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ORIGINAL PAPER

Concentration dependent effect of GsMTx4 on mechanosensitive channels of small conductance in *E. coli* spheroplasts

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Abstract The spider peptide GsMTx4, at saturating concentration of 5 μM, is an effective and specific inhibitor for stretch-activated mechanosensitive (MS) channels found in a variety of eukaryotic cells. Although the structure of the peptide has been solved, the mode of action remains to be determined. Because of its amphipathic structure, the peptide is proposed to interact with lipids at the boundaries of the MS channel proteins. In addition, GsMTx4 has antimicrobial effects, inhibiting growth of several species of bacteria in the range of 5–64 μM. Previous studies on prokaryotic MS channels, which serve as model systems to explore the principle of MS channel gating, have shown that various amphipathic compounds acting at the protein–lipid interface affect MS channel gating. We have therefore analyzed the effect of different concentrations of extracellular GsMTx4 on MS channels of small conductance, MscS and MscK, in the cytoplasmic membrane of wild-type *E. coli* spheroplasts using the patch-clamp technique. Our study shows that the peptide GsMTx4 exhibits a biphasic response in which peptide concentration determines inhibition or potentiation of activity in prokaryotic MS channels. At low peptide concentrations of 2 and 4 μM the gating of the prokaryotic MS channels was hampered, manifested by a decrease in pressure sensitivity. In contrast, application of peptide at concentrations of 12 and 20 μM facilitated prokaryotic MS channel opening by increasing the pressure sensitivity.

Keywords MscS · MscK · Patch-clamp · *Grammostola* peptide · Amphipath · Biphasic response

Abbreviations

MS	Mechanosensitive channels
WT	Wild-type
SACs	Stretch-activated channels
MscL	Mechanosensitive channel of large conductance
MscS	Small conductance
MscK	Small conductance, K^+ dependent
MscM	Mini-conductance
POPG	Palmitoyloleoylphosphoglycerol
POPC	Palmitoyloleoylphosphocholine
PE	Phosphoethanolamine
PG	Phosphoglycerol
PC	Pressure cycle

Introduction

Mechanosensitive (MS) channels are present in all domains of life: animal, plants, fungi and bacteria. They are involved in a variety of intracellular processes, including osmotic and cell volume regulation. In eukaryotes membrane stretch can activate stretch-activated channels (SACs), well studied in heart, astrocytes, smooth and skeletal muscles (Hamill and Martinac 2001; Bowman et al. 2007). Prokaryotes also host channels in their cytoplasmic

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membrane that are activated by membrane stretch; here they function as receptors for membrane tension and serve as osmotic emergency valves (Levina et al. 1999; Martinac 2004). Although, they differ from the eukaryotic channels in structure and sequence they largely share the functional properties that are characteristic for mechanosensory transduction. The appearance of MS channels early in evolution suggests an overall conserved mechanism (Kloda and Martinac 2002) by which tension, curvature and/or changes in the trans-bilayer pressure profile in the membrane are sensed by the proteins and transduced into a cellular response. The unifying principle recently proposed (Kung 2005) suggests that changing the pressure profile in the lipid bilayer by introducing hydrophobic mismatches/membrane curvature at the protein–lipid interface is responsible for MS channel gating.

Over the past years MS channels of prokaryotes served as model systems to study the general principles underlying the ability of biological systems to detect mechanical and osmotic forces (Kung 2005; Perozo et al. 2002). Through electrophysiological characterization of *E. coli* membrane patches four discrete MS channel activities have been described: mechanosensitive channel of large conductance (MscL), small conductance (MscS), small conductance, K^+ dependent (MscK) and mini-conductance (MscM) (Martinac 2004). They all gate at different membrane tension, with more tension required for larger conducting channels. Basic functional properties of MscS and MscK have been investigated previously in *E. coli* spheroplasts (Martinac et al. 1987; Cui et al. 1995; Berrier et al. 1996; Levina et al. 1999; Li et al. 2002): gating threshold, conductance, abundance, ion dependency, deactivation, and response to activators/inhibitors. The addition of amphipaths, i.e., compounds that have hydrophobic and hydrophilic regions, to the lipid bilayer, will induce spontaneous channel gating in eukaryotes and prokaryotes even in the absence of tension (Martinac et al. 1990; Lin and Rydqvist 1999; Patel et al. 2001; Qi et al. 2005). By intercalating into the membrane, they are likely to produce local stress provoking a conformational change in the channel protein. In contrast, compounds that inhibit MS channel activity are rare and their mechanism of action often not fully understood (Hamill and McBride 1996).

Until the purification and characterization of the peptide GsMTx4 from the venom of the tarantula *Grammostola spatulata* (now genus *Phixotricus*) (Suchyna et al. 2000) no specific pharmacological agents of SACs had been known. Since then, various studies elucidated how effective and most of all specific this peptide acts as an inhibitor for this type of MS channels (Oswald et al. 2002; Gottlieb et al. 2007). Structurally the peptide is a member of the inhibitory cysteine knot peptide superfamily (Zhu et al. 2003). Three specific disulfide bonds in a 34 amino acid peptide forms a compact structure with a net charge of +5. The peptide is amphipathic having a hydrophobic core and face that suggests a role in lipid binding (Suchyna et al. 2004). Its mechanism of action on MS channels is not yet fully understood. A direct stereo-chemical interaction via a lock and key mechanism can be excluded, because the enantiomer peptide was equally active (Suchyna et al. 2000, 2004). These studies (Suchyna et al. 2000, 2004) have also demonstrated that GsMTx4 resides near the protein–lipid interface of both endogenous eukaryotic channels and gramicidin channels. Conductance through the pore in the presence of GsMTx4 is affected in a manner consistent with the peptide charge positioned within the Debye length of conducting ions.

GsMTx4 has been shown to bind to anionic lipids with a higher affinity than to zwitterionic ones (Jung et al. 2006; Posokhov et al. 2007). It is also able to partition into the membrane by interacting with the nonpolar parts, reaching penetrations of ~0.7–0.9 nm from the lipid centre (Nishizawa and Nishizawa 2007; Posokhov et al. 2007). To affect channel gating, the peptide must be in close proximity, within 1 nm of the channel's boundary lipids. In this context, the nature of the individual lipids surrounding the protein would have an important role in such an interaction. Furthermore, a recent result from molecular modeling points out that the peptide changes the local shape of the membrane by intercalating into the lipid bilayer, pulling both leaflets closer together (Nishizawa and Nishizawa 2007). Apart from being helpful in the investigation of cellular processes and the understanding of diseases (Bode et al. 2001; Bowman et al. 2007), GsMTx4 could also help elucidate the mechanism by which tension in the membrane is transduced into protein structural changes.

To date the effect of the peptide has not been tested on prokaryotic MS channels. Recent work (Jung et al. 2006) demonstrated antimicrobial effects on the growth of various bacterial species, but the concentration range for antimicrobial activity was much higher than the inhibiting concentration for eukaryotic channels. The antimicrobial activity of the peptide may lie in its ability to modulate MS channels directly. We therefore tested the effect of the peptide on MS channels in giant spheroplasts of *E. coli*. To compare the electrophysiology of GsMTx4 to its antimicrobial activity (Jung et al. 2006) this work was done in wild-type (WT) bacteria. Recent work has shown that protein and lipid content of the bacterial membrane can alter channel response (Å. Wieslander and D. Daley, University of Stockholm, personal communication, 2006). Hence, the channels were recorded in their native membrane environment, WT *E. coli* giant spheroplasts, taking into account that the presence of native lipids at the lipid–protein interface may be critical for the effect of GsMTx4.

In our study on MscS and MscK, the prokaryotic channels of small conductance, we found an inhibitory effect of the extracellularly applied peptide GsMTx4 at lower concentrations requiring more tension to gate the channels. In contrast, higher concentrations of the peptide had a stimulating effect, requiring less tension for the channel activation. These findings support the idea that GsMTx4 acts as an anti-microbial peptide by altering the gating properties of mechanosensitive channels.

Materials and methods

E. coli spheroplasts

Experiments were performed on giant spheroplasts from WT *E. coli* strain AW737 using the previously described method (Martinac et al. 1987). Thus recordings are from chromosomally encoded WT channels in their natural lipid environment. Data were collected from several independent preparations.

GsMTx4

The purified spider toxin GsMtx4 has been obtained after the procedure previously described in Ostrow et al. (2003).

Electrophysiology

Channel activity was recorded on excised inside-out patches from giant spheroplasts bathed in standard solution with either 250 mM KCl or NaCl (supplemented with 90 mM $MgCl_2$, 5 mM HEPES, pH 7.2), representing the cytoplasmic medium. Standard pipette solution was 200 mM chloride salt of K^+ or Na^+, respectively, supplemented with 40 mM $MgCl_2$, 5 mM HEPES, adjusted to pH 7.2 with the main cation hydroxide. Standard pipette solution without peptide represented the control periplasmic conditions. GsMTx4 was applied via the patch pipette in final concentrations of 0.5, 2, 4, 12 and 20 μM, in standard pipette solution. Gigaseal formation between pipette and cytoplasmic membrane of spheroplasts was achieved with the aid of −30 mV pipette voltage and a minimum of suction to the pipette. The tip of the recording pipette was kept free of peptide to facilitate formation of gigaseals.

Recordings were obtained as a function of applied suction via the patch pipette at a pipette voltage of −30 mV. Pressure was applied in steps of −10 mmHg, starting at apparent 0 mmHg pressure and each pressure was clamped for at least 45 s during the pressure cycle. In KCl solution pressure was increased until the current across the membrane reached saturation. The first pressure cycle (average duration: 12 min) started 20 min after seal formation to give the peptide sufficient time to reach the pipette tip and act on the patch membrane, which takes several minutes (Suchyna et al. 2004). If seals were stable this first cycle was followed by a second and third pressure cycle lasting approx. 30 min per cycle including lag-times of 20 min between recordings. During the experiments carried out in the presence of Na^+ ions (pipette and bath solution free from K^+) pressure steps were applied until we observed the initial full MscS channel openings. Under K^+ conditions where activity was saturated, data gathered from the first pressure cycle were used to determine opening threshold of MscK channel. The pressure inducing first full openings was labeled as the activation threshold. Single channel conductance was recorded between −50 and +50 mV to determine the I–V relation in the presence of the various peptide concentrations.

All data were acquired using an Axonpatch amplifier and a Digidata 1440 digitizer in conjunction with pClamp10 software (Axon Instruments, Foster City, CA, USA) at a sampling rate of 5 kHz and filtering of 1 kHz. Pipettes were pulled (Flaming/Brown micropipette puller, Model P-87, Sutter Instruments, Novato, CA, USA) from Borosilicate glass microcapillary pipettes (Drummond Scientific Company, Broomall, PA, USA) to a bubble number of 3.3–3.6 corresponding to a resistance of 2.5–2.8 MΩ in standard solutions of KCl or NaCl. Data were analyzed using the software ClampFit10 and Origin10.

Open probability Po from recordings of saturated channel current in KCl were fitted with a two-state Boltzmann distribution as previously described (Hamill and Martinac 2001). The derived parameters α, $p_{1/2}$ and ΔG_0 are shown as mean ± SE and were analyzed for significant differences between control and peptide treatments using the Student's *t* test.

Results

Properties of *E. coli* MS channels

The effect of the peptide GsMTx4 was analyzed on inside-out patches from WT spheroplasts of *E. coli*. To distinguish between the two channels of small conductance, MscS and MscK, we applied the measure of single channel conductance, K^+ sensitivity, threshold pressure for activation, channel abundance and appearance of deactivation/saturation (i.e., declined/sustained channel activity upon prolonged pressure). The results from these control experiments are summarized in Table 1 and the characteristics correspond to those described previously for MscS and MscK (Martinac et al. 1987; Cui et al. 1995; Levina et al. 1999; Li et al. 2002; Akitake et al. 2005; Sotomayor et al. 2007). The channels MscL and MscM are also found in the

Table 1 Control parameters for MscK and MscS

Channel protein	Leading cation	Conductance (nS ± SE)	Activation threshold (mm Hg)	Rel. abundance (%)	Activity
MscS	Na^+	−ve 0.89 ± 0.02 +ve 1.15 ± 0.03	~ −70	~100	De-activation
MscK	K^+	−ve 0.61 ± 0.02 +ve 1.01 ± 0.02	~ −20	~75	Saturation

Data were obtained from excised inside-out patches from giant spheroplasts, prepared from WT strain *E. coli* AW737 (for details see "Materials and methods"). The values for the control parameters obtained in this study confirm the parameter values found previously in the references stated. References on MscS: Levina et al. (1999), Akitake et al. (2005), Sotomayor et al. (2007); references on MscK: Martinac et al. (1987), Cui et al. (1995), Li et al. 2002

cytoplasmic membrane of *E. coli*. However, MscL requires a very high negative hydrostatic pressure (> −130 mmHg) for activation and therefore did not contribute to the membrane conductance. MscM is a low abundance membrane protein (~20% of patches, ≤3 per patch) and due to its lower conductance (1/3 of MscS/K) it is clearly distinguishable from MscS and MscK.

In recording solution with KCl both channels MscK and MscS are detectable and we applied the single channel conductance and the threshold activation to identify these channels (see recording traces in Figs. 1, 2c). Due to the channels' characteristics (Table 1) the response of MscK to GsMTx4 was studied in solution with 200 mM KCl while the response of MscS, due to its gating activity in the absence of K^+ ions, was recorded in the presence of 200 mM NaCl, respectively.

Single channel conductance in the presence of GsMTx4

We first determined the single channel conductance of MscS and MscK in the presence of various peptide concentrations, shown in Fig. 1. Analysis of the I–V relation at a voltage range between −50 and +50 mV revealed that neither peptide concentration applied changed the conductance for MscS (Fig. 1a) or MscK (Fig. 1b) significantly. The conductance for the single channels remained at values observed in our control recordings and as obtained previously for MscS and MscK, respectively (Cui et al. 1995; Li et al. 2002).

Threshold pressures for MscS and MscK gating activity

MscS shows profound inactivation during an increase in applied pressure; hence, a description of the data with a

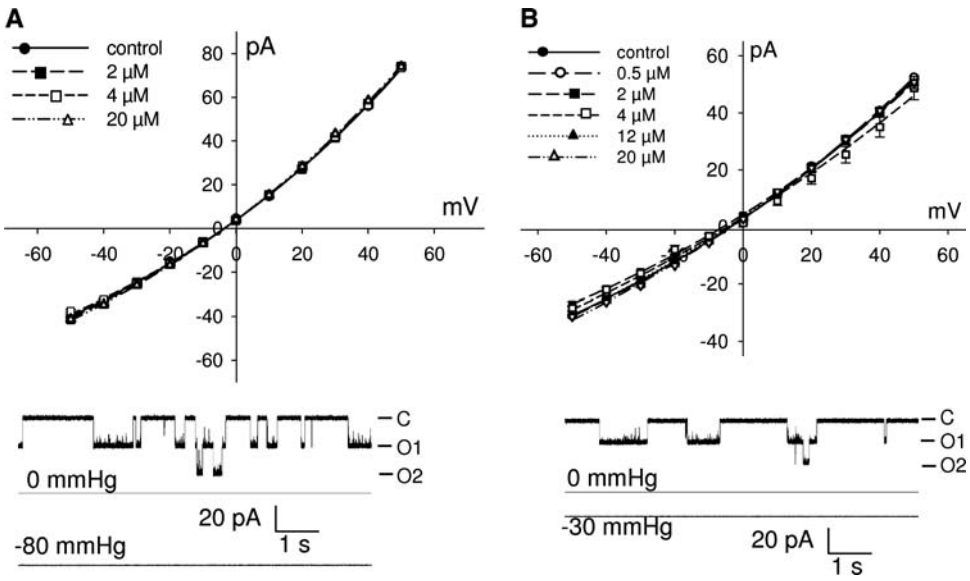

Fig. 1 Current–voltage relations in MscS and MscK. **a** *Top* Current–voltage relation of MscS (obtained in Na^+ recording solution) in the presence of 2, 4, and 20 μM GsMTx4 in the pipette compared to control conditions (no GsMTx4). **a** *Bottom* Representative recording trace of MscS under control conditions at −30 mV pipette voltage on inside-out patches. Gating occurred at a pressure stimulus of −80 mmHg. **b** *Top* Current–voltage relation of MscK (obtained in K^+ recording solution) in the presence of 0.5, 2, 4, 12, and 20 μM GsMTx4 in the pipette compared to control conditions. **b** *Bottom* Representative recording trace of MscK under control conditions at −30 mV pipette voltage on inside-out patches. Gating occurred at a pressure stimulus of −30 mmHg. Both sets of data are derived from at least 3 repetitions per treatment, on at least two independent spheroplast populations. '*C*' denotes the channels' closed state, and 'O_n' the open state of the respective channel (*n*) contributing to the total current obtained in the presented recording

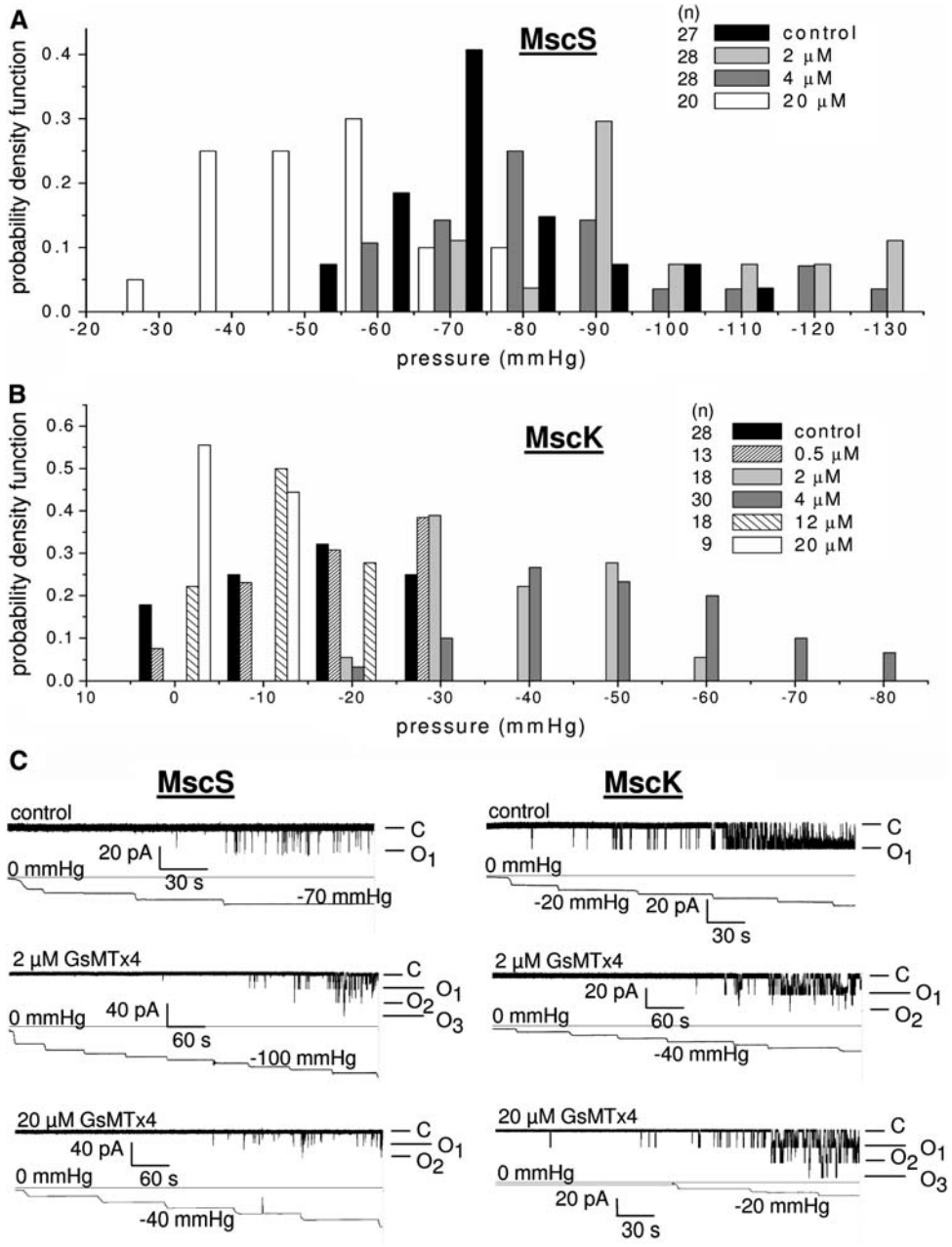

Fig. 2 Probability density functions for activation thresholds of MscS and MscK. **a** Threshold pressures for activation of MscS in the presence of 2, 4, and 20 μM GsMTx4 compared to control conditions. Plotted is the probability density as a function of the activation threshold for channel activity against the respective pressure, obtained during the first pressure cycle (see "Materials and methods" for details). **b** Threshold pressures for activation of MscK in the presence of 0.5, 2, 4, 12, and 20 μM GsMTx4 compared to control conditions. Plotted is the probability density as a function of the activation threshold for channel activity against the respective pressure, obtained during the first pressure cycle (see "Materials and methods" for details). **c** *left* Representative recording traces for activation thresholds of MscS as shown in **a** under control conditions and in the presence of 2 and 20 μM GsMTx4 with −30 mV pipette voltage applied. **c** *right* Representative recording traces at −30 mV pipette voltage for activation thresholds of MscK as shown in **b** under control conditions and in the presence of 2 and 20 μM GsMTx4. (*n*) states the number of traces contributing to the values for the probability density in each experiment, recorded from at least two independent spheroplast preparations. '*C*' denotes the channels' closed state, and 'O_n' the open state of the respective channel (*n*) contributing to the total current obtained in the presented recording

two-state Boltzmann distribution, for which saturation is required, could not be applied (see recording traces in Fig. 2c, left panel). We therefore compared the negative hydrostatic pressure at which MscS will first show gating activity with the threshold values in the presence of the GsMTx4 concentrations. Figure 2a shows the relative abundance of a threshold pressure obtained in (*n*) numbers of recordings plotted as probability density function against

the negative hydrostatic pressure applied on patches in NaCl solution. The lower peptide concentrations of 2 and 4 μM shifted the activation threshold to higher pressure while the higher peptide concentration of 20 μM reduced the pressure needed to activate MscS compared to control conditions. Additionally, in the presence of the low concentrations 2 and 4 μM about 25% of patches did not show any channel activity. This was not observed in any of the control patches or those exposed to 20 μM peptide.

Likewise, we analyzed MscK activity in KCl solution and observed similar results (Fig. 2b, c, right panel). The low peptide concentration of 0.5 μM did not change the activation threshold of MscK compared to control patches. However, in the presence of 2 and 4 μM peptide the activation threshold was shifted significantly to higher pressure. Again, we observed the reverse effect in the presence of higher peptide concentrations; 12 and 20 μM shifted the activation threshold to lower pressures compared to control patches. Spontaneous gating activity was detected in control conditions as previously reported (Cui et al. 1995) and in the presence of 0.5 and 12 μM, and highly abundant with 20 μM peptide, but never in patches exposed to 2 or 4 μM GsMTx4.

Boltzmann analysis of MscK gating

MscK shows increasing activity with increasing pressure stimulus until saturation of current is achieved which corresponds to 100% open channels. We therefore collected data for Boltzmann analysis on this channel and observed a 'training effect' in the same patch along the three pressure cycles applied. Figure 3a shows representative two-state Boltzmann distributions (*line*) and Po values from recorded traces (*symbols*) of each of the 3 pressure cycles plotted versus negative hydrostatic pressure. An example of this phenomenon is displayed from control experiments (Fig. 3a, top plot), and in the presence of 2 μM (Fig. 3a, centre plot) and 20 μM (Fig. 3a, bottom plot), respectively, but was observed throughout all peptide concentrations tested. The traces show that the curves of the second and third pressure cycles are shifted to a lower pressure when compared to the first cycle experienced by the patch. This indicates that the sequential application of pressure cycles facilitates opening of the channels in a patch. This 'training effect' on the channel gating is underlying the effect of the peptide over the sequential pressure cycles (see later in this section), which need to be applied, as the peptide requires several minutes to act (Suchyna et al. 2000).

The peptide's effect during the second pressure cycle (~60 min after seal formation) was plotted as two-state Boltzmann distributions (*line*) using Po values from recorded traces (*symbols*) (Fig. 3b). The Boltzmann distribution in the presence of 0.5 μM GsMTx4 was similar to control conditions. However, the curves and recording traces for 2 and 4 μM peptide showed a shift toward higher pressures whereas those for 12 and 20 μM were shifted toward lower pressures. Figure 3c shows the respective recording traces of all peptide concentrations tested.

We analyzed three parameters (α, $p_{1/2}$, ΔG_0) derived from the Boltzmann analysis (Martinac et al. 1987, 1990; Hamill and Martinac 2001) on MscK channel gating for the various peptide concentrations; α determines the slope of the Boltzmann fit (Fig. 4a, top plot), $p_{1/2}$ the pressure at which the channel is open half the time (Fig. 4a, bottom plot), and ΔG_0 the free energy difference between the channels open and closed state (Fig. 4b). The meaning of ΔG_0 in the context of this study differs from its usual definition in thermodynamics. This has to do with the fact that mechanosensitive channels respond to mechanical forces along the plane of the cell membrane (membrane tension), and not pressure perpendicular to it (Gustin et al. 1988; Sachs and Sokabe 1990; Sokabe et al. 1991; Zhang and Hamill 2000). The free energy (ΔG) is a linear function of membrane tension, such that: $\Delta G = t \times \Delta A - \Delta G_0$, where ΔG_o is the difference in free energy between the closed and open conformations of the channel in the absence of the externally applied membrane tension. ΔA is the difference in membrane area occupied by an open or closed channel at a given membrane tension, and $t \times \Delta A$ is the work required to keep a mechanosensitive channel open by external mechanical force at the open probability of $0 < Po < 1$ (Howard et al. 1988). ΔA is the parameter which determines the mechanosenitivity of channel gating such that the larger ΔA the more sensitive is the channel to membrane tension.

The value for α (Fig. 4a, top) shows a significant increase in the presence of 2 μM GsMTx4 and a significant decrease with 12 and 20 μM peptide in the pipette, whereas 0.5 μM peptide had no effect. The values for α in the presence of 4 μM peptide had no effect in the first and the second pressure cycle but showed a decrease in the third pressure cycle as observed for the highest peptide concentrations tested (Fig. 4a, top).

The $p_{1/2}$ values show the same tendency as seen for the slope values (Fig. 4a, bottom). In the presence of 2 and 4 μM peptide $p_{1/2}$ was increased significantly compared to both the control conditions and the presence of the high peptide concentrations 12 and 20 μM. In turn 0.5 μM, as well as 12 and 20 μM peptide did not change $p_{1/2}$ in the second and third pressure cycle compared to $p_{1/2}$ in control experiments. Only in the first pressure cycle, we recorded a significant decrease of $p_{1/2}$ in the presence of 20 μM GsMTx4 (Fig. 4a, bottom). This pattern is similar to the results obtained from the threshold experiments (Fig. 2): inhibition of channel activity by 2 and 4 μM of peptide can be overcome by an increase of pressure stimulation and the high peptide concentrations facilitate channel gating.

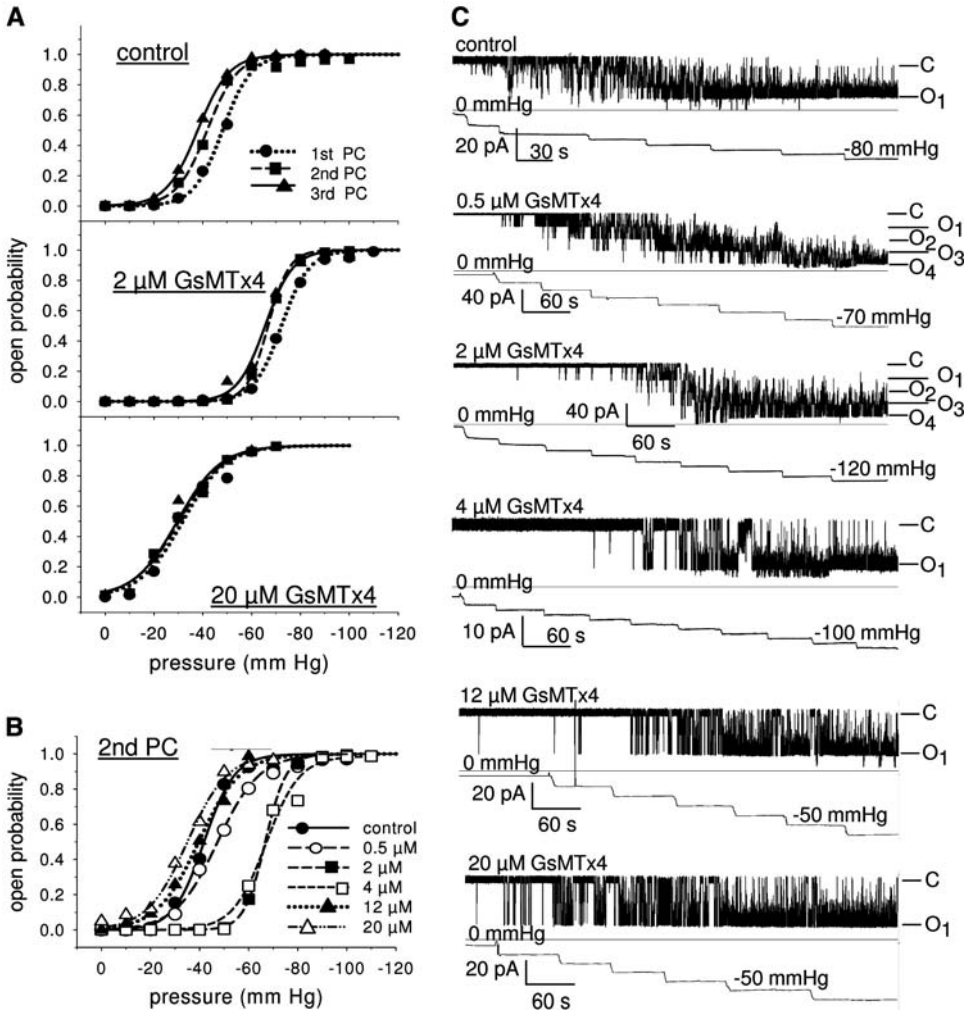

Fig. 3 Representative Boltzmann fits and recording traces from MscK activity during patch 'training' and as a result of the peptide GsMTx4. **a** Training effect on MscK activation during the application of three pressure cycles (*PC*), plotted as the channel open probability (*Po*) against the stimulus negative hydrostatic pressure. For each treatment, the curves represent a typical Boltzmann fit (*lines*) on representative Po values (*symbols*) derived from recording traces for control, 2 and 20 μM GsMTx4 as respective examples. The curves' shifts toward lower activation pressure could be seen under all conditions (see Results for details). **b** Dual effect of GsMTx4 on MscK activation during the second pressure cycle (*PC*), plotted as the channel open probability (*Po*) against the stimulus negative hydrostatic pressure. For each treatment the curves combine a typical Boltzmann fit (*lines*) on representative Po values (*symbols*) derived from recording traces for control, and 0.5, 2, 4, 12, and 20 μM GsMTx4. **c** Representative recordings traces at −30 mV pipette voltage from which individual Po values (*symbols*) fitted Boltzmann distributions (*lines*) shown in **b** were extracted. '*C*' denotes the channels' closed state, and 'O_n' the open state of the respective channel (*n*) contributing to the total current obtained in the presented recording

The most interesting results observed for the different peptide concentrations were the changes to the free energy ΔG_0 (Fig. 4b), which clearly show the biphasic effect of the peptide. The lowest peptide concentration tested (0.5 μM) had no influence on ΔG_0. GsMTx4 at 4 μM induced a significant increase in the free energy ΔG_0 during the first and second pressure cycle while at 2 μM this was observed in all three pressure cycles. Taken together these results show that channel gating is hampered. In contrast, the two high peptide concentrations (12 and 20 μM) showed a significant decrease in free energy required during all pressure cycles. This decrease of the free energy between the open and the closed state clearly shows that with high peptide concentrations channel gating is facilitated. Consistent with this was the observation that forming and maintaining of a gigaseal was very difficult (seal loss during the first pressure cycle was approximately 40%, data not shown), indicating that high concentrations of GsMTx4 hinder this process.

Particularly puzzling at first was that the initially inhibiting effect of 2 and 4 μM GsMtx4 became less pronounced with increasing time of exposure, during the three pressure cycles. The tendency was most clear in the presence of 4 μM peptide, in which the values for all three Boltzmann parameters were decreasing toward the values obtained for

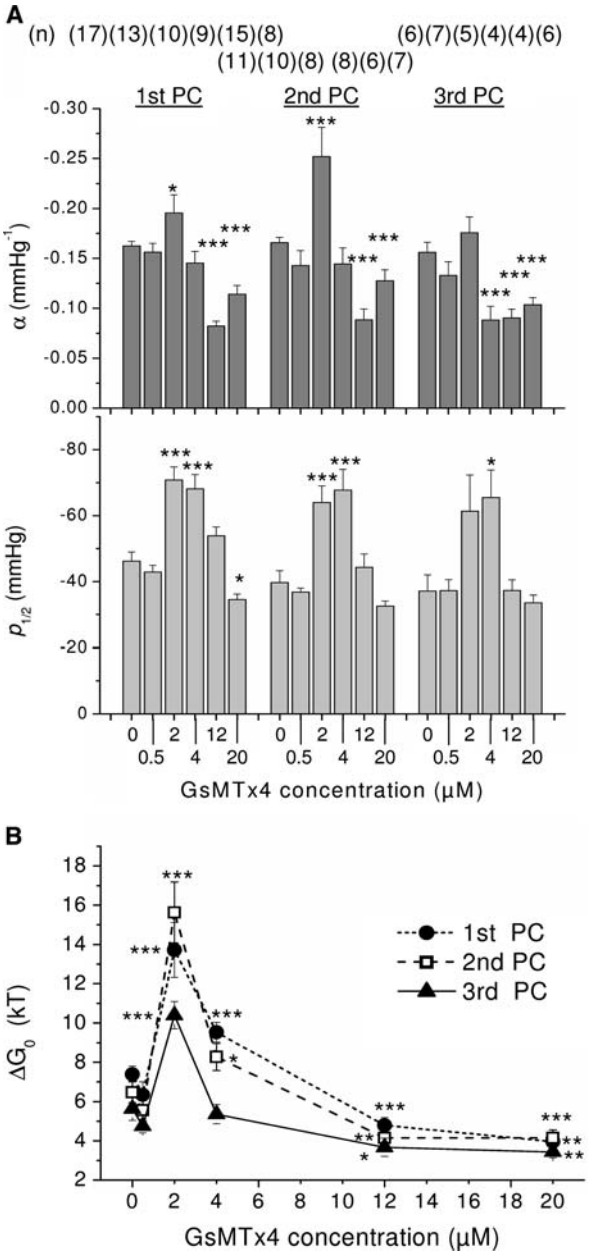

Fig. 4 Comparison of Boltzmann parameters of MscK under control conditions and in the presence of GsMTx4. The parameters were derived from Boltzmann distributions (Hamill and Martinac 2001) fitted on Po data for MscK (as seen in Fig. 3b), obtained in the presence of different concentrations of GsMTx4 along the three pressure cycles (*PC*) applied (see "Materials and methods" for detail). **a** *Top*: The curves' slopes α (=1/ln[Po/1−Po]) are plotted against the different GsMTx4 concentrations present during the three pressure cycles. A bottom: The pressure, at which the channel is open 50% of the time, termed as $p_{1/2}$ is plotted against GsMTx4 concentrations present in each experiment along the three pressure cycles. **b** ΔG_0 as the free energy difference between the channels open and closed state is plotted against GsMTx4 concentration present in the experiments during the application of three sequential pressure cycles (*PC*). Data are shown as mean ± SE. To test for statistical differences between control and the individual peptide conditions we applied the Student's *t* test; with (*n*) numbers of independent recordings and levels of significance $*P < 0.05$; $**P < 0.01$; $***P < 0.005$. Pressure cycles are abbreviated with PC

12 and 20 μM peptide. This may either be a result of the 'training effect' (Fig. 3a) or due to the accumulation of the peptide in the membrane over time, which would reverse the inhibitory effect normally seen at low concentrations.

Discussion

MS channels in WT *E. coli*

Our study was carried out on native channels in giant spheroplasts from WT *E. coli*, because there is growing evidence that lipid environment and protein abundance influence channel gating. Previously, a lipid mutant *E. coli* strain was engineered, in which phosphatidylethanolamine is replaced by monoglucosyldiacylglycerol (Wikström et al. 2004). Recent investigations on protein abundance revealed that the ratio among the MS channels in this strain and other *E. coli* strains with modified lipid composition is significantly different from the one obtained in strains with a WT lipid assortment (Å. Wieslander and D. Daley, University of Stockholm, personal communication, 2006). Additionally, we acquired preliminary data from patch-clamp analysis on MS channels in this lipid mutant: MscS and MscK channel function was hampered, as number of active channels and response to pressure were reduced. A detailed study on these observations will be published elsewhere. Moreover, channel characteristics in liposomes of different protein-to-lipid ratios show great diversity (Akitake et al. 2005; Vasquez et al. 2007) and differ from those in cells. The sensitivity of MS channels to their lipid environment is especially critical when studying the effect of a peptide, whose predicted mechanism of action is on the lipid bilayer or at the protein–lipid interface. Consequently, we chose to analyze GsMTx4 interaction with channels in the native cytoplasmic membrane of the WT strain of *E. coli*, to exclude the influences of an altered lipid environment possibly found in *E. coli* strains with manipulated MS channel content. Having conducted the experiments in WT *E. coli* strain also permits the discussion of our results in the context of the previously demonstrated antimicrobial activity of the peptide GsMTx4 (Jung et al. 2006; see below).

GsMTx4 interaction with the lipid bilayer

The ability of GsMTx4 to bind to lipid surfaces was first suggested by the NMR structure of GsMTx4, which has amphipathic characteristics (Bowman et al. 2007; Gottlieb et al. 2007). The peptide has since been shown to bind to vesicles. Using a fluorescence-quenching technique (Posokhov et al. 2007), Ladokhin and colleagues showed that GsMTx4 penetrates into the lipid bilayer approximately 9 nm from the membrane midpoint. This is similar to other

peptides that possess amphipathic qualities. For example hanatoxin-1 (a gating modifier of K^+ channel) inserts ~0.9 nm from the bilayer centre as detected by bromine labeled Tryptophan residue at position 30 (Phillips et al. 2005). GsMTx4 has a preference for vesicle composition (Posokhov et al. 2007) because the free energy of GsMTx4 partitioning is more favorable for palmitoyloleoylphosphoglycerol (POPG) containing vesicles than for pure palmitoyloleoylphosphocholine (POPC) vesicles and the peptide has a 400-fold higher affinity to anionic lipids like POPG than to zwitterionic POPC (Jung et al. 2006). This is likely due to the peptide's net positive charge since the negative charges at the headgroups of anionic lipids and the hydrophobic surface with the nonpolar regions in the membrane (Bowman et al. 2007; Gottlieb et al. 2007). However, an analysis of the electrostatic properties of the peptide-vesicles equilibrium complex demonstrated a little dependency on the electrostatic properties, suggesting a more complex interchange of hydrophobic and electrostatic interactions (Posokhov et al. 2007).

Most recently, a molecular modeling approach predicted two binding modes for GsMTx4, a 'deep' and a 'shallow' mode (Nishizawa and Nishizawa 2007). In the 'deep' binding mode, peptide penetration was estimated to be 0.5 nm from the bilayer centre. The 'deep' binding mode seems to be mediated by a strong interaction of the positive charges from Lysine residues 8 and 28 with the carbonyl chains of the lipids, drawing both leaflets together. This resulted in significant thinning of the membrane. The ability of the peptide to interact with the charged lipid headgroups was viewed as the 'shallow' mode of binding and involved bending of only the outer leaflet. However, which mode is energetically favored would strongly depend on the headgroups and the acyl chains of the lipids involved.

These findings on the dual mode of GsMtx4 binding are an important feature of the peptide's interaction with the membrane and may be related to the biphasic effect of the peptide found in this study. This context will be discussed in the following paragraphs.

Mode of actions of GsMTx4 on MS channel gating

We have demonstrated that GsMTx4, a specific inhibitor of eukaryotic SACs, also affects the gating of the prokaryotic MS small conductance channels in a concentration dependent manner. Interestingly, for both MscS and MscK we observed the biphasic gating response. When the peptide was applied to the bilayer outer leaflet at concentrations of 2–4 μM, channel activity was inhibited, while at the higher concentrations of 12–20 μM channel activity was potentiated.

Previous studies on eukaryotic channels regarding the influence of GsMTx4 on single channel conductance showed no effect or only minor changes (Bowman et al. 2007; Suchyna et al. 2000, 2004). The fact that single channel conductance of prokaryotic MS channels did not significantly change in the presence of any of the peptide concentrations is a further indication that GsMTx4 is not a pore blocker. Rather our data demonstrate that the peptide acts as a gating modifier, because the dose response shifts to the right or left depending on peptide concentration. The simplest explanation for this process is that the peptide modulates the protein-membrane interface without direct interaction with the protein channel itself (Bowman et al. 2007; Gottlieb et al. 2007). The question remains how peptide concentration translates into opposing gating effects.

The inhibition of channel function in low peptide concentration may occur due to action of the peptide near the channel protein, possibly in a 'shallow' mode proposed by Nishizawa and Nishizawa (2007). During gating, the channel undergoes conformational changes: it thins as it widens. The boundary lipids may also thin under tension of the membrane, coupling the gating to hydrophobic mismatches as previously proposed (Perozo et al. 2002; Kung 2005; Perozo 2006). In the presence of low peptide concentrations at these specific sites, this process may be disturbed. A possible reason why the peptide shifts from the shallow to the deep binding mode at high concentrations, may lie in the increased energy costs if many peptide molecules bind to the membrane surface. These energy costs may be reduced if the translocation of the peptide to the deep binding mode is facilitated.

At high concentrations, the peptide has the opposite effect on channel activity, effectively pre-stressing both channels, which facilitated channel opening in response to tension in the membrane and provoked more spontaneous openings in the absence of pressure. The energy necessary for the state transition of MscK channel is significantly lower than in control experiments. The presence of high peptide concentration may allow an alternative mode of interaction, for example a 'deep' binding mode (Nishizawa and Nishizawa 2007). Assuming that the peptide also acts along the interface, it could facilitate transition from the close to open state as previously proposed (Hamill and Martinac 2001). Energy cost arises due to hydrophobic mismatches between lipids and channel protein, which will be compensated by local changes in bilayer thickness. The proposed penetration of the peptide into the lipid bilayer as calculated by MD simulation may facilitate this transition.

The ability of GsMTx4 to affect the hydrophobic mismatch of channels was shown for the gramicidin channel (Suchyna et al. 2004). GsMTx4 increases the lifetime of gramicidin channels of various lengths, which is attributed to the peptide's ability to modulate the hydrophobic mismatch that allows proper alignment of the subunits. This effect is associated with GsMTx4 being near the channel because the conductance through the channel was affected.

Another explanation of the biphasic, concentration dependent effect of the peptide in this study is the peptide's selective affinity to different lipids (Jung et al. 2006; Posokhov et al. 2007). The cytoplasmic membrane of *E. coli* is a heterogeneous lipid environment (Heller et al. 1987) and it may be possible that the channels live in different lipids. The peptide could then produce a change in gating based on these differences. Moreover, the different affinity of the peptide to individual lipid species may provoke a resorting or rearrangement of certain types of lipids in the channels' environment. Occurrence of lipid species sorting could also underlie the regularly observed 'training effect', which is similar to the potentiation of channel activity at high concentrations of GsMTx4.

Concentration dependent dual effects have been reported previously for other compounds that act on the lipid bilayer. Several studies have shown that submillimolar concentrations of Gd^{3+} block eukaryotic and prokaryotic MS channels (Hamill and McBride 1996). However, further investigations on *E. coli* MS channels revealed that Gd^{3+} acts as a channel stimulator at low concentrations around 20 μM (Cui et al. 1995). Gd^{3+} is also proposed to act by binding to lipids and altering the mechanical properties of the membrane rather than acting on the protein specifically (Ermakov et al. 1998). A more recent example for a dual effect is the inhibition and stimulation of TREK1 by PIP2 at the inner leaflet (Chemin et al. 2007). In this study, the authors argue for an effect by the phospholipids around this eukaryotic MS channel. Curiously, GsMTx4 inhibits these channels when applied to the inner leaflet (Gottlieb, P.A., Bowman, C., and Sachs, F., unpublished results) but not when applied to the outer leaflet (E. Honoré, Institut de Pharmacologie Moléculaire et Cellulaire, personal communication, 2005). Clearly how channels are gated will determine their responsiveness to agents such as GsMTx4. Currently, we are measuring the peptide effect on purified MscS reconstituted into artificial liposomes with variations in lipid content and ratio to better understand the role of lipids on the kinetics of this mechanosensitive channel.

Antimicrobial activity of GsMTx4

The study on overnight growth rates of Gram-negative and Gram-positive bacteria (Jung et al. 2006) also revealed antimicrobial activity of GsMTx4. The authors proposed that the mechanism by which growth of WT *E. coli* was altered by the peptide could be via affecting MS channels or by altering membrane packing. These ideas are supported by the present study on MS channel activity in spheroplasts from WT *E. coli*. Interestingly, the study mentioned above (Jung et al. 2006) revealed that the minimum effective concentration was much lower for Gram-positive (0.5–8 μM) than for Gram-negative bacteria (8–64 μM). Several differences in these two groups of bacteria might account for this observation. The species are discriminated by the fact that Gram-negative bacteria have an additional membrane on top of the peptidoglycan layer. This might scavenge some of the peptide, making a 'higher' concentration necessary to reach the cytoplasmic membrane. They also have a dissimilar lipid composition of their cytoplasmic membranes (Heller et al. 1987). The outer bilayer leaflet of *E. coli* as a representative for Gram-negative bacteria is predominantly inhabited by zwitterionic PE (phosphoethanolamine) with some anionic PG (phosphoglycerol), whereas the membrane of Gram-positive bacteria almost exclusively hosts PG-type lipids (Heller et al. 1987), to which the peptide has a higher affinity (Jung et al. 2006; Nishizawa and Nishizawa 2007). Due to this interaction GsMTx4 may also serve as a lead compound for the synthesis of future components active as antimicrobial drugs.

It is still a matter of discussion whether all MS channels share the principle of gating via bilayer tension, i.e., changes in the pressure profile/curvature of the lipid bilayer due to the introduction of hydrophobic mismatches at the protein-lipid interface (Hamill and Martinac 2001; Kung 2005). Thus, studying the influence of a complex compound like the peptide GsMTx4 on the behavior of prokaryotic MS channels may also contribute to our understanding of the molecular process behind mechanosensory transduction.

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