Proceedings of the Indiana Academy of Science
(1987) Volume 97 p. 143-156.

The Role of Ion Channels in Plant Protoplasts

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ABSTRACT

Plant hormones such as abscisic acid (ABA) regulate growth and a variety of physiological responses, but their mechanism of action is not well-defined. We employed patch voltage-clamp to study a voltage-dependent. TEA^+ sensitive K^+ channel in *Vicia faba* guard cell protoplasts and an ethacrynic acid and Zn^{++-} sensitive Cl^- channel in *Asclepias tuberosa* suspension cell protoplasts. Single channel K^+ conductance in *Vicia faba* guard cell protoplasts was 65 ± 5 pS and the mean open time was 1.25 ± 0.30 msec at 150 mV. While auxins and 2,4-D had no effect, micromolar levels of ABA increased the mean open time of *Vicia faba* K^+ channels and caused bursts of openings that were sufficient to account for ABA-dependent stomatal closure. In independent experiments *in vivo* measurements of stomatal aperature were used to demonstrate that ABA-dependent stomatal closure could be inhibited by EGTA or nifedipine. *Asclepias tuberosa* suspension cell derived protoplasts had K^+ and Cl^- channels with single channel conductances of 40 ± 5 pS and 100 ± 17 pS respectively. Mean mean open time for K^+ channels was 4.5 ± 0.4 msec at 100 mV, while Cl^- channels had a mean open time of 8.8 ± 1.2 msec and were blocked by Zn^{++} and ethacrynic acid. In contrast to *Vicia faba* K^+ channels, Cl^- channels in *Asclepias tuberosa* were blocked by micromolar levels of ABA, but again were not affected by auxins or 2,4-D.

Introduction

Plant hormones such as abscisic acid (ABA) regulate developmental events and physiological responses (5,7,12,15), but the factors linking hormonal signals and intracellular events are not well-defined. Ions cross cell membranes through quasi-permanent aqueous pores, referred to as ion channels, that may be gated by transmembrane electric field changes (voltage-dependent channels), or activated by one or more external stimuli (agonist-activated channels). In animal cells, hormones affect receptors coupled to ion channels, with subsequent ion fluxes or voltage shifts regulating intracellular second messengers. Binding proteins for ABA exist in various cells (1) and plant cells have been shown to contain cation and anion-selective channels by conventional voltage-clamp (2,3,11,13,26,30). There is also considerable recent evidence for Ca^{++}-dependent second messenger systems in a variety of plant tissues (1,5,14,18,19,20). Until recently the cell wall hindered direct studies of ion channels in plants, but patch-clamp of 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 20-30 pS (27), large enough to produce the net K^+ fluxes associated with ABA-dependent stomatal closure (15,28). Channels carrying maleate and K^+ were seen in vacuoles from barley leaf mesophyll protoplasts (9), while TEA^+-sensitive and TEA^+-insensitive K^+ channels with conductances of 100 pS and 30 pS respectively were observed in protoplasmic droplets from *Chara corallina* (11).

One important question is whether the ion channels observed in patch-clamped protoplasts are directly involved in significant physiological responses of plant tissues. To

resolve this, we measured the effect of ABA on stomatal aperature and guard cell protoplast diameter and tested the ability of the well-known Ca^{++} channel blocking agents EGTA and nifedipine to inhibit ABA-dependent changes in guard cell volume in *Vicia faba*. We then employed patch voltage-clamp to study two different, plant hormone-sensitive ion channels. The activity of a voltage-dependent K^+ channel in *Vicia faba* was enhanced by micromolar levels of ABA, whereas ABA blocked the Cl^- channel in *Asclepias tuberosa*. Demonstration of a qualitatively distinct hormone specificity suggests membrane-bound ion channels have a significant role in plant cell physiology.

Methods

Plant Protoplasts

Cell suspension culture-derived protoplasts were obtained from callus of *Asclepias tuberosa* (4) maintained on a rotary shaker in liquid Murashige-Skoog medium (17), with 5.0 mg/L adenine, 0.5 mg/L 2,4-dichlorophenoxyacetate (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, ref. 4) under fluorescent ceiling lights at 25 °C. To obtain protoplasts, 6-14 day old suspension cultures were first filtered through 400 um sterile gauze, and then centrifiged at 5000 rpm for 10 minutes. The pellet was then resuspended and digested for 4-12 hours at 25 °C (on a reciprocal shaker) in an equivolume (25 ml) solution of 0.5% Macerase and 1.0% Cellulysin (Calbiochem) in 350 mM mannitol, 350 mM sorbitol, 6 mM $CaCl_2$, 0.7 mM $NaHPO_4$ and 3 mM MES as pH = 5.8. Protoplasts were washed, filtered through sterile gauze (100 um and 40 um) 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).

Guard cell protoplasts were isolated from leaves of broad bean, *Vicia faba*. Leaflets from 3 week old plants were rinsed 3 times in a basal medium (BM) containing 550 mM sorbitol and 5 mM MES adjusted to pH 5.8 with KOH (33). All solutions were filter-sterilized using 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 (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 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 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, few mesophyll protoplasts were present in the final suspension.

Stomatal Aperature

Stomatal aperature in *Vicia faba* was measured at 400× with a Nikon inverted phase-contrast microscope equipped with an ocular micrometer. Epidermal peels were obtained from the same leaves as used for protoplast isolation, with the peels subsequently floated in Ca^{++}-free distilled water in the dark for 30 minutes, and then exposed to light and CO_2-free, humidified air. Stomatal aperature was measured after 2-3 hours. Either ABA alone or ABA plus an inhibitor were then added to the bathing solution, with stomatal aperature redetermined 2-3 hours later. In each experiment 8-10 cells were measured, the data being expressed in terms of mean ± standard error. All differences noted were significant with $p < 0.01$ (Student's t-test).

Patch-Clamp Recording

Single channel recordings and whole-cell voltage-clamp were achieved using standard techniques (ref. 8, shown schematically in Figure 1). Patch pipettes were made us-

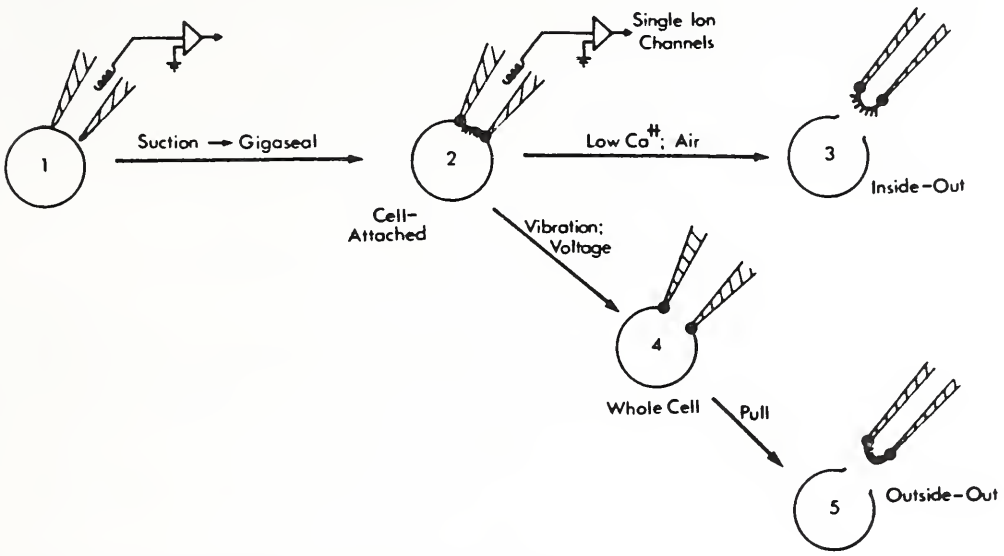


FIGURE 1. The principles of patch voltage-clamp.

ing a Kopf puller and had resistances of 8-12 Megohm when filled with the recording solution. Patch pipettes were gently brought into contact with protoplasts until resistance increased to 50-100 Megohm, with suction then applied to the pipette interior to promote high resistance seal (10-50 Gigohm) formation. 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^{++} concentration.

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 (bathing) 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 $CaCl_2$ unless otherwise noted and experiments were performed at room temperature of 22-25 °C. 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 intervals via a Teckmar Labmaster board (Axon Instruments) by an IBM PC-XT, controlled using the program pCLAMP (Axon Instruments). Membrane potentials are given assuming that the protoplast exterior was a zero voltage. Note that in the whole-cell clamp and outside-out excised patch configurations positive pipette potentials correspond to membrane deplorizations, while in both the cell-attached and inside-out excised patch configurations positive pipette potentials correspond to 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 80-90% 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 (deplorizations 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 biionic conditions using Tris as an impermeable cation substitute and gluconate as an impermeable anion, with the selectivity ratios estimated after correction for ion activity (10). 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 abscisic acid (Sigma Chemical), Zn^{++} and ethacrynic acid (Sigma Chemical) were used at 10-100 uM. Some solutions were rendered Ca^{++} free using an EGTA buffer solution with $[Ca^{++}] = 10^-$ 8M, while nifedipine (Sigma Chemical) was used at 100 uM. All data are expressed as means ± standard errors for 5-8 independent experiments.

Results

Ion Channels in Vicia faba

In whole-cell patch-clamp *Vicia faba* guard cell protoplasts had voltage-dependent outward currents for depolarizations greater than 50-60 mV (Figure 2A). These currents were completely inhibited when 10 mM TEA^+ was added to the bathing solution and when Cs^+ was substituted for K^+ in the patch pipette. Time-dependent activation of outward current in *Vicia faba* was slow compared to outward membrane currents in nerve or muscle cells, with half-activation times of 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 these currents was linear, reversing 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. These data provide an estimated of 20:1 for the K^+/Na^+ selectivity ratio.

Figure 2B shows typical single channel K^+ currents for *Vicia faba* in an inside-out patch held at +100 mV before, during and after application of 10 uM ABA. Under control conditions there was one high-conductance channel and one or more channels of lower conductance that we were unable to resolve. Extrapolation of single-channel currents for the large channel yielded a reversal potential of −81 ± 7 mV when the pipette solution was 100 mM NaCl and the bath solution was 100 mM KCl, suggesting this was indeed a K^+-selective channel. Single channel K^+ conductance averaged 65 ± 5 pS in 7 patches and was voltage-independent. As for whole-cell currents, single K^+ channels were blocked by 10 mM TEA^+ (Figure 2B, traces 10-12) and could not be observed with Cs^+ in the patch pipette.

Exposure of *Vicia faba* guard cell protoplasts to 10 uM ABA increased the K^+ channel activity. Not only did K^+ channels open more frequently, but many channel openings occured in bursts lasting 50-100 msec (traces 6-10 in Figure 2B). ABA also increased mean open time within a burst. Under control conditions mean open time was 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. In the presence of ABA a satistical analysis restricted to well-separated openings yielded mean open times of 1.20 ± 0.24 msec at +80 mV and 2.05 ± 0.22 msec at +150 mV, all significantly longer than the control values. In contrast to ABA, the plant hormones kinetin (100 uM) and indole-3-acetic acid (IAA; 100 uM) had no effect on whole-cell or single-channel K^+ currents.

Ion Channels in Asclepias tuberosa

Two voltage-dependent conductances were observed in *Axclepias tuberosa* protoplasts. In 15-20% of the protoplasts examined depolarizations of 40-150 mV activated time-dependent outward membrane currents in symmetrical solutions containing 100 mM KCl (Figure 3), while hyperpolariztions had no effect. In symmetrical 100 mM KCl solutions the instantaneous current-voltage curve reversed at 5 ± 4 mV. When the external solution was 100 mM NaCl and the pipette contained 100 mM KCl the average reversal

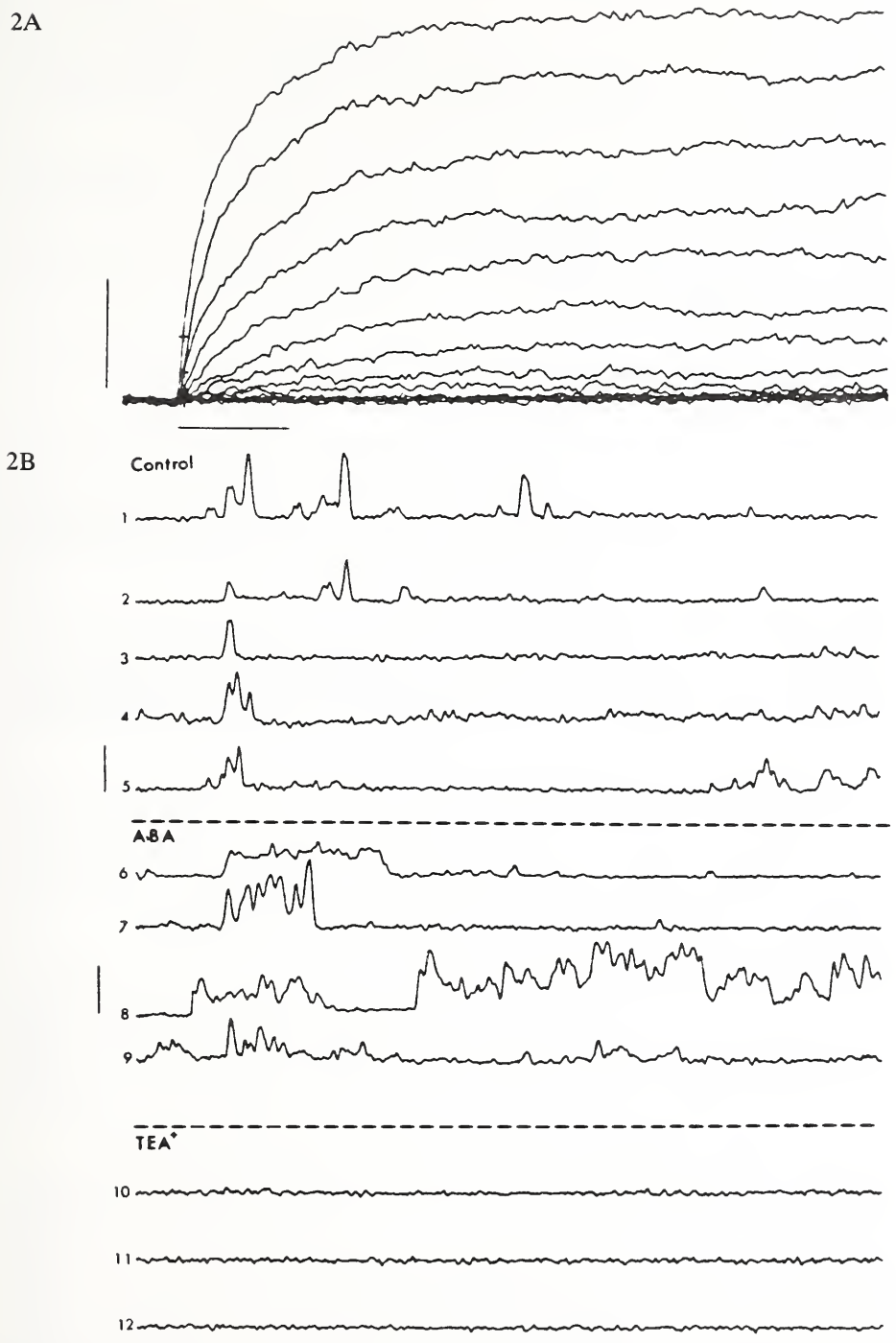


FIGURE 2. Part A shows whole-cell outward K^+ currents in *Vicia faba* guard cell protoplasts for depolarizations of 10-150 mV (10 mV increments). The pipette solution was 100 mM KCl, 2 mM $CaCl_2$ buffered with 5 mM Hepes to pH 5.8, while the bath contained 100 mM NaCl. Current and time calibrations 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. Numbers refer to consecutive traces acquired in an event-triggered mode.

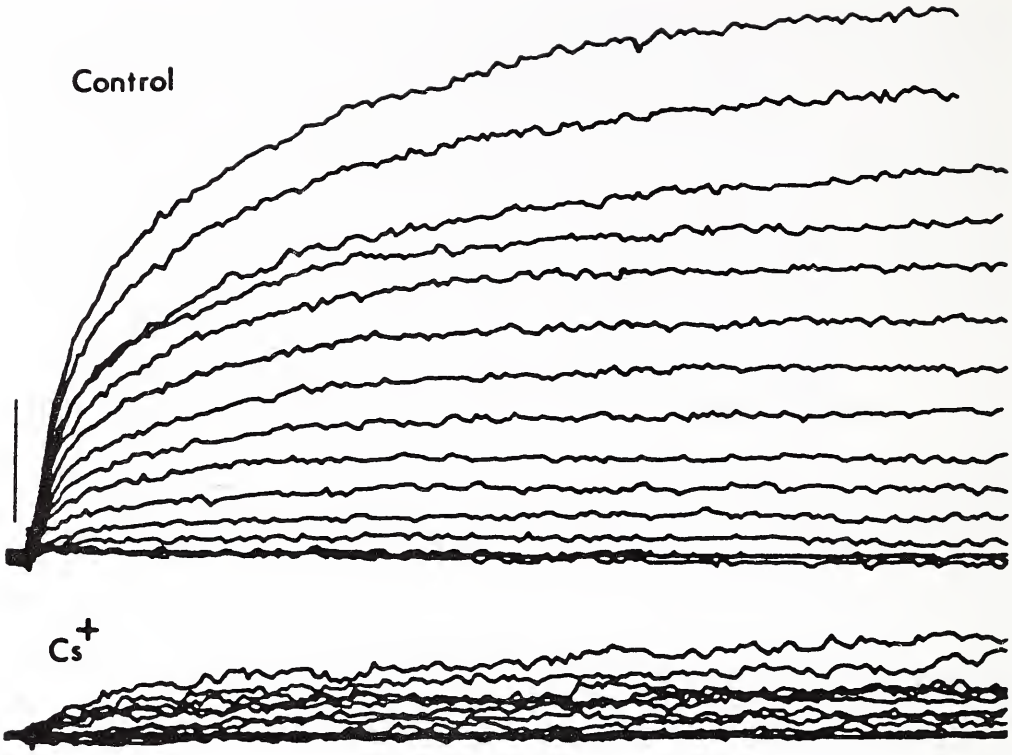


FIGURE 3. Whole-cell outward K^+ currents (upward deflections) in *Asclepias tuberosa* protoplasts for voltage steps of 10-150 mV (10 mV increments) before and after addition of sufficient Cs^+ to yield a final concentration of 10 mM. The pipette and both solutions were 100 mM KCl, 2 mM $CaCl_2$ buffered with 5 mM Hepes to pH 5.80 ± 0.05. Current and time calibrations are 100 pA and 10 msec respectively.

potential was −68 ± mV, whereas a value of 54 ± 6 mV was obtained in 100 mM KCl when the patch pipette contained 100 mM NaCl. The calculated K^+/Na^+ selectivity ratio for the *Asclepias tuberosa* K^+ channel was approximately 15:1. Half-activation times were 300-350 msec at + 50 mV and 180-200 msec at + 130 mV, also similar to the time course of K^+ currents in *Vicia faba* guard cell protoplasts. Outward currents were blocked by TEA^+ but, in contrast to *Vicia faba*, 100 uM ABA had no effect.

Figure 4A shows single channel K^+ currents in *Asclepias tuberosa* for an inside-out patch held at a potential of + 100 mV. Extrapolation of single-channel currents yielded a reversal potential in symmetrical 100 mM KCl of 2 ± 5 mV, −72 ± 7 mV when the pipette (external) solution was 100 mM NaCl and the bathing (internal) solution was 100 mM KCl, and 56 ± 5 mV when the bath solution was 100 mM NaCl and the pipette contained 100 mM KCl. Selectivities derived from these data were 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 ABA, IAA or 2,4-D. Single K^+ channel conductance averaged 40 ± 5 pS in 7 different patches, with a mean open time of 4.5 ± 0.5 msec.

Approximately 80% of *Asclepias tuberosa* protoplasts examined lacked time-dependent outward K^+ currents. Instead hyperpolarizations of 50-150 mV elicited a voltage-dependent inward currents in symmetrical 100 mM KCl or CsCl (Figure 5). In symmetrical 100 mM KCl or NaCl solutions, or with 100 mM KCl in the pipette and 100 mM NaCl in the bath, the reversal potential averaged −3 ± 6 mV. When gluconate

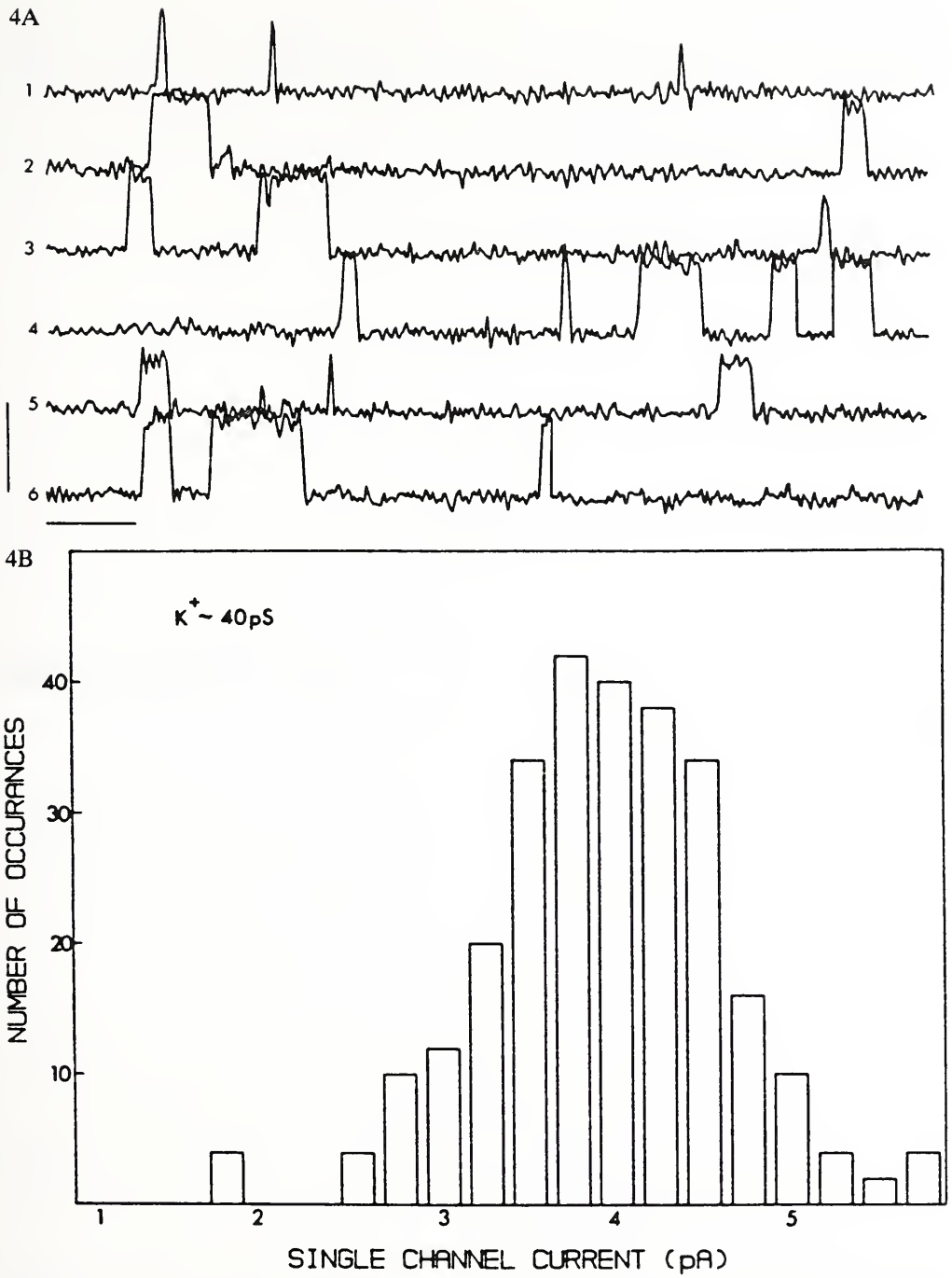


FIGURE 4. Part A shows single channel outward K^+ currents in an inside-out patch held at a potential of +100 mV relative to the grounded bathing solution. The pipette and bath solutions were 100 mM KCl and 2 mM $CaCl_2$ buffered with 5 mM Hepes to pH 5.80 ± 0.05. Current and time calibrations are 4 pA and 10 msec repsectively. The numbers refer to consecutive traces acquired in an event-triggered mode. Part B shows an amplitude histogram for *Asclepias tuberosa* K^+ channels with data from 310 events divided into 0.25 pA bins.

was used as an impermeable anion the reversal potential was 78 ± 6 mV with 100 mM Na^+ gluconate in the bathing solution and NaCl in the pipette, while it was −75 ± 5

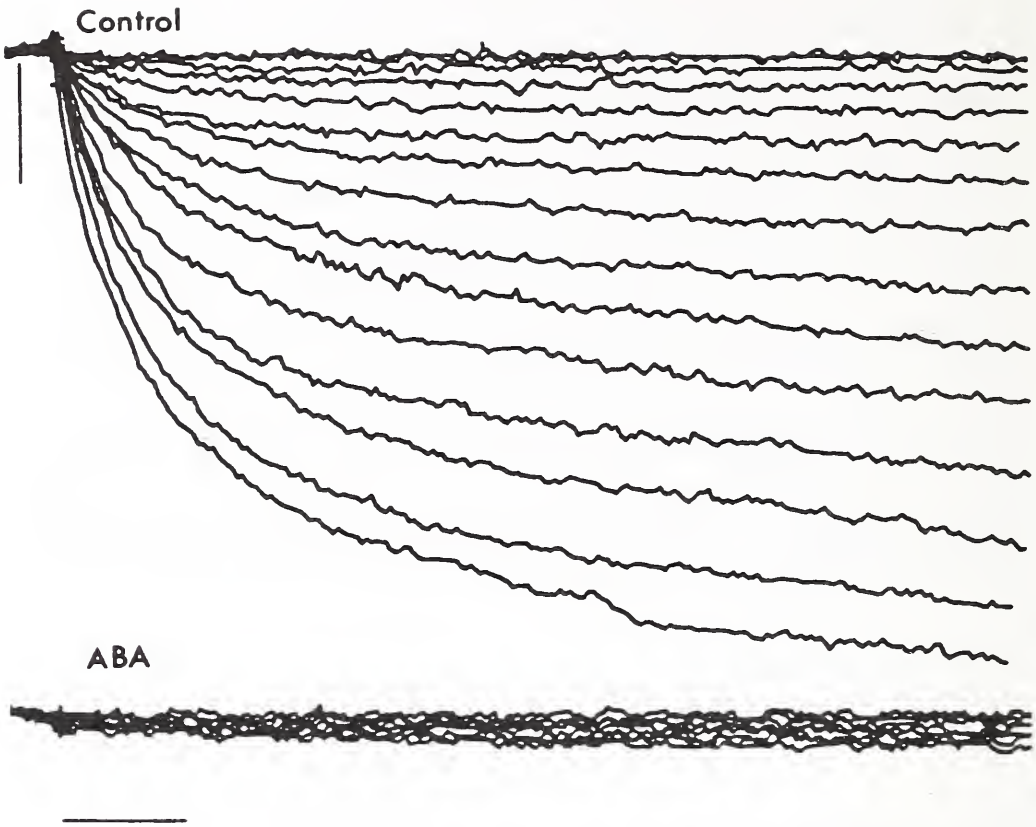
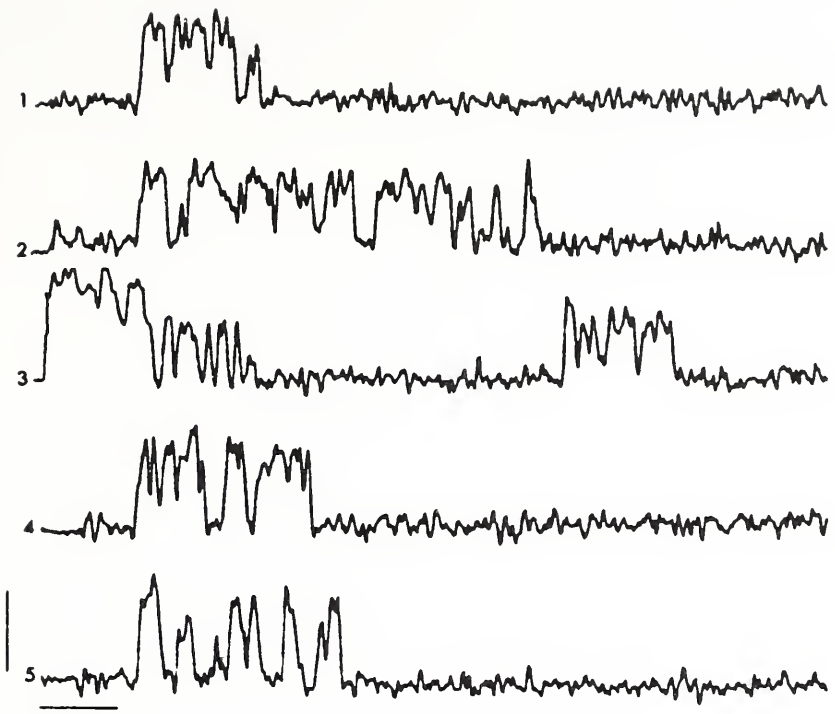


FIGURE 5. Whole-cell inward Cl^- currents in *Asclepias tuberosa* protoplasts for voltage steps of -10 to -150 mV (10 mV increments) before and after addition of 100 uM abscisic acid (ABA). The pipette and bath solutions were 100 mM CsCl and 2 mM $CaCl_2$ buffered with 5 mM Hepes to pH 5.80 ± 0.05. Current and time calibrations are 100 pA and 20 msec.

mV with 100 mM K^+ gluconate in the pipette and a bathing solution of 100 mM KCl. Such data unambiguously identifies these as Cl^- currents, and also defines the Cl^-/cation selectivity ratio as about 20:1. Inward Cl^- currents were inhibited by 10 uM Zn^{++} and 100 uM ethacrynic acid, both known to block anion channels in several animal cells (10). Application of the plant hormones IAA and 2,4-D to the bathing solution at a final concentration of 100 uM) had no effect on these Cl^- currents, whereas 100 uM ABA suppressed them in 6 of 10 cells examined.

Figure 6A illustrates single channel Cl^- currents in *Asclepias tuberosa* for an excised inside-out patch at a potential of -100 mV (traces have been inverted to show inward currents upward for comparison with single-channel K^+ currents). Extrapolation of single-channel currents gave reversal potentials comparable to those for whole-cell data. As for whole-cell Cl^- currents, activity was inhibited by Zn^{++} (Figure 6B). In contrast to single-channel K^+ currents in *Asclepias tuberosa*, single-channel Cl^- currents in the majority of protoplasts were quite nonuniform, suggesting there may be one or more open states. Figure 7 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 6 such patches averaged 100 ± 17 pS. Application of 100 uM ABA decreased the mean open time of *Asclepias tuberosa* Cl^- channels, but the hormones IAA and 2,4-D had no effect.

6A



6B

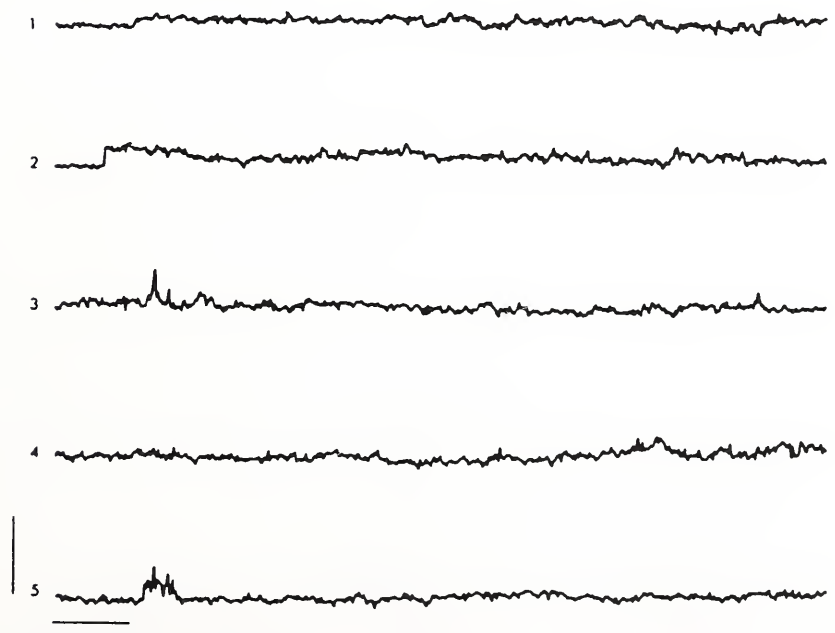


FIGURE 6. Part A shows single channel inward Cl^- currents (shown as upward, reversing the usual convention for visual comparison with the single-channel K^+ currents of Figure 2A) during consecutive traces in an excised inside-out patch held at a potential of -100 mV in symmetrical 100 mM KCl. Part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

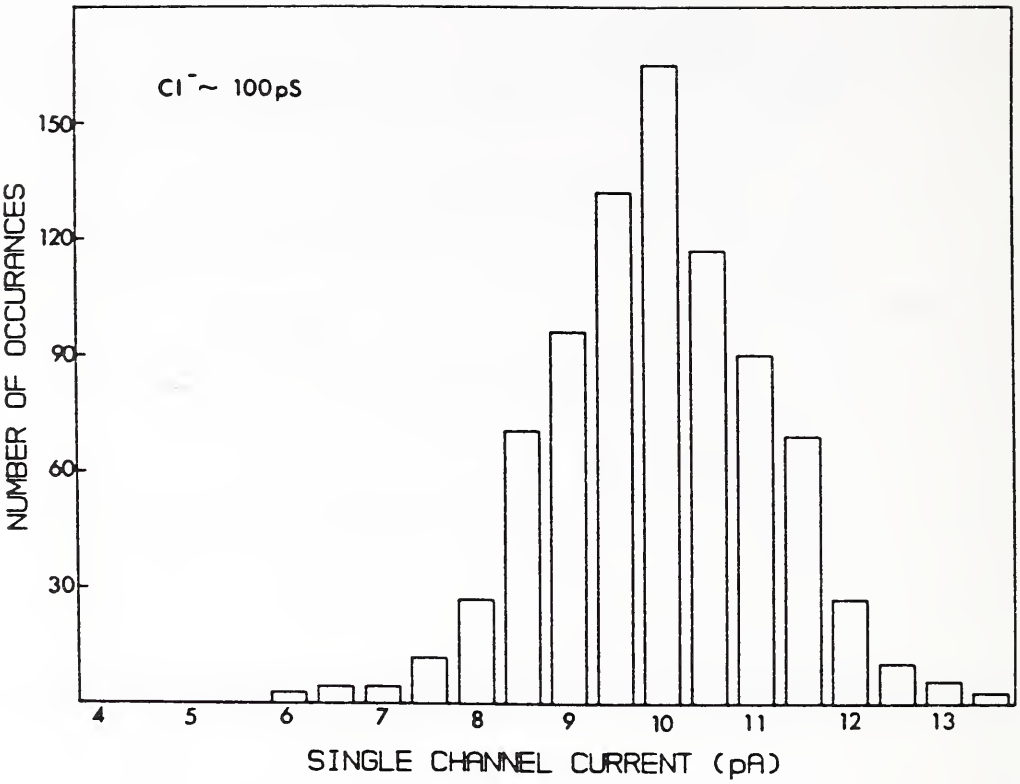


FIGURE 7. The amplitude histogram for Cl^- channels with data from 717 events divided into 0.5 pA bins.

In-Vivo Measurements of Stomatal Aperature

Figure 8 summarizes the results of *in-vivo* stomatal aperature measurements. Exposure of epidermal peels to light in a moist, CO_2-free atmosphere caused the aperature of stomatal pores to increase by $+59.3 \pm 7.3\%$ ($n = 115$) over a period of 2-3 hours. Addition of 10 uM ABA decreased average stomatal aperature by $-37.2 \pm 4.3\%$ ($n = 43$) from the maximal values seen following light exposure, with slightly larger decreases seen in the presence of the well-known K^+ ionophore valinomycin ($-42.8 \pm 4.8\%$; $n = 30$).

In another series of experiments the plant hormone ABA was added in the presence of 100 uM nifedipine, a Ca^{++} channel blocker, or with the external free Ca^{++} reduced to less than 1 uM with an EGTA buffer. Under such conditions no significant change in stomatal aperature could be observed (average changes of $-2.4 \pm 2.2\%$ and $+2.8 \pm 1.5\%$ respectively).

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. The fact that ABA, but not IAA or 2,4-D, increased the number of single-channel events, prolonged channel open time and induced long-lasting bursts of channel openings suggests that the *Vicia faba* K^+ channel may mediate guard cell responses to environmental factors such as water stress. Increases in channel activity, and thus K^+ permeability, by ABA would facilitate K^+ efflux, leading to osmotically-driven water loss and stomatal closure.

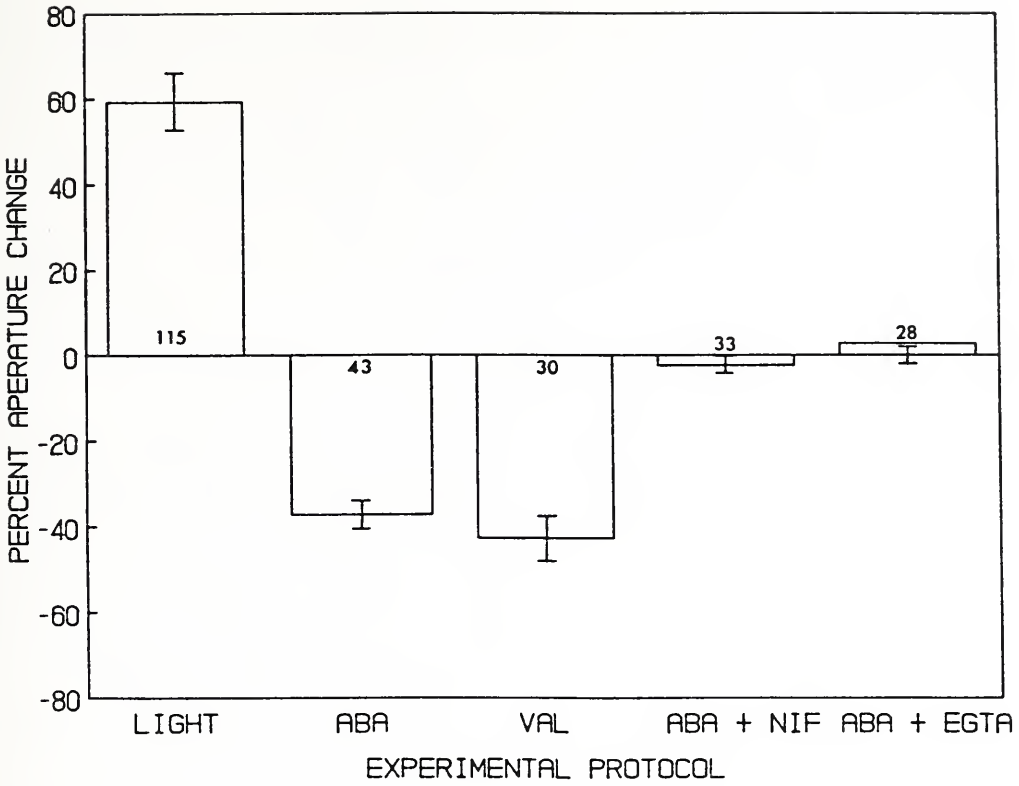


FIGURE 8. Effects of stimuli on stomatal aperature in epidermal peels from *Vicia faba*. The number of observations is given in the lower portion of each group.

One hypothetical model that might explain ABA-dependent regulation of guard cell volume is shown in the upper portion of Figure 9. As for stimulus-secretion coupling in animal cells, increases in intracellular [Ca^{++}] are fundamental. ABA is assumed to interact with a membrane receptor that regulates a Ca^{++} channel (labelled #1). The resulting Ca^{++} influx, perhaps augmented by Ca^{++}-induced Ca^{++} release from internal stores, opens a Ca^{++}-activated K^+ channel either directly, or via an intracellular second messenger. An alternative explanation, shown as #4 in Figure 9, would have ABA interacting with the cell membrane to cause internal Ca^{++} release without the involvement of a Ca^{++}-selective ion channel. However, the fact that the effects of ABA on stomatal aperature were inhibited by removal of external Ca^{++} and by application of nifedipine tends rule out this mechanism.

A possible role for K^+ channels in light-induced swelling in *Vicia faba* guard cell protoplasts is shown in the lower portion of Figure 9. It is assumed that light activates an unknown second messenger system (labelled #1) which, in turn, activates an active H^+/K^+ pump (#2). However, instead of a Ca^{++}-activated K^+ channel being opened, K^+ channels are assumed to close, preventing any short-circuiting of the increase in intracellular [K^+] necessary to create the osmotic gradient necessary for guard cell swelling. In this regard it is interesting that valinomycin and other K^+ ionophores promote stomatal closure and block the normal response to light.

In contrast to *Vicia faba*, a majority of *Asclepias tuberosa* protoplasts contained only Cl^- channels. Single-channel Cl^- conductance was 100 ± 17 pS, though several lower conductance levels were also apparent in the raw data. Cl^- channel activity could be completely blocked by Zn^{++}, ethacrynic acid and by ABA. The role of K^+ and Cl^-

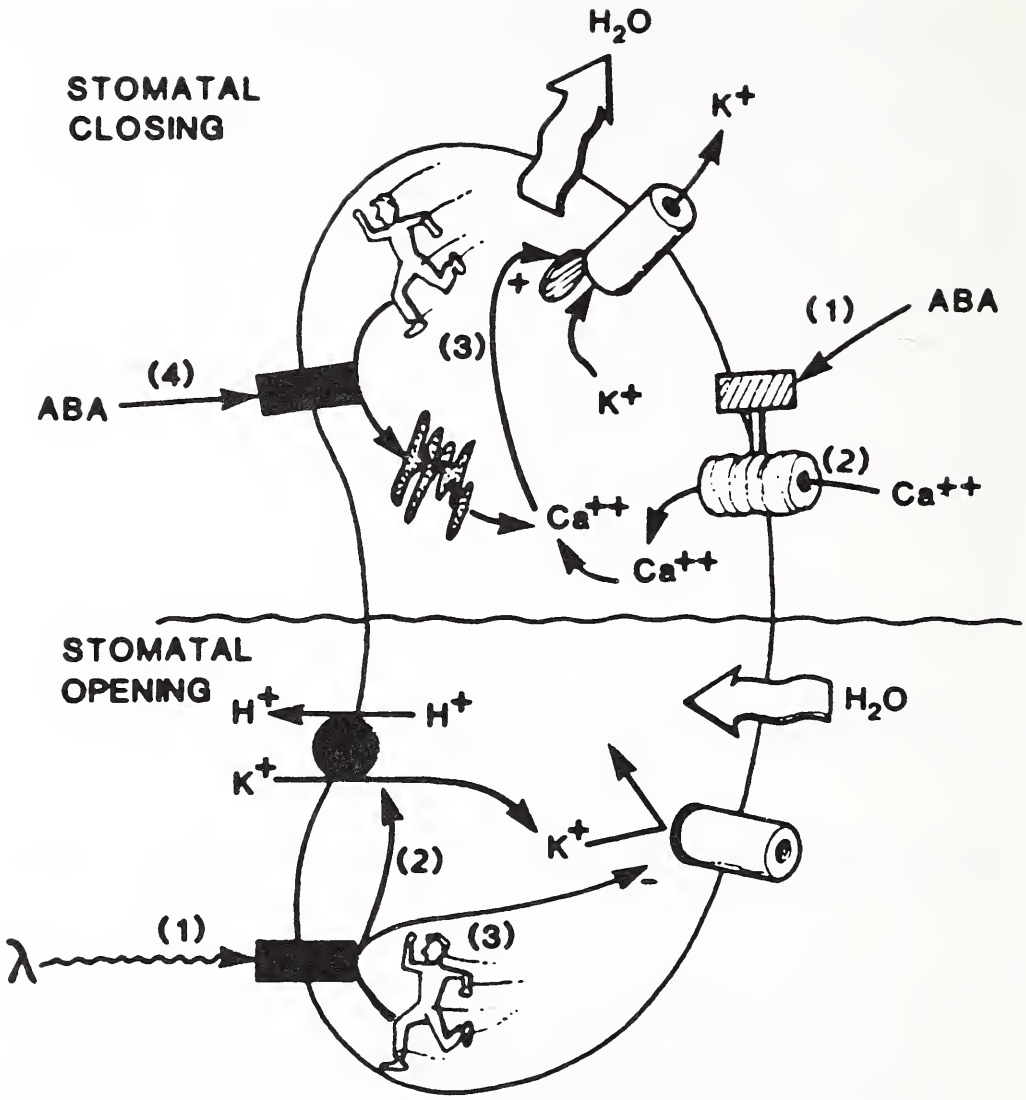


FIGURE 9. A model of ion channel regulation of guard cell volume.

channels in protoplasts derived from long-term suspension-cell cultures of *Asclepias tuberosa* is unknown, however the selective inhibition of a Cl^- channel by ABA raises the possibility that anion-selective channels may also be involved in the responses of plant cells to hormones.

Acknowledgement

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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