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Extrinsic charge movement in the squid axon membrane

Effect of pressure and temperature

R. Benz*, F. Conti, and R. Fioravanti**

Istituto di Cibernetica e Biofisica, Consiglio Nazionale delle Ricerche, I-16032 Camogli, Italy

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Abstract. The absorption of the lipophilic anions dipycrilamine (DPA^-) and tetraphenylborate ($TPhB^-$) by the lipid matrix of the squid axon membrane, and the kinetics of their translocation, were studied by the charge pulse relaxation technique. The axons were treated with tetrodotoxin (TTX) and 4-aminopyridine to block the ionic currents responsible for nerve excitation. At high enough concentrations of absorbed ions ($\sim 10^{-12}$ mol/cm^2) the membrane voltage relaxation following a brief current pulse consisted mainly of two exponential components, whose time constants and relative amplitudes were used for estimating the translocation rate constant, K, and the density of absorbed ions, N. These measurements were performed at different hydrostatic pressures in the range 1–100 MPa ($\sim$ 1,000 atm), and at different temperatures in the range 5° C–20 °C. Both K and N were found to be little affected by pressure. The pressure dependence of K indicated that the translocation of lipophilic ions across the nerve membrane involves activation volumes of the order of 5 cm^3/mol. In all experiments the passive membrane resistance was little affected by pressures up to 80 MPa. However, above 100 MPa it fell dramatically to low values, presumably because of phase separation phenomena between the membrane components. The temperature dependence of K, both for DPa^- and $TPhB^-$, implied an activation energy for ion translocation of the order of 60 kJ/mol, close to that measured in artificial lipid bilayers.

It is concluded that the lipid bilayer structure of the nerve membrane is not modified by pressures below 80 MPa and that the intramembrane movements of relatively small charged groups cannot account for the large activation volumes involved in the gating of ionic channels.

Key words: Nerve, membrane, axon, pressure, activation volume

Introduction

Measurements of the kinetics of lipophilic ion translocation across artificial lipid bilayers have been extensively used for membrane structure studies (Andersen and Fuchs 1975; Benz et al. 1976; Benz and Läuger 1977; Benz and Cros 1978; Benz and Gisin 1978; Andersen et al. 1978). Most of these investigations have been performed using the charge pulse method. This method allows the calculation of the concentration of adsorbed ions, N, and the translocation rate constant, K, from the relaxation of the membrane potential following a sudden charging of the membrane capacitance by a brief current pulse (Benz and Läuger 1976; Benz et al. 1976). These parameters provide information about membrane properties such as surface charge, dipole potential and membrane thickness, which have a strong influence on N and K (Benz and Läuger 1977; Benz and Cros 1978; Benz and Gisin 1978; Andersen et al. 1978). Recently, lipophilic ions have also been successfully used for probing the structure of nerve membranes (Benz and Conti 1981; Benz and Nonner 1981; Fernandez et al. 1983), providing information about the intrinsic asymmetry potential, the composition, and the thickness of the lipid matrix of these membranes.

Hydrostatic pressure has long been known to have marked effects on nerve and muscle physiology (Ebbecke and Schaefer 1935; Grundfest 1936; Spyropoulos 1957a, b; for a review, see Wann and Macdonald 1980). Recently, the pressure dependen-

* Permanent address: Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz, Federal Republic of Germany, recipient of a fellowship by EMBO

** To whom reprint requests should be sent

ce of ionic currents in squid giant axons has been extensively analysed. Interpretation in terms of direct effects of pressure on the rate constants of the gating of ionic channels has been used to obtain estimates of the apparent volume changes involved in the conformational transitions of these membrane proteins (Henderson and Gilbert 1975; Conti et al.1982a, b). This interpretation is based upon the assumption that high pressures do not modify the properties of the lipid matrix in which the ionic channels are embedded. Measurements of the effect of pressure upon the absorption and translocation rate of lipophilic ions in the nerve membrane can test this assumption, and provide information about the possible origin of the estimated volume changes. Indeed, the translocation of lipophilic ions simulates several aspects of the intramembrane charge movements which accompany the gating of ionic channels (Almers 1978; Benz and Nonner 1981; Benz and Conti 1981; Fernandez et al. 1983), and its pressure dependence can tell us if the movement of gating charges might per see, involve significant activation volumes.

In this paper we report that the properties of axon membranes containing extrinsic lipophilic ions are little affected by pressures up to 1,000 MPa, which is evidence that the lipid matrix of the axon membrane is not modified by high pressure and that the intramembrane movement of charged groups involves only small activation volumes. We also report measurements of the temperature dependence of lipophilic ion translocation, which yields information on the activation energy of this phenomenon in the squid axon membrane.

We conclude from our results that the strong pressure dependence of ionic currents in squid axons is unlikely to result from hindrance of gating charge movements. The latter appear to be only one aspect of a large structural change undergone by the ionic channels during their transitions between closed and open states. A preliminary report on part of this work has already been presented (Benz et al. 1983).

Materials and methods

The experiments were performed on giant axons dissected from the hindmost stellar nerve of the squid *Loligo vulgaris,* available in Camogli. The axons were placed in a perspex chamber, specially designed for measurements at high hydrostatic pressure, as described by Conti et al. (1982a).

Measurements of membrane potential relaxations following charge pulses followed standard procedures described in detail by Benz and Conti (1981). In preliminary experiments the active currents of the axons were abolished by adding 300 nM tetrodotoxin (TTX) to the extracellular bath (artificial sea water: 450 mM NaCl; 10 mM KCl, 50 mM $CaCl_2$; 1 mM TrisCl; pH 7.8) and by perfusing the axons intracellularly with a standard solution containing sucrose, potassium phosphate buffer with 300 mEq/l K^+, and 20 mM tetraethyl ammonium chloride, pH 7.3. In these experiments the lipophilic ions (Fluka, Buchs, Switzerland) were added both to the intracellular and to the extracellular perfusate at concentrations in the range $1-3 \times 10^{-7}$ M. However, considering the long time required for the absorption of lipophilic ions and the additional difficulties involved in the experiments at high hydrostatic pressure, most of the later measurements were performed on unperfused axons. In this case the active potassium currents were abolished by extracellular addition of 10 mM 4-aminopyridine (4-AP) (Yeh et al. 1976) and the lipophilic ions were added intracellularly by depositition on the Pt-Pt black wire, used as the intracellular current electrode, several films of a 10^{-4} M solution of the lipophilic ion in ethanol. In these experiments we did not measure the steady-state membrane potential with a separate voltage electrode, but this simplification was of no consequence because a small uncertainty on its value has little effect on the interpretation of the data (Benz and Conti 1981). Voltage relaxations were measured at the resting potential, estimated to be around -40 mV, i.e., $10-15$ mV less negative than normal because of the addition of 10 mM 4-AP (Yeh et al. 1976).

The pressurizing system, using vaseline oil as 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.

The temperature near the axon was continuously monitored with a thermistor. Most measurements were performed at 13° C. Temperature variations during measurements at different pressures were kept within 1° C, by allowing some time for thermal reequilibration of the oil bath after each pressure change. Since the temperature coefficient of lipophilic ion kinetics is quite low (Benz et al. 1976; Benz and Conti 1981; this paper) no temperature correction was made to the data. For the measurements of

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

the activation energy for lipophilic ion translocation the temperature was varied between 5° C and 22° C at a rate of about 0.5 °C/min, presumably yielding a negligible temperature gradient across the axon.

The charge pulse relaxation measurements were performed as previously described (Benz and Conti 1981). A fast pulse generator (Philips PM 5712) was connected to the internal current electrode via a diode with reverse resistance larger than 10^{12} Ω. The voltage relaxation following a charge pulse was boosted with a voltage amplifier (Burr Brown 3551J) and measured with a digital oscilloscope (Nicolet Explorer III). The time resolution of the apparatus was about 2 μs. Analysis of the data was carried out with a Hewlett Packard 9825 table computer (with plotter).

Theory

The theory of the movement of extrinsic charges across the squid axon membrane has been given in full detail in a previous publication (Benz and Conti 1981). Here we will only summarize the basic assumptions and list the simplified equations which allow the calculation of the transport parameters from our present experimental data.

It is assumed that the extrinsic charges are located in free energy wells at the two membrane-solution interfaces, with a total concentration per unit membrane surface, N. At the asymmetry membrane potential (which is close to the resting potential for the experiments reported in this work; Benz and Conti 1981), the lipophilic ions are equally distributed between the two interfaces, and cross the single energy barrier separating the two wells with the same rate constant, K, in either direction. Under these conditions, the shape of the barrier has no influence on the description of the charge pulse experiments as long as they are performed in the limit of low voltage ($V \ll 25$ mV; Benz and Zimmermann 1983). Finally, the exchange of lipophilic ions between the membrane and the aqueous phase is neglected within the time of a relaxation measurement because it is rate-limited by diffusion (Benz et al. 1976). Also, it has characteristic times much longer than the passive time constant of the squid axon membrane ($\tau_M = R_M C_M$, where R_M and C_M are the membrane specific resistance and capacitance, respectively).

In a charge pulse experiment the system is at equilibrium at any time, $t < 0$, and the membrane capacitance is charged instantaneously at $t = 0$ to produce a voltage increment, V_0. The decay of this voltage perturbation, $V(t)$, is described by a double exponential equation:

$$V(t)/V_0 = a_1 \exp(-\lambda_1 t) + a_2 \exp(-\lambda_2 t), \quad (1)$$

where a_1, $a_2 = 1\text{-}a_1$, λ_1, and λ_2 are functions of K, N, and τ_M. The inverse relations, which allow the estimation of the latter parameters from the experimental results, are given by (see Eqs. (16)–(25) of Benz and Conti (1981), with $\alpha_2 = 1$, $\bar{\nu} = 0$, and z = 1):

$$K = (a_2\lambda_1 + a_1\lambda_2)/2, \quad (2)$$

$$N = 2RTC_M (\lambda_1 + \lambda_2 - 2K - \lambda_1\lambda_2/K)/F^2K, \quad (3)$$

$$\tau_M = 2K/\lambda_1\lambda_2, \quad (4)$$

where R is the gas constant, T is the temperature, and F is the Faraday constant.

Results

Pressure dependence of the kinetics of lipophilic ion movement

In a previous study of the kinetics of lipophilic ion movement in the squid axon membrane (Benz and Conti 1981), it was found to be difficult to establish equilibrium for the partition of lipophilic ions between the membrane and the aqueous phase. When introduced only in the extracellular solution the extrinsic ions were only slightly adsorbed on the membrane in a reasonable time, whereas intracellular perfusion yielded different results from axon to axon, presumably depending on the different extent of enzymatic removal of axoplasm. In this study the measurements were started after the axon, impaled with the internal current electrode, had been kept for 1 h in the extracellular bath containing the extrinsic ions. An estimated 50 pmol of lipophilic ion had been deposited on the internal current electrode. If these ions had uniformly redistributed themselves throughout the axoplasm, their final intracellular concentration would have been around 6 μM. However, most of the ions were presumably stripped away right at the cut end of the axon where the electrode was introduced, and the concentration near the axon membrane was kept much lower by diffusion limitations and a continuous loss of extrinsic ions into the Schwann cell layer and the remaining connective tissue. Nevertheless, the quasi-stationary concentration of lipopholic ions obtained within the axon membrane after about 1 h was high enough to produce a distinct membrane-voltage relaxation, due to the fast redistribution of extrinsic charges, whose kinetics could be measured as a function of pressure. This relaxation has a much larger amplitude than that associated with the movement of intrinsic charges which is detectable in the absence of lipophilic ions (Benz and Conti 1981).

Figure 1 shows the influence of hydrostatic pressure on the voltage decay, following brief charge pulses of 50 ns duration, in an axon treated with DPA^-. The experiment was performed according to the following protocol. First we took a control measurement at normal pressure (0.1 MPa); then the pressure was raised and the measurement repeated at 20 MPa. After return to normal pressure another control was taken and the same procedure was repeated to test higher pressures up to 100 MPa in steps of 20 MPa. The four traces in Fig. 1A were obtained at 0.1 MPa, 40 MPa, 80 MPa, and 100 MPa. Figure 1B shows all the control traces, from the initial one to that obtained after return from 100 MPa. It is obvious that increasing the hydrostatic pressure slows down the voltage relaxation process in a reversible manner.

The equations given in the Theory section allow a quantitative description of the above effects in terms of the parameters which characterize the absorption and the movement of the extrinsic ions in the squid axon membrane. The results of such an analysis for the experiment illustrated in Fig. 1 are summarized in Table 1. Increasing pressure from 0.1 MPa to 100 MPa produced a gradual decrease of K from about 10 ms^{-1} to about 7 ms^{-1}. The change in N was less systematic. While it seemed to decrease by 30%–40% at 80 and 100 MPa, it appeared to be little affected or even slightly increased at 20, 40, or 60 MPa. This variability probably arises from two major sources of error in the estimate of N. Firstly, as already discussed in Materials and methods, the amount of membrane absorbed ions can undergo slow changes, independently of pressure. Secondly, N has a much stronger dependence than K on membrane potential (Benz and Conti 1981), and Eq. (3) could be a poor approximation if resting potential variations of the order of 10 mV are produced by

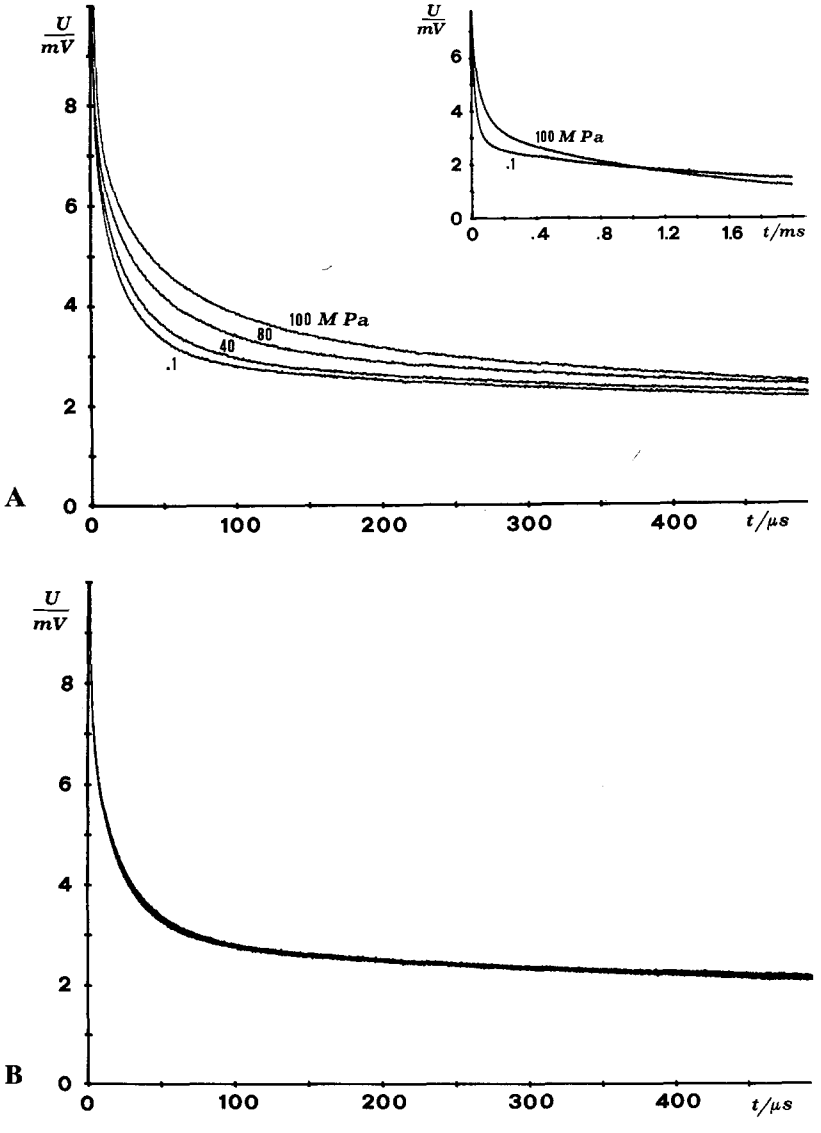

Fig. 1 A and B. Pressure dependence of the membrane potential relaxation after a charge pulse of 50 ns duration, in an axon treated with DPA^-. In **A** the relaxation curves measured at the indicated pressures are shown on an expanded time scale, while the inset shows a longer sample of the relaxations measured at atmospheric pressure (0.1 MPa) and at 100 MPa. **B:** Six superimposed control records obtained at 0.1 MPa in the same experiment before, between, and after five different successive pressurizations. The concentration of membrane absorbed DPA^- was estimated in the order of 1 pmol/cm (see Table 1). Temperature: 14° C

Table 1. Analysis of the experiment illustrated in Fig. 1, according to Eqs. (1)–(4)

P/MPa	$\lambda_1^{-1}/\mu s$	λ_2^{-1}/ms	a_1	$K/[\text{ms}]^{-1}$	$N/\text{pmol} \cdot \text{cm}^{-2}$	τ_M/ms
0.1	23.5	3.13	0.56	9.5	1.24	1.40
20	23.9	3.27	0.56	9.3	1.26	1.45
0.1	24.2	3.29	0.53	9.7	1.13	1.55
40	27.1	3.50	0.52	8.9	1.09	1.68
0.1	22.8	3.42	0.54	10.2	1.16	1.59
60	30.2	3.19	0.49	8.5	0.95	1.64
0.1	23.0	3.35	0.54	10.2	1.14	1.57
80	36.9	3.17	0.47	7.3	0.85	1.71
0.1	24.3	3.53	0.53	9.8	1.11	1.68
100	42.9	2.07	0.41	7.0	0.65	1.25
0.1	25.2	3.24	0.52	9.6	1.07	1.57

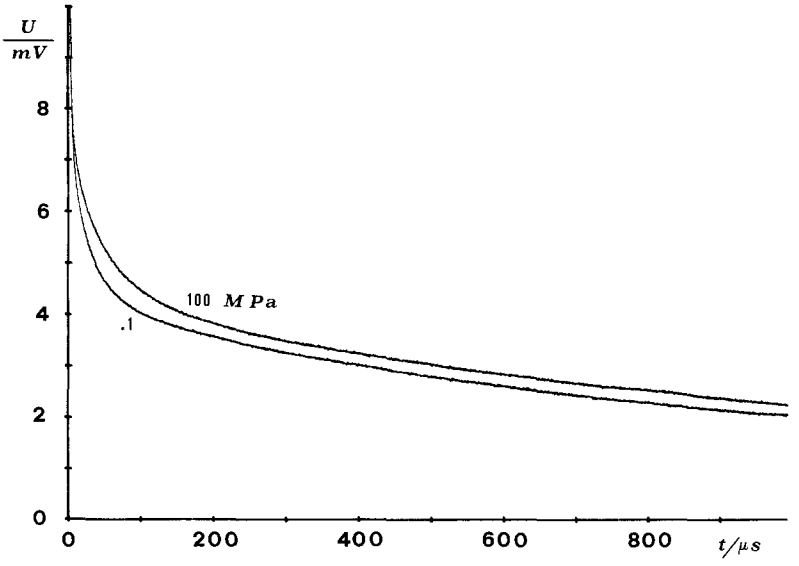

Fig. 2. Charge pulse relaxation curves measured at atmospheric pressure and at 100 MPa from an axon with a concentration of absorbed DPA^- about half of that of the axon of Fig. 1. Temperature: 13° C

pressure changes. Finally, changes in N brought about by pressure-induced changes in the absorption coefficient are expected to take a long time to occur and therefore necessarily depend on the duration of the pressurization period. This time could vary considerably in different experiments, usually being longer for higher pressures.

The passive membrane time constant was estimated from the relaxation data to be practically independent of pressure, having in the experiment of Fig. 1 values in the range of 1.4–1.7 ms. It has to be noted, however, that a dramatic decrease of τ_M usually occurred in the pressure range 100–120 MPa. This decrease, due to a breakdown of membrane resistance, seems similar to that previously observed by Spyropoulos (1957b), except that this author reports that this phenomenon occasionally occurs at much lower pressures. Also in our experiments the drop in R_M did not seem to occur at any sharp transition point. For example, the decrease in τ_M observed at 100 MPa in the experiment in Table 1 could suggest a gradual beginning of such a phenomenon. After breakdown the high membrane resistance could be slowly recovered within 10 min if the axons were rapidly taken to atmospheric pressure. However, if the axon was kept for several minutes above the critical pressure range, τ_M dropped to values of the order of 100 μs and only a very poor recovery was obtained at atmospheric pressure. It seems obvious that this phenomenon is associated with a dramatic irreversible change of the structure of the axon membrane, possibly due to phase separation within the lipid or between lipids and membrane proteins.

In general, the effect of pressure on the interaction of DPA^- with the squid axon membrane was rather small. At 100 MPa, the rate constant of DPA^- translocation across the membrane never decreased below 65% of its value at atmospheric pressure and the apparent influence of pressure on DPA^- absorption was even smaller. This conclusion was not dependent on the amount of adsorbed lipophlic ions. Figure 2 shows voltage relaxation curves measured at 0.1 MPa and 100 MPa from an axon with a total

concentration of absorbed DPA^- about one half of that of the axon in Fig. 1. The analysis of these data showed that in going from 0.1 MPa to 100 MPa, K decreased from 11 to 7.7 ms^{-1}, whereas N decreased from 0.64 to 0.52 pmol · cm^{-2}.

An overall description of the effects of pressure, P, on DPA^- treated axons is given in Fig. 3. The figure shows plots of the changes in K and N at various hyperbaric pressures, relative to the mean values measured before and after pressurization. The data points refer to 38 measurements on six different axons. The straight lines are least-squares fits to the data based on the interpretation of the pressure effect in terms of Eq. (6) (see Discussion), which yield an estimate of the activation volume for DPA^- translocation of 5.4 $cm^3 \cdot mol^{-1}$, and an apparent reaction volume for DPA absorption of 4.7 $cm^3 \cdot mol^{-1}$.

It has been shown that the membrane structure has similar effects on the kinetics of $TPhB^-$ and DPA^-, apart from the fact that $TPhB^-$ has a K value about 40 times smaller (Benz et al. 1976). Thus, it is no surprise that the kinetics of $TPhB^-$ transport through the squid axon membrane is affected by pressure as little as that of DPA^-. Figure 4 shows charge pulse measurements performed with an axon treated with $TPhB^-$. The control at 0.1 MPa was taken about 1 h after $TPhB^-$ was added to the axon from outside (300 nM) and inside (40 pmol in a volume of about 8 µl). The other two measurements shown in Fig. 4 were taken at later times at 60 MPa

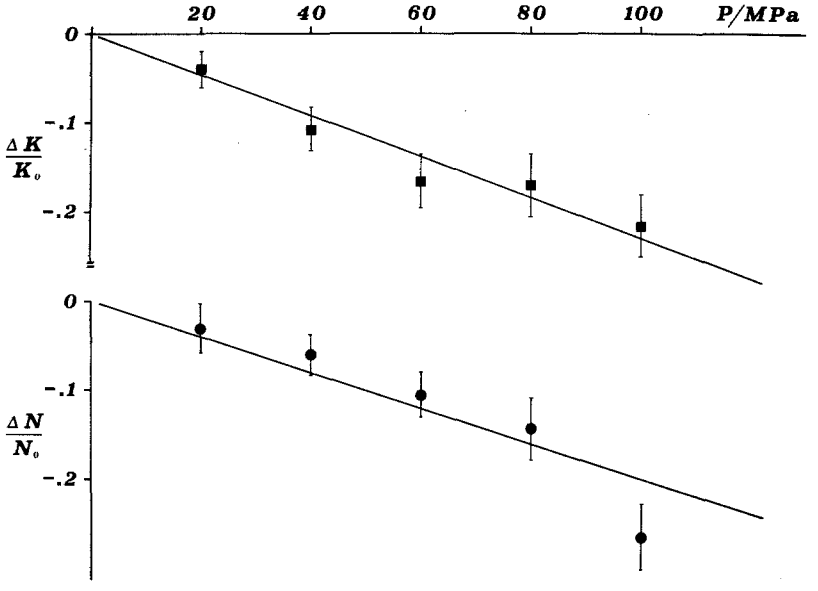

Fig. 3. Pressure dependence of the parameters which characterize the interaction of DPA^- with the squid axon membrane. K_0 and N_0 are the mean estimates of the translocation rate constant and concentration of absorbed ions at atmospheric pressure before and after any measurement at pressure P. ΔK and ΔN are the changes of K and N measured during pressurization. The data points and the vertical bars give the mean ± standard deviation from six measurements at 20 MPa, nine at 40 MPa, nine at 60 MPa, nine at 8 MPa, and five at 100 MPa. The data were obtained from six different axons at an average temperature of 13° C. The straight lines are least squares fits according to Eq. (6), yielding an activation volume of 5.4 cm^3/mol for DPA^- translocation and an apparent reaction volume of 4.7 cm/mol for DPA^- absorption

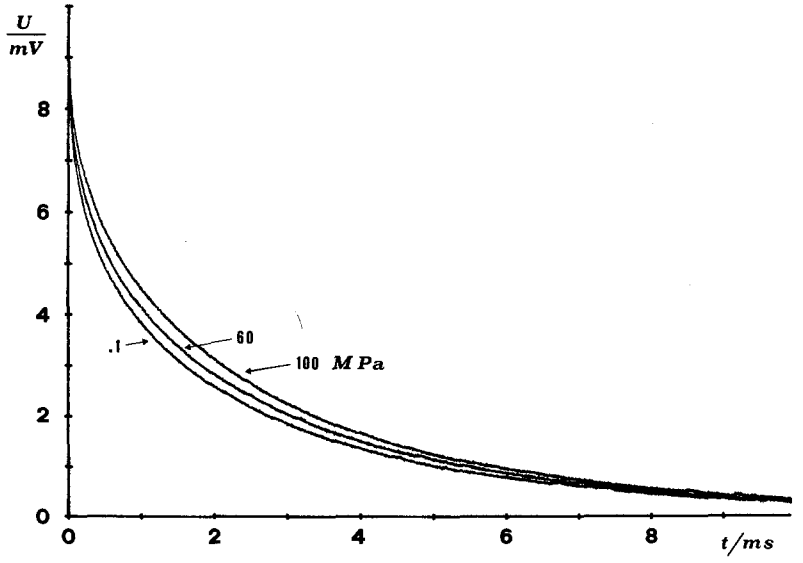

Fig. 4. Pressure dependence of the charge pulse relaxation curves in an axon treated with $TPhB^-$. Experimental conditions are similar to those described in the legend of Fig. 1. The concentration of membrane absorbed $TPhB^-$ was estimated to be of the order of 0.3 pmol/cm^2 (see Table 2). Temperature: 13° C

Table 2. Analysis of the experiment illustrated in Fig. 4, according to Eqs. (1)–(4)

P/MPa	λ_1^{-1}/ms	λ_2^{-1}/ms	a_1	K/[ms]$^{-1}$	N_t/pmol · cm^{-2}	τ_M/ms
0.1	0.69	4.3	0.34	0.52	0.31	3.1
20	0.70	4.4	0.34	0.51	0.32	3.1
40	0.79	4.2	0.35	0.45	0.30	3.0
60	0.81	3.5	0.37	0.44	0.28	2.5
0.1	0.68	3.7	0.37	0.51	0.33	2.6
60	0.87	3.8	0.35	0.42	0.26	2.8
80	1.0	3.5	0.33	0.38	0.20	2.7
100	1.2	3.7	0.35	0.32	0.18	2.8
0.1	0.71	3.8	0.36	0.50	0.31	2.7

and 100 MPa, respectively. Table 2 shows the data for K, N, and τ_M, obtained from the analysis of the whole sequence of measurements performed during this experiment. It is also seen in this case that the effect of pressure is small and reversible. Increasing pressure from 0.1 MPa to 100 MPa decreased K and N by about 30%–35%. Similar results were obtained in three other experiments with $TPhB^-$ treated axons.

Temperature dependence

Vaseline oil and water undergo small temperature changes during the compression and decompression phases of our experiments. To test if these changes significantly affected our measurements we performed a number of charge pulse experiments at normal pressure and different temperatures on axons treated with both lipophilic ions, DPA^- and $TPhB^-$. The results for the temperature dependence of K are summarized in Fig. 5 by an Arrhenius plot of the ratio $K(T)/K^*$, where $K(T)$ and K^* are the estimates of the translocation rate constant measured in the same axon at temperature T and at 13° C. Going up and down in temperature in the same preparation yielded fully reversible changes of $K(T)$. It is seen that these effects were small both for DPA^- and for $TPhB^-$. The straight line which gives a best fit for the data of Fig. 5 corresponds to an activation energy of about 63 kJ/mol (15 Kcal/mol). As described in Materials and Methods, temperature variations between measurements at different pressures were kept within 1° C. Figure 5 shows that such variations could not have a large influence on the results given in the previous section, which were not corrected for temperature effects. This was also justified by the finding that relaxation data taken at different times during a prolonged pressurization (up to 10 min) did not show any significant systematic drift. The activation energy obtained from the data of Fig. 5 for both lipophilic anions compares very well with that measured in lipid

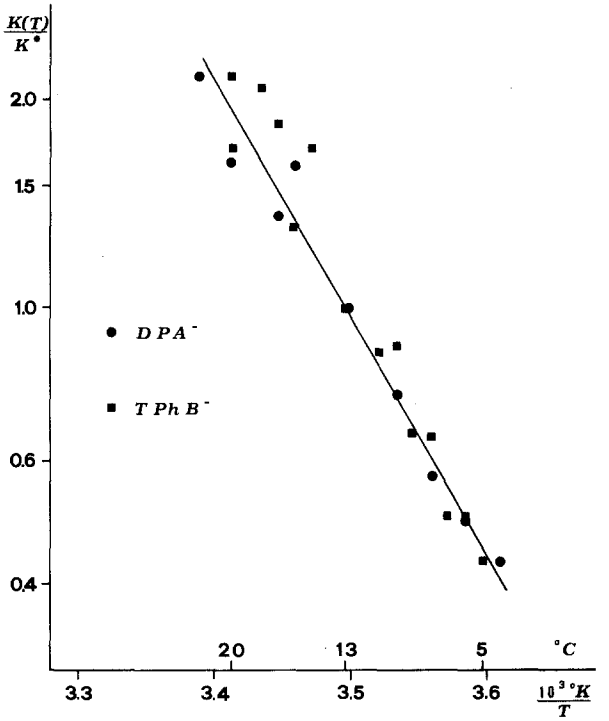

Fig. 5. Temperature dependence of the kinetics of lipophilic ion translocation across the squid axon membrane. K^* is the estimated value of the translocation rate constant at the reference temperature of 13° C. The straight line is the least-squares fit of the Arrhenius plot, yielding an activation energy of 63 kJ/mol

bilayer membranes. The temperature dependence of the translocation rate constants of DPA^- and $TPhB^-$ in dioleyl-phosphatidylcholine bilayers correspond to activation energies of about 40 kJ/mol. (Benz et al. 1976), and 50 kJ/mol (Benz, unpublished results).

Discussion

The results that we have presented further confirm the idea that there is no basic difference in the physical environment experienced by extrinsic lipophilic ions in lipid bilayers or in a biological

membrane (Benz and Conti 1981; Benz and Nonner 1981). Quantitative differences in the ion translocation rate constants measured in either membrane system are most probably merely due to a different thickness of the hydrocarbon core, which is much thicker in artificial lipid bilayers containing solvent than in a solvent-free lipid bilayer structure such as that expected to occur in a natural membrane. Indeed a strong dependence of the translocation rate for lipophilic ions on membrane thickness is predicted on purely electrostatic grounds by the image force model (Parsegian 1969; Neumcke and Läuger 1969; Benz and Läuger 1977). Thus, the hydrocarbon regions of nerve membranes and artificial lipid bilayers appear to be identical as far as the adsorption of lipophilic ions is concerned. This is in apparent contrast with the results of Conrad and Singer (1979), who found an abnormally low adsorption of other amphipatic compounds into biological membranes and conclude that the latter have an abnormally high surface pressure.

Within the simple theoretical framework adopted in this paper the translocation of a lipophilic ion across the membrane is described as a first-order reaction with identical forward and backward rates, K. According to Eyring's theory of absolute reaction rates (Johnson et al. 1974), the pressure dependence of K can then be expressed as:

$$K(P) = K(P_0) \exp[-\Delta V^{\neq}(P - P_0)/RT], \qquad (5)$$

where $\Delta V^{\neq}$ is the transient volume change (activation volume) per mole, produced during the movement of a lipophilic ion from one of its equilibrium positions to the top of the intermediate free energy barrier. For small relative changes such as those observed in this work, Eq. (5) becomes:

$$[K(P) - K(P_0)]/K(P_0) = -\Delta V^{\neq}(P - P_0)/RT, \qquad (6)$$

which justifies the fit of the data of Fig. 3A with a straight line. That fit yielded a positive activation volume of 5.4 cm^3/mol. A similar estimate, whithin the experimental error, was obtained from $TPhB^-$ data. These activation volumes are about one order of magnitude smaller than those which describe the pressure dependence of the kinetics of ionic currents across the squid axon membrane (Conti et al. 1982a, b).

A similar interpretation of the pressure dependence of lipophilic ion absorption is in principle possible, but it would be inappropriate for the fit of our present data. The exchange of lipophilic ions between membrane and aqueous phase is very slow, because it is diffusion-controlled (Benz et al. 1976; Jordan and Stark 1979). Even if the absorption coefficient was strongly pressure-dependent, it is unlikely that our estimates of N for relatively short pressurization times give a true reflecton of this effect. The straight line through the data of Fig. 3B would correspond to a positive volume of DPA^- absorption of 4.7 cm^3/mol, but the above considerations and the relatively large errors which may be involved in the estimate of N discourage us from placing any confidence in this figure.

Concerning the passive electrical properties of the squid axon membrane, our present data confirm previous results (Spyropoulos 1975b; Conti et al. 1982a) which show very small changes of membrane resistance and capacitance at pressures below 60 MPa. Indeed, we found no significant effect of pressure on the membrane time constant, τ_M, for pressures up to 80 MPa. At higher pressures, generally only above 100 MPa, a breakdown in the resistivity of the squid axon membrane was observed, suggesting the triggering of phase separation phenomena. A similar effect has been described in the early work of Spyropoulos (1957b), except that this author reports having frequently observed it in the pressure range 40–80 MPa.

Temperature did not have a large influence on the kinetics of lipophilic ions. Increasing the temperature from 5° C to 20° C increased K by a factor of 4, yielding an estimated activation energy of about 60 kJ/mol which is comparable to that measured in lipid bilayer membranes (Bruner 1975; Benz et al. 1976; Benz unpublished results). The difficulty in establishing partition equilibrium between membrane and aqueous phase prevented a reliable estimate of the free energy change associated with the adsorption of lipophilic ions to the squid axon membrane. Qualitatively, however, the decrease of N with increasing temperature was consistent with the observation that the adsorption of lipophilic ions on a lipid bilayer membrane is associated with a net increase in entropy (Bruner 1975; Benz et al. 1976).

The effect of hydrostatic pressure upon the electrical properties of the nerve membrane can provide direct information on the structure and dynamics of the macromolecules which govern its ionic permeability changes. Using the most simple hypothesis, the pressure dependence of the kinetics of ionic currents is a direct consequence of the fact that channel proteins change their effective volume during a conformational transition, and the pressure effect provides quantitative estimates of these volume changes (Conti et al. 1982a, b). This interpretation is now strongly supported by the present study of the effects of pressure on the structure of the lipid matrix containing the channel proteins. This paper provides evidence that the relatively low pressures, which produce large changes in the kinetics of ionic

channels, do not alter to any comparable degree the properties of the membrane lipid, as probed by the transmembrane mobility of lipophilic ions. The results reported here also have implications for the possible interpretation of the origin of the activation and reaction volumes involved in the opening and closing of ionic channels. For example, it seems safe to rule out a major role of changes in electrostriction of the solvation shell of charged (gating) groups. Such changes could occur as a consequence of the movement of these groups within the lipid matrix between regions of different polarity. However, if this effect was important, it should be most pronounced in the case of the translocation of lipophilic ions across the whole membrane, often used in the past as a realistic model for gating charge movements.

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References

Almers W (1978) Gating currents and charge movements in excitable membranes. Rev Physiol Biochem Pharmacol 82: 96–182

Andersen OS, Feldberg S, Nakadomari H, Levy S, McLaughlin S (1978) Electrostatic interactions among hydrophobic ions in lilid bilayer membranes. Biophys J 21: 35–70

Andersen OS, Fuchs M (1975) Potential energy barriers to ion transport within lipid bilayers. Studies with tetraphenylborate. Biophys J 15: 795–830

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 Membr Biol 59: 91–104

Benz R, Cros D (1978) Influence of sterols on ion transport through lipid bilayer membranes. Biochim Biophys Acta 506: 265–280

Benz R, Gisin BF (1978) Influence of membrane structure on ion transport through lipid membranes. J Membr Biol 40: 293–314

Benz R, Läuger P (1976) Kinetic analysis of carrier-mediated ion transport by the charge-pulse technique. J Membr Biol 27: 171–191

Benz R, Läuger P (1977) Transport kinetics of dipicrylamine through lipid bilayer membranes. Effects of membrane structure. Biochim Biophys Acta 468: 245–258

Benz R, Nonner W (1981) Structure of the axolemma of frog myelinated nerve: Relaxation experiments with a lipophilic probe ion. J Membr Biol 59: 127–134

Benz R, Zimmermann U (1983) Evidence for the presence of mobile charges in the cell membrane of *Valonia ultricularis*. Biophys J 43: 13–26

Benz R, Conti F, Fioravanti R (1983) Pressure dependence of lipophilic ion translocation across the squid axon membrane. Atti VI Congresso SIBPA-VII Congresso GNCB, Camogli, pp 33–34

Benz R, Läuger P, Janko K (1976) Transport kinetics of hydrophobic ions in lipid bilayer membranes. Charge pulse relaxation studies. Biochim Biophys Acta 455: 701–720

Bruner LJ (1975) The interaction of hydrophobic ions with lipid bilayer membranes. J Membr Biol 22: 125–141

Chandler WK, Meves H (1965) Voltage clamp experiments on internally perfused giant axons. J Physiol (London) 180: 788–820

Conrad MJ, Singer SJ (1979) Evidence for a large internal pressure in biological membranes. Proc Natl Acad Sci USA 76: 5202–5206

Conti F, Fioravanti R, Segal JR, Stühmer W (1982a) Pressure dependence of the sodium currents 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 Drücke auf den Aktionsstrom von Muskeln und Nerven. Pfluegers Arch Ges Physiol 236: 678–692

Fernandez JM, Taylor RE, Bezanilla F (1983) Induced capacitance in the squid giant axon. J Gen Physiol 82: 331–346

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

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

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

Jordan PC, Stark G (1979) Kinetics of transport of hydrophobic ions through lipid membranes including diffusion polarization in the aqueous phase. Biophys Chem 10: 273–287

Neumcke B, Läuger P (1969) Nonlinear electrical effects in lipid bilayer membranes. Biophys J 9: 1160–1170

Parsegian A (1969) Energy of an ion crossing a low dielectric membrane. Solutions of four relevant electrostatic problems. Nature (London) 221: 844

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

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

Yeh JZ, Oxford GS, Wu CH, Narahashi T (1976) Dynamics of aminopyridine block of potassium channels in squid axon membrane. J Gen Physiol 68: 519–535