Vol. 145, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1987 Pages 284-290

EFFECTS OF ABSCISIC ACID ON K^+ CHANNELS IN *VICIA FABA* GUARD CELL PROTOPLASTS

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Received April 8, 1987

Potassium channels were resolved in *Vicia faba* guard cell protoplasts by patch voltage-clamp. Whole-cell currents and single K^+ channels had linear instantaneous current-voltage relations, reversing at the calculated Nernst potential for K^+. Whole cell K^+ currents activated exponentially during step depolarizations, with half-activation times of 400-450 msec at +80 mV and 90-110 msec at +150 mV. Single K^+ channel conductance was 65 ± 5 pS with a mean open time of 1.25 ± 0.30 msec at 150 mV. Potassium channels were blocked by internal Cs^+ and by external TEA^+, but they were insensitive to external 4-aminopyridine. Application of 10 uM abscisic acid increased mean open time and caused long-lasting bursts of channel openings. Since internal and external composition can be controlled, patch-clamped protoplasts are ideal systems for studying the role of ion channels in plant physiology.

Plant hormones regulate growth and physiological responses, but their mechanism of action is unknown. Binding proteins for abscisic acid (ABA) exist in various cells (1) and there is much indirect evidence that plant hormones affect membrane permeability (2-7). Cation and anion selective ion channels have been demonstrated in plant cells by conventional and patch voltage-clamp (8-18), and plant hormones were shown to affect nonspecific membrane permeability and ion channels in model systems (19-21). *Vicia faba* guard cell K^+ channel conductance is large enough to produce net fluxes sufficient to account for ABA-dependent stomatal closure (17,22). In bilayers composed of several types of natural and synthetic phospholipids, ABA enhanced permeability to urea and erythritol (19,20,23), potassium (24), chloride (25) and praeseodymium (26), with ABA proposed to interact at membrane defects (27). In related studies, ABA enhanced phospholipid vesicle aggregation (20), increased electrical conductivity of planar bilayers (28) and increased water flux into root tissue (29-32). In this study we employed whole-cell and patch voltage-clamp to demonstrate a voltage-dependent, ABA-sensitive K^+ channel in *Vicia faba* guard cell protoplasts.

0006-291X/87 $1.50

METHODS

Guard cell protoplasts were derived from leaves of broad bean, Vicia faba. Bean seeds were soaked overnight and planted in Pro-Mix BX (Hummert Seed Co.) and grown at 23 °C under 24 hour light in a growth chamber. Leaflets from 3 week old plants were cleaned in 3% calcium hypochlorite plus several drops of Tween 80 for 10 minutes and rinsed 3 times in basal medium (BM) containing 550 mM sorbitol and 5 mM MES adjusted to pH 5.8 with KOH (33). All solutions were filter-sterilized through 0.22 um filters (Millipore). Leaflets were vacuum infiltrated for 3 minutes with 0.8% macerase (Calbiochem) and 1.0% dextran sulfate in BM and incubated in this solution at 25 °C for 20 minutes on a reciprocal shaker. Epidermal peels from the abaxial leaf surface were taken under a sterile hood and incubated in 0.5% macerase, 1.0% cellulysin (Calbiochem) and 0.5% dextran sulfate in BM for 12 hours on a reciprocal shaker (set at low speed) at 25 °C. To purify guard cells, the incubation medium was filtered through a 70 um nylon mesh to remove debris. Cells were centrifuged at 2000 rpm for 10 minutes in a clinical centrifuge, washed in BM and recentrifuged at 2000 rpm for 3 minutes twice. The last pellet was resuspended in a minimum amount of a solution containing 100 mM NaCl, 300 mM sorbitol, 2 mM $CaCl_2$ and either 0.7 mM $NaHPO_4$ or 5 mM MES adjusted to pH 5.8. Although protoplasts from mesophyll and epidermal cells were released during digestion, only a few mesophyll protoplasts were present in the final suspension.

Single channel recordings and whole-cell voltage-clamp were achieved using standard techniques (34). Patch pipettes with resistances of 8-12 Megohms were brought into contact with protoplasts until resistance increased to 50-100 Megohm, with suction then promoting high resistance seal (10-50 Gigohm) formation. Whole cell currents could be reliably recorded in about 25% of the cells examined, but Gigaseals were achieved only 5-10% of the time. Soft soda-lime glass (200 lambda, 2mm capillary pipettes, Clay-Adams Scientific) gave the best results. Once high resistance seals were formed, inside-out excised patches could be obtained. The internal (pipette) solution was 100 mM KCl or CsCl, buffered with 5 mM $NaHPO_4$ or Hepes to pH 5.8. The external solution was 100 mM NaCl, again at pH 5.8. Both solutions contained 2 mM $CaCl_2$ and experiments were performed at room temperature. Currents were filtered at 3-10 KHz by a 4 pole Bessel filter, monitored with a List EPC-7 patch-clamp and sampled at 33 usec via a Teckmar Labmaster board. Single-channel data was plotted as amplitude and duration histograms, with conductance and mean open times determined by pCLAMP (Axon Insturments). Series resistance was 80-90% compensated and capacity currents subtracted by the analog circuitry in the EPC-7. Leak currents were subtracted using a divided pulse (P/5) protocol, with reference pulses in regions where there was no ion channel activation or inactivation. Reversal potentials were determined under biionic conditions using Tris as an impermeable cation and selectivity ratios estimated after correction for ion activity. In some experiments concentrated solutions of tetraethylammonium (TEA^+) and 4-aminopyridine (4-AP) were added to give a final concentration of approximately 10 mM, while 10 uM abscisic acid (Sigma Chemical) was applied using a microperfusion pipette placed 20-30 microns from the patch-clamped protoplast. Potentials are given assuming the protoplast exterior to be at zero voltage, and data is expressed as means ± standard errors for 5-8 independent experiments.

RESULTS

Under whole-cell patch-clamp Vicia faba guard cell protoplasts exhibited a voltage-dependent outward current, with an activation threshold of 50-60 mV (Figure 1A). Outward currents were completely inhibited when 10 mM TEA^+ was added to the bathing solution and when Cs^+ was substituted for K^+ in the patch

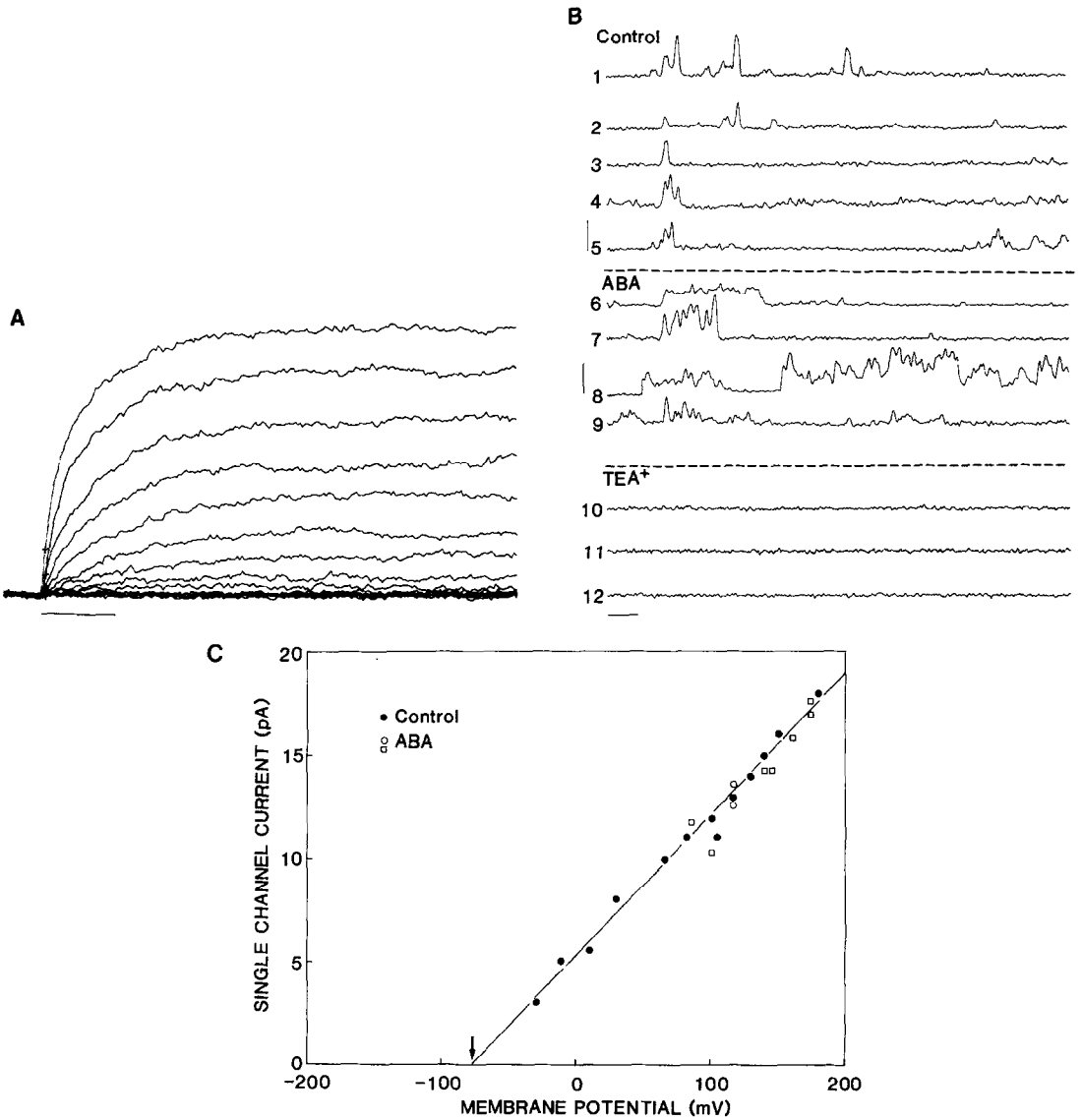

Figure 1. Part A shows whole-cell outward K^+ currents in Vicia faba guard cell protoplasts for depolarizations of 10-150 mV (10 mV increments). The pipette solution was 100 mM KCl, 2 mM $CaCl_2$ buffered with 5 mM Hepes to pH 5.8, while the bath contained 100 mM NaCl. Current and time calibrations are 100 pA and 500 msec respectively. Part B shows single channel K^+ currents in an inside-out patch held at a potential of +100 mV relative to the grounded bathing solution before (control), during, and after (recovery) application of 10 uM ABA. Current and time calibrations are 5 pA and 10 msec respectively. The numbers refer to consecutive traces acquired in an event-triggered mode. Part C shows the single-channel current-voltage relation obtained under the same conditions. The solid line is the best least-squares fit for a single-channel conductance of 65 ± 5 pS.

pipette, but were not affected by the external application of the K^+-channel blocker 4-aminopyridine (10 mM). The time-dependent activation of outward current was characterized by half-activation times, with typical values being 400-450 msec at +80 mV, 200-225 msec at +100 mV, and 90-110 msec at +150 mV.

In symmetrical 100 mM KCl solutions the instantaneous current-voltage relation for the time-dependent outward whole-cell current was linear and reversed at a potential of 2 ± 4 mV. When the external solution was 100 mM NaCl and the pipette contained 100 mM KCl the reversal potential was -80 ± 4 mV, giving a K^+/Na^+ selectivity ratio of approximately 20:1.

Outward whole-cell K^+ currents did not inactivate during depolarizing pulses as long as 2.5 seconds, but when guard cell protoplasts were held at a potential of 100 mV for 20-40 seconds the magnitude of the outward current was reduced by 50-70% with no change in its time course. At a holding potential of 200 mV, outward currents could be abolished, while a holding potential of -100 mV increased the steady-state outward current by 30-50%, suggesting the presence of a slow inactivation process.

Single-channel currents could be observed in the cell-attached mode and in excised inside-out patches. Figure 1B shows typical single channel K^+ currents in an inside-out patch held at +100 mV (pipette potential of -100 mV relative to the grounded bath) before (control), during (labelled ABA), and after (recovery) application of 10 uM ABA. Under control conditions (traces labelled 1-5) there was one high-conductance channel and one or more channels of lower conductance that we were unable to resolve. In some cases (end of trace 5), activity appeared in bursts that could not be characterized.

Extrapolation of single-channel currents corresponding to the large channel measured at holding potentials from -12 mV to +175 mV yielded a reversal potential of -81 ± 7 mV when the pipette (external) solution was 100 mM NaCl and the bath (internal) solution was 100 mM KCl, both in control conditions and in the presence of ABA (Figure 1C), suggesting that this was indeed a K^+-selective channel. Single channel K^+ conductance averaged 65 ± 5 pS in 7 patches and was independent of voltage over the range -12 mV to +150 mV. As for whole-cell currents, single K^+ channels were insensitive to 10 mM 4-aminopyridine, but were blocked by 10 mM TEA^+ (Figure 1B, traces 10-12) and could not be observed when Cs^+ was present in the patch pipette. Figure 2A shows the interval histograms obtained from the same patch as in Figure 1C. Prior to ABA, mean open time was voltage-dependent, averaging 0.60 ± 0.15 msec at +80 mV, 0.80 ± 0.12 msec at +100 mV, and 1.25 ± 0.18 msec at +150 mV.

Following addition of 10 uM ABA there was a dramatic increase in the number of channel openings, with many occuring in bursts lasting 50-100 msec and sufficiently complex that open times could not be accurately characterized (traces 6-10 in Figure 1B). Analysis restricted to only well-separated single openings yielded mean open times of 1.20 ± 0.24 msec at +80 mV and 2.05 ± 0.22 msec at +150 mV, with the corresponding interval histogram illustrated in Figure 2B. It should be noted, however, that the histogram in the presence of

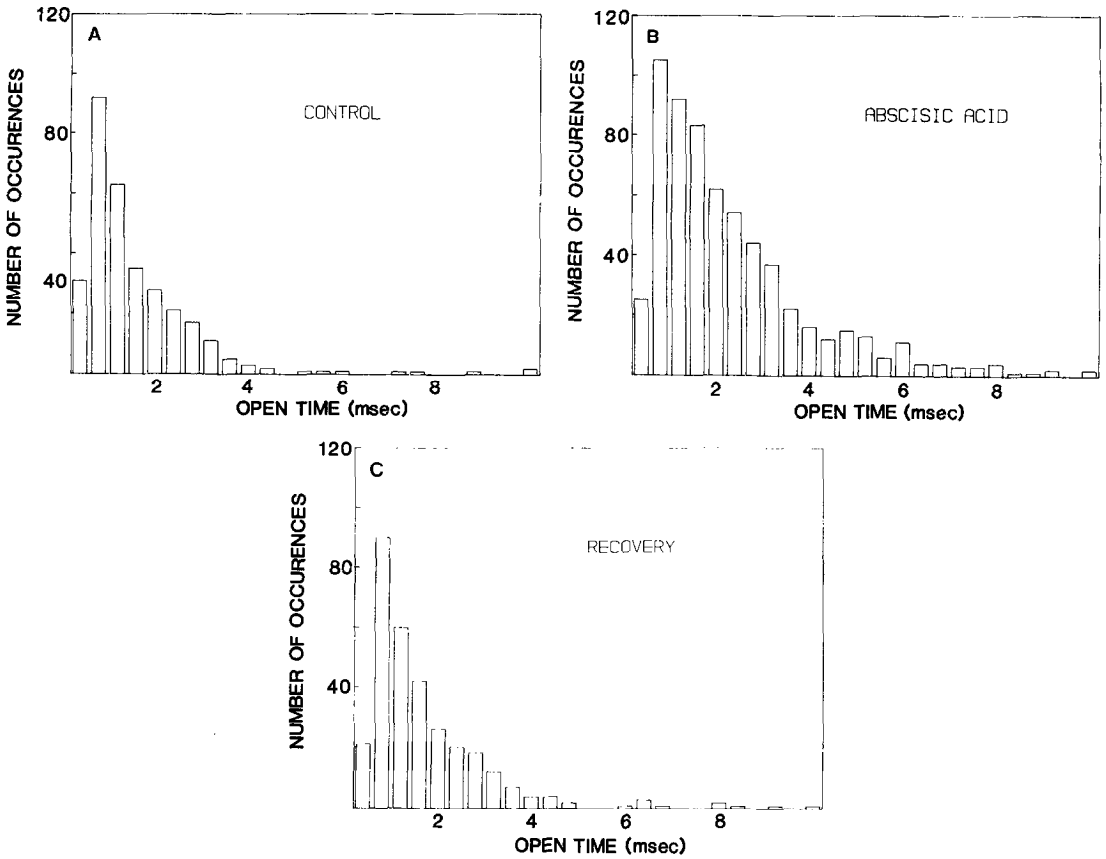

Figure 2. Duration histograms for single-channel K^+ currents in an excised inside-out patch held at a potential of 120 mV before (control), during and after (recovery) from 10 uM ABA.

ABA was not strictly exponential with an increased proportion of longer openings. The changes induced by ABA were completely reversible, as shown by the histogram in Figure 2C. The number of channels appearing during a 4 second period of continuous data acquisition also was increased by ABA. At +120 mV, 264 events were recorded before ABA, 375 in the presence of 10 uM ABA, and 189 during the recovery interval. While tested in only a few cells, there appeared to be no effect of the plant hormones kinetin (100 uM) and indole-3-acetic acid (IAA; 100 uM) on either whole-cell or single-channel K^+ currents.

DISCUSSION

Vicia faba guard cell protoplasts have voltage-dependent, ABA-sensitive K^+ channels with a single-channel conductance of 65 ± 5 pS and open time of 1.25 ± 0.30 msec at +100 mV. These K^+ channels were not affected by the K^+ channel blocker 4-aminopyridine, but were blocked by internal Cs^+ and external 10 mM TEA^+. The single-channel conductance observed here is twice that seen in a prior study (17), which also reported a mean open time at +60 mV of 10 msec,

though the 20:1 K^+/Na^+ selectivity obtained here is comparable. It may be that choice of glass or another variable affects these parameters, or that one of the smaller K^+ channels present in the records of Figure 1A corresponds to these observations. Nevertheless, the fact that ABA increased the number of single-channel events and mean open time, and induced dramatic long-lasting bursts of activity, suggests that the K^+ channel we observed here is physiologically significant. In particular, enhancement of K^+ permeability by ABA would tend to facilitate K^+ efflux and thus stomatal closure.

Effects on ion channels could contribute to intracellular plant hormone actions, as substantial evidence suggests a role for Ca^{++}-dependent second messengers in plants (35,36). For example, an ABA-dependent increase in K^+ permeability could activate or inhibit second messenger systems if these K^+ channels leak sufficient Ca^{++} to cause a threshold increase in intracellular Ca^{++}, or cause Ca^{++} release from plasma-membrane-associated or intracellular binding sites (37). In any case, the results reported here point out the potential usefulness of patch-clamped protoplasts as model systems for studying plant physiology.

(Supported by National Science Foundation Grant BNS 86-96095 to C.L.S., by an Indiana Academy of Sciences Grant to K.J.W., and by the Project Development Program of Indiana University)

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