

Expression of osmotically responsive cationic channel TRPV4 in the endolymphatic sac

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Abstract

The immunohistochemical expression pattern and the physiological role of transient receptor potential vanilloid (TRPV) 4 in the endolymphatic sac were investigated. TRPV4 was expressed predominantly in the apical membrane of the mitochondria-rich cells and cell volume regulation by TRPV4 was observed in a tissue culture of the rat endolymphatic sac. TRPV4 was also present in the endolymphatic sac of both patients with vestibular schwannoma and with Meniere's disease. TRPV4 is assumed to play a role in cell and fluid volume regulation in the human endolymphatic sac as an osmoreceptor.

Key word:

Endolymphatic sac , Transient receptor potential vanilloid 4 , Meniere's disease ,
Osmolarity

Introduction

In recent years, the interest in mechanosensitive ion channels has been growing and in such mechanosensitive ion channels, transient receptor potential vanilloid 4 (TRPV4) is recognized as an osmoreceptor [O'Neil and Heller, 2005]. TRPV4, a member of the transient receptor potential (TRP) superfamily, is a Ca^{2+} permeable non-selective cationic channel activated by hypotonicity and plays a functional role in fluid and cell-volume regulation [Liedtke, 2005; Becker et al., 2005].

Regarding the inner ear, TRPV4 is known to be present in the guinea pig [Takumida et al., 2005] and is essential for hearing in *Drosophila* [Kim et al., 2003] and mice [Tabuchi et al., 2005]. However, except in the cochlea, the role of TRPV4 has not been established in the inner ear, especially in fluid regulating parts of the inner ear such as the endolymphatic sac. In addition, although osmolarity is involved in the fluid regulation in the endolymphatic sac and in the pathogenesis of Meniere's disease [Godlowski, 1972; Klockhoff and Lindblom, 1966], how the endolymphatic sac epithelium senses osmolarity changes is also poorly understood.

The human endolymphatic sac is assumed to be the most responsible site of the pathogenesis of endolymphatic hydrops and Meniere's disease [Hallpike and Cairns, 1938; Zechner G and Altmann F, 1969; Schindler RA et al., 1979; Arenberg IK and Norback DH, 1981], and if TRPV4 is present in the human endolymphatic sac, it is

speculated that TRPV4 is related to the pathogenesis of Meniere's disease in addition to cell volume and fluid regulation.

In the present study, three independent experiments were conducted in the rat and human endolymphatic sac to understand the role of TRPV4 under normal conditions and in Meniere's disease. First of all, expression patterns of TRPV 4 in the rat endolymphatic sac were investigated by an immunohistochemistry.

In general, when cells swell owing to osmosis in a hypotonic condition, cells recover their original volume in continued presence of the osmotic stress by an active reduction of cell volume which is called regulatory volume decrease [Lang et al., 1998]. It has been proven that TRPV4 controls regulatory volume decrease in airway epithelial cells [Arninges et al., 2004], and keratinocyte cell lines [Becker et al., 2005]. Thus, to investigate whether TRPV4 controls regulatory volume decrease in the endolymphatic sac, cell volume measurements using tissue culture of rat endolymphatic sac were conducted. After these experiments in the rat endolymphatic sac, expression patterns of TRPV4 were also investigated in human endolymphatic sacs obtained from patients with vestibular schwannoma and Meniere's disease by an immunohistochemistry. The data presented here demonstrates a novel association between TRPV4 and the endolymphatic sac.

Materials and Methods

Tissue culture of the endolymphatic sac

Rats (postnatal day 4) were anaesthetized by sodium pentobarbital (0.4 mg/g body mass) and then decapitated. Temporal bones were removed immediately and placed in cold (4°C) HEPES-buffered saline with Hank's balanced salt solution (138mMNaCl, 5mMKCl, 0.5mM MgCl₂, 1.3mM CaCl₂, 5mM glucose, 10mM HEPES, pH 7.2). The entire endolymphatic sac was removed from the temporal bone, opened at the edge of the distal portion of the endolymphatic sac, and mounted flat on a culture slide coated with 20µl Cell Tek (Becton Dickinson Labware, USA) diluted 1:5 and covered with a 300µl culture medium consisting of Minimum Essential Medium with D-valine (MEM D-Val) to suppress fibroblast growth, supplemented with 10% fetal calf serum (FCS), 10mMHEPES, 100IU/ml penicillin and 2mM glutamine. Cultures were kept in a 5% CO₂ atmosphere at 37°C for up to 2 days. The morphology of the culture was monitored by differential interference contrast (DIC) infrared light microscopy. Detailed surface morphology of the epithelia was obtained by scanning electron microscopy. The coverslips with the explants were fixed with a 2.5% glutaraldehyde, 0.1M sodium cacodylate buffer for 120 min, postfixed in 1% osmium tetroxide for 60 min, washed, dehydrated, gold-coated according to the standard procedures, and were

examined using a Hitachi 500 scanning electron microscope.

Immunostaining procedure for the rat endolymphatic sac.

Cells grown on glass coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. The cells were washed in PBS, permeabilized with 0.1 % Triton X-100 in PBS for 3 min. Specimens were incubated with a rabbit polyclonal antibody to TRPV4 (Alomone Labs Ltd., Jerusalem, Israel) at a dilution of 1:200 in PBS at 4°C overnight in a humidified chamber. The TRPV4 antibody was originally raised against a synthetic peptide representing the carboxyl-terminal sequence of TRPV4, and then subjected to affinity purification with the peptide antigen (CDG HQQ GYA PKW RTD DAP L). Specimens were then incubated with anti-rabbit Cy3-conjugated secondary antibody (DIANOVA, Japan) for 1 hr, washed, and mounted. Then, immunostained specimens were analyzed with a confocal laser microscope (LSM 510, Zeiss, Germany). Images were digitally captured and then analyzed using imaging software provided and downloaded from Zeiss. Control samples consisted of rat kidney tissue known to contain TRPV4. Technique controls were performed for each specimen using the same method but omitting the use of the primary antibody.

Measurement of Cell Volume

Cells were loaded with fluorescent probe calcein (Molecular Probes, Japan), and were excited at 490nm. Emitted fluorescence was measured at 510nm. Prior to each experiment, initial fluorescence changes under isotonic conditions were recorded and later used to calculate fluorescence drift correction. Fluorescence intensity changes induced by anisosmotic challenges were monitored as an index of relative cell volume change with an Olympus X51 microscope. Readings were taken every 30s for 10 min. The total calcein fluorescence intensity was measured by Universal Imaging MetaMorph software. The cross-sectional area was assumed to be proportional to the cell volume and was expressed as a relative value to control conditions ($t < 0$ seconds). Relative volume (V_t/V_0) was calculated as $(V_t/V_0) = [(F_t/F_0) - f_b] / (1 - f_b)$; where V_t is the cell volume at time t and V_0 is the initial cell volume. F_0 is the fluorescence of cells in isotonic solution and F_t is the fluorescence intensity at time t . f_b is the background fluorescence. Data are presented as mean \pm SD. Significant difference between individual groups was tested by using analysis of variance. Cell volume was measured in isotonic solutions first and then measured in the following solutions: hypotonic solutions, Ca^{2+} -free hypotonic solutions, and hypotonic solutions containing Gd^{3+} .

Hank's balanced salt solution was used as an isotonic solution. Hypotonicity was achieved by the addition of distilled water, lowering the osmolarity from 300 mOsm to 200 mOsm. GdCl_3 (Sigma Japan) was used at a final concentration of 100 μM prepared in Hank's balanced salt solution. Modified Hank's balanced salt solution (138mMNaCl, 5mMKCl, 0.5mM MgCl_2 , 1.0mM EGTA, 5mM glucose, 10mM HEPES, pH 7.2) was used for Ca^{2+} -free experiments. Measurements of cell volume changes were performed in both the mitochondria-rich cells and the ribosome-rich cells and whether cells measured were the mitochondria-rich cells or the ribosome-rich cells were determined by whether shapes of the cells were round or polygonal [Qvortrup K et al., 1994; Kumagami et al., 1998].

Human endolymphatic sac

Samples of endolymphatic sac tissue were obtained from 6 patients who had undergone a translabyrinthine removal of vestibular schwannoma and 6 patients with Meniere's disease during endolymphatic sac surgery.

Patient Data; vestibular schwannoma patients

Regarding the 6 patients with vestibular schwannoma, their ages ranged from 34 to

62 years, with a mean of 48.8 years. All the patients with vestibular schwannoma were unilaterally affected and their pure tone audiometry showed various degree of sensorineural hearing loss on their affected ears ranging from 60 dB to total deafness, with an average of 76.5 dB. The diameters of their tumors were all within 3.0 cm and the histopathological diagnosis was schwannoma in all 6 cases.

Patient Data; Meniere's disease patients

In the present study, all 6 patients with Meniere's disease could be classified as definite cases diagnosed in accordance with the diagnostic scale of the 1995 American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS) for Meniere's disease [Committee, 1995]. The age of the patients in this group ranged from 32 to 49 years with an average of 39.3 years. The duration of the disease ranged from 2 to 4 years with an average of 2.6 years. All the patients were unilaterally affected, showing sensorineural hearing loss on their affected ears and intractable vertigo attacks.

Specimen Collection

In the patients with vestibular schwannoma, almost all the intraosseous portion of the endolymphatic sac was excised. In the patients with Meniere's disease, a part of

the intermediate portion of the endolymphatic sac was biopsied. The purpose of the present study was explained to each patient and informed consent was obtained before the surgeries. All procedures were performed in accordance with the guidelines of the Declaration of Helsinki.

Tissue preparation for immunohistochemistry in human endolymphatic sac.

Immediately after removal the endolymphatic sac was washed with saline to remove blood and bone dust. Each sample ranged in length from 2 to 3 mm. The specimens were shock-frozen in a liquid nitrogen-cooled isopentane and were stored at -70°C. Proceeding this, 4- μ m-thick cryostat sections were made. The sections were then serially sectioned as parallel as possible to the long axis of the endolymphatic sac, fixed, and mounted on a glass slide.

Immunostaining procedure for the human endolymphatic sac.

Sections were immersed in 1.5% Triton X-100 in phosphate buffered saline (PBS) at pH 7.2 for 5 min, followed by immersion in 3% H₂O₂ for 5 min, and blocked with 5% normal goat serum in PBS for 1 hour. A polyclonal TRPV4 which was originally raised against a synthetic peptide representing the carboxyl-terminal sequence of TRPV4 was used.

The antibody was subjected to affinity purification with the peptide antigen (CDG HQQ GYA PKW RTD DAP L). The specimens were incubated with the antibody at a dilution of 1:200 in PBS at 4°C overnight in a humidified chamber. Then, the sections were incubated overnight with the first antibody at a dilution of 1:200 in PBS at 4°C overnight in a humidified chamber, washed three times with 0.0075% Brij 35 in PBS, and reacted with horseradish peroxidase (HRP)-goat anti-rabbit IgG for 1h. After the slides were washed with 0.0075% Brij 35 in PBS, HRP sites were visualized with 3,3'-diaminobenzidine-4HCl (DAB)/ H₂O₂. For negative control, normal rabbit IgG or normal rabbit serum was used instead of the first antibody, respectively, in every experiment. For laser microscopic imaging, the same procedure was employed as in the rat endolymphatic sac.

Results

Tissue culture of the rat endolymphatic sac

Outgrowth of epithelial-like cells of the explant is observed in culture. Cells corresponding to ribosome-rich cells were polygonally shaped and flat whereas cells corresponding to mitochondria-rich cells were round in culture. The two cell types could be easily distinguished by shape and surface morphology by a differential

interference contrast (DIC) infrared light microscopy (Figure 1A-C). In the tissue culture of the rat endolymphatic sac, basic ultrastructures of the epithelium were preserved well. The mitochondria-rich cells having round shape and the ribosome-rich cells having polygonal and flat shape were observed by a scanning electron microscopy (Figure 2A-B).

Immunostaining of TRPV4 of the rat endolymphatic sac in culture

Moderate-strong staining of TRPV4 was observed in both the ribosome-rich cells and the mitochondria-rich cells, however, strong staining of TRPV4 was predominantly observed in the apical membrane of the mitochondria-rich cells (Figure 3A-C).

Cell volume measurement

Although strong staining of TRPV4 was more predominant in the mitochondria-rich cells than in the ribosome-rich cells, cell volume measurements were performed in both the mitochondria-rich cells and the ribosome-rich cells. Reduction of extracellular osmolality led to a rapid increase in cell volume of round-shaped cells which were recognized as mitochondria-rich cells followed by regulatory volume decrease. The mitochondria-rich cells in culture reached a maximum volume of 25-35% above control

cells in isotonic conditions. A decrease of cell volume by regulatory volume decrease started about 180 seconds after exposure to hypotonic solutions. After 360 seconds, 90% of the observed cells had completed regulatory volume decrease and most of them regained their original volume. Replacement of the medium alone did not affect cell volume, however, treatment with 100 μ M Gd³⁺ solution blocked regulatory volume decrease, and no subsequent regulatory volume decrease occurred after the initial swelling under Ca²⁺-free solutions (Figure 4). In the ribosome-rich cells, regulatory volume decrease in hypotonic solution was observed to be the same as the mitochondria-rich cells. However, in ribosome-rich cells, cells showing a weak inhibition of regulatory volume decrease in Gd³⁺ and Ca²⁺-free hypotonic solutions coexisted with cells showing no inhibition of regulatory volume decrease (Figure 5). Thus, compared to the result of the mitochondria-rich cells, the ribosome-rich cells showed a lesser inhibition of cell volume decrease by 100 μ M Gd³⁺ or Ca²⁺-free solution on average.

Immunostaining of TRPV4 of the endolymphatic sac in human

Moderate to strong staining of TRPV4 in the epithelium of the endolymphatic sac obtained from all 6 patients with vestibular schwannoma was demonstrated in light

microscopic observations (Figure 6A). No degeneration of the endolymphatic sac epithelium was observed in the 6 patients with vestibular schwannoma. Regarding Meniere's disease, in 2 patients whose epithelial cells were preserved well, TRPV4 was observed in the epithelium (Figure 6B). However, it was difficult to determine whether the endolymphatic sac had degeneration of the epithelium or was not adequately obtained and fixed (Figure 6C) in 4 cases. In the kidney tissue of rats selected as control specimens, positive staining was demonstrated and staining without the primer antibody showed negative findings.

Discussion

Our results indicate that TRPV4 has a functional role as an osmosensor in the endolymphatic sac. TRPV4 expression in the rat endolymphatic sac is more predominant in the apical membrane of the mitochondria-rich cells than in the ribosome-rich cells. The mitochondria-rich cells protrude into the lumen of the endolymphatic sac and the apical membrane faces endolymph. Based on this, in the endolymphatic sac, the mitochondria-rich cells should sense an osmotic gradient of endolymph by TRPV4.

In the human keratinocyte cell line (HaCaT) that produces TRPV4 endogenously,

although during swelling and volume regulation, a strong Ca^{2+} influx is measured, regulatory volume decrease is blocked by Gd^{3+} and is diminished in a Ca^{2+} - free solution [Becker et al., 2005]. Since Gd^{3+} is described as an inhibitor of TRPV4 [Becker et al., 2005] as well as stretch-activated channel [Caldwell et al., 1998], Gd^{3+} was used as an inhibitor of TRPV4 also in the present study. In the endolymphatic sac, regulatory volume decrease of mitochondria rich-cells is clearly inhibited by Gd^{3+} in all measurements. Thus, TRPV4 would be a likely candidate for mediating regulatory volume decrease in the mitochondria-rich cells. Since the mitochondria-rich type cells in the endolymphatic sac are suggested to have several ion channels and transporters such as pendrin or vascular H^{+} -ATPase [Peters et al., 2002; Duo et al., 2004], the mitochondria-rich type cells are suggested to play an important role in ion and pH regulation of endolymph. In addition to the above ion channels and pumps, it is assumed that mitochondria-rich cells maintain a proper cell function in that TRPV4 responds to osmolality changes and regulates cell volume.

To the contrary, regulatory volume decrease is not always inhibited by Gd^{3+} in ribosome-rich cells. Based on the results of immunostaining, TRPV4 is present in mitochondria-rich cells predominantly and thus, seems to function constitutively. Contrary to this, in ribosome-rich cells, since TRPV4 is not expressed in all cells,

TRPV4 is assumed to be conditionally expressed and exhibit a functional role. The expression patterns of TRPV4 are similar to the rat kidney collecting duct. In the rat kidney collecting duct, intercalated cells having abundant mitochondria exhibit greater TRPV4 expression than principal cells with abundant ribosomes absorbing water [Tian et al., 2004; Cohen, 2005].

Considering the cellular mechanism of cell volume regulation by TRPV4 in the endolymphatic sac, as regulatory volume decrease was inhibited in a Ca^{2+} free solution, Ca^{2+} also plays an important role in cell volume regulation associated with TRPV4. In the present study, regulatory volume decrease of cells in the endolymphatic sac, especially mitochondria-rich cells were inhibited by Ca^{+} -free solutions. Although cytosolic Ca^{2+} of the endolymphatic sac epithelium was not measured, the influx of Ca^{2+} accompanied by activation of TRPV4 in hypotonicity would seem to be essential for regulatory volume decrease in the endolymphatic sac epithelium expressing TRPV4.

Our study revealed not only a physiological role of TRPV4 in the rat endolymphatic sac, but also the presence of TRPV4 in the human endolymphatic sac. TRPV4 is also involved in the synthesis of vasopressin [Mizuno et al., 2002], secretion [Liu et al., 2006], fluid viscosity changes and ciliary function [Andrade et al., 2005]. In the human endolymphatic sac, hyaluronan [Dankwardt-Lilieström et al., 1994] is

synthesized, natriuretic peptide receptors are present [Dornhoffer et al., 2002], and a natriuretic peptide production was suggested in the rat endolymphatic sac [Qvortrup K et al., 1996]. Since TRPV4 exhibits a functional role in the endolymphatic epithelium to changes of osmolality, TRPV4 may participate in the synthesis of hyaluronan and natriuretic peptide which are related to osmoregulation. This should be further investigated.

In the present study, and also in the endolymphatic sacs obtained from patients with Meniere's disease, staining of TRPV4 was observed and TRPV4 seems to play a role in Meniere's disease. Regarding the pathological findings of the endolymphatic sac of patients with Meniere's disease, the small size of the endolymphatic sac [Hebbar et al. 1991], perisaccular fibrosis [Schindler et al., 1979], degeneration of the epithelium [Schindler, 1979; Kumagami 1990], and saccitis [Danckwardt-Lillieström et al., 1994] have been reported. However, the knowledge of the ultrastructure of well-preserved human endolymphatic sac is still limited [Lim D and Glasscock MI, 1981; Danckwardt-Lillieström et al., 2000] and findings of the endolymphatic sac in Meniere's disease remain controversial [Wackym et al., 1990]. In the present study, as the sample size was small and the number of well-preserved endolymphatic sac obtained from patients with Meniere's disease was limited, it was difficult to distinguish

degeneration from a traumatized piece of the endolymphatic sac. Thus, we could not reach a conclusion on essential roles of TRPV4 in Meniere's disease. Therefore, further studies analyzing human endolymphatic specimen adequately obtained from patients with Meniere's disease are required.

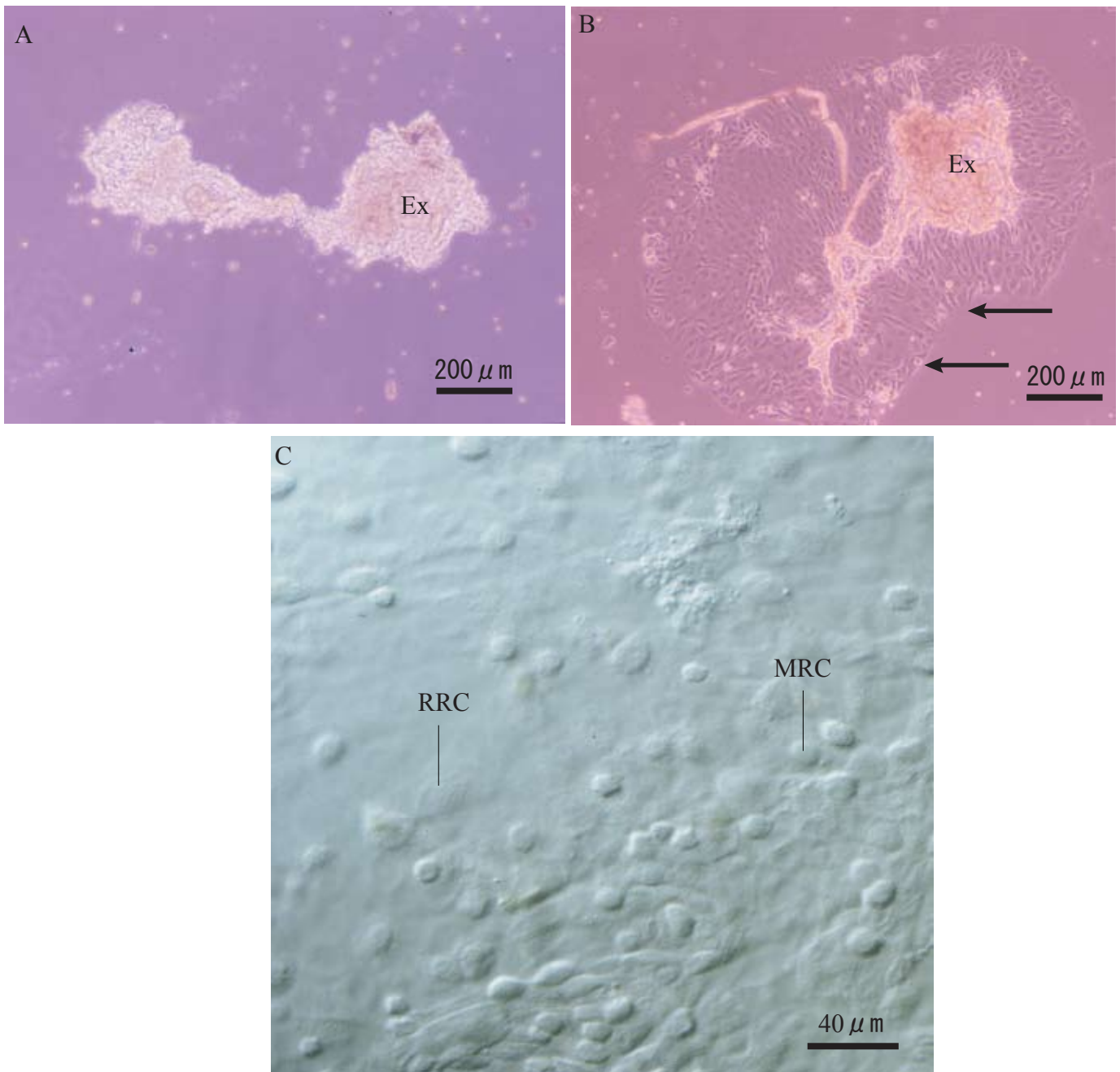


Figure 1A-C. A surface view of tissue culture of the rat endolymphatic sac.

A. Infrared light microscopic image of an explant of the rat endolymphatic sac (Ex). The intraosseous portion of the rat endolymphatic sac was cultivated.

B. Overview of the endolymphatic sac after 2 days in culture. Outgrowth of epithelial-like cells of the explant is observed in culture (arrow).

C. Infrared light microscopic images of individual cells from the intermediate portion of the endolymphatic sac in culture. Two cell types can be distinguished by shape and surface morphology. Cells corresponding to ribosome-rich cells (RRC) are polygonally shaped and flat whereas cells corresponding to mitochondria-rich cells (MRC) are round.

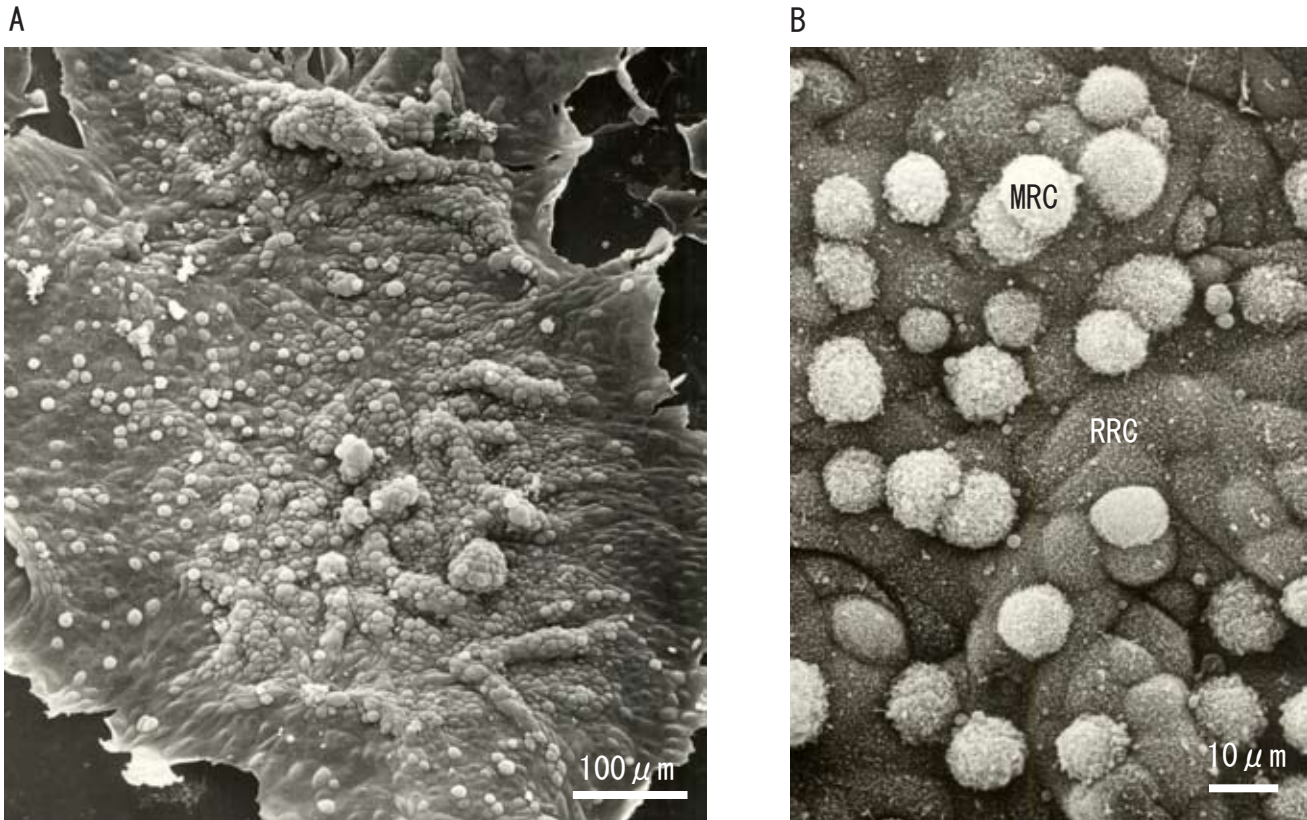


Figure 2A-B. Scanning electron micrograph of cultured rat endolymphatic sac.

A. A whole view of rat endolymphatic sac after 2 days in culture.

B. Polygonally shaped ribosome-rich cells (RRC) and round mitochondria-rich cells (MRC) having numerous microvilli and protruding into the lumen can be identified at higher magnification. The cells are similar to adult native rat endolymphatic sac.

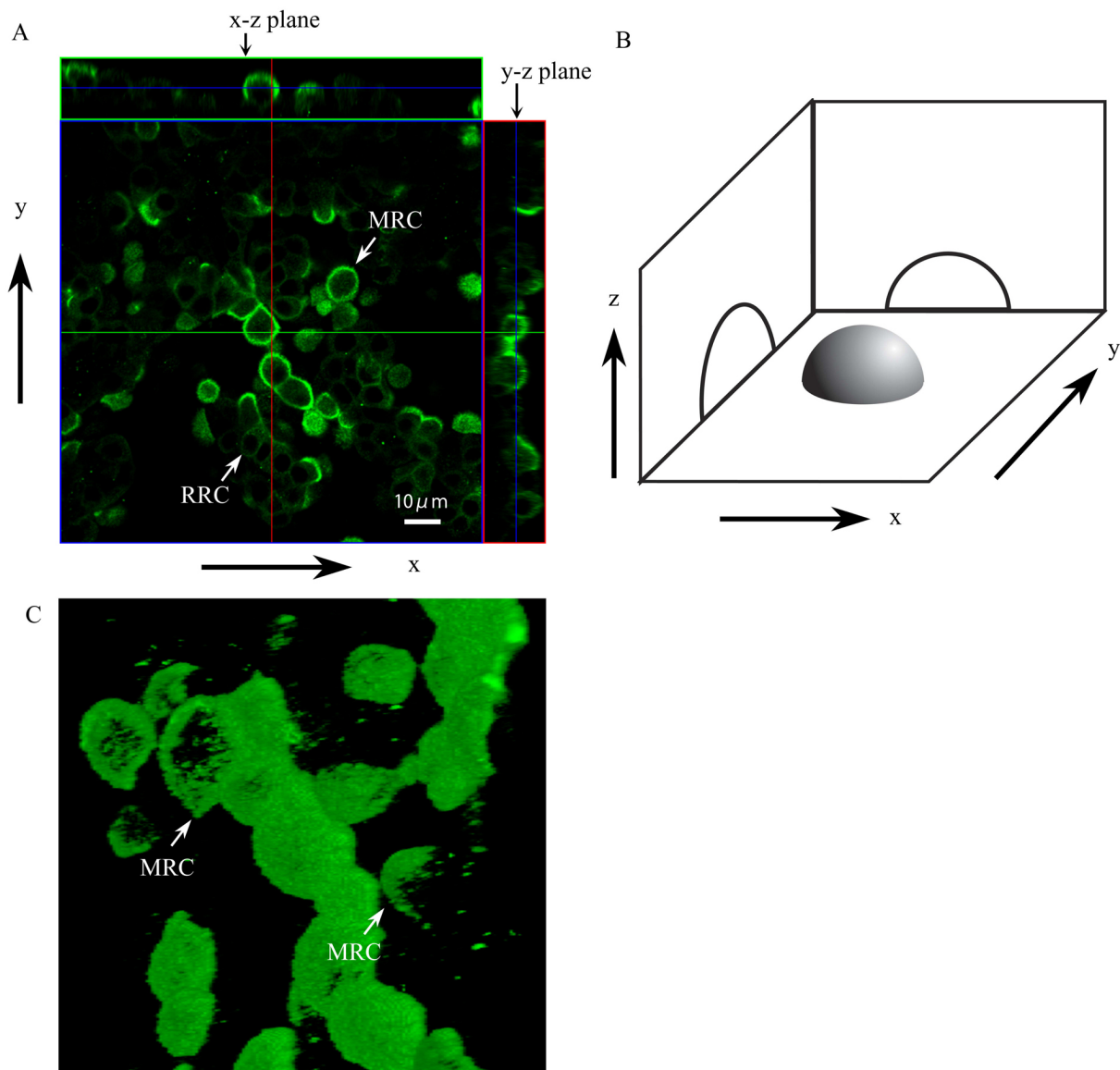


Figure 3A-C. Localization of TRPV4 in the rat endolymphatic sac.

A. A confocal laser microscopic observation of immunofluorescence staining of TRPV4 in the cultured rat endolymphatic sac. The surface view of the rat endolymphatic sac in culture is indicated. The image shows a single x-y plane taken slightly above the basal membrane (center) and the cross-section in x-z and y-z planes, respectively (top and right). TRPV4 is clearly localized to the plasma membrane. TRPV4 is predominantly expressed in the apical membrane of the mitochondria rich cells which are relatively round in shape (MRC). However, in polygonally shaped epithelial cells assumed to be the ribosome-rich cells (RRC), fluorescence of TRPV4 is limited in some cells. RRC = ribosome-rich cell. MRC = mitochondria-rich cells.

B. A scheme explaining the views projected from x-z and y-z planes.

C. A view of 3-dimensional reconstruction of the confocal laser microscopic image. Fluorescence of TRPV4 is predominantly present in the mitochondria-rich cells as the fluorescence which covers the surface of the hemisphere.

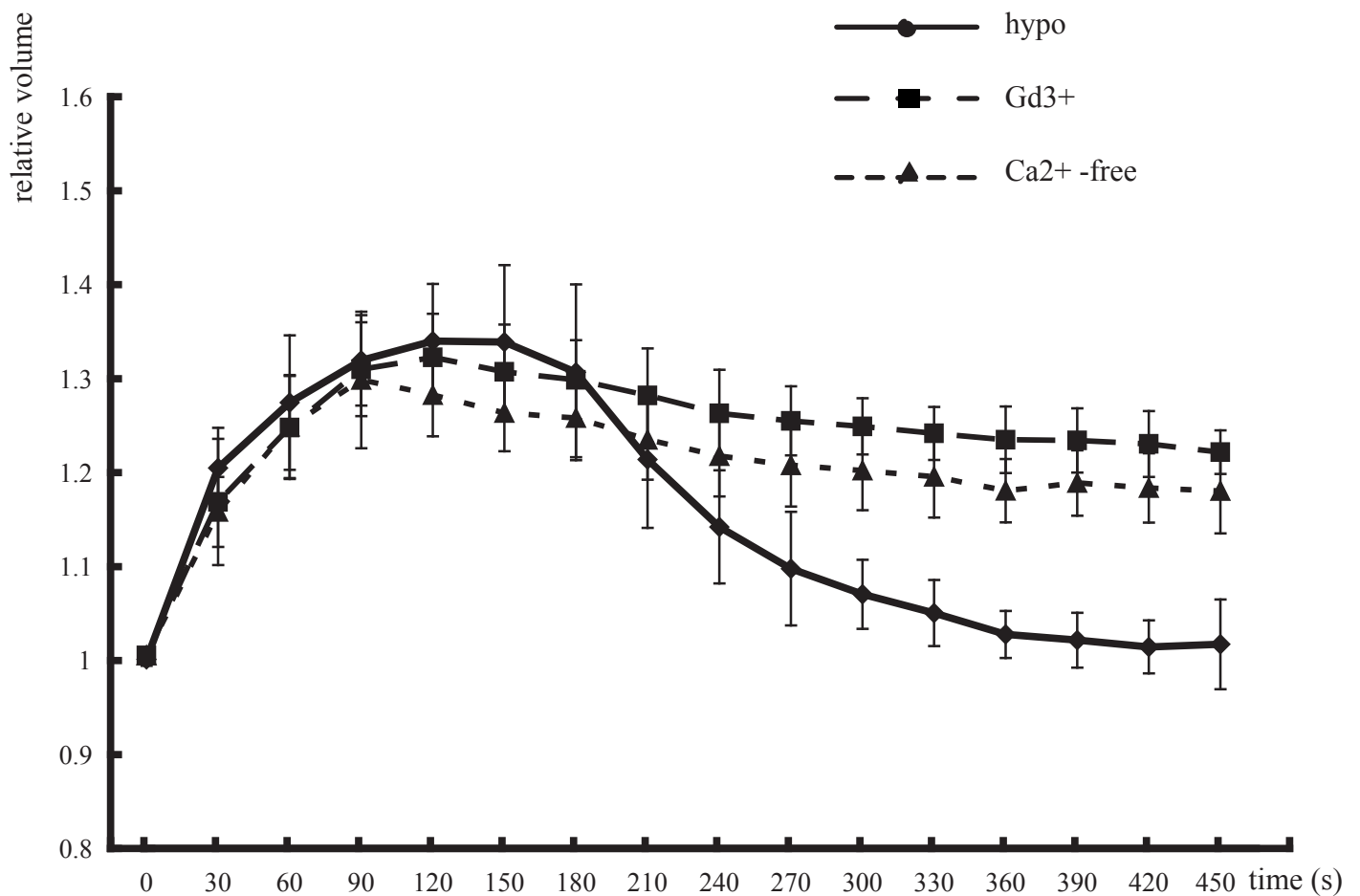


Figure 4. Measurements of relative volume changes of the mitochondria-rich cells.

Relative volume changes of the mitochondria-rich cells were measured when the cells were exposed to hypotonic medium, $100 \mu\text{M}$ Gd^{3+} or Ca^{2+} -free solution. Reduction of extracellular osmolarity led to a rapid increase in cell volume of the rat endolymphatic sac cells (hypotonic, filled circles, $n=20$) followed by regulatory volume decrease. Treatment with $100 \mu\text{M}$ Gd^{3+} (filled triangles, $n=20$) or Ca^{2+} -free solution (open squares, $n=20$) inhibited the volume decrease response and cell volume stayed elevated. TRPV4 is essential for regulatory volume decrease.

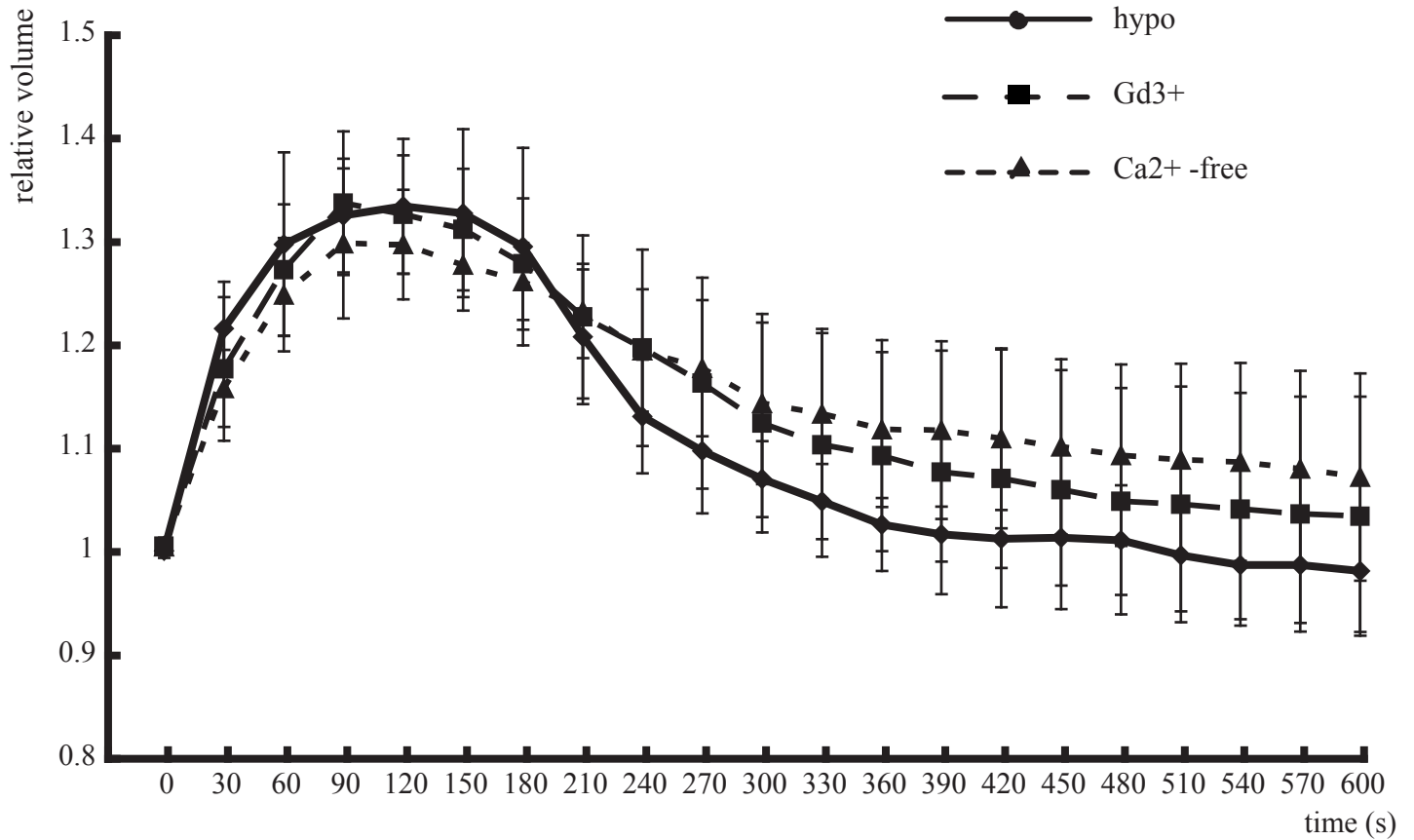


Figure 5. Measurements of relative volume changes of the ribosome-rich cells.

Relative volume changes of the ribosome-rich cells were measured when the cells were exposed to hypotonic medium, 100 μ MGd3+ or Ca2+ -free solution. Reduction of extracellular osmolarity led to a rapid increase in cell volume of the rat endolymphatic sac cells (hypotonic, filled circles, n=20) followed by a regulatory volume decrease. Treatment with 100 μ MGd3+ (filled triangles, n=20) or Ca2+ -free solution (open squares, n=20) inhibited the volume decrease response and cell volume stayed elevated in some cells. However, some cells did not respond to treatment with 100 μ MGd3+ or Ca2+ -free solution. Thus, compared to the result of the mitochondria-rich cells, the ribosome-rich cells showed a lesser inhibition of cell volume decrease by 100 μ MGd3+ or Ca2+ -free solution on average.

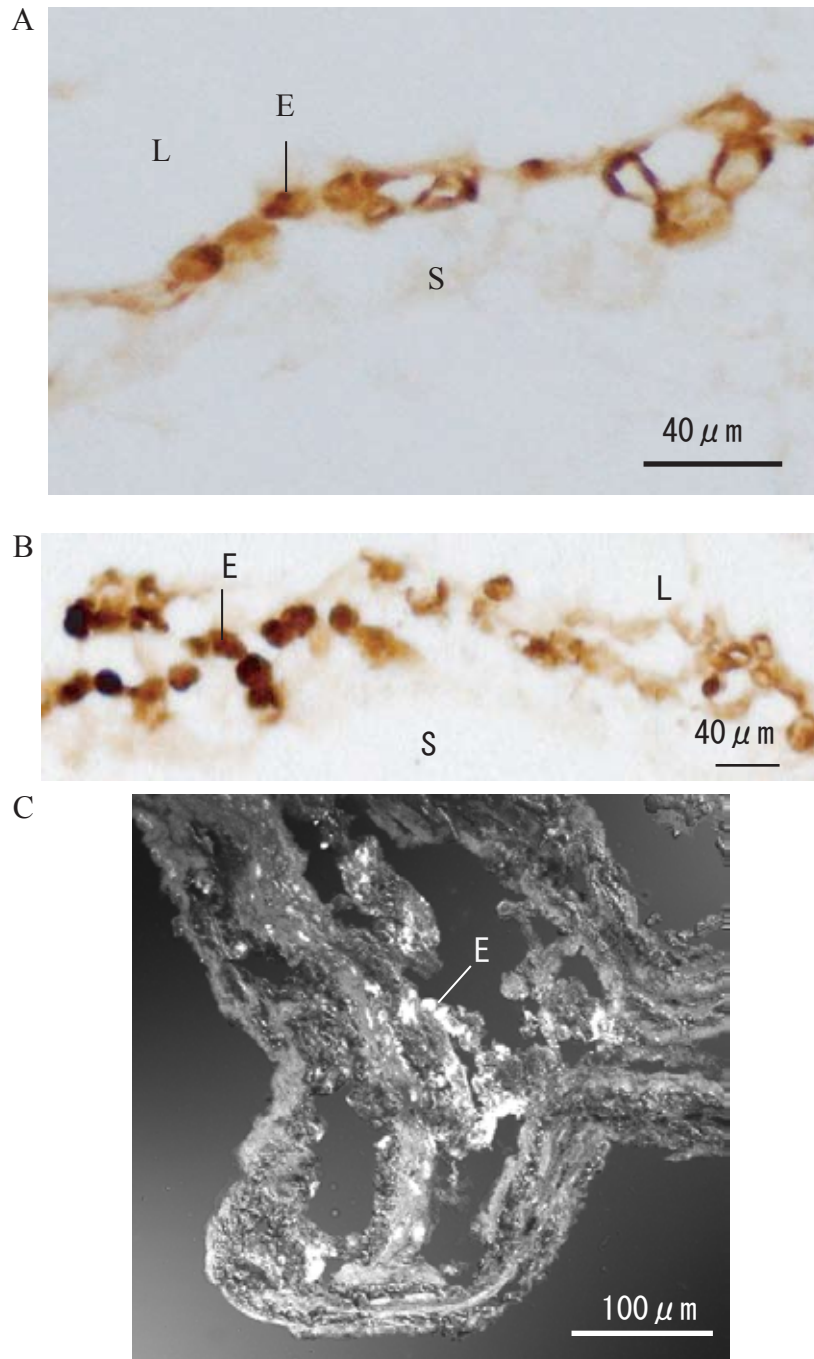


Figure 6A-C. Localization of TRPV4 in human endolymphatic sac.

A. Staining of TRPV4 in the endolymphatic sac obtained from a patient with vestibular schwannoma. Moderate to strong staining in the epithelium of the endolymphatic sac is observed. L = lumen of the endolymphatic sac. E = endolymphatic sac epithelium. S = subepithelial layer.

B. Staining of TRPV4 in the endolymphatic sac obtained from a patient with Meniere's disease. Moderate to strong staining in the epithelium of the endolymphatic sac as observed in a patient with Meniere's disease. In this case, the epithelium was preserved.

C. A confocal laser microscopic observation of immunofluorescence staining of TRPV4 in the endolymphatic sac obtained from a patient with Meniere's disease. The confocal laser microscopic image is combined with DIC imaging. Although immunofluorescence of TRPV4 seems to be limited in the remaining epithelium, it is difficult to determine whether the endolymphatic sac has degeneration of the epithelium or was not adequately obtained and fixed.

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