Original Article

Acidic and Basic pH Effect in Two Cytoplasmic and Endoplasmic Reticulum Luminal Spaces on Chloride Channel Electrophysiological Behavior

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Abstract

Background: In regard of chloride channel electrophysiological behavior importance in cellular homeostasis maintenance, some of diseases appearance because of chloride channels impairment, also reports of synchronization between chloride channels impairment and misadjusted pH and that presumably acid or basic pH in cytoplasmic and endoplasmic reticulum luminal spaces are effective on this behavior, current study was performed.

Materials and Methods: Research was performed by experimental method. Vesicles from rat liver tissue endoplasmic reticulum were extracted and assessed in 30 samples in 6 groups. Electrophysiological behaviors of channels were measured in control, acidic and basic pH in cis and Trans environments and according of channel conductance and Po this behavior was determined and judged statistically. Data were filtered at 1 kHz and stored at a sampling rate of 10 kHz for offline analysis by PClamp9. Statistical analysis was performed based on Markov noise free single channel analysis.

Results: Channel conductance was 72 pS and its current – Voltage relation curve was linear. Channel has Voltage dependent behavior and has grater Po in positive Voltages. Channel conductance in acidic pH remained at 72 pS as of control situation. Channel Po was not changed. In basic pH these findings were also repeated. Also, in cis and Trans spaces these behaviors were sawed.

Conclusion: It seems that in pH stream from 6 to 8.5, current channel electrophysiological behavior could be important in endoplasmic reticulum and cellular homeostasis maintenance especially in positive ion such as calcium ion accumulation situation in cytoplasm.

Keywords: Endoplasmic reticulum, Chloride channel, Hepatocyte, Acidic pH, Basic pH

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Introduction

It well known that chloride channels have many important homeostatic functions in the cell including membrane potential determining, cellular volume regulation, and transepithelial fluid secretion pH regulation and calcium ion homeostasis[1, 2]. These channels provide counter-ion balancing during proton

exchange in vesicles loading or acidification as in calcium ion release and uptake[3]. Indicated that there is pH=8 in luminal space of endoplasmic reticulum (ER) which can disturb calcium ion homeostasis in this organelle by sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) pump inhibition[4]. Because of chloride channels impact on pH adjustment, these channels affect many pH-related phenomenon such as protein synthesis and folding, vesicle loading, conduction of vesicles in right destination, phospholipid composition arrangement different cellular membranous compartments, and calcium ion homeostasis[5, 6].

There are some scientific reports about apoptosis occurrence because of cytoplasmic acidosis and calcium ion homeostasis disruption[7, 8]. Due to intracellular acidification, apoptosis can occur by caspase dependent or nondependent routes[9]. This acidification-induced apoptosis is accompanied with degenerative diseases such as neurodegenerative diseases[10]. Chloride channels impairment is indicated in some diseases such as cystis fibrosis, ostepetrosis, macular degeneration, muscular myotonia, renal stones, and hyperecplexia[11]. In some of these diseases chloride channels impairment is accompanied by pH disadjustment. Beside of diseases mentioned above pH disadjustment is indicated in some of other diseases including cancer. Regulation of pH is important in both physiological and pathological situations. It is demonstrated that cancer cells established a changed pH gradient. This new pH gradient provides ability of fast growth and protein synthesis, invasion, migration and metastasis[12, 13].

Chloride channels dysfunction involved in some heart disease including hypertrophy, arrhythmia, ischemia, and heart frailer[15-20]. Research on chloride channels has begun from 1979[21] which scientists have identified Voltage-gated chloride channel in 1980[22]. In addition, researchers have cloned CFTR gene in 1989[23]. Today we know that there are five families of chloride channels including ligand-gated chloride channels, Voltage-gated chloride channels (CLCs), Cystic fibrosis transmembrane conductance regulator (CFTR), calcium activated chloride channels (CaCCs) and intracellular chloride channels (CLIC)[18]. These channels are distributed all over the cell. From cellular compartments we choice ER for research because of its fundamental homeostatic functions and its structural-functional relation with other cellular organelles and plasma membrane. It is indicated that mitochondrial separation from ER can cause calcium homeostasis disruption and apoptosis induction[24-26]. ER apoptosis induction by cytoplasmic acidosis, ER stress effect in physiopathology of heart and neuro degenerative disease and direct relation between ER homeostasis disruption and tumorigenesis are the important reasons for our choice[27-30]. Biophysical, pharmacological, and regulatory agents can affect electrophysiological behavior of ion channels. It is obvious that precise recognition of these agents could be very beneficial in identification of channel physiology and pathophysiology so in related disease treatment.

Ultimately in regard of synchronization between pH regulation and chloride channels function in the cell also, probability of cytoplasmic and luminal pH effect on electrophysiological behavior of chloride channel, current study was performed in Shahid Beheshty university of medical science.

Methods

HEPES, Trizma Base (2-amino-2-[hydroxymethyl]-1,3-propanediol), sucrose, imidazole, pyrophosphate and potassium chloride were purchased from Sigma (St. Louis, MO, USA) and n-Decane and hydrochloric acid was obtained from Merck (Darmstadt, Germany). Salt and solvent were analytical grade (Sigma, St. Louis, MO, USA). Animal experiments were conformed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Shahid Beheshti University of Medical Sciences (Tehran, Iran).

ER proteins extraction: Male Wistar rats, weighting 180–200 grams, were used for ER extraction. Hepatic endoplasmic reticulum vesicles were extracted by the method of Kan et al.[31]. Rats were anesthetized by ether, and the livers were rapidly removed and homogenized in 50 ml ice-cold sucrose (0.25 M) solution at 2850 rpm using a potter homogenizer (Potter-Elvehjem Homogenizer, Iran). The homogenate was centrifuged at 8700 ×g for 13 minutes. The supernatant was centrifuged at 110,000

×g at 4°C for 60 min (Beckman model J-21B, USA). The pellet was gently resuspended in 9 ml ice-cold 2 M sucrose by a glass homogenizer to obtain a homogenous suspension. Subsequently, in sucrose gradient conditions, the suspension was centrifuged at 300,000 ×g for 60 min, and the obtained pellet was dissolved in 20 ml sucrose 0.25 mM + imidazole 3 mM + Na pyrophosphate 0.5 mM. The solution was then centrifuged three times at 140,000 ×g for 40 min. The obtained pellet (rough endoplasmic reticulum microsomes microsomes) was dissolved in 1 ml sucrose 0.25 mM + imidazole 3 mM at a final concentration of 7 mg/ml. Rough microsomes were stored in 10-µl aliquots in 250 mM sucrose/3 mM imidazole (pH 7.4) at -80°C until use.

Lipid preparation: L-α-phosphatidylcholine (L-α-lecithin) was extracted from fresh egg yolk by the procedure described by Singleton et al.[32]. The endoplasmic reticulum membrane was relatively enriched in the neutral zwitterionic phospholipids having large polars head groups such as L-α-phosphatidylcholine.

Planar lipid bilayers and vesicle fusion: Experiments were performed by using black (bilayer) lipid membrane technique. Planar phospholipid bilayers were formed in a 300 µm-diameter hole drilled in a Delrin partition, which separated two chambers, cis (cytoplasmic side) and trans (luminal side). Chambers contained 4 ml KCl 200 mM cis/50 mM trans. Under these conditions, there will be a net movement of water across the bilayer from trans to cis face. Vesicles in the pre-fusion state will swell if water enters the lumen across the bilayer[33]. Cis and Trans solutions contained 10 µM Ca2+. The pH on both sides was adjusted to 7.4 with Tris–HEPES. Planar phospholipid bilayers were painted using a suspension of L-α-lecithin in Decane at a concentration of 25 mg/ml. The indication of the thickness of the bilayer membrane formed across the hole was obtained by monitoring capacitance. A low frequency (1-10 Hz) and a low amplitude (5-20 mV peak-to-peaks) triangular wave were used. Typical capacitance values ranged from 200 to 300 pF. Fusion of the vesicles was initiated mechanically by gently touching the bilayer from the cis face using a small stainless steel wire of 150 µm diameter, on the tip of which a small drop of the vesicle-containing solution was deposited.

Electrical recording: BC-525D amplifier (Warner Instrument, USA) in the voltage clamp mode was used to amplify the current and to control the voltage across the bilayer through Ag/AgCl electrodes. The cis electrode was set to a command voltage relative to the Trans electrode which was grounded.

PH adjustment in luminal (Trans) and cytoplasmic (cis) faces: After single-channel recording of channel in neutral pH (7.4 in cis and Trans solution) recording was repeated in pH of 6 and 8.5 in cis and Trans environment. Acidic pH was prepared by adding HCl and basic pH was obtained by addition of KOH in both cis and trans chambers.

Data analysis: The recordings were filtered at 1 kHz with a four-pole Bessel low-pass filter, digitized at a sampling rate of 10 kHz and stored on a personal computer for off-line analysis by PClamp9 (Axon Instruments Inc, USA). Significant difference between control and acidic or basic pH was assessed by Student T-Test. The results were expressed as means ± standard error of the means (SEM) and $P< 0.05$ values considered significant.

Results

Biophysical properties of ion channel: Our results indicated a chloride channel in ER with conductance of 72 pS when the *Trans* chamber was voltage-clamped relative to the *cis* chamber, which was grounded. Figure 1 shows single-channel currents recorded at various holding potential conditions (50/200 mM KCl *trans/cis*) at various holding potentials (-60 mV to +50 mV) following incorporation of rat ER membrane vesicles into planar bilayers. Sing-channel current voltage relationship was illustrated in Figure 3. A Zero-current potential value close to +30 mV, the equilibrium potential expected for chloride ions under the prevailing ionic conditions was indicated. Furthermore the reverse close to +30 mV indicated unidirectional reconstitution of the channel into bilayer membrane.

The channel gating behavior was voltage dependent with decreased amounts at increasingly negative potential values. The current-voltage (I-V) relation was linear and the slope conductance was 72 pS with positive reversal potential close to +30 mV that illustrates anionic selectivity of this channel under

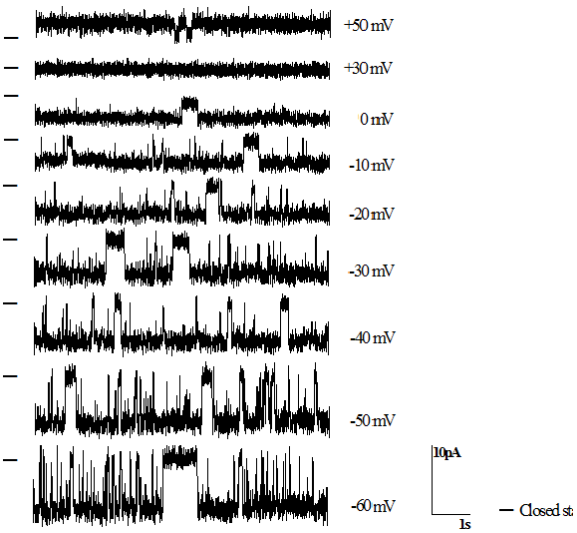

Figure 1. Single-channel currents recorded at various holding potential conditions (50/200 mM KCl trans/cis) at various holding potentials (-60 mV to +50 mV). Data are mean ± S.E. (n = 6). The – indicates the closed state.

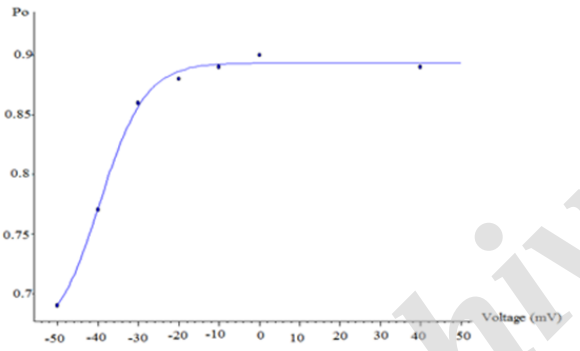

Figure 2. The average steady-state of open probability values as a function of the holding potential for full open conducting state obtained from sex different experiments.

these conditions. The open probabilities (Po) of this channel at various holding potentials and its fitted curve were showed in figure 2. The Po curve was fitted by Boltzmann Z-delta equation.

Pharmacological properties of the ion channel: According to Figure 4 and Figure 5, this channel was blocked by addition of either 300 µm NPPB or ،،1mM DIDS to cytoplasmic face (cis chamber). Also as indicated in Figure 6 and 7, current study indicated that this channel has no responsiveness to environment pH differences. There was no significant difference in electrophysiological properties of channel including conductance or gating behavior in natural (7.4), acidic (6.5) or basic

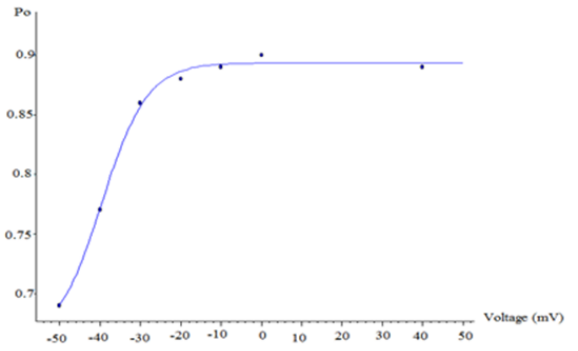

Figure 2. The average steady-state of open probability values as a function of the holding potential for full open conducting state obtained from sex different experiments.

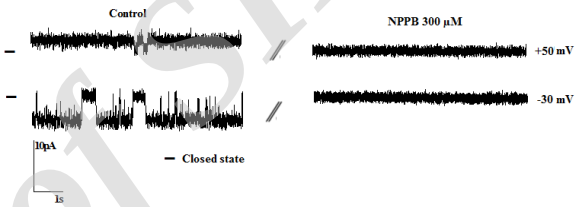

Figure 4. The effects of NPPB on channel activity at different voltages. Channel activities were completely inhibited after the addition of 300µm NPPB to cis face. Closed levels are indicated by –.

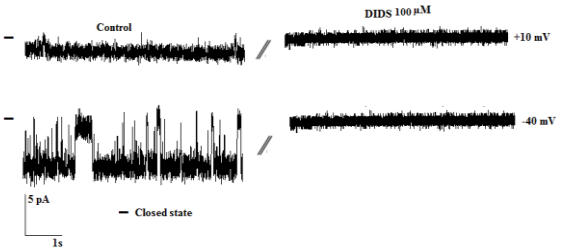

Figure 5. The effect of DIDS on channel activity is indicated at +10 and -40 mV. Channel activity was inhibited by addition of 1mM DIDS to cis chamber. Closed level is indicated by –.

(8.5) pH. This no responsiveness to pH was appeared in both luminal (Trans) and cytoplasmic (cis) environments. On the other hand by adding of 50 mM phosphate ion to cis chamber there was elucidated that this ion has no significant effect on electrophysiological behavior of current chloride channel.

Discussion

Research was indicated that acidic or basic pH in

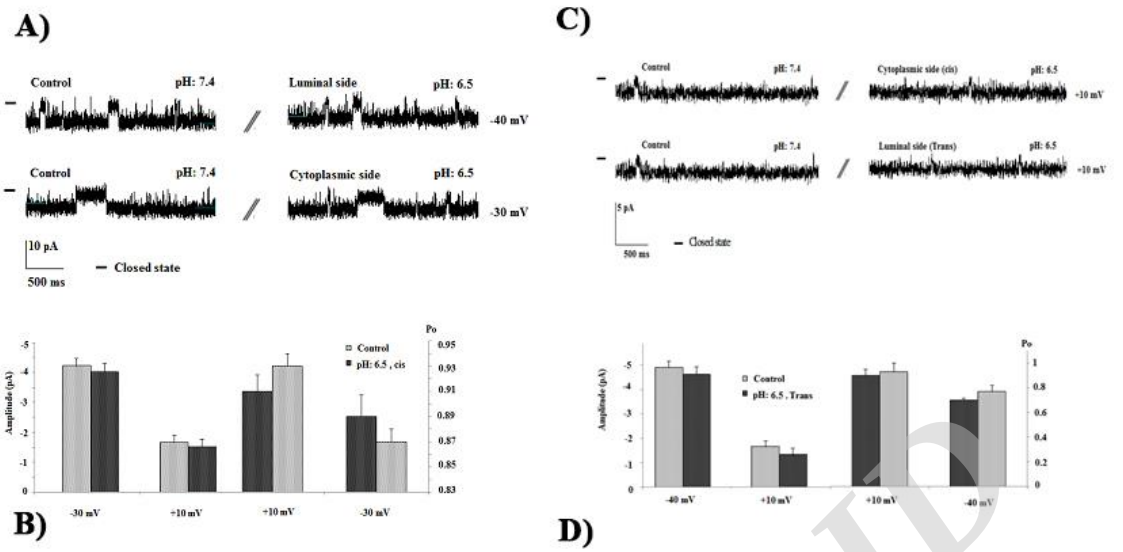

Figure 6. The effect of acidic pH on channel activity is indicated at -30, -40 mV and +10 mV. Either in cytoplasmic (cis) or in luminal (Trans) environment there are no acidic pH effects on electrophysiological activity of this chloride channel. Data are mean ± S.E. (n = 4). Closed level is indicated by–.

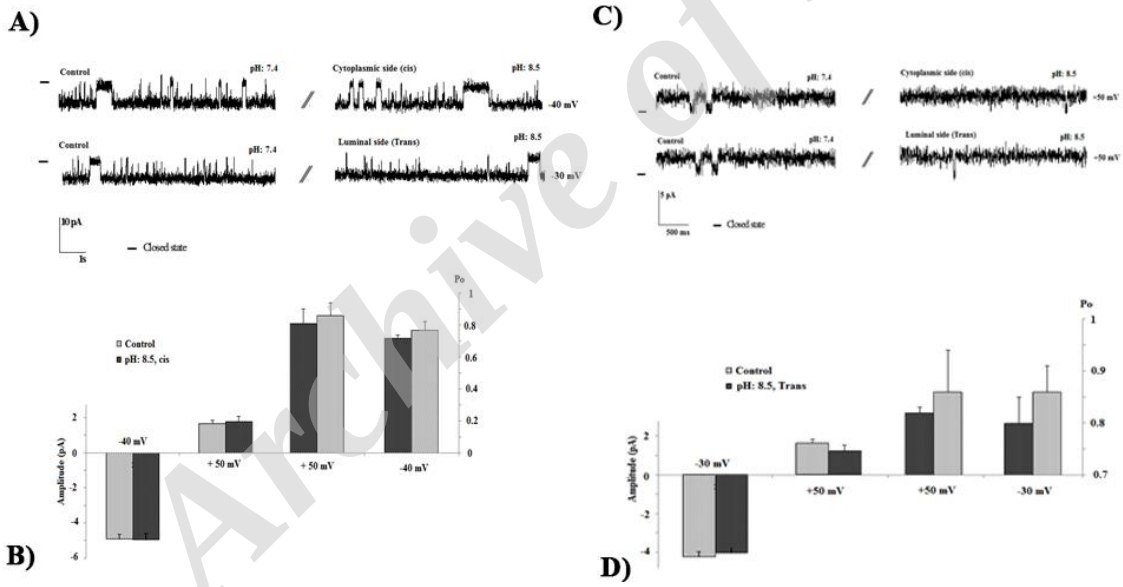

Figure 7. The effect of basic pH on channel activity is indicated at -30, -40 mV and +50 mV. Either in cytoplasmic (cis) or in luminal (Trans) environment there are no basic pH effects on electrophysiological activity of this chloride channel. Data are mean ± S.E. (n = 4). Closed level is indicated by–.

either cis (cytoplasmic) and Trans (luminal) spaces had not affect electrophysiological behavior of current chloride channel including conductance and open probability (Po) and these values are equal to theirs control amounts. In pH stream from 6 to 8.5 there was no effect of proton or hydroxyl ion on channel gating or conductance. One probability is the impact of membrane surface charges on pH changes buffering. The other probability of non pH sensitivity of current chloride channel can be referred to absence of proton affecting site in related subunit of channel structure. Our results are limited to pH stream of 6 to 8.5 and probability of channel pH sensitivity is not rejected beyond this limitation.

It is previously indicated that among chloride channels only CFTR, CLIC1, CLC-2, CLC-3, CLC-4, CLC-5 and CLC-7 are expressed in hepatocyte[34]. On the other hand, there is demonstrated that rat hepatocyte

expresses a lot of CLC-4[35]. Because our channel inhibited by DIDS so it could not belong to CFTR or CLIC families[36, 37]. It seems that belonging of our study chloride channel to CLC family is more probable. Among hepatocyte CLCs, CLC-2 is specially located in plasma membrane and is not belonging to ER[38]. CLC-5 has more proton/chloride exchanger function also; CLC-7 requires Osteopetrosis-associated transmembrane protein 1 (Ostm-1) co-expression to be functional[39]. CLC-4 has more expression in rat hepatocyte and was indicated in ER[40]. Beside of great structural similarity between CLC-3 and CLC-4, the precise identification of our study channel category requires more investigation[41].

Previous studies have been indicated that among ion channels only calcium-activated chloride channels (CaCCs), inward rectifier potassium channels (Kir), acid-sensing ion channels (ASIC), N-Methyl-D-aspartate (NMDA) receptors, transient receptor potential (TRP) and CLCs are pH sensitive[42]. In addition, studies were indicated CFTR and mitochondrial Voltage-dependent anion channel (VDAC) pH sensitivity[43-45]. Scientists were demonstrated that CFTR senses intracellular pH directly. Due to increase or decrease in MgATP affinity to CFTR nucleotide binding domain 2 (NBD2), its electrophysiological function was activated in intracellular acidic pH and decreased in intracellular basic pH respectively[43]. VDAC closing in intracellular acidosis during ischemia prevents cellular apoptosis induction[44]. It is demonstrated that pH effect on CaCCs is indirect and is by the aim of pH affecting calcium channels[46]. As mentioned above, Voltage gated chloride channels are pH sensitive. Researchers were indicated that CLC-2 is inhibited by extracellular acidic or basic pH. They were argued that inhibition of channel function in basic pH is because of direct occlusion of channel pore by hydroxyl ion or Voltage- dependent gating curve displacement due to changes in membrane surface charges. Also, they were suggested that inhibition in acidic pH is because of protonation derived fixation of gating machinery moving part[47]. Beside of probability of current channel belonging to CLCs family, it has no pH sensitivity. We speculate that presumably channel separation from its native situation and only using of L-α-phosphatidylcholine (L-α-lecithin) for artificial membrane formation could be effective in difference of current channel behavior. On the other hand the probability of pH sensitivity beyond pH stream from 6 to 8.5 is not rejected. In current study increase of acidic or basic pH beyond mentioned pH stream was caused bilayer membrane instability and theirs data were not reliable. Current study suggests that non pH sensitivity in pH stream of 6 to 8.5 could be related to fixation of gating machinery moving part due to protonation or change of membrane surface charges. According to channel greater open probability during positive Voltages it seems that channel has more activation in depolarized situations such as positive charges accumulation as indicated in calcium homeostasis disturbances so this channel could be important in apoptosis regulation[48-51].

Previous studies were bolded pH importance in some cellular phenomenon such as phagocytosis, protein synthesis, folding and degradation, vesicle loading and conducting in right destinations[53, 53]. On the other hand, basic pH establishment is favorable during cellular growth and differentiation[54]. Beside of such physiological situations, in some of pathological conditions including cancer a new pH gradient is established. Due to this ability, cancer cells can increase their growth, invasion, migration, metastasis, and drug resistance[55-57]. It is obvious that all of agents contributing in new pH gradient establishment could be important drug targets in cancer treatment[58-59]. Because of current channel functionality in pH stream of 6 to 8.5, overexpression of this channel is probable in cancer so more investigation about this hypothesis is suggested. Also, in some diseases there is synchronization between chloride channels impairment and pH disadjustment[11]. It is presumably useful to examine this channel overexpression in such diseases treatment.

In conclusion our study indicate that hepatocyte endoplasmic reticulum chloride channel has no pH sensitivity in either cytoplasmic or luminal spaces. This could be from buffering effect of membrane surface charges during proton or hydroxyl ion concentration changes, or could be from fixation of gating machinery moving part during protonation. According to current channel more open probability in

positive Voltages seems to be physiologically important in depolarized situations as accumulation of calcium ion and in apoptosis regulation. Ultimately our study suggests more investigations about this channel as an important drug target in diseases related to changed pH gradient including cancer also, in diseases of chloride channels impairments are accompanied by pH regulation disturbances.

Conclusion

Current research indicated that in pH stream from 6 to 8.5, this 72 pS chloride channel electrophysiological behavior could be important in endoplasmic reticulum and cellular homeostasis maintenance especially in positive ion such as calcium ion accumulation situation in cytoplasm.

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