Mechanosensitive ion channels have been documented in a variety of cells belonging to Eukarya and Eubacteria. We report the novel finding of two types of MS ion channels in the cell membrane of the halophilic archaeon Haloferax volcanii, a member of the Archaea that comprise the third phylogenetic domain. The two channels, MscA1 and MscA2, differed in their kinetic properties with MscA1 exhibiting more frequent open-closed transitions than MscA2. Both channels have large conductances that rectify between −40 mV and +40 mV where the conductance of MscA1 ranged from 380 to 680 picoSiemens, whereas MscA2 ranged from 850 to 490 picoSiemens. Both channels were blocked by submillimolar gadolinium. In addition, the channels of either membrane vesicles or detergent-solubilized membrane proteins remained functional upon reconstitution into artificial liposomes, a result that indicates that these channels are activated by mechanical force transmitted via the lipid bilayer alone. Subsequently a 37-kDa protein corresponding to the MscA1 channel activity was purified via the lipid bilayer alone. Subsequently a 37-kDa protein corresponding to the MscA1 channel activity was purified. With the possible functional similarity to bacterial MS channels, our finding of MS channels in Archaea emphasizes the ubiquity and importance of these channels in all domains of the evolutionary tree.

According to the recent revision the universal phylogenetic tree is composed of three domains: Eukarya, Eubacteria, and Archaea (formerly archaeabacteria) (1–6). From this scheme archaeabacteria, which are prokaryotes like eubacteria, constitute an intermediary domain between eubacteria and eu- karyotes, and although prokaryotes, archaeabacteria are neither phylogenetically closer to eubacteria or to eukaryotes (7). As a distinct group of microorganisms Archaea comprise several different families of cells adapted to environments of certain habitats characterized by extreme temperatures such as in ocean hydrothermal vents, or high salt concentrations as occur in the Dead Sea (3, 8).

The existence of ion channels in cell membranes of different organisms belonging to the eubacterial and eukaryotic phylogenetic domains has been well documented. In contrast, the existence of ion channels in cell membranes of Archaea was only recently documented with the discovery of porin-like channels in the archaeabacterium Haloferax volcanii (formerly Halobacterium volcanii) (9). In the present study we report the finding of two types of MS ion channels in the plasma membrane of the same microorganism that seem to share many properties of the described bacterial MS1 ion channels (10–18).

The finding of this class of channels in the cell membrane of an archaeon demonstrates that MS channels, as well as porins, are present in organisms belonging to all domains of the evolutionary tree and indicates the importance of these types of membrane pores in the phylogeny of ion channels.

EXPERIMENTAL PROCEDURES

Isolation of the Cell Envelope—Cells of H. volcanii were grown and membranes prepared as described previously (9). Cells were cultured in nutrient rich media (in mM: 3350 NaCl, 170 MgCl2, 200 MgSO4, 6 CaCl2, 26 KCl, 6.6 NaHCO3, 5.4 NaBr, plus 5 g of yeast extract per liter) until the absorbance A600 was 1.0, washed three times by centrifugation with phosphate-buffered saline, and the pellet resuspended in 2 mM MgSO4, 5% sucrose, 100 mM NaCl, DNase (20 μg/ml), 50 mM NaH2PO4, pH 7.6, and passed twice through a French press at 8,000 psi (Amicon, SLM Instruments, Inc.). The resulting suspension was centrifuged at 9,000 rpm for 20 min (Avanti J-25, Beckman) to eliminate cell debris. The supernatant was centrifuged for 25 min at 90,000 rpm (Beckmann TL-100). The pellet containing the membrane vesicle fraction was then resuspended in 10 mM HEPES-KOH, pH 7.0, and protein content was determined using the DC protein assay (Bio-Rad). Aliquots of the suspension were stored at −20 °C for further use. Alternatively, cell membrane extract following French pressing was layered on top of a discontinuous sucrose gradient (25 and 60%) and spun down for 16 h at 25,000 rpm (SW28 Beckman rotor). Two major membrane fractions were collected as a “pink band” at the 25%/60% interface and a “black pellet.” The supernatant was centrifuged for 25 min at 90,000 rpm (Beckmann TL-100). The pellet containing the membrane vesicle fraction was then resuspended in 10 mM HEPES-KOH, pH 7.0, and protein content was determined using the DC protein assay (Bio-Rad). Aliquots of the suspension were stored at −20 °C for further use. Alternatively, cell membrane extract following French pressing was layered on top of a discontinuous sucrose gradient (25 and 60%) and spun down for 16 h at 25,000 rpm (SW28 Beckman rotor). Two major membrane fractions were collected as a “pink band” at the 25%/60% interface and a “black pellet.” The protein content was measured as above. Aliquots (50 μl) were stored at −20 °C until required.

Membrane SOLubILization and Protein Purification—Membrane fraction (pink band) was solubilized in 200 mM KCl, 10 mM HEPES-KOH, pH 7.0, buffer containing 100 mM octylglucoside and incubated for 30 min. Alternatively, membrane fractions after French press were washed by centrifugation in a high salt wash solution (700 mM NaCl, 10 mM Tris, pH 8.0) followed by a low salt wash (1 mM EDTA, 10 mM Tris, pH 8.0). For the subsequent steps either octylglucoside or Triton X-100 were used. The resulting suspension was resuspended in 1% Triton X-100 or 50 mM octylglucoside and cut with ammonium sulfate (at approximately 7% saturation in Triton X-100 or 17.5% in octylglucoside). The supernatant was collected after centrifugation at 90,000 rpm for 20 min (Beckman TL-100) and dialyzed overnight at 4 °C against 10 mM Tris, pH 8.0, with either 0.5% Triton X-100 or 25 mM octylglucoside. The dialyzed fraction was concentrated on a Centricon 10 (Amicon), and the proteins were separated by preparative electrophoresis and electrophoresis in the presence of Triton X-100 or octylglucoside. The gel system consisted of a 4% stacking gel (pH 6.8) and a 9% resolving gel (pH 8.8) prepared as used in SDS-polyacrylamide gel electrophoresis, except that both gels contained 0.5% Triton X-100 or 25 mM octylglucoside. The protein fraction was mixed at a ratio of 2:1 with a buffer containing 10% glycerol, 65 mM Tris, pH 8.8, and 0.005% bromphenol blue before loading onto the gel. The electrophoretic separation was carried out at a constant current of 60 mA for 8 h. The resolving gel was cut in three parts identical in size, and the proteins were eluted from each part of the gel including the stacking gel using the Electro Eluter (Model 422, Bio-Rad) with a buffer of 192 mM glycine, 25 mM Tris containing 0.2% Triton X-100 or 25 mM octylglucoside. The electroeluted proteins were concentrated using Centricon 10 tubes (Amicon) and stored at 4 °C for

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§ To whom correspondence should be addressed. Tel.: 618-9346-2986; Fax: 618-9346-3468; E-mail: bmartinae@receptor.pharm.uwa.edu.au.

The abbreviations used are: MS, mechanosensitive; pS, picoSiemens.
**FIG. 1.** Activity of MS channels from *H. volcanii* at a pipette holding potential of +20 mV. The left panels show the activity of the channel tentatively called MscA1 since it was the first conductance observed. Some patches showed activity of a second MS channel (right panels) tentatively named MscA2. A, mechanosensitive channel activity from reconstituted native membranes. B, activity of MS channels from membranes following detergent solubilization and reconstitution. Note the reduced activation pressures compared with the reconstituted channels from native membranes.

**FIG. 2.** Relationship between solubilized membrane channel open probability (Po) and applied pressure for MscA1 and MscA2 at a holding potential of +20 mV. A, sample 10-s recordings from the same patch for each channel at several pressures, demonstrating the change in channel activity with applied pressure. B, the Po estimated from the recordings shown in A are plotted versus negative pressure (○), whereas the smooth curve is the line of best fit of the Boltzmann distribution estimated from the data as described (12).

Further use in addition we introduced another step of purification before the electrophoresis. The membrane fractions solubilized in 25 mM octylglucoside were cut with ammonium sulfate (20%) and applied to a phenyl-Sepharose 6 fast flow column. The column was washed with 50 mM phosphate buffer (pH 6.8) containing 25 mM octylglucoside and 20% ammonium sulfate and then eluted by a linear ammonium sulfate gradient (20 to 0%). Active fractions were concentrated (Centricon 10, Amicon) and loaded onto a SDS-polyacrylamide gel to extract the proteins by electroelution as described above.

**Liposome Preparation and Protein Reconstitution—** Proteoliposomes were made essentially according to the method used for reconstitution of the large MS ion channel (MscL) of *Escherichia coli* (19). Alternatively, the solubilized protein fractions were incubated with azolectin liposomes (protein to lipid ratio of 1:100 or 1:30) for 30 min at room temperature and Bio-Beads SM-2 (Bio-Rad) were added (400 mg of Bio-Beads per ml of protein solution for 1% Triton X-100 or 50 mM octylglucoside) to remove the detergent. The suspension was rocked for 3–4 h at room temperature and then the Bio-Beads were allowed to settle. The supernatant was ultracentrifuged for 25 min at 90,000 rpm (TL-100, Beckman), and the resulting pellet was resuspended in 20–50 μl of 10 mM HEPES-KOH, pH 7.0. Aliquots of the liposomes were spotted onto glass slides and dehydrated/rehydrated as described to yield giant liposomes used for patch-clamp experiments.

**Electrophysiology—** For each experiment, a 2-μl aliquot of proteoliposomes was placed in a 800-μl chamber containing recording solution (in mM: 200 KCl, 40 MgCl2, 10 HEPES, pH 7.2, with KOH). Micropipettes of borosilicate glass (100 μm microcapillaries, Sigma) were standardized by routinely testing a sample pipette by submersion in absolute ethanol and measuring the pipette bubble number (20), which was typically in the range 3.2–3.4. Pipettes were filled with recording solution. Membrane patches were obtained from unilamellar blisters from collapsing liposomes (21), and in some cases a brief application of suction (<20 mm Hg) was applied to form seals with resistances in the range 10–50 GΩ.

Negative pressure (suction) was applied by syringe to the back of the pipette to activate MS ion channels (12). Pressure and current were recorded simultaneously using the pCLAMP6 program and Digidata 1200 A/D converter (Axon Instruments). Mechanosensitive ion channel currents were recorded by applying continuous pipette voltage either as voltage steps or voltage ramps in the range of ±60 mV. The channel currents were observed once a threshold of applied pressure was achieved. Since the threshold activation pressure for bacterial MS channels reconstituted in liposomes is often reduced (but approaches those characteristic of *E. coli*), only patches so “trained” were used in estimations of Boltzmann characteristics.

**Data Analysis—** Single channel currents were estimated either from amplitude histograms or using a cursor measurement following subtraction of the baseline. Threshold activation pressures were taken as the pressure at which the first channel opening to the full-level conductance was observed in a previously untested patch. Data are expressed as means and standard errors unless otherwise noted.

**RESULTS**

Reconstitution of native membrane vesicles yielded several ion channel types, predominantly exhibiting porin-like channel behavior (9). With slight modification of this method of Besnard, Martinac, and Ghazi (9), the presence of porin-like channel activity was minimized. Isolated liposome patches were examined for the presence of MS ion channels of similar type to those characteristic of *E. coli*; namely activated by mechanical tension in the lipid bilayer alone. Two separate MS channel conductances (MscA1 and MscA2) were observed in liposomes incorporated with native membrane vesicles (Fig. 1A). As well

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*Note:* This text is a continuation of the previous document, providing additional information and experimental details about the study of mechanosensitive ion channels in Archaea. The figures (Fig. 1 and Fig. 2) illustrate the activity and characteristics of the MscA1 and MscA2 channels, respectively, under various experimental conditions. The methods for liposome preparation, protein reconstitution, and electrophysiological recording are described, along with data analysis techniques used to interpret the results. The results section discusses the identification and characterization of MS channels in *H. volcanii*, highlighting the unique properties and activation pressures of these channels compared to those observed in bacterial systems. Further experiments and observations are also presented, with a focus on the comparison of channel behavior between archaeal and bacterial systems, emphasizing the distinct characteristics of archaeal MS channels, particularly in the context of their mechanosensitivity and pressure activation.

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*Note:* The text contains scientific details regarding the experimental setup, data analysis, and the interpretation of results, which are essential for understanding the study of MS channels in Archaea. The figures and experimental procedures are integral to the presentation of the research findings, providing visual and quantitative support for the described experimental outcomes.

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*Note:* The document includes a detailed methodology for preparing liposomes and reconstituting MS channels from native membranes, as well as a comprehensive description of the electrophysiological recordings and data analysis techniques. The results section highlights the unique properties of MS channels in *H. volcanii*, including their pressure-dependent activation and the comparison with bacterial MS channels.

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A. C. Le Dain, unpublished observation.
as observation in separate patches, on occasion both channel types were observed in the same patch. It was also possible to observe multiple channels of a given type in a particular patch. Both archaeal MS channel conductances were activated by application of negative pressure (suction) with a threshold activation pressure required for an e-fold change in open probability and is the reciprocal of the slope of the plot of pressure versus the natural log of Po/(1 − Po). Data are mean ± S.E. with the number of patches indicated in parentheses. Only patches previously demonstrated to contain MS channels were used for parameter estimation (see “Experimental Procedures”).

**Table 1**

| Voltage (mV) | $P_{0.5}$ MscA1 (mm Hg) | $1/s$ | $P_{0.5}$ MscA2 (mm Hg) | $1/s$ |
|------------|----------------|------|----------------|------|
| −20        | 35 ± 6 (3)     | 1.79 ± 0.51 (3) | 45 ± 4 (3)     | 1.62 ± 0.35 (3) |
| +20        | 32 ± 4 (4)     | 2.85 ± 0.77 (4) | 41 ± 3 (3)     | 1.35 ± 0.14 (3) |

as observation in separate patches, on occasion both channel types were observed in the same patch. It was also possible to observe multiple channels of a given type in a particular patch. Both archaeal MS channel conductances were activated by application of negative pressure (suction) with a threshold activation pressure required for an e-fold change in open probability and is the reciprocal of the slope of the plot of pressure versus the natural log of Po/(1 − Po). Data are mean ± S.E. with the number of patches indicated in parentheses. Only patches previously demonstrated to contain MS channels were used for parameter estimation (see “Experimental Procedures”).

**DISCUSSION**

In the present study we report the discovery of two novel types of MS channels in the cell membrane of the archaeon *H. volcanii*. In analogy to the bacterial MS channels (18, 24) we named the archaeal channels MscA1 and MscA2 and have found that both channels have many properties in common with bacterial MS channels but exhibit certain differences. When compared with *E. coli* MS channels, MscS (12) and MscL (19, 25), which have a pressure sensitivity of ~5 mm Hg per e-fold change in open probability, MscA1 and MscA2 exhibited an increased sensitivity of 1.4–2.9 mm Hg per e-fold change in open probability. Second, unlike MscS, which is voltage-dependent (26), the archaeal MS channels do not show such significant voltage dependence (Table 1). However, both of the archaeal MS channels possess large conductances in the range of several hundreds of picosiemens and seem to have a very low or minimal selectivity for cations over anions (data not presented), properties in common with bacterial MS channels (16, 18, 26, 27). In addition, both archaeal channels were blocked by gadolinium at concentrations comparable to those reported to block bacterial MS channels (19, 23). Significantly, MscA1 and MscA2 can be solubilized by detergents and reconstituted in artificial liposomes whereupon they remained fully functional when examined by the patch-clamp technique. This channel property is identical to that of the MscL, which allowed this ion channel to be date the only cloned MS channel whose mechanosensitivity has been unambiguously documented (17, 28). Furthermore, this functional property suggests that the archaeal channels belong to a family of MS channels that are activated by the mechanical force transmitted exclusively via the lipid bilayer (16, 17). Interestingly, the archaeal MS channels possess a unique characteristic in that their conductive...
properties alter in a nonlinear fashion with the pipette voltage, i.e. they rectify such that with the pipette voltage changing between −60 and +60 mV, the conductance of MscA1 decreases whereas that of MscA2 increases. At present the importance of this channel property for the physiology of the H. volcanii remains unclear.

In our view, the significance of discovering MS channel activity in the cell membrane of an archaeal cell is manifold. First, from an evolutionary perspective, this finding documents the ubiquity and importance of MS channels in the three domains of the phylogenetic tree. This channel ubiquity raises the question of the physiological function of these channels in prokaryotes. In bacteria, MS channels have been proposed to play a role in osmoregulation and more explicitly in the release of osmolytes (23). When cultivated in high osmolality media, bacteria are capable of accumulating high concentrations of osmolytes (or osmosticants) such as trehalose or glycine betaine, the role of which is to counterbalance the external osmolality. Upon a sudden shift to lower osmolality media, these organic compounds are excreted (29–31). Although the role of bacterial MS channels in osmolyte efflux is not proven, it is probable given the fast kinetics of this process (30, 31). Moreover, the role of bacterial MS channels in the efflux of molecules of this size would be consistent with their apparently anomalous conductance; for example in the case of the MscL, molecules as large as polyamines readily pass through the pore (27). The study of osmoregulation is less advanced in archaea but it is noteworthy that accumulation of osmosticants has been documented in methanogens (32) and recently in halophilic archaea (33). Although excretion of these species upon osmotic downshock has yet to be documented, it is highly plausible that archaeal MS channels play a similar role to that postulated for bacterial channels.

Second, the mechanism of gating the archaeal channels by the mechanical force transmitted by the lipid bilayer alone, supports the idea that mechanosensitivity may have evolved several times during evolution (28). Taking into account the two recognized mechanisms of mechanosensitivity (34), the present finding suggests that gating of MS channels according to the bilayer model (35–37) may represent a more general mechanism than that described by the tethered model (38–41).

Finally, in view of a comparative analysis of *Eukarya*, *Eubacteria* and *Archaea*, our finding demonstrates that *Archaea*, or at least their halophilic branch, harbor eubacterial-like rather than eukaryote-like MS channel-type proteins in their cellular membranes. The fact that archaeal MS channels appear electrophysiologically similar to their bacterial counterparts is consistent with the idea that the prokaryotic *Archaea* proteins involved in transport are closer in function to those of *Eubacteria* rather than *Eukarya* (3, 42). Although at present it is not known if the protein structure will prove to be as related, it is unlikely since one of the identified proteins has a molecular weight at least twice that of the MscL monomer. Elucidation of the physiological role of MS channels in *Archaea* as well as their molecular relatedness to bacterial MS channels will be the focus of further study.

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40. Howard, J., Roberts, W. M., and Huds有很大一部分是关于MS通道在Archaea中的发现及其生理功能的讨论。这些发现对了解这些微生物的生理学性质有重要意义。