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Pressure dependence of sodium gating currents in the squid giant axon

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Abstract. Asymmetric displacement currents, I_g, were measured in squid axons at different hydrostatic pressures, P, up to 60 MPa. Potassium and sodium currents were abolished by intracellular Cs^+ and TEA^+, by extracellular Tetrodotoxin (TTX), and by Na^+ substitution with $Tris^+$. The time course of Ig became progressively slower with increasing pressure, and the amplitude decreased. With appropriate scaling in time and amplitude, I_g records at any given P could be made to superimpose very well with those obtained at atmospheric pressure. The same scaling factors yielded a good superposition of all records obtained for voltage steps to membrane potentials in the range −30 to +42 mV. The ratio between the amplitude and time factors was larger than unity and increased with P, indicating a progressive decrease (up to 35% at 60 MPa) of the total charge displaced, Q, with no significant change in its voltage dependence. The time-scaling factor increased exponentially with P, as expected if all the steps involved in the opening of a sodium channel, and producing a major charge redistribution, have the same activation volume, $\Delta V_g^{\pm} \sim 17$ cm^3/mol. This value is roughly one-half of that characterizing the pressure dependence of sodium current activation, suggesting that some late, rate-limiting step in the opening of sodium channels has a large activation volume without being accompanied by an easily detected charge movement.

Part of the decrease of Q with pressure could be attributed to an increase in sodium inactivation. However, we cannot exclude the possibility that there is a reversible reduction in the number of fast activating sodium channels, similar to the phenomenon that has been reported to occur at low temperatures (Matteson and Armstrong 1982).

Key words: Sodium channel, nerve, gating currents, pressure, activation volumes

Introduction

The most obvious physiological relevance of studies of nerve excitability at high hydrostatic pressures is the understanding of some of the complex symptoms which comprise the high pressure nervous syndrome (Bennett 1975; Brauer 1975). However, as was clearly stated by early investigators (Ebbecke and Schaefer 1935; Grundfest 1936; Spyropoulos 1957a, b) the pressure dependence of nerve and muscle physiology can be used to gain information about the physical chemistry of membrane excitation phenomena (Wann and Macdonald 1980; Macdonald 1984). Indeed, starting with the work of Henderson and Gilbert (1975) on squid axons, studies of the effects of pressure on voltage-clamped nerves (Harper et al. 1981; Conti et al. 1982a, b) have provided new insights into the molecular mechanisms underlying the functioning of ionic channels. In particular, Conti et al. (1982a, b) have attempted to directly relate the pressure dependence of the sodium and potassium currents in squid axons to volume changes accompanying the structural changes of the respective ion-selective channels.

Following the demonstration that nerve excitability is modulated by membrane potential, Hodgkin and Huxley (1952) predicted that the increase in membrane ionic permeabilities should be accompanied by displacement currents. In terms of the modern notion of ionic channels, the same argument implies that at least some of the conformational changes which transform a membrane channel from an impermeable structure into an ionic pore involve a redistribution of polar groups, which significantly

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changes the energy of interaction with the intramembrane electric field. First successfully recorded by Armstrong and Bezanilla (1973), these charge redistributions, or gating currents, have become one of the most important tools for studying sodium channels in nerve membranes (for recent reviews, see: Armstrong 1981; French and Horn 1983; Keynes 1983). Gating current provide additional insight into the operation of ionic channels, because they also appear for those conformational transitions which do not lead directly to the opening of a channel. On the other hand, the various steps involved in the opening of a sodium channel are not necessarily similar and do not necessarily bring about identical charge redistributions (Armstrong and Gilly 1979).

The aims of the present study on the effect of hyperbaric pressures on sodium gating currents were twofold. Firstly, to show, as assumed by Conti et al. (1982a), that pressure has a direct influence on the thermodynamic properties of sodium channels, independently of whether or not these structures are actually used as ionic pathways. Secondly, to investigate the possibility that different steps in the activation of sodium channels can be discriminated on the basis of their activation volumes, as revealed by their different pressure dependence. Our results show that the kinetics of gating currents are markedly affected by pressure, but less so than the sodium currents. We will argue, therefore, that the structural changes which bring about measurable gating currents do not constitute all the steps in the gating of sodium channels. Some late, rate-limiting step, involving the largest activation volume, is not associated with an appreciable gating current. We also demonstrate a reversible decrease of the total gating charge with pressure, part of which may be related to the "sleepy channels" effect seen at low temperatures (Matteson and Armstrong 1982).

A preliminary report of gating current measurements in squid axons exposed to high hydrostatic pressure has been presented (Stühmer et al. 1983).

Materials and methods

Axon preparation

The experiments were performed on giant axons dissected from the hindmost stellar nerve of the squid, *Loligo vulgaris,* available in Camogli. The axons were thoroughly cleaned of all surrounding small fibres for a length of about 3.5 cm and placed in a Perspex chamber similar to that described by Conti et al. (1982a). The chamber consisted of a floor and two movable lateral walls, one of which had an L-shaped cross-section and formed the ceiling of the compartment containing the axon. A hole through the ceiling, containing a small glass tube, established free communication between the extracellular fluid and the oil bath in which the whole chamber was immersed during the measurements made under pressure. Two Perspex partitions were inserted, perpendicular to the longitudinal axis of the axon, at the two ends of the compartment before closing the chamber. The partitions (1 mm thick) had small grooves, slightly larger than the axon diameter, which were filled with silicone grease. The partitions were gently pressed upon the axon to prevent fluid exchange between the extracellular solution and the oil bath. During preliminary operations, the lateral walls of the chamber were kept widely separated, allowing free access to the axon.

The axons were perfused intracellularly according to the technique of Tasaki et al. (1962), as modified by Rojas and Ehrenstein (1975). The purpose of perfusion was to abolish potassium currents, by replacing the intracellular potassium with cesium and by injecting Tetraethylammonium (TEA). Therefore there was no need to perfuse for long periods or to use proteolytic enzymes. Normally, the whole axon length was perfused for about 4 min with the following solution: 300 mM CsF, 40 mM TEA-phosphate, 350 mM sucrose, 20 mM *Tris*-phosphate; pH 7.2. The perfusion was discontinued by completely withdrawing the inlet glass pipette while maintaining a constant flow. Thereafter, the cut end of the axon tended to collapse along about 1 mm of its length. It then dried out spontaneously, apparently providing a good seal for the remaining portion of the axon. The extracellular solution was initially natural sea water. After testing for normal sodium currents, it was changed to a sodium-free solution with the following composition: 460 mM *Tris*Cl; 50 mM $CaCl_2$; 1 μM TTX*; pH 7.8. The temperature, T, near the axon, was continuously monitored with a thermistor and regulated by keeping the pressure vessel in a thermostatted bath. All measurements were performed between 9° C and 14° C. Temperature variations, during one experiment at different pressures, were kept within 1° C by allowing some time for thermal re-equilibration after each pressure change. When needed, kinetic data were corrected for small temperature changes, by assuming a Q_{10} value of 3 (Kimura and Meves 1979).

Electrodes

A major technical improvement over the apparatus of Conti et al. (1982a) concerned the voltage measuring electrodes. Both the intracellular and the extracellular voltage electrodes consisted of glass pipettes

* TTX – tetrodotoxin

with a small portion of the tip filled with asbestos fibres forced into position mechanically and sealed to it by gentle heating. This very effectively prevented any convective movement of solutions in and out of the electrode, yielding a stable liquid-junction potential, which did not change appreciably during the application of hydrostatic pressures up to 100 MPa. The intracellular pipette was a long capillary about 50 μm in diameter, filled with 0.5 M KCl, and containing a floating Pt-Pt black wire (20 μm in diameter) along the whole length up to the asbestos tip. It was assembled together with the internal current electrode, a Pt-Pt black wire of 80 μm diameter, in a "piggy-back" fashion (Chandler and Meves 1965). When it was in its final position, the intracellular electrode assembly was first fixed to the chamber with dental sticky wax and then disconnected from the manipulator with which it had been inserted. The extracellular pipette was 0.5 mm in diameter and was filled with the extracellular solution. Through the small hole in the ceiling of the chamber, it was permanently positioned in such a way that, in the closed configuration, the tip of the electrode was less than 0.5 mm from the top centre of the axon. The filling solutions of both pipettes were in free communication with the pressure-transmitting oil (through short segments of polyethylene tubing) and with Ag-AgCl electrodes sealed into small Perspex holders.

The extracellular current electrodes were three Pt-Pt black foils (5.3 mm × 5 mm) fixed to each lateral wall. In the closed chamber configuration the two sets of electrodes were 3 mm apart. Teflon septa (50 μm) mechanically separated the central foil from the two lateral ones, serving as electrical guards. The septa protruded 1 mm into the solution, virtually in contact with the axon, and considerably increased the resistance between central and guard electrodes. This improved the efficacy of the guard system, thereby decreasing the extrinsic noise introduced by the virtual ground amplifier.

Pressure apparatus

The pressurizing system, using vaseline oil as the pressure-transmitting medium, was similar to that described by Conti et al. (1982a). It consisted of a pressure bomb (internal cylindrical volume of 100 cm^3, 25 mm cross-sectional diameter) capable of withstanding pressures up to 150 MPa, a hand-driven hydraulic pump, and a pressure gauge with 0.1 MPa sensitivity and 160 MPa full scale[1]. This apparatus, including the electrical connections through the pressure bomb, was obtained from Nova Swiss Werk (Zürich, Switzerland), to our own design.

Stimulation and recording apparatus

We used a standard voltage-clamp system with lateral guards (Moore and Cole 1963). Because gating currents have a much less pronounced voltage dependence than sodium currents, the dc gain of the main amplifier (AD 46 J, Analog Devices) was kept small (100–300) in order to allow a more effective compensation of the series resistance, R, and a consequent improvement in the speed of the voltage-clamp circuit. In several experiments, a direct estimate of R was obtained from the voltage response to a square current stimulus and we found that up to 90% R compensation could be introduced without producing undesired oscillations in the voltage-clamp step response. The rise time of the actual voltage across the axolemma was probably not improved very much because of intrinsic limitations related to the complex morphology of the periaxonal space (Benz and Conti 1981). In any case, the amount of R compensation did not seem to have an appreciable influence on the gating currents measured.

The sequence of pulses required for the measurement of asymmetric displacement currents was generated by a microcomputer (AIM 65, Rockwell International) programmed according to various pulse protocols. The microcomputer also generated the signals necessary to synchronize the operation of a digital data acquisition system. Most of the linear component of current records was subtracted before acquisition, using a four-component analog transient generator. The data were sampled at 100 kHz with a 12-bit analog to digital converter and accumulated in a shift register buffer. After completing the acquisition of one record, which usually consisted of 256 of 512 sampled points, the content of the shift registers was transferred to an FM tape recorder.

Various pulse protocols were used. The P/4 protocol (Armstrong and Bezanilla 1977) was used in preliminary experiments. Later, a slightly more complex protocol was found to be more convenient. It consisted of: (i) N_c-positive control pulses of 30 mV amplitude, each preceded by a prepulse which brought the membrane potential from the normal holding level, $E_H = -70$ mV, to the control level, $E_c = -150$ mV; (ii) N_c-negative control pulses of −30 mV preceded by the same prepulse; (iii) a sequence of N_1, N_2, . . . N_n test pulses from E_H to various membrane potential levels, E_1, E_2, . . . E_n; (iv) repetition of (i); (v) repetition of (ii). The average difference between records obtained in (i) and (ii) or

1 The pressure values quoted in this paper are given by approximating 1 atm with 0.1 MPa. The pressure gauge was calibrated in atm units

in (iv) and (v) was taken as being equivalent to the response to a positive control pulse of 60 mV amplitude. Whenever the average control records before and after the test pulses were found to be significantly different the experiment was discontinued. After appropriate scaling, the average control record obtained from series (i), (ii), (iv), and (v) was used in the off-line analysis to subtract the symmetric component of the current records measured during the test pulses in (iii). The numbers N_c, N_1, $N_2 \ldots N_n$ were chosen so that the noise of any average test record was at least as large as that of its appropriately scaled control record. The advantages of the above protocol were twofold. Firstly, it exploited the same control sequences for the subtraction of the linear responses to several test depolarizations. Secondly, by taking the difference between positive and negative control records we removed possible systematic artefacts in the baseline, developed during the prepulse from E_H to E_c. The off-line analysis was performed with a PDP11/40 computer.

Results

Asymmetric displacement currents, I_g, produced by various step depolarizations at normal pressure are shown in Fig. 1A. The records were obtained by averaging the responses from four successive runs using the protocol described above. The amplitude, time course and voltage dependence of these currents are characteristic of the non-linear capacitive responses which have already been identified by several authors as being associated with the gating of sodium channels in the squid axon (see, for example, Armstrong and Gilly 1979). The measurements illustrated in Fig. 1A were started at least 20 min after intracellular and extracellular perfusion of the axon, with potassium-free solutions, so that most of the potassium channels were probably non-functional (Chandler and Meves 1970; Almers and Armstrong 1980). Thus, although small components of I_g likely to be associated with the gating of potassium channels have recently been identified (Gilly and Armstrong 1980; Bezanilla et al. 1982), we shall assume that the I_g records reported in this work are free from such contributions and refer to our measurements as those of sodium gating currents.

The three series of records shown in sections B–D of Fig. 1 were measured at later times, after the hydrostatic pressure had been raised to 30 MPa, after a further increase to 60 MPa, and after returning to atmospheric pressure, respectively. The overall qualitative picture that emerges from Fig. 1 is that raising the pressure slows the time course of sodium gating currents and decreases their amplitude in a fully reversible manner. As discussed later, it is also apparent that the slowing of I_g at high pressure does not fully compensate for the amplitude decrease, so that increasing pressure results in a decrease of the area under the I_g records, i.e., in a reversible loss of mobile gating charge.

The gating charge movements associated with the repolarization of the axon, after a positive voltage step, were affected by pressure in a similar way. However, a detailed analysis of the pressure dependence of the "off" gating currents will not be pursued in this work. Such analysis would necessarily require a detailed discussion of the effect of pressure on sodium inactivation and on the related phenomenon of gating charge immobilization (Armstrong and Bezanilla 1977), which is beyond the scope of this paper.

Effect on kinetics

For a quantitative characterization of the effect of pressure on gating current kinetics we adopted a straightforward approach, based on the direct comparison of I_g records obtained at different pressures, without assuming any particular analytical description of their time course. This method is illustrated in Fig. 2, where four of the records of Fig. 1A (0.1 MPa) are shown together with the corresponding records of Fig. 1C (60 MPa) after scaling to approximately the same amplitude. In the same figure, the high pressure data are also replotted after compression of the time axis, in order to obtain the best superposition with the data at atmospheric pressure. It is clearly seen that an appropriate selection of time and amplitude scaling makes the superposition quite good. This result is not trivial, because a similar attempt to obtain a simple description of the effect of temperature was not successful (Bezanilla and Taylor 1978). The amplitude and time-scaling factors, θ_I and θ_t, were calculated by computer using a three-parameter fitting program, which allowed for a slight difference in the steady-state asymptote of the two records being compared. The mismatch was practically zero for the data of Fig. 2, and it was generally no more than a few percent of the peak I_g amplitude.

The values of θ_t used in Fig. 2 are fairly independent of voltage. This result was confirmed in several experiments, performed at various hyperbaric pressures, on 12 different axons. Figure 3A shows plots of the mean θ_t values obtained as a function of membrane potential, E, for $P = 40$ MPa, and $P = 60$ MPa. The data show no obvious systematic change with E. The same conclusion is even better supported by the analysis of Fig. 3B, which plots the mean ($\pm$ SEM) of the ratio between θ_t (E, P), measured at

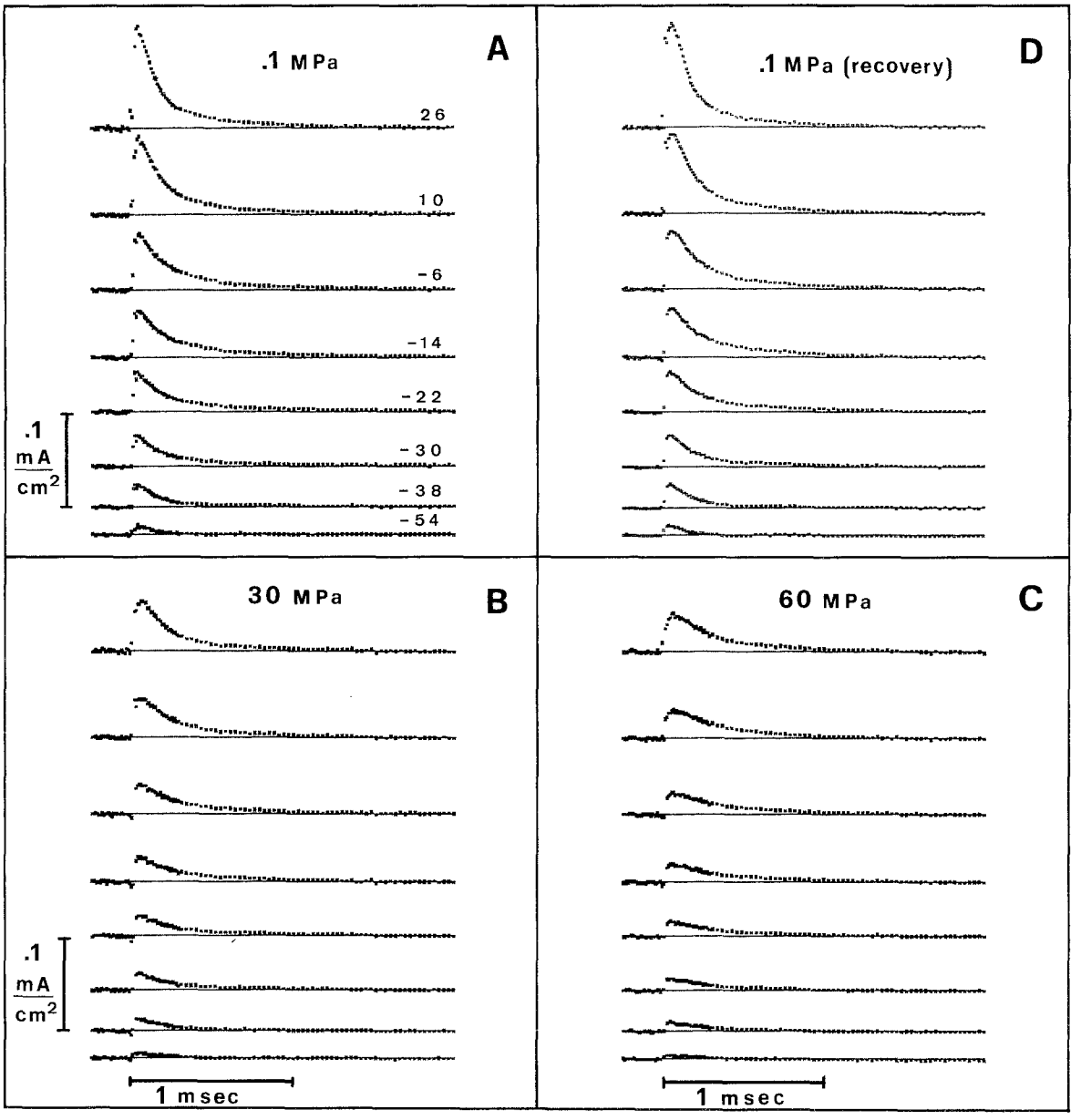

Fig. 1A–D. Gating currents associated with steps of membrane potential from $E_H = -70$ mV to the various test levels indicated, and measured at different hydrostatic pressures. The records were obtained from the kind of pulse protocol described in the text, with: $N_c = 10$; $N(-54) = 20$; $N(-38) = 18$; $N(-30) = 14$; $N(-22) = 10$; $N(-14) = 6$; $N(-6) = 6$; $N(10) = 6$; $N(26) = 5$. Control pulses were applied from a prepulse level of -150 mV. The records at atmospheric pressure (**A**) are averages obtained from four successive runs of the above protocol; those in **B** (30 MPa) are averages from two runs; and those in **C** (60 MPa) and **D** (recovery at 0.1 MPa) are averages from three runs. Same amplitude and time scale for all records. Temperature: 11° C

pressure P for voltage steps to E, and θ_t (26, P), measured in the same experiment for steps to $E = 26$ mV. The latter value of membrane potential was chosen as reference because the various protocols used throughout the present study included test pulses to $E = 26$ mV. The data in Fig. 3B were derived from a total of 134 different measurements of θ_t (E, P) in the voltage range -30 to $+42$ mV. It is seen, that for this range, systematic variations of θ_t with voltage did not exceed 6%.

The pressure dependence of θ_t is illustrated in Fig. 4. Here, all measurements of θ_t (E, P) at any given pressure and for $E \geqslant -30$ mV were pooled to yield an average estimate, θ_t (P), which is plotted on a semilogarithmic scale as a function of P. The straight line drawn in Fig. 4 is the best fit to the data according to Eq. (1)

$$\ln \theta_t(P) = \Delta V_g^{\neq} (P - P_0)/RT, \qquad (1)$$

where $P_0 = 0.1$ MPa, R is the gas constant, and $\Delta V_g^{\neq}$

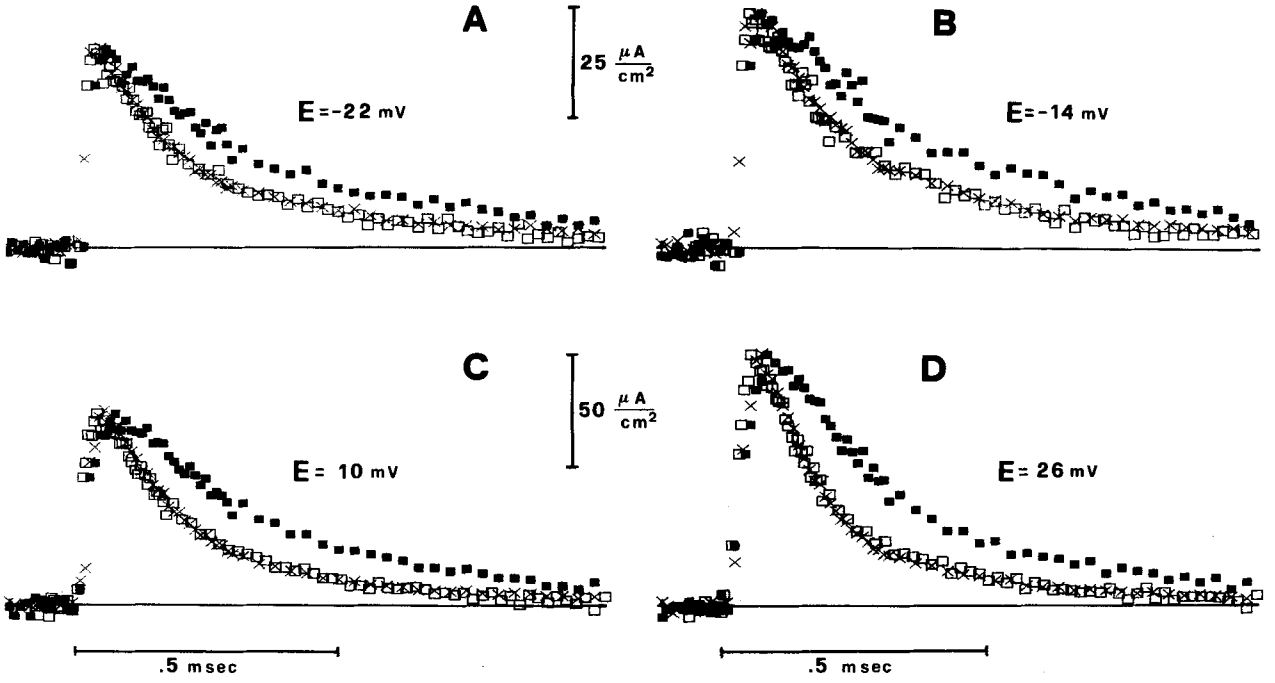

Fig. 2A–D. Pressure dependence of gating current kinetics. Four of the records of Fig. 1A (0.1 MPa) are shown on an expanded time and amplitude scale (×), together with the corresponding records of Fig. 1C (60 MPa) further amplified by θ_I to have approximately the same peak amplitude (■). The records at 60 MPa are also replotted as (□) after having been compressed in the time axis by the factor, θ_t. The following values were used for θ_I and θ_t, respectively: 2.6 and 1.6 in **A**; 2.53 and 1.6 in **B**; 2.66 and 1.7 in **C**; 2.69 and 1.7 in **D**. The amplitude scales refer to the data at atmospheric pressure

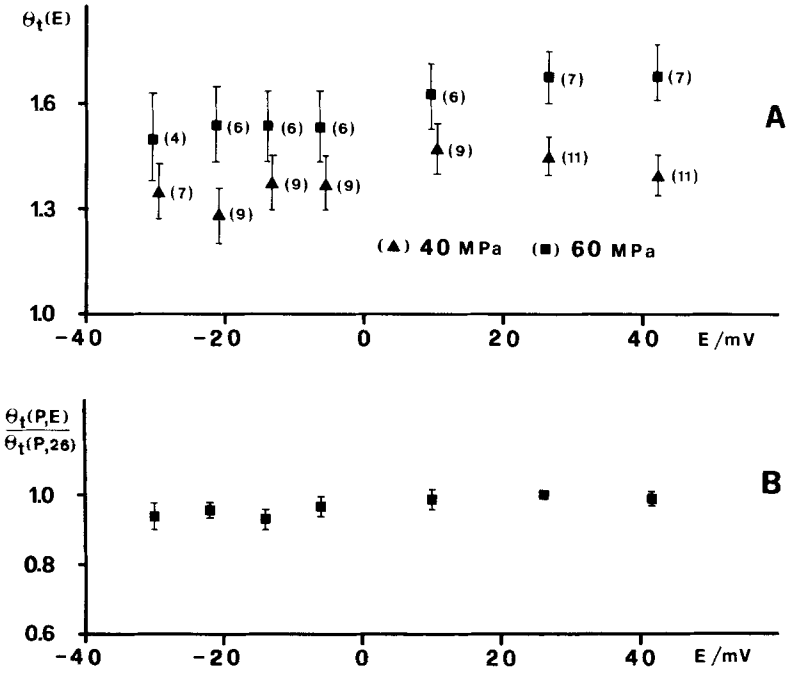

Fig. 3. A: Voltage dependence of the factorial change of gating current kinetics, θ_t, produced by raising the pressure from normal (0.1 MPa) to 40 MPa (▲) or to 60 MPa (■). The data are given as mean and standard deviations from the number of measurements indicated in parenthesis. **B:** Plot of the average ratio between θ_t values measured at any voltage E and at 26 mV, for pressures in the range of 10–60 MPa. Error bars refer to standard deviations of the mean

is the apparent activation volume of all the molecular processes which make a significant contribution to I_g measurements. The fit of the data to Eq. (1) yields a value of $\Delta V_g^{\pm}$ of about 17 cm^3/mol, one-half of the apparent activation volume which describes the slowing effect of pressure upon the rise time of sodium currents, I_{Na} (Conti et al. 1982a).

For a more direct comparison, the latter effect is illustrated in Fig. 5, showing the rising phases of I_{Na} for $E = -22$ mV and for $E = 8$ mV, recorded from

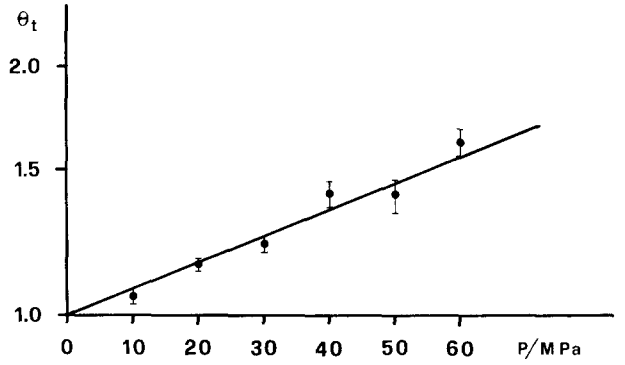

Fig. 4. Pressure dependence of the factorial change of gating current kinetics, θ_t. Each data point is the mean value (± SEM) measured for membrane potentials in the range of −30 to 42 mV. The straight line is the best fit of the data according to Eq. (1) and corresponds to an apparent activation volume for the voltage-gated transitions of the sodium channel of 17 cm^3/mol

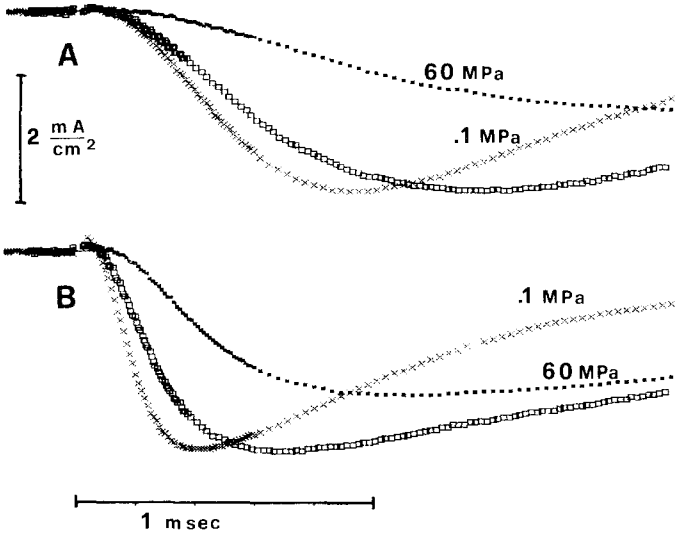

Fig. 5A and B. Sodium currents produced by step depolarizations from $E_H = -70$ mV to −22 mV **(A)**, and to 8 mV **(B)**, measured in the same axon at normal pressure (×) and at 60 MPa (■). The open squares are the high pressure data magnified 1.8 times **(A)**, or 1.4 times **(B)**, and compressed in time by the average factor 1.6 which describes the slowing of gating currents at 60 MPa. The axon was perfused with CsF and TEA-phosphate to abolish potassium currents. The extracellular medium was artificial sea water containing 450 mM NaCl. Linear capacity and leakage currents were subtracted using control pulses from −70 to −120 mV. Temperature: 10° C

the same axon at 0.1 MPa and 60 MPa. The data are similar to those reported by Conti et al. (1982a), except that the axon of Fig. 5 was perfused intracellularly with Cs^+ and TEA^+, which completely abolished the potassium currents. The records at 60 MPa are also shown after being scaled in amplitude, so that their peak values equalled those at 0.1 MPa, and compressed in time by a factor of 1.6, i.e., the mean θ_t of the gating currents at 60 MPa. It is clear that this factor is far too small to produce the superposition of the I_{Na} rising phases. It could be shown that the time compression factor required for this purpose would be about 2.05 for the records at −22 mV, and about 2.45 for those at 8 mV [2]. Thus, increasing pressure markedly decreases the overlap between the time course of I_g and I_{Na} records. By extrapolating this result it appears that, if one could raise the hydrostatic pressure to much higher values without producing phase separation phenomena (Benz et al. 1984), conditions would be created in which detectable gating currents have subsided before I_{Na} becomes significant. It seems unavoidable to conclude from this argument that the opening of sodium channels must involve some final step which contributes very little to I_g but has the largest activation volume. This should be taken as a serious caveat against equating the whole complex behaviour of sodium channels with the phenomenology of their gating currents.

Effect on gating charge

The total charge, Q, displaced as a consequence of a step depolarization from the holding potential, $E_H = -70$ mV, was measured by numerical integration of I_g records over a time interval of about 2.3 ms. An example of such data, obtained from the records of Fig. 1A and 1C, is shown in Fig. 6. The smooth lines are least-squares fits according to Eq. (2), which would be adequate to describe the distribution between two possible states of molecular membrane structures which differ by an effective transmembrane charge displacement of z electronic charge units, and which are equally populated at the membrane potential, E_0.

$$Q = Q_{max}\left\{\frac{1}{1+\exp\{(E-E_0)zF/RT\}} - \frac{1}{1+\exp\{E_H-E_0)zF/RT\}}\right\}, \quad (2)$$

where F is the Faraday constant. The two curves fitting the data at 0.1 and at 60 MPa were obtained for approximately the same values of the parameters E_0 and z (−27.5 mV and 1.6 at 0.1 MPa; −25.5 mV and 1.5 at 60 MPa), but for Q_{max} values of 27 nC/cm^2 and 17 nC/cm^2, respectively. Thus, it appears that the

2 Notice, however, that the factor 1.6 yields a quite good superposition in the very early turn-on of I_{Na}, so that the larger time compression needed to match the steepness of the rising phases would create a mismatch in the initial delays (Conti et al. 1982a). Our present data suggest that such a mismatch is related to the different effect of pressure on I_g and on I_{Na} kinetics

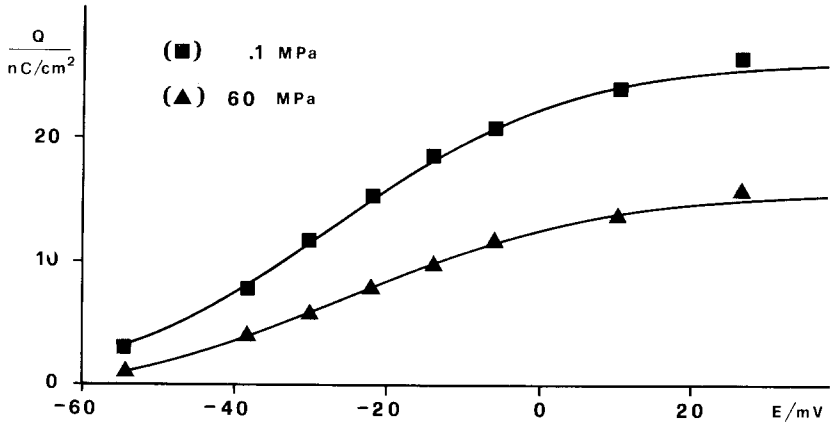

Fig. 6. The effect of pressure on the activation curve of the total gating charge movement produced by step depolarizations from −70 mV to various membrane potential levels, E. The data points were obtained from the numerical integration of the I_g records of Fig. 1A, C. The theoretical curves are best fits of the data to Eq. (2) using the following parameters: $Q_{max} = 27$ nC/cm², $E_o = -27.5$ mV, and $z = 1.6$ at 0.1 MPa; $Q_{max} = 17$ nC/cm², $E_o = -25.5$ mV, and $z = 1.5$ at 60 MPa

major effect of pressure on Q is a voltage-independent reduction, which is of the order of 37% in the case of Fig. 6. This reduction is also demonstrated directly by the analysis which we illustrated in Fig. 2, because it roughly coincides with the ratio of the amplitude and time-scaling factors used to obtain the superposition of normal and high pressure records. Indeed, provided the scaling operation is correct, it is obvious that the ratio between θ_t (P, E) and θ_I (P, E) yields the Q factorial change, θ_Q (P, E) produced on going from normal pressure to pressure P. Thus, the computer analysis of the experimental records using the scaling procedure provided an alternative and more direct estimate of θ_Q (P, E), which we believe is also less subject to errors arising from drifts and noise in the I_g baseline.

The data of Figs. 2 and 6 indicate that θ_Q (P, E) is voltage-independent. A full statistical analysis of the results from 12 different axons confirms this conclusion, as shown in Fig. 7. Plotted here are estimates of θ_Q (P, E) relative to the estimate obtained from the same experiment at the reference membrane voltage, $E = 26$ mV. It is seen that such relative values do not significantly depart from unity over the whole range of membrane potentials between −30 mV and +42 mV.

The decrease of Q with pressure was confirmed in all experiments. Figure 8 is a plot of the average factorial decrease of Q with pressure obtained by pooling, as θ_Q (P), all estimates in the voltage range −30 to +42 mV. The straight line is a least-squares fit of the data according to Eq. (3)

$$\ln \theta_Q(P) = (P - P_0)\,\Delta V^*/RT\,. \qquad (3)$$

The value of the apparent reaction volume, ΔV^*, which fits the data of Fig. 8 is −10cm³/mol. It appears that a fraction of the sodium channels is forced, by pressure, to undergo a transition to a state in which fast, voltage-induced, gating currents are prevented. In this case ΔV^* represents the net volume decrease

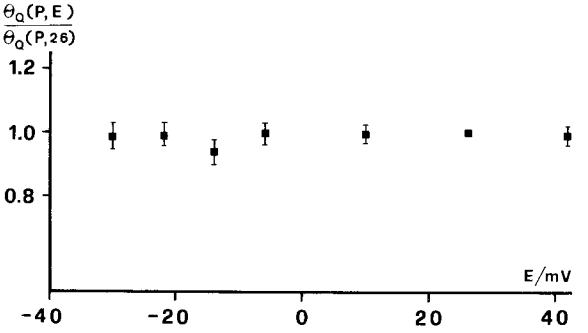

Fig. 7. Voltage dependence of the decrease in total gating charge with increasing pressure. The data are the mean ratios (± SEM), at any pressure in the range 10–60 MPa, between the factorial decrease of gating charge, θ_Q, at membrane potential, E, and at the reference voltage of 26 mV

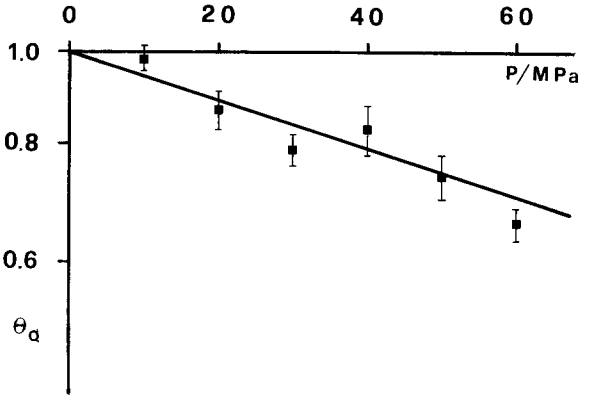

Fig. 8. Pressure dependence of the factorial decrease of mobile gating charge, θ_Q, produced by hyperbaric pressures. The data are mean values (± SEM) of measurements at membrane potentials in the range −30 to 42 mV. The straight line is the best fit of the data according to Eq. (3) and corresponds to a reaction volume for charge immobilization of −10 cm³/mol

associated with the transition to this "immobilized state", one which may be simply related to classical sodium inactivation or, to the "sleepy state" induced by low temperatures (Matteson and Armstrong 1982).

Discussion

It is evident that pressure has profound effects on the gating currents which accompany the conformational changes of sodium channels. These effects are qualitatively similar to, and of the same order of magnitude as, those produced on the ionic currents, leaving little doubt that the latter effects originate from a direct influence of pressure on the thermodynamics of the sodium channel structure, independently of whether sodium currents flow or not. The kinetics of gating currents have a much larger pressure dependence than expected from changes in the viscosity of the lipid matrix of the squid axon membrane, as probed by the transmembrane mobility of relatively small hydrophobic ions such as dipicrilamine or tetraphenylborate (Benz et al. 1984). This discourages any view of the conformational transitions of sodium channels as simple movements, within the membrane, of one or a few charged groups or dipoles. More realistically, it appears that these transitions involve rearrangements of considerable portions of the channel structure, and that the effective charge redistribution detected with I_g measurements represents only a part of the whole process.

The quantitative comparison of the effects of pressure on I_g and I_{Na} reveals some important differences which may be useful to the interpretation of the gating mechanism of sodium channels. First, we consistently find that gating charge movements are slowed by pressure to a lesser extent than the opening of sodium channels, as revealed by I_{Na}. The only simple way to explain this difference is to assume that a final step, which is rate-limiting for the opening of a sodium channel (at least at large depolarizations), has an activation volume about twice as large as the preceding steps, without appearing as a significant component of the charge redistribution. The observation that I_{Na} can be slowed down much less than I_g is not unique. For example, substitution of D_2O for normal water slows I_{Na} in squid axons by a factor of 1.4 (Conti and Palmieri 1968) without appreciably modifying I_g (Meves 1974), a phenomenon also observed and extensively studied in Mixycola axons (Schauf and Bullock 1979).

That the final, most pressure sensitive, step involves no charge redistribution cannot be definitely established from our data. For example, our results are qualitatively consistent with the kind of model proposed by Armstrong and Gilly (1979), which includes a final slow opening step, if we assume that the contributions to I_g due to the charge movement during this step have been somehow overlooked in our analysis because of measurement sensitivity limitations. Such a model can qualitatively account for the different pressure dependence of the activation curves of gating charge and sodium currents. As reported by Conti et al. (1982a), increasing pressure produces an appreciable shift in the depolarizing direction of the sodium activation curve, whereas no such systematic shift was observed in our present study for the voltage dependence of gating charge. The discrepancy can be explained by assuming that only the final opening step (assumed to play a major role in I_{Na} and to make a minor contribution to I_g) involves a small net volume increase, so that at high pressure the voltage at which this step has equal backward and forward rate constants is slightly more positive.

If one wishes to interpret the gating of sodium channels according to the parallel activation model recently proposed by Keynes (1983), on the basis of a dissection of I_g records into inactivating and non-inactivating components (Greef et al. 1982), one would have to add to such a kinetic scheme some rate-limiting, highly pressure-sensitive, final opening step. Such a step could be identified, for example, with the coupling reaction between inactivating and non-inactivating subunits.

The other unexpected finding of the present investigation is the decrease with pressure of the maximum displaced charge, Q_{max}. The changes observed were fully reversible and could not be reasonably attributed to any artefactual cause. We cannot exclude the possibility that some slow gating charge movement (associated for example, with the slow step hypothesized by Armstrong and Gilly 1979) became so slow at high pressures as to be lost in the baseline noise of our records. However, the direct evidence that the prolongation of the early time course of I_g records does not compensate for their decrease in amplitude shows unambiguously that the fast charge displacement is also decreased by pressure. Part of the observed decrease of Q with pressure might be attributed to an increase in sodium inactivation. In the present study, the gating currents were measured from a holding potential of -70 mV. According to the data of Conti et al. (1982a) the fast sodium inactivation at -70 mV is increased by almost 10% when the pressure is raised to 60 MPa, and it is conceivable that the slow sodium inactivation (Rudy 1978) is also slightly increased by pressure. It is possible, however, that this effect does not account for the whole 35% mean decrease of Q observed at 60 MPa.

It seems unlikely that the mobile charge abolished by pressure is not associated with channel gating, in view of the evidence presented by Armstrong and Croop (1982) and because it is implausible that such stray charge would have the same voltage dependence and the same time course as the persisting

gating charge. Thus, it seems that one must conclude that increasing pressure decreases the availability of sodium channels which are capable of undergoing fast conformational changes.

To account for the fraction of Q reduction which might not be attributable to an increase in sodium inactivation it could be proposed that some sodium channels become "sleepy" at high pressure, as they do at low temperature (Matteson and Armstrong 1982). The contribution to I_g from these channels, with a time constant of several milliseconds, would have been easily lost in the baseline of our records.

The above conclusion seems to be in contrast to the negligible pressure dependence of the maximum peak sodium conductance, g^p_{Na}, reported by Conti et al. (1982a). However, it should be stressed that the latter result does not necessarily imply that the number of fast activating sodium channels is independent of pressure; mainly because, in view of the fact that sodium inactivation is slowed by pressure much more than sodium activation (Conti et al. 1982a) (cf. Fig. 5), g^p_{Na} measurements give a biased indication of the pressure dependence of the maximum sodium conductance, $\bar{g}_{Na}$. On the other hand, should accurate measurements demonstrate that $\bar{g}_{Na}$ is decreased by pressure significantly less than Q, one would have to conclude that the single sodium channel conductance is increased by pressure, a result with interesting consequences for the interpretation of the mechanism of ionic flow through an open sodium channel.

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References

Almers W, Armstrong CM (1980) Survival of K permeability and gating currents in squid axons perfused with K-free media. J Gen Physiol 75: 61–78

Armstrong CM (1981) Sodium channels and gating currents. Physiol Rev 61: 644–683

Armstrong CM, Bezanilla F (1973) Currents related to the movement of the gating particles of the sodium channels. Nature 242: 459–461

Armstrong CM, Bezanilla F (1977) Inactivation of the sodium channel. II. Gating current experiments. J Gen Physiol 70: 567–590

Armstrong CM, Croop RS (1982) Simulation of Na channel inactivation by thiazin dyes. J Gen Physiol 80: 641–662

Armstrong CM, Gilly WF (1979) Fast and slow steps in the activation of Na channels. J Gen Physiol 74: 691–711

Bennett PB (1975) The high pressure nervous syndrome. In: Bennet PB, Elliot DH (eds) The physiology and medicine of diving and compressed air work. Bailliere Tindall, London, pp 248–263

Benz R, Conti F (1981) Structure of the squid axon membrane as derived from charge-pulse relaxation studies in the presence of absorbed lipophilic ions. J Membrane Biol 59: 91–104

Benz R, Conti F, Fioravanti R (1984) Extrinsic charge movement in the squid axon membrane: effect of pressure and temperature. Eur Biophys J 11: 51–59

Bezanilla F, Taylor RE (1978) Temperature effects on gating currents in the squid giant axon. Biophys J 23: 479–484

Bezanilla F, White MM, Taylor RE (1982) Gating currents associated with potassium channel activation. Nature 296: 657–659

Brauer RW (1975) The high pressure nervous syndrome: animals. In: Bennett PB, Elliott DH (eds) The physiology and medicine of diving and compressed air work. Bailliere Tindall, London, pp 231–247

Chandler WK, Meves H (1965) Voltage clamp experiments on internally perfused giant axons. J Physiol (London) 180: 799–820

Chandler WK, Meves H (1970) Sodium and potassium currents in squid axons perfused with fluoride solutions. J Physiol (London) 211: 623–652

Conti F, Palmieri G (1968) Nerve fiber behavior in heavy water under voltage-clamp. Biophysik 5: 71–77

Conti F, Fioravanti R, Segal JR, Stühmer W (1982a) Pressure dependence of the sodium current of squid giant axon. J Membr Biol 69: 23–34

Conti F, Fioravanti R, Segal JR, Stühmer W (1982b) Pressure dependence of the potassium currents of squid giant axon. J Membr Biol 69: 35–40

Ebbecke V, Schaefer H (1935) Über den Einfluß hoher Drucke auf den Aktionsstrom von Muskeln und Nerven. Pfluegers Arch Ges Physiol 236: 678–692

French RJ, Horn R (1983) Sodium channel gating: models, mimics, and modifiers. Annu Rev Biophys Bioeng 12: 319–356

Gilly WF, Armstrong CM (1980) Gating current and potassium channels in the giant axon of the squid. Biophys J 29: 485–492

Greeff NG, Keynes RD, van Helden DF (1982) Fractionation of the asymmetry current in the squid giant axon into inactivating and non-inactivating components. Proc R Soc Lond B 215: 375–389

Grundfest H (1936) Effects of hydrostatic pressure upon the excitability, the recovery and the potential sequence of frog nerve. Cold Spring Harbor Symp Quant Biol 5: 179–187

Harper AA, Macdonald AG, Wann KT (1981) The action of high hydrostatic pressure on the membrane currents of *Helix* neurones. J Physiol (Lond) 311: 325–339

Henderson JV, Gilbert DL (1975) Slowing of ionic currents in the voltage clamped squid axon by helium pressure. Nature 258: 351–352

Hodgkin AL, Huxley AF (1952) A quantitative description of membrane current and its application to conduction and excitation in nerve. J Physiol (London) 177: 500–554

Johnson FJ, Eyring H, Stover B (1974) The theory of rate processes in biology and medicine. Wiley, New York

Keynes RD (1983) Voltage-gated ion channels in the nerve membrane. Proc R Soc Lond 220: 1–30

Kimura JE, Meves H (1979) The effect of temperature on the asymmetrical charge movement in squid giant axons. J Physiol (London) 289: 479–500

Macdonald AG (1984) The effects of pressure on the molecular structure and physiological functions of cell membranes. Philos Trans R Soc Lond B304: 47–68

Matteson DR, Armstrong CM (1982) Evidence for a population of sleepy sodium channels in squid axon at low temperature. J Gen Physiol 79: 739–758

Meves H (1974) The effect of holding potential on the asymmetry currents in squid giant axons. J Physiol 243: 847–867

Moore JW, Cole KS (1963) Voltage-clamp technique. In: Nastuk WL (ed) Physical techniques in biological research, Vol 6: Electrophysiological methods, part B. Academic Press, New York

Rojas E, Ehrenstein G (1975) Voltage clamp experiments with potassium as the only internal and external cation. J Cell Comp Physiol 66: 71–77

Rudy B (1978) Slow inactivation of the sodium conductance in squid giant axons. Pronase resistance. J Physiol 283: 1–21

Schauf CL, Bullock JO (1979) Modifications of sodium channel gating in *Muxicola* giant axons by deuterium oxide, temperature, and internal cations. Biophys J 27: 193–208

Spyropoulos CS (1957a) Responses of single nerve fibers at different hydrostatic pressures. Am J Physiol 189: 214–218

Spyropoulos CS (1957b) The effects of hydrostatic pressure upon the normal and narcotizated nerve fiber. J Gen Physiol 40: 849–857

Stühmer W, Conti F, Inoue I (1983) The effect of hydrostatic pressure on Na gating currents of squid giant axons. Biophys J 41: 54a

Tasaki I, Watanabe A, Takenaka T (1962) Resting and action potential of intracellularly perfused squid giant axon. Proc Natl Acad Sci USA 48: 1177–1181

Wann KT, Macdonald AG (1980) The effects of pressure on excitable cells. Comp Biochem Physiol 66A: 1–12