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Pressure Dependence of the Sodium Currents of Squid Giant Axon

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Summary. The effects of hydrostatic pressures up to 62 MPa upon the voltage-clamp currents of intact squid giant axons were measured using mineral oil as the pressure transmitting medium. The membrane resistance and capacitance were not appreciably affected over the whole range of pressures explored. The predominant effect of pressure is to slow the overall kinetics of the voltage-clamp currents. Both the early (Na) currents and the delayed (K) ones were slowed down by approximately the same time scale factor, which was in the range of 2 to 3 when pressure was increased from atmospheric to 62 MPa.

Finer details of the effects, most evident at moderate depolarizations, are: the apparent initial delay in the turn-on of Na currents is increased by pressure *less* than is the phase of steepest time variation, and the later decay is slowed *more* than is the rising phase. The initial time course of the currents at high pressures can be made to overlap with that at normal pressure by a constant time compression factor, Θ_m, together with a small, voltage-dependent delay.

In a given axon, Θ_m was fairly independent of voltage, and it increased exponentially with pressure according to an apparent activation volume, $\Delta V^{\pm}$, ranging between 32 and 40 cm^3/mole. $\Delta V^{\pm}$ tended to decrease with increasing temperature. Contrary to what is observed for moderate or large depolarizations, the kinetics of Na inactivation produced by conditioning prepulses of -50 or -60 mV was little affected over the whole range of pressures explored.

Inferences about the pressure dependence of the steady-state Na activation were made from the comparison of the plots of early peak currents, I_p, versus membrane potential, E. The Na reversal potential, E_{Na}, and the slope of the plots near E_{Na} did not change significantly with pressure, but the peak Na conductance *vs.* E relationship was shifted by about $+9$ mV upon increasing pressure to 62 MPa. Steady-state Na inactivation, h_∞, was slightly affected by pressure. At 62 MPa the midpoint potential of the $h_\infty(E)$ curve, E_h, was shifted negatively by about 4 mV, while the slope at E_h decreased by about 38%.

Under the tentative assumption that pressure directly affects the gating of Na channels, the Na activation data follows a simple Hodgkin-Huxley scheme if the opening of an *m gate* involves an activation volume of about 58 $Å^3$ and a net volume increase of about 26 $Å^3$. However, a self-consistent description of the totality of the effects of pressure on Na inactivation cannot be obtained within a similar simple context.

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Introduction

High hydrostatic pressures have been long known to produce marked modifications of nerve excitability (Ebbecke & Schaefer, 1935; Grundfest, 1936). The work of Spyropoulos (1957*a*, *b*) on single myelinated and unmyelinated nerve fibers led him to conclude that the major effect of high pressures was the prolongation of the duration of the nerve action potential. More recently, Henderson and Gilbert (1975) studied the changes of voltage-clamp currents in squid giant axons subjected to gas (helium) pressures of up to 21 MPa and Harper, Macdonald and Wann (1981) performed similar studies on *Helix* neurones using hydrostatic (oil) pressures. A general review of the literature related to the effects of pressure on excitable cells has been published by Wann and Macdonald (1980).

The dependence on pressure of the kinetics of a simple chemical reaction yields a direct measurement of the volume change, $\Delta V^{\pm}$, associated with the formation of the activated (transition) state of the reaction (Johnson, Eyring & Polissar, 1954; Johnson, Eyring & Stover, 1974; Le Noble, 1967; Asano & Le Noble, 1978). Measurements of activation volumes provide useful information to discriminate between possible complex molecular reaction pathways such as those involved in enzymic catalysis (Low & Somero, 1975).

The currently accepted view of the process of nerve excitation is that the underlying changes in ionic membrane permeability involve structural modifications of specialized membrane components which form, in certain particular conformational states, ion-selective aqueous pores spanning the membrane (Armstrong, 1975*b*; Hille, 1975). The most direct evi-

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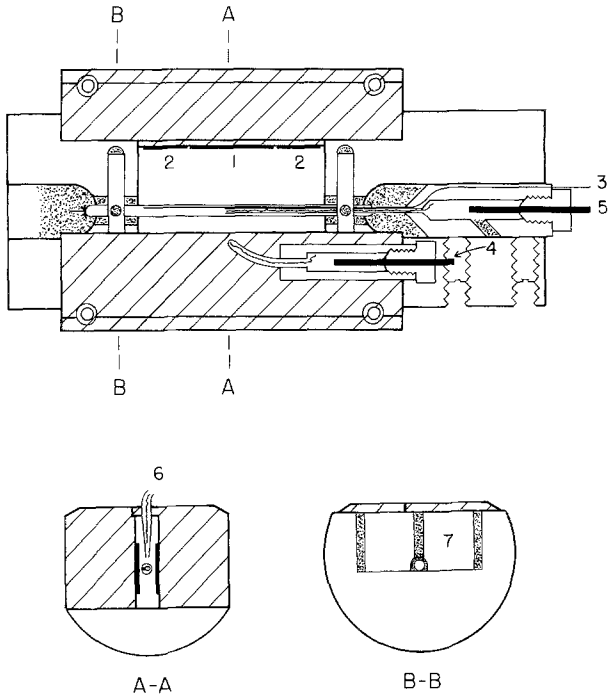

Fig. 1. Schematic drawing of chamber, electrodes, and axon, approximately to scale with radial dimension of axon and internal electrodes exaggerated. Top view shows covers and attached lateral walls widely separated, as they are during manipulation and impalement of an axon. The internal electrode assembly is shown in its final, rigidly fixed position. Cross sections show closed configuration of the chamber as it is when within the pressure bomb. The cover-walls are held tightly in place by screws passing through holes in the cover, top view, and into chamber body. Hatched areas denote movable cover and lateral walls; strippled regions denote silicone grease. (*1*) Pt/Pt black external current measuring electrode; (*2*) Pt/Pt black external guard electrodes; (*3*) internal current wire; (*4*) Ag/AgCl external reference electrode; (*5*) Ag/AgCl internal voltage electrode; (*6*) polyethylene external voltage probe; (*7*) Perspex partition

dence for the discrete nature of ionic channels in nerve is the observation of single channel events (Conti & Neher, 1980; Sigworth & Neher, 1980; Horn, Patlak & Stevens, 1981). According to this picture, the kinetic parameters of the Hodgkin-Huxley (HH) equations (Hodgkin & Huxley, 1952*a*) are related to the rate constants of channel isomerizations; the effects of pressure might yield information regarding the activation volumes of these conformational changes. The experiments of Henderson and Gilbert (1975) were indeed aimed at such a characterization, but the use of helium gas as the pressure transmitting medium severely limited the accuracy and quantitative reproducibility of the results and added the possible complication of specific effects of helium *per se* (Macdonald, 1975; Mastrangelo, Trudell & Cohen, 1978).

In this and the following paper (Conti, Fioravanti, Segel & Stühmer 1982) we present the results of an investigation of the reversible effects on the ionic currents of the squid giant axon of hydrostatic (oil) pressures up to 62 MPa. The present paper concentrates on the pressure dependence of the early phase of voltage-clamp currents, associated with the transitions of the Na channels.

A brief report of these experiments, based on the analysis of analog data from several axons, has been presented elsewhere (Conti, Fioravanti, Segal, Stühmer, 1980). The present work contains the detailed analysis of six experiments in which nerve stimulation and data acquisition were performed digitally with the aid of a microprocessor.

Materials and Methods

Nerve Preparation and Electronics

The experiments were performed on giant axons of the squid *Loligo vulgaris*, available in Camogli. The intact axons, completely freed of all adherent small nerve fibers, were mounted in a Perspex chamber, illustrated in Fig. 1. During preliminary operations, the chamber was placed within a special holder which was aligned with the manipulator used for impalement of the axon by the intracellular electrodes. These were of the piggy-back type (Chandler & Meves, 1965), consisting of a platinized Pt wire (80 μm diameter and 18 mm exposed length) and a glass pipette (50–80 μm OD) filled with 0.5 M KCl and containing a floating 20- μm platinized Pt wire. The external voltage electrode consisted of a small diameter polyethylene tube filled with seawater; its opening was located less than 1 mm from the central axon surface. Both the intracellular pipette and the extracellular tubing were connected to reference Ag–AgCl electrodes. The external current electrodes were platinized Pt foils, glued to the two lateral walls of the inner chamber space. Two central foils (5 × 9 mm) were connected to a virtual ground to measure axon currents, while the four lateral foils (5 mm × 4.5 mm) served as guard electrodes (Moore & Cole, 1963). After impalement of the axon, the Perspex shank of the internal electrode assembly was tightly fixed to the chamber and disconnected from the manipulator. At the two ends where the axon emerged from the measuring chamber, it passed through small grooves within movable Perspex partitions. These were sealed with silicone grease to prevent displacement of the extracellular fluid by the hydraulic oil. Then, the lateral walls and covers of the chamber were drawn together. In the final configuration, the axon extended 20 mm along the central axis of a parallelepiped with 7 × 2 × 20 mm linear dimensions, filled with artificial seawater of the following composition: 450 mM NaCl, 10 mM KCl, 50 mM $CaCl_2$, and 1 mM Tris Cl; pH 7.8.

The voltage-clamp circuit followed standard schemes (Moore & Cole, 1963), including compensation of the resistance in series with the axolemma (Hodgkin, Huxley & Katz, 1952). The passive (linear) properties of the axon were characterized by the voltage response to square pulses of currents. A transient linear response simulator was used to subtract analogically most of the capacitive response of the axon. The resulting response was then usually filtered with a 10 kHz bandwidth low-pass filter.

Pressure Apparatus

After testing the physiological state of the preparation with a series of voltage-clamp pulses, the chamber-electrode assembly was dis-

connected from its holder and immersed gently in the mineral oil which filled the pressure vessel. This was essentially a cylinder with inner diameter of 17.4 mm and outer diameter of 25.4 mm. It and the remainder of the hydraulic pressurizing system were very similar to that used by Segal (1977), consisting of commercially available components (Autoclave Engineers, Erie, Pa.). Electrical connections between the axon chamber and the electronic apparatus were made by electrical feedthroughs (Conax, Buffalo, N.Y.). A valve located at the highest point of the bomb allowed removal of residual air. In the final arrangement the sealed cylinder containing the chamber and the axon was rotated to the horizontal position.

A hand-driven pump was employed to pressurize the system. By manipulating the valves of the apparatus it is possible to change the pressure within 150 msec but we chose to produce slower, gradual increments – 20 MPa within times of the order of 30 sec – in order to avoid mechanical artifacts, to which the voltage electrodes seemed particularly susceptible. Pressure was continuously monitored with a Bourdon tube pressure guage with 0.17 MPa accuracy.

The effect of pressure on electrode potentials was tested periodically by placing the electrodes in the chamber without the axon, and those electrodes showing a change of potential difference of more than 0.5 mV at the highest pressures employed were discarded. The best indication, albeit indirect, that electrode artifacts during actual experiments must have been small was the observation that the reversal potential of the Na currents did not change by more than 3 mV in those experiments in which the axon showed full recovery of its initial physiological state after return to atmospheric pressure. However, we cannot totally exclude the presence of artifacts of this order of magnitude. For this reason, not much confidence can be placed in the measurements of absolute membrane potential shifts of the order of a few mV.

The temperature in the vessel was regulated to a fixed value during the experiment by circulating thermostated water around the pressure vessel. In preliminary experiments the temperature near the axon was monitored continuously with a thermistor and we found that, following a rapid pressure change of 62 MPa, it rose by 2 to 3 °C (due to adiabatic heating), but it returned to the original value within about 2 min. Temperature re-equilibration was particularly fast in our system because of the very large surface-to-volume ratio of the pressure vessel. For comparison, it should be noted that the pressure vessels used by Harper et al. (1981) had a cross-sectional area 25 times larger than ours. In all the experiments discussed in this work, electrical recordings were not made until 3 min had elapsed following a pressure change.

Stimulation and Data Acquisition

Voltage-clamp current measurements were made using automatic sequences of clamp pulses of variable amplitude and duration, separated by intervals of 0.5 to 2 sec, generated by an 8-bit digital-to-analog converter controlled by a microprocessor. Three different pulse routines were used for the study of: (i) the activation-inactivation time course of Na currents for various pulse potentials, E_p, in the range of -40 to $+80$ mV; (ii) the steady-state Na inactivation in the range $-100 \leqq E \leqq -30$ mV; (iii) the time course of Na inactivation as a function of the duration, t_{pp}, of prepulses to -60 or -50 mV. The first routine included a series of 13 depolarizing pulses with fixed duration (2 to 6 msec, depending on temperature and pressure) from a holding membrane potential, E_H, of -80 mV. The second routine contained a sequence of eight double pulses, the second pulse bringing the membrane potential to -10 mV for a fixed duration (~ 2 msec), while the prepulse level, E_{pp}, varied between -100 and -30 mV in steps of 10 mV and lasted about 150 msec. The third routine consisted of a similar double pulse sequence with prepulse levels fixed at -50 or -60 mV and durations, t_{pp}, increasing roughly exponentially with the pulse index from 0 to 24 msec.

The voltage-clamp currents were converted into sequences of 256 or 512 digital data blocks by an analog-to-digital converter (12 bit) and stored temporarily in a shift register buffer with a total capacity of $12 \times 1{,}024$ bits. At the end of each data block acquisition, the content of the buffer was transferred to an FM tape recorder for subsequent off-line analysis.

Data Analysis

The data were formally analyzed according to the HH equations. Four parameters were extracted for the characterization of the early phase of each voltage-clamp current record: (i) the time to the peak, t_p; (ii) the amplitude of the peak current, I_p; (iii) the time to the first inflection point, t_f; (iv) the time derivative of the current at t_f; i_f. These parameters were extracted from least squares fits with cubic parabolas of the appropriate section of the early currents. The values of I_p, measured as a function of E_{pp} or t_{pp}, were fitted with HH theoretical curves using programs for the least squares fit of exponentials.

Results

Passive Membrane Properties

The linear properties of the axons were measured from the change in membrane potential caused by square hyperpolarizing current-clamp pulses through the central region of the axon superposed on the dc membrane currents which held the membrane potential in the range of -80 to -100 mV. Two types of current pulses were used: (i) short (50 μsec) pulses of large amplitude (50 μA, equivalent to 0.22–0.32 mA/cm^2) to characterize the membrane capacitance, C_m, and the impedance in series with it and (ii) long (15 msec) pulses of small amplitude (1 μA) to characterize the dc membrane resistance, R_m, and the time constant $R_m C_m$. Whenever the effects of pressure were fully reversible, none of these parameters was appreciably changed upon increasing pressure up to 62 MPa, in fair agreement with Spyropoulos (1957*b*), who found large decreases in R_m only at higher pressures. In two experiments, we observed a progressive decrease of R_m, which was not obviously related to pressure but was merely the sign of deterioration of the preparation. In these cases, too, C_m was not changed appreciably. Considering the accuracy of measurements of C_m derived from Polaroid pictures, we can conclude that any change of C_m upon increasing pressure to 62 MPa was smaller than 3%.

General Effect of Pressure on Voltage-Clamp Currents

The overall effect of hydrostatic pressure upon the voltage-clamp currents of a squid giant axon is illustrated in Fig. 2. This shows the responses of a single axon to six different step depolarizations from a holding potential of -80 mV, measured at atmospheric

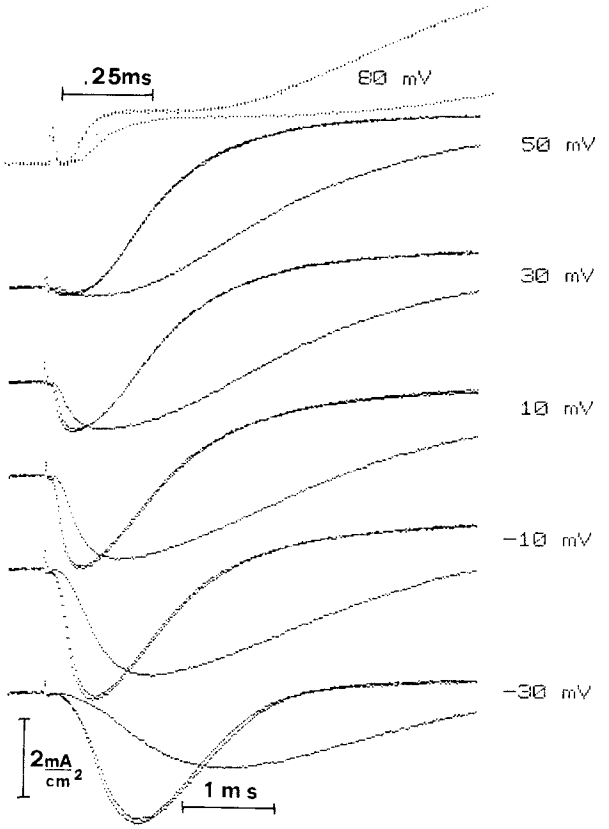

Fig. 2. Voltage-clamp responses of a single axon to the indicated depolarizations from a holding potential of −80 mV obtained at normal pressure, at 62 MPa, and again at normal pressure following decompression. The currents at high pressure are clearly distinguishable from those at atmospheric pressure by the much slower time course of the former. The control responses after decompression are distinguished, at the four smaller depolarizations, by their slightly smaller inward currents. The amplitude calibration is the same for all curves. The time calibration at the bottom of the figure pertains to all curves except those at 80 mV, for which a different calibration is given at the top of the figure. The data were recorded digitally with a 10 µsec sampling period. Axon 1, temperature: 10 °C

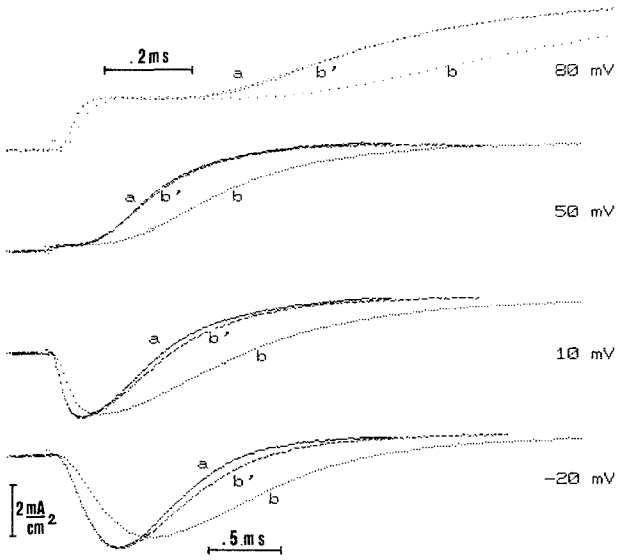

Fig. 3. Voltage-clamp responses of a single axon to the indicated step depolarizations from a holding potential of −80 mV obtained at normal pressure (*a'*) and at 41 MPa (*b*). The data of curves *b* are also replotted as curves *b'* after having been multiplied by an amplitude scaling factor, compressed in time by a time scaling factor, and slightly delayed. The amplitude scaling factors are: 1.13 at −20 mV; 1.05 at 10 mV; 1.00 at 50 mV; 1.00 at 80 mV. The time scaling factors are 1.52 at −20 mV; 1.61 at 10 mV; 1.59 at 50 mV; and 1.59 at 80 mV. The delays used are 30 µsec at −20 mV; 25 µsec at 10 mV; 0 µsec at 50 mV; and 15 µsec at 80 mV. (*See* text for a more detailed description.) Axon 3, temperature: 15 °C

pressure, at 62 MPa, and again after decompression. All responses were measured at 10 °C. It is seen that the changes induced at the highest pressures explored in the present study are fully reversible. The control measurements shown in the figure were made 10 min after the return to normal pressure, and further control measurements after an additional 10 min did not reveal any obvious change. However, the same measurements performed only 3 min after decompression showed that the recovery of normal kinetics was 5 to 10% incomplete, most likely because of incomplete temperature re-equilibration. Figure 2 shows that the primary effect of pressure is to slow all phases of the voltage-clamp currents to about the same degree. The currents following step depolarizations to 50 mV are mainly carried by K ions, and the analysis of this component of membrane current will be described in more detail separately (Conti et al., 1982). However, it is important to stress here that pressure does not increase the overlap between Na and K currents, since both components are little affected in amplitude, but both are slowed down to about the same extent.

Independent of the assumption of any particular analytical expression for the time course of the voltage-clamp currents, a direct and simple characterization of their modifications by hydrostatic pressure can be obtained as illustrated in Fig. 3. This figure shows records of responses to step depolarizations ($E_p = -20$ mV, 10 mV, 50 mV, 80 mV; $E_H = -80$ mV) in a single axon at 15 °C, at atmospheric pressure (*a*) and at 42 MPa (*b*). Each response at 42 MPa is also shown (as *b'*) after having been scaled in amplitude by a factor close to unity (1 to 1.13), compressed in time by a factor ranging between 1.52 and 1.61, and finally slightly delayed by 15 to 30 µsec. It is seen that the curves labelled *b'* superimpose almost exactly upon the records at normal pressure except for a small but significant mismatch in the falling phases of the inward currents for $E_p = -20$ mV and $E_p = 10$ mV. Thus, apart from a small change

in the peak inward currents at small depolarizations, which will be discussed in more detail later, the data of Fig. 3 show three distinct features of the effect of pressure upon the kinetics of Na currents: (i) a major slowing down of the rising phase; (ii) a smaller prolongation of the delay; (iii) a slightly more pronounced effect on the decay phase, associated with inactivation. These individual features are discussed below in some detail.

Effect on τ_m

The method of analysis illustrated in Fig. 3 was applied only to three series of standard voltage-clamp curves, obtained from different axons at 42 or at 62 MPa. In all other cases, the early phase of the Na currents was characterized with three time parameters, obtained from least squares fits of the appropriate segments of the responses with cubic parabolas. These parameters are: (i) the time to the peak early current, t_p; (ii) the time to the inflection point, t_f; (iii) the ratio between the peak current, I_P, and the time derivative of the current at the inflection point, $t_1 = I_p/\dot{I}_f$. According to the HH equations, and assuming zero Na activation at the holding membrane potential, each of these three parameters is the product of the Na activation time constant τ_m, times a function of the ratio, τ_h/τ_m, where τ_h is the time constant of Na inactivation.

However, since t_p, t_f and t_1 have a very weak dependence on τ_h/τ_m, their changes reflect mainly changes in τ_m. Furthermore, both t_1 and the difference, $t_p - t_f$, are independent of possible delays (both positive or negative) in the onset of the HH kinetics of Na current. Since t_1 is least sensitive to changes in τ_h/τ_m, it was used to characterize the changes in τ_m produced by changes in pressure, while the comparison between the pressure dependence of $t_p - t_f$ and that of t_1 was used to gain some insight into possible changes in τ_h/τ_m.

In the three experiments for which the data were directly analyzed according to the method of Fig. 3, we found that the ratio of the t_1 values measured at different pressures for depolarizations in the range of -10 to $+20$ mV provided the time scaling factor that produced the best superposition of the corresponding data, as judged by eye. In this range the t_1's were practically independent of voltage for all experiments. For larger depolarizations the accuracy of the t_1 estimates was much reduced, but the direct comparison of the responses as in Fig. 3 showed that the same time scaling factor gave an excellent description of the effect of pressure.

For depolarizations smaller than $E_p = -20$ mV the effect of pressure upon t_1 and/or upon the time scaling factor appeared to depend more on membrane potential. In most cases the increase of τ_m with pressure appeared to be smaller, but the data in this voltage range had a large scatter because of two probable artifacts: spatial nonuniformities of the voltage-clamp system, which are always difficult to rule out completely in good axons at small depolarizations; and small dc shifts of electrode potentials which would produce the largest effects at low depolarizations where the voltage dependence of τ_m is steepest.

Due to the above limitations the only reliable quantitative description of our present data concerns the pressure induced change in the characteristic time constant of Na activation for $E_p \geqq -20$ mV. This change was estimated from the mean ratio, Θ_m, in the range $-20 \leqq E_p \leqq 10$ mV, between the t_1 values measured at various pressures and the respective values at atmospheric pressure. The data are reported in Table 1, where the values in parentheses refer to Θ_m estimates with respect to the control measurements after decompression. Observe the fair agreement between these values and those out of parentheses, which are relative to the initial measurements at atmospheric pressure. However, the control values are generally slightly lower, indicating a small systematic bias in the direction to be expected if the control

Table 1. Change with pressure of the characteristic rise time, t_1, of early currents[a]

Axon	T (°C)	P (MPa)	$t_1(P)/t_1(0.1)$	Partial means
6	5	21	1.68 (1.38)	1.53
1	10	21	1.37 (1.42)	1.30
2	10	21	1.34 (1.05)	
5	15	21	1.22 (1.20)	1.21
Mean	10	21	1.33 (±0.19)	
4	5	42	2.02 (1.58)	1.99
6	5	42	2.39 (1.96)	
1	10	42	1.98 (1.57)	1.75
2	10	42	1.92 (1.51)	
3	15	42	1.62 (1.43)	1.61
5	15	42	1.71 (1.68)	
Mean	10	42	1.78 (±0.28)	
6	5	62	3.34 (2.74)	3.04
1	10	62	2.21 (2.11)	2.47
2	10	62	3.12 (2.45)	
3	15	62	2.58 (2.27)	2.43
5	15	62	2.45 (2.41)	
Mean	11	62	2.57 (±0.40)	

[a] The data in column 4 give the ratio between t_1 values measured at pressure P and those measured at atmospheric pressure (0.1 MPa) before pressurization (data out of parentheses) and after decompression (data in parentheses); mean (±SD). Column 5 gives average values of $t_1(P)/t_1(0.1)$ measured at the three different temperatures indicated in column 2.

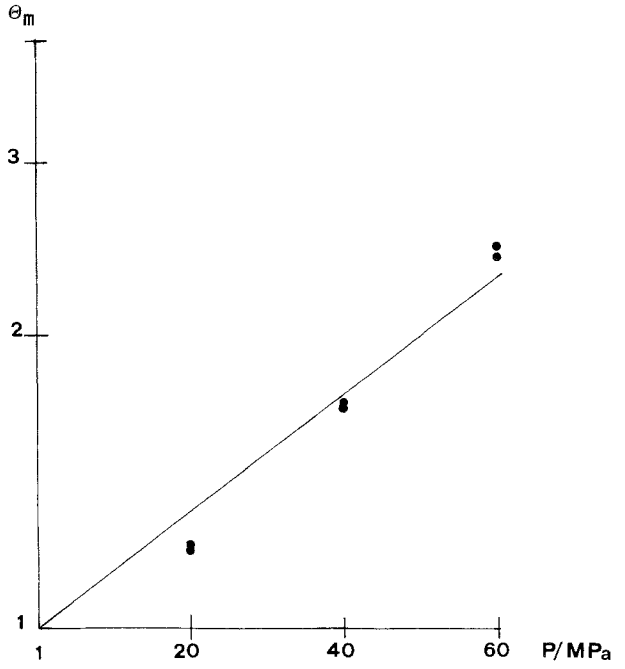

Fig. 4. Plot of the mean factoral change with pressurization of the characteristic time constant, t_1, of the rising phase of inward currents in the range of $-20 \leqq E_p \leqq 10$ mV. Θ_m is the mean value of $t_1(P)/t_1(0.1)$ measured at different pressures, P, and referred to the initial (upper points) and final measurements at normal pressure. Axon 5; temperature: 15 °C. The straight line was drawn according to Eq. (4), with $\Delta V^{\pm} = 32$ cm^3/mole

measurements were made at a slightly lower temperature. Figure 4 is a semilogarithmic plot of Θ_m as a function of pressure, P, for one experiment in which pressure was increased to 21, 42, and 62 MPa, and then returned to normal. The straight line was drawn through the data according to the best fit of the equation:

$$\ln \Theta_m = P\Delta V^{\pm}/RT \tag{1}$$

where R is the gas constant, T is absolute temperature and the apparent activation volume, $\Delta V^{\pm}$, is equal to 32 cm^3/mole, yielding an e-fold change of Θ_m for a pressure change of 73.6 MPa. Similar fits for the mean values of Θ_m at the three different temperatures explored in this work, given in the last column of Table 1, yield activation volumes of 30 cm^3/mole at 15 °C, 35 cm^3/mole at 10 °C, and 44 cm^3/mole at 5 °C. Owing to the limited amount of data at different temperatures, we do not want to emphasize too much the temperature dependence of $\Delta V^{\pm}$. The overall means for all the data of Table 1 yield a $\Delta V^{\pm}$ of about 35 cm^3/mole.

Effect on the Initial Delay

If the initial value of the activation parameter, m_o, is not zero, then the HH equations predict that t_p and t_f are both decreased by the same amount, t_o, given by:

$$t_o = \tau_m \ln(1/(1-m_o/m_\infty)) \tag{2}$$

where m_∞ is the steady-state Na activation at the clamp potential. Equation (2) shows that a time scaling equal to the factor by which τ_m is affected by pressure would not properly account for the change in t_o if the ratio m_o/m_∞ is changed by pressure. However, the voltage dependence of the corrective delay needed in order to obtain superposition of the early Na currents at different pressures was not fully consistent with Eq. (2). Thus, although we have no data on the actual value of m_o at -80 mV, we expected from the shift of the activation curves discussed later than m_o should, if anything, decrease upon increasing pressure. Consequently, the corrective delay should vanish, or even reverse, upon increasing the depolarization to levels for which $m_\infty = 1$, and it should do so independent of pressure. In our measurements the corrective delay did indeed decrease with increasing E_p, from values in the range of 50 to 100 μsec to values of the order of 15 to 20 μsec, but it never vanished, even for $E_p = 80$ mV. Furthermore, we found that the corrective delay increased when the holding potential, E_H, was changed from -60 to -100 mV, again in contrast to Eq. (2) which predicts that $t_o \to 0$ for $m_o \to 0$ (at large holding hyperpolarizations). A holding potential dependent delay in the onset of the HH type of kinetics of Na currents has been described by Keynes and Rojas (1976). If such a delay is increased by pressure less than τ_m is, then it would account for the corrective delay described here.

Kinetics of Na Inactivation

The time course of the inactivation of Na currents for $E_p > -40$ mV could not be measured with any reasonable accuracy from the voltage-clamp currents because of the presence of K currents. However, some clear inference about the relative effect of pressure on activation and inactivation kinetics can still be obtained from our data: for $E_p \geqq -30$ mV the process of Na inactivation is slowed by pressure at least as much or slightly more than that of Na activation.

A direct demonstration of this conclusion is obtained from inspection of the data of Fig. 3 for $E_p = -20$ mV and $E_p = 10$ mV. It is seen that the time compression of the responses at 42 MPa which produced a good superposition of their early phases (for $t \leqq t_p$) with those of the responses at normal pressure failed to produce as good an overlap of the falling phase of the inward currents, showing that this phase is slowed down by pressure more than the early one.

Since the rise in K conductance might contribute substantially to the decay of inward current, this result could also be interpreted as the consequence of a more pronounced slowing effect of pressure upon K currents than upon Na currents. However, in this particular axon the same time scaling factor described equally well the slowing down of both Na and K currents, a feature which was fairly well reproduced in most axons. Furthermore, as shown in the next paper (Conti et al., 1982), the amplitude of the late K current is, if anything, increased by pressure, which could only produce an apparent acceleration of the decay of Na currents. Therefore, the data of Fig. 3 force us to conclude that Na inactivation at $E_p = -20$ mV or $E_p = 10$ mV is slowed down by pressure slightly more than is Na activation, while at $E_p = 80$ mV the two processes are affected by pressure to a comparable degree.

Confirming the above conclusions was the invariable finding in all experiments that $t_p - t_f$ was increased by pressure more than was t_1. Since $t_p - t_f$ is a much more steeply increasing function of τ_h/τ_m than is t_1, the result implies that τ_h/τ_m is increased by pressure.

In apparent contrast to the above results, τ_h measured for conditioning membrane potentials of -60 and -50 mV, according to the classical method of Hodgkin and Huxley (1952b), showed very little dependence on pressure, as was reported by Harper et al. (1981) for *Helix* neurons. This is illustrated in Fig. 5 which shows semilogarithmic plots of peak current, I_p, as a function of the duration of prepulses at -50 mV from a holding potential of -80 mV. The data is from two different axons at atmospheric pressure and at 62 MPa, at 10 °C. To emphasize the exponential decay of peak currents for increasing prepulse duration, t_{pp}, the data are plotted as: $(I_p(t_{pp}) - I_p(\infty))/(I_p(0) - I_p(\infty))$. The values of $I_p(0)$ and $I_p(\infty)$ were determined in each case from the direct least squares fit of $I(t_{pp})$ according to the equation:

$$I_p(t_{pp}) = I_p(\infty) + \{I_p(0) - I_p(\infty)\} \exp(-t_{pp}/\tau_h). \quad (3)$$

The data of Fig. 5 show that in one experiment τ_h decreased (from 6.7 to 4.7 msec) upon increasing pressure to 62 MPa, while in the other experiment τ_h changed in the opposite direction (from 5.1 to 5.7 msec). The lack of a systematic change of τ_h with pressure was confirmed in all six axons analyzed for $E_{pp} = -50$ mV and for $E_{pp} = -60$ mV.

In conclusion, from the overall analysis of our Na inactivation kinetics data, it appears that the influence of pressure on τ_h is strongly voltage dependent, being quite small for $E \leqq -50$ mV and at least as large as that on τ_m for $E \geqq -20$ mV.

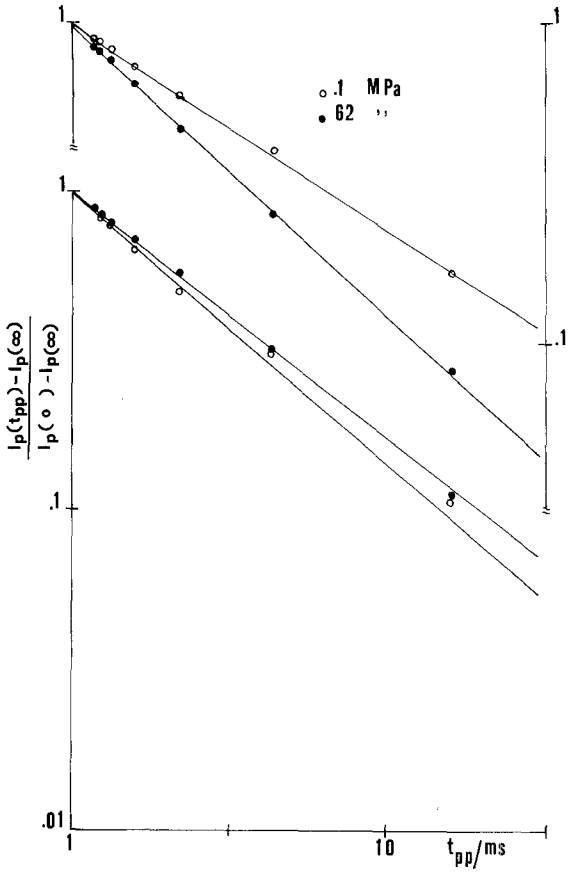

Fig. 5. Effect of pressure on the time constant of Na inactivation, τ_h, following prepulses to -50 mV of variable duration, t_{pp}. The figure shows semilogarithmic plots of the peak Na current, I_p, as a function of t_{pp}, at atmospheric pressure and at 62 MPa. The data are plotted as $\{I_p(t_{pp}) - I_p(\infty)\}/\{I_p(0) - I_p(\infty)\}$. $I_p(0)$ and $I_p(\infty)$ were obtained from the least squares fit of the original I_p data according to Eq. (6). The data fitted by the two lower straight lines are from axon 2 ($T = 10$ °C) and give τ_h values of 5.1 msec at atmospheric pressure and 4.7 msec at 62 MPa

Steady-State Na Activation

In addition to its main effect of slowing all the kinetics of the ionic currents, pressure increased to the highest levels explored in this work produced an appreciable change in the steady-state properties of the Na channels. Figure 6*A* shows peak Na currents as a function of voltage, E, of a single axon at 10 °C, measured at normal pressure, at 42, and at 62 MPa. $I_p - E$ plots such as those of Fig. 6*A* were characterized in each experiment by estimates of: (i) the maximum peak inward current, I_p^{max}; (ii) the potential at which the maximum is attained, E_{max}; (iii) the reversal potential of the early currents, E_{Na}; (iv) the slope of the $I_p - E$ characteristic near E_{Na}. The first two of the above parameters were obtained from the least squares fit of the I_p data around E_{max} with a cubic parabola; the last two were obtained from a similar fit of the I_p data near E_{Na}.

Table 2. Pressure dependence of steady-state characteristics of activation and inactivation of Na currents[a]

Axon	P (MPa)	T (°C)	$I_p^{max}(P)/I_p^{max}(0.1)$	$E_{max}(P)-E_{max}(0.1)$ (mV)	$G_p^{max}(P)/G_p^{max}(0.1)$	$E_h(P)-E_h(0.1)$ (mV)	$U_h(P)/U_h(0.1)$
6	21	5	0.95	6.5	1.02	−2.5	1.15
2	21	10	0.97	1.5	1.06	−2.0	1.10
1	21	10	0.97	2.0	1.04	−0.5	1.07
5	21	15	1.03	2.0	1.12	−1.5	1.10
Mean	21	10	0.98 (±0.03)	3.0 (±2.5)	1.06 (±0.04)	−1.5 (±1.0)	1.10 (±0.03)
4	42	5	0.67	7.0	0.85	−5.5	1.34
6	42	5	0.61	12.5	0.85	−7.0	1.43
1	42	10	0.91	5.5	1.06	−2.0	1.24
2	42	10	0.88	6.5	1.07	−1.0	1.26
3	42	15	0.97	2.5	1.07	−2.0	1.15
5	42	15	0.97	6.0	1.18	−2.5	1.22
Mean	42	10	0.84 (±0.16)	6.5 (±3.5)	1.02 (±0.13)	−3.5 (±2.5)	1.27 (±0.10)
6	62	5	0.47	14.0	0.70	−10.0	2.3
1	62	10	0.76	7.0	0.92	−2.0	1.43
2	62	10	0.76	12.0	1.07	−3.0	1.51
3	62	15	0.83	7.0	0.96	−3.5	1.39
5	62	15	0.85	5.5	1.08	−2.5	1.41
Mean	62	11	0.73 (±0.15)	9.0 (±3.5)	0.95 (±0.15)	−4.0 (±3.0)	1.61 (±0.39)

[a] All data is reported as mean change (±SD) at elevated pressure with respect to the initial and final measurement at 0.1 MPa (atmospheric pressure).

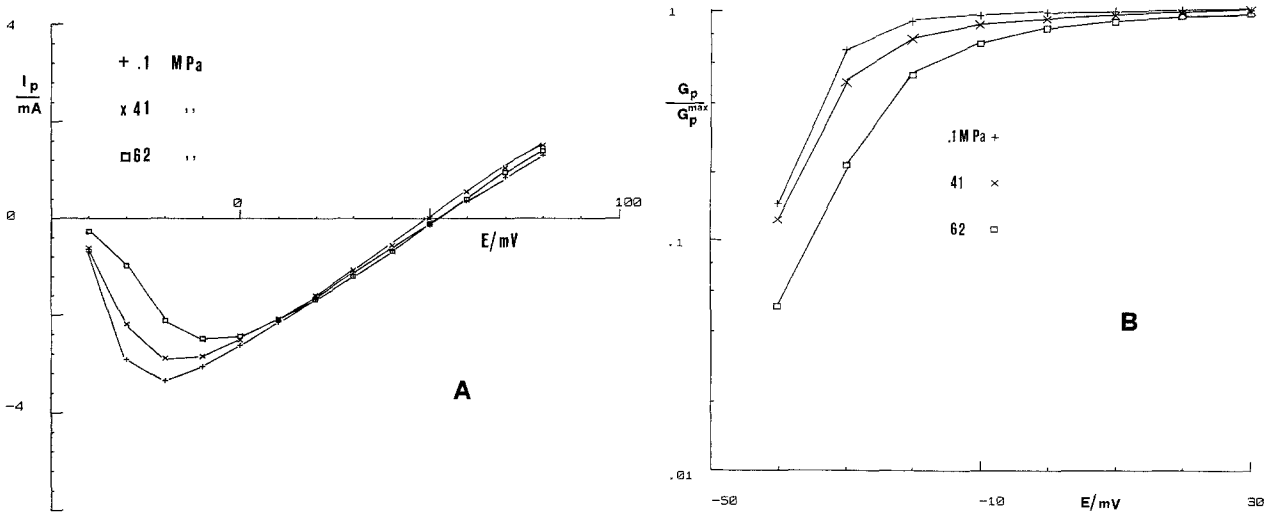

Fig. 6. Effect of pressure upon the voltage dependence of Na activation. (*A*): Peak Na currents, I_p, as a function of pulse potential, E, for step depolarizations from a holding potential of −80 mV, measured in a single axon at normal pressure, at 41 MPa, and at 62 MPa. (*B*): Semilogarithmic plots of the relative peak Na conductance, G_p/G_p^{max}, calculated from the data in *A*. Note the shift toward more positive values of the membrane potential, E_{max}, for which I_p is maximum, and the corresponding shift of the G_p curves in *B*. Axon 2; temperature: 10 °C

In the experiment of Fig. 6*A* E_{Na} and G_p^{max} were practically unchanged by pressure, while I_p^{max} at 42 and at 62 MPa was about 12 and 24% lower than at atmospheric pressure, respectively. This effect is qualitatively similar to but much less pronounced than that reported by Henderson and Gilbert (1975). Furthermore, the reduction in I_p observed by these authors was fairly independent of voltage, while it is clear from our data that the reduction of I_p at high pressures occurs mainly at small depolarizations

so that E_{max} is shifted significantly toward larger depolarizations at the higher pressures. These effects were confirmed in all experiments.

The results are summarized in Table 2 in terms of the elevated pressure with respect to the measurements at atmospheric pressure before and after pressurization. In two experiments (axons 4 and 6) the effects observed were generally more pronounced. However, these measurements are likely to have been affected by larger experimental errors because the recovery of the initial peak currents upon return to atmospheric pressure was poorer (85% in axon 4 and 65% in axon 6). While the data on the shift of E_{max} is subject to the largest relative error, there is a clear suggestion that pressure shifts the Na activation curves. The shift increases, roughly linearly with pressure and can account, *per se,* for the observed decrease of I_p^{max}. This interpretation is confirmed by the direct comparison of the plots of peak Na conductance, $G_p = I_p/(E - E_{Na})$, versus membrane potential at different pressures, shown in Fig. 6*B*. We have not attempted a more quantitative analysis of the above effects in terms of the HH Na activation parameter, m, because we have made no direct measurements of τ_h/τ_m, and we have shown that this parameter may vary significantly with pressure.

It should be mentioned that the effects shown in Fig. 6 might have been exaggerated by series resistance artifacts, which produce apparent shifts in the depolarizing direction whenever the amplitude of the peak currents is decreased by some cause (Kimura & Meves, 1979). However, to so attribute the shifts of E_{max} would be inconsistent with our finding that G_p^{max} is unchanged. Furthermore, with the series resistance compensation used in our system, decreasing temperature shifted E_{max} negatively (as reported by Kimura & Meves, 1979) although I_p decreased with a Q_{10} of 1.4–1.5 as reported for intact axons by Cole (1968) and by Wang, Narahashi and Scuka (1972).

Steady-State Na Inactivation

Following the standard procedure introduced by Hodgkin and Huxley (1925*b*), the steady-state Na inactivation was measured from the amplitude of the peak inward currents produced by step deploarizations to a membrane potential of −10 mV from conditioning membrane potentials, E_c, in the range of −100 to −40 mV maintained for about 150 msec preceding the test pulse. The amplitudes of the peak currents were least squares fitted with the theoretical expression:

$$I_p(E_c)/I_p(-\infty) = h_\infty(E_c) = 1/(1+\exp\{(E_c - E_h)/U_h\}). \quad (4)$$

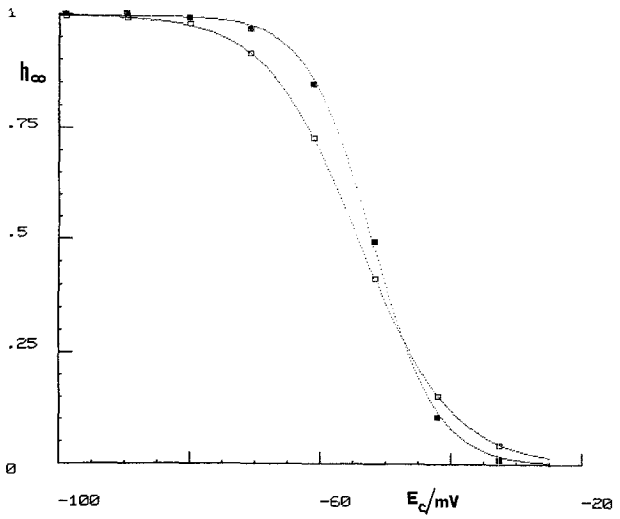

Fig. 7. Effect of pressure on steady-state Na inactivation, h. The data points are the peak Na currents for step depolarizations to −10 mV from conditioning prepulses of 150 msec duration to E_c, normalized to their estimated asymptotic value, $I_p(-\infty)$, for large negative E_c. $I_p(-\infty)$ was −3.2 mA/cm² at atmospheric pressure and −2.6 mA/cm² at 62 MPa. The theoretical curves were drawn according to Eq. (7) for: $E_h = -52$ mV, $U_h = 5$ mV at normal pressure (0.1 MPa); and $E_h = -54$ mV, $U_h = 7$ mV at 62 MPa. Axon 1; temperature: 10 °C

Figure 7 shows h_∞ data from the same axon as in Fig. 2 obtained at normal pressure (0.1 MPa) and at 62 MPa. The same measurements repeated after decompression yielded h_∞ data virtually identical to that shown in the figure for 0.1 MPa. The lines drawn through the data are the theoretical fits according to Eq. (4). It is seen that pressure shifted E_h to more negative values by about 2 mV and produced an increase of about 40% in U_h, manifested by a decrease in the steepness of the voltage dependence of h_∞. These effects were reproduced qualitatively in all experiments. The data are reported in the last two columns of Table 2 as mean changes at elevated pressure with respect to the initial and final measurements at normal pressure. It is seen that the E_h data have a large relative error, which is understandable because the shifts in E_h are small and comparable to possible artifactual electrode potential shifts. The data is consistent with a roughly linear decrease of E_h with increasing pressure. The increase of U_h with pressure appears to be more pronounced at lower temperatures, but we cannot be too confident of this point because the data at 5 °C is limited, and the two axons studied at 5 °C are also those which showed the poorest recovery after decompression.

Discussion

One of the tasks of the present work was to demonstrate that the kinetic and steady-state properties of

the currents in the squid axon can be varied in a reversible and reproducible way as a function of pressure in the range of 0.1 to 62 MPa (1 to 612 Atm). Compared to previous, similar efforts, our experiments are free of possible side effects consequent to the use of gas as the pressure transmitting medium (Henderson & Gilbert, 1975), and we have been able to extend the analysis of voltage-clamp currents beyond the range of 21 MPa explored by Henderson and Gilbert (1975) in squid axons and by Harper et al. (1981) in snail neurons. With our technique we obtained a rather complete characterization of the Na currents at several pressures in the same axon, with excellent recovery of the original responses upon return to atmospheric pressure. The "immediate" effects of pressure can be observed within less than 1 min after compression and were measured quantitatively within a few minutes after allowing for a fair temperature re-equilibration. The latter process was relatively fast, owing to the small inner volume of the pressure vessel which was barely large enough to accommodate a cylindrical axon chamber of about 3 cm^2 cross section. For comparison, it should be noted that in the experiments of Henderson and Gilbert (1975) raising the He pressure from 0.1 to 21 MPa took about 30 min, and in those of Harper et al. (1981), who used a pressure vessel with more than 100 cm^2 inner cross section, a maximum oil pressure of 21 MPa was attained in about 20 min, and it produced a temperature increase of 1.5 °C, lasting 1.5 hr.

Qualitatively, the major effects of pressure that we have observed in this work, i.e., the slowing down of the turn-on kinetics of Na currents, agree with those reported by Henderson and Gilbert (1975) and by Harper et al. (1981). Quantitatively, the reversible effects we have observed are about 50% smaller than those reported by the others and correspond to apparent activation volumes at the lower end of the range of values reported by Henderson and Gilbert (1975). Furthermore, our interpretation of the effects of pressure upon the kinetics of Na inactivation disagrees with the conclusion reached by Henderson and Gilbert (1975) and by Harper et al. (1981). Their conclusion was based upon the observation of a decrease in peak Na currents which was fairly independent of voltage (Henderson & Gilbert, 1975) and of no significant change in τ_h as measured with small conditioning depolarizations (Harper et al., 1981). In our experiments the change in the peak Na currents at 21 MPa were very small and consistent with the direct observation that the inactivation of Na currents was slowed down at least as much as was the rising phase. In agreement with Harper et al. (1981), we also did not find any systematic effect of pressure upon τ_h measured at -60 and -50 mV, but we found that Na inactivation above -20 mV is slowed by pressure at least as much as is Na activation, indicating the the effect of pressure on τ_h is strongly voltage dependent.

Changes in the passive impedance of the membrane are slight when the pressure is increased up to 62 MPa, in fair agreement with the data of Spyropoulos (1975*b*)[1]. An upper limit to the change in membrane capacitance can be set at about 3% for 62 MPa, based upon the accuracy of reading our Polaroid records. Thus, a possible difference between the lateral and perpendicular compressibility of the lipid bilayer matrix of the axon membrane (Stamatoff et al., 1978), should lie within this limit. This result, and the apparent absence of discontinuities in the changes produced by pressures upon the active ionic currents, suggest that these changes do not involve abrupt modifications of the nerve membrane structure. Further support of this view is provided by the finding that the kinetics of the translocation of lipophilic ions across the squid axon membrane (Benz & Conti, 1981) are little affected at pressures up to 62 MPa (R. Benz, F. Conti & R. Fioravanti, *unpublished*).

Thus, although we cannot exclude the possibility that high pressures produce more complex modifications of the ionic channels and of their lipid surrounds, it is conceivable that the pressure dependence of Na currents merely depends upon finite volume changes accompanying the isomerization of Na channels. A possible simple theoretical framework for this interpretation is given in the Appendix. Within such a context, the pressure dependence of τ_m at fairly large depolarizations (for which $\alpha_m \gg \beta_m$ and $\tau_m \sim 1/\alpha_m^a$) would be consistent with a simple HH scheme if the opening of *m gates* involves an activation volume, $V_m^{\pm} - V_m^c$ of 35 cm^3/mole (58 $Å^3$ per *m gate*). Likewise, the positive shift of the Na activation curves would imply that the opening of an *m gate* produces a net volume increase, $V_m^o - V_m^c$, of about 26 $Å^3$. The latter estimate is obtained from Eq. (A6) assuming a shift of Na activation of about 3 mV for a pressure increase of 21 MPa (Table 2) and an effective charge per *m gate*, q_m of 1.2 ec (from a mean of the estimates by Meves and Vogel (1973), Keynes and Rojas (1974), and Kimura and Meves (1979).

A self-consistent description of the effects of pressure upon Na inactivation is not possible within a similar simple framework. The pressure dependence

[1] However, Spyropoulos (1975*b*) reports that R_m was "often" strongly reduced between 40 and 80 MPa, whereas we found no evidence of drastic R_m changes associated with pressures up to 62 MPa

of τ_h at large depolarizations (for which $\beta_h \gg \alpha_h$ would imply that the closing of an *h gate* involves an activation volume, $V_h^h - V_h^o$, larger than 58Å^3. On the other hand, since τ_h is little affected by pressure when α_h and β_h are comparable ($E = -50$, -60 mV) α_h should have a pressure dependence opposite to that of β_h or at least no pressure dependence at all. Either case would imply an increase of h_∞ with pressure, contrary to what is observed (Fig. 7). Further difficulties arise in the interpretation of the decrease in steepness of the $h_\infty(E)$ curves with increasing pressure. This effect could be accounted for by Eq. (6) only by postulating some *ad hoc* mechanism by which the effective charge of the *h gates*, q_h, is decreased by pressure.

It is interesting to note that the effects of increasing pressure on the h_∞ curves are similar to those produced by decreasing temperature (Wang et al., 1972; Kimura & Meves, 1979). Quantitatively, as confirmed by comparing our own measurements at 5 and at 15 °C, an increase of pressure by 62 MPa produced roughly similar changes in E_h and U_h as a temperature decrease of 10 °C.

Another observation which is not easily explained with the simple HH model is that the effect of pressure on the early phase of the sigmoidal rise of Na currents is smaller than on the steepest phase. As discussed earlier, such differential effects cannot be solely accounted for by a change in the steady-state value of m at the holding potential. Unless we abandon the simple idea of describing our data in terms of activation and reaction volumes involved in the gating of Na channels, the above difficulties can be removed only by giving up some of the simple features of the HH scheme. For this purpose, sequential models, such as those suggested by Armstrong and Bezanilla (1977) and by Armstrong and Gilly (1980) on the basis of gating current measurements, might be more appropriate. By postulating that inactivation occurs only after an advanced state of activation, and that the sequence of steps leading to full activation does not obey the simple m^3 law, these models predict that τ_h depends on all the rate constants of the intermediate steps leading to activation; the relationship between τ_h and h_∞ is consequently much more complex. The conclusion reached by Henderson and Gilbert (1975) and by Harper et al. (1981), that τ_h is unaffected by pressure while τ_m is considerably increased, would argue strongly against a sequential coupling of the activation and inactivation processes. However, our present data doe not confirm their conclusion, as discussed already.

Further detailed studies of the nerve membrane and lipid bilayers are needed to make us confident that the apparent activation volumes reported here are directly related to the molecular mechanism of the gating of Na channels. Thus, it seems premature at present to speculate on whether the estimated $\Delta V^\pm$ values arise from structural changes of the Na channel itself or from the change in the degree of exposure to water of channel groups which modify the density of adherent water by electrostriction (Low & Somero, 1975).

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Appendix

The Expected Pressure Dependence of HH Channels

The influence of pressure on the rate and equilibrium constants of chemical reactions is well understood on the basis of fundamental thermodynamic principles and Eyring's theory of absolute reaction rates (Johnson et al., 1954; 1974). We state here the expected pressure dependence of the kinetic and equilibrium properties of ionic channels obeying the HH equations (Hodgkin & Huxley, 1952*a*) under the simple assumption that the channels and their surrounds are not modified by pressure.

For a simple two-state channel undergoing a first order isomerization between closed, C, and open, O, states:

$$C \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} O; \tag{A1}$$

the pressure dependence of the reaction rates, α and β, is given by (Johnson et al., 1954; 1974):

$$-RT\,\partial \ln \alpha/\partial P = \partial \Delta G_f^\pm/\partial P = V^\pm - V^c \tag{A2}$$

$$-RT\,\partial \ln \beta/\partial P = \partial \Delta G_b^\pm/\partial P = V^\pm - V^o \tag{A3}$$

$$-RT\,\partial \ln(\beta/\alpha)/\partial P = V^c - V^o \tag{A4}$$

where $\Delta G_f^\pm$ and $\Delta G_b^\pm$ are, respectively, the free energies of activation for the forward ($C \to O$) and backward reactions; ($V^\pm - V^c$) or ($V^\pm - V^o$), is the volume change produced by the transition of $N_o (= 6.02 \times 10^{23})$ channels from the closed, or open, state to the activated state of the reaction, and $V^c - V^o$ is the volume change produced by the transition of N_o channels from the open to closed state. Under the assumption that $V^o - V^o$ does not change significantly with pressure – which is very plausible for reactions in solution and for the relatively narrow pressure range of our concern – Eq. (A4) becomes

$$\beta/\alpha = K(T, E) \exp\{P(V^o - V^c)/RT\} \tag{A5}$$

where $K(T, E)$ is the equilibrium constant of reaction (A1) at low pressure and at membrane potential, E. In the first order approximation, the explicit dependence of K on membrane potential is expressed, by defining an *effective* electric charge, q, by:

$$\beta/\alpha = \exp\{[q N_o (E + E_o) + (V^o - V^c) P]/RT\} \tag{A6}$$

where E_o is the membrane potential for which there is equal probability of occupancy of the two channel states at $P = O$ and at the given temperature. Equation (A6) shows that the effect of pressure on the equilibrium between open and closed channels is equivalent to a change in E_o, i.e., to a shift in the channel activation curve along the voltage axis. Such a shift will be in

the depolarizing direction if the opening of a channel is favored by a depolarization ($q<0$), but it is accompanied by a volume increase. In this case pressure would antagonize the channel opening, and larger depolarizations would be needed to overcome the effect.

The observed kinetics of axonal ionic currents are more complicated than predicted by a two-state model, and the HH description of these currents implies eight or five channel states for Na and K channels, respectively. However, the HH equations, in fact, make use of only four independent transition rates (α_m, β_m, α_h, and β_h) for Na channels and only two transition rates (α_n and β_n) for K channels, according to a scheme in which the various channel isomers correspond to different configurations of four channel subunits, each undergoing independent simple isomerizations of type (A1). Accordingly, one expects that Eqs. (A2)–(A6) are still adequate to describe the effects of pressure on the HH processes of Na activation, m, of Na inactivation, h, or of K activation, n.

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