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A slow anion channel in guard cells, activating at large hyperpolarization, may be principal for stomatal closing

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Slowly activating anion channel currents were discovered at micromolar 'cytoplasmic' Ca^{2+} during patch-clamp measurements on guard-cell protoplasts of *Vicia faba* and *Xanthium strumarium*. They activated at potentials as low as −200 mV, with time constants between 5 and 60 s, and no inactivation. The broad voltage dependence exhibited a current maximum near −40 mV. The single-channel open time was in the order of seconds, and the unitary conductance was 33 ps, similar to that of the already described 'quick' anion channel of guard cells. Because of its activity at low potentials, the slow anion channel may be essential for the depolarization of the plasmalemma that is required for salt efflux during stomatal closing.

Anion channel; Patch clamp; Stomatal closure; Voltage dependence

1. INTRODUCTION

Plants attempt to minimize water loss with respect to carbon uptake by opening their stomata primarily during periods favoring photosynthesis and closing them when darkening sets in or water stress develops. Stomatal opening is caused by an accumulation of salts of K^+ in the guard cells and an ensuing increase in their volume. The basic mechanisms involved in the opening process appear to be better known than those primary in the controlled loss of cations and anions from the guard cells during closing. Anion channels, available for efflux, were discovered in these cells; however they permitted passage of anion currents of sufficient magnitude only at plasmalemma potentials positive to −100 mV [1,2]. For salt loss to occur, K^+_{out} channels must open in addition; they do so at still higher potentials, above −40 mV [3]. A depolarization of the plasmalemma above this threshold will be necessary to bring about stomatal closure. Potentials in this range are above the equilibrium potential of K^+ (in guard cells of wide open stomata estimated to be as low as −120 mV). Auxiliary hypotheses, like the opening of Ca^{2+} channels, were invoked to account for the necessary collapse of membrane polarization [2,4]. Now it seems that a newly discovered type of anion channel may provide this key function. Besides being active at potentials as low as −200 mV, this channel differs from the anion channel described earlier [1,2] by slow activation kinetics. We shall refer to it as the 'slow anion channel' (SLAC) of the guard-cell plasmalemma, in contrast to the 'quick anion channel' (QUAC) described before [1,2].

We used guard cell protoplasts of *Vicia faba* and *Xanthium strumarium* for our investigation. *V. faba* has been the species most frequently used for patch-clamp measurements on guard cells, whereas much is known about stomatal behavior in whole leaves from studies with *X. strumarium*. We preferred to use *X. strumarium*, and we verified the results obtained with material from *V. faba*.

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Abbreviations: EGTA, Ethylene glycol bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid; HEDTA, *N*-Hydroxyethylethylenediaminetriacetic acid; MES, 2-[*N*-morpholino]ethanesulfonic acid; Tris, Tris [hydroxymethyl]aminomethane.

2. MATERIALS AND METHODS

2.1. *Plants and protoplast preparation*

Plants of *Xanthium strumarium* L. were grown in a greenhouse, hydroponically in clay pebbles (Lecaton, 4–8 mm; nutrient solution: Flory 9 from Planta, Regenstauf, Germany, 1 $g \cdot l^{-1}$, with an addition of 0.24 $g \cdot l^{-1}$ of $CaSO_4$). Natural light was supplemented by light from metal vapor lamps (Osram Power Star HQI TS 250 or 400/D, 350 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the top of the plants). Flowering was prevented by additional light for one hour in the middle of the night. Day/night temperatures were 22/18°C. The fourth leaf above the cotyledons from four-week-old plants was used. Plants of *Vicia faba* L. were cultivated in the same environment, but in soil.

Epidermal fragments were obtained by mincing the leaves in a blender [5]. For the subsequent enzymic digestion the epidermal fragments were shaken at 20°C with an amplitude of 2 cm and a frequency of 1.7 Hz for 2–3 h in 1 mM $CaCl_2$, 10 mM Na-ascorbate and containing 0.02% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan), 2% (w/v) Cellulase Onozuka R-10 (Yakult Honsha, Tokyo, Japan), 2% (w/v) BSA, π = 600 $mosmol \cdot kg^{-1}$ for *X. strumarium* and π = 400 $mosmol \cdot kg^{-1}$ for *V. faba*, adjusted with sorbitol.

2.2. *Patch-clamp measurements [6]*

Voltage pulses were applied and currents measured by an EPC-7 patch clamp amplifier (List-electronic, Darmstadt, Germany). The results were filtered by an eight-pole Bessel filter (Frequency Devices, Haverhill, Massachusetts, USA), digitized by an ITC 16 interface (Instrutech, Elmont, NY, USA) and stored on hard disc (SY Quest Technology, Fremont, CA, USA) by a Mega Atari ST4 (Atari, Sunnyvale, CA, USA) or after digitization by a VR 10 interface (Instrutech) on video tape. Data were analysed by patch-clamp software from Instrutech.

Unless otherwise stated, solutions contained Cs^+ instead of K^+ to prevent concealment of the anion currents by currents of K^+. (K^+ channels are virtually impermeable to Cs^+.)

All pipette solutions for the whole-cell measurements were 5 μM with respect to free Ca^{2+}, adjusted with $CaCl_2$ and HEDTA [7]. The osmolalities were 600 mosmol·kg^{-1} of all media used with *X. strumarium* and 400 mosmol·kg^{-1} for those for *V. faba*, adjustment with sorbitol. Other components of the solutions are given in the figure legends.

3. RESULTS

3.1. *Slow-anion-channel currents*

Evidence for the presence of slow anion channels appeared in whole-cell currents of guard-cell protoplasts of *V. faba* and *X. strumarium*. Starting from holding potentials of −160 mV (*V. faba*), and −220 or −200 mV (*X. strumarium*), repetitive pulses of 90 s duration were applied in increments of 20 mV to cover the voltage range from −240 mV to +60 mV. Fig. 1 shows an example acquired with *X. strumarium*. Exponential functions were fitted to the activation curves; time constants, τ, were obtained and, by extrapolation to infinity, the steady-state currents. At −180 mV, the time constant was 5 s, and it increased with membrane potential, U_m, to 16 s at −20 mV. These values applied to cells characterized by Cl^--currents of about 40 pA at U_m = −100 mV. In cells exhibiting currents of twice this magnitude, activations occurred even more slowly, and the time constant approached 1 min. For comparison, the time constant for the quick-anion-channel current was 18 ms at U_m = −40 mV, where the current maximum was located. In some cases, it was necessary to fit a sum of two *e*-functions to the activation curves. For example, at U_m = −20 mV, three out of five examples listed had a faster component (τ = 7 s, additional current of 12 pA). The slow-anion-channel currents did not show signs of inactivation during the 90-s pulses, in contrast to the behavior of the quick-channel currents [2].

It proved virtually impossible to apply pulse programs from holding potentials below −220 mV. The seals of the protoplasts became increasingly unstable upon each return to the holding potential.

Current–voltage relationships were constructed from the extrapolated currents. Figure 2 shows a combination of seven normalized examples. All individual curves intersect the abscissa at the equilibrium potential of Cl^- (+40 mV); the currents recorded prove to be carried by Cl^-. Therefore, the minima of the curves indicate maxima of anion efflux. These maxima were

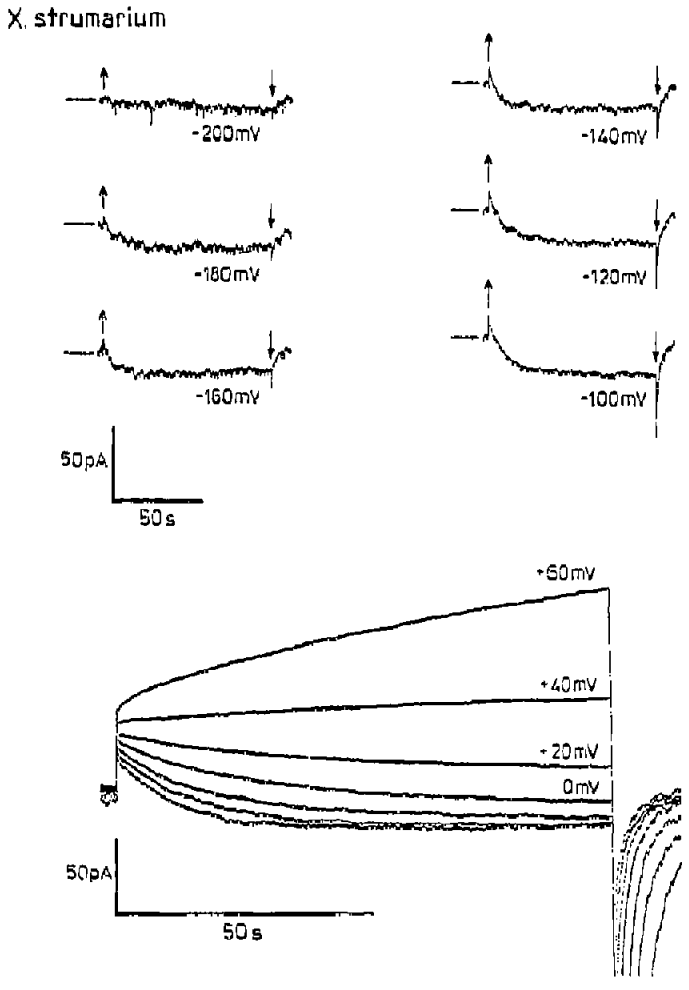


Fig. 1. An example of the activation kinetics of the slow-anion-channel current of a guard-cell protoplast of *X. strumarium*. After 60 s at a holding potential of −220 mV, pulses of 90 s duration were applied in 20-mV steps, ranging from −200 to −100 mV (top), and from −50 to +60 mV (bottom, expanded time scale!). External solutions were 30 mM CsCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM MES; internal solutions were 150 mM CsCl, 5 μM free Ca^{2+}, 2 mM free Mg^{2+}, 10 mM MgATP, 10 mM HEDTA, 10 mM HEPES.

situated between U_m = −40 mV and −20 mV. The appearance of Cl^- currents at large hyperpolarizations was remarkable.

3.2. *Quick-anion-channel currents*

Quickly activating anion channels were discovered in guard cells of *V. faba* [1,2]. Corresponding currents appeared also in *X. strumarium*, when we applied voltage ramps to guard cells of both species. The curves intersected the abscissa at voltages slightly below the equilibrium potential of Cl^- (40 mV). In guard-cell protoplasts of *V. faba*, the maximum Cl^- current occurred between −42 and −45 mV. In *X. strumarium* it passed between −53 and −55 mV.

3.3. *Single-channel recordings*

Quick anion channels. Activities of Cl^- channels with short open times were detected in guard-cell protoplasts

of *X. strumarium*. These records resemble those presented for *V. faba* [1] and therefore are not shown. Even the unitary conductance of 36 ps was similar to that of *V. faba* (39 ps). The mean open time was 2.9 ms at a potential of −90 mV and 4.1 ms at a potential of −70 mV.

Slow anion channels. In outside-out patches single chloride channel activities appeared. Open times were in the order of 1 s, and the single-channel conductance was 33 ps (Fig. 3A,B). The open probability of the slow channels increased at less negative potentials, so that only 1 or 2 channels were closed at those voltages. Because we could not determine with sufficient confidence the number of active channels in the outside-out patches, an accurate estimation of the mean open time was not possible.

4. DISCUSSION

The solutions in our patch pipettes were micromolar with respect to Ca^{2+}, corresponding to a high level of cytoplasmic Ca^{2+}. Under this condition, activities of the slow anion channel (SLAC) appeared in the plasmalemma of guard cells of *V. faba* and *X. strumarium*. They were characterized by large voltage- and current-dependent activation-time constants, τ, of the whole-cell current, which extended from seconds to a minute. The mean open time of the single slow channel was in the order of seconds. The 'quick' anion channel (QUAC) in the same cell type of both species was explicitly different, with τ in the order of 20 ms and a mean open time of 3–4 ms. Both channel types contrasted also in their voltage dependencies, SLAC displaying activity down to −200 mV, and very likely even lower, whilst QUAC was active above −100 mV. On the other hand, the coincidence of the anion current maxima between −60 and −20 mV as well as the similarity of the unitary conductances of the two types of anion channel were astonishing (SLAC 33 ps, QUAC 36 ps). One cannot suppress the notion that both channel types might be related to each other. The single-channel conductance

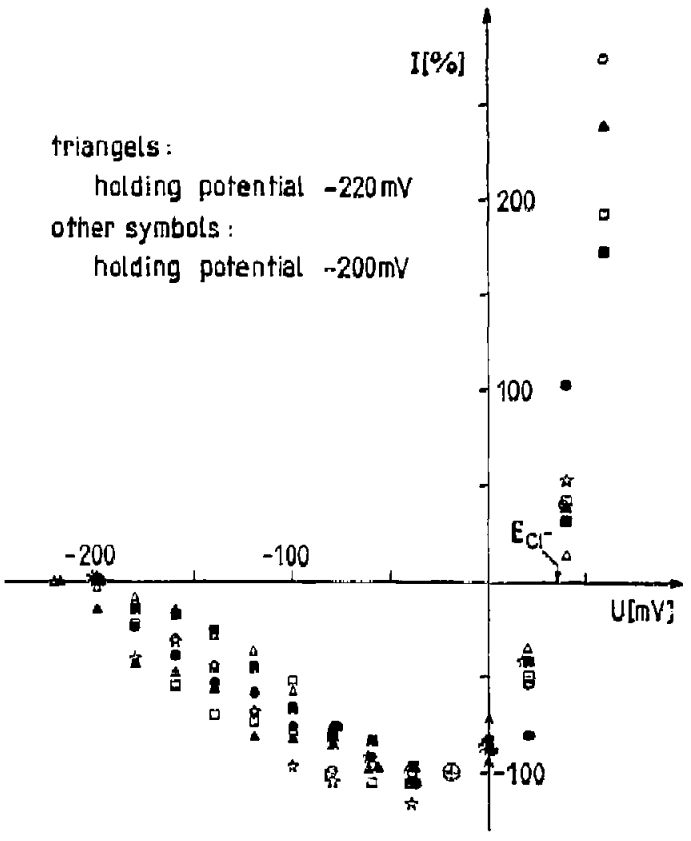
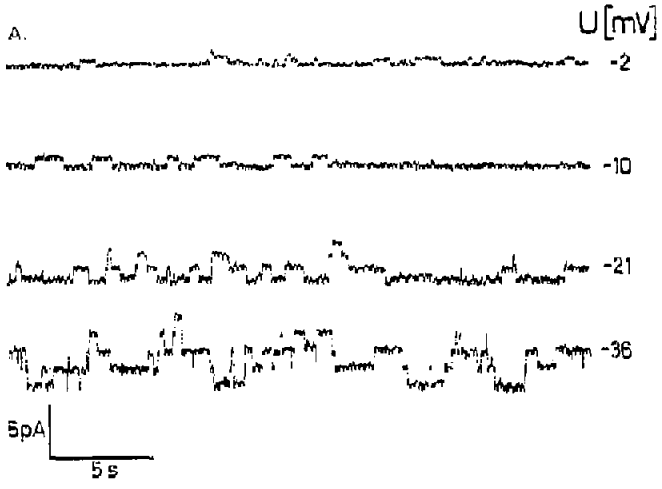


Fig. 2. Current–voltage relationship of the slow-anion-channel current. Series of seven measurements (one example shown in Fig. 1). Currents obtained by extrapolation to infinity of exponential functions fitted to the activation curves. Normalized to currents at −20 mV. The mean current at this potential was 83 ± 46 pA, corresponding to a current density of 12 μA cm^{-2}. E_{Cl^-} = equilibrium potential of Cl^-. Same solutions as in Fig. 1.



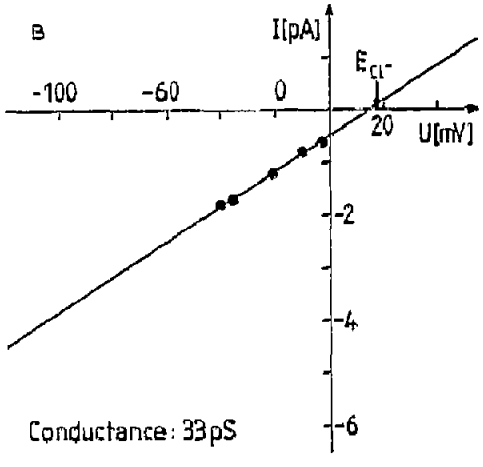


Fig. 3. Recordings of single-channel activity and current–voltage relationship of slow anion channels in guard-cell protoplasts of *X. strumarium*. (A) Activity in an outside-out patch. At least four channels were active in this patch (trace at −36 mV). Note that the duration of these records was 28 s. (B) Single-channel current–voltage relationship of the slow anion channel. External solutions were 30 mM KCl, 20 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM MES; internal solutions were 150 mM KCl, 2 mM $MgCl_2$, 2 mM MgATP, 1 mM EGTA, 10 mM HEPES.

of SLAC was certainly different from the one of 1 ps which Schroeder and Keller estimated to apply to a slow anion channel. They concluded on this channel from current records, called it 'S-type anion channel' and hypothesized, in a recent article, it may be involved in stomatal closing [8]. The information presented in [8] does not suffice to judge whether the S-type channels are identical with our SLAC or not. On the other hand we recognize manifestations of SLAC in a report by Schroeder and Hagiwara [9]. We can demonstrate that the instantaneous activations of anion currents shown there (their Fig. 3) were in fact effects of SLAC activation which already had occurred at the holding potential of +48 mV; and the observed 'wash-out' would be a deactivation of the SLAC currents in our interpretation. Similarly, slow deactivations appearing in current records of abscisic acid-treated guard cells after a voltage jump from −55 to −150 mV [10] may also have been expressions of SLAC.

As to the likely function of SLAC, one has to consider the occurrence of its activity at high 'cytoplasmic' Ca^{2+} concentrations. As was explained in the Introduction, stomatal closing needs depolarization of the plasmalemma from a potential below the diffusion potential of K^+ to one above. Stoppage or shorting of the H^+ pump alone will not suffice to accomplish this; anion efflux through the activated SLAC will be required to take U_m above the K^+ equilibrium potential. The following sequence of events appears as a plausible one leading to stomatal closing: in general, an initiating rise of Ca^{2+} occurs in the cytoplasm of guard cells, as summarized in [10] (but see also [11]). This causes activation of SLAC and blockage of K^+_{in} channels. The result is a depolarization to voltages above −40 mV, at which simultaneous efflux of anions and cations can occur [1–3]. As to the role of the quick channels (QUAC), their capacity to activate to very large conductances at a potential around −40 mV and to inactivate with a half-time of 10 s would allow rapid ion fluxes and stomatal responses and provide for fine control. SLAC did not show inactivation; it would assure sustained salt release.

It will be necessary to determine the anion specificity of SLAC and the dependence of its activity on a sliding scale of intracellular Ca^{2+} concentration. Responses to inhibitors will be used to recognize a possible relationship between QUAC and SLAC.

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