

# Single acetylcholine receptor channel currents recorded at high hydrostatic pressures

(single channel/pressure/activation volume)

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**ABSTRACT** A technique for performing patch-clamp experiments under high hydrostatic (oil) pressure is described. The method allows the transfer of whole cells or membrane patches in a recording configuration into a pressure vessel, where pressure can be increased up to 60 MPa ( $\approx$  600 bar). We have studied in this way the pressure dependence of single acetylcholine receptor channels in excised "outside-out" membrane patches from cultured rat muscle cells. In the range of 0.1 to 60 MPa the open channel conductance in 140 mM NaCl solutions did not vary by more than 2%, which implies that the translocation of sodium ions through the channel pore does not involve steps with significant activation volumes. At high acetylcholine concentrations (20  $\mu$ M) bursts of single-channel activity allowed measurements of the mean open and mean closed times of the channel. Pressurization to 40 MPa increased both mean open and mean closed times giving apparent activation volumes of about 59 and 139  $\text{Å}^3$ , respectively. This implies a net volume increase of 80  $\text{Å}^3$ , associated with the transition from the agonist-free state to the open state of the channel, which may be partially associated with the agonist-binding step. All the observed pressure effects were reversible. The activation volumes for the gating of acetylcholine receptor channels are comparable to those of sodium and potassium channels in the squid giant axon, suggesting that there is some basic common mechanism in the operation of ion-channel proteins.

Hydrostatic pressure is a standard tool for studying chemical reactions, because reaction rates and equilibria can be strongly dependent on this thermodynamic parameter (1). The effects of pressure on biological molecules (2) must be ultimately responsible for the profound physiological effects of hyperbaric conditions on living organisms and isolated tissues (3, 4). Pressure-dependent properties have been already observed for the proteins providing the sodium and potassium pathways in squid giant axons and snail neurons (5–8) and for the pores formed by alamethicin in artificial bilayers (9). We describe here an extension of the patch-clamp technique (10–12) that allows patch-clamp recordings at high hydrostatic pressures. This method gives access to a broad field of physiological and biophysical investigations on the effects of pressure on ion channels in small mammalian cells. As a first example of such an application we report here studies on the pressure dependence of the nicotinic acetylcholine receptor (AcChoR) channels in isolated membrane patches from cultured muscle cells.

The AcChoR channels are well characterized under atmospheric conditions (10–16). They mediate the transmission of electrical signals across the synaptic cleft, so that the pressure dependence of their properties might play a major role in the complex phenomenology underlying the "high pres-

sure nervous syndrome." Under normal conditions the ion flow through the AcChoR pore yields an electrical current on the order of 2 pA lasting for most events between 5 and 50 ms. These values define the minimal resolution required from the electrical recording system.

## MATERIALS AND METHODS

**Cell Preparation.** Our studies were carried out on cultured embryonic muscle cells of rat. Muscles of newborn Wistar rats were dissociated and stored in culture. After 3 days the cells were treated with colchicine in order to destroy the cytoskeleton (13). Between day 4 and day 10 in culture the now-round myoballs could be used for the experiments.

The ionic concentrations of the standard bath solution used in most experiments were as follows: NaCl, 140 mM; MgCl<sub>2</sub>, 2 mM; KCl, 2.8 mM; CaCl<sub>2</sub>, 1 mM; and Hepes(-NaOH), 10 mM. The standard pipette solution had the following composition: CsCl, 140 mM; MgCl<sub>2</sub>, 2 mM; EGTA(-NaOH), 10 mM; and Hepes(-NaOH), 10 mM. Both solutions were adjusted to pH 7.2 using NaOH.

**Patch-Clamp Apparatus.** The major modification of the apparatus described by Hamill *et al.* (12) concerned the pipette-holder, which was designed in order to permit the transport of an excised patch of membrane from the preliminary test dish to a pressure bomb, as shown in Fig. 1a. The outer part of the holder could be slid up and down along the inner part. A small glass cup, attached to the sliding tube by means of a swiveling steel hanger, was used as a container for the saline reservoir in the final measuring configuration. The external bath electrode was an Ag/AgCl wire dipped in this glass container. After establishing a "gigaseal" between the pipette and the cell membrane under microscope control (12), the patch was excised into an "outside-out" configuration. The pipette tip was then moved into the glass cup without exposing the patch to air. At this stage, the whole assembly could be raised out of the culture dish, and the solution in the glass cup could be changed to the test solution containing the agonist. All these manipulations were performed with the patch under electrical control—the patch-pipette being constantly connected to the input stage of the patch-clamp amplifier through a low-noise flexible BNC cable (Suhner, Taufkirchen, F.R.G.). Thus, before inserting the "flying-patch" into the pressure bomb, it could be ascertained that the patch contained functional AcChoR channels.

Abbreviations: AcChoR, nicotinic acetylcholine receptor; AcCho, acetylcholine.

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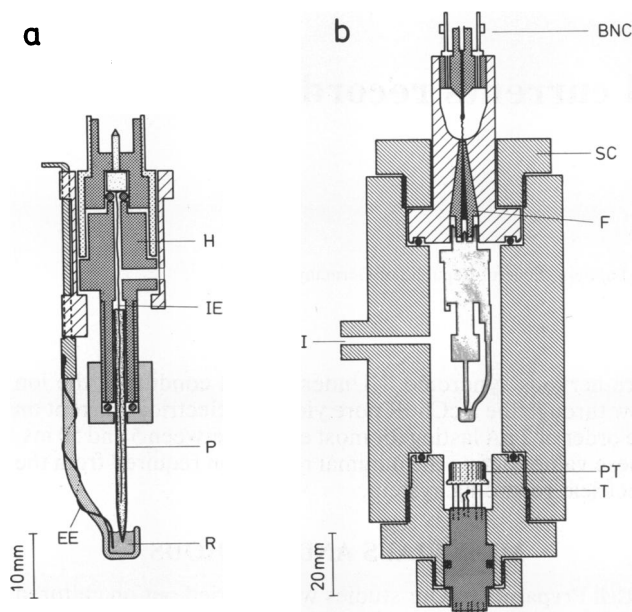


FIG. 1. (a) Cross-section of a flying-patch holder. The inner part (H) that holds the patch pipette (P) and acts as a guide for the internal Ag/AgCl electrode (IE) is identical to the holders used for standard patch-clamp measurements (12). The outer part can be twisted and displaced vertically in order to move the pipette tip into the small container for the saline reservoir (R). The external bath electrode (EE) is an Ag/AgCl wire dipped in this glass container. (b) Cross-section of the pressure vessel. F, Teflon isolated high-pressure electrical feed-through connector; SC, stainless steel screw; I, oil inlet; T, thermistor; PT, pressure transducer.

The control of the membrane potential and the measurement of membrane currents were performed with an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, F.R.G.). The background noise of the whole recording system was  $\approx 0.2$  pA<sub>rms</sub> for a bandwidth of 3 kHz ( $-3$  dB). For good, stable patches the noise did not change appreciably upon pressurization. Continuous recordings of patch currents were preliminarily stored in digital form on a video tape. The data were subsequently replayed through a low-pass 8-pole Bessel-filter (generally,  $-3$  dB cut-off frequency at 500 Hz), and the relevant events were sampled and stored digitally on a PDP 11/23 computer using a CATCH software program similar to that described by Sigworth (17).

**Pressure-Apparatus.** The pressure vessel was built out of stainless steel and is shown in Fig. 1b. At the beginning of the experiment, the pipette holder was inserted into a Teflon isolated high-pressure electrical feed-through connector inserted in the movable cover of the vessel. Upon placing the cover in position the flying-patch holder was immersed in pure paraffin oil that was used as pressure-transmitting medium, and the vessel was then sealed with a screw. Oil was pressed into the vessel with a hand-driven pump (Nova-Swiss) through a thin steel capillary. Pressures up to 70 MPa could be reached by pumping a few milliliters of oil. To minimize mechanical disturbances and to avoid transient temperature variations that were too large, an absolute rate of pressure change of  $<1$  MPa/sec was used. At pressures above 40 MPa membrane patches frequently became unstable; therefore, in most experiments this limit was not exceeded. Due to the compressibility of paraffin oil, the temperature inside the chamber could rise by up to  $4^\circ\text{C}$  for relatively fast compressions to 40 MPa, but for slow compression rates the temperature increase was less than  $1.5^\circ\text{C}$  within 60 sec from the end of pressurization. Temperature and pressure were measured by a thermistor and by a pressure transducer placed at the bottom of the pressure

vessel. The temperature could be regulated by circulating a thermostatically controlled water/glycol mixture in plastic tubing coiled several times around the vessel.

## RESULTS

**Pressure-Independence of Single-Channel Conductance.** Fig. 2 shows recordings of single-channel events obtained from the same membrane patch at atmospheric pressure and at 30 MPa. It is apparent that the mean amplitude of the single-channel current is not appreciably influenced by pressure, whereas the mean lifetime of open and closed states increases upon pressurization.

Fig. 3 shows a quantitative statistical analysis of the single-channel current amplitudes obtained from the same experiment as in Fig. 2, for openings having durations longer than 5 ms. The amplitude histograms at constant membrane potential are not significantly affected by pressure. Within the range of 0.1 to 60 MPa in all our measurements on 20 different patches the mean current amplitude never changed more than the standard error of the amplitude distribution. The single-channel  $I$ - $V$  relationship, obtained from amplitude histograms at different voltages is shown in Fig. 3c for a membrane patch studied at three different pressures. In the range of  $-70$  to  $-20$  mV the slope conductance was in this case about 29 pS and was independent of pressure.

To investigate the effects of pressure at different sodium concentrations, two membrane patches were studied using a low sodium solution 43 mM NaCl/2 mM MgCl<sub>2</sub>/2.8 mM

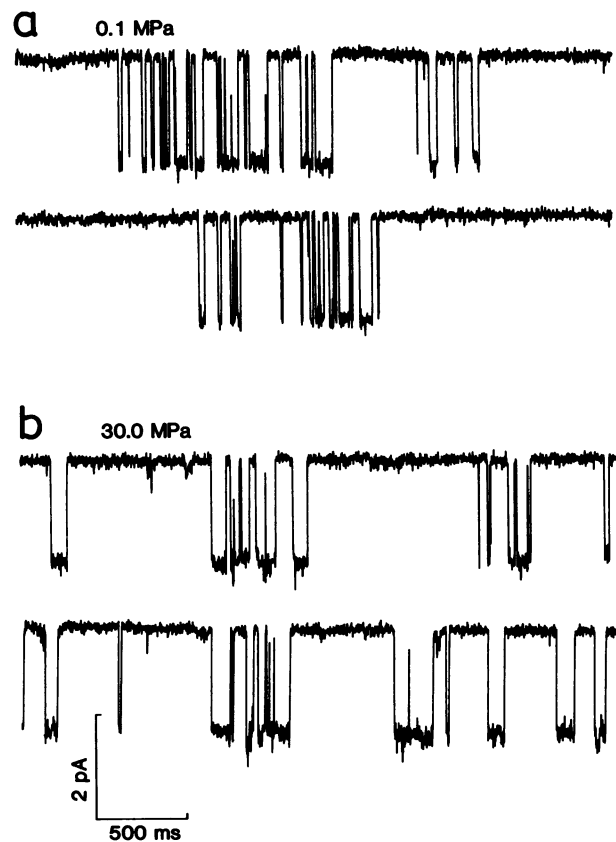


FIG. 2. Openings of individual extrasynaptic AcChoR channels appear in bursts at desensitizing AcCho concentrations ( $20 \mu\text{M}$  in this case). (a) Bursts from an outside-out membrane patch recorded at atmospheric pressure and  $12.7^\circ\text{C}$ . (b) Recordings from the same patch at 30.0 MPa and  $13.3^\circ\text{C}$ . In both a and b the membrane potential was  $-80$  mV. The single channel conductance is unchanged upon increasing pressure, whereas the lifetimes of the open and shut states are markedly prolonged.

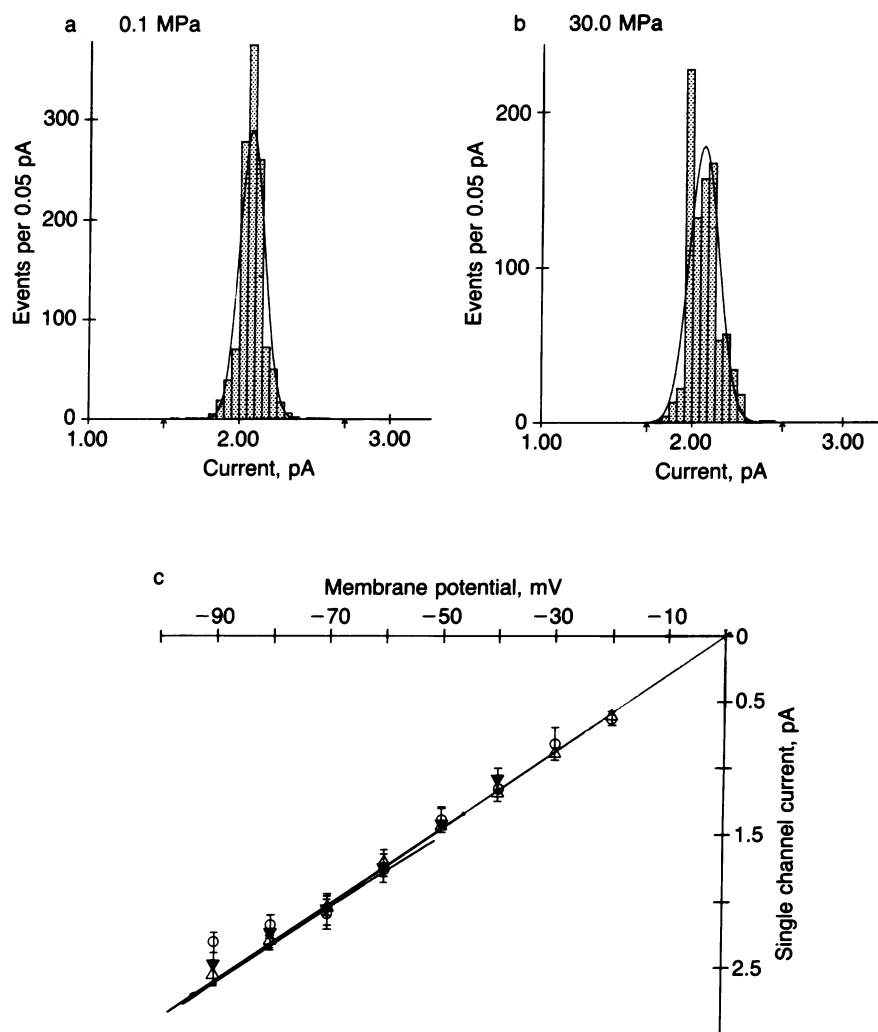


FIG. 3. Histograms of single-channel current amplitudes at a fixed membrane potential were fitted by Gaussian distributions. The data shown in *a* (1199 events) and *b* (888 events) were obtained from the same patch and under the same conditions described here for *a* and *b*, respectively. After correction for the slight temperature difference, the mean single-channel currents referred to 12.7°C at -80 mV were estimated at  $2.12 \pm 0.08$  pA and  $2.07 \pm 0.10$  pA at 0.1 MPa and at 30 MPa, respectively. (c) Current-voltage relationship for the open AcChoR channel, obtained in one patch from the analysis of several amplitude histograms at different membrane potentials and for three different pressures. Each data point gives the mean current from an amplitude histogram. Least squares fits of the data up to -70 mV by straight lines yielded approximately the same slope conductance at 0.1 MPa ( $\circ$ ,  $29.6 \pm 1.2$  pS), at 20 MPa ( $\Delta$ ,  $28.0 \pm 0.4$  pS), and at 40 MPa ( $\nabla$ ,  $28.4 \pm 1.7$  pS). All data were corrected to the same temperature ( $T = 13.3^\circ\text{C}$ ) using a measured  $Q_{10}$  for single-channel currents of 1.34.

KCl/2 mM CaCl<sub>2</sub>/10 mM Hepes on both sides of the membrane. Under these conditions the single-channel conductance was about 15 pS at 23°C, but again it showed no significant pressure sensitivity.

**Pressure Dependence of Open-Close Kinetics.** The gating kinetics of AcChoR channels were studied using high acetylcholine (AcCho) concentrations (20  $\mu\text{M}$ ). As in the case shown in Fig. 2, the channel openings under these conditions occurred in bursts separating desensitized periods (14). Individual open and closed times were measured semi-automatically and analyzed following the criteria discussed by Colquhoun and Sakmann (16). The closed-time histograms were fitted with single exponentials within time ranges where contributions from brief closures (Nachschläge) and from dwellings in desensitized states could be ignored. This is shown in Fig. 4*a* and Fig. 4*b* for the same membrane patch at 0.1 and 30 MPa. The mean closed time,  $\tau_c$ , was markedly increased by high pressure. At temperatures and pressures at which the mean duration of the Nachschläge could be estimated, a criterion of minimal interference was used for disregarding these interruptions in the measurement of the open times. Open-time histograms obtained in this way were also fitted by single exponentials, as shown in Fig. 4*c* and *d*. The mean open time,  $\tau_o$ , was also increased by pressure, although to a lesser extent than  $\tau_c$ .

By interpreting  $\tau_c$  and  $\tau_o$  as reciprocal of the rate constants of a first-order reaction:

$$\text{closed} \xrightleftharpoons[\beta^*]{\alpha^*} \text{open}, \quad [1]$$

these pressure dependencies can be described by apparent activation volumes,  $\Delta V_c^\ddagger$  and  $\Delta V_o^\ddagger$ , associated respectively with the opening and with the closing of an AcChoR channel and defined by (18):

$$\Delta V_c^\ddagger = RT \frac{\partial \ln \tau_o}{\partial P} = \frac{RT}{P} \frac{\ln \tau_o(P)}{\tau_o^0} \quad [2]$$

$$\Delta V_o^\ddagger = RT \frac{\partial \ln \tau_c}{\partial P} = \frac{RT}{P} \frac{\ln \tau_c(P)}{\tau_c^0} \quad [3]$$

where  $\tau_o^0$  and  $\tau_c^0$  are the values of  $\tau_o$  and  $\tau_c$  measured at normal pressure. Table 1 gives estimates of  $\Delta V_c^\ddagger$  and  $\Delta V_o^\ddagger$  obtained from 20 independent measurements. In only six cases the amount of burst-like activity from single channels was sufficient to allow a reliable estimate of  $\tau_c$  and  $\Delta V_o^\ddagger$ ; in the three experiments at temperatures above 22°C the high frequency of closures that were too short to be resolved prevented reliable estimates of  $\tau_o$  and  $\Delta V_c^\ddagger$ .

The data of Table 1 show no obvious systematic dependence on pressure, justifying the use of apparent activation volumes to describe the effect of this parameter on channel kinetics,  $\Delta V_c^\ddagger$  appears also to be fairly independent of temperature, whereas  $\Delta V_o^\ddagger$  shows a tendency to increase at higher temperatures. However, more data would be needed to support the latter conclusion. The difference  $\Delta V = \Delta V_o^\ddagger - \Delta V_c^\ddagger$  yields the mean volume change characterizing the transition of an AcChoR channel from the closed states with mean lifetime  $\tau_c$  to the open states with mean lifetime  $\tau_o$ . We found on the average:  $\Delta V_o^\ddagger = 139 (\pm 11) \text{ \AA}^3$ ;  $\Delta V_c^\ddagger = 59 (\pm 4)$

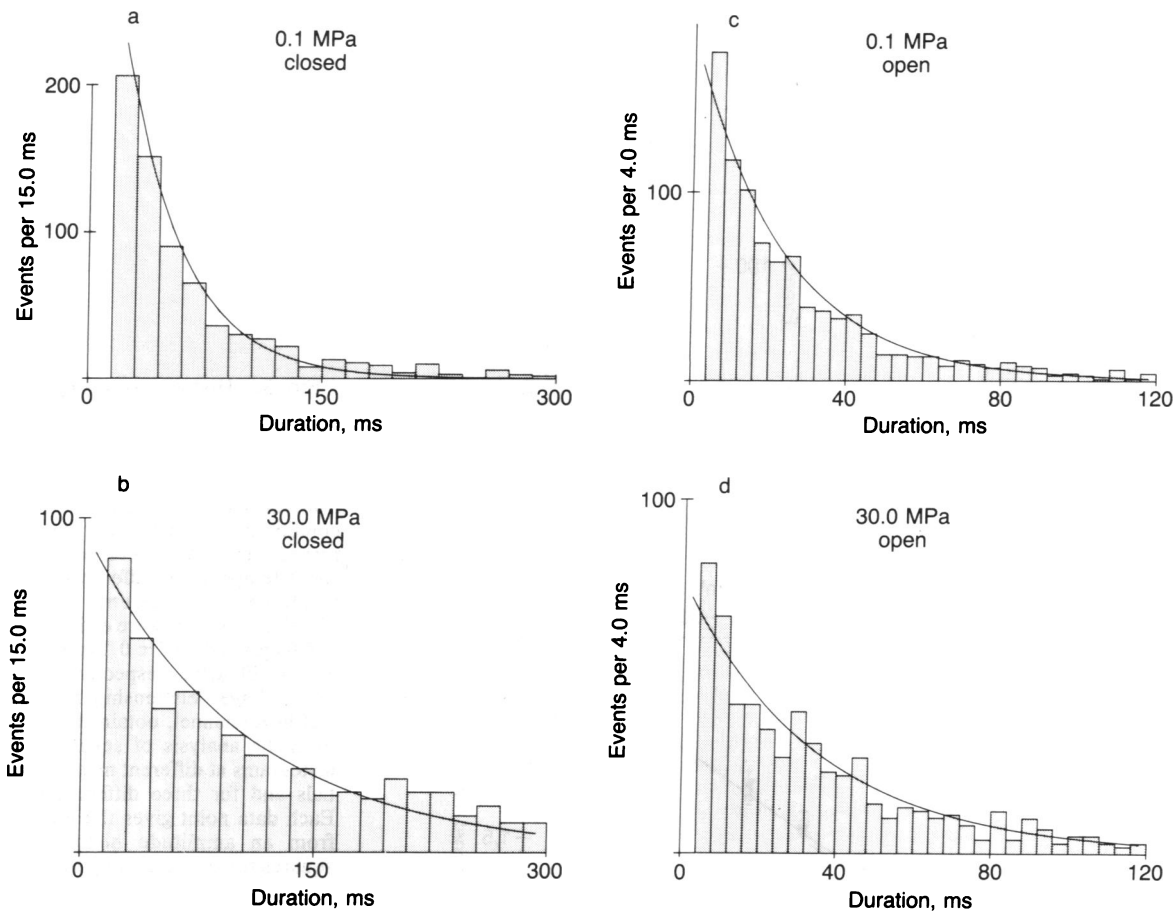


FIG. 4. Lifetime histogram analysis of the experiment of Fig. 2. (a)  $\tau_c = 37.70$  ms,  $12.7^\circ\text{C}$ , 696 events; (b)  $\tau_c = 103.78$  ms,  $13.3^\circ\text{C}$ , 540 events; (c)  $\tau_o = 23.70$  ms,  $12.7^\circ\text{C}$ , 905 events; (d)  $\tau_o = 35.00$  ms,  $13.3^\circ\text{C}$ , 576 events. The pressure dependence of the mean open time,  $\tau_o$ , is described by an apparent activation volume,  $\Delta V_o^\ddagger = 60 \text{ \AA}^3$ . The apparent activation volume describing the pressure dependence of the mean closed time is  $\Delta V_c^\ddagger = 142 \text{ \AA}^3$ .

$\text{\AA}^3$ ; and  $\Delta V = 80 (\pm 12) \text{ \AA}^3$ . The positive sign of  $\Delta V$  implies that the equilibrium between the various channel states is shifted by pressure toward the closed configurations.

Table 1. Activation volumes of the closing and opening transition at various temperatures and pressures

Temperature, $^\circ\text{C}$	Pressure, MPa	$\Delta V_c^\ddagger, \text{ \AA}^3$	$\Delta V_o^\ddagger, \text{ \AA}^3$
8.3	38	67.8	—
8.7	41	69.6	—
8.7	41	82.4	—
9.0	39	62.8	—
9.4	40	39.8	—
10.3	40	54.8	—
10.3	40	40.1	—
12.7	30	73.7	—
13.3	30	59.9	141.9
13.6	41	35.2	—
14.2	40	93.1	—
14.7	34	47.9	136.0
14.7	34	56.5	87.9
18.4	40	48.7	—
18.6	42	70.8	—
18.7	60	64.4	—
20.8	41	45.6	—
22.8	40	—	158.9
22.8	40	—	152.5
29.5	33	—	159.0
Mean		59.4 ( $\mp$ 3.9)	139.4 ( $\mp$ 11.0)

Because patches always contained more than one channel, we could not determine the pressure dependence of the interburst periods.

**Temperature Dependence of the AcChoR Channel Properties.** In order both to test the method of analysis and obtain correction factors for the small temperature variations occurring in pressure experiments, the effect of temperature alone on the single-channel conductance,  $\gamma$ , and on the gating kinetics of AcChoR was measured separately in 15 different patches. Arrhenius plots of  $\gamma$  in the range of 10 to  $35^\circ\text{C}$  were well fitted by a single  $Q_{10}$  of  $1.34 (\pm 0.02)$  in agreement with earlier data from rat myotubes (19) and from chick myotubes (20). In this temperature range both  $\tau_o$  and  $\tau_c$  showed a  $Q_{10}$  around  $3.0 (\pm 0.3)$ . Sigworth (19) found a  $Q_{10}$  of 3.4 for  $\tau_o$  for the same preparation with application of lower agonist concentrations (0.3–2  $\mu\text{M}$ ).

## DISCUSSION

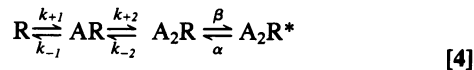
From our analysis of the amplitude of single-channel currents it appears that the process of ion permeation through an open AcChoR channel is totally insensitive to pressure, as is the case for the alamethicin pores incorporated into lipid bilayers (9). Both the rate of entry of the ions into the pore and that of exit seem to be little affected by pressure.

The energy profile inside the AcChoR pore can be described approximately by two energy barriers and one binding site (13). Under the conditions of most of our experiments (e.g., see Fig. 3), where the ionic concentration of monovalent cations is high, the site is most of the time

saturated (21), and the influx at negative membrane potentials is expected to be rate limited by the jump over the energy barrier on the cytoplasmic side (22). We can exclude that this step involves any significant activation volume. Our measurements at lower sodium concentrations, where the rate of entry of the ions into the pore should influence the open channel conductance significantly, indicate that the entry step is also pressure independent.

The apparent activation volumes that describe the pressure dependence of the gating kinetics of the AcChoR channel have the same sign and are comparable in size to those found for the sodium and potassium channels of axonal membranes (5, 6). The estimated mean volume change of about  $80 \text{ \AA}^3$  during the activation of an AcChoR channel is also consistent with the positive reaction volume found by Sauter *et al.* (23) for the binding of AcCho to *Torpedo* membranes. Both the binding of AcCho and the pore opening may involve volume changes because of modifications in water exposure of various regions of the receptor protein or because of compressions and expansions of the protein itself.

Unfortunately, due to the limited resolution of our present recordings we cannot dissect the contributions to the observed volume changes that arise from the binding reaction from those associated with the conformational transition that opens the pore. Characterizing the AcChoR-channel kinetics with the two parameters  $\tau_c$  and  $\tau_o$  amounts to describing the pore-opening process according to Eq. 1 with  $\tau_o = 1/\alpha^*$  and  $\tau_c = 1/\beta^*$ . The relationship between  $\alpha^*$  and  $\beta^*$  and the rate constants of the more detailed and realistic reaction scheme (15):



is obtained by assuming fast equilibrium between states R (receptor) and AR (AcCho-receptor) and between states  $A_2R$  and  $A_2R^*$ . Then:

$$\alpha^* = \frac{\alpha k_{-2}}{\alpha + \beta} \quad \text{and} \quad [5]$$

$$\beta^* = \frac{k_{+2}k_{+1}[A]^2}{(k_{-1} + k_{+1}[A])} \quad [6]$$

According to Eyring's theory of absolute reaction rates (18) the pressure dependence of each individual rate constant in (2) is characterized by a true activation volume,  $\Delta V^\ddagger$ , which is the volume change needed in order to overcome the free enthalpy barrier associated with the specific reaction step. However, the apparent activation volumes describing the pressure dependencies of  $\alpha^*$  and  $\beta^*$  are expected to be weighted functions of several  $\Delta V^\ddagger$ s and depend also on the relative probability of the various channel states.

The effects described here, as well as those observed for ionic channels in nerves (5–8, 24) are readily appreciable only above 10 MPa. Therefore, they are unlikely to play a major role in the physiology of the high pressure nervous syndrome, which can develop in whole organisms at pressures as low as

6 MPa (3). We must therefore conclude that the most crucial site that is responsible for the syndrome is at the presynaptic level. According to Parmentier *et al.* (25) and Ashford *et al.* (26), pressure has very drastic effects on the release of synaptic vesicles. The study of specialized secretory cells, which can be easily accomplished with the technique described here, should allow a complete characterization of these phenomena.

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