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# Plasmalemmal, Voltage-Dependent Ionic Currents from Excitable Pulvinar Motor Cells of *Mimosa pudica*

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**Summary.** Plasmalemmal ionic currents from excitable motor cells of the primary pulvinus of *Mimosa pudica* were investigated by patch-clamp techniques. In almost all of the enzymatically isolated protoplasts, a delayed rectifier potassium current was activated by depolarization, while no currents were detected upon hyperpolarization. This sustained outward current was reversibly blocked by Ba and TEA and serves to repolarize the membrane potential. Outward single channel currents that very likely underly the macroscopic outward potassium current had an elementary conductance of ≈20 pS. In addition, in a few protoplasts held at hyperpolarized potentials, depolarization-activated transient inward currents were observed, and under current clamp, action potential-like responses were triggered by depolarizing current injections or by mechanical perturbations. The activation characteristics of both inward currents and spikes showed striking similarities compared to those of action potentials *in situ*.

**Key Words** delayed rectifier potassium current · action potential · *Mimosa* · pulvinar motor cells · plasmalemma · patch clamp

## Introduction

In common with pulvinar motor cells found in many other species, cortical parenchymal cells from the primary pulvinus of *Mimosa pudica* (Fig. 1*A*) undergo slow spontaneous turgor changes with an endogenous rhythm synchronized mainly by dark/light transitions (*for review*, Satter, 1979). In addition to these slow nyctinastic movements, rapid movements associated with action potentials can be triggered in *M. pudica* by a variety of stimuli, including mechanical pressure, light-on (with blue light being most efficient), a discrete drop in temperature, and membrane depolarization (*for review*, Roblin, 1979). Clearly then, the perception and transduction of such signals are likely to involve changes in membrane conductances (Stoeckel & Takeda, 1990), although cytoskeletal contractile proteins may be secondarily involved (Fleurat-Lessard et al., 1988). As changes in plasmalemmal and vacuolar ionic fluxes are obviously the basic driving force underlying both rapid and slow turgor movements (*for reviews,* Hill & Findlay, 1981; Okazaki & Tazawa, 1990), we describe here the characterization of plasmalemmal ionic currents in *M. pudica* pulvinar protoplasts that very probably play an important role in rapid movement.

In *Mimosa*, not only cells of the cortical parenchyma in the pulvini are excitable, but as well, propagation of action potentials along the primary and secondary petioles and in the stem has been long known (Umrath, 1937). For example, intracellular microelectrode measurements (Sibaoka, 1962), associated with iontophoretic dye injections (Samejima & Sibaoka, 1983) in the primary petiole revealed the presence of excitable cells in the parenchyma of the protoxylem, in the parenchyma of the phloem, and among companion cells of the phloem. In other higher plants, rapid movements presumably associated with action potentials have been described, for example in pulvini of other sensitive species and in the leaf blade of the Venus fly-trap *Dionaea muscipula* (*for review*, Sibaoka, 1969). Note that action potentials have also been detected in plants where no spectacular movement occurs (Pickard, 1973).

The ionic basis of action potential generation in plants is best characterized in large excitable green algae (like *Chara, Nitella, Nitellopsis*): an initial calcium influx triggers the opening of calcium-sensitive chloride channels and the resultant depolarization associated with chloride efflux then in turn results in activation of voltage-dependent potassium channels, leading to membrane repolarization (Mullins, 1962; Beilby, 1982; Lunevsky et al., 1983). In higher plants, similar ionic currents are generally thought to be involved in action potential generation, although supporting data in the literature are rather indirect and less detailed. For example, in *Aldrovanda* (Iijima & Sibaoka, 1985) and in *Dionaea* (Hodick

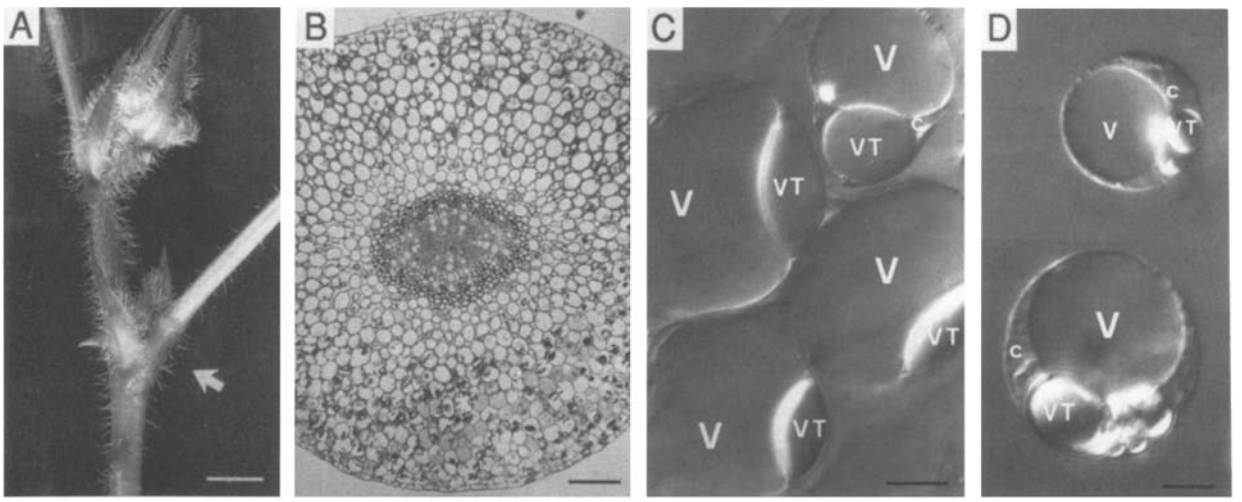


**Fig. 1.** The plant *Mimosa pudica* and isolated pulvinar protoplasts. (*A*) The primary pulvinus (*arrow*) at the base of the youngest well-expanded leaf. (*B*) Transverse section showing the motor tissue around the vascular bundle. The lower (abaxial) half was used for preparing protoplasts. (*C*) Nomarski interference contrast view of a partly-digested slice of motor tissue with protoplasts *in situ* visible. (*D*) Isolated protoplasts of motor cells used for patch-clamp analysis observed as in (*C*). (*V*: central vacuole; *C*: cytoplasmic layer; *VT*: tannin containing vacuole). Scale bars represent 5 mm (*A*), 350 $\mu$m (*B*) and 12 $\mu$m (*C,D*).

& Sievers, 1988), the resting potential varied with external K concentrations, whereas the action potential peak was dependent on the external Ca concentration. While intracellular recordings of action potentials have been obtained in the primary pulvinus of *Mimosa* (Abe & Oda, 1976; Samejima & Sibaoka, 1982), the nature of the ionic currents involved is not well documented. Nevertheless, the rapid movement in *Mimosa* is known to be associated with macroscopic KCl efflux (Samejima & Sibaoka, 1980; Kumon & Suda, 1984).

We report here our analysis of the electrophysiological behavior of isolated pulvinar protoplasts from *Mimosa*. Notably, a depolarization-activated delayed rectifying outward K current that undoubtedly serves to repolarize the membrane potential is characterized. As well, we present data demonstrating mechanically and electrically evoked action potential-like responses and depolarization-activated macroscopic inward currents. These currents probably underly the rapid movements in *Mimosa*. Part of our results have been presented elsewhere in an abbreviated form (Stoeckel & Takeda, 1989*b*).

## Materials and Methods

### Plants and Isolation of Protoplasts

Plants were cultivated from seeds in greenhouses at 20–30°C where daylight was supplemented with artificial light or in a culture chamber at 22–24°C with 14 hr/day artificial illumination (30 $\mu E.m^{-2}.s^{-1}$). The youngest well-expanded leaf from plants having 8 to 20 leaves was isolated. The motor tissue from the lower half of the primary pulvinus (Fig. 1*A,B*) was cut using a razor blade into 2 or 3 fine slices, and rinsed twice with the enzymatic digestion medium (*see below*). Only the lower (abaxial) half of the pulvinus was used (the cells therein are known to be excitable and it is the volume change of these motor cells which results in rapid movement), as some question exists as to the excitability of the cells in the upper half (which plays the role of a spring). Usually, about ten half-pulvini were incubated in 2 ml of the digestion solution for 2–4 hr at 28–32°C. The digestion medium contained 3–4% cellulase (Cellulase Onozuka R10 from Yakult Honshu, Japan or Caylase 345 from Cayla, Toulouse, France), 1% macerozyme (Macerozyme Onozuka R10 from Yakult Honshu, Japan) and 0.05–0.2% pectolyase (Pectolyase Y23 from Seishin Pharmaceutical, Japan) dissolved in the normal extracellular bath solution used during patch-clamp experiments. The extracellular solution usually contained (in mM): 600 mannitol, 8 $CaCl_2$, 25 MES; the pH was adjusted to 5.5 with KOH, and the K concentration was ≈6 mM (osmolarity was ≈720 mOsmol/kg). After digestion, the protoplasts were centrifuged (30 min at 100 × *g*) on a discontinuous Ficoll gradient (20%, 10%, 0% in extracellular solution). Protoplasts collected at the 10%–0% interface were kept on ice until used and remained viable for at least 6–8 hr after isolation.

Protoplasts obtained under these conditions varied in size, with a diameter up to 60 $\mu$m. As observed with phase contrast or Nomarski optics (Fig. 1*C,D*), their intracellular organization appeared similar to that previously described *in situ* (Fleurat-Lessard & Roblin, 1982). A large transparent central vacuole and smaller birefringent tannin vacuoles were apparent. In the thin cytoplasmic layer, randomly distributed spindle-like chloroplasts were visible.

### Electrophysiology

Conventional patch-clamp techniques (Hamill et al., 1981) were used, as in previous studies (Stoeckel & Takeda, 1989*a,b*), with macroscopic currents being recorded in the whole-cell configura-

tion and single channel currents from isolated outside-out patches. Whole-cell current-clamp recording was also carried out. Pipettes were made from thin-wall borosilicate (Vitrex BRI/E) capillaries which had resistances of 20–40 MΩ when filled with normal internal "KCl" solution, which usually contained (in mM): 150 KCl, 10 or 100 HEPES/KOH (pH 7.2), 10 EGTA, 0.57 $CaCl_2$ (pCa ≈ 8), and usually, 2–5 MgATP was also included (osmolarity was adjusted to ≈760 mOsmol/kg with mannitol). The presence or absence of MgATP had no significant, systematic influence on the results. Two other internal pipette solutions were also used, with *inter alia* 16 or 70 mM KCl and the remaining K being added as a phosphate mixture buffered at pH 7.2: "high phosphate" (in mM: 76 $K_2HPO_4$, 20 $KH_2PO_4$) and "low phosphate" (in mM: 34 $K_2HPO_4$, 9 $KH_2PO_4$) solutions, respectively. The normal external bath solution is given above. When external K concentrations were varied, KCl was added and mannitol was reduced in an iso-osmotic fashion. Pipettes were sometimes coated with beeswax and/or dipped in Sigmacote (Sigma) to reduce associated capacitance. Seal resistances were at least several GΩ, and sometimes took several minutes to form (Schroeder, 1988). Cell capacitance and series resistance were compensated for using the inbuilt circuitry of the patch amplifier (List EPC-7). Data were acquired on FM tape (Racal Store 4) for later off-line analysis (using a Nicolet digital oscilloscope or a Goupil 386DX lab computer with a CED 1401 interface and CED software). Capacity transients and linear leak were in general not subtracted. Experiments were made in a 2-ml bath, usually without perfusion, at room temperature using a microscope equipped with phase contrast optics (Nikon Diaphot) and a green filter.

Data analyzed from a total of 312 cells are presented here.

## Results

### Delayed Rectifier, Outward K Current

With normal external bathing and internal KCl pipette solutions, step depolarization produced outward-going, whole-cell currents whose activation was more rapid for larger voltage pulses (Fig. 2*A*). The threshold for activation of these outward going currents was about −50 mV (Fig. 2*B*). The protoplasts had a high input resistance, typically ≥20 GΩ, with only a small linear leak current being seen for hyperpolarizing voltage steps from a −80 mV holding potential (Fig. 2*B*). No difference in outward current behavior was observable when holding potentials of either −80 or −180 mV were used (Fig. 3*A*), other than a more rapid decay of inward tail currents for −180 mV holding potentials (Fig. 3*A* inset). The outward current was maintained, showed no apparent inactivation for step durations up to 7 sec (Fig. 3*C*).

Tail current reversal analysis (Fig. 4) in external solutions with different KCl concentrations revealed a high selectivity for K over Cl, with the interpolated reversal potentials (Fig. 4*B*) approximately following a Nernstian relation over a wide range of K concentrations (Fig. 4*A*). When Na was substituted

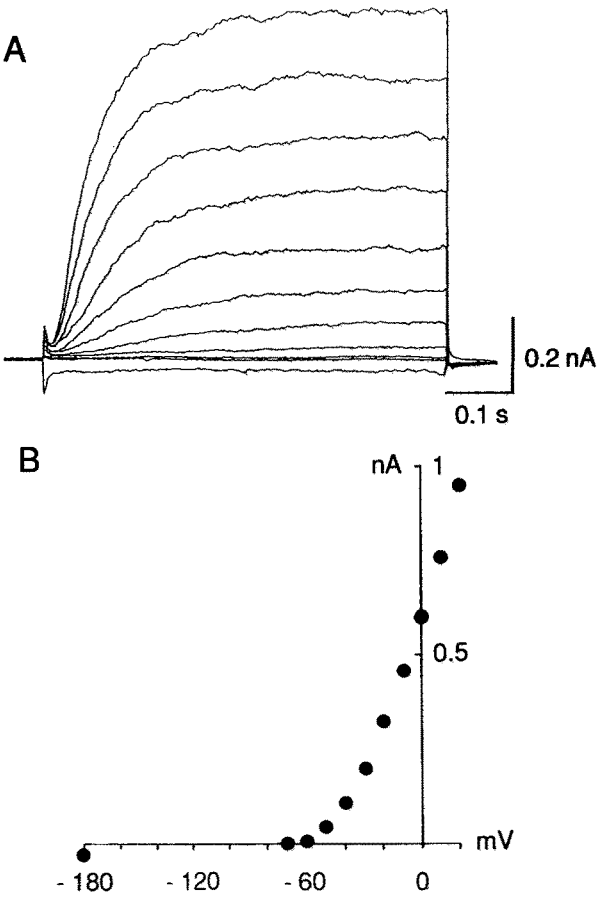


**Fig. 2.** (*A*) Depolarization-activated outward whole-cell currents obtained for 10 successive 600 msec long test pulses (starting from −70 mV, in 10 mV steps, to 20 mV) in an isolated pulvinar protoplast from *Mimosa*. Holding potential, −80 mV. A single hyperpolarizing step to −180 mV is included. (*B*) Corresponding peak current-voltage (*I/V*) relationship. Note the delayed, outward-rectifying nature of this current. Non leak subtracted; outward current is plotted upwards. Normal external bath and internal KCl pipette solutions.

for K in the internal pipette solution (in mM: 150 NaCl; 10 HEPES/NaOH, pH 7.2; 10 EGTA, 0.57 $CaCl_2$; pCa ≈ 8), no outward currents were activated by depolarizing steps ($n$ = 5), indicating either an exceedingly small Na permeability for these channels or as known for some types of K channels, a channel-blocking effect of internal Na. However, in external solutions containing *inter alia* 6 mM K or 6 mM Na + 1.5 mM K with a high Na internal pipette solution, depolarizing voltage steps which did not trigger outward currents elicited inward tail currents upon repolarization to the holding potential of −80 mV ($n$ = 4). In one cell, these inward tail currents increased dramatically when the external K concentration was increased to 50 and 100 mM. In another cell, when K in the internal pipette solution was

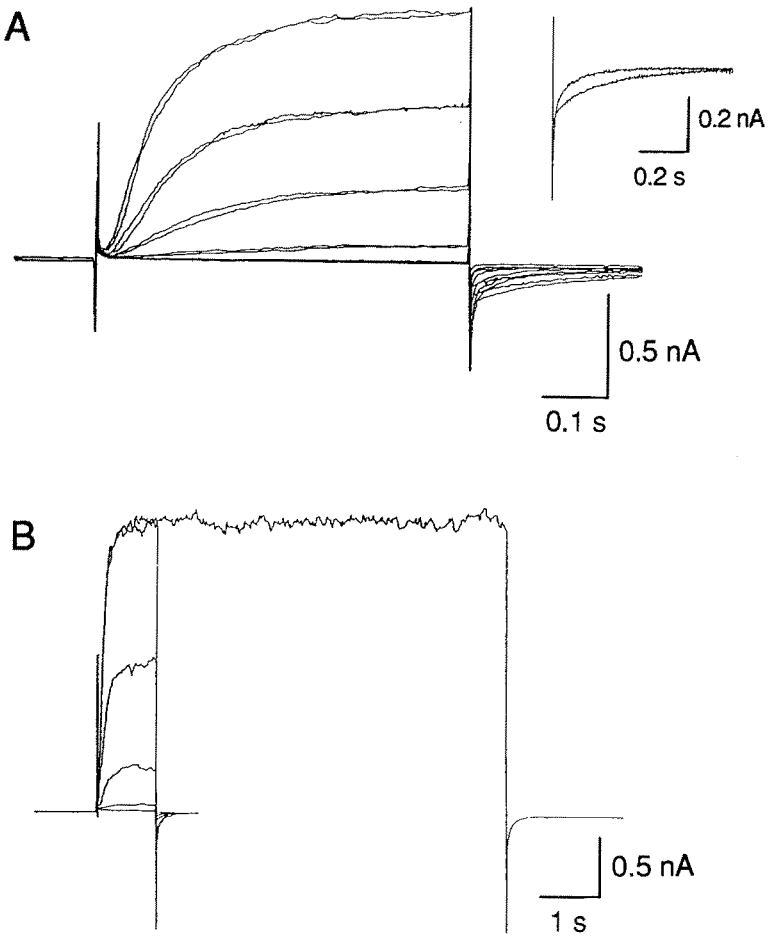


**Fig. 3.** Outward currents (*A*) are not influenced by different holding potentials of −80 and −180 mV (600 msec steps to −40, −20, 0, 20 and 40 mV). Inset shows tail currents for steps to 40 mV from the two different holding potentials with the more rapid tail currents being associated with the −180 mV holding potential. (*B*) No inactivation is observed for long-lasting test pulse durations (1 sec long to −40, −20, 0, 20 and 40 mV from a −80 mV holding potential) and a 7-sec long step to 40 mV). Different cells in *A* and *B*; normal external bath and internal high phosphate pipette solutions.

replaced by Cs, no outward currents were elicited upon depolarization.

Upon bath application, both Ba (10 and 20 mM; $n > 20$) and TEA (20–25 mM; $n = 5$) induced an essentially complete and reversible block of the depolarization-activated outward current (Fig. 5*A,B*).

Single channel currents from outside-out patches ($n = 10$) were activated upon depolarization (Fig. 6*A*). An average single channel conductance of 18 ± 4 pS was found with an extrapolated reversal potential more negative than −60 mV with internal KCl pipette solutions, as illustrated in Fig. 6*B*, where a single channel slope conductance of 19 pS and an extrapolated reversal potential of −73 mV were determined. The threshold for these outward currents was close to −50 mV, quite similar to that observed for macroscopic outward currents (Fig. 2) and no inward currents were observed at more hyperpolarized voltages. Due to the size of the patch and/or the high density of channels in the membrane, more than one single channel current opening was usually present in isolated patches, and thus kinetic analysis was not attempted.

## Current-Clamp Analysis

In protoplasts under current clamp, hyperpolarizing current pulse injections caused large, slowly rising membrane hyperpolarizations, consistent with passive RC membrane behavior, while in contrast, symmetrical depolarizing current pulses induced smaller depolarizations characterized by an initial change in voltage followed by a pronounced inward relaxation for the larger pulse amplitudes (Fig. 7*A*). To test the hypothesis that the outward going, delayed rectifier K current described above was responsible for shunting the membrane voltage during large depolarizing current injections, 20 mM Ba was added to the bath. As illustrated, symmetrical current pulses provoked symmetrical, passive RC voltage responses, and depolarizations to large positive membrane potentials were obtained easily with Ba in the bath (Fig. 7*B*).

A second effect of external Ba was apparent under current clamp. Under control conditions in cells clamped using an internal KCl pipette solution, the zero current (resting) potential was on average

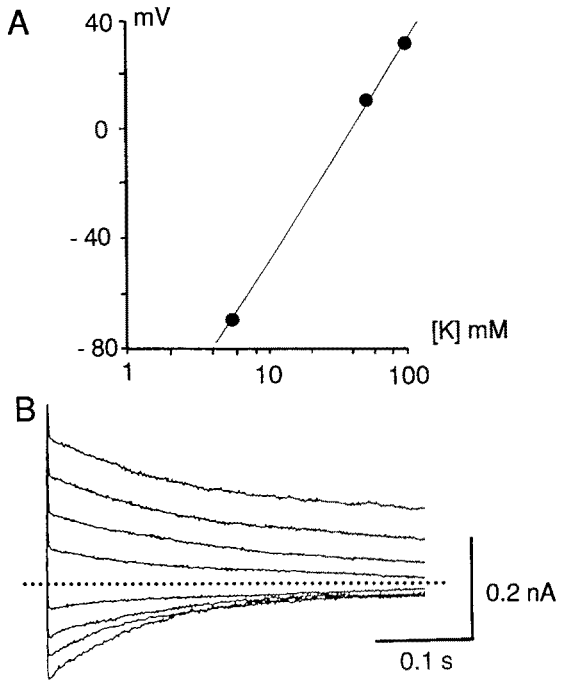


**Fig. 4.** (*A*) Tail current reversal potentials depend on extracellular K concentration. (*B*) Family of tail currents for return potentials of −40 to −70 mV and −90 to −130 mV (in 10 mV steps) for a test pulse to 20 mV. Holding potential −80 mV; normal external bath and internal KCl pipette solutions.

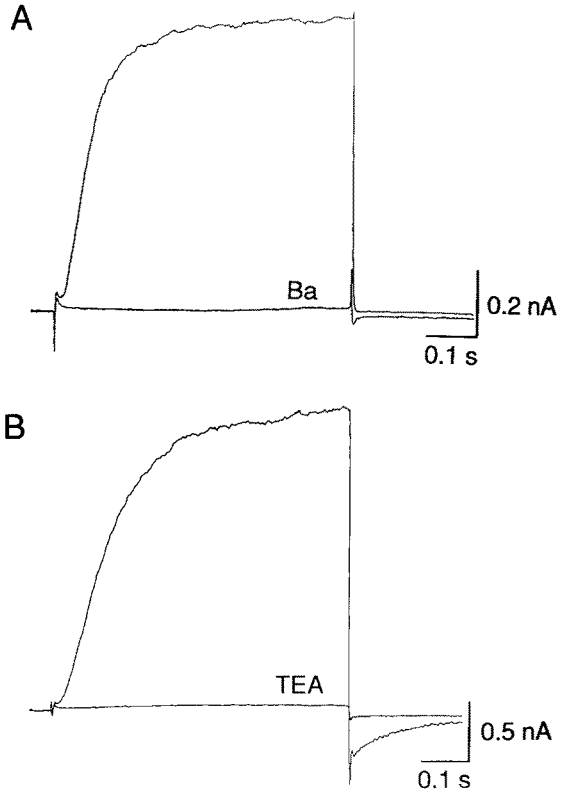


**Fig. 5.** The outward delayed rectifier K current is blocked by external Ba (*A*) and by external TEA (*B*). Superimposed traces for test pulses to 20 and 40 mV (in *A* and *B*, respectively) from a −80 mV holding potential ±20 mM Ba or 20 mM TEA. Separate cells; normal external bath solution and internal KCl (*A*) and high phosphate (*B*) pipette solutions.

$-60 \pm 12$ mV ($n = 54$), not far from the K equilibrium potential (which was calculated using K concentrations to be −85 mV) and following bath addition of Ba to a final concentration of ≈20 mM, a rapid and reversible depolarization of the membrane potential was seen (*see* Fig. 8*A*; Fig. 13*A*). Note that occasionally, with internal pipette solutions containing high phosphate concentrations, resting membrane potentials much more hyperpolarized than the equilibrium potentials for any of the ions present in the solutions appeared, sometimes only transiently, presumably due to the electrogenic activity of the plasmalemmal H-ATPase. An additional difference was apparent in the presence of Ba at very hyperpolarized membrane potentials: irregularly-shaped depolarizations having a fast onset and a slower offset sometimes occurred (Fig. 8*B*; *cf.* Fig. 7*B*). A somewhat similar effect of low concentrations (10 μM) of external Ba has been described in *Eremosphaera viridis* (Thaler et al., 1987), in this case apparently due to the activation of Ca-sensitive K channels. However, our fast, irregular depolarizations at hyperpolarized potentials were much more prominent with increasing Ba concentrations, thus most probably not being associated with an increased K conductance. Furthermore, note that in *Eremosphaera*, external Ba concentrations in the mM range blocked these transient potentials.

When the resting (zero current) potential was hyperpolarized to large negative values using small steady holding currents, voltage responses to depolarizing current pulse injections having a characteristic RC time course were observed for small pulse amplitudes, whereas a distinct repolarizing process was apparent for larger current pulses (Fig. 9*A*). As well, upon cessation of current injection, a shoulder appeared, probably as a result of inward K tail current (*cf.* Fig. 3*B*). After the addition of 20 mM Ba to the bath, both the repolarizing deflection during the current pulses and the shoulder after the current pulses disappeared (Fig. 9*B*), strongly suggesting that these two effects are linked with the K current described above. Note that the plateau responses for the largest current steps in the presence of Ba (Fig. 9*B*) are suggestive of the activation of an additional conductance.

## Action Potential-Like Responses and Inward Currents

In a small number of protoplasts ($n = 9$), large depolarizing current pulses elicited all-or-none, over-

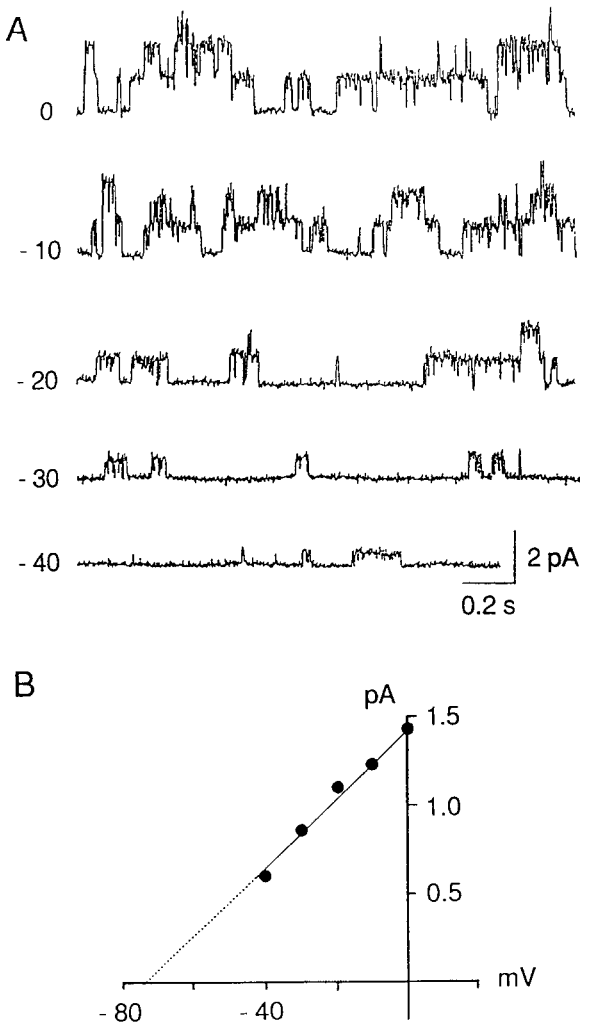


**Fig. 6.** (*A*) Single channel currents recorded from an isolated outside-out patch (numbers indicate the patch membrane potentials in mV; filter 400 Hz). (*B*) The corresponding *I/V* curve for this patch has an extrapolated reversal potential at −73 mV and a slope conductance of 19 pS. Normal external bath and internal KCl pipette solutions.

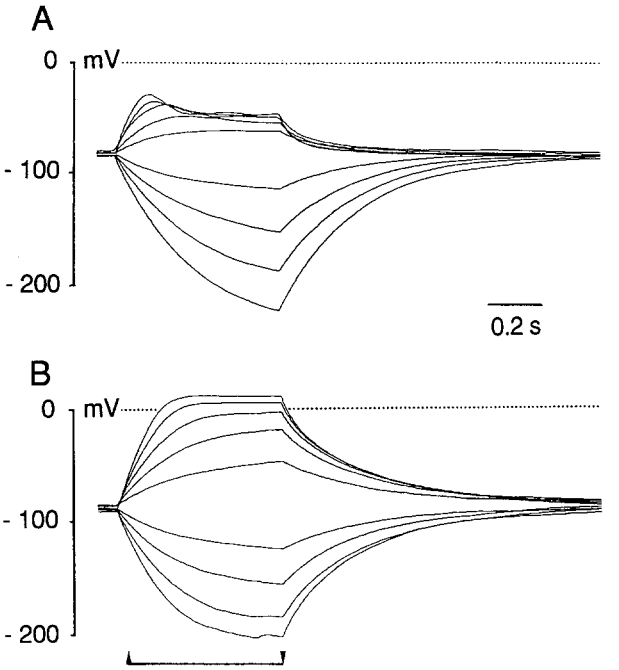


**Fig. 7.** Membrane potential responses in the same cell under current clamp to current steps (±10, 20, 30 and 40 pA; and 50 pA) from a resting membrane potential of about −80 mV, in control conditions (*A*) and after addition of 20 mM Ba to the bath (*B*). Note that the depolarizing voltage responses are shunted in control once the outward K current is activated (*A*) and that they become symmetrical in the presence of Ba (*B*). Normal external bath and internal high phosphate pipette solutions.

shooting spike-like voltage responses under current-clamp (using initially hyperpolarized membrane potentials) as illustrated in Fig. 10. This behavior was clearly different from the passive membrane behavior observed in the rest of the protoplasts studied. The initiation of the spike-like response was associated with a clear increase in the rate of change of membrane voltage (Fig. 10). The threshold for activation was quite variable, being between about −90 and −20 mV (Fig. 10*A*,*B*). A clear shoulder of voltage was also seen upon cessation of current pulses, as mentioned above (Fig. 9*A*).

Under whole-cell voltage-clamp using a −180 mV holding potential, step depolarization resulted in the activation of small transient inward currents in a few cells ($n = 6$), as illustrated in Fig. 11. In four of these cells, further depolarization also evoked the outward-going K current described above. In this particular protoplast, no voltage-dependent currents were observed for steps to −60 and −40 mV; a pure inward current was seen at −20 mV and outward currents were then also activated for larger depolarizations (Fig. 11). In two of the six protoplasts, both action potential-like responses and inward currents were observed. These inward currents and spike-like responses were extremely difficult to characterize, firstly due to the very low percentage of excitable protoplasts, secondly, because of their long absolute refractory period (*see below*), and lastly to the rather transient presence of these responses in the 13 excitable protoplasts studied. For these reasons, it was not possible to establish *I/V* relationships. Nevertheless, the threshold for inward current activation was between −20 and 0 mV in five of the six cells, and was −80 mV in the sixth cell.

Successive depolarizing current injections of the same amplitude produced variable voltage responses which were dependent on the time interval between stimuli. As illustrated in Fig. 12*A*, the initial

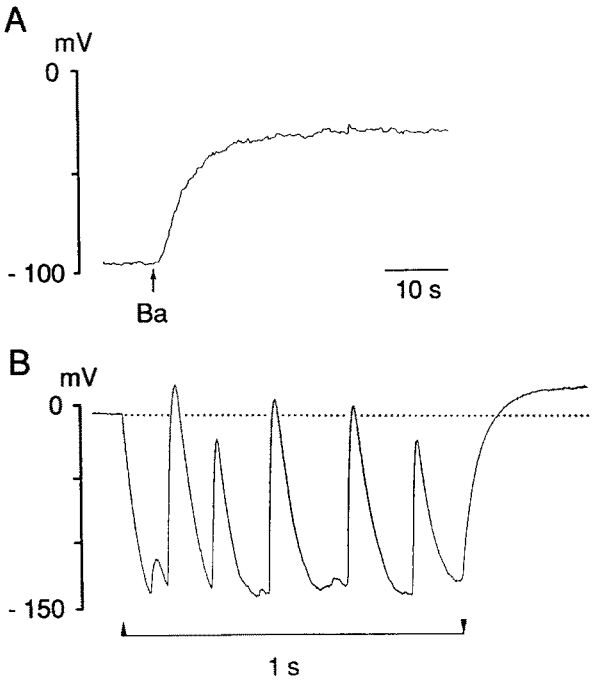


**Fig. 8.** Under current clamp, external Ba (20 mM) application rapidly depolarizes the resting membrane potential (*A*). The depolarization was reversible. In some cells, another effect of external Ba (20 mM) observed during large hyperpolarizing current steps is the appearance of fast, transient depolarizations (*B*). The membrane potential was initially polarized to about 0 mV; note the overshoot in the voltage response at the offset of the current step. Separate cells; normal external bath solution and internal KCl (*A*) and high phosphate (*B*) pipette solutions.

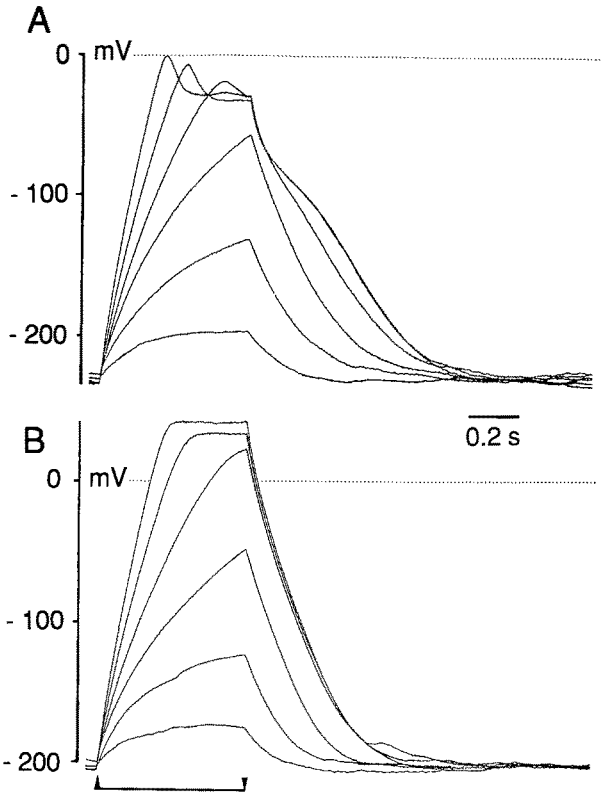


**Fig. 9.** (*A*) Depolarizing current pulses (amplitudes of 5, 10, 15, 20, 25 and 30 pA; 600 msec long) evoke characteristic RC voltage responses under current clamp from a hyperpolarized membrane potential (note the activation of the outward K current shunts the voltage responses and that a pronounced shoulder occurs at the largest current step offset, probably due to a large inward K tail current). In the same cell, after addition of 20 mM Ba to the bath, the repolarizing shunt disappears, as well as the previously observed shoulder (*B*). The voltage plateaus seen in (*B*) for the largest current steps are indicative of the presence of an additional conductance (or possibly, a residual K conductance). Normal external bath and internal high phosphate pipette solutions.

action potential-like response (trace labeled *1*) was obtained 2 min after a previous spike. With stimulation using the same current step amplitude (as for *1*) and ≈1-min interstimulus intervals, action potential initiation was delayed (Fig. 12*A*; trace labeled *2*) and then abolished (trace labeled *3*). In Fig. 12*B*, the initial action potential response (trace labeled *1*) was evoked 2 min after the preceding spike; the same stimulus applied 1 min later was ineffective in triggering a spike (trace labeled *2*), while after an additional delay of 1.3 min, a slightly larger current step was again effective (trace labeled *3*). A similar dependence of inward current amplitude on interstimulus interval was found under voltage clamp. Inward currents evoked by successive voltage steps to 0 and 20 mV separated by 30 sec are shown in Fig. 12*C,D* (traces labeled *1*); when the interstimulus interval was 10 sec, the inward currents were much smaller for the same voltage commands (Fig. 12*C,D*; traces labeled *2*) while the outward K current was unaffected (Fig. 12*C*).

In fact, spike-like responses were detected initially while we were looking under current clamp at the effects of external Ba on the membrane potential. To obtain a final concentration of 20 mM Ba in the 2-ml bathing solution, 40 μl of 1 M Ba stock solution was added to the bath drop by drop, using an Eppendorff pipette. As can be seen in Fig. 13*A*, the addition of drops of the Ba solution provoked artifactual disturbances of the membrane voltage (partly due to the introduction of the hand-held Eppendorf pipette within the Faraday cage). In addition, a clear spike-like response was observed before the depolarizing action of Ba on the resting potential (*cf.* Fig. 8*A*) became apparent (Fig. 13*A*). It was possible to reproducibly elicit spike responses that were closely similar in form in the same protoplast by successive applications of drops (Fig. 13*B*). We first thought that the addition of drops of a concentrated (1 M) Ba solution close to the protoplast under study created

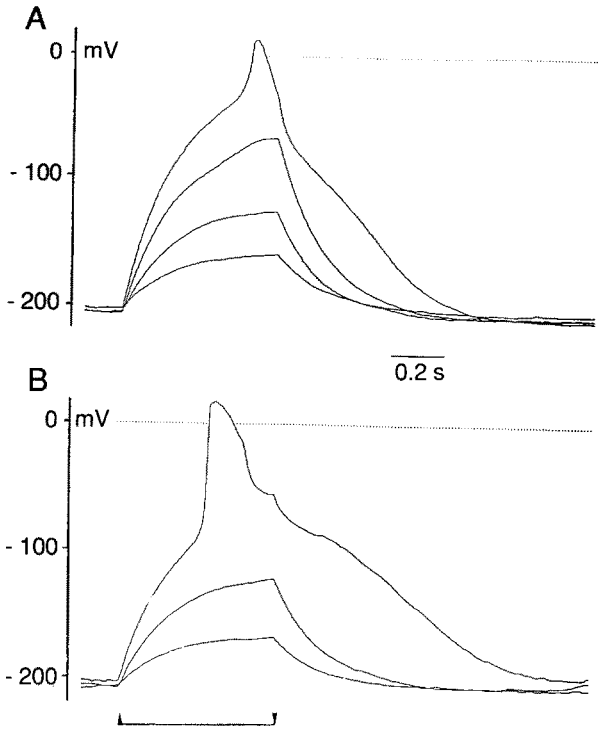


**Fig. 10.** Under current clamp, depolarizing current steps activate all-or-none, spike-like voltage responses in some protoplasts. (*A*) Successive 600 msec long current steps having amplitudes of 5, 10, 15 and 20 pA were given from a hyperpolarized holding potential of about −200 mV. The threshold for the spike-like response was about −40 mV. (*B*) In the same cell, a second series of current steps (successively 5, 10 and 15 pA in amplitude) triggered a spike but with a much lower threshold of about −90 mV. Note the voltage shoulder apparent on the offset of the current pulses triggering the spikes. Normal external bath and internal high phosphate pipette solutions.

an osmotic stress responsible for triggering the spike-like response. However, we succeeded in reproducing this effect in a total of seven cells using drops of the external medium, other iso-osmotic ionic solutions, and even distilled $H_2O$. Thus, the spikes do not seem to be linked to a specific effect of Ba nor to an osmotic shock, but perhaps to a mechanical disturbance of the medium surrounding the protoplast. In the same protoplast, spikes elicited both electrically and by drops have approximately the same shape (Fig. 13*C*). Note that both electrically and "mechanically" evoked spikes and inward currents share the following characteristics: activation from quite hyperpolarized holding potentials, requirement for long interstimulus intervals for successive responses, and are most often observed using high K phosphate internal solutions in cells spontaneously maintaining large hyperpolarized resting potentials.

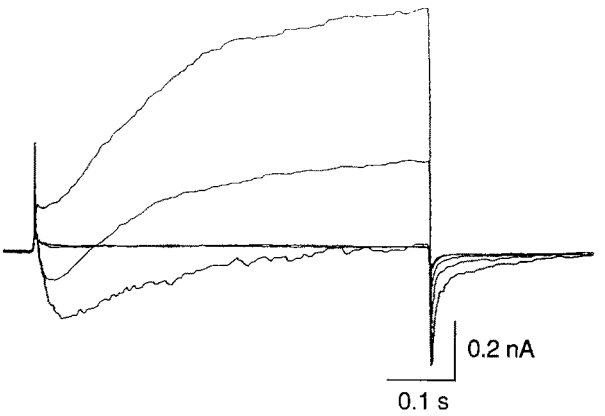


**Fig. 11.** Under voltage clamp, depolarizing steps evoked inward currents in some protoplasts. Holding potential was −180 mV; 600 msec long steps to −60, −40, −20, 0 and 20 mV. The steps to −60 and −40 mV elicited only small baseline leak currents. The step to −20 mV triggered only inward current, while the depolarizating steps to −20 and 0 mV also cause activation of the delayed, outward rectifier K current. Normal external bath and internal low phosphate/KCl pipette solutions.

### Inward Single-Channel Currents Under Whole-Cell Voltage-Clamp

In many cells ($n > 20$), under whole-cell voltage-clamp, inward single-channel currents were infrequently observed at hyperpolarized membrane potentials (Fig. 14*A*). In all cases, the presence of the outward, delayed rectifying K current allowed unambiguous verification that these single channel currents were obtained in the whole-cell recording configuration. In two cells where it was possible to obtain *I/V* relationships, the slope conductance was 110 pS (Fig. 14*B*), with an extrapolated reversal potential of −25 mV (which does not correspond closely with any of the calculated equilibrium potentials for the ionic species present in the solutions used: $E_K = -85$ mV, $E_{Cl} = 56$ mV, $E_{Ca} \geq 170$ mV). The frequency of occurrence of these currents was extremely low under whole-cell recording and thus, perhaps not surprisingly, they were not observed in isolated patches under the same experimental conditions. This rarity may be due to a very low number of channels and/or to particular activation requirements not being met with our experimental protocols and thus, a detailed analysis of these currents was not possible.

## Discussion

The patch-clamp technique (Hamill et al., 1981), as used in studies of the plasmalemma of protoplasts or of the tonoplast of vacuoles, has permitted sig-

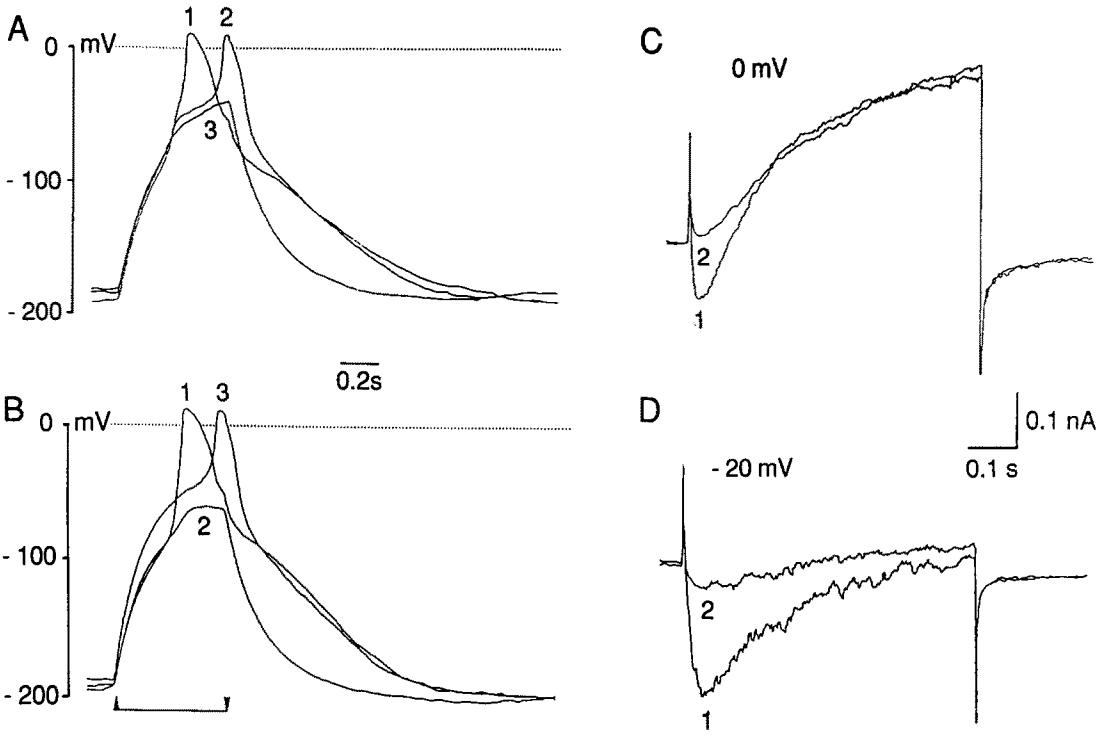


**Fig. 12.** Both depolarization-triggered spikes under current clamp (*A,B*) and inward currents under voltage clamp (*C,D*) are sensitive to the interstimulus interval. (*A*) Membrane potential responses under current clamp to successive 14 pA depolarizing current steps, given after intervals of 2 min (trace labeled *1*) and 1.5 min (*2*) and 1 min (*3*). (*B*) In the same cell, voltage responses to 14 pA current steps after intervals of 2 min (trace labeled *1*) and 1 min (*2*), and after a 18 pA step (*3*) given 1.3 min later. (*C,D*) Currents under voltage clamp evoked from a holding potential of −180 mV for successive command steps to 0 mV (*C*) and to 20 mV (*D*), separated by interstimulus intervals of ≥30 sec (traces labeled *1*) and of 10 sec (traces labeled *2*). Normal external bath and internal high phosphate pipette solutions (*A–D*).

nificant advances in the understanding of the molecular mechanisms controlling membrane ion permeability in higher plant cells (Takeda, Kurkdjian & Kado, 1985; Kado, Kurkdjian & Takeda, 1986; Hedrich & Schroeder 1989; Hedrich, Stoeckel & Takeda, 1990*b*; Stoeckel & Takeda, 1990; Tester, 1990). Aside from the obvious advantages of minimizing penetration-associated leakage conductances and allowing precise control over the composition of solutions on both sides of the membrane, the technique avoids uncertainties which arise due to the compartmentalization of plant cells (i.e., the series organization of the plasmalemma and the tonoplast, especially prominent in differentiated cells) and to the intercellular electrical connections formed by plasmodesmata in intact tissue. However, it should be noted that making protoplasts may induce many changes in the properties of plant cells, due to the osmotic shock involved in this procedure and/or to the action of the enzymes at the plasmalemma. As well, numerous plasmodesmata in areas where the cell wall is thinner are found in pulvinar motor tissue, and thus damage may also occur due to the disruption of these intercellular connections.

## Outward, Delayed Rectifier Potassium Current

A delayed, outward rectifying K conductance activated by depolarization is the most prominent membrane response observed in isolated pulvinar protoplasts from *Mimosa pudica*. Many of the characteristics of this current (block by external Ba and TEA and by internal Na) are similar to those found for other outward, delayed K rectifiers, both in animal (Hille, 1992) and plant cells. For example, in protoplasts from excitable, trap-lobe motor cells of *Dionaea muscipula*, an outward, delayed rectifying K current activated by depolarization and blocked by external TEA and Ba was described (Iijima & Hagiwara, 1987). Single channel conductances of 3–4 pS in symmetrical 30 mM K solutions were found. A similar voltage-dependent, outward K current has been characterized in protoplasts from non-excitable, pulvinar motor cells of *Samanea saman* (Moran et al., 1988; Moran, Fox & Satter, 1990). The single channel conductance ranged from 15 to 40 pS for external K concentrations between 5 and 125 mM. In protoplasts from guard cells of *Vicia*

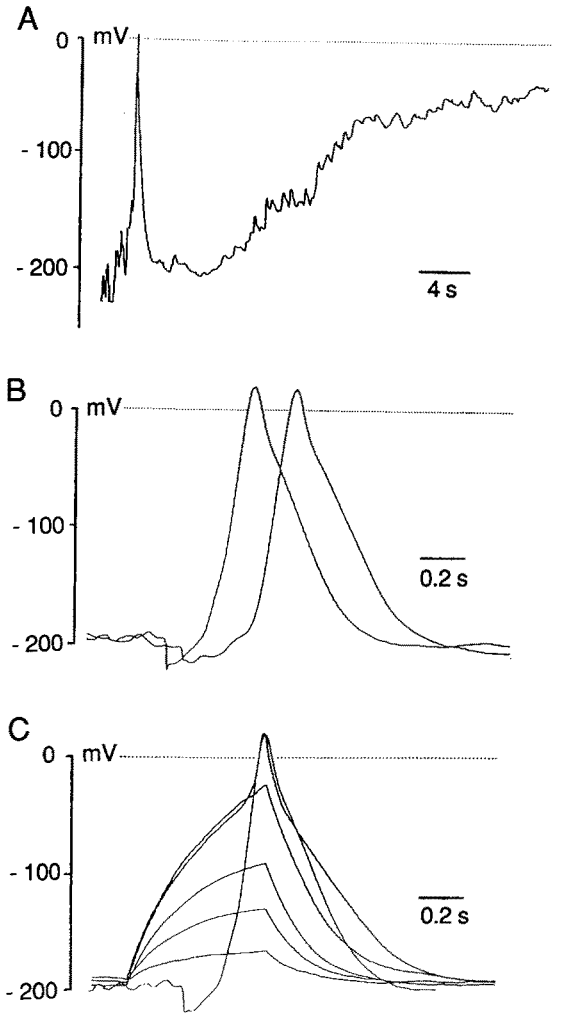


**Fig. 13.** (*A*) Under current clamp in some cells, drop-wise addition of 40 $\mu$l of $BaCl_2$ (1 M solution) to the bath elicits a rapid spike-like depolarization followed by a slower, long-lasting depolarization of the resting potential. (*B*) Superimposed spikes triggered in another cell by successive addition of drops of $H_2O$. (*C*) A family of voltage responses to depolarizing current steps (5, 10, 15 and twice, 20 pA in amplitude given every 10 sec; note only the first 20 pA step triggered a spike) in the same cell as in (*B*), with a spike evoked by a drop of $H_2O$ superimposed (the peaks of the spikes were aligned). Normal external bath and internal high phosphate pipette solutions.

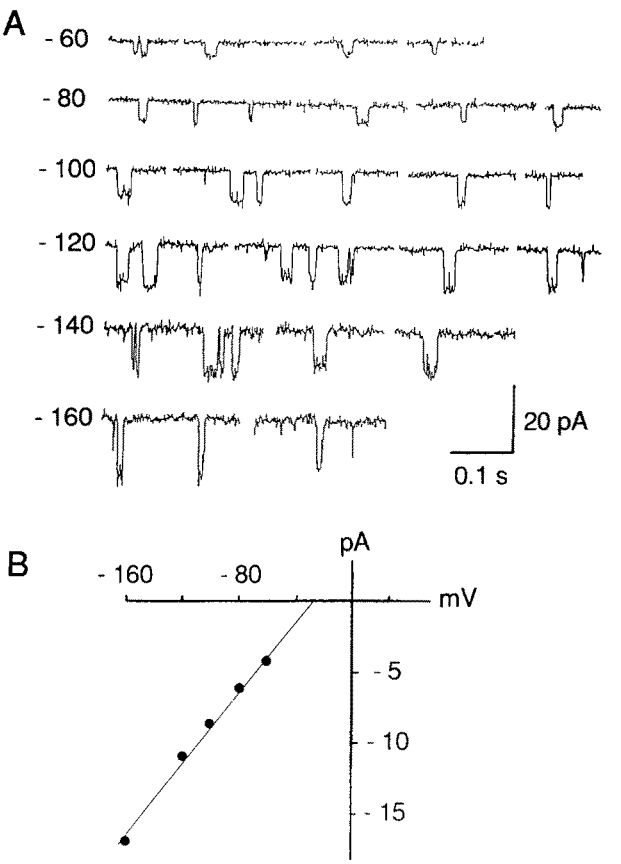


**Fig. 14.** Inward single channel currents observed in the whole-cell recording configuration. (*A*) Selected examples of inward currents at the indicated membrane potentials (*B*) Corresponding *I/V* relationship with an extrapolated zero current potential near −25 mV and a slope conductance of 110 pS. Normal external bath and internal KCl pipette solutions; filter 700 Hz.

*faba* (Schroeder, Raschke & Neher, 1987; Schroeder, 1988, 1989; Schroeder & Hagiwara, 1989), a depolarization-activated, outward rectifying K current having sigmoid activation and voltage-dependent tail current deactivation kinetics has been well characterized. This current is blocked by external TEA and Ba. A single channel conductance of 20 pS was found in symmetrical 105 mM K solutions. In protoplasts from guard cells of *Zea mays* (Fairley-Grenot & Assmann, 1992), a similar outward K rectifier was reported. In protoplasts derived from the cotyledons of *Amaranthus* seedlings (Terry, Tyerman & Findlay, 1991), an outward delayed rectifying K current was reported and a single channel conductance of ≈30 pS in symmetrical 100 mM K solutions was found. A similar K current was described in protoplasts of suspension-cultured corn cells (Ketchum & Poole 1990, 1991; Fairley, Laver & Walker, 1991). This current was inhibited by external TEA, Ba, Cs, quinine and charybdotoxin. In protoplasts from suspension-cultured *Asclepias tuberosa* cells (Schauf & Wilson, 1987), an outward time-dependent, depolarization-activated K current blocked by external Cs and TEA was observed. The outward currents were absent with Cs or Tris in the internal solution, and a single channel conductance of 40 pS was found. Thus, as is evident, an outwardly directed, delayed rectifier K current activated by depolarization is a most commonly found feature of protoplasts.

In *Mimosa* and in other sensitive plants, the

transmembrane potential of excitable cells reaches very depolarized levels during the action potential (Abe & Oda, 1976; Samejima & Sibaoka, 1982), thus leading to activation of the delayed K rectifier. As is clearly seen from our current-clamp data, this outward current results in membrane repolarization. This K efflux very likely represents a major part of the total ion fluxes which drive rapid movements (Samejima & Sibaoka, 1980; Kumon & Suda, 1984), and more generally, turgor regulation (Hill & Findlay, 1981; Schroeder & Hedrich, 1989). On the other hand, in nonexcitable cells, membrane depolarization is known to be an early response to various environmental stimuli, for example, during elicitation (Pelissier et al., 1986) or wound responses (Julien et al., 1991). As well, in certain cell types, stretch-activated channels permeant to Ca or Cl have been described (Falke et al., 1988; Cosgrove & Hedrich, 1991; Moran, 1991), and their activation by mechanical stresses or by changes in turgor would presumably lead to a sustained depolarization. If sufficient, the resultant activation of the delayed K rectifier would cause membrane repolarization and K efflux.

### Action Potentials and Depolarization-Activated Inward Currents

In marked contrast, in a clearly minor fraction of the protoplasts studied, action potential-like responses were produced by depolarization and/or mechanical stimulation. This is similar to the *in situ* physiology of these motor cells, where action potentials can be elicited by a variety of stimuli, including depolarization and mechanically applied pressure (*for review*, Roblin, 1979). The underlying depolarization-triggered, transient inward currents, although observed only in a few cells, shared a number of common characteristics: a quite negative holding potential (about $-180$ mV) was necessary; the activation threshold was between $-20$ and 0 mV in five of the cells (in one case, threshold was around $-80$ mV) and there was a long refractory period ($\geq$30 sec). *In situ*, excitable motor cells of the pulvinus have very hyperpolarized resting potentials (Abe & Oda, 1976; Samejima & Sibaoka, 1982), as is the case for excitable cells in the parenchyma of the vascular bundle of the primary petiole (Sibaoka, 1962). In the petiole and in the primary pulvinus, action potentials are followed by a long absolute refractory period, lasting more than 10 times the duration of the spike (Umrath, 1937).

We have no direct evidence accounting for either the low frequency of occurrence or the transitory presence of the inward currents and the spike-like responses in any given responsive cell. All the protoplasts were obtained from the abaxial cortical parenchyme of primary pulvini (Fig. 1*A*), all of which displayed normal mechanical sensitivity in the intact plant. Although the protoplasts used were somewhat variable in size ($\approx$35–50 $\mu$m in diameter), by eye, their overall morphology and intracellular organization (Fig. 1*D*) were not obviously different at 300$\times$ under phase contrast optics. Nevertheless, a remote possibility may be that not all cells found in the tissue used to obtain protoplasts are excitable. More likely is that excitability is lost either during protoplast preparation which involves osmotic shock, rupture of plasmodesmata and exposure to enzymes or is inhibited by our experimental conditions. Note that under whole-cell recording, the cell is perfused by the pipette internal solution, and that a putative factor necessary for maintaining excitability may have been effectively diluted out, as in another patch-clamp study of protoplasts from excitable motor tissue (Iijima & Hagiwara, 1987), where similarly, only an outward, delayed rectifying K current was observed. A last possibility could be related to variability in the physiological status of individual plants, as even though all plants used displayed normal mechanical sensitivity, inward currents and spike-like responses were observed preferentially during two time periods (March–April and August).

All the inward currents and spike-like responses (in a total of 19 cells) were observed using a K phosphate pipette internal solution containing MgATP (*see* Materials and Methods). However, due to the low frequency of these events, it is not possible to make any real correlation with this particular internal solution. Nevertheless, certain characteristics of this solution may be favorable for action potential generation, as found in perfused tonoplast-free cells of *Chara australis* where low internal Cl concentrations (<30 mM) were required for excitability (Shimmen & Tazawa, 1980). Measured values of internal Cl in *Characea* are between 10–30 mM (Tazawa, Kishimoto & Kikuyama, 1974). When we used a high KCl internal pipette solution, no spike-like responses or inward currents were ever observed, although in a few cases ($n = 5$; *not shown*) under current clamp, depolarizing current injections induced the development of another type of depolarizing conductance, which was stable and long-lasting (up to 2 sec).

In most cases, "excitable" protoplasts were also characterized by a very negative resting membrane potential of about $-180$ mV (as measured in current-clamp), more hyperpolarized than any equilibrium potential for the ions present in our solutions. This suggests that the activity of the plasmalemmal H-ATPase may be favored by the use of

the high K phosphate, MgATP-containing internal pipette solution, although again, the very low numbers of cells having this behavior preclude any definitive interpretation. Note that our protoplasts can have very large input resistances (*cf.* Fig. 10) and that any small electrogenic pump current would be shunted by leakage conductances associated with whole-cell recording that are greater than the resting input conductance of intact cells. Alternatively, the few cells displaying large negative resting potentials may be representative of those relatively unaffected by the procedures involved in protoplast preparation. Another peculiarity associated with high phosphate internal solutions was that the threshold for activation of the outward K rectifier was shifted to more depolarized potentials, $\approx -20$ mV (*cf.* Fig. 3*A,B*) compared to $\approx -50$ mV in internal KCl solutions (Fig. 2), and secondly, that the apparent tail current reversal potential was also shifted rightwards, as is evident from the large inward tail currents at $-80$ mV (Fig. 5*B*). A possible explanation for this rightward shift may be that $E_K$ is less hyperpolarized than would be expected from calculation using the apparent K concentrations in the phosphate solutions (which was even higher than in the KCl solutions), as the K activity measured by a K-selective electrode was lower than expected (perhaps due to partial dissociation of the K phosphate salts).

In any case, it was thus unfortunately possible to characterize in detail neither the current-voltage relationship nor the nature of the charge carrier for the transient inward current. An anionic inward current having a similar activation threshold was described in *Vicia faba* guard cell protoplasts (Keller, Hedrich & Raschke, 1989; Hedrich, Busch & Raschke, 1990*a*), although there was apparently not the same long absolute refractory period nor the necessity for large hyperpolarized holding potentials. A similar *I-V* relationship has been described for the inward Ca-activated Cl current which likely underlies action potential generation in *Characeae* (Shiina & Tazawa, 1987; Okihara et al., 1991). In motor cells of *Samanea saman*, a preliminary description has been made of a short-lived, depolarization-activated inward current, tentatively ascribed to Ca influx (Moran & Satter, 1989). Also, in protoplasts of suspension-cultured corn cells, an unidentified, inward current with a similar threshold was reported for one cell (Fairley et al., 1991). As for the nature of the permeant ionic species responsible for the inward current described here, only Cl, $PO_4$, Ca and H are possible candidates. Since the Cl equilibrium potential was usually near 0 mV for the solutions used in this set of experiments, the presence of robust inward currents in this potential range would seemingly exclude Cl as the charge carrier, although this would be surprising if indeed true (*cf.* Hill & Findlay, 1981). Another somewhat unlikely possibility would be $PO_4$ efflux through putative Cl channels. By comparison with animal cells (Hille, 1992), the observed inward current threshold in our cells could fit with the activation of a voltage-dependent Ca current, as has been tentatively suggested for *Samanea* motor cells (Moran & Satter, 1989). Lastly, in certain molluscan neurones, depolarization to potentials more positive than $\approx -10$ mV results in an increase in membrane H conductance (Thomas & Meech, 1982; Byerly, Meech & Moody, 1984). As the external solution pH is 5.5 and the internal solution pH is 7.2, such an increase in H conductance, if present, would give rise to an inward H current. Clearly, more work is required in order to discriminate between these various possibilities.

In higher plants, action potentials have been described in various organs of several species, mostly associated with the induction of rapid movements (*for review*, Sibaoka, 1969), but not always (*for review*, Pickard, 1973). However, while net ion efflux of K and Cl are largely believed to drive osmotically based movements (Hill & Findlay, 1981), almost no evidence is available directly demonstrating the ionic nature of the underlying inward current in higher plant cells. Thus in *Mimosa*, after action potential-associated rapid movements, efflux of K and Cl has been measured in the medium surrounding the pulvinus (Samejima & Sibaoka, 1980; Kumon & Suda, 1984) and blockers of K and Cl channels inhibit movement (Roblin & Fleurat-Lessard, 1987). A reduced spike amplitude was observed when external Cl was increased in the medium surrounding excitable cells of the primary petiole and pulvinus (Samejima & Sibaoka, 1982). In other species, action potentials are also known to be sensitive to Ca. For example, in *Aldrovanda vesiculosa*, increasing external Ca shifted the peak depolarization of the action potential (Iijima & Sibaoka, 1985), while in *Dionaea* leaves, action potentials of sensory and mesophyll cells are strictly dependent on external Ca (Hodick & Sievers, 1988). A reasonable inference would be that in *Mimosa* pulvinar cells, Ca-sensitive Cl channels are responsible for generating the inward current associated with action potential activity and the resultant rapid movement (Okazaki & Tazawa, 1990).

Whatever the eventual identity of the inward current charge carrier, it is clear that isolated pulvinar motor cells from *Mimosa* are a valuable experimental model for the analysis of mechanisms involved in signal perception and membrane transduction. It will be of interest to characterize further the properties of these excitable cells compared to

other cells responding to similar environmental signals but which are known to be nonexcitable, for example pulvinar motor cells from *Samanea saman* (Satter et al., 1988) or stomatal guard cells (MacRobbie, 1988; Schroeder & Hedrich, 1989). As we have only occasionally observed mechanically or stretch-activated currents (*not shown*) in our cells and such activity likely being an inherent property of sensitive plants, further investigation of these phenomena obviously is merited. If inward depolarizing current results from the activation of such mechanically sensitive channels, this may provide the link between a mechanical stimulus and the triggering of action potentials in motor cells which drive rapid movements. Finally, of particular importance will be the direct demonstration of the exact role of transmembrane Ca currents in regulating changes in cell volume (Okazaki & Tazawa, 1990).

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