Rat Merkel Cells Are Mechanoreceptors and Osmoreceptors

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Abstract

Merkel cells (MCs) associated with nerve terminals constitute MC-neurite complexes, which are involved in slowly-adapting type I mechanoreception. Although MCs are known to express voltage-gated Ca2+ channels and hypotonic-induced membrane deformation is known to lead to Ca2+ transients, whether MCs initiate mechanotransduction is currently unknown. To answer to this question, rat MCs were transfected with a reporter vector, which enabled their identification. Their properties were investigated through electrophysiological studies. Voltage-gated K+ (Kv), Ca2+ and Ca2+-activated K+ (KCa) channels were identified, as previously described. Here, we also report the activation of Ca2+ channels by histamine and their inhibition by acetylcholine. As a major finding, we demonstrated that direct mechanical stimulations induced strong inward Ca2+ currents in MCs. Depolarizations were dependent on the strength and the length of the stimulation. Moreover, touch-evoked currents were inhibited by the stretch channel antagonist gadolinium. These data confirm the mechanotransduction capabilities of MCs. Furthermore, we found that activation of the osmoreceptor TRPV4 in FM1–43-labeled MCs provoked neurosecretory granule exocytosis. Since FM1–43 blocks mechanosensory channels, this suggests that hypo-osmolarity activates MCs in the absence of mechanotransduction. Thus, mechanotransduction and osmoreception are likely distinct pathways.

Introduction

The sense of touch is not fully understood in mammals [1]. The slowly adapting type I mechanoreceptor (SAI) formed by the Merkel cell (MC)-neurite complex is critical for shape and texture discrimination [2]. SAI is concentrated at touch sensitive areas of the skin, such as fingertips, lips, touch domes and vibrissal outer root sheath in rodents (for review see [3, 4]). However, since previous work has produced conflicting results, it is still unclear whether MCs are able to initiate mechanotransduction by themselves [5, 6]. Mechanotransduction requires stimulation of mechanosensitive proteins, the opening of ion channels and the subsequent activation of nerve terminals, which generate action potentials. For MCs, electrophysiological evidence has demonstrated the presence of L-type (Ca2.1,2), P/Q-type (Ca2.1) and N-type (Ca2.2) voltage-gated Ca2+ channels and the role of Ca2+-induced Ca2+ release (CICR) in the evocation of robust intracellular Ca2+ transients [7, 8, 9]. Consecutive synaptic transmission to somatosensory neurons was bolstered by tight connections with nerve terminals, which were observed by confocal imaging and ultrastructural studies [10, 11]. Furthermore, essential components of the synaptic machinery were detected [12, 13, 14]. However, direct mechanical stimulation previously failed to activate quinacrine-labeled MCs [7]. Fluorescent dyes like quinacrine or FM1–43 were successfully used to identify MCs in epidermal cell cultures [15]. Unfortunately, quinacrine inhibits some ion channels and Ca2+ uptake in neuroendocrine cells [16, 17]. FM1–43 is a useful tool for studying neuropeptide secretion and membrane trafficking [18]. It was also found to be an efficient blocker of mechanosensory ion channels in sensory cells, such as neurons and hair cells [19, 20]. Therefore, although these dyes specifically label MCs in epidermal cell cultures, their biological effects have to be considered. Hence, a remaining challenge is the identification of rare functional MCs among predominant keratinocytes.

To overcome this problem, Lumpkin et al. generated transgenic mice in which the enhancer of the neural transcription factor mouse atonal homolog1 (Math1) drove the expression of GFP [21]. In the epidermis, Math1 is specifically expressed by MCs. Fluorescence-activated cell sorting enabled isolation of a population of cells constituted by 85 to 95% MCs. Evaluation of this purified cell population indicated that MCs express presynaptic proteins [12] and that hypotonic-induced membrane deformation initiated Ca2+ signaling [22] modulated by Ca2+-activated K+ (BKCa) channels [23].

As MCs express the osmoreceptor TRPV4 (transient receptor potential vanilloid type 4) [22, 24] and because its activation by hypotonic solution or the phorbol derivative 1,2-PDD (1,2-phorbol-
12, 13-didecanoate) also leads to \( \text{Ca}^{2+} \) influx [25,26,27], we addressed the hypothesis that different pathways can be initiated in hypotonic-stimulated MCs.

Based on the strategy used by Lumpkin et al., we identified the enhancer of Math1 in the rat genome by sequence alignment. This rat sequence was fused into a \( \beta \)-galactosidase expression vector system. Epidermal cells from rat footpads were isolated and then transfected with the engineered vector in order to permit identification of functional MCs. To determine whether MCs respond to both mechanical and osmotic stimuli, we electrophysiologically analyzed direct mechanical stimulations of MCs in an osmotic medium. We then used hypotonic solutions or 4\( \mu \)PDD to stimulate MCs in the absence of mechanotransduction. For this last experiment, we labeled MCs with the mechanosensory channel inhibitor FM1–43, which allowed us to follow membrane stimulation of MCs in the absence of mechanotransduction. Our electrophysiological recordings confirmed previous data on MCs: they express \( \text{Ca}^{2+} \), \( K^+ \) and \( \text{Ca}^{2+} \)-activated \( K^+ \) channels. In addition, we found that MCs responded to histamine and acetylcholine (ACh). More interestingly, we demonstrated that MCs express stretch-channels capable of transducing the strength and the length of a mechanical stimulation. We also showed that hypotonicity or exposure to 4\( \mu \)PDD was sufficient to induce neurosecretory granule exocytosis from FM1–43-labelled MCs. Taken together, these results confirm mechanotransduction properties of MCs and support the hypothesis that dense-core granule exocytosis is linked to events other than touch, such as hypotonicity and TRPV4 activation. To conclude, we hypothesize that mechanotransduction and osmoreception differentially activate MCs. Mechanotransduction may initiate synaptic release while osmoreception could induce dense-core granule exocytosis.

**Results**

**Transfection of Merkel Cells**

In order to define a suitable transfection protocol to transiently transfect MCs, the first tests were carried out on whiskers. This model appeared more appropriate than footpads because MCs are more numerous in whiskers and they are located specifically at the upper part of the outer root sheath, as demonstrated by anti-cytokeratin 20 immunostaining (Figure 1a). This localization is due to MCs allowed us to assess the suitability of our vector, because coupled \( \beta \)-galactosidase labeling and immunostaining was very difficult. In our experimental conditions, the best results were obtained with Tranfast. Using this reagent, most Math1-driven \( \beta \)-galactosidase-expressing cells were observed at the expected location (Figure 1b). We failed to detect transfected MCs cells following transfection by DEAE-dextran/chloroquine, Lipofectamine or Nanofectine. A low number of \( \beta \)-galactosidase-expressing cells were observed when we used Lipofectamine LTX or PEI. Unfortunately, as the number of MCs per whisker varied considerably, the yield of transfected cells compared to the total number of MCs could not be assessed.

As electrophysiological studies required isolated cells, epithelial cells from rat footpads were preferentially used. Basal and suprabasal epithelial cells were dissociated as described in the Materials and Methods section. Immunofluorescence analyses against CK20 showed that MCs usually represent 2.37\% \( \pm 1.67\% \) of the cultured epithelial cells (n=3). Retrieved cells were transfected with pMath1-\( \beta \)-galactosidase using Tranfast. \( \beta \)-Galactosidase staining enabled us to distinguish MCs from other cell types (Figure 2). Briefly, 1.35\% \( \pm 0.73\% \) (n=3) of the dissociated epithelial cells appeared blue, indicating that an average of 57\% of MCs were transfected by this approach.
depending on the number of channels that were recorded at the same time. The activation kinetics and the permeation properties are consistent with L-type Ca\(^{2+}\) channels. After a 3-min incubation with 10 μM Ruthenium red (RR), an inhibitor of Ca\(^{2+}\) channels, inward currents were fully inhibited, which suggested that inward currents were mainly carried by Ca\(^{2+}\) channels (Figure 3c). Ca\(^{2+}\) channels usually induce signal transmission and depolarization through Ca\(^{2+}\) transients in excitatory cells. They are also known to trigger the CICR pathway in MCs [9].

MCs Produce Voltage Activated K\(^{+}\) Channels

Outward currents were detected in 34% (17/50) of the recorded MCs. These currents were generally (14/17) delayed slow activated K\(^{+}\) currents with no spontaneous inactivation. They usually began to activate at -40 mV and reached a maximal activation state at +80 mV for an average conductance of 60 nS (Figure 4a, b; n = 7). Tetraethylammonium (TEA), a classical inhibitor of voltage-activated K\(^{+}\) channels, was added to the medium at a final concentration of 10 mM and records were compared to control conditions. Without TEA, we detected non-inactivated outward currents, which activated at -40 mV and reached a maximum activation state at +80 mV for a conductance of 90 nS. Three min after the addition of TEA, we observed non-inactivated outward K\(^{+}\) currents activated at -30 mV with a maximum activation state at +40 mV for a conductance of 50 nS. The amplitude of the currents at +120 mV was decreased to 35%
Ca\textsuperscript{2+} Channels in MCs Are Modulated by Acetylcholine and Histamine

ACh has pleiotropic roles in basic physiological functions. In the skin, ACh is known to regulate intracellular Ca\textsuperscript{2+} concentration via nicotinic (nAChR) and muscarinic (mAChR) receptors [20]. To investigate its effect on MCs, we added ACh (10 \textmu M) to transfected MCs and measured Ca\textsuperscript{2+} currents after 2 min. In tested MCs, exposure to ACh almost entirely inhibited Ca\textsuperscript{2+} currents and led to a faint remaining inward current (Figure 6, n = 4). This inhibition suggested a signal transduction event through M2 or M4 mAChR [29].

Histamine H3 receptor (H3R) reduces inflammation and nociception. H3R has been recently described on MCs [30], but its function has not been defined in these cells. The addition of histamine (300 \textmu M) induced inward Ca\textsuperscript{2+} currents and led to a faint remaining inward current (Figure 6, n = 4). This inhibition suggested a signal transduction event through M2 or M4 mAChR [29].

Ca\textsuperscript{2+} currents after 2 min. In some records (3/17), outward currents with stronger amplitudes of approximately +130 to +150 mV were observed (Figure 5a, b). In these cases, the average conductance sharply increased from 115±36 nS to 900±190 nS (n = 3). The pattern of the current-voltage relationship indicated both K\textsuperscript{+} and Ca\textsuperscript{2+} currents, as indicated by merge modelling (Figure 5c). This increased intracellular Ca\textsuperscript{2+} concentration coupled to rectifying K\textsuperscript{+} channels suggested the involvement of previously described BKCa channels [23]. No voltage activated Na\textsuperscript{+} channels or fast inactivated Ca\textsuperscript{2+} channels (T type) were evidenced.

MCs Have Mechanosensory Stretch-Sensitive Channels

Previous investigations of the mechanosensory properties of MGs produced conflicting results [3,31,32]. Because direct mechanical stimulation failed to activate MCs [7], most authors used indirect hypo-osmotic stimulation to explore their mechanosensory properties [22,27]. Here, we tested direct mechanical stimulation of MCs by applying controlled suction through a patch pipette, which was monitored by a pressure gauge. A stimulation of 200 mmHg induced a strong transient depolarizing current in MCs. This inward current was not delayed (or delayed by less than 100 ms) and had amplitude of 40 nA. It was followed by a marked depolarizing sustained current lasting as long as the suction. The resting potential was restored at the end of the stimulation (Figure 8). We assessed membrane currents in MCs when mechanical stimulations were increased. MCs respond to a first stimulation of 100 mmHg by a stable depolarizing current of 2.2 nA, which was maintained during the four seconds of stimulation. The increase in pressure to 200 mmHg induced a much more significant depolarizing current of 3.5 nA, which was stable over the 11-second stimulation (Figure 9). Thus, MCs were able to give electrophysiological responses to mechanical stimulations performed over 15 seconds without accommodation and in a strength-dependent manner.

Gadolinium(III) (Gd\textsuperscript{3+}) is an inhibitor of non-selective cationic stretch channels. In order to confirm the presence of such channels

![Figure 5. Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels in MCs.](image)

In three recordings, a sharp increase of outward current was detected, suggesting the presence of BKCa channels, which have already been described in MCs. (a) Representative voltage-dependent currents activated in response to a series of depolarizing pulses. (b) The current-voltage curve demonstrated a marked increase of the conductance around +130 mV, suggesting the involvement of different currents. (c) In order to determine whether these currents correspond to both Ca\textsuperscript{2+} and K\textsuperscript{+} currents, the two current-voltage curves from a previous recording were added. The pattern of the forecast current to voltage relationship (black squares) corresponded to the recorded currents.

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Figure 6. ACh inhibits inward currents in MCs. (a) Representative inward Ca\(^{2+}\) currents without an inactivating component (top traces) recorded in the control condition from transfected MCs of rat touch dome. These currents were almost entirely inhibited after two min of exposure to ACh (10 μM) (bottom traces). (b) Steady-state current densities were plotted as a function of voltage in normal conditions (black squares) and after stimulation with ACh (grey triangles) (n = 4).

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Figure 7. Histamine induces inward currents in MCs. The histamine H3 receptor was identified at the surface of MCs. Its activation induced inward Ca\(^{2+}\) currents in MCs. Representative current densities recorded during a series of depolarizing steps in control conditions (a, c: top traces). Inward slow activating Ca\(^{2+}\) currents (a) or outward fast activating K\(^{+}\) currents (b) were identified as revealed by the current-voltage relationships (b, d: black squares). In both conditions, the addition of histamine (300 μM) induced inward Ca\(^{2+}\) currents without inactivating components (a, c: bottom traces) as demonstrated by the current-voltage relationships (b, d: grey triangles).

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Figure 8. Suction initiates spontaneous inward currents in MCs. MCs are believed to function as a mechanoreceptors. To determine whether they respond to touch, suction (200 mmHg) was applied to transfected MCs from a rat footpad. Suction induced a strong, non-delayed, inward transient current. The decrease of inward currents at the second part of the recording is explained by the time required to stop the stimulation.

don MCs, Gd\(^{3+}\) was applied at 100 µM. Voltage-dependent currents were recorded before and after mechanical stimulation. Before suction, slow inward currents were detected. When we applied a suction of −100 mmHg with an imposed potential, a marked augmentation of the current intensity was observed (+175±13%, n = 3). The current to voltage relationship revealed an increased inward conductance with permeability enhancement of 34.1±4.8% (Figure 10a, b). In the presence of Gd\(^{3+}\), suction up to 180 mmHg did not increase inward currents. In fact, inward currents were not modified to any degree. Therefore, Gd\(^{3+}\) inhibited the response to suction by blocking non-selective cationic stretch activated channels (Figure 10c, d). The current flowing through these channels should be essentially a Ca\(^{2+}\) current, considering the equilibrium potential of the current voltage relationship. Hence, MCs respond to deformation by producing slowly adapting Gd\(^{3+}\)-sensitive mechanosensory channels that induced depolarization following activation.

Discussion

The mechanotransduction properties of MCs in the MC-neurite complex remain controversial [5,6]. Recently, increasing evidence has demonstrated the importance of Ca\(^{2+}\) signaling to induce depolarization of MCs stimulated by hypo-osmolarity [9,22,23]. In this study, we described a process by which to transiently transfect rat MCs. By performing electrophysiological recordings, we report the presence of voltage-activated Ca\(^{2+}\) channels in MCs. These findings confirm recently published data. Histamine and acetylcholine modulated the activation of Ca\(^{2+}\) channels in MCs. Importantly, we provide evidence that MCs act as mechanoreceptors because direct mechanical stimulation induced sustained strength-dependent depolarizations. The inhibition of these currents by Gd\(^{3+}\) confirmed the presence of cationic non-

Figure 9. MCs transduce the strength and the length of the stimulation. A 100 mmHg-suction was applied to transfected MCs. Stimulation induced inward currents sustained throughout the stimulation (about 15 sec). An increase in the strength of the stimulation after 4 sec led to increased inward currents.
selective stretch activated channels on MCs. Finally, we demonstrated that hypo-osmolarity led to neurosecretory granule exocytosis, possibly through the activation of TRPV4 in FM1–43-labeled MCs. Taking into account that FM1–43 inhibits mechanotransducer channels [19,20], our results suggest that MCs act as mechanoreceptors and osmoreceptors.

Recent reports extensively described the expression, production and activation of N-, P/Q- and L-type Ca\textsuperscript{2+} channels, K\textsuperscript{+} and KCa channels, as well as voltage-activated currents in MCs [7,23]. In this work, similar currents were recorded and no Na\textsuperscript{+} current was identified, which confirmed the gathered electrophysiological data on MCs from touch domes, whisker follicles and footpads of rodents. Although Piskorowski et al. detected inward Ca\textsuperscript{2+} currents in half of the MCs (14/30) and after inhibition of masking K\textsuperscript{+} currents, in our model, we observed a majority of inward currents (33/50), while K\textsuperscript{+} and KCa currents were more sparse (14/50 and 3/50, respectively). The use of RR to inhibit Ca\textsuperscript{2+} channels generally did not allow disclosure of K\textsuperscript{+} currents in our experiments, which suggests that Ca\textsuperscript{2+} currents are not masking K\textsuperscript{+} currents. This finding is consistent with data for polarized cells and agrees with published immunostainings, which detected specific Ca\textsuperscript{2+} channels on microvilli or close to the nerve terminal. Conversely, KCa channels were found on the whole plasma membrane [23], but these channels were probably not activated when Ca\textsuperscript{2+} currents were blocked.

Ca\textsuperscript{2+} channels are involved in cell signalling, enzyme activation and neuropeptide release. Receptors to ACh had not yet been described on MCs. However, ACh is known to inhibit the Ca\textsuperscript{2+} current of N and P/Q type channels in neurons via the activation of presynaptic muscarinic M2 [34] and M4 receptors [29]. Moreover, ACh signalling often regulates neuropeptide release in neuroendocrine cells expressing VIP, like MCs [35,36], through the activation of mAChR [37]. Hence, the expression of mAChR is highly probable on MCs. We provide electrophysiological evidence that AChRs are present on MCs and that their activation inhibits Ca\textsuperscript{2+} channels. As we also found that ACh inhibits VIP release in swine MC [36], this evidence provides a strong argument in favour of considering ACh as a modulator of MC secretory functions via mAChR. This finding also supports the involvement of N or P/Q type Ca\textsuperscript{2+} channels. No effect was observed on delayed K\textsuperscript{+} channels and no inward current was induced by ACh. These data suggest that ACh acts through muscarinic M2 receptors. However, a precise identification of the

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**Figure 10. Touch-evoked inward currents are inhibited by Gadolinium.** Gd\textsuperscript{3+} is a stretch-channel inhibitor. Once added to the medium (100 mM), Gd\textsuperscript{3+} inhibited inward currents induced by suction, supporting the presence of stretch channels in MCs. (a) Representative inward Ca\textsuperscript{2+} currents recorded in control conditions (top traces) and following the application of suction (bottom traces). (b) Current-voltage relationships demonstrating increased depolarizations during suction (grey triangles) compared to control conditions (black squares). (c) Recorded inward currents in control conditions (top traces) were not modified by suction when cells were pre-exposed to Gd\textsuperscript{3+} (bottom traces). (d) Steady-state current densities during the depolarizing step were plotted as a function of membrane potential.

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**Figure 11. FM1–43 stained neurosecretory granules of MCs.** FM1–43 is a fluorescent dye and a permeant blocker of mechanosensory channels, which stained the neurosecretory granules of neurons, hair cells, and MCs. (a) MCs are concentrated in rat footpads as demonstrated by CK20immunostaining. (b) Intraperitoneal injection of FM1–43 at 3 mg/kg in the rat allows for identification of MCs in the basal layer of the epidermis of rat footpads. Scale bars: 50 \(\mu\)m.
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receptor type remains to be carried out. Furthermore, ACh appears to act as a neurotransmitter in MCs, similar to its role in some neuroendocrine cells that coproduce VIP and ACh [37] in the central nervous system.

The histamine H3 receptor was previously described at the surface of MCs [30]. This receptor mainly reduces neuromediator release in the brain [38] and peripherally inhibits inflammation and nociception [39]. In this study, we found that histamine induced the depolarization of rat MCs, rather than the inhibition of inward currents. We previously found that histamine increased VIP release from MC [36]. Therefore, we hypothesize that other activating histamine receptors are present in MCs. Otherwise, the H3 receptor may act differentially in MCs. Activation of MCs may be partly mediated by the release of histamine from cutaneous mast cells and because neuropeptides like VIP or CGRP, which are secreted by MCs, are known to modulate inflammatory processes, this finding might suggest putative immunomodulatory functions of MCs [33,36]. This latter hypothesis is bolstered by the increased density in MCs observed in the context of inflammatory skin diseases [40,41,42,43].

The SAI formed by the MC-neurilect complex is critical for shape and texture discrimination [2]. However, despite evidence of synaptic capabilities [11,12], successive analyses failed to confirm the mechanotransduction properties of MCs. Recently, it was demonstrated that cell swelling induced membrane deformation-initiated Ca2+ signaling in MCs. Unfortunately, this finding was not sufficient to establish the mechanotransduction properties of MCs because MCs express hypotonic-activated ion channels. Eleven receptors of the TRP superfamily were identified in MCs [22] and most of them react to several stimuli [33]. Moreover, a latency of 200 µsec was found for the SAI [44], while cell swelling activated MCs with a latency of 11 seconds. In our study, we applied mechanical stimuli by suction in iso-osmotic medium at neutral pH. Our results revealed touch-evoked inward currents that were not delayed. Observed depolarizing currents were strength-dependent and maintained throughout the stimulation without accommodation. Suction performed during voltage-gated current recordings allowed us to identify stretch channels on the current-voltage relationship, mostly carrying Ca2+. Finally, these currents were inhibited by Gd3+, a cationic non-selective stretch channel inhibitor. Taken together, these results firmly demonstrated that MCs are mechanoreceptors and strongly support the idea that MCs initiate mechanotransduction in the MG-neurilect complex.

After establishing that MCs are mechanoreceptors, we tested whether MCs react to hypo-osmolarity without mechanical stimulation. Given that cell swelling induces membrane deformation, we labeled MCs with FM1–43, which is known to inhibit mechanotransducer channels. In addition, FM1–43 can be used to follow neurosecretory granule trafficking [18]. Here, we showed that hypo-osmolarity led to MC degranulation. This result links hypotonicity to neuroendocrine function. In addition, we stimulated the osmoreceptor TRPV4 by a pharmacological agonist to avoid cell swelling. TRPV4 is a cationic channel known to respond to hypo-osmolarity, acidification and membrane deformation [25,45,46,47].

Although hypotonic-evoked inward currents were not impaired in TRPV4-null mice, we demonstrated here that activation of this receptor was sufficient to initiate neurosecretory granules movement (Figure 12). Since analyses revealed a decrease of 61% (n = 33) in fluorescence intensity, a release of neurosecretory granules is suggested. Therefore, TRPV4 activation must induce neuropeptides release. This result was confirmed by the increased amount of VIP released observed in 4µ-PDD stimulated swine MC [36]. Thus, TRPV4 can be linked to osmoreception in MCs; however, other ion channels probably assume this function as well. Moreover, because Gd3+ was also found to inhibit TRPV4 [48], it can be hypothesized that TRPV4 also acts in mechanotransduction.

In summary, we have shown that MCs are mechanoreceptors and osmoreceptors. Mechanoreception involves Ca2+ signalling in MC and may be associated with synaptic release. Osmoreception is more related to neurosecretory functions with a longer latency than mechanoreception. Hence, these results support our recent study in swine MC in which we provide evidence of two distinct secretory pathways in regard to their Ca2+ dependency [30]. Furthermore, we demonstrated the participation of TRPV4 in osmoreception, although other ion channels are also likely to act as osmoreceptors. In addition, as Ca2+ channels are modulated by histamine and ACh, putative regulatory functions are likely in the cutaneous pathophysiological processes. Therefore, MCs fully belong to the somatosensory system, but still remain neuroendocrine cells that have a role in skin biology.
Materials and Methods

Animal Care

Experiments were conducted in accordance with French government policies (Services vétérinaires de la Santé et de la production animale, Ministère Français de l’Agriculture) and designed in accordance with recommendations of the regional ethical committee and the European Community directive no 86/609. Experiments were permitted by departmental agreement no A29-019-3. Male Wistar Rats were housed in the same place at 23°C, with a 12-h day light and fed ad libitum with standard rat pellets and had free access to water. Young male Wistar rats, from 5 to 15 days old, were used for this study.

Cell Culture

The epidermal layer of the rat footpad was separated from the dermis by enzymatic digestion. Briefly, the tissue was incubated with dispase (15 U/mL, 37°C; Gibco, Paisley, UK) for 2 hours. Basal and suprabasal cells were dissociated from the epidermal layer by digestion with 0.05% trypsin-EDTA (Lonza, Walkersville, MD). The number of cells recovered and their viability were determined by haemocytometer counting using the trypan blue exclusion method. Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM)/Ham’s F12 Medium (Lonza, Walkersville, MD) supplemented with 5% fetal calf serum (FCS) (PAA Laboratories, Pasching, Austria) and Normocin™ (Invivogen, San Diego, CA) at 100 µg/mL as an antibiotic. The outer root sheaths of young rat whisker pads were dissected under a binocular microscope after a 1 hour of digestion with dispase (15 U/mL) and collagenase IV (100 U/mL) (Sigma, St. Louis, MO).

Immunostaining

Tissues or cells were fixed for 10 to 30 min in 4% paraformaldehyde (pH 7.4), washed 3 times in PBS and incubated for 10 min in PBS containing 0.5% Triton X-100, followed by a 15-min incubation in PBS, 5% FCS and 0.1% Tween 20 at room temperature. Preparations were exposed to monoclonal mouse anti-cytokeratin 20 (CK20) antibody (1:25; Progen GmbH, Heidelberg, Germany) overnight at 4°C. After rinsing, the preparations were exposed to polyclonal goat anti-mouse IgG conjugated to TRITC (1:300; Sigma) diluted in PBS 0.1% Tween 20 and 1% FCS.

Live-Cell Neurosecretory Granules Imaging

FM1–43 (3 mg/kg body weight) (Sigma) diluted in PBS was injected into rats intraperitoneally. Animals were sacrificed 24 hours later and skin biopsies were laid down on slides without fixation for analysis. Cells were stimulated by hypotonic solution for 10 min in PBS containing 0.5% Triton X-100, followed by a 15-min incubation in PBS, 5% FCS and 0.1% Tween 20 at room temperature. Preparations were exposed to monoclonal mouse anti-cytokeratin 20 (CK20) antibody (1:25; Progen GmbH, Heidelberg, Germany) overnight at 4°C. After rinsing, the preparations were exposed to polyclonal goat anti-mouse IgG conjugated to TRITC (1:300; Sigma) diluted in PBS 0.1% Tween 20 and 1% FCS.

Vector Construction

Searches in rat for sequences similar to Math1 enhancers (GenBank: AF218258 [49]) were carried out using the BLAST program [30]. Two highly conserved domains, displaying 92 and 94% of homology to the sequences in human and separated by 80 nucleotides, were identified within a 1.51 kb sequence located 5 kb downstream of the Math1 coding region on chromosome 4q31. This sequence was amplified by PCR using the Pfu DNA polymerase (Promega, Madison, WI) and the following oligonucleotides, flanked respectively by EcoRI and XhOl sites: 5’ CGGAAATTTCCAGGTTCCAGCAATGAGTTTGCC 3’ and 5’ CGCTCGAGGCTCAGCCTAGGCTTTGCTTGGC 3’. Thirty PCR cycles were performed with a denaturing temperature of 92°C for 30 sec, an annealing temperature of 60°C for 1 min and an amplification at 74°C for 2 min. The PCR product was purified, digested with EcoRI and XhoI and ligated into a pCMV-β-galactosidase vector that had been cut with the same restriction enzymes. In the resultant vector, pMath1-β-galactosidase, the CMV promoter was replaced by the Math1 enhancer.

Transfection

The day before transfection, 1×105 epidermal cells or 20 whisker pads were seeded in 35 mm culture dishes containing medium supplemented with FCS without antibiotic. Six transfection reagents were tested. All reagents were used following the recommendations of the provider (Table 1). Briefly, (1) Cells were exposed for 30 min to 2 µg of plasmid and 500 µg/mL of DEAE-dextran (Sigma) in 200 µL of DMEM/F12. Subsequently, 700 µL of 80 µM chloroquine (Sigma) was added to the medium. A DMSO shock (DMSO 10% in DMEM/F12) was performed 5 hours later for 2 min and then the medium was changed. (2) Lipofectamine LTX (Invitrogen, Karlsruhe, Germany) was diluted in 200 µL of the DNA solution (2.5 µL of reagent per 1 µg of plasmid) and, after 30 min, the solution was added to the culture medium. (3) In the third trial, 10 µL of Lipofectamine (Invitrogen) and 2 µg of plasmid were diluted each in 100 µL of medium. After 30 minutes, the two solutions were pooled and added to the culture medium 10 min later in a final volume of 1 mL. (4) Alternatively, 6.4 µL of Nanofectine® (PAA Laboratories, Pasching, Austria) and 2 µg of DNA were each diluted in 100 µL of medium. After 5 min, the solutions were pooled. After a 20-min incubation, the solution was added to the culture medium. (5) DNA (1 µg) was added to 4.4 µL of polyethylenimine (PEI) (Sigma) and the mixture was diluted to a volume of 100 µL of DMEM/F12. The diluted mixture was incubated for 15 min prior to addition to the culture medium. (6) Twelve-µL of Transfast (Promega) and 2 µg of plasmid were each diluted in 100 µL of medium. After 5 min, the solutions were pooled and incubated for 10 min. The culture medium was then replaced by the DNA-media.

Table 1. Transfection reagents used to transfect MCs.

| Transfection Reagents | Amount used | plasmid | Final volume |
|-----------------------|-------------|---------|--------------|
| DEAE-dextran          | 100 µg      | 2 µg    | 200 µL       |
| Lipofectamine LTX     | 2.5 µL      | 1 µg    | 200 µL       |
| Lipofectinine         | 10 µL       | 2 µg    | 200 µL       |
| Nanofectine           | 6.4 µL      | 2 µg    | 200 µL       |
| Polyethyleneimine     | 4.4 µL      | 1 µg    | 100 µL       |
| Transfast             | 12 µL       | 2 µg    | 200 µL       |

Brief descriptions of the transfection protocols. Data were collected two days after transfection. Transfection reagents were evaluated by determining the number of blue cells seen in the presence of X-gal on the vibrissal outer root sheath.
Transfast solution. After 2 to 3 hours, 1 mL of DMEM/F12 was added to the cells.

β-Galactosidase Staining

Two days after transfection, cellular β-galactosidase activity was assessed at 37 °C using a reaction mixture composed of 1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal), 4 mM K3Fe(CN)6, 4 mM K4Fe(CN)6 and 2 mM MgCl2 in DMEM/F12. All chemicals were purchased from Sigma. The first cells appeared blue within 30 min. Electrophysiological recordings were performed on the same day.

Electrophysiological Analyses

The culture medium was replaced with an osmotic buffer (300 mosm) containing HEPES buffer (50 mM), NaCl (130 mM), KCl (3 mM), CaCl2 (4 mM) and glucose (10 mM) with the pH adjusted to 7.4 by NaOH addition. Transmembrane ionic currents were recorded from blue cells corresponding to MCs using a macro-patch clamp technique [51] that has been previously described [32]. Briefly, pipettes were pulled and heat polished from 1.5 mm borosilicate glass (Clark Electromed, USA) with a DMZ-Universal puller (Zeitz Instruments, Germany). Resistance of the pipette was monitored using a multimeter. Pipettes were filled with standard saline solution. Voltage pulses were delivered to the cells and current recordings were processed via a GeneClamp 500B amplifier and a CV-5-100U headstage (Axon Instruments) connected to a microcomputer through a 12-bit A/D/D-A interface (CED 1401+; Cambridge Electronic Design Ltd., UK). Voltage-clamp protocols and data acquisition were performed with WinWCP V3.2.5 (Whole Cell Program, J. Dempster, Strathclyde University, UK). Currents were low-pass filtered at 5 kHz and digitized at 40 kHz. We systematically checked Giga-seal and compensated for any observed leaky currents. The maximum conductance was calculated from the slope of the current to voltage relationship. TEA was used at 10 mM, ACh and RR were used at a final concentration of 10 μM. Histamine was used at a final concentration of 300 μM, Gadolinium (Gd3+) was used at 100 μM, and 4αPPD was used at 1 μM. All of these chemicals were supplied by Sigma. Suction was controlled by a pressure gauge. Mean values were compared by statistical tests (Student’s t-test or Mann-Whitney as appropriate) after checking the normality of distribution. A significant difference was assumed for p values <0.05. Error bars show the standard error of the mean (SEM).

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Author Contributions

Conceived and designed the experiments: JBP NL LM. Performed the experiments: NB JPP LM. Analyzed the data: NB JPP NL UP NR GD CC LM. Contributed reagents/materials/analysis tools: NB JPP NL UP NR GD CC LM. Wrote the paper: NB JPP NR CC LM.

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