

Research at channel level on the effect of LaCl_3 on Radish (*Raphanus sativus* L.) vacuolar membrane

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Abstract We recorded slow vacuolar (SV-type) channel currents of Radish vacuoles successfully for the first time by using the whole-vacuolar patch-clamp recording mode. SV-type currents would increase and threshold potentials of activation would shift towards more negative values with the increase of concentrations of cytosolic Ca^{2+} . When 2.5 mmol/L LaCl_3 and 4 mmol/L EGTA were added to bath solutions, SV-type currents were suppressed remarkably. Then adding LaCl_3 with different concentrations to pipette solutions, we found that LaCl_3 with higher concentrations ($>4 \times 10^{-7}$ mol/L) had a strong inhibitory effect on SV-type currents, while LaCl_3 with lower concentrations ($\leq 4 \times 10^{-7}$ mol/L) promoted channel currents. This promoting effect provides an important basis at channel level for researching further the effects of rare earth on physiological activities of plants and the production-increase effects of rare earth fertilizers on crops.

Keywords: whole-vacuolar patch-clamp recording, SV-type channel current, cytosolic Ca^{2+} , La^{3+} .

Vacuoles are an important compartment of mature plant cells, many epiphytes and algae (except for prokaryotes). The large central vacuole, as the storage organelle of plants, may account for 90% of the total cell volume. Recent investigations by patch-clamp techniques have identified ion channels and pumps as pathways for the movement of ions and metabolites^[1].

Ion channels play very important roles in growth development and the process of cell responding to the outside^[2]. Many studies have indicated that the SV-type channel is a cation selective one with poor selectivity among monovalent cations (K^+ , Na^+ , Cs^+) and divalent cations (Ca^{2+} , Mg^{2+} , Ba^{2+}), and that this voltage- and time-dependent channel can be activated by cytosolic Ca^{2+} ^[3-6]. In the absence of cytosolic Mg^{2+} , the SV-type channel can not be activated unless the concentration of cytosolic Ca^{2+} is at least up to 10 $\mu\text{mol/L}$. Based on these results, a simplified model for the SV-type channel has been proposed. There are two binding sites on the cytosolic side: a high-affinity calcium binding site which is not activated by Mg^{2+} and a low-affinity binding site which can be occupied by either Mg^{2+} or Ca^{2+} ; meanwhile

on the vacuolar luminal side there is also a binding site which binds Ca^{2+} and is inhibitory^[7].

Large quantities of data have shown that La^{3+} has antagonistic action to Ca^{2+} and blocking effect on calcium channels^[8-11]. However, it has not been reported yet that patch-clamp techniques are used to study how La^{3+} affects SV-type channel currents. Since La^{3+} and Ca^{2+} share many similar chemical properties^[12] and cytosolic Ca^{2+} can activate the SV-type channel, can cytosolic La^{3+} activate it? Can La^{3+} be transported into vacuoles by this type of poor cation selective channel? Focusing on these problems, we used the whole-vacuolar patch-clamp mode to record SV-type channel currents on the ubiquitous Radish vacuoles which were easy to be separated. We also studied its calcium-dependence and the effects of different cytosolic and luminal La^{3+} concentrations on the channel currents. It should be noted that the effect of luminal La^{3+} with low concentrations on the channel currents provides a powerful proof for researching further the applied amounts of rare earth elements in plants.

1 Materials and methods

(i) Isolation of Radish vacuoles. Radish vacuoles were isolated from fresh taproots grown in the field^[1, 13]. A slice of storage tissue was cut off with a razor blade and the surface rinsed with some drops of bath solution to wash the vacuoles extruded directly into the recording chamber.

(ii) Patch clamp experiments. Patch-clamp pipettes were prepared from soft glass capillaries (BJ03, Beijing), and pulled on a multi-stage programmable puller. Giga- Ω seals between electrode and the vacuolar membrane (above 10 G Ω) were obtained by gentle suction. Vacuoles were voltage-clamped by using CV 203BU HEADSTAGE (Axon Instruments), currents were amplified by using an amplifier (Digidata 1200), and data were acquired by using Software PCLAMP 6.0. Data were analyzed and then figures were plotted with Clampfit (Axon Instruments) and Software MICROCAL-ORIGIN (5.0).

(iii) Preparation of experimental solutions. The standard solutions used in patch-clamp experiments were composed of 10 mmol/L KCl, 2 mmol/L MgCl_2 , 2 mmol/L EGTA and 5 mmol/L MES-Tris, pH 5.5 in pipette solutions (luminal side)^[14], and 100 mmol/L KCl, 10 mmol/L HEPES-Tris, 4 mmol/L EGTA, pH 7.3 in bath solutions (cytosolic side). The respective concentrations of CaCl_2 and LaCl_3 which were added to pipette solutions and bath solutions will be expounded later in this note. Osmolalities of pipette solutions and cytosolic solutions were adjusted to 580 and 450 mmol/kg respectively by the addition of D-sorbitol.

2 Results

(i) The voltage-dependent SV-type channel currents.

Employing a conventional experimental method^[1,13], we recorded very steady SV-type channel currents of Radish vacuoles for the first time (fig. 1(a)). Membrane potential was stepped from -40 to +180 mV in 20-mV increments from a holding potential of 0 mV. The episode duration was 4.1 s. Bath solutions contained 100 mmol/L KCl and 10 mmol/L HEPES-Tris, pH 7.3. For the composition of pipette solutions, please see "Materials and methods". The same experiments were repeated ($n > 20$ vacuoles) and the results were similar, but there was a tiny difference in currents. Fig. 1(b) shows the current-voltage relationship with its data acquired from experiments performed as in (a). After taking the average of these SV currents ($n = 20$) which were recorded in the same condition, we concluded that only when the minimum activation potentials of SV-type channel were (25 ± 5) mV, could the currents pass. The characteristic of SV-type channel was called voltage-dependence.

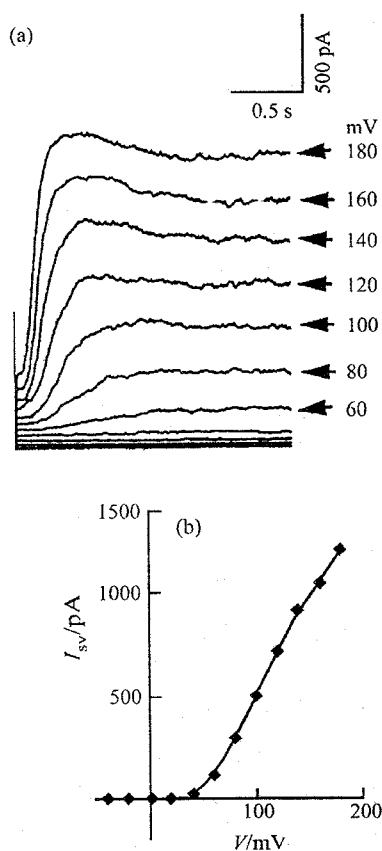


Fig. 1. The voltage-dependent SV-type channel (a) and current-voltage relationship curve (b)

(ii) The calcium dependence of SV-type channel. Cytosolic Ca^{2+} can activate SV-type channel, which is a distinct feature of the channel. When the holding potential was 0 mV and the membrane potential was +180 mV, the respective curves were recorded at varying free cytosolic Ca^{2+} concentrations of 10, 50, 100, 500 $\mu\text{mol/L}$, 1, 2 and 4

mmol/L (fig. 2(a)). At each concentration, the data were taken based on the average values in 5 separate vacuoles at least. Fig. 2(b) shows the free Ca^{2+} -SV current relationship with the membrane potential (+180 mV). The data were acquired from the experiments performed as in fig. 2(a). Bath solutions contained 100 mmol/L KCl, 4 mmol/L EGTA and 10 mmol/L HEPES-Tris, pH 7.3. Free Ca^{2+} concentrations were 10 $\mu\text{mol/L}$ (3.97 mmol/L), 50 $\mu\text{mol/L}$ (4.04 mmol/L), 100 $\mu\text{mol/L}$ (4.1 mmol/L), 500 $\mu\text{mol/L}$ (4.5 mmol/L), 1 mmol/L (5 mmol/L), 2 mmol/L (6 mmol/L) and 4 mmol/L (8 mmol/L). The data in brackets were the total CaCl_2 concentrations in bath solutions, which were calculated with the MC5 software after taking chelator, ionic strength, acidity and environmental temperature into consideration. Osmolalities of bath solutions were adjusted to 450 mmol/kg by the addition of D-sorbitol. See "Materials and methods" for the composition of pipette solutions. According to fig. 2, the SV-type channel has a strong calcium dependence. In the absence of cytosolic Mg^{2+} , cytosolic Ca^{2+} at less than 10 $\mu\text{mol/L}$ did not activate SV currents. And the currents would increase with the increase of free Ca^{2+} concentrations.

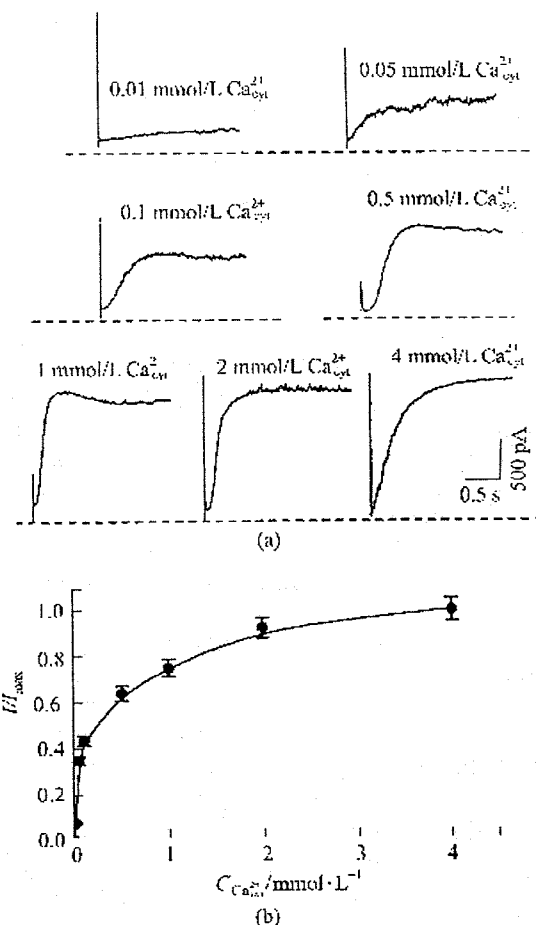


Fig. 2. The calcium-dependent character of SV-type channel in Radish vacuoles.

(iii) The inhibitory effect of cytosolic LaCl_3 at high concentrations on SV-type channel currents. LaCl_3 at high concentrations can inhibit SV-type channel currents and thus decreases ion permeability of Radish root vacuolar membrane. As shown in fig. 1, in the absence of EGTA and CaCl_2 in bath solutions, SV-type channel could be activated. When the storage tissue was cut during the isolation of Radish vacuoles, some vacuoles were damaged on the vacuolar luminal side and calcium ions permeated to bath solutions, which led to the increase of free cytosolic Ca^{2+} concentrations and the activation of SV-type channel. When we added 4 mmol/L EGTA and 2.5 mmol/L LaCl_3 to the above bath solutions, we observed that the maximum activation currents decreased from 1640 pA (in the control) to 539 pA after 5 min (fig. 3(a), (b)). This was the reflection of the inhibitory effect of La^{3+} on SV channel currents. The inhibition rate was up to 67.1%. The same experiments were performed repeatedly ($n > 10$ vacuoles) and the results were similar. On an average, the inhibition rate reaches 60%—75%. Yet it is necessary to point out that it is difficult to make a quantitative study on the action mechanism of La^{3+} to SV-type channel. Because the concentration of free Ca^{2+} permeating to bath solutions is rather high (sometimes up to micromolar concentration), La^{3+} is much more capable of binding EGTA than Ca^{2+} , and EGTA binds large quantities of lanthanum ions while binding hardly calcium ions. It is difficult to make a quantitative study on the action mechanism of La^{3+} to SV-type channel. Therefore, in this note, we only make a qualitative discussion on the effect of high concentration LaCl_3 on this channel.

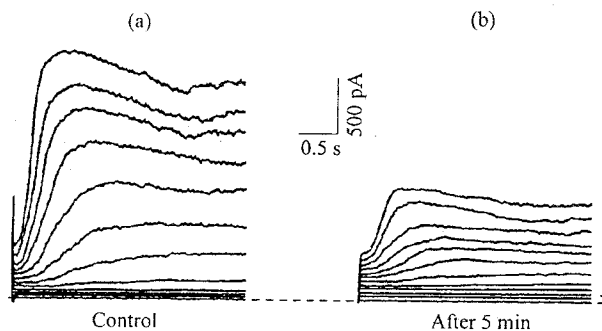


Fig. 3. The inhibitory effect of LaCl_3 with higher concentrations in bath solutions on SV-type channel currents.

(iv) Effect of LaCl_3 at different concentrations in pipette solutions on SV-type channel currents. After the addition of LaCl_3 at different concentrations to pipette solutions, the effect of La^{3+} on luminal vacuolar side on SV currents was observed. When the membrane potential was +180 mV, the respective curves were shown at different luminal La^{3+} concentrations of 4×10^{-10} , 4×10^{-9} , 4×10^{-8} , 4×10^{-7} , 4×10^{-6} , 4×10^{-5} and 4×10^{-4} mol/L (fig. 4(a)). At each concentration, the data were the average of those recorded for at least 5 separate vacuoles. Fig. 4(b) shows the relationship curve of SV-type current

vs. the logarithm of luminal LaCl_3 concentration. The data were obtained from the experiments performed as in fig. 4(a).

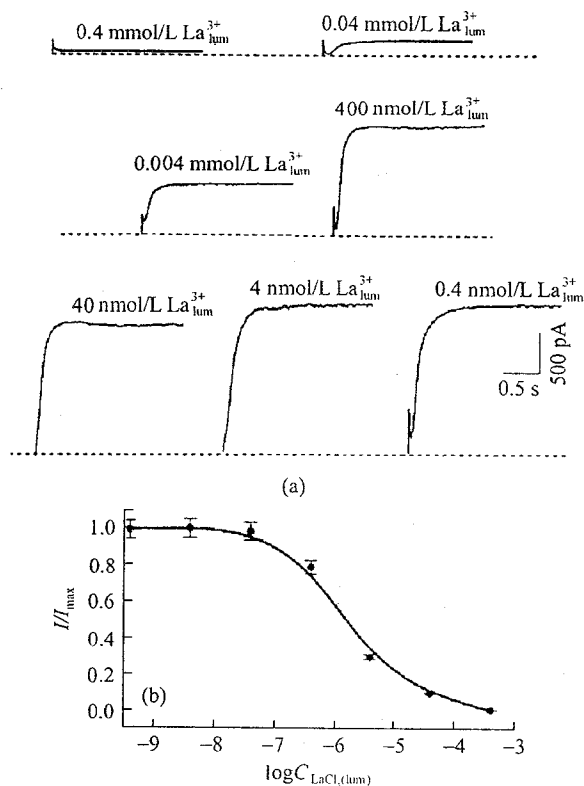


Fig. 4. Effect of LaCl_3 at different concentrations in pipette solutions on SV-type channel current.

Bath solutions were composed of 100 mmol/L KCl, 4 mmol/L EGTA, 2 mmol/L (6 mmol/L) CaCl_2 and 10 mmol/L HEPES-Tris (The figure in bracket was the total concentration of added CaCl_2), with pH 7.3. Pipette solutions contained 10 mmol/L KCl, 2 mmol/L MgCl_2 , 5 mmol/L MES-Tris and LaCl_3 solution at different concentrations of 4×10^{-10} , 4×10^{-9} , 4×10^{-8} , 4×10^{-7} , 4×10^{-6} , 4×10^{-5} and 4×10^{-4} mol/L with pH 5.5. By adding D-sorbitol, osmolalities of bath solutions and pipette solutions were adjusted to 450 and 580 mmol/kg respectively. As shown in fig. 4(b), the average value of SV-type currents was 1522 pA in the absence of luminal La^{3+} . In contrast, SV-type currents increased when the concentration of LaCl_3 became lower than or equal to 4×10^{-7} mol/L. On the average, the currents reached the maximum of 2300 pA when the concentration was decreased to 4×10^{-8} mol/L and the currents hardly changed when the concentration continued to decrease gradually; while when the concentration was more than 4×10^{-7} mol/L, the currents were inhibited to different degrees and at the concentration of 4×10^{-4} mol/L the currents were almost inhibited completely. According to these results,

we conclude that LaCl_3 at low concentration in pipette solutions can promote SV-type currents while LaCl_3 at high concentration can inhibit the currents.

3 Discussion

It was found in our study that without any chelator free calcium ions permeating to bath solutions are capable enough to activate SV-type channel and activation currents are different in intensity. According to the relationship curve of current- Ca^{2+} concentration, we can estimate the concentration of permeating free Ca^{2+} which sometimes can reach the scale of micromolar concentration.

The study on calcium dependence of SV-type channel indicates that the channel will be activated when the concentration of free cytosolic Ca^{2+} is more than $10 \mu\text{mol/L}$, and with the concentration increasing, channel currents will increase and activation potentials will shift towards more negative values, which accords with ref. [7]. A striking feature of SV-type channel is its activation by cytosolic Ca^{2+} . Free Ca^{2+} and channel proteins on the cytosolic calcium binding site combine to form a kind of binding-protein which can activate SV-type channel.

The study on the action mechanism of LaCl_3 at high concentrations on the cytosolic side to SV-type channel shows that LaCl_3 has a strong inhibitory effect on SV-type channel. Based on the above results, we deduce the action mechanism of this process as follows. Since the complexing ability of La^{3+} is much stronger than that of Ca^{2+} , the concentration of free cytosolic Ca^{2+} is far more than that of La^{3+} . When the whole-vacuolar patch-clamp mode is just formed, free Ca^{2+} and channel proteins combine on the cytosolic Ca^{2+} binding site to form binding-proteins which activate SV-type channel. The more the calcium ions permeate to bath solutions, the larger the channel currents become and the lower the activation potentials are. Then La^{3+} combines with channel proteins, which makes the protein conformation deformed, the SV-type channel blocked and the currents inhibited greatly. As a result, ion permeability of Radish root vacuolar membrane is decreased.

The study on the action mechanism of luminal LaCl_3 to the SV-type channel shows that La^{3+} at low concentrations promotes the channel currents while La^{3+} at high concentrations inhibits the channel currents. Just as we know, compared with Ca^{2+} , La^{3+} has bigger ion radius, higher charge density and higher affinity. Therefore, when the concentration of La^{3+} is more than $4 \times 10^{-7} \text{ mol/L}$, La^{3+} is prior to combine with channel proteins, which brings about the deformation of protein conformation, the block of SV-type channel and the inhibition of the currents. These results support strongly the view that La^{3+} can block many channels and it has antagonistic action to Ca^{2+} . But it is worth noting that SV-type currents increase instead of decrease when the concentration of La^{3+} is lower than or equal to $4 \times 10^{-7} \text{ mol/L}$ in pipette solutions. As to

the action mechanism of the new result, it still needs to be studied in detail.

It is not difficult to conclude that the inhibitory effect of La^{3+} on SV-type channel has a close relation with its concentration. La^{3+} at high concentrations mainly takes an inhibitory effect on the function and the activity of vacuoles, while La^{3+} at low concentrations promotes SV-type currents. This study provides an important basis at channel level for researching further the effects of rare earths on physiological activities of plants and the production-increase effects of rare earths fertilizers on crops. Anyway, the research on the action mechanism of rare earth to physiological activities of plants is a very complicated process, which needs to be studied and solved from different angles and with many different methods.

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