Ionic transport in the plasma membrane of carrot protoplasts from embryogenic cell-suspension cultures

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Abstract Ionic transport properties of protoplasts obtained from embryogenic carrot suspension cells were studied by the patch-clamp technique. In the whole-cell configuration, carrot protoplasts presented macroscopic time-dependent outward currents, showing kinetics of activation which did not depend appreciably on the amplitude of the stimulus. Time- and voltage-dependent whole-cell inward rectifying currents as well as instantaneous non-selective currents were also observed. Both time-dependent inward and outward currents are carried by potassium ions. In a cell-attached configuration, two types of single-channel signals, displaying conductances of 10 and 17 pS, were observed; the instantaneous 10 pS channel was also present in outside-out excised patches.

Key words: Ionic channel; Protoplast; Carrot cell

1. Introduction

In plant cells, potassium fluxes through voltage-dependent K^+ channels lead to long-term changes in intracellular K^+ concentration which influence plant nutrition, enzyme activities, turgor pressure, etc. Ion channels have been identified in cultured cells [1–3] of tobacco, corn and *Arabidopsis*, in wheat and barley roots [4–6], in pulvinar [7] as well as barley aleurone [8] cells. As a consequence of the important role they play in the stomatal system, they have also been intensively studied in guard cells (*Vicia faba* and *Zea mays*) [9–15]. Besides the relevant function played in regulating the osmolarity and turgor in guard cells or the selective uptake of ions in roots, ion channels are likely to play an important role also in other processes, such as hormonal signal transduction. It has been reported that, in guard cells, ABA regulates a non-specific Ca^{2+} channel [16]. In the same cells, it has been shown that external auxins modulate the voltage-gating of fast anion channels (GCAC1) [17] and control the activity of K^+ channels [18]. Recently, it has been reported that auxin modulates an anion channel which is present in tobacco cell suspensions (TSAC) [19]. For these reasons, in the present work the patch-clamp technique [20,21] as applied to plant cells [22,9] was used to study the transport properties mediated by ionic channels in carrot (*Daucus carota* L.) protoplasts. This system might be particularly interesting because it not only responds to auxin but because auxin, given at different times, determines both the acquisition of totipotency and the arrest of the embryogenic process.

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2. Materials and methods

Carrot suspension cells (*Daucus carota* cv. 'S. Valery') were cultivated in B5 medium (Flow) supplemented with 0.5 mg/l (2.3 μM) 2–4D and 0.25 mg/l (1.1 μM) 6-benzylaminopurine (BAP) [23]. Cells in the exponential phase, 3 days after transfer to fresh medium, were used to prepare protoplasts. Cells (~2 g) were centrifuged at 200 × *g* to remove culture medium; then they were incubated at 30°C for 3 h (in the dark) in a 30 ml enzyme solution containing 50 mM sodium citrate buffer, 0.4 M mannitol, 2% cellulase 'Onozuka' R10 (Yakult Biochemicals, Japan) and 1% macerozyme 'Onozuka' R10. Protoplasts were filtered through 50 μm mesh and collected after washing 2 × with a solution of 0.3 M mannitol and 0.1 M $CaCl_2$ by centrifugation (5 min, 100 × *g*) [24].

Immediately before starting patch-clamp experiments, a few microliters of cell suspension were added to the Petri dish recording-chamber containing ~1 ml of bath solution. Protoplasts were stored at 4°C in the dark and then transferred to the Petri dish were they could be patched for a few hours. Glass cover-slips were extensively rinsed in distilled water, ethanol, methanol and occasionally coated with poly-L-lysine (then stored at −20°C) to enhance protoplast adhesion to the glass cover-slips mounted in the Petri dish.

The ionic current was monitored with an Axon 200 current voltage amplifier interfaced to an Instrutech A/D/A board (Instrutech, Elmont, NY). An Atari 4MegaST personal computer was used to generate the stimulation protocols and to store the digitised current records on the computer hard-disk. Single-channel recordings were also occasionally stored on a video cassette recorder equipped with a PCM Sony, modified according to [25]. Current records were low-pass filtered with a 4-pole filter Kemo VBF8 (Kemo, Beckenham, UK). Current records were analysed off-line either with a 486 MS-DOS-compatible system or a MacIntosh Quadra 950.

Patch pipettes were pulled from Kimax 51 glass tubing, coated with Sylgard (Dow-Corning) and fire polished immediately before use. In the standard bath solution, after fire-polishing, pipettes had a series resistance on the order of 5 MΩ. The mean protoplast capacitance per unit area, measured by the compensation circuitery of the current amplifier, was 0.7 ± 0.3 μF/cm^2.

The standard ionic solutions (mM) were in the bath: KCl 98, $CaCl_2$ 1, Hepes 10, Sorbitol 410, pH 7, in the pipette: KCl 10, KF 90, $MgCl_2$ 5, Hepes 10, EGTA 1, Sorbitol 460, pH 7.4.

3. Results

Time-dependent outward currents were observed in about 40% of the carrot protoplasts when depolarising step voltages were applied through the cell membrane. This result is shown in Fig. 1A where current records elicited by step voltages from 0 to +150 mV, from a holding potential of −50 mV, are reported. Outward currents occasionally showed moderate (<10%) inactivation (not shown). The steady state of the current, elicited by each step potential, was measured to construct the current–voltage characteristics shown in Fig. 1B. Usually, a stationary state was reached within a few hundreds ms with an activation time constant which was only slightly affected by the amplitude of the applied step potential, $\tau = 60 \div 80$ ms (Fig. 1C).

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The application of a depolarising voltage followed by a second stimulus, V_{tail}, allowed us to measure the instantaneous tail currents mediated by the outward channel in a wide range of voltages. The evaluation of peak tail current is simpler in whole-cell patches where only outward currents are present (like the one reported in Fig. 2A). The tail current reversed at potentials of about 0 mV in symmetric potassium solutions and asymmetric chloride (panel B), thus, indicating that the depolarisation-induced currents are carried by potassium selective channels. Stepping to lower depolarising potentials the outward channel deactivated exponentially, while the time constant of deactivation increased as a function of V_{tail} (panel C).

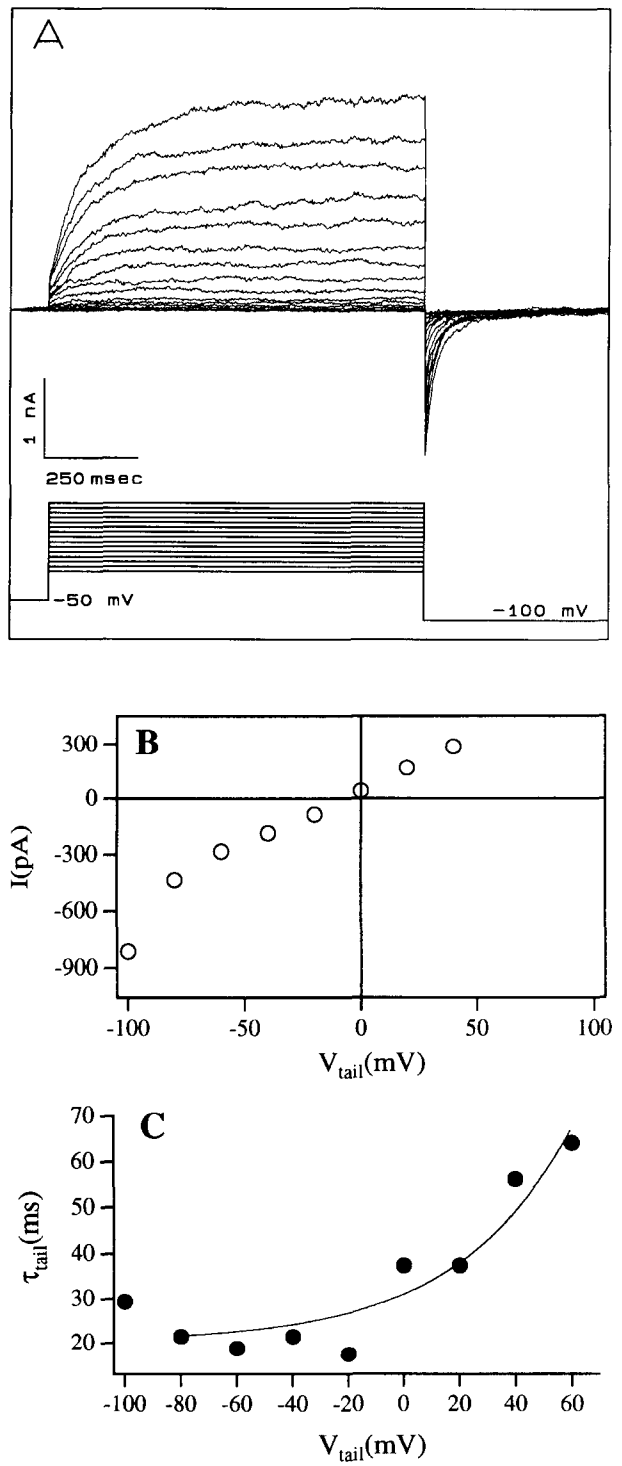

Fig. 1. (A) Typical outward currents elicited by depolarising voltage pulses ranging from 0 to +150 mV (10-mV steps) from a holding potential of −50 mV; tail potentials to −100 mV. The lower inset represents the potential protocol. (B) The steady-state outward current was plotted as a function of the step potential. (C) Time constant of activation plotted as a function of the step voltage. Standard ionic solutions.

In standard ionic solutions (i.e. at neutral pH), only 15% of the protoplasts displayed inward currents at hyperpolarising potentials, usually more negative than −150 mV; however, we also verified (Fig. 3A,B) that acidic pH decreased the activation threshold and the time-dependent inward currents were already elicited at potentials more negative than −100 mV. Double-pulse tail currents, measured in the same solutions used in Fig. 3, showed that the reversal potential (+23 mV) was coincident with the calculated Nernst potential for potassium (+26 mV), thus, suggesting that the outward current as well as the inward time-dependent current are carried by K^+.

In about 20% of the protoplasts which were successfully patched, immediately after the break-in both hyperpolarising and depolarising potentials elicited large currents (Fig. 4) that differ from the other macroscopic currents described above. These currents activated instantaneously upon application of the transmembrane potential, displayed linear I–V characteristics and partial inactivation at hyperpolarising potentials but did not show any ion-selectivity. When present, this fast current completely obscured the time-dependent components which could be revealed after the addition to the bath of 1 mM $LaCl_3$ which, partially and reversibly, inhibited the large instantaneous current [1].

To verify if these currents are actually due to the openings of ionic channels, single-channel recordings were obtained by excision of membrane patches or in the on-cell configuration. Typical traces recorded in these two configurations, showing single-channel transitions, together with the corresponding current–voltage characteristics, are reported in Fig. 5 and 6. Two different types of channel openings can be observed in Fig. 5 (on cell configuration); one type presented a single-channel conductance of 10 ± 1 pS and long lasting transitions elicited by both negative and positive transmembrane potentials; the second channel type (conductance = 17 ± 2 pS) was characterised by bursts of events which occurred instantaneously upon application of step potentials hyperpolarising the cell (positive in the pipette) and inactivated within a few hundred milliseconds. Four current records obtained at $V = -170$ mV in excised outside-out cell-free patches are reported in Fig. 6A to demonstrate that single-channel openings appear instantaneously upon application of the step potential. The kinetic properties and the conductance (i.e. a mean open time of several hundred milliseconds and a single-channel conductance of 10 pS) of this channel were very similar to those of the smaller channel shown in Fig. 5. The current–voltage characteristics in Fig. 6B show data points only at $V < 0$ mV but similar transitions of comparable amplitudes were also observed at depolarising potentials. These signals were not analysed in detail because several openings overlapped. Nevertheless, the occurrence of single-channel events and the mean current traces obtained by averaging several single-channel records (inset of panels B in Figs. 5 and 6) strongly support the hypothesis that these two channel types

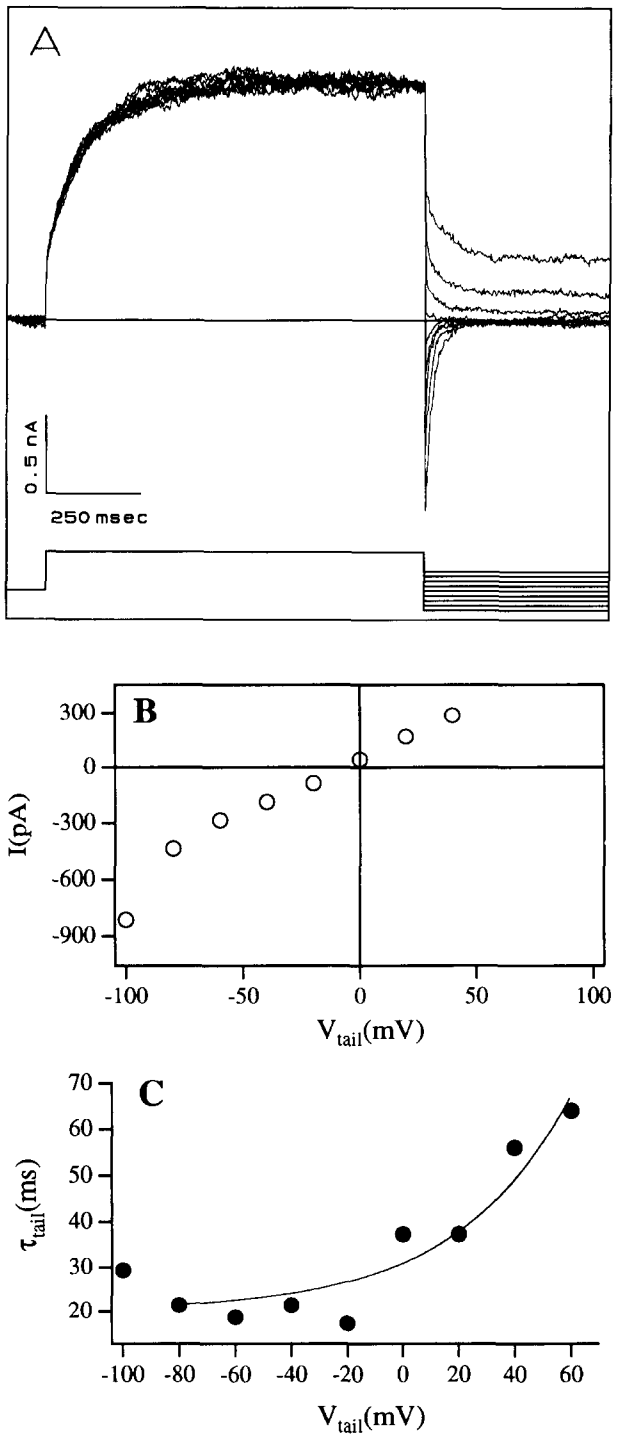

Fig. 2. (A) Tail currents elicited by a step voltage to + 100 mV from a holding potential of − 50 mV. Tail potentials from − 100 mV to + 60 mV in 20-mV steps. The reversal potential, V_{rev} ~0 mV, suggests that the channel is potassium selective. The lower inset illustrates the potential protocol. Same solutions as in Fig. 1. (B) The peak current was plotted as a function of V_{tail}. (C) Voltage dependence of outward current time constant of deactivation. The continuous line represents an exponential best fit to the data points comprised between − 80 and + 60 mV. Standard ionic solutions.

are responsible for the fast current observed in the whole-cell configuration.

4. Discussion

Our results show that both outward and inward time- and voltage-dependent channels are present in the plasma membrane of carrot suspension cells.

The activation and the current–voltage characteristics of potassium selective outward currents exhibited properties similar to those already reported in other protoplasts obtained from either plant tissues [10,11,4] or cultured cells [3,26]. The activation and deactivation time course of the current were fitted by single-exponential functions, thus, indicating the existence of only one open and closed state. Particularly, the time course of activation (Fig. 1C) showed a moderate increase (< 20% in 100 mV range) as the potential of the stimulus increased, while the time constant of the deactivating tail currents (Fig. 2C) did not change appreciably at negative potentials but increased with depolarising tail potentials. The increment of the activation time constant at higher depolarising potentials seems to be a characteristic peculiar to our cells; in fact, both in *V. faba* and *Z. mays* guard cell protoplasts [14] as well as in protoplasts from cell suspension cultures (e.g. tobacco cells [3] and corn shoots [27]), the activation time course of the outward currents

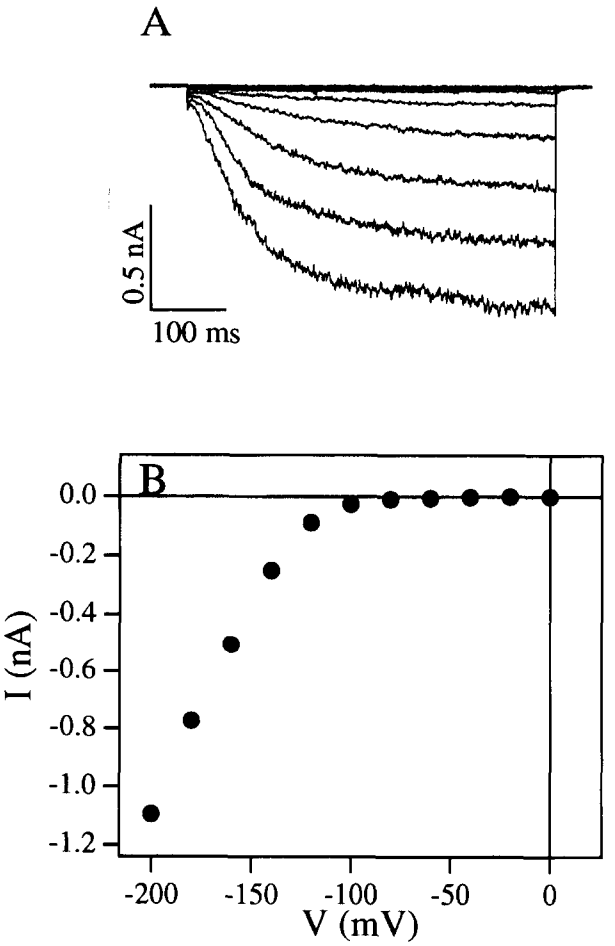

Fig. 3. (A) Inward currents elicited by hyperpolarising voltage pulses from 0 mV to − 200 mV (20-mV steps). Holding and tail potentials were 0 mV and 0 mV, respectively. Bath solution (mM): KCl 10, KGluconate 90, $CaCl_2$ 0.1, $MgCl_2$ 2, MES 10, Sorbitol 384, pH 5.5. Pipette solution (mM): KCl 10, $MgCl_2$ 2, Hepes 10, EGTA 10, Sorbitol 554, KOH 22 to pH 7. (B) Current–voltage characteristics of the traces shown in A).

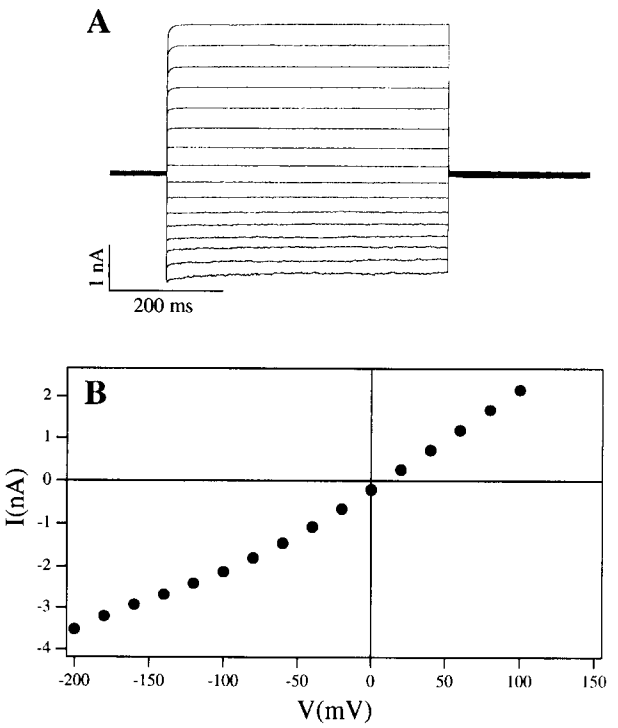

Fig. 4. (A) Large fast-activating currents, often present immediately after the break into the cell, are shown together with the corresponding current–voltage characteristics (panel B). No P/4 procedure to correct for leakage and capacitive currents was used. Bath solution (mM): KCl 200, $CaCl_2$ 1, $MgCl_2$ 5, MES 10, Sorbitol 276, pH 6. Pipette solution as in Fig. 3.

slightly decreases as the potential of the stimulus increases. Also protoplasts isolated from *Arabidopsis thaliana* suspension cells presented a voltage-dependent reduction of the activation time constant as the depolarising potential increased (our qualitative evaluation from reference [28]).

On the other hand, the increase of the deactivation time constant of our suspension cells is in agreement with the increase observed in tobacco cultured cells [3] and in guard cells [29], but opposite to that measured again in guard cells (*Vicia* and *Zea*) by other authors [14]. Different working conditions (e.g. different solutions and temperature) may also contribute to change the kinetics of the system [28].

At neutral pH (standard ionic solutions), the inward current was only present in a small percentage of the cells tested. Moreover, it usually ran down with time, thus, indicating that cytoplasmic factors are possibly needed to maintain this current. Furthermore, usually the current–voltage relationships of inward currents in carrot protoplasts showed an atypically large threshold for activation (at hyperpolarising potentials of around −150 mV) when compared with the corresponding threshold of similar currents in other protoplasts. This difference may depend on properties which are intrinsic to our cell line as well as on the composition of the ionic solutions. Indeed, we verified that at acidic pH values (i.e. 5.5) in the bath, the threshold of activation of the time-dependent potassium inward currents decreased substantially; moreover, in this case, outward currents were usually absent.

In accordance with observations performed in other protoplasts, also in carrot cells the inward current was also blocked by the addition in the external bath solution of 1 mM Cs^+ (not shown), a typical blocker of potassium channels in animal and plant cells [30–32].

In standard ionic solutions, the protoplasts analysed displayed the following current types: about 40% only time-dependent outward currents, 15% only time-dependent inward currents, 25% both outward and inward time-dependent currents and finally about 20% non-time-dependent currents. We know that two kinds of cell types are present in our cell culture: (1) proliferating cells and (2) PEM (pro-embryogenic-masses) which represent the first stage of embryo development. Our results could reflect this type of heterogeneity. We plan to purify the two cell types to identify different behaviours with respect to the ionic currents.

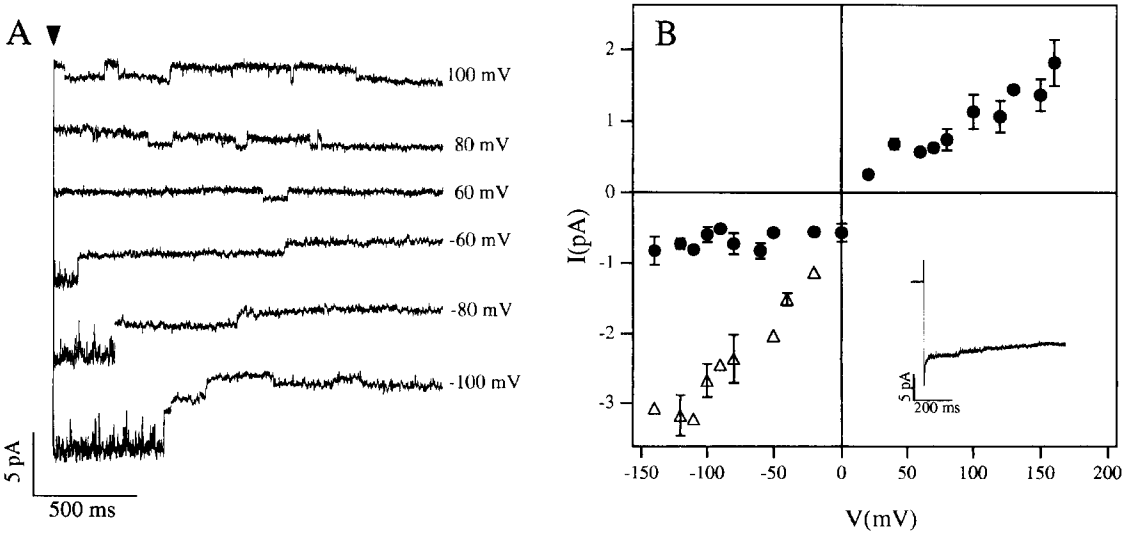

Fig. 5. (A) Typical single-channel recordings in on-cell patches. Pipette and bath solutions were (mM): KCl 100, $CaCl_2$10, MES 10, sorbitol 460, pH 6. The applied potential refers to the bath. The arrow pointing to the vertical line indicates where the transmembrane potential was applied. For the sake of clarity, a few data points were omitted to remove the capacitance transient. (B) Current–voltage characteristics of the two channel types shown in panel A; the inset shows the mean current obtained averaging 15 traces at $V = -100$ mV.

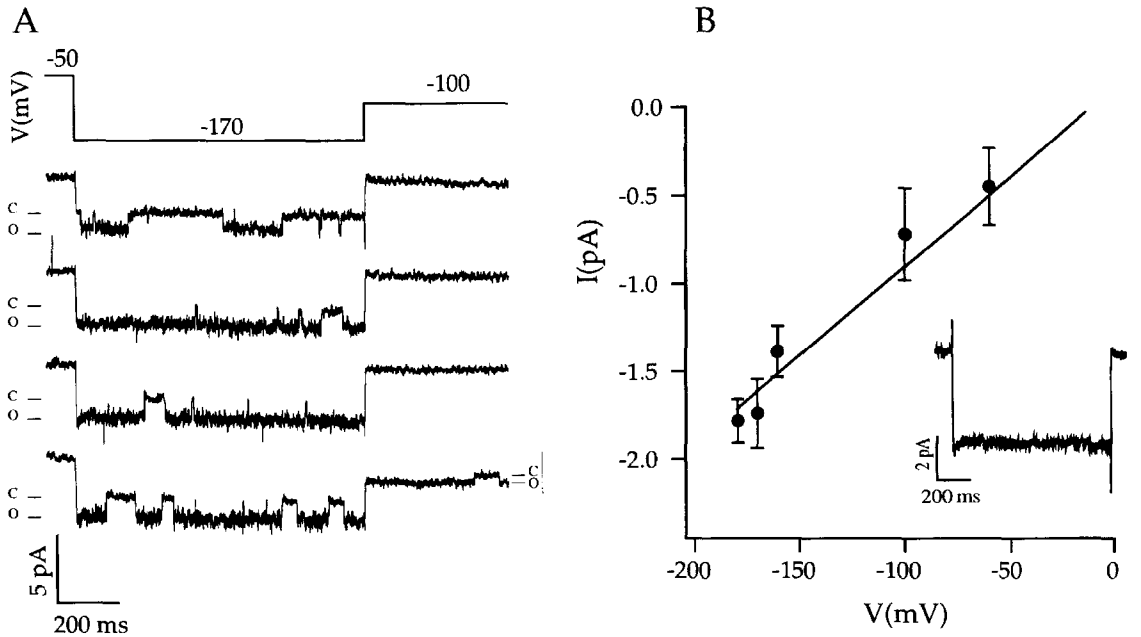

Fig. 6. (A) Single-channel recordings obtained from an excised outside- out patch. Voltage steps to −170 mV elicited step-like signals which can occasionally be observed on the tail potential at −100 mV and also at less negative potentials. Holding potential −50 mV. The upper inset shows the potential protocol. O and C indicate the open and closed state, respectively. Note (right side of the fourth trace) close–open transitions at $V = -100$ mV. (B) Current–voltage relationship of the channel shown in panel A. Ionic solutions as in Fig. 1, with the exception of the internal concentration of $MgCl_2$ = 2 mM. The inset shows the mean current obtained averaging 30 traces at $V = -170$ mV.

The large fast-activating non-selective currents which often appeared immediately after the break-in, were reminiscent of similar currents also observed in other plant protoplasts and alternatively ascribed to anion channels activated by cytosolic calcium [11], to slow anion selective channels [33] in guard cell protoplasts, to calcium selective channels in maize suspension cells [1,4] and to putrescine/chloride currents in *A. thaliana* [34]. Fast-activating single-channel events, which presumably correspond to this current, were identified in carrot cells both in the cell-attached and excised-patch configuration (see Figs. 5 and 6). We suggest that the two channel types correspond to the large instantaneous current elicited by both negative and positive potentials and to the inactivating component present at hyperpolarising potentials in Fig. 4, respectively. There may be different explanations for the fact that the fast current was partially and reversibly (also several times) inhibited by the addition to the bath of 1 mM lanthanum: (I) lanthanum might block the fast channel, acting similarly to other metal ions or polications which have been shown to inhibit other channel types [1,21]; (II) as an alternative, lanthanum could regulate the gating properties of the fast channel [35,36] acting either at the extraplasmatic side or (if it diffuses into the cell) at the cytoplasmic side of the membrane. It is possible that lanthanum simulates the action of cytoplasmic polications or other native factors regulating the kinetics of the channel, i.e. small organic units or accessory proteins associated to the channel.

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