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A mechanically activated ion channel is functionally expressed in the MrgprB4 positive sensory neurons, which detect stroking of hairy skin in mice

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Abstract

Mas-related G-protein coupled receptor B4 (MrgprB4) has been reported to be expressed in the dorsal root ganglion (DRG) neurons which detect stroking of hairy skin of mice. However, the mechanisms by which the MrgprB4 positive (+) neurons respond to adequate stimulus remain unsolved as it was also reported that electrophysiological analysis of cultured MrgprB4+ neurons did not reveal responses to mechanical stimuli. Contrary to the observation, however, in this study we show that the MrgprB4+ neurons functionally express a mechanically activated channel using DRG neurons dissociated from genetically-modified mice whose MrgprB4+ neurons express a red fluorescent protein. Hypotonicity-induced cell swelling increased intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) of MrgprB4+ neurons. The $[\text{Ca}^{2+}]_i$ increases were prevented by extracellular Ca^{2+} removal and by applications of nonselective Piezo channel blockers. Patch clamp analysis revealed that the MrgprB4+ neurons exhibited rapidly-adapting mechanically-activated currents. The MrgprB4+ neurons were stained with anti-Piezo2 antibody. These results raise the possibility that the MrgprB4+ neurons directly detect the stroking-like stimuli of hairy skin.

Keywords: Mas-related G-protein coupled receptor member B4; Dorsal root ganglion neuron; Swelling-induced Ca^{2+} influx; Rapidly-adapting mechanically-activated currents; Piezo2

1. Introduction

The Mas-related G-protein coupled receptors (Mrgpr) are predominantly expressed in a subset of small-diameter dorsal root ganglion (DRG) neurons and can be used as a marker for specific functionally distinct sensory neurons [1-3]. Among the Mrgprs, MrgprB4 has been reported to be expressed in the C-fiber somatosensory neurons which were activated by gentle stroking with a brush on the hairy skin of mice *in vivo* [3,4]. Behavioral testing indicated that the excitation of the MrgprB4 positive (+) neurons was positively reinforcing [3]. However, there has been no data showing that the MrgprB4+ neurons are *per se* mechanosensitive *ex vivo* [3] as Liu *et. al.* mentioned: “Electrophysiological analysis of MrgprB4+ neurons in culture has thus far failed to reveal responses to any thermal or mechanical stimuli tested” [4]. Therefore, the mechanisms by which the MrgprB4+ neurons respond to adequate stimulus are still unknown.

Piezo1 and Piezo2 are mechanically activated cation channels [5,6] and have been revealed to mediate responses to mechanical stimuli in many types of cells [5,7-10]. In DRG neurons, though Piezo1 is not expressed, Piezo2 mediates the rapidly-adapting mechanically-activated (RA-MA) currents, which are characterized by their rapid decay time constants under whole-cell patch clamp conditions [5,7]. Furthermore, Piezo2 has been reported to be responsible for the mechanosensitivity of many A β low-threshold mechanoreceptors

involved in innocuous touch sensation [7]. However, the expression of Piezo2 in the MrgprB4+ neurons has not been clarified.

A simple method to evaluate the mechanosensitivity of cells is an application of hypotonic solutions, which results in cell swelling, under the measurement of intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) [10]. However, the responses to the cell swelling alone cannot be used as evidence of direct mechanosensitivity as the cell swelling also causes cytosolic alterations [10]. Another method is a transient membrane indentation by a piezo-electrically driven glass probe under whole-cell patch clamp conditions, by which the RA-MA currents have been measured [10]. The recent discoveries that the cell swelling potentiates the RA-MA currents [11] will also make it more reliable to evaluate the mechanosensitivity mediated by the Piezo channels.

Contrary to the observation mentioned in the literature [4], in the current study, we demonstrate that the MrgprB4+ neurons functionally express at the least a mechanosensitive channel. We utilized genetically-modified mice whose MrgprB4+ neurons express a red fluorescent protein [3] in order to identify such neurons. We evaluated the mechanosensitivity of the cultured MrgprB4+ neurons using two types of mechanical stimuli, the hypotonic stimuli and membrane indentations by a glass probe. We also examined the expression of Piezo2 in the MrgprB4+ neurons using immunostaining.

2. Material and methods

2.1 Animals

Animal care and experimental protocols were approved by the institutional animal care and use committee in the Graduate School of Veterinary Medicine, Hokkaido University. The B6N.129S1-Mrgprb4^{tm3(cre)And}/J mice (homozygotes) were obtained from the Jackson Laboratory (Bar Harbor, ME). In these mice, the membrane-tagged tdTomato (mtdTomato), a red fluorescent protein, is constitutively expressed in MrgprB4+ DRG neurons as the entire open reading frame of *Mrgprb4* gene was replaced by an mtdTomato-2A-NLSCre-Frt-PGK-Neomycin-FRT cassette [3]. Male and female adult mice older than 8 weeks of age were used.

2.2 Cell preparation

Thoracic DRGs were dissociated from mice euthanized by CO₂ inhalation. The DRGs were enzymatically digested with collagenase type II (Worthington, Lakewood, NJ) and DNase (Roche, Basel, Switzerland) and subsequently with trypsin (Worthington). After trituration, dissociated cells were plated on poly-L-lysine (Sigma-Aldrich, St Louis, MO) coated coverslips and cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with

fetal bovine serum (Moregate Biotech, Blimba, Australia or Thermo Fisher, Waltham, MA), and penicillin/streptomycin (Thermo Fisher). For the patch clamp experiments, the coverslips were coated with poly-L-lysine and laminin 511-E8 (iMatrix-511, Nippi, Tokyo, Japan). The cells were used 16–32 h after plating.

2.3 Immunocytochemistry

The neurons were fixed by incubation in PLP fixative (2% paraformaldehyde, 75 mM lysine, 37 mM sodium phosphate, 10 mM sodium periodate) for 3 hours at 4°C. The fixed neurons were permeabilized in 0.1% Triton X-100 solution (MP Biomedicals, Santa Ana, CA). The nonspecific bindings of IgG were blocked by incubations in image-iT signal enhancer (Thermo Fisher scientific) and subsequently in a solution containing 5% normal goat serum and 0.2% bovine serum albumin. The neurons were incubated with an anti-Piezo2 rabbit antibody (#sc-84763, Santa Cruz Biotechnology, Dallas, TX) or the anti-Piezo2 antibody which was treated with its antigen. The neurons were further incubated with an Alexa 488-conjugated F(ab')₂ fragment of goat anti-rabbit IgG(H+L) (Thermo Fisher) and DAPI. Images were captured with a confocal laser scanning microscope (LSM700, Carl Zeiss, Oberkochen, Germany).

2.4 Intracellular Ca^{2+} imaging

Intracellular Ca^{2+} imaging was conducted as has been described previously [12] with certain modifications. The neurons were incubated with 10 μ M fura-2 acetoxymethyl ester (Dojindo, Kumamoto, Japan) and 0.002% cremophor EL (Sigma-Aldrich) in the standard extracellular solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH = 7.4). Fura-2 fluorescence was measured using an inverted microscope (Diaphot 300, Nikon, Tokyo, Japan) with a fluorescence ratio imaging system (AQUACOSMOS, Hamamatsu Photonics, Shizuoka, Japan). The MrgprB4⁺ neurons were identified by the red fluorescence of mtdTomato. The neurons were illuminated at 340 and 380 nm for 61.1 ms at 2 s intervals and the fluorescence signals (F_{340} and F_{380} , respectively) were detected at 510 nm. The intracellular calcium concentration ($[Ca^{2+}]_i$) was calculated using the following equation [13].

$$[Ca^{2+}]_i = K_d \times (R - R_{min}) / (R_{max} - R) \times F_{380 free} / F_{380 bound}$$

$K_d = 259$ nM at 20 °C [14]. R is F_{340}/F_{380} . The minimum ratio (R_{min}) and the maximal ratio (R_{max}) were determined by measurements as described elsewhere [13,15]. $F_{380 free}/F_{380 bound}$ is the ratio of F_{380} of R_{min} to that of R_{max} [13]. The hypotonic stimuli were imposed by lowering the extracellular NaCl concentration to 90 mM (192 milliosmolar, measured with a Vapro Vapor Pressure 5600 (Wescor Inc., Logan, UT)) from 140 mM (the standard extracellular solution, 292 milliosmolar). All experiments were performed at room temperature.

2.5 Whole-cell patch-clamp recordings

The methods used for measurements of whole-cell currents were similar to those described previously [16]. The pipette solution was composed of 130 mM CsCl, 10 mM HEPES, 10 mM EGTA, 4 mM MgCl₂, 2 mM ATP disodium salt, and 70 mM sucrose (pH = 7.4). The sucrose was added in order to make the osmolality of the pipette solution comparatively hypertonic (363 milliosmolar) [11]. The extracellular solution was ordinarily the standard extracellular solution. In most experiments, the membrane currents were recorded at a holding potential of -84 mV, which was corrected for the liquid junction potential measured. The whole-cell currents were filtered at 2 kHz, and sampled at 5 kHz. Leak currents before mechanical stimuli were subtracted from the current traces. The cell capacitance of mtdTomato⁺ cells was 29.1 ± 0.8 pF (n = 21). The series resistance was 6.5 ± 0.5 M Ω (n = 21).

To apply mechanical stimuli, the membranes of DRG cell bodies were displaced by a heat-polished glass pipette. The probe was positioned at an angle of 45° to the surface of a coverslip and its movement was driven by a Clampex 8 controlled piezo-actuator (PZ38, piezosystem jena, Jena, Germany). Prior to each application of mechanical stimuli, the probe was placed just above the plasma membrane of a cell body without making obvious contact. This initial position was defined as 0 μm . The contact of the probe with the membrane was

monitored by the movement of the membrane when the probe was moved horizontally. The probe had a velocity of 0.5 $\mu\text{m}/\text{msec}$ during the displacement. A series of mechanical steps in 1 μm increments up to 8 μm was applied every 5 sec. When mechanical stimuli by 8 μm displacements were repeated, they were applied at 10-second intervals.

2.6 Statistical analysis

All average results are presented as mean \pm standard error. Statistical significance was evaluated using Student's two-tailed t test or Dunnett's test as appropriate. A value of $p < 0.05$ was considered significant.

3. Results

3.1 Hypotonic stimuli increases $[\text{Ca}^{2+}]_i$ of MrgprB4+ neurons.

We firstly evaluated the mechanosensitivity of the MrgprB4+ neurons by measuring the $[\text{Ca}^{2+}]_i$. The percentage of the MrgprB4+ neurons, which were identified by the fluorescence of mtdTomato (Fig. 1A), in the dissociated DRG neurons was small ($2.7 \pm 0.3\%$, $n = 4$ preparations from 4 mice). The average cell diameter of the live MrgprB4+ neurons was $26.9 \pm 0.2 \mu\text{m}$ ($n = 103$). These values are consistent with those reported elsewhere [4]. The application of a hypotonic solution (90 mM NaCl) increased $[\text{Ca}^{2+}]_i$ of the MrgprB4+ neurons (Fig. 1B and

1C) and simultaneously induced cell swelling (Fig. 1B, inset). When the osmolality was maintained at a normal level by adding 100 mM mannitol, the increase in $[Ca^{2+}]_i$ was negligible (Figs 1B and 1C). Therefore, the increase in $[Ca^{2+}]_i$ by application of the 90 mM NaCl solution is considered to be induced not by the reduction in the NaCl concentration but by the reduction in the osmolality.

3.2 Hypotonicity-induced $[Ca^{2+}]_i$ increase is blocked by Piezo channel blockers but not by TRP channel antagonists.

The hypotonicity-induced $[Ca^{2+}]_i$ increase was markedly decreased by removal of the extracellular Ca^{2+} (Figs 2A and 2B), indicating that the $[Ca^{2+}]_i$ increase was mediated by influx of Ca^{2+} . The hypotonicity-induced $[Ca^{2+}]_i$ increase was significantly inhibited by 10 μ M ruthenium red, 30 μ M gadolinium chloride, and 20 μ M FM1-43. These compounds have been reported to inhibit Piezo channels [5,6,17] although they are not specific blockers. Ruthenium red is also known as an inhibitor of some transient receptor potential (TRP) channels [18]. Among the ruthenium red sensitive TRP channels, TRPV4 was reported to be a swelling-activated channel in mouse DRG neurons [19] though there is also a contradictory report [20]. In the current study, the hypotonicity-induced $[Ca^{2+}]_i$ increase in the MrgprB4+ neurons was not inhibited by 1 μ M HC067047, a TRPV4 selective antagonist [21] (Fig. 2C).

Among other ruthenium red sensitive channels, TRPV2, TRPA1, and TRPV1 were also reported to mediate the $[Ca^{2+}]_i$ increase induced by changes in extracellular osmolality [22-25]. However, the hypotonicity-induced $[Ca^{2+}]_i$ increase in the MrgprB4+ neurons was neither inhibited by 100 μ M tranilast, a TRPV2 antagonist [23], nor by 10 μ M HC030031, a TRPA1 selective antagonist [12] (Fig. 2D). Consistent with the report that MrgprB4+ neurons did not express TRPV1 [4], 1 μ M capsaicin, a TRPV1 agonist [25], did not increase $[Ca^{2+}]_i$ of the MrgprB4+ neurons (Supplementary Figure S1) and was unable to evoke whole-cell currents (Supplementary Figure S2). These results suggest that the MrgprB4+ neurons functionally express a swelling-activated Ca^{2+} -permeable channel, possibly mediated by Piezo2 channel.

3.3 MrgprB4+ neurons exhibit rapidly-adapting mechanically-activated (RA-MA) currents.

Next, we evaluated the mechanosensitivity of the MrgprB4+ neurons with another type of mechanical stimulus, focal deformation of the plasma membrane by an electrically driven mechanical probe, under whole-cell patch clamp recordings. We used a hypertonic pipette solution as it has been reported that Piezo2 mediated RA-MA currents were potentiated by osmotic swelling using hypertonic pipette solutions [11]. The mtdTomato+ cells exhibited negligible RA-MA currents in the initial measurements but larger currents in later measurements when the cells were swollen (Figs. 3A-C, and Supplementary Fig. S3A) decay time constants of

the currents measured 5 and 10 min after establishment of whole-cell configuration were less than 10 msec (Supplementary Fig. S3B), which is a commonly-used criterion of RA-MA currents [5]. The decay time constants were gradually increased with time (Supplementary Fig. S3B), consistent with the observation in the literature [11]. The average of the RA-MA current densities measured from the mtdTomato⁺ cells ($26.9 \pm 0.2 \mu\text{m}$) was approximately half of that measured from large diameter ($40.8 \pm 1.7 \mu\text{m}$) mtdTomato negative cells, but the difference was not statistically significant (Supplementary Fig. S3C). The reversal potential of the RA-MA currents measured from the mtdTomato⁺ cells was near 0 mV (Supplementary Fig. S3D). The replacement of extracellular Na⁺ and K⁺ with N-methyl-D-glucamine (NMDG) eliminated the RA-MA currents and the application of 100 mM Ca²⁺ recovered them (Fig. 3D). These results indicate that the RA-MA currents were mediated by a Ca²⁺-permeable nonselective cation channel similar to Piezo channels [5,26]. The RA-MA currents were inhibited by Piezo channel blockers, 10 μM ruthenium red, 30 μM gadolinium chloride, and 20 μM FM1-43 [5,6,17] (Fig. 3E).

3.4 MrgprB4⁺ neurons express Piezo2 protein.

Finally, we examined the expression of Piezo2 in the MrgprB4⁺ neurons using immunocytochemistry. The dissociated mtdTomato⁺ cells were stained with an anti-Piezo2

antibody (Figs 4A and B). The staining was attenuated when the antibody was preincubated with its immunizing peptide (Fig. 4A). There were larger cells which showed a higher intensity of Piezo2 staining than the mtdTomato⁺ cells. (Fig. 4B) This is consistent with the results that MrgprB4⁺ neurons exhibited smaller RA-MA currents than large diameter neurons (Fig. 3F). The staining in the nuclei (Figs 4A and 4B) were presumably a nonspecific staining as it was also observed in the cells lacking the staining of Piezo2 around the plasma membrane (Fig. 4B).

4. Discussion

These results of both experiments using two types of mechanical stimuli revealed that dissociated MrgprB4⁺ neurons functionally express at the least a mechanically activated channel. We think that the RA-MA currents in the MrgprB4⁺ neurons are most likely mediated by Piezo2 for the following reasons. First, the physiological and pharmacological characteristics of the RA-MA currents were similar to those mediated by Piezo2. Second, MrgprB4⁺ neurons were stained with anti-Piezo2 antibody. Finally, there is accumulating evidence that RA-MA currents in DRG neurons are mediated by Piezo2 [5,7,11]. The RA-MA current densities in the MrgprB4⁺ neurons were relatively smaller than those of the mtdTomato⁻ neurons although the difference was not statistically significant. This is probably due to the lower expression level of Piezo2. However, we also cannot deny the possibility that the difference is due to other

unknown components such as interacting proteins of Piezo2. Piezo channels have also been reported to mediate the responses to hypotonic stimuli [9,27]. Therefore, Piezo2 is the first candidate for the channel which mediates the hypotonicity-induced $[Ca^{2+}]_i$ increase in the MrgprB4⁺ neurons. The results of the pharmacological analyses support the possibility. However, experiments with knock down or knock out of *Piezo2* in the MrgprB4⁺ neurons are necessary to fully prove the contribution of Piezo2 to the mechanosensitivity of the MrgprB4⁺ neurons.

The reason the functional expression of the mechanically activated channel in the MrgprB4⁺ neurons has not been reported might be simply due to the low expression level of Piezo2 in the MrgprB4⁺ neurons. When we used a typical isotonic pipette solution, the RA-MA currents were small similar to the initial currents shown in Fig.3B (data not shown). Therefore, unless using hypertonic pipette solutions, the small RA-MA currents in the MrgprB4⁺ neurons might tend to be missed. However, we also cannot deny the possibility that differences in experimental conditions, such as culture conditions, influenced the expression of Piezo2 in the MrgprB4⁺ neurons.

As a mechanism of the swelling-induced potentiation of RA-MA currents, it was suggested that the number of Piezo2 channels in the plasma membrane was augmented by the increases of static plasma membrane tension [11]. The reason the initial currents in the

MrgprB4⁺ neurons were small may be because the plasma membrane tension of cell bodies of the cultured MrgprB4⁺ neurons was not so high as to increase the number of Piezo2 protein in the plasma membrane. Concerning physiological conditions *in vivo*, we have to consider the situation of peripheral projections of the MrgprB4⁺ neurons under the skin. It may depend on the transportation of Piezo2 to the periphery and the plasma membrane tension of the projections whether Piezo2 is expressed in the plasma membrane of the projections of the MrgprB4⁺ neurons. Further studies are needed to clarify whether the expression level of Piezo2 in the peripheral projections of MrgprB4⁺ neurons is enough to depolarize the membrane to threshold for triggering an action potential through its activation by mechanical stimuli on hairy skin *in vivo*.

Though MrgprB4 is thought to be a G-protein coupled receptor, its endogenous ligands and physiological roles in the MrgprB4⁺ neurons are still unknown. However, the genetically-modified mice which were used in this study are unsuitable for the experiments to reveal the physiological significance of MrgprB4. That is because in the mice the open reading frame of *Mrgprb4* is knocked out and replaced with the gene encoding mtdTomato so that the MrgprB4⁺ neurons of the mice do not express MrgprB4 but express mtdTomato instead. Though the mice were also used in the study which revealed the physiological function of the MrgprB4⁺ neurons [3], the lack of MrgprB4 might attenuate the Piezo2 expression in the

neurons as the knockout of *Mrgprd* was reported to decrease excitability in cutaneous polymodal nociceptors [28]. Further studies are needed to clarify the role of signal transduction through MrgprB4 in the modulation of the mechanosensitivity of the MrgprB4+ neurons.

In this study, we demonstrated that the dissociated and cultured MrgprB4+ neurons showed mechanosensitivity. This finding suggests the possibility that the MrgprB4+ neurons are directly activated by the stroking-like mechanical stimuli on hairy skin *in vivo*. However, there is also another possibility that the MrgprB4+ neurons are indirectly activated by transmitters secreted from cells in the skin, e.g. by ATP released from keratinocytes [29] as the MrgprB4+ neurons were reported to respond to ATP [4]. To clarify the mechanisms by which the MrgprB4+ neurons are activated *in vivo*, we need further *in vivo* experiments using, for example, conditional knock-out mice, lacking Piezo2 specifically in the MrgprB4+ neurons. However, the findings presented in this study may serve as a foundation to design further investigations because they raise the possibility that the MrgprB4+ neurons directly respond to the stroking-like stimuli on hairy skin.

5. Conclusion

In conclusion, this study provided evidence that the cultured MrgprB4+ neurons functionally express a mechanically activated channel, which is presumably Piezo2. These novel findings

may prove of benefit in understanding the mechanisms by which mammals detect stimuli that lead to tactile pleasant sensation in hairy skin.

Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DRG, dorsal root ganglion; EGTA, ethylene glycol tetraacetic acid; MrgprB4, Mas-related G-protein coupled receptor member B4; mtdTomato, membrane-tagged tdTomato; NMDG, N-methyl-D-glucamine; RA-MA, rapidly-adapting mechanically-activated; TRP, transient receptor potential

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Figure Legends

Fig. 1. Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of the MrgprB4 positive (+) neurons was increased by the hypotonic stimuli. (A) An image of a fixed dorsal root ganglion (DRG) neuron exhibiting the fluorescence of mtdTomato (magenta) with confocal microscopy. Nuclei were stained with DAPI (cyan). (B) A representative data showing that $[\text{Ca}^{2+}]_i$ of a MrgprB4+ neuron, which was identified with the fluorescence of mtdTomato, was gradually increased by a hypotonic stimulus (NaCl (90)). An addition of 100 mM mannitol to the low NaCl solution prevented the $[\text{Ca}^{2+}]_i$ increase (NaCl (90) Mannitol (100)). The rapid increase in $[\text{Ca}^{2+}]_i$ by the application of an isotonic 60 mM KCl solution (KCl (60)) indicates that the cell was an excitable cell. (inset) The images of the cell captured by an excitation wavelength at 340 nm. The time when the images were captured are shown in parentheses. Area of the cell was increased to 122.5% of the original after the hypotonic stimulus and mostly returned to the original by the perfusion of the isotonic solution (100.3%). (C) A summary of the $[\text{Ca}^{2+}]_i$ increase ($\Delta[\text{Ca}^{2+}]_i$) for 2 minutes. The basal increase in $[\text{Ca}^{2+}]_i$ under the normal condition (NaCl (140)) was obtained from the $[\text{Ca}^{2+}]_i$ between 1 min and 3 min. $n = 5$. $**p < 0.01$ vs. NaCl (140).

Fig. 2. Hypotonicity-induced $[Ca^{2+}]_i$ increase was inhibited by removal of extracellular Ca^{2+} or addition of nonselective Piezo channel blockers. (A) Representative traces of $[Ca^{2+}]_i$ of MrgprB4⁺ neurons. Hypotonic stimulus was applied for 2 minutes using a solution containing 90 mM NaCl (a, control). The hypotonicity-induced $[Ca^{2+}]_i$ increase was diminished in the absence of extracellular Ca^{2+} (b, Ca (0), using 5 mM EGTA) or in the presence of 10 μ M ruthenium red (c, RR), 30 μ M gadolinium chloride (d, Gd), or 20 μ M FM1-43. (B) Summary of the increase in $[Ca^{2+}]_i$ for 2 minutes. $n = 6$ or 7 . $**p < 0.01$ vs. Control. (C) The hypotonicity-induced $[Ca^{2+}]_i$ increase in the presence of 1 μ M HC067047, a TRPV4 antagonist, was not significantly different from that in the presence of vehicle (0.003% DMSO, Control). $n = 6$ each. (D) The hypotonicity-induced $[Ca^{2+}]_i$ increases in the presence of 100 μ M tranilast, a TRPV2 antagonist, or 10 μ M HC030031, a TRPA1 antagonist, were not significantly different from that in the presence of vehicle (0.1% DMSO, Control). $n = 5$ or 7 .

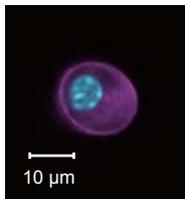
Fig. 3. MrgprB4⁺ neurons exhibit rapidly-adapting mechanically-activated (RA-MA) currents. (A) Pictures of an mtdTomato⁺ cell, a patch pipette (left), and a glass probe (right). They were taken before the seal formation (a and b), and 5 min (c) and 20 min (d) after establishment of whole-cell configuration. Bright field images (a, c, and d) and the fluorescence of mtdTomato

(b) are shown. Bars, drawn at the right bottom of each pictures, indicate 25 μm . (B) Whole-cell currents at -84 mV when mechanical stimuli (0 to 8 μm for 400 msec) were applied to the mtdTomato⁺ cell shown in A. The currents were measured as early as possible (Initial), 5, 10, 15, and 20 min after establishment of whole-cell configuration. (C) A time course of change in current densities evoked by 8 μm displacement of the glass probe. $n = 8$ each. (D) (Left) Representative currents measured in the standard extracellular solution (Na), the Na⁺- and K⁺-free solution (NMDG), and the 100 mM CaCl₂ solution (Ca). (Right) A summary of current amplitude normalized to the value in the standard extracellular solution (Na). $n = 3$ or 4. ** $p < 0.01$. (E) (Left) Representative currents in the presence of 10 μM ruthenium red (RR) (a), 30 μM gadolinium chloride (Gd) (b), or 20 μM FM1-43 (c) or prior to the applications of these compounds (Control). (Right) Summaries of current amplitude normalized to the value of controls. The currents were recovered after wash (Wash). $n = 5$ each. ** $p < 0.01$.

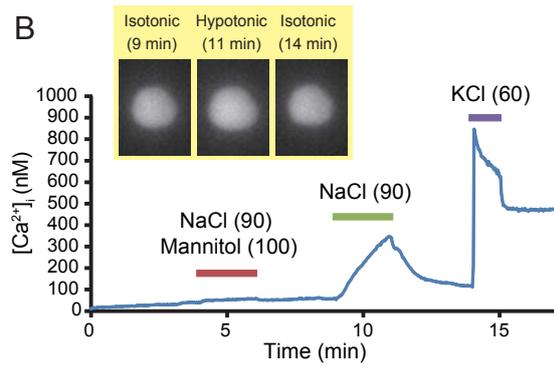
Fig. 4. MrgprB4⁺ neurons express Piezo2 protein. (A) Dissociated mtdTomato⁺ cells which were incubated with an anti-Piezo2 antibody (left) or an antigen-preabsorbed anti-Piezo2 antibody (right). Intensity of Piezo2 staining (green, top left) in mtdTomato⁺ (magenta, middle) cells was attenuated when the cells were incubated with an antibody which was preabsorbed with its immunizing peptide (top right). Nuclei were stained with DAPI (cyan, middle). The

differential interference contrast (DIC) images are shown at the bottom. (B) Lower magnification images for the comparison of the expression level of Piezo2 among the DRG neurons. The arrow indicates an mtdTomato⁺ cell. The arrowheads indicate cells lacking the staining of Piezo2 around the plasma membrane.

A



B



C

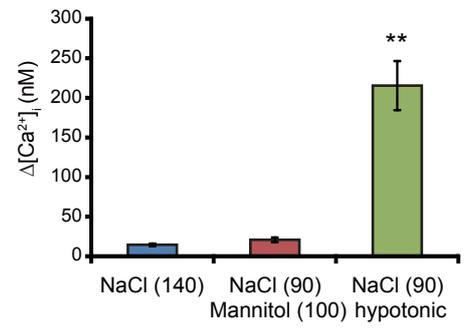


Figure 1 Yamaguchi S

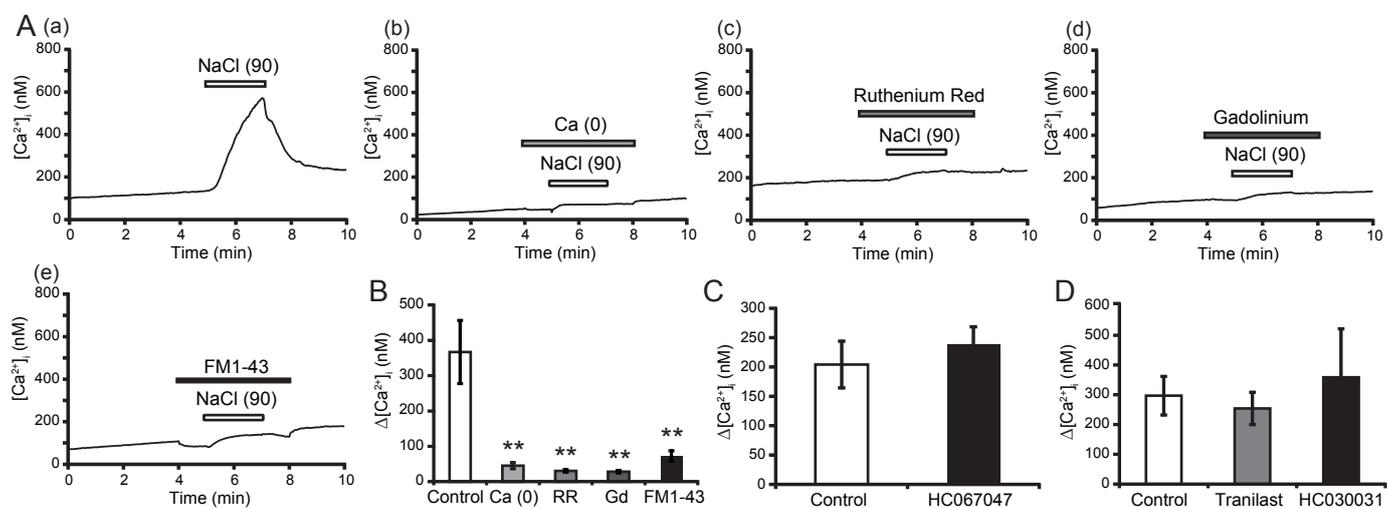


Figure 2 Yamaguchi S

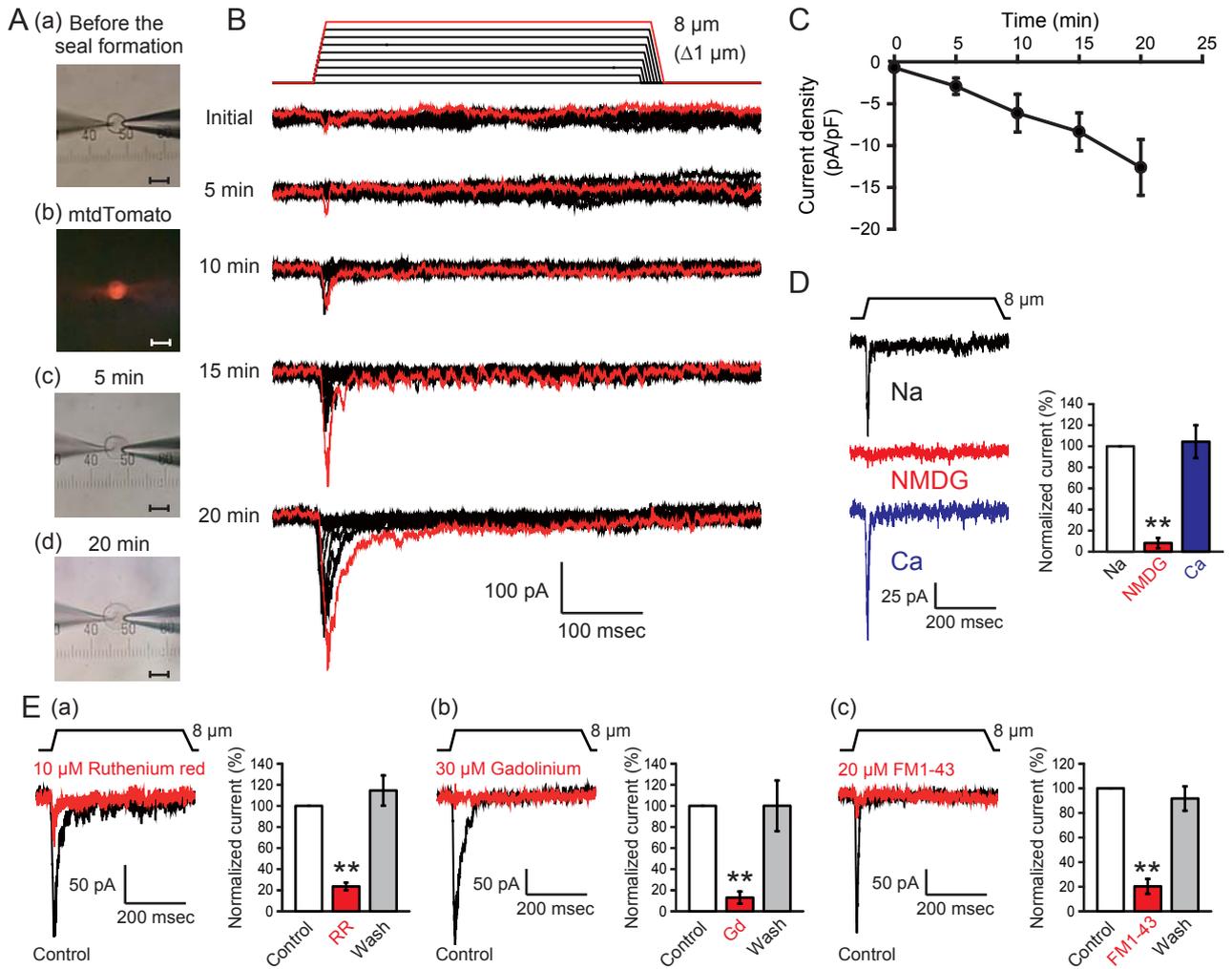


Figure 3 Yamaguchi S

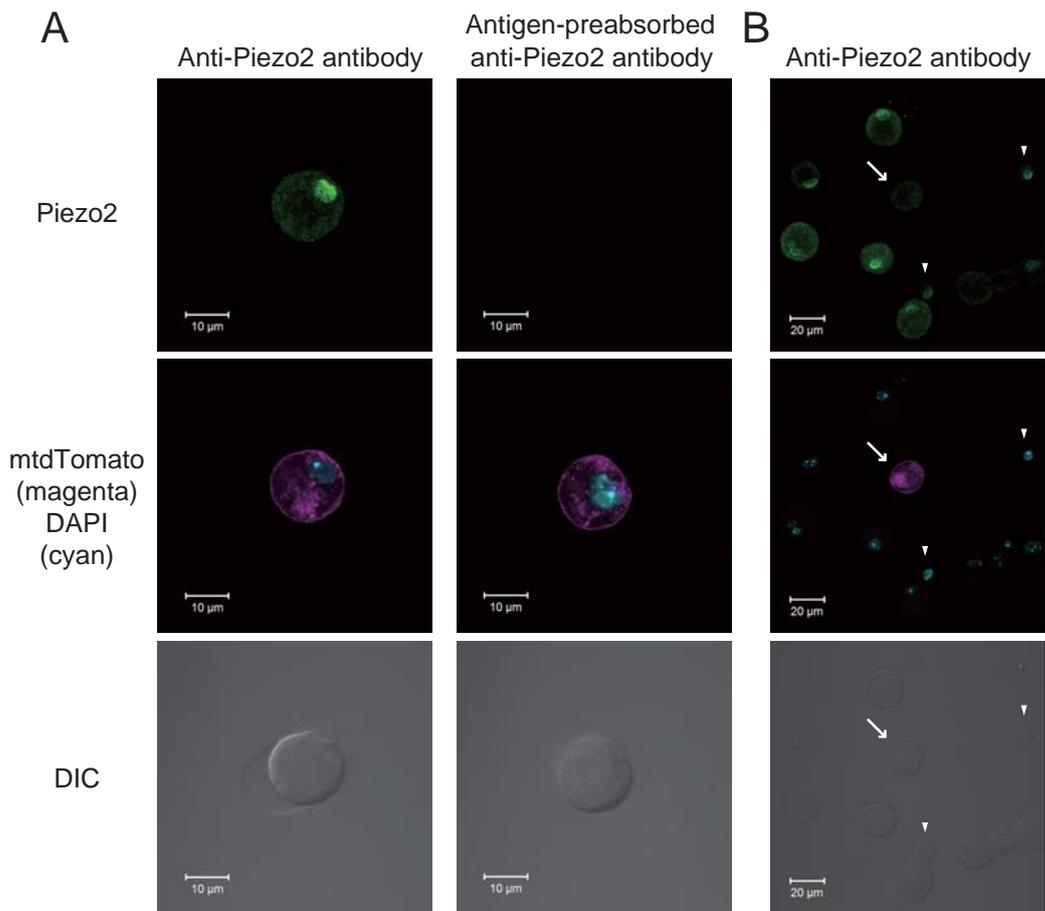
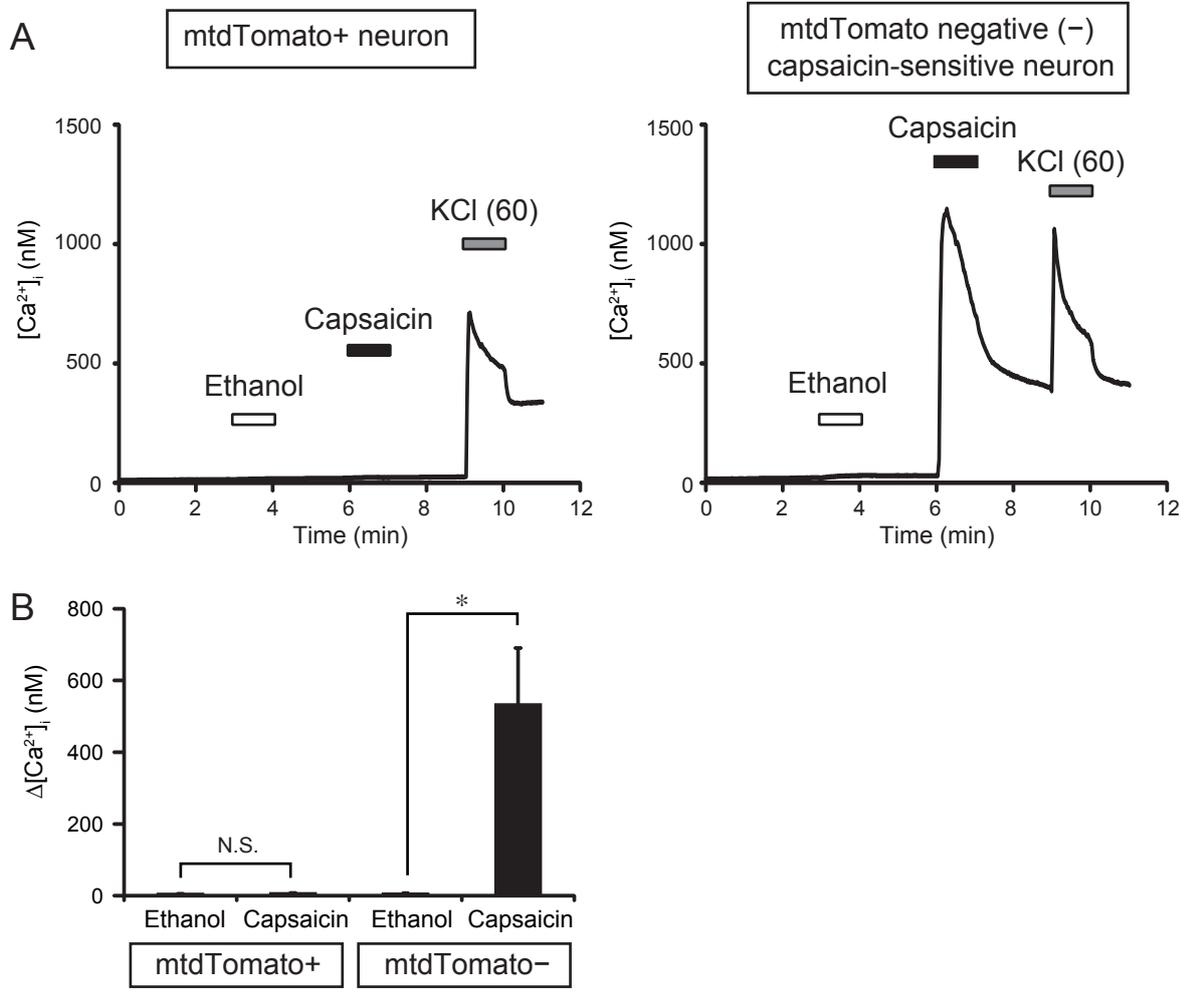
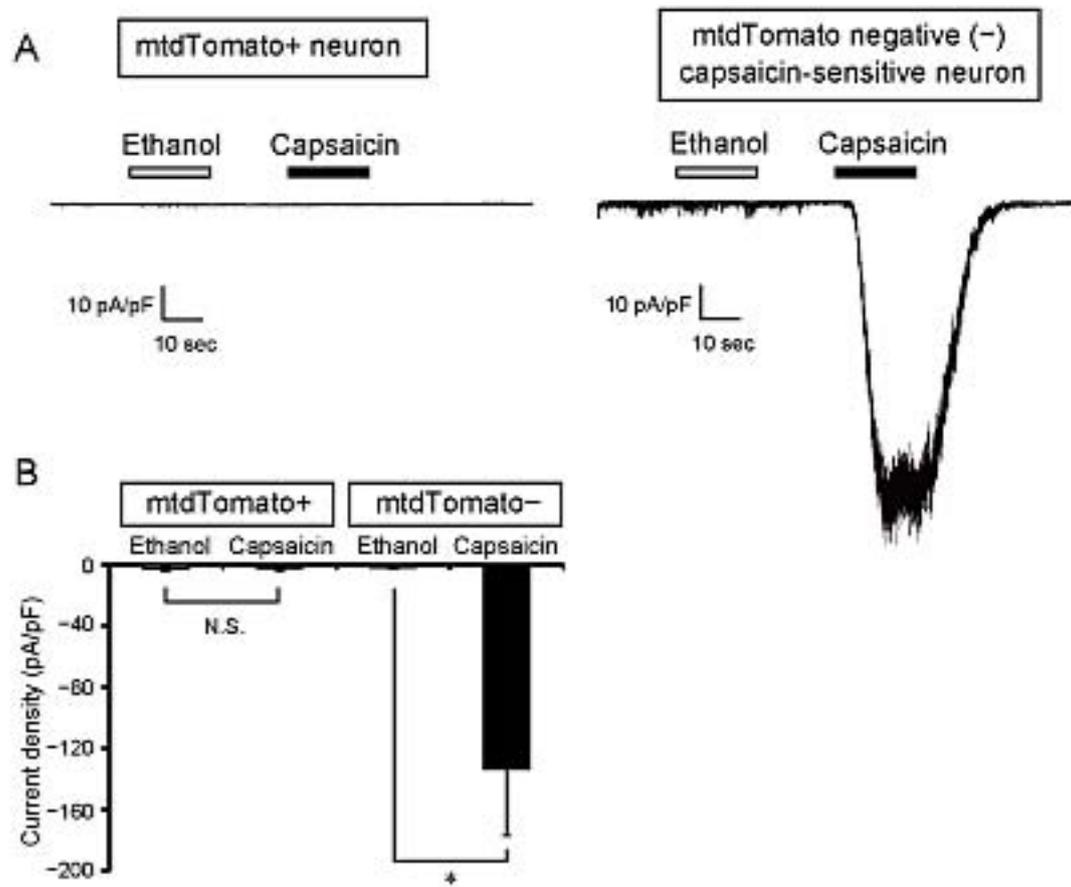


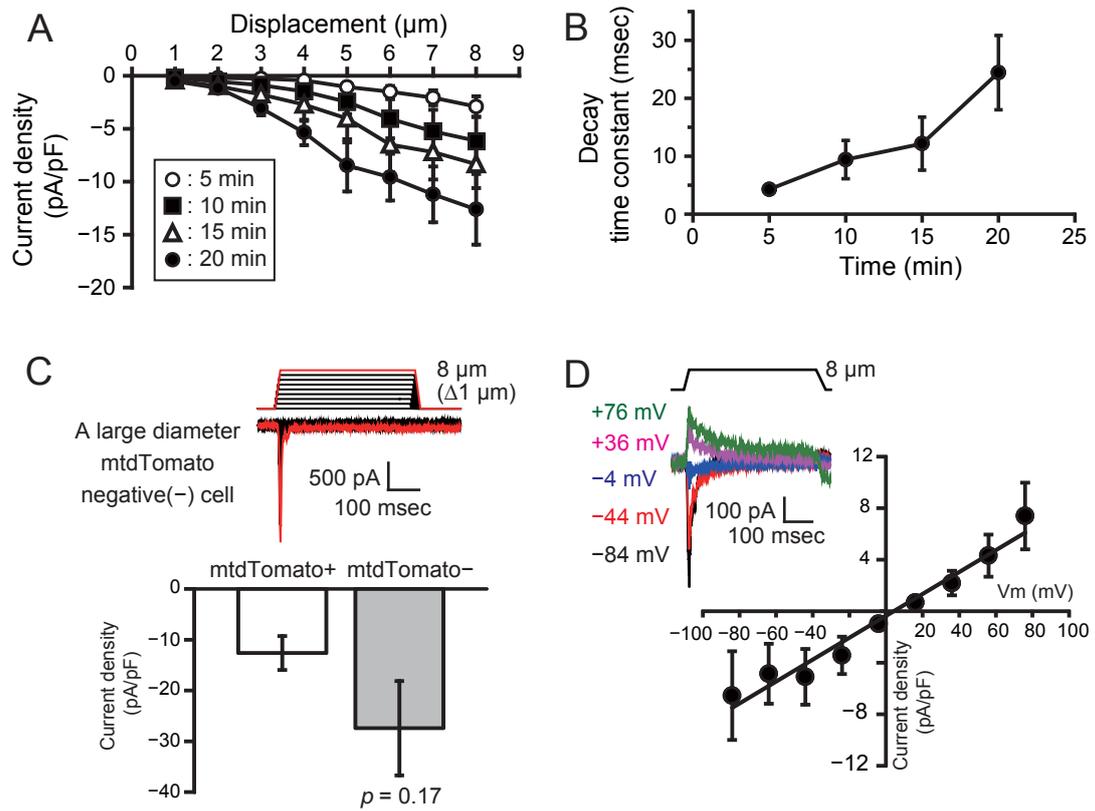
Figure 4 Yamaguchi S



Supplementary Figure S1. Yamaguchi S



Supplementary Figure S2. Yamaguchi S



Supplementary Figure S3. Yamaguchi S

Supplementary Figure Legends

Supplementary Figure S1.

Capsaicin does not increase intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$ of the MrgprB4⁺ neurons. (A) Representative traces of $[\text{Ca}^{2+}]_i$ of a MrgprB4⁺ (mtdTomato⁺) neuron (left) and a mtdTomato negative (–) capsaicin-sensitive neuron (right). Capsaicin (1 μM), its solvent (0.01% ethanol), and KCl (60 mM) were applied for 1 min. (B) A summary of the $[\text{Ca}^{2+}]_i$ increase ($\Delta[\text{Ca}^{2+}]_i$) during applications of ethanol and capsaicin for 1 min. Shown are mean \pm S.E. $n = 4$ and 5. $*p < 0.05$

Supplementary Figure S2

Capsaicin does not evoke membrane currents in the MrgprB4⁺ neurons. (A) Typical traces of whole-cell currents at -84 mV recorded from a MrgprB4⁺ (mtdTomato⁺) neuron (left) and a mtdTomato negative (–) capsaicin-sensitive neuron (right). Capsaicin (1 μM) and its solvent (0.01% ethanol) were applied for 20 sec. The intracellular pipette solution was an isotonic Cs-Cl rich solution which contains no sucrose. (B) A summary of the current densities evoked by applications of ethanol and capsaicin. Shown are mean \pm S.E. $n = 6$ and 7. $*p < 0.05$

Supplementary Figure S3.

Characterizations of rapidly-adapting mechanically-activated (RA-MA) currents in the MrgprB4⁺ neurons. (A) Current densities evoked by 1-8 μm displacements at 5 (open circles), 10 (squares), 15 (triangles), and 20 min (filled circles). $n = 8$ each. (B) A time course of change in decay time constants, which were calculated by single-exponential fitting of the decay of currents evoked by 8 μm displacement. The time constant in the initial measurements is not shown because the currents were too small to be fitted properly in many cases. $n = 8$ each. (C) (Upper) Typical whole-cell currents measured from a large diameter (42 μm) mtdTomato negative (–) cell. (Lower) Comparison of RA-MA current densities measured from mtdTomato⁺ cells and large diameter (40.8 \pm 1.7 μm) mtdTomato[–] cells at 20 min. When the decay time constant at 5 min was less than 10 msec, the currents were considered to be RA-MA currents. $n = 8$ each. (D) A current-voltage relationship of RA-MA currents measured from mtdTomato⁺ cells ($n = 4$ each). (Upper left) Representative RA-MA currents measured at –84 mV, –44 mV, –4 mV, +36 mV, and +76 mV. The currents were measured at least 10 min after establishment of whole-cell configuration to ensure usable current values.