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Microscopic Elements of Electrical Excitation in *Chara*: Transient Activity of Cl^- Channels in the Plasma Membrane

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Abstract. The plasma membrane of *Chara corallina* was made accessible for patch pipettes by cutting a small window through the cell wall of plasmolyzed internodal cells. With pipettes containing Cl^- as Ca^{2+} or Ba^{2+} (50 or 100 mM), but not as Mg^{2+} or K^+ salt, it was possible to record in the cell-attached mode for long periods with little channel activity, randomly interspersed with intervals of transient activation of two Cl^- channel types (cord conductance at +50 mV: 52 and 16 pS, respectively). During these periods of transient channel activity, variable numbers (up to some 10) of the two Cl^- channel types activated and again inactivated over several 100 msec in a coordinated fashion. Transient Cl^- channel activity was favored by voltages positive of the free running membrane voltage (> −45 mV); but positive voltage alone was neither a sufficient nor a necessary condition for activtion of these channels. Neither type of Cl^- channel was markedly voltage dependent. A third, nonselective 4 pS channel is a candidate for Ca^{2+} translocation. The activity of this channel does not correlate in time with the transient activity of the Cl^- channels. The entire set of results is consistent with the following microscopic mechanism of action potentials in *Chara,* concerning the role of Ca^{2+} and Cl^- for triggering and time course: Ca^{2+} uptake does not activate Cl^- channels directly but first supplies a membrane-associated population of Ca^{2+} storage sites. Depolarization enhances discharge of Ca^{2+} from these elements (none or few under the patch pipette) resulting in a local and transient increase of free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) at the inner side of the membrane before being scavenged by the cytoplasmic Ca^{2+} buffer system. In turn, the transient rise in $[Ca^{2+}]_{cyt}$ causes the transient activity of those Cl^- channels, which are more likely to open at an elevated Ca^{2+} concentration.

Key words: Cell-attached patch-clamp technique—(single) Cl^- channel activity—Voltage dependence—Action potential—*Chara*—Kinetics

Introduction

The *Characean* algae are a classical experimental system used to investigate electrical excitation in plant membranes. In the past, the ionic nature of the action potential has been systematically investigated with conventional voltage-clamp methods (*for review, see* Beilby, 1984*b*). Other than in animal cells, the upstroke of the action potential in plant cells does not appear to be caused by only one type of depolarizing current but by an ensemble of not yet readily separated events (Beilby, 1984b).

From the time dependence of the biphasic depolarization current under voltage-clamp conditions, Beilby and Coster (1979 *a,b*) and Beilby (1984*b*) argued that a voltage-activated inward Cl^- current is the prime mechanism for the depolarization. A second current, probably carried by Ca^{2+}, lags behind the Cl^- current in a voltage-dependent manner. The delay for the onset of the second current shortens with decreasing polarization of the membrane. This current, therefore, would only become active at a potential close to its reversal voltage so that its contribution to the upstroke of the action potential would be negligible (Beilby & Coster, 1979, *a,b*; Beilby, 1984*b*).

In contrast, Lunevsky and co-workers (1983) provided evidence that in other, closely related *Characeae, Nitella and Nitellopsis,* a putative Ca^{2+} current precedes the Cl^- current. They hypothesized that a transmembrane Ca^{2+} current increases $[Ca^{2+}]_{cyt}$, and consequently activates Ca^{2+}-sensitive Cl^- channels. The resulting Cl^- inward current is then believed to amplify the depolarization in the second phase of the action potential upstroke. This hy-

pothesis has been supported by the demonstration of Ca^{2+}-activated Cl^- currents at the plasma membrane of *Nitellopsis* (Shiina & Tazawa, 1988). The *Chara* plasma membrane being excitable only in the presence of external Ca^{2+} ($[Ca^{2+}]_o$) (Findlay & Hope, 1964) and in the absence of Ca^{2+} channel blockers (Beilby, 1984*a*; Tsutsui et al., 1986, 1987 *a,b*; Shiina & Tazawa, 1987) has also emphasized a key role for Ca^{2+} influx in the triggering of electrical membrane excitation in *Characean* algae. Indeed, Ca^{2+} inward current may even be responsible for the entire depolarization during excitation of tonoplast-free cells because in these cells Cl^- efflux remained undetected during action potentials (Kikuyama et al., 1984).

The patch-clamp technique now allows us to investigate the current components as well as the cascade of events during electrical membrane excitation at the control level of single channel activity, an approach already applied in animal cells (Mazzanti & DeFelice, 1987, 1988; Lang & Ritchie, 1990).

Previous patch-clamp investigations of the *Chara* plasma membrane have documented the presence of Cl^- channels: Coleman (1986) and Laver (1991) detected, with patch pipettes attached to the plasma membrane of *Chara* cells, Cl^- channels with different conductance levels. The open probability of one channel increased after clamping the membrane to more negative test voltages (Coleman, 1986). As for the other Cl^- channels, there was no indication from the kinetic properties about a possible role in membrane excitation (Coleman, 1986; Laver, 1991). In a recent study, two Ca^{2+}-sensitive Cl^- channels from excised, inside-out patches of the plasma membrane of *Chara* were investigated (Okihara et al., 1991). One channel revealed similar voltage-dependent activation and complex conductance levels to the one described by Coleman (1986). The second, a 25 pS Cl^- channel (in 100 mM Cl^- (bath): 20 mM Cl^- (pipette)) had a biphasic Ca^{2+}-dependent open probability with a maximum at 1 μM $[Ca^{2+}]_{cyt}$. P_o, furthermore, was a function of the membrane voltage and opened most frequently in a range between -80 and -100 mV. The distinct Ca^{2+} and voltage dependence make this current a likely candidate for the proposed Ca^{2+}-activated Cl^- current in the upstroke of the *Characean* action potential (Okihara et al., 1991).

We aimed in the present patch-clamp study to further investigate the ensemble of currents and the cascade of events which determine the voltage-dependent macroscopic kinetics of a *Chara* action potential on a single channel level. With $CaCl_2$ or $BaCl_2$ in the pipette, we observed in the cell-attached mode, at voltages positive of the free running membrane voltage, a transient activation of some ten Cl^- channels in the plasma membrane. The activation of transient Cl^- currents is discussed with respect to membrane excitation.

Materials and Methods

Plant Material and Experimental Protocol

Chara corallina Klein *ex* Wild was grown in tap water under daylight conditions. Young internodal cells (usually the second youngest cell) were separated from adjacent cells and plasmolyzed in two steps over a period of approximately 1 hr in experimental solution (in mM: 10 KCl, 1 $CaCl_2$, 5 (N-[2-Hydroxyethyl]-piperazine- N′-[2-ethanesulfonic acid]) (HEPES)/2.5 KOH; pH 7.5, 320 sorbitol).

The plasma membrane was made accessible for patch-clamp pipettes by cutting a small window into the cell wall (Laver, 1991). For this operation, a normal patch-clamp pipette was advanced onto the cell wall in an area where the protoplast had withdrawn from the wall. Continuous "rubbing" with the pipette tip along the axis of the internodal cell produced a small cut of several hundred μm. These cuts were large enough to advance a fresh electrode through the wall onto the plasma membrane. Only cells with vigorous cytoplasmic streaming (*see* Results) and without any obvious sign of damage were considered for experiments.

Electrodes were pulled from filamented borosilicate glass (Hilgenberg, FRG) and had usually a resistance of approximately 10 MΩ in symmetrical 100 mM KCl solution. Regardless of the electrode filling, seals of about 2 to 50 GΩ were attained by applying a small negative pressure to the pipette. In the present investigation, electrodes generally contained various, unbuffered Cl^- salt solutions with a pH of about 5.8–6. The relevant liquid junction voltages occurring under these conditions were obtained empirically and the actual membrane voltages corrected appropriately.

The concentration of ions in the *Chara* cytoplasm is reported as (in mM): K^+ 115; Na^+ 3; Mg^{2+} 3; Cl^- 4 to 10; Ca^{2+} 2×10^{-4} (Beilby & Blatt, 1986; *references in* Coleman, 1986; Okihara & Kiyosawa, 1988). For cell-attached recordings with the standard pipette solution (P) of 50 mM $CaCl_2$ the equilibrium voltages were calculated as (in mV): $E_{Ca^{2+}}$, + 160; E_{Cl^-}, range -81 to -59. K^+, Na^+, Mg^{2+} and anions other than Cl^- were not included in the standard pipette solution and the respective equilibrium voltages are therefore plus (cations) or minus infinity (anions).

All chemicals were obtained from Sigma (St. Louis, MO). All solutions, i.e., external bathing medium and pipette solution were filtered (0.2 μm).

Electrical

Ionic current measurements across membrane patches were carried out according to the conventional method described by Hamill et al. (1981) using a commercial patch-clamp amplifier (model E/M-EPC7, List, Darmstadt, FRG). Data were digitized and stored with a digital audio processor and video recorder system (Bertl & Gradmann, 1987).

Data were analyzed using the hardware and software facilities of a commercial computer-based system (pCLAMP, Axon Instruments, Foster City, CA). Current records were replayed from the video system, filtered appropriately (between 100 and

500 Hz corner frequency) through an 8-pole Bessel filter (model LPBF-08D, NPI, Tamm, FRG) and sampled at a rate 5 times the filter corner frequency.

Current frequency distributions were constructed from digitized current records. When the frequency peaks were clearly separated, the open probability of the channels was obtained from the respective peak areas of the open and closed state frequency distributions.

All data on membrane currents are, if not stated otherwise, obtained from experiments in the cell-attached mode. All potentials therefore represent the negative pipette voltage plus the mean estimated free running membrane voltage (V_M) of −45 mV (*see* Results).

The V_M of plasmolyzed internodal cells was estimated with the "K^+ anesthesia method" (Shimmen, Kikuyama & Tazawa, 1976). Cells were therefore electrically separated in two halves by a vaseline junction. While one half was bathed in the standard experimental solution containing 10 mM KCl, the other half was in an identical solution but with 100 mM KCl and a lower sorbitol concentration to achieve isosmotic conditions across the length of the cell. The voltage across the two solution pools was measured with Ag/AgCl electrodes and a custom-built electrometer. The potential difference is an approximation of the free running membrane voltage because of the low membrane resistance in 100 mM KCl.

Abbreviations

V_R, resting transmembrane voltage; G, conductance; E, equilibrium voltage; P_o, open probability; $[Ca^{2+}]_{cyt}$, cytoplasmic concentration of free Ca^{2+}; $[Ca^{2+}]_o$, extra-cellular Ca^{2+} concentration

Results

Basic Parameters of the Experimental System

Free Running Membrane Voltage

The resting voltage (V_R) of plasmolyzed *Chara* internodes bathed in (mM) 10 KCl, 1 $CaCl_2$ 5 HEPES/KOH and pH 7.5 was −45 ± 3 mV (n = 6) when measured with the K^+ anesthesia method (Shimmen et al., 1976). Very similar voltages (−43 ± 4 mV; n = 19) were obtained when V_R was determined with the patch-clamp amplifier in the current-clamp mode.

Long-term measurements with the former technique showed that plasmolyzed cells with an open cell wall maintained, under these detailed conditions, very stable membrane voltages with variations of less than ±2 mV over a recording period of 60 min (n = 4). The stable membrane voltage makes the cells very suitable for patch-clamp recordings in the cell-attached mode over long periods of time.

Rate of Cytoplasmic Streaming

The cytoplasmic streaming rate in *Characean* algae is an indirect measure for $[Ca^{2+}]_{cyt}$ (Williamson & Ashley, 1982; Okazaki & Tazawa, 1986). Measurements of the rate of cytoplasmic streaming from five cells gave values of 61 ± 4 $\mu m \cdot sec^{-1}$ for control cells and 58 ± 6 $\mu m \cdot sec^{-1}$ after plasmolysis and cell wall opening. Thus, the experimental preparation had no perceivable effect on the streaming rate and therefore, presumably, on $[Ca^{2+}]_{cyt}$. Hence, the experimental system of plasmolyzed *Chara* internodes is in a state closely resembling intact, turgescent *Chara* internodal cells used in previous whole-cell voltage-clamp experiments (Beilby & Coster, 1979 *a,b*; Beilby, 1984*b*).

Plasma Membrane Currents

From the data obtained with space-clamped intact *Chara* cells (Beilby & Coster, 1979*a*; Lunevsky et al., 1983) we anticipated an activation of channels for Cl^- and Ca^{2+} as the ensemble of currents underlying electrical membrane excitation. To support the detection and identification of the two currents under investigation, we used the respective ions in a high pipette concentration (P: 50 to 100 mM). This operation reverses the direction of the Cl^- flux during an action potential from the characteristic efflux (in artificial pond water) into an influx (from the pipette) but should not affect the excitability of the cells (Beilby & Coster, 1979*a*; Lunevsky et al., 1983). Ca^{2+} in the pipette was in some experiments replaced by Ba^{2+} to further increase the conductance of a putative Ca^{2+} channel (e.g., Hille, 1992) and block unwanted K^+ channel activity (Tester, 1988).

Figure 1 illustrates current records of single open channels obtained from a cell-attached experiment with 50 mM $CaCl_2$ as pipette electrolyte. The channel records present selected traces from various holding voltages showing the rare switching of the two dominant ion channels observed under these conditions (channel fluctuations only observed at voltages $> V_R$; P_o at voltages $< V_R$ for both conductances $\ll 0.01$). The single channel conductance found in comparable experiments was independent of whether Cl^- was present as $CaCl_2$ or $BaCl_2$ in the pipette (*data not shown*). The current/voltage (*I/V*) relation of the open channel currents from Fig. 1 is illustrated in Fig. 5, which identifies the two channels as Cl^- translocators (for details *see below*).

Transient Activity of Cl^- Channels

In the majority of comparable experiments with $CaCl_2$ or $BaCl_2$ in the pipette, these two types of

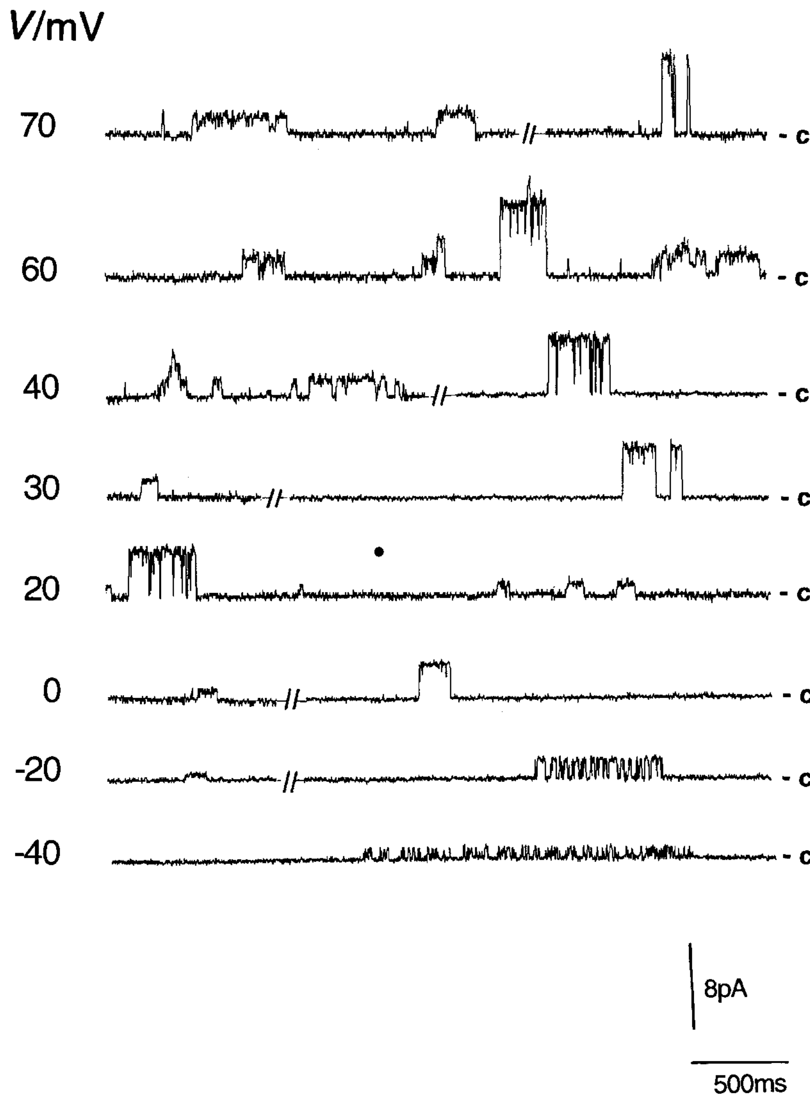

Fig. 1. Unitary open channel currents from two different types of channels in a cell-attached patch. The open probability of the two conductance levels was low at all voltages ($P_o < 0.01$) so that the traces here are only selected examples of channel fluctuations. The baseline *c* and the holding membrane voltages are indicated along the current traces. P: 50 mM $CaCl_2$. Sample rate 500 Hz, filter 100 Hz.

channels exhibited a unique mode of activity: long periods with a low open probability were irregularly interspersed with sections where variable numbers of channels activated and inactivated in a coordinated fashion. One example is illustrated in Fig. 2. In the given example (P: 100 mM $BaCl_2$) the membrane was clamped in two consecutive steps from a negative voltage (1st, −60 mV; 2nd, −100 mV) to +20 mV. Upon the steps to +20 mV, there was at first a period with very little channel activity (for details *see below*). After holding the membrane for 3.6 min (1st) or 16.3 sec (2nd) at +20 mV, a transient and repetitive activation of large currents was recorded.

A closer inspection of the current transients (called Cl^- transients; marked by Roman numerals) revealed that they resulted from a coordinated activation and inactivation of a large number of single channels. The composition of one Cl^- transient is detailed in Fig. 3 and Table 1. In this case the whole amplitude of the illustrated current transient was the result of a maximum of five small current fluctuations (1.6 pA) and 1 larger current fluctuation (4.7 pA). The same analysis for the remaining Cl^- transients, recorded in the same patch and at the same test voltage, is shown in Table 1. The composition as well as the number of identical channels switching within the time frame of the events varied considerably: while the small current fluctuation was present in all events, the occurrence of the larger one was variable.

Cl^- transients recorded under these conditions could be much larger than those shown in Figs. 2 and 3 (*compare* Figs. 8 and 10). Peak currents due

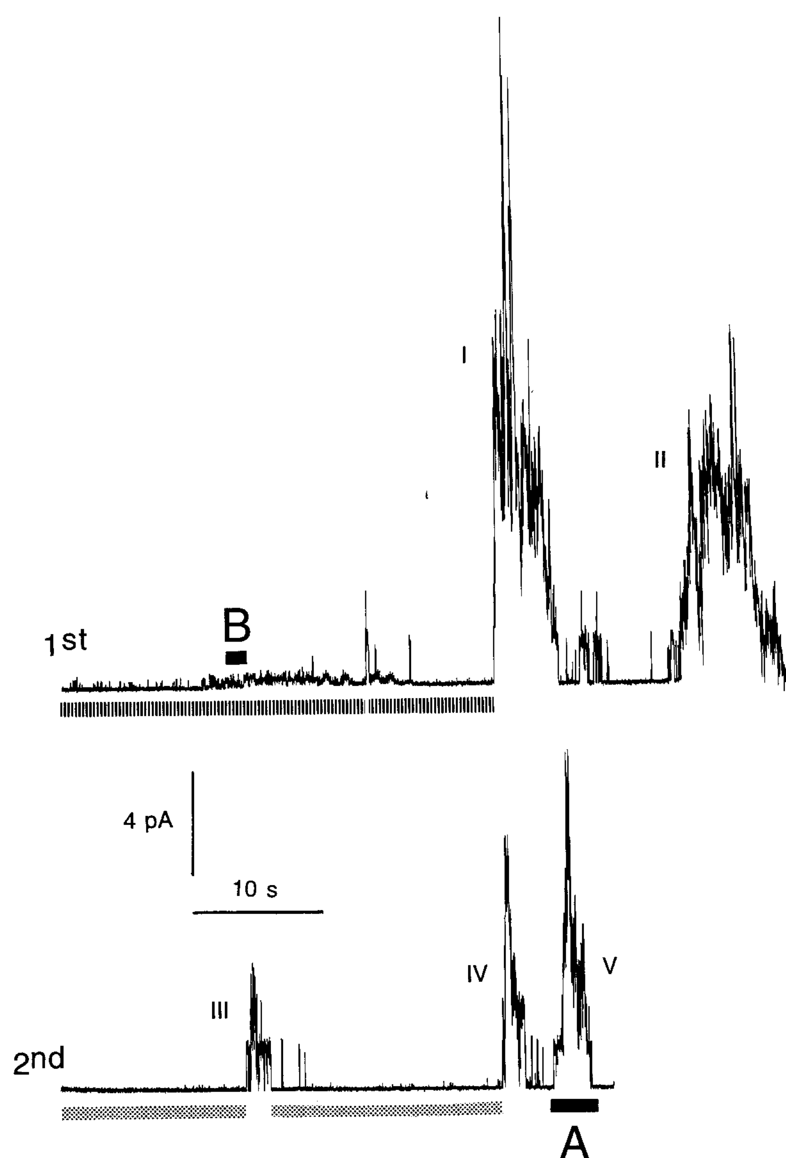

Fig. 2. Transient activity of multiple channels in cell-attached patch. Current records including periods with transient channel activity were obtained in a patch clamped to +20 mV with 100 mM $BaCl_2$ as pipette electrolyte. Sample rate 100 Hz, filter 500 Hz. The periods with transient channel activity are marked by Roman numerals and a blowup of one period labeled *A*, detailing the single channel composition, is illustrated in Fig. 3. The patch was clamped in two consecutive steps from −60 (1st) and −100 mV (2nd) to +20 mV.The illustrated current traces start at 186.5 and 0.1 sec after stepping for the first (1st) and the second time (2nd) to +20 mV, respectively. A second time period, marked *B*, is detailed in Fig. 6 showing the activity of a small current of about 0.3 pA preceding periods with transient Cl^- channel activity. The hatched and stippled bars along the current traces indicate the periods of data collection for the probability density histogram shown in Fig. 7.

to transient channel activity of up to 150 pA were recorded (P: 100 $CaCl_2$) when holding the membrane at +20 mV. In those cases, the entire current could not be dissected into individual components as done for Table 1. However, for a conservative estimate, there were approximately 50 open channels within one patch assuming that the 1.4 and 4.7 channels are present in equal numbers. If these large transient currents arose from multiple copies of the small channel (e.g., as in Fig. 2, Table 1) the channel density could approach 100 channels per patch. Thus, considering the patch area approximately 5 to 10 μm^2 in electrodes with resistance ≤ 10 MΩ (Mazzanti & DeFelice, 1988) a maximum channel density of ≥10 to 20 Cl^- channels/μm^2 is estimated.

Channel/Current Identification

Cl^- transients similar to those shown in Fig. 2 were also found at other membrane voltages. Figure 4 illustrates the current traces from an experiment using 50 mM $CaCl_2$ as pipette electrolyte, showing that the transient current reversed sign at a voltage between −20 and −100 mV. An analysis of clearly resolvable current steps found within transient currents in different patches revealed again two conductances with open channel amplitudes (open points in *I/V* relation, Fig. 5) which were comparable to those switching singularly.

A regression line through the data points of the open channel *I/V* relation provides a reversal voltage for the two current levels at −65 mV and a cord

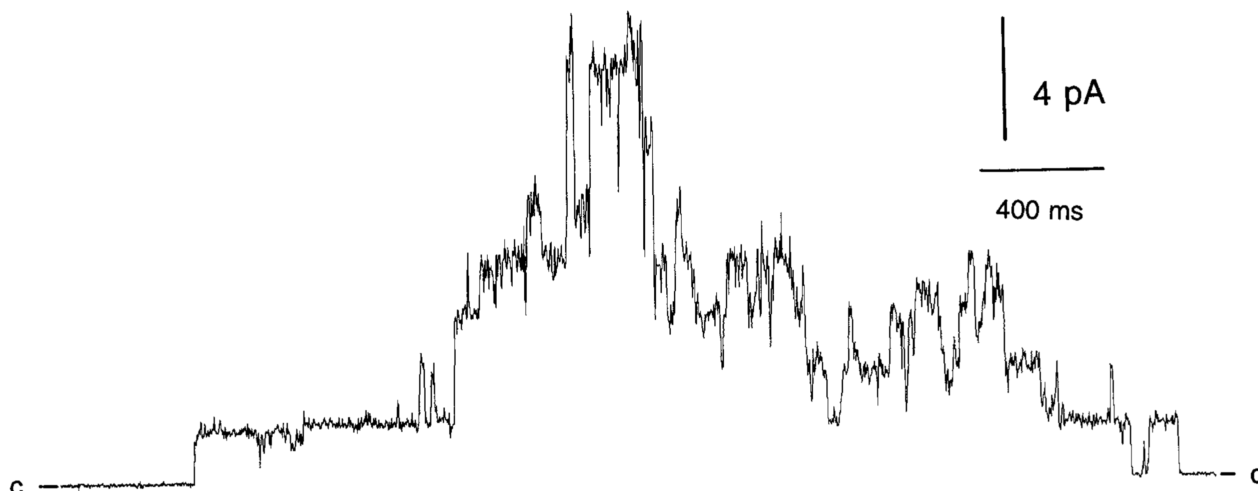

Fig. 3. Blowup of the current record from the time period marked as *A* in Fig. 2, which contains a section with transient current activity. The baseline *c* is indicated along the current trace. The resolvable step sizes show the switching of five channels with an open channel amplitude of 1.6 pA and 1 channel with an open channel amplitude of 4.7 pA.

Table 1. Dissection of total currents during transient periods of activity into two groups of open channels[a]

Number of open channels	Event no.				
	I	II	III	IV	V
n	15	7	3	6	5
m	3	1	0	0	1

[a] *n* = 1.6 pA units and *m* = 4.7 pA units. Roman numerals crossreference with numbers at the current traces in Fig. 2.

conductance at +50 mV of 16 pS for one channel type and of 52 pS for the other one (Fig. 5). From the reported cytoplasmic Cl^- concentrations in *Chara* of 4 to 10 mM (*see* Materials and Methods) and the ionic composition of the pipette electrolyte, the reversal voltage for Cl^- currents is calculated to be between −83 (−100) and −60 (−78) mV for 50 (or 100) mM Ca/$BaCl_2$. This agrees well with the experimentally obtained reversal voltages, and shows that the transiently activating currents are carried by Cl^- ions. In accordance with this notion, the cord conductance at +50 mV of the two channel types increased 1.3-fold for a twofold increase in Cl^- concentration in the pipette, and the estimated reversal voltage shifted negatively by about 12 mV (*data not shown*).

Other Channels Outside Time Frame of Transient Current Spikes

The current records illustrated in Figs. 4 and 10 show also considerable, nontransient single channel activity. This observation is representative for 10% of the experiments conducted under these conditions. An *I/V* relation of the open channel amplitudes identified this channel also as a Cl^- channel (*data not shown*). These Cl^- channels had a slightly smaller conductance (G = 12 pS) than the transiently activating channels and, in addition, a distinctly different voltage-dependent kinetics: the open probability increased rapidly in an ordinary voltage-dependent manner and was high at voltages positive of V_R (e.g., P_o = 0.4 at +10 mV) with no transient pattern of activity. At this point, it cannot be decided whether this channel is in fact a translocator distinct from the transiently activating 16 pS channel or if it reflects an additional open state distinct from the two states with low or transiently high channel activity.

Furthermore, a very small current of some 100 fA was occasionally detected (three experiments) (Fig. 6). Even though the unitary current was very small, long closed times with a small noise level allowed an unequivocal determination of the sign of the current. The current voltage relation of the open channel currents gave a reversal voltage at approximately −15 mV and a conductance of 4 pS (Fig. 6*B*). This reversal voltage does not match the equilibrium voltage of any of the relevant ions (*see* Materials and Methods) and must therefore reflect a channel with low selectivity. With respect to the ionic composition of the *Chara* cytoplasm and the pipette electrolyte, the current *may* carry K^+ and Ca^{2+}.

To investigate whether the activation of Cl^- transients was correlated with the activity of the small channel—possibly as a result of net Ca^{2+} influx—we measured the open probability of the 4 pS channel during time periods preceding Cl^- transients (Fig. 2). Figures 2 and 7 show that in the first

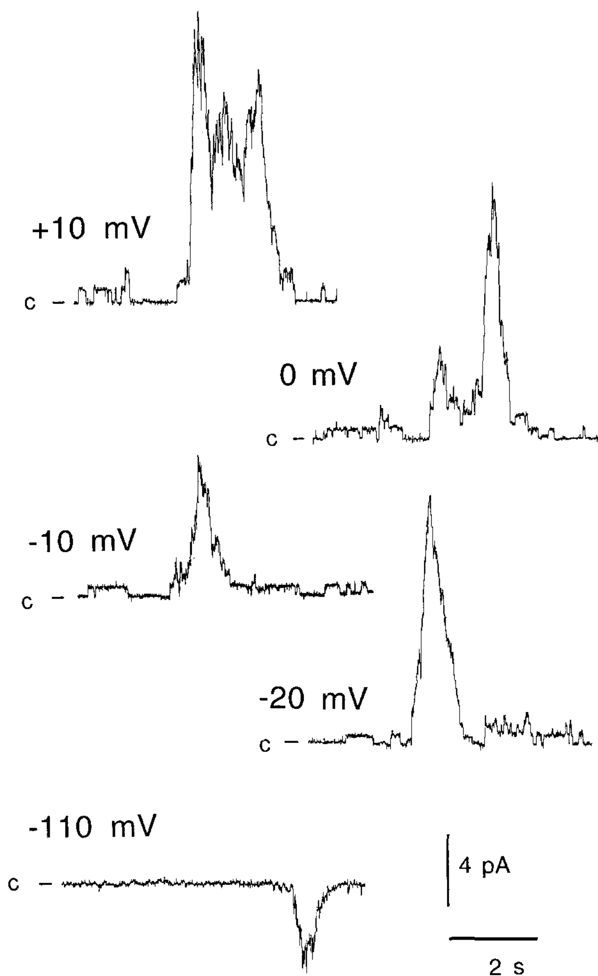

Fig. 4. Transient current activity at various membrane voltages in one cell-attached patch. The baseline *c* and the holding membrane voltages are indicated along the current traces. P: 50 mM $CaCl_2$. Sample rate 500 Hz, filter 100 Hz. Note that the current reversed sign at a voltage between −20 and −110 mV.

voltage step to +20 mV there was indeed considerable activity of the small 4 pS channel prior to the occurrence of the Cl^- transients. But channel activity was hardly detectable in the period preceding the activation of Cl^- transients in the second voltage step (Figs. 2 and 7). The lack of a temporal correlation between the Cl^- channels and the 4 pS channel is further supported by the small channel being active also at more negative voltages—with an even larger driving force for a putative Ca^{2+} influx—but this did not lead to Cl^- channel activity (e.g., −100 mV, Fig. 6).

Conditions for Transient Cl^- Channel Activity

Ionic Conditions and Configuration

The experimental conditions used are summarized in Table 2. Cl^- transients were only observed when $CaCl_2$ or $BaCl_2$ was present in considerable concentration (P: 50 or 100 mM). Under these conditions, transient channel activity was observed in 9 and 5 cells with Ca^{2+} or Ba^{2+}, respectively.

When Ca^{2+} was present in the pipette at concentrations ≤5 mM, transient currents were not recorded (n = 15) (*see also* Laver, 1991). Similarly, with 100 mM $MgCl_2$ as pipette electrolyte, it was not possible to evoke comparable transient Cl^- channel activity (n = 5) at any test voltage.

In patches showing Cl^- transients (P: $BaCl_2$ or $CaCl_2$ ≥ 50 mM), the ability of the membrane to activate these currents even at extreme test voltages (−200 to +100 mV), was completely lost (n = 5) after excising the patch and exposing the cytoplasmic side to the cell bathing solution (in mM: 10 KCl, 1 $CaCl_2$, 5 HEPES/KOH, pH 7.5, 320 sorbitol).

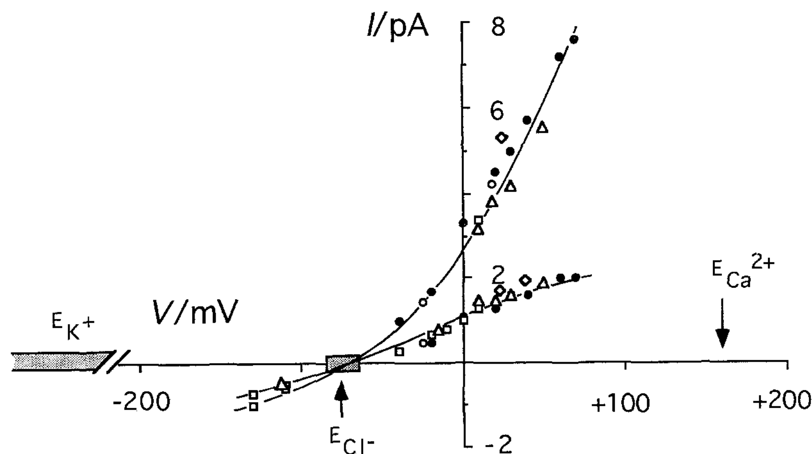

Fig. 5. Current-voltage (*I/V*) relation of open channels; currents from Fig. 1 (filled symbols) and from single channels detected within transient current events (open symbols). Each open symbol represents transiently active open channel currents in a different patch with either 50 mM $CaCl_2$ (□, ◇) or $BaCl_2$ (△, ○) as pipette electrolyte. The lines represent a fit (by eye) through the pooled data points, indicating a reversal voltage for both currents at approximately −65 mV. This is in the range of voltages expected for the Cl^- equilibrium voltage (E_{Cl^-}, indicated by stippled bar) calculated with the assumption that the cytoplasmic Cl^- concentration is between 4 and 10 mM (Coleman, 1986; Okihara & Kiyosawa, 1988).

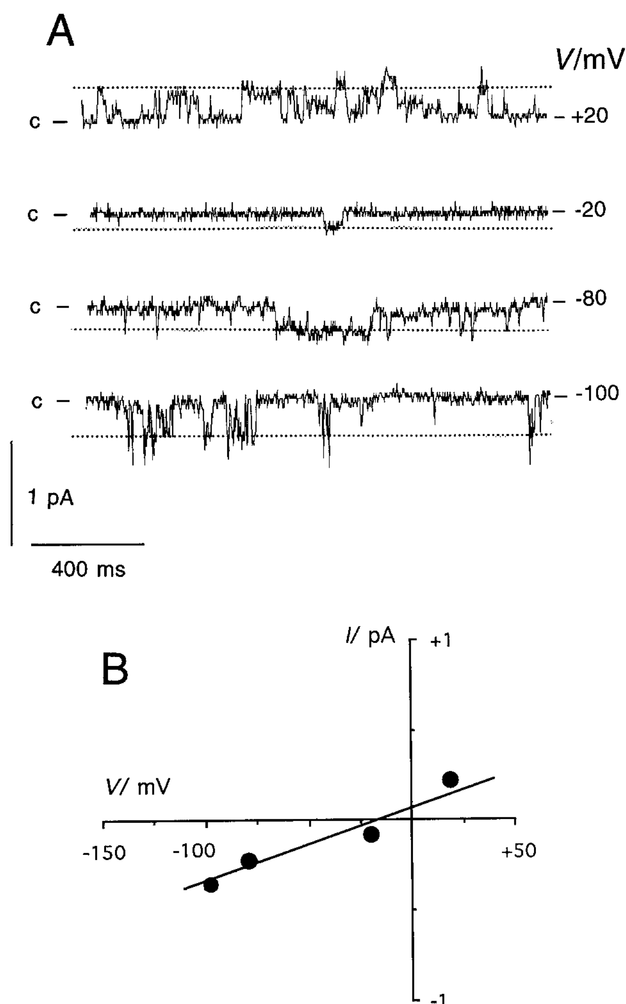

Fig. 6. Nonselective, low conductance channel. Unitary currents from a cell-attached patch (100 mM $BaCl_2$ in pipette) (*A*) and current/voltage relation of open channel currents (*B*). Sample rate 2.5 kHz, filter 500 Hz. The current records are from the same patch as in Fig. 1, and the trace for +20 mV corresponds to the time period marked *B* in this figure. Baseline *c* and the clamped membrane voltages are indicated along the current traces; the open channel amplitude is indicated by the dotted line. The linear regression through the data points gives a reversal voltage of −15 mV and a conductance of 4 pS.

Voltage Dependence

Figure 8 illustrates the voltage dependence for the activation of Cl^- transients. In this experiment (P: 50 mM $BaCl_2$) the membrane was clamped to various voltages in the sequence indicated. Holding voltages ≤ +20 mV resulted in an increasing frequency of Cl^- transients, but channel activation was again halted after clamping the patch back to a more negative voltage.

The frequency of transient current occurrence as a function of the holding potential is shown for four individual cells in Fig. 9. A feature common to all the cells was that membrane voltages positive of approximately −45 mV increased the probability of Cl^- transients. However, the actual voltage dependence varied greatly between patches. The unbroken curve in Fig. 9 represents a Boltzmann distribution fit to the data points. The theoretical distribution gives a half-maximal voltage for Cl^- transient activation at +63 ± 2 mV and a charge valency of 1.

A comparable relationship was found when analyzing the closed times between Cl^- transients as a function of voltage. These times shortened as the voltage was clamped ≥ −45 mV (*data not shown*).

Figures 4 and 9 also illustrate the possibility of Cl^- transients at negative voltages of V_R. These observations were rare and only observed in experiments where the membrane was subjected to alternating voltage pulses and where Cl^- transients had been activated in the preceding, positive going voltage pulse. Due to the limited number of observations, this correlation has not been analyzed in detail.

Transient Cl⁻ Channel Activity at Activating Voltages Appears Randomly

The occurrence of Cl^- transients in response to activating (positive going) voltage pulses was not markedly correlated with the time after the voltage steps. When four different patches (P: 50 mM $CaCl_2$) were subjected to the same voltage pulse from −110 to −10 mV it took between 0.55 and 6.3 sec before the first periods with transient Cl^- channel activity were detectable. The variability in lag times was even more pronounced in experiments with $BaCl_2$. Here, the first transient Cl^- channel activity occurred between 4 sec and 3.6 min after holding the test voltage of different patches at +20 mV.

A comparable variability for lag times was also found within any one cell tested. Figure 10 illustrates an example for a cell (P: 50 mM $CaCl_2$) challenged with 10-sec long pulses between −130 and +10 mV. In 8 out of 15 pulses the step to positive voltage activated transient Cl^- channel activity. The time when transient channel activity appeared was irregularly distributed over the total pulse time (Fig. 10). Furthermore, there was no detectable relation between the time for holding the patch at an activating voltage and the number of channels active, i.e., the mean current conducted during periods of transient channel activity.

Figures 8 and 10 also illustrate that, at an appropriate test voltage, a repetitive but irregular appearance of Cl^- transients was recorded. The random succession of transient activity can be subjected to

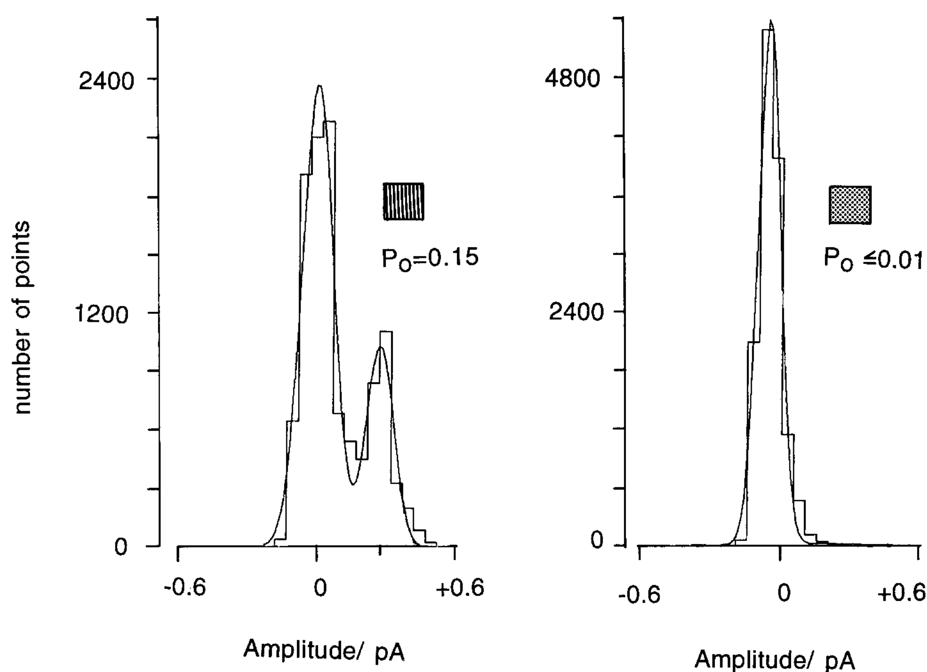

Fig. 7. Probability density histograms of low conductance (4 pS) channel. Data were digitized at 500 Hz (filter 100 Hz) for 31 sec during the 1st (|||) and 31.2 sec during the 2nd [░] clamp step to +20 mV prior to transient Cl$^-$ channel activity. The large peaks reflect the baseline noise. The small peak in the first histogram results from the open channel current of the 4 pS channel active during the 1st period of data collection. The peak is absent in the histogram from data collected during the 2nd period. Data were fitted by the sum of two Gaussians. The fit parameters are for the 1st (and 2nd) time interval: baseline (in pA); 0 ± 0.068 (0 ± 0.048), channel (in pA); 0.27 ± 0.059 (0.36 ± 0.3). The open probability (P_o) was calculated from the areas under the Gaussians with the assumption that the patch contained two channels (*see* Fig. 6 at −100 mV).

Table 2. Ion composition of pipette solutions used for cell-attached measurements at *Chara* plasma membrane[a]

Pipette solutions	Current transients detectable	(*o*/*p*)
50 or 100 $BaCl_2$	+	(5/7)
50 or 100 $CaCl_2$	+	(9/14)
100 $MgCl_2$	−	(0/5)
1 or 5 $CaCl_2$, 100 KCl ±5 HEPES/KOH, pH 5.8–7.5	−	(0/15)

[a] Cl$^-$ transients were detected (+) in *o* patches out of a total of *p* patches (*o*/*p*) investigated.

a statistical test (*for details see* Fatt & Katz, 1952). The characteristics of random time series is that the probability for the appearance of any one event is independent of its history. For an interval Δt, which is very small compared to the mean interval T, the probability for the occurrence of at least one event is $\Delta t/T$. With increasing intervals for t, P increases exponentially according to the equation $P = 1 - \exp(-t/T)$. Accordingly, the intervals between successive periods of transient Cl$^-$ channel activity in a very large number of observations (total number N), is expected to exhibit an exponential distribution if the activation occurs at random. The frequency of occurrence n of any interval between t and $t + \Delta t$ should follow the equation $n = N \Delta t/T \exp(-t/T)$. The hypothesis was tested by measuring the time gaps between the end of one Cl$^-$ transient and the start of the following event. To increase the available number of observations, data from three comparable experiments were pooled. However, to check that the frequencies of observations were comparable between different experiments, the same analysis was also carried out independently for any one experiment, giving comparable results. Figure 11 illustrates the distribution of intervals between successive periods of transient Cl$^-$ channel activity after grouping data into bins of 0.5 sec. The distribution can be described by the simple exponential equation $n = N \Delta t/T \exp(-t/T)$. Replotting the data of the total number of intervals with a duration smaller than t against t shows that the data follow the predicted curve for distribution ($P = 1 - \exp(-t/T)$ with a mean time interval T of 1.2 sec, which suggests that Cl$^-$ transients follow in a random succession.

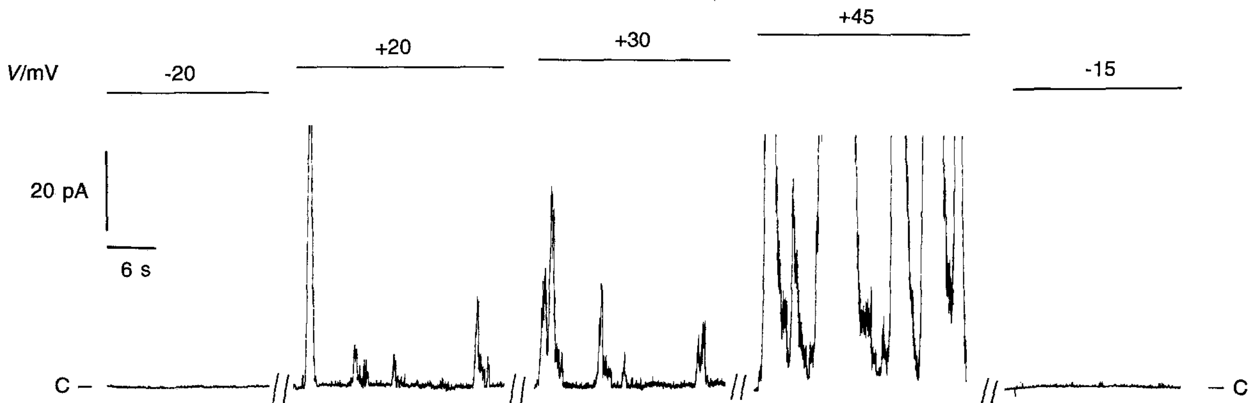

Fig. 8. Clamp voltages positive of V_R increased the probability of Cl^- transients. One cell-attached membrane patch (50 mM $BaCl_2$ in pipette) was clamped for some seconds to the sequence of the membrane voltages indicated. The current responses (after subtraction of leak current; note maximum currents at +45 mV are out of range) exhibited a frequent occurrence of transient Cl^- channel activity only at voltages positive of −20mV. The baseline *c* is indicated along the current traces.

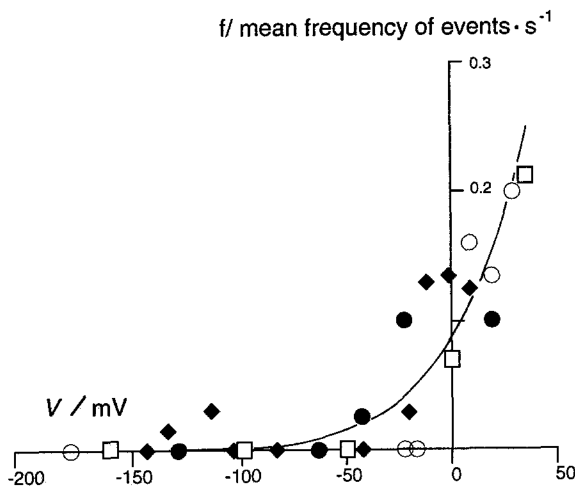

Fig. 9. Frequency of transient Cl^- channel activity as a function of the clamp voltage. In four different patches (pipette: (○, □) 50 mM $BaCl_2$, (◆, ●) 50 mM $CaCl_2$) the mean frequency *f* (number of observations per total time of recording) was estimated as a function of the holding voltage. For this analysis, distinct peaks reflecting current transients were counted over recording periods exceeding 1 min. The unbroken curve represents a Boltzmann distribution fit to the data. The mean frequency was calculated as follows: $f = \{1 + \exp[(zF/RT)\,(V_{1/2} - V)]\}^{-1}$ where z is the activation charge valency, F is the Faraday constant, T the absolute temperature, $V_{1/2}$ is the voltage of half-maximal activation and V the clamped membrane voltage. In the present case z = 1 and $V_{1/2}$ = 63.2 mV.

Activation/Inactivation Kinetics of Cl^- Transients

Within the same patch and at the same holding voltage, the peak currents of individual Cl^- transients could vary over one order of magnitude, while the duration of Cl^- transients was limited to a narrow range. In one representative example (V = +20 mV, P: 50 mM $BaCl_2$) the peak currents of 16 measured Cl^- transients ranged from 4.2 to 83.3 pA. At the same time, the total duration of these Cl^- transients varied only between 0.85 and 2.2 sec. (mean 1.5 ± 0.5 sec).

A general pattern for Cl^- transients was that the rising time (from the onset of the transient to the peak) was faster than the trailing time (from the peak back to the baseline). In the same 16 Cl^- transients the rising time (mean: 0.5 ± 0.2 sec) was at an average 2.5 ± 1.8 times shorter than the trailing time (mean: 0.96 ± 0.4 sec). The same general pattern was also observed in other patches, and there appeared to be no obvious voltage dependence for the activation/inactivation kinetics (*see* Fig. 4).

Discussion

The important result of the present study is that intact *Chara* cells contain a unique mode for channel activation/inactivation which generates transient conductance of Cl^- currents (termed Cl^- transients). For the interpretation of Cl^- transients the following results need to be considered: (i) Cl^- transients are caused by ensembles of two types of Cl^- channels which are abundant (10 to 20 channels/μm^2) in the plasma membrane. (ii) Under resting conditions these channels have a small but definite open probability (<0.01) which is not markedly voltage dependent. (iii) Individual Cl^- transients vary in size over one order of magnitude but have a limited range of durations (with a rising time about two times shorter than the trailing time). (iv) Voltages

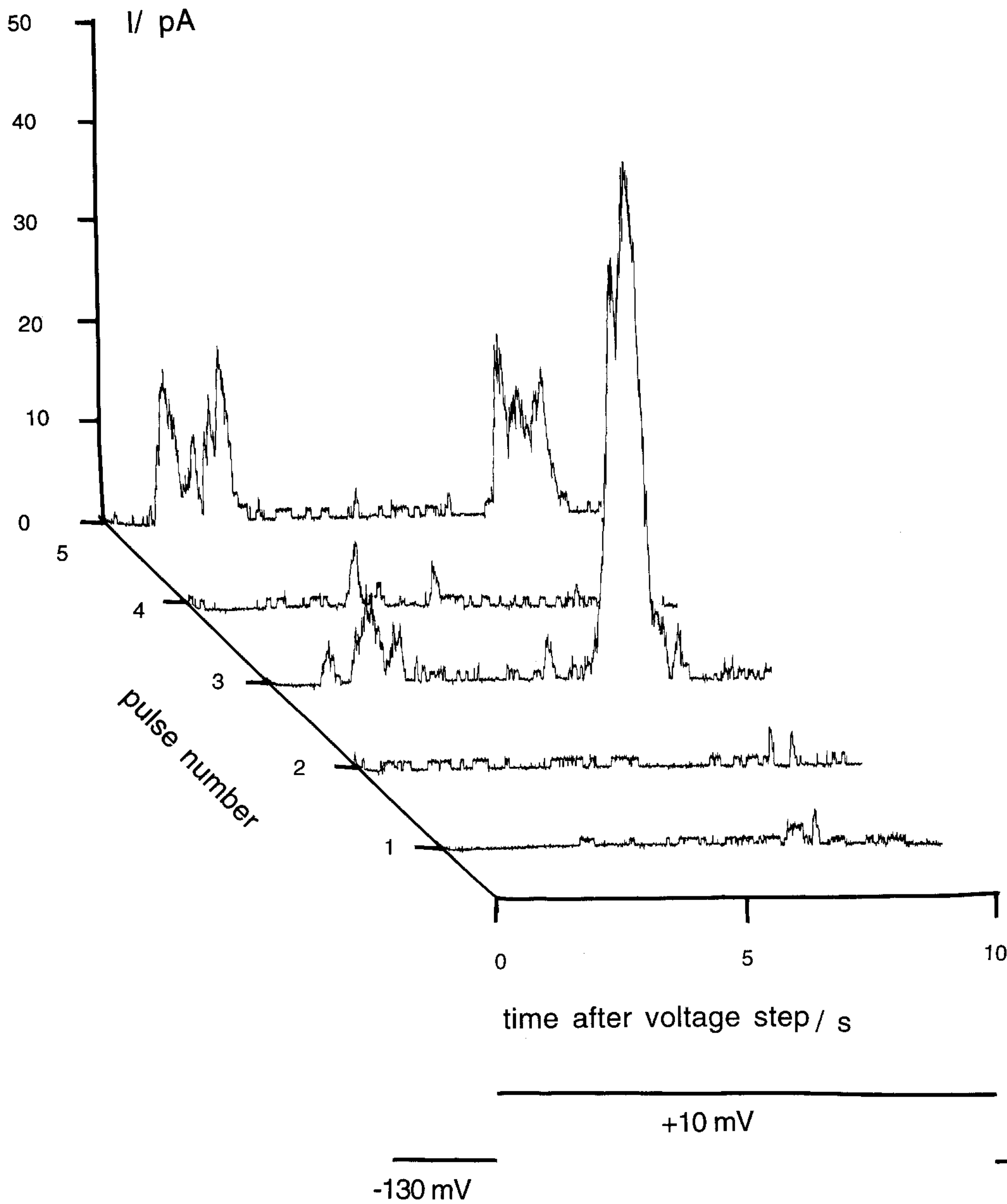

Fig. 10. Pattern of Cl^- transient activation after voltage steps. Identical clamp steps to an activating voltage result in an irregular activation of transient Cl^- channel activity. Illustrated are examples of five consecutive current traces at +10 mV recorded during the course of a voltage-clamp protocol with a total of 15 alternating, 10-sec long pulses between −130 and +10 mV; cell-attached, P: 50 mM $CaCl_2$. 500 Hz sample rate, filter 100 Hz.

more positive than V_R facilitate a transient rise in open probability, although voltage is neither a necessary nor a sufficient condition for Cl^- transient activation. (v) The termporal gaps between Cl^- transients follow an exponential distribution, indicating a stochastic nature of the frequency. (vi) $[Ca^{2+}]_o$ or $[Ba^{2+}]_o$ on the extrafacial side of the membrane is essential for Cl^- transients and cannot be replaced by Mg^{2+} or K^+. (vii) Nonselective, low conductance (4 pS) channels with variable open probability may mediate entry of Ca^{2+}, although their activity does not show a temporal correlation with the Cl^- transients.

A Mechanism for Cl^- Transients Involving Membrane-Associated Storage Sites for Ca^{2+}

The experimental findings are consistent with a mechanism which uses membrane-associated stor-

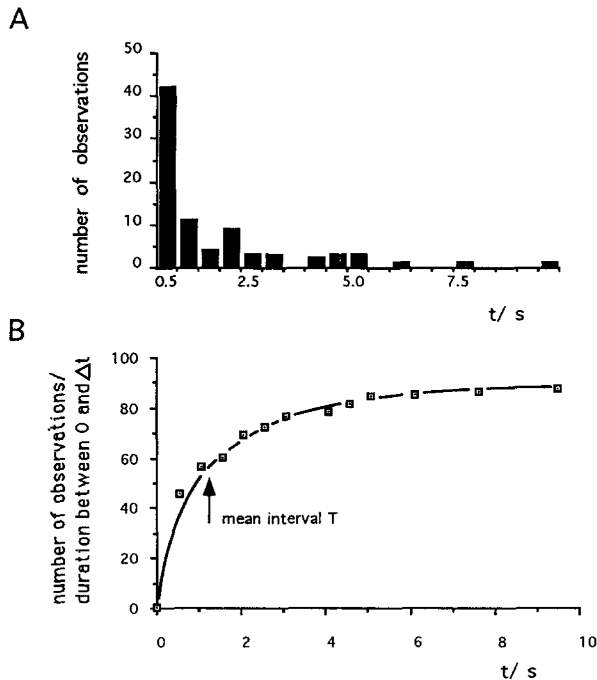

Fig. 11. Distribution of time gaps between periods of transient Cl^- transients. (*A*) Frequency distribution histogram; (*B*) replot of the same data showing the total number of observed intervals with durations smaller than t as a function of t. Data were obtained by measuring the time gaps between successive periods with transient channel activity. Data are pooled from three individual patches showing considerable transient Cl^- channel activity when clamped to +10 mV (P: 50 mM $CaCl_2$ (2 patches) or 50 mM $BaCl_2$ (1 patch)) including a total of 84 time gaps. The line in *B* presents a theoretical curve of the distribution, $y = N(1 - \exp(-t/T))$, where N is the total number of observations, and T (indicated by arrow, $T = 1.2$) gives the mean interval time.

age sites for Ca^{2+}. To accommodate the expressed data, these stores can be readily supplied with Ca^{2+} from the pipette solution *via* the nonselective 4 pS channel or by an undetected (even smaller) Ca^{2+} channel of random activity. Individual stores eventually discharge accumulated Ca^{2+}, a process hastened by voltages positive of V_R. The resulting local increase of $[Ca^{2+}]_{cyt}$ activates Ca^{2+}-dependent Cl^- channels in the vicinity of the vesicles, causing the affected ensemble of Cl^- channels to conduct the observable currents. These currents decay again as the local excess of Ca^{2+} enters the known Ca^{2+} buffering system of *Chara* (Hayama & Tazawa, 1980; Kikuyama & Tazawa, 1983), which operates with a time constant ≤1 sec (Williamson & Ashley, 1982). The individual relationship between the amount of Ca^{2+} released from stores and the size of the affected ensemble of Cl^- channels determine the magnitude of the observed Cl^- transients.

A comparable mechanism, similarly based on Ca^{2+} discharge from membrane-associated Ca^{2+} stores, has been shown as the functional basis of spontaneous transient outward currents in smooth muscle cells (Xiong, Kitamura & Kuriama, 1992). The view that a comparable system may also work in plant cells is fostered by reports which demonstrate or postulate Ca^{2+} storage sites in association with the plasma membrane (Saunders, 1981; Kikuyama & Tazawa, 1983; and further references in this paper).

A simpler mechanism for Ca^{2+} and voltage-mediated channel activity has already been postulated for the action potential in *Characean* cells (Lunevsky et al., 1983). According to this proposal, Ca^{2+} would enter the cytoplasm directly through voltage-gated Ca^{2+} channels without an intermediate storage step and, as a result, activate Ca^{2+}-dependent Cl^- channels. This simplification must be rejected here because there is no temporal correlation between the activity of the 4 pS channel, which is the only detected candidate for Ca^{2+} translocation, and Cl^- transients. But more importantly, the long and variable lag times between an activating voltage step and the onset of transient Cl^- channel activity are also inconsistent with this view.

A more complex mechanism for Ca^{2+} and voltage-mediated channel activity has been demonstrated in mammalian neuron cells for the activation of K^+ channels (Gorman & Thomas, 1978; Hille, 1992). According to this, Ca^{2+} enters the cell in response to positive voltage steps, but is rapidly buffered via binding to proteins and Ca^{2+} export from the cytoplasm. Following this rationale, Cl^- transients in *Chara* would lag behind a voltage step and only occur if these mechanisms can no longer cope with an excessive net load of Ca^{2+} entry. This explanation can also be rejected here. Diffusion assisted by cytoplasmic streaming would simply not allow a *local* (in the patch area) buildup of $[Ca^{2+}]_o$ or $[Ba^{2+}]_o$ in the giant size alga over minute-long clamp periods.

Similarities between Transient Cl^- Currents and Measure Excitation in *Chara*

A number of the present findings parallel those associated with electrical excitation in intact *Chara* cells: (i) while transient channel activity is due to the opening of two different Cl^- channels, the excitation currents recorded in intact *Chara* cells under voltage clamp have a large Cl^- current component (Findlay & Hope, 1964; Beilby & Coster, 1979*a*; Lunevsky et al., 1983; Beilby, 1984*b*). The two types of Cl^- channels, therefore, are most likely the pathway for the large efflux of Cl^- associated with the action

potential (Kikuyama et al., 1984). (ii) Like the transiently active Cl^- channels, the transporter of the Cl^- component of the excitation current is not a rectifier but is able to pass inward and outward current depending on the thermodynamic gradient (Beilby & Coster, 1979*a*; Beilby, 1984*b*). (iii) Similar to the voltage-facilitated occurrence of Cl^- current spikes, positive voltage also increases the likelihood of membrane excitation (Beilby & Coster, 1979*a*). But positive membrane voltage is not the primary trigger for membrane excitation; action potentials can also occur at very negative voltages without preceding membrane depolarization (Thiel, MacRobbie & Hanke, 1990). Furthermore, even at extremely positive voltages, membrane excitation is inhibited if Ca^{2+} is absent from the external medium (Findlay & Hope, 1964) or when putative Ca^{2+} channels are blocked by Ca^{2+} channel inhibitors such as La^{3+} or verapamil (Beilby, 1984 *a,b*; Tsutsui et al., 1987 *a,b*). (iv) As a further parallel, the Cl^- transients as well as the Cl^- component of the excitation current and the rise in Cl^- efflux are transient phenomena characterized by a fast rise and a somewhat slower decay. In the case of whole-cell excitation currents and of the Cl^- efflux (Beilby & Coster, 1979*b*; Lunevsky et al., 1983; Kikuyama et al., 1984) the rise is about 2 to 5 times faster than the decay. Similarly, Cl^- transients in membrane patches rise about 2–3 times faster than they decay. Similar to the Cl^- transients, the Cl^- component of excitation currents in *Chara* is only moderately voltage dependent (Beilby & Coster, 1979*a*; Lunevsky et al., 1983). (v) Finally, based on aequorin light emission, Ca^{2+} measurements in the cytoplasm of tonoplast-free *Chara* cells have given evidence for a biphasic Ca^{2+} signal during membrane excitation (Kikuyama & Tazawa, 1983). The very first rise in $[Ca^{2+}]_{cyt}$ was apparently not due to influx across the plasma membrane but was more likely a consequence of Ca^{2+} release from internal membrane located stores. Furthermore, these stores were apparently refilled by Ca^{2+} entry across the plasma membrane. These observations compare favorably with the interpretation for the activation of transiently activating Cl^- channels.

Profound differences between the present data and those obtained for excitation currents in space-clamped *Chara* cells are: First, in space-clamped *Chara* cells, voltage step protocols positive of the excitation threshold voltage always result in a fast and conservative "excitation current" response. Second, the activation of action potentials in space-clamped cells requires much less extracellular Ca^{2+} than the transient Cl^- channel activity induced in patches (Beilby, 1984*b*). Finally, in the present study it appeared that Cl^- transients required either Ca^{2+} or Ba^{2+} in the pipette, but transients were not observed with Mg^{2+}. On the other hand, there are contrasting reports in the literature about the role of divalent cations for membrane excitation in intact *Characean* algae (Findlay & Hope, 1964; Beilby, 1984*b*; Tsutsui et al., 1987*c*).

The discrepancy between the activation kinetics of Cl^- channels and the whole-cell excitation current upon positive voltage steps can be explained by the difference of membrane area considered. In the patch-clamp experiment only a very small membrane area (approximately 5 to 10 μm^2) is clamped to a voltage where excitation becomes more likely. However, the entire membrane area, even of a small *Chara* leaf cell, is more than 10^6 times larger than the membrane under the patch electrode. Thus, if the same membrane depolarization which was applied to membrane patches were to be imposed on the entire membrane area—as done in space-clamp experiments—the likelihood of early activation of 'responsive' membrane areas would greatly increase. The whole-cell current obtained under voltage clamp, therefore, would always reflect the activation of the most rapidly responding membranes areas. The observed variability of the current response of any one membrane area to depolarizing voltage steps may account for the considerable scatter typically encountered in recording of action currents under space voltage clamp (Beilby & Coster, 1979*b*) and Cl^- efflux in excited *Characeae* (Kikuyama et al., 1984).

The difference in area could also explain the need for the high $[Ca^{2+}]_o$ concentrations in the patch-clamp approach. With lower $[Ca^{2+}]_o$ it may still be possible, although less likely, to evoke the transient activation of Cl^- channels in membrane patches. Again, the likelihood would greatly increase when the entire membrane area is considered. Furthermore, based on the $[Ca^{2+}]_{cyt}$ measurements during excitation (Kikuyama & Tazawa, 1983) it can be speculated that high $[Ca^{2+}]_o$ supports a rise in Ca^{2+} concentration in the putative internal storage sites and in the consequent amount of Ca^{2+} release into the cytoplasm. Thus, as a result, the amount of Ca^{2+} released (and Cl^- channels activated) could be expected to increase with increasing $[Ca^{2+}]_o$.

In conclusion, transient Cl^- currents reflect microscopic events during an action potential at the *Chara* plasma membrane. Action potentials are not due to a simple voltage-dependent activation of either Cl^- or Ca^{2+} channels. Neither does a voltage-gated Ca^{2+} current trigger an action potential due to an immediate rise in cytoplasmic Ca^{2+}. Based on the present statistical approach, the macroscopic Cl^- excitation currents and Cl^- efflux in intact

Chara cells are explained by superposition of many microscopic events.

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