Luminal Chloride-dependent Activation of Endosome Calcium Channels

PATCH CLAMP STUDY OF ENLARGED ENDOSOMES

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Although Ca$^{2+}$ release from early endosomes (EE) is important for the fusion of primary endosomes, the presence of an ion channel responsible for releasing calcium from the EE has not been shown. A recent proteomics study has identified the TRPV2 channel protein in EE, suggesting that transient receptor potential-like Ca$^{2+}$ channels may be in endosomes. The submicron size of endosomes has made it difficult to study their ion channels in the past. We have overcome this problem by generating enlarged EE with the help of a hydrolysis-deficient SKD1/VPS4B mutant in HEK293 cells. Here we report the first patch clamp recording of a novel endosome calcium channel (ECC) in these enlarged EE. The ECC shows a similar pharmacology to that of the TRPV2 channel. In addition, the ECC has a unique chloride-dependent regulation; it is inhibited by the endosome luminal chloride with a $K_{50}$ of 82 mm.

A number of TRP-type calcium channels have been localized to intracellular vesicles; however, their physiological roles in situ have remained largely uncharacterized. The recent identification of the TRPV2 channel protein in an early endosomal fraction (1) has raised the possibility of characterizing the physiological roles of the TRP-like channel in the native environment of the endosome membrane. To achieve this goal, it would be necessary to apply the patch clamp technique directly to endosome membranes. Past attempts at such studies have been unsuccessful, mainly because of the submicron size of endosomes. We have been able to increase the size of the endosome membrane by introducing a hydrolysis-deficient mutant of SKD1/VPS4B (E235Q) into HEK293 cells. Overexpression of SKD1/VPS4B (E235Q), which disrupts endosome trafficking (2), leads to the formation of large endosomes (3–6 μm in diameter) in HEK293 cells. Using these enlarged EE, we have made the first patch clamp recordings of a novel endosome calcium channel (ECC). This ECC was sensitive to >500 μM La$^{3+}$ inhibition as reported in other TRP family channels, but was activated by 100 μM La$^{3+}$ and by 200 μM 2-aminoethoxydiphenyl borate (2-APB), whereas being insensitive to TRPV1 activator capsaicin. Surprisingly ECC activity was affected by the endosome luminal chloride ion concentration ([Cl$^{-}$]$_{lum}$). [Cl$^{-}$]$_{lum}$ inhibited ECC with a $K_{50}$ of 82 mm.

EXPERIMENTAL PROCEDURES

HEK293 cells stably expressing the tetracycline-inducible SKD1/VPS4B (E235Q) gene (3) were treated with tetracycline 4–6 h prior to the experiments. GFP-tagged SKD1/VPS4B (E235Q)-expressing cells were used to demonstrate co-localization of GFP-SKD1/VPS4B (E235Q) with endocytosed rhodamine sulfate. c-myc-SKD1/VPS4B (E235Q)-expressing cells were used to show Lysotracker Green negative staining in the rhodamine sulfate containing enlarged endosomes and were also used in patch clamp experiments.

Enlarged endosomes of sizes from 3–6 μm were visually identified and dissected manually with a glass electrode. Under the phase contrast microscope, enlarged endosomes were viewed as phase-bright (see Fig. 1C, circled). To facilitate slicing of the plasma membrane with an electrode, cells with enlarged endosomes, closer to the edge of cells, were used because cell thickness increased toward the center. The electrode was pressed against the cell vertically and then was quickly pulled away from the cell to slice the cell membrane. Plating the cells on poly-l-lysine-treated cover glass prevented the cells from detaching from the cover glass. Multiple attempts (2–3 times) were required to free the enlarged endosomes from the cell. When successful, enlarged endosomes were seen as phase-dark spheres. Isolation was successful for 20–30% of attempts. Compared with cell membrane blebs, the surface of isolated enlarged endosomes looked slightly rough as if the surrounding structures such as actin filaments were still connected. The cell membrane blebs were usually bigger than enlarged endosomes, and their surface looked smooth.

The patch pipette resistance was 5–6 megOhms. Following giga seal (>5 Gohm) formation, ion channel activities were examined in inside-out configurations. The whole endosome capacitance was 298.06 ± 149.03 femtofarad ($n = 4$), which corresponds to an EE diameter of 3–6 μm, if the lipid unit membrane capacitance is assumed to be 1 microfarad/cm$^2$. An Ag/AgCl agar bridge was used as a ground electrode. The Ag/AgCl agar bridge eliminated a junction potential caused by varying Cl$^{-}$ in the bath solution. The solutions used were (in...
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A pipette solution (KCl 30, K2SO4 60, MgCl2 0.1, EGTA 0.5, HEPES 10, pH 7.20); a Na+-based solution (NaCl 145, KCl 5, CaCl2 1.8, MgCl2 1, HEPES 10, pH 7.20); a K+-based solution (KCl 150, MgCl2 0.1, HEPES 10, pH 7.20); and a K+-based low Cl− solution (KCl 30, K2SO4 60, MgCl2 0.1, HEPES 10, pH 7.20). For the chloride effects, [Cl−] was varied by mixing KCl 150 and K2SO4 75 in different ratios. Patch clamp data were analyzed using Clampfit program (Axon Instruments), OriginPro 7 (OriginLab), and Sigmaplot 9.0 (Systat Software Inc.).

RESULTS

Using artificial bilayer reconstitution, the biophysical and pharmacological properties of intracellular TRP channels from the endoplasmic reticulum membrane (4, 5) and the lysosomal membrane (6) have been examined. TRPV5 and TRPV6 localization has been seen in rab11 positive recycling endosomes (7). However, little is known about their functional properties in native endosomal membranes. Recently the TRPV2 channel protein was identified in rab5 positive early endosomes in IL4/PGE2-treated J774 cells.

Newly endocytosed primary endosomes undergo Ca2+-dependent fusion to become early endosomes (EE) (8, 9). Various reports suggest that the EE itself function as the Ca2+ source (10–12). Although Ca2+ release through ion channels in EE has not been shown, the presence of TRPV2 protein in EE (1) and in intracellular vesicles (13, 14) suggests that the ECC could be a TRP-like channel.

Newly formed EE membranes are exposed to two distinct environments. One environment is the EE lumen, which contains an endocytosed extracellular high Cl− and high Ca2+ solution, and the other is the cytosolic environment, which contains low Cl− and low Ca2+. The difference in ionic compositions between the two leads to a time-dependent release of Cl− and Ca2+ ions from the EE (15, 16). CIC type Cl− channels (i.e. CIC-5) are reported to regulate the Cl− movement. They are localized in the EE, and a disruption of the CIC-5 gene causes a dramatic slowing of endocytosis and reduced endosome acidification (17, 18). On the other hand, no study has been reported describing the existence and/or the characteristics of an endosome Ca2+ channel.

To explore such a possibility, a patch clamp study of the native EE membrane is essential; however, the submicron size of and thus lack of direct access to the EE membrane have previously prevented such an approach. We have overcome this difficulty by generating enlarged EE (∼3–6 μm in diameter) with the help of a hydrolysis-deficient SKD1/VPS4B (E235Q) (2) in HEK293 cells (3). The SKD1/VPS4B AAA + ATPase regulates membrane trafficking at the early endosome junction between lysosomes and recycling endosomes (2, 3, 19). HEK293 cells expressing SKD1/VPS4B (E235Q) formed enlarged EE vacuoles, which were visible under a phase contrast microscope (Fig. 1C). When the cells were incubated with media containing rhodamine sulfate (Rhod), these enlarged EE accumulated the Rhod (Fig. 1A). This suggests that newly formed primary endosomes continue to fuse to the enlarged EE even after expression of SKD1/VPS4B (E235Q). These Rhod containing enlarged EE were not stained by the acidotropic dye, Lysotracker Green, even after 40 min of incubation with the Rhod containing solution (Fig. 1B). Some Rhod containing endosomes escaped the SKD1/VPS4B (E235Q) effect and matured to become lysosomes (stained by Lysotracker Green, Fig. 1B, circled). This suggests that SKD1/VPS4B (E235Q) inhibits the maturation of enlarged EE, and thus the enlarged EE do not acquire lysosomal-like acidity.

From these results we concluded that these visible large vacuoles represent EE. We then developed a procedure to manually isolate the Rhod containing enlarged EE using a glass pipette (see Fig. 1C). This allowed us to use them for patch clamp studies.

FIGURE 1. A, endocytosed Rhod sulfate accumulated in a GFP-SKD1/VPS4B (E235Q)-expressing large endosome. An enlarged EE is shown surrounded by GFP-SKD1/VPS4B (E235Q) (left, green). Endocytosed rhodamine accumulated in this large EE (middle). Merging of the GFP and Rhod images is shown (right). Arrowheads show co-localization of GFP-SKD1/VPS4B (E235Q) and Rhod accumulation. B, a large endosome induced by c-myc-SKD1/VPS4B (E235Q) expression was not acidic. Acidic Lysotracker Green positive vesicles are shown (left). Lysotracker did not accumulate in this enlarged endosome (surrounded by arrows); however, Rhod did (middle). Endosomes following a normal trafficking path to lysosomes displayed co-localization of Rhod and Lysotracker (yellow; circled by dotted line; right); C, the process of manual dissection of an enlarged EE (circled in yellow) is shown (left and middle). Cells were incubated with Rhod previous to dissection. The isolated EE contained Rhod, which was visible under UV light (right). (Scale bars, 10 μm for A and B; 20 μm for C.)
Endosome Calcium Channel—The enlarged EE were electrically quiet in the on-endosome and inside-out excised patch clamp configuration. Reports describing changes in the EE luminal Cl\(^{-}\) and Ca\(^{2+}\) concentrations show that i) within 60 s of EE formation, luminal Cl\(^{-}\) is released to the cytosol (15); and ii) a slow loss of luminal Ca\(^{2+}\) to the cytosol follows (16). For that reason, we explored whether a reduction in [Cl\(^{-}\)]\text{lum} might affect ECC activity. By reducing chloride concentrations in the bath from 150 to 30 mM, a physiological change in [Cl\(^{-}\)]\text{lum} might affect ECC activity. By reducing chloride concentrations in the bath from 150 to 30 mM, a physiological change in [Cl\(^{-}\)]\text{lum} might affect ECC activity.

Acidic pH Activates the Early Endosome Cation Channel—It has previously been shown that the lysosomal cation channel, MCOLN1, (6) and another intracellular cation channel, PKD2, are both inhibited by acidic pH (4, 5). In contrast, as shown in Fig. 3, C and D, ECC activity was enhanced 2–3-fold (n = 8) when the luminal surface of EE excised patch membranes was exposed to pH 5.4 and 6 (the intact EE pH is reported to be 6.2 (8, 9, 15, 21)). Having demonstrated that ECC activity is regulated by both Cl\(^{-}\) concentration and pH separately, we next examined how the two together would affect ECC single channel currents (Fig. 3, E and F). Under control conditions (150 mM KCl, neutral pH), ECC activity was rarely seen (open probability, P\(_o\) = 0.06). Following a change to low Cl\(^{-}\) and pH 6 conditions, the single channel current was activated and P\(_o\) increased to 0.49. Changing the solution to restore Cl\(^{-}\) concentration, whereas keeping the pH low, resulted in reduced ECC activity (P\(_o\) = 0.33). This confirmed that ECC could be activated by

\( P_{\text{Ca}}[\text{Ca}^{2+}] / P_{\text{K}}[\text{K}^{+}] = \{\exp(E_{\text{rev}}/F/RT)\} \{\exp(E_{\text{rev}}/F/RT) + 1\} / 4 \)

(Eq. 1)

where P\(_{\text{K}}\), K\(^{+}\) permeability; F, Faraday constant; T, temperature, K\(^{\circ}\); R, gas constants.

The Early Endosome Cation Channel Is Ca\(^{2+}\) Permeable—To demonstrate whether this endosome channel was a Ca\(^{2+}\) channel, Ca\(^{2+}\) permeability was examined. The E\(_{\text{rev}}\) shifted from 0 mV (150 mM symmetrical K\(^{+}\); n = 8) to \(-10 \pm 2\) mV, when K\(^{+}\) was replaced with Ca\(^{2+}\) in the bath solution (150 mM K\(^{+}\) pipette/150 mM Ca\(^{2+}\) bath; n = 3). A Ca\(^{2+}\) carrying inward current was clearly seen and was reversibly inhibited by 1.5 mM La\(^{3+}\) (Fig. 3, A and B). From the difference in E\(_{\text{rev}}\), the relative calcium permeability (P\(_{\text{Ca}}\)) was determined to be P\(_{\text{Ca}}\) / P\(_{\text{K}}\) = 1.9 (20) (see Equation 1).

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ECC and TRP Channel Pharmacology—Because TRP family channels (22), i.e. growth factor regulated calcium channel (TRPV-2) (13), PKD2 (4, 5), and MCOLN1 (6) are found in intracellular membranes, known TRP inhibitors such as ruthenium red (RuR), amiloride, and La\(^{3+}\) were tested for their ability to affect ECC activity. Inhibition of ECC by 0.5–1 mM La\(^{3+}\) are shown in Figs. 2A and 3A (n = 6). Amiloride (∼1 mM) reversibly inhibited ECC activity (50–80% maximum inhibition; Fig. 4, A–C). RuR also reversibly inhibited ECC (70% inhibition with 40 μM) (Fig. 4, D and E).

We noticed that the ECC current was potentiated upon application of a low concentration of La\(^{3+}\). Because addition of 100 μM La\(^{3+}\) has previously been shown to potentiate the TRPC5 current (23), the possibility that 100 μM La\(^{3+}\) could act as an ECC activator was examined (Fig. 4, F and G). Before the addition of La\(^{3+}\), ECC activities were relatively quiet and were only induced at negative voltages (Fig. 4F, left). The addition of 100 μM La\(^{3+}\) resulted in the induction of ECC currents. The effect was clearly evident at positive voltages (n = 3; Fig. 4, F (right) and H). Macroscopic ECC currents were also potentiated by 1.5-fold with 100 μM La\(^{3+}\) (3 out of 5 experiments).

To pharmacologically evaluate the identity of ECC, the effect of 2-APB, a specific activator of TRPV1–3 channels (24), was examined. Addition of 200 μM 2-APB further potentiated, by more than 2-fold, ECC currents that had been previously activated by 100 μM La\(^{3+}\) (n = 2; Fig. 4, H and I). The TRPV1-specific activator capsaicin had no effect in concentrations, as high as 5 μM (n = 3) on ECC currents, which still could be activated by 100 μM La\(^{3+}\) (not shown).

TRPV2 Presence in HEK293 Cells—Because the pharmacological characteristics of ECC are closer to those of TRPV2,
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DISCUSSION

Importance of ECC in Endocytosis
—Within 60 s of endocytosis the EE [Cl$^-$]$_{lum}$ is reduced to $\sim$20 mM (15). This reduction in the EE [Cl$^-$]$_{lum}$ is likely to be a consequence of the activation of voltage-dependent Cl$^-$ channels (25) in the EE membranes (i.e. CIC-5). Negatively charged membrane proteins, which have no effect on a large volume in the extracellular space, can readily affect charge distributions in small primary endosomes (15). These negative charges, which create a Donnan potential in the endosome lumen (15, 21), not only become a driving force for Cl$^-$ to exit the endosomes but also affect the direction of the voltage gradient through the membrane. The plasma membrane separates extracellular space (0 mV) from cytosolic space (negative resting membrane potential). After endocytosis, this plasma membrane becomes an endosome membrane and separates the more negatively charged endosomal luminal space from the cytosol (see above and also Fig. 5). Consequently the charge gradient through the membrane reverses during the endocytosis. Cl$^-$ channels in the endosome membrane sense this reversed voltage gradient as an activation stimulus. Masking these negative charges with acidic pH or poly-L-lysine treatment prevented [Cl$^-$]$_{lum}$ reduction (15), suggesting that the Donnan potential could be the cause of chloride channel activation prior to [Cl$^-$]$_{lum}$ reduction. This charge-masking treatment also inhibited acidification of endosomes (15). Significantly reduced endosome acidification and a dramatic slowing of endocytosis were also observed when the CIC-5 endosome chloride channel gene was disrupted (17, 18). Gerasimenko et al. (16) have reported that the luminal-free [Ca$^{2+}$] falls in acidifying endosomes. Low extracellular Ca$^{2+}$ (200 $\mu$M) significantly inhibited the acidification of endosomes without affecting endocytosis (16), which suggests that acidification of endosomes is also dependent on the efflux of Ca$^{2+}$ from endosomes. Thus it appears that without a reduction (or efflux) of luminal Cl$^-$ and Ca$^{2+}$, the endosomes do not acidify. Acidification, vesicle

FIGURE 5. I, chronological events that lead to ECC activation are summarized (A–E). Purple and green ovals indicate ECC and Cl$^-$ channels; green and red circles indicate Cl$^-$ and Ca$^{2+}$ ions, respectively. Large blue circles indicate negatively charged membrane proteins that are responsible for the Donnan potential. A, extracellular space; B, newly formed primary endosomes (charges in the vesicle lumen are highly negative); C, activation of the Chloride channel and consequential Cl$^-$ release; D, activation of ECC by low Cl$^-$; E, Ca$^{2+}$ released from vesicles. Na$^+$, K$^+$-ATPase and V-type H$^+$-ATPase were omitted from the drawings for the sake of simplicity. II, flow chart of endosome acidification and possible targets that inhibit acidification. Steps A–E in I correspond to steps a–e in II.
fusion, and endosome maturation are closely related (8, 9). Because primary endosomes fuse to early endosomes in a Ca\(^{2+}\)-dependent manner, if one assumes that this Ca\(^{2+}\) efflux from the primary endosomes is responsible for the endosome fusion, then the role of the low Cl\(^{-}\) and pH-activated Ca\(^{2+}\) release becomes significant (Fig. 5). One interpretation of this data is that local Ca\(^{2+}\) release through ECC controls endosome fusion and maturation. Reports describing the importance of EE luminal calcium (10–12) and intravesicular calcium release (26) in EE fusion support this notion.

**Is ECC the TRPV2 Channel?**—TRPV2 was so far the only cation channel protein that was detected in the proteomics study of the rab5 positive early endosomal fraction suggesting its relative abundance in early endosomes. Our RT-PCR experiment detected TRPV2 message in HEK293 cells (Fig. 4); therefore it is possible that TRPV2 may be responsible for the ECC current. ECC was inhibited by \(~500\ \mu M\) to \(1 \text{ mM} \) La\(^{3+}\), RuR, and amiloride (as are other TRP channels) and was potentiated by acidic pH, 100 \(\mu M\) La\(^{3+}\), and 200 \(\mu M\) 2-APB. TRPV2 was shown to form a heteromultimer with TRPV1 and/or TRPV3 (14). All three TRPV1–3 are potentiated by 2-APB (24). The insensitivity of ECC to capsaicin, a specific TRPV1 activator, eliminates the possibility of TRPV1 involvement in the ECC current. The concentration used for 2-APB was higher than the reported EC\(_{50}\) for both TRPV2 and TRPV3; thus we cannot rule out the involvement of TRPV3. However, the reported biophysical properties of TRPV3 (22) are very different from those of TRPV2. The conductance of TRPV3 (170 picosiemens) is approximately twice as high as that of ECC (50–60 picosiemens), and the Ca\(^{2+}\) selectivity of TRPV3 \((P_{\text{Na}}/P_{\text{Ca}} = -10)\) is far larger than that of ECC \((P_{\text{Ca}}/P_{\text{K}} = 1.9)\). Approximately TRPV4–6 are not likely to be the ECC because they are insensitive to 2-APB (24). TRPM6 is another TRP channel that is activated by 2-APB in the \(\mu M\) range (27). However, it is not likely that TRPV2 will form a heteromultimeric channel complex with the structurally distant TRPV6.

There are no reports of potentiation of TRPV3 channels by 100 \(\mu M\) La\(^{3+}\), but it has been seen in TRPC4 and TRPC5 (24). Similar to ECC, 100 \(\mu M\) La\(^{3+}\) potentiates TRPC4 and TRPC5, but millimolar concentration of La\(^{3+}\) inhibits them.

Finally the relative Ca\(^{2+}\) permeability of the ECC to K\(^{+}\) or Na\(^{+}\) \((P_{\text{Ca}}/P_{\text{K}} = 1.9)\) was slightly smaller but comparable with that of TRPV2 \((P_{\text{Ca}}/P_{\text{Na}} = 2.94)\) (28). The pharmacological similarity between the ECC and the TRPV2, together with the proteomics study (1), suggests that the ECC is likely a product of the TRPV2 channel or a closely related TRP gene family channel.

In conclusion, the successful patch clamp analysis of genetically enlarged endosomes revealed a novel endosome calcium channel. This approach allows us to examine ion endosomes in the native membrane environment leading to a better understanding of their physiological role in whole endosome function.

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