Effects of Hydrostatic Pressure on Membrane Processes

Sodium Channels, Calcium Channels, and Exocytosis

STEFAN H. HEINEMANN, FRANCO CONTI, WALTER STÜHMER, and ERWIN NEHER

From the Max-Planck-Institut für Biophysikalische Chemie, Am Fassberg, D-3400 Göttingen-Nikolausberg, Federal Republic of Germany

ABSTRACT A patch-clamp study under high hydrostatic pressure was performed by transferring cells or membrane patches into a pressure vessel (Heinemann, S. H., W. Stühmer, and F. Conti, 1987, *Proceedings of the National Academy of Sciences*, 84:3229–3233). Whole-cell Na currents as well as Ca currents were measured at pressures up to 40 MPa (~400 atm; 1 MPa = 9.87 atm) in bovine adrenal chromaffin cells. Ca currents were found to be independent of pressure within experimental resolution. The mean amplitude and the gating kinetics of Na currents were affected by <20% at 10 MPa. This lack of a pronounced effect is surprising since the high-pressure nervous syndrome (HPNS), a disorder at high pressures known to result from impaired nervous transmission, manifests itself at pressures as low as 5 MPa. The results show that ion channels involved in transmission cannot be implicated in HPNS. However, when exocytosis was studied at high pressure by monitoring the cell capacitance (Neher, E., and A. Marty, 1982, *Proceedings of the National Academy of Sciences*, 79:6712–6716), more drastic effects were seen. The degranulation evoked by dialyzing the cell with 1 μM free Ca^{2+} could be slowed by a factor of 2 by application of 10 MPa. The same effect was observed for the degranulation of rat peritoneal mast cells stimulated with 40 μM of the GTP analogue GTP-γ-S. According to these results, the process of exocytosis is the most likely site at which hydrostatic pressure can act to produce nervous disorders. Furthermore, we demonstrate that pressure can be a useful tool in the investigation of other cellular responses, since we were able to separate different steps occurring during exocytosis owing to their different activation volumes.

Address reprint requests to Dr. Walter Stühmer, Max-Planck-Institut für Biophysikalische Chemie, Am Fassberg, D-3400 Göttingen-Nikolausberg, Federal Republic of Germany. Dr. Heinemann's present address is Dept. of Physiology, Yale University School of Medicine, New Haven, CT 06510. Dr. Conti's permanent address is Dept. of Physics, Istituto di Cibernetica e Biofisica, CNR, Università di Genova, I-16146 Genoa, Italy.

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INTRODUCTION

Many types of excitable cells are sensitive to changes in hydrostatic pressure (for review, see Wann and Macdonald, 1980). The effects of pressure on biological molecules (Heremans, 1982) must ultimately be responsible for the profound physiological effects of hyperbaric conditions on living organisms and isolated tissues (e.g., Brauer, 1975; Macdonald, 1984). Some types of ion channels have already been shown to have pressure-dependent properties, most notably a slowing down of gating kinetics of Na (Henderson and Gilbert, 1975; Conti et al., 1982*a*, 1984; Harper et al., 1982) and K channels (Conti et al., 1982*b*; Harper et al., 1982) in neurons and of alamethicin pores in artificial bilayers (Bruner and Hall, 1983). The patch-clamp technique allows electrical access to small cells (Hamill et al., 1981). Recently, this technique was extended to allow experiments to be performed at high pressure. Single-channel events of the acetylcholine (ACh) receptor channel of rat muscle could be recorded at pressures up to 60 MPa (Heinemann et al., 1987). While pressure did not affect the single-channel conductance, the kinetics were slowed down by a factor of ~2 during pressurization to 40 MPa. However, this and the other effects mentioned above cannot explain the profound physiological effects observed in whole organisms. As already pointed out by Parmentier et al. (1981) and Ashford et al. (1982), more pronounced effects of pressure are detectable in synaptic transmission. A reduction in the frequency of the spontaneous release of transmitter of ~50% owing to a pressure of 5 MPa was observed in frog neuromuscular junction, and 10 MPa yielded a 50% decrease in the amplitude of the excitatory postsynaptic potential in *Aplysia californica* ganglion. These and other results from single ACh receptor channels (Heinemann et al., 1987) indicate that the most pressure-sensitive site must be at the presynaptic level. This led us to investigate exocytosis directly at high pressure. Exocytosis is accompanied by an increase in membrane area, which can be measured as an increase in electrical cell capacitance.

Since there were no data available on the effect of pressure on voltage-gated ion channels in mammalian cells, and Otter and Salmon (1985) concluded indirectly that a Ca channel in *Paramecium* is inhibited by 10 MPa, we also measured Na and Ca currents in bovine chromaffin cells at various pressures.

MATERIALS AND METHODS

Cell Preparation

Chromaffin cells from the medulla of bovine adrenal glands were isolated and cultured as described by Fenwick et al. (1982*a*) and Marty and Neher (1982). The cells were plated on untreated glass coverslips. Cells could be used for only 4 d after preparation, since for the pressure experiments they had to be transferred into a pressure vessel (see below), and older cells could not be detached from the glass without damage.

Rat peritoneal mast cells were prepared by peritoneal lavage as described by Fernandez et al. (1984) and Almers and Neher (1985). They were also plated on glass and could be used for experiments within ~1–10 h after preparation.

High-Pressure Apparatus

Pieces of coverslips with cells were transferred into culture dishes filled with the extracellular saline. Loose, floating cells were selected for the experiments. Gigaseals were

achieved according to Hamill et al. (1981). The patch pipettes had resistances between 2 and 3 MΩ in the salines described below. In the cell-attached configuration, the pipette was lifted up without yielding an outside-out patch since the cells did not stick to the glass bottom. For the measurements of ionic currents, whole-cell configurations were established by a suction pulse that ruptured the patch membrane. For all other measurements, cells were kept in the cell-attached configuration at this stage. As described by Heinemann et al. (1987), the tip of the pipette was then transferred into a small glass cup attached to the pipette holder and filled with saline. The cup also carried an Ag/AgCl wire as a reference electrode. The pipette holder was plugged into a connector on the inside of the lid of a pressure vessel. The electrical connection with the patch-clamp amplifier (EPC-7, List-Medical, Darmstadt, Federal Republic of Germany) was established by a Teflon pressure feed-through in the lid and a low-noise cable (Suhner, Herisau, Switzerland). In this way, the assembly with the cell under investigation could be transferred into the pressure bomb, which was filled with pure paraffin oil acting as the pressure-transmitting medium.

Pressure could be raised by means of a hand-driven pump (Nova-Swiss, Zurich, Switzerland). In this configuration, whole-cell current recordings as well as measurements of cell capacitance could be easily performed at pressures up to 40 MPa.

The temperature inside the bomb was regulated with a heat-exchanger coil wrapped around the bomb. Temperature and pressure measurements were performed with electrical devices placed at the bottom of the pressure bomb. Values for both parameters were sampled and stored digitally during the experiments.

Capacitance Measurements

As described by Neher and Marty (1982), the increase in cell membrane area that accompanies exocytosis can be monitored by measuring the electrical capacitance of the cell membrane using a phase-sensitive signal detector. In chromaffin and mast cells, stimulated secretion can produce a severalfold increase in membrane area, which is easily viewed through an optical microscope (Fernandez et al., 1984). The phase-sensitive signal detection is performed with a so-called lock-in amplifier. It generates a sinusoidal voltage signal (18 mV rms and 795 Hz), which is used as command input for the voltage clamp. The resulting current serves as an input for the lock-in amplifier. The amplifier determines the magnitude of the sinusoidal component of the input signal at two mutually orthogonal phase angles. The values of the phase angles can be set with a phase-shifter provided by the lock-in amplifier.

For the calculation of the cell capacitance, C_M, one needs to assume a certain equivalent circuit diagram. Like Neher and Marty (1982), we approximated the complex impedance of the pipette-cell assembly by C_M and a membrane conductance, G_M, in parallel plus a conductance in series, G_s. The lock-in signals, together with the information on leak current and holding potential, allow the determination of the values of the three elements. A BASIC-23 program run on a PDP 11/23 computer (Digital Equipment Corp., Marlboro, MA) sampled the real and imaginary part of the impedance given by the lock-in amplifier, the leak current given by the EPC-7 current monitor, the pressure, P, and temperature, T, at rates between 0.2 and 0.5 s^{-1}. In the offline analysis, the holding potential was given manually, and C_M, G_s, and G_M were calculated and displayed together with P and T as a function of time (see Figs. 3 and 4), as detailed by Lindau and Neher (1988).

The calculations of the elements of the equivalent circuit required that, contrary to previous uses of the lock-in amplifier (Neher and Marty, 1982), the cell capacitance was not compensated. Furthermore, the phase angle had to be adjusted such that a purely conductive or capacitive load gave exactly a 0 or 90° signal, respectively. In the cell-attached configuration under the final experimental conditions (with the patch-clamp

holder already mounted in the pressure bomb), the lock-in output for $R_e(Z)$ and $I_m(Z)$ was set to zero using the offset potentiometer of the lock-in amplifier and the C_{fast} potentiometer of the EPC-7, respectively.

The experiments at high pressure brought about some complications. Rupturing of the membrane patch in order to reach the whole-cell configuration could not be done outside the pressure bomb for two reasons. First, the capacitance of the recording system changed when we immersed the pipette-holder into the oil-filled pressure vessel, mainly because a surface layer of solution, which is always present on the pipette, is removed by the oil. This would give an additional artifact in the capacitance measurements. Second, exocytosis starts immediately after patch rupture and very often goes to completion within a few minutes. The investigation of the pressure sensitivity of the full time course of release would not be possible. Therefore, patch rupture can only be done when the assembly is ready for the measurement.

We therefore transferred the cell into the pressure vessel in the cell-attached configuration, closed the bomb, chose the hydrostatic conditions, and adjusted the lock-in amplifier before we finally broke the membrane patch. This had to be done with a voltage pulse, since suction could no longer be applied after the pipette-holder had been transferred into the pressure vessel.

For patch rupture, we needed hyperpolarizing pulses of −500 to −1,000 mV for 2–25 ms, depending on the seal resistance. For cells with very tight seals (100 GΩ), −500 mV and 2 ms were sufficient. In many cases, it was not possible to achieve a series conductance >100 nS with this technique. Although a series conductance of 50 nS allows the correct determination of the cell capacitance, there is no reliable control over the influx of pipette solution into the cells. Therefore, we only considered experiments with series conductances of at least 150 nS.

Stimulation of Cell Degranulation

Chromaffin Cells. After rupture of the membrane patch, the pipette solution diffuses into the cell within a few seconds. Therefore, degranulation of chromaffin cells was evoked by dialyzing cells in this way with a pipette solution containing 1 μM free Ca^{++}. Cells were voltage-clamped to −70 mV in order to prevent influx of Ca^{++} through voltage-gated Ca channels. Since the total amount of capacitance increase and the time course of degranulation are strongly temperature dependent (Penner and Neher, 1986), we set the temperature of the pressure bomb to ~30°C, yielding a total increase of about two- to threefold over the initial cell capacitance.

Solutions (in millimolar): external: 140 NaCl, 2.8 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES-NaOH; internal: 20 NaCl, 135 K-glutamate, 4 $MgCl_2$, 9 $CaCl_2$, 10 EGTA-KOH, 10 HEPES-KOH, 0.5 ATP.

Mast cells. Fusion of mast cell granules was stimulated by loading the cells with the GTP analogue GTP-γ-S and ATP, giving a total capacitance increase of about three- to fourfold over the initial cell capacitance at room temperature. Since the time course of degranulation is much faster than in chromaffin cells, experiments were carried out in the temperature range between 22 and 25°C. The holding potential was set in most cases to −25 mV.

Solutions (in millimolar): external: 140 NaCl, 2.5 KCl, 5 $MgCl_2$, 2 $CaCl_2$, 10 HEPES-NaOH; internal: 20 NaCl, 135 K-glutamate, 4 $MgCl_2$, 10 HEPES-KOH, 0.5 ATP, 0.04 GTP-γ-S, or, additionally, 6 $CaCl_2$, 9 EGTA-KOH.

Voltage-Clamp Experiments

We measured both Na and Ca currents in bovine chromaffin cells. The methods are described by Fenwick et al. (1982*b*). Here we briefly summarize the procedure.

Stimulation with voltage pulses and sampling and storage of the measured ionic currents was performed with a computer program written in BASIC-23 run on a PDP 11/23 computer. This program automatically provided linear leak and transient capacitance subtraction by a P/4 pulse procedure and averaging. Before sampling, currents were filtered by an eight-pole low-pass filter with the cutoff frequencies indicated below.

Na currents. The holding potential for the measurement of Na currents was set to −90 mV. Depolarizing voltage pulses between −30 and +65 mV for 5–10 ms elicited Na currents. Ca currents were blocked by addition of 1 mM $CoCl_2$ to the external solution. Data were sampled at 40-μs intervals. The peaks were fitted with a cubic function in order to determine the minima. The resulting peak current-voltage relationships (*I-V* relationships) were fitted with a function derived from the Hodgkin-Huxley (1952) formalism of Na channel gating:

$$I_{peak} = g_{max}(E - E_{rev})\, P(E), \quad (1)$$

where E_{rev} is the reversal potential for Na currents, g_{max} is the maximal conductance, and $P(E) = m^3h$ is the probability of finding a channel open.

The voltage dependence of Na channel inactivation was determined by applying conditioning pulses of variable potential and 30 ms duration, which preceded a test voltage pulse to 10 mV. Peak currents plotted against voltage, the h_∞ relationship, was fitted with Eq. 2:

$$h_\infty = I(E)/I(\infty) = 1/\{1 + \exp[(E - E_{½})/\alpha]\}. \quad (2)$$

$E_{½}$ is the half-inactivating potential and α is the steepness of the voltage dependence. We estimated the kinetics of the activation process, τ_m, by measuring time during which the individual current records show the maximal steepness, which corresponds to the inflection point. A measure for τ_h, the time constant of channel inactivation, was derived from an exponential fit to the declining current phase.

Solutions (in millimolar): external: 140 NaCl, 2.8 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 1 $CoCl_2$, 10 HEPES-NaOH; internal: 80 CsF, 60 CsCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES-NaOH, 10 EGTA-NaOH.

Ca currents. For Ca currents, the holding potential was set to −80 mV. Currents were evoked by depolarizing pulses between −10 and +30 mV, lasting 30 ms. *I-V* relationships were constructed from the Ca plateau currents. Na channels were blocked by addition of tetrodotoxin (TTX) to the external medium; K currents were suppressed by TEA and CsCl in the pipette solution.

Solutions (in millimolar): external: 140 NaCl, 2.8 KCl, 2 $MgCl_2$, 5 $CaCl_2$, 10 HEPES-NaOH, 20 μg/ml TTX; internal: 120 CsCl, 2 $MgCl_2$, 1 $CaCl_2$, 1 ATP, 10 HEPES-NaOH, 11 EGTA-NaOH, 20 TEA-Cl. The pH of all solutions described in this article was adjusted to 7.2 using NaOH or KOH.

Ion pair dissociation in water may be accompanied by volume decreases of the order of up to 25 cm^3/mol (Asano and Le Noble, 1978), so that pH and pCa decreases may occur at high pressures. We estimated these effects, which amount to <0.4 units at 40 MPa and are therefore insignificant.

RESULTS

Whole-Cell Na Currents

In order to determine the contribution of Na channels to pressure effects on the synaptic transmission in mammals, we performed experiments on bovine chromaffin cells.

Fig. 1*A* shows whole-cell Na currents at different pressures. At 40 MPa, the current amplitudes and the kinetics of the currents are visibly affected. The current amplitude, however, did not recover completely after returning to atmospheric conditions. This loss of active channels after application of high pressure was seen in most of our experiments. In some cases, a full recovery of peak current could be achieved by waiting 30 min after the return. Since the reduction of peak current is superimposed on a rundown of Na currents, occurring even at atmospheric conditions, we do not want to stress this point too much and will merely summarize the overall effect on peak current after application of pressure, including a normal rundown. A step to 20 MPa produces a 13 (±4)% loss ($N = 5$); a step to 40 MPa produces a 32 (±2)% loss ($N = 15$).

Fig. 1*B* (trace *a*) illustrates that channel kinetics are slowed down by pressure in a fully reversible manner. Current traces at +5 mV membrane potential are scaled in amplitude in order to match the maximal currents. The traces at 0.1 MPa (continuous lines) match exactly in the rising phase. In Fig. 1*B* (trace *b*), the time scale of the high-pressure trace (41 MPa) has been compressed by a factor of 1.4 in order to make the rising phase of the currents match. Clearly, the inactivation phase is more affected by pressure than the activation phase. The activation volume, ΔV, describing the pressure dependence of a time constant, is determined by:

$$\ln(\Theta) = P\Delta V/(kT), \tag{3}$$

where Θ is the ratio of the time constants at high and normal pressure, k is Boltzmann's constant, and T is the absolute temperature. In the case of Fig. 1*B*, the factorial change of 1.4 in the kinetics of the Na current corresponds to an apparent $\Delta V = 33$ Å^3 (~20 cm^3/mol) associated with Na activation. At −5 to 40 mV, we determined an average of $\Delta V = 39$ (±2) Å^3 for this experiment.

Activation volumes describing the channel function in a more detailed manner have to be associated with individual rate constants of channel gating. We did not perform a complete kinetic analysis of Na currents, but in some experiments we estimated τ_m and τ_h as described in the Methods. A rough estimate for the pooled pressure dependences is as follows: for 17.6°C: $\Delta V(\tau_m) = 37.4$ (±5.9) Å^3 ($n = 4$) and $\Delta V(\tau_h) = 61.5$ (±7.8) Å^3 ($n = 3$); for 23.3°C: $\Delta V(\tau_m) = 26.3$ (±2.8) Å^3 ($n = 3$) and $\Delta V(\tau_h) = 39.6$ (±3.6) Å^3 ($n = 3$). However, we have to note that the activation volumes show a voltage dependence, as also reported by Conti et al. (1982*a*) for squid giant axons.

In Fig. 1*C*, the *I-V* relations of the Na channels at different pressures are compared. The reversal potential is not affected by pressure, whereas in this case the activating phase of the *I-V* curve is shifted in the hyperpolarizing direction by 10 mV at 40 MPa. The steady state inactivation, however, does not show such a shift (Fig. 1*D*). $E_{½}$, the potential of half-inactivation, is constant within a scatter of ±1 mV. We found shifts, on the average, of −1.8 mV ($n = 17$) for E_{peak} and −2.1 mV ($n = 9$) for $E_{½}$ after application of 40 MPa.

The shift in the voltage dependence of Na channels in chromaffin cells is a serious problem. As described by Marty and Neher (1983), a voltage shift in peak current and h_∞ of approximately −10 mV develops between 1 and 30 min

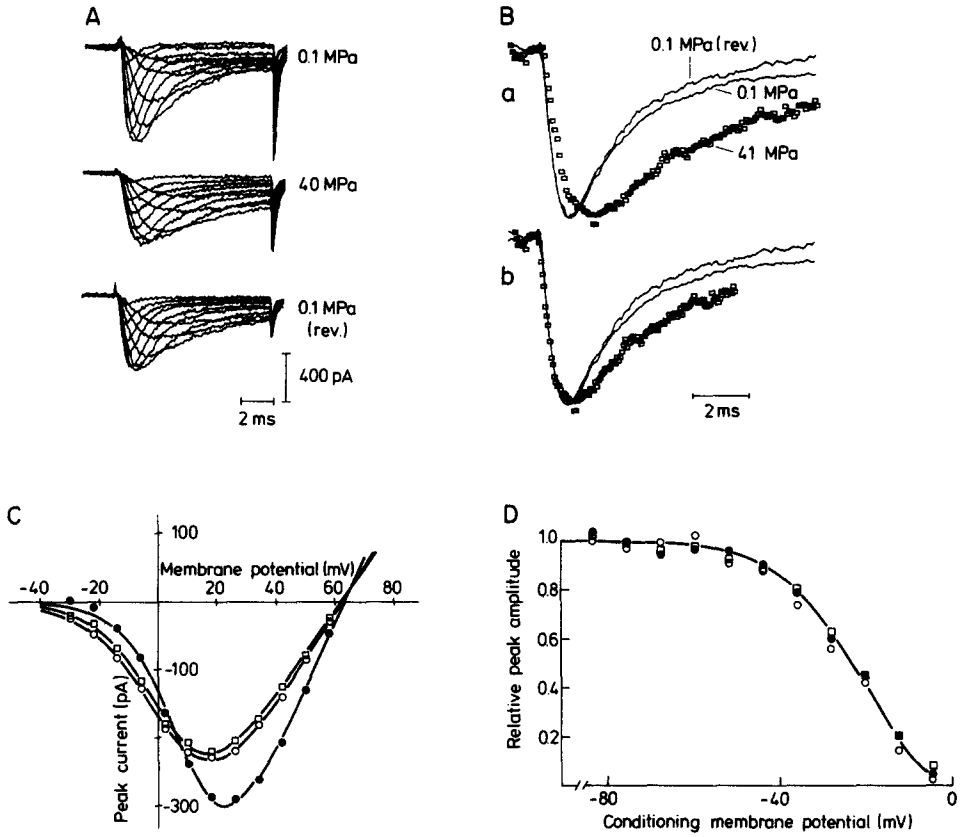

FIGURE 1. Na currents. (*A*) Na currents evoked by depolarising pulses to −14 through +58 mV at 0.1, 40, and again at 0.1 MPa. (*B*) (Trace *a*) Current traces at +5 mV test potential at different pressures are scaled in amplitude. Continuous curves: 0.1 MPa, the amplitude of the control trace after pressure application, is scaled by a factor of 1.43; squares: 41 MPa, scaled by a factor of 1.67 in amplitude. Note that the rising phases of pre- and post-pressure controls agree closely; at high pressure, the time course is slowed down. In order to get an estimate of the slowing, we plotted the high-pressure trace with a compressed time scale in trace *b*. The amplitude scale was the same as in *a*. With a scaling factor of 1.4, the rising phase of the low- and high-pressure traces can be made to fit; the inactivation phase, however, is slowed even further. A time scaling of ~2 would be needed in order to superimpose the falling phase. Temperature: 18.4, 17.9, and 17.3°C. (*C*) *I-V* relationships are plotted on the same scale. There is a voltage shift between 0.1 MPa (filled circles: E_{peak} = 22.0 mV, $E_{½}$ = 11.2 mV) and 40 MPa (squares: E_{peak} = 16.0 mV, $E_{½}$ = 3.8 mV) and a loss of current (−576 and −431 pA). Reversing the pressure to 0.1 MPa (open circles: E_{peak} = 18.0 mV, $E_{½}$ = 4.7 mV, I_{peak} = −452 pA) does not change the voltage dependence and the peak amplitude any further. Temperatures: 17.4, 17.9, and 17.0°C. (*D*) Steady state Na channel inactivation measured by application of conditioning pulses to different potentials from the holding potential of −90 mV is not affected by pressure. 0.1 (filled circles), 40 (squares), 0.1 (rev., open circles) MPa. The curve shown was drawn by eye. The values for $E_{½}$, which were obtained by fitting the curves with Eq. 2, are −23.7, −23.3, and −25.7 mV.

after rupture of the patch. Because of this disturbing effect, we could not determine whether a voltage shift caused by pressure, as reported by Conti et al. (1982*a*) for squid axons, also exists in chromaffin cells.

Whole-Cell Ca Currents

Ca currents, measured in the whole-cell configuration, show a characteristic rundown. Fenwick et al. (1982*b*) reported an irreversible loss of Ca channels after patch rupture. Since this rundown is accelerated by internal Ca^{++}, we used a free concentration of 10^{-8} M ([Ca]/[EGTA] = 1:11). Under these conditions, the loss of Ca currents is expected to develop with a half-decay time of ~25 min. Channel kinetics seem not to be affected by this rundown phenomenon.

Pressure experiments were designed to be completed as quickly as possible in order to meet these circumstances. Only five records of current responses to different step depolarizations were recorded at each pressure. By increasing the

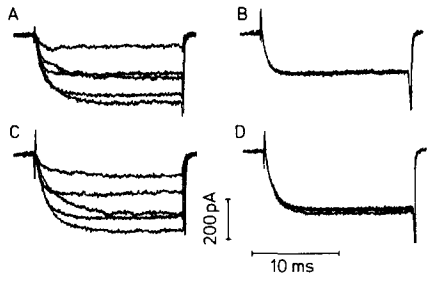

FIGURE 2. Ca currents. (*A*) Ca currents evoked by depolarizing pulses starting from a holding potential of −80 mV to −10 through +30 mV at atmospheric conditions. Panel *B* shows currents of the same cell at 40 MPa. There is no detectable effect of pressure on the Ca currents. This is seen more clearly in *C* and *D*. Currents at 0.1, 20, and 40 MPa at +20 (*C*) and +30 (*D*) mV are plotted superimposed without scaling. In *C*, the three traces cannot be distinguished; in *D*, the plateau current at 40 MPa is slightly larger. Since kinetics and maximal current are not changed in this experiment, the upper limit for a pressure effect on Ca currents is given by the temperature excursion, which is 2.1°C in this case (23.8°C at 0.1 MPa, 25.9°C at 40 MPa). Capacitive artifacts were blanked out. The cutoff filter frequency was 3 kHz; the access resistance of the whole-cell clamp was 2.5 MΩ.

pressure up to 40 MPa in 5-MPa increments, one complete experiment could be performed in <10 min. However, a more severe limitation to the speed at which the measurements could be taken arose from the necessity of keeping the temperature excursions produced by the pressure changes within a maximum accepted range of 2°C.

Fig. 2, *A* and *B*, shows Ca currents at different pressures. Pressure has no significant effect on the overall shape of the individual records. The voltage dependence is not shifted systematically either and the experiments were fully reversible. In Fig. 2, *C* and *D*, current traces at +20 and +30 mV, recorded at0.1, 20, and 40 MPa, are shown superimposed. Neither the kinetics nor the plateau currents are affected appreciably by pressure. In order to get an upper limit of pressure effects on Ca currents, temperature excursion must also be taken into account. Assuming that a pressure change of 40 MPa compensates exactly for a temperature increase of 2°C, we arrive at a limit for the apparent activation volume of 20 $Å^3$, assuming a temperature coefficient, Q_{10}, of 3.

Exocytosis

In contrast to the observations on ionic currents, we found that exocytosis is affected by pressures as low as 5 MPa. This can be seen both in chromaffin cells and mast cells, although the details of these responses are different in the two preparations.

Chromaffin Cells

Exocytosis in chromaffin cells was evoked by an increase in internal free Ca^{++}, a natural stimulant. Fig. 3*A* shows the time course of release at atmospheric conditions. To determine the effect of pressure on exocytosis, we performed the

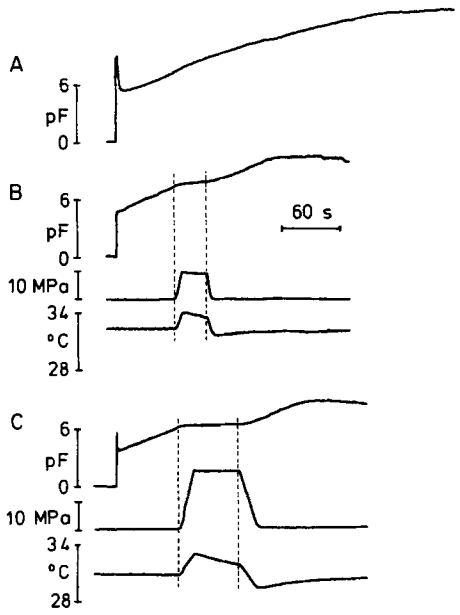

FIGURE 3. Degranulation of chromaffin cells. (*A*) Cell capacitance plotted as function of time at atmospheric pressure and 30°C. Fusion of vesicles with the plasma membrane was evoked by dialyzing the cells with 1 μM free Ca^{++}. The whole-cell configuration (initial step increase in capacitance) was achieved by application of voltage pulses, which are also responsible for the initial artifacts in *A* and *B*. (*B*) The first trace shows the time course of cell capacitance while a pressure step of 10 MPa was applied (pressure on second trace). The temperature excursion arising from pressure change is plotted below. Pressure abruptly slows the release. Decompression enhances the release again. (*C*) The same kind of experiment as in *B*, but with a pressure step to 20 MPa. In this case, the effect is even more drastic. Pressure almost inhibits the exocytosis completely. Cells were voltage-clamped to −70 mV and the series conductance was ~200 nS.

same kind of experiment at 20 MPa. When the pressure was held at this level for ~3 min after changing to the whole-cell configuration, the release process was slowed down, and the cells did not reach the same final capacitance as they did in the control experiments. This suggests that an irreversible loss of release capability is taking place during this time, which may be caused by the dialysis of an essential compound from the cell interior.

In order to circumvent this problem of wash-out, we elicited release under atmospheric conditions and applied pressure during the degranulation process for short periods of only 1–2 min. These experiments are shown in Fig. 3, *B* and

C. Pressure steps to 10 MPa reduced the speed of degranulation by ~50%. Temperature (bottom trace) is seen to increase transiently by ~2°C after pressure application of 20 MPa. The effects observed are somewhat underestimated, because degranulation is hastened at higher temperatures. We estimated the

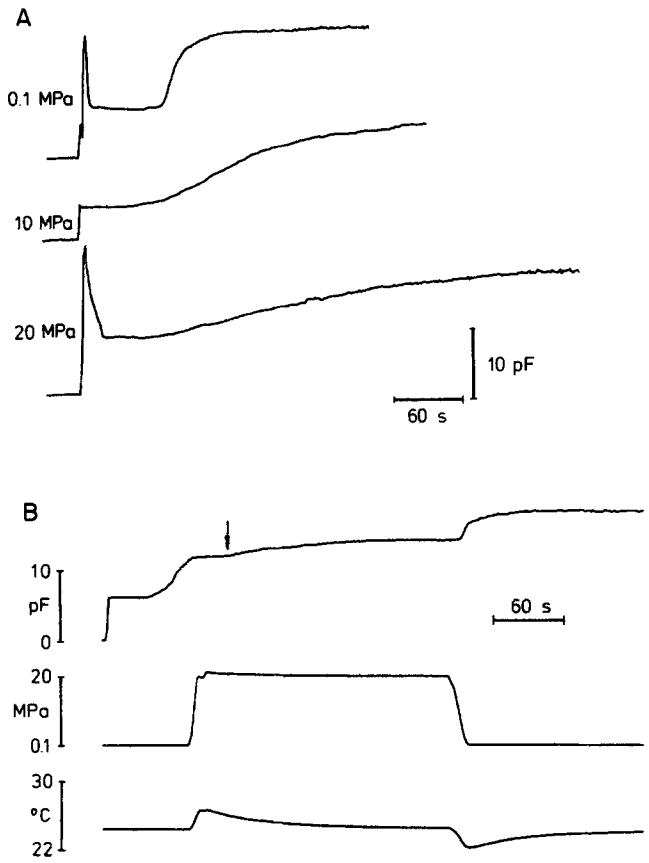

FIGURE 4. Degranulation of mast cells. (*A*) Cell capacitance is plotted as function of time at 0.1, 10, and 20 MPa. Fusion of mast cell granules was evoked by dialyzing the cells with 40 μM GTP-γ-S. The step in capacitance at the beginning of each trace indicates the cell capacitance, which appears in the measurement at the moment of patch rupture. The artifacts in the first and third traces arose from transient leaks generated by the voltage pulses, which were given in order to break the patches (see Fig. 3). Pressure was applied ~15 s before patch rupture. Temperature: 23–24°C. (*B*) Time course of cell capacitance (first trace) while a pressure step of 20 MPa was applied (second trace). In the third trace, the temperature excursion arising from pressure changes is plotted. Pressure abruptly slowed the release. After a "second delay" (arrow), however, the cell capacitance started to increase again with a time course similar to experiments under continuous high pressure (see *A*). Decompression strongly enhanced the release for a short period. Experiments were performed without Ca buffer in the internal solution. Cells were voltage-clamped to −25 mV and the series conductance was on the order of 100 nS.

activation volume by comparison of slopes in the capacitance increase before, during, and after the pressure application. For seven experiments with pressures between 10 and 20 MPa, we found $\Delta V = \sim 400$ Å^3, corresponding to a decrease in slope by ~85% at 20 MPa.

Mast Cells

In Fig. 4*A*, time courses of mast cell degranulation stimulated by 40 μM GTP-γ-S are compared at different pressures. The characteristic delay before the rising phase did not depend on pressure; nor could we detect a drastic loss of release potency as seen in chromaffin cells. The kinetics of release were estimated by measurement of the time elapsed between 10 and 90% of capacitance increase. According to this measure, we found an apparent activation volume of 390 (±57) Å^3 as an average of eight experiments.

The experiments shown in Fig. 4*A* were carried out without a Ca^{++} buffer in the internal solution. Since the degranulation of mast cell is accelerated by internal Ca^{++}, we repeated the same experiments with 0.5 μM free Ca^{++}, buffered with EGTA. In this way, we could rule out a possible pressure dependence of internal Ca^{++} concentration. In seven experiments, we could not find a significant difference from the previous experiments without Ca buffer.

We also performed experiments using pressure steps lasting for 1–3 min as in the case of chromaffin cells. Besides a slowing down of the process of degranulation, two further peculiarities are visible (see Fig. 4*B*). After application of 20 MPa pressure, the degranulation is suppressed almost completely, corresponding to an activation volume on the order of at least 1,000 Å^3. However, after another delay (indicated by the arrow in Fig. 4*B*), the release process resumes again, with kinetics as expected from the experiments of Fig. 4*A*. This delay was on the order of 30 s. Going back to atmospheric conditions evokes an overproportional, sudden increase in capacitance that settles within a few seconds.

DISCUSSION

The cause of HPNS is known to reside in the nervous system, probably in the synaptic transmission. In this study, we have investigated the pressure dependence of the main presynaptic membrane processes involved in nervous transmission. We considered the following steps: (*a*) transmission of action potentials that are mediated by the gating of Na^+- and K^+-selective channels; (*b*) Ca^{++} influx through voltage-gated Ca channels; and (*c*) secretion of transmitter by Ca-stimulated fusion of vesicles with the plasma membrane. All of these contributions, with the exception of K channels, were measured in chromaffin cells, a mammalian preparation that bears many similarities to neuronal cells. As an additional test of possibly different mechanisms of exocytosis, we also investigated the degranulation of rat mast cells.

Ionic Currents

K currents have already been investigated in squid giant axons (Conti et al., 1982*b*) and *Helix* neurons (Harper et al., 1982). Na currents have been already extensively investigated in squid giant axons (Henderson and Gilbert, 1975;

Conti et al., 1982*a*, 1984) and in *Helix* neurons (Harper et al., 1982). These authors found a pressure dependence of Na channel activation kinetics on the order of 45 Å^3 at 15°C. The results we obtained in chromaffin cells compare well with the findings in squid giant axon by Conti et al. (1982*a*). A systematic loss of peak current of ~30% after application of 40 MPa was observed (16% in squid axon). The activation volumes associated with τ_m, however, were consistently smaller than those found in Na channels of squid. This can be explained by the temperature effect observed by Conti et al. (1982*a*). Our results, obtained between 16 and 24°C, fitted well the trend of decreasing activation volumes at higher temperatures. Combining these results with the data obtained in squid giant axons, we estimate a temperature dependence for the apparent activation volume of −2.3 (±0.2) Å^3/°C. In addition, we could show that the channel inactivation, represented by τ_h, is more affected by pressure than τ_m. This was suggested by the squid data, but could not be determined directly because of excessive outward currents.

Voltage-gated Ca currents were less accessible to high-pressure experiments in the past. Otter and Salmon (1985) reported an inhibition of a Ca influx in *Paramecium* at pressures of 10 MPa. Since these results were obtained indirectly and the Ca channels involved in nervous transmission in mammals are probably quite different, we measured Ca currents in chromaffin cells. We estimated 20 Å^3 at 25°C as an upper limit for an apparent activation volume of kinetics and plateau current. The constancy of whole-cell Ca currents under high pressure suggest that the single-channel conductance is not markedly affected by pressure, in agreement with findings regarding other types of ion channels (acetylcholine receptor channel: Heinemann et al., 1987; Na channel: Conti, F., and S. H. Heinemann, unpublished data; alamethicin pore: Bruner and Hall, 1983).

It is quite likely that the kinetics of Ca channels in chromaffin cells are more sensitive to pressure at lower temperatures, as is the case for Na channels, but we can certainly exclude that compression to 20 MPa affects the gating of Na and Ca channels significantly in the physiological temperature range of mammals. Therefore, it is highly unlikely that the ionic channels investigated at high pressure play a major role in high-pressure disorders such as HPNS.

Vesicle Fusion

The pressure effect on the entire time course of degranulation in mast cells corresponds to an apparent activation volume of 390 (±57) Å^3. Estimates for chromaffin cells were on the same order. Exocytosis is mediated by a rather long chain of biochemical reactions, diffusion processes, and, finally, the fusion of vesicles with the cell membrane. The release experiments in mast cells show two different phases in the pressure response. Immediately after changes in pressure, the release is strongly inhibited. After a delay, however, this inhibition decreases to a level as measured by continuous pressure application. This finding suggests that one of the very last steps in the reaction chain, which is not rate-limiting on a long time scale, is very pressure sensitive. Pressure application inhibits exocytosis as long as the early steps in the reaction chain cannot overcompensate the slowing down of the last one. The overcompensation is seen after decompression as a sudden, overproportional capacitance increase.

The highly pressure-sensitive step may be the fusion of vesicles with the membrane, whereas the other steps like the mobilization of vesicles and their association with the membrane may be described by the apparent activation volume of 390 $Å^3$.

Parmentier et al. (1981) and Ashford et al. (1982) found that a hydrostatic pressure of 10 MPa reduces the amplitude of evoked excitatory postsynaptic potentials by ~50% and the frequency of spontaneous release by 75%. They expected the release process to be the pressure-sensitive site. We have shown directly that the exocytosis has a large pressure coefficient as compared with ionic channels. Apparent activation volumes of 390 $Å^3$ fit well with the data on excitatory postsynaptic potentials.

Man's diving range is limited by neurological disorders manifesting themselves at ~6 MPa, corresponding to a depth of 600 m. It is very likely that a suppression or slowing down of transmitter release is responsible for this limitation, although we cannot exclude participation of other effects. The known hyperexcitability of nervous tissue may reflect a depression of inhibitory transmitter release, since the pressure sensitivity of ionic channels is small.

For deep-sea animals, the activation volumes we have determined might have important implications. For instance, a different composition of the lipids forming the cell membranes could be an adaptation to counteract the effects of compression on membrane fluidity (Wann and Macdonald, 1980).

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REFERENCES

Almers, W., and E. Neher. 1985. The Ca signal from fura-2 loaded mast cells depends strongly on the method of dye-loading. *FEBS Letters.* 192:13–18.

Asano, T., and W. J. Le Noble. 1978. Activation and reaction volumes in solution. *Chemical Reviews.* 78:407–489.

Ashford, M. L. J., A. G. Macdonald, and K. T. Wann. 1982. The effects of hydrostatic pressure on the spontaneous release of transmitter at the frog neuromuscular junction. *Journal of Physiology.* 333:531–543.

Brauer, R. W. 1975. The high pressure nervous syndrome: animals. *In* The Physiology and Medicine of Diving and Compressed Air Work. P. B. Bennet and D. H. Elliot, editors. Balliere Tindall, London. 231–247.

Bruner, L. J., and J. E. Hall. 1983. Pressure effects on alamethicin conductance in bilayer membranes. *Biophysical Journal.* 44:39–47.

Conti, F., R. Fioravanti, J. R. Segal, and W. Stühmer. 1982*a*. Pressure dependence of the sodium currents of squid giant axon. *Journal of Membrane Biology.* 69:23–34.

Conti, F., R. Fioravanti, J. R. Segal, and W. Stühmer. 1982*b*. Pressure dependence of the potassium currents of squid giant axon. *Journal of Membrane Biology.* 69:35–40.

Conti, F., I. Inoue, F. Kukita, and W. Stühmer. 1984. Pressure dependence of sodium gating currents in the squid giant axon. *European Biophysics Journal.* 11:137–147.

Fenwick, E. M., A. Marty, and E. Neher. 1982*a*. A patch-clamp study of bovine chromaffin cells and of their sensitivity to acetylcholine. *Journal of Physiology.* 331:557–597.

Fenwick, E. M., A. Marty, and E. Neher. 1982*b*. Sodium and calcium channels in bovine chromaffin cells. *Journal of Physiology.* 331:599–635.

Fernandez, J. M., E. Neher, and B. D. Gomperts. 1984. Capacitance measurements reveal stepwise fusion events in degranulating mast cells. *Nature.* 312:453–455.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv.* 391:85–100.

Harper, A. A., A. G. Macdonald, and K. T. Wann. 1982. The action of high hydrostatic pressure on the membrane currents of *Helix* neurons. *Journal of Physiology.* 311:325–339.

Heinemann, S. H., W. Stühmer, and F. Conti. 1987. Single AChR-channel currents recorded at high hydrostatic pressures. *Proceedings of the National Academy of Sciences.* 84:3229–3233.

Henderson, J. V., and D. L. Gilbert. 1975. Slowing of ionic currents in the voltage clamped squid axon by helium pressure. *Nature.* 258:351–352.

Heremans, K. 1982. High pressure effects on proteins and other biomolecules. *Annual Review of Biophysics and Bioengineering.* 11:1–21.

Hodgkin, A. L., and A. F. Huxley. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *Journal of Physiology.* 117:500–544.

Lindau, M., and E. Neher. 1988. Patch-clamp techniques for time-resolved capacitance measurements in single cells. *Pflügers Archiv.* In press.

Macdonald, A. G. 1984. The effects of pressure on the molecular structure and physiological functions of cell membranes. *Philosophical Transactions of the Royal Society of London, Series B.* 304:47–68.

Marty, A., and E. Neher. 1983. Tight-seal whole-cell recording. *In* Single-Channel Recording. B. Sakmann and E. Neher, editors. Plenum Publishing Corp., New York. 107–122.

Neher, E., and A. Marty. 1982. Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. *Proceedings of the National Academy of Sciences.* 79:6712–6716.

Otter, T., and E. D. Salmon. 1985. Pressure-induced changes in Ca^{2+}-channel exitability in *paramecium. Journal of Experimental Biology.* 117:29–43.

Parmentier, J. L., B. B. Shrivastav, and P. B. Bennet. 1981. Hydrostatic pressure reduces synaptic efficiency by inhibiting transmitter release. *Undersea Biomedical Research.* 8:175–183.

Penner, R., and E. Neher. 1986. Intracellularly injected tetanus toxin inhibits exocytosis in bovine adrenal chromaffin cells. *Nature.* 324:76–78.

Wann, K. T., and A. G. Macdonald. 1980. The effects of pressure on excitable cells. *Comparative Biochemical Physiology.* 64A:141–147.