Activation of plasma membrane voltage-dependent calcium-permeable channels by disruption of microtubules in carrot cells

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Abstract Plasma membrane-bound voltage-dependent calcium channels may couple the perception of an initial stimulus to a regulated pathway for calcium influx. The activities of these channels have been shown to be very low and highly unstable but may be recruited by large-predepolarizing pulses, according to a process referred to as recruitment. By combining pharmacological and electrophysiological approaches, we demonstrate in the present paper that the cytoskeleton plays an important role in the regulation of the activity and stability of voltage-dependent calcium channels during whole-cell patch-clamp experiments on carrot protoplasts. Whereas drugs affecting the organization of the microfilament network have no measurable effect, the manipulation of the microtubule network elicits important changes. Thus, the addition of colchicine or oryzalin, which are known to disrupt microtubule organization, leads to a 6–10-fold increase in calcium channel activities and half-life. In contrast, stabilization of the microtubules by taxol has no effect on any of these parameters. The data obtained suggest that interactions of microtubules and voltage-dependent calcium channels by either direct or indirect mechanisms inhibit channel activities and decrease their half-life. In contrast, the disruption of the network overcomes such an inhibitory effect and allows the activation of calcium channels. It is speculated that under normal physiological conditions these protein-protein interactions may work in a reversible manner and contribute to signal transduction in higher plants.

Key words: Plant calcium channel; Cytoskeleton

1. Introduction

Changes in cytosolic Ca^{2+} concentration of higher plant cells have been shown to sense many environmental, pathogenic and hormonal signals by modulating calcium-dependent cellular activities (for review see [1]). Since they couple the perception of an initial stimulus to a regulated pathway for calcium influx into plant cells, plasma membrane-bound voltage-dependent calcium-permeable channels (VDCCs) may be important targets in cell signalling [2–4]. While their existence has been inferred from indirect approaches for years [5], the characterization of depolarization-activated Ca^{2+} channels located in the plasma membrane has been established only recently [6–10]. So far, measurements of voltage-operated calcium movements either through plasma membrane derived vesicles or by application of whole-cell patch-clamp technique to isolated protoplasts have established that voltage-dependent inward calcium currents can be activated by physiologically occurring depolarizations in different plants [6–8,10]. Basically, the VDCCs became activated at membrane potentials positive to −140 mV and maximum currents occurred at approx. −80 to −100 mV [8]. Such properties make VDCCs optimally suited for higher plant systems for which resting potentials are in the range of −140 to −180 mV and where plasma membrane depolarizations positive to −120 mV have been shown to take place as early response to various stimuli [11–13]. However, activities of VDCCs were measurable in approx. 2–3 carrot protoplasts out of 10 only and decreased rapidly following the establishment of whole-cell recordings [8,9]. Attempts to stabilize and/or to enhance calcium currents have shown that large transient pre-depolarizations of the plasma membrane potentiate the activity of carrot calcium channels [9]. Thus, predepolarizing pulses positive to 0 mV induced not only the recovery of calcium channel activities but also the activation of initially quiescent channels by a process referred to as recruitment. Recruitment of quiescent channels may allow flexibility to plant cells to gate the flow of calcium in response to a number of depolarizing stimuli [9].

In addition to variations in membrane potential and changes in cytosolic concentration of second messengers, early responses to biotic and abiotic stimuli involve generally the reorganization of cytoskeleton, the complex protein meshwork that structures and organizes the cytoplasm of eukaryotic cells. Interestingly, recent studies on both animal and plant systems have suggested that components of the cytoskeleton are likely to be involved in the regulation of several membrane transport proteins such as voltage-sensitive Na^+ and Ca^{2+} channels, anion exchangers, Na^+-K^+-ATPases and H^+-ATPases in animal cells [14,15] and potassium and calcium channels in higher plant cells [16–20]. Drugs which interact with specific proteins of the cytoskeleton have been useful tools in understanding the regulation of a wide variety of cellular functions mediated by the cytoskeleton. These drugs generally act by stabilizing or disrupting microfilaments or microtubules that constitute two distinct networks in plant cells and their use has recently allowed to demonstrate that cytoskeletal elements participate in stomatal aperture regulation [20]. We have used such a pharmacologically based approach to address the potential effect of cytoskeletal elements on the activity/stability of VDCCs. In the present paper, we show that colchicine, an anti-mitotic agent known to disrupt cortical microtubules in carrot cells without affecting their viability [21,22] is able on the one hand to increase radiolabelled calcium influx into carrot protoplasts and to enhance and stabilize calcium currents during whole-cell patch-clamp experiments on carrot protoplasts on the other. Consistently, oryzalin, a compound known to have similar effects to colchicine on microtubule organization at lower concentrations [23]

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also exerts identical effects in terms of calcium current intensities and stability. The data obtained suggest that disruption of microtubules leads to the activation and stabilization of plasma membrane voltage-dependent calcium channels in carrot cells. Therefore, the organization of the cytoskeleton may contribute, by regulating calcium channel activities, to Ca^{2+}-dependent signal transduction in higher plant cells.

2. Materials and methods

2.1. Carrot protoplast preparation

Protoplasts were isolated from carrot (*Daucus carota* L.) cells by enzymatic digestion of the cell wall according to a procedure described previously [24,25] modified as follows. Cell wall digestion was performed by overnight incubation of carrot cells with low concentrations of hydrolytic enzymes at 20°C, e.g. 0.4% caylase 345 (Société Cayla, Toulouse, France) and 0.01% pectolyase Y23 (Sheishin Pharmaceutical Co., Tokyo, Japan). When appropriate, 1 mM colchicine (final concentration) was added to the digestion medium. Protoplast viability was estimated by using fluorescein diacetate.

2.2. $^{45}CaCl_2$ uptake

Calcium uptake by carrot protoplasts was determined as described [24]. Briefly, protoplasts (10^6 per ml) were preincubated in 25 mM HEPES/KOH buffer, pH 6.7 with 700 mM mannitol, 5 mM KCl and 1 μM valinomycin for 10 min at 20°C. Calcium uptake was initiated by adding 0.1 mM $CaCl_2$ and 2.3 μCi/ml of $^{45}CaCl_2$ to give a specific activity of 23 mCi/mmol in the assay medium. At the indicated times, 100-μl aliquots were filtered through HAWP Millipore filters and the filters were washed three times with 2 ml of ice-cold 20 mM Tris-HCl buffer, pH 7.5, containing 100 mM $MgCl_2$ and 500 mM mannitol. The radioactivity remaining on the filters was measured in a liquid scintillation spectrometer. Experiments were performed on six independent protoplast preparations.

2.3. Immunocytochemistry

Cytoplasmic microtubule networks were immunochemically stained for light microscopy according to a procedure already described [26,27]. Briefly, carrot protoplasts were fixed for 2 h with 2% paraformaldehyde and 0.03% glutaraldehyde in 50 mM PIPES buffer, pH 6.9, containing 5 mM EGTA, 5 mM $MgSO_4$, 5% DMSO and 0.03% Nonidet p40 (PIPES buffer). After washing with PIPES buffer, protoplasts were embedded in 1% agarose and layered on poly(L-lysine)-precoated microscope slides. Protoplasts were permeabilized with 0.25% Triton X-100 in PIPES buffer during 30 min. After washing, the aldehyde groups were reduced with $NaBH_3CN$ (1 mg/ml) in 0.01 M phosphate-buffered saline at pH 8.

The specimens were incubated at room temperature successively with 5% normal goat serum prepared in Tris-buffered saline at pH 7.6 for 30 min and with monoclonal anti-β-tubulin (Amersham) at 1:250 dilution, overnight. Fluorescein isothiocyanate linked sheep anti-mouse IgG (Amersham) was then added at a 1:50 dilution. Control experiments were performed by omission of the first antibody. Stained microtubules were observed by using a Leitz Laborlux 12 microscope equipped with epifluorescence optics.

2.4. Electrophysiological investigations

During patch-clamp recordings, protoplasts were bathed at 20°C in an external medium containing 450 mM sorbitol, 30 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 1.6 mM $Ca(OH)_2$, pH 6.7. The pipette solution which equilibrated with the cytoplasm contained 500 mM sorbitol, 10 mM Mg-ATP, 5 mM $Tris_2$-EGTA, 2 mM $MgCl_2$, 10 mM HEPES-Tris pH 7.2, with 80 μM colchicine or other drugs when specified. Whole-cell patch-clamp experiments were performed as described by Hamill et al. [28]. Gigaohm resistance seals between pipettes, pulled from Kimax-51 capillaries (Kimble Glass, Inc., Owens, IL) and protoplast membranes were obtained with gentle suction, leading to the whole-cell configuration. Recordings were performed and low pass-filtered with an RK 300 amplifier (Biologic Instrument, Claix, France). The application of voltage protocols and subsequent data analysis were performed using a Lab Master DMA interface (Axon Instruments, Foster City, CA) and the patch-clamp software pCLAMP 5.5.1 (Axon Instruments). Membrane potentials were corrected for liquid junction potentials as described by Ward and Schroeder [29].

3. Results

To study the effect of cytoskeletal elements on Ca^{2+} transport through the plasma membrane of carrot cells, we tested drugs known to interfere with the cytoskeleton organization, for their action on $^{45}CaCl_2$ uptake by carrot protoplasts. Under our experimental conditions, taxol, a compound known to stabilize microtubules in a polymerized state, had no effect on calcium influx into carrot protoplasts at a concentration of 50 μM when added during either the preparation of protoplasts or the uptake experiments (data not shown). In contrast, the addition of 1 mM colchicine while preparing protoplasts by overnight digestion at 20°C resulted in a significant stimulation of $^{45}CaCl_2$ influx without affecting protoplast viability (Fig. 1). Thus, the time-dependent Ca^{2+} uptake by carrot protoplasts prepared in the presence of 1 mM colchicine was 40–80% over the control experiments, 6 min after the initiation of calcium influx.

Immunocytochemical staining of microtubules showed that control protoplasts displayed randomly oriented cortical microtubules as already described [30] (Fig. 2A). In contrast, most of the treated protoplasts had their cortical microtubules completely disrupted. Only some remaining short pieces of strands still attached to the plasma membrane or insulated on the cortical area were detectable (Fig. 2B). The results suggest that the complete disruption of the microtubule network is necessary to stimulate the uptake of radioactive calcium by carrot protoplasts. The pathway of calcium entry regulated by the organization of microtubules has been addressed by application of the whole-cell patch-clamp technique to carrot protoplasts.

Whole-cell patch-clamp experiments were performed on

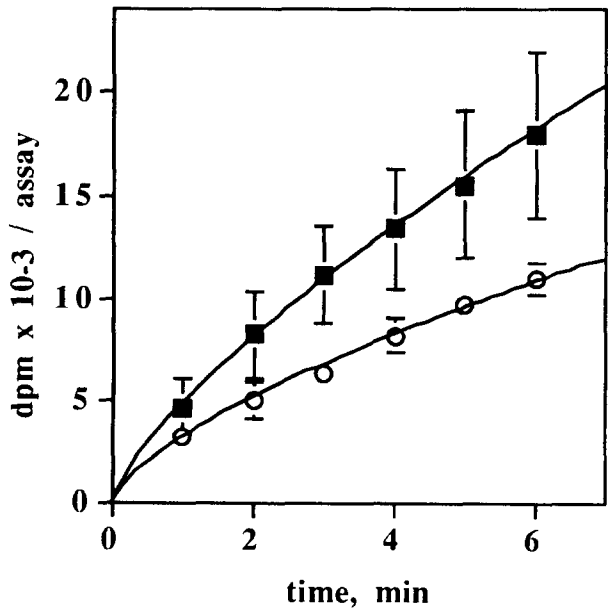

Fig. 1. Uptake of $^{45}CaCl_2$ by carrot protoplasts. Carrot cell suspension cultures were digested overnight in the absence (○) or presence (■) of 1 mM colchicine. The plot shows representative results of experiments performed on six independent protoplast preparations and error bars represent ± standard deviation.

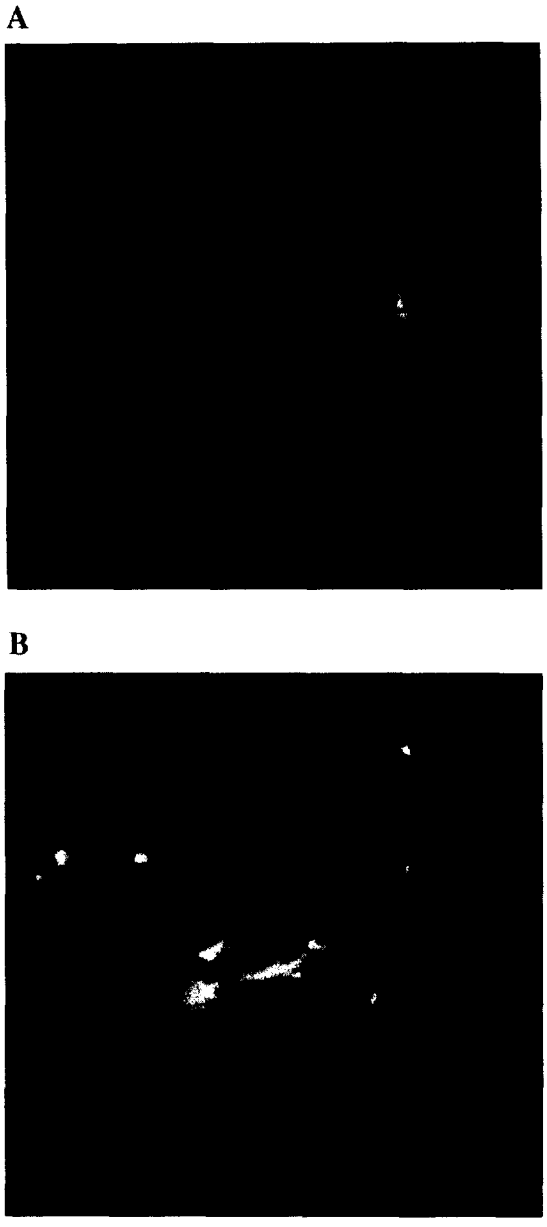

Fig. 2. Immunodetection of β-tubulin of carrot protoplasts. The protoplasts were prepared overnight in the absence (A) or presence (B) of 1 mM colchicine. The bar is 4 μm.

carrot protoplasts under conditions which allow the distinction of voltage-dependent Ca^{2+} channels from other ion channel types [8,9]. Regardless of the treatments, depolarizing voltage ramps from a holding potential of −160 to +50 mV led to the activation of inward calcium currents as previously detailed [8,9]. As expected, peak currents measured 3 min after the establishment of the whole-cell configuration were very low, e.g. 35 pA (± 17 pA, n = 20), in untreated protoplasts (Fig. 3A). In contrast, when 1 mM colchicine was added during the preparation of carrot protoplasts, whole-cell calcium currents increased significantly (Fig. 3C). Under these experimental conditions, peak calcium currents measured 3 min after the establishment of whole-cell configurations were approx. 400 pA (± 105 pA, n = 3), i.e. 10-fold higher than in the control. Colchicine treatment did not alter either the activation potential and the peak current potential or the reversal potential of voltage-dependent currents (Fig. 3, compare A and C). Moreover, the data established that the observed increase of $^{45}CaCl_2$ uptake by carrot protoplasts treated by colchicine is, at least partially, due to activation of plasma membrane-bound VDCCs.

The whole-cell patch-clamp technique allows direct manipulation of the cytoplasmic content by adding putative regulatory molecules to the pipette solution. Accordingly, the whole-cell patch-clamp technique was applied to carrot protoplasts prepared in the absence of colchicine but with a patch pipette solution containing 80 μM colchicine. As shown in Fig. 3B, the characteristics of VDCCs remained unchanged. However, inward calcium currents were significantly greater than those measured in the absence of the drug in the patch pipette. Thus, peak currents reached 74 pA (± 19 pA, n = 6), 3 min after the establishment of whole-cell configurations. Consequently, direct addition of colchicine at low concentration to the cytoplasm elicits activation of plasma membrane VDCCs confirming that the observed effects are mainly due to the action of the drug on the intracellular milieu.

In addition to their low activities, VDCCs are very unstable following the establishment of the whole-cell configuration [8,9]. As shown in Fig. 4A, calcium-permeable channel activities decreased dramatically with time following the establishment of whole-cell configurations and were hardly detectable upon depolarizing ramps performed 6 min after obtaining the whole-cell configuration (ramp 3, Fig. 4A). Addition of 80 μM colchicine to the pipette solution led not only to an increase in calcium currents as anticipated but also to a significant stabilization of voltage-dependent calcium channel activities (Fig. 4B). A slow decrease occurred progressively but, in contrast to control protoplasts, most of the initial activity

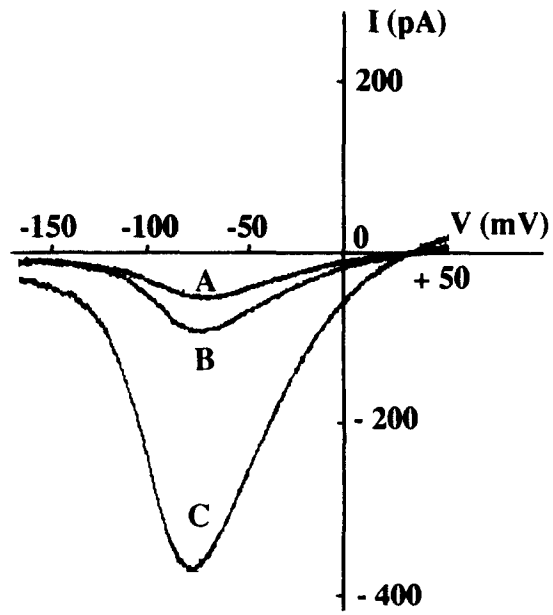

Fig. 3. Activation of voltage-dependent calcium-permeable channels of carrot cells by colchicine. The protoplasts were prepared overnight in the absence (A,B) or presence (C) of 1 mM colchicine. Whole-cell currents were recorded during a voltage-ramp between −160 and +50 mV, 3 min after the establishment of the whole-cell configuration, in the absence (A,C) or presence (B) of 80 μM colchicine in the pipette solution.

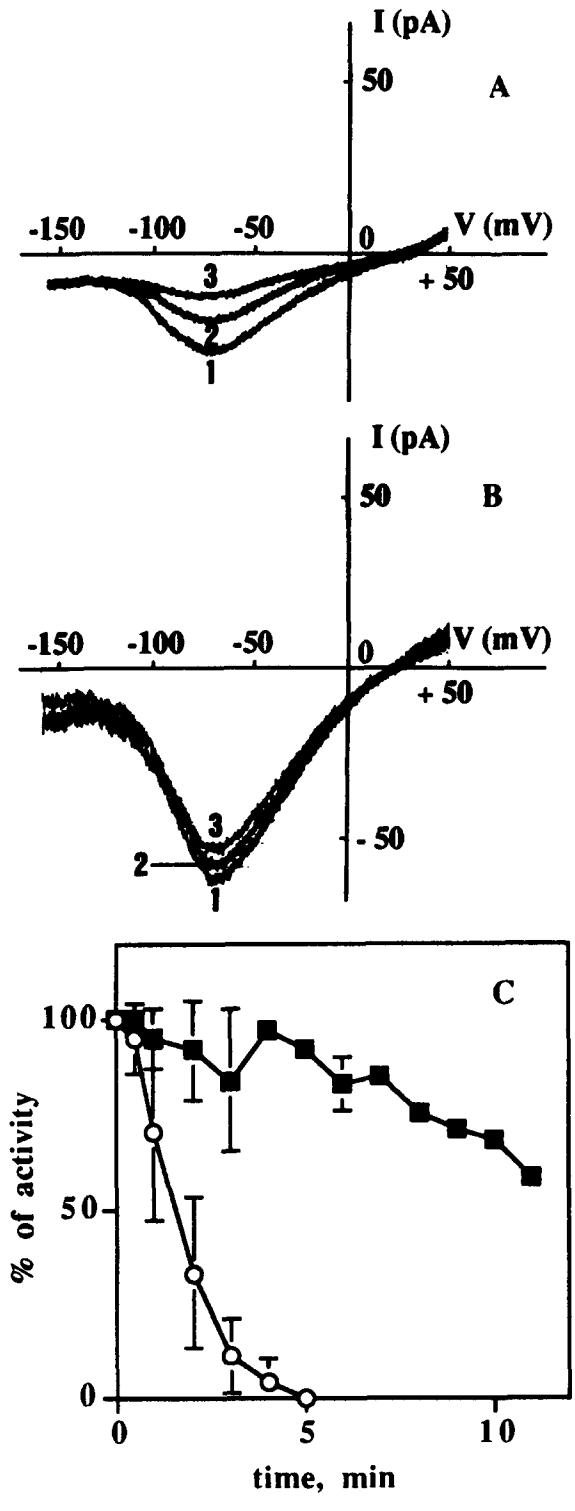

Fig. 4. Stabilization over time of voltage-dependent calcium channel activities by colchicine. 3 min after the establishment of the whole-cell-configuration, calcium currents were recorded during voltage-ramps between −160 and +50 mV performed every minute (from ramp 1 to ramp 3), in the absence (A) or presence (B) of 80 μM colchicine in the pipette solution. (C) The currents measured at the peak current of voltage-ramps applied every minute, 3 min after the establishment of the whole-cell configuration, are expressed as a percentage of initial activities and plotted as a function of time. Whole-cell patch clamp experiments were performed in the absence (○) or presence (■) of 80 μM colchicine in the pipette solution.

(85%) was maintained 6 min after the establishment of the whole-cell configuration (ramp 3, Fig. 4B). The stabilizing effect of colchicine is illustrated by data reported in Fig. 4C representing the loss of VDCCs activities with time. It appears that in the absence of drug the relative half-life of VDCCs was 1.5 min (±0.5 min, $n=12$). In the presence of 80 μM colchicine the relative half-life shifted to 9 min (±5 min, $n=4$), i.e. 6 times longer compared to the control. These protoplasts still displayed measurable calcium channel activities 22 min (±5 min, $n=4$) after the establishment of the whole-cell configuration.

Substitution of colchicine by oryzalin led to the same types of results in terms of current intensities and stability (Fig. 5). This compound, being highly permeant, was directly added to the bath solution during whole-cell patch-clamp recordings. Under these conditions, 5 μM oryzalin induced a considerable increase in voltage-dependent calcium currents (Fig. 5A). Thus, currents measured at the peak current potential, 3 min after the establishment of the whole-cell configuration, reached 142 pA (±59 pA, $n=4$), 4-fold higher than in control protoplasts (Fig. 5A, compare traces 1 and 2). In addition, oryzalin induced significant stability of voltage-dependent calcium channel activities (Fig. 5B). The relative half-life was 9 min (±1 min, $n=4$). Oryzalin (5 μM) exerted a more pronounced effect on VDCCs than significantly higher concentrations of colchicine (80 μM). Such efficacy has been established concerning the relative effect of the two drugs on the organization of plant microtubules that have been shown to be less sensitive to colchicine than to oryzalin by at least one order of magnitude in terms of concentration [23].

In contrast, stabilization of microtubules by adding 50 μM taxol to the pipette solution had no measurable effects on voltage-dependent calcium currents (data not shown).

Due to the limited permeability of the plasma membrane to some compounds altering the organization of actin microfilaments, the other main components of cytoskeleton, we have taken advantage of the patch-clamp technique to add them directly in the pipette solution. Neither cytochalasin D (20 μM) and the protein profilin (3.6 μg/ml) which stabilize microfilaments, nor phalloidin (100 μM) which disrupts their organization had any effect on current intensity or stability (data not shown). Consequently, the observed effects of cytoskeletal components on plasma membrane-bound VDCCs seem to be mainly due to microtubule organization and not to microfilaments.

4. Discussion

Several lines of evidence have demonstrated the existence of voltage-dependent calcium-permeable channels that can be activated by physiologically occurring depolarizations of plant plasma membrane [6–10]. The activation of these channels could result in massive entry of Ca^{2+} into the cell which would become lethal if not regulated [8]. Therefore, understanding the mechanisms allowing control of VDCC activities is crucial. We have previously shown that most of VDCCs are inactive in carrot cells but may be recruited upon large predepolarizations of the plasma membrane [9]. Recruitment of VDCCs could be a means to modulate channel activities in intensity and duration, but the mechanisms underlying this process remained poorly defined. The data described in the present paper provide experimental evidence demonstrating

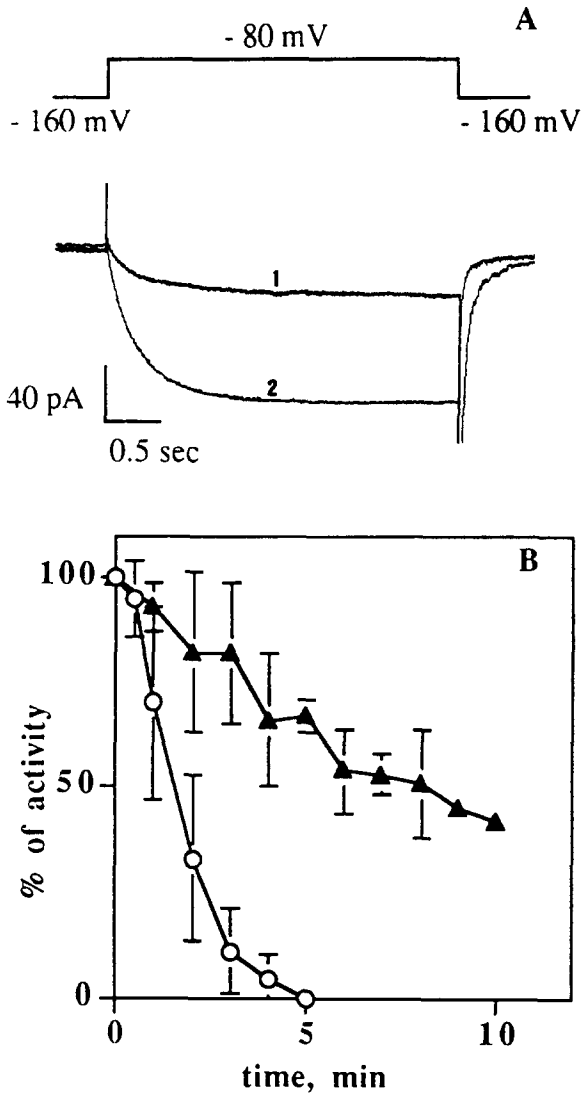

Fig. 5. Activation and stabilization of voltage-dependent calcium channel activities by oryzalin. (A) Voltage-dependent inward calcium currents were recorded 3 min after the establishment of the whole-cell configuration by depolarization of the plasma membrane from the holding potential at −160 mV to the peak current potential at −80 mV. The top inset shows the voltage pulse protocol. Trace 1, control protoplast. Trace 2, protoplast incubated with 5 μM oryzalin. (B) The currents measured at the peak current of voltage-ramps applied every minute, 3 min after obtaining the whole-cell configuration, are expressed as a percentage of initial activities and plotted as a function of time. (○) Control protoplasts. (▲) Protoplasts incubated with 5 μM oryzalin.

that cytoskeletal elements may be involved at least partially in these modulatory mechanisms.

By combining pharmacological and electrophysiological approaches, we have shown that drugs acting on the organization of microfilaments have no effect on channel activities and stability. In contrast, disruption of the microtubule network, the other main cytoskeletal component, led to a dramatic increase in channel activities and life-time. As already reported [23], oryzalin was more effective than colchicine at micromolar concentrations. Attempts to study the dose-response effects of oryzalin on the activation/stabilization of VDCCs led to an 'all or none'-type of results with a threshold concentration of approx. 5 μM oryzalin corresponding to a concentration inducing significant disorganization of the microtubule meshwork.

Interestingly, both large pre-depolarizations of the plasma membrane and microtubule disruption led to a 10-fold stimulation of VDCC activities. However, pre-depolarizing treatments led to the reversible recruitment of VDCCs whereas microtubule disruption significantly increases the life-time of the activated channels. The possibility is that the association of microtubules with the plasma membrane by a still unknown mechanism tends to inhibit VDCCs. The complete disruption of the network by colchicine or oryzalin might therefore dramatically activate the channels. If the effects of pre-depolarization are linked to microtubule organization, it may be speculated that the manipulation of the membrane potential will control the association of microtubules with the plasma membrane. In this way, several reports indicate that components of the plant cytoskeleton are presumably involved in the regulation of calcium transport during mechanical signal transduction processes in higher plants [16,19]. There are also strong indications suggesting that cortical microtubules are involved in mediating the transduction of external stimuli such as temperature, gravity, wounding and growth substances, processes where calcium has been implicated as a second messenger [31–33].

At present, it is not clear whether microtubules directly interact with VDCCs and the influence of microtubules associated proteins cannot be ruled out. However, a regulatory effect of nucleotides bound to microtubules seems improbable since we have shown that manipulating the GTP/GDP content of carrot cells has no effect on calcium channel activity and stability [8].

In animal systems, microtubules have been shown to modulate the activity and stability of VDCCs but in the opposite direction. Thus, the dissociation of microtubules to tubulin by colchicine increased the probability of VDCCs existing in a closed state, whereas the stabilization of microtubules by taxol increased the open state probability of channels [14]. Therefore, it seems that the activation of animal VDCCs involves the formation of channel clusters which could be maintained by microtubules [14]. In contrast, in carrot protoplasts the dissociation of putative inhibitory microtubule-linked elements from the plasma membrane seems to be involved in mechanisms of activation of calcium-permeable channels.

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