Characterization of a Light-Controlled Anion Channel in the Plasma Membrane of Mesophyll Cells of Pea

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In leaf mesophyll cells of pea (Pisum sativum) light induces a transient depolarization that is at least partly due to an increased plasma membrane conductance for anions. Several channel types were identified in the plasma membrane of protoplasts from mesophyll cells using the patch-clamp technique. One of these was an anion channel with a single-channel conductance of 32 picosiemens in symmetrical 100/100 KCl solutions. In asymmetrical solutions the reversal potential indicates a high selectivity for Cl\(^-\) over K\(^+\) at high cytoplasmic Cl\(^-\). At negative membrane voltages the channel openings were interrupted by very short closures. In the open channel conductance several substrates were identified. At a cytoplasmic negative logarithm of Ca concentration higher than 6.3, no channel openings were observed. When the protoplast was illuminated in the cell-attached configuration, at least one channel type had a higher opening probability. This channel can tentatively be identified as the above-described anion channel based on conductance and the characteristic short closures at negative membrane potentials. This light activation of the 32-picasiemens anion channel is a strong indication that this channel conducts the light-induced depolarizing current. Because channel activity is strongly Ca\(^{2+}\)-dependent, a role of cytoplasmic Ca\(^{2+}\) concentration changes in the light activation of the conductance is discussed.

The stimulation of growth of dicotyledonous leaves by light involves at least two photoreceptors, phytochrome and a blue-light receptor (Van Volkenburgh et al., 1990). Although photosynthesis does enhance the growth rate, light can stimulate growth in the absence of photosynthesis (Van Volkenburgh and Cleland, 1990). Expansion of a dicotyledonous leaf, consisting of several distinct tissues, demands closely matched responses of the different cell types. Experimental and theoretical studies provide evidence that the epidermis of the leaf and the stem control the change in growth rate in response to environmental stimuli. But, without an appropriate response of mesophyll and venial tissue, this would lead to an unbalanced growth of the whole organ. Integration of tissue growth can be based on one of two mechanisms: either the stimulus is perceived by one tissue and the response of other tissues is subsequently regulated by signals emitted by the first cell type, or the environmental stimulus is perceived by all tissue types that respond appropriately in an autonomous way.

Illumination increases the growth rate of isolated leaf epidermal strips in pea (Pisum sativum; results not shown), but it also affects the processes that are assumed to be involved in cell expansion in mesophyll cells, including extracellular acidification and potassium uptake (Van Volkenburgh and Cleland, 1990). Therefore, it is likely that perception and transduction of stimuli also occur in isolated mesophyll cells. Redistribution of ions across the plasma membrane is essential during the growth of cells. Extrusion of protons into the apoplast to activate cell wall-loosening processes and uptake of potassium to adjust the osmotic potential of the cytoplasm are pivotal processes in the cell extension.

Changes in ionic fluxes may be reflected as changes in the membrane potential. In pea leaves light induces a large (up to 60 mV), transient depolarization of the membrane potential. This depolarization originates in the mesophyll cells. Increasing the external chloride concentration diminishes the size of the depolarization, indicating a role for Cl\(^-\) efflux (Elzenga et al., 1995). In protoplasts isolated from leaf mesophyll, a voltage-dependent anion conductance is one of the dominating conductances (Elzenga and Van Volkenburgh, 1997). In the present study single-channel patch-clamp techniques in isolated patches or CAPs were used to characterize an anion channel in mesophyll cells. In the CAP configuration, illumination of the protoplast increases the open probability of this channel, a property that is expected if this conductance is involved in the light-induced depolarization observed in mesophyll cells.

**MATERIAL AND METHODS**

Seeds of pea (Pisum sativum) (Argenteum mutant) were obtained from the Plant Genetic Resources Unit (U.S. Department of Agriculture/Agriculture Research Service, New York Service Agricultural Experiment Station, Geneva, NY). The seeds were germinated on wet filter paper and grown in soil in a growth cabinet (model E54, Percival Scientific, Boone, IA) under a light regime of 14 h light/10 h dark at 20°C (fluence rate 180 μmol m\(^{-2}\) s\(^{-1}\) provided by cool-white fluorescent tubes [Philips, Eindhoven, The Netherlands]) or under greenhouse conditions.

Abbreviations: BTP, bis-Tris propane; CAP, cell-attached patch; \(E_{\text{rev}}\), reversal potential; i/o, inside out; o/o, outside out; pCa, negative logarithm of Ca concentration; PVC, polyvinylchloride; SITS, 4-acetoamido-4-isothiocyanostilbene-2,2'-disulfonic acid; THF, tetrahydrofuran.

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Anion Concentration Measurements at the Mesophyll Surface

The anion concentration at the surface of the leaf mesophyll was measured by placing an ion-selective and a reference electrode gently on a leaf from which the epidermis had been removed, exposing the mesophyll cells. The surface of the mesophyll was wetted with 100 μL of a solution containing 0.5 mM CaCl₂, 10 mM Hepes/BTP, pH 7.0, and 1 mM Suc. This solution ensured electrical contact between the ion-selective electrode and the reference electrode. The leaf was illuminated from below through the glass slide on which it was placed. Light was provided by a Philips 7748S 24 V 250 W incandescent lamp through a fiberglass light guide. The light intensity was measured with a quantum sensor (type SKP 215, Skye Instruments, Powys, UK) at the level of the leaf tissue. The light intensity was modified by applying neutral-density filters.

Anion-Selective Electrode

The anion-selective electrode was made according to Chaniotakys et al. (1988) using a PVC matrix membrane containing 6% of the ionophore 5,10,15,20 meso-tetraphenyl-21 H, 23 H-porphin manganese III chloride (Sigma), 28% PVC, 1% lipophilic cation methyl triphenyl phosphonium bromide (Sigma), and 65% of the solvent 2-nitrophenyl octyl ether (Fluka). A11 ingredients were was modified by applying neutral-density filters. PVC tube was filled with a solution containing 100 mM NaCl and 10 mM Tris/H₂SO₄, pH 7.4.

PVC tube was filled with a solution containing 100 mM NaCl and 10 mM Tris/H₂SO₄, pH 7.4. A silver/silver chloride wire was placed in the filling solution. The sensitive electrode, which made contact with the experimental solution, was floated on a buffered enzyme solution (1.7% [w/v] Cellulysin [Calbiochem, La Jolla, CA], 0.2% [w/v] Pectolyase Y-23 [Seishin, Tokyo, Japan], 0.2% [w/v] BSA [Sigma; in some experiments this was omitted], 2.325% [w/v] Gamborg B5 [GIBCO-BRL], 2 mM CaCl₂, 10 mM Mes/KOH, pH 5.5 [Schroeder, 1988], and mannitol to adjust the osmolarity to 610 mOsm) for 5 min at 30°C on a rotary shaker at 50 rpm. The tissue was transferred to a wash solution (identical to the enzyme solution except that cellulase, cellulysin, pectolyase, and BSA were omitted), transferred after 5 min to a second wash, and transferred after another 5 min to the bath solution. In this solution, which has an osmolarity of 240 mOsm, the protoplasts swelled and were released from the tissue. The protoplasts were allowed to settle on the glass bottom of the measuring cuvette.

Electrophysiology

Spherical protoplasts with a sharply defined plasma membrane and tonoplast were selected for seal formation and patch-clamp experiments; standard patch-clamp techniques were used (Hamill et al., 1981). Patch pipettes were pulled from 50-μL micropipettes (VWR Scientific, West Chester, PA) on either a PB-7 (Narishige, Greenvyl, NY) or a 3P-A (List-Medical, Darmstadt, Germany) pipette puller. To reduce pipette capacitance, the tip and shank were dipped in polystyrene (Q-dope), GC Electronics, Rockford IL., which was allowed to harden for at least 2 h before use. The tips of the electrode were fire-polished immediately before use. Tip resistance was typically between 3 and 9 MΩ, depending on the solution composition and tip geometry. The bath solution contained 1 mM CaCl₂, 2 mM MgCl₂, 10 or 100 mM KCl, 10 mM Hepes-BTP, pH 7.0, and mannitol to adjust the osmolarity to 240 mOsm. The standard pipette solution contained 2 mM MgCl₂, 100 mM KCl, and 10 mM Hepes-BTP, pH 7.0. The osmolarity was adjusted to 290 mOsm with mannitol. For pCa values between 7 and 5, 1 mM BAPTA was included. The amount of CaCl₂ added was calculated from an algorithm using the protonation and Ca²⁺-binding constants from Martel and Smith (as cited in Harrison and Bers, 1987) and adjusted for the ionic strength (Scharff, 1979, eq. 13). The Ag/AgCl reference electrode was connected to the bath via a salt bridge filled with the pipette solution. Electrode offsets were compensated electronically with the circuitry on the patch-clamp amplifier. Junction potentials were both calculated (Barr, 1994) and measured, and were found to be less than 2.5 mV and not corrected for in the analyses.

Seal formation was monitored by measuring the current induced by a 100-Hz 1-mV square pulse from a function generator. Single-channel currents were low-pass-filtered at 3 kHz (3-dB corner frequency) with either an 8-pole Bessel-type filter (type 902, Frequency Devices, Haverhill, MA) or an 8-pole Butterworth-type filter (type 3340-41, Krohn-Hite, Avon, MA). Currents were monitored with a current-voltage converter (EPC-7, Adams and List, New York), digitized by an A/D converter (custom-made based on a Labmaster ADC board [Scientific Solutions Inc., Solon, OH] or type CED 1401 [Cambridge Electronic Design, Cambridge, UK]) at a sample frequency of 10 kHz and stored on a PC hard drive. All experiments were performed in voltage-clamp mode. Command voltage protocols and A/D conversion were under software control (pClamp, Axon Instruments, Foster City, CA; with the Labmaster ADC or CED patch-clamp software with the CED 1401 ADC).

The curves fitted to the I-V plot are two-state, class I kinetic models, as described by Hansen et al. (1981) and Gradmann et al. (1987). Fitting was done using programs based on simplex algorithms written in Turbo Pascal.
Light was provided by the 20-W incandescent microscope lamp (type 7388 ESB M/30, Philips, Eindhoven, The Netherlands); the light intensity that was measured with a type SKF 215 quantum sensor (Skye Instruments, Powys, UK) at the level of the cells was 50 \mu\text{mol} \text{m}^{-2} \text{s}^{-1}.

In the experiments where the effect of light on the open probability was determined in the CAPs, the mean open state probability \(NP_o\) of the channels was calculated from the relation \(NP_o = I/i\), where \(I\) is the time-averaged current, \(N\) is the number of channels in the patch, \(i\) is the amplitude of the unitary current, and \(P_o\) is the probability of a channel being open. This method can be used without knowledge of the total number of channels in the patch, but is only applicable when the changes in \(P_o\) can be measured in one and the same patch. Because the results of several patches were combined for the determination of the effect of \([\text{Ca}^{2+}]\) on \(P_o\), we fitted the all-point histograms of the current traces to a binomial distribution with the number of channels and \(P_o\) as free-running parameters.

**RESULTS**

This study on the role of light in activation of anion channels was initiated by the observation that illumination induces a transient depolarization of the membrane (Elzenga et al., 1995). The effect of changes in the external \(K^+\) and \(\text{Cl}^-\) concentration on the size of the depolarization indicates that at least part of the depolarization is due to an increased efflux of \(\text{Cl}^-\). The conclusion that light induces an increased efflux of \(\text{Cl}^-\) is confirmed by the rise in the anion concentration at the surface of the mesophyll cells exposed after the epidermal layer has been removed (Fig. 1). The rate of increase of the anion concentration is dependent on the light intensity and can be totally blocked by 10 \mu\text{M} of DCMU, an inhibitor of photosynthesis.

**Light Stimulates a Channel in the CAP Mode**

Figure 2A displays single-channel current traces from a CAP on a pea mesophyll protoplast. The patch contained at least three channels that change activity depending on the light conditions. The changes in open probability \(P_o\) are shown in Figure 2C. Light induces about a 3-fold increase in \(P_o\) of the channel (dark 0.10 \pm 0.04 and light 0.34 \pm 0.06, \(n = 5\)). From the traces 4 and 5 and Figure 2C, it is clear that the increase in activity takes more than 5 s to occur and that full activation is not reached within 20 s. The solution in the pipette contained (in mM) 100 KCl, 2 MgCl\(_2\), 1 CaCl\(_2\), 10 Hepes/BTP, pH 7, and 75 mannitol. The solution bathing the protoplast contained 10 KCl, 2 MgCl\(_2\), 10 CaCl\(_2\), 10 Hepes/BTP, and 175 mannitol. The pipette voltage was clamped at +75 mV, which means in the CAP configuration that the membrane potential of the patch is hyperpolarized to a value 75 mV more negative than the native membrane potential of the protoplast. Although it is generally possible with these mesophyll protoplasts to create an isolated patch configuration, the patches that were kept in the CAP configuration fairly long to study light-dependent channel activity almost never remained intact in subsequent attempts to create an i/o patch. Therefore, direct measurements of the \(E_{\text{rev}}\) to determine the channel type could not be made. Identification of the channel was done indirectly by comparing the conductance and channel opening and closing characteristics in separate CAP and isolated patch experiments.

**Characterization of a Cl\(^-\) Channel in the Isolated-Patch Configuration**

In Figure 3A the current traces at different holding voltages of one of the channel types recorded in isolated patches is shown. In Figure 3B the current-voltage relation of the same channel type is shown for different ion compositions at both the cytoplasmic and extracellular sides of the membrane patch. A characteristic for this channel is that at positive membrane potentials the channel openings...
are long-lasting, and that at negative membrane potentials the openings are interrupted by short closures, giving the traces a “noisy” appearance (Fig. 3, A and C). Curves fitted to the data were used to determine the $E_{rev}$ of the open channel current for the different solution compositions. In asymmetric solutions the $E_{rev}$ closely matched the theoretical equilibrium potential for Cl$^-$ with a high cytoplasmic Cl$^-$ concentration (106 mM Cl$^-_{cyt}$, 18 mM$^{-1}$ Cl$^-_{apo}$; fitted $+38$ mV, theoretical $+40$ mV). At a low cytoplasmic Cl$^-$ concentration the $E_{rev}$ from the fit differs from the one predicted (18 mM Cl$^-_{cyt}$, 108 mM$^{-1}$ Cl$^-_{apo}$; fitted $-27$ mV, theoretical $-40$ mV). However, the relatively large scatter of the single-channel current at negative membrane voltages could be the reason for this discrepancy. From the $E_{rev}$ at the high cytoplasmic Cl$^-$ concentration and using the Goldmann-Hodgkin-Katz equation, the selectivity of the channel for Cl$^-$ over K$^+$ is calculated to be $>28$. The conductance of the channel is maximally about 32 picasiemens in the voltage range measured, slightly rectifying at positive voltages. The selectivity of the channel for Cl$^-$ is in agreement with the blocking effect of SITS on the channel, as is shown in Figure 4. SITS, at a concentration of 0.5 mM, reduces the number of channel openings without affecting the single-channel conductance.

**Identification of the Light-Activated Channel as the Cl$^-$ Channel**

In Figure 3B the relation between the current and the holding potential of the light-activated channel in the CAP configuration is also indicated. A definitive identification of the light-activated channel is not possible in this way, because the membrane potential of the protoplast and the ionic composition of the cytoplasm are not precisely known. However, the current-voltage relation of the CAP light-activated channel found in all of the experiments was within the range of the current-voltage relation of the Cl$^-$ channel in the isolated-patch experiments. A further feature of the Cl$^-$ channel in the isolated patch configuration, noticeable upon close inspection of the traces in Figure 3A, was the presence of several subconductance states. In Figure 5A these subconductance levels are indicated in more detail to show that at least five open channel conductance levels can be distinguished. Several subconductance levels were also seen in the CAP light-activated channel (Fig. 5B).

**The Cl$^-$ Channel Activity Is Ca$^{2+}$-Dependent**

The channel opening of the Cl$^-$ channel in the isolated patches was strongly dependent on the Ca$^{2+}$-concentration at the cytoplasmic side of the membrane patch. Current traces of a single patch at two different Ca$^{2+}$ concentrations are displayed in Figure 6A. The open probability is (close to) zero at [Ca$^{2+}$] of 0.1 μM, increasing with increasing [Ca$^{2+}$] with one-half-maximal activation at about 0.5 μM (Fig. 6B).

**DISCUSSION**

In mesophyll cells of pea leaves light induces a 2- to 3-min-long, transient depolarization that is at least partly...
Single-Channel Analysis of Light-Controlled Anion Conductance

Figure 3. Current-voltage relation of anion channel in the plasma membrane of leaf mesophyll cells of pea. A, Single-channel recording of o/o patch at different holding potentials. The holding potential in mV is indicated at the left of the individual traces. The closed-channel current is indicated by the horizontal bar at the right of the traces. Composition of bath solution was 1 mM CaCl₂, 2 mM MgCl₂, 100 mM KCl and 10 mM Hepes-BTP, pH 7.0. The pipette solution was 2 mM MgCl₂, 100 mM KCl, 10 mM Hepes-BTP, pH 7.0, and 1 mM BAPTA pCa 6. Mannitol was added to adjust the osmolarity to 240 mOsm in the bath solution and to 290 mOsm in the pipette solution. B, Current-voltage relation of single open-channel currents in 100/100, 10/100, and 100/100 KCl. The different KCl concentrations lead to the Cl⁻ concentration on the cytoplasmic and apoplastic sides, as indicated in the inset. For other ions in the solutions, see “Materials and Methods.” The curves fitted to the I-V plot are two-state, class I kinetic models. The relation between the holding potential (thus not taking into account the membrane potential of the protoplast) and the open-channel current of the light-activated channel recorded in CAP configuration is also indicated. C, Single-channel recording of o/o patch at −60 mV (top trace) and a CAP (bottom trace, holding potential as in Fig. 2). The closed-channel current is indicated by the horizontal bar at the right of the traces.

Due to an anion efflux (Elzenga et al., 1995). In light the extracellular concentration of anions in the apoplast increases in a light-intensity-dependent way (Fig. 1). Both results can be understood as the result of light-activation of an anion-selective channel. The dependence of both reactions on the light intensity is very similar, with one-half-maximal responses for depolarization and Cl⁻ efflux at 66 and 55 μmol m⁻² s⁻¹, respectively. At a light intensity of 100 μmol m⁻² s⁻¹ the lag time between the start of illumination and the onset of the depolarization is 5 s, whereas the light-induced change in the extracellular Cl⁻ concentration can be observed within 10 s.

In CAPs of mesophyll protoplasts light activates one channel with several conductance levels or activates several channel types. The time course of channel activation, 5 s before first activation and 20 to 30 s before full activation, is in the same order of magnitude as the time course of the onset of depolarization and Cl⁻ efflux. In the dark at least one of the components, which is characterized by fast openings and closings, is strongly reduced; a channel or conductance with slower kinetics and a reduced current is the most frequently seen (Fig. 2, traces 2-4). This component cannot easily be identified with one of the open-channel current levels seen in the light. It could be a different channel type, but could also be a subconductance state, which is induced in the dark because of the different kinetics of the channel in the light and in the dark (Thomine et al., 1995).

The light-activated channel characterized by short closings is most likely anion-selective. As direct determination of the selectivity of a channel requires control over the composition of the solution on both sides of the membrane; this can only be achieved in the isolated patch (either the i/o or o/o) configuration. Determining whether a channel is light-activated requires maintaining the patch in the CAP configuration for approximately 15 min (a dark-light-dark cycle). With the protoplasts used, we were unable to go from the CAP to the isolated patch configuration and maintain a stable seal when the CAP configuration had lasted that long. In isolated patches that were obtained immedi-
Figure 5. Multiple conductance levels of leaf mesophyll anion channel. A, Single-channel recording of the anion channels in an i/o patch at a holding potential of +60 mV. The different levels of the current through the channel indicative for multiple conductance levels are indicated by thin, horizontal lines. The bottom line is the current level without a channel opening (closed level). Note that excursions to the closed level occur from different open levels. The medium composition was as described in "Materials and Methods," with 100 mM KCl in both the bath and pipette solution. B, Single-channel recording of the anion channels in a CAP. The different levels of the current are indicated as in A. See Figure 2 for details on holding potential and medium composition. C and D, All point histograms of the current traces in A and B, respectively. The current is normalized to the most frequently measured single-channel current level.

Immediately after a gigaΩ seal had formed, one of the channels frequently found was selective for anions. The similarity in theoretical equilibrium potential for Cl⁻ and the measured £E_{rev} (Fig. 3) and the effect of the anion channel blocker SITS both indicate a selectivity for anions of this channel in isolated patches (Fig. 4). The I-V relation of this channel displayed a slight rectification, the currents seemingly saturate at negative membrane voltages. This relation could be fitted with a three-state enzyme kinetic model (Hansen et al., 1981; Gradmann et al., 1987); alternatively, the apparent saturation could be due to unresolved fast closures (Klieber and Gradmann, 1993).

The identification of the light-activated channel as the anion-selective channel is based on three characteristics. First, the conductance of the light-activated channel falls within the range of the anion channel in isolated patches, assuming that the cytoplasmic Cl⁻ concentration is around 100 mM. Second, the light-activated channel displays similar short closures as those that were displayed by the channel in the isolated patch at negative membrane voltages (Fig. 3C). Third, the light-activated channel has several conductance levels, as has the anion channel in the isolated patches (Fig. 5). The only other abundant conductance in isolated patches was a K⁺-selective channel that did not resemble the light-activated channel. It had a three-fold higher conductance in symmetrical 100 mM KCl, its I-V relation did not show the apparent saturation at negative membrane voltages, nor did it have the "noisy" appearance at negative voltages.

The light-activated anion channel does not resemble any of the previously reported channels responding to light. It is different from the light-activated channel in Arabidopsis mesophyll cells, which is cation-selective (Spalding et al., 1992) and from the Ca²⁺-activated K⁺ channel in Mougeotia, which is sensitive to red/far-red changes (Lew et al., 1990) and activates after a very long lag time.

The Ca²⁺-dependence of channel activity (Fig. 6) in the isolated patches might be relevant for the mechanism of light activation. Transient red light-induced rises in cytoplasmic Ca²⁺ have been described to occur in Triticum (Shacklock et al., 1992). These changes take place in minutes and most likely function in a signal transduction chain. In photosynthetically active cells light will, however, lower the cytoplasmic Ca²⁺ concentration by accumulating Ca²⁺ in the chloroplasts, which has been described for Nitellopsis (Miller and Sanders, 1987). Lowering the cytoplasmic Ca²⁺ concentration would decrease the open probability of the anion channel and lead to a reduced Cl⁻ efflux. This does not agree with our observations on the light-induced change in extracellular anion concentration. Although in some experiments the rate of change was highest in the first minute after the light was turned on and diminished after several minutes in the light, the rate did not change with time in the light in the majority of the experiments (results not shown).

Although the Ca²⁺-dependence of the anion channel activity does allow for a role of cytoplasmic Ca²⁺ in light activation, other light-dependent processes that influence the anion channel activity could be more significant. Changes in the cytoplasmic ATP concentration can influence channel kinetics, as described for potassium channels in Arabidopsis mesophyll cells (Spalding and Goldsmith, 1993) and anion channel conductance in Arabidopsis hypocotyl epidermis cells (Thomine et al., 1995).

The change in channel activity is not due to light-induced changes in the membrane potential, because differences in the activity between light and dark could be
observed at pipette potentials ranging from 0 to +80 mV (results not shown), whereas the light-induced membrane potential changes observed in situ in mesophyll cells were less than 25 mV with similar ion composition of the medium bathing the cells (Elzenga et al., 1995).

Light-induced acidification of the apoplast of bean leaf mesophyll cells is essential for leaf extension growth (Van Volkenburgh and Cleland, 1980). Prolonged acidification demands that the extruded, positively charged protons are carried through the production of organic acids (Davies, 1986). Charge-balancing extruded protons solely by cation influx will thus tend to increase the osmolarity of the cytoplasm. Although this is a condition that is beneficial when rapid cell expansion occurs, some circumstances where proton extrusion occurs will demand only a part of the increase in osmolarity that K\(^+\) influx would provide. In these circumstances, K\(^+\) influx could be a cellular mechanism to control external pH, membrane voltage, and the osmotic potential of the cell contents.

**Figure 6.** Effect of cytoplasmic Ca\(^{2+}\) concentration on the open probability of the leaf mesophyll anion channel. A, Single-channel recordings of one isolated i/o patch exposed at the cytoplasmic face to different Ca\(^{2+}\) concentrations. The bath and pipette solutions were as indicated in Figure 2A, except for the pCa values. The closed-channel current level is indicated by the horizontal line to the right of each trace. The membrane potential was clamped at +40 mV. B, Relation between cytoplasmic Ca\(^{2+}\) concentration and open probability. The data represent the mean and SD of three independent experiments with a total of 30 s of recording.

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**LITERATURE CITED**


