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Anion Channels in *Chara corallina* Tonoplast Membrane: Calcium Dependence and Rectification

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Abstract. Tonoplast K^+ channels of *Chara corallina* are well characterized but only a few reports mention anion channels, which are likely to play an important role in the tonoplast action potential and osmoregulation of this plant. For experiments internodal cells were isolated. Cytoplasmic droplets were formed in an iso-osmotic bath solution according to a modified procedure. Ion channels with conductances of 48 pS and 170 pS were detected by the patch-clamp technique. In the absence of K^+ in the bath solution the 170 pS channel was not observed at negative pipette potential values. When Cl^- on either the vacuolar side or the cytoplasmic side was partly replaced with F^-, the reversal potential of the 48 pS channel shifted conform to the Cl^- equilibrium potential with similar behavior in droplet-attached and excised patch mode. These results showed that the 48 pS channel was a Cl^- channel. In droplet-attached mode the channel rectified outward current flow, and the slope conductance was smaller. When *Chara* droplets were formed in a bath solution containing low (10^{-8} M) Ca^{2+}, then no Cl^- channels could be detected either in droplet-attached or in inside-out patch mode. Channel activity was restored if Ca^{2+} was applied to the cytoplasmic side of inside-out patches. Rectification properties in the inside-out patch configuration could be controlled by the holding pipette potential. Holding potential values negative or positive to the calculated reversal potential for Cl^- ions induced opposite rectification properties. Our results show Ca^{2+}-activated Cl^- channels in the tonoplast of *Chara* with holding potential dependent rectification.

Key words: Patch-clamp — *Chara* — Tonoplast — Ca^{2+}-activated — Cl^- channels — Rectification

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Introduction

Characean cells provide a convenient experimental material to study membrane (plasma membrane and tonoplast) transport processes (Koppenhöfer & Schramm, 1974; Hope & Walker, 1975). The vacuole in *Chara* internodal cells occupies much of the cell volume (about 95%) and can be accessed by several ways (Kamiya & Kuroda, 1957; Takeshige, Tazawa & Hager, 1988). The tonoplast origin of the envelope membrane of cytoplasmic droplets prepared from internodal cells has long been known (Sakano & Tazawa, 1986; Lühring, 1986). Relatively few types of channels are reported in the tonoplast, so far only K^+ and Cl^- channels have been identified. There are extensive reports in the literature dealing with the K^+ channels (Bertl, 1989; Tyerman & Findlay, 1989; Laver, 1990; Laver & Walker, 1991; Laver, 1992; Tyerman, Terry & Findlay, 1992; Klieber & Gradmann, 1993), but only a few articles mention (Kikuyama, 1986; Homblé & Fuks, 1991) and only one partly characterizes a Cl^- channel (Tyerman & Findlay, 1989). A 30 pS Cl^- channel was reported in droplet-attached configuration that showed rectification. In detached patches, channel activity could only be observed for brief periods, during which ion substitution experiments revealed a permeability sequence of $NO_3^- > Cl^-$ > aspartate (76:33:1). It was suggested that some factor or critical concentration of an ion was required for sustained activity of the Cl^- channels (Tyerman & Findlay, 1989).

Cytoplasmic Ca^{2+} has been shown to modulate ion-transport proteins in plant cells (Hedrich & Neher, 1987; Schroeder & Hagiwara, 1989). In the cytoplasmic compartment of resting Characean cells, free Ca^{2+} is maintained at a concentration of less than a micromolar (range 0.1–0.56 μM) while the vacuolar compartment contains >100 μM (Williamson & Ashley, 1982). Transient in-

creases in the Ca^{2+} concentration in the cytoplasm ($[Ca^{2+}]_{cyt}$) of *Chara* cells control the activity of several ion channels present in the plasma membrane (Lühring, 1985; Okihara et al., 1991; Homann & Thiel, 1994; Johannes, Crofts & Sanders, 1998; Thiel & Dityatev, 1998) and K^+ channels in the tonoplast (Laver, 1990; Laver, 1992; Tyerman et al., 1992). K^+ channels in the *Chara* tonoplast are activated by micromolar $[Ca^{2+}]_{cyt}$, an activation that is found to be only weakly voltage dependent (Laver & Walker, 1991). Kikuyama (1986) reported that in a solution lacking Ca^{2+} and containing Ba^{2+}, Mg^{2+} or Mn^{2+}, *Chara* cells lost their excitability in time, resulting in the depletion of the tonoplast action potential (AP). It was assumed that $[Ca^{2+}]_{cyt}$ played an essential role in the generation of an AP at the tonoplast and that Ca^{2+} might activate Cl^- channels, as was also suggested by Lunevsky et al. (1983). More recent Ca^{2+}-influx measurements showed that during the AP, the $[Ca^{2+}]_{cyt}$ can exceed the resting level by a factor 740–2000 in intact *Chara* cells (Reid, Tester & Smith, 1997).

In the present study the patch-clamp technique was applied to the tonoplast surrounding cytoplasmic droplets of *Chara*. By using Cs^+ containing solutions the K^+ channels could be excluded from the measurements, as Cs^+ ions are not able to pass the K^+ channels. Cl^- channels were identified in both droplet-attached and excised patches. We found that they are sensitive to Ca^{2+} present at the cytoplasmic side of the membrane. The effects of the anion channel blocker Zn^{2+} on Cl^- channels, Ca^{2+} depletion by EGTA and voltage dependent kinetics of the channels were also studied.

Materials and Methods

Plant Culture and Droplet Isolation

Chara corallina was cultured at 25°C in aquaria filled with partially deionized water, under illumination (14-hr light/10-hr dark) with fluorescent (OSRAM Dulux S, UK) lamps. The soil in one part of the aquaria was periodically (after 8 to 12 weeks) replaced with sterilized fresh forest soil. For droplet isolation internodal cells were separated from the neighboring cells (cell length 5–7 cm, cell diameter 0.7–1 mm) and intracellular perfusion was carried out for droplet isolation, using a modification of the method of Tazawa, Kikuyama and Shimmen (1976). In short, the cell was blotted dry with a tissue paper and laid on a Parafilm (American Can Company, Greenwich, CT) covered glass slide. Turgor reduction in air resulted in wilting of the cell within 3 to 5 min, due to water evaporation; 20 μl of bath solution was pipetted on both ends of the cell and each cell end was removed with a scissor. The glass was carefully tilted back and forth in such a way that the bath medium flowed through the cell entering the open ends. Shearing forces caused the pouring out of the vacuole and streaming cytoplasm and formation of cytoplasmic droplets surrounded by tonoplast. The inside part of the droplet contains cytoplasm (Sakano & Tazawa, 1986; Lühring, 1986). With this procedure, droplets with diameters in the range of 20 to 200 μm could be collected in an Eppendorf tube for storage. 10–20 μl of this suspension could be transferred to the experimental chamber. Subsequently, 480 μl bath solution was added carefully avoiding turbulence. The larger droplets with diameters of 60 μm or more were preferred for experiments as they were sticking onto the glass allowing perfusion of the chamber.

Solutions

The chemicals used were purchased from Merck (Darmstadt, Germany), Sigma Chemical (St. Louis, MO), Boehringer (Mannheim, Germany), and Gibco-BRL (Gaithersburg, MD). Salt solutions were prepared from 0.1 or 1 M stock solutions with MilliQ water and filtered (Millipore S.A., Molsheim, France, type 0.22 μm) before use. The pH was adjusted with HEPES/Tris buffer (pH 7.3–7.4). The composition of solutions present at each side of the membrane is indicated in the legends to the figures. To block the native K^+ channels and allow Cl^- fluxes, Cs^+ and Cl^- were used as the major salt components in most of the experiments.

Free-Ca^{2+} concentrations in the presence of a single chelating ligand were computed using the program Ligandy (developed by P. Tatham and B. Gomperts, University College, London). During experiments, perfusion of the experimental chamber was achieved using bath perfusion (*about 200 μl/min*) and waste withdrawal at equal rates, the volume of the bath (*±500 μl*) remaining unchanged.

Pipettes

Patch-pipettes with internal filaments (Clark Electromedical Instruments, Pangbourne Reading, RG8 7HU-England, type GC150TF-15) were pulled with a Narishige two-stage puller (Tokyo, Japan, Model PB-7) and fire-polished to give a tip resistance of about 9–14 MΩ when filled with experimental solutions. Seal resistances of about 5 to 30 GΩ were easily achieved and this stable contact of the pipette with the membrane persisted upon excision of the patch and perfusion of the bath.

Single Channel Recording

Currents were recorded at room temperature (23 ± 2°C) with an Axopatch 200 Amplifier (Axon Instruments, Foster City, CA) using conventional voltage-clamp technique according to Hamill et al. (1981). Data acquisition, analysis and control of the amplifier were performed with pCLAMP 6.0 in combination with a TL-1 interface (Axon Instruments). Signals were digitized at 2 to 10 kHz and filtered at 1 or 2 kHz with a 4-pole lowpass Bessel filter. The pipette potential was measured with respect to the bath potential (ground). Junction potentials were balanced before the formation of each gigaseal. In case of droplet-attached experiments, the real pipette potential value is the value of the resting droplet membrane potential of about +11 mV (cytoplasmic side regarded to the bath, Tyerman & Findlay, 1989) added to the pipette clamp voltage (V_p). Whole-droplet configuration was achieved from droplet-attached configuration by application of a short suction pulse to the pipette, or sometimes occurred spontaneously. The pipette could be withdrawn from this configuration without loosing the gigaseal contact with the membrane, thus forming outside-out patches. Excising a patch from the droplet-attached configuration resulted in inside-out patch. Outward Cl^- currents are cytoplasmic-side directed and are graphically represented as downward (for droplet-attached and inside-out patches) or upward deflections (for outside-out patches).

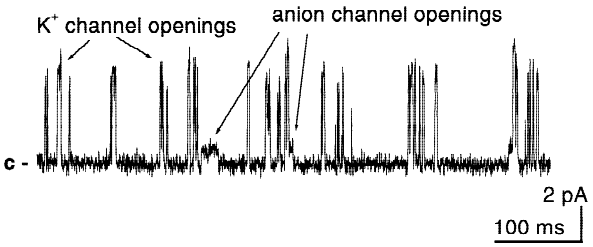

Fig. 1. Single K^+ and anion channels coexist in the tonoplast of *Chara.* Droplet-attached configuration, pipette potential: +20 mV. The closed (*c*) state of the K^+ and anion channel is indicated. *Bath solution:* 155 mM CsCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES/Tris, pH 7.4; *Pipette solution:* 155 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES/Tris, pH 7.4.

Statistics

Mean values ± SEM are given, with number of droplets used for experiments, *N*. Student's *t*-test was used for statistical evaluation of the data ($p < 0.05$).

Results

K^+-and Anion Channels Coexist in the Tonoplast of *Chara*

In droplet-attached and detached patches the appearance of large-conductance channels and small-conductance channels could be observed. Figure 1 shows a trace of opening and closing events obtained from a tonoplast patch in droplet-attached configuration at +20 mV pipette potential. From the current-voltage relationships two different single channel conductances could be identified, 170 pS and 37 pS for the high and low conductance channels, respectively. According to Lühring (1986), Laver and Walker (1987), Tyerman and Findlay (1989) the 170 pS channel is likely to be a K^+ channel and the 37 pS channel is likely to be a Cl^- channel. The high conductance K^+ channel and the 37 pS channel could be observed together in a patch.

The ion gating and permeation kinetics of the tonoplast K^+ channel was studied in detail before (Lühring, 1986; Laver & Walker, 1987; Laver, 1992; Tyerman et al., 1992). It was reported that Cs^+ blocks K^+ permeation in the channel (Pottosin, 1992; Klieber & Gradmann, 1993). We have also found that in the presence of divalent cations (*not shown*) and/or Cs^+ the K^+ channel does undergo flickering when it is in the open state. Klieber and Gradmann (1993) reported that under bi-ionic conditions, Cs^+ on one side does not affect the K^+ (saturation) currents from the opposite side. Indeed, Cs^+ being present in the bath or in the pipette did not influence K^+ channel openings in the positive or, respectively, negative range. Figure 2 illustrates this with traces recorded

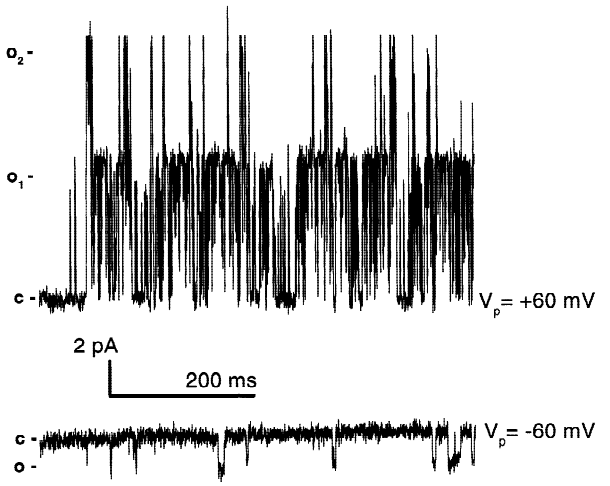

Fig. 2. With Cs^+ in the bath and K^+ in the pipette no outward K^+ current can be detected. Droplet-attached configuration. Pipette potential (V_p): +60 and −60 mV. Closed (*c*) and open states of the small conductance (*o*) and of K^+ channel (o_1 and o_2) are indicated. *Bath:* 155 mM CsCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES/Tris, pH 7.4; *Pipette:* 155 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES/Tris, pH 7.4.

in droplet-attached configuration with Cs^+ present in the bath. At least two K^+ channels were present at +60 mV, opening with high frequency. Flickerings in the open state of the channel also can be observed. In these recordings openings of the small conductance channel could not be resolved. However, as expected at −60 mV no K^+ channels could be detected, only the small conductance channel appeared at negative pipette potential values without K^+ channel activity "contamination."

Characterization of the Small Conductance Channel

Selectivity

To further identify the current carried by the small conductance channel we replaced the K^+ in the pipette and bath solutions by Na^+ and Cs^+. The Nernst equation was used to calculate the reversal potential values (E_{rev}) for Cl^- ions, using the concentrations of the ions on the outside and inside membrane surface (Fig. 3*a* and *b*). With asymmetrical Cl^- concentrations (lowered pipette or bath Cl^- concentration), the detected channels were selective to Cl^-, since the measured reversal potential values (E_{rev}: −24.3 ± 2.2, $N = 4$ and +23.6 ± 1.4, $N = 9$, respectively) were close to the expected theoretical values of the Cl^- equilibrium potential (Fig. 3*c*). Tyerman and Findlay (1989) also described this high selectivity of the small conductance channels for Cl^- ions in the tonoplast of *Chara.*

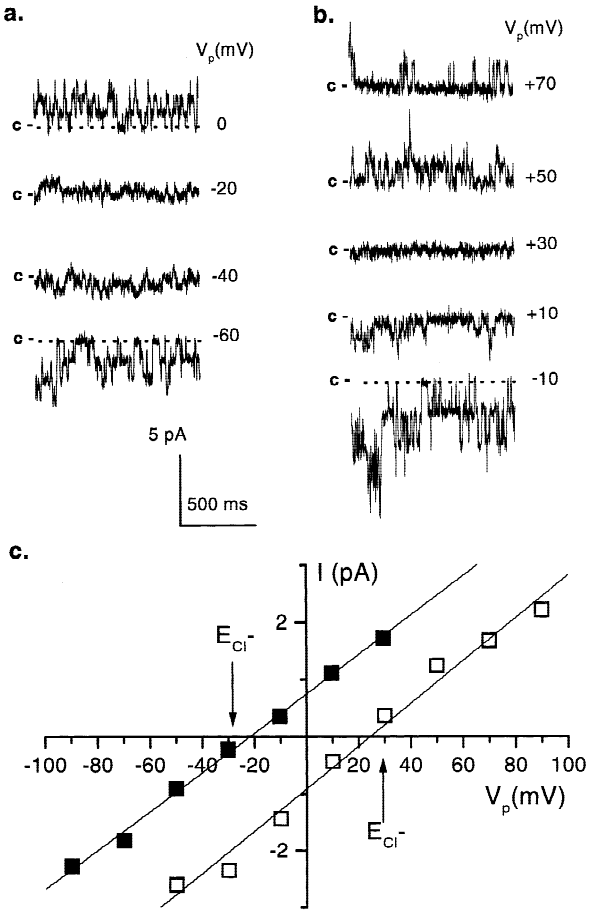

Fig. 3. Shifting the reversal potential for Cl^- ions in inside-out patch configuration (representative traces from *a* and *b* are shown, *c* = closed channel). (*a*) From a holding pipette potential of −30 mV voltage pulses (V_p) between −90 and +50 mV with 10 mV increments were applied for inside out membrane patches, and the current traces recorded ($N = 4$). Calculated Nernst potential for Cl^- ions (E_{Cl^-}): −29.1 mV. *Bath solution:* 106 mM NaCl, 50 mM CsCl, 1 mM $CaCl_2$, 5 mM HEPES/Tris pH 7.4; *Pipette solution:* 106 mM NaF, 50 mM CsCl, 1 mM $CaCl_2$, 5 mM HEPES/Tris pH 7.4; (*b*) From a holding pipette potential of +30 mV voltage pulses (V_p) between +90 and −50 mV with 10 mV increments were applied, and the current traces recorded ($N = 9$). Calculated Nernst potential for Cl^- ions (E_{Cl^-}): +29.1 mV. The same solutions as in *a*, but exchanged. (*c*) Single-channel current-voltage relationships for currents from experiments shown in *a* and *b*.

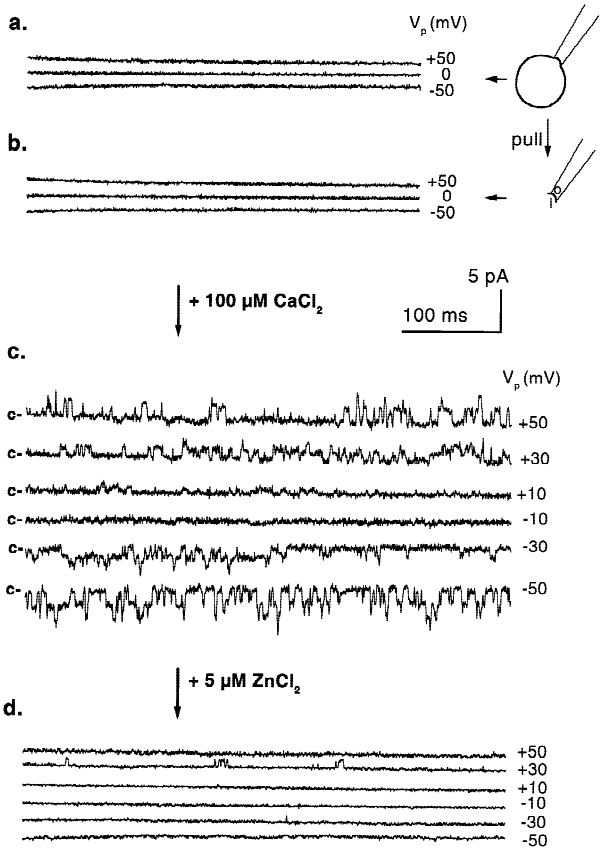

Fig. 4. Cl^- channels are activated by calcium from the cytoplasmic side. *Chara* droplets were formed in a *bath* solution containing 140 mM CsCl, 5 mM HEPES/Tris, pH 7.4, 10^{-8} M free Ca^{2+} (adjusted with 500 µM EGTA); *Pipette:* 140 mM CsCl, 5 mM HEPES/Tris, pH 7.4, and 150 µM $CaCl_2$, E_{rev} for Cl^- ions = 0 mV). The pipette potential was stepped to values as indicated from a holding potential of 0 mV. Scale similar for all traces. (*a*) Droplet-attached mode. (*b*) Inside-out configuration. (*c*) Single-channel openings recorded after bath perfusion with 100 µM $CaCl_2$. The single-channel slope conductance calculated from this particular recording was 37 pS (*c* = closed channel). (*d*) Effect of the anion channel blocker Zn^{2+} on the activity of the channels (recording was started 5 sec after applying the blocker): 5 µM $ZnCl_2$ almost completely blocked Cl^- permeation.

Activation of the Channel from the Cytoplasmic Side by Ca^{2+} and Blocking with Zn^{2+} Ions

When using Cs^+ as the only monovalent cation, no single channel openings could be detected in solutions containing low Ca^{2+} (buffered with EGTA). Figure 4*a* shows current traces recorded in droplet-attached configuration, with no channel openings in a voltage range of −50 to +50 mV. After patch excision still no currents could be detected (Fig. 4*b*). Addition of $CaCl_2$ to the bath instantaneously activated single channels. The activation persisted for minutes over a whole voltage range between −60 and +60 mV, without inactivation in the presence of Ca^{2+} (Fig. 4*c*). In this experiment the conductance of the activated single-channel was about 37 pS, suggesting that the Cl^- channel was activated. Upon Ca^{2+}-activation a 37 pS (30%) or a 48 pS (70%) conductance dominated the records ($N = 16$). The appearance of the channel did not depend on the fact that Ca^{2+} was present or absent (chelated by EGTA) in the pipette. Chelating Ca^{2+} in the bath by applying EGTA perfusion ($N = 3$), (*not shown*) reversibly abolished channel activity. The EGTA-effect was instantaneous. Zn^{2+} is a well-known blocker of anion channels (Stanfield, 1970; Keller, Hedrich & Rasche, 1989; Hille, 1992). Addition of $ZnCl_2$ to

the bath resulted in a complete block of the channel. The time needed for total inhibition was variable. In three cases the block occurred instantaneously (Fig. 4*d*), but we also detected gradual lowering of the open probability (P_o) value (open times became shorter and long closed states were present, the complete block resulting after 5 to 10 min). In five cases 5 to 10 μM $ZnCl_2$ was able to block the channel activity completely. In one experiment 30 μM $ZnCl_2$ was not effective.

The Ca^{2+}-dependence of the Cl^- current was tested in experiments performed in the absence or in the presence of different concentrations of $[Ca^{2+}]_{cyt}$ (ranging between 150 nM and 5 mM). With 100 μM $CaCl_2$ in the pipette, changes in $[Ca^{2+}]_{cyt}$ revealed a dependence of the channel P_o on the $[Ca^{2+}]_{cyt}$ (Fig. 5*a*). No considerable Cl^--conductance was present up to 0.75 μM $[Ca^{2+}]_{cyt}$, then P_o of the channel increased with elevated $[Ca^{2+}]_{cyt}$ in a concentration-dependent manner, with a median effective concentration ($[EC_{50}]$) of about 2×10^{-4} M at +40 and 1×10^{-4} M at −40 mV, respectively (Fig. 5*b*). The single channel current amplitude was not affected by changes in the $[Ca^{2+}]_{cyt}$, except that in some cases putative substates (48 pS and conductances smaller than 37 pS) of the Cl^- channel were present. An interesting point is the apparent transient activation of the channel by calcium. In approximately 30% of the cases, this transient character could be detected in the inside-out patch configuration: after an initial burst activity in the patch (with traces showing 2–3 open levels) lasting for some seconds, channel activity reached a steady-state level which lasted for minutes. In some experiments steady-state channel activity in the patch persisted for more than one-half hour, without inactivation. Because of the short initial burst kinetics and transient character of the channel activity, no attempt was made to fit the plotted mean P_o values, detected at −40 and +40 mV.

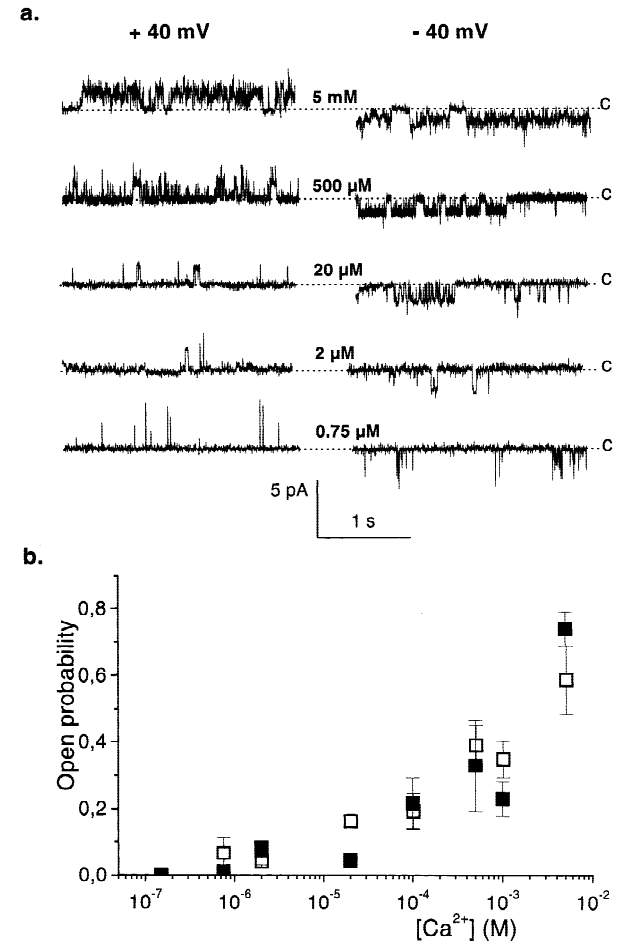

Fig. 5. Chloride channel activity depends on $[Ca^{2+}]_{cyt}$. (*a*) Single channel activity, recorded from inside-out patches bathed by 0.75, 2, 20, 500 μM and 5 mM Ca^{2+}, at V_ps of +40 or −40 mV. Current traces at the indicated concentrations were recorded from different experiments, during which perfusion was omitted (*c* = closed channel, 2.5-sec long sequences are shown). (*b*) Steady-state channel open probability (P_o) values for the 37 pS channel at −40 mV (open square) and +40 mV (solid square) pipette potential (error bars represent SEM, each point represents 3–5 determinations, each in a separate patch) were calculated from all-points histograms generated from 30 to 50-sec long traces. 48 pS conductance openings often appeared together with the smaller conductance values. All openings were taken into account, considering that different current levels are states of the same channel.

Ca^{2+} is Not Able to Activate from the Vacuolar Side

Excision of the patch to outside-out mode gave rise to a transient activation of Cl^- channels present in the patch ($N = 4$). 2 μM Ca^{2+} in the pipette was already able to activate the channels in the outside-out patch configuration. Fig. 6*a* shows activation due to 150 μM $CaCl_2$ present in the pipette solution. The conductance of the single-channel in this particular case was 48 pS. The channels slowly inactivated, the activation in outside-out mode being transient (Fig. 6*b*). Addition of $CaCl_2$ to the bath did not result in Cl^- channel activity recruitment (Fig. 6*c*). A number of experiments (with at least 20 independent inside-out patches) were performed as shown in the Fig. 4*a* and *b*, except that the pipette solution (vacuolar side of the membrane patch) contained different concentrations of Ca^{2+}, with the highest tested concentration being 1 mM. None of these experiments resulted in the activation of the channels after excision of the patch. These findings indicated that Ca^{2+} was not acting on the vacuolar side of the membrane.

In inside-out patch configuration inactivation of the Cl^- channel in the presence of high $[Ca^{2+}]$ (ranging 0.1–1 mM) was scarce (*see above*, Fig. 5). However, in outside-out configuration, the channels fully inactivated after a relatively high initial activity in the presence of $CaCl_2$ at the cytoplasmic pipette side (Fig. 6*b* and *c*). The time course of the inactivation shows, that it happens

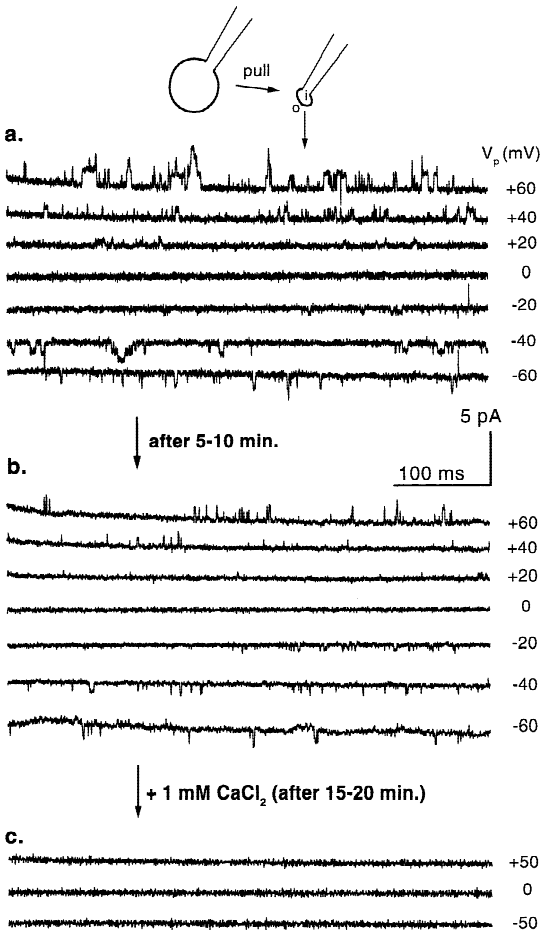

Fig. 6. Cl^- channels are not activated by Ca^{2+} from the vacuolar side. Scale similar for all traces. (*a*) A short suction applied to the pipette in droplet-attached configuration resulted in whole-droplet configuration. Excising the patch to outside-out configuration gave rise to a transient activation of Cl^- channels. Upward and downward deflections represent channel openings. (*b*) These channels slowly inactivated in time. (*c*) Addition of $CaCl_2$ to the bath did not result in Cl^- channel activity recruitment. *Bath:* 140 mM CsCl, 5 mM HEPES/Tris, pH 7.4, 10^{-8} M free Ca^{2+} (adjusted with 500 μM EGTA); *Pipette:* 140 mM CsCl, 5 mM HEPES/Tris, and 150 μM $CaCl_2$, pH 7.4.

within 15–20 min from the start of an experiment (Fig. 7). Inactivation persisted while recording conditions were constant. Channel activity and open time decreased, (Fig. 6) resulting in a gradually decreasing open probability (P_o). Apparently, the single-channel conductance was not changed. It cannot be excluded that inactivation is caused by the desensitization of the Ca^{2+}-activated channel. Alternatively, some peripherally bound proteins which might have extra control functions and are present in the bath after droplet isolation might be lost (for instance, in the outside-out patch configuration they may be washed out more easily by the pipette solution), and the mechanism that confers sensitivity to Ca^{2+} becomes labile.

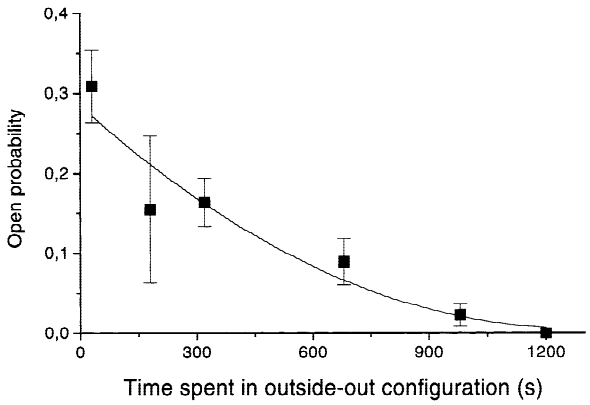

Fig. 7. Cl^- channels inactivate in outside-out patch configuration with 150 μM Ca^{2+} at the cytoplasmic side. Cl^- channel P_o values were plotted against time. The pipette potential was stepped to +40 mV from a holding potential value of 0 mV. E_{rev} for Cl^- ions = 0 mV. The tendency of the decay is indicated by the fitted curve. (Error bars show SEM, $N = 4$). In one experiment channels were inactivated within 180 sec. Similar results were obtained with −40 mV pipette potential.

Two Types of Cl^- Channel Activity

To investigate the voltage dependence of the single channel currents, the pipette was voltage clamped to potential values ranging from −40 to +40 mV (or −50 to +50 mV) from a pipette holding potential of 0 mV. In experiments started shortly after droplet isolation, very often rectification of the Cl^- conductance could be seen in droplet-attached configuration (Fig. 8*a*). The slope conductance calculated from the current-voltage relationship of this channel at negative pipette potentials had a value of about 37 pS (37.3 ± 1.9, $N = 10$, Fig. 8*b*). This rectification in some cases (about 5%) was not present anymore in droplets 1–2 hr after isolation.

The rectification ceased when the membrane patch was excised in a bath solution with identical Cl^- content as the pipette solution (Fig. 8*c*). In the inside-out and outside-out (*not shown*) patch configuration the amplitude of the single-channel current showed a linear voltage relationship with a mean slope conductance of 48 pS (48.1 ± 0.8, $N = 11$, Fig. 8*b*). This finding suggested that the reason for the strong rectification was the asymmetrical Cl^- concentration in the pipette (high) and droplet (low), as was also concluded by Tyerman and Findlay (1989). The absence of rectification in "old" droplets could then be explained by the fact that Cl^- concentration in such droplets became higher, as Cl^- concentrations in the bath, droplets and pipette gradually reached equilibrium. However, our experiments with asymmetrical Cl^- in excised patch configuration did not show rectification in 72% of the cases ($N = 18$, Fig. 3).

Holding Potential Dependent Rectification of the Cl^- Channel

To further investigate the rectification we tested the idea that the holding membrane potential could influence the

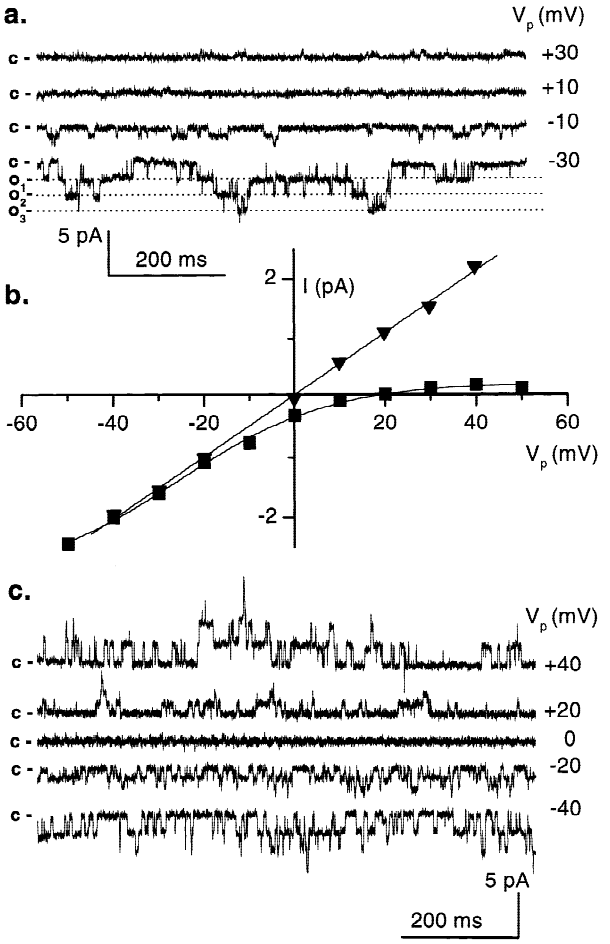

Fig. 8. Two types of Cl^- channel activity. From a holding pipette potential of 0 mV the pipette potential was stepped to values as indicated. Scale similar for all current traces (*c* = closed channel, *o* = open channel). Current traces shown in *a* and *c* were recorded from different experiments. *Bath and pipette solution:* 130 mM CsCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES/Tris, pH 7.4. (*a*) Current traces recorded in droplet-attached configuration. At −30 mV current levels of superimposed individual channels were detected, and numbered continuously according to the number of detected levels. (*b*) Current-voltage relationships. In droplet-attached configuration the single-channel slope conductance (G_{slope}) was 37 pS (37.3 ± 1.9, $N = 10$) and showed rectification. In inside-out patch configuration the current-voltage relationship was linear, G_{slope}: 48 pS (48.1 ± 0.8, $N = 11$). (*c*) Single-channel activity in inside-out patch configuration with linear current-voltage relationship.

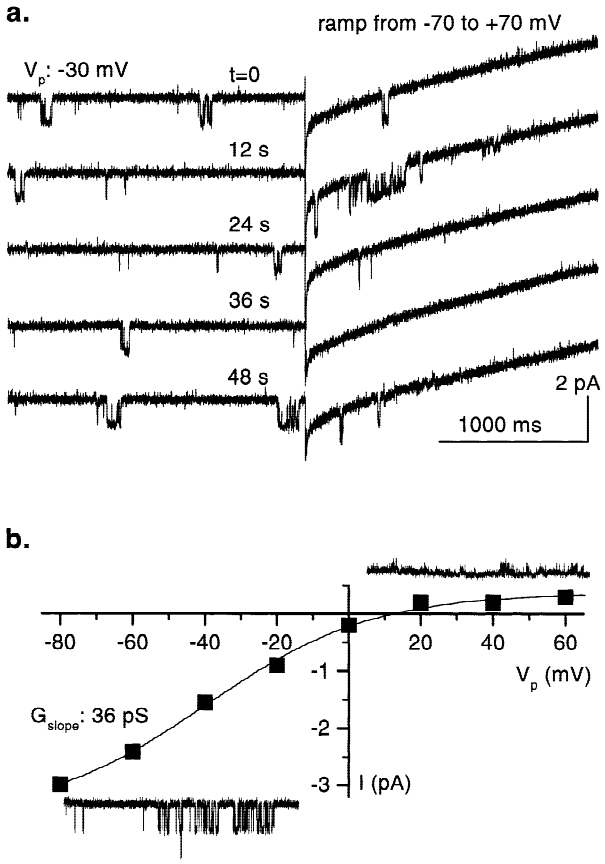

Fig. 9. Negative holding potential dependent rectification of the Cl^- channel in inside-out patch configuration. Time scale similar for all traces. (*a*) The pipette voltage (V_p) was held constant at least 2 min before the start of a protocol. Interepisode times before each ramp were 10-sec long. A 2-sec long recording with channel activity (at −30 mV) before the ramp is shown. Five consecutive episodes are presented. Downward deflections represent openings. (*b*) Single-channel current-voltage relationships obtained from inside-out patches; G_{slope} = 36 pS (35.6 ± 1.1, $N = 4$). Voltage steps from −80 to +60 mV with 20 mV increments were applied from a holding pipette potential of −30 mV. Short traces of channel activity recorded at steps −60 and +60 mV are inserted. *Bath:* 140 mM CsCl, 1 mM $CaCl_2$, 5 mM HEPES/Tris, pH 7.4; *Pipette:* 140 mM CsCl, 150 μM $CaCl_2$, 5 mM HEPES/Tris, pH 7.4, E_{rev} for Cl^- ions = −0.4 mV.

behavior of the channel. Different holding pipette potential values were applied to the excised membrane patches before and between voltage-step and ramp protocols. Figures 9 and 10 show single-channel activity before and during ramp protocols. Previous experiments showed that when the membrane patch was held at a voltage value corresponding to that of the calculated E_{rev} for Cl^- ions, then channel activity could be detected both in the positive and negative range (Fig. 8*c*), with a linear single-channel current-voltage relationship (Fig. 8*b*). However, holding the membrane patch at voltage values different from the calculated E_{rev} for Cl^- ions (at −50, −30, +30 or +50 mV in the case of $E_{rev} = 0$), dramatically changed the behavior of the channels. Figure 9*a* shows current traces at a holding potential of −30 mV and subsequent ramps from −70 to +70 mV. Channel activity was present preceding ramps at −30 mV, and showed rectification during ramps, with much larger outward currents than inward currents. Similar experiments using step protocols instead of ramp protocols showed the same, as illustrated in Fig. 9*b*. The current-voltage relationship of inside-out patches gave a slope conductance value at the negative range of about 36 pS (35.6 ± 1.1, $N = 4$). This value is not significantly different

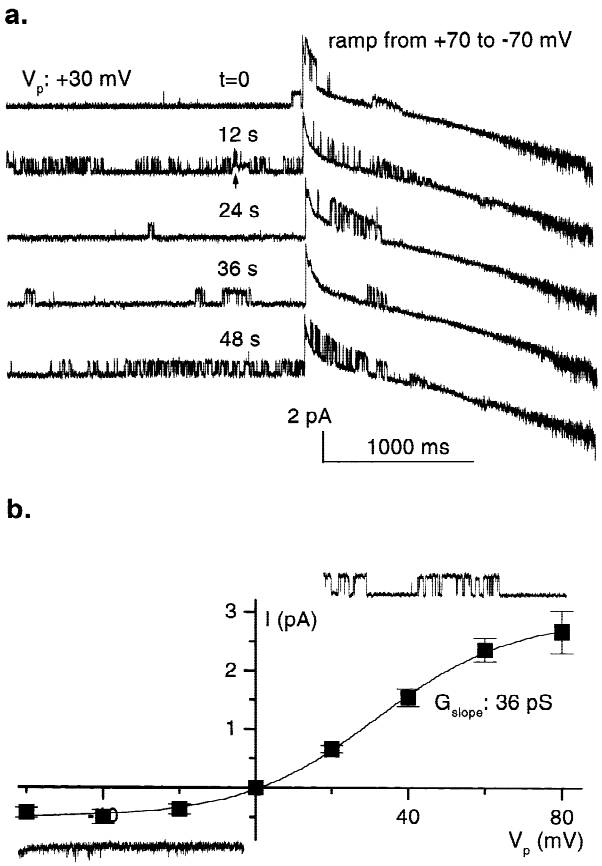

Fig. 10. Positive holding potential dependent rectification of the Cl^- channel in inside-out patch configuration. Time scale similar for all traces. (*a*) V_p was held constant at least 2 min before the start of a protocol. Interepisode times before each ramp were 10-sec long. A 2-sec long recording with channel activity (at +30 mV) before the ramp is shown. Five consecutive episodes are presented. Upward deflections represent openings. (*b*) Single-channel current-voltage relationships obtained from inside-out patches; G_{slope} = 36 pS (35.8 ± 3.5, N = 7, error bars indicate SEM). Voltage steps from +80 to −60 mV with 20 mV increments were applied from a holding pipette potential of +30 mV. Short traces of channel activity recorded at steps +60 and −60 mV are inserted. *Bath:* 140 mM CsCl, 150 μM $CaCl_2$, 5 mM HEPES/Tris, pH 7.4; *Pipette:* 140 mM CsCl, 150 μM $CaCl_2$, 5 mM HEPES/Tris, pH 7.4, E_{rev} for Cl^- ions = 0 mV.

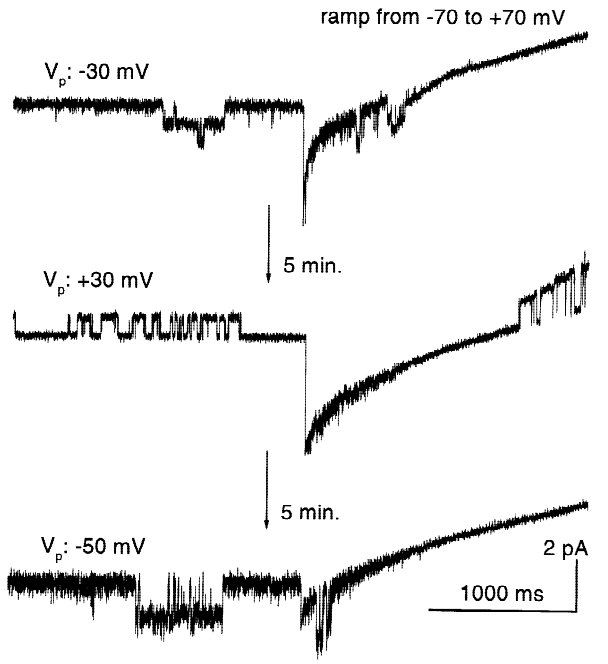

Fig. 11. Shifting the rectification for Cl^- channel in inside-out patch configuration. For each case a 2-sec long recording with channel activity (with holding pipette potential values of −30, +30, and −50 mV) is shown before the ramp. During the 2-sec long ramps the voltage changed from −70 to +70 mV. Downward and upward deflections at negative, respectively positive pipette potential values are channel openings. *Bath:* 140 mM CsCl, 5 mM HEPES/Tris, 10 μM $CaCl_2$, pH 7.4; *Pipette:* 140 mM CsCl, 5 mM HEPES/Tris, 150 μM $CaCl_2$, pH 7.4, E_{rev} for Cl^- ions = 0 mV.

from the conductance value detected in the droplet-attached configuration. When the holding pipette potential was positive (+30 mV, Fig. 10) Cl^- channels displayed an inverse behavior, with larger inward than outward currents, and sometimes showing flicker block in the negative range. The current-voltage relationship (Fig. 10*b*) gave a slope conductance value in the positive range of about 36 pS (35.8 ± 3.5, N = 7). Moreover, when the holding potential was changed from negative to positive (to +30 mV), and the patch was held long enough at this new voltage value (at least 3–5 min), the rectification could be inverted, recording negligible current amplitudes in the negative range, and clear channel openings in the positive range. The transition from outward to inward rectifying occurs gradually: the number of openings decreased in the negative range of the ramp, and openings became shorter (spikes). Many times the membrane noise increased in the negative range. Concomitantly, the channels started to become active in the positive range. The time needed for full transition was variable, spikes still could appear in the negative range after an apparent full transition (approx. 5–10 min). Channels were affected again if the pipette holding potential was "switched back" to negative values (−30 or −50 mV, Fig. 11). The transition from inward to outward rectifying showed similar characteristics as described for the opposite transition. Surprisingly, we could not trigger this holding potential dependent rectification in outside-out patches, in three consecutive experiments.

Under these experimental conditions (with different pipette holding potential values), very often a second, smaller conductance channel appeared in the same membrane patch, sometimes superimposing with the Cl^- conductance channel (Fig. 10*a*, second trace, arrow). Although, we do not exclude that a channel with smaller conductance (17 and sometimes 25 pS) than the above

described can also reside in the membrane, subconductance levels of the same channel entity might also be present in the membrane patch. Cooperative subunit phenomena and a model recently presented by McCulloch, Laver and Walker (1997), showed subconductance states of the *Chara* plasma membrane anion channel.

Discussion

The Ca^{2+}-dependence of vacuolar channels has been reported for many plant cells, providing a general mechanism for the regulation of ion transport to and from plant vacuoles. In higher plants several types of ion channels mediate ion fluxes from vacuole to cytoplasm (for reviews *see* Maathuis et al., 1997 and MacRobbie, 1998). The ubiquitous slow vacuolar (SV) and the *Vicia faba* vacuolar (VK) channels are activated by Ca^{2+} and show cation selectivity. However, the ionic selectivity of the SV channel is controversial regarding permeability to anions. At least, in *V. faba* guard cell vacuoles the SV channel is highly cation selective (Allen & Sanders, 1996), and cannot provide a significant pathway for physiological anion release from vacuoles (Pei et al., 1996). A candidate for providing a more general mechanism for anion uptake in higher plants would be the kinase-dependent vacuolar chloride (VCL) channel (Pei et al., 1996).

In freshwater giant algae such as *Chara* and *Nitella*, the plasma membrane AP is always accompanied by a tonoplast AP, which is chemically triggered (Findlay, 1970; Lunevsky et al., 1983). It was suggested that Ca^{2+} is required for the regulation of Cl^- channels in the tonoplast, being the most probable candidate for inducing the tonoplast AP in vivo (Kikuyama, 1986).

Our work demonstrates that Ca^{2+}-modulated Cl^- channels do exist in the membrane of *Chara corallina* droplets. Ca^{2+} present in the solutions activates these channels from the cytoplasmic side, apparently without the need of other cytoplasmic factors. Cl^- channels in this membrane are difficult to study, because they are masked by massive K^+ channel activity, but in some circumstances it was possible to detect coexisting channels. K^+ channel activity was blocked if Cs^+ was used in the solutions as the only monovalent cation. In this way we were able to identify single Cl^- conducting channels in the range of 37–48 pS. When the cytoplasmic side of the membrane faced a low $[Ca^{2+}]$ solution (10^{-8} mM, buffered with EGTA), Cl^- channel activity was not present in the membrane patch, but could be restored by $CaCl_2$-perfusion of the cytoplasmic side. The physiological concentration for the elevated free cytoplasmic Ca^{2+} during an action potential was reported to be 6.7 μM (Williamson & Ashley, 1982). The sometimes observed variation in the Ca^{2+}-sensitivity of the Cl^- channels did not allow us to fully characterize the nature of the Ca^{2+} interaction with the channel pore, only the $[EC_{50}]$-values were determined when holding the membrane patch at −40 and +40 mV pipette potential. The channels appear to be at least partly activated in the submicromolar range of $[Ca^{2+}]_{cyt}$, but their activation value seems to be higher than that reported for plasma membrane Cl^- channels (being 1 μM, Okihara et al., 1991). The interpretation of the dose-response relationship (Ca^{2+}-dependence of channel activity) was made more complicated by the fact that when present, the 48 pS state of the channel showed similar P_o values (0.25–0.35) in the presence of 20 μM to 1 mM $[Ca^{2+}]_{cyt}$.

Generally, the conductance increased after patch excision, but we have also found that the excised channel had a lower conductance value upon Ca^{2+}-activation, suggesting that other regulatory mechanisms might also influence the channel conductance.

Although, the here presented Cl^- channels share some common properties with other Ca^{2+} modulated Cl^- channels from the plasma membrane of higher plants, the rectification seems to be a distinctive feature of *Chara* tonoplast Cl^- channels. Depending on the holding potential, Ca^{2+}-dependent Cl^- channels exhibit linear or nonlinear current-voltage relationships since channel conductance during steps and ramps was dependent on the holding potential applied before and between step and ramp protocols. This suggests that during and after the AP, the membrane potential dependent conductance of the tonoplast Cl^- channels may help to stabilize the membrane potential. This mechanism might also regulate Cl^- fluxes between the vacuole and cytoplasm during osmostress conditions when long-lasting tonoplast membrane potential shifts occur.

Holding (membrane) potential dependent rectification of *Chara* tonoplast Cl^- channels might represent a novel mechanism for long-term regulation of ion current kinetics in such giant cells. We will focus on further investigation of the mechanism of rectification, the influence of pH and other factors on the Cl^- channels of the *Chara* tonoplast.

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