Identification and characterization of the single channel function of human mucolipin-1 implicated in mucolipidosis type IV, a disorder affecting the lysosomal pathway

Janice M. LaPlante[a], John Falardeau[b], Mei Sun[c], Marie Kanazirska[a], Edward M. Brown[a], Susan A. Slaugenhaupt[b], Peter M. Vassilev[a,*]

[a] *Division of Endocrinology, Diabetes and Hypertension and Membrane Biology Program, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, 221 Longwood Ave., Boston, MA 02115, USA*
[b] *Molecular Neurogenetics Unit, Massachusetts General Hospital and Harvard Institute of Human Genetics, Harvard Medical School, Boston, MA 02115, USA*
[c] *Center for Prostate Disease Research, Rockville, MD 20852, USA*

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Abstract Mucolipin-1 (MLN1) is a membrane protein with homology to the transient receptor potential channels and other non-selective cation channels. It is encoded by the *MCOLN1* gene, which is mutated in patients with mucolipidosis type IV (MLIV), an autosomal recessive disease that is characterized by severe abnormalities in neurological development as well as by ophthalmologic defects. At the cellular level, MLIV is associated with abnormal lysosomal sorting and trafficking. Here we identify the channel function of human MLN1 and characterize its properties. MLN1 represents a novel Ca^{2+}-permeable channel that is transiently modulated by changes in $[Ca^{2+}]$. It is also permeable to Na^+ and K^+. Large unitary conductances were measured in the presence of these cations. With its Ca^{2+} permeability and modulation by $[Ca^{2+}]$, MLN1 could play a major role in Ca^{2+} transport regulating lysosomal exocytosis and potentially other phenomena related to the trafficking of late endosomes and lysosomes.

Key words: Mucolipin; Mucolipidosis; Lysosome; Exocytosis; Channel; Late endosome

1. Introduction

Most of the stages of the endocytotic/exocytotic cycles, including exocytosis of lysosomes, are Ca^{2+}-regulated phenomena. Ca^{2+} release from late endosomal/lysosomal luminal compartments is required for fusion of these organelles [1–3] but the channels underlying this process remain elusive. Lysosomal channels may also contribute to the Ca^{2+}-triggered exocytosis of lysosomes in secretory and other cells. A potential candidate for such functions is mucolipin-1 (MLN1), a membrane protein encoded by the *MCOLN1* gene that we and others recently cloned and found to be mutated in patients with mucolipidosis type IV (MLIV) [4–6]. MLIV is an autosomal recessive disease [7–9] that is characterized by severe abnormalities in neurological development as well as by ophthalmologic defects. It usually presents during the first year of life with severe mental retardation and delayed motor milestones. MLIV is unique among the mucolipidoses in that it appears to result from a disruption in sorting and/or transport along the late endocytotic pathway [10–12]. MLN1 shares sequence homology and membrane topological features with the transient receptor potential channels and other non-selective cation channels [13–18]. Here we identify the channel function of human MLN1 and characterize its properties.

2. Materials and methods

2.1. Expression of MLN1 in oocytes

MCOLN1 cDNA (GenBank accession number AF287269) was synthesized by reverse transcription of total RNA from normal human fibroblasts. The 2025 bp sequence includes a 1740 bp open reading frame. The cDNA was cloned into the mammalian expression vector pSV-Sport1 (Gibco BRL). A C-terminal green fluorescent protein (GFP) fusion construct was generated by the in frame insertion of the GFP domain from pEGFP-C1 into the pSV-MLN1 construct. Specific details of the plasmid construction are available from the authors. Capped RNA was synthesized from linearized pSV-MLN1 templates using the mMessage mMachine In Vitro Transcription Kit (Ambion) and modified with the Poly(A)Tailing Kit (Ambion). Oocytes at stage V–VI were isolated from *Xenopus laevis* and injected with 50 nl H_2O containing 50 ng of MLN1 cRNA. Equal amounts of H_2O were injected into control oocytes.

2.2. Fluorescence measurements and detection of MLN1 expression in oocytes

The MLN1 protein was visualized in oocytes by GFP fluorescence. Fluorescent images were collected and analyzed using a Zeiss microscope with fluorescence attachment, digital camera and ImagePro software. The lysosome red fluorescent probe, Lysotracker DND99 (Molecular Probes), was used to assess the distribution of lysosome-related structures in oocytes. The oocytes were treated for 15 min with 50 nM of the probe immediately before freezing and slicing them.

2.3. Electrophysiological measurements

Patch-clamp methodologies were employed for measurement of single channel currents using cell-attached or excised membrane patches [17,18]. Patch pipettes were filled with a solution containing in mM (unless otherwise specified): 100 KCl, 0.1 $CaCl_2$, 10 HEPES, pH 7.5. When filled with this external solution, the pipette tip resistances were typically 5–10 MΩ. Seals with resistances of >5 GΩ were employed in single channel experiments, and currents were measured with an integrating patch-clamp amplifier. Single channel currents were filtered at 3–10 kHz through an 8-pole Bessel filter. The bath solution

*Corresponding author. Fax: (1)-617-732 5764.
E-mail address: pvassilev@rics.bwh.harvard.edu (P.M. Vassilev).

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contained, unless otherwise specified: 100 mM KCl, 0.1 μM $CaCl_2$, 10 mM HEPES, and 5 mM EGTA, pH 7.5.

2.4. Data acquisition and analysis

Voltage stimuli were applied and currents digitized (50–200 μs per point) and analyzed using a PC, a Digidata converter, and programs based on pClamp. The probability of channel opening (P_o) was calculated from 1–2 min segments of current records. The Goldman–Hodgkin–Katz equation was used to calculate the permeability ratios between Na^+ (P_{Na}) and K^+ (P_K): $E_{rev} = RT/F \ln [P_{Na}/P_K]$, where E_{rev} is the change in reversal potential measured when K^+ is replaced by Na^+ in the pipette solution; R, T, and F are the gas constant, absolute temperature, and Faraday constant, respectively.

2.5. Fluorescence method for measuring the release of the lysosomal enzyme, N-acetyl-β-D-glucosaminidase (NAG)

The method is based on measuring the fluorescence of the enzyme substrate, 4-methyl-umbelliferyl-*N*-acetyl-β-D-glucosamine, as previously described [19]. The amount of NAG released for each condition is expressed as % of the total content of NAG in the cells determined after their lysis using 0.1% Triton X-100.

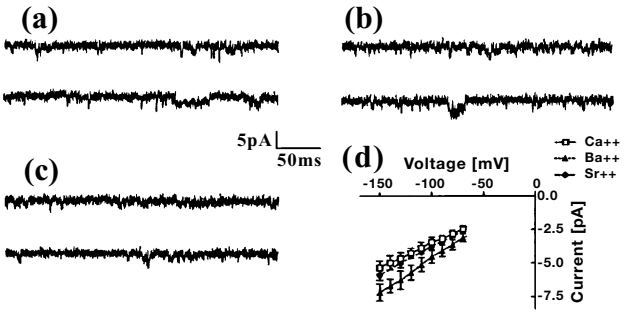

Fig. 2. Divalent cation currents mediated by MLN1. MLN1 single channel currents were recorded in the presence of 100 mM $CaCl_2$ (a), $BaCl_2$ (b) and $SrCl_2$ (c) in the external pipette solution, respectively. The current traces were taken at −90 mV (top traces) or −120 mV (bottom traces) in panels a, b and c. The current–voltage relations for Ca^{2+} (open squares), Sr^{2+} (closed circles), and Ba^{2+} (closed triangles) are shown in panel d.

3. Results and discussion

We have isolated and assembled a full-length *MCOLN1* cDNA, encoding MLN1, and expressed it in *Xenopus* oocytes. Ionic currents were measured in oocytes 3–5 days after injections with synthetic RNA (cRNA) encoding full-length MLN1.

Although there are no published reports on the localization of endogenous MLN1 in mammalian cells, it is presumed that the protein should be mainly expressed in lysosomal and late endosomal membranes, given the cellular defects in MLIV. Thus we first examined the levels and cellular distribution of MLN1 in oocytes using GFP-tagged MLN1. We injected GFP-tagged MLN1 cRNA into oocytes and 3–5 days later prepared frozen sections of the oocytes and visualized them using fluorescence microscopy. There were GFP-labeled MLN1-containing fluorescent structures in the cytoplasm of many of the oocytes that co-localized with lysosomes stained with the lysosome-selective probe Lysotracker DND99, but a substantial number of the oocytes also showed peripheral distribution of MLN1 in clusters near or on the plasma membrane (see Fig. 5). The appearance of a fraction of MLN1 on the plasma membrane may be due to overexpression, or perhaps to constitutive lysosomal exocytosis, a phenomenon that has been previously described in both secretory and non-secretory cells [19–21].

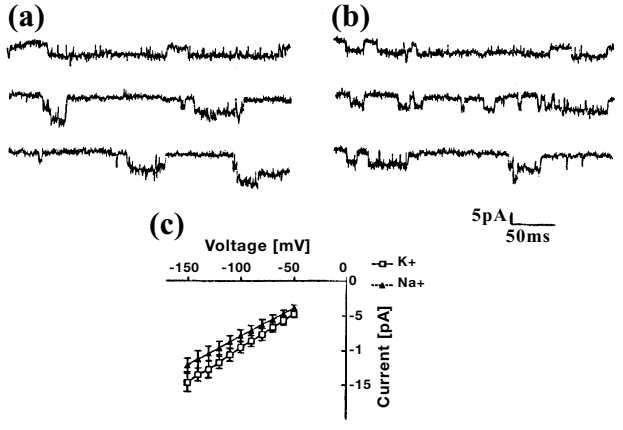

Fig. 1. Monovalent cation currents in oocytes injected with MLN1 cRNA. Current traces were recorded in cell-attached patches in the presence of 100 mM KCl (a) or 100 mM NaCl (b) in the pipette solution. Downward deflections represent channel openings. The current records were taken at −80 mV (top traces), −100 mV (middle traces) or −120 mV (bottom traces) in panels a and b. Current–voltage relations in the presence of 100 mM KCl (open squares) or 100 mM NaCl (closed triangles) in the pipette solution are shown in panel c.

In single channel studies using 100 mM KCl (Fig. 1a) or NaCl (Fig. 1b) in the pipette solution we observed distinctive, relatively long channel openings and closings in MLN1-expressing oocytes ($n = 85$). The amplitudes of the inward currents increased linearly at more negative voltages but the amplitudes of the outward currents at positive voltages (not shown) were much smaller than the inward currents. Such activity was absent in water-injected oocytes ($n = 54$) and oocytes expressing other proteins ($n = 82$). The unitary conductances determined from the slopes of the current-voltage relations (Fig. 1c) were 92.4 ± 10.2 pS ($n = 15$) for K^+ and 82.6 ± 8.5 pS ($n = 15$) for Na^+ in the presence of 100 mM of either of the cations. The slope conductances and reversal potentials were determined in the presence of K^+ on one side of excised patches and Na^+ on the other side (i.e. under biionic conditions) to characterize the selectivity and relative permeabilities of the MLN1 channels. The permeability ratio, P_{Na}/P_K, was 0.94 ± 0.04 ($n = 5$). To assess the contribution of anion fluxes to the MLN1 channel currents, we varied the content of anions and cations in the solutions on both sides of the inside-out patches and found that Cl^- influx or efflux does not contribute to the observed currents.

We treated the oocytes with actinomycin D (10 μg/ml), a widely used approach to distinguish cloned from endogenous channels, and observed no effects on the MLN1 channel activities. All of these data support the notion that the observed channel activity does not result from upregulation or modulation of channels endogenous to the oocytes.

We also studied MLN1 single channel currents in the presence of divalent cations. The channel activity usually occurred in bursts of brief openings (Fig. 2a–c). The current amplitudes measured at defined voltages (Fig. 2a–c) and the current–voltage relationships (Fig. 2d) for Ca^{2+} differ slightly from those for Sr^{2+} and Ba^{2+}. The Ca^{2+} conductance, measured over the linear voltage range (−70–120 mV), 34.4 ± 3.5 pS ($n = 6$), was

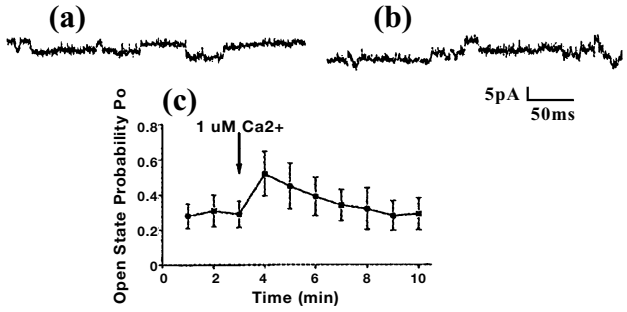

Fig. 3. Modulation of MLN1 channels by changes in cytosolic Ca^{2+} (Ca_i). Current traces recorded at −110 mV in cell-attached patches of untreated oocytes (a) are compared with those in oocytes pretreated for 15 min with 10 μM ionomycin (b). Changes in the channel open state probability (*Po*) (±S.E.M., $n=4$) mediated by elevation of Ca_i from 0.1 to 1 μM in inside-out patches excised from untreated oocytes are shown in panel c.

slightly smaller than that of Sr^{2+} (37.2 ± 2.9 pS, $n=4$) but that of Ba^{2+} was the largest, 44.6 ± 4.2 pS, $n=6$.

Although some of the described properties of the MLN1 channel are similar to those of the polycystin L (PCL) and polycystin-2 (PC2) channels that we have previously characterized [17,18] there are some noteworthy differences. The membrane topology of MLN1 is similar to these recently cloned members of the polycystin family of proteins, most strikingly to PC2 with its long extracellular loop between the first and second transmembrane domains. The long openings of the MLN1 channels are comparable to those of PCL. However, the unitary conductance of the latter is nearly 40% larger than that of MLN1. The conductance and kinetics of the MLN1 channel also differ from those of PC2 in that PC2 has a substantially higher conductance for K^+ than for Na^+, while MLN1 has similar conductances for both cations. Moreover, the MLN1 channel does not display flickery burst kinetics, a property characteristic of the PC2 channel [18].

The observation of MLN1 channel activity on the plasma membrane, despite the presence of a late endosomal/lysosomal targeting motif in MLN1, is not completely unexpected. Although one possible explanation may be related to overexpression of MLN1 in the oocytes, many studies on mast cells and other specialized secretory cells have shown that a portion of the lysosomes that are near the cell surface can be exocytosed constitutively as well as in a Ca^{2+}-dependent manner. In recent studies it was found that this is probably a ubiquitous phenomenon and that a similar lysosomal exocytosis occurs in fibroblasts as well as epithelial and other non-secretory cells [19–21]. The exocytotic process in these non-secretory cells is also stimulated by increases in intracellular Ca^{2+}, and the lysosomes behave as Ca^{2+}-regulated exocytotic vesicles. Lysosomal exocytosis was triggered by treating fibroblasts and other cells with Ca^{2+} ionophores such as ionomycin as well as with receptor agonists and other agents inducing Ca^{2+} influx or release from intracellular stores, thereby increasing the level of cytosolic Ca^{2+} (Ca_i) [19]. It was shown that at least 10–20% of the lysosomes in fibroblasts are exocytosed and that not only are their luminal contents released in the extracellular spaces, but integral membrane proteins specific for the lysosomes also appear on the plasma membrane during this process [19]. It is possible that, like these integral membrane proteins, MLN1 can also reside, at least transiently, on the plasma membrane (see Fig. 5).

To investigate this possibility we tested the effects of ionomycin on MLN1 channel activities and found that the activity of the MLN1 channels was higher in cell-attached patches of oocytes pretreated with this Ca^{2+} ionophore than in those of untreated oocytes (Fig. 3a,b). Ionomycin was used at the same concentrations and under similar conditions as in previous studies on cultured cells [19]. In cell-attached patches, however, the ionomycin-induced Ca^{2+} influx could cause stimulation of intracellular messengers and related signaling pathways with potential impact on the activity of the MLN1 channels. Therefore, we carried out studies in inside-out patches where [Ca^{2+}] on the cytosolic sides of the excised patches could be controlled. An elevation in [Ca^{2+}] on the intracellular sides of the patches from 0.1 to 1 μM mediated a transient increase in the open state probability (*Po*) (Fig. 3c) suggesting that the MLN1 channel can be modulated at least transiently by changes in Ca_i. *Po* usually returned to the baseline level 3–5 min after this rise of channel activity. In some patches only one channel was activated, while in other patches more than one channel current level was observed, particularly during the transient phase of activation. We estimated the average number of channel current levels observed during the different phases of channel modulation by changes in Ca_i and found a transient increase after elevation of Ca_i.

To explore the contribution of increased recruitment of channels to the plasma membrane during activation, we then compared the MLN1 channel activities in inside-out patches excised from untreated oocytes with those from ionomycin-pretreated oocytes (Fig. 4a,b). These studies were conducted in the presence of basal [Ca^{2+}] on the cytosolic patch sides, 0.1 μM. In patches of untreated oocytes usually only one or two MLN1 channel current levels were observed (Fig. 4a), while two or three channels were frequently available in patches from oocytes treated with ionomycin (Fig. 4b). *Po* was slightly higher in patches of ionomycin-pretreated than

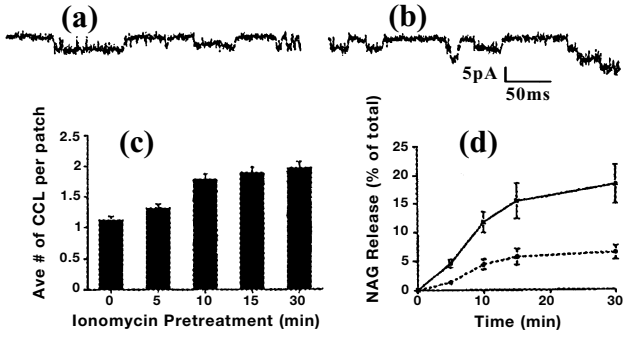

Fig. 4. Effects of the Ca^{2+} ionophore, ionomycin, on MLN1 channel and lysosomal exocytosis. Current traces taken at −110 mV in the presence of 0.1 μM Ca_i in inside-out patches excised from untreated oocytes (a) or from oocytes pretreated for 15 min with 10 μM ionomycin (b). The differences between the average numbers of channel current levels (CCL) (±S.E.M., $n=25$) observed in inside-out patches excised from untreated oocytes (0) or oocytes treated for 5, 10, 15 and 30 min with 10 μM ionomycin as marked under each bar are shown in panel c. Only patches containing at least one channel current level were analyzed. Excised patches that did not contain functioning channels were not taken into account due to the uncertainties about potential channel rundown phenomena. The amount of NAG (±S.E.M., $n=5$) released into the bath solution from untreated oocytes (dashed line) and from oocytes treated for 5, 10, 15 and 30 min with 10 μM ionomycin (solid line) is shown in panel d. The released amount of NAG is expressed as % of its total content in the cells as explained in Section 2.

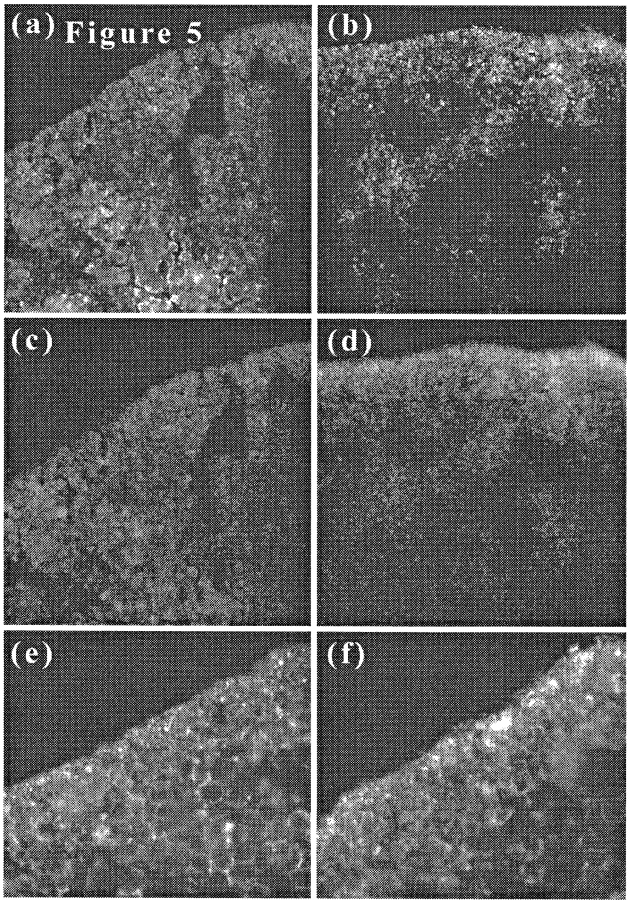

Fig. 5. Translocation of MLN1-containing lysosomes to plasma membrane stimulated by ionomycin. Frozen sections of the oocytes were prepared 5 days after the injection and the distribution of the GFP fluorescence (a,b) and of the Lysotracker (c,d) in untreated (a,c) or ionomycin-treated (10 μM for 15 min) oocytes (b,d) was observed. The same area of an untreated oocyte is shown in panels a and c, and the same area of an ionomycin-treated oocyte is shown in panels b and d. The magnification for all the images shown in a–d is 100×. The images in panels e and f have been taken at a higher magnification, 400×. They show the distribution of GFP-labeled MLN1 in untreated oocytes (e) and in oocytes treated for 15 min with 10 μM ionomycin (f). Photos are representative of at least six oocytes from three different batches for each of the conditions.

in untreated oocytes (data not shown) but the average number of channel current levels increased substantially with prolonged treatments with ionomycin (Fig. 4c). These results, particularly those shown in Fig. 4b,c, strongly suggest that the Ca^{2+} ionophore promotes MLN1 translocation to the plasma membrane. To investigate whether this process is associated with translocation of the lysosomes toward the cell periphery resulting in fusion with the cell membrane and release of the lysosomal content during exocytosis, we measured the amount of NAG released from the oocytes (Fig. 4d). NAG is a lysosome-specific enzyme whose activity has previously been characterized in *Xenopus* oocytes [22]. The release of this enzyme, which was assessed using a fluorescent substrate, was stimulated by pretreatment with ionomycin (Fig. 4d) confirming that the Ca^{2+} ionophore promotes the exocytosis of the MLN1-containing lysosomes. Similar to the electrophysiological data shown in Fig. 4c the amount of the released lysosomal enzyme increased substantially with prolonged treatments with ionomycin (Fig. 4d).

In support of these data we found an increased distribution of GFP-tagged MLN1 at the plasma membrane after ionomycin pretreatment (Fig. 5a,b). This pretreatment also stimulated the translocation to the plasma membrane of the lysosomes stained with the red fluorescent probe, Lysotracker DND99 (Fig. 5c,d). In many areas the GFP-tagged MLN1 co-localized with the Lysotracker-stained lysosomal structures in the cytoplasm or at the plasma membrane. The fluorescence images displayed in Fig. 5e,f that have been taken at a higher magnification confirm that ionomycin stimulates substantially the translocation of the GFP-labeled MLN1 to the plasma membrane.

The MLN1 channels may be involved in previously described Ca^{2+} transport across lysosomal membranes [23] as well as in Ca^{2+}-dependent fusion between the lysosomal and plasma membrane during the exocytotic stage of the membrane trafficking process. It has been shown that lysosomal exocytosis plays a role in membrane resealing related to wound healing [20], and it is likely that it also functions in the removal of cellular debris near sites of cellular damage and in membrane repair. It is possible that disturbance in these processes could be implicated in both corneal opacification [24] and achlorhydria [25], two hallmarks of MLIV. Further studies are necessary to clarify the potential role of MLN1 in lysosome trafficking toward cell periphery and to verify whether its deficiency can explain the juxtanuclear distribution of the lysosomes observed in fibroblasts and other cells of patients with MLIV.

Additional functional characterization of MLN1 could also help to assess its potential contribution to Ca^{2+} release from late endosomes/lysosomes, a step required for the fusion between these organelles that is likely disturbed in MLIV. The fusion between endosomes and lysosomes preceding the delivery of lipids and other materials to the latter type of catabolic organelles depends on a local source of Ca^{2+}, their luminal compartments. Numerous studies have shown that the fusion between yeast vacuoles, which represent the lysosomal equivalent in yeast, as well as between mammalian late endosomes and lysosomes occurs in several stages, including priming and docking [1,2], but the final phase of membrane fusion can only be triggered when Ca^{2+} is transported from the organellar lumen to the cytosol leading to modulation of calmodulin and probably other Ca^{2+}-binding proteins. It has been suggested that the transport of Ca^{2+} across the membranes of these organelles is mediated by an as yet unidentified Ca^{2+}-permeable channel(s) or other specific types of transporters [1–3]. Activation of the high conductance MLN1 channels in these membranes could produce large increases in Ca^{2+} in the cytosolic spaces between the organelles, thus triggering their fusion.

With its high K^+ conductance MLN1 may also contribute to K^+ transport across the membranes of the Ca^{2+}-containing organelles. Previous reports support the importance of K^+ influx pathways in the endoplasmic reticulum (ER) that are coupled to Ca^{2+} release as part of a highly cooperative ion exchange mechanism [26,27]. Counter-currents carried by K^+ probably maintain the electroneutrality of the ER membrane system during Ca^{2+} release. The MLN1 channel could play a similar role in counter-current mechanisms in endosomes and lysosomes.

Thus MLN1 represents a novel Ca^{2+}-permeable channel related to endosomal/lysosomal trafficking. MLN1 may take

part in the control of Ca^{2+}-dependent stages of the endocytotic/exocytotic cycles as well as in pathophysiological processes involving lysosomal aggregation, proteolysis and storage. Abnormalities in each of these processes could be implicated in the pathogenesis of MLIV.

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