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Supporting Information

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Rationally designed chemical modulators convert a bacterial channel protein into a pH-sensory valve

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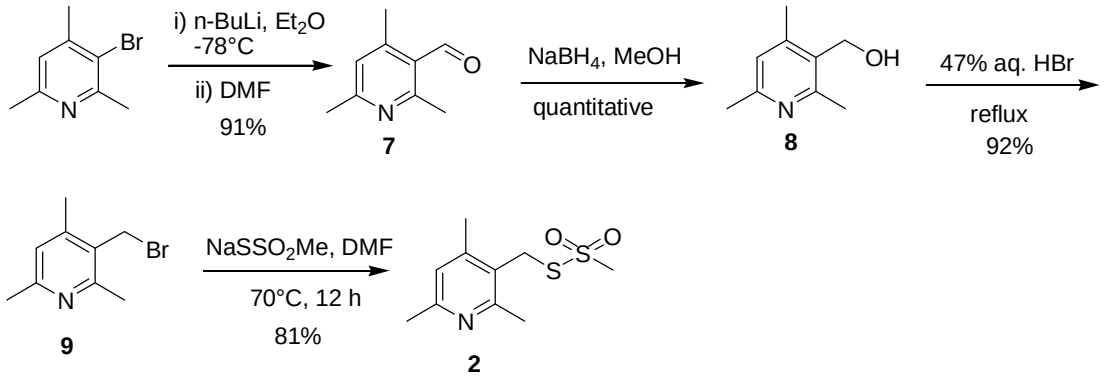
1. Experimental procedures

Chemical synthesis of the compounds

4-(Bromomethyl)pyridine hydrobromide (1)
Compound **1** was prepared from 4-pyridinemethanol according to a known procedure[1].

S-(2,4,6-Trimethylpyridin-3-yl)methyl methanesulfothiolate (2)
Compound **2** was prepared from 3-bromo-2,4,6-trimethylpyridine[2] using the following sequence of the reactions.



2,4,6-Trimethylpyridine-3-carbaldehyde (7)
To the solution of 3-bromo-2,4,6-trimethylpyridine[2] (3g, 15 mmol) in the Et_2O (50 ml) was added at -78°C under N_2 n-BuLi (10.3 ml of 1.6 M, 16.5 mmol) and the mixture stirred at the same temperature for 1 h. Then DMF (1.55 ml, 20 mmol) was added and the reaction mixture was allowed to warm to r.t. After stirring for 30 min at r.t. water (10 ml) was added, the organic layer separated and the aqueous layer extracted with Et_2O (3x20 ml). Combined organic extracts were dried over Na_2SO_4, filtered, evaporated and purified by chromatography (Silica-gel, hexane:ethylacetate / 3:1) to yield 2,4,6-trimethylpyridine-3-carbaldehyde (2.05 g, 91%) as a colorless oil which solidified upon standing.
^{1}H NMR (400 MHz, $CDCl_3$) d 2.41 (s, 3H), 2.46 (s, 3H), 2.68 (s, 3H), 6.81 (s, 1H), 10.58 (s, 1H),
^{13}C NMR (100.6 MHz, $CDCl_3$) d 20.25 (q), 23,06 (q), 24.62 (q), 124.49 (d), 125.85 (s), 150.02 (s), 160.57 (s), 161.81 (s), 192.09 (d)
MS (EI): 149 [M+]; HRMS calcd. For $C_9H_{11}NO$ 149.0841, found 149.0854

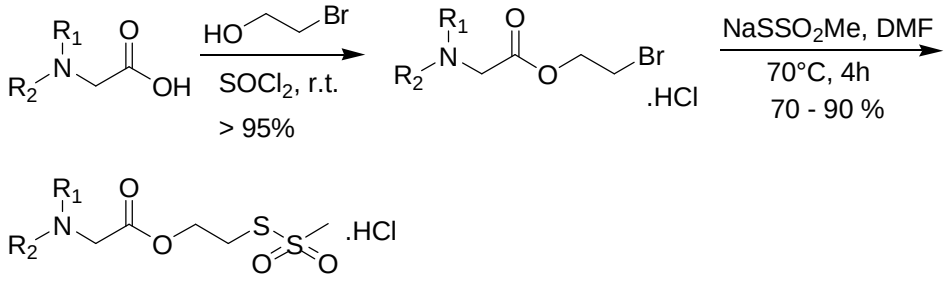
(2,4,6-Trimethylpyridin-3-yl)methanol (8)
$NaBH_4$ (0.45 g, 12 mmol) was slowly added to a solution of 2,4,6-trimethylpyridine-3-carbaldehyde (1.5 g, 10 mmol) in methanol (20 ml) cooled in an ice-bath. After the addition was completed, the cooling bath was removed and the reaction mixture was stirred 30 min at r.t. The reaction was quenched with water (50 ml), methanol was evaporated and the residue extracted with Et_2O (3x50 ml). The combined organic extracts were dried over Na_2SO_4, filtered and the solvent evaporated to give (2,4,6-trimethylpyridin-3-yl)methanol (1.52 g, quantitative) as a colorless oil which solidified upon standing.
(400 MHz, $CDCl_3$) d 2.33 (s, 3H), 2.38 (s, 3H), 2.46 (s, 3H), 3.46 (bs, 1H), 4.66 (s, 2H), 6.78 (s, 1H)
^{13}C NMR (100.6 MHz, $CDCl_3$) d 18.98 (q), 21.87 (q), 23.86 (q), 58.22 (t), 123.28 (d), 129.53 (s), 147.29 (s), 156.56 (s), 156.74 (s)
MS (EI): 151 [M+]; HRMS calcd. For $C_9H_{13}NO$ 151.0997, found 151.1009

3-(Bromomethyl)-2,4,6-trimethylpyridine (9)
(2,4,6-Trimethylpyridin-3-yl)methanol (457 mg, 3 mmol) was dissolved in HBr (10 ml of a 47% aqueous solution) and heated at reflux for 8 h. After cooling, the mixture was neutralized with 10M aq. NaOH while kept in an ice cooling bath until a precipitate appeared and subsequently a sat.aq. $NaHCO_3$ solution was added. The resulting mixture was extracted with CH_2Cl_2, dried over Na_2SO_4 and the solvent evaporated at temperature below 30°C to give 3-(bromomethyl)-2,4,6-trimethylpyridine as a white solid (595 mg, 92%) which is unstable and has to be used as soon as possible in the next step.
^{1}H NMR (400 MHz, $CDCl_3$) d 2.31 (s, 3H), 2.41 (s, 3H), 2.55 (s, 3H), 4.48 (s, 2H), 6.80 (s, 1H)
^{13}C NMR (100.6 MHz, $CDCl_3$) d 18.68 (q), 22.00 (q), 24.09 (q), 28.05 (t), 123.33 (d), 127.09 (s), 146.73 (s), 156.67 (s), 157.40 (s)
MS (EI): 213, 215 [M+]; HRMS calcd. For $C_9H_{12}N^{81}Br$ 215.0133, found 215.0134

S-(2,4,6-Trimethylpyridin-3-yl)methyl methanesulfothiolate (2)
3-(Bromomethyl)-2,4,6-trimethylpyridine (214 mg, 1 mmol) was dissolved in DMF (2ml) and sodium methanethiosulfonate (134 mg, 1 mmol)) was added. The mixture was heated at 70°C for 12 h, cooled, poured into water (10 ml) and extracted with Et_2O (3x10ml). The combined organic extracts were dried over Na_2SO_4, filtered, evaporated and purified by chromatography (Silica-gel, hexane:ethylacetate / 3:1 followed by pure ethylacetate) to yield S-(2,4,6-trimethylpyridin-3-yl)methyl methanesulfothiolate (3) (133 mg, 54%) as a white solid.
^{1}H NMR (200 MHz, $CDCl_3$) d 2.37 (s, 3H), 2.44 (s, 3H), 2.60 (s, 3H), 3.31 (s, 3H), 4.44 (s, 2H), 6.84 (s, 1H)
^{13}C NMR (75.4 MHz, $CDCl_3$) d 19.28 (q), 22.42 (q), 24.02 (q), 35.82 (t), 50.44 (q), 122.63 (s), 123.62 (d), 147.41 (s), 156.99 (s), 157.50 (s)
MS (EI): 245 [M+]; HRMS calcd. For $C_{10}H_{15}NO_2S_2$ 245.0544, found 245.0546

2-Methanesulfonylsulfanyl-ethyl ester of glycine and its N alkylated derivatives (3-5)

These compounds were prepared using a two step sequence starting from glycine, N-methylglycine and N,N-dimethylglycine hydrochloride.



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General procedure for synthesis of 2-bromo-ethyl ester of glycine and its N-alkylated derivatives

Glycine or its N-alkylated derivative in the form of the free compound or as the HCl salt (20 mmol) was suspended in 2-bromoethanol (14.3 ml, 200 mmol) and the mixture cooled to 0°C. Thionyl chloride (1.8 ml, 25 mmol) was added dropwise and the reaction mixture was stirred at room temperature until a clear solution was obtained. The resulting solution was poured into 200 ml of ether; the precipitated solid was filtered, washed with ether and dried in vacuo. In the case that only oil separated instead of precipitate, ether was decanted, the oil washed with ether (2x 100 ml) and all the residual solvents were removed in vacuo. The oil usually solidified upon standing overnight at 4°C. The compound is obtained as HCl salt. Yield is over 95%, purity is over 98%.

Amino-acetic acid 2-bromo-ethyl ester; hydrochloride

^{1}H NMR (300 MHz, D_2O) d 3.66 (t, *J* = 5.6 Hz, 2H), 3.97 (s, 2H), 4.58 (t, *J* = 5.6 Hz, 2H)
^{13}C NMR (100.6 MHz, DMSO) d 30.35 (t), 39.58 (t), 65.19 (t), 167.41 (s)
MS (EI): 181, 183 [M+]

Methylamino-acetic acid 2-bromo-ethyl ester; hydrochloride

^{1}H NMR (300 MHz, D_2O) d 2.81 (s, 3H), 3.67 (t, *J* = 5.6 Hz, 2H), 4.07 (s, 2H), 4.60 (t, *J* = 5.6 Hz, 2H)
^{13}C NMR δ (100.6 MHz, DMSO) d 30.39 (t), 32.69 (q), 48.00 (t), 65.29 (t), 166.49 (s)
MS (EI): 195, 197 [M+]; HRMS calcd. For $C_5H_{10}NO_2{}^{81}Br$ 196.9874, found 196.9871

Dimethylamino-acetic acid 2-bromo-ethyl ester; hydrochloride

^{1}H NMR (300 MHz, D_2O) d 3.00 (s, 6H), 3.67 (t, *J* = 5.6 Hz, 2H), 4.21 (s, 2H), 4.60 (t, *J* = 5.6 Hz, 2H)
^{13}C NMR (100.6 MHz, DMSO) d 30.13 (t), 42.73 (q), 55.18 (t), 64.98 (t), 165.27 (s)
MS (EI): 209, 211 [M+]; HRMS calcd. For $C_6H_{12}NO_2{}^{79}Br$ 209.0051, found 209.0060

General procedure for synthesis of 2-Methanesulfonylsulfanyl-ethyl ester of glycine or its N alkylated derivatives (3-5)

The salt of 2-bromo-ethyl ester of glycine or its N-alkylated derivative (10 mmol) was dissolved in DMF (10 ml) and sodium methanethiosulfonate (1.47g, 11 mmol) was added. The mixture was heated at 70°C for 4 h, the solid precipitate was filtered and DMF was evaporated in vacuo. The residue was dissolved in a small amount of boiling acetonitrile (20 ml), the mixture filtered, and the solvent evaporated in vacuo. Products (4-6) obtained as HCl salt. Yield 70 – 90%. Compounds may contain small amount <5% NaCl. Solid products can be recrystallized from acetonitrile-ether or ethanol-ether.

Amino-acetic acid 2-methanesulfonylsulfanyl-ethyl ester; hydrochloride (3)

^{1}H NMR (300 MHz, D_2O) d 3.57 (s, 3H), 3.59 (t, *J* = 5.9 Hz, 2H), 3.99 (s, 2H), 4.60 (t, *J* = 5.9 Hz, 2H)
^{13}C NMR (100.6 MHz, DMSO) d 33.88 (t), 35.77 (t), 50.10 (t), 63.54 (t), 167.23 (s)
HRMS calcd. for

Methylamino-acetic acid 2-methanesulfonylsulfanyl-ethyl ester; hydrochloride (4)

^{1}H NMR (300 MHz, D_2O) d 2.82 (s, 3H), 3.57 (s, 3H), 3.59 (t, *J* = 5.9 Hz, 2H), 4.08 (s, 2H), 4.60 (t, *J* = 5.9 Hz, 2H)
^{13}C NMR (100.6 MHz, DMSO) d 32.69 (q), 34.01 (t), 48.00 (t), 50.24 (q), 63.78 (t), 166.63 (s)
MS (EI): 227 [M+]; HRMS calcd. For $C_6H_{13}NO_4S_2$ 227.0286, found 227.0279

Dimethylamino-acetic acid 2-methanesulfonylsulfanyl-ethyl ester; hydrochloride (5)

^{1}H NMR (300 MHz, D_2O) d 3.02 (s, 6H), 3.57 (s, 3H), 3.59 (t, *J* = 5.9 Hz, 2H), 4.23 (s, 2H), 4.61 (t, *J* = 5.9 Hz, 2H)
^{13}C NMR d
^{13}C NMR (100.6 MHz, DMSO) d 34.05 (t), 43.35 (q), 50.33 (q), 55.95 (t), 63.96 (t), 165.72 (s)
MS (EI): 241 [M+]; HRMS calcd. For $C_7H_{15}NO_4S_2$ 241.0442, found 241.0439

[(4,5-Dimethoxy-2-nitro-benzyloxycarbonyl)-methyl-amino]-acetic acid 2-methanesulfonylsulfanyl-ethyl ester (6)

Methylamino-acetic acid 2-methanesulfonylsulfanyl-ethyl ester; hydrochloride **4** (53 mg, 0.2 mmol) was dissolved in 1 ml of DMF and 6-nitroveratrylchloroformate (61 mg, 0.22 mmol) and pyridine (0.1 ml) were added. After overnight stirring the mixture was poured into water (1 ml) and extracted with ethyl acetate (3x 3 ml). All the solvents were evaporated in vacuo and the product purified by chromatography (Silica-gel, hexane:ethylacetate / 1:1). Yield 56 mg (60%) of **6**. The product is on the NMR timescale a mixture of two isomers due to restricted rotation around CO-N bond in the carbamate moiety. Measurements at two different frequencies (300 MHz and 400 MHz) allows to distinguish between two close absorption

peaks belonging to different isomers and peaks splitted due to a coupling. Variable temperature measurement shows coalescence at higher temperatures.
^{1}H NMR (300 MHz, $CDCl_3$, 20°C) d 3.01, 3.09 (2s, 3H), 3.35 (s, 3H), 3.40 (t, *J* = 6.0 Hz, 2H), 3.93, 3.94 (2s, 3H), 4.00, 4.01 (2s, 3H), 4.06, 4.08 (2s, 2H), 4.44 (t, *J* = 6.0 Hz, 2H), 5.51, 5.56 (2s, 2H), 6.96, 7.01 (2s, 1H), 7.68, 7.71 (2s, 1H)
^{13}C NMR (100.6 MHz, $CDCl_3$, 20°C) d 34.79, 34.88, 35.66, 36.36, 50.53, 50.84, 50.90, 50.97, 56.52, 56.86, 63.20, 63.26, 64.69, 64.84, 108.25, 109.67, 110.59, 128.12, 128.31, 139.64, 139.73, 148.13, 148.21, 153.80, 155.61, 156.35, 169.21, 169.32
MS (EI): 466 [M+]; HRMS calcd. For $C_{16}H_{22}N_2O_{10}S$ 466.0715, found 466.0729

Protein Isolation. Wild-type[3] or G22C[4] MscL with C-terminal 6His-tag was expressed in the *mscL*-knockout strain PB104[5], using the pB10a[3] expression vector. A 10 l culture was grown in a batch fermentor at 37°C in Luria-Bertani (LB) medium containing 100 µg/ml ampicillin. Protein expression was induced at mid-log phase by 1 mM isopropyl-ß-D-thiogalactopyrapyranoside (IPTG) (Sigma) and bacteria were grown for two more hours. The cells were passed twice through a French Press at 15,000 psi and membrane fractions were isolated by differential centrifugation.[5] Membrane vesicles (25 g wet weight) were suspended in 25 mM Tris-Cl, pH 8.0 and stored at -80 °C. For protein isolation, typically, 3 g wet weight of membrane vesicles were solubilized in 30 ml extraction buffer (10 mM Na_2HPO_4/ NaH_2PO_4, pH 8.0, 300 mM NaCl, 35 mM imidazole, 2% (v/v) Anapoe®-X-100 (oxidant free Triton X-100)(Anatrace). The solubilized fraction was cleared by centrifugation (40000 rpm, 45 min, 4 °C) and incubated under gentle rotation (1 h, 4 °C) with 4 ml nickel-nitriloacetic acid (Ni-NTA) metal-affinity matrix equilibrated with extraction buffer. The matrix was transferred into a 1.5 by 10 cm column and washed with 25 ml of wash buffer (extraction

buffer with 1% Anapoe®-X-100) and 15 ml of histidine buffer (10 mM Na_2HPO_4/ NaH_2PO_4, 300 mM NaCl, 50 mM histidine and 0.2% Anapoe®-X-100) with a flow rate of 0.5 ml/min. MscL was eluted with 10 ml elution buffer (histidine buffer containing 235 mM histidine). Typical isolations yielded 3.4 mg of MscL protein (Bradford assay) which was >98% pure as analyzed by SDS-PAGE and N-terminal sequencing.

MscL Modification. MscL was isolated as described above. Compounds **2**-**6** were dissolved in a buffer composed of 10 mM Na-phosphate (pH 8), 150 mM NaCl and 1 mM EDTA. 500 µl detergent solubilized MscL (0.3 mg/ml) was mixed with 40 mM final concentration of the desired compound, and incubated 15 min at room temperature. The modified protein was separated from the free modulator by passing the mixture through a pD10 desalting column (Amersham Biosciences) that was pre-equilibrated with 10 mM Na-phosphate (pH 8), 150 mM NaCl and 0.1 % Anapoe®-X-100.

In the case of compound **1,** MscL was modified during the last step of the isolation procedure, while the protein was still attached to the Ni-NTA column using the following procedure. After the first washing step, the column was washed again with extraction buffer without imidazole. A freshly prepared solution of compound **1** at a final concentration of 1 mg/ml in the same buffer was then added to the matrix and incubated at 4 $^{\circ}$C overnight. Finally, the excess modulator was removed in another washing step and the protein was eluted as in the isolation procedure. Modified proteins were subjected to

electrospray ionization mass spectrometry (ESI-MS) and a liposomal efflux assay to determine whether modification was successful and complete.

Eelectrospray ionization mass spectrometry (ESI-MS). 500 µl of protein samples (0.1-0.3 mg/ml) were incubated with 100 mg wet weight of Bio-Beads SM-2 adsorbents (Bio-Rad) at 40 °C for 45 min to remove the detergent. The supernatant was incubated at 60 °C for 30 min to further precipitate the protein, cooled on ice and centrifuged at 14,000 rpm for 15 min at 4 °C. The pellet was washed twice with 2 ml of ice-cold sterile water. Finally, the supernatant was removed and the pellet was dissolved in 300 µl 50 % formic acid and 50 % acetonitrile shortly prior to ESI-MS analysis at the mass spectrometry core facility at the Department of Pharmacy of the University of Groningen.

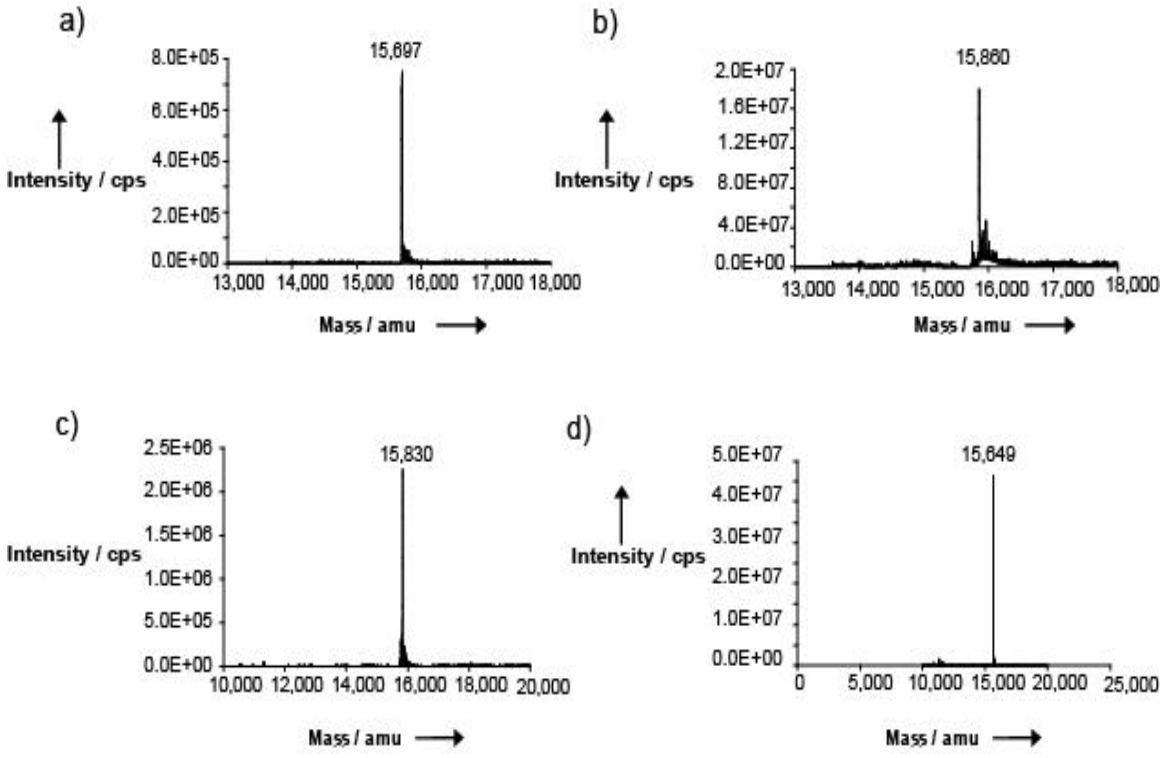


Figure 1. The ESI-MS results of a) unlabeled MscL-G22C, b) MscL-G22C modified with compound **2**, c) compound **3** and d) wild-type MscL treated with

compound **3** are given in the following figures. No unmodified protein signal is present in the spectra of the modified proteins, indicating that the modification was complete.

Membrane Reconstitution. The protein was incorporated into synthetic lipid membranes consisting of either 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC) (for compound **1, 2**), or DOPC, cholesterol and 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene Glycol)2000] (DSPE-PEG-2000) in a molar ratio of 70:20:10, respectively, (for compounds **3**-**6**) using a detergent-mediated reconstitution procedure.[6] Pre-warmed DOPC-Cholesterol_DSPE-PEG-2000 liposomes (20 mg/ml) were extruded 11 times through a 400 nm pore size polycarbonate filter (Avestin) and the resulting large unilamellar vesicles were titrated at 60 °C with Anapoe®-X-100 until saturation. The detergent-destabilized liposomes were then mixed with the modified protein, solubilized in the same detergent, at a 1:120 (wt:wt) protein to lipid ratio and incubated at 60 °C for 45 min. For the efflux experiments, 1 volume of calceine (Na-salt) (Sigma) was added to the mixture to a final concentration of 50 mM. For patch clamp experiments, 1 volume of 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 150 mM NaCl was added to the lipid-protein mixture. Detergent removal was achieved by incubating the mixture with 200 mg wet weight of Bio-Beads SM-2 adsorbents (Bio-Rad) at 4 °C for several hours.

In the case of reconstitution of the protein into DOPC liposomes the same procedure was followed except that all the reactions were carried out at room temperature instead of 60 °C.

Calceine efflux assay. It has been shown that the small, charged molecule, [2-(trimethylammonium)-ethyl]-methanethiosulfonate) ($MTSET^+$), can reach C22 position of the protein in its native membrane from the periplasmic side, even if the channel is in its closed form, and that reaction with this reagent leads to spontaneous channel openings as evidenced in patch clamp analysis.[4] This charge-induced channel opening of MscL-C22 in the absence of tension was used as the basis for a fluorescent efflux assay to analyze the channel activity at ensemble level.

First, unmodified MscL-C22 channels were incorporated into synthetic lipids in the presence of calceine, as explained above. Then, the liposomal fraction was separated from the free calceine by Sephadex G50 size exclusion column chromatography. A 10-15 µl volume of the liposomal fraction was placed in 2 ml iso-osmotic buffer (10 mM sodium phosphate, pH 8, 150 mM NaCl, 1 mM EDTA), and the activity of the channels was followed by measuring the increase in fluorescence upon calceine dequenching after its release through the channel in response to the addition of $MTSET^+$ to a final concentration of 1 mM. The experiments were performed at room temperature but no differences were observed with experiments at 37 °C. Fluorescence was monitored at 520 nm (excitation at 490 nm) in an SLM 500 spectrofluorometer. Release was expressed as a percentage relative to the maximal possible value obtained after lysing all liposomes by the Anapoe®-X-100 detergent added to a final concentration of 8 mM. Under iso-osmotic conditions, MscL-G22C-containing liposomes did not release any fluorescent

dye (Figure 2, ?) in the absence of $MTSET^+$. However, as soon as 0.01 volume of a $MTSET^+$ solution to reach a final concentration of 1 mM was added to a duplicate sample, a sharp increase in fluorescence was observed (Figure 2, ¦). Liposomes without embedded MscL-G22C (Figure 2, X) or containing the wild-type MscL (Figure 2, ?) did not show any release activity, either in the presence or absence of $MTSET^+$, in agreement with our mass spectrometry results. Therefore, we conclude that in proteoliposomes, cysteine at the 22nd amino acid position of MscL can be modified specifically and the resulting channel activity can be followed in iso-osmotic buffers as a fluorescence signal in a straightforward calceine efflux assay.

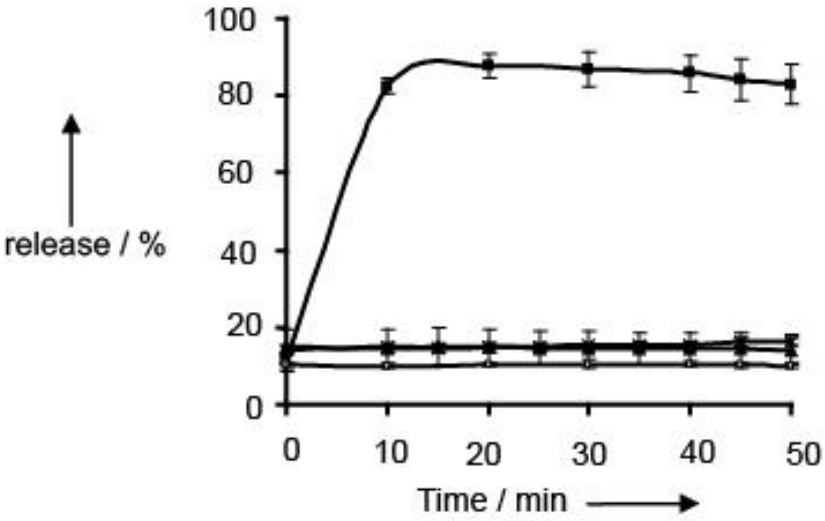


Figure 2. When the protein is reconstituted in synthetic lipids containing a fluorescent dye, induction of channel openings with $MTSET^+$ leads to release of the dye and an increase in fluorescence (¦). In the absence of $MTSET^+$ the same proteoliposomes do not release any dye (?). Liposomes containing wild-type channels (?) and liposomes without protein (X) also do not respond to $MTSET^+$.

In the calceine efflux experiments, the response to $MTSET^+$ was also used as a method to quantify the efficiency of the chemical modification in each experiment by adding $MTSET^+$ into preparations of modified MscL-G22C. If modification was complete (as indicated by the mass spectrometry results), the addition of $MTSET^+$ did not result in any additional activity in the fluorescence, whereas $MTSET^+$ addition led to an increase in fluorescence if the modification was not complete. Even though it is not clear at this moment how many charges should be present at the homo-pentameric occlusion site to induce spontaneous channel openings of MscL-G22C, we found the mass-spectrometry and efflux assay results to be in complete agreement. Therefore a sample, fulfilling both criteria (i.e. absence of a signal for unmodified protein in ESI-MS and no activity in efflux assays upon addition of $MTSET^+$), was considered as fully modified.

In case of pH compounds, the protein was first modified as explained above and after the removal of the excess compound by a Sephadex G25 column chromatography step, the modified protein was reconstituted in the liposomes.

Time dependence of calcein efflux assay. The time dependence of pH-induced MscL activity is given in Figure 3. As soon as the proteoliposomes interact with the buffer, the release starts and completed within 30 minutes.

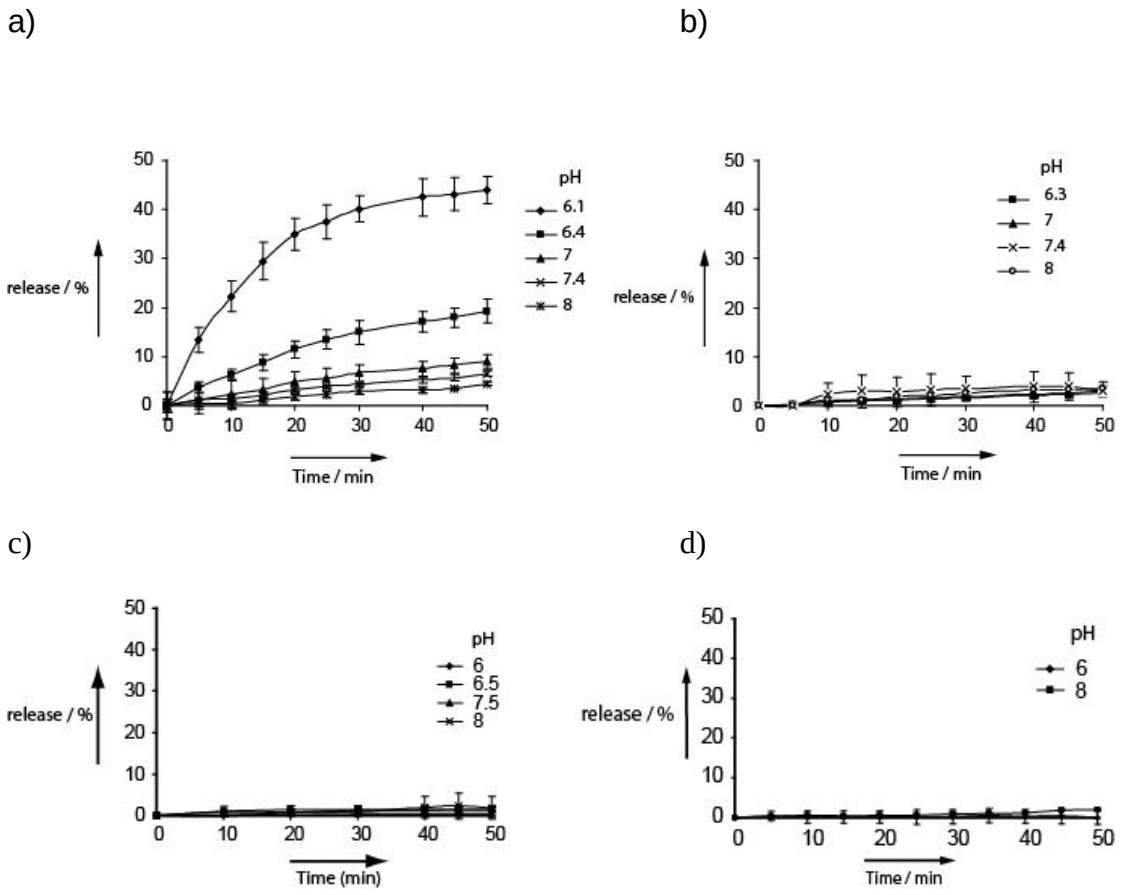


Figure 3. Time dependence of pH-induced channel opening. a) MscL-G22C-modified with compound **5**, b) liposomes without any protein, c) MscL-G22C unlabeled, d) Wilde-type MscL (with C-terminal his-tag). Error bars indicate the standard deviation from at least 3 independent experiments.

Comparison of pH-modulators with each other. In order to compare the performance of different compounds with each other, $MTSET^+$ was used as an internal reference in each efflux experiment and the activity of MTS compounds were normalized to that of $MTSET^+$. The activity depends on the charge status and the hydrophobicity of the channel constriction at a particular pH. Even though it is difficult to know the exact situation within the pore, we calculated the hydrophobicities for compounds attached to cysteine methyl ester using partition coefficient octanol / water (clogP) as implemented in

Chem3D, version 8.0. As indicated in figure 4, compound **2** with the highest calculated hyrophobicity (0.17), gave the lowest activity. Its pKa is also the lowest (7.10) so that dropping the pH low enough to charge the whole subunits was not possible within the experimental conditions. Compound **5**, on the other hand, gave the highest activity within the pH compounds and its hydrophobicity (0.38) is much lower than that of compound **2. I**ts pKa is low enough to keep the channel closed at high pH's and high enough to allow lowering the pH within the experimental conditions to charge more subunits. See the main text for further discussion.

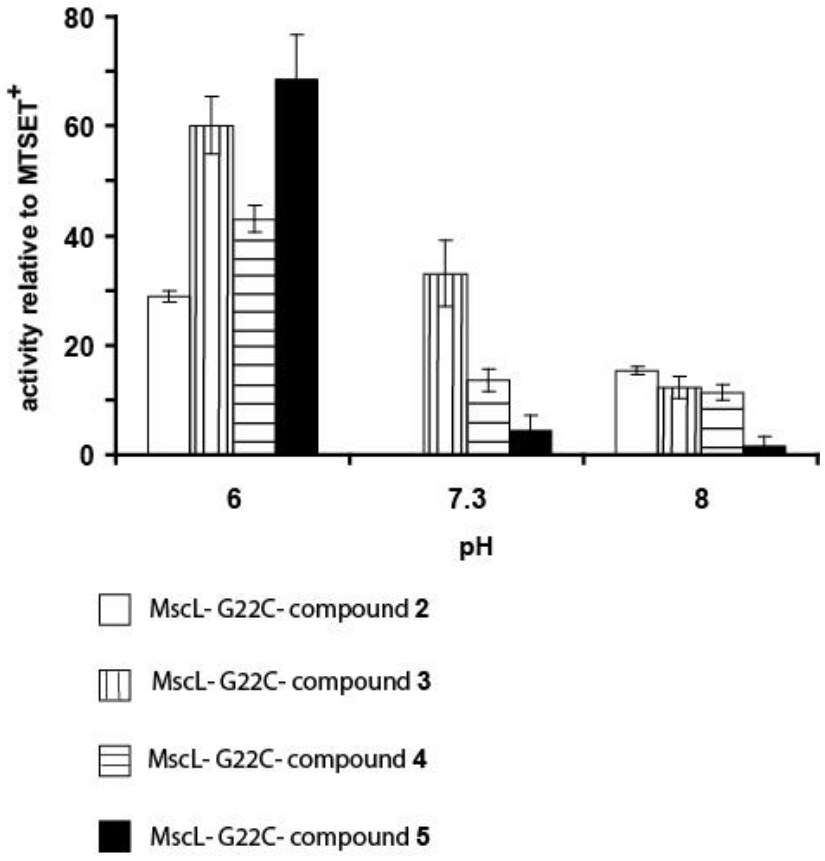
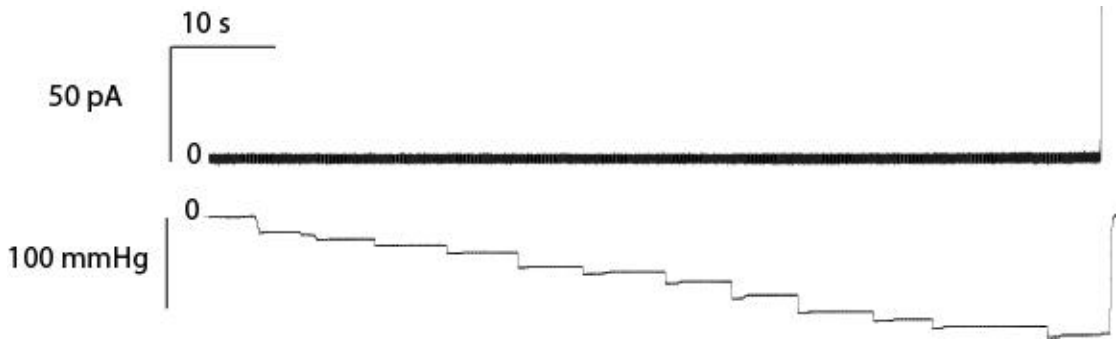


Figure 4. Comparison of MTS pH modulators on the basis of their relative activity with respect to the control compound $MTSET^+$.

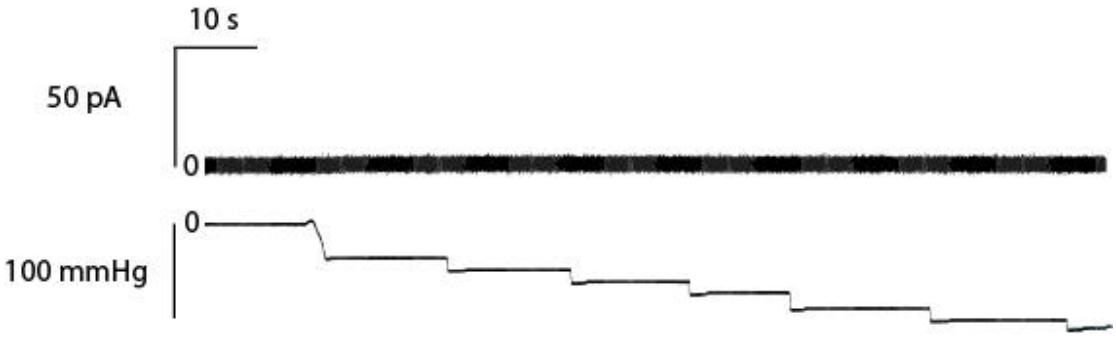
Patch Clamp. Giant proteoliposomes[7] and giant *E. coli* spheroplasts[8] for patch clamp measurements were prepared as described. Pipettes with a resistance of 2.5-4 MO were pulled from 100 µl borosilicate capillaries in a Sutter 97/IVFmicropipette puller. Single channel traces were recorded at 20 mV in a bath-pipette buffer composed of either 200 mM KCl, 90 mM $MgCl_2$, 10 mM $CaCl_2$, 5 mM HEPES, at desired pH for spheroplasts, or in 200 mM KCl, 40 mM $MgCl_2$, 5 mM HEPES at different pH's for giant liposomes. Data were amplified and filtered at 10 kHz using an Axopatch 1D amplifier, sampled at 33kHz in a Digidata 1322A digitizer and analyzed with pCLAMP8 software.

The patch clamp of control samples for pH-induced activity is given in Figure 4. As shown by the efflux assay, liposomes without any protein, liposomes with wild type or liposomes with unmodified protein didn't give any activity in response to pH. Liposomes with channels required tension to open at any pH. Reconstituted unmodified G22C was very difficult to patch since the tension required to open the channel was close to the limit for rupture of the patch. Only a few patches with a small number of channels just prior to rupture could be obtained at any pH for these sample (data not shown).

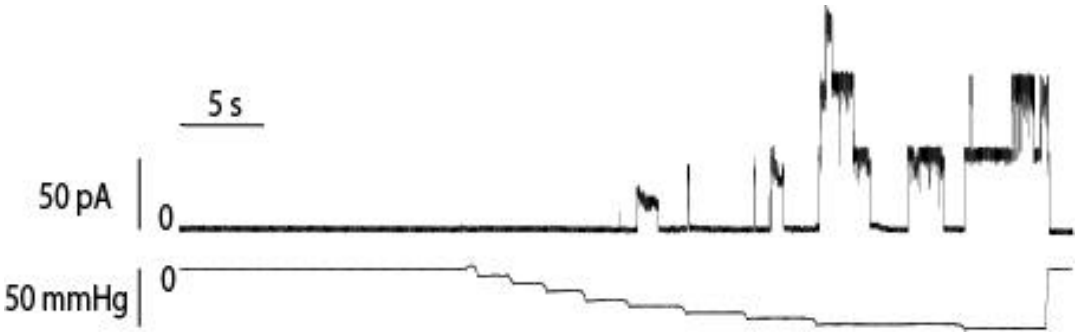
a)



b)



c)



d)

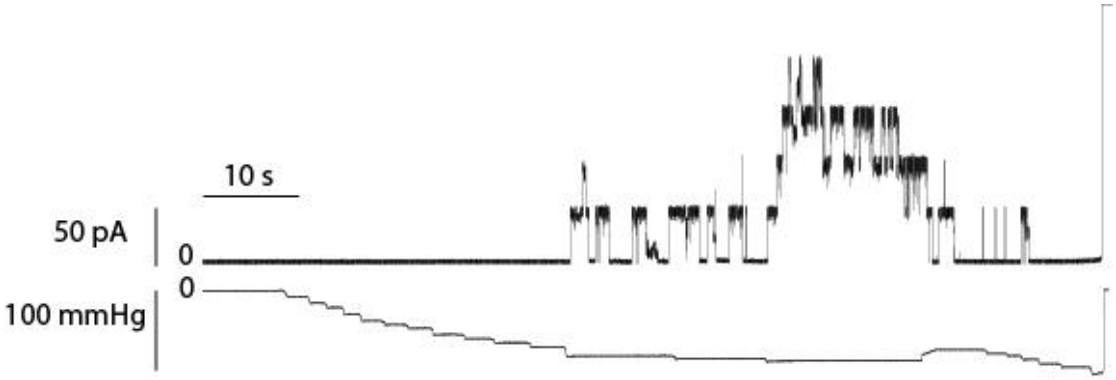


Figure 5. Patch clamp analyses of control samples. a) Liposomes at pH 7.45. b) Liposomes at pH 6. Liposomes were reconstituted by using the same procedure for proteoliposomes except that protein elution buffer was added in stead of the MscL protein. c) Wild-type MscL at pH 7.45 and d) at pH 6 (for each sample at least 3 individual measurements were performed)

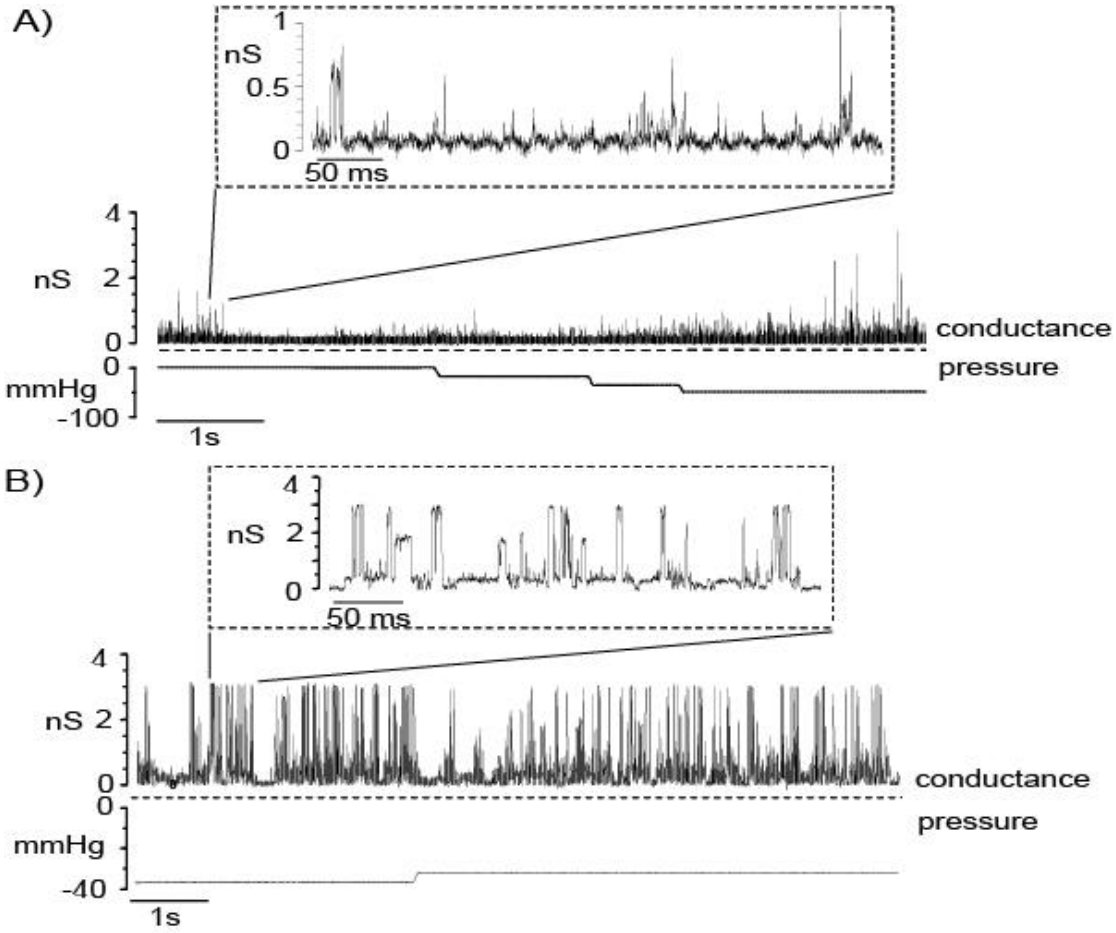
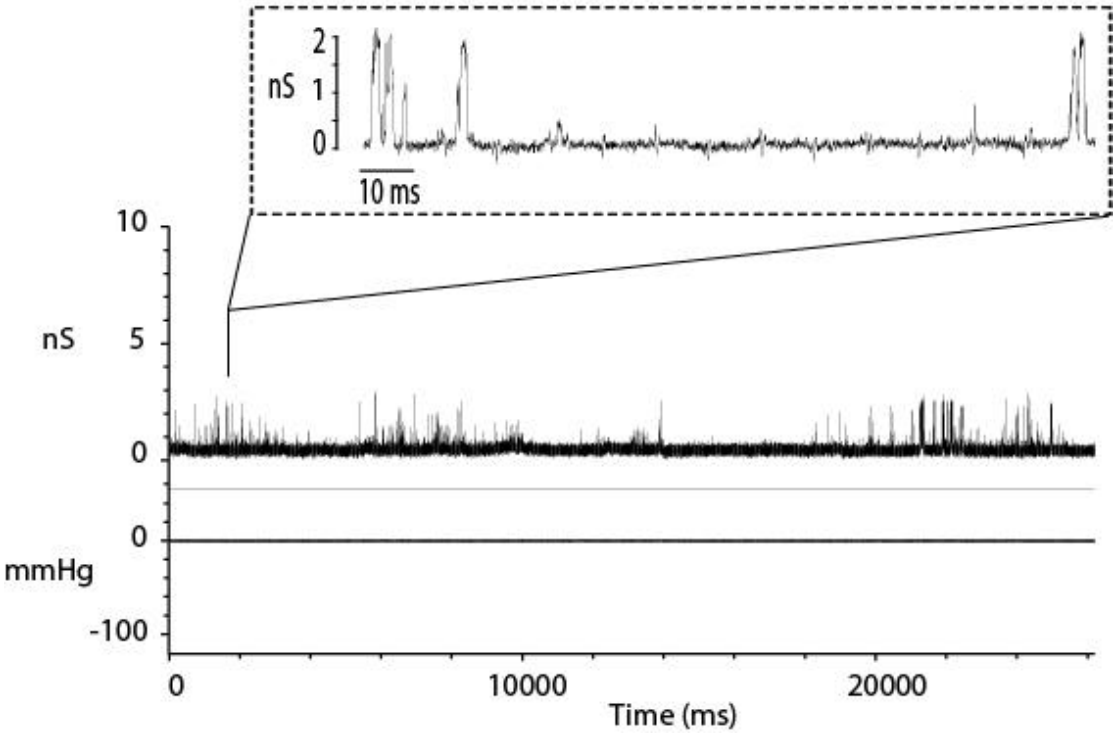


Figure 6. Single molecule level analyses of MscL-G22C modified with compound **1** in patch clamp. A) At pH 5.2 the channel opened even in the absence of applied tension to its subconducting states. Application of tension led to higher and even full conducting states. B) At pH 7.2, the channel needed applied tension to open. Typical channel openings in each trace are indicated in an enlarged form in dashed boxes.

a)



b)

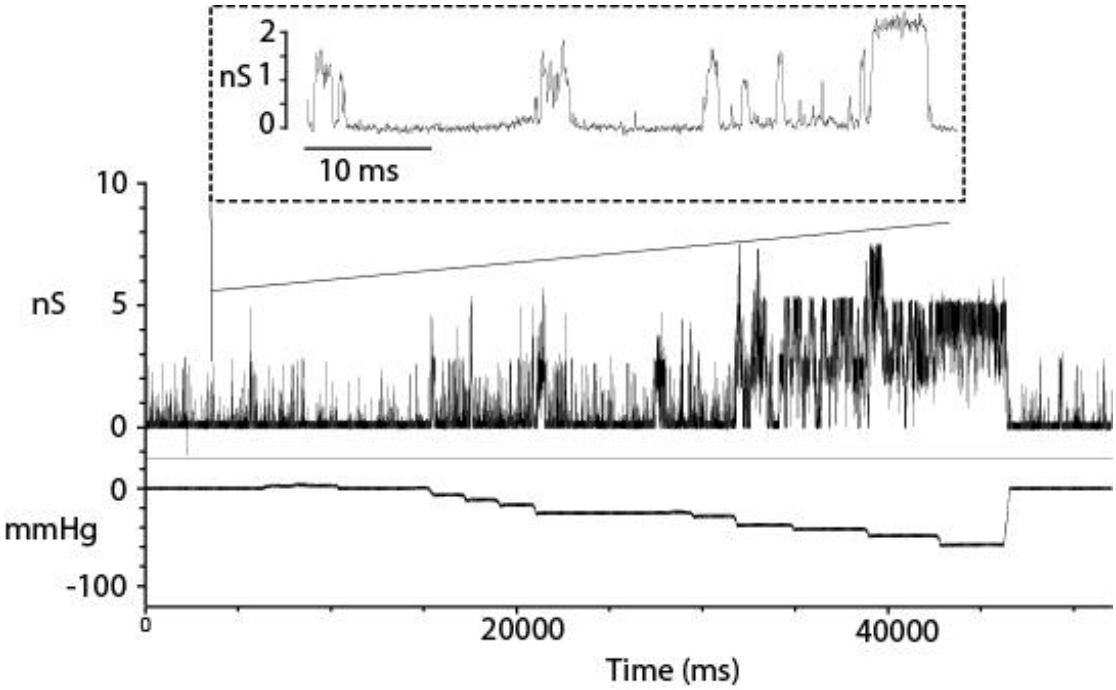
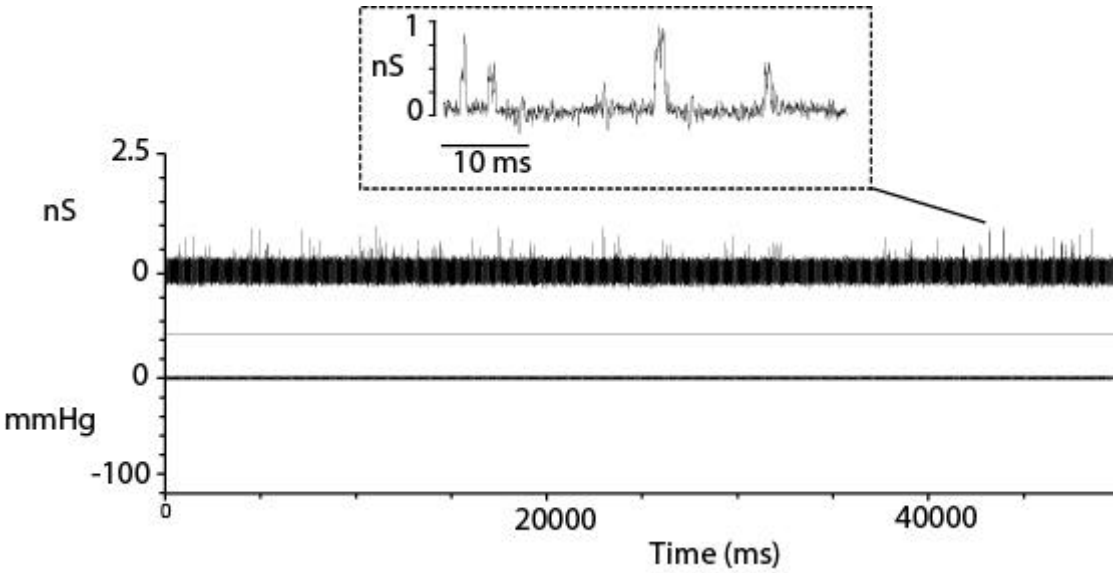


Figure 7. Single molecule level analyses of MscL-G22C modified with compound **3** in patch clamp**.** a) at pH 7.45 (9 individual measurements), b) at pH 6 (12 individual measurements)

a)



b)

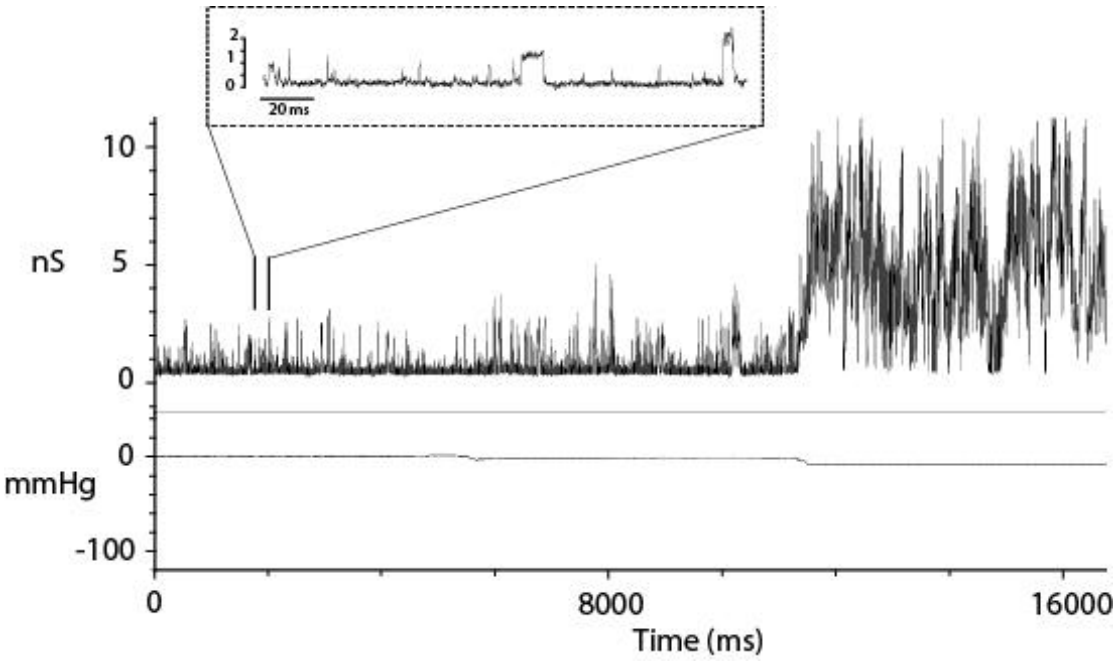


Figure 8. Single molecule level analyses of MscL-G22C modified with a) modulator - **4** (10 individual measurements) and , b) modulator -**5** (8 individual measurements) in patch clamp at pH 6. No channels could be opened at pH 7.45 (minimum 10 individual measurements per sample)

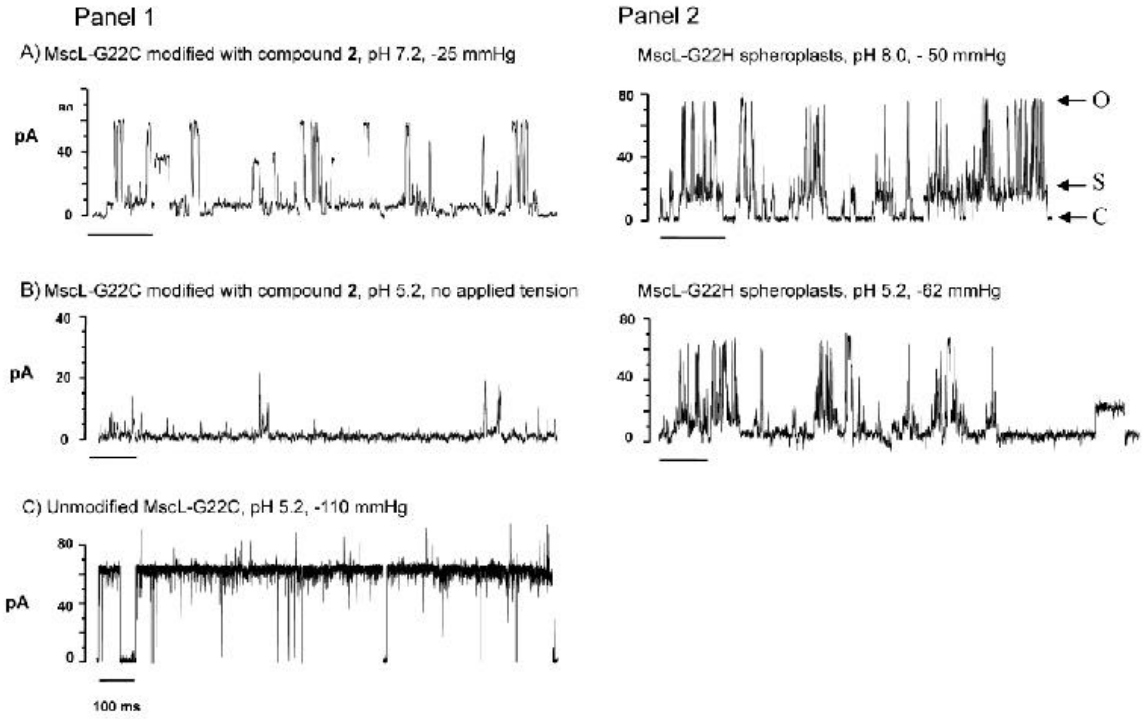


Figure 9. Comparison of the channel kinetics of MscL-G22H and MscL-G22C modified with compound **1**. MscL-G22H is a natural pH-sensing mutant of MscL and analyzed here in spheroplasts. At all the pHs tested, MscL G22H required some tension in order to open however the tension requirement was lower at low pH (panel 2, A and B) when histidines are mainly in the charged form. At any pH, the channel visited a stable substate (S). The chemically modified MscL-G22C reconstituted in DOPC liposomes also showed such a preference for a substate (panel 1, A and B). At high pH, this channel required tension to open but at low pH the modified channel opened in the absence of applied tension (panel 1, B). Whereas the unmodified channel required high tension to open (panel 1, C). In this case the preference for a subconducting state over the full open state was also not observed. Therefore by modification with pH-modulators, it is possible to open the channel by a drop in pH, instead of applying tension.

References

[1] R. L. Bixler, C. Niemann, *J. Org. Chem*. **1958**, *23*, 575-584.

[2] P. Mal, U. Lourderaj, Parveen, P. Venugopalan, J. N. Moorthy, N. Sathyamurthy, *J. Org. Chem.* **2003,** *68*, 3446-3453.

[3] S.I. Sukharev, P. Blount, B. Martinac, F.R. Blattner, C. Kung, *Nature* **1994**, *368*, 265–268.

[4] K. Yoshimura, A. Batiza, M. Schroeder, P. Blount, C. Kung, *Biophys. J.* **1999**, *77*, 1960-1972.

[5] P. Blount, S.I. Sukharev, P. C. Moe, M. J. Schroeder, H. R. Guy, C. Kung, *EMBO J.* **1996**, *15*, 4798–4805.

[6] J.L. Rigaud, B. Pitard, D. Levy, *Biochim. Biophys. Acta* **1995**, *1231*, 223-246.

[7] A. H. Delcour, B. Martinac, J. Adler, C. Kung, *Biophys. J.* 1989, *56*, 631-636.

[8] B. Martinac, M. Buechner, A. H. Delcour, J. Adler, C. Kung, *Proc. Natl. Acad. Sci.* USA. **1987**, *84*, 2297–2301.