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ARTICLE

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# Effect of high hydrostatic pressure on the bacterial mechanosensitive channel MscS

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**Abstract** We have investigated the effect of high hydrostatic pressure on MscS, the bacterial mechanosensitive channel of small conductance. Pressure affected channel kinetics but not conductance. At negative pipette voltages (corresponding to membrane depolarization in the inside-out patch configuration used in our experiments) the channel exhibited a reversible reduction in activity with increasing hydrostatic pressure between 0 and 900 atm (90 MPa) at 23°C. The reduced activity was characterized by a significant reduction in the channel opening probability resulting from a shortening of the channel openings with increasing pressure. Thus high hydrostatic pressure generally favoured channel closing. Cooling the patch by approximately 10°C, intended to order the bilayer component of the patch by an amount similar to that caused by 50 MPa at 23°C, had relatively little effect. This implies that pressure does not affect channel kinetics via bilayer order. Accordingly we postulate that lateral compression of the bilayer, under high hydrostatic pressure, is responsible. These observations also have implications for our understanding of the adaptation of mechanosensitive channels in deep-sea bacteria.

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## Introduction

Mechanosensitive (MS) ion channels function as molecular mechanoelectrical transducers by converting mechanical stimuli into electrical or chemical signals in living cells (Sachs and Morris 1998; Hamill and Martinac 2001; Sukharev and Corey 2004). They have been discovered in organisms of different phylogenetic origins; plants, fungi, mammalian cells, bacteria and archaea (Sachs 1988; Morris 1990; Martinac 2004), which indicates their early evolutionary origin. The activity of bacterial MS channels has been extensively studied in giant spheroplasts (Martinac et al. 1987) and in reconstituted membrane fractions of *Escherichia coli* (Berrier et al. 1989; Delcour et al. 1989), which harbours several types of MS channels in its cellular envelope (Berrier et al. 1996; Martinac 2004).

One of these is MscS, an MS channel of small conductance, which displays voltage dependence characterized by 15 mV per e-fold change in the channel open probability and increased activity with membrane depolarization (Martinac et al. 1987). As for other prokaryotic MS channels (Hamill and Martinac 2001; Martinac 2004), tension produced by stretching the membrane bilayer is sufficient to gate this channel (Martinac et al. 1990). The tertiary structure of MscS from *E. coli* was solved by X-ray crystallography to a 3.9-Å resolution (Bass et al. 2002). It comprises a homoheptamer in which each subunit comprises three transmembrane segments, TM1, TM2 and TM3, and there is a large cytoplasmic region. The TM3 helices line the channel pore, whereas the TM1 and TM2 helices are thought to be the sensors for membrane tension and voltage.

High hydrostatic pressure, a thermodynamic intensity parameter like temperature, is widely used to investigate

the structure of proteins and lipid bilayers (Balny et al. 2002). Pressures over the range of several 100 MPa unfold and denature proteins. These hydrated states occupy less volume than the native state and are thus energetically favoured, in accordance with Le Chatelier's principle. All multimeric proteins have been found to dissociate to subunits at high pressure (Macdonald 2001b). In contrast, lipid bilayers undergo a bulk compression which reflects their anisotropic structure; lateral compression is marked and in some cases results in a thickening of the compressed structure (Braganza and Worcester 1986; Beney et al. 1997). Phase changes occur at high pressure, producing states which do not occur in normal conditions (Czeslik et al. 1998). Spectroscopic measurements show that, in general, the order of lipid bilayers is increased by 100 MPa by an amount equal to that caused by a reduction in temperature of approximately 17°C (Cossins and Macdonald 1989). In addition to providing unique information about the equilibrium state of large molecules and assemblies, high hydrostatic pressure is also used to study reaction kinetics (Balny et al. 2002).

High pressure has been used to study the functioning of a number of ion channels and other membrane proteins. In such cases we are concerned with a protein which is partly solvated by lipid and the question arises of whether pressure acts via the lipid bilayer or via the intraprotein and aqueous–protein interactions. In the case of the uncoupled ATPase from dog kidney and fish gill, the enzyme's activity is reduced as the order of the boundary lipid increases (Gibbs 1997). Ion channels under high pressure, on the other hand, seem to be affected by pressure acting on their protein structure directly. For example, classical voltage clamp experiments, carried out at high pressure on squid axon and other favourable cells, reveal a slowing of the gating kinetics underlying the action potential which is not compatible with bilayer order (Kendig et al. 1993). High pressure slows gating current rather less than it slows the opening of the Na and K channels (Conti et al. 1984). The clearest example of pressure perturbing an intraprotein interaction in an ion channel is provided by the work of Meyer and Heinemann (1997) on N-type inactivation in K channels. This process involves a tethered peptide ball diffusing and associating with protein subunits to occlude the channel pore. High pressure slows inactivation, opposing the association (binding) reaction, and favours the more hydrated, dissociated, state, which occupies less volume. High pressure can also activate certain channels, as in the case of the BK channel, in which an endogenous inhibitor is postulated to be dissociated by pressure (Macdonald 1997, 2001a). For reviews of high-pressure work on ion channels see Macdonald (2002a, 2002b).

In this paper, we report our first experiments studying the effect of high hydrostatic pressure on MscS in giant spheroplasts of *E. coli* using a development of Heinemann's "flying-patch" patch-clamp technique (Heinemann et al. 1987; Macdonald and Martinac 1999). We can cite at least three reasons for the work. First, the functioning of this channel is intimately related to its boundary lipid and it is a reasonable, if general, prediction that high pressure should affect gating by perturbing the protein–bilayer interaction. The very large heptameric protein structure which projects into the cytosol is also likely to be susceptible to high pressure and, third, this is the first pressure study of a MS channel and one which is also present in deep-sea organisms which live naturally at pressures of up to 100 MPa (e.g. *Methanococcus jannashii*) (Kloda and Martinac 2001; Martinac and Kloda 2003).

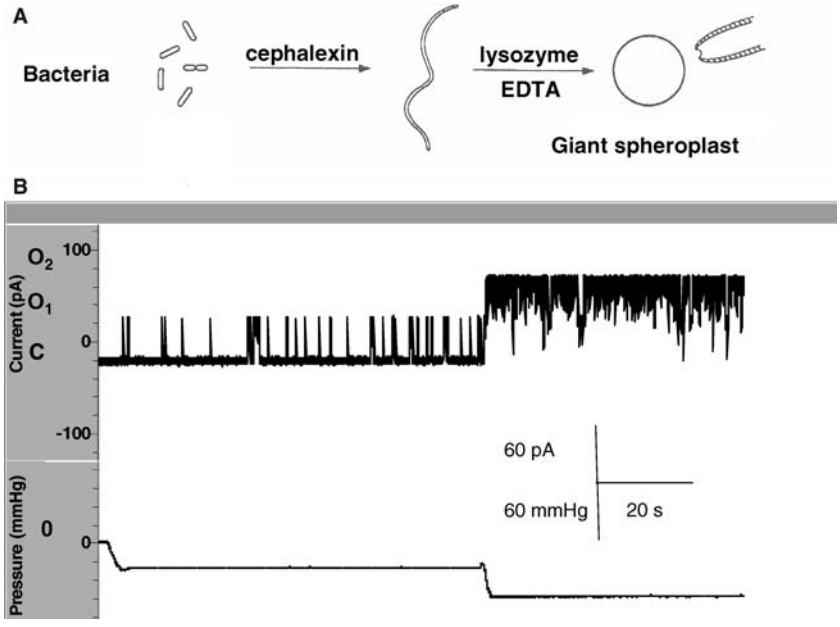


**Fig. 1** MscS activity in giant spheroplasts. **A** Method of study of bacterial mechanosensitive channels in giant spheroplasts (adapted from Sukharev et al. 1997). **B** Current trace of *E. coli* MscS recorded from an inside-out excised patch. The pipette voltage was +40 mV. Two channels were active in the particular patch. *C* denotes the closed state, while $O_1$ and $O_2$ denote the open state of the channel.

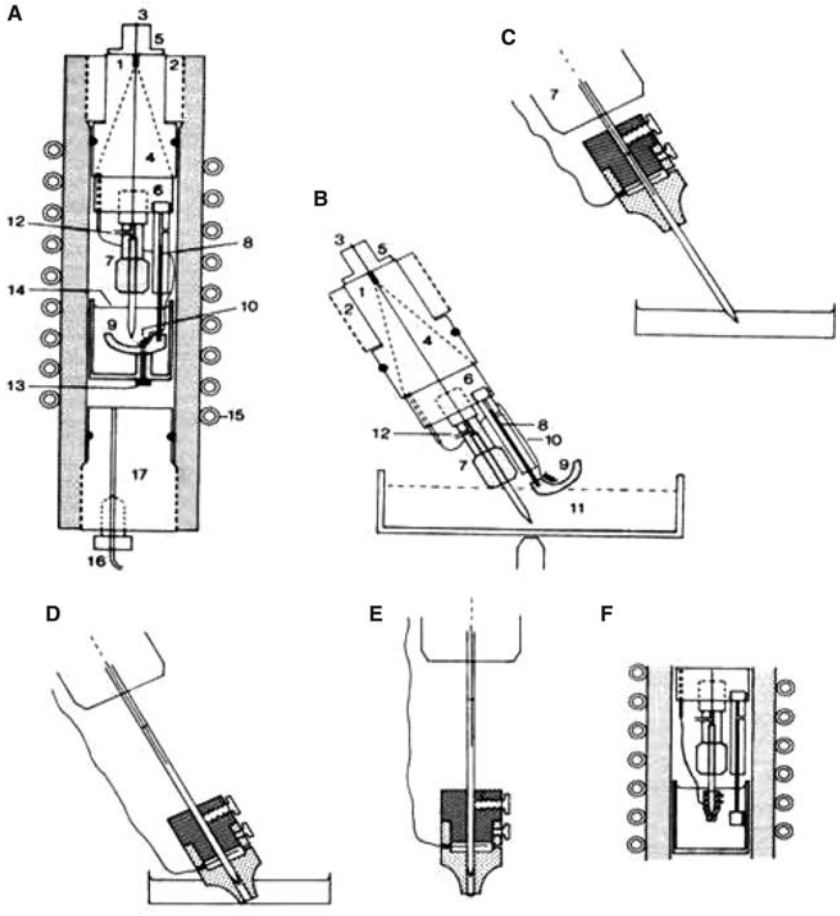


**Fig. 2** Apparatus for patch-clamp recording at high hydrostatic pressure. **A**, **B** The apparatus and the method of transferring the patch to the pressure vessel, as in Macdonald (1997). **A** The pressure vessel has an internal diameter of 29 mm. *1* End closure, *2* retaining ring, *3* electrical lead connecting patch to the amplifier, *4* Teflon insulation, *5* Teflon plug-socket, *6* extension of *4*, supporting *7* the pipette holder and *8* the mechanical sliding support for *9* the transfer dish, *10* bath electrode, *12* suction line, disconnected prior to mounting the electrode in the pressure vessel, *13* bolt which secures the "heat shield" beaker, *14* interface between the bath solution in the heat shield and liquid paraffin, *15* copper tubing for temperature control, *16* high-pressure capillary which connects the vessel to the high-pressure pump and gauge, *17* lower end closure. **B** The patch pipette mounted in its holder *7*, which is supported by the pressure vessel end closure *1*, connects via *5* to the patch amplifier (not shown). The whole assembly is mounted on a Leitz mechanical micromanipulator (not shown). A giant Petri dish *11* contains a reference electrode and spheroplasts and is mounted on the substage of an inverted microscope. The inside-out patch was formed by air exposure as previously described (Martinac et al. 1987) and then the transfer dish *9* was swung underneath the electrode tip, allowing the whole assembly to be transferred to the pressure vessel without interrupting the electrical patch-clamp circuit. En route the heat shield beaker was attached. This provided 7 ml of aqueous solution and protected the patch from the heat generated when the paraffin was pressurized, to a maximum of 90 MPa (Macdonald, 1997). **C**–**F** The modification to the apparatus which facilitates the transfer of the excised patch to the pressure vessel. **C** The pipette holder *7* is shown enlarged with the polymethacrylate sleeve device shown *cross-hatched and stippled*. It comprises two parts enclosing a chamber containing the fine wire bath electrode. A nylon screw in the upper part grips the shaft of the patch pipette. **D** After an inside-out patch has been formed, the sleeve is lowered to enclose the tip of the pipette and the capillary ensures that an electrical path connects the patch to the bath electrode in the chamber, above. **E** The device may then be lifted clear of the dish and the whole assembly mounted in the pressure vessel, shown in **F**. Note that the modified heat shield beaker in **A** is now supported by *8*. (Reproduced with permission from Macdonald and Martinac 1999.)

## Materials and methods

### Bacterial strains and preparation of giant spheroplasts

*E. coli* strain AW737 was used for the preparation of giant spheroplasts as previously described (Ruthe and Adler 1985; Martinac 1987). Briefly, bacteria were grown at 37°C in Luria–Bertani (LB) broth containing 10 g/l Bacto-tryptone, 5 g/l yeast extract and 5 g/l NaCl until the cells had reached the mid log phase (optical density of 0.4–0.5) at 595 nm. An amount of 3 ml of 37°C bacteria was put in the 27-ml flask filled with LB broth supplemented with 180 μg cephalexin and was left shaking on a 42°C shaker. After approximately 2 h *E. coli* cells grew into long filaments (100–150-μm long), which could readily be converted into giant spheroplasts (Fig. 1A) by addition of 200 μg/ml lysozyme and 6 mM/l EDTA to the bacterial filaments suspended in 0.8 M sucrose solution.

### Electrophysiological recording at high hydrostatic pressure

Single channel currents were filtered at 2 kHz, digitized at 5 kHz and analysed using pCLAMP6 data acquisition and analysis software (Axon instruments, USA). Current recordings were viewed with the Axoscope for Windows program (Axon Instruments, USA). Suction applied to the patch-clamp pipette at atmospheric pressure to check for the presence of MscS in spheroplast patches was measured with a piezoelectric pressure transducer (Omega Engineering, USA). We used voltage to activate MscS in a high-pressure chamber in which it could not be activated by membrane tension. Details of the apparatus for patch-clamp recording at high hydrostatic pressure are described in the legend of Fig. 2. The experiments were conducted at 23°C. Initially a nominal pressure of approximately 0.1 MPa provided a control baseline. High pressure was then usually applied in steps of 5–20 MPa, with pressure increasing at a rate of approximately 10 MPa/min at each step. The temperature of the hydraulic oil (medicinal liquid paraffin) increased significantly but that of the beaker of recording solution (Fig. 2F) increased far less. Mock-up experiments in which a thermistor replaced the patch electrode showed that a typical compression to 50 MPa increased the temperature of the solution in the beaker by 1.4°C, and equilibration to within 0.5°C of the precompression temperature occurred in 11 min (data not shown). Decompression caused similar but inverse changes in temperature. The time course of these changes dictated the selection of data recording.

Separate experiments were carried out in which the transfer procedure was used to plunge the patch assembly into cooled (approximately 13°C) liquid paraffin at normal atmospheric pressure (Fig. 6). This treatment was intended to order the lipid bilayer to an extent similar to 50 MPa at 23°C. Recording solutions contained 200 mM KCl, 40 mM $MgCl_2$ and 5 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES), pH 7.2 (pipette) and 250 mM KCl, 90 mM $MgCl_2$ and 5 mM HEPES, pH 7.2 (bath).

For pressure experiments HEPES was replaced by tris(hydroxymethyl)aminomethane (Tris) buffer, since the pH of Tris is unaffected by the pressure used here (Disteche 1972), whereas it is highly sensitive to temperature (see the leaflet with the Sigma Tris for the temperature coefficient). All recordings were obtained from inside-out excised membrane patches containing between one and three active channels (Fig. 1B).

## Results

All data shown here are from a single experiment, which allowed for a most complete and consistent characterization of the MscS activity at different hydrostatic pressures obtained from a single patch. More than ten successful pressure experiments were carried out in this study, with all of them demonstrating the same effect of the high pressure on the channel behaviour as in the single experiment shown here. The life of a spheroplast patch is typically quite long and fortunately pressure did not seem to curtail it. The number of channels remained unchanged irrespective of the pressure applied (Fig. 3).

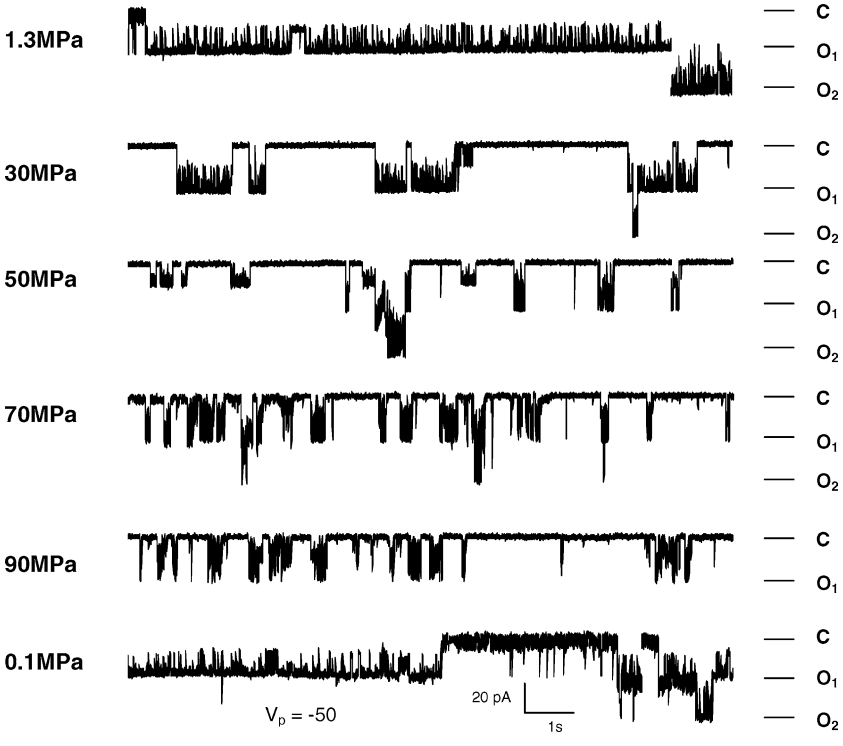


**Fig. 3** MscS activity at different hydrostatic pressures. Single channel currents recorded at different hydrostatic pressures (indicated on the *left side* of each current trace). The pipette voltage was –50 mV. Note the reversibility of the effect of high pressure

The effect of pressure on channel activity resulted in a decrease in the duration of a single channel opening, resulting in an overall decrease in channel open probability (Fig. 4A). Decompression reversed the pressure effect. In the particular experiment quantified in this study we were able to apply different levels of high pressures several times ($n = 3$ or 4) upon decompression, which further confirmed the reversibility of the pressure effect. A semilogarithmic plot of the channel open probability versus pressure (Fig. 4B) revealed an exponential relationship between open probability and high pressure, allowing the calculation of the volume change on going from the open to the closed state (see "Discussion").

High pressure had no effect on single channel conductance, although the channels tended to gate predominantly at subconducting levels as the pressure was increased (Fig. 5). An interesting feature of the channel gating consisted of it frequently visiting an approximately 50% conductance level from the fully open level at atmospheric pressure. One of the characteristics of the MscS of *E. coli* is to gate at subconducting levels at negative pipette potentials corresponding to depolarization of the spheroplast membrane (Martinac et al. 1987), with the 50% subconducting level being frequently visited (Dobson et al. 2002). With increase in hydrostatic pressure the channel most frequently gated at the 50% subconducting level, visited from the open as well as the closed state (Fig. 5, asterisk).

Finally, a series of experiments were carried out at atmospheric pressure, in which the effects of lowering temperature by approximately 10°C on the channel activity were examined. Overall, the channel activity was little affected, showing the same characteristic long openings at low temperature comparable to the openings recorded at 23°C (Fig. 6).

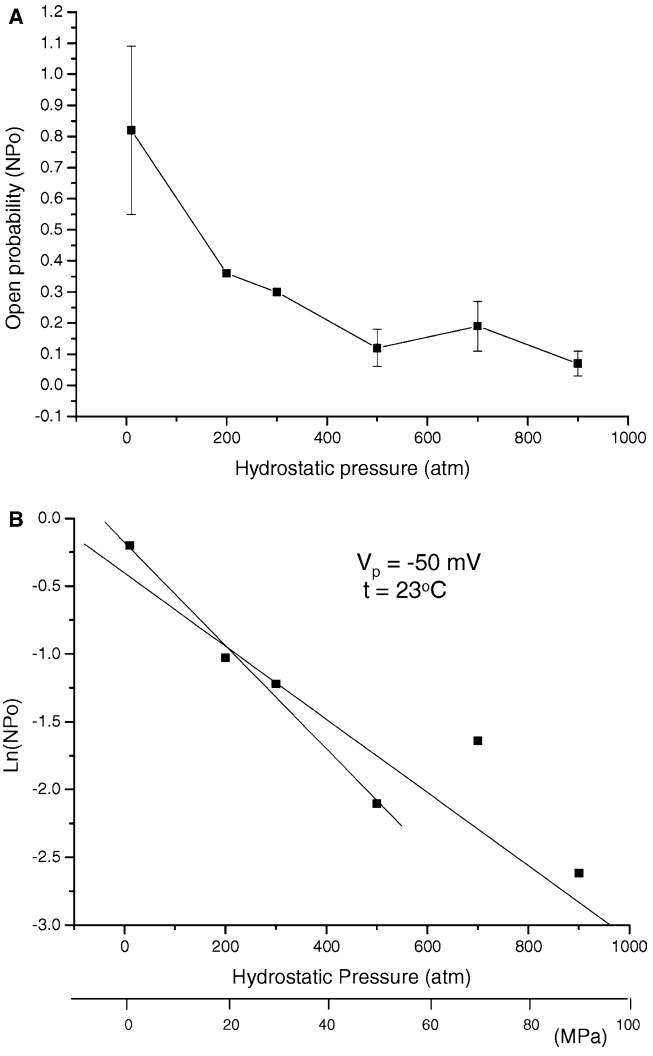


**Fig. 4** Channel open probability against hydrostatic pressure. **A** Open probability $NP_o$ against hydrostatic pressure was calculated from a single experiment. Channel activity was recorded several times ($n = 3$ or 4; mean ± standard deviation) at all pressures except for data obtained at 200 and 300 atm ($n = 1$). **B** Semilogarithmic plot of $NP_o$ versus hydrostatic pressure replotted from **A** shows a linear relationship (see "Discussion"). Note the increase in the number of channel openings as the hydrostatic pressure increased above 50 MPa, suggesting an additional effect of hydrostatic pressure on the channel in addition to lateral compression of the bilayer causing an overall decrease in the channel open probability. The experimental points were fitted from (1) 0–50 MPa, giving a change in volume $\Delta V$ of −94 ml/mol (i.e. 155 Å$^3$ per channel molecule) and (2) 0–90 MPa, giving $\Delta V$ of −61 ml/mol (i.e. 110 Å$^3$ per channel molecule)

## Discussion

Previous work has shown that MscS is activated by membrane bilayer tension and by voltage (Martinac et al. 1987; Sukharev 2002). These two factors combine additively and are thought to cause very similar molecular movements. We used this channel property to study the effects of high hydrostatic pressure on single MscS activity in inside-out excised patches of giant spheroplasts in a high-pressure chamber in which the channel could not be activated by membrane tension. High hydrostatic pressure affected the channel kinetics such that it favoured the closed state, resulting in a reduction of the channel open probability with increase in hydrostatic pressure. Channel conductance remained unaffected, although the channel exhibited frequent gating at subconducting levels as the pressure was increased. This finding is in agreement with results of other studies of ion channels in which pressure was found to manifest a variety of kinetic effects but no change in conductance (see "Introduction").

Generally, the channel open probability decreased with an increase in hydrostatic pressure. As this probability is an equilibrium state we may treat it as an equilibrium constant. This varies with pressure according to the classical thermodynamic relationship $\mathrm{d}\ln K/\mathrm{d}p = -\Delta V/RT$, where $K$ is the equilibrium constant, $\Delta V$ is the volume change across the equilibrium, $p$ is pressure and $R$ and $T$ are the gas constant and temperature, respectively. Over the range 1–50 MPa (Fig. 4B) we calculated a value of $\Delta V = -94$ ml/mol,

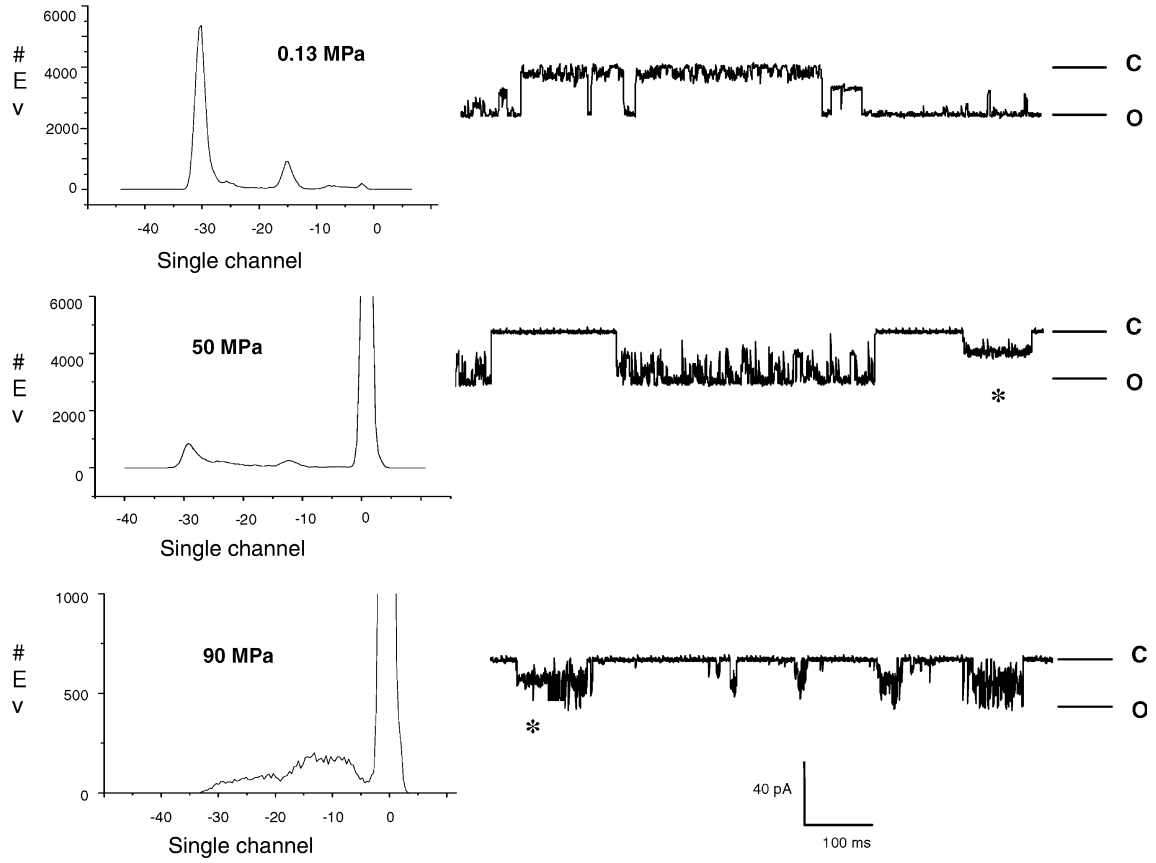


**Fig. 5** Amplitude histograms of single MscS currents at different pressures. Current histograms at three different hydrostatic pressures were obtained from the traces shown in Fig.3. Note the increased tendency of the channel gating at subconducting levels with increase in hydrostatic pressure (*asterisk*)

which divided by Avogadro's number ($6.023\times10^{23}$) gives a change in volume, per channel molecule, of $-155$ Å$^3$ in going from the open to the closed state (see legend of Fig. 4). An average of several experiments carried out over a similar range of high hydrostatic pressures gave a volume change of $147\pm108$ Å$^3$ ($n=3$) per MscS molecule. Like all reaction volumes this volume change is very small in comparison with the volume of the main molecule of interest, here the MscS heptamer. Its volume is approximately $6.0\times10^5$ Å$^3$, calculated by approximating the channel to a cylinder 80 Å in diameter and 120-Å long. (Bass et al. 2002). The $\Delta V$ of $-94$ ml/mol (Fig. 4b) arises in the reaction system as a whole: channel protein, lipid bilayer, aqueous solvent and the ions present. Can we relate $\Delta V$ to what we know of the channel's structure?

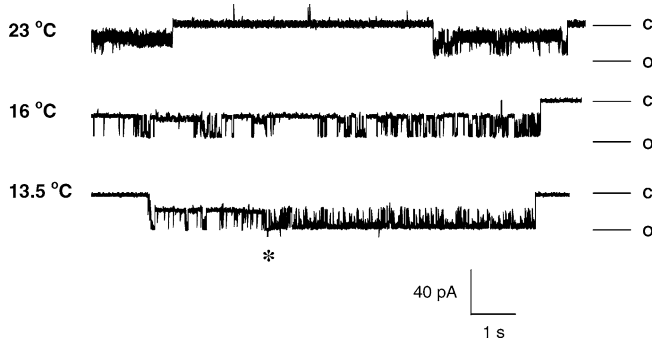


**Fig. 6** Effect of lowering temperature on the channel activity. MscS current traces recorded at 23, 16 and 13.5°C. The pipette voltage was $-60$ mV. Note frequent channel gating at a subconducting level, and the change in the gating mode at 13.5°C (*asterisk*). The change between gating modes is characteristic of MscS gating transitions at depolarizing voltages of 60 mV and above (corresponding to pipette voltages of $-60$ mV and lower in the inside-out excised patch configuration) (Dobson et al. 2002)

Although we cannot exclude the possibility that the MscS heptamer itself is directly affected by the high pressure we prefer to interpret our results in terms of the properties of the associated lipid bilayer. There are two good reasons for this. First, the pressure we used is relatively low to cause major effects in the protein component of the channel, but we cannot rule out the possibility of subtle effects. Second, the bilayer will undoubtedly "feel" the effect of the whole range of pressures we used and therefore as a sensitive component in the system it deserves close examination. The exact nature of the spheroplast membrane bilayer is not known but we can be confident that under high pressure its order will increase: 50 MPa has an effect similar to cooling by 10°C (Cossins and Macdonald 1989). A significant amount of lateral compression will also occur, and the thickness of the bilayer may increase, depending

on the constituent lipids. It is of considerable interest that cooling the channel 10°C did not mimic the effect of 50 MPa, so we conclude that bilayer order is not a factor here. However the lateral compression of the bilayer is an appealing factor as, intuitively, it seems to be intimately involved in the lateral expansion of the channel area as it adopts the open state. It has previously been shown that minute changes in bilayer thickness (approximately 1 Å) are sufficient to stabilize the closed or open conformation of MscL, the bacterial MS channel of large conductance, depending on the thickness of the bilayer (Hamill and Martinac 2001; Perozo et al. 2002). Since MscS is also activated by bilayer tension (Martinac et al. 1990; Sukharev 2002), it should also be sensitive to bilayer thickness (i.e. hydrophobic mismatch between bilayer thickness and the hydrophobic portion of the channel facing the bilayer) in a way similar to MscL. The calculated $\Delta V$ may thus reflect a change in the volume of the MscS–bilayer complex as it undergoes its open–closed transitions, with the closed state occupying less volume owing to the bilayer component. The negative sign of $\Delta V$ indicates that the closed state of the channel is thermodynamically favoured at high pressures, since work has to be done when the channel opens and expands laterally. The tendency of the channel to gate more frequently at subconducting levels as hydrostatic pressure increased (Figs. 3, 5) is an intriguing complication, which seems in no way inconsistent with this view.

It is worth noting that deep-sea organisms, particularly bacteria and archaea, possess MS channels which would normally function at pressures up to 100 MPa. Homeoviscous adaptation of the membrane lipids to high pressure has been described in similar organisms (DeLong and Yayanos 1985; Bartlett 2002). Others, e.g. *M. jannashii* are, surprisingly, in normal laboratory use. The work described here could be profitably extended to such organisms, as the adaptation of channel proteins to high pressure is an important aspect of understanding the molecular adaptations of deep-sea organisms (Cossins and Macdonald 1989; Somero 1990; Bartlett 2002). However, the primary conclusion from our preliminary study is that high hydrostatic pressure and temperature changes provide a promising approach to the analysis of the thermodynamics of MS channel gating.

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