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# Ion channels in the vacuoles of the seagrass *Posidonia oceanica*

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**Abstract Voltage-dependent ionic channels were investigated by the patch-clamp technique in the vacuolar membrane from the leaves of the seagrass *Posidonia oceanica*. Vacuoles extruded from the meristematic white part of the leaves displayed rectifying slow currents which activated in several seconds at positive potentials and deactivated at negative voltages within a few hundreds of ms. Like the *S*low *V*acuolar (SV) channel already identified in the tonoplast of terrestrial plants, the SV voltage-dependent channel of *Posidonia* leaves was activated by micromolar concentrations of $Ca^{2+}$ and was equally permeable to $K^+$ and $Na^+$. The single-channel conductance of the *Posidonia* SV-type channel was 106 ± 12 pS (in symmetric 400 mM $K^+$). In the same ionic solutions, another channel, occasionally observed in vacuoles from the green part of the leaves, displayed a single-channel conductance of 47 ± 4 pS. To our knowledge, this is the first electrophysiological characterization of ion transport pathways in *Posidonia*, a marine plant of crucial importance for the ecology of the Mediterranean sea.**



*Key words:* *Posidonia oceanica*; Seagrass; Patch clamp; Ion channel

## 1. Introduction

In the plant kingdom, in the class of monocotyledoneae, seagrasses (family of Zoosteraceae) take a unique place because of their ability to grow completely submerged in sea water. The seagrass species *Posidonia oceanica* (L.) Delile grows exclusively in the Mediterranean sea where it is the dominating species in waters of 2–30 m depth. Meadows of *P. oceanica* are sites of high primary production; they provide shelter for the growth of fishes and invertebrates, their leaf-blades modulate the water flow above the sediment and, at high irradiance, they provide oxygen to the water column [1,2].

Several morphological adaptations have been described in these halophythes that enable them to live in an environment with high salinity, low light levels, hydrostatic pressure, wave action and slow diffusion of inorganic carbon species [3]. We wondered whether the physical constraints of the marine environment would be reflected in differing physiological and biophysical properties of the transporters or, owing to a common ancestral origin, these marine plants would display properties similar to those of terrestrial plants. Because of the difficulty of growing *P. oceanica* in culture for prolonged periods and of obtaining protoplasts and vacuoles from seagrasses [4], until now, extensive research on these topics has been restricted to in situ or whole-plant studies. The mechanisms of nutrient uptake, ion homeostasis and uptake of inorganic carbon by seagrasses are not well understood [5] and are subject to considerable debate [6]. On the other hand, in all terrestrial plants investigated so far, ion-selective channels present in the plasma and vacuolar membranes were observed to play a fundamental role in these processes. Following the characterization of ionic channels in the vacuoles and protoplasts of terrestrial plants [7–9], we applied the patch-clamp technique to *P. oceanica* vacuoles obtained from the basal region of the leaves.

## 2. Materials and methods

*Posidonia oceanica* was collected from the Mediterranean sea and maintained in natural sea water for up to 1 month. Patch-clamp experiments were performed on the vacuoles of *Posidonia* leaves harvested from 3 different meadows in the Mediterranean sea, maintained in oxygenated natural sea water and used within a few weeks of the collection. Two meadows are located on the western coast at a distance of 30 and 70 km from Genoa, the third site is located 200 km (Livorno area) from the other two meadows. Whenever possible, experiments were performed on the same day or within a few days of the plant harvesting.

Vacuoles from *Posidonia* leaves were extruded into the patch-clamp recording chamber by slicing the leaves in standard bath solution (see below) supplemented with 1 mM dithiothreitol. Unless otherwise indicated, experiments were performed on vacuoles obtained from the white meristematic part of the leaves. The ion transport properties of the tonoplast were studied using the patch-clamp technique in the whole-vacuole, excised-patch and vacuole-attached configurations. Access to the vacuole interior was gained by breaking the membrane under the patch pipette by short (~700 μs) voltage pulses up to ~1 V.

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). A Macintosh personal computer running the software 'Pulse' (Heka Electronic, Lambrecht, Germany) was used to generate the stimulation protocol and to store the digitized current records on the computer hard-disk. Single-channel recordings were also stored on a video cassette recorder equipped with a digital PCM (Sony 501ES, Nordmende V5005) modified according to Bezanilla [10]. Current records were low-pass filtered with a 4-pole filter KemoVBF8 (Kemo, Beckenham, UK). Analyses of single channels were performed off line; single-channel openings were analyzed by constructing current histograms that were fit with Gaussian distributions.

The standard bath solution was (in mM) KCl 400, $MgCl_2$ 5, $CaCl_2$ 0.1, Tris-MES 10, pH 7.2; the standard pipette solution was identical to the bath except that it contained 1 mM $CaCl_2$. The osmotic pressure of the pipette and bath solutions was adjusted to ~1200 mOsm by the addition of appropriate amounts of sorbitol. According to the sign convention on endomembranes proposed by Bertl et al. (1992), the potential difference across the vacuolar membrane ($V$) is calculated as $V = V_{cytosol} - V_{vacuole}$ [11,12]. This convention implies that positive currents represent cation flow out of the cytosol.

The liquid junction potentials (LJP) were always less than 1 mV, measured according to the procedure described by Neher [13,14]. Nernst potentials were calculated taking into account the activity coefficient of the ionic solutions [15]. The applied voltage was corrected for series resistance when the estimated error was greater than 5 mV.

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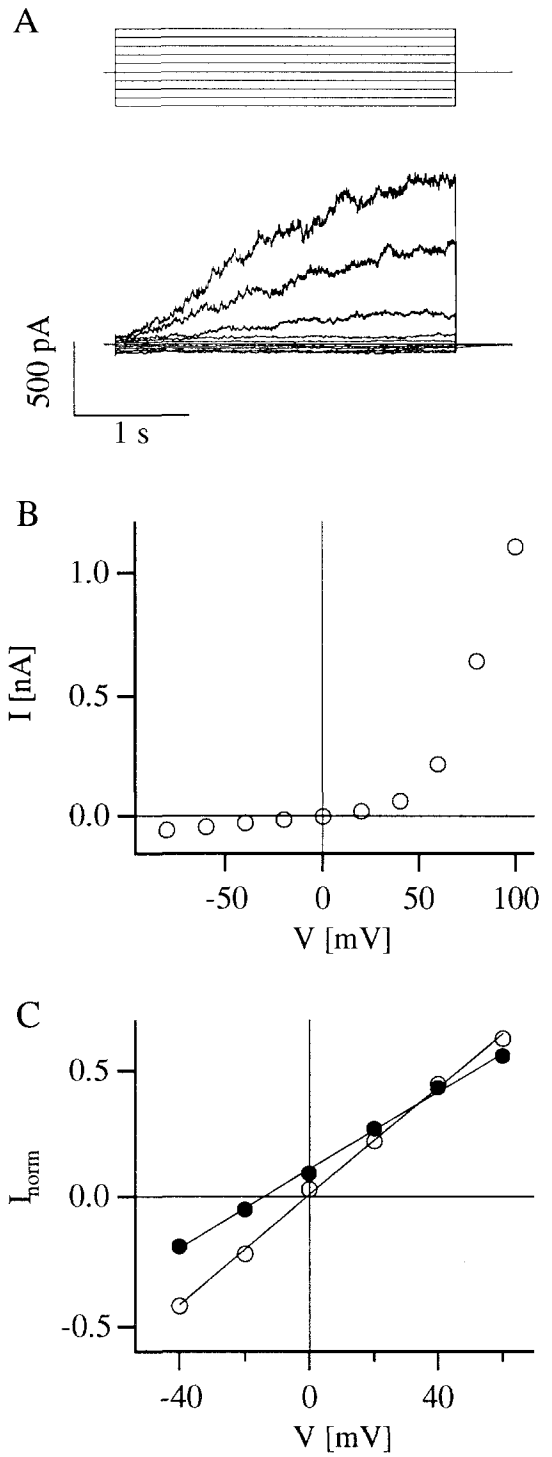


Fig. 1. Macroscopic SV currents in the vacuoles of *Posidonia* leaves. (A) Macroscopic whole-vacuole currents elicited by a series of voltage steps ranging from −80 mV to 100 mV in 20 mV steps. Holding and tail potentials were at 0 mV. The upper trace illustrates the potential protocol. (B) Current-voltage characteristic (of data in A) obtained by plotting the steady-state current at each test pulse. The pipette and the bath contained symmetric standard bath solutions. (C) Normalized instantaneous tail currents versus voltage in symmetric (○) (standard ionic solutions) and asymmetric (●) solutions (the bath contained the standard 400 mM KCl solution while the pipette contained 200 mM KCl). Step potentials to +100 mV; tail potentials ranging from +60 mV to −40 mV in 20 mV steps. Holding potential, 0 mV. The reversal potential, $V_{rev} = -14$ mV, suggests that the channel is potassium selective ($V_{rev}(K^+) = -15.6$ mV and $V_{rev}(Cl^-) = +15.1$ mV).

## 3. Results

In the whole-vacuole configuration, positive membrane potentials (≥ +40 mV) applied to *Posidonia* vacuoles (obtained from the white part of the leaf) elicited macroscopic outward currents which displayed a marked voltage dependence and time-dependent activation (Fig. 1A,B). The time course of the voltage-dependent currents resembles that of the *S*low *V*acuolar (SV) channels [7], characteristic of the tonoplast of terrestrial plants. *Posidonia* currents displayed slow activation kinetics with half-times of the order of seconds (in Fig. 1, $t_{1/2}(act) = 1.1$ s at $V = +100$ mV) and instantaneous tail currents that relaxed within hundreds of ms (e.g. $\tau(deact) = 150$ ms at $V_{Tail} = -50$ mV following a $V_{Step} = +100$ mV). Instantaneous tail currents demonstrated that the channel *I–V* characteristic is linear in the voltage range investigated. In asymmetric solutions (Fig. 1C), the reversal potential, $V_{rev} = -14$ mV, was very close to the Nernst potential for $K^+$ ($V_{K^+} = -15.6$ mV). This observation indicates that potassium is the major permeant ion through the SV-type channel of *Posidonia* tonoplast, in these solutions. Therefore, the current is carried by $K^+$ entering the vacuole.

However, *Posidonia* channels did not discriminate among monovalent cations. Indeed the substitution of potassium with an equal concentration of sodium (Fig. 2A) did not appreciably affect the macroscopic current amplitude, the kinetics of activation, or the voltage dependence of the channel. Fig. 2B also shows that in NaCl solutions SV currents activated very steeply at membrane potentials ≥ +40 mV. Instantaneous tail currents (measured after the application of step potential to +80 mV) reveal that sodium permeates the channel equally well as potassium ($V_{rev} = -2$ mV in the presence of 400 mM NaCl in the bath and 400 mM KCl in the pipette).

We also demonstrated that a drastic decrease of the cytoplasmic $Ca^{2+}$ concentration, from 0.1 mM to nominally zero calcium (~20 nM), reversibly abolished the time-dependent macroscopic currents (Fig. 3). Application of a bath solution containing 1 mM EGTA inhibited the outward current very efficiently within a few seconds; after returning to the control solution (containing 0.1 mM $Ca^{2+}$ solution) the recovery of the current was fast and nearly complete.

Due to the slow kinetics of activation, it was also possible to resolve single-channel openings in the macroscopic currents at critical membrane potentials where just a few channels were statistically expected to be open. Fig. 4 shows such single-channel transitions extracted from the macroscopic currents already shown in Fig. 2. In the upper trace of Fig. 4, the magnified current trace elicited by a pulse to +40 mV shows openings of at least four channels. In the same figure, the bottom trace is a magnification of the tail current observed at −40 mV after a voltage pulse to +50 mV; at least six different conductance levels, due to the successive closures (deactivation) of six channels, can be identified.

This figure clearly demonstrates that the macroscopic SV-type current is due to the consecutive opening of several individual channels. We determined that the conductance of the elementary transitions shown in Fig. 4 (400 mM external NaCl and standard internal solution) is ~100 pS. Similar traces were also observed in the presence of symmetric KCl solutions (data not shown).

Consistent with the estimation derived from Fig. 4, experiments performed on the same vacuole, first in the vacuole-

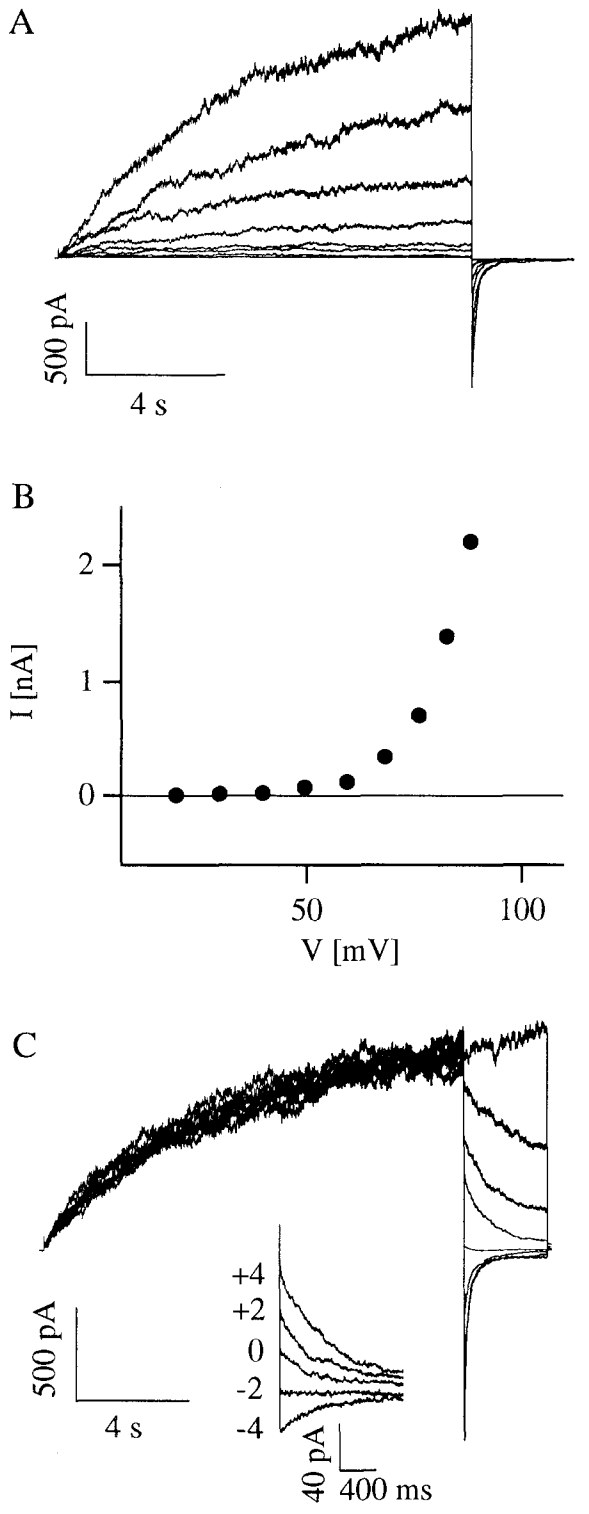


Fig. 2. *Posidonia* SV channel is permeable to $Na^+$. (A) Macroscopic whole-vacuole $Na^+$ currents elicited by a series of voltage steps ranging from +20 mV to +88 mV. The bath solution was (in mM): NaCl 400, $MgCl_2$ 5, $CaCl_2$ 0.1, Tris-MES 10, pH 7.2. Standard pipette solution. Holding potential, 0 mV; tail potential, −40 mV. (B) Current-voltage relation similar to that obtained in the presence of $K^+$ (see Fig. 1). (C) Instantaneous tail currents elicited by tail potentials ranging from +80 to −80 mV in 20 mV steps; step potential to +80 mV. The inset shows tail currents of the same experiment at a much higher resolution (from +4 to −4 mV, steps of 2 mV). Tail currents reverse at around −2 mV.

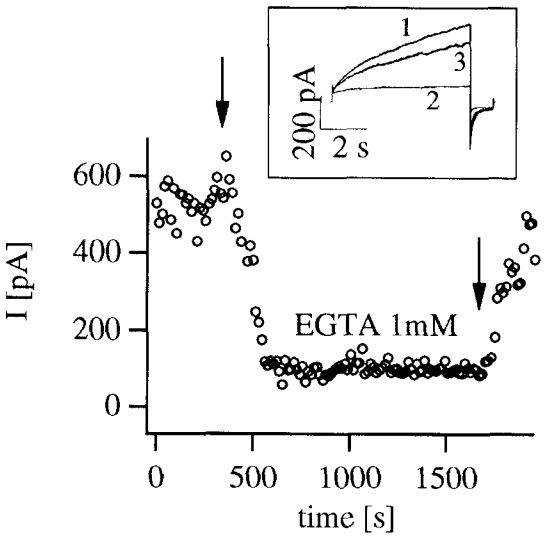


Fig. 3. *Posidonia* SV channels are regulated by cytoplasmic calcium. Slow vacuolar currents were drastically reduced when the calcium concentration in the bath was decreased from 0.1 mM to nominally zero calcium by perfusion with a solution identical to the control (standard solution) but containing 1 mM EGTA. Data points represent the mean value of the last ≈100 ms of each current record measured in the presence and in the absence of calcium; the downward arrows indicate the beginning (left arrow) and the end (right arrow) of EGTA perfusion. The same solutions used in Fig. 2. The inset shows the average current traces observed in control conditions (curve 1, average of 30 records), in the presence of EGTA (curve 2, average of 30 records) and in recovery conditions (curve 3, average of 10 records). Holding potential 0 mV, applied potential +80 mV, tail potential −40 mV. The interval between two consecutive pulses was 15 s.

attached (Fig. 5A) and then in the excised patch configuration (Fig. 5B), showed single-channel conductances of comparable amplitude (102 ± 16 pS and 106 ± 12 pS, respectively). The single-channel openings elicited by positive potentials displayed a delay typical of the SV-type channels, as shown from records in Fig. 5A,B. Further, the macroscopic current could be reconstructed by averaging 18 consecutive single-channel records elicited by a step potential to +50 mV (inside-out patch configuration, Fig. 5D). Therefore, the macroscopic currents shown in Figs. 1–3 are due to the superposition of several slow-activating channels.

Finally, we report that we were occasionally able to record ion channels in membranes of vacuoles extruded from the green part of *Posidonia* leaves which displayed a lower single-channel conductance of 47 ± 4 pS.

## 4. Discussion

We were able to record reliable channel activity both in the whole cell and excised patch configuration in ≈25% of the patches with seal resistances of several gigaohms. In some other cases the channel activity could not be clearly resolved or we observed a rapid run down of the current after the excision of the patch. This low rate could not be correlated with the location of the *Posidonia* meadow, or with the time elapsed from plant harvesting. Since the experiments reported in this paper were performed exclusively during late fall and winter, it is possible that seasonal parameters regulating the plant growth affect channel expression and as a consequence

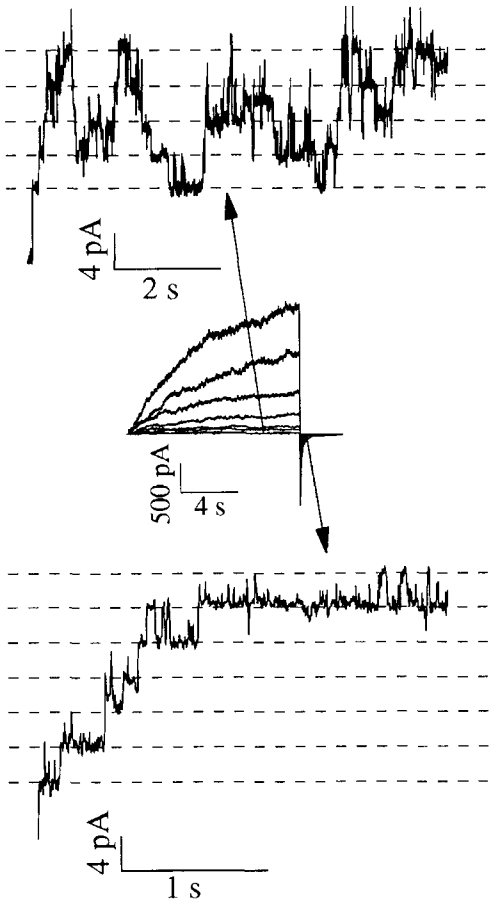


Fig. 4. Slow currents of *Posidonia* leaves are due to the opening of single channels. The middle traces display the same macroscopic currents already shown in Fig. 2A while the upper and lower traces represent two of these records at higher magnification. The upper trace shows the activation of 4 channels (≈4 pA) elicited by a step voltage to +40 mV. In the lower trace, at least 6 channels (evoked by a step potential to +50 mV) deactivated when the tail membrane potential was driven to −40 mV. The same conditions as in Fig. 2.

the rate of success for observing ion channels. Alternatively, the rapid run down of channel activity observed in some experiments suggests the possibility that some regulatory agents, present inside the vacuole or in the cytoplasm, could be dialyzed after the break-in or during vacuole extrusion as proposed by other authors [9,16].

The selectivity and the activation time of the SV current in *Posidonia* vacuoles are comparable to those of similar currents in the vacuoles of terrestrial plants in the presence of similar concentrations of cytoplasmic calcium [12,17,18], a modulator of SV channel activation and kinetics [7,9].

The SV-type channel in vacuoles of *Posidonia* leaves shows a conductance appreciably lower (by a factor 2) than the conductance of similar SV channels in terrestrial plants. As an example, in 400 mM KCl conductances of ≈270 pS were

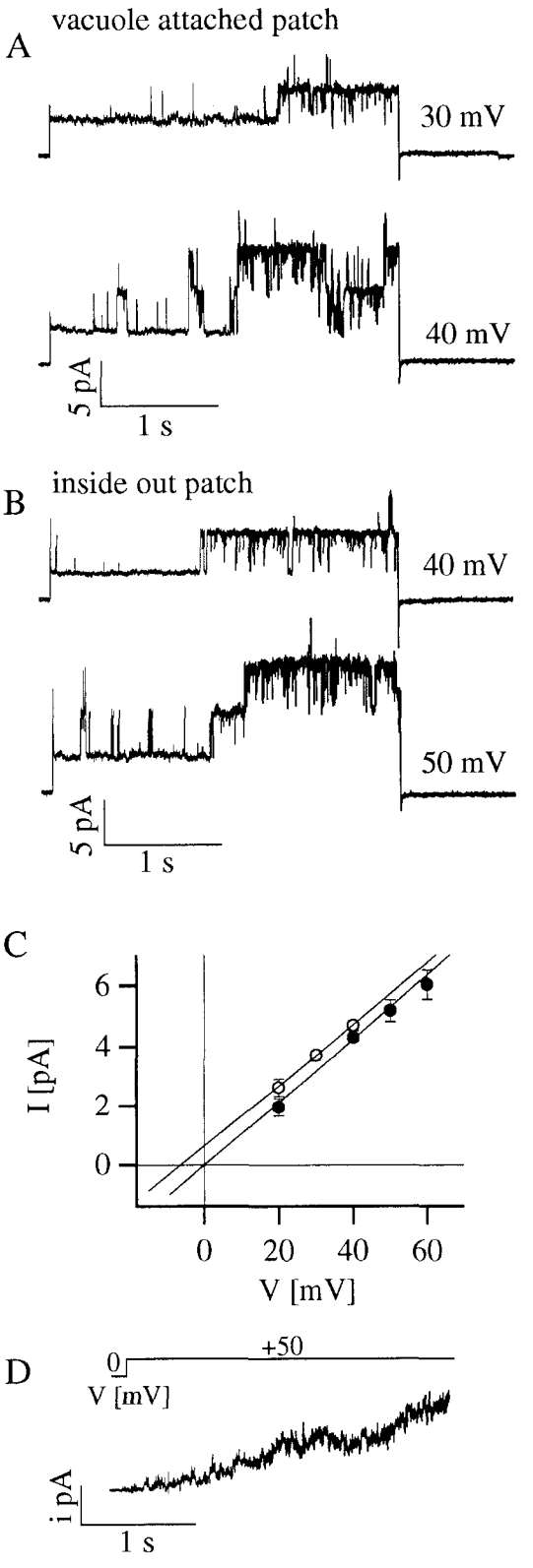


Fig. 5. Current transitions representative of slow vacuolar single-channel openings. (A) Currents were recorded in the on-vacuole configuration. Current steps show the delay in activation typical of the SV channel. Symmetrical solutions (in mM): KCl 400, $MgCl_2$ 5, $CaCl_2$ < 40 μM, Tris-MES 10, pH 7.2. (B) Single-channel currents recorded in the excised inside-out configuration. (C) Current-voltage relation indicates a single-channel conductance of 102 ± 16 and 106 ± 12 pS in the on-vacuole (empty circles) and inside-out configuration (filled symbols), respectively. (D) Mean current, i (in arbitrary units), obtained averaging 18 consecutive single-channel traces like those shown in panel (B). The upper profile represents the potential protocol. Data were corrected for capacitive and leak currents.

measured in sugar beet vacuoles [12] while values between 70 and 200 pS were observed in tonoplasts from different plants at $K^+$ concentrations ranging from 50 to 200 mM [7–9,12,14,17–19]. This difference may be due to a different structure of the pore of the slow vacuolar channel in marine plants or to an early saturation (or maximum, see [12]) of the conductance as a function of the concentration of the permeant ion in terrestrial plants. This could reflect a physiological adaptation of the plant to the marine environment in which ions are relatively abundant and constant.

We also observed that the reversal potential and the single-channel conductance of the SV-type channel did not differ significantly before (cell-attached mode) and after the excision of the patch. This observation suggests that the vacuole contains approximately the same ionic concentration present in the pipette and that the run-down possibly affects the opening probability but does not change the single-channel amplitude.

In conclusion, with the exception of the smaller single-channel conductance, the ion transport properties of SV channels present in *Posidonia* tonoplast are highly reminiscent of the properties of terrestrial plants. This is not surprising from an evolutionary point of view since seagrasses are believed to have evolved from terrestrial plants which returned to the sea by gradual progressive steps and acclimation [1]. Interestingly, the relatively low selectivity of the *Posidonia* SV channel could explain the correspondent low selectivity and the otherwise inexplicable $Na^+$ permeation [17] of the correspondent SV channel present in the vacuole of terrestrial plants. It may be that this is a characteristic of ancestral plants which has been conserved in terrestrial plants.

Since very little is known about *Posidonia* ion-transport systems, we may only speculate on possible roles played by the SV channel identified in this paper. Possibly, this channel is crucial in seagrasses owing to the high salt concentration of sea water. Indeed, in order to be able to grow, plant cells must have turgor pressure and therefore the osmotic pressure must be higher inside the cell than outside. This would imply very large salt concentrations in the cytoplasm and a consequent inhibition of enzyme activity, unless ions are accumulated inside the vacuole which, in turn, cooperate to balance cell volume. Therefore, the SV-type channel could be an efficient transport system to drive cations inside the vacuole when cytosolic calcium increases within the micromolar range. Similarly to terrestrial plants, vacuolar transmembrane potentials (necessary to drive $K^+$ and $Na^+$ out of the cytoplasm, i.e. inside the vacuole), could be regulated by other transporters, like $H^+$-ATPase and/or other channels, which presumably exist in *Posidonia* vacuole or cell membrane. As an example, in terrestrial plants a reduced efficiency of $H^+$-ATPase could participate in evoking relatively more positive potentials which are able to activate the SV-type channel driving cations into the vacuole.

To our knowledge this is the first electrophysiological characterization of an ion channel in the tonoplast of *Posidonia oceanica*, a plant playing a fundamental role in the ecology of the Mediterranean sea. Knowledge of the biophysical properties of the proteins controlling ion transport is important for a clearer understanding of the processes which regulate the growth and the reaction of this Mediterranean endemic species towards biotic and abiotic pollutants.

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