Ion channels of intact young root hairs from *Medicago sativa*

François Bouteau*<sup>a</sup>, Anne-Marie Pennarun*<sup>a</sup>, Armen Kurkdjian<sup>b</sup>, Monique Convert<sup>a</sup>, Daniel Cornel<sup>a</sup>, Michèle Monestiez<sup>a</sup>, Jean-Pierre Rona<sup>a</sup>, Ulrike Bousquet<sup>a</sup>

<sup>a</sup> Electrophysiologie des membranes (LPCMSP), université Paris-7, CASE 7069, 2, place Jussieu, 75251 Paris cedex 05, France

<sup>b</sup> Institut des sciences végétales, Centre national de la recherche scientifique, Bât. 22, avenue de la Terrasse, 91198 Gif-Sur-Yvette cedex, France

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Abstract — Root hairs are a primary site for nutrient absorption and for initiation of signalling processes linked to variations of the root environment: plant-microbe interactions or abiotic changes. In many of these cases, the earliest detectable response is the modification of plasma membrane transports, detected through alteration of the electrical membrane potential. In spite of this, root hairs have not been extensively used in electrophysiological research so far. Problems with cell shape and current coupling are often prohibitive for microelectrode voltage-clamp on intact root hairs. In the present study, these difficulties have been overcome and the ion channel currents are described for young root hairs from alfalfa seedlings (*Medicago sativa* cv Sitel).

Electrophysiological and pharmacological studies indicated an inward rectifying K<sup>+</sup> time-dependent current. This current was sensitive to tetraethylammonium and Cs<sup>+</sup> (10 mM each). Two other currents never shown in root hairs were described: an outward rectifying time-dependent K<sup>+</sup> current, inhibited by tetraethylammonium and Cs<sup>+</sup> (10 mM each) allowing K<sup>+</sup> efflux under strong depolarizations and an instantaneous inward current identified as an anion current, inhibited by 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid and anthracene-9-carboxylic acid (100 μM each). These results should contribute to the understanding of root hair development and of signalling processes in *M. sativa* root hairs. © 1999 Éditions scientifiques et médicales Elsevier SAS

Ion channels / root hairs / alfalfa / single-electrode voltage-clamp

A9C, anthracene-9-carboxylic acid / dSEVC, discontinuous single-electrode voltage-clamp technique / E<sub>cmt</sub>, activation potential / E<sub>m</sub>, resting membrane potential / E<sub>r</sub>, reversal membrane potential / IAC, instantaneous inward anion current / IRKC, inward rectifying K<sup>+</sup> current / I-V, current-voltage relationships / NA, niflumic acid / IAA-94, R(+)-methylindazole; indanyloxyacetic acid 94 / NPPB, 5-nitro-2-(3-phenylpropyl amino)-benzoic acid / ORKC, outward rectifying K<sup>+</sup> current / SBS, standard buffer solution / SITS, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid / TEA, tetraethylammonium chloride / TPA, tetrathylammonium chloride / V<sub>h</sub>, holding membrane potential

1. INTRODUCTION

Root hairs are differentiated epidermal cells which, by increasing the absorbing surface of the root, play a major role in the uptake of nutrients and water [32]. Root hairs also behave like sensors able to perceive signals from their environment [32]. Changes in plasma membrane transports represent an early response of plant cells to various signals, such as hormones [8, 17], elicitors [31, 41], toxins of bacterial origin ([25] and references therein), wounding signals such as systemin [29, 30], light and other environmental signals [22]. The response to these signals is mediated by ion fluxes (mainly H<sup>+</sup>, Cl<sup>-</sup>, Ca<sup>2+</sup> and K<sup>+</sup>), which are activated or inhibited (see [47] for reviews). Several reports have described anion currents [34, 48] and cation currents [9, 23, 48] in various root cells but only a few reports describe ion channel currents specific to root hairs. Gassmann and Schroeder [13] reported the presence of a K<sup>+</sup> inward rectifying current activated at hyperpolarized membrane voltages in wheat root hair protoplasts, using the patch-clamp technique. Lew [19] also recorded inward K<sup>+</sup> currents (elicited within 50 ms) in *Arabidopsis thaliana* intact root hairs using the double-barrelled microelectrode voltage-clamp technique.
Our aim was to identify the major ion channel currents in *Medicago sativa* root hair plasma membrane in situ. Microelectrode voltage-clamp technique is now widely used for guard cells [2, 12, 33] but it raises the problem of space clamping and current coupling when applied to root hairs [20, 28]. In spite of this, we succeeded in describing several currents which were assumed to exist but, to our knowledge, had never been shown in root hairs. These results should contribute to the understanding of root hair development and of signalling processes such as those implicated in plant-microbe interactions or during environmental changes.

2. RESULTS

The value of the resting electrical membrane potential ($E_m$) of root hairs, using SBS, was $-136 \pm 17$ mV ($n = 125$). In SBS (0.1 mM K$^+$), the cell wall potential (potential measured just before impalement when the microelectrode enters in contact with the root hair wall) is around $-40$ mV (not shown); thus, the calculated wall K$^+$ concentration was estimated to be 0.4 mM due to the presence of the cell wall and the Donnan effect [14]. Assuming 70 mM K$^+$ in the cytosol [7] and 0.4 mM K$^+$ in the apoplast, the estimated $E_K$ was about $-130$ mV, thus near $E_m$.

2.1. Time-dependent outward rectifying current (ORKC)

The time-dependent activation of outward currents was only observed in about 4% of recordings. In these cases, a current activates upon depolarization of the membrane (figure 1 A, C). The kinetics of the current activation was sigmoidal and showed a clear voltage dependency (figure 1 B). The activation potential ($-96 \pm 18$ mV, $n = 13$, $P < 0.05$) is significantly more positive than the $E_m$. This current is inhibited in 2 to 3 min by 10 mM TEA or Cs$^+$ (figure 1 D, E), known to inhibit rectifying K$^+$ channels. These results are in favour of the K$^+$ nature of this time-dependent outward rectifying current, thus called ORKC for outward rectifying K$^+$ current.

2.2. Time-dependent inward rectifying current (IRKC)

When root hairs were bathed in SBS, a typical inward rectification, showing a net time dependence upon hyperpolarization, could be observed when the membrane potential was previously clamped to 100 mV (figure 2 A). The time-dependent activation of the inward current was not generally observed with hyperpolarizing pulses applied from $V_h = E_m$ (figure 2 B). However, the deactivation of the current at positive voltages to $-80$ mV indicates the closure of the channels. Thus, a part of the channels should be opened at $E_m$ and explain the instantaneous current upon hyperpolarization from $V_h = E_m$ (figure 2 B). When activated, this current does not inactivate within 20 s (data not shown). The steady-state I-V relationship shows a threshold value close to $E_m$ and $E_K$ for this inward rectifying time-dependent current ($-129 \pm 18$ mV, $n = 20$). The analysis of the deactivation of the current at 100 mV (figure 2 D) showed the increase of the current with the highest amplitude of the hyperpolarization confirming the voltage gating of the channels. At voltages positive to $E_K$, the intensity is low, but the
current is still measurable, indicating as seen on the I-V curve that some channels could remain open and that they could function in the outward direction depending on $E_m$ value and on the electrochemical gradient.

Upon increasing the external K$^+$ concentration from 0.1 to 10 mM, the amplitude of the inward rectifying current increased and the time-dependence appeared even at $V_h = E_m$ (figure 2 E). The values of $E_{rev}$ follow those of $E_K$ (figure 2 F); the major ion species crossing the channels should be K$^+$. TPA (200 μM), a well known K$^+$ channel inhibitor, was tested in the presence of 10 mM K$^+$ in the bathing medium (figure 2 E). TPA reduced the time-dependent
inward rectifying current elicited at –200 mV as does cesium (10 mM) (figure 2 G) or TEA (10 mM) (see figure 3). All these results suggest the K⁺ nature of the time-dependent inward current, therefore named IRKC for inward rectifying K⁺ current.

In fact, only 6 % of the cells displayed only the IRKC. The other recordings showed, associated with the IRKC, an instantaneous inward current even when \( V_h \) was positive to the IRKC activation potential (figures 3 A, 4 A). The amplitude of this instantaneous inward current represents 82 ± 13 % (\( n = 40 \)) of the total current in SBS (ranging from 100 to 57 %).

Figure 3 A shows an example of recordings displaying a large inward current showing the two components in presence of 10 mM KCl. A net time-dependent current was recorded, corresponding to about 45 % of the total current. Using the TEA (10 mM), we could discriminate a large instantaneous current (figure 3 B) from the time-dependent current (figure 3 C) in the total inward current. The TEA exclusively reduced the time-dependent component.
(figure 3 B). The analysis of the I-V curves (figure 3 D) indicates a positive voltage threshold for the total current, and a voltage threshold around −100 mV for the current corresponding to the difference between the steady-state current and the instantaneous current. This difference corresponds to the time-dependent current and its reduction by TEA is responsible for the reduction of the total current (figure 3 D). The TEA-induced inhibition, the voltage threshold near −100 mV and the time dependence of the current suggest that this component corresponds to the IRKC.

### 2.3. Instantaneous inward current

An important feature of this inward current was its instantaneous kinetics. Furthermore, this current showed a 'rectifying-like' feature with positive threshold voltages (figures 3 D, 4 D). These threshold voltage values were clearly different from the IRKC activation potential and far from the estimated $E_K$ and $E_{Ca}$ (>> 100 mV), suggesting an anionic nature for this current. However, in our conditions, we cannot exclude that a part of this current could be carried by Ca$^{2+}$ with an activation threshold more negative than $E_{Ca}$.

The use of 10 mM TEA-Cl (figure 3) allows the reduction of the total current, in inhibiting the IRKC component as indicated before, but it also increases the external chloride concentration leading to a negative shift in the threshold voltage value of the total inward current (inset in figure 3 D) in the way expected for $E_{Cl^{-}}$ and thus for an anion current. The activation potential of this current was highly variable in SBS. However, the negative shift in the activation potential value of the total inward current upon external chloride addition (e.g. figure 3 E) was systematically observed when TEA-Cl or Cs-Cl were used. Attempts to further increase the external chloride concentration were unsuccessful because of osmotic effects leading to impalements which were not stable.

Thus, to further characterize these instantaneous currents, we used anion channel blockers. The addition of STS (100 μM) decreased drastically the whole current in 2 to 3 min (figure 4 A, B). This decrease was mainly due to the irreversible inhibition of the instantaneous inward current (figure 4B). Figure 4 C shows the instantaneous part of the current which disappeared. Interestingly, an inward time-dependent current remained, not affected by STS, which was probably the IRKC (figure 4 B). The I-V curve analysis (figure 4 D) for the total current and for the difference between steady-state and instantaneous currents, allows to discriminate between the IRKC component (threshold voltage around −100 mV) and the instantaneous component imposing its positive threshold voltage upon the total current. This analysis confirmed that the reduction of the total current was mainly due to the reduction of the instantaneous component (figure 4 D).

In the same way, we tested another anion channel blocker A9C (100 μM). It also reduced the whole current within a few minutes reaching about 60 % of inhibition at −200 mV (figure 4 E), but the total current reduction was mainly linked to the instantaneous current inhibition and not to the low amplitude IRKC component reduction.

The other anion channel inhibitors tested, Zn$^{2+}$ (200 and 400 μM), NPPB (20 μM), niflumic acid (250 μM) and IAA-94 (100 μM), were inefficient in inhibiting the instantaneous inward current (not shown). Taking all these results together, we favour the hypothesis of an anionic nature for the instantaneous inward current, thus called IIAC for instantaneous inward anion current.

### 2.4. Root hair protoplast currents

We worked with root hair protoplasts (symplastically isolated) with the aim of discriminating the putative part of coupling currents in the recordings obtained with intact root hairs. The time-dependent kinetics and negative activation thresholds suggested a K$^+$ nature for the outward and inward currents seen in figure 5 A and B, respectively. The instantaneous activation and the positive activation threshold recorded for the inward current in figure 5 C suggested an anionic nature for this current. Thus, the three main ionic currents described on intact root hairs (IRKC, ORKC and IIAC) were also recorded on protoplasts indicating that they could not be explained by coupling currents.

### 3. DISCUSSION

Root hairs are a primary site for the initiation of plant-microbe interactions and for nutrient absorption in plants [11, 32]. The aim of this paper is to describe the main ionic channel currents present in *M. sativa* root hairs as near as possible to physiological conditions. Therefore, we used dSEVC which allows to work with intact root hairs from seedlings (cf. Methods). The fact that they keep their cellular characteristics could be of primary importance for the regulation of membrane transport [26, 44].

The shape of root hairs raised the problem of space clamping [28] but the choice of young short root hairs avoided any behaviour as electric cables. Another
question addressed, when working with cells integrated in an intact tissue, concerns the extent of current leakage through the plasmodesmata connections with neighbouring epidermal cells and underlying cortical cells [20]. A cytological argument was developed, using the injection of fluorescent dyes, to demonstrate that the symplastic connections between root epidermal cells of *A. thaliana* [101] are progressively ruptured during the process of differentiation, probably due to a decrease in the number of plasmodesmata or to their plugging [5]. Nevertheless, in *A. thaliana* root hairs, the coupling ratio was shown to be highly variable, ranging from 0 to about 90%, and decreased with differentiation [20]. The presence of coupling current could lead to the overestimation of the current. However, the coupling current through plasmodesmata was shown to be ohmic in root hairs [20]. Similarly, in animal cells, coupling currents through gap junctions were demonstrated to be ohmic so that they were subtracted together with leak during analysis [40]. Our recordings, before any leak was subtracted, showed ohmic conductances between 200 MΩ and 1 GΩ. These values appeared higher than those observed in *A. thaliana* [20]. Thus, for *M. sativa* root hairs, the putative coupling currents were thought to be weak or nil. Furthermore, to examine the implication of putative coupling currents on the electrical signature of root hairs, we used isolated root hair protoplasts. The different current kinetics observed for intact root hairs could also be recorded in protoplasts indicating that these currents could not be attributed to coupling currents.

The profiles of current recordings in *M. sativa* root hairs were variable in shape and intensity even for root hairs belonging to the same seedling. This heterogeneity is due to the simultaneous activities of several ionic channels. Such a variability of currents was already emphasized for plant cells by different authors working with the patch-clamp technique ([23] and references therein).

Time-dependent outward rectifying currents were identified as K⁺ currents (ORKC), from their voltage dependent sigmoidal activation [36] and their inhibition by TEA and Cs. This current was not frequently observed in our conditions where the external pH was low. This could reflect a sensitivity to pH as was shown for other plant outward rectifying K⁺ channel currents which are decreased with the acidification of external pH [33, 43]. The activation potential of ORKC was always much more positive than *Eₘ*. Thus, this channel should not be able to play a role in the repolarization, after a slight depolarization, as was proposed for root cells by Maathuis and Sanders [24]. In the case of *M. sativa* root hairs, this regulation could be attributed to the slight outward functioning of the IRKC (see below). The ORKC could play a role in repolarization, after a strong depolarization. This is the first description of an outward rectifying K⁺ current in root hairs despite the frequent description of K⁺ efflux in the root hair zone in response to elicitor [41] or Nod factor [7] which induce membrane depolarizations.

Few results have been obtained to date on an inward rectifying K⁺ current from root hairs. Lew [19] described an inward potassium current in *A. thaliana* growing root hairs. This result was confirmed by Gassmann and Schroeder [13] in wheat root hairs. In *M. sativa*, we identified a time-dependent inward rectifying current as a K⁺ current (IRKC), owing to its sensitivity to the external K⁺, to the classical K⁺ channel inhibitors (TPA, TEA and Cs⁺) and to the activation potential values (close to *Eₘ*) at which inward functioning is observed. These characteristics

![Figure 5. Different current types exhibited by protoplasts released from root hair tips; A, time-dependent outward rectifying current and current-voltage relationship drawn from the steady-state currents; B, time-dependent inward rectifying current and current-voltage relationship drawn from the steady-state currents; C, instantaneous inward current and current-voltage relationship drawn from currents at 10 ms.](image)
are typical of the presence of inward rectifying K⁺ channels [35]. Thus, the IRKC of *M. sativa* root hairs could correspond to the low affinity system described in several materials [23, 38], and particularly in wheat root hairs [13]. Moreover, the presence of open channels at Eₜ₉ in *M. sativa* is in agreement with the growing status of young root hairs. The IRKC from *M. sativa* root hairs seems also able to exhibit a slight outward directed activity that disappears towards positive potential values. Such a behaviour was observed in guard cells [3, 38] and for K⁺ channel expressed in *Xenopus* oocytes [46], but not in wheat root hairs [13] nor, more generally, in root cells [24]. In *M. sativa* root hairs in situ, the IRKC, showing an outward functioning, could play a dual role, i.e. one of K⁺ uptake and one of a sharp membrane potential regulation for low amplitude variations around Eₜ₉.

In addition to the time-dependent K⁺ currents, we observed an instantaneous inward current (IIAC) which was assumed to be anionic, owing to its activation threshold far from Eₖ and Eₐ₉, its sensitivity to external chloride concentrations and to the anion channel blockers SITS and A₉C. The permeation of various anions, nitrate, chloride or malate was reported for plant anion channels [15, 37] but, contrary to the various anions, nitrate, chloride or malate was reported for plant anion channels [15, 37] but, contrary to the activation in response to an hypo-osmotic shock induced when the plantlets were placed in the bathing medium from the solid medium presenting an unknown matrix potential. Further study would be necessary to better characterize this anion channel corresponding to the current described here. The use of patch-clamp technique on root hair protoplasts should be adequate for these investigations; unfortunately, until now our attempt to seal root hair protoplasts have been unsuccessful (Kurkdjian, unpubl. results).

In conclusion, these results show that dSEVC provides a fine system for electrophysiological studies of root hairs even if they highlight the limits and difficulties of microelectrode voltage-clamp and pharmacological approaches in physiological conditions, i.e. when several currents coexist in a cell connected to the plant. The analysis of the ion currents across the plasma membrane of young intact root hairs from *M. sativa* seedlings has allowed us to discriminate potassium currents, as well as an anion current. Thus, we confirmed the presence of the inward K⁺ channel currents and described outward K⁺ channel currents and inward anion channel currents (anion efflux) which were assumed to exist in view of recent ion selective microelectrodes measurements [7], but had never been demonstrated. These results should contribute to the understanding of root hair development and of signalling processes in *M. sativa*.

4. METHODS

4.1. Plant material

Alfalfa seeds (*Medicago sativa* cv. Sitel) were sterilized as previously described [39]. The seeds were deposited on solid media (Bacto agar 10 g L⁻¹ from Difco, Detroit, MI) containing the standard bathing solution (SBS) used for measurement: 5 mM Mes, 1 mM CaSO₄ and 0.1 mM KCl buffered at pH 6.0 (5 mM Tris). The growth conditions for plantlets and root hairs were previously described [18]. Whole plantlets (about 30 h old after seed deposition) were mounted on slides and constantly perfused with SBS. The experiments were carried out at room temperature. Young growing root hairs, 5 to 20 μm long (zone 1 according to Heidstra et al. [16]) were chosen because they were reported to be the more sensitive to Nod factor in terms of electrical membrane

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response [18]. Root hair protoplasts were prepared using an enzyme cocktail. Plantlets were exposed to 1% Cellulase RS and 0.1% Pectolyase Y23 diluted in a medium containing 1 mM CaSO₄, 300 mM sorbitol and 25 mM Mes adjusted to pH 5.5 with KOH (the final K⁺ concentration was about 4 mM). The cell wall apices are first digested at the tip of root hairs thus allowing the protoplast release [4]. Protoplast release was followed under the microscope and achieved within a few minutes (figure 6B). The protoplasts were then washed with the enzyme free medium. At this stage, most of them detached from the root hair ghosts.

4.2. Electrophysiology

Voltage-clamp measurements were carried out using a discontinuous single voltage-clamp microelectrode technique [10] to record the whole-cell currents from intact root hairs and isolated root hair protoplasts. These protoplasts were immobilized by means of a microfunnel controlled by a pneumatic micromanipulator (de Fonbrune, Ets. Beaudoin, Paris, France). In this technique, both current passing and voltage recording use the same microelectrode. Interactions between the two tasks are prevented by multiplexing (sampling frequency 1.5 to 3 kHz), which ensure that the voltage recorded by the microelectrode tip is sampled and saved only after the current-induced voltage drop across the microelectrode has decayed to a negligible value. A specific software (pCLAMP5.5, Axon Inst., Foster City, CA, USA) drives the electrometer. Microelectrodes were made from borosilicate capillary glass (Clark GC 150F, Clark Electromedical, Pangbourne Reading, UK) pulled on a vertical puller. Tip diameters were about 0.5 μm and electrical resistances were 50–100 MΩ in the buffer solution. They were filled with 600 mM KCl. The electrode was connected to an electrometer (Axoclamp 2A, Axon Instruments, Foster City, CA, USA; input impedance 10¹² Ω), which allowed us to compensate for the resistance and the capacitance of the microelectrode. These two parameters were systematically checked before and after microelectrode impalement. In dSEVC, using an electrometer from Axon Instruments, the technique used for measurements allows one to check electrical parameters of the microelectrode even upon impalement. When the variation of these parameters is too large, i.e. the time constant of the microelectrode increases considerably indicating that the microelectrode is plugged [10], the measurements were rejected. Voltage and current were displayed on a dual input oscilloscope (Gould 1425, Gould Instruments Ltd, Hainault, UK), digitalized with a PC computer that was fitted with an acquisition board (Labmaster TL 1, Scientific Solution Inc., Solon, OH, USA). Due to technical limitations linked to the time sharing protocol for clamping, the first point where current activation can be measured is about 10 ms, following the application of a voltage step. The opening or the closure of the channels was obtained as indicated in the figures with protocols of 20-mV steps, during 500 or 1000 ms, with a resting phase of 500 or 1000 ms at the holding potential. The reversal membrane potential (E_{rev}) for K⁺ was determined from a tail current analysis. For this, the membrane was clamped to 100 mV (V_h) and then the voltage was directly stepped to −200 mV to activate the inward current; this was followed by steps ranging from −180 to −40 mV, in 10-mV increments, to deactivate the channels. The reversal potential (E_{rev}) was determined at the point of intersection of the two curves obtained.

Figure 6. Young root hairs and protoplasts of M. sativa. The seeds were germinated according to the protocol described in Methods. The plantlets were left for 6 h in a vertical position for root hairs to grow. A, Root hairs; B, protoplasts released from young root hair tips, after 3 min incubation with enzyme cocktail. Bar scales = 10 μm.

from the initial and steady-state currents measured and plotted against the voltage steps [2]. We systematically checked to ensure that root hairs were correctly clamped by comparing the protocol voltage values with the ones really imposed. Only a small percentage of root hairs failed to display a linear relationship between theoretical and measured potentials. These root hairs were then abandoned.

4.3. Effectors and chemicals

Fresh solutions of ion channel inhibitors were prepared from stock solutions just before use. The chemicals were all analytical grade (Sigma). TPA, TEA, CsCl, and ZnCl₂ were prepared as 100 mM stock solutions in double distilled H₂O; SITS were dissolved as 20 mM stock solutions in double distilled H₂O, adjusted to pH 7.0 with 1 N NaOH. A9C, NA, IAA-94 and NPPB were prepared as 20 mM stock solutions in ethanol.

4.4. Presentation of data

Kinetics are given as single measurements, representative of at least three equivalent tests carried out under the same conditions. For current kinetics and I-V curves, the leak resistance was subtracted when necessary. For IRKC analysis, the instantaneous current was subtracted from the steady-state current to ensure that only the time-dependent current was considered. Whenever possible, data are given as means ± SD.

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