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Ca^{2+} and nucleotide dependent regulation of voltage dependent anion channels in the plasma membrane of guard cells

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Using the patch-clamp technique we discovered that the voltage dependent anion channels in the plasma membrane of guard cells are activated by a rise in cytoplasmic Ca^{2+} in the presence of nucleotides. Upon activation, these anion channels catalyse anion currents 10–20 times higher than in the inactivated state, thus shifting the plasma membrane from a K^+ conducting state to an anion conducting state. Prolonged stimulation by depolarizing voltages results in the inactivation of the anion current ($t_{1/2}$ = 10–12 s). We suggest that activation of the anion channel by Ca^{2+} and nucleotides is a key event in the regulation of salt efflux from guard cells during stomatal closure.

Key words: anion channel/Ca^{2+} and nucleotides/guard cells/patch clamp/plasma membrane

Introduction

Terrestrial plants exchange CO_2 and H_2O with the atmosphere by turgor-operated valves, the stomata, located in the epidermis. At night or under water stress the stomatal pores close in order to minimize water loss with respect to CO_2 fixation (Raschke, 1979).

Stomatal closure is the result of a release of potassium, chloride and malate through potassium and anion channels in the plasma membrane of the two pore-forming guard cells (Schroeder *et al.*, 1984; Hedrich and Schroeder, 1989; Keller *et al.*, 1989).

In addition to light, CO_2 and plant hormones (Raschke, 1979) alterations in cytoplasmic $[Ca^{2+}]$ that follow changes in extracellular $[Ca^{2+}]$ have been shown to affect stomatal movement (DeSilva *et al.*, 1985; MacAinsh *et al.*, 1990; Gilroy *et al.*, 1990). Low external $[Ca^{2+}]$ favours stomatal opening and high $[Ca^{2+}]$ stomatal closure.

Evidence is accumulating that K^+ influx channels participating in stomatal opening are inhibited by elevated cytoplasmic Ca^{2+} (Schroeder and Hagiwara, 1989). On the other hand the regulation of the release of K^+ and anions during stomatal closure is not yet clear.

The patch-clamp technique was used to study the regulation of anion channels in the plasma membrane of guard cell protoplasts of *Vicia faba*. Ionic currents were recorded in the whole-cell and outside-out patch configuration of the patch-clamp technique (Hamill *et al.*, 1981). Anion channels were characterized by their responses to calcium ions, nucleotides and changes in membrane potential, and with respect to their kinetic properties.

Results

Ca^{2+} and nucleotides activate anion channels

Figure 1A shows whole-cell currents during a voltage ramp extending from −200 to +80 mV with the pipette ('cytoplasmic') solution containing 150 mM KCl, 1 mM EGTA, 2 mM MgATP and with 30 mM KCl and 0.1 mM $CaCl_2$ in the extracellular medium. Under these conditions the predominant conductance resulted from voltage dependent K^+ channels (Schroeder *et al.*, 1987). Upon hyperpolarization (less than −100 mV) K^+ influx channels were activated whereas depolarizing voltages (greater than −20 mV) elicited K^+ efflux. Additionally, the opening of an anion efflux channel could be recognized around the reversal potential of K^+ (between −80 and −20 mV, Figures 1Aa, 3 and 4A). Sometimes, during repetitive voltage ramps, a spontaneous increase in anion channel activity emerged (Figure 1A, b). When the extracellular $[Ca^{2+}]$ was raised to 10 mM, or external K^+ was fully replaced by Ca^{2+}, we were able to stimulate anion currents reproducibly (Figure 1B, b). Alternate perfusions of the bath with solutions high (b) or low in Ca^{2+} (a) reversibly activated and inactivated the anion channels in a predictable manner.

To determine whether increase in external $[Ca^{2+}]$ is able to activate anion channels directly or through elevation of the cytoplasmic Ca^{2+} level we used several intracellular Ca^{2+}-buffer concentrations. The cytoplasm was allowed to equilibrate with pipette solutions containing 0.1, 1 and 10 mM EGTA. With high extracellular $[Ca^{2+}]$, activation of anion currents could only be elicited in cells equilibrated with 0.1 or 1 mM EGTA (Figure 2A). External Ca^{2+} failed to activate the channel when the cytoplasmic $[Ca^{2+}]$ was buffered to nanomolar levels (10 mM cytoplasmic EGTA).

These results indicate that activation of anion channels was caused by a rise in cytoplasmic Ca^{2+} rather than in external Ca^{2+}. They also imply that under whole-cell conditions the buffering capacity of the physiological Ca^{2+} chelator within the cytoplasm corresponds to EGTA equivalents of between 1 and 10 mM.

Since cytoplasmic Ca^{2+} was found to be essential for the activation of anion channels above the residual level, the question arose of whether cytoplasmic factors, such as nucleotides (known to control anion channels in other cell types; Ming *et al.*, 1989), were also involved.

If channel activation requires the presence of both Ca^{2+} and nucleotides, then the response to an increase in extracellular Ca^{2+} should depend on the intracellular level of ATP or other nucleotides. Indeed, when MgATP was excluded from the pipette solution, only background anion currents were measured, even when extracellular $[Ca^{2+}]$ was elevated to 40 mM (Figure 2B, a). ATP-free conditions were established by allowing 0.3 U/ml hexokinase plus 10 mM glucose to equilibrate with the cytoplasm. This

scavenging of ATP was also used to keep ATP generated by mitochondria and chloroplasts at a very low level.

Further evidence for the involvement of nucleotides was gained from experiments using non-hydrolysable nucleotides. Both adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) and GTPγS were able to support Ca^{2+}-dependent activation of anion channels (Figure 2A). These results indicate that the nucleotide acceptor involved in the activation process can be classified as 'non-specific' with respect to nucleotides.

With high external Ca^{2+} and nucleotides present in the pipette, anion channels did not activate immediately after establishing the whole-cell configuration. Generally, it took 1−5 min with the cell held at the resting level of −120 to −180 mV (Blatt, 1987) to activate fully the anion channels under these conditions, probably because of the time required for the nucleotides to diffuse to their site of action (Pusch and Neher, 1989).

Selectivity

In our previous paper on the identification of anion channels in guard cells (Keller *et al.*, 1989), the anion current representing the 'inactivated state' showed a permeability sequence of $NO_3^- > Cl^- > malate^{2-}$.

To test whether malate, the other physiologically relevant anion in guard cells (Raschke, 1979), besides Cl^-, was also carried by anion channels in the activated state, patch-clamp experiments were conducted with solutions containing salts of non-permeating cations in combination with chloride or malate. After replacement of K^+ by the K^+ channel blockers Cs^+ or TEA^+ (tetraethylammonium) on the

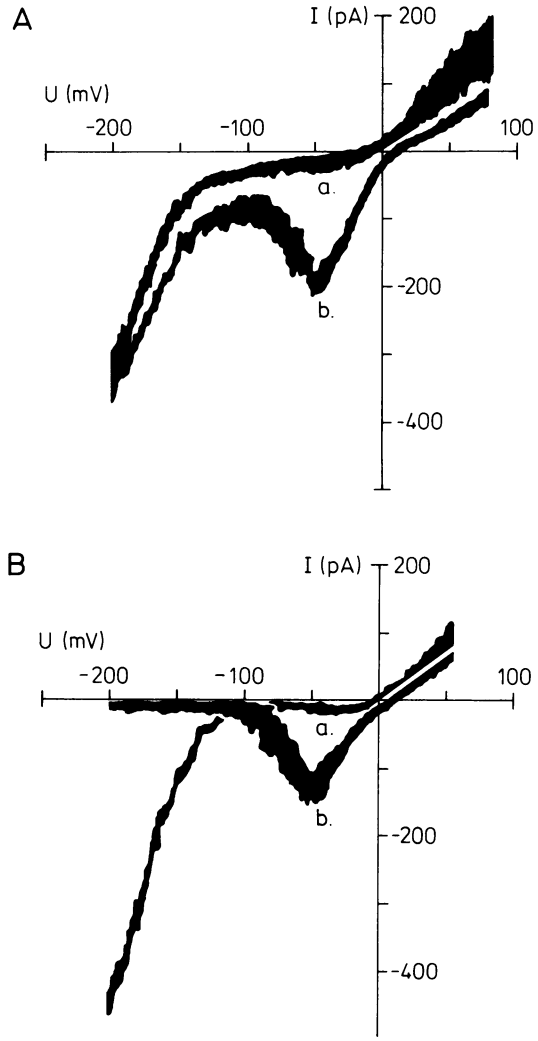

Fig. 1. Voltage and Ca^{2+} dependence of potassium and anion currents across the plasma membrane of guard cell protoplasts. Currents were elicited by voltage ramps from −200 to +80 mV at 5 mV/100 ms. (**A**) In the presence of 0.1 mM extracellular Ca^{2+}, transmembrane ion fluxes were dominated by K^+ influx (below −100 mV) or K^+ efflux currents (above −20 mV) (**a**). Spontaneous activation (non-predictable) of 'background' anion channels in the range between −80 and −20 mV led to accelerated anion efflux (**b**). (**B**) Elevation of extracellular Ca^{2+} from 0.1 mM (**a**) to 40 mM (**b**) reproducibly activated anion currents. Note, that K^+ influx was absent when K^+ (30 mM) was replaced by Ca^{2+} (40 mM). Appearance and decay of K^+ influx current was used to indicate that exchange of K^+ by Ca^{2+} (and vice versa) was complete during bath perfusion. Each current−voltage curve represents at least four identical experiments.

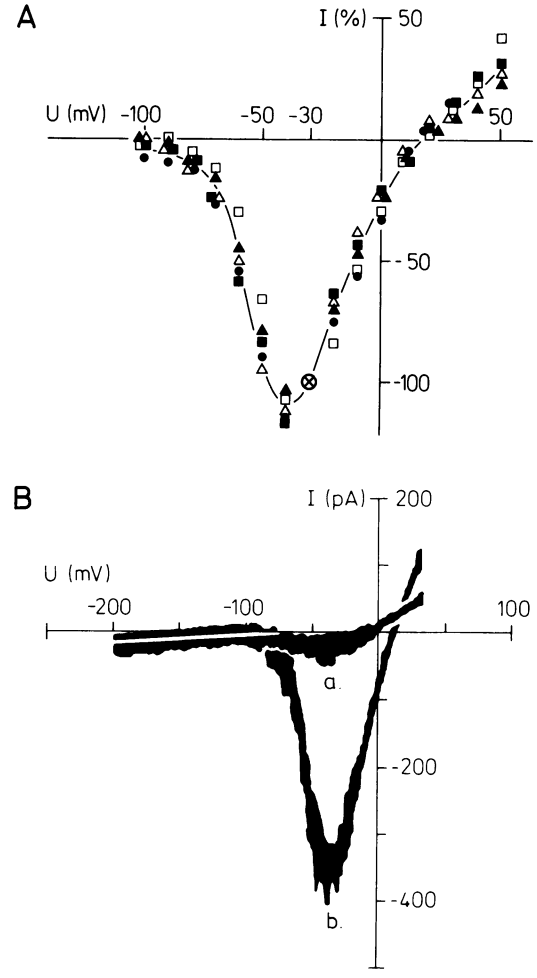

Fig. 2. (**A**) Current−voltage relation of anion channel activity as affected by various nucleotides, their concentrations, and by the cytoplasmic Ca^{2+} buffer capacity. Nucleotides and EGTA were applied through the patch pipette. Open symbols represent anion currents in the presence of 0.1 mM EGTA whereas closed symbols were recorded with 1 mM EGTA. (□) 2 mM ATP, (△) 10 mM ATP, (▲) 100 μM ATPγs, (■) 100 μM GTPγs, (●) 200 μM GTPγs. Each symbol sequence represents three or four identical experiments. Current−voltage curves were normalized with respect to the currents recorded at −30 mV. (**B**) Activation of anion channels by Ca^{2+} and ATP. Current−voltage relation of anion currents in the activated and inactivated state. (**a**) Suppression of the anion current in the absence of ATP (scavenged by hexokinase and glucose) from the cytoplasm. (**b**) Activation of anion current by a rise in $[Ca^{2+}]$ (see Figure 1B) in the presence of ATP. Hexokinase (0.3 U/ml) and glucose (10 mM) were applied through patch pipettes. Other solutions were identical with those mentioned in Figure 1B, b.

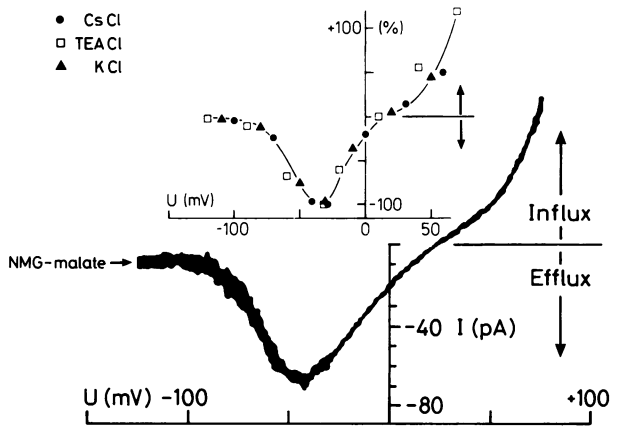

Fig. 3. Ionic identification of inward and outward currents upon activation by Ca^{2+} and nucleotides under bionic conditions. **Lower trace**: outward and inward malate fluxes through the anion channels in the activated state. **Inset**: outward and inward whole-cell anion currents carried by Cl^-. Chloride currents were normalized with respect to the currents monitored at −30 mV. Pipette solutions: 150 mM NMG-malate (lower, continuous trace), (▲) 150 mM KCl, (●) 150 mM CsCl, (□) TEA-Cl, in each case in addition to 200 μM $GTP_\gamma S$, and 0.1 mM EGTA. Other solutions were identical with those mentioned in Figure 1B, b. Each sequence of symbols represents a record of three or four identical experiments. Current−voltage curves were normalized with respect to currents obtained at −30 mV.

cytoplasmic side of the plasma membrane, the current−voltage relation of the activated anion channel was basically not altered (Figure 3, inset). Similar results were obtained when KCl was substituted by NMG (*n*-methylglucamine)-malate (Figure 3, lower trace). In solutions containing 154 mM Cl^- in the cytoplasm and 84 mM Cl^- in the bath the zero current potential could be determined to be +15−18 mV, the Nernst potential for chloride (Figure 2A, b and B, and Figure 3, inset). We never observed anion currents in the presence of glutamate or gluconate. These results indicate that depolarization activated anion currents (outward and inward currents) were basically carried by chloride and malate. Thus the anion channel in the activated state seems to possess the same anion selectivity as the one responsible for the background anion conductance (Keller *et al.*, 1989).

Upon activation the peak current of malate and chloride efflux increased several-fold, from 15−50 pA to 150−600 pA in the case of chloride and from 6−8 pA to 100−150 pA for malate. Changes in the anion gradients were found not to shift the peak current potential significantly. Whereas the background conductance was barely sufficient to account for the observed salt release from guard cells (Van Kirk and Raschke, 1978) the conductance in the activated state exceeds the requirement for stomatal closure several-fold.

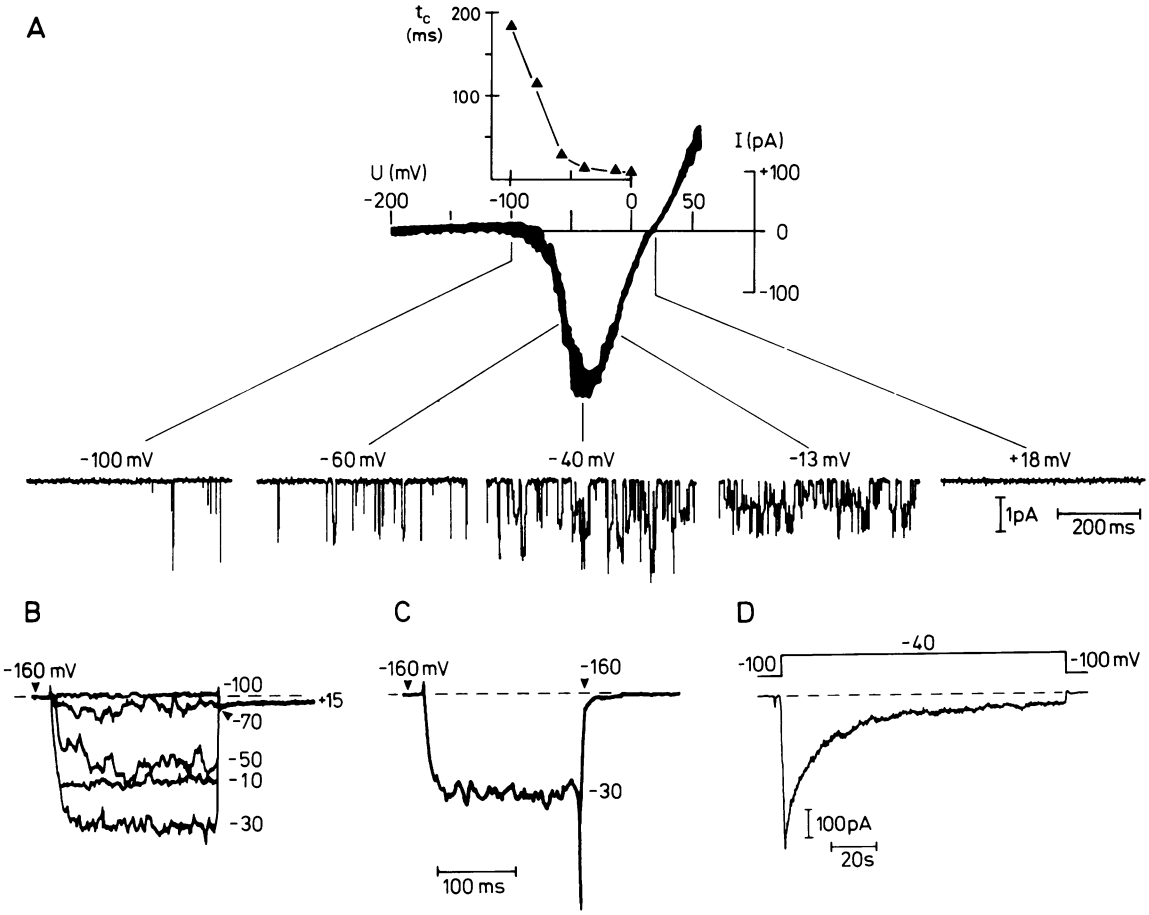

Fig. 4. Voltage and time dependent activity of anion channels recorded in whole cells and outside-out patches. (**A**) Current—voltage relation of activated whole-cell anion currents during a voltage ramp from −200 to +60 mV. Closed times (tc) of single anion channels (**inset**) recorded at voltages indicated. Note that in the presence of a chloride gradient (154 mM in the cytoplasm and 84 in the bath) the currents reversed around the equilibrium potential for chloride (+18 mV). (**B**) Anion currents generated by 200 ms voltage pulses from a holding potential of −160 mV to −100, −70, −50, −30 and −10 mV; each followed by a pulse to +15 mV. (**C**) Tail current resulting from a voltage pulse to −30 mV followed by a pulse to −160 mV. (**D**) Inactivation of anion channels resulting from prolonged stimulation. Decay of the anion current recorded during a voltage step from a holding potential of −100 mV to −40 mV.

Voltage and time dependent activity of anion channels

The kinetic properties of the anion channels were studied with a series of depolarizing voltage pulses (Figure 4B and C). Currents activate with a half-rise time of >30 ms (at −70 mV) and <10 ms (at −10 mV). The anion current peaked in the range of −30 to −40 mV (Figure 4B). This current−voltage relation was identical with that obtained by voltage ramps from −200 to +60 mV (Figure 4A). When the membrane potential was stepped from a holding level of −160 mV to −30 mV and back to the holding potential (Figure 4C) an instantaneous rise in anion current, caused by the increase in the driving force for anion efflux, was followed by a rapid and complete deactivation (<20 ms).

Single-channel analysis using outside-out patches (Figure 4A, single-channel traces) demonstrated that under steady-state conditions deactivation of anion currents at membrane potentials negative to about −30 mV was caused by an increase of the closed times of the anion channels (Figure 4A, upper graph).

Besides the voltage dependent activation and deactivation described above, anion channels also showed time dependent inactivation during prolonged stimulation. When the membrane potential was clamped to the peak current potential for extended times the anion current declined exponentially, with a half-time of 10−12 s (Figure 4D). The residual current (maximal inactivation) was of the same order of magnitude as the anion currents in the inactivated (background) state. However, anion currents could be fully restored when after inactivation the membrane potential was held negative to −100 mV for a period of ≥1 min. Partial or even total recovery of the anion current could also be obtained when the membrane was clamped to voltages positive to the anion equilibrium potential. Whether these holding potentials induce or facilitate Ca^{2+} entry, nucleotide binding, or voltage dependent alterations in protein structure needs further elucidation.

Discussion

These results support the idea that anion currents are activated upon a rise in cytoplasmic Ca^{2+} through a Ca^{2+} and nucleotide dependent process. The sites of action are unknown, but it is tempting to speculate that regulation occurs at the channel level (Rossie and Catterall, 1987).

Whereas currents through K^+ channels did not inactivate with time (Schroeder, 1988), prolonged stimulation of the anion channel fully reversed the activation. The shift in the state of activation of the anion channel was accompanied by a change in the reversal potential of the whole-cell current (Figure 2B). In the activated state the anion permeability was dominating. During inactivation of the anion channel the K^+ permeability increased and generally dominated the whole-cell conductance. Transient activation and inactivation of anion channels may provide a potential mechanism for excitability in response to sudden changes in the water relations (Raschke, 1970) and other stimuli (Johnsson *et al.*, 1976).

In conclusion, volume decreases of guard cells as induced by an increase in extracellular Ca^{2+} (DeSilva *et al.*, 1985) can be elicited by a rise in cytoplasmic Ca^{2+} (MacAinsh *et al.*, 1990; Gilroy *et al.*, 1990). Ca^{2+} influx depolarizes the membrane (Felle, personal communication) and thereby may trigger the release of potassium and anions (Keller *et al.*, 1989). We showed that the activation state of the anion channel as depending on Ca^{2+} and nucleotides may determine both the magnitude and time course of salt release.

Material and methods

Protoplast isolation and patch-clamp recordings

Guard cell protoplasts were enzymatically isolated from 2−3 week old leaves of the broad bean, *V.faba* (Hedrich *et al.*, 1989). Patch pipettes were sealed against the plasma membrane to study ion fluxes in the whole-cell configuration and in outside-out patches (Hamill *et al.*, 1981).

Current measurements were made with an EPC-7 patch-clamp amplifier (List-electronic Darmstadt, FRG), low-pass filtered at 1 or 2 kHz with an eight-pole Bessel filter. Data were digitized (VR10, Instrutec Inc., USA), stored on video tape and analysed using patch-clamp software of Instrutec Inc. on a Mega Atari ST4.

Solutions

Guard cell protoplasts were exposed to 150 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 2 mM MgATP, 10 mM HEPES/MES pH 7.2 in the pipette, and 30 mM KCl (or 40 mM $CaCl_2$ to activate anion channels), 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mm TRIS/MES pH 5.5 in the bath.

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