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Properties of Single K^+ and Cl^- Channels in *Asclepias tuberosa* Protoplasts[1]

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ABSTRACT

Potassium and chloride channels were characterized in *Asclepias tuberosa* suspension cell derived protoplasts by patch voltage-clamp. Whole-cell currents and single channels in excised patches had linear instantaneous current-voltage relations, reversing at the Nernst potentials for K^+ and Cl^-, respectively. Whole cell K^+ currents activated exponentially during step depolarizations, while voltage-dependent Cl^- channels were activated by hyperpolarizations. Single K^+ channel conductance was 40 ± 5 pS with a mean open time of 4.5 milliseconds at 100 millivolts. Potassium channels were blocked by Cs^+ and tetraethylammonium, but were insensitive to 4-aminopyridine. Chloride channels had a single-channel conductance of 100 ± 17 picosiemens, mean open time of 8.8 milliseconds, and were blocked by Zn^{2+} and ethacrynic acid. Whole-cell Cl^- currents were inhibited by abscisic acid, and were unaffected by indole-3-acetic acid and 2,4-dichlorophenoxyacetic acid. Since internal and external composition can be controlled, patch-clamped protoplasts are ideal systems for studying the role of ion channels in plant physiology and development.

Plant hormones regulate developmental events and physiological responses (5, 7, 12, 15), but factors linking hormonal signals and intracellular events are not well defined. In animals, hormones affect receptors coupled to transmembrane ion channels, with subsequent fluxes or permeability-dependent voltage shifts altering intracellular second messenger systems. Plant cells have been shown to contain both cation and anion-selective channels by conventional voltage-clamp (2, 3, 11, 13, 26, 30), and there is evidence of Ca^{2+}-dependent second messengers (1, 5, 14, 18–20). Plant hormones also affect nonspecific membrane permeability and ion channels (23, 24, 29).

Until recently cell walls hindered direct biophysical studies of ion channels in plants, but patch-clamp of enzymically isolated protoplasts has made it possible to resolve single ion channels (16, 27). *Vicia faba* guard cells were shown to have K^+ channels with a conductance of 20 to 30 pS[2] (27), large enough to produce net K^+ fluxes sufficient to account for ABA-dependent stomatal closure (15, 28). In a related study, K^+-selective cyclic polyethers inhibited stomatal opening, presumably by short-circuiting K^+ channels normally present (12). Four distinct channels were observed in wheat protoplasts including a 35 to 40 pS voltage-dependent channel and a larger 160 to 180 pS channel (16), the latter having a conductance similar to Ca^{2+}-activated K^+ channels in animal cells. Channels carrying maleate and K^+ were seen in vacuoles from barley leaf mesophyll protoplasts (9), while TEA^+-sensitive and TEA^+-insensitive K^+ channels with single-channel conductances of 100 and 30 pS, respectively, have been observed in protoplasmic droplets from *Chara corallina* (11).

We used whole-cell and excised-patch voltage-clamp to demonstrate voltage-dependent TEA^+ and Cs^+ sensitive K^+ channels, as well as Zn^{2+} and ABA-sensitive Cl^- channels in protoplasts derived from *Asclepias tuberosa* suspension cell cultures. The presence of hormone-responsive ion channels in an experimental situation that permits control of internal and external composition should prove ideal for studying the role of ion channels in plant physiology and development.

MATERIALS AND METHODS

Cell suspension cultures were derived from callus of *Asclepias tuberosa* by methods previously described (4, 21). Suspension cultures were maintained on a rotary shaker in liquid Murashige-Skoog medium (17), supplemented with 5.0 mg/L adenine, 0.5 mg/L 2,4-D, 0.5 mg/L benzyl adenine, 0.1 gm/L myoinositol, 0.4 gm/L casein hydrolysate, and 2% sucrose (pH 5.8, see Ref. 4). Cultures were maintained under fluorescent ceiling lights at 25°C by transferring 5 ml aliquots to 20 ml fresh medium every 1 to 2 weeks. Rapidly growing cultures were obtained by transferring cells to fresh medium more often. To obtain protoplasts, 6 to 14 d old cultures were filtered through 400 μm sterile gauze, centrifuged at 5000 rpm for 10 min, and the pellet resuspended and digested for 4 to 12 h at 25°C (on a reciprocal shaker) in an equivolume (25 ml) solution of 0.5% Macerase and 1.0% Cellulysin (Calbiochem) dissolved in 350 mM mannitol, 350 mM sorbitol, 6 mM $CaCl_2$, 0.7 mM $NaHPO_4$, and 3 mM MeS (pH 5.8). Protoplasts were washed, filtered through sterile gauze (100 and 40 μm), and resuspended in 100 mM saline (NaCl, CsCl, or Cs^+ glutamate, 250 mM mannitol, 250 mM sorbitol, 2 mM $CaCl_2$, 0.7 mM $NaHPO_4$, pH 5.8).

Single channel recordings and whole-cell voltage-clamp were achieved using the techniques of Hamill *et al.* (8). Patch pipettes were made using a Kopf puller with a 9 to 10 mm initial pull at 17.5 amp and a second pull at 12.0 to 12.5 amp. After fire-polishing, these pipettes had resistances of 8 to 12 Megohm. Patch pipettes were gently brought into contact with protoplasts until resistance increased to 50 to 100 Megohm, with suction then applied to the pipette interior to promote high resistance seal (10–50 Gigohm) formation. Protoplasts were extremely delicate. While whole cell currents could be reliably recorded in about 25% of cells examined with seal resistances of 100 to 500 Megohm, Gigaseals were only achieved 5 to 10% of the time. Soft soda-lime glass (200 μL, 2 mm capillary pipettes, Clay-Adams Scientific) gave the best results. Digestion time did not

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[2] Abbreviations: pS, picosiemens; TEA^+, tetraethylammonium; pA, picoamperes.

affect Gigaseal formation, but protoplasts seemed least fragile 1 to 3 h after isolation. Once a high resistance seal was formed, patch pipettes could be pulled away from the cell, giving excised patches in inside-out or outside-out conformations, depending on the Ca^{2+} concentration (8).

The internal (pipette) solution was 100 mM KCl, CsCl, K^+ gluconate, or Na^+ gluconate buffered with 5 mM $NaHPO_4$ or Hepes to pH 5.80 ± 0.05. The external solution was 100 mM NaCl, CsCl, Na^+ gluconate, or K^+ gluconate, again at pH 5.80 ± 0.05. Internal and external solutions contained 2 mM $CaCl_2$ and experiments were performed at room temperature. Currents were filtered at 3 to 10 kHz by a 4 pole Bessel filter, monitored with a List EPC-7 patch-clamp and sampled at 33 μs intervals via a Teckmar Labmaster board (Axon Instruments) by an IBM PC-XT, controlled using the program pCLAMP (Axon Instru-

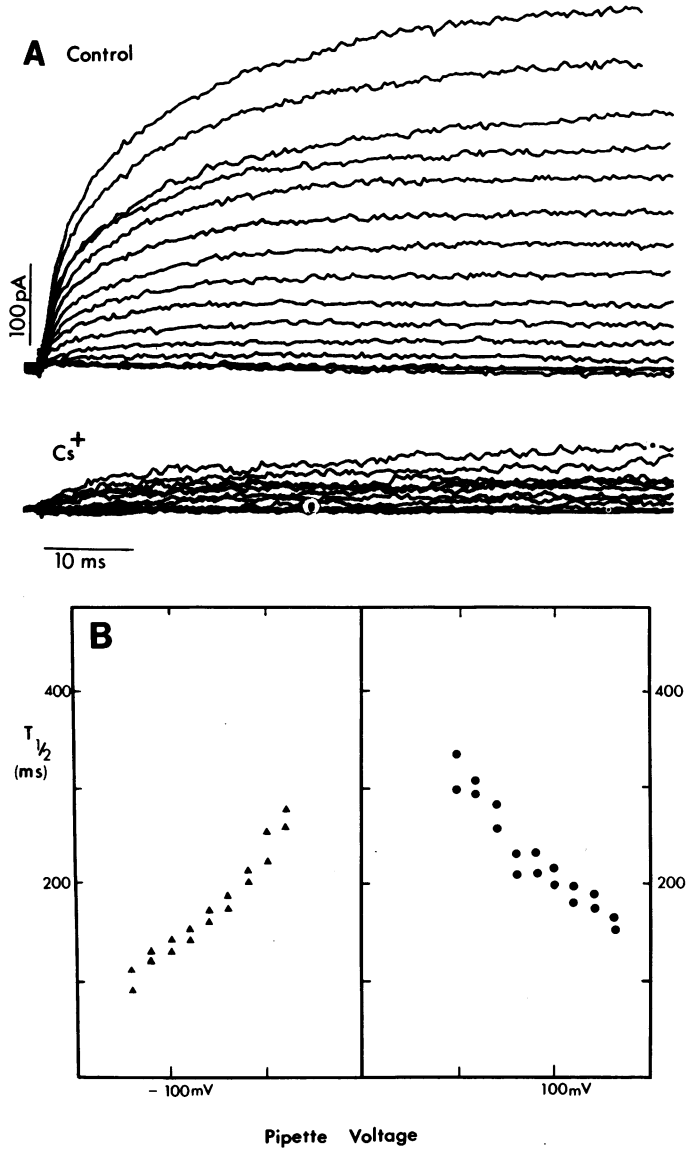

FIG. 1. A, Whole-cell outward K^+ currents (upward deflections) in *A. tuberosa* protoplasts for voltage steps of 10 to 150 mV (10 mV increments) before and after addition of sufficient Cs^+ to yield a final concentration of 20 mM. The pipette and bath solutions were 100 mM KCl, 2 mM $CaCl_2$ buffered with 5 mM Hepes to pH 5.80 ± 0.05. Current and time calibrations are 100 pA and 10 ms, respectively. B, Half-times for K^+ activation during depolarizing steps (●) and inactivation during hyperpolarizations (▲).

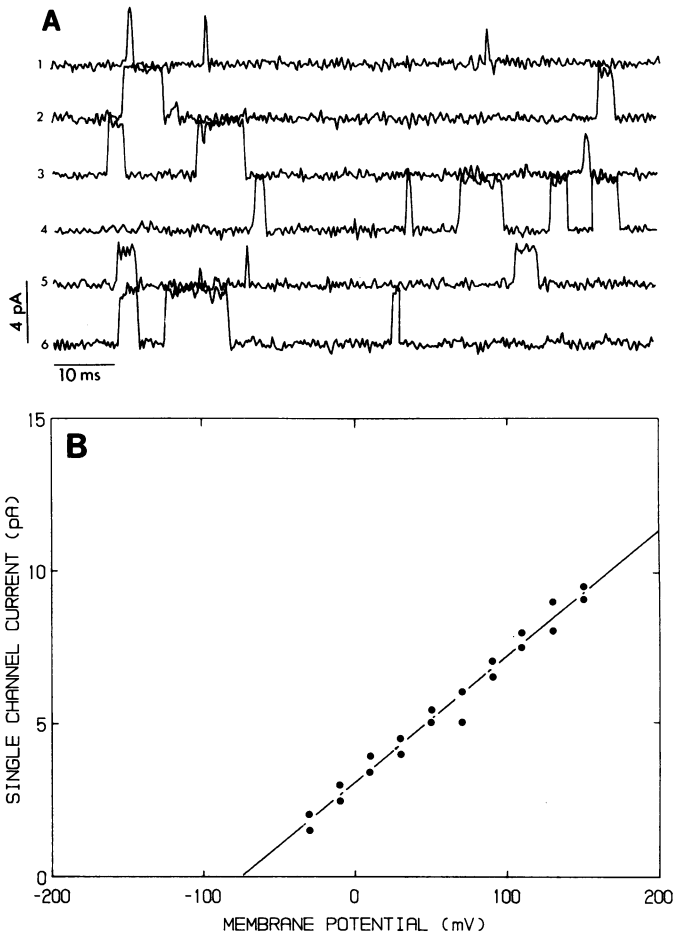

FIG. 2. A, Single channel outward K^+ currents in an inside-out patch held at a potential of +100 mV relative to the grounded bathing solution. The pipette and bath solutions were 100 mM KCl and 2 mM $CaCl_2$ buffered with 5 mM Hepes to pH 5.80 ± 0.05. Current and time calibrations are 4 pA and 10 ms, respectively. The numbers refer to consecutive traces acquired in an event-triggered mode. B, Current-voltage curve for single K^+ channels in two patches with an external (pipette) solution of 100 mM NaCl and an internal (bathing) solution of 100 mM KCl. The solid line corresponds to a conductance of 40 pS.

ments). Membrane potentials are given assuming the protoplast exterior to be at zero voltage. Note that in the whole-cell clamp and outside-out excised patch configurations positive pipette potentials correspond to membrane depolarizations, while in cell-attached and inside-out excised patch configurations positive pipette potentials correspond to membrane hyperpolarizations.

Single-channel data was analyzed and plotted as amplitude and duration histograms, with conductance and mean open times determined by pCLAMP. Series resistance was 80 to 90% compensated and capacity currents subtracted by the analog circuitry in the EPC-7. Whole-cell currents were corrected for linear leakage current by a P/5 protocol. In this protocol, currents were first measured for full-sized voltage steps (± P) in regions where current-voltage relations were nonlinear (depolarizations activated outward K^+ current and hyperpolarizations inward Cl^- currents), then measuring currents using voltage steps scaled to one-fifth the size (± P/5) using a holding, or reference potential in a range where the current-voltage relation was linear and there was no apparent ion channel activation or inactivation. Reversal potentials were determined under bionic conditions using Tris as an impermeable cation substitute and gluconate as an impermeable anion, with the selectivity ratios estimated after correction for ion activity as detailed by Hille (10). In some experi-

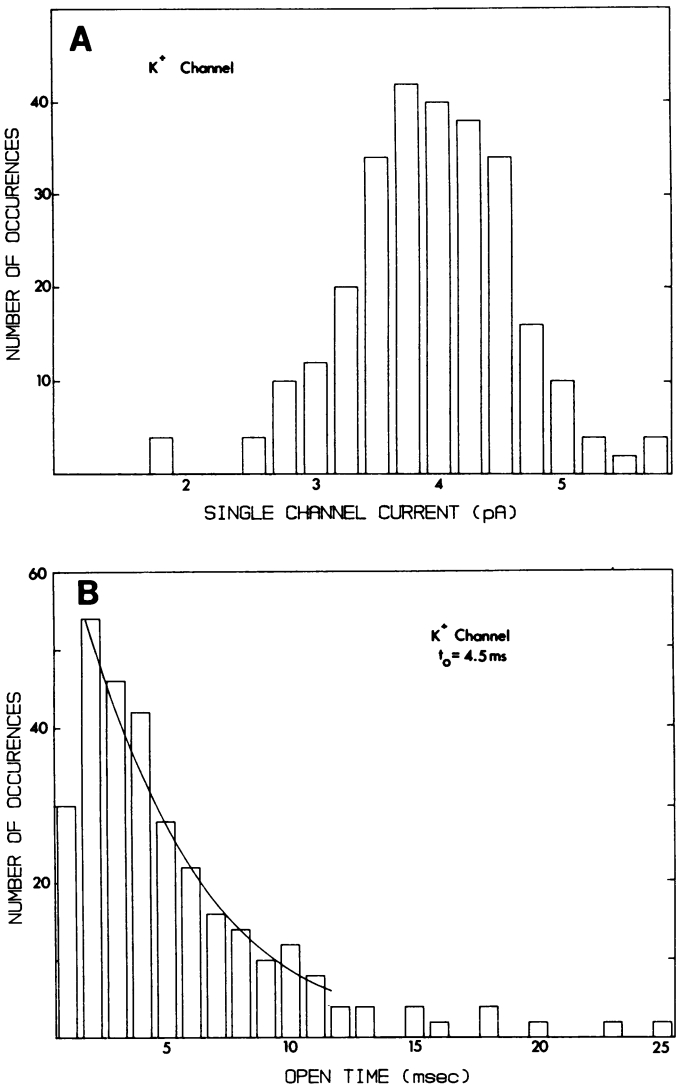

FIG. 3. A, Amplitude histogram for *A. tuberosa* K^+ channels with data from 310 events divided into 0.25 pA bins. B, Interval histogram from the same patch as in part A, the line representing a mean open time of 4.5 ± 0.5 ms.

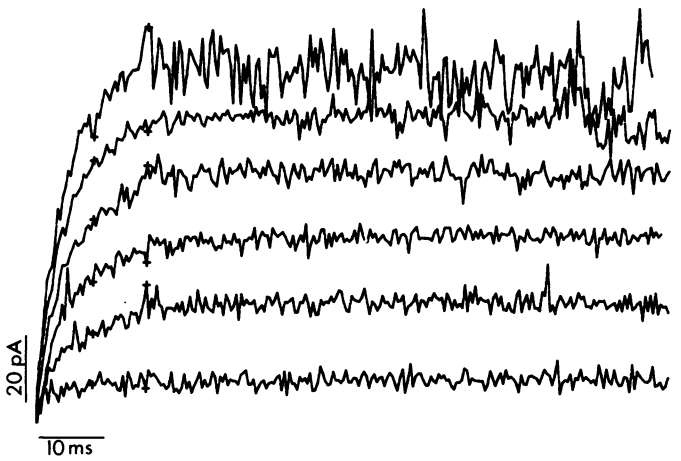

FIG. 4. Whole-cell outward K^+ currents in a *A. tuberosa* protoplast having a relatively low channel density for voltage steps of 20 to 120 mV (20 mV increments). The pipette and bath solutions were 100 mM KCl, 2 mM $CaCl_2$. Current and time calibrations are 20 pA and 10 ms, respectively.

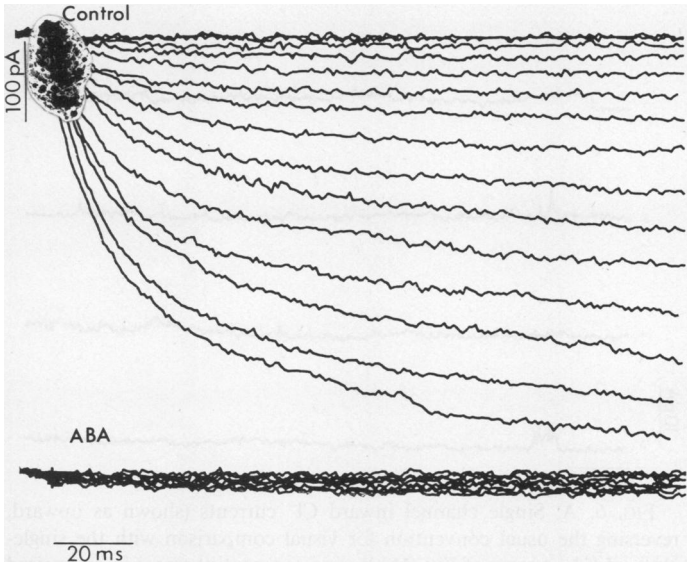

FIG. 5. Whole-cell inward Cl^- currents in *A. tuberosa* protoplasts for voltage steps of − 10 to −150 mV (10 mV increments) before (control) and after (ABA) addition of 100 μM abscisic acid (ABA). The pipette and bath solutions were 100 mM CsCl (to eliminate K^+ current) and 2 mM $CaCl_2$ buffered with 5 mM Hepes to pH 5.80 ± 0.05. Current and time calibrations are 100 pA and 20 ms.

ments concentrated solutions of TEA^+ and 4-aminopyridine (4-AP) were added to give a final concentration of approximately 10 mM, while abscisic acid (Sigma Chemical), Zn^{2+} and ethacrynic acid (Sigma Chemical) were used at 10 to 100 μM. All data are expressed as means ± standard errors for 5 to 8 independent experiments.

RESULTS

Voltage- and Time-Dependent K^+ Currents. Two voltage-dependent conductances were characterized in *Asclepias tuberosa* suspension-cell protoplasts using the whole-cell configuration. In 15 to 20% of protoplasts, patch-clamped depolarizations of 40 to 150 mV activated time-dependent outwardly rectifying membrane currents in symmetrical solutions containing 100 mM KCl (Fig. 1A; note voltages are given relative to the resting potential). At the resting potential the conductance is low and for hyperpolarizations all currents were time-independent and linear, but normally could not be resolved due to the baseline noise. Half-activation times were voltage-dependent, ranging from 300 ms for voltage steps of +50 mV to 180 msec at +130 mV (filled circles, Fig. 1B). When a protoplast was depolarized to activate outward currents and then repolarized to negative membrane potentials there was time-dependent, generally nonexponential inactivation with half-times that ranged from 300 ms at −30 mV to 100 ms at −120 mV (filled triangles, Fig. 1B). Outward currents were inhibited when Cs^+ (lower portion, Fig. 1A) and TEA^+ (not illustrated) were added to the bathing solution, but were not affected by 4-aminopyridine. Application of 100 μM ABA, IAA and 2,4-D (again to the bathing solution) had no detectable effect on either outward or inward whole-cell currents in these cells.

Outward currents in *A. tuberosa* protoplasts are carried largely by K^+. In symmetrical 100 mM KCl solutions the instantaneous current-voltage relation for the time-dependent outward whole-cell current was linear and the current reversed at a potential of 5 ± 4 mV (mean ± SE for six cells). When the external solution was 100 mM NaCl and the pipette contained 100 mM KCl the current-voltage relation was again linear and the average reversal

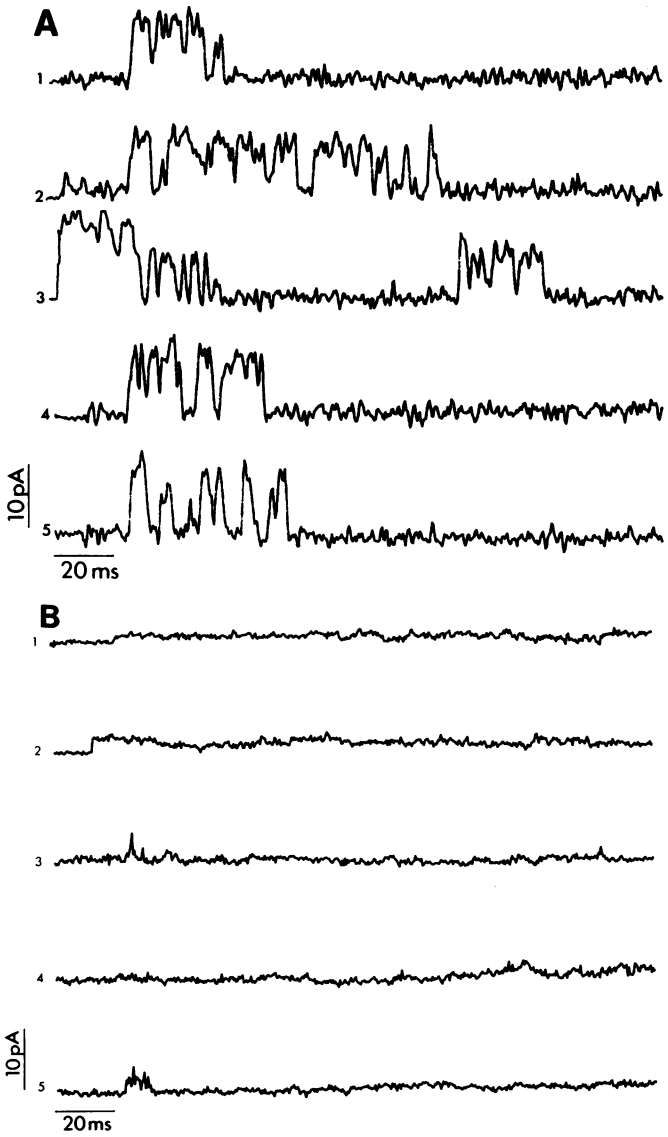

FIG. 6. A, Single channel inward Cl^- currents (shown as upward, reversing the usual convention for visual comparison with the single-channel K^+ currents of Fig. 2A) during consecutive traces in an excised inside-out patch held at a potential of −100 mV in symmetrical 100 mM KCl. B, Similar single-channel data obtained following addition of 10 μM Zn^{2+}. Current and time calibrations are 10 pA and 20 ms.

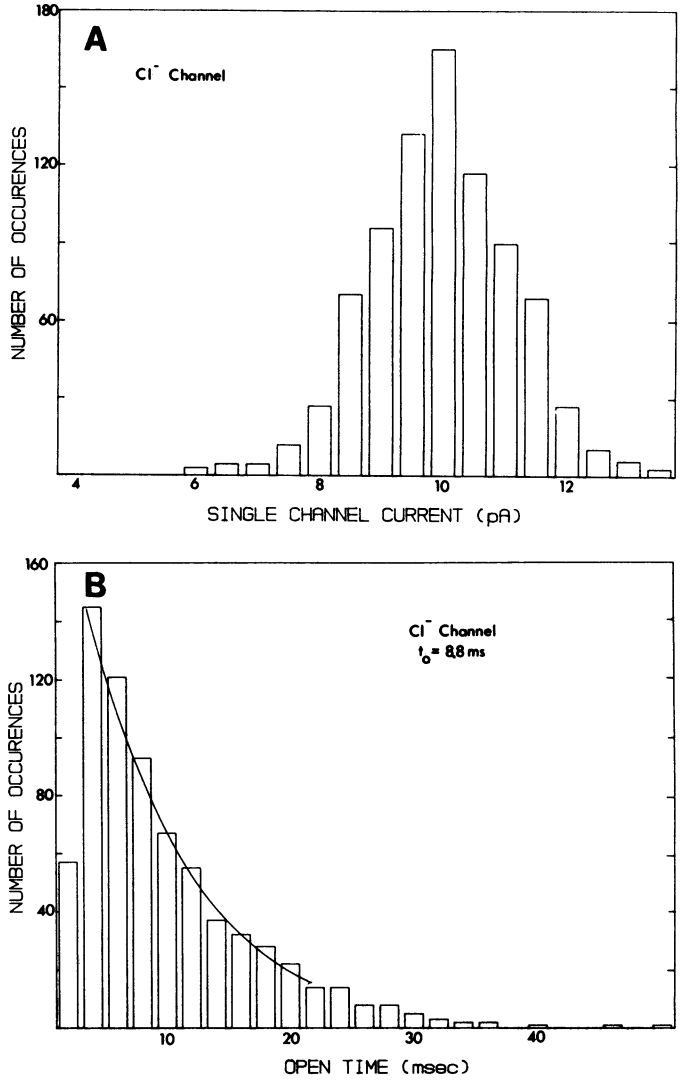

FIG. 7. A, Amplitude histogram for Cl^- channels with data from 717 events divided into 0.5 pA bins. B, Corresponding interval histogram, the line representing a mean open time of 8.8 ms.

potential was −68 ± 6 mV (mean of five different protoplasts), whereas a value of 54 ± 6 mV was obtained from five cells bathed in 100 mM KCl when the patch pipette contained 100 mM NaCl. Since protoplast diameter averaged 42 ± 5 μm, the interior can be assumed to have been replaced by the pipette solution in a matter of seconds for whole-cell patch-clamp. The selectivity ratio for K^+:Na^+ calculated from these reversal potentials was 15:1 with internal KCl and 8.5:1 with internal Na^+. When cells were examined using pipette and bath solutions containing only Cs^+ or Tris, the only outward currents seen were linear and time-independent.

Outward whole-cell currents did not inactivate during depolarizing pulses as long as 1.25 s, but when protoplasts were held at a potential of 100 mV for 10 to 20 s the magnitude of the outward current was reduced by 40 to 60%, but there was no change in the half-activation times at corresponding membrane potentials. At 200 mV outward currents could be abolished, while holding a potential of 100 mV usually increased the steady state outward current by 20 to 30%. Outward currents in *Asclepias* thus have a slow inactivation process resembling some K^+ channels in animal cells (9).

Single-channel K^+ currents could be observed in the cell-attached mode, in excised inside-out patches and, following patch destruction, in the outside-out configuration. Figure 2A illustrates typical single channel K^+ currents in an inside-out patch held at a potential of +100 mV (pipette potential of −100 mV relative to the grounded bathing solution). Extrapolation of the single-channel currents measured at several different holding potentials yielded a reversal potential in symmetrical 100 mM KCl of 2 ± 5 mV (mean ± SE in four experiments), −72 ± 7 mV when the pipette (external) solution was 100 mM NaCl and the bathing (internal) solution was 100 mM KCl (illustrated for two of five patches in Fig. 2B), and 56 ± 5 mV when the bath solution was 100 mM NaCl and the pipette contained 100 mM KCl. The selectivities derived from these data are the same as those obtained from whole-cell currents. As for whole-cell currents, single-channel K^+ currents were blocked by TEA^+, but were unaffected by 4-aminopyridine, ABA, IAA, or 2,4-D. Application of TEA^+ caused a decrease in mean open time, but no change in single-channel conductance.

Figure 3A shows a typical amplitude histogram with data from

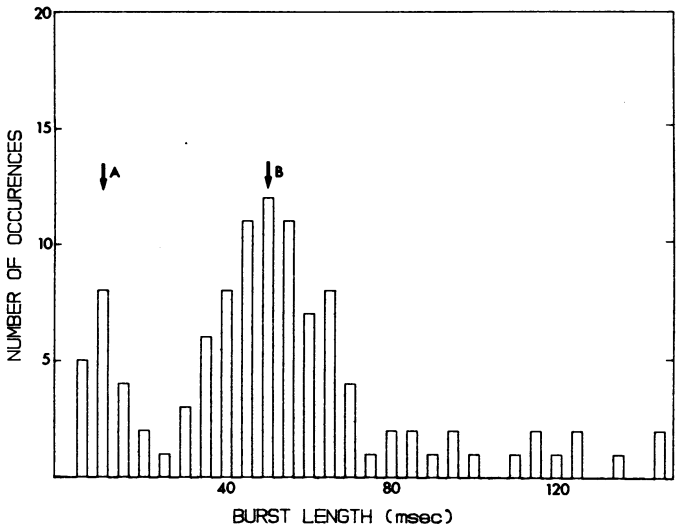

FIG. 8. Results of analyzing burst duration in a total of 144 records. While in some cases (arrow A) only one to two openings occurred, the majority of bursts lasted an average of 50 ± 14 ms, with some in excess of 100 ms.

310 events divided into 0.25 pA bins. Single K^+ channel conductance averaged 40 ± 5 pS in seven different patches. Figure 3B presents the interval histogram from the same patch as in part A, the line representing a mean open time of 4.5 ± 0.5 ms. It was more difficult to define a mean closed time because, when data were acquired for longer than a few seconds, the frequency of single-channel openings progressively decreased. The time-dependent slow inactivation present in whole-cell currents thus appears to have a parallel in the behavior of single channels. The number of single K^+ channels present in a single patch (areas of 5–10 μm^2) and steady state outward currents both varied substantially when the protoplasts from a single digestion were compared to one another. Some whole-cell outward currents were smooth, suggesting channel densities greater than 100/cell, while currents in others (with seal resistances of several hundred Megohm) became increasingly noisy on depolarization (Fig. 4), consistent with lower channel densities.

Asclepias Tuberosa Cl^- Currents. In 80% of protoplasts examined membrane hyperpolarization, not depolarization, elicited a time- and voltage-dependent activation in symmetrical 100 mM KCl or CsCl (Fig. 5). Currents measured in response to depolarizations were small, linear, and usually time-independent, although in some cells, there was a partial inactivation that usually could not be well resolved. Inward whole-cell currents could be inhibited by addition of Zn^{2+} to an estimated final concentration of 10 μM and, in some cases, by 100 μM ethacrynic acid. Both these compounds have been shown to block certain classes of anion-selective ion channels in animal cells (10). The activation time constants in these cells ranged from 500 ms at −50 mV to 150 ms at −100 mV. Application of IAA and 2,4-D to the bathing solution (final concentrations of 100 μM) had no detectable effect on inward current, but 100 μM ABA suppressed them in 6 of 10 cells examined.

In symmetrical 100 mM KCl or NaCl, as well as with 100 mM KCl in the pipette and 100 mM NaCl in the bathing solution, instantaneous current-voltage relations for the whole-cell currents were linear. The reversal potential was −3 ± 6 mV (mean ± SE for eight cells). When gluconate was used as an impermeable anion, the reversal potentials were 78 ± 6 mV with 100 mM Na^+ gluconate in the bathing solution and equimolar NaCl in the pipette, and −75 ± 5 mV with 100 mM K^+ gluconate in the pipette and a bathing solution containing 100 mM KCl, giving a Cl^-/cation selectivity ratio of about 20:1. Other anions were not tested.

Figure 6A illustrates single channel Cl^- currents in an excised inside-out patch held at a potential of −100 mV (pipette potential of 100 mV relative to the grounded bathing solution). Note that these traces have been inverted to show inward currents, and thus channel openings, upward for better comparison with the single-channel K^+ currents. Extrapolation of single-channel currents by methods similar to those in Figure 2B gave a reversal potential in symmetrical 100 mM KCl of 2 ± 5 mV and 76 ± 4 mV when the pipette (external) solution was 100 mM K^+ gluconate and the bathing (internal) solution contained 100 mM KCl. The reversal potential was −80 ± 6 mV when the bath was 100 mM K^+ gluconate and the pipette contained 100 mM KCl. As for inward whole-cell currents, single-channel activity was inhibited by Zn^{2+} (Fig. 6B; the increase in current at the beginning of trace 2 was an artifact).

In contrast to single-channel K^+ currents which had a single, well defined open conductance in all cells, single-channel Cl^- currents in the majority of protoplasts were less uniform in amplitude, suggesting the presence of one or more incompletely resolved subconductance states (trace 3, for example). Figure 7A shows the amplitude histogram for Cl^- channels from a patch with relatively uniform openings, with data from 717 events divided into 0.5 pA bins. Single channel conductance in six such patches averaged 100 ± 17 pS. Figure 7B illustrates the corresponding interval histogram, the line representing a mean open time of 8.8 ± 0.7 ms. As for whole-cell Cl^- currents, IAA and 2,4-D had no effect when applied to the bathing solution. Application of 100 μM ABA decreased mean open time, but did not change the single-channel conductance.

In contrast to single K^+ channels, *Asclepias* Cl^- channels tended to open in bursts (Fig. 6A), behavior that could either arise from transitions among subconductance states or interactions between separate channels. The result of analyzing burst duration in a total of 144 records is shown in Figure 8. While in some cases (arrow A) only 1 to 2 openings occurred, the majority of bursts lasted an average of 50 ± 14 ms, with some in excess of 100 ms. The occurrence and average duration of bursts were not changed when brief pulses of suction were applied to the patch pipette, and were unaffected by cation substitution, TEA^+, or Cs^+. Application of Zn^{2+} reduced burst duration, while increasing the time between bursts and single openings in a given burst. No slow inactivation was evident in whole-cell or single-channel Cl^- currents.

DISCUSSION

Asclepias tuberosa protoplasts contain two different ion channels. Potassium channels were activated by depolarizations, while Cl^- channels were activated by hyperpolarizations. Single K^+ channels had a single, well defined, open-state conductance of 40 ± 5 pS, mean open time of 4.5 ms, and were blocked by Cs^+ and TEA^+ with no change in single-channel conductance. Shroeder *et al.* (27) reported a single-channel conductance of 37 pS and mean open time of 10 ms at +60 mV for K^+ channels in guard cell protoplasts from *Vicia faba*, and also noted that the K^+/Na^+ selectivity ratio was increased when cytoplasmic K^+ was replaced by Na^+. The K^+-selective channels in *Asclepias* protoplasts characterized here appear to be similar to those in *V. faba*.

Single Cl^- channels were less consistent. The largest conductance averaged 100 ± 17 pS with a corresponding mean open time of 8.8 ms. Several lower conductance levels were apparent in the raw data, but could not be unambiguously analyzed. Cl^- channel activity could be completely blocked by Zn^{2+}, ethacrynic acid, and by the inhibitory plant hormone ABA, but not by IAA or 2,4-D. At partially inhibitory levels, mean open time was decreased but single-channel conductance remained unchanged, again suggesting an all-or-none block.

In protoplasmic droplets from *Chara corallina* there were two types of ion channels (11). The larger, activated by hyperpolarization, had a conductance of 100 pS, a mean open time of 100 to 200 ms, and a TEA^+ sensitivity reminiscent of many K^+ channels in animal cells (10). Depolarizations, on the other hand, activated a TEA^+-insensitive 30 pS ion channel whose reversal potential was near the resting potential. *Triticum aestivum* protoplasts had four categories of ion channels, often coexisting in a single patch (16). The most voltage-dependent channel had a conductance of 35 to 40 pS, similar to that of K^+ channels in *Asclepias*. Another had a single-channel conductance of 70 to 100 pS, in the range observed for Cl^- channels in *Asclepias*, but its selectivity was not studied. In *Triticum* some currents had time constants of 100 ms to several seconds and were unaffected by La^{3+}, suggesting a possible Cl^- selectivity (16).

While many properties of protoplasts resemble behavior seen in intact plant cells (6, 22, 25), the physiological role of K^+ and Cl^- channels in protoplasts derived from long-term suspension-cell cultures of *A. tuberosa* is unknown. However, the selective inhibition of a Cl^- channel by ABA observed here raises the possibility that anion-selective channels may be important targets for inhibitory plant hormones.

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