Redox agents regulate ion channel activity in vacuoles from higher plant cells

Armando Carpaneto, Alberto M. Cantù, Franco Gambale*

Istituto di Cibernetica e Biofisica, Via De Marini 6, I-16149 Genoa, Italy

Abstract The ability of redox agents to modulate certain characteristics of voltage- and calcium-activated channels has been recently investigated in a variety of animal cells. We report here the first evidence that redox agents regulate the activation of ion channels in the tonoplast of higher plants. Using the patch-clamp technique, we have demonstrated that, in tonoplasts from the leaves of the marine seagrass *Posidonia oceanica* and the root of the sugar beet, a variety of sulphydryl reducing agents, added at the cytoplasmic side of the vacuole, reversibly favoured the activation of the voltage-dependent slow vacuolar (SV) channel. Antioxidants, like dithiothreitol (DTT) and the reduced form of glutathione, gave a reversible increase of the voltage-activated current and faster kinetics of channel activation. Other reducing agents, such as ascorbic acid, also increased the SV currents, although to a lesser extent in comparison with DTT and glutathione, while the oxidising agent chloramine-T irreversibly abolished the activity of the channel. Single channel experiments demonstrated that DTT reversibly increased the open probability of the channel, leaving the conductance unaltered. The regulation of channel activation by glutathione may correlate ion transport with other crucial mechanisms that in plants control turgor regulation, response to oxidative stresses, detoxification and resistance to heavy metals.

© 1999 Federation of European Biochemical Societies.

Key words: Patch-clamp; Redox reaction; Glutathione; Ion channel; Plant vacuole

1. Introduction

In several cell types both extracellular and cytoplasmic factors modulate ionic transport and affect other functional properties of ion channels such as the kinetics, activation and/or inactivation. Sites important for the maintenance of the protein structure reside in the covalent disulphide bridges between two cysteine residues of the same polypeptide chain or different chains [1]. The tripeptide glutathione is the most important molecule participating in the reduction of cysteine-cysteine bridges in the cell cytoplasm; it is present in the majority of eukaryotic and prokaryotic cells where it participates in gene expression, phytochelatin synthesis and redox reactions (for a recent review see [2]). Recently, the influence of redox agents on channels of animal cells has received growing interest because it has been demonstrated that redox compounds regulate important ion channel mechanisms such as channel gating, inactivation and permeation in several cell types including neurones [3–6].

Since the effects induced by redox agents on ion channels of

higher plant cells have not been previously studied, we investigated whether reducing or oxidising compounds, acting on thiol groups, affect the transport properties of vacuoles obtained from the leaves of the marine plant *Posidonia oceanica* and the taproot of sugar beet.

2. Materials and methods

Vacuoles were extruded into the recording chamber by slicing the sugar beet taproots [7] and the white meristematic part of *P. oceanica* leaves [8] in their standard bath solution supplemented with 1 mM DTT. Currents were measured in the whole-vacuole or the (cytoplasmic-side-out) excised-patch configurations. Transmembrane voltages and ionic currents were controlled and monitored with a List EPC7 current-voltage amplifier interfaced with an Instrutech AD/DA board (Instrutech, Elmont, NY). The sign convention on endomembranes was adopted.

The standard solutions (in mM) were: for *P. oceanica* (identical in the bath and the pipette) KCl 400, MgCl$_2$ 5, CaCl$_2$ 0.1, HEPES 25, pH 7.2, Π = 900 mOsm by addition of sorbitol; for sugar beet KCl 150, MgCl$_2$ 2, HEPES 15, pH 7.2, with CaCl$_2$ equal to 0.1 mM in the bath and 1 mM in the pipette; Π was adjusted to values slightly larger than the osmotic pressure of the taproot.

The bathing medium was changed by a 'fast perfusion system' based on two perfusion pipettes (diameter ∼ 30 μm) respectively filled with the bath solution and the bath solution plus the redox agent. A piezoelectric manipulator allowed us to alternatively perfuse the vacuole with one or the other pipette in a few milliseconds. Alternatively, a peristaltic pump, together with a gravity-driven 'slow perfusion system', allowed us to smoothly change the whole bath solution in about 2 min. Experiments performed using one or the other perfusion procedure did not display quantitatively different effects on the steady state of the currents.

3. Results

Utilising patch-clamp techniques, we have previously demonstrated [8] that the tonoplast of *P. oceanica* leaves expresses a voltage-dependent slow vacuolar (SV) channel [7] with characteristics very similar to those of terrestrial plant vacuoles. Fig. 1 shows whole-vacuole currents in the presence and absence of the reducing agent dithiothreitol (DTT) in the bath solution. It can be seen that in the absence of DTT the current amplitude of *P. oceanica* and sugar beet vacuoles is approximately 15% and 50% of the value observed in the presence of DTT, respectively (Fig. 1a). The decrease of the current, upon removal of DTT, is a reversible process, recovering within ∼ 15 min after washout; in contrast, patched vacuoles that were maintained for long times (≥ 15 min) in the absence of DTT displayed a recovery of the current that decreased progressively and irreversibly. Consistently, the presence of DTT prevented the run-down of the current with the age of the vacuole preparation, and stable SV currents could be elicited from sugar beet vacuoles incubated up to 24 h from the isolation in a bath solution containing 1 mM DTT.

*Corresponding author. Fax: (39) (10) 6475500.
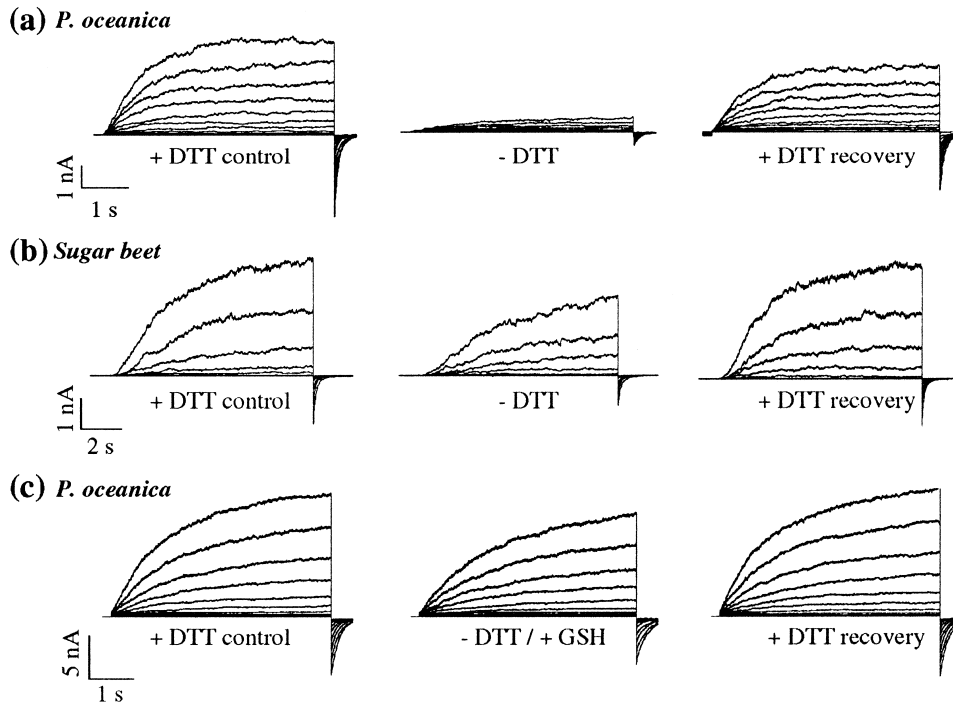E-mail: gambale@icb.ge.cnr.it

Fig. 1. DTT increases *P. oceanica* and sugar beet SV currents. a: Left: Whole-vacuole currents elicited, in the presence of 1 mM DTT, by a series of voltage steps from −30 mV to 50 mV in 5 mV increments; holding potential −30 mV. Middle: Currents elicited when DTT was removed from the bath solution. Right: Recovery. b: Also sugar beet vacuoles display a significant increase of SV current on the addition of 1 mM cytoplasmic DTT. Voltage steps incremented by 10 mV from +10 mV to 80 mV; holding potential 0 mV. c: GSH (middle) induces current increase comparable to that observed in the presence of DTT (left/control, right/recovery) in *P. oceanica*. Holding potential 0 mV, voltage steps from +10 mV to +100 mV in 10 mV increments. Standard ionic solutions. Currents were filtered at a minimum of 100 Hz and sampled at 500 Hz. In all panels tail potentials to −50 mV.

The effects induced by other redox agents added at 1 mM concentration in the external standard solution are shown in Table 1; while β-mercaptoethanol was ineffective in increasing SV current, the reduced form of the sulphydryl reagent glutathione (GSH) elicited a relevant current increase. Fig. 1c shows that the amplitude of the current in the presence of 1 mM GSH is comparable to that observed in the presence of DTT, and therefore much larger than that measured when DTT was not present in the bath (Fig. 1a).

To quantify the increase of the SV current mediated by DTT, the normalised (with respect to the condition DTT = 1 mM) steady-state current (I_{nor}) of the *P. oceanica* vacuole is plotted as a function of DTT concentration in Fig. 2a. It can be observed that I_{nor} increases with DTT concentration up to 8-fold. In vacuoles from both plants, DTT and GSH also decreased the time constants of current activation as illustrated in Fig. 2b) where the half activation time, $t_{1/2}$, of

P. oceanica current is plotted as a function of DTT concentration. The faster activation of the channel may be due to any modification of the protein that controls the transition of the channel from the closed to the open state.

The complete and irreversible inhibition of *P. oceanica* current (Table 1) on addition of chloramine-T (Ch-T, a compound that specifically oxidises exposed methionine and cysteine residues [9]) also supports the hypothesis that the reduction of sulphydryl groups plays a major role in the increase of SV current. The vacuoles did not recover the current decrease induced by Ch-T even after a 20 min wash in 1 mM DTT. On the other hand, ascorbic acid, a reducing agent in hydroxylation reactions, increased the current, although to a lesser extent compared with DTT and GSH.

The current increase induced by the addition of DTT is a very fast process that occurs within a few seconds of DTT treatment. This is illustrated in Fig. 2c, which shows the de-

Table 1
Increase and inhibition of *P. oceanica* current by redox agents

Redox agent	$I_{agent}/I_{control}$ (mean ± S.E.M.)	N vacuoles	n measurements	Properties
Dithiothreitol	8.3 ± 0.6	11	29	Reversible (within 15 min)
Glutathione	6.3 ± 0.5	3	11	Reversible
Ascorbic Acid	3.3 ± 0.5	2	8	Reversible
β-Mercaptoethanol	1.0 ± 0.2	2	5	Reversible
Chloramine-T	0	3	3	Irreversible

Reducing agents increase the SV current to different extents while the oxidising agent Ch-T inhibits the current totally and irreversibly. $I_{agent}/I_{control}$ represents the ratio between the steady-state current elicited by 1 mM concentration of different agents added to the control external solution (I_{agent}) and the steady-state current obtained in the external standard solution without any redox agent ($I_{control}$).
N and n represent the number of tested vacuoles and the total number of measurements done, respectively. *P. oceanica* standard solutions with 1 mM internal $CaCl_2$. Voltage pulse to +80 mV; holding potential 0 mV.

crease and the increase of the steady-state current when the vacuole was exposed to solutions that did and did not contain DTT by using a fast perfusion procedure. The current decay and recovery were fitted by single exponential functions with time constants of ~ 2 s. An estimation of the time constants describing the increase (τ_{inc}) and decrease (τ_{dec}) of the current respectively on the addition and the removal of DTT in *P. oceanica* gave $\tau_{inc} = 2.6 \pm 0.8$ s ($n = 6$) (mean $\pm$ S.E.M.; $n =$
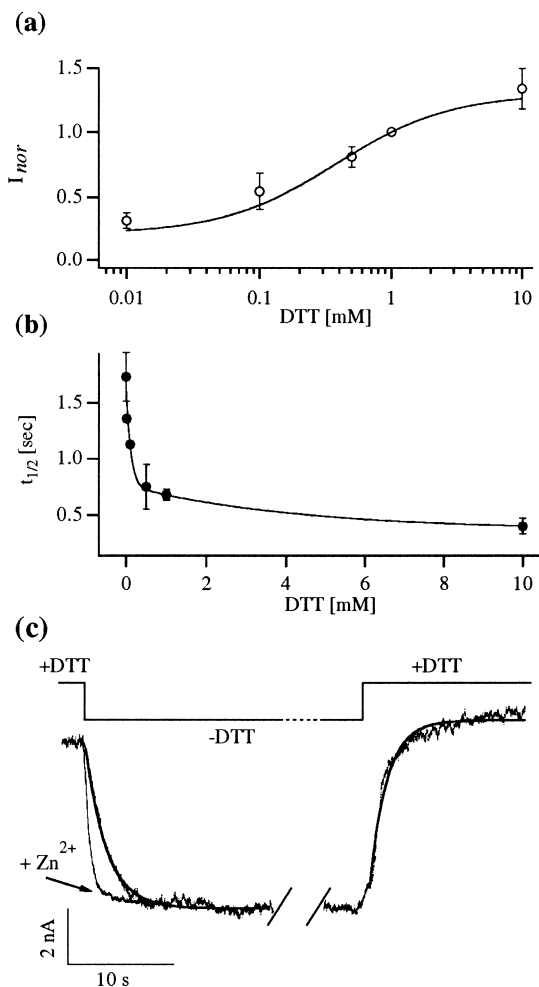

(a)

(b)

(c)

Fig. 2. Reversible current increase and kinetic of activation in *P. oceanica* vacuoles as a function of DTT concentration. a: Normalised current, $I_{nor} = I(DTT)/I(DTT = 1$ mM$)$, plotted together with the best fit obtained by the Michaelis-Menten function $I_{nor} = k_0 + k_1[DTT]/([DTT] + K_m)$ (continuous line), where $k_0 = 0.20 \pm 0.04$, $k_1 = 1.09 \pm 0.14$ and $K_m = 0.38 \pm 018$ mM. b: Time constant of half activation, $t_{1/2}$, as a function of DTT concentration. Step potentials to +40 mV or to +80 were applied from a holding potential of 0 mV. The amplitude of the step potential did not affect $t_{1/2}$ appreciably. The continuous line through the experimental data points has no theoretical meaning. c: Typical reversible decrease/increase of *P. oceanica* current observed on the removal/addition of DTT by the fast perfusion procedure. The increase and decrease of the steady-state current followed an exponential course with time constants of ~ 2 s. Steady-state voltage at +80 mV. The arrow indicates the decrease of the current (appropriately scaled) induced by the addition of 1 mM ZnCl$_2$; applied voltage +100 mV. Note that the inhibition of the current induced by Zn^{2+} is reported uniquely to show the velocity of the fast perfusion system. The upper profile represents the presence/absence of 1 mM DTT.
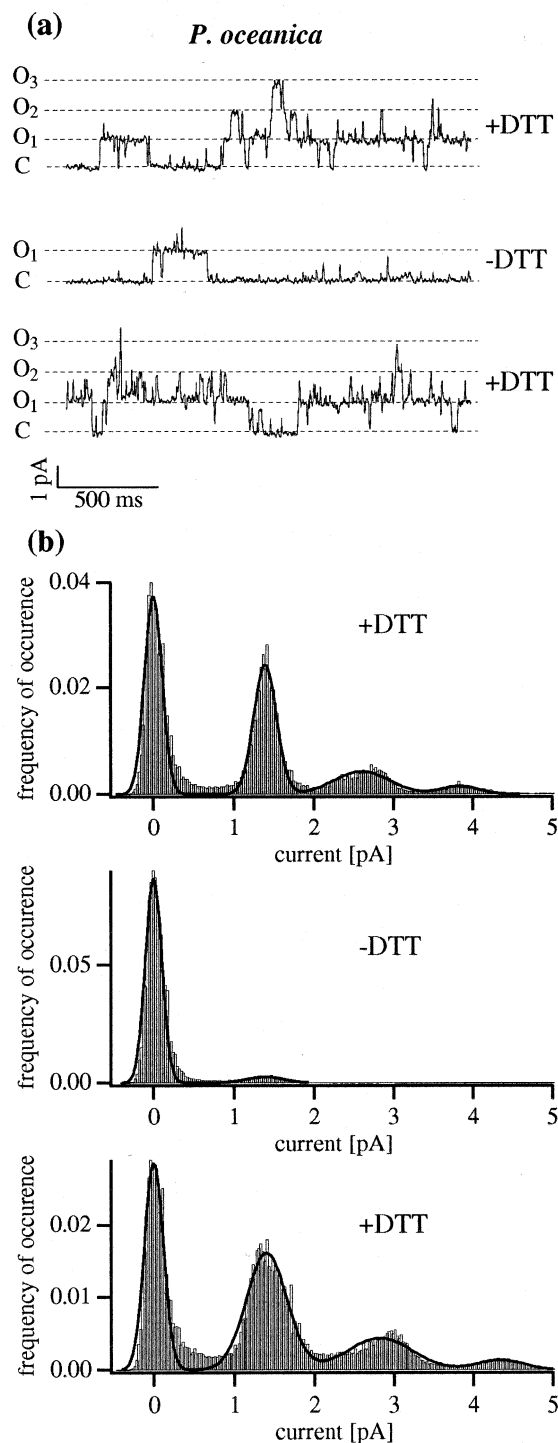

(a)   *P. oceanica*

(b)

Fig. 3. DTT increases the open probability of SV channels in *P. oceanica* vacuoles. a: Upper trace: Representative single channel openings observed in the presence of 1 mM DTT. The closed state (C) and the different open levels (O$_1$, O$_2$, O$_3$ etc. due to the opening of 1, 2, 3 etc. channels) are indicated by dotted lines. The middle trace shows the single channel openings recorded from the same patch without DTT. Lower trace: Recovery. Applied potential +10 mV, sampling time 1 ms, filter 300 Hz. b: Frequency of occurrence of the different current levels exemplifies the probability of finding 0, 1, 2, 3 channels open in the presence (upper histogram) or absence of DTT (middle histogram). The lower histogram represents the recovery. Histograms were derived from strip records lasting at least 60 s. The bin size was 0.025 pA.

number of experiments) and $\tau_{dec} = 2.0 \pm 0.4$ s ($n = 10$). Sugar beet vacuoles displayed comparable time constants.

Note that the time necessary to step from the bath solution plus reducing agent to the bath solution (and vice versa) is in the order of a few hundred milliseconds; we verified the velocity of the perfusion system in a few control experiments where (with no DTT in the pipettes and the bath) one of the two perfusion pipettes contained the bath solution and the other was loaded with an identical solution containing 1 mM Zn^{2+}, a well-known blocker [10] of the SV type channels (Fig. 2c).

To determine whether the effects of reducing agents depend on the probability of the channel to be open or on variations of the channel conductance, we analysed excised cytoplasmic-side-out patches containing a few channels. Fig. 3 demonstrates that the open probability of the channel in *P. oceanica* vacuoles markedly decreased when 1 mM DTT was removed from the solution. Up to four current levels can be observed in the current traces (Fig. 3a) and histograms (Fig. 3b) when DTT was present in the bath solution, while only one open level was observed without DTT. Upon the removal of DTT, the probability of a channel to be open decreased approximately 2-fold in sugar beet vacuoles (from 0.04 to 0.02) and almost 10-fold in *P. oceanica* vacuoles (from 0.10 to 0.01). These variations are comparable to the reduction of the macroscopic currents observed after removal of DTT in the whole-vacuole configuration (Fig. 1).

Conversely, the presence of the reducing agent did not affect the channel conductance, γ, since in *P. oceanica* vacuole $\gamma_{pos} = 131 \pm 13$ pS ($n = 345$) (mean $\pm$ variance, $n =$ number of channels) in the presence of DTT and $\gamma_{pos} = (128 \pm 21)$ pS ($n = 96$) without DTT; similarly in sugar beet vacuoles, in the presence of DTT, $\gamma_{sb} = 94 \pm 4$ pS ($n = 185$) while, without DTT, $\gamma_{sb} = 96 \pm 2$ pS ($n = 69$).

Finally, we found that DTT added to the solution of the patch pipette did not prevent the decrease of the current when it was removed from the bath solution; therefore, the increase of the current mediated by reducing agents is not due to permeation of DTT into the vacuole. Also, variations in the concentration of lumenal calcium (from 0.1 to 1 mM) did not affect the current increase induced by DTT.

4. Discussion

To date, the effects of redox compounds on ion channels have been studied only in animal cells [3–6] or yeast [9]; here we report the first evidence that also in higher plant vacuoles the functional properties of the SV channels are regulated by redox compounds that possibly act on sulphydryl groups of cysteine residues located at the cytoplasmic side of the vacuole. We wish to underline that the effects of redox compounds shown in yeast [9] refer to a channel that is different from the SV channel since it opens at negative voltages and drives cations into the vacuole.

Clearly reducing agents increase the open probability of the SV channel; as already suggested for calcium-activated channels in animal cells [3], this increase may depend on modifications of the sites that control channel activation through the binding of Ca^{2+} ions to the channel. On the other hand, a precise measurement of the effects induced by DTT on the voltage-dependent characteristics of the channel is difficult because the activation curve saturates at very high transmembrane voltages, i.e. much larger than +100 mV; this fact does not allow a reliable evaluation of both $V_{1/2}$ and the gating charge. Experiments are in progress to quantify modifications of the calcium, voltage and pH characteristics of the SV channel in the presence of reducing agents. This could open new perspectives that will clarify the physiological role of this protein in plant vacuoles.

Since the increase of the vacuolar current mediated by glutathione occurs at concentrations of physiological significance [11], the modulation of the SV channel may be subsidiary to other physiological processes not only as cell volume and turgor regulation but also as vacuolar segregation of xenobiotic compound, such as harmful halides, heavy metal ions and herbicides [11,12].

Finally, while reducing agents like DTT are currently added to solutions used to obtain vacuoles and protoplasts investigated by electrophysiological techniques, their effects on plant channels seem to be underestimated in laboratory practice. Therefore, our results may have important methodological implications for the interpretation of some electrophysiological data in plant systems.

References

[1] Glazer, A.N. (1970) Annu. Rev. Biochem. 39, 101–130.
[2] Noctor, G., Arisi, A.C.M., Jouanin, L., Kunert, K.J., Rennenberg, H. and Foyer, C.H. (1998) J. Exp. Bot. 49, 623–647.
[3] Wang, Z.W., Nara, M., Wang, Y.X. and Kotlikoff, M.I. (1997) J. Gen. Physiol. 110, 35–44.
[4] Elliot, S.J. and Koliwad, K.K. (1998) Microcirculation 4, 341–347.
[5] Stephens, G.J., Owen, D.G. and Robertson, B. (1996) Pflügers Arch. 431, 435–442.
[6] Koliwad, K., Elliot, S.J. and Kunze, D.L. (1996) J. Physiol. 495, 37–49.
[7] Hedrich, R. and Neher, E. (1987) Nature 329, 833–835.
[8] Carpaneto, A., Cantù, A.M., Busch, H. and Gambale, F. (1997) FEBS Lett. 412, 236–240.
[9] Bertl, A. and Slayman, C.L. (1990) Proc. Natl. Acad. Sci. USA 87, 7824–7828.
[10] Hedrich, R. and Kurkdjian, A. (1988) EMBO J. 7, 3661–3666.
[11] Rea, P.A., Li, Z.S., Lu, Y.P., Drozdowicz, Y.M. and Martinoia, E. (1998) Annu. Rev. Plant Physiol. 49, 727–760.
[12] Ward, J.M. and Schroeder, J.I. (1994) Plant Cell 6, 669–683.