Exocytosis from chromaffin cells: hydrostatic pressure slows vesicle fusion

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Pressure affects reaction kinetics because chemical transitions involve changes in volume, and therefore pressure is a standard thermodynamic parameter to measure these volume changes. Many organisms live in environments at external pressures other than one atmosphere (0.1 MPa). Marine animals have adapted to live at depths of over 7000 m (at pressures over 70 MPa), and microorganisms living in trenches at over 110 MPa have been retrieved. Here, kinetic changes in secretion from chromaffin cells, measured as capacitance changes using the patch-clamp technique at pressures of up to 20 MPa are presented. It is known that these high pressures drastically slow down physiological functions. High hydrostatic pressure also affects the kinetics of ion channel gating and the amount of current carried by them, and it drastically slows down synaptic transmission. The results presented here indicate a similar change in volume (activation volume) of 390 ± 57 Å³ for large dense-core vesicles undergoing fusion in chromaffin cells and for degranulation of mast cells. It is significantly larger than activation volumes of voltage-gated ion channels in chromaffin cells. This information will be useful in finding possible protein conformational changes during the reactions involved in vesicle fusion and in testing possible molecular dynamic models of secretory processes.

1. Hydrostatic pressure allows measurements of activation volumes

Stepwise changes in thermodynamic variables can yield information helpful to gain insight into an unknown mechanism. For example, changes in temperature allow conclusions about the energetics of chemical reactions. Another global fundamental thermodynamic variable, albeit often neglected, is pressure. The dependence of kinetics on pressure of a simple chemical reaction allows a direct measurement of the volume change associated with the formation of the transient activated state of the reaction (∆V*) [1]. In other words, it is possible to measure changes in volume as cubic Ångström per molecule (a microscopic quantity) during transitions from one state to another simply by measuring changes in reaction rates induced by changes in external pressure (both are macroscopic quantities). As the activated transition states have generally a larger volume than the initial or final state, an increase in pressure leads to a slowing-down of reaction kinetics, given that an increase in volume has to work against pressure (which tends to decrease volume). Physiological chemical processes, such as the gating of voltage-dependent ion channels or the fusion of vesicles necessary for exocytosis, are affected by pressure and therefore it is possible to estimate activation volumes, and also to discriminate between possible complex molecular reactions pathways leading to ion flow through channels or vesicle fusion.

2. Effect of hydrostatic pressure on ion channels

(a) Extrasynaptic acetylcholine receptors

As an example, figure 1 compares single-channel records obtained from extrasynaptic acetylcholine receptors recorded at atmospheric pressure (0.1 MPa)
and 30 MPa [2]. A significant slowing-down of gating kinetics can be seen. However, the conductance of the channel did not significantly change. After a temperature correction to 13.3 °C for an increase of less than 2 °C due to compression, using the appropriately scaled amount of change known in reaction rate produced by a change in temperature of 10 °C (Q10) of 1.34 for single-channel currents, the conductance was 29.6 ± 1.2 pS at 0.1 MPa, and 28.4 pS ± 1.7 pS at 40 MPa. Analysing close-times and open-times histograms at various pressures, and correcting for temperature effects, activation volumes for the closed and open state associated with closing (ΔVc) and opening (ΔVo) the acetylcholine channel of 139 ± 11 Å³ and 59 ± 4 Å³, respectively, were obtained. This gives a difference in volume between the closed and open states, ΔVo of 80 ± 12 Å³. As closing the pore involves a larger volume than opening it, increased pressure favours the closed state. It should be noted that these results include the activation volumes of binding and unbinding of acetylcholine. Also, activation volume in molecular terms is rather abstract, as it includes, for example, changes in packing of water associated with the changes in exposure of charges to an aqueous environment.

(b) Effects of pressure on other ion channels

Many types of excitable cells are sensitive to pressure [3]. Hydrostatic pressure has profound effects on drug action, particularly general anaesthetics. Organisms living in oceans as well as divers are subject to large changes and/or high hydrostatic pressures. So in addition to being a useful tool, pressure is a parameter that needs to be considered in physiology. Most types of voltage-gated ion channels have pressure-dependent properties, mostly a slowing-down of gating kinetics. The ionic currents [4–6], and gating currents [7] of voltage-gated sodium channels have been extensively studied. Voltage-gated sodium channels from squid giant axons slow their activation kinetics by a factor of about 1.6 at 62 MPa, giving a ΔVc of about 58 Å³, and a ΔVo of 26 Å³ between the closed and the open state [6]. Marine animals are exposed to such pressures at depths of 7000 m, at which squids, among other living organisms, have been reported. Seven thousand metres is about one-half of the maximal ocean depth. Comparing the slowing-down of kinetics for the gating currents and the corresponding sodium currents, it becomes clear that higher pressures slow down the ionic currents more than the corresponding gating currents. This means that in addition to an increase in volume during gating, the opening of the ion conduction pathway requires additional conformational rearrangements that significantly contribute to the total activation volume, as gating currents precede the opening of the ion channel pore. The measured ΔVo was 28 Å³. The unavoidable conclusion is that gating and the channel opening transition are separate phenomena, with the latter involving larger molecular rearrangements that carry little or no charge rearrangements [7].

Voltage-gated potassium channels in neurons have a similar slowing-down of kinetics as voltage-gated sodium currents [4,5,8,9]. Potassium channels from squid giant axons slow their activation kinetics by a factor of about 1.9 at 41 MPa, giving a ΔVo of about 60 Å³ [9]. The voltages of activation and inactivation of most ion channels slightly shift by a few tens of millivolts to the right or left with pressure, and the activation volumes are slightly different at various test potentials. In addition, the conductance for most channels studied so far (except for the voltage-gated calcium channel CaV1.2, see below) does not significantly change. For example, alamethicin pores in artificial bilayers do not significantly alter their conductance at pressures of up to 100 MPa, while kinetic analyses reveal an activation volume in the order of 100 Å³ [10]. These results indicate that ions do not change their molecular volume while transiting through the conduction pore, which is consistent with current theories of ion permeation in which the hydration shell of permeant ions is replaced by fixed charges within the permeation pore. This exchange is not expected to cause significant conformational changes, and hence will not lead to changes in activation volumes. However, the closed–open conformational transition of an ion channel involves large molecular rearrangements.

Voltage-gated Ca²⁺ channels are relevant for secretion as they trigger exocytosis. Heinemann et al. [11] measured effects of pressure on voltage-gated sodium and calcium channels in bovine chromaffin cells. The activation volumes obtained for sodium currents (ΔVc = 33 Å³) were similar to those reported by Conti et al. [6] for sodium channels in squid giant axons. For Ca²⁺ currents, an upper limit for the activation volume of 20 Å³ was obtained, with hardly any effect on current amplitude. However, Aviner et al. [12] did see a drastic slowing-down of activation kinetics with pressure of recombinant CaV1.2 and CaV3.2 channels in Xenopus oocytes, with concomitant strong effects on current amplitudes. They also carefully studied inactivation kinetics at various pressures as well as the dependence on voltage of activation, inactivation and conductance. Most interesting is the observation that CaV1.2 currents increased by 70 ± 32% at 5 MPa, while currents through CaV3.2 channels decreased by 27 ± 5% at the same pressure. For CaV1.2, a
\[ \Delta V \text{ of 756 } \text{Å}^3 \] was obtained, meaning that although activation of this current will be slowed down dramatically with pressure, the increased \( \text{Ca}^{2+} \) influx could well account for effects of the High-Pressure Nervous Syndrome observed in deep-sea divers. High-Pressure Nervous Syndrome is observed at pressures larger than about 1 MPa and is characterized by severe impairment of basic physiological functions, including motor and cognitive impairments [13–17].

3. Effect of hydrostatic pressure on secretion
Several reports [18,19] have pointed out that severe pressure-dependent effects are detectable in synaptic transmission. A reduction in the frequency of the spontaneous release of transmitter in the frog neuromuscular junction by a factor of two occurred at pressures as low as 5 MPa. In addition, the amplitude of the excitatory postsynaptic potential in ganglia of \textit{Aplysia californica} was reduced to one-half at 10 MPa. These results clearly point to a very pressure-sensitive presynaptic event involved in vesicle fusion.

The strong dependence of \( \text{CaV}1.2 \) currents on pressure, together with the effects described above for other ion currents, cannot explain the various severe physiological effects observed in whole organisms under pressure. One possible explanation is that other voltage-gated \( \text{Ca}^{2+} \) channels in the presynaptic terminal, particularly those directly involved in presynaptic \( \text{Ca}^{2+} \) entry (P/Q, N or R type), are affected differently. In fact, the opposite behaviour with pressure observed in \( \text{CaV}1.2 \) and \( \text{CaV}3.2 \) indicate that different calcium channel types react very differently to pressure. Therefore, the release processes of extra-somatic and somatic vesicle fusion are different in terms of activation volumes. Additionally, binding of \( \text{Ca}^{2+} \) to molecules regulating the vesicle-release and the reuptake mechanisms in extra-somatic and somatic secretion might have very different activation volumes. See Daniels & Grossman [20] for a comprehensive review.

There are many studies reporting how osmotic pressure affects vesicle fusion and retrieval kinetics, but these forms of pressures mainly induce lateral stress on the membrane, similar to stretching the membrane in two dimensions. The magnitude of this pressure is very limited, as otherwise the membrane would rupture. A pressure as low as 0.3 KPa is able to split endocytic events into two pathways, one pressure-insensitive and another strongly pressure sensitive [21], probably because endocytosis involves a decrease in membrane surface, which will be contrasted by the forces stretching the membrane. By contrast, hydrostatic pressure, which acts in three dimensions, compresses all components isotropically, and therefore can be studied at several tens of megapascal in living matter. Only this kind of pressure application allows a calculation of activation volumes, although these volumes are apparent and have to be considered as molecular rearrangements.

The patch-clamp technique [22] makes it possible to record capacitance changes accompanying fusion of single vesicles with the cell membrane. This is attained by measuring the time course of the total impedance of the cell, which includes the imaginary part. Towards this aim, the complex impedance is measured by imposing a small sinusoidal voltage to the cell while measuring the elicited current. Using a lock-in amplifier, very small changes in phase and amplitude between the voltage excitation and the current response can be detected at low noise levels [11]. Changes in the real part of the impedance correspond to changes in the equivalent resistance and shifts in phase correspond to changes in capacitance. By designing a special high-pressure recording chamber (figure 2), patch-clamp experiments have

![Figure 2. (a) Cross section of the flying-patch holder. The reservoir and external assembly can be extended and turned while establishing the whole-cell configuration in a standard patch-clamp set-up. Once the cell is patched and detached from the culture dish, the reservoir assembly is placed carefully under the pipette and cell being patched. The flying-patch holder can then be removed from the head stage and carefully inserted into the pressure chamber (b), which is filled with paraffin oil (via I, oil inlet). Adapted from Heinemann et al. [2].](http://rstb.royalsocietypublishing.org/)

\[ \text{holder} \]
\[ \text{internal electrode} \]
\[ \text{pipette} \]
\[ \text{reservoir} \]
\[ \text{pressure transducer} \]
\[ \text{thermistor} \]
\[ \text{BNC} \]
\[ \text{I} \]

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been performed at pressures up to 60 MPa, which corresponds to 592 times normal atmospheric pressure or to the pressure experienced at \( \approx 5900 \) m under the surface of the ocean. The high-pressure chamber consists of a patch-pipette holder connected to a pressure feed-through that can be placed on the manipulator of a conventional patch-clamp set-up. Attached to the holder is a reservoir with a ground electrode, which will replace the external recording chamber while transiting the patch to the high-pressure chamber and performing recordings. The procedure has a surprisingly high rate of successful transfers of patches or cells attached to the pipette into the high-pressure chamber. Once immersed into paraffin oil and after closure of the chamber, patches were very stable and lasted for up to 1 h. Vibrations do not affect the assembly, the electrical shielding is extremely good given the high-voltage pulse used to obtain the whole-cell configuration at a holding potential of \( \approx 60 \) MPa, can also be used.

Paraffin oil was chosen as the medium transmitting pressure to the patch-clamp assembly, as besides being a very good insulator, it is practically incompressible.

Due to the complication of having to measure changes in capacitance from a cell under high pressure, vesicle fusion had to be induced after the patch-clamp assembly was inside the high-pressure chamber. This was achieved by first establishing the whole-cell configuration in chromaffin cells already in the tight-seal cell-attached configuration, and then being immersed in oil and positioned in the sealed high-pressure chamber. As under these conditions suction could not be applied to rupture the membrane patch, brief high-voltage pulses were applied to the pipette electrode, which will replace the external recording chamber per se. Note that this procedure excludes any pressure effect due to changes in kinetics of calcium channels, and therefore gives a direct readout of pressure effects on vesicle fusion.

Figure 3 shows measurements during secretion in chromaffin cells at various pressures. Figure 3a depicts the time course of total capacitance at atmospheric pressure, starting just before obtaining the whole-cell configuration at a holding potential of \(-70 \) mV. Just after rupture of the patch, an artefact due to the high-voltage pulse can be seen (seen also in figure 3c), thereafter the step in capacitance corresponding to the addition of the cell capacitance can be seen in the record. Immediately after obtaining the whole-cell configuration, the steady increase in capacitance due to vesicle fusion induced by \( 1 \mu \text{M} \text{Ca}^{2+} \) in the pipette solution equilibrating with the internal solution of the cell can be seen. Figure 3b shows a similar experiment in which a step in pressure to 10 MPa was applied (middle trace, dotted lines) while vesicles were being fused. The concomitant pressure and change in temperature (lower traces) were recorded. Note the significant decrease in vesicle-fusion rate during the pressure step. The same procedure is seen in figure 3c, but with a pressure step to 20 MPa. This pressure is sufficient to practically completely inhibit vesicle fusion. The unavoidable increase in temperature will tend to underestimate the direct pressure effect as the rate of vesicle fusion is increased at higher temperatures. Heinemann et al. [11] estimated the activation volume of vesicle fusion in bovine chromaffin cells to be \( \approx 400 \) Å\(^3\), and therefore roughly an order of magnitude larger than the effect on voltage-gated ion channels, except for \( \text{Ca}_{1.2} \) channels.

From the above, it can be concluded that, at least in chromaffin cells, the \( \text{Ca}^{2+} \)-dependent vesicle-fusion process is strongly pressure dependent by itself. The data obtained from the experiments mentioned will be helpful in testing parameters defined in molecular dynamic models regarding this particular process for compatibility and in comparing these to vesicle-fusion models of other cells.

**Ethics statement.** For this review, no experiments were performed. The use and care of animals in the experiments from which the data was taken for this review followed the guidelines of the German and Italian laws on animal protection, and appropriate ethical approval and licences had been obtained.

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