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Calcium channel antagonists induce direct inhibition of the outward rectifying potassium channel in tobacco protoplasts

Sébastien Thomine[a,*], Sabine Zimmermann[a], Bert Van Duijn[b], Hélène Barbier-Brygoo[a], Jean Guern[a]

[a]*Institut des Sciences Végétales, CNRS, F-91198 Gif-sur-Yvette Cedex, France*
[b]*Institute of Molecular Plant Sciences, Clusius Laboratory, 2333 AL Leiden and Department of Physiology, Leiden University, 2300 RC Leiden, The Netherlands*

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Abstract

Verapamil, nifedipine and bepridil, three antagonists of L-type calcium channels in animal cells, were shown to induce direct inhibition of outward rectifying potassium current in *Nicotiana tabacum* cv. Xanthi protoplasts with IC_{50} of 5 μM, 5 μM and 1 μM, respectively. In the outside-out configuration, verapamil reduced the open probability of the ion channel responsible for the outward rectifying potassium conductance. Verapamil also blocked the outward rectifying potassium conductance in protoplasts from the *N. tabacum* cv. Bright Yellow cell line. Thus, studies using these molecules to demonstrate the involvement of calcium channels in plant physiological responses should be regarded with caution.

Key words: Patch clamp; Potassium outward rectifier; Verapamil; Nifedipine; Bepridil; Tobacco protoplast

1. Introduction

Outward rectifying potassium channels have been described in a number of plant cell types and species [1–6]. Pharmacological characterization of these channels can provide tools to discriminate between different channel types [7], to achieve their biochemical isolation [8] and to determine their physiological roles. The role of the potassium outward channel can be easily understood for cells performing rapid volume changes, such as guard cells or pulvini cells [4,5]. However, for other cell types a regulatory function of cell volume or membrane potential has been often assumed without experimental arguments. Well characterized ion-channel effectors could be used to study the possible place of an ion channel in more integrated responses of plant cells and whole plants.

Ion channel inhibitors with a well described action on animal channels have been widely used to study plant cell physiology assuming that ion channels would have the same properties in plants and animals. In particular, verapamil, nifedipine and bepridil have been applied as specific inhibitors of calcium channels in a number of studies [9–12]. However, it is known that L-type calcium channel antagonists can also interact with animal sodium and potassium channels [13]. Furthermore, it has recently been shown that these drugs can inhibit outward rectifying potassium channels in *Amaranthus* protoplasts [14]. The data we present in this paper give evidence that inhibitors of calcium channels in animal cells such as verapamil, nifedipine and bepridil can block delayed outward potassium rectifiers in protoplasts from tobacco (*Nicotiana tabacum*), a model plant widely used in physiological, molecular and biochemical studies.

2. Materials and methods

2.1. Cell culture and protoplast isolation

Two cell strains of tobacco (*Nicotiana tabacum*) have been used: one originated from mesophyll protoplasts (cv. Xanthi) and the other originated from stem medulla parenchyma (cv. Bright Yellow). The Xanthi cells were maintained in Gamborg's B5 medium containing 1 μM 2,4-di-chlorophenoxyacetic acid (2,4-D) and 60 nM kinetin, under light at 26°C, with a passage every 7 days. Cell cultures 2–5 days after transfer were used for the isolation of protoplasts by cell-wall degrading enzymes. Cells were collected by centrifugation (5 min, 300 rpm) of 8 ml of the suspension (2 ml packed cell volume) and resuspended in 10 ml of the filtrated enzyme solution (pore size 0.22 μm) consisting of 100 mM $CaCl_2$, 5 mM $MgCl_2$, 150 mM mannitol (450 mOsm), 10 mM MES/Tris (pH 5.6) supplemented with 1% cellulase (Onozuka R 10)

*Corresponding author. Fax: (33) (1) 69 82 37 68.

Abbreviations: E_X, equilibrium potential for ion X; IC_{50}, concentration inducing 50% inhibition; pS, picosiemens; V_m, applied membrane potential; BAPTA, 1,2-bis (2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; EGTA, ethylene glycol bis (2-aminoethylether)-*N*,*N*,*N*′, *N*′-tetracetic acid; MES, 2-(*N*-morpholino) ethanesulfonic acid; Tris, Tris (hydroxymethyl) aminomethane.

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and 0.04% pectolyase (Y-23, Seishin Pharmaceutical). Incubation of the tobacco cells was continued for 75 min in the dark at 26°C. Protoplasts were collected by filtration (pore diameter 104 μm) to remove the cell aggregates and by centrifugation (5 min, 350 rpm). Subsequently protoplasts were resuspended in the bath solution (see composition below) for patch clamp experiments. The Bright Yellow cells were grown and treated as described in Van Duijn et al. [6].

2.2. Electrophysiological investigations

Patch clamp experiments were performed as described by Hamill et al. [15] on freshly isolated tobacco protoplasts. Pipettes (5–10 MΩ in standard bath solution) were pulled from Kimax-51 capillaries (Kimble glass, Inc.). Gigaohm resistance seals between sylgard-coated pipettes and protoplast membranes could be obtained with gentle suction leading to the whole cell configuration. During measurements, the protoplasts were maintained in a bath solution containing either (mM) 1 or 10 K-glutamate supplemented with 5 $CaCl_2$, 5 $MgCl_2$, 10 MES/Tris (pH 5.6). Patch pipettes were filled with (mM) 100 K-glutamate, 2 $MgCl_2$, 10 Tris/MES (pH 7.2), supplemented with 5 mM MgATP. Unless specifically indicated, the free calcium concentration was buffered to 1 μM with 5 mM EGTA as calculated with 'Ligandy' program (P. Tatham and B. Gomperts, University College, London). All solutions were adjusted to 450 mOsm with mannitol. Equilibrium potential for K^+ was calculated from ionic activities. Stock solutions (10 and 1 mM) of verapamil, nifedipine and bepridil were prepared in DMSO. The final concentration of DMSO during experiments never exceeded 0.5% (v/v). At this concentration, DMSO had no effect by itself on the potassium current ($n = 4$). For current measurements the EPC 7 patch clamp amplifier (List-Electronic) was used with a low-pass filter (1 kHz). Application of voltage programs and handling of the data were performed by TL-1 DMA Interface and patch clamp software pCLAMP 5.5.1. (Axon Instruments, Inc.). Membrane potentials were corrected for liquid junction potentials according to Neher [16]. The fitting of the activation curves and the single channel data analysis were carried out with the help of pCLAMP software.

3. Results

3.1. Identification of an outward rectifying conductance at the plasma membrane of Xanthi tobacco protoplasts

Tobacco protoplasts were studied using the patch clamp technique in the whole-cell configuration. Voltage pulses from a resting potential of −85 mV toward potentials between −145 mV and +95 mV were applied to the membrane (Fig. 1A, inset). For pulses toward potentials more hyperpolarized than −75 mV, no current could be observed (Fig. 1A,B). When the potential was stepped to values more depolarized than −75 mV, delayed outward currents activated with sigmoidal kinetics (Fig. 1A,B) reflecting an increase in membrane permeability due to the opening of voltage-dependent ion channels ($n = 22$). The activation was faster as the voltage increased, showing a voltage-dependent feature of this current (Fig. 1C, $n = 18$).

The reversal potential of the current, as determined by the tail current method, was −81 ± 8 mV ($n = 12$). This potential is close to the equilibrium potential for K^+ ($E_K = -107$ mV) in comparison with the equilibrium potentials for the other permeant ions in solution ($E_{Cl} = -20$ mV, $E_{Ca} = +99$ mV, $E_H = +102$ mV, $E_{Mg} = +12$ mV) indicating that the channels involved are selective for potassium. Furthermore, increasing the external potassium concentration from 1 mM to 10 mM shifted the reversal potential to −52 ± 6 mV ($n = 6$) which corresponded to E_K (−53 mV).

The tobacco outward potassium rectifying conductance displays pharmacological features typical for potassium current. Fig. 2 shows that barium, an inhibitor of potassium channels [1,4,5,17], applied in the bath at 5 mM reduced the current amplitude at +85 mV by 86 ± 2% ($n = 4$). Additionnaly, TEA was previously shown to have inhibitory effect on the potassium outward current in Bright Yellow protoplasts [6].

3.2. Inhibition of the outward potassium current by calcium channel blockers

Three molecules known to inhibit voltage-dependent calcium channnels in animal cells [13] verapamil, nifedipine and bepridil, were tested on the outward rectifying potassium conductance of the Xanthi tobacco protoplasts. Fig. 1B–D shows the inhibition of the potassium current upon external addition of 5 μM verapamil. The steady state inhibition was achieved after 2 to 5 min depending on the inhibitor concentration. In agreement with observations on L-type calcium channels [13], the inhibition was stronger for depolarized membrane potentials (Fig. 1B). At 75 mV, 5 μM verapamil blocked 54 ± 7% ($n = 4$) of the potassium outward current. The activation kinetics of the current were unchanged after inhibition, as reflected by the measurement of activation time constants before and after inhibition (Fig. 1C). Similar results were obtained when verapamil was tested on the outward potassium current of protoplasts from the Bright Yellow cells [6]. In these protoplasts, 10 μM verapamil induced a 57 ± 16% decrease in the potassium outward currents at +100 mV ($n = 4$).

Nifedipine and bepridil, two other molecules known for their inhibitory effect on L-type voltage-dependent calcium channels from animal cells and structurally unrelated to verapamil [13], were able to inhibit the outward rectifying potassium conductance of Xanthi protoplasts in a similar way as verapamil. In all three cases, the inhibition occurred within some minutes and left the activation kinetics unchanged. All three were active in the micromolar range but the IC_{50} was significantly lower for bepridil (1 μM, Fig. 3C) than for verapamil and nifedipine (5 μM, Fig. 3 A,B).

The reversibility of the inhibition was tested by perfusing the protoplasts with inhibitor-free medium. Under these conditions 60% to 100% of the potassium current could be recovered within some minutes after bepridil ($n = 4$) or nifedipine inhibition ($n = 3$), but the effect of verapamil could not be reversed.

Considering these results, the question can be raised whether the inhibitors tested act directly at the potassium channels, as shown by Terry et al. [14]. If the potassium channels are activated by cytosolic calcium as suggested by Ketchum and Poole [11] in corn cells, the block of a putative inward calcium current by the calcium channel

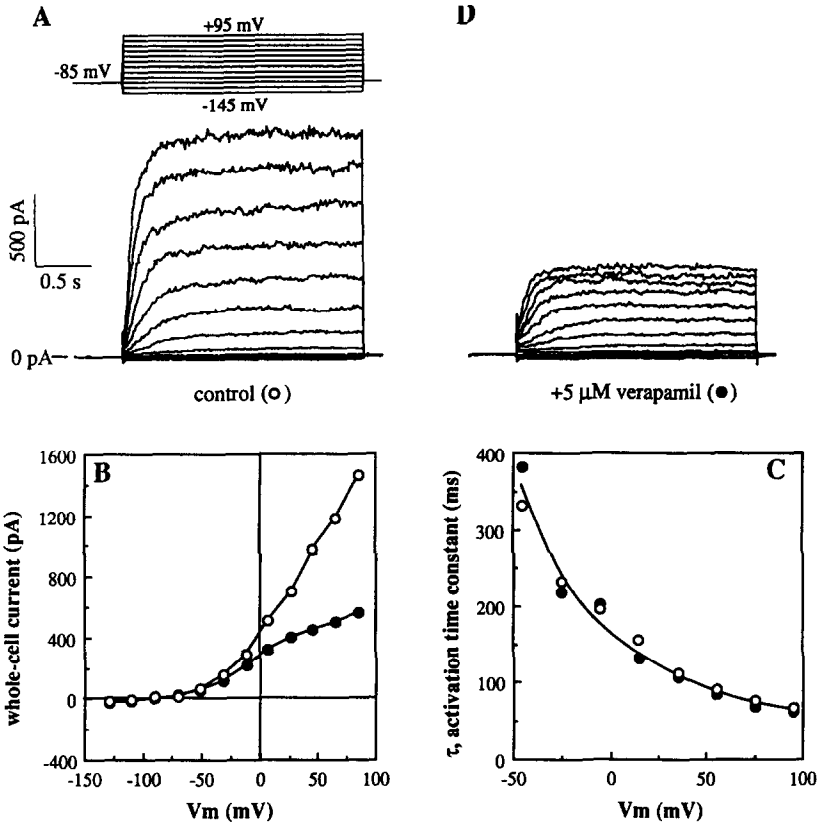

Fig. 1. Effect of verapamil on the whole-cell current of Xanthi tobacco protoplasts. Current response before and 5 min after the addition of 5 μM verapamil to the bath solution was measured using pipette (intracellular) and bath (extracellular) solutions (1 mM K^+) as described in section 2. (A and D) Plasma membrane current response upon voltage pulses of 2 s ranging from − 145 mV to +95 mV (20 mV steps) from a holding potential of − 85 mV as shown in the top inset in A. (B) Current–voltage relationship before (open circles) and after verapamil addition (closed circles) from the currents displayed in A and D. (C) The sigmoidal current activation was fitted by a power function ($I = (A - B_{exp}(-t/\tau))^2$) [22] where τ is the activation time constant plotted against the voltage for current response before (open circles) and after verapamil addition (closed circles).

antagonists could result in an inhibition of the potassium current by depleting internal calcium. According to this hypothesis, buffering the internal calcium concentration to a high value should prevent the inhibition by calcium channel blockers. Most of the inhibition experiments (closed circles in Fig. 3) were carried out using an internal solution (pipette solution) with a calcium concentration buffered to 1 μM. Similar inhibition rates could be obtained with a calcium concentration raised to high unphysiological values (100 μM, n = 5, data not shown).

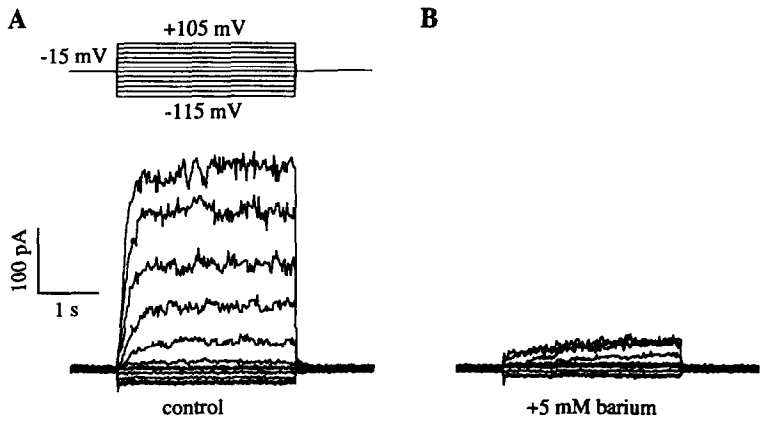

Fig. 2. Effect of barium ions on the whole-cell current of Xanthi tobacco protoplasts. Plasma membrane current responses upon voltage pulses of 2 s ranging from −115 mV to +105 mV (20 mV steps) from a holding potential of −15 mV (top inset) was measured before and 3 min after addition of 5 mM barium in the bath solution, using pipette (intracellular) and bath (extracellular 10 mM K^+) solutions as described in section 2.

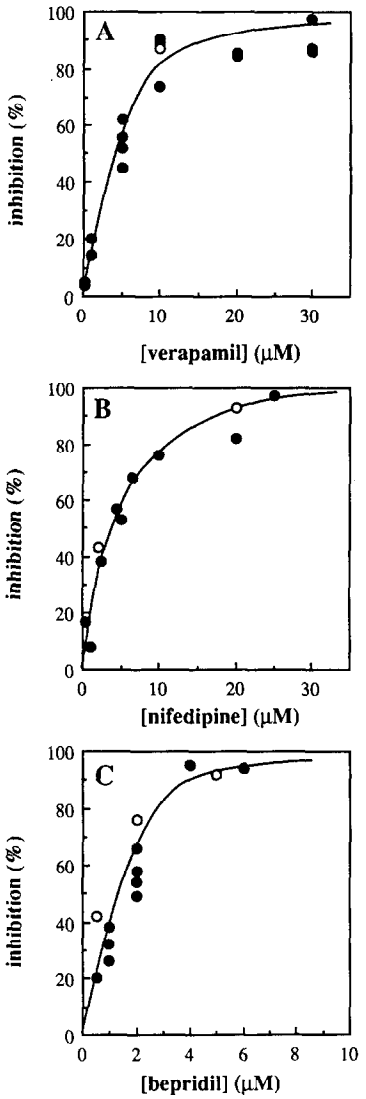

Fig. 3. Dose-response curves for verapamil (A), nifedipine (B) and bepridil (C). Note the different scale for bepridil concentrations (C). The inhibition was calculated as a percentage of the control current response for +75 mV after leak correction. The curves were fitted by eye. A, B and C show the results of 11, 6 and 11 experiments, respectively. The solutions used were the same as for Fig. 1 for most of the experiments (closed circles). However, some data (open circles) were obtained in a bath solution containing (mM) 50 $CaCl_2$, 5 $MgCl_2$, 10 Tris/MES (pH 5.5), mannitol (450 mOsm) and with a pipette solution containing (mM) 150 KCl, 2 $MgCl_2$, 0,1 EGTA, 10 MgATP, 10 Tris/MES (pH 7.2), mannitol (450 mOsm).

Furthermore, when calcium was completely depleted from the intracellular solution by adding 10 mM BAPTA in the pipette solution, the potassium outward current could still be observed (n = 6, data not shown) and was still sensitive to calcium channel antagonists (n = 2, data not shown). These results suggest that the calcium channel blockers inhibit the potassium channel more likely directly rather than via a block of putative calcium channels.

3.3. Inhibition of single outward rectifying potassium channels by verapamil

The ion channel responsible for the whole-cell outward potassium current could be studied in outside-out patches from Xanthi protoplasts. The mean amplitude of outward currents through single channels was determined for potentials between −5 mV and +75 mV in 10 mM potassium glutamate (Fig. 4A). The reversal potential for this channel (−47 mV, Fig. 4A) was close to the reversal potential of the whole-cell current (E_{rev} = −52 mV in 10 mM K^+). No single channel activity was detected for more negative potentials, i.e. single channels activated in the same potential range as the whole cell current. The single channel conductance was about 10 pS (Fig. 4A).

Fig. 4B and C show the inhibition of single outward rectifying potassium channels upon external addition of 10 μM verapamil in the outside-out configuration. Analysis of 2-min-long recordings before and after addition of verapamil revealed a decrease in the single channel open probability from 10.4% to 1.2%. Thus, the inhibition by verapamil was characterized by a decrease in the open probability whereas the single channel amplitude was unchanged. The inhibition rate of the single channel activity induced by 10 μM verapamil was 91 ± 4% (n = 3). This correlates with the inhibition observed on whole-cell outward potassium currents (Fig. 3A). Inhibition of single outward potassium channels in isolated patches provides evidence that the calcium channel antagonists act directly on the outward rectifying potassium channel.

4. Discussion

The outward potassium conductance shown here on protoplasts from Xanthi tobacco cell culture displays the same current/voltage relationship and activation kinetics as the one already described by Van Duijn et al. [6] using protoplasts from the Bright Yellow tobacco cell strain. Thus, this channel appears ubiquitous in tobacco suspension cultured cells derived from tissues as different as leaf mesophyll and stem medulla parenchyma. A conductance with strikingly identical properties has been described in *Vicia faba* mesophyll protoplasts [18]. Apart from this, many delayed outward rectifying conductances with slightly different kinetic properties have been described in a number of different cell types [2–5]. The broad distribution of this conductance emphasizes the necessity for determining its role in plant cells.

Our results show that three calcium channel antagonists inhibit directly the outward rectifying potassium channel in tobacco protoplasts from cell suspension cultures. With IC_{50} in the micromolar range, they are among the most potent outward rectifier potassium channel blockers known for plants. Inhibitory effects of

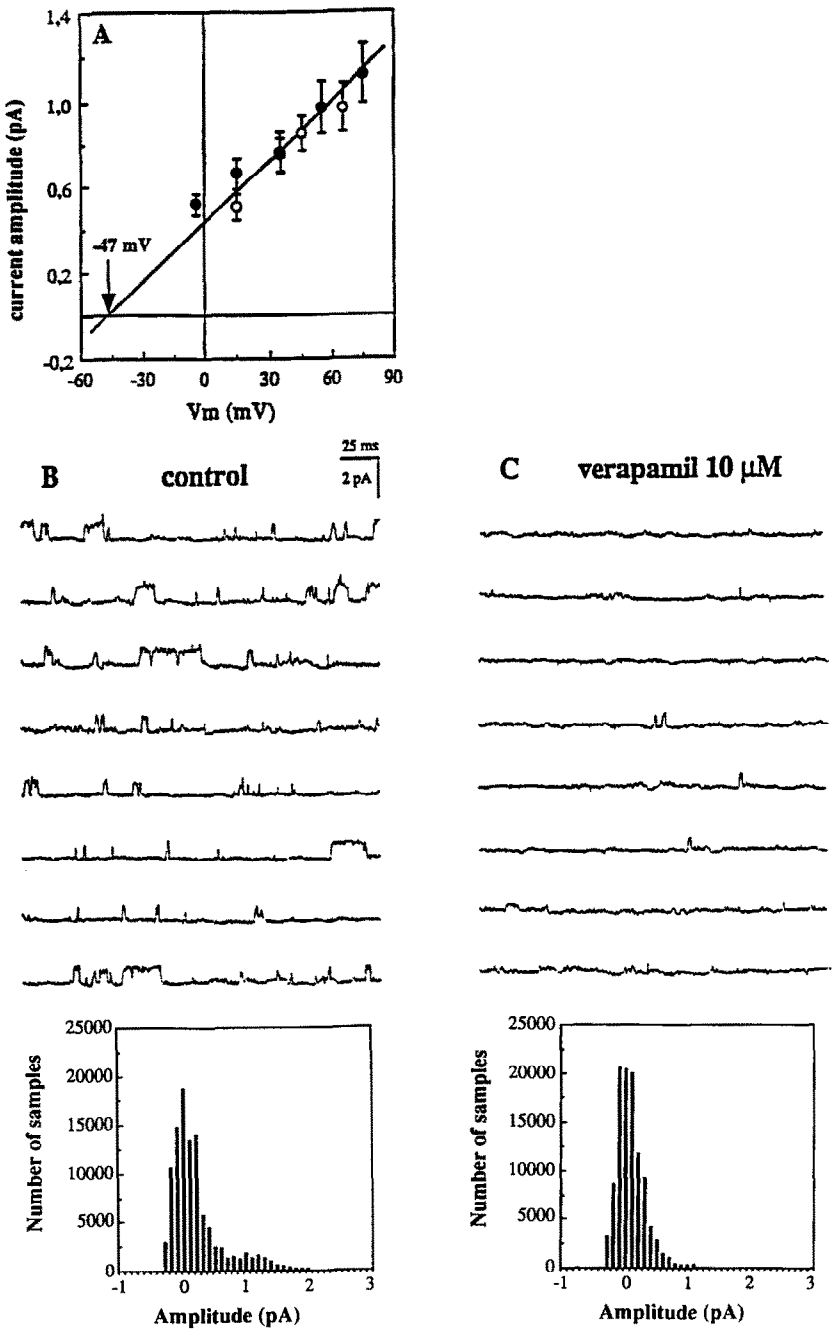

Fig. 4. Single channel properties of outward conducting potassium channels. (A) Current–voltage relationship of the outward potassium channel. Recordings from two independent outside-out patches (different symbols) were analysed in order to determine the amplitude of single channel currents for potentials between −5 mV and +75 mV. For solutions see section 2 (bath here 10 mM K^+). (B and C) All-point amplitude histograms from 51 s long recordings and single potassium channel activity before (B) and 2 min. after (C) external addition of 10 µM verapamil. The membrane potential was hold at +30 mV. Solutions as indicated (Fig. 3, open circles).

calcium channel blockers in the same concentration range have been already observed in *Amaranthus* protoplasts on outward rectifying potassium channels [14]. In the case of verapamil, we have analysed the mechanism of inhibition at the single channel level. We show that verapamil induces a decrease in the open probability of the channel consistant with the inhibition of the whole cell current, whereas the single channel amplitude remains unchanged. Further studies will show if this pharmacological property of outward rectifying potassium channels is more general in the plant kingdom.

These results raise questions concerning the use of 'calcium channel antagonists' to probe for the involvement of calcium channels in physiological processes. Indeed, the observed effects of these inhibitors in plant cells are not necessarily connected with the involvement of calcium channels, but may be the consequence of the inhibition of potassium efflux. Our results do not exclude

that voltage dependent calcium channels exist in higher plant cells and that these are also sensitive to the calcium channel blockers. In carrot protoplasts, calcium channel antagonists from the phenylalkylamine series and bepridil block calcium influx [19] and calcium permeable channels can be reconstituted from calcium antagonist binding proteins [8] providing evidence for the occurrence of calcium channels in higher plant plasma membrane.

The results presented here allow pharmacological comparison between plant outward rectifying potassium channels and animal L-type calcium channels and suggest that structural relationships may exist between the two channel types. On one hand, they have a different behaviour concerning barium. Whereas the L-type calcium channel is more permeable to barium than to calcium [7], the outward rectifying potassium currents in plant cells are blocked by barium. However, it is known that the change of a single amino acid in the pore region of the nicotinic receptor can modify the selectivity between monovalent and divalent cations [20]. On the other hand, the ability of three inhibitor molecules, belonging to distinct chemical families [13], to inhibit directly potassium channels when applied in the micromolar range suggests that these channels carry specific binding sites for each inhibitor as do L-type calcium channels from animal cell membranes. Hence, plant outward rectifying potassium channels and animal voltage-dependent calcium channels could share common structural elements, in particular binding sites for antagonists. This suggests that they may have evolved from a common ancestral gene [21].

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