J. Membrane Biol. 163, 183–191 (1998)

The Journal of
Membrane
Biology

Transient Activity of Excitatory Cl^- Channels in *Chara:* Evidence for Quantal Release of a Gating Factor

G. Thiel[1], **A.E. Dityatev**[2]

[1]A. v Haller-Institut für Pflanzenwissenschaften, Abt. Biophysik der Pflanzen. Untere Karspüle 2, 37073 Göttingen, Germany
[2]Institut für Biosynthese Neuronaler Strukturen, Zentrum für Molekulare Neurobiologie. UKE, Universität Hamburg, Germany

Received: 27 October 1997/Revised: 26 February 1998

Abstract. In attached patches on the plasma membrane of nonexcited *Chara corallina* cells, randomly activating, transient Cl^- currents with variable amplitudes were recorded. The peak amplitudes of these currents could be grouped into distinct populations with approximately equidistant mean peak currents. Generally, the mean current of the smallest population measured about half of the distance between the means of subsequent populations. Currents of the smallest population occurred most frequently at all voltages; the frequency of observations decreased with increasing amplitudes of the currents. At all voltages transient currents from different populations were similar in duration with the exception of the smallest currents, which lasted only 0.6 times as long as larger currents. Furthermore, transient currents were most frequent at positive voltages, but once initiated at a positive conditioning pulse they were also observed during subsequent pulses to negative voltages. The results are consistent with the idea that *Chara* contains Ca^{2+} stores in the vicinity of the plasma membrane, which are indirectly filled from the external medium. Upon quantal Ca^{2+} discharge from adjacent stores, a process independent of membrane voltage, the concentration of Ca^{2+} in the cytoplasm increases transiently. Depending on the number of discharging stores, distinct numbers of Ca^{2+}-stimulated Cl^- channels activate, giving rise to the macroscopic excitatory Cl^- current in these cells.

Key words: Action potential — *Chara* — Patch clamp — Quantal release — Quantal analysis — Cl^- channel gating

Correspondence to: G. Thiel

Introduction

In the giant alga *Chara corallina* depolarization beyond a critical threshold triggers an action potential (AP) (*see* Beilby & Coster, 1979). Underlying this short lasting depolarization is a transient rise in the permeability of the plasma membrane to Cl^- (Thiel, 1995; Beilby & Coster, 1979). The repolarization, a consequence of the return of the Cl^- permeability back to the low resting level, is further aided by an additional transient but delayed rise in K^+ permeability (Homann & Thiel, 1994; Thiel, 1995).

In patch clamp recordings in the cell-attached configuration on the plasma membrane of electrically excited *C. corallina* cells, one type of K^+ channel and two types of Cl^- channels are found to be transiently activated during the course of excitation (Thiel, Homann & Gradmann, 1993; Homann & Thiel, 1994). Activation and inactivation of these channels during an AP progresses with kinetics similar to the respective changes in ion permeability during excitation (Homann & Thiel, 1994; Thiel, 1995). So, changes in ion permeability during an AP can largely be explained on the basis of a transient rise of Cl^- and K^+ channel activity. In terms of gating, the K^+ channel active during an AP was found to rectify outwardly (Thiel, Homann & Plieth, 1997) which suggests that its transient activity during the AP is solely due to AP-associated voltage changes. The gating properties of the Cl^- channels turned out to be more complex. Activation of these channels required Ca^{2+} in the pipette medium and was favored by depolarizing voltages. However, voltage alone was not sufficient to trigger channel activity (Thiel et al., 1993). Hence, Cl^- channel activity could not be explained by an immanent voltage sensitivity of the channels. For the same reason, an earlier proposed direct coupling of voltage-dependent Ca^{2+}

influx and with a subsequent activation of Ca^{2+}-sensitive Cl^- channels (Lunevsky et al., 1983) cannot account for the gating of Cl^- channels observed in patch-clamp recordings (Thiel et al., 1997). As an alternative model it was therefore proposed (Thiel et al., 1993), that Ca^{2+} stores in the vicinity of the membrane are supplied with Ca^{2+} via plasma membrane resident channels and that these stores randomly discharge Ca^{2+}, a process reinforced by positive voltage. The resulting local rise in cytoplasmic Ca^{2+} (Ca^{2+}_{cyt}) may then activate Ca^{2+} sensitive Cl^- channels in the vicinity of the release site until Ca^{2+}_{cyt} is buffered back to the resting level. This hypothesis has further been fostered by recent measurements of Ca^{2+}_{cyt} with the fluorescent dye fura-2. In this study, the quenching of fura-2 fluorescence during an AP by Mn^{2+} suggested Ca^{2+} release together with Mn^{2+} from internal stores (Plieth et al., 1997).

In the present study the transient Cl^- currents in cell-attached patches were further analyzed. A frequency distribution of peak Cl^- currents exhibited distinct, roughly equally spaced populations, suggesting a quantal composition of the current. This could reflect an activation of Cl^- channels due to a quantal release of Ca^{2+} from stores.

Materials and Methods

Plant Material and Solutions

Chara corallina Klein ex Wild was grown as described previously (Thiel et al., 1993). Internodal cells were stored one day before experiments in standard artificial pond water (in mM: 0.5 $CaCl_2$, 0.1 KCl, 1 NaCl, 5 (N-[2-Hydroxyethyl]- piperazine- N′-[2-ethanesulfonic acid]) (HEPES)/NaOH, pH 7.5). Ionic currents across the plasma membrane were measured as reported previously (Thiel et al., 1993). *Chara* internodal cells were plasmolyzed in a solution containing 3 or 10 mM KCl, 1 mM $CaCl_2$, 5 mM N-[2-Hydroxyethyl]-piperazine-N′-[2-ethanesulfonic acid (HEPES)/KOH, pH 7.5 and 320 mM sorbitol. In regions where the protoplast had withdrawn from the cell wall, a small window was cut into the wall allowing access to the plasma membrane (Thiel et al., 1993). For current recordings in the cell-attached configuration, patch pipettes contained 50 mM $CaCl_2$. The stated membrane voltage is the sum of the empirically determined liquid junction voltage of −30 mV and the free running membrane voltage of −45 mV measured in cells kept under the same conditions (Thiel et al., 1993). In some experiments (e.g., Fig. 1) the membrane voltage was measured simultaneously with patch currents as described in Homann and Thiel (1994). Currents were replayed from videotape filtered with 100 Hz (8-pole Bessel filter, npi, Tamm, Germany) and sampled at 500 Hz. For high temporal resolution (Fig. 1 inset) data were filtered with 1 kHz and sampled at 4 kHz. Peak currents were measured as the difference between the baseline and the maximum current reached during periods of transient channel activity. Occasionally, large current transients exhibited multiple distinct peaks. In these cases we considered only the first current maximum reached within 600 msec of current rise from the baseline and/or the last maximum obtained within 1100 msec of decay back to the baseline. These time frames were based on the conserved kinetics of current transients with a distinct singular peak (Fig. 4, Thiel, 1995). An analysis of quantal size and a statistical test of significance of quantal distribution were performed as described in detail previously (Dityatev et al., 1994; Bolshakov et al., 1997).

Abbreviations

V_M, membrane voltage; AP, action potential; mpc, mean peak current; $InsP_3$, myo-Inositol 1,4,5-trisphosphate

Results

Transient Cl^- Currents have a Quantal Composition

Transient Cl^- currents were evoked in cell-attached patches upon clamping the patch to depolarizing voltages. An example for a continuous recording with a train of transient Cl^- currents at a constant patch voltage of −19 mV (pipette voltage +55 mV; cell membrane voltage −74 mV) is shown in Fig. 1. The blow ups illustrate that transient channel activity lasted about 1 sec; during these periods the currents typically rose to a discrete maximum in about 300 to 500 msec and then decayed more slowly to the basal current. As in previous studies the small transient currents can be resolved at higher magnification as discrete fluctuations of two types of Cl^- channels (Thiel et al., 1993).

At first glance, these discrete transients seem to be similar in duration but variable in amplitude. To further analyze the composition of these transient Cl^- currents, the amplitudes (i.e., peaks) of the discrete transient currents (in the present example $n = 122$) were measured and plotted as a frequency distribution (Fig. 2*A*). The histogram exhibits distinct maxima with roughly equally spaced intervals between the mean amplitudes of increasing orders of populations. The distribution could be fitted by a sum of Gaussian curves, with mean peak currents (mpc) and variances (in brackets) at 6.2 (3.5), 18.9 (4.3), 33.5 (5.9), 49.3 (6.1), 67.4 (7) and 87 (8.1) pA.

A comparable pattern of peak current distribution with distinct maxima was found in all 10 patches in which more than 30 discrete current peaks in each patch were measured at a constant V_M between −20 and +20 mV. To allow comparison of peak current distributions obtained from different patches and at different voltages the mean and amplitude of the first peak were scaled to unity. The respective mean peak current distributions obtained from these patches are summarized in Fig. 2*B*.

One prominent feature of the frequency distributions is that mpcs appear to be approximately equidistant. Only the interval between 0 current and the mean of the first order population is about half that of the following intervals. In the example given in Fig. 2, the intervals between zero current and the first order mpc and between

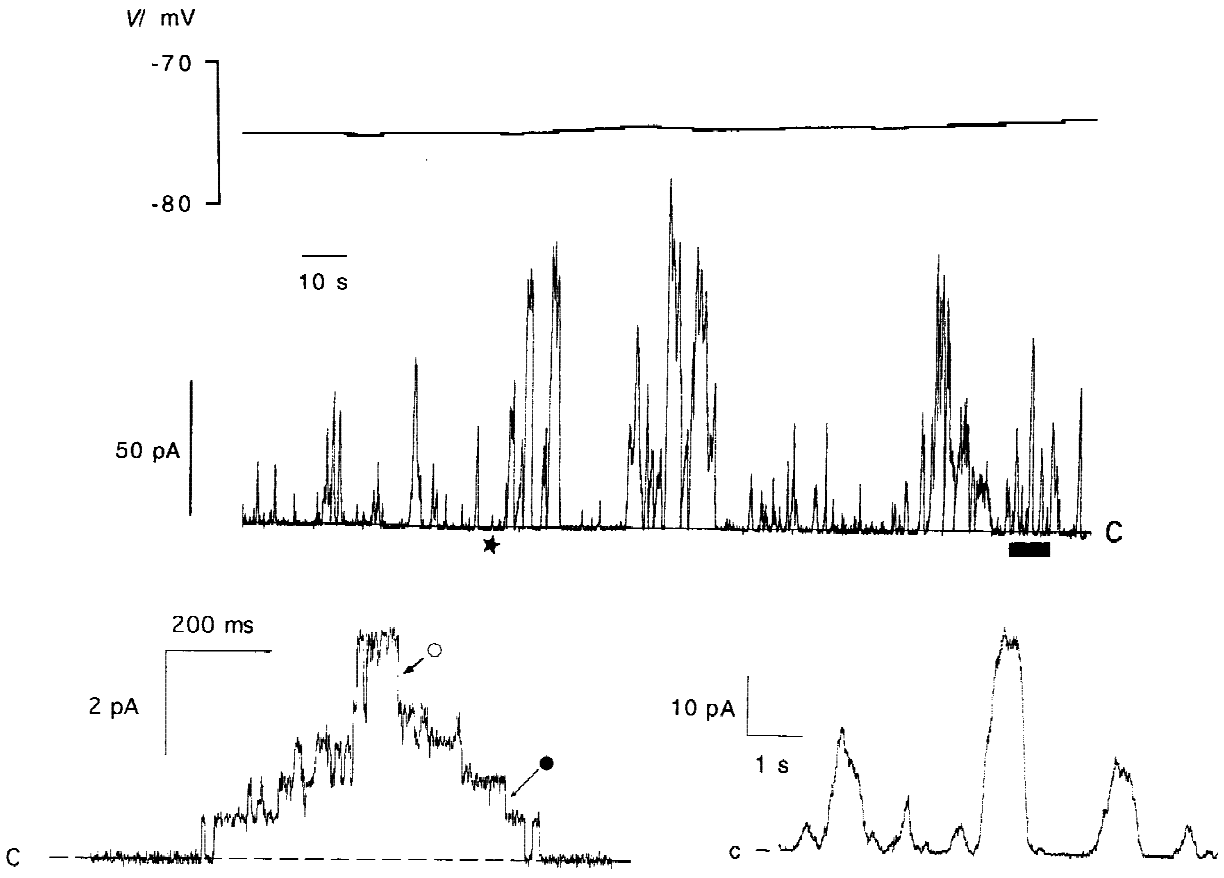

Fig. 1. Continuous recording of transient Cl^- currents across the plasma membrane of *Chara corallina* at constant membrane voltage. Patch current record (lower trace) obtained in a cell-attached patch at a V_M of −19 mV (*c.* −74 mV cell voltage plus 55 mV pipette voltage) with 50 mM $CaCl_2$ as pipette electrolyte. Trajectory of simultaneously recorded cell voltage is shown in upper trace. Cell bathed in 3 mM KCl, 1 mM $CaCl_2$, 5 HEPES/KOH, pH 7.5 and 320 mM sorbitol. (*Inserts*) Blow ups of current trace marked by solid bar (right panel) or by star (left panel). Arrows mark examples for fluctuation of 17 (●) and 38 (○) pS Cl^- channel generally associated with excitation (Homann & Thiel, 1994). c marks baseline with channels closed.

subsequent mpcs, respectively, are 6.2, 13.7, 14.6, 15.8, 18 and 20 pA. The same pattern of peak distributions between subsequent mpcs were found in all other patches analyzed (Fig. 2*B;* 9). On average, the intervals between higher order populations were 2.1 ± 0.2 (n = 10) times larger than the mean current of the first order population.

Small numbers of observations generally bear the hazard that sampling or binning artifacts introduce false peaklike distributions. Therefore, to test first the real quantal size and second the statistical significance of quantal components in the frequency distributions, the latter were analyzed by a method based on spectral analysis (Dityatev et al., 1994). Figure 3 illustrates treatment of one data set in which 129 discrete peak currents from one patch (measured at −20 mV) are expressed as cumulative probability density function. The cumulative function could be fitted by an 8th order polynomial. The derivative of the polynomial was used as an envelope of experimental probability density function (PDF) of peak currents. The latter was computed by Parsen's method as a sum of Gaussian functions each having peak current amplitude as its mean and standard deviations of 0.5 pA. A subtraction of the envelope from experimental PDF provided a periodic function that was analyzed by means of spectral analysis (Fig. 3*C*). From the reciprocal of the frequency corresponding to the maximum of the spectra density, a quantal size, Q, representing the interval between peaks, was estimated to be 7.35 pA for the present data set. To determine the significance of the peaks, 150 new data sets were generated using the polynomial fit to the experimental data set given in Fig. 3*A*. These data sets evidently were derived from distributions resembling the distribution of the measured peak currents, but had no peaks. For each of the simulated data sets, the maximum of the spectral density was computed and compared with the maximum, S_{max}, calculated for the experimental data set. The fraction of data sets in which the maximum of the spectral density exceeds S_{max} is taken as significant level, *P*, of the hypothesis that the experimental data set is from a continuous unimodal dis-

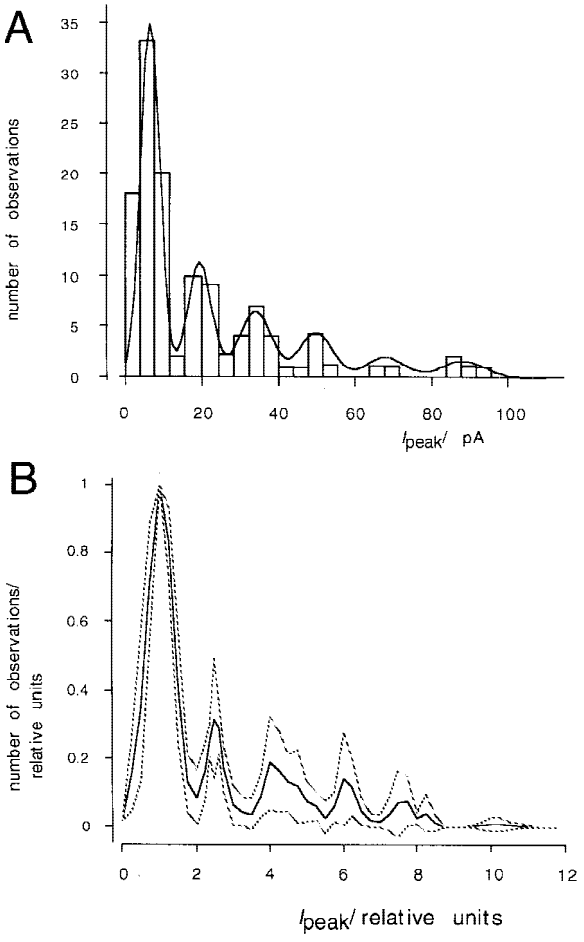

Fig. 2. Frequency distribution of peak amplitudes of transient Cl^- currents (*A*) Histogram of discrete peak currents from Fig. 1. Gaussian curves (solid line) fitted to peak current distribution (*B*) Mean peak current distribution. Transient currents were measured in ten patches at voltages between −20 and +30 mV. Histograms of the respective peak current distribution as in *A* were fitted by Gaussian curves. Mean (solid line) ± SD (dotted lines) of Gaussian curves obtained by scaling mean and amplitude of the first peak to unity.

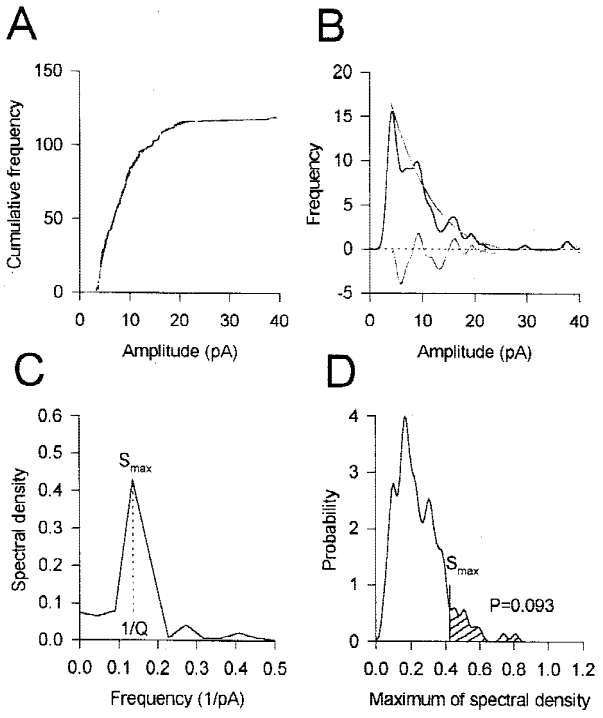

Fig. 3. Spectral analysis of peak currents measured in one patch at −20 mV as in Fig. 1. (*A*) Cumulative probability density function of experimental data (steps) and polynomial fit (smooth line). (*B*) Experimental probability density function and derivative of polynomial from *A*. The lower curve represents the difference between the probability density function and the derivative of the polynomial. (*C*) Spectral density of the difference curve in *B*. The maximum of the spectral density function (Smax) corresponds to the quantal size, Q, of 7.35 pA. (*D*) Distribution of maxima of spectral densities for 150 simulated samples with distribution described by 8th order polinomial given in *A*. The ratio of data samples having maxima bigger than S_{max} to the total number provides the level of significance, $P = 0.093$.

tribution. In the present case a significance of 0.093 was obtained suggesting that the distribution is not unimodal but of quantal nature (Dityatev et al., 1994).

When analyzed in this way in three out of five data sets having more than 100 measurements, *P* was smaller than 0.1, the mean *P* for all five sets was 0.18 ± 0.06. For comparison, 50 data sets were simulated without peaks (10 sets of simulated data per set of experimental data) but with the same standard deviation and the same shape as the distribution of the experimental data. As was expected from the definition of the level of significance, only 5 out of 50 simulated data sets had *P* smaller than 0.1. The mean *P* of the simulated data was 0.5 ± 0.04 and thus higher than that of the experimental data. This underlines, that the quantal distribution of the experimental data is real and not an artifact of sampling.

Transient currents are due to the activity of two types of Cl^- channels (Thiel et al., 1993). This means that the quantal current of 7.35 pA obtained in Fig. 3 from a patch at −20 mV is in the extreme case composed of either 8 channels of the 17 pS type of 4 of the 38 pS type channels. To further compare the channel composition of quantal events from different patches the quantal current at 0 mV was obtained. The latter was determined by extra- or intrapolating between the Cl^- reversal voltage (−78 mV, Homann & Thiel, 1994) and the quantal current obtained at the respective membrane voltages. On average, such a unitary quantal current measures 9.9 ± 2.5 pA ($n = 8$ patches).

Identification of individual populations of transient Cl^- currents allows comparison between transients from different populations with respect to their activation and inactivation kinetics. To address this question, discrete consecutive events were recorded at +10 mV, scaled to the peak and grouped according to their size. Figure 4 illustrates the average of 10 transients belonging to the

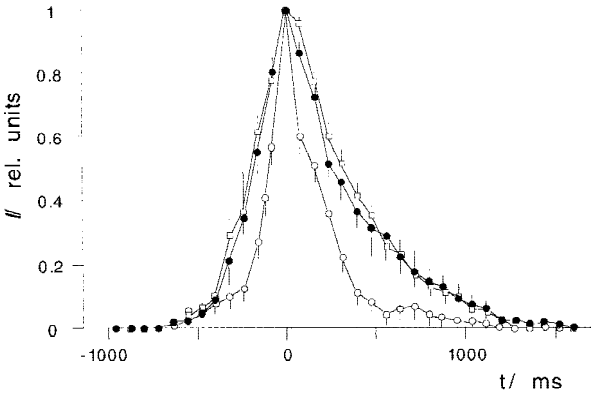

Fig. 4. Temporal analysis of transient Cl^- currents belonging to different order populations. Transient Cl^- currents were recorded at +10 mV. From the distribution of peak currents, transients were separated into distinct populations as in Fig. 2. The mean ± SE was obtained over 10 consecutive transient Cl^- currents belonging to the first (○), second (●) and third (□) order population respectively. The peak of each transient current was scaled to unity and used to align the average.

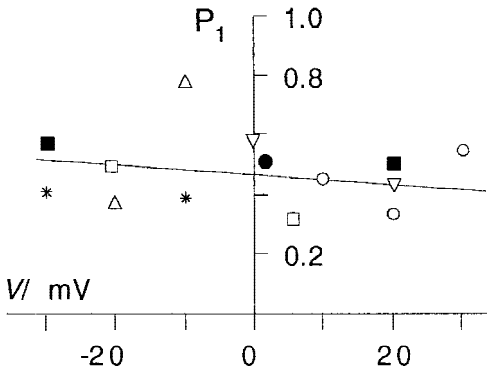

Fig. 5. Relative probability, P_1, of a transient current to belong to the first order population. Frequency distributions of discrete transient Cl^- current amplitudes were obtained at different voltages. The total number of observations was set to unity and the relative contribution of observations belonging to the first population was estimated. Different symbols represent data from different patches.

first, second and third order population of peak currents respectively. Notably, the mean first order transient has a similar asymmetric shape as the higher order transients: the rising time (time between 10% and 90% of peak) is in average 2.5 ± 1.1 ($n = 10$) times faster than the decline time. However, the duration of the singular transient, i.e., the time spent above half of the peak amplitude, is 1.6 times shorter than that of the higher order transients. The duration of current transients belonging to the next order population is only marginally longer. On average, the half width of the first, second and third order population was 292 ± 100, 458 ± 65 and 508 ± 84 msec ($n = 10$) respectively (Fig. 4).

Effect of V_M on Transient Cl^- Currents

The distribution of peak currents in Fig. 2*A* shows that 58% of the discrete transient Cl^- currents are, under the given conditions, of unitary size. To test whether this distribution is affected by V_M, the probability for a transient current to be of unitary size was determined in patches at different voltages. In Figure 5, the respective probability is plotted *vs.* the V_M. Apparently, V_M does not affect the probability for a transient current to be of unitary size either in different patches or within one patch. This observation is confounded by the fact that a minority of the transient currents exhibited multiple peaks (Fig. 1). These transient currents with multiple peaks were however not considered in the frequency distribution which gives an underestimate of the real numbers of events. Nonetheless, an estimation for the probability for multiple peaks did not exhibit an appreciable divergence between different voltages (*data not shown*). Hence, the distribution of singular peak currents sufficiently represents the real distribution of transient Cl^- currents.

To further the analysis of the effect of voltage on the kinetics of individual transient Cl^- currents, a series of discrete transients was recorded at +20 mV and −50 mV. Ten subsequent current transients of unitary size at each V_M were scaled to the current peak and averaged (Fig. 6). The graph of averaged unitary transients at both V_M did not show any effect of voltage on the shape of these transients. At both V_M, the transients were asymmetric displaying a rapid rise time but a slower decay rate (*compare* Thiel et al., 1993).

The present data suggest that quantal release of a gating factor determines Cl^- channel activation. Since activation of Cl^- transients is promoted by positive V_M (Thiel et al., 1993) it had to be examined, therefore, whether voltage will trigger the final release of the gating factor or whether voltage only plays a secondary role in a reaction step preceding the final release of this factor. To elucidate the role of voltage, a patch was subjected to a pulse protocol with 10 sec long test V_M alternating between −100 and +10 mV in a cycle. The example current responses in Fig. 7 show the pattern of transient Cl^- currents observed at the two test V_M. As expected from previous work (Thiel et al., 1993), Cl^- transients are frequently activated at the positive test V_M (trace b) but are absent at negative voltages. However, using the alternating puls protocol transient currents could also be detected at the negative V_M (trace *c*), but only under the condition that the prepulse to the positive voltage had already elicited transient Cl^- currents. For control, the same patch was clamped for several min at −100 mV without observing a transient current (*not shown*). If the activation of transient Cl^- currents upon pulses to −100 mV (0.037 sec^{-1}) had been independent from the prepulse, this operation should have elicited 14 or 7 tran-

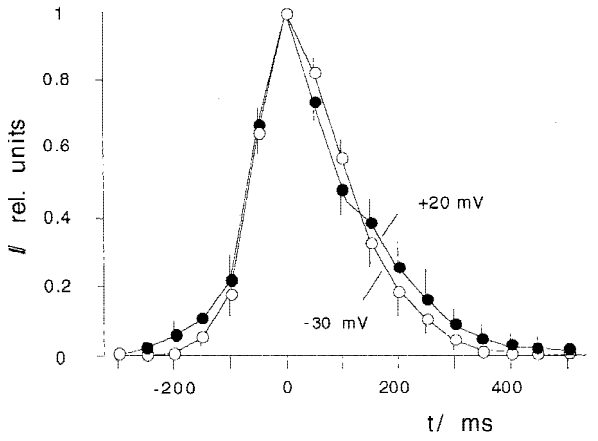

Fig. 6. Temporal analysis of transient Cl^- currents as a function of V_M. Transient Cl^- currents were recorded at −30 mV (○) and +20 mV (●). From the distribution of peak currents at each voltage, transients were separated into distinct populations as in Fig. 2. From 10 consecutive transients belonging to the first order population the mean and standard error was obtained. The peak of each transient current was scaled to unity and used to align the average.

sient currents while holding the patch for 6.5 and 3.5 min at −100 mV respectively.

The apparent prerequisite for the activation of transient Cl^- currents at negative V_M on transient Cl^- channel activity at a preceding positive prepulse is further shown in Fig. 7*B*. In this plot, the frequency of the occurrence of transient Cl^- currents at negative V_M is plotted against the frequency for the occurrence of transient Cl^- currents at the preceding positive V_M. The results show, that activation of transient Cl^- currents at positive V_M favored activity also at negative voltages. However, activity at positive V_M did not per se cause activity at negative voltages. Hence, voltage alone is neither at positive nor at negative voltages, the final trigger for quantal release of the relevant gating factor.

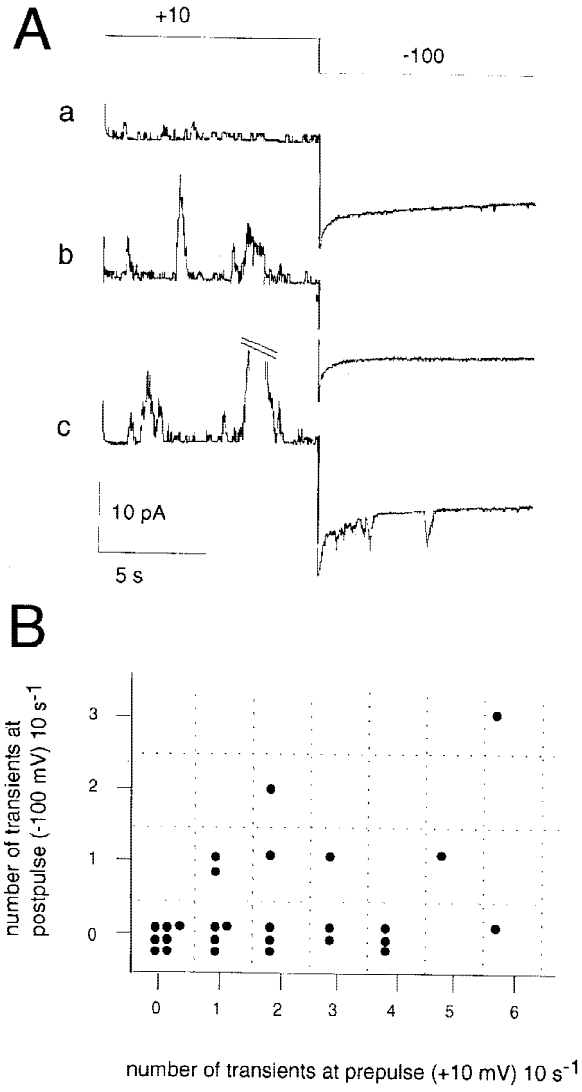

Fig. 7. Activation of transient Cl^- currents at positive prepulse voltage facilitates transient Cl^- channel activity also at negative voltage. (*A*) current recording from a cell-attached patch illustrate examples of the spectrum of current responses evoked by alternating clamp voltages between +10 and −100 mV; (*trace a*) No transient Cl^- currents at both voltages; (*trace b*) transient Cl^- currents only at positive voltage, (*trace c*) transient Cl^- currents at both voltages. (*B*) Summary of data from 27 pulses. The points represent the numbers of transient Cl^- currents observed at −100 mV as a function of the number of transient Cl^- currents at the respective prepulse to +10 mV. Each point represents data from one pair of pulses.

Discussion

Transient activity of two types of Cl^- channels is the elementary event underlying the electrically stimulated AP in *Chara* cells (Homann & Thiel, 1994; Thiel, 1995). Previous work has shown that transient activity of these Cl^- channels in a membrane patch is stimulated by positive V_M and requires Ca^{2+} but not Mg^{2+} in the external medium (Thiel et al., 1993). The present experiments now describe that the amplitudes of transient Cl^- currents are distributed into distinctly spaced populations. With support from a statistical analysis of the peak current distributions, the data suggest that the channel activity underlying membrane excitation is evoked by quantal release of a gating factor. This is the first evidence for a control of ion channel activity in plants by a quantal stimulus.

There is substantial evidence in *Chara* for Ca^{2+} release from internal stores (Beilby, 1984; Thiel et al., 1990; Kikuyama & Tazawa, 1983; Kikuyama, Shimada & Hiramoto, 1993; Tazawa et al., 1994; Thiel et al., 1993, 1997; Plieth et al., 1997) and furthermore for a

Ca^{2+}_{cyt}-stimulated activation of Cl^- channels (Shiina & Tazawa, 1987, 1988; Okihara et al., 1991). On this background, the present data are consistent with the idea that *Chara* cells contain — adjacent to the internal surface of the membrane — Ca^{2+} stores. These stores are filled with Ca^{2+} from the external medium. Upon local and random discharge of Ca^{2+} from the stores, a process that is independent from V_M, Ca^{2+}_{cyt} increases transiently giving rise to a voltage-independent activation of Ca^{2+}-stimulated Cl^- channels. The quantal composition apparent in the peak current distributions now underlines that Ca^{2+} is discharged not spatially uniform but in integer quantals.

This interpretation can further be justified on the following grounds. Positive V_M favors the activation of transient Cl^- currents in *Chara* (*see* also Thiel et al., 1993). But from the present data it becomes evident, that V_M has no perceivable effect on the shape of the individual transients. Voltage also has no obvious impact on the quantal composition of the transients. In other words, the number of quanta of released Ca^{2+} is not determined by the V_M, neither are activation and inactivation of the Cl^- channels affected by voltage. Furthermore, while the activation of transient Cl^- currents is most improbable at negative V_M (Thiel et al., 1993), transient Cl^- currents can nevertheless be observed even at negative V_M under conditions in which transient Cl^- currents had been activated at a preceding positive V_M. So, it can be concluded that positive V_M has no impact on the final step of Ca^{2+} release from the stores and also not on the parameters governing activation and inactivation of the Cl^- channels. The stimulating role for positive V_M must hence be associated with an earlier step in a serial sequence promoting Ca^{2+} release. In this respect, it would be consequent to suggest that positive V_M promotes the filling of the internal stores, because positive V_M shortens the time between individual transient Cl^- currents (Thiel et al., 1993). This view is consistent with the finding that the postulated internal stores involved in excitation can be refilled via Ca^{2+} influx from the external medium in a time dependent manner (Plieth et al, 1997; Kikuyama & Tazawa, 1983).

The population of the smallest mpc should reflect activation of one ''quantum'' of Cl^- channels. Previously, it was shown that small transient Cl^- currents are composed of a mixture of 17 pS and 38 pS Cl^- channels (Thiel et al., 1993; Homann & Thiel, 1994). Considering that the unitary population of mpc at 0 mV is about 10 pA, it can be estimated, that a singular Ca^{2+} discharge activates in the extreme case either 7 channels of the 17 pS type or 3 of the 38 pS type. Considering a mean patch area of 10 μm^2 (Thiel et al., 1993) a uniform discharge of 1 quantum of Ca^{2+} would cause a peak Cl^- current of 1 Am^{-2}. For comparison, conventional space clamp recordings of the Cl^- excitation current have estimated a mean Cl^- peak current of approximately 5 A m^{-2} (Homann & Thiel, 1994). Since both the cell attached- and the whole cell measurements were carried out with 50 mM $CaCl_2$ in the external medium, it must be assumed that a uniform single quantum discharge of Ca^{2+} is not sufficient to activate an adequate number of Cl^- channels to explain the large transient currents during excitation.

According to the view of a quantal release of a gating factor as a trigger for Cl^- channels, it should be expected that larger current transients are composed of the sum of unitary size transients. However, in all distribution histograms, the mean currents of the second order transient and the intervals between subsequent transients were larger than the sum of two mean currents of the first order transient. This phenomenon could be explained by a model in which one Ca^{2+} release site functions as a nucleation point for stimulating release from adjacent sites. This would lead to the summation of Ca^{2+}_{cyt} when discharges in sufficiently short intervals from adjacent sites. An exemplary one-dimensional solution to this synergistic action is given in Fig. 8. Assuming a Gaussian Ca^{2+}_{cyt} concentration profile around a virtual single Ca^{2+} release site (unitary amplitude; 1.5 μm variance; i.e., Nelson et al., 1995), the effect of Ca^{2+} discharge from multiple sites (here 5 sites) can be conceived to overlap if the release sites are in sufficient proximity (here 1.5 μm gap between sites). The resulting summation in Ca^{2+}_{cyt} concentration is illustrated in Fig. 8*B*. Considering now further that Cl^- channels require a minimum concentration of Ca^{2+}_{cyt} for activation (Shiina & Tazawa, 1988; Okihara et al., 1991) the number of equally distributed Ca^{2+} sensitive Cl^- channels activated upon release from either a single or from multiple sites will be proportional to the area in excess of the threshold concentration under the Gaussian curve or under the sums of Gaussian curves respectively. By choosing in the present example a Ca^{2+}_{cyt} threshold for Cl^- channel activation at 39% of the peak amplitude for a unitary Ca^{2+} release, the effective areas under the Gaussian curves are 3, 5, 7 and 9 times that of the area due to singular release. This ratio is similar to that obtained between the unitary peak current and higher order mpcs (Fig. 8*C*). It could hence offer an explanation for the unusual distribution. Notably, the same ratios of areas can be obtained with other combinations of threshold values and gaps between release sites. The hyperbolic relation between both parameters given two asymptotic values: (i) single sites can not be more than 4 μm apart and (ii) the threshold level can not be smaller than 35.5% of the maximum Ca^{2+} release achieved by a single release. As yet, this one-dimensional model is simplistic but it becomes more plausible in the context of the finding that single Ca^{2+} release sites in *Xenopus* oocytes are in comparable proximity and that release from one site triggers a quasi-immediate release from adjacent sites if

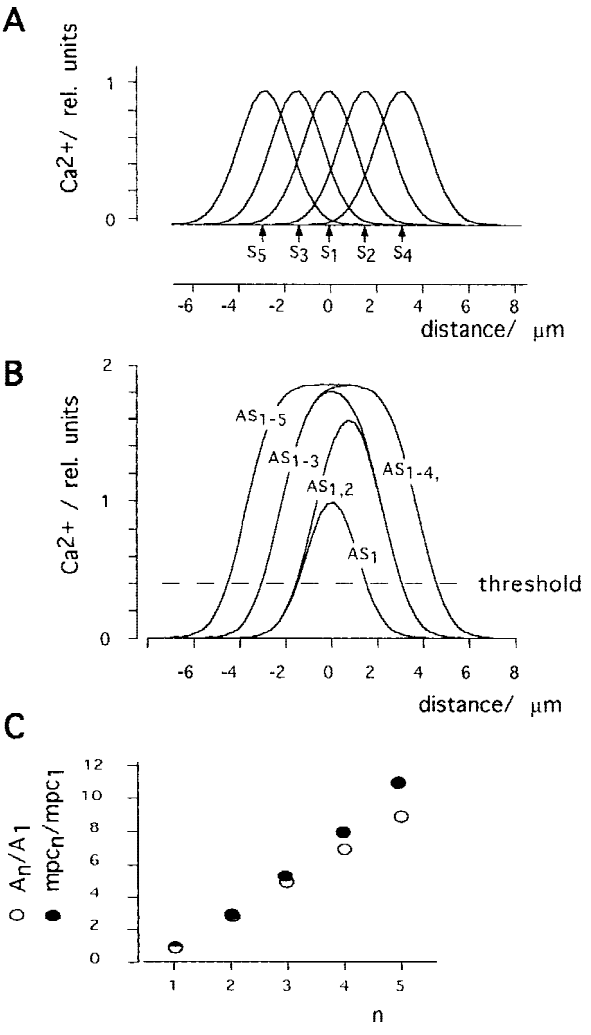

Fig. 8. Model to explain synergistic effect of putative simultaneous Ca^{2+} release from adjacent sites on the gating of Ca^{2+} sensitive Cl^- channels. (*A*) Release of one quantum of Ca^{2+} from each of 5 virtual point sources (S1–S5) at 1.5 μm distance gives rise to overlapping Gaussian concentration profiles for Ca^{2+} with a unitary amplitude and a variance of 1.5 μm around each release site (*see* Nelson et al., 1995). (*B*) Gaussian concentration profile for release of Ca^{2+} from one site (AS_1) and sum of Gaussian concentration profiles ($AS_1 + AS_n$) caused by concomitant release of Ca^{2+} from *n* sites (1–5) respectively. Dashed line represents imaginary threshold of Ca^{2+} concentration that must be exceeded in order to activate Cl^- channels. Effective Ca^{2+} concentration for Cl^- channel activation is calculated as area (*A*) under the sum of Gaussian curves in excess of threshold level. (*C*) Relative increase of area (in excess of threshold) under Gaussian curves (○) for multiple site release (A_n) over single site release (A_1) and relative increase of higher order mps (mpc_n) over smallest mpc (mpc_1) (●) (mpc data from Fig. 2*B*).

the distance is smaller than 6–7 μm (Yao, Choi & Parker, 1995).

The activity of Ca^{2+}-stimulated ion channels is generally tightly correlated with the concentration of cytoplasmic Ca^{2+} (Hille, 1992). Hence, in the present case the amplitudes of transient Cl^- currents should provide an indirect measure for the mechanism of local Ca^{2+}-release. In this context it can be concluded from the present data that the activation of transient Cl^- currents is not due to an activation of a Ca^{2+} channel. The stochastic open life time of channels (Hille, 1992) should cause an exponential distribution of the amount of released Ca^{2+} and hence an exponential distribution of the amplitudes of transient Cl^- currents. The finding of a Gaussian distribution requires an explanation for Ca^{2+}-release other than through a stochastically operating Ca^{2+} channel. A similar conclusion was drawn from the amplitude distribution of single Ca^{2+} puffs in *Xenopus* oocytes, a phenomenon which was also not consistent with Ca^{2+} release through stochastically operating Ca^{2+} channels (Yao et al., 1995).

Transient membrane currents as a consequence of spatially restricted sparklike Ca^{2+}-release events similar to those assumed here, have recently been described in a number of animal cell types (e.g., Benham & Bolton, 1986; Jafri et al., 1992; Nelson et al., 1995; Lipp & Niggli, 1996; Thorn, Moreton & Berridge, 1996). They appear to comprise a general feature of $InsP_3$ signaling in different types of cells (Yao et al., 1995). In this context, it can be expected that relevant stimuli, such as positive voltage initiate global Cl^- channel activation in *Chara* cells during an AP by stimulating the frequency of localized sparklike Ca^{2+} release from internal stores. In neurones, a voltage-sensitive Ca^{2+} channel at the plasma membrane acts as an indirect voltage sensor for Ca^{2+} release from internal stores (Chavis et al., 1996). In analogy, also in *Chara* a small voltage-induced Ca^{2+} influx may be functionally coupled to the release of Ca^{2+} from internal stores. The presence of $InsP_3$-sensitive Ca^{2+} stores in *Chara* (Thiel et al., 1990), the known Ca^{2+} sensitivity of $InsP_3$-triggered Ca^{2+} release (*rev.* Berridge, 1993), and the quantal nature of $InsP_3$-induced Ca^{2+} release (Muallem, Pandol & Becker, 1989; Parker & Ivorra, 1990) offer a plausible link between Ca^{2+} influx across the plasma membrane and Ca^{2+} release from internal stores during the AP in *Chara.*

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 523). I am grateful to Dr. H. Lühring (Jülich) for help with the manuscript and to Drs. D. Gradmann and E. Neher (Göttingen) for helpful suggestions.

References

Benham, C.D., Bolton, T.B. 1986. Spontaneous transient outward current in single visceral and vascular smooth muscle cells of rabbit. *J. Physiol.* **381:**385–406

Beilby, M.J. 1984. Calcium and action potentials. *Plant Cell and Environment* **7:**415–421

Beilby, M.J., Coster, H.G.L. 1979. The action potential in *Chara corallina.* II Two activation-inactivation transients in voltage clamps of the plasmalemma. *Austr. J. Plant Physiol.* **6:**323–335

Berridge, M.J. 1993. Inositol trisphosphate and calcium signaling. *Nature* **361:**315–325

Bolshakov, V.Y., Golan, H., Kandel, E.R., Seigelbaum, S.A. 1997. Recruitment of new sites of synaptic transmission during the cAMP-dependent late phase of LTP at CA3-CA1 synapses in the hippocampus. *Neurone* **19:**635–651

Chavis, P., Fagni, L., Lansman, J.B., Bockaert, J. 1996. Functional coupling between ryanodine receptor and L-type calcium channels in neurons. *Nature* **382:**719–722

Dityatev, A.E., Kozhanov, V.M., Gapanovich, S.O., Clamann, H.P. 1994. Quantal analysis based on spectral methods. *Pfluegers Arch.* **429:**22–26

Jafri, M.S., Vajda, S., Pasik, P., Gillo, B. 1992. A membrane model for cytosolic calcium oscillations. *Biophys. J.* **63:**235–246

Hille, B. 1992. Ionic channels of excitable membranes. Sinauer Associates, Sunderland, MA

Homann, U., Thiel, G. 1994. Cl^- and K^+ channel currents during the action potential in *Chara.* Simultaneous recording of membrane voltage and patch current. *J. Membrane Biol.* **141:**297–309

Kikuyama, M., Tazawa, M. 1983. Transient increase of intracellular Ca^{2+} during excitation of tonoplast-free *Chara* cells. *Protoplasma* **117:**62–67

Kikuyama, M., Shimada, K., Hiramoto, Y. 1993. Cessation of cytoplasmic streaming follows an increase of cytoplasmic Ca^{2+} during action potential in *Nitella. Protoplasma* **174:**142–146

Lipp, P., Niggli, E. 1996. A hierarchical concept of cellular and subcellular Ca^{2+}-signaling. *Prog. Biophys. Molec. Biol.* **65:**265–296

Lunevsky, V.Z., Zherelova, O.M., Vostrikov, I.Y., Berestovsky, G.N. 1983. Excitation of Characeae cell membranes as a result of activation of calcium and chloride channels. *J. Membrane Biol.* **72:**43–58

Muallem, S., Pandol, S.J., Beeker, T. 1989. Hormone-evoked calcium release from intracellular stores is a quantal process. *J. Biol. Chem.* **264:**205–212

Nelson, M.T., Cheng, H., Rubart, M., Santana, L.F., Bonev, A.D., Knot, H.J., Lederer, W.J. 1995. Relaxation of arterial smooth muscle by calcium sparks. *Science* **270:**633–637

Okihara, K., Ohkawa, T., Tsutsui, I., Kasai, M. 1991. A calcium-dependent and voltage-dependent chloride-sensitive anion channel in the Chara plasmalemma: a patch-clamp study. *Plant Cell Physiol.* **32:**593–602

Parker, I., Ivorra, I. 1990. Localized all-or-none calcium liberation by inositol trisphosphate. *Science* **250:**977–979

Plieth, C., Sattelmacher, B., Hansen, U.-P., Thiel, G. 1998. The action potential in Chara- Ca^{2+} release from internal stores visualized by Mn^{2+}-induced quenching of fura-dextran. *The Plant Journal* **13:** 167–175

Shiina, T., Tazawa, M. 1987. Ca^{2+}-activated Cl^- channel in the plasmalemma of Nitellopsis obtusa. *J. Membrane Biol.* **99:**135–146

Shiina, T., Tazawa, M. 1988. Ca^{2+}-dependent Cl^- efflux in tonoplast-free cells of *Nitellopsis obtusa. J. Membrane Biol.* **106:**135–139

Tazawa, M., Yoko-o, T., Mimura, T., Kikuyama, M. 1994. Intracellular mobilization of Ca^{2+} and inhibition of cytoplasmic streaming induced by transcellular osmosis in internodal cells of *Nitella flexilis. Plant Cell Physiol.* **35:**63–72

Thorn, P., Moreton, R., Berridge, M. 1996. Multiple, coordinated Ca^{2+} release events underlie the inositol trisphosphate-induced local Ca^{2+} spikes in mouse pancreatic acinar cells. *EMBO J.* **15:**999–1003

Thiel, G., MacRobbie, E.A.C., Hanke, D.E. 1990. Raising the intracellular level of inositol 1,4,5-trisphosphate changes plasma membrane ion transport in Characean alga. *EMBO J.* **9:**1737–1741

Thiel, G., Homann, U., Gradmann, D. 1993. Microscopic elements of electrical excitation in *Chara:* transient activity of Cl^- channels in the plasma membrane. *J. Membrane Biol.* **134:**53–66

Thiel, G. 1995. Dynamics of chloride and potassium currents during the action potential in *Chara* studied with action potential clamp. *Eur. Biophs. J.* **24:**85–92

Thiel, G., Homann, U., Plieth, C. 1997. Ion channel activity during the action potential in *Chara:* new insight with new techniques. *J. Exp. Bot.* **48:**609–622

Yao, Y., Choi, J., Parker, I. 1995. Quantal puffs of intracellular Ca^{2+} evoked by inositol triphosphate in *Xenopus* oocytes. *J. Physiology* **482:**533–553