Cooperative Block of the Plant Endomembrane Ion Channel by Ruthenium Red

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ABSTRACT Effects of ruthenium red (RR) on the slow Ca^{2+}-activated Ca^{2+}-permeable vacuolar channel have been studied by patch-clamp technique. Applied to the cytosolic side of isolated membrane patches, RR at concentrations of 0.1–5 μM produced two distinct effects on single channel kinetics, long lasting closures and a flickering block of the open state. The first effect was largely irreversible, whereas the second one could be washed out. The extent of flickering block steeply increased ($z\delta = \sim 1.35$) with the increase of cytosol-positive voltage, dragging RR into the channel pore. At least two RR ions are involved in the block according to Hill coefficient $n = \sim 1.30$ for the dose response curves. The on-rate rate of the drug binding linearly depended on the RR concentration, implying that one RR ion already plugged the pore. The blocked state was further stabilized by binding of the second RR. This stabilization was in excess of that predicted by independent binding as the dependence of unblocking rate on RR concentration revealed. A cooperative model was therefore employed to describe the kinetic behavior of RR binding. At zero voltage the half-blocking RR concentration of 36 μM and the bimolecular on-rate constant of 1.8×10^8 M^{-1} s^{-1} were estimated.

INTRODUCTION

Ruthenium red (RR) is a synthetic crystalline inorganic polycationic dye originally used in electron microscopy for staining of cells and tissues due to its strong binding to phospholipids, fatty acids, and mucopolysaccharides (Luft, 1971). It has a bulk valence of 6 and linear structure, given by formula $[(NH_3)_5Ru\text{-}O\text{-}Ru(NH_3)_4\text{-}O\text{-}Ru(NH_3)_5]^{6+}$, with an approximate thickness of 8 Å and a length of 15 Å (Gomis et al., 1994). This compound has been first shown to inhibit the mitochondrial Ca^{2+} transport (Moore, 1971). Since the work on Ca^{2+} release channels from sarcoplasmic reticulum (SR), RR was considered as a "classical" inhibitor of Ca^{2+}-induced Ca^{2+} release (Smith et al., 1985, 1988). Consequently, RR has been widely used as a diagnostic tool to reveal the mobilization of Ca^{2+} from intracellular stores, particularly from the endoplasmic reticulum (ER). This was anticipated, for example, in recent studies on Ca^{2+} signaling in plant cells (Price et al., 1994; Bauer et al., 1998). On the other hand, there is emerging evidence that RR at the same (submicromolar to micromolar) concentration range also effectively inhibits some plasma membrane Ca^{2+} and Ca^{2+}-permeable channels both in animal and plant cells (Gomis et al., 1994; Hamilton and Lundy, 1995; White, 1996; Piñeros and Tester, 1997; Malécot et al., 1998; Ma and Michel, 1998). This study showed that RR, at concentrations used to suppress Ca^{2+}-induced Ca^{2+} release, inhibited Ca^{2+}-permeable channel from plant non-ER membrane, the so called SV (slow vacuolar) channel. The rapid block was caused by at least two RR ions, bound deep within the channel pore in a cooperative manner.

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MATERIALS AND METHODS

Plant material and solutions

Fresh *Beta vulgaris* (whole plants) were obtained twice a week from a local market and kept at 4°C in the darkness before use. Vacuoles were isolated mechanically from the tap root slices as described previously (Dobrovinskaya et al., 1999). The osmotic pressure of the vacuolar sap was verified by a cryoscopic osmometer (Osmomat 030, Gonotec GmbH, Berlin, Germany) and that of experimental solutions was adjusted accordingly by sorbitol. Bath solution contained (in mM): 100 KCl, 0.1 $CaCl_2$, 15 HEPES-KOH (pH 7.5), and the patch pipette-filling one was the same but 0.1 mM $CaCl_2$ was substituted by 5 mM EGTA. Solutions containing ruthenium red (RR), ammoniated ruthenium oxychloride, were prepared from 3 mM stock solution. All chemicals were analytical grade (Sigma Chemical Co, St. Louis, MO). The RR batch contained 10.3% RR as determined by absorption at 534 nm.

Patch-clamp measurements and analyses

Patch pipettes were pulled from Kimax-51 capillaries (Kimble, Toledo, OH), with a final resistance after fire polishing of 4–5 MΩ when filled by standard pipette solution. Current measurements were performed using an Axopatch 200A integrating patch-clamp amplifier (Axon Instruments, Foster City, CA). Single channel measurements were done on excised outside-out (cytoplasmic side of the membrane faces the bath) patches. The convention of current and voltage was according to Bertl et al. (1992), i.e., the sign of voltage refers to the cytosolic side, and positive (outward) currents represent an efflux of cations into the vacuole. The records were filtered at 10 kHz by a low-pass Bessel filter, digitized using a DigiData 1200 Interface (Axon Instruments), and recorded directly on a hard disk of an IBM-compatible PC. For analyses the records were additionally filtered at 2 kHz. In selected cases (Fig. 1 *a*) the cutoff filter frequency was set to 1 kHz, or, for the measurements of mean single channel currents, filtering at 67 Hz was used to dump the fast flickering caused by RR. Analyses were carried out using the pClamp 6.0 software package (Axon Instruments).

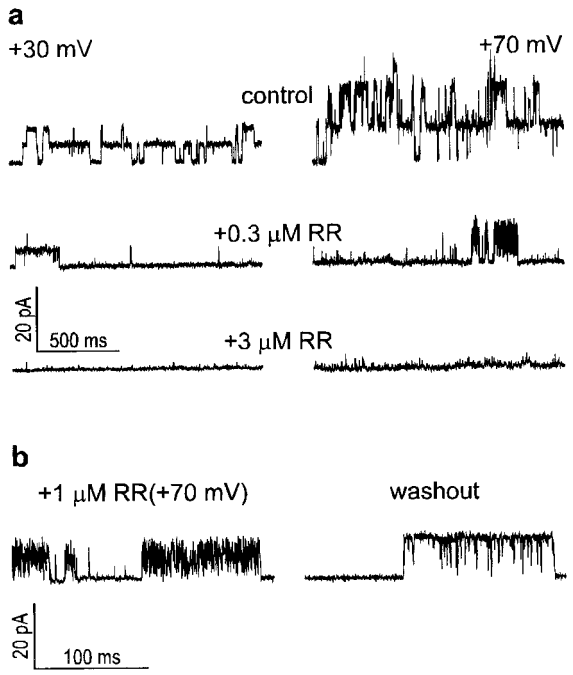


FIGURE 1 Two different modes of ruthenium red action on the slow vacuolar channel. Ruthenium red applied to the cytosolic side of the isolated tonoplast patch caused long-lasting channel closures and rapid voltage-dependent block of the channel open state (*a*). (*b*) The rapid voltage-dependent block was reversible. Original records were filtered at 1 kHz (*a*) and 2 kHz (*b*).

RESULTS

Two modes of RR action on the SV channel

Dual effects of RR on single Ca^{2+}-activated Ca^{2+}-permeable SV channels of the vacuoles from the red beet storage tissue cells are illustrated in Fig. 1 *a*. At concentration 0.1–1 μM RR produced long term closures, reducing the open probability by about 10-fold as an average, whereas at 3–5 μM RR only few percent or none of the initial activity remained ($n = 30$ separate cytosolic-side-out patches). This effect was largely irreversible, and also a large scattering of its magnitude in individual patches facing the same concentration of cytosolic RR was observed. Hence, no systematic study of the dose dependence was possible. The effect showed little if any voltage dependence as particularly a typical recording in Fig. 1 *a* evidenced. In parallel, RR caused a rapid flickering block of the channel open state, increasing at increased positive holding potential, e.g., traces at 70 mV in Fig. 1 *a* compared to those at 30 mV. The latter effect was reversible (Fig. 1 *b*) and the kinetics of block could be resolved at 2 kHz. Therefore, we have studied the mechanism of the SV channel fast block by RR.

Voltage dependence of the fast block by RR

The voltage dependence of fast block of the SV channel was generally studied on separate cytosolic-side-out patches facing desired RR concentration in the bath. To maintain the stable activity of the SV channels in the range of potentials 30–120 mV, the concentration of cytosolic Ca^{2+} as high as 100 μM was required (Dobrovinskaya et al., 1999). Before the application of RR simultaneous openings of up to 10 individual SV channels could be detected in typical tonoplast patch. The experiment was designed in such a way that in a RR-treated patch single channel activity could be monitored most of the time, with a little contribution of double channel openings. Hence, to test the higher concentration of RR the patches with higher channel activity in control conditions were selected; vice versa, the lower concentrations were tested on patches originally containing few active channel copies. A comparison of the fast closed-open kinetics in a typical patch containing single SV channel under the control conditions and in another patch treated by 0.5 μM RR is presented in Fig. 2. In both cases the increase of positive voltage caused increased flickering of the single channel currents. In control conditions this effect was caused mainly by a voltage-dependent block by cytosolic Ca^{2+} (I. I. Pottosin, unpublished results). Application of RR caused a drastic increase of the frequency of fast closures at high positive potentials ($\geq$70 mV). Hence, the contribution of fast closures observed under control conditions was insignificant in RR-treated patches, and, as in control conditions, the distributions of closed and open times within bursts of single channel activity could be fairly fitted by monoexponential functions (Fig. 2 *b*). As can be seen from the example of dwell-time analysis at 100 mV, RR (0.5 μM) caused a 14.5-fold decrease of mean open time, and mean closed time increased by a factor of 1.7 (Fig. 2 *b*). Similar analysis has been performed for a set of holding voltages with different RR concentrations. We took advantage of the slow kinetics of the SV channel, so the closures lasting $\geq$10 ms caused by intrinsic channel gating have been ignored and only closed-open transitions within bursts of activity were analyzed. In all cases the resulting closed and open time distributions could be well described by monoexponential functions. Voltage dependence of mean closed and open times in the presence of 0.5 μM RR is given in Fig. 3 *a*. An approximately symmetric exponential decay of the mean open time and rise of the mean closed time was observed. The probability of finding the channel in the unblocked state, P_o, steeply decreased with the increase in potential. Two approaches were used to quantify the voltage dependence of P_o. First, P_o has been calculated as a relative time spent in the open unblocked state:

$$P_o = \frac{\tau_{open}}{(\tau_{open} + \tau_{closed})} \quad (1)$$

where τ_{open} and τ_{closed} are mean open and closed times within a burst at given potential, respectively. In the alternative approach single channel records were additionally low-pass filtered at 67 Hz, and the mean open level current amplitudes have been measured and taken relative to the control current. Both approaches provided reasonably con-

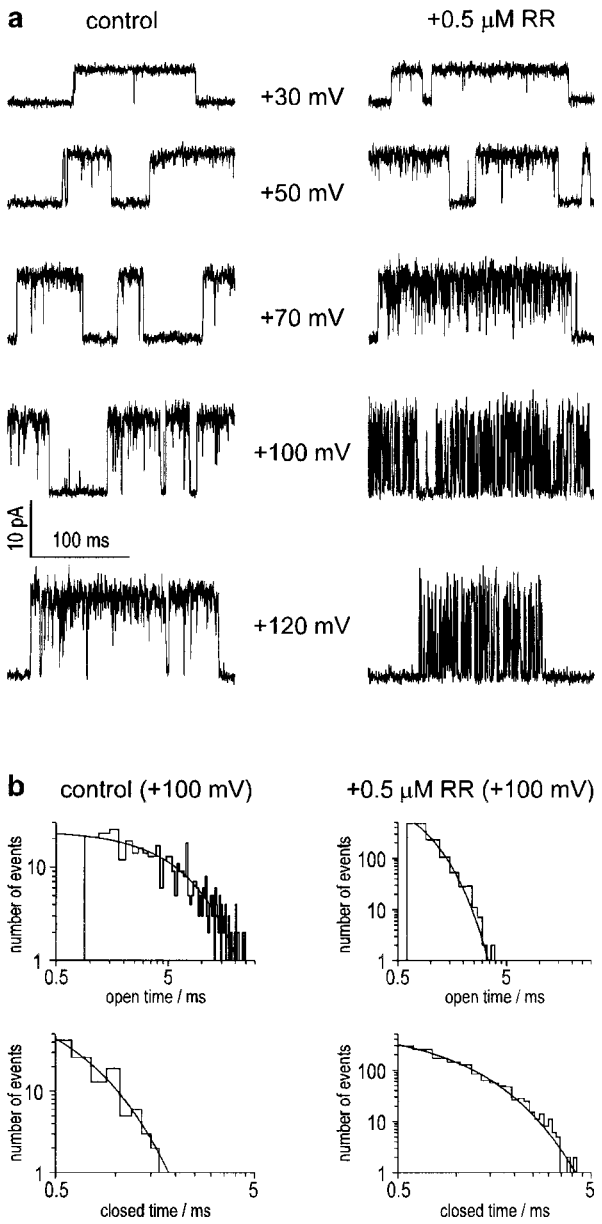


FIGURE 2 Rapid kinetics of the voltage-dependent SV channel block by ruthenium red. Original records were filtered at 2 kHz. The extent of rapid block steeply increased with the increase of voltage (*a*). Dwell-time distributions (*b*) for the non-blocked channel (control) and for the one exposed to 0.5 μM ruthenium red (RR). For each experiment 15 s of single channel record at 100 mV, the short stretch of which is presented in (*a*) was analyzed. The long lasting closures separating the bursts of activity were removed from the analysis. The rest of the counted closed and open events could be described by monoexponential function (*solid lines*) with characteristic times of 6.40 ms and 0.37 ms for open and closed distributions in control, and 0.44 ms and 0.64 ms for those in the presence of RR, respectively. Note that there was a more than 10-fold increase in the number of counted events in dwell-time distributions upon application of RR compared to control.

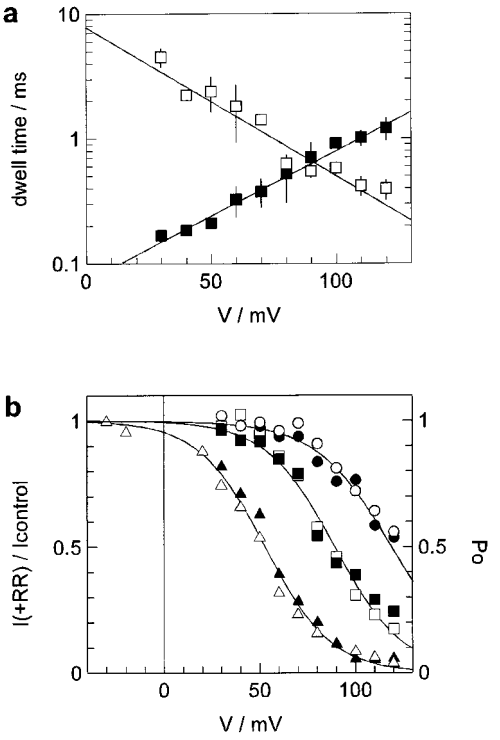


FIGURE 3 Voltage dependence of the fast block of SV channel by ruthenium red. Characteristic dwell times in the presence of 0.5 μM RR (*a*). Open (□) and closed (■), each point is a mean of at least three separate determinations (mean ± S. D.) plotted against voltage. *Solid lines* are monoexponential fits; mean open time $\tau_o(V) = 7.8 \pm 1.9$ ms × exp(−0.027 ± 0.003 V/mV) and mean closed time $\tau_c(V) = 0.072 \pm 0.006$ ms × exp(0.024 ± 0.01 V/mV). (*b*) The open channel probability within a burst of activity for 0.1 μM (●), 0.5 μM (■), and 3 μM (▲) RR was calculated as relative time spent in the open state (Eq. 1). Alternatively, the time-averaged single channel currents within bursts of activity were measured and taken relative to the control (*open symbols*). The data sets obtained by both of these methods were fitted to the Woodhull (1973) model of voltage dependent block (Eq. 2, *solid lines*), yielding zero voltage dissociation constants, $K_d(0)$, of 41 ± 13, 74 ± 25, and 63 ± 10 μM, and the value of effective blocking charge movement across the transmembrane potential drop, $z\delta$, of 1.30 ± 0.10, 1.43 ± 0.10, and 1.47 ± 0.07 for 0.1, 0.5, and 3 μM RR, respectively.

sistent sets of data points (Fig. 3 *b*). The sets of data points at 0.1, 0.5, and 3 μM RR (Fig. 3 *b*) were fitted by the Woodhull (1973) equation of the voltage-dependent block, assuming that RR binds to a single site within a channel and does not permeate:

$$P_o = \{1 + ([RR]/K_d(0))\exp(z\delta VF/RT)\}^{-1} \qquad (2)$$

where $K_d(0)$ is dissociation constant at zero voltage, z is the valence of blocker, δ is the electrical distance to the binding site, V is membrane voltage, and F, R, and T have their usual meanings. Fits of the voltage dependence at additional RR concentrations yielded the following values of $K_d(0)$ μM ($z\delta$): 72 ± 29 μM (1.29 ± 0.10), 88 ± 31 μM (1.28 ± 0.09), 75 ± 27 μM (1.39 ± 0.12), 91 ± 43 μM (1.40 ± 0.17), and 44 ± 11 μM (1.32 ± 0.14) at 0.2, 0.3, 1.0, 2.0 and 5.0 μM RR, respectively.

Concentration dependence of the RR block

We selected three holding potentials of 50, 70, and 100 mV, where the concentration dependence of the RR block was

analyzed. Examples of single channel recordings at 70 mV and different RR concentrations are shown in Fig. 4 *a*. At RR concentration >5 μM the channel openings became too brief and only partly resolved at 2 kHz, resulting in an apparent decrease of mean open channel amplitude, hence a substantial portion of closed-open transitions was missed (results not shown). Therefore, we have restricted ourselves to the concentration range of 0.1 to 5 μM RR. The dose dependence of the RR effect on the open probability at three holding potentials is presented in Fig. 4 *b*. In all three cases the dose dependence was steeper compared to that predicted by simple Michaelis-Menten type of binding (*dashed lines*). The data points at 50, 70, and 100 mV could be well described by curves with a Hill coefficient of 1.30 as an average (Fig. 4 *b*, *solid lines*). Hence, at least two RR ions participated in the blocking reaction.

Additional evidence for binding of multiple RR ions was obtained from the analysis of the concentration dependence of dwell times. As it is predicted by simple binding mechanism, the blocking rate (reciprocal mean open time, $1/\tau_{open}$) was linearly dependent on the [RR] (Fig. 5 *a*). Therefore, the first RR bound already produced a nonconductive (blocked) state of the channel, although the unblocking rate ($1/\tau_{closed}$) decreased with the increase of [RR], and this decrease was more prominent at a higher voltage (Fig. 5 *b*). If RR were bound to a single site within a channel pore at a time, the unblocking rate would depend solely on membrane voltage and not on RR concentration.

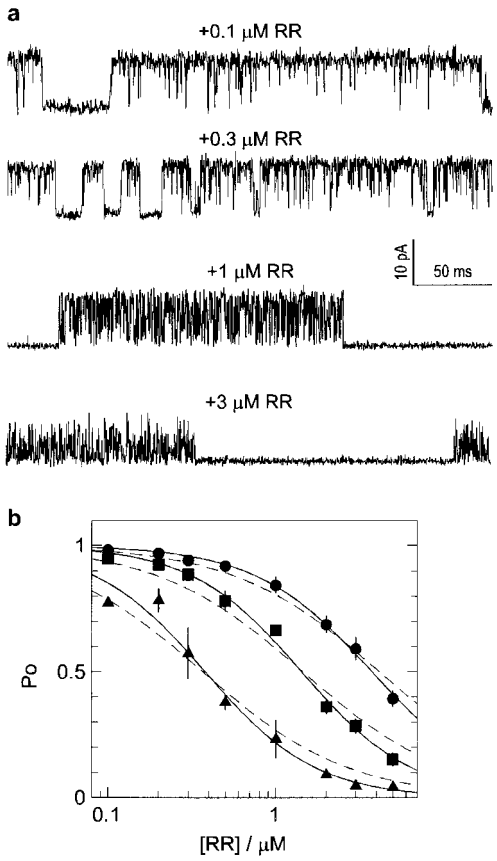


FIGURE 4 Concentration dependence of the ruthenium red block. Examples of single channel recordings (filtered at 2 kHz) at holding potential of 70 mV and different concentrations of RR are presented in *a*. (*b*) The open probability within a burst of activity was calculated (Eq. 1) on the basis of characteristic closed and open times, obtained from dwell-time distributions at 50 mV ([●), 70 mV (■), and 100 mV (▲). Data are presented as mean ± S.D. (n = 3–5). *Solid lines* are best fits of a data by the Hill equation, with a K_d of 3.77 ± 0.11, 1.43 ± 0.06, and 0.38 ± 0.03 μM, and Hill coefficients of 1.26 ± 0.05, 1.32 ± 0.07, and 1.32 ± 0.14 for 50, 70, and 100 mV, respectively. For the comparison, same data sets were fitted by a simple Michaelis-Menten type (Hill coefficient, n = 1) dose dependence. The probability that the data sets at 50, 70, and 100 mV could be described equally fair by either of the two equations (F-test) was 0.12, 0.13, and 0.23, respectively, which indicates the preference of the cooperative model.

DISCUSSION

Ruthenium red as Ca^{2+} antagonist

In this work we have observed two distinct modes of RR action on the SV channel, an irreversible inhibition, reflected by long-term closures, and rapid voltage-dependent flicker-block of the open channel, which was largely reversible. The two modes of block, the irreversible and the reversible, could be compared with the effects of RR on the sarcoplasmic reticulum Ca^{2+} release channel. When the channel was activated by Ca^{2+} or nucleotides, RR mainly caused an irreversible long-lasting closure of the channel (Smith et al., 1985, 1988), whereas in ryanodine-modified channel, which becomes locked in the open subconductance state, the fully reversible rapid block by cytosolic RR dominated (Ma, 1993). Dual effects of RR on the SV channel might be related to the dual role of Ca^{2+} as the channel unique activating agent (Pottosin et al., 1997) and as a permeant ion (Ward and Schroeder, 1994). Here we have studied in detail the rapid channel block by RR, which may be attributed to a direct effect on the ion-conducting pathway. The nature of the slow irreversible block by RR and its possible relation to the mechanism of the channel activation by Ca^{2+} is a purpose of future studies.

Ruthenium red, besides its binding to phospholipids and fatty acids (Luft, 1971), reportedly affected the function of many calcium-binding proteins (Masuoka et al., 1990; Amann and Maggi, 1991) and of various Ca^{2+} channels. It was not surprising, therefore, to find here that RR also affected large conductance Ca^{2+}-permeable SV channel of plant vacuolar membrane. It would be emphasized, however, that vacuolar site of action of RR yet has not been suspected, although RR block of Ca^{2+} oscillations in plant cells was taken as evidence for Ca^{2+} mobilization from the endoplasmic reticulum (Bauer et al., 1998). The SV channel is blocked in a voltage-dependent manner by some organic dyes such as quinacrine and 9-amino-acridine (Weiser and Bentrup, 1993) and by polyamines (Dobrovinskaya et al., 1999), with half-blocking concentrations at 100 mV ranging from 0.3 to ~100 μM compared to 0.38 μM for RR found in this study (Fig. 4). For the comparison, the half-blocking

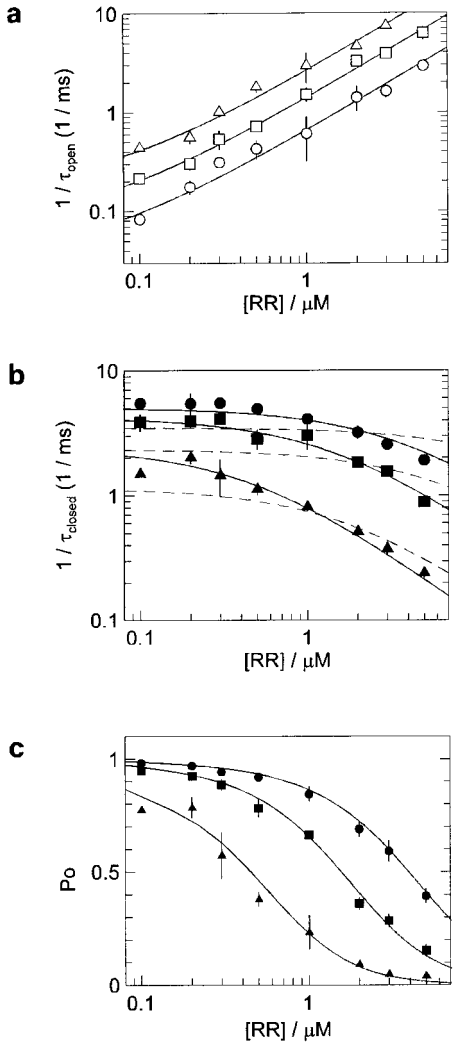


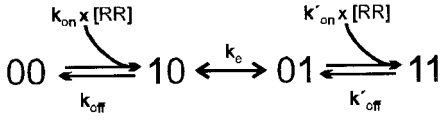
FIGURE 5 Dose dependence of the on- and off-rates of ruthenium red block and of the probability of unblocked state. Data are presented as mean ± S.D. (n = 3–5). (*a*) The reciprocal of the mean open time is linearly dependent on concentration. Double- logarithmic plot was selected for a better resolution of data points at low RR concentrations. Holding voltage was 50 mV (○), 70 mV (□), and 100 mV (△). *Solid lines* through the data points are obtained by linear regression, with slope factors of 0.63 ± 0.07 $\mu M^{-1}ms^{-1}$ (50 mV), 1.35 ± 0.07 $\mu M^{-1}ms^{-1}$ (70 mV), and 2.52 ± 0.23 $\mu M^{-1}ms^{-1}$ (100 mV). (*b*) Off-rate of RR block decreased with the increase of concentration. The reciprocal mean closed times at 50 mV (●), 70 mV (■), and 100 mV (▲) were plotted against RR concentration. The data points for all three potentials were fitted simultaneously by Eq. 7 (*solid lines*), fixing the bimolecular on-rate constants (k_{on}) to the values of slope factors at correspondent potentials in *a*, and assuming two identical binding sites (k_e = 1). Simultaneous fit yielded the following parameter values: α = 8.2 ± 1.3 and k_{off} rates (in ms^{-1}) of 10.1 ± 0.53 (50 mV), 8.5 ± 0.51 (70 mV), and 4.9 ± 0.33 (100 mV). For the comparison, the best fits by Eq. 7 assuming non-cooperative binding (α = 1) are shown (*dashed lines*). (*c*) The probability to be unblocked at 50 mV (●), 70 mV (■), and 100 mV (▲), calculated on the basis of dwell times, was replotted from Fig. 4 *b*. *Solid lines* are predictions of Eq. 6, with the same values of parameters α, k_{on}, and k_{off} as ones resulted from the fitting of dose dependence of dwell times in *a* and *b* at corresponding potentials.

cytosolic RR concentrations for the SR ryanodine receptor and for the cyclic-nucleotide gated channels of 0.22 and ~0.1 μM (at 100 mV), respectively, were reported (Ma, 1993; Ma and Michel, 1998). In sarcoplasmic reticulum (SR), RR inhibited also alternative (non-ryanodine receptor) Ca^{2+} release pathway, albeit at higher concentrations (Sukhareva et al., 1994). Also the mitochondrial Ca^{2+} uptake is blocked at higher (micromolar) RR concentrations compared to the block of ryanodine receptor-mediated Ca^{2+} release from SR (Griffiths, 1997). External RR caused a voltage-independent block of all types of Ca^{2+} channels in chromaffin cells (K_d = 7 μM; Gomis et al., 1994), N- and P- type (but not L-type) Ca^{2+} channels in brain synaptosomes (K_d = 2–6 μM and 70–250 μM; Hamilton and Lundy, 1995), L-type cardiac Ca^{2+} channels (K_d = 0.8 μM; Malécot et al., 1998), and with an affinity at low micromolar range (irreversibly) blocked Ca^{2+} channels from plasma membrane of the wheat root (Piñeros and Tester, 1997). It could be concluded that RR is one of the most efficient inhibitors of the SV channel discovered so far, and compared to the effects on ion channels from animal cells, the potency of the RR block of the SV channel is on the top of the list. The activity of Ca^{2+}-activated Ca^{2+}-permeable SV channel, which is an ubiquitous component of vacuoles in higher plant cells, is almost completely suppressed by RR at concentration of a few micromolar (Fig. 1 *a*), the range of concentrations commonly used to abolish Ca^{2+} release from endoplasmic reticulum. That is why the use of RR as a diagnostic inhibitor of Ca^{2+} release in plants deserves additional precautions.

Mechanism of rapid RR-induced block in the SV channel

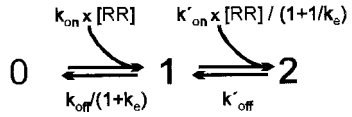
Experimental results on the kinetics of rapid block of the SV channel by RR could be summarized as follows. 1) Assuming the Woodhull (1973) model of the voltage dependent block and single-site binding, RR appears to block the SV channel by entering the pore to an electrical distance δ = ~0.23, based on $z\delta$ = 1.36 as an average and a valence of 6+. 2) Analysis of the dose-dependence of block (Fig. 4 *b*) yielded a Hill coefficient of ~1.3, which implies that at least two RR ions are involved. 3) The reciprocal of the channel mean open time (blocking rate) linearly depended on [RR] (Fig. 5 *a*). Thus, already the first RR ion entering the channel pore plugged it. 4) The unblocking rate decreased with the increase of [RR]. The most economic explanation of this fact is that with the increased [RR] increased the probability of double occupancy of the channel pore by RR, each ion blocking the pore. Consequently, the blocked state is additionally stabilized and the channel becomes unblocked only when both blocking RR ions are removed.

To explain the dose-dependent kinetics of the RR block, the following blocking mechanism was considered:



(3)

Where 00 is fully unblocked open state, and 10 and 01 are single- and 11 double-blocked states, respectively. This scheme could be further reduced,



(4)

lumping 10 and 01 together, where new states 0 = 00, 1 = 01 + 10, and 2 = 11. The reciprocal of the open time will be linearly depended on [RR]:

$$1/\tau_{open} = k_c + k_{on}[RR] \approx k_{on}[RR] \quad (5)$$

where k_c was introduced to explain the channel closures in the absence of [RR]. Substituting k'_{on}/k'_{off} by $\alpha k_{on}/k_{off}$, where α describes the relative stabilization of the binding of the second RR by the presence of the first one ($\alpha = 1$ independent binding, $\alpha < 1$ or $\alpha > 1$ negative or positive cooperativity) the probability of the unblocked state (0) according to the Eq. 4 could be expressed as following:

$$P_o = \{1 + k_{on}[RR](1 + k_e)/k_{off} + (k_{on}[RR]/k_{off})^2 k_e \alpha\}^{-1} \quad (6)$$

And combining Eqs. 1, 5, and 6 yielded the following expression for the unblocking rate:

$$1/\tau_{closed} = (k_{off}/(1 + k_e))/(1 + k_{on}[RR]\alpha/(k_{off}(1 + 1/k_e))) \quad (7)$$

Simultaneous fitting of the dose dependence of unblocking rate at 50, 70, and 100 mV (Fig. 5 *b*) by Eq. 7 yielded $\alpha = \sim 8$, i.e., binding of the first RR ion favors the binding of the second one (positive cooperativity). The blocked state was therefore over-stabilized. Alternative fits under the assumption of independent binding ($\alpha = 1$) poorly explained the data points in Fig. 5 *b*. Eq. 3 describes sequential binding mechanism, i.e., RR entering from the bulk solution always bind to the same site. However, an alternative parallel scheme, assuming that the both binding sites are simultaneously accessible, provided solutions mathematically equivalent to Eqs. 5–7 (results not shown). Though, for any scheme an over-stabilization of the double-blocked state was required for the explanation of the steep dose dependence of the open probability (Figs. 4 *b* and 5 *c*) and of the dependence of the unblocking rate on RR concentration (Fig. 5 *b*).

The rapid block of the SV channel by RR, applied to the cytosolic side, steeply increased when voltage is made more positive. As it became clear that at least two RR ions are involved, a simple Woodhull equation of the voltage-dependent block is no longer applicable. We have refitted, therefore, the data in Fig. 3 *b* by an equation similar to Eq. 6, introducing the voltage dependence ($z\delta$) of the k_{on}/k_{off} ratio and assuming a cooperative binding ($\alpha = 8.2$) of two RR ions to equivalent binding sites located in parallel at the same electrical distance, δ. This resulted in the value of $z\delta = 0.95 \pm 0.01$ for an individual RR ion compared to 1.40 ± 0.05 obtained by conventional fit to the Woodhull equation, assuming a single binding site (Fig. 3 *b*) or the values of δ of 0.16 and 0.23, respectively. A parallel location of two RR ions in the pore is the simplest explanation of both concentration and voltage dependence. It may be more accurate to suppose that the two RR ions are located at a different depth and the two binding sites also differed by their affinity, but on the basis of the data presented in this study, justification of more complex models was hardly possible. Apparently, depending on the choice of structural interpretation, the electrical distance left by RR on the way to the deepest binding site within the channel pore may be between 0.16 (both ions located at the same depth) and 0.23 (second ion does not contribute to the voltage dependence). The effective electrical distance of 0.16 or up to 0.23 traversed by the long RR ion carrying two positively charged groups at the ends and one in the middle might reflect the average of displacements of these charges along the transmembrane voltage drop. Notably, the effective electrical distance in case of RR was close to that reported for the SV channel block by cytosolic spermidine and spermine, linear polyamines with a similar charge distribution and a length, comparable to RR (Dobrovinskaya et al., 1999).

The resting transtonoplast voltage difference seems to follow K^+ equilibrium potential, which is close to zero under normal physiological conditions (Bethmann et al., 1995). Therefore, we attempted to approximate the values of block parameters at zero voltage (Fig. 6). Extrapolation to zero voltage yielded $K_d(0) = \sim 36$ μM. The mechanism of the SV channel block by RR qualitatively resembles that reported for ryanodine modified SR Ca^{2+} release channel (Ma, 1993). For the latter also at least two RR ions are involved in block. The block of SR Ca^{2+} release was less voltage-dependent, the slope of log K_d was -0.011 mV^{-1}

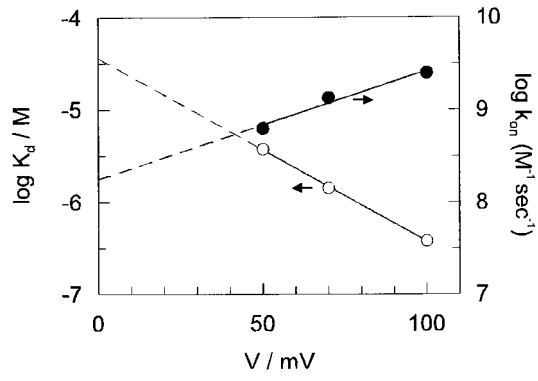


FIGURE 6 Estimates of parameters of ruthenium red block at zero voltage. Dissociation constants at 50, 70, and 100 mV have been obtained from dose-response curves in Fig. 4 *b* fitted by the Hill equation. Resulting log K_d values were plotted against voltage (○); extrapolation of linear fit (slope -0.0199 ± 0.0005 mV^{-1}) to zero voltage yielded log $K_d(0)$ of -4.44 ± 0.04 ($K_d(0) = 36 \pm 3$ μM). The values of k_{on} were obtained from linear fits presented in Fig. 5 *a*, and extrapolation of log k_{on} (●, slope 0.0118 ± 0.020 mV^{-1}) to zero voltage yielded a value of 8.25 ± 0.15 ($k_{on}(0) = 1.77 \pm 0.77 \times 10^8$ M^{-1} s^{-1}). Standard errors for log K_d and log k_{on} values were smaller than the symbol size.

compared to $-0.020\ mV^{-1}$ for the SV, which was mainly caused by lesser voltage dependence of the on-rate constant. Whereas the mean residence times of RR within the pore of SV and SR Ca^{2+} release channels were close to each other, the on-rates differed by almost one order of magnitude. For the SV channel the value of bimolecular on-rate constant at zero voltage $k_{on}(0) = 1.8 \times 10^8\ M^{-1}\ s^{-1}$ was estimated (Fig. 6) compared to approximately $1.1 \times 10^9\ M^{-1}\ s^{-1}$ for the SR Ca^{2+} release channel (Ma, 1993). The value of the on-rate constant for RR in the SV channel was nevertheless at the upper limit reported for the diffusion of various organic blockers (Hille, 1992). It could be an overestimate, however, as due to the attraction by negative charges of phospholipids and/or the channel protein itself, the local concentration of RR in the proximity of the channel mouth could be substantially higher compared to that in bulk solution.

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REFERENCES

Amann, R., and C. A. Maggi. 1991. Ruthenium red as a capsaicin antagonist. *Life Sci.* 49:849–856.

Bauer, C., C. Plieth, O. Popescu, U.-P. Hansen, W. Simonis, and G. Schönknecht. 1998. Strontium-induced repetitive calcium spikes in a unicellular green alga. *Plant Physiol.* 117:545–557.

Bertl, A., E. Blumwald, R. Coronado, R. Eisenberg, G. P. Findlay, D. Gradmann, B. Hille, K. Köhler, H.-A. Kolb, E. A. C. MacRobbie, G. Meissner, C. Miller, E. Neher, P. Palade, O. Pantoja, D. Sanders, J. I. Schroeder, C. Slayman, R. Spanswick, A. Walker, and A. Williams. 1992. Electrical measurements on endomembranes. *Science*. 258: 873–874.

Bethmann, B., M. Thaler, W. Simonis, and G. Schönknecht. 1995. Electrochemical potential gradients of H^+, K^+, Ca^{2+}, and Cl^- across the tonoplast of the green alga *Eremosphaera viridis. Plant Physiol.* 109: 1317–1326.

Dobrovinskaya, O. R., J. Muñiz, and I. I. Pottosin. 1999. Inhibition of vacuolar ion channels by polyamines. *J. Membr. Biol.* 167:127–140.

Gomis, A., L. M. Gutierrez, F. Sala, S. Viniegra, and J. A. Reig. 1994. Ruthenium red inhibits selectively chromaffin cell calcium channels. *Biochem. Pharmacol.* 47:225–231.

Griffiths, E, J. 1997. Ruthenium red as an inhibitor of mitochondrial Ca^{2+} uptake in single adult rat cardiomyocytes. *J. Physiol.* 504:96P.

Hamilton, M. G., and P. M. Lundy. 1995. Effect of ruthenium red on voltage-sensitive Ca^{2+} channels. *J. Pharmacol. Exp. Ther.* 273: 940–947.

Hille, B. 1992. Ionic Channels of Excitable Membranes, Ed. 2. Sinauer Associates, Sunderland, Massachusetts.

Luft, J. H. 1971. Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanism of action. *Anat. Res.* 171:347–368.

Ma, J. 1993. Block by ruthenium red of the ryanodine-activated calcium release channel of skeletal muscle. *J. Gen. Physiol.* 102:1031–1056.

Ma, J., and W. C. Michel. 1998. Drugs affecting phospholipase C- mediated signal transduction block the olfactory cyclic nucleotide-gated current of adult zebrafish. *J. Neurophysiol.* 79:1183–1192.

Malécot, C., V. Bito, and J. A. Argibay. 1998. Ruthenium red as an effective blocker of calcium and sodium currents in guinea-pig isolated ventricular heart cells. *Br. J. Pharmacol.* 124:465–472.

Masuoka, H., M. Ito, T. Nakano, M. Naka, and T. Tanaka. 1990. Effects of ruthenium red on activation of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase. *Biochem. Biophys. Res. Commun.* 169:315–322.

Moore, C. L. 1971. Specific inhibition of mitochondrial Ca^{2+} transport by ruthenium red. *Biochem. Biophys. Res. Commun.* 42:298–305.

Piñeros, M., and M. Tester. 1997. Calcium channels in higher plant cells: regulation and pharmacology. *J. Exp. Botany*. 48:551–577.

Pottosin, I. I., L. I. Tikhonova, R. Hedrich, and G. Schönknecht. 1997. Slowly activating vacuolar channels can not mediate Ca^{2+}-induced Ca^{2+}-release. *Plant J.* 12:1387–1398.

Price, A. H., A. Taylor, S. J. Ripley, A. Griffiths, A. J. Trewavas, and M. R. Knight. 1994. Oxidative signals in tobacco increase cytosolic calcium. *Plant Cell.* 6:1301–1310.

Smith, J. S., R. Coronado, and G. Meissner. 1985. Sarcoplasmic reticulum contains adenine nucleotide-activated calcium channels. *Nature.* 316: 446–449.

Smith, J. S., T. Imagawa, J. Ma, M. Fill, K. Campbell, and R. Coronado. 1988. Purified ryanodine receptor from rabbit skeletal muscle is the calcium-release channel of sarcoplasmic reticulum. *J. Gen. Physiol.* 92:1–26.

Sukhareva, M., J. Morrissette, and R. Coronado. 1994. Mechanism of chloride-dependent release of Ca^{2+} in the sarcoplasmic reticulum of rabbit skeletal muscle. *Biophys. J.* 67:751–765.

Ward, J. M., and J. M. Schroeder. 1994. Calcium-activated K^+ channels and calcium-induced calcium release by slow vacuolar ion channels in guard cell vacuoles implicated in the control of stomatal closure. *Plant Cell*. 6:669–683.

Weiser, T., and F.-W. Bentrup. 1993. Pharmacology of the SV channel in the vacuolar membrane of *Chenopodium rubrum* suspension cells. *J. Membr. Biol.* 136:43–54.

White, P. J. 1996. Specificity of ion channel inhibitors for the maxi cation channel in rye root plasma membrane. *J. Exp. Botany*. 47:713–716.

Woodhull, A. M. 1973. Ionic blockage of sodium channels in nerve. *J. Gen. Physiol.* 61:687–708.