MCAs in Arabidopsis are Ca2+-permeable mechanosensitive channels innately sensitive to membrane tension

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MCAs in *Arabidopsis* are Ca^{2+}-permeable mechanosensitive channels innately sensitive to membrane tension

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Mechanosensitive (MS) ion channels respond to mechanical stress and convert it to electric and ionic signals that activate appropriate cellular mechanisms. Although the force-sensing mechanisms of MS channels remain obscure, the following have been proposed: activation by force from membrane lipids and activation by force delivered from associated proteins. Five MS channel families have been identified to date in plants, including the *Arabidopsis thaliana* Mid1-Complementing Activity (MCA) channel; however, their activation mechanisms have not yet been elucidated in detail. We herein demonstrated that the MCA2 channel is a Ca^{2+}-permeable mechanosensitive channel that is directly activated by membrane tension. The N-terminal 173 residues of MCA1 and MCA2 were synthesized *in vitro*, purified, and reconstituted into artificial liposome membranes. Ca^{2+} fluorometry demonstrated that liposomes reconstituted with MCA1(1-173) or MCA2(1-173) mediated Ca^{2+} influx. The patch-clamp technique revealed that the application of pressure to the membrane reconstituted with MCA2(1-173) elicited channel currents. This channel was also activated by voltage. Blockers for mechanosensitive channels inhibited stretch, but not voltage, activation. Since MCA proteins are found exclusively in plants, these results suggest that MCA represents a plant-type MS channel that opens directly with membrane stretch.

Introduction

MS channels are integral membrane proteins that comprise a gated pore to mediate the flux of ions across membranes in response to extrinsic mechanical stress, such as touch, wind, gravity, osmotic pressure, and pathogen invasion, as well as intrinsic mechanical stress, including cell division and cellular growth[1,2]. Despite their

importance in responses to mechanical stress, the precise mechanisms for the gating of the MS channel remain unclear. However, the following two general mechanisms have been proposed and widely accepted: the force-from-lipids mechanism[3,4], in which MS channels act as direct mechanosensors of tension in the lipid bilayer; and the force-from filaments mechanism[5,6], in which the extracellular matrix and/or cytoskeleton act as mediators of the force that pulls MS channels open.

Five families of MS channels have been reported to date in plants: Mid1-Complementing Activity (MCA) proteins[7,8], MscS-like (MSL) proteins[9,10], Two Pore Potassium (TPK) channels[11], Reduced hyperosmolality-induced $[Ca^{2+}]_i$ increase (OSCA) channels[12] evolutionally related to DUF221[13,14], and Piezo channels[15]. MCA channels are unique in terms of structural and evolutional aspects. Arabidopsis MCA proteins have only a single transmembrane segment and assemble into a homotetramer to constitute a channel[16-18], while the subunits of other plant MS channel families have multiple transmembrane segments and form multimers, including dimers (TPK), trimers (Piezo), pentamers (OSCA), and heptamers (MSL)[19-21]. Genes encoding MCA proteins are found exclusively in plants[7,22], while those of other plant MS channel homologs are found in bacteria, protists, fungi (MSL), and animals (Piezo, OSCA, and TKP)[9,11,12,15,23,24]. MCA1 and MCA2 are involved in the response to gravity in Arabidopsis hypocotyls[25]. Moreover, MCA1 is required for perceiving the hardness of soil and hypoosmotic stress[7]. The N-terminal 173 residues, including the transmembrane segment and EF-hand-like motif, are sufficient for Ca^{2+} uptake activity when expressed in yeast cells[17]. Therefore, a Ca^{2+}-permeable pore is likely be composed of the N-terminal 173 residues.

The demonstration of direct activation by membrane tension requires the functional reconstitution of purified channels into an artificial lipid bilayer and activation by membrane stretch. Arabidopsis OSCA1.2 has been shown to be inherently mechanosensitive in this type of experiment[26]. MSL, Piezo, and TKP in plants have not been shown to be inherently mechanosensitive, although their counterparts in bacteria and animals are inherently mechanosensitive. The present study was performed to investigate whether MCA, which is unique to plants, has independently gained the capacity to be activated directly by membrane tension. To this end, we synthesized and MCA1 and MCA2 *in vitro*, reconstituted them into liposomes, and examined the Ca^{2+} permeability and electrophysiological properties. Here, we report a C-terminally truncated form of the MCA2 protein synthesized and purified *in vitro* acts as an inherent Ca^{2+}-permeable mechanosensitive channel that directly senses membrane tension to open.

Results

In vitro synthesis of MCA1 and MCA2 proteins

MCA1 and MCA2 proteins were synthesized using a liposome-supplemented wheat germ cell-free translation system[27]. We initially attempted to synthesize and purify full-length MCA1 and MCA2 proteins fused C-terminally with a 6xHis tag, designated MCA-6H and MCA2-6H, respectively; however, since they underwent extensive aggregation, we were unable to solubilize them during subsequent purification steps. Therefore, we synthesized MCA1(1-173)-6H and MCA2(1-173)-6H (Fig. 1, line 1) and found that both proteins aggregated to a markedly lesser extent. Note that, to avoid lengthy names of the constructs, the abbreviation of 6xHis, 6H, was not added to

the constructs in the main text hereafter. The synthesized proteins were solubilized with 1% *n*-dodecyl-β-D-maltopyranoside (DDM) (Fig. 1, line 2) and applied to a nickel-nitrilotriacetic acid (Ni-NTA) column for one-step affinity purification. The eluate (Fig. 1, line 4) was then subjected to PD-10 desalting column chromatography to change the elution solution to a buffer suitable for the reconstitution of channels on liposomes. The purified proteins in the final preparation migrated in SDS-PAGE gels with a mobility corresponding to a molecular mass of approximately 19 kDa, consistent with the expected protein size (Fig. 1, lane 5). The final preparation had no visible contaminant protein, except for a small amount of a protein with a slightly slower mobility, which always appeared with the same abundance ratio to the 19-kDa protein during the purification steps, suggesting that the slower mobility protein is a consequence of its anomalous SDS-PAGE behavior often caused by altered detergent binding to membrane proteins[28].

C-terminally truncated MCA1 and MCA2 are permeable to Ca^{2+}

Previous studies indicated that both MCA1(1-173) and MCA2(1-173) are permeable to Ca^{2+} in yeast cells[17]. Therefore, we investigated whether the two proteins synthesized *in vitro* permeated Ca^{2+} when incorporated into liposomes (hereafter referred to as MCA1(1-173) and MCA2(1-173) liposomes). The control group included liposomes prepared with the cell-free protein synthesis reaction products from the empty vector (vector liposome) and liposomes incubated without reaction products (pure liposome). Liposomes were loaded with the fluorescent Ca^{2+} indicator, fluo4, and fluorescence was monitored when $CaCl_2$ was added to the bath solution.

The bath solution initially contained 1 mM EGTA, which chelates Ca^{2+}. When the $CaCl_2$ concentration was increased to 0.5 mM, a small increase in fluorescence was detected in the suspension of MCA1(1-173) and MCA2(1-173) liposomes (Fig. 2a). An elevation to 1.5 mM $CaCl_2$ resulted in a larger increase in fluorescence. A still further elevation to 2.5 mM did not evoke a marked increase in fluorescence. In contrast, only a small and gradual increase in fluorescence was observed in the vector and pure liposomes. The significantly larger increases observed in fluorescence in MCA1(1-173) and MCA2(1-173) liposomes than in control liposomes indicated an elevation in Ca^{2+} concentrations by the influx of Ca^{2+} through MCA1(1-173) and MCA2(1-173). The largest increase between 0.5 and 1.5 mM was consistent with the 1:1 stoichiometry of the binding of Ca^{2+} to EGTA.

The site-directed mutagenesis of Asp-21, which is present in the transmembrane segments of both MCA1(1-173) and MCA2(1-173), showed that the D21N mutation resulted in the loss of Ca^{2+} uptake when expressed in yeast cells[17]. To clarify whether this defect *in vivo* correlated with Ca^{2+} influx into liposomes, we generated MCA1(1-173)D21N and MCA2(1-173)D21N by cell-free synthesis. When MCA1(1-173)D21N and MCA2(1-173)D21N were reconstituted into liposomes, the changes observed in fluorescence upon increases in the bath Ca^{2+} concentration almost coincided with those in the pure liposome (Fig. 2b). Based on the results that a Ca^{2+} concentration increase was observed in MCA1(1-173) and MCA2(1-173) liposomes, but not in control liposomes, and that the increase was not observed in MCA1(1-173)D21N and MCA2(1-173)D21N liposomes, we suggest that both MCA1(1-173) and MCA2(1-173) have the ability to mediate Ca^{2+} influx.

C-terminally truncated MCA2 is an inherently mechanosensitive channel

The channel activities of MCA2(1-173) were examined using a patch clamp technique. A large blister of a lipid bilayer was generated from the liposomes reconstituted with MCA2(1-173). When +160 mV was applied to the membrane excised from the blister, sparse square wave currents with a constant amplitude were detected (Fig. 3a). When the holding potential was raised to +180 mV or higher, we observed increases in the frequency of the occurrence of current as well as the unit amplitude. This current was not observed when liposomes were prepared from the reaction product from an empty vector. The amplitude of currents nearly doubled when two square wave currents appeared to overlap (Fig. 3d). Since current followed a two-state model of open and closed states, MCA2(1-173) should form an ion channel.

When the holding potential was changed between –200 mV and + 200 mV, channel openings were only observed at potentials larger than +100 mV and the open probability increased with voltage (Fig. 3b). Unit current increased almost linearly between +140 mV and +200 mV and the slope appeared to be shallower at lower potentials (Fig. 3c). Slope conductance changes with voltage have also been reported for other channels[29].

The mechanosensitivity of MCA2(1-173) was examined by the application of negative pressure through the patch pipette. Channel activity was not detected in the absence of pressure at +100 mV; however, when negative pressure was gradually increased, channel events were initiated (Fig. 3d). Channel opening appeared to occur more frequently at a high pressure. The resting level of activity was resumed when pressure was released. Pressure dependence was obtained by sectioning the current trace according to the pressure range with a bin of 10 mmHg. Open probability markedly

increased at pressures between 30 and 70 mmHg (Fig. 3e). Examinations at higher pressures were not possible because of membrane breakage.

These results indicated that the channel activity of MCA2(1-173) increased with voltage and membrane stretch. We then investigated whether voltage and stretch activation are separatable. We initially tested gadolinium, which affects mechanosensitive channel activation by changing the physical properties of the lipid bilayer[30]. When gadolinium was present in the bath solution, pressure application did not markedly increase channel activity over that in its absence (Fig. 4a). Pressure dependence confirmed that gadolinium suppressed activation by pressure at concentrations of 10 and 100 μM (Fig. 4b). In contrast, voltage activation was largely unaffected by gadolinium (Fig. 4c). We then examined the effects of the spider venom toxin, GsMT4, which affects the interaction between the lipid bilayer and mechanosensitive channels[31]. GsMT4 also affected stretch activation, but not voltage activation (Fig. 4d, e). Thus, stretch activation of MCA2(1-173) can be blocked separately from voltage activation and would require physical interaction with lipid bilayer.

MS channels that are activated by membrane tension can be activated by altering the intrinsic membrane curvature[32,33]. We applied lysophosphatidylcholine, an inverted cone-shaped lipid, to the patch membrane reconstituted with MCA2(1-173) and found that channel activity increased without the application of pressure (Fig. 4f).

Discussion

The results showing an increase in channel activity with pressure, inhibition by mechanosensitive channel blockers, and activation by changing the intrinsic membrane

curvature support the idea that MCA2(1-173) is a MS channel that is directly activated by membrane tension without the help of accessory components, such as a cytoskeleton and an extracellular material. Direct activation by membrane tension has also been reported for other MS channels; however, MCA2(1-173), which constitutes a homotetramer as a subunit with a single transmembrane segment, represents the simplest form of MS channels. Since the transmembrane α helix of MCA2 is amphipathic, the hydrophilic side including Asp-21 may assemble to form a channel pore and the hydrophobic side interacts with membrane lipids[17]. The failure to detect the influx of Ca^{2+} in liposomes reconstituted with D21N MCA2(1-173) supports this view.

MCA2(1-173) was activated by a voltage of 120 mV or higher, which we assume to correspond to a membrane potential of –120 mV or lower *in vivo*. This threshold is above the resting potential recorded in mesophyll cells (–160 to –180 mV) and root cells (–180 mV) of *Arabidopsis*[34,35]. However, the spontaneous opening of MCA2(1-173) at the resting potential is unlikely to occur because MCA2(1-173) passes Ca^{2+}, which activates various targets. We speculate that the threshold may be lower or voltage activation may be suppressed in full-length MCA2 or *in vivo*. Since pressure activated MCA2(1-173) at voltages at which voltage activation did not occur, it is possible that a natural key signal to open MCA2(1-173) is membrane tension.

The absence of channel currents at negative voltages in MCA2(1-173) indicates strong rectification in which current flows only in one direction. The mechanism for rectification has been studied extensively in inward rectifier potassium channels. The rectification in inward rectifiers is not a result from an inherent property of channel itself. Rather, rectification is due to channel blockade by cations, such as Mg^{2+} and polyamines[36] (Lu et al, 2004). Mg^{2+} in the patch clamp solution used in this study is

possible to contribute to the rectification. The observation of rectification also indicates that MCA2(1-173) proteins were incorporated into the liposome lipid bilayer in a unidirectional orientation and not in a directionally fifty-fifty manner. The directional incorporation into liposomes is not surprising since membrane proteins including ion channels have been known to be incorporated in a uniform direction when the dehydration/rehydration method is adopted for proteoliposome preparations. For instance, a bacterial mechanosensitive channel, MscS, is incorporated in a 100% right-side-out configuration[37]. Various ion channels such as TREK-1, KirBAC1.1, and TRPV1 show rectification when reconstituted into liposomes, indicating that these channels are incorporated into membrane in a unidirectional manner[38-40].

It currently remains unclear why Ca^{2+} flowed into liposomes in fluorescence experiments in the absence of pressure and voltage. Since the majority of liposomes that form after 30 minutes of sonication have a diameter smaller than 100 nm[41], the curvature of liposomes may be sufficient to activate MCA2(1-173) because changes in the membrane curvature induced by the application of lysophosphatidylcholine activated MCA2(1-173) (Fig. 4f).

In summary, we showed that artificially synthesized MCA2(1-173) proteins formed an MS channel in liposome membranes *in vitro*. Purified MCA1(1-173) proteins may exhibit similar activity because the two truncated proteins represented essentially the same activity in the *in vitro* assay system (Fig. 2) and in yeast cells[17]. This is the first study to demonstrate that plant-specific membrane proteins self-assemble to become an active ion channel. Consequently, the coiled-coil and PLAC8 motifs, which are absent from MCA1(1-173) and MCA2(1-173), do not appear to be necessary for the formation and activity of MS channels and, thus, may be involved in the cellular

modulation of MCA channels because they are present in the cytoplasmic side[18]. Therefore, the present study will serve as a starting point for obtaining a deeper understanding of plant MS channels in terms of structural and regulatory viewpoints.

Methods

***In vitro* synthesis and purification of MCA proteins.** MCA1, MCA2, and their derivatives were prepared as follows. We explained the procedure for the MCA2(1-173) protein as a representative for simplicity. MCA2(1-173) fused C-terminally with a 6xHis tag (-MGSHHHHHH), designated MCA2(1-173)-6H, was synthesized *in vitro* using the ProteoLiposome Expression Kit (CellFree Sciences Co., Ltd, No. CFS-TRI-PLE, Ehime, Japan) according to the manufacturer's protocol. A DNA fragment encoding MCA2(1-173)-6H was inserted between the *Eco*RV and *Not*I restriction sites of the *in vitro* expression vector, pEU-E01-MCS (CellFree Sciences Co., Ltd.). A translation reaction was conducted at 15˚C for 20 h in the presence of asolectin liposomes. The crude ProteoLiposome fraction containing synthesized MCA2(1-173)-6H proteins was isolated by centrifugation at 20,000*g* at 4˚C for 10 min, and washed four times with PBS by centrifugation. The final precipitate of the PBS wash from a 1-ml translation reaction mixture was suspended in 1.5 ml of solubilization buffer (50 mM sodium phosphate buffer, pH 8.0, 0.3 M NaCl, 1% DDM, and 50 mM imidazole), and centrifuged as described above. A portion (0.5 ml) of the supernatant (a total of 1.5 ml) was applied three times to a Ni-NTA-Spin Column (QIAGEN, No. 31014, Hilden, Germany), and the column was washed with 0.6 ml of solubilization buffer four times. MCA2(1-173)-6H proteins were eluted from the column with 0.6 ml of elution buffer (50 mM sodium phosphate buffer, pH 8.0, 0.3 M NaCl, 1% DDM, 0.3 M imidazole),

and the buffer was changed to MOPS buffer (5 mM MOPS-KOH, pH 7.2, 0.2 M KCl, 1% DDM) using a PD-10 column (GE Healthcare Japan, No. 17-0851-01, Tokyo, Japan). The amount and purity of proteins were assessed by SDS-PAGE followed by silver staining. Typically, 60~80 μg of the pure MCA2(1-173)-6H protein was obtained from a 1-ml translation reaction mixture.

The same procedure was applied to the purification of MCA1(1-173)-6H, MCA1(1-173)D21N-6H, and MCA2(1-173)D21N-6H.

Measurement of Ca^{2+} influx into liposomes

Synthesized MCA1 and MCA2 were reconstituted into liposomes as described previously[42,43]. Twenty milligrams of L-α-phosphatidylcholine (Sigma, P5638) was dissolved with 0.5 mL of chloroform and the film was dried for one hour onto a glass test tube with nitrogen gas. The lipid film was kept in a vacuum for more than one hour. One milliliter of dehydration/rehydration (D/R) buffer (200 mM KCl, 5 mM MOPS-KOH, pH 7.2) was added and the suspension was vortexed until no particles were visible. The suspension was clarified with a bath sonicator at 30ºC for 10 minutes.

The channel preparation was mixed with 9.2 mg lipid at a protein-lipid ratio of 1:1,000. The mixture was supplemented with EGTA-KOH, pH 7.4 (1 mM final concentration), n-octyl-β-D-glucoside (1%), and Fluo 4 (0.03 μM). Approximately 400 mg of Bio-Beads SM-2 Adsorbents (BioRad, Hercules, CA, USA) prehydrated in wash buffer (D/R buffer supplemented with 1 mM EGTA-KOH, pH7.4) was added to the sample and the mixture was incubated for 3 hours. The liquid fraction was taken and incubated with fresh Bio-Beads overnight. The fluorescent dye that was not incorporated in liposomes was removed by exchanging the buffer with wash buffer

using a desalting column (Zeba Spin Desalting Column, 7K MWCO, Thermo Scientific, Rockford, IL, USA).

The fluorescence of Fluo4 incorporated into liposomes was measured with a fluorescence spectrometer (PerkinElmer LS55, Waltham, MA, USA). Excitation and emission wavelengths were 495 and 527 nm, respectively (slit width: 5 nm). Ca^{2+} concentrations were increased by sequentially adding $CaCl_2$ solution. Liposomes were lysed by adding 1% (final concentration) n-octyl-β-D-glucoside at the end of measurements to obtain a measure of the total amount of Fluo-4 and normalize the measurements.

Patch clamp experiments on liposomes

A lipid suspension was prepared as described above. The channel preparation was mixed with 5 mg lipid at a protein-lipid ratio of 1:1,000. n-Octyl-β-D-glucoside was added to yield a final concentration of 1%. The sample was incubated at 4ºC on a seesaw shaker for one hour. Approximately 400 mg of Bio-Beads prehydrated in D/R buffer was added to the sample and incubated for 3 hours. The liquid fraction was taken and incubated with fresh Bio-Beads overnight. The liquid fraction was ultracentrifuged at ~160,000*g* for 60 minutes. The pellet was suspended in 50 µL dehydration buffer consisting of 10 mM MOPS-KOH, pH 7.2, and 5% ethylene glycol. The suspension was spotted on an 8-well slide and dehydrated under a vacuum at 4ºC overnight. Between 10 and 20 µl of D/R buffer was placed onto the spots and kept in a moist chamber for more than one hour. The rehydrated preparation was placed in patch clamp buffer (200 mM KCl, 90 mM $MgCl_2$, 10 mM $CaCl_2$, 5 mM Hepes, pH 6.0) and allowed to form blisters.

The blister membrane was caught onto a glass pipette and excised from the blister. The pipette contained the same buffer as the bath solution. The holding potential was controlled and the current was amplified with a patch clamp amplifier (Axopatch 200B, Axon Instruments, Union City, CA, USA). Current recordings were filtered at 2 kHz and digitized at 5 kHz using a Digidata 1322A interface with pCLAMP 9 software (Axon Instruments). Negative pressure was applied to the patch membrane through the pipette using a High-Speed Pressure Clamp-1 apparatus (HSPC-1; ALA Scientific Instruments, Westbury, NY, USA).

Statistical analysis

Two-sided *t*-test was applied where statistical analysis was performed. Equal variance was not assumed.

Data availability

The data that support all experimental findings of this study are available within the paper.

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Author Contributions

H.I. conceived the research. K.Y., K.I. and H.I. designed the experiments. K.I. performed the experiments of protein synthesis and purification. K.Y. performed the experiments of fluorescence measurements and patch-clamp analyses. K.Y., K.I. and H.I. analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Figure legends

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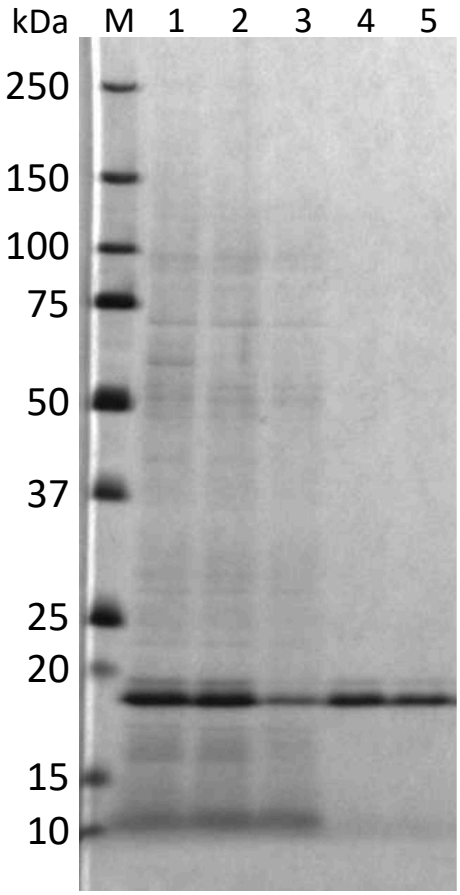

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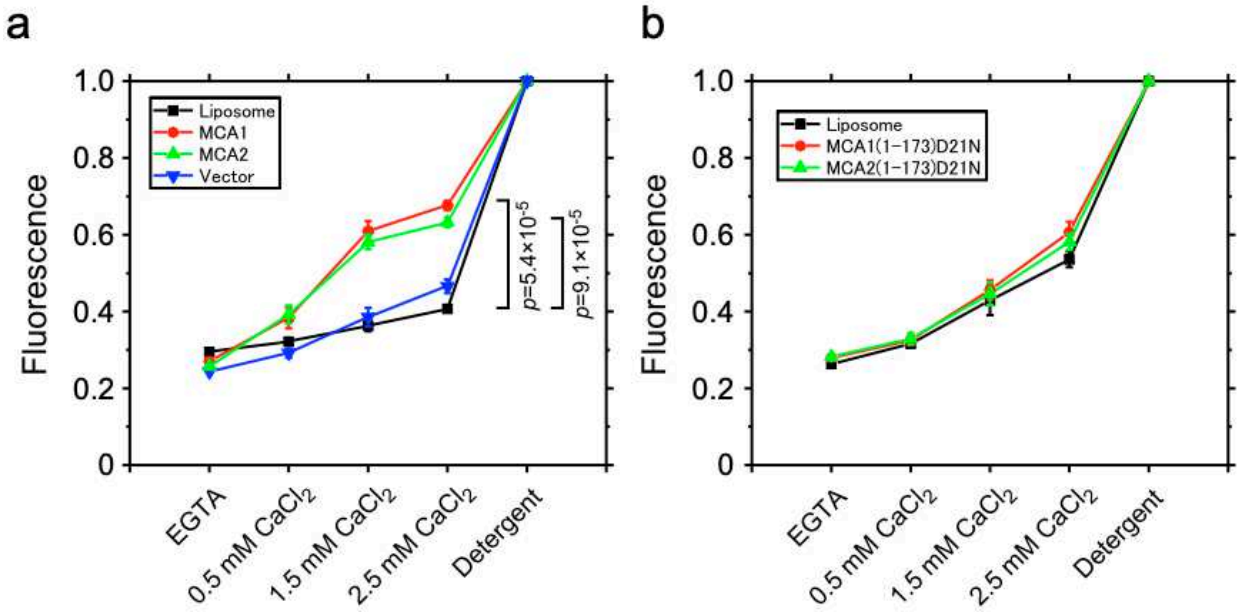

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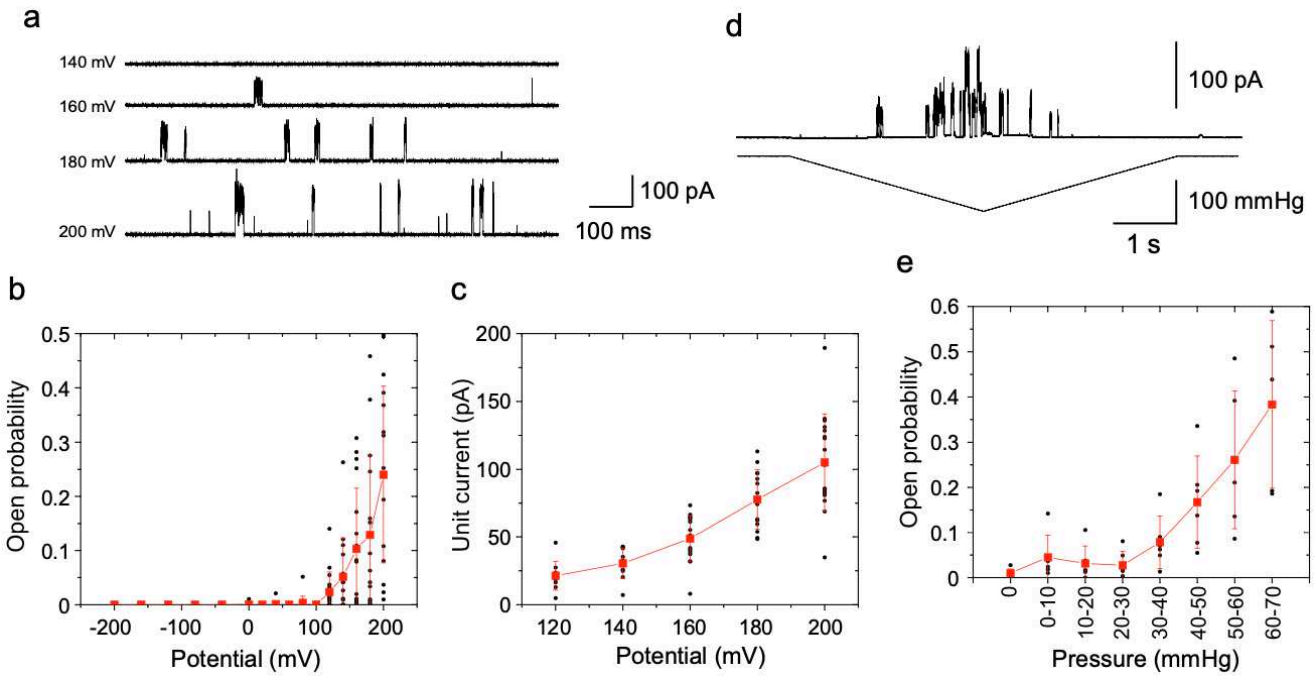

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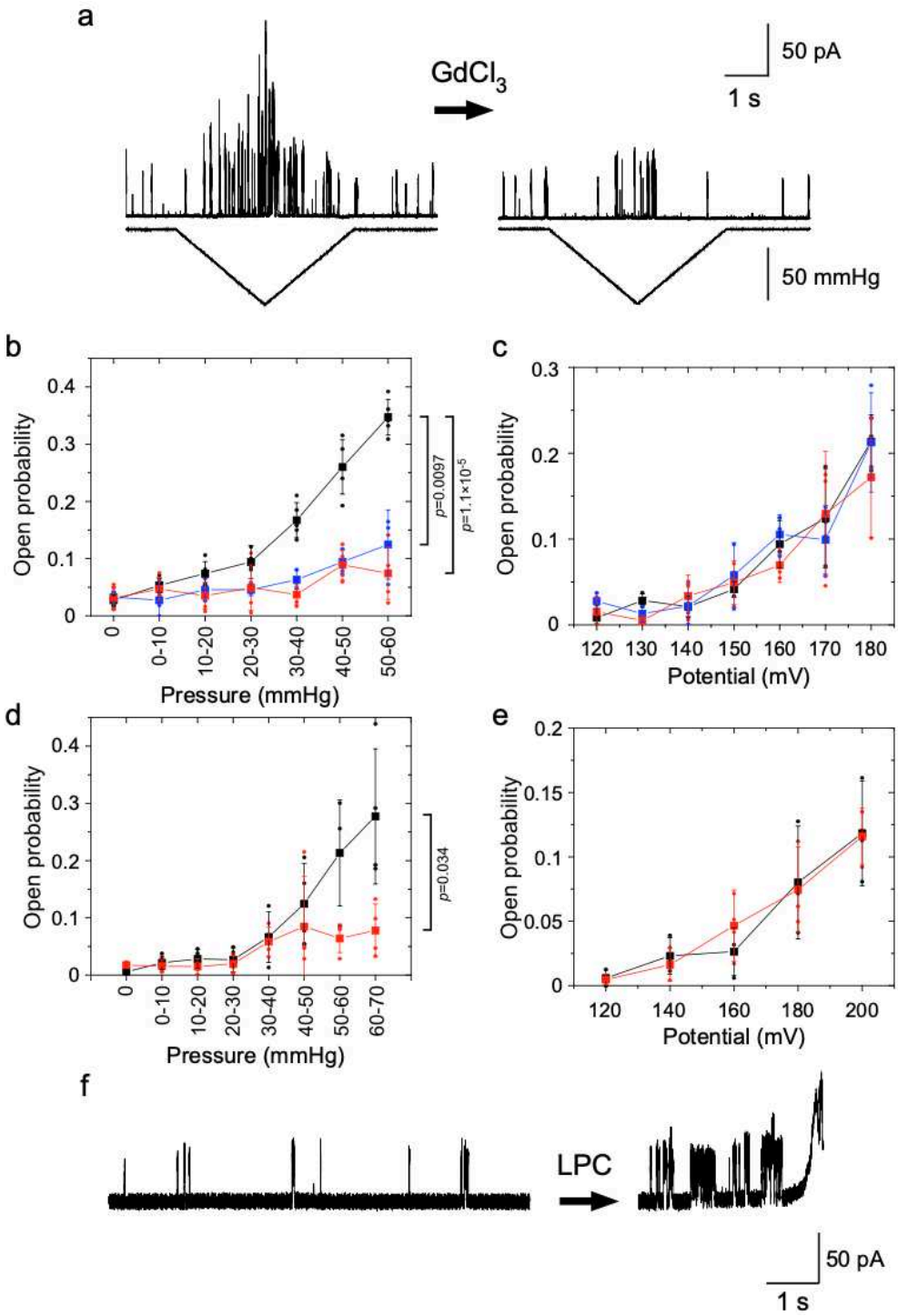

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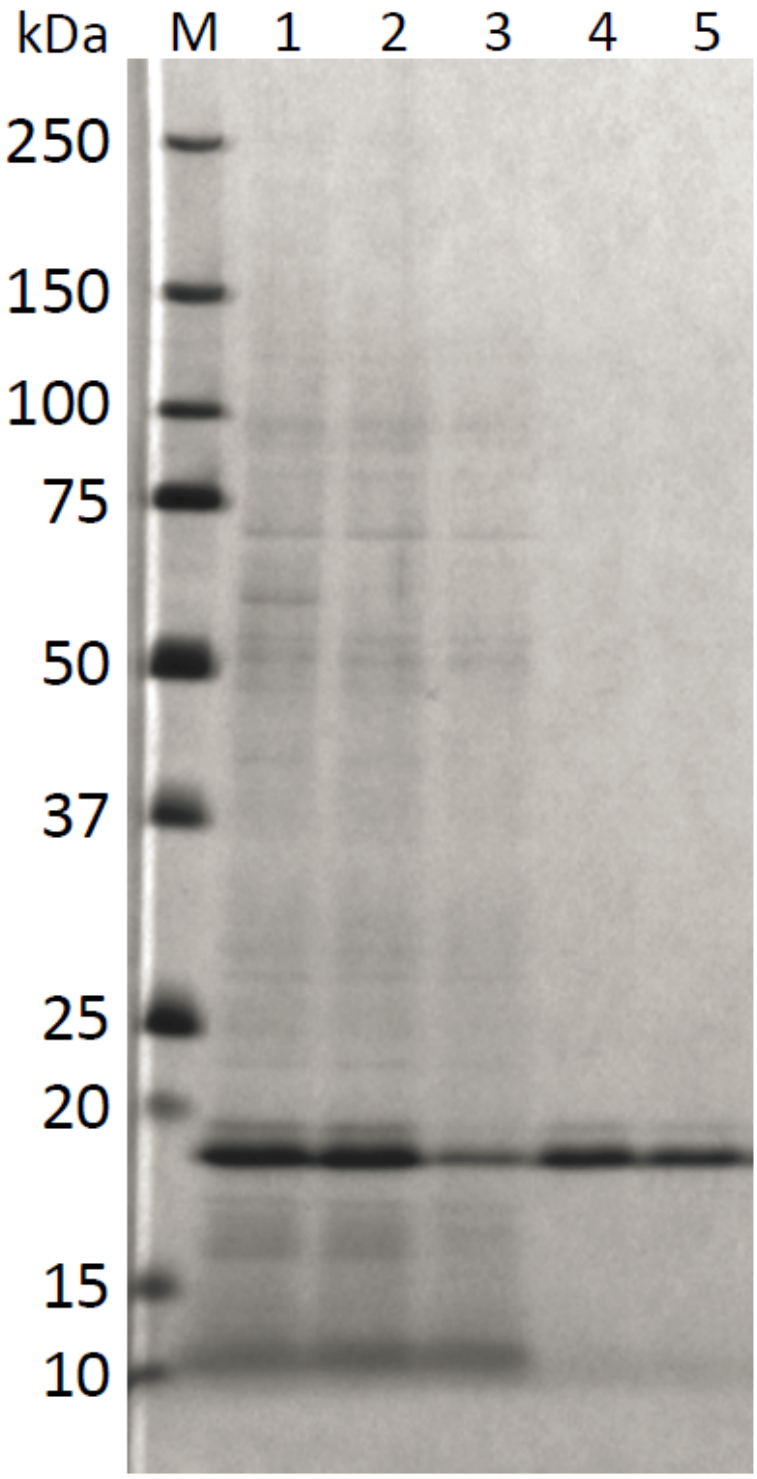
Figures



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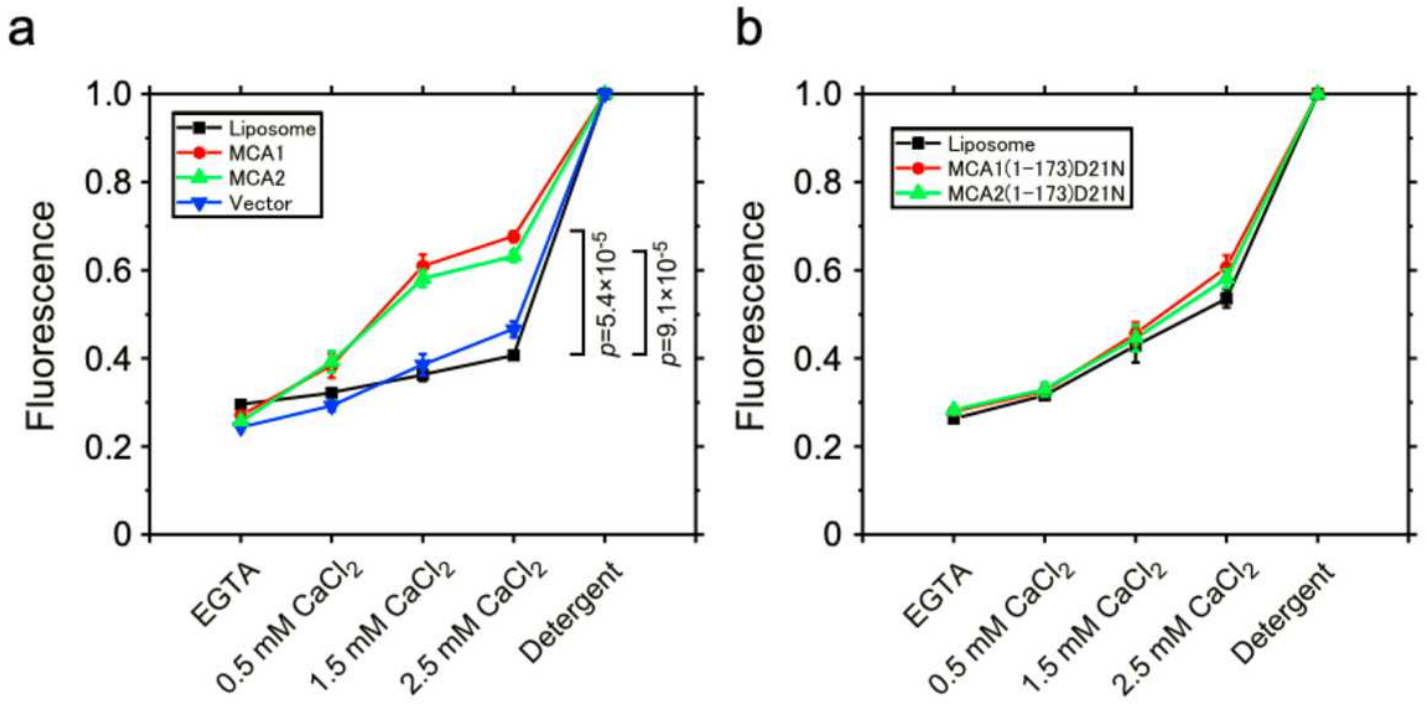

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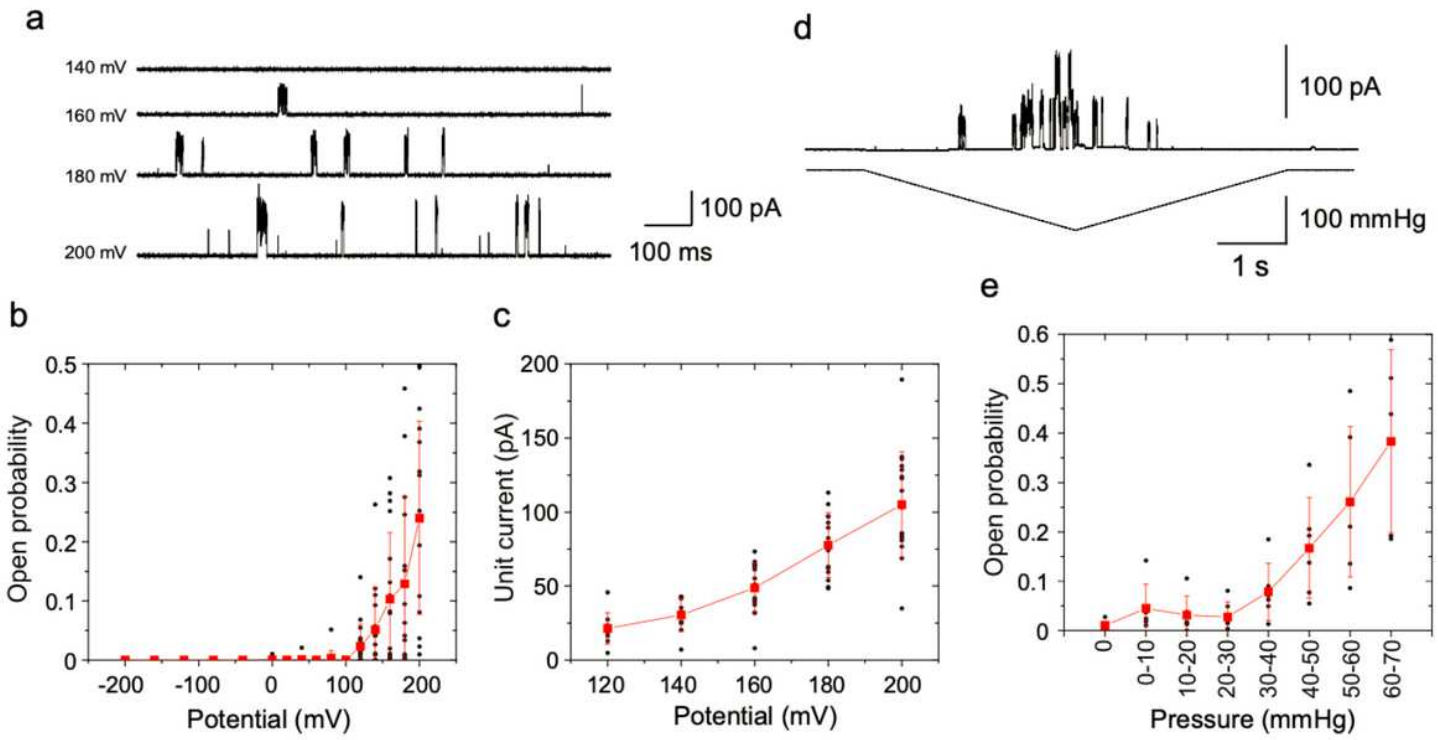

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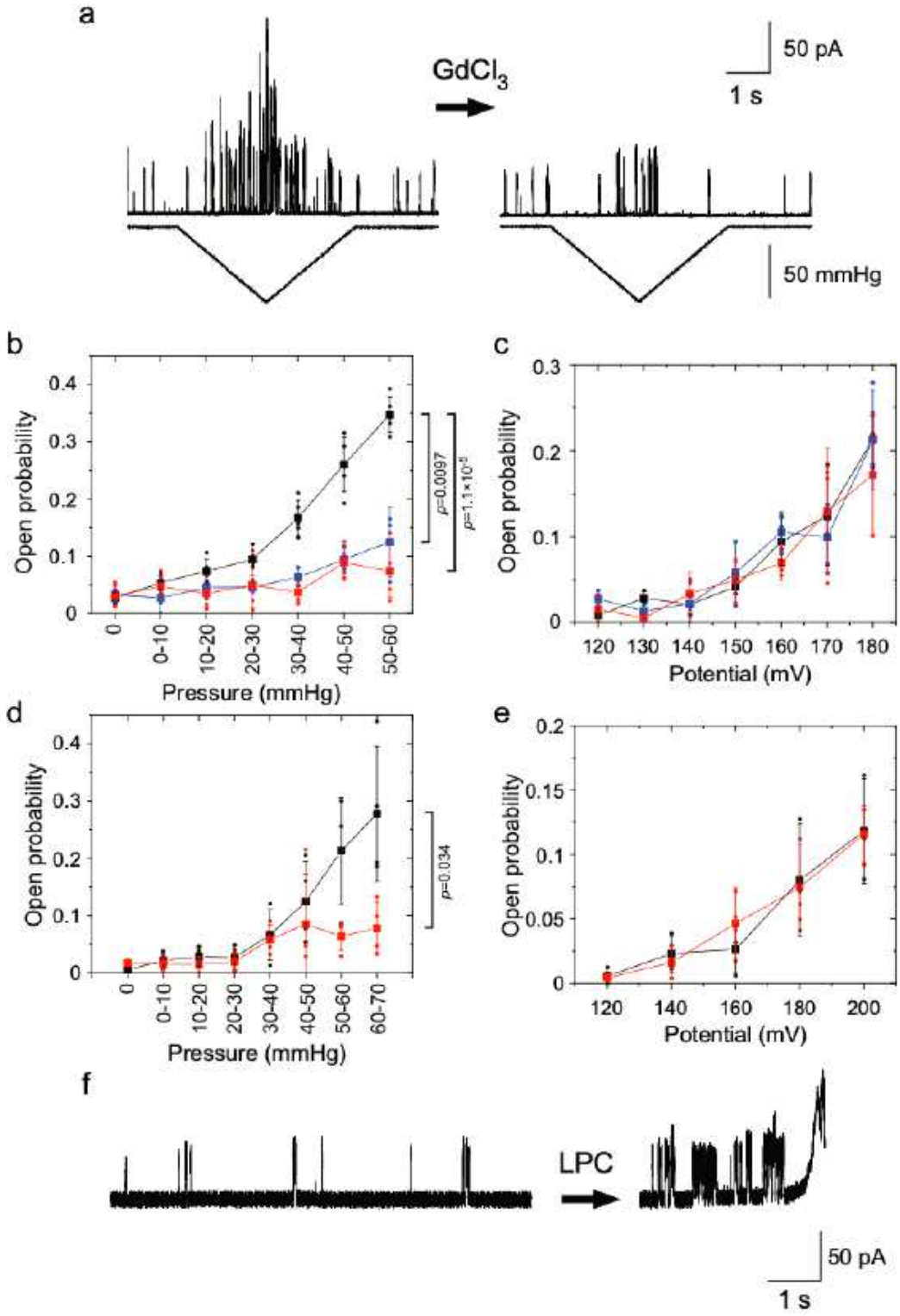

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