightly different kinetics and different selectivities are present. The current near the apparent "reversal" potential would represent the sum of inward current through channels with one reversal potential and outward current through channels with another reversal potential. Thus, if relative to normal channels, aconitine-modified channels have lower permeability to sodium, higher permeability to NH_4 , and slightly faster activation kinetics, then the reversal potential will be time dependent and will be more positive at early times in NH_4 and more negative at early times in sodium.

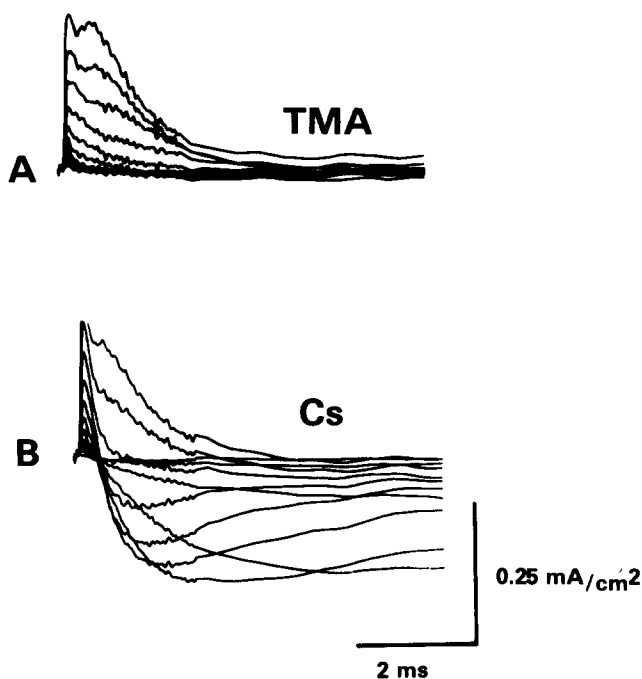


FIGURE 9. Currents in Cs and TMA. A. Currents from an aconitine-treated fiber with all external Na replaced by TMA ion. Only outward currents are observed. B. Upon washing out the TMA solution with Cs Ringer inward sodium channel currents are seen. Before aconitine treatment only outward currents were observed in both TMA and Cs Ringer. Muscle 56, 4.4°C.

Cesium, which does not pass through normal sodium channels, is relatively permeant after aconitine treatment. Fig. 9 shows currents from a fiber bathed first in TMA and subsequently in Cs Ringer. In TMA solution, only outward currents are seen, which indicates that aconitine-modified sodium channels are not measurably permeable to TMA ions. Upon washing out the TMA Ringer with Cs Ringer, inward currents are observed, which demonstrates that Cs ions are permeant through aconitine-modified sodium channels. These inward currents measured in Cs solution are blocked by TTX. Small inward currents occasionally observed in TMA Ringer and generally observed in methylammonium Ringer do not inactivate and are not blocked by TTX and therefore probably arise from nonlinearities in the leakage current.

Block of Sodium Channels by H⁺ and TTX Is Unaffected by Aconitine

Fundamental to a model that has been proposed for the sodium channel selectivity filter is a negative site with which ions associate in their traverse of the pore. In this model, the field strength of this site determines in part the selectivity of the sodium channel (Hille, 1975*a, b*). Since it has also been hypothesized that this same site is where protons and TTX bind to produce channel block (Woodhull, 1973; Hille, 1975*a, b, d*), it is important to determine whether aconitine alters the affinity of blocking site in the process of changing channel selectivity.

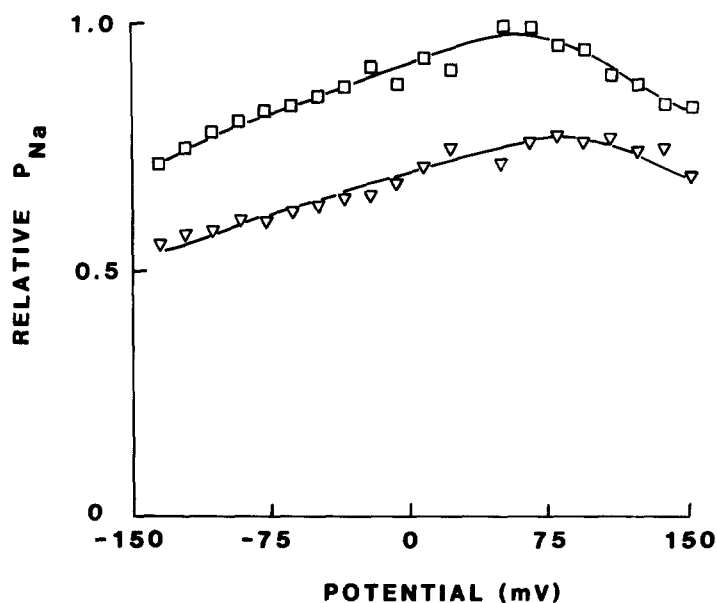


FIGURE 10. Isochronal tail permeabilities at normal and low pH after aconitine. Prepulses to +75 mV were followed by test pulses from -150 to +165 mV. Relative permeabilities were calculated by the constant-field relationship from currents measured 60 μ s after the steps to the test potentials. The curve through the pH 7.4 control points (squares) was drawn by eye and entered into the computer. The curve through the pH 5.1 points (triangles) is the identical control curve scaled by a voltage-independent $pK = 4.51$, and shifted 24 mV in the depolarizing direction to account for the change in kinetics caused by the low-pH solution.

In previous studies of proton block two different measures of the number of channels free from block have been used. Traditionally, peak sodium currents have been measured (Hille, 1968; Woodhull, 1973). However, this method requires that several assumptions be made about the effect of pH on channel kinetics. Less dependent on such assumptions is block determined from sodium current tails (Campbell, 1982). Fig. 10 illustrates block of sodium channel tail currents after aconitine treatment. Isochronal tail permeabilities determined

at pH 7.4 and 5.1 are shown. Prepulses to +75 mV were interrupted at the peak of current and the voltage was stepped to test potentials from -135 to +150 mV. Permeabilities were calculated from currents measured 60 μ s after the steps to test potentials. The smooth curve through the pH 5.0 points was obtained by scaling the control curve assuming voltage-independent block with an apparent pK of the blocking site of 4.51. This is close to the voltage-independent pK of 4.68 measured previously in normal muscle fibers at pH 5.0 (Campbell, 1982) and suggests that proton block of muscle sodium channels is not altered by aconitine.

It has been suggested that TTX blocks sodium channels by binding to the selectivity filter (Hille, 1975*c, d*). Thus, it might be expected that channels with modified selectivity might also have altered TTX sensitivity. Block of sodium channel currents in NH_4 Ringer was tested at four toxin concentrations in five fibers. The average K_d of toxin binding was 3.24 ± 1.29 (mean \pm SD, 17 measurements), which is close to values previously reported in frog nerve and muscle (Almers and Levinson, 1975; Jaimovich et al., 1976; Hahin and Strichartz, 1981). Additional support for the hypothesis that the K_d of toxin block is not changed by aconitine is lent by the observation that in individual fibers, block of inward NH_4 currents at -60 mV (which should exclusively represent currents through modified channels) was virtually identical to block of outward currents at +75 mV (which should have included currents through both modified and unmodified channels).

DISCUSSION

Kinetic Effects of Aconitine

The effects of aconitine on the kinetics of frog muscle sodium channels are similar to those previously described for myelinated nerve with an important difference. In agreement with results from nerve, aconitine shifts the voltage dependence of sodium channel activation 40-50 mV in the hyperpolarized direction and decreases the peak sodium channel conductance (Schmidt and Schmitt, 1974; Mozhayeva et al., 1976). In contrast to results from nerve (Schmidt and Schmitt, 1974; Mozhayeva et al., 1976; Mozhayeva et al., 1980), inactivation of aconitine-modified sodium channels in muscle is complete for depolarizations beyond -60 mV. This result is always observed both in sodium Ringer and in the various substituted Ringer solutions. It seems likely that this represents a difference between sodium channels in nerve and muscle rather than an artifact such as poor voltage control of a region of the T system. Thus, although activity in the T system might cause transient increases and decreases in the total current measured, if muscle sodium channels failed to inactivate, as they do in nerve, one would nevertheless expect a steady state sodium current after such transients. Such steady state currents are not seen in muscle. A similar nerve-muscle difference is seen with the venom from the scorpion *Leiurus quinquestriatus*: inactivation in nerve is slowed and incomplete (Koppenhöfer and Schmidt, 1968*a, b*), whereas inactivation in muscle is slowed but continues to completion (Catterall, 1979).

These results imply that either the sodium channels of frog nerve and muscle are structurally different or that some other difference between nerve and muscle cells modulates the response of sodium channels to these toxins.

The observation that, at a given voltage, aconitine-modified sodium channels open faster, with less sigmoidal delay than normal sodium channels, is also consistent with previous results from node (Schmidt and Schmitt, 1974). The usefulness of a detailed kinetic analysis of sodium conductance changes in aconitine-treated muscle is problematic because of the existence of two populations of channels that are not separable by pharmacological means. Thus, the records from aconitine-treated muscle shown in Figs. 4B–D contain various proportions of current through modified and normal sodium channels. Qualitatively, however, it is possible to say that the apparent shifts seen in the voltage dependence of h_{∞} (Fig. 3) and τ_h are consistent with the idea that at negative potentials aconitine slows the rate of return from the inactivated state.

Decreased Peak Conductance after Aconitine

Simultaneous with the appearance of sodium channels with modified kinetics and selectivity is an aconitine-induced decrease in peak sodium conductance. This decrease may be due to loss of a large fraction of channels caused by the aconitine treatment. However, since additional aconitine treatment does not result in further diminution of the conductance measured at -50 mV, such a progressive loss of channels seems unlikely. Alternatively, aconitine may reduce single-channel conductance without altering channel number, as has been observed in sodium channels modified by batrachotoxin (Khodorov et al., 1981) and by trimethyloxonium ion (Sigworth and Spalding, 1980). Without a measure of single-channel conductance it is not yet possible to choose between these alternatives.

Altered Sodium Channel Selectivity

The present results are consistent with previous determinations of relative permeability of aconitine-modified sodium channels in node calculated from shifts in the reversal potential. In nerve, aconitine-modified channels exhibit incomplete inactivation. Thus, in nerve, it is possible to determine independently the relative permeability of modified channels by making the current measurements after normal channels have inactivated. This is not possible in muscle; however, the relative size of currents at -50 mV, where few unmodified channels should be open, is consistent with the node results.

Hille (1971, 1972, 1975*a, b*) has shown how selectivity might be accomplished in a singly occupied channel. In this model, exclusion of impermeant ions is caused by molecular sieving at the narrowest constriction of the channel called the selectivity filter. In the present experiments, the largest ion found to be permeant to modified channels is the normally impermeant Cs. The slightly larger methylammonium ion does not contribute inward current through aconitine-modified sodium channels. In Hille's model of the sodium channel selectivity filter, the impermeability of the channel to Cs and methyl-

ammonium is explained by the hypothetical 3-Å width of the channel, which is too narrow to admit the 3.38-Å-diam Cs ion and the 3.6-Å methylammonium ion. Taking this approach, one would conclude that aconitine increases the minimum bore of the channel to between 3.4 and 3.6 Å.

Block of Modified Channels

In the selectivity filter model, the permeability sequence of ions that pass through the filter is determined by the height of the energy barrier traversed in passing through this constriction. This energy barrier depends in part on the energy of association of the ion with a negative site, presumably an ionized carboxylic acid, which is hypothesized to be part of the selectivity filter structure. This same negative site has been presumed to be where protons bind when blocking the channel (Woodhull, 1973). Since one way in which selectivity might be altered is by changing the field strength of this site, altered selectivity might be expected to be accompanied by altered proton block. For example, even if the only change in the hypothetical selectivity filter were to widen the bore by 0.4–0.6 Å, the degree of hydration and the electrostatic influence of nearby structures would be expected to be altered and thereby change the parameters of proton block.

Contrary to the prediction above, the results illustrated in Fig. 10 argue that block of sodium tail currents in muscle is not significantly altered by aconitine. Rather, in both normal (Campbell, 1982) and aconitine-treated muscle, block of tail currents at pH 5 is independent of voltage with an apparent pK of ~4.5–4.7. This observation suggests that either the proton blocking site is not intimately involved in channel selectivity, or at least that some other site, which is modified by aconitine, is also essential for determining channel selectivity.

In contrast to the present results, previous reports in frog nerve have concluded that aconitine substantially alters proton block of sodium channels (Naumov et al., 1979a; Mozhayeva et al., 1982b). Before considering an explanation for this discrepancy, it is first necessary to discuss the two different methods that have been used to measure block. When determined from the reduction of peak currents, the reduction of current at low pH has generally been found to be dependent on the voltage at which it is measured: the amount of reduction at any pH decreases with increasingly positive potential (Woodhull, 1973; Ulbricht and Wagner, 1975; Campbell and Hille, 1976; Carbone et al., 1978). The original interpretation of this apparent voltage dependence of block was that the proton binding site is partway through the membrane voltage drop, and as a result the access of protons to the site is dependent on membrane voltage (Woodhull, 1973). In frog muscle, however, this interpretation has been called into question by the observation that proton block, determined from tail currents, is independent of voltage (Campbell, 1982). This voltage-independent block is consistent with the idea that the site of block is at or near the outside of the sodium channel, where it does not experience the membrane field.

In frog nerve, Naumov et al. (1979a) found that block of aconitine-modified

channels did not exhibit the voltage dependence seen in block of sodium current peaks in untreated nerve. They found the degree of block of aconitine-modified channels at pH 4.77 to be equivalent to a pK of the blocking site of 4.5, nearly identical to the block of tail currents reported here at pH 5. They concluded that aconitine had altered the blocking site. However, an alternative explanation is suggested by the block of tail currents in normal and aconitine-treated muscle. In particular, tail current experiments in muscle have demonstrated that the voltage-dependent reduction of peak sodium currents in untreated muscle is accounted for by altered kinetics at low pH: at increasingly depolarized potentials, the number of channels open at the peak of current increases more steeply at low pH than it does at normal pH (Campbell, 1982). If this is also true in frog nerve, then the elimination of voltage-dependent block seen by Naumov et al. (1979a) may actually be a consequence of the aconitine effect on channel kinetics: the altered kinetics may simply reveal the voltage-independent block that is normally obscured by the effect of pH on the number of channels open at the peak sodium current.

More recently, Mozhayeva et al. (1982a, b) have reported block of tail currents to be somewhat voltage dependent both before and after aconitine treatment. Their present model of proton block of frog node has two blocking sites: one that does and one that does not exhibit voltage-dependent binding. They suggest that their results are consistent with the idea that aconitine modifies only the voltage-dependent site. However, their graphs of relative tail conductances vs. voltage have nearly identical shape before and after aconitine (which implies little change in voltage dependence). Their two-site model apparently has not yet been fitted to their tail current results from nerve and therefore a more detailed comparison with my results is difficult.

Currents through aconitine-modified channels in muscle are blocked by TTX with a K_d of 3 nM. This is nearly identical to values previously reported for frog nerve (Hahin and Strichartz, 1981) and frog muscle (Almers and Levinson, 1975; Jaimovich et al., 1976) and suggests, in agreement with previous results (Mozhayeva et al., 1976), that aconitine modifies sodium channel kinetics and selectivity without altering TTX binding. These results support the conclusion that the TTX binding site is separate from the site where selectivity takes place. Spalding (1980) reached the same conclusion from results in trimethyloxonium (TMO)-treated muscle fibers. TMO, which methylates carboxyl groups, greatly reduces the sensitivity of sodium channels to TTX without altering the relative permeability of the channel to several cations.

The Relationship between Gating and Selectivity

The functions of selectivity and gating are conceptually distinct, but there is no *a priori* reason to invoke separate channel structures to account for the two functions. The all-or-none behavior of currents through single sodium channels (Sigworth and Neher, 1980) and the insensitivity of selectivity to the kinetic state of the channel (Chandler and Meves, 1965) only require that the transition between the open- and closed-channel configurations is fast relative

to the time resolution of single-channel current measurements, and that there is a single open configuration of the channel interior. Although not conclusive, the evidence that aconitine (Mozhayeva et al., 1977), grayanotoxin I (Narahashi and Seyama, 1974; Hironaka and Narahashi, 1977), veratridine (Ulbricht, 1969; Naumov et al., 1979b), and batrachotoxin (Khodorov, 1979) all simultaneously alter kinetics and selectivity of sodium channels suggest that the two functions may be structurally related.

I wish to thank Dr. K. Beam for helpful discussions throughout this work, and Drs. Beam and R. Hahin for commenting on the manuscript. I thank Ms. Patty Hardy for assistance in preparing the manuscript.

Initial experiments were carried out with support from NS 07474 to Dr. W. K. Chandler and a postdoctoral fellowship from the Muscular Dystrophy Association. Supported by the Muscular Dystrophy Association and NS 15400.

Received for publication 18 January 1982 and in revised form 14 July 1982.

REFERENCES

- ALMERS, W., and S. R. LEVINSON. 1975. Tetrodotoxin binding to normal and depolarized frog muscle and the conductance of a single channel. *J. Physiol. (Lond.)* **247**:483-509.
- BRISMAR, T. 1977. Slow mechanism for sodium permeability inactivation in myelinated nerve fibre of *Xenopus laevis*. *J. Physiol. (Lond.)* **270**:283-297.
- CAMPBELL, D. T. 1976. Ionic selectivity of the sodium channel of frog skeletal muscle. *J. Gen. Physiol.* **67**:295-307.
- CAMPBELL, D. T. 1982. Do protons block sodium channels by binding to a site outside the pore? *Nature (Lond.)*. In press.
- CAMPBELL, D. T., and B. HILLE. 1976. Kinetic and pharmacological properties of the sodium channel of frog skeletal muscle. *J. Gen. Physiol.* **67**:309-323.
- CARBONE, E., R. FIORAVANTI, G. PRESTIPINO, and E. WANKE. 1978. Action of extracellular pH on Na⁺ and K⁺ membrane currents in the giant axon of *Loligo vulgaris*. *J. Membr. Biol.* **43**:295-315.
- CATTERALL, W. A. 1979. Binding of scorpion toxin to receptor sites associated with sodium channels in frog muscle. *J. Gen. Physiol.* **74**:375-391.
- CHANDLER, W. K., and H. MEVES. 1965. Voltage clamp experiments on internally perfused giant axons. *J. Physiol. (Lond.)* **180**:788-820.
- HAHIN, R., and L. GOLDMAN. 1978. Initial conditions and the kinetics of the sodium conductance in *Myxicola* giant axons. I. Effects on the time-course of the sodium conductance. *J. Gen. Physiol.* **72**:863-877.
- HAHIN, R., and G. R. STRICHARTZ. 1981. Effects of deuterium oxide on the rate and dissociation constants for saxitoxin and tetrodotoxin action: voltage-clamp studies on frog myelinated nerve. *J. Gen. Physiol.* **78**:113-139.
- HILLE, B. 1968. Charges and potentials at the nerve surface: divalent ions and pH. *J. Gen. Physiol.* **51**:221-236.
- HILLE, B. 1971. The permeability of the sodium channel to organic cations in myelinated nerve. *J. Gen. Physiol.* **58**:599-619.
- HILLE, B. 1972. The permeability of the sodium channel to metal cations in myelinated nerve. *J. Gen. Physiol.* **59**:637-658.
- HILLE, B. 1975a. Ionic selectivity, saturation and block in sodium channels: a four-barrier

- model. *J. Gen. Physiol.* **66**:535–560.
- HILLE, B. 1975*b*. Ionic selectivity of Na and K channels of nerve membranes. In *Membranes—A Series of Advances*. Vol. 3: Dynamic Properties of Lipid Bilayers and Biological Membranes. G. Eisenman, editor. Marcel Dekker, Inc., New York. 255–323.
- HILLE, B. 1975*c*. The receptor for tetrodotoxin and saxitoxin; a structural hypothesis. *Biophys. J.* **15**:615–619.
- HILLE, B. 1975*d*. An essential ionized acid group in sodium channels. *Fed. Proc.* **34**:1318–1321.
- HILLE, B., and D. T. CAMPBELL. 1976. An improved vaseline gap voltage clamp for skeletal muscle fibers. *J. Gen. Physiol.* **69**:265–293.
- HIRONAKA, T., and T. NARAHASHI. 1977. Cation permeability ratios of sodium channels in normal and grayanotoxin treated squid axon membranes. *J. Membr. Biol.* **31**:359–381.
- HODGKIN, A. L., and A. F. HUXLEY. 1952*a*. Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J. Physiol. (Lond.)*. **116**:449–472.
- HODGKIN, A. L., and A. F. HUXLEY. 1952*b*. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol. (Lond.)*. **117**:500–544.
- JAIMOVICH, E., R. A. VENOSA, P. SHRAGER, and P. HOROWICZ. 1976. Density and distribution of tetrodotoxin receptors in normal and detubulated frog sartorius muscle. *J. Gen. Physiol.* **67**:399–416.
- KHODOROV, B. I. 1979. Further analysis of the mechanisms of action of batrachotoxin on the membrane of myelinated nerve. *Neuroscience*. **4**:1315–1330.
- KHODOROV, B. I., B. NEUMCKE, W. SCHWARZ, and R. STÄMPFLI. 1981. Fluctuation analysis of Na channels modified by batrachotoxin in myelinated nerve. *Biochim. Biophys. Acta.* **648**:93–99.
- KOPPENHÖFER, E., and H. SCHMIDT. 1968*a*. Die Wirkung von Skorpiengift auf die Ionenströme des ranvierischen Schnürrings. I. Die Permeabilitäten P_{Na} und P_K . *Pflügers Arch. Eur. J. Physiol.* **303**:133–149.
- KOPPENHÖFER, E., and H. SCHMIDT. 1968*b*. Die Wirkung von Skorpiengift auf die Ionenströme des ranvierischen Schnürrings. II. Unvollständige Natrium-Inaktivierung. *Pflügers Arch. Eur. J. Physiol.* **303**:150–161.
- MOZHAYEVA, G. N., A. P. NAUMOV, and YU. A. NEGULYAEV. 1976. Effect of aconitine on some properties of sodium channels in the Ranvier node membrane. *Neurophysiol. (Kiev)*. **8**:152–160.
- MOZHAYEVA, G. N., A. P. NAUMOV, and YU. A. NEGULYAEV. 1982*a*. Interaction of H^+ ions with acid groups in normal sodium channels. *Gen. Physiol. Biophys.* **1**:5–19.
- MOZHAYEVA, G. N., A. P. NAUMOV, and YU. A. NEGULYAEV. 1982*b*. Interaction of H^+ ions with acid groups in aconitine-modified sodium channels. *Gen. Physiol. Biophys.* **1**:21–35.
- MOZHAYEVA, G. N., A. P. NAUMOV, YU. A. NEGULYAEV, and E. D. NOSYREVA. 1977. Permeability of aconitine-modified sodium channels to univalent cations in myelinated nerve. *Biochim. Biophys. Acta.* **466**:461–73.
- MOZHAYEVA, G. N., A. P. NAUMOV, and E. D. NOSYREVA. 1980. Some features of kinetic and steady-state characteristics of aconitine-modified sodium channels. *Neurophysiol. (Kiev)*. **12**:612–618.
- NARAHASHI, T., and I. SEYAMA. 1974. Mechanism of nerve depolarization caused by grayanotoxin I. *J. Physiol. (Lond.)*. **242**:471–487.
- NAUMOV, A. P., YU. A. NEGULYAEV, and E. D. NOSYREVA. 1979*a*. Change in sodium channel acid group affinity for hydrogen ions under the action of aconitine. *Dokl. Akad. Nauk. SSSR.* **244**:229–233.

- NAUMOV, A. P., YU. A. NEGULYAEV, and E. D. NOSYREVA. 1979b. Change of selectivity of sodium channels of nerve fiber treated with veratrine. *Tsitologia*. **21**:692-696.
- SCHMIDT, H., and O. SCHMITT. 1974. Effect of aconitine on the sodium permeability of node of Ranvier. *Pflügers Arch. Ges. Physiol.* **349**:133-148.
- SIGWORTH, F. J. 1980. The conductance of sodium channels under conditions of reduced current at the node of Ranvier. *J. Physiol. (Lond.)*. **307**:131-142.
- SIGWORTH, F. J., and E. NEHER. 1980. Single Na⁺ channel currents observed in cultured rat muscle cells. *Nature (Lond.)*. **287**:447-449.
- SIGWORTH, F. J., and B. C. SPALDING. 1980. Chemical modification reduces the conductance of sodium channels in nerve. *Nature (Lond.)*. **283**:293-295.
- SPALDING, B. C. 1980. Properties of toxin-resistant sodium channels produced by chemical modification in frog skeletal muscle. *J. Physiol. (Lond.)*. **305**:485-500.
- ULBRICHT, W. 1969. The effect of veratridine on excitable membranes of nerve and muscle. *Ergeb. Physiol. Biol. Chem. Exp. Pharmacol.* **61**:18-71.
- ULBRICHT, W., and H.-H. WAGNER. 1975. The influence of pH on equilibrium effects of tetrodotoxin on myelinated nerve fibres of *Rana esculenta*. *J. Physiol. (Lond.)*. **252**:159-184.
- WOODHULL, A. M. 1973. Ionic blockage of sodium channels in nerve. *J. Gen. Physiol.* **61**:687-708.