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Channel-mediated K^+ flux in barley aleurone protoplasts

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Abstract. Gibberellic acid (GA_3) stimulates K^+ efflux from the barley (*Hordeum vulgare* L. cv. Himalaya) aleurone. We investigated the mechanism of K^+ flux across the plasma membrane of aleurone protoplasts using patch-clamp techniques. Potassium-ion currents, measured over the entire surface of the protoplast plasma membrane, were induced when the electrochemical gradient for K^+ was inward (into the cytoplasm). The magnitude and voltage-dependence of this inward current were the same in protoplasts treated with GA_3 and in control protoplasts (no GA_3). Inward currents activated by negative shifts in the membrane potential (E_M) from the Nernst potential for K^+ (E_K) showed membrane conductance to be a function of the electrochemical gradient (i.e. $E_M - E_K$). Single-channel influx currents of K^+ were recorded in small patches of the plasma membrane. These channels had a single-channel conductance of 5–10 pS with 100 mM K^+ on the inside and 10 mM K^+ on the outside of the plasma membrane. Single-channel currents, like whole-cell currents, were the same in protoplasts treated with GA_3 and control protoplasts. Voltage-gated efflux currents were found only in protoplasts that had been incubated without GA_3. We conclude that K^+ influx in the aleurone is mediated by channels and these membrane proteins are not greatly effected by GA_3.

Key words: Aleurone (protoplasts) – Gibberellin and K^+ fluxes – *Hordeum* (channel-mediated K^+ flux) – Patch-clamp technique – Plasma membrane – Potassium ion (channels)

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Abbreviations and symbols: E_K = Nernst potential for K^+; E_M = membrane potential; E_{rev} = reversal potential; GA_3 = gibberellic acid; K_i = concentration of K^+ inside the cell; K_o = concentration of K^+ outside the cell; R = gas constant; S = conductance (siemens); T = temperature (°K); λ_i = ionic activity coefficient for internal (cytoplasmic) solution; λ_o = ionic activity coefficient for external medium

Indroduction

The barley aleurone is widely used as a model system for studying hormone action and secretion in plants (for reviews, see Jones 1985; Jacobsen and Chandler 1987). We here report work that utilizes the barley aleurone to study the molecular mechanisms of K^+ transport across the plasma membrane. The aleurone may be regarded as a digestive gland that responds to gibberellic acid (GA_3) by synthesizing and secreting acid hydrolases (chiefly α-amylase) and by releasing K^+ and other inorganic cations into the extracellular solution (Eastwood and Laidman 1971; Jones 1973). Hydrolase secretion has been extensively studied, but little is known about cation release or how it is regulated. The efflux of K^+ that is stimulated by GA_3 begins 8 h after treatment with GA_3 and continues at a constant rate for 10 h, thereby reducing the K^+ content of the cell from 6.8 pmol/cell to 3.8 pmol/cell while raising the K^+ concentration of the incubation medium from a few micromolar to several millimolar (Jones 1973). This K^+ efflux is sensitive to metabolic poisons and to inhibitors of protein synthesis, but the mechanism by which K^+ flux occurs is not understood (Jones 1973). The discovery of outward rectifying K^+-selective channels in the plasma membrane of *Vicia faba* guard-cell protoplasts and in protoplasts of other higher plants (Schroeder et al. 1984; Moran et al. 1986; Iijima and Hagiwara 1987; Schauf and Wilson 1987b) has shown that K^+ efflux in these cells could be mediated by K^+ channels. In addition, voltage- and time-dependent inward K^+-channel currents have been described in guard-cell protoplasts (Schroeder et al. 1987). Detailed analysis of this inward K-current has provided direct evidence of its importance during stomatal opening (Schroeder 1988). The purpose of our study was to determine if K^+ flux in the aleurone is also mediated by ion

channels in the plasma membrane, and to determine the effect of GA_3 on channel activity.

In order to determine if K^+ channels are involved in K^+ flux across the aleurone plasma membrane, we measured K^+ currents in whole aleurone protoplasts and in small patches of plasma membrane using the patch-clamp technique (Hamill et al. 1981). This technique allows precise measurement of ion flux under conditions where the membrane potential and the ion concentration gradient across the plasma membrane are well defined.

Using aleurone protoplasts that are capable of responding to GA_3, we found K^+-selective, inward (i.e. into the cytoplasm) currents in protoplasts whether or not they had been treated with GA_3. The activation of these inward currents varied with the Nernst potential for K^+ (E_K) in a fashion similar to the inward rectifier of animal cells (see, for example, Hagiwara and Jaffe 1979). Measurements of single-channel activity in small patches of the aleurone-protoplast plasma membrane show that the K^+-selective currents seen in whole aleurone protoplasts are mediated by single ionic channels in the plasma membrane and that these channels are not greatly modified by treatment with GA_3 under the imposed recording conditions.

Material and methods

Protoplast preparation and purification. Protoplasts were prepared as described by Jacobsen et al. (1985) and modified by Bush et al. (1986). Briefly, de-embryonated grains of barley (*Hordeum vulgare* L.) cv. Himalaya (Department of Agronomy, Washington State University, Pullman, USA; 1985 harvest) were cut in half along the suture, surface-sterilized, and allowed to imbibe 10 mM arginine in a N_2 atmosphere for 24 h. The starchy endosperm was removed from the aleurone layer and the isolated layer was incubated for 2 d in a N_2 atmosphere in 4.5% Onozuka R-10 cellulase (Yakult Pharmaceutical, Nishinomiya, Japan) in Gamborg's B-5 medium (Gamborg et al. 1968). After 48 h, the Onozuka cellulase was replaced by incubation medium containing modified Gamborg's B-5, 350 mM mannitol, and 20 mM $CaCl_2$ with or without 5 μM GA_3. Protoplasts were incubated for up to 60 h, during which time they did not re-form their cell wall but underwent normal vacuolation (Bush et al. 1986) and, if treated with GA_3, secreted amylase. Only those protoplasts that underwent vacuolation were chosen for electrical measurements. These protoplasts were chosen because this change in the cells is correlated in GA_3-treated protoplasts with the period just after maximal enzyme secretion, when any effects of the hormone on membrane proteins should have occurred. Typically, these highly vacuolate cells were 30–40 μm in diameter and had one or a few large central vacuoles and a layer of peripheral cytoplasm (Fig. 1 A).

A necessary step in the patch-clamp technique is the formation of a high-resistance electrical seal with the patch pipette. Although some (roughly 25%) aleurone protoplasts taken directly from their incubation medium will form such a seal, more will do so if they are first purified by flotation on a sucrose or Nycodenz (Nyegaard and Co., Oslo, Norway) discontinuous gradient. Both types of discontinuous gradients consisted of three steps, all of which contained 10 mM dimethylglutaric acid, pH 4.5, and 10 m $CaCl_2$ and were adjusted to 1100 mOsM with sorbitol. The most dense step contained either sucrose (1 M) or Nycodenz (5 g in 5 g H_2O); the middle step contained sucrose (600 mM) plus sorbitol (300 mM) or Nycodenz (3 g in 5 g water). The lightest step in both gradients was sorbitol (1 M). Protoplasts were separated from the incubation medium by allowing them to settle in the bottom of a conical 1.5-ml tube. After 30 min, the incubation medium was removed and the protoplasts resuspended in 1 ml of the heaviest step. The two lighter steps were quickly layered on top of the gradient. Protoplasts banded at the interface between the two lightest steps after 10 min at $1 \cdot g$.

Electrical measurements. The electrogenic flux of ions (i.e. current) across the aleurone plasma membrane was measured using standard patch-clamp techniques (Hamill et al. 1981). Current was measured across a small patch of membrane containing one or a few ionic channels, using the cell-attached (Fig. 1 B) and outside-out patch configurations (Fig. 1 D). Current across the entire surface of the plasma membrane was measured using the whole-cell configuration (Fig. 1 C).

A high-resistance electrical seal between the patch pipette and the plasma membrane formed most readily in areas of the membrane with a relatively thick layer of cytoplasm underneath (Fig. 1 A). Seals were formed using procedures developed for guard-cell protoplasts (Schroeder 1988). Briefly, patch pipettes made from thin-walled Kimax (Kimble, Toledo Oh., USA) glass capillaries that were coated with Sylgard (Sakmann and Neher 1983) and fire-polished were pressed against the membrane, and a small amount of the protoplast (plasma membrane plus underlying cytoplasm) was drawn into the pipette by gentle mouth-suction on the pipette (Fig. 1 b). The formation of the electrical seal was monitored by measuring the current induced by a 6-ms voltage pulse from 0 mV first to −20 mV and then to +20 mV (with respect to the reference electrode in the solution bathing the protoplasts). A 15- to 20-GΩ seal formed within 10–30 min. The formation of the electrical seal without rupturing the plasma membrane produced the cell-attached configuration (Fig. 1 B). The whole-cell configuration (Fig. 1 C), in which current flow across the entire surface of the protoplast plasma membrane could be measured, was derived from the cell-attached configuration (Fig. 1 B) by rupturing the plasma membrane within the patch pipette by suction on the pipette. In the whole-cell configuration, the pipette solution diffuses into the cell, and the chemical and electrical gradients are known.

The relationship between whole-cell current and membrane potential was characterized using two types of voltage-pulse protocols. Current-voltage relations were determined from steady-state currents induced by 1.25-s voltage pulses (Hille 1984). The membrane potential, E_M, was held at 0 mV (with respect to the reference electrode in the solution bathing the cells) and pulsed first to negative (i.e. hyperpolarizing) potentials and then returned to 0 mV. This pulse protocol was repeated at 2-s intervals in voltage increments of 20 mV. The membrane potential at which an activated whole-cell current had zero net current (i.e. E_{rev}) was determined using a double-pulse protocol (Hodgkin and Huxley 1952). This protocol consisted of a large negative voltage pulse from 0 mV, which activated the inward current, followed by another pulse to a less-negative potential, which deactivated the inward current. The deactivation of the inward current resulted in a transient change in whole-cell current to a new steady-state current. This double-pulse protocol was repeated at 2-s intervals, each time increas-

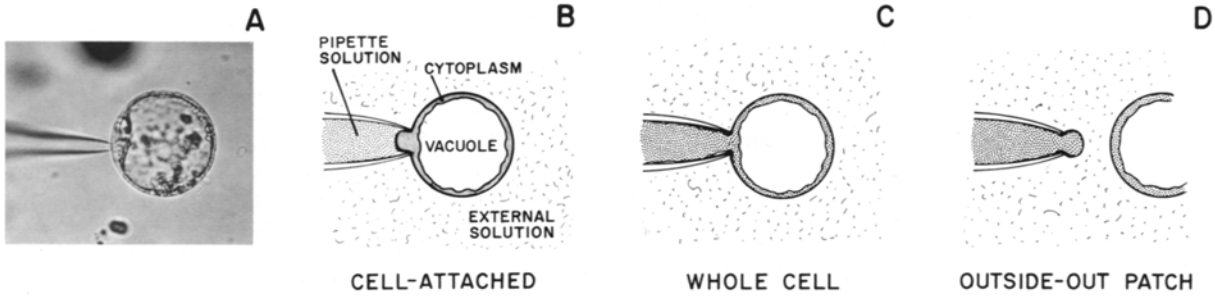

Fig. 1 A–D. Patch-clamp configurations used to measure current across a small patch of the aleurone plasma membrane (**A, B, D**) or the entire membrane surface (**C**). **A** Photomicrograph of a barley aleurone protoplast in cell-attached configuration. The protoplast had been incubated in GA_3 and shows typical cell structure: a large central vacuole and a peripheral layer of cytoplasm. **B** Diagram of the cell-attached configuration. An electrical seal forms between the plasma membrane and the patch pipette after a small portion of the cell is pulled into the pipette by suction. The membrane remains intact and the solution in the pipette does not mix with the cytoplasm. **C** Diagram of the whole-cell configuration, which is derived from the cell-attached configuration by rupturing the plasma membrane. This allows the ionic components of the pipette solution and the cytoplasm to mix. **D** Diagram of the outside-out patch which is derived from the whole-cell configuration by pulling the pipette away from the protoplast. The excised membrane reseals with the pipette solution on the inside of the membrane

ing the second voltage pulse until the transient current changed its direction from a decay of inward current to a decay of outward current. E_{rev} was that potential at which there was no decay of either inward or outward current. Leakage currents were not subtracted from the current-voltage relations.

In order to compare the whole-cell current of two protoplasts, current was expressed per unit surface area of the membrane. The capacitive transients produced by the charging of the membrane during voltage pulses provided an estimate of 0.95 $\mu F \cdot cm^{-2}$ for the specific membrane capacitance. Membrane surface area was routinely estimated from measured capacitive transients using this specific membrane capacitance.

Whole-cell and single-channel currents were measured using an EPC-7 patch-clamp amplifier (List-electronic, Darmstadt, FRG). The EPC-7 also voltage-clamped the membrane at the desired voltage, measured whole-cell capacitance, and corrected for the series resistance at the pipette-cell junction. The signal from the patch-clamp amplifier was displayed on an oscilloscope and digitally recorded on videotape. Recordings on videotape were analysed using a PDP11/73 computer (INDEC, Sunnyvale, CA., USA). For analysis of single-channel recordings, the signal from the EPC-7 was filtered at 800 Hz using a low-pass filter with 8-pole Bessel characteristics.

The compositions of the solutions in the external solutions and in the patch pipette were designed to approximate physiological conditions for Ca^{2+}, Mg^{2+}, ATP, and pH. Solutions bathing the protoplasts during electrical measurements contained $CaCl_2$ (1 mM); $MgCl_2$ (2 mM); 2-(N-morpholino)-ethanesulfonic acid (Mes, 10 mM, pH 5.6); sorbitol (1100 mOsM); and either potassium glutamate (1, 10, or 100 mM), KCl (1, 10 or 100 mM), or sodium glutamate (10 or 100 mM). Solutions could be changed by perfusion using peristaltic pumps while electrical recordings were being made. The volume of the bath solution was approx. 200 µl and the rate of perfusion was 0.5–1 $ml \cdot min^{-1}$. Solutions used to fill the pipette contained ethylenglycol-bis-(β-aminomethyl ether)-N,N,N′,N′-tretraacetic acid (EGTA, 1 mM to insure low Ca^{2+}); $MgCl_2$ (2 mM); N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes, 10 mM, pH 7.2); sorbitol (1100 mOsM); and either potassium glutamate (10, 100 or 500 mM) or KCl (10 or 100 mM). Glutamate was chosen as the counter ion for K^+ because it does not readily permeate the plasma membrane (Schroeder et al. 1984).

The electrochemical gradient for K^+ was defined in the standard way (Nobel 1970, chptr. 3) as the difference between the membrane potential and the Nernst potential for K^+, (i.e., $E_M - E_K$). The Nernst potential was calculated by the standard relation, RT/F ln (λ K_o/λ_i K_i), where R is the gas constant, T is temperature in °K, F is the Faraday constant, K_o and K_i are the concentration of K^+ in the external medium (K_o) and in the cytoplasm (K_i), and λ_o and λ_i are the ionic activity coefficients for K^+ in the external medium (λ_o) and cytoplasm (λ_i). Activity coefficients were calculated for each experimental solution using the Davies equation (Sposito 1981, chptr. 2). Experimentally determined values for the junction potential arising at the solution interface between pipette and bath were used to correct E_M. Membrane conductance (expressed in siemens, S) was calculated in the standard way as the ratio between current and membrane potential.

Results

Whole-cell K^+ currents. In order to determine whether electrical currents could be measured across the plasma membrane under conditions that would promote K^+ flux, we examined aleurone protoplasts in the whole-cell configuration with 100 mM K^+ in the cytoplasm and 10 mM K^+ in the external solution. We found voltage-dependent currents were activated across the plasma membrane when the membrane potential was shifted from the holding potential (Fig. 2). In protoplasts that had not been treated with GA_3, voltage pulses more negative than the resting potential (−45 mV; Fig. 2) induced inwardly directed current, whereas voltage pulses more positive than the resting potential produce outwardly directed currents (Fig. 2B). Protoplasts treated with GA_3 also showed inward current in response to hyperpolarizing voltage pulses but, in contrast to those not treated with

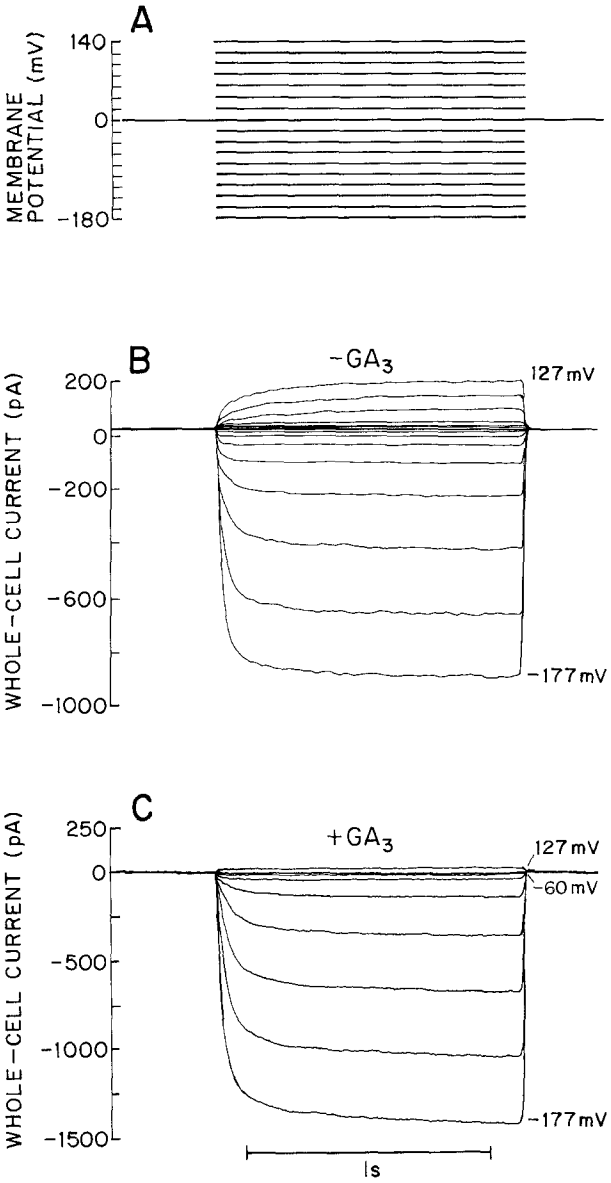

Fig. 2A–C. Recordings of imposed membrane potential (**A**) and the resulting whole-cell currents (**B, C**) in barley aleurone protoplasts incubated without GA_3 (**B**) and with GA_3 (**C**). The whole-cell configuration was established with 100 mM potassium glutamate in the cytoplasm and 10 mM potassium glutamate in the external solution. Negative values for current indicate current influx (i.e. into the cytoplasm) and positive currents indicate efflux. **A** Superposition of multiple recordings of changes in the membrane potential. The membrane potential was held at 0 mV and pulsed first to −177 mV for 1.25 s and then returned to 0 mV. The cycle was repeated 16 times and each time the pulse from 0 mV was incremented by +20 mV. **B** Superposition of consecutive recordings of whole-cell current induced by changes in the membrane potential shown in **A** for an aleurone protoplast incubated without GA_3. The whole-cell capacitance was 49 pF and the resting potential was −45 mV. **C** Superposition of consecutive recordings of whole-cell current induced by imposed membrane potentials of −177 to −60, 0 and +127 mV for an aleurone protoplast incubated with GA_3. The whole-cell capacitance was 54 pF and the resting potential was −50 mV

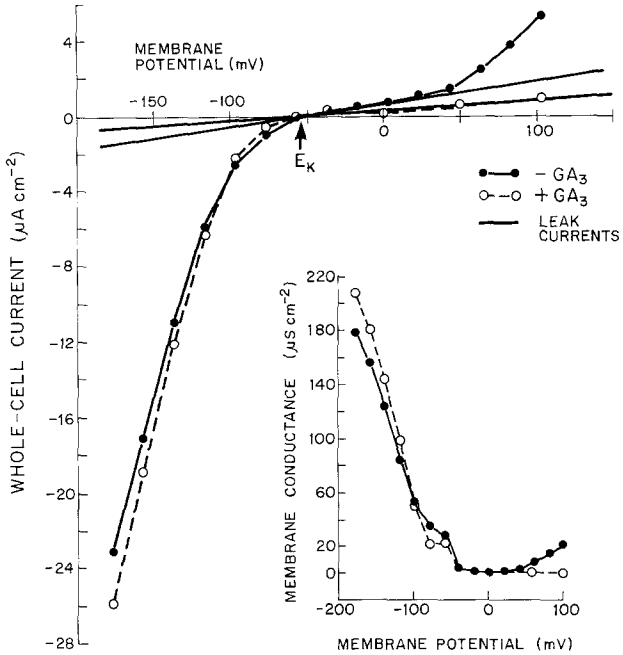

Fig. 3. The effect of GA_3 on current flow across the barley aleurone plasma membrne and on membrane conductance as a function of the imposed membrane potential. The data are calculated from the traces shown in Fig. 2. Whole-cell currents and membrane conductance are expressed per unit area of membrane. Membrane conductance was calculated after subtracting the ohmic leak current. The leak current was calculated by linear regression

GA_3, showed very little outward current in response to depolarizing voltage pulses (Fig. 2C).

The inward current seen in protoplasts of both plus- and minus-GA_3 treatments was stable and was found in every protoplast examined, including those that had been incubated without GA_3 for more than 120 h (data not shown). The time course of activation of the inward current was also similar in the two treatments (Fig. 2B, C). During the 1.25-s voltage pulses there was no apparent deactivation of the whole-cell inward current.

The absolute magnitude of the inward current varied between cells in proportion to the size of the cell but not as a function of treatment with GA_3 (Fig. 3). When whole-cell currents were expressed as current flux (i.e. current per 1 cm^2 of membrane), the magnitudes of the inward current as a function of the applied E_M in protoplasts incubated with and without GA_3 were remarkably similar (Fig. 3). In addition, both treatments showed outward currents that were linearly dependent on E_M between −57 and +35 mV when 100 mM K^+ was in the cytoplasm and 10 mM K^+ was in the external solution (Fig. 3). The whole-cell conduc-

tance in this region was typically 5–10 $\mu S \cdot cm^{-2}$. The selectivity of the current in this linear region of the current-voltage relation was not studied. Rather, we assumed that this linear current represented leak current at the junction of the patch pipette with the cell, and subtracted its calculated value from the measured value of current when calculating conductance (Fig. 3, inset).

Membrane conductance was strongly dependent on E_M, a characteristic of K^+ flux through voltage-gated ionic channels (Fig. 3). The inward conductance varied with voltage in a similar way for protoplasts incubated with and without GA_3 (Fig. 3). In protoplasts that were not treated with GA_3, outward currents also showed a voltage-dependent conductance that activated at +25 mV (Fig. 3).

With 100 mM potassium glutamate on the cytoplasmic side and 10 mM potassium glutamate on the outside of the protoplast, the E_M that produced zero net current was −47 to −50 mV in both plus- and minus-GA_3 treatments. This E_{rev} is close to −52 mV, the E_K for these solutions. This indicates that K^+ is the ion that carries the whole-cell currents.

In order to confirm that the whole-cell currents were K^+ currents, we measured E_{rev} as a function of the concentration of potassium glutamate in the external solution. If K^+ carries the current, E_{rev} should equal E_K. Using a double-pulse procedure (Fig 4A), E_{rev} was measured with 100 mM potassium glutamate in the cytoplasm and 1, 10 or 100 mM potassium glutamate in the external solution. Typically, the activation and steady-state level of current induced by the first voltage pulse of the double-pulse procedure was stable (Fig. 4B). The deactivation of the inward current induced by the second voltage pulse (a step to a more positive membrane potential) was slow and required several hundred milliseconds for completion (Fig. 4B). E_{rev} was identified by the absence of this slow transient (Fig. 4B). With 100 mM potassium glutamate in the cytoplasm and 10 mM potassium glutamate in the external solution, E_{rev} was −47 mV (Fig. 4B). With 100 mM potassium glutamate on both sides of the plasma membrane, E_{rev} was 0 mV, which is also the E_K. Changing the K^+ concentration in the external solution to 1 mM changed E_{rev} to −100 mV, again close to E_K, which was −105 mV in these solutions (Fig. 5A). These reversal potentials were not changed when Cl^- was substituted for glutamate (data not shown), indicating that glutamate and chloride flux across the plasma membrane had no major influence on the reversal potential of the whole-cell current.

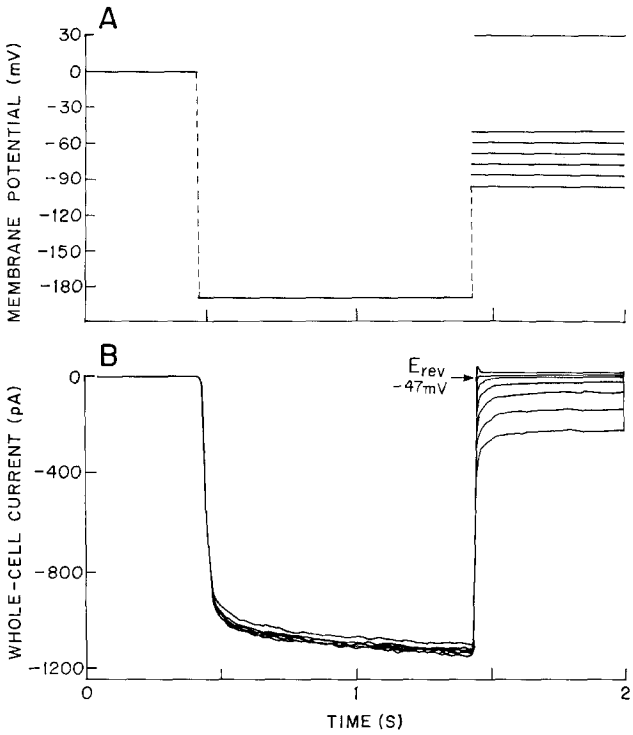

Fig. 4A, B. Determination of the reversal potential of voltage-dependent inward current for GA_3-incubated barley aleurone protoplasts in the whole-cell configuration. The cytoplasm contained 100 mM potassium glutamate and the external solution contained 10 mM potassium glutamate. **A** Seven consecutive tracings of the protoplast membrane potential. The membrane potential was changed using a double-pulse procedure from 0 mV to −180 mV and then stepped to a higher potential (−97 mV to 37 mV). **B** The activation of the whole-cell current and subsequent deactivation induced by the double-pulse membrane potential in **A**

A second effect of lowering K_o was an overall reduction in the inward current (Fig. 5A). Lowering K_o, shifted the current-voltage relation along the voltage axis in accordance with a shift in E_K (Fig. 5A). This is seen most clearly by the similarity in membrane conductances with 100 mM potassium glutamate in the cytoplasm and 1, 10 or 100 mM potassium glutamate in the external solution (Fig. 5B). Similarly, changes in K_i from 100 to 500 mM had no major effect on the conductance of the whole-cell current (data not shown). Over the range of K_i and K_o we tested, the inward current was dependent upon the difference between E_M and E_K and was only slightly dependent on K_i and K_o.

The measurements of reversal potential support the hypothesis that K^+ carries the current seen in whole-cell recordings. Therefore, we determined the selectivity of the current for K^+ by replacing the K^+ in the external solution with Na^+.

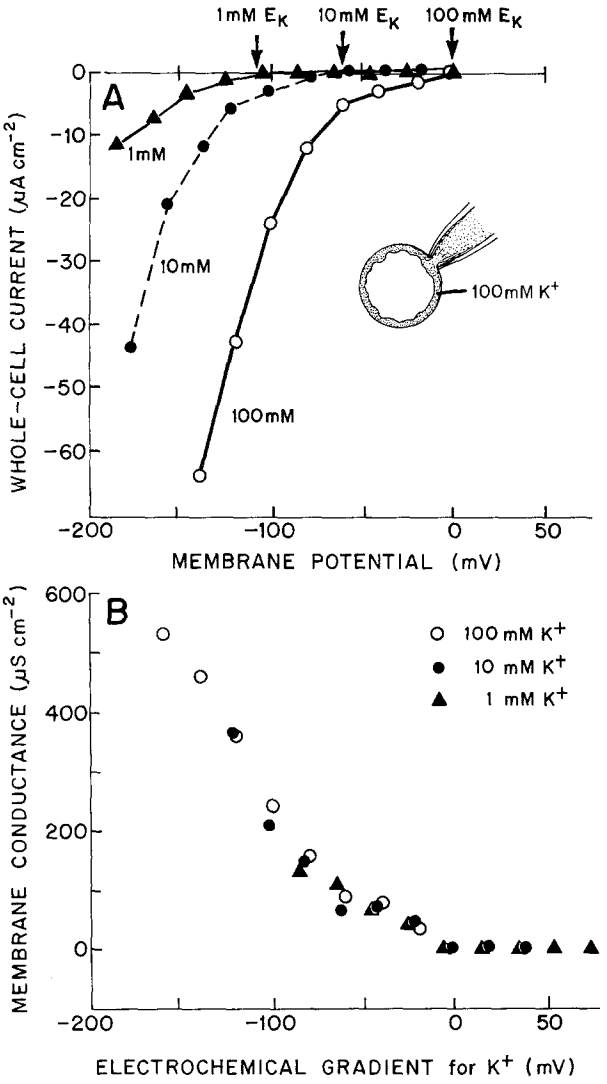

Fig. 5A, B. The effect of the concentration of K^+ in the external solution on the whole-cell current-voltage relation (**A**) and non membrane conductance (**B**) for barley aleurone protoplasts incubated in GA_3. **A** The relation between whole-cell current and membrane potential for protoplast with 1, 10, or 100 mM potassium glutamate in the external solution. The Nernst potentials, E_K, are indicated by the *arrows*. **B** Whole-cell membrane conductance for the data shown in **A**. Membrane conductance was calculated as a function of the electrochemical potential ($E_M - E_K$)

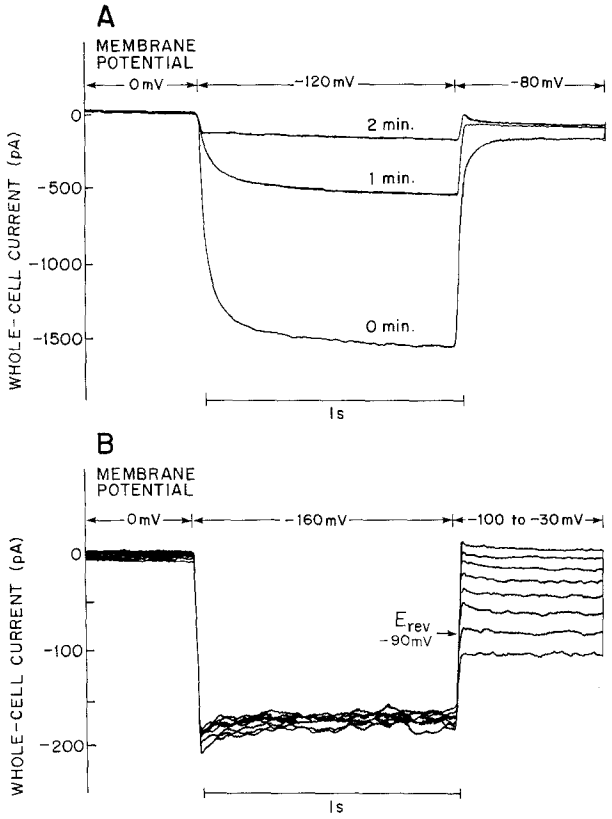

Fig. 6A, B. Specificity of the whole-cell inward current in barley aleurone protoplasts for K^+ compared to Na^+, as shown by the decline in the magnitude of the whole-cell current, and in E_{rev} during exchange of 100 mM sodium glutamate for 10 mM potassium glutamate in the external solution. **A** Inward currents induced by changes in the membrane potential using a double-pulse protocol in which the membrane potential was pulsed first to −120 mV and then to −80 mV. Recordings of current measured in 10 mM potassium glutamate before perfusion with 100 mM sodium glutamate (0 min) and 1 and 2 min after perfusion with 100 mM sodium glutamate. **B** Determination of the reversal potential (E_{rev}) by a double-pulse protocol after the external solution was changed to 100 mM sodium glutamate. The membrane potential was pulsed to −160 mV and then to more positive potentials (−100 to −30 mV)

Whole-cell currents were first measured with 100 mM potassium glutamate in the cytoplasm and 10 mM potassium glutamate in the external solution using a double-pulse procedure. The first pulse to −140 mV resulted in a large inward current which decayed to a new level when the voltage was stepped up to −80 mV (Fig. 6A). The inward transient induced by the second voltage pulse indicates that −80 mV is more negative than the reversal potential of the current, which is consistent with the data on E_{rev} shown above.

When the external solution was changed from 10 mM potassium glutamate to 100 mM sodium glutamate by perfusion, both the steady-state current induced by the first voltage pulse and the transient current induced by the step change to the second voltage pulse were altered. As Na^+ replaced K^+ in the external solution (1- and 2-min trace, Fig. 6A), the steady-state current was reduced and the transient current was abolished. When Na^+ had completely replaced K^+, the inward current was further reduced and the transient current

shifted direction from inward to outward, indicating that Na^+ induced a shift to a more negative E_{rev} (Fig. 6A, 2-min trace). The final E_{rev}, as measured by a double-pulse procedure, was −90 mV (Fig. 6B). The relative selectivity of the plasma membrane for K^+ over Na^+ was 35:1 as calculated using the Goldman equation (Nobel 1970, chptr. 3) and the measured reversal potential.

Single channels in the plasma membrane. The magnitude and voltage dependence of the whole-cell currents indicates that they are carried by ionic channels in the plasma membrane. In order to test whether K^+-currents were carried by channels, we measured the activity of single ion channels using the cell-attached configuration. In this configuration, the protoplast remains intact and currents are measured across the small patch of membrane where the pipette is sealed. The patch of plasma membrane is voltage clamped from the outside so that, in contrast to the whole-cell configuration, positive applied voltages hyperpolarize and negative voltages depolarize the membrane.

Recordings of single-channel activity made in the cell-attached configuration showed voltage-dependent influx and efflux currents. Applied positive voltages produced inward currents whose magnitude and duration increased with increasing positive voltages (Fig. 7A). These influx channels showed large and rapid current fluctuations in the open state (Fig. 7A). The size of the current in the influx channel was linearly proportional to the applied voltage in the range of +30 to +100 mV (Fig. 7B). The single-channel conductance was calculated to be 35 pS with 100 mM K^+ in the pipette and did not differ in protoplasts treated with and without GA_3 (Fig. 7C).

In order to measure the E_{rev} of inward single-channel current, single-channel recordings were also made on outside-out patches of membrane. The single-channel currents in these patches varied linearly with the membrane potential (Fig. 7B) and had an E_{rev} potential of −47 mV (Fig. 7B). This close correspondence between the E_K and E_{rev} indicates that the current through these single channels is carried by K^+. The single-channel conductance under these conditions was 5–10 pS (Fig. 7B).

Outward single-channel currents were found in aleurone membranes when patches of membrane in the cell-attached configuration were depolarized by negative voltage pulses and single-channel efflux currents were activated (Fig. 8A). These efflux channels were usually found in protoplasts that were not treated with GA_3 but not in protoplasts treated with GA_3, a result that is consistent with the whole-cell currents. The efflux channels, like whole-cell efflux currents, were quite labile. Single-channel events at a given membrane potential tended to decrease with time. The single-channel conductance with 100 mM potassium glutamate in the pipette was 33 pS (Fig. 8).

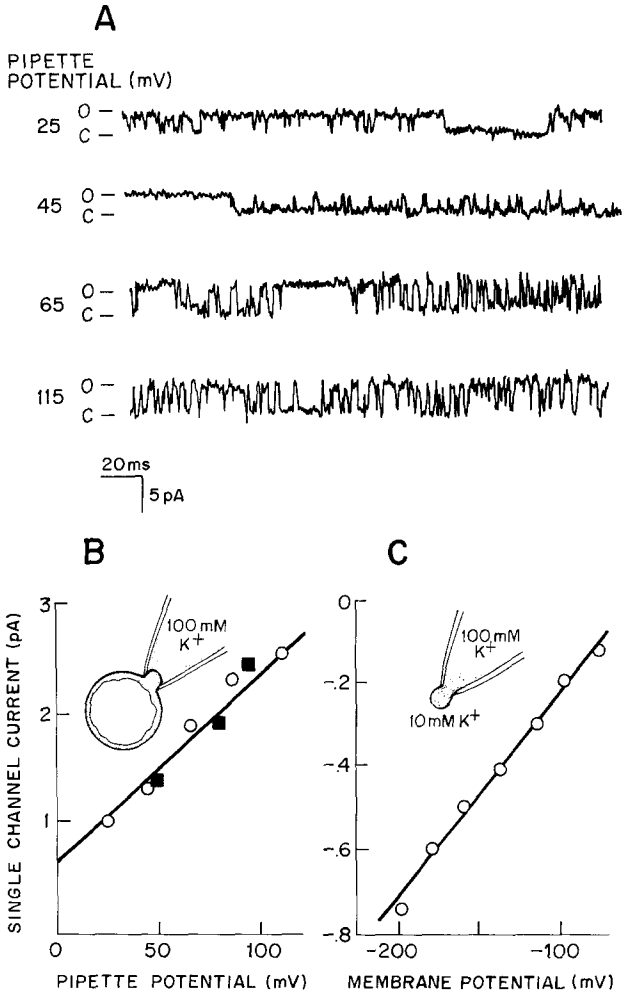

Fig. 7A–C. Recordings of inward channel activity in barley aleurone protoplasts (**A**) and the corresponding single-channel current-voltage relation in cell-attached mode (**B**) and in an outside-out patch (**C**). **A** Single-channel recordings of influx currents measured in the cell-attached configuration with 100 mM potassium glutamate in the pipette. Protoplasts had been incubated in GA_3. In this configuration, positive current values indicate influx. Recordings of current are shown for the channel in the open (*O*) and in the closed (*C*) state induced by hyperpolarization of the membrane by the indicated pipette potential. **B** Current-voltage relation for single influx channels recorded in the cell-attached mode for cells incubated with (■) or without (○) GA_3. The pipette contained 100 mM potassium glutamate and the bath contained 10 mM potassium glutamate. **C** Current-voltage relationship for single-channel currents for an outside-out patch with 100 mM potassium glutamate in the pipette solution and 10 mM potassium glutamate in the bath solution. In this configuration, negative current values indicate influx

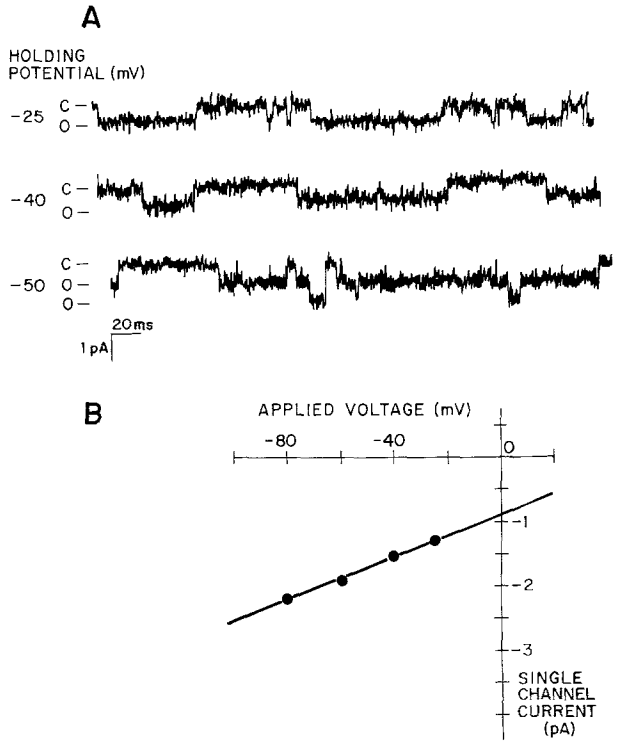

Fig. 8A, B. Recordings of single-channel efflux currents (**A**) and the single-channel current-voltage relation (**B**). **A** Single-channel recordings of efflux currents in a protoplast in cell-attached configuration with 100 mM potassium glutamate in the pipette. Single-channel currents are shown in the open (*O*) and closed (*C*) position induced by the indicated holding potential in the pipette. Protoplasts were incubated without GA_3. In this configuration, negative pipette potentials depolarize the membrane and negative current values indicate efflux. **B** Current-voltage relation for single-channel efflux currents in a protoplast under the conditions described in **A**

Discussion

We have shown that the passive flux of K^+ across the plasma membrane of barley aleurone protoplasts produces current that is mediated by single channels. Since K^+ currents and single-channel activity were found in aleurone protoplasts incubated both in the presence and in the absence of GA_3 (Figs. 2, 3, 6B), we conclude that channel-mediated K^+ flux is a constitutive feature of the aleurone plasma membrane and is not induced by GA_3. This means that GA_3 does not stimulate the efflux of K^+ from the aleurone cell simply by incorporating K^+ channels in the plasma membrane. In fact, GA_3 appeared to reduce channel-mediated K^+ efflux (Figs. 2, 3). This leads us to speculate that GA_3 may stimulate K^+ efflux by altering E_M or K_i.

Because aleurone cells do not grow or undergo large reversible changes in volume, they differ from the other higher-plant cells in which K^+ channels have been described (Schroeder et al. 1984; Moran et al. 1986; Iijima and Hagiwara 1987; Schauf and Wilson 1987b). Nevertheless, the channel-mediated K^+ currents that we found in the aleurone are similar to those described in cells whose volume does change. The whole-cell K^+ currents that we measured in aleurone protoplasts, like those in protoplasts of *Vicia faba* (Schroeder et al. 1987) and *Dionaea muscipula* (Iijima and Hagiwara 1987) were voltage- and time-dependent (Figs. 2, 3). The magnitude and direction of K^+ currents in the aleurone were determined by the electrochemical gradient for K^+ (Fig. 4B) as would be expected for K^+-flux mediated by channels. The membrane potential at which the inward current reversed (E_{rev}) was always within a few millivolts of E_K (Figs. 3, 4B, C). Moreover, the whole-cell K^+ currents we observed were highly selective for K^+ over Na^+ and anions (Fig. 5).

The increase of the inward conductance of the aleurone plasma membrane for K^+ as as a function of $(E_M - E_K)$ is qualitatively similar to the inward rectifier of some animal cells. Inward conductance in the aleurone, like that in the egg-cell membrane of *Mediaster* (Hagiwara et al. 1976), activate close the E_K with a steep voltage dependence when E_M is more negative than E_K (Figs. 3A, 5A, B). Our data indicate that, unlike the inward rectifier in animals in which conductance depends on E_M and K_o, conductance in the aleurone may be a function of the total electrochemical gradient (Fig. 5B). However, additional studies on the effect of K_i on conductance are needed to determine if, as in *Mediaster*, conductance is independent of K_i (Hagiwara et al. 1976).

The single-channel activity we measured in aleurone protoplasts indicates that the whole-cell currents were mediated by channel molecules. The single-channel inward current seen in outside-out patches of aleurone membrane (Fig. 7) linearly extrapolated to zero current when E_M equalled E_K, and on this basis we concluded that the channels were K^+ channels. The reversal potential of the single-channel current measured in cell-attached mode cannot be determined with precision since neither K_i nor E_M of the intact cell is known. However, it is possible to estimate that K_i is 150–250 mM from measurements of total cell K^+ in aleurone layers (calculated from Jones 1973). Assuming a membrane potential close to E_K, the single-channel K^+ current should have an E_{rev} of 0 to -25 mV for the conditions we used in the attached mode. This is close to the observed values and therefore these data are consistent with our

conclusion that K^+ carries the single-channel current.

The inward current-voltage relation of single channels in either cell-attached our outside-out patches was ohmic (i.e. current was linearly proportional to voltage) with a single-channel conductance of 5–10 pS with 100 mM K^+ inside and 10 mM K^+ outside of the membrane (Fig. 7). This single-channel conductance is slightly lower than that reported for protoplasts of guard cells of *Vicia faba* (Schroeder et al. 1984, 1987), but is similar to that of outward rectifying K^+ currents reported for trap-lobe cells of *Dionaea muscipula* (Iijima and Hagiwara 1987). Gibberellic acid had no effect on the single-channel conductance of the inward K^+ channel. Since GA_3 also had no effect on the current-voltage relation of the whole-cell inward current, we conclude that the hormone did not alter the density of the inward channels. Using the observed single-channel conductance and assuming an open-channel probability of unity for the observed maximum whole-cell conductance (Fig. 5B), it is possible to estimate a lower limit for channel density of 0.1–0.2 channels $\cdot \mu m^{-2}$.

Our data do not clearly indicate whether K^+ efflux is mediated by ion channels. Efflux currents with voltage-gated conductances were greatly reduced in protoplasts treated with GA_3 and were only found at large depolarizing voltages in protoplasts not treated with GA_3 (Fig. 3). Since single-channel efflux currents were not found in GA_3-treated protoplasts using the cell-attached configuration, we presume that the absence of whole-cell outward currents is not an artifact of the whole-cell configuration. Efflux currents that were not voltage-gated but that were essentially ohmic were found in all protoplasts when E_M was close to E_K (Fig. 3). It is likely that most of this ohmic current is not channel-mediated but represents nonphysiological leak at the pipette-cell junction (Iijima and Hagiwara 1987). Nevertheless, the possibility that a small amount of channel-mediated efflux current exists around E_K cannot be dismissed. The efflux currents tended to be labile as previously observed for efflux currents in guard-cell protoplasts (Schroeder 1987). Moreover, only a small amount of efflux current would acount for the K^+ efflux induced by GA_3. Using the measured reduction of the K^+ content of the aleurone, it is possible to calculate that a steady-state current of 0.2 $\mu A \cdot cm^{-2}$ (i.e. whole-cell current less than 10 pA; compare Fig. 2) through a K^+-selective channel would account for all of the observed K^+ efflux. Thus a small but physiologically important, channel-mediated efflux current could be masked by the ohmic leak currents around E_K. Further experiments will be required to establish if K^+ efflux is mediated by channels.

Conclusions

The aleurone plasma membrane contains voltage-gated K^+ channels with properties that are similar to those described for protoplasts (see, for example, Schroeder et al. 1987) and to the inward rectifier of animal cells. The action of these single-channel molecules results in large, whole-cell K^+ currents that should insure that the resting membrane potential of the aleurone cell is close to E_K. Although GA_3 greatly stimulates K^+ efflux, it has little detectable effect on channel-mediated currents. Our data indicate, therefore, that GA_3 must stimulate efflux by raising the membrane potential to a value more positive than E_K.

It is reasonable to assume that GA_3 stimulates K^+ efflux from aleurone cells by altering either the concentration of K^+ in the cytoplasm or the membrane potential. Aleurone cells contain large stores of insoluble potassium phytate that are sequestered in the aleurone grains (Liu and Pomeranz 1975). Gibberellic acid has been shown to stimulate phytase activity in the aleurone, resulting in the release of inorganic ions (Gabard and Jones 1986; Stewart et al. 1988). A direct effect of GA_3 on E_M may be envisioned by a variety of mechanisms including an increase in conductance for another ion (Schauf and Wilson 1987a; Ephritikhine et al. 1987). These potential actions of GA_3 should be testable using the aleurone protoplast. The ability of the aleurone protoplast to respond to the plant hormones GA_3 and abscisic acid (Jacobsen and Chandler 1987), its longevity and its unusually abundant cytoplasm make it particularly well-suited for electrophysiological studies using patch-clamp electrodes.

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