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A Patch-Clamp Study of Ion Channels in Protoplasts Prepared from the Marine Alga *Valonia utricularis*

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Abstract. The giant marine alga *Valonia utricularis* is a classical model system for studying the electrophysiology and water relations of plant cells by using microelectrode and pressure probe techniques. The recent finding that protoplasts can be prepared from the giant "mother cells" (Wang, J., Sukhorukov, V.L., Djuzenova, C.S., Zimmermann, U., Müller, T., Fuhr, G., 1997, *Protoplasma* **196:**123–134) allowed the use of the patch-clamp technique to examine ion channel activity in the plasmalemma of this species. Outside-out and cell-attached experiments displayed three different types of voltage-gated Cl^- channels (VAC1, VAC2, VAC3, *V*alonia *A*nion *C*hannel 1,2,3), one voltage-gated K^+ channel (VKC1, *V*alonia K^+ *C*hannel 1) as well as stretch-activated channels. In symmetrical 150 mM Cl^- media, VAC1 was most frequently observed and had a single channel conductance of 36 ± 7 pS ($n = 4$) in the outside-out and 33 ± 5 pS ($n = 10$) in the cell-attached configuration. The reversal potential of the corresponding current-voltage curves was within 0 ± 4 mV ($n = 4$, outside-out) and 9 ± 7 mV ($n = 10$, cell-attached) close to the Nernst potential of Cl^- and shifted towards more negative values when cell-attached experiments were performed in asymmetrical 50:150 mM Cl^- media (bath/pipette; E_{Cl^-} −20 ± 7 mV ($n = 4$); Nernst potential −28 mV). Consistent with a selectivity for Cl^-, VAC1 was inhibited by 100 μ*M* DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid). VAC1 was activated by a hyperpolarization of the patch. Boltzmann fits of the channel activity under symmetrical 150 mM Cl^- conditions yielded a midpoint potential of −12 ± 5 mV ($n = 4$, outside-out) and −3 ± 6 mV ($n = 9$, cell-attached) and corresponding apparent minimum gating charges of 15 ± 3 ($n = 4$) and 18 ± 5 ($n = 9$). The midpoint potential shifted to more negative values in the presence of a Cl^- gradient.

VAC2 was activated by voltages more negative than E_{Cl^-} and was always observed together with VAC1, but less frequently. It showed a "flickering" gating. The single channel conductance was 99 ± 10 pS ($n = 6$). VAC3 was activated by membrane depolarization and frequently exhibited several subconductance states. The single channel conductance of the main conductance state was 36 ± 5 pS ($n = 5$). VKC1 was also activated by positive clamped voltages. Up to three conductance states occurred whereby the main conductance state had a single channel conductance of 124 ± 27 pS ($n = 6$).

In the light of the above results it seems to be likely that VAC1 contributes mainly to the Cl^- conductance of the plasmalemma of the turgescent "mother cells" and that this channel (as well as VAC2) can operate in the physiological membrane potential range. The physiological significance of VAC3 and VKC1 is unknown, but may be related (as the stretch-activated channels) to processes involved in turgor regulation.

Key words: *Valonia* — Protoplasts — Patch clamp — Ion channels — Stretch-activated channels — Voltage gating

Introduction

The giant marine algae have been proven in the last eight decades to be extremely useful cells for studying the electrical membrane properties as well as membrane transport and its regulation by turgor pressure (Osterhout, 1924; Gutknecht, 1966; 1967; Findlay et al., 1971; Novak & Bentrup, 1972; Zimmermann & Steudle, 1974; Hope & Walker, 1975; Lainson & Field, 1976; Zimmermann, 1978; Davis, 1981; Wendler, Zimmermann & Bentrup, 1983; Benz & Zimmermann, 1983; Beilby,

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1989; Wang et al., 1991, 1997*a*,*b;* Bisson & Kirst, 1995). However, the interpretation of the electrophysiological (and pressure) data obtained on giant algae is not always straightforward because of the placement of the microelectrodes into the vacuole that occupies more than 97% of the cell volume. Thus, separate measurements of the electrical and transport properties of the tonoplast and the plasmalemma arranged in series were not possible despite many efforts (Lainson & Field, 1976; for exceptions *see* Freudling & Gradmann, 1979; Davis, 1981).

The individual electrical properties of the two membranes could be measured very recently by Wang et al. (1997*a*) and Ryser et al. (1999) in turgescent giant cells of *Valonia utricularis* and *Ventricaria ventricosa*, respectively, by using a vacuolar perfusion technique with integrated microelectrodes for charge pulse relaxation experiments. Separate permeabilization of the tonoplast and the plasmalemma and subsequently performed electrophysiological measurements were achieved by addition of the pore-forming antibiotic nystatin to the vacuolar or external perfusion solutions.

This approach has suggested—among other things—that the tonoplast of *V. utricularis* and *V. ventricosa* is multifolded and that the conductance of the plasmalemma of the cells of these two species is much higher than that of the tonoplast. The plasmalemma conductance was apparently governed mainly by Cl^- in the case of *V. utricularis*, whereas K^+ played a more dominant role in *V. ventricosa.*

In this communication, we applied the patch-clamp technique to protoplasts produced from the "mother cells" of *V. utricularis* (Wang et al., 1997*b*) in order to identify the transport elements of the plasmalemma in more detail and to reject or to support previous conclusions. These protoplasts could easily be obtained when the protoplasmic content of turgorless "mother cells" was squeezed out in sea water or in solutions suitable for patch-clamp studies. They were formed within 2 hr and regenerated very rapidly to walled cells with a central vacuole which grew to large-sized cells over the following months. Besides this, there was evidence (Wang et al., 1997*b*) that the membrane of the protoplasts contained exclusively plasmalemma material from the "mother cells." Problems arising from large central vacuoles and cell walls do not exist provided that newly formed protoplasts are used immediately for patch-clamp studies (after short treatment of the protoplasts with enzymes for removal of cell wall fibrils). Thus, these protoplasts seemed to be ideal objects for patch-clamp studies.

Patch-clamp measurements in the outside-out and cell-attached configuration performed here showed that three voltage-gated Cl^- channels and at least one voltage-dependent K^+ channel as well as stretch-activated channels exist in the membrane of *Valonia* protoplasts. These findings seem to correlate with the results obtained from the electrophysiological studies on perfused and nonperfused "mother cells."

Materials and Methods

Plant Material

Cells of *Valonia utricularis* were collected from the rocky coast of Ischia, Gulf of Naples, Italy. They were cultivated in 40 l perspex containers in *N*atural *S*ea *W*ater (NSW, 1120 mosmol kg^{-1}, pH 8.1) at 16°C (289 K) under a 12 hr light/dark regime (2 × 36 W Fluora lamps, Osram, München, Germany).

Preparation of Protoplasts

The preparation of protoplasts from giant "mother cells" was described in detail by Wang et al. (1997*b*). Briefly, cylindrical cells with a length of 10–30 mm and a diameter of 3–5 mm were taken. They were dried gently with filter paper and exposed to air. The cells plasmolyzed within about 5 min. Then, one end of the plasmolyzed cell was cut off and the protoplasmic content was squeezed out in the same hypotonic patch-clamp medium which was used later for the studies in the cell-attached and outside-out patch configuration, respectively (*see below*).

After 20 min incubation in the particular patch-clamp medium, the irregularly shaped, green-colored protoplasmic aggregates were washed two times to remove cell wall fragments and other cellular components from the medium. The protoplasts became spherical after about 2.5 hr. Usually, hundreds of protoplasts exhibiting diameters between 30–300 μm could be produced from a single "mother cell." Surprisingly, the protoplasts regenerated to walled cells after about 24 hr and grew in the following months despite the unphysiological media used for their preparation.

Even though it was possible to obtain giga-seals with these spherical protoplasts just after rounding up, the rapid regeneration of the cell wall (*see* Wang et al., 1997*b*) prevented routine measurements. Therefore, in most of the experiments the protoplasts were pretreated for 30 min at 30°C (303 K) with an enzyme cocktail containing (in % w/v) 0.5 cellulase, 0.1 pectinase, 0.5 hemicellulase, 0.1 lysozym and 1.5 bovine serum albumine.

All chemicals and enzymes (*see also below*) were purchased from Sigma (Sigma Chemical, St. Louis, MO).

Patch-Clamp Conditions

Standard patch-clamp methods were used in the cell-attached and outside-out configurations (Hamill et al., 1981). Seal resistances were in the range of 1–3 GΩ. The patch pipettes consisted of borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany). The external diameter of the capillaries was 1.5 mm and the wall thickness 0.25 mm. They were pulled on a two stage vertical puller (L/M-3P-A, List-Medical, Darmstadt, Germany).

All bath and pipette media were filtered through a 0.22 μm filter before use. Different external and pipette media were used in cell-attached and outside-out experiments to reveal the ion specificity of the channels. For recordings in (almost) symmetrical Cl^- media, the protoplasts were prepared and bathed in B1 medium containing (in mM) 10 KCl, 100 NaCl, 10 $CaCl_2$, 10 $MgCl_2$ and 10 HEPES/BTP (N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)/1,3-Bis[tris-(hydroxymethyl)-methylamino]propane). The osmolality and pH of this

Table 1. Composition of the media used in this study

Ions	Media (concentrations in mM)					
	P1	P2	P3	B1	B2	B3
K^+	115	10	115	10	100	0
Na^+	10	100	10	100	10	110
Cl^-	145	150	30	150	50	150
Ca^{2+}	55×10^{-6}	10	55×10^{-6}	10	10	10
Mg^{2+}	10	10	10	10	10	10
HEPES	10	10	10	10	10	10

medium was about 290 mosmol kg^{-1} and 8.1, respectively. The patch pipettes were filled with P1 medium containing (in mM) 115 KCl, 10 NaCl, 0.1 $CaCl_2$, 10 $MgCl_2$, 10 HEPES/BTP and 1 EGTA (Ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid), free Ca^{2+} concentration 55 nM, osmolality 291 mosmol kg^{-1}, pH 7.0.

The equilibrium Nernst potential for Cl^- between the P1 pipette solution and the B1 medium was close to zero because of nearly equal concentrations of Cl^- on both sides of the membrane.[1] This situation is characterized below as "symmetrical 150 mM Cl^-."

To simulate the Cl^- gradients in the "mother cells" (Wang et al., 1991), the following media were used in cell-attached experiments (in mM): external B2 medium, 100 K^+-gluconate, 10 NaCl, 10 $CaCl_2$, 10 $MgCl_2$ and 10 HEPES, osmolality 290 mosmol kg^{-1}, pH 8.1; pipette P2 medium, 10 KCl, 100 NaCl, 10 $CaCl_2$, 10 $MgCl_2$ and 10 HEPES, osmolality 290 mosmol kg^{-1}, pH 8.1. Under asymmetrical 50:150 Cl^- (B2/P2) conditions the Nernst potential for Cl^- was shifted to +28 mV (pipette relative to bath).

In outside-out experiments, Cl^- gradients were established by using the following media (in mM): external B3 medium, 110 NaCl, 10 $CaCl_2$, 10 $MgCl_2$ and 10 HEPES/BTP; osmolality 290 mosmol kg^{-1}, pH 8.1; pipette P3 medium, 115 K^+-glutamate, 10 NaCl, 0.1 $CaCl_2$, 10 $MgCl_2$, 1 EGTA and 10 HEPES/BTP, free Ca^{2+} concentration 55 nM, osmolality 291 mosmol kg^{-1}, pH 7. The composition of these asymmetrical 150:30 media (B3/P3) led to a Nernst potential of −41 mV for Cl^- (pipette relative to bath). For clarity the composition of the media used in the various experiments is summarized in Table 1.

For inhibition of Cl^- channels, DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) was used. This channel inhibitor was applied at a final concentration of 100 μM by perfusing the bath with B1 medium to which an appropriate aliquot of a stock solution (50 mM resolved in 1 ml Dimethyl sulfoxide (DMSO)) had been added. The final concentration of DMSO was 1%. DIDS was purchased from Fluka (Neu-Ulm, Germany).

Performance of Patch-Clamp Experiments

When a giga-seal had been established, the patch was regularly scanned for channel activity in the cell-attached configuration. If possible, patch-clamp experiments in the outside-out configuration were performed subsequently. The combination of cell-attached and outside-out recordings turned out to be a useful strategy to study ion channels in protoplasts of *V. utricularis.* The reason for this was that direct information about the selectivity and the voltage dependence of ion channels could only be obtained from excised patches, whereas measurements in the cell-attached configuration reflected more closely the transport processes in the intact cell. Moreover, a comparison of channel activity in cell-attached and outside-out patches led under some circumstances to conclusions about the resting membrane potential of the protoplasts and the ionic relations in the cytosol as well (*see below*).[2]

Results

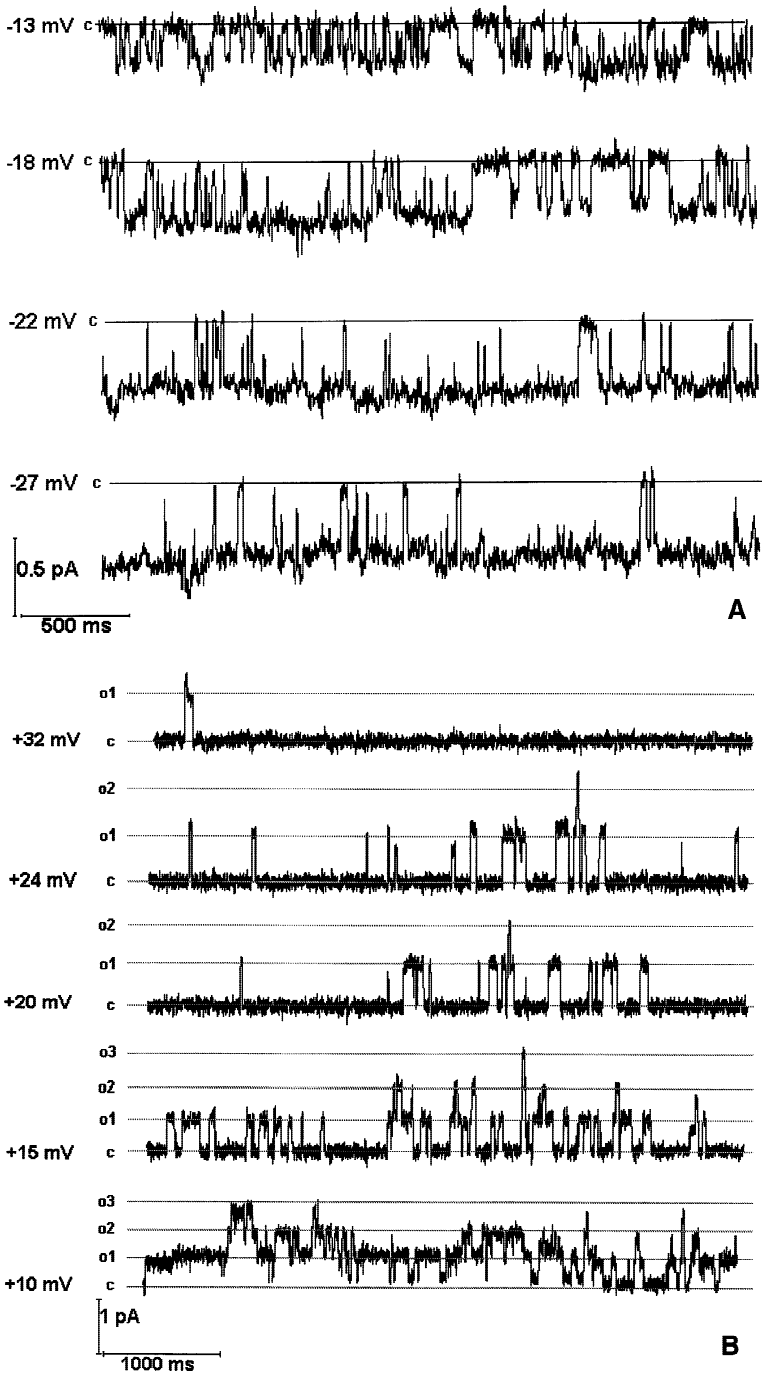
The Voltage-dependent Anion Channel VAC1

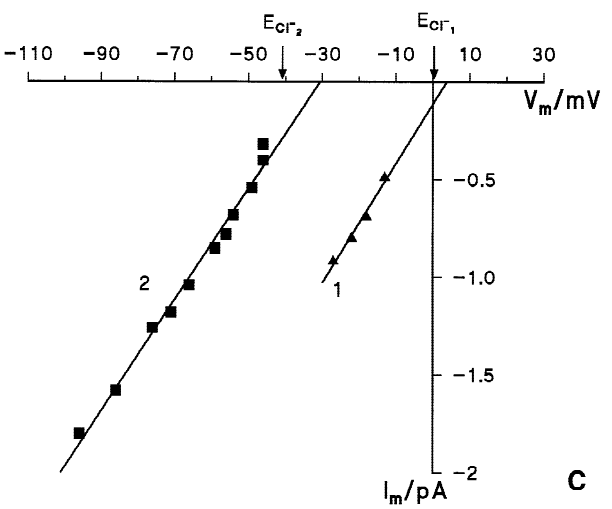
Typical current traces recorded over a range of clamped voltages between −13 and −27 mV by using the outside-out configuration are shown in Fig. 1*A.* Recordings were made at symmetrical 150 mM Cl^-, but under asymmetrical Na^+/K^+-conditions (B1/P1 media). The current fluctuations indicated gating of a single ion channel. It is obvious that the channel activity depended strongly on the transmembrane voltage, i.e., that the channel activity increased towards lower (negative) membrane potentials. Figure 1*C* (curve 1) displays the corresponding single channel current-voltage relationship. Extrapolation shows that the current-voltage curve apparently reverses near zero (0 ± 4 mV, $n = 4$), i.e., at the Nernst potential for Cl^-. Corresponding cell-attached experiments are shown in Fig. 1*B.* The recordings were carried out under the same symmetrical bath and pipette media as above. It is clear that under these conditions at least three channels were activated (*see below*). Outward currents are only shown because it was not possible to resolve single channel inward currents, probably due to the high activity of the channels in the cell-attached patches (for exception *see* Fig. 2, curve in the absence of DIDS). In contrast, positive openings above the reversal potential were seldom in outside-out patches due to the general low activity of the channel in this configuration. From the corresponding current-voltage curve (Fig. 1*D*, curve 1) similar values for the reversal potential of the channels (in average +9 ± 7 mV, $n = 10$) were extrapolated. These findings are expected for a Cl^--selective conductance and thus justified to denote this channel as VAC1 (*V*alonia *A*nion *C*hannel *1*).

The single channel conductance calculated from current-voltage curves performed in the outside-out and

[1] The Nernst potentials were always calculated under the assumption that the composition of the cytosolic medium was identical to the bath and that the membrane potential of the protoplast was approximately zero. For justification of this assumption, *see below.*

[2] Note that the signs of the pipette currents, I_p, and the pipette command potentials, V_p, have been reversed where necessary. Thus the usual convention applies to all measurements independent of the patch configuration used (Terry, Tyerman & Findlay, 1991); potentials are from the point of view of the cytoplasmic side of the membrane, and outward current is taken as positive charge moving out or negative charge moving into the cytoplasm.





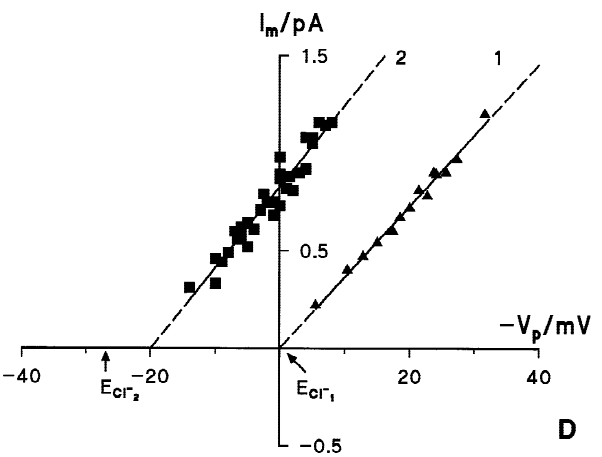


Fig. 1. Single channel recordings of VAC1 in the outside-out (*A*) and in the cell-attached configuration (*B*) by using 150 mM symmetrical Cl^- media (B1/P1). Current-voltage relations derived from these recordings are shown in (*C*) and (*D*), respectively, (curves 1), together with current-voltage relations of VAC1 in the presence of a Cl^- gradient (curves 2) with 150:30 mM Cl^- media (B3/P3) in (*C*) and 50:150 mM Cl^- media (B2/P2) in (*D*). In (*A*), the closed (*c*) state of the channel is marked for clarity by a continuous line. Only one channel was active in this patch. In (*B*), the closed (*c*) and 3 open (*o*) states are marked with dotted lines. $E_{Cl^{-1}}$ and $E_{Cl^{-2}}$ are the Nernst potentials for symmetrical and asymmetrical Cl^-, respectively; V_p is the pipette potential; its reverse value is equal to the membrane potential V_m (*see* footnote 2).

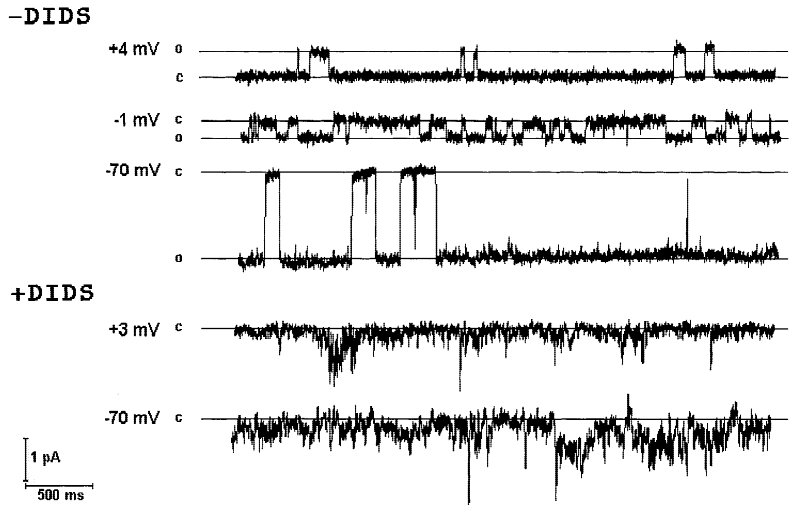


Fig. 2. Typical single channel recordings of VAC1 in protoplasts of *V. utricularis* at different clamped membrane potentials in the absence (upper traces) and in the presence (lower traces) of the inhibitor DIDS (100 μM). Measurements were performed in symmetrical 150 mM Cl^- media (B1/P1) by using the cell-attached configuration (same patch). Closed (*c*) and open (*o*) states of VAC1 are indicated by continuous lines.

cell-attached configuration (*see* Fig. 1*C* and *D*, curves 1) agreed also very well (36 ± 7 pS, $n = 4$ and 33 ± 5 pS, $n = 10$, respectively).

In the giant "mother cells" large Cl^- gradients exist between the cytoplasm and the external sea water (Gutknecht, 1966; Wang et al., 1991). Thus, in order to relate the activity of the VAC1 to the electrophysiology of the "mother cells" it was of interest to study the function of VAC1 in the presence of a Cl^- gradient. However, patch-clamp experiments in Artificial Sea Water failed because (i) giga-seals were not obtained in sea water despite much effort and (ii) replacement of the (osmotically adapted) B1 medium by sea water always led to a breakdown of the giga-seal.

Therefore, protoplasts were produced and subjected to cell-attached patch-clamp measurements in asymmetrical 50:150 mM Cl^- media (B2/P2). As shown in Fig. 1*D* (curve 2), the current-voltage relationship of VAC1 shifted towards more negative values when a Cl^- gradient was established across the membrane. The current-voltage curve reverses at −20 ± 7 mV ($n = 4$), i.e., at slightly less negative values than expected from the Nernst potential (−28 mV). In contrast to the cell-attached configuration, outside-out experiments in asymmetrical 150:30 mM Cl^- media (B3/P3) were very difficult to perform in the presence of a Cl^- gradient. The reason for this is unknown. Despite these difficulties we obtained one outside-out experiment from which the current-voltage curve could be extracted (Fig. 1*C*, curve 2). In this particular case, the reversal potential was −31 mV, i.e., more positive than the Nernst potential of Cl^- (−41 mV). This finding might be taken as evidence that other cations or anions contributed partly to the electrical conductance of VAC1 in the presence of a Cl^- gradient.

However, the response of VAC1 to the anion channel inhibitor DIDS gave additional support for the assumption that this channel contributed predominantly to the Cl^- conductance of the plasmalemma of *V. utricularis.* Data obtained from a cell-attached patch (one of the very few ones with low channel activity, *see below*) are shown in Fig. 2. When 100 μM DIDS was added to the bath, channel activity declined rapidly (within a few seconds). In the presence of the inhibitor, the traces became "noisy" and, in turn, opening and closing events could not be resolved any more. Similar observations were made with two other patches.

In order to elucidate in more detail whether VAC1 was activated in the range of the resting membrane potential of the "mother cell" (*see below*) we determined the channel activity as a function of the clamped voltage both in the outside-out and the cell-attached configuration. The channel activity is defined by n^*p_o, where n is the number of channels in a patch (Fig. 1*A* and *B*) exhibiting an opening probability p_o. The channel activity, n^*p_o, is given by (Almers, 1978):

$$n^*p_o = \frac{n}{1 + \exp\left(-\frac{z \cdot F}{RT}(V - V_{1/2})\right)} \quad (1)$$

with V = the clamped membrane potential, $V_{1/2}$ = the voltage at which the activity was half-maximum (i.e., the midpoint potential), z = the apparent minimum gating charge, F = Faraday constant, R = gas constant and T = temperature in absolute values.

Figure 3 shows the channel activity as a function of the clamped voltage for four independent outside-out (*A*) and for two independent cell-attached measurements (*B*). Each data point in the figure is based on recordings of at least 30 sec duration in the steady state. The values were obtained by random variation of the clamped membrane voltage to exclude possible rundown effects on channel activity.

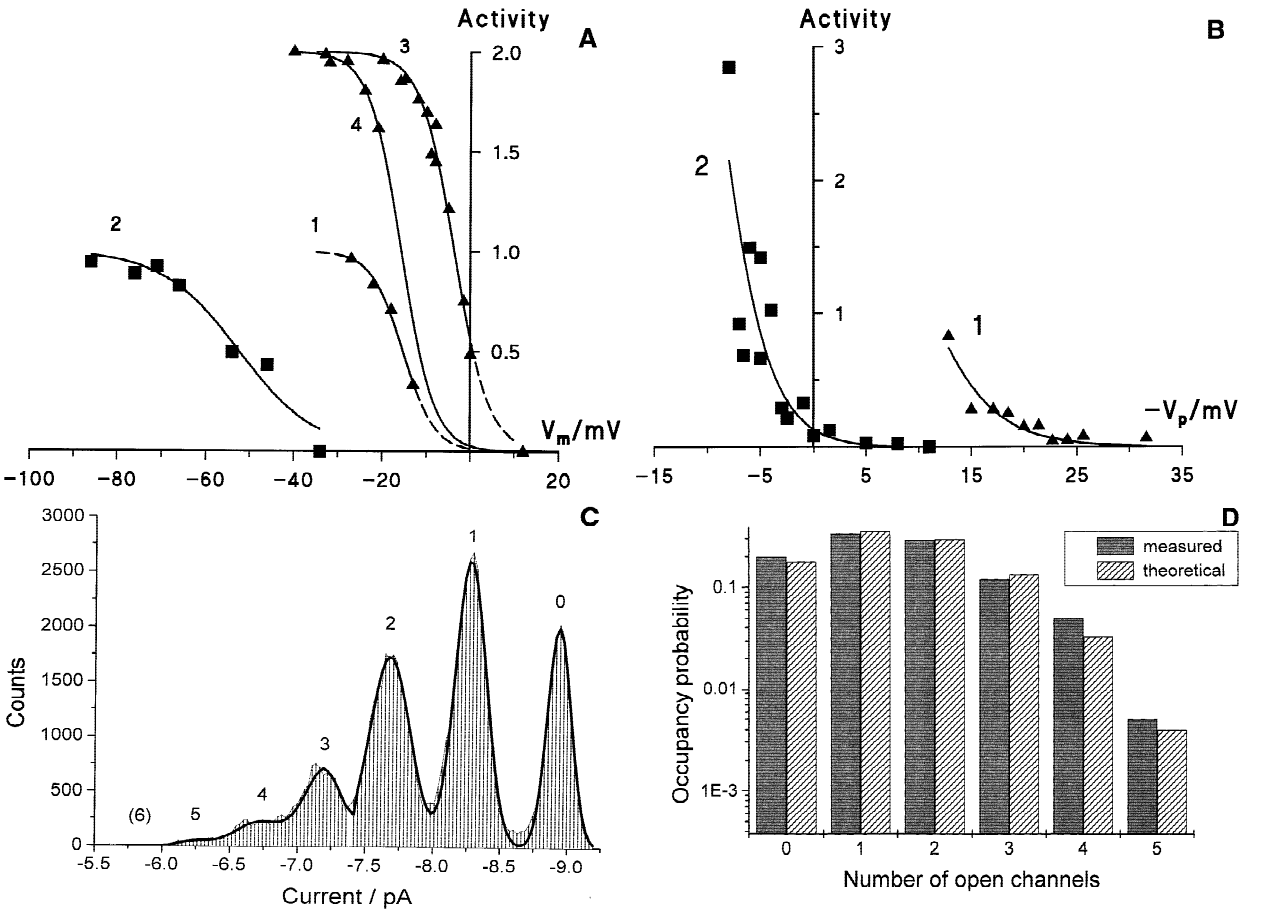


Fig. 3. Analysis of channel activity ($n^* p_o$) of VAC1 in the outside-out (*A*) and in the cell-attached configuration (*B–D*). In (*A*) and (*B*), channel activity is plotted as a function of the clamped membrane potential. The data points of curves 1, 3 and 4 in (*A*) and of curve 1 in (*B*) represent measurements in symmetrical 150 mM Cl^- media (B1/P1), whereas the data points of curves 2 in (*A*) and (*B*) were recorded in asymmetrical 150:30 mM Cl^- media (B3/P3 media) and 50:150 mM Cl^- media (B2/P2), respectively. Data were fitted with Eq. 1 (continuous lines). Midpoint potentials ($V_{1/2}$) obtained from the best Boltzmann fits were (±95% confidential range): *A*: −15 ± 8 mV (curve 1); −52 ± 5 mV (curve 2); −4 ± 1 mV (curve 3) and −16 ± 2 mV (curve 4). *B*: +9 ± 2 mV (curve 1) and −9 ± 2 mV (curve 2). Note that in (*A*) only one channel was active in curve 1 and 2, whereas two channels were observed in curve 3 and 4. Channel activity in *A* and *B* was calculated from all-point histograms. An example is given in *C*. The data points were extracted from a cell-attached experiment performed at $-V_p = -6$ mV. The peak numbers (0–6) indicate the number of channels that were simultaneously active at a given current level. Number "6" is given in brackets because the peak was too small to be fitted properly. According to Jackson (1985) and Draber et al. (1993), data were fitted by superposition of Gaussian distributions (solid line). The relative area below each peak is a measure of the probability of the number of channels which were simultaneously in the open state. The calculations of the probability of the number of simultaneously open channels from the Gaussian distributions is given in (*D*) together with the theoretical values by assuming a binominal distribution for independent voltage gating of the channels. It is evident that the probability values determined experimentally agree well with the values calculated theoretically.

According to Fig. 3*A*, an activity of only one or two Cl^- channels could be recorded in the outside-out configuration. When the experiments were performed in symmetrical 150 mM Cl^- (curves 1, 3 and 4), a hyperpolarization of the patch by only 20 mV was needed to increase the channel activity from zero to its maximum value. Fitting the Boltzmann equation to these data yielded values for the apparent minimum gating charge of the channel of 15 ± 3 ($n = 4$) and an estimate of −12 ± 5 mV for the midpoint voltage, $V_{1/2}$, at which p_o was half-maximal. Corresponding calculations and fitting of the channel activity for the outside-out patch performed in the presence of a Cl^- gradient (Fig. 1*C*, curve 2), yielded a gating charge of 7 and a midpoint voltage of −52 mV (curve 2 in Fig. 3*A*). This means that the threshold voltage for channel activation obviously shifted together with the Nernst potential of Cl^- towards more negative values. Simultaneously, the voltage dependence of the channel activity was apparently slightly less compared to symmetric Cl^- conditions.

In cell-attached patches, analysis of the channel activity was limited to a narrow voltage range (Fig. 3*B*), i.e., slightly negative in relation to the threshold potential for channel activation. A total number of 5 or 6 VAC1

channels were estimated to be activated by a hyperpolarization of the membrane[3]. Single channel activity could not be resolved properly in most patches when more than 3 channels were permanently active. The total number of voltage-activated channels, *n*, in a patch could be deduced from the number of peaks in all-point histograms (Fig. 3*C*). The validity of this approach could be tested by fitting Gaussian curves to the peaks and by calculating the relative area below each peak. This parameter is a measure of the probability of the number of channels which were simultaneously in the open state. Calculations show (Fig. 3*D*) that the probability of simultaneously open channels determined experimentally agreed well with the probability calculated theoretically by assuming a binominal distribution (for the mathematical treatment, *see* Jackson, 1985; Draber, Schultze & Hansen, 1993).

Fitting of the voltage dependence of channel activity (Fig. 3*B*) by using Eq. 1 yielded for symmetrical 150 mM Cl^- a midpoint potential, $V_{1/2}$, of -3 ± 6 mV ($n = 9$) and a gating charge of 18 ± 5; for asymmetrical 50:150 mM Cl^- media (B2/P2) the corresponding values for the midpoint voltage and gating charge were -15 ± 7 mV ($n = 3$) and 17 ± 4, respectively.

As expected for a Cl^--selective channel, a good agreement was found for the reversal potentials of VAC1 in the cell-attached configuration and the Nernst potentials of Cl^-, calculated under the assumptions that the resting potential of the protoplast was zero and that there was no Cl^- gradient across the membrane. These assumptions were supported by measurements of the resting potential in the whole-cell configuration, which preceded the formation of the cell-attached configuration. The resting potentials were determined directly after the whole-cell configuration had been established. They were $+1 \pm 5$ mV ($n = 33$) in symmetrical 150 mM Cl^- (B1/P1) and -1 ± 5 mV ($n = 29$) in asymmetrical 30:150 mM Cl^- (B3/P3) conditions. Generally, VAC1 displayed also similar voltage dependence in cell-attached and outside-out experiments. The ionic composition of the cytosol was apparently more or less equal to that of the bath solution, at least with respect to Cl^- (even though the measurements were performed about 2–4 hr after preparation of the protoplasts in the respective patch-clamp media).

Stretch-Activated Channel(s)

When a gentle suction was applied to a patch in the cell-attached configuration, additional channel activity was elicited in about 10% of the patches. Underpressure was varied between 60 and 220 mM H_2O. In Fig. 4*A* an experiment performed in asymmetrical 150:30 mM Cl^- media (B3/P3) was selected which did not show any background channel activity. This facilitated the analysis of the stretch-activated current (in contrast to traces where VAC1 activity was present before underpressure application).

Upon suction, current fluctuations originating from channel activity started within 100 msec. Opening and closing events could well be identified for this patch as indicated for the +96 mV-trace in Fig. 4*A*. When suction was released, channel activity ceased immediately. The corresponding current-voltage relationship is shown in Fig. 4*B* (curve 1). The reversal potential was intrapolated to be +46 mV and was thus close to the Nernst potential for Cl^- (+41 mV). This finding indicated that the channel was Cl^- selective. The single channel conductance for this experiment was determined to be 22 pS.

However, it has to be noted that there were other experiments which showed less agreement between the reversal potential and the Nernst potential for Cl^-. Moreover, single channels could often not be resolved even in the absence of background activity. Similar observations were made for symmetrical 150 mM Cl^-. The current-voltage relationship of such an experiment is given in Fig. 4*B* (curve 2). Inspection of the curve shows that the reversal potential was with +20 mV less positive than under asymmetrical Cl^- conditions, but more positive than the Nernst potential (0 mV). The single channel conductance was determined to be 16 pS for this experiment.

The above data apparently indicate that the stretch-activated channel was either not exclusively selective for Cl^- or that the stretch-activated channels did not show uniform properties.

Other Channels in the Plasma Membrane

Besides VAC1 and the stretch-activated channel(s), three other channel types could be identified that only occurred in cell-attached patches. One of these channels was always seen together with VAC1, but could clearly be distinguished from this channel because of its large conductance and "flickering" mode of gating. An experiment performed in asymmetrical 150:30 mM Cl^- media (B3/P3) is given in Fig. 5*A*. The small current steps (marked by the dotted lines) clearly resulted from multiple VAC1 activity. Consistent with this, the corresponding current-voltage relationship (Fig. 5*B*, curve 1) intersected the voltage axis at +40 mV, i.e., close to the Nernst potential of Cl^- (+41 mV). In contrast, the large openings, interrupted by brief closures (*see* Fig. 5*A*, arrows) were apparently associated with a different type of channel. The amplitude of these "large" single channel

[3] The occurrence of a larger number of VAC1 channels in the cell-attached configuration (compared to the outside-out configuration) indicated that the channel activity in the intact protoplast was apparently enhanced by an internal factor that was lost by patch excision.

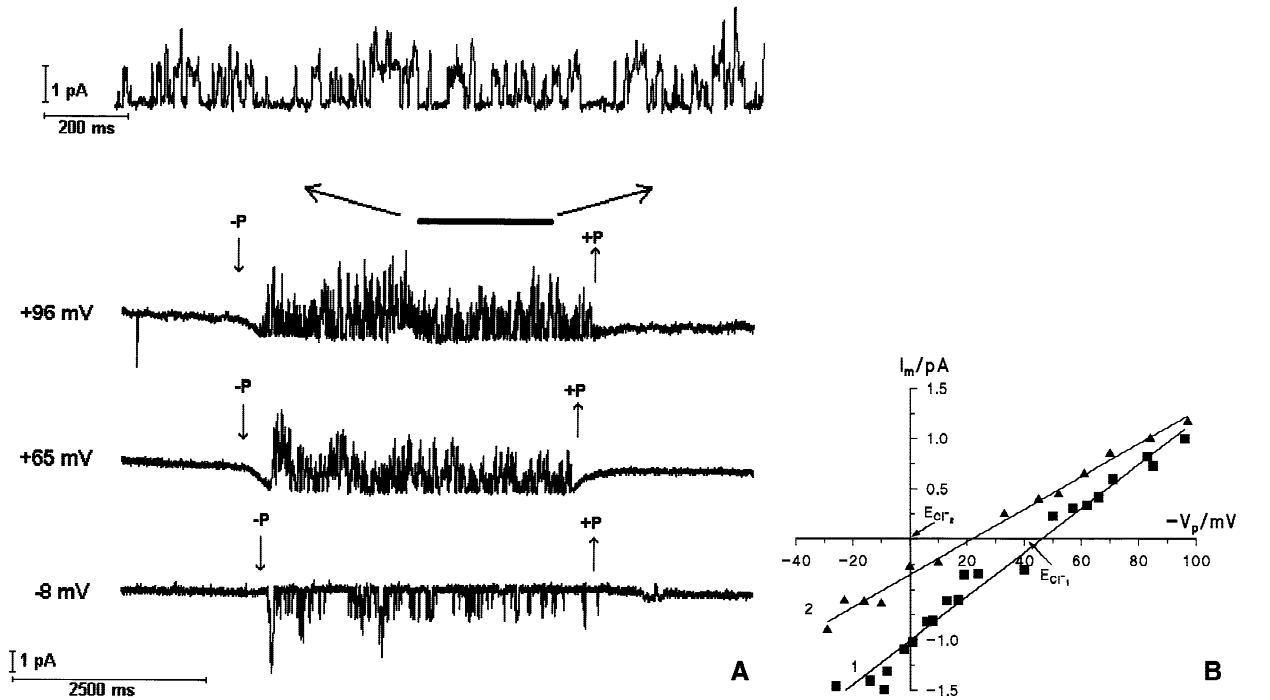


Fig. 4. Current traces of stretch-activated channels in the membrane of protoplasts of *V. utricularis* (*A*) and current-voltage curves (*B*) recorded in the cell-attached configuration. The data in (*A*) and the corresponding calculated current-voltage curve 1 in (*B*) were recorded in asymmetrical 150:30 mM Cl^- media (B3/P3). The curve 2 in (*B*) was performed in symmetrical 150 mM Cl^- media (B1/P1). Stretch-activated channels were induced by temporary application of suction to the patch (about 160 mm H_2O); the presence of suction is denoted by upwardly directed and the absence by downwardly directed arrows. $E_{Cl^-}1$, $E_{Cl^-}2$ and $-V_p$ have the same meaning as explained in the legend to Fig. 1. Note that a part of the upper trace in (*A*) is given at an enlarged scale at the top of the figure (indicated by the horizontal black bar) in order to demonstrate that single channel currents could clearly be resolved.

currents was somewhat variable. From the corresponding current-voltage relationship (Fig. 5*B*, curve 2), a single channel conductance of 85 pS was calculated. The reversal potential (+59 mV) differed from that of VAC1, but was close to the Nernst potential of Cl^- (+41 mV). Thus, in the light of this experiment, it seemed that the current through this channel was mainly mediated by Cl^-.

However, in 6 other experiments performed in symmetrical 150 mM Cl^- media, the reversal potential shifted to +12 ± 11 mV (*data not shown*); the single channel conductance was 99 ± 10 pS. The difference in the values of the reversal and the Nernst potential indicated that the conductance of the channels was determined by Cl^-, but not exclusively. Despite this, the channel was termed VAC2.

While VAC1 and VAC2 were activated by membrane potentials that were more negative than the Nernst potential for chloride, a further voltage-dependent channel was found that required positive voltages for activation. An example recorded at symmetrical 150 mM Cl^- media (B1/P1) is shown in Fig. 6. It is evident that in this case the channel activity was increased by a depolarization of the patch (Fig. 6*A*). Moreover, this channel was characterized by the regular appearance of a subconductance state (*see* arrows) that was not found with VAC1 and VAC2. From the corresponding current-voltage relationship of the main conductance state of this channel (Fig. 6*B*, curve 1) a reversal potential of −4 mV and a conductance of 34 pS were extracted (on average the conductance was 28 ± 8 pS and the reversal potential +1 ± 12 mV, $n = 4$). For asymmetrical 50:150 mM Cl^- media (B2/P2), the current-voltage relationship shifted to more negative values as demonstrated in Fig. 6*B* (curve 2). The reversal potential was −25 ± 5 mV ($n = 5$) and thus close to the Nernst potential of Cl^- (−28 mV). This channel was named VAC3. The main open state of VAC3 at 50:150 mM Cl^- (B2/P2) had a conductance of 36 ± 5 pS ($n = 5$).

The only channel that was apparently permeable for K^+ was also triggered by positive voltages (Fig. 7). For the experiment shown in Fig. 7*A*, three different conductance states could be identified. The current-voltage relationships calculated for these states are given in Fig. 7*B*. It is evident that the currents reverse close to the Nernst potential of K^+ (+61 mV). The conductances of the three states were calculated to be 58, 159 and 290 pS, respectively. The analysis of 6 other patches demonstrated that the state with a reversal potential of +56 ± 13 mV and a conductance of 124 ± 27 pS was dominant.

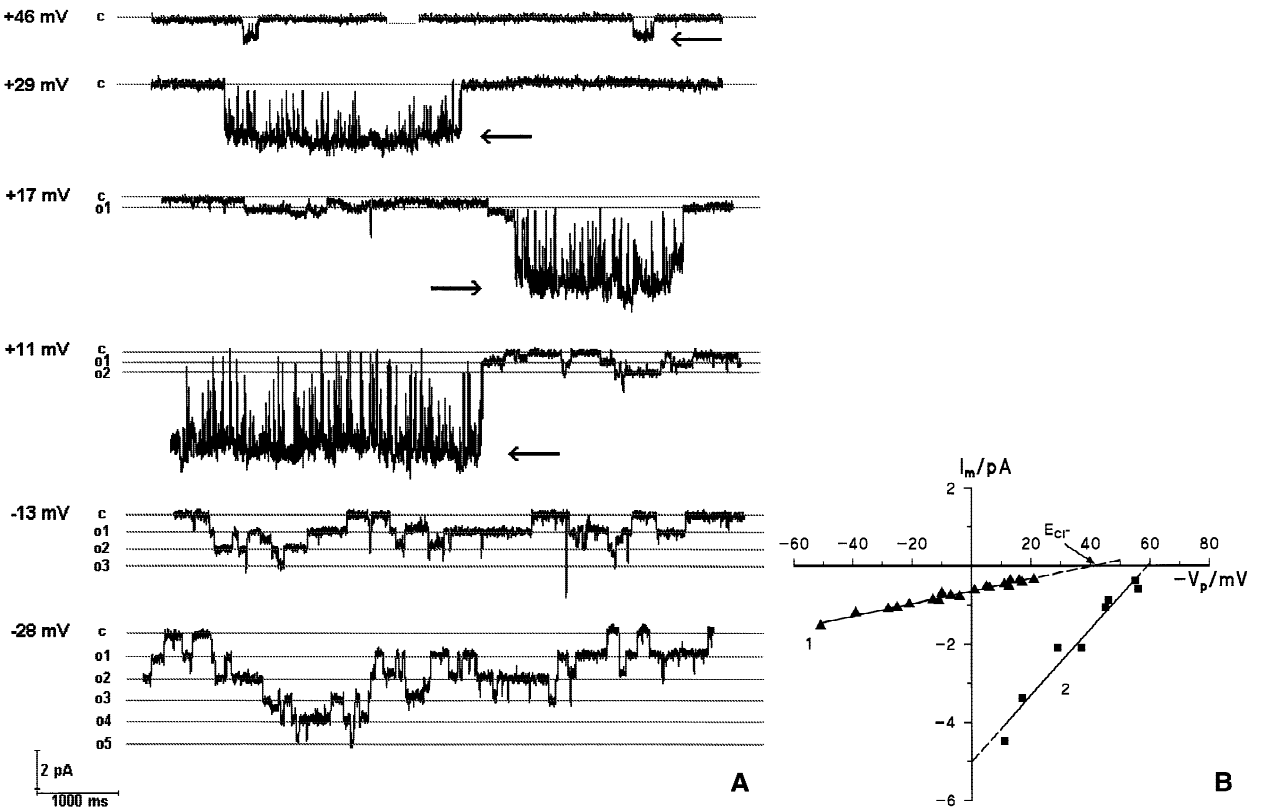


Fig. 5. Current traces (*A*) and the corresponding current-voltage curves (*B*) measured on protoplasts of *V. utricularis* at different clamped membrane potentials which show simultaneous activity of two Cl^--sensitive channels (VAC1 and VAC2) in the same patch. Measurements were performed in asymmetrical 150:30 mM Cl^- media (B3/P3) using the cell-attached configuration. Dotted lines indicate VAC1 activity (including a maximum of five simultaneously active channels). Arrows denote openings of the separate channel VAC2. Curve 1 in (*B*) represents the current-voltage relations of VAC1 and curve 2 that of VAC2. E_{Cl^-} and $-V_p$ have the same meanings as in Fig. 1. Note that a VAC2 activity was not recorded at negative voltages. For further explanation, *see* text.

In these patches, the multistate channel fluctuations were also always observed. The conductances of these states were about 15 pS or a multiple of it. Reversal potentials close to the Nernst potential of K^+ indicated a high selectivity for K^+ compared to Cl^- and Na^+. Therefore, the channel was termed VKC1 (*V*alonia *K*$^+$ *C*hannel *1*).

Plotting of the channel activity as a function of the clamped voltage showed (inset in Fig. 7*B*) that the channel activity increased with increasing positive membrane potentials. Fitting of the data by using Eq. 1 yielded a midpoint voltage of +185 mV and a gating charge of 3.

It is interesting to note that VKC1 was only observed in cell-attached patches when symmetrical 150 mM Cl^- media (B1/P1) were used. Under these conditions, the K^+ gradient across the membrane was opposite to that observed in the giant "mother cells" and this may be related with the finding that the channel was activated outside of the resting membrane potential range of the "mother cells" (*see below*).

Discussion

There are only a few reports of patch-clamp studies on ion channels of the plasmalemma of marine algae in the literature. Bertl & Gradmann (1987) and Bertl, Klieber & Gradmann (1988) prepared protoplasts from *Acetabularia mediterranea* and demonstrated a voltage-gated K^+ channel with slow kinetics in these protoplasts. White, Smahel & Thiel (1993) incorporated plasma membrane isolated from this alga into planar lipid bilayers and found both voltage-dependent K^+ and Cl^- channels with different single channel conductances. Cl^- channels and a putative K^+ channel have also been found in protoplasts of *Chaetomorpha coliformis* (Findlay & Findlay, 1995). Nonselective stretch-activated channels apparently exist in *Fucus* eggs as demonstrated by cell-attached patch-clamp experiments after removal of cell wall with laser-microsurgery (Taylor et al., 1996).

The plasma membrane of protoplasts of *V. utricularis* apparently also contained three Cl^--specific channels and at least one specific K^+ channel as well as stretch-activated channels. A summary of the channels described in this study is given in Table 2. Particularly the finding of a strongly voltage-gated Cl^- channel (VAC1) is consistent with results obtained from charge pulse experiments on the turgescent giant "mother cells" with impaled microelectrodes (Wang et al., 1991). The

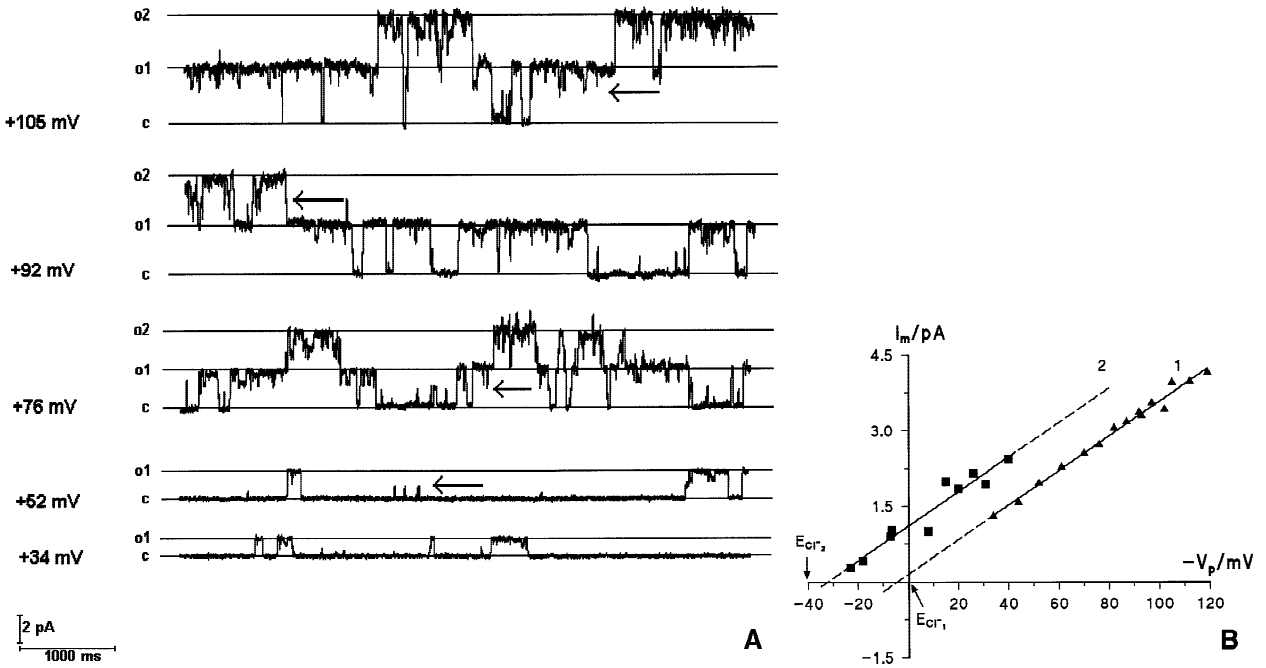


Fig. 6. Current recordings (*A*) and current-voltage curves (*B*) of a depolarization-activated anion channel (VAC3) recorded in the cell-attached configuration. Measurements were carried out in symmetrical 150 mM Cl^- media (B1/P1; *A* and the corresponding curve 1 in *B*) and in asymmetrical 50:150 mM Cl^- media (B2/P2; curve 2 in *B*). The continuous lines in (*A*) indicate the closed (*c*) and two main open (o1 and o2) states. E_{Cl^-1}, E_{Cl^-2} and $-V_p$ have the same meanings as in Fig. 1. Note the occurence of subconductance levels (arrows). Note further that in (*B*) only current amplitudes of the main open state were plotted. For further details, *see* text.

microelectrode measurements have clearly shown[4] that the conductance of the plasmalemma was predominantly determined by Cl^- and to a smaller extent by K^+.

However, the question is whether VAC1 and the other channels found here have physiological significance. For this, it is important to demonstrate that the channels could operate in the membrane potential range measured in the turgescent "mother cells." The vacuolar membrane potential of *V. utricularis* (i.e., the sum of the potentials dropping across the tonoplast and the plasmalemma) was in average with about +5 mV slightly positive (Steudle & Zimmermann, 1974; Wang et al., 1991) and depended on turgor pressure and external Cl^-. At a constant turgor pressure of about 0.3 MPa the vacuolar membrane potential depended strongly on the external Cl^- concentration. It became more and more positive when Cl^- was replaced successively by MES^- (Wang et al., 1991) and it decreased with increasing turgor pressure (up to 0.4 MPa) to more negative values. Usually a large variability of the vacuolar membrane potential at higher turgor pressure was observed (between −5 and −40 mV; Steudle & Zimmermann, 1974).

Separation of the electrical properties of the individual membranes in perfused cells by addition of nystatin to the bath or to the perfusion medium (Wang et al., 1997*a*) showed (C. Ryser and U. Zimmermann, *unpublished data*) that the potential of the plasmalemma of *V. utricularis* was in the order of about −25 to −30 mV at a turgor pressure of about 0.1 MPa. Similar values for the plasmalemma potential were found for *Ventricaria ventricosa* (Ryser et al., 1999). More negative values (about −70 mV) were reported by Gutknecht (1966) and Davis (1981). Gutknecht (1966) measured the membrane potential of aplanospores of *V. ventricosa*, whereas Davis (1981) placed a microelectrode into the thin cytoplasmic layer of giant "mother cells" of this species. The agreement of the data of Davis and Gutknecht for *V. ventricosa* appears to be rather compelling, but aplanospores may not necessarily reflect the membrane properties of the giant walled "mother cells" and cytoplasmic microelectrode recordings may be subject to misinterpretations (*see above*). Thus, it seems at the present stage of information that the plasmalemma potential recorded in cells with nystatin-permeabilized tonoplast reflects most likely the correct value of this electrical membrane parameter.

[4] However, it should be noted that the contribution of Cl^- and K^+ to the total conductance of the membrane may be subjected to some variations due to seasonal and culture conditions. This can be seen both by charge pulse experiments as well as by patch-clamp studies.

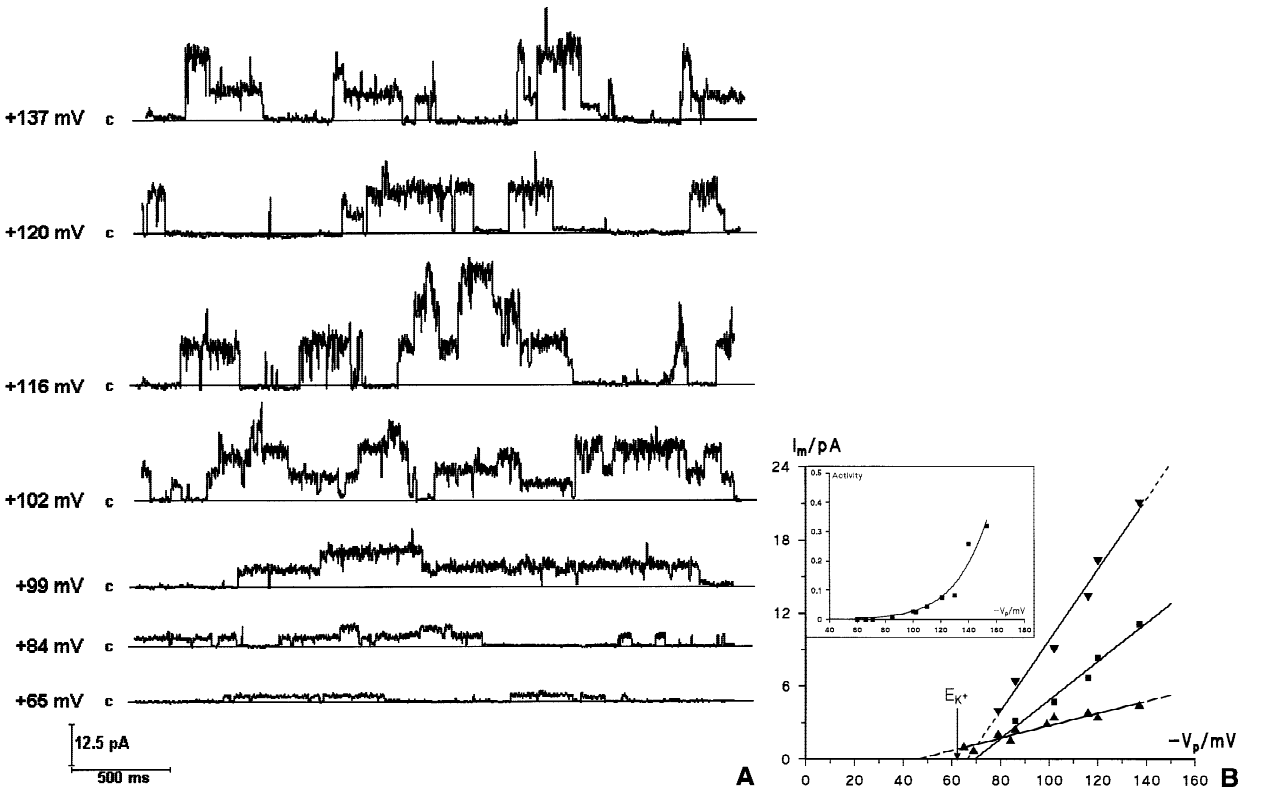


Fig. 7. Current recordings (*A*) of a multistate K^+-selective channel (VKC1) measured in symmetrical 150 mM Cl^- media (B1/P1) using the cell-attached configuration. The current-voltage curves in (*B*) were calculated for three distinct levels seen in (*A*). Inset: The channel activity is plotted as a function of the clamped voltage by taking the data from (*A*). Data points were fitted by using Eq. 1 (continuous line). Note that the closed state (*c*) in (*A*) is indicated by a continuous line and that in (*B*) E_{K^+} is the Nernst potential of K^+.

Table 2. Summary of the channels observed in protoplasts of *V. utricularis*

		Channel type				
		VAC1	VAC2	VAC3	VKC1	stretch
Cell-attached	Conductance [pS]	33 ± 5 (n = 10)	99 ± 10 (n = 6)	28 ± 8 (n = 4)	124 ± 27 (n = 6)	19
	Voltage-dependence	Activated by hyperpolarization	Activated by hyperpolarization	Activated by depolarization	Activated by depolarization	None
Outside-out	Conductance [pS]	36 ± 7 (n = 4)	—	—	—	—
	Voltage-dependence	Activated by hyperpolarization	Activated by hyperpolarization	Activated by depolarization	Activated by depolarization	None

A potential of −25 to −30 mV would be close to the Nernst potential of Cl^-. This is calculated to be about −37 mV if we assume that the cytosolic Cl^- concentration is similar to that of *V. ventricosa* (138 mM; Gutknecht, 1966) and that the corresponding value of the artificial sea water is 596 mM.

The agreement of the experimental findings with the Nernst potential of Cl^- suggests that VAC1 and also VAC2 (which was less frequently observed than VAC1, but also voltage-dependent) were active in the range of the physiological plasmalemma potentials. Thus, these two channels could provide well for the Cl^- conductance of the plasmalemma of the giant "mother cells," even at different Cl^- concentrations (*see* Wang et al., 1991 and above). Because of its strong voltage-dependence (Fig. 3), VAC1 seems particularly to be a very good candidate to keep the membrane potential in a narrow voltage range. At a membrane potential of −30 mV, about 50%

of the VAC1 channels will be in the open state. This value can be estimated from the Boltzmann curve in Fig. 3*B* which was fitted to the data measured in asymmetric 50:150 mM Cl^- media (B2/P2) by using the cell-attached configuration (but note that the Cl^- gradient under natural conditions may be somewhat steeper). As mentioned above, the Nernst potential of this solution for Cl^- is −28 mV and thus close to that of the "mother cells" bathed in sea water. Note that the dominance of a Cl^- conductance as in *V. utricularis* is rather unusual for plant cells (Hope & Walker, 1975).

Additional support for the assumption that VAC1 contributes mainly to the Cl^- conductance of the plasmalemma of the turgescent "mother cells" of *V. utricularis* came from the observation that both VAC1 and the plasmalemma conductance of the "mother cells" could be inhibited by 100 μM DIDS (Fig. 2 and Spieß et al., 1993).

In contrast to the apparent involvement of VAC1 (and possibly of VAC2) in the plasmalemma conductance of the "mother cells," it is difficult to answer the question about the physiological role of the Cl^--selective channel VAC3 and of the K^+-selective channel VKC1. VAC3 was activated by membrane depolarization, i.e., the activity increased in response to positive (nonphysiological) membrane voltages. VKC1 was operating at K^+ gradients which were opposite to those observed in the "mother cells." It is conceivable that VAC3 could serve as an additional "security valve" to prevent pronounced excursions of the membrane potential into the positive voltage range once a threshold value is exceeded (*see also* Terry et al., 1991; Tyerman, 1992). Such situations may occur under natural conditions when the "mother cells" (which exhibit a pronounced turgor pressure regulation; Zimmermann, 1978) are exposed to strongly diluted sea water for a short time. Similarly, VKC1 may also be activated during (extreme) osmoregulation and turgor pressure regulation processes if we assume that turgor pressure and turgor pressure gradients modified the potential-dependence of this K^+-selective channel. Apart from the pressure-dependence of the potential and resistance of the total membrane barrier in the "mother cells" (Steudle & Zimmermann, 1974; Wang et al., 1997*a*), it is also well-known (Zimmermann & Steudle, 1974; Zimmermann, 1978) that the K^+ influx decreased dramatically within increasing turgor pressure, whereas the K^+ efflux increased correspondingly.

The occurrence of the stretch-activated channel (which was at least partly selective for Cl^- and voltage-independent) can apparently be taken as a hint that turgor-dependent transport elements contribute to the membrane conductance of turgescent cells both under steady-state conditions and during turgor pressure regulation (Cosgrove & Hedrich, 1991; Garill, Tyerman & Findlay, 1994; Shepherd, Beilby & Heslop, 1999).

Obviously, the experiments reported here share the general problem of patch-clamp studies on plant protoplasts, i.e., that the interpretation of the data is not completely straightforward because the transport properties of the membranes are investigated in the turgorless state (McCulloch & Beilby, 1997). There are a few experiments in which good agreement has been found between the electrical membrane properties of walled cells and protoplasts (Blatt, 1991; Grabov & Blatt, 1997). Despite this, ion transport may be affected by protoplast formation. The limitations of patch-clamp studies are also apparent by the finding that most of the channel activity was lost after patch excision. This may be due to a cytosolic factor that activates the channels and is lost when cell-free patches are formed. Similar effects can be envisaged when the cells are transferred into the turgorless state and/or when the outer surface charge of the plasmalemma is changed upon removal of the cell wall. An additional (general) problem arises in the case of the formation of protoplasts from giant algal cells (Menzel, 1988; Nawata, Kikuyama & Shihira-Ishikawa, 1993), i.e., that some inclusion of tonoplast material might occur during the spontaneous formation process and may be present for some time in the newly formed cells. Even though there was no evidence for incorporation of tonoplast material into the membrane surrounding the protoplasts (Wang et al., 1997*b*) we cannot exclude definitely such effects, and thus the possibility that the Cl^- channels, which occurred at less frequency than VAC1, and the K^+ channel must be assigned to the tonoplast, although it would be surprising not to find any K^+ channels in the plasma membrane of a turgor regulating species like *V. utricularis* (Chamberlin & Strange, 1989).

These and the other open questions can be solved if protoplasts can be prepared from regenerated, walled protoplasts because the external membrane of the regenerated cells should be free of tonoplast constituents. Enzymatic and/or mechanical removal of the very tight walls is not possible at present (as is also the case for the "mother cells"). However, it is conceivable that conditions can be found under which the wall of regenerated protoplasts can be degraded. Further progress is also expected if the patch-clamp technique can be applied to immobilized protoplasts in which a turgor pressure can be built up (Scheurich, Schnabl & Zimmermann, 1980; Schnabl, Scheurich & Zimmermann, 1980). Such developments should allow one to arrive at an integrated view of the membrane transport properties derived from patch-clamp studies on protoplasts and electrophysiological studies on walled cells.

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