Ca^{2+}-Induced Activation and Irreversible Inactivation of Chloride Channels in the Perfused Plasmalemma of *Nitellopsis obtusa*

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Abstract. Experiments were carried out on the algal cells with removed tonoplast using both continuous intracellular perfusion and voltage clamp on plasmalemma. The transient plasmalemma current induced by depolarization disappeared upon perfusion with the Ca^{2+}-chelating agent, EGTA, since the voltage-dependent calcium channels lost their ability to activate. Subsequent replacement of the perfusion medium containing EGTA by another with Ca^{2+} for clamped plasmalemma (-100 mV) induced an inward Cl^- current which showed both activation and inactivation. The maximal amplitude of the current at $[Cl^-]_{in} = 15$ mmol/l (which is similar to that in native cells) was approximately twice that in electrically excited cell *in vivo*. The inactivation of Cl channels in the presence of internal Ca^{2+} was irreversible and had a time constant of 1—3 min. This supports our earlier suggestion (Lunevsky et al. 1983) that the inactivation of Cl channels in an intact cell (with a time constant of 1—3 s) is due to a decrease in Ca^{2+} concentration rather than to the activity of their own inactivation mechanism. The Cl channel selectivity sequence was following: $Cl^- \gg CH_3SO_4^- \approx K^+ \gg SO_4^{2-}$ ($P_K/P_{SO_4} \sim 10$). Activation of one half the channels occurs at a Ca^{2+} concentration of 2×10^{-5} mol /l. Sr^{2+} also (though to a lesser extent) activated Cl channels but had to be present in a much more higher concentration than Ca^{2+}. Mg^{2+} and Ba^{2+} appeared ineffective. Ca^{2+} activation did not, apparently, require participation of water-soluble intermediator including ATP. Thus, Cl channel functioning is controlled by Ca^{2+}-, Sr^{2+}-sensitive elements of the subplasmalemma cytoskeleton.

Key words: *Characeae* — Perfused plasmalemma — Chloride channel — Activation-inactivation by Ca^{2+} — Subplasmalemma cytoskeleton

Introduction

In our earlier studies we have determined how electrical excitation in *Characeae* cells develops (Berestovsky et al. 1976; Lunevsky et al. 1977, 1983). Both, the plasmalemma and tonoplast were found to have similar ionic channels. When the voltage on any membrane exceeds the excitation threshold, Ca channels become

Table 1. Composition of various perfusion media containing EGTA

	Medium		
	Cl	Low Cl	MS
EGTA	3	3	3
KCl	150	15	–
KCH_3SO_4	–	–	150
Tris-HCl	5	–	–
HEPES	–	1	1
Sucrose	–	270	–
pH	7.4	7.1	7.1

Concentration is given in mmol/l.

activated and Ca^{2+} ions enter the cytoplasm either from the external medium or from the vacuole. Ca^{2+} ions activate the Cl channels in both membranes regardless of the voltage on the membrane. Ca channels become inactivated and the Cl channels close. Moreover, depolarization of plasmalemma activates K channels (Kitasato 1973; Sokolik and Yurin 1981) which produce an outward K^+ current. The inward Ca^{2+} and Cl^- currents (with their activation-inactivation kinetics) combine with the outward K^+ current to produce action potential. The existence of a fast (cation) component in the transient inward current was also demonstrated by Beilby and Coster (1979).

The properties of Ca channels have been studied rather comprehensively in living *Characeae* cells (Lunevsky et al. 1977, 1980, 1983; Volkov et al. 1979) as well as in experiments with the reconstitution of single channels in planar lipid membranes (Aleksandrov et al. 1976; Aleksandrov 1983; Lunevsky et al. 1980; Volkova et al. 1980). Properties of the Cl channels are less known. In particular, following questions have not yet been solved. (i) Do Cl channels possess an own inactivation mechanism or does their inactivation result from a decrease of cytoplasmic free Ca^{2+} ions due to Ca^{2+} adsorption by proteins and uptake by mitochondria, vacuole, etc. (Lunevsky et al. 1983)? (ii) What is the relationship between the concentration of free Ca^{2+} and the number of open channels? According to our estimates (Lunevsky et al. 1983), the Ca^{2+} concentration in cytoplasm reaches during the action potential generation 2×10^{-5} mol/l (being $< 10^{-6}$ mol/l at rest). (iii) Is the interaction of Ca^{2+} ions with Cl channels direct or is it mediated by a chain of biochemical reactions.(iv) Is ATP necessary for activation of Cl channels?

In the present study the above questions were investigated on giant cells of the fresh-water alga *Nitellopsis obtusa*. The tonoplast was removed. The cells were continuously perfused and the plasmalemma was set up for voltage clamp. This

Table 2. Composition of CA-Mg-EDTA (EGTA) buffer used to maintain low level of free Ca^{2+}

Ca^{2+}	$(2-3)\times10^{-3}$	$(6-9)\times10^{-3}$	$(2-3)\times10^{-2}$	$(0.8-1.0)\times10^{-1}$
$Ca(OH)_2$	4.5	0.22	0.86	2.0
$MgCl_2$	1.05	1.05	1.05	1.05
EGTA	5	–	–	–
EDTA	–	5	5	5

Concentration is given in mmol/l, pH 7.1.

approach allowed a control of the intracellular concentrations of Ca^{2+} and other substances near the plasmalemma.

Materials and Methods

Algae, *Nittellopsis obtusa*, were collected from the Valdai lake during September and stored in aquaria filled with artificial pond-water containing (in mmol/l): KCl 0.1; NaCl 1.0; $CaCl_2$ 0.5; pH 0.8—8.1. Experiments were carried out on internodal cells of 0.7 to 0.8 mm in diameter.

Sucrose was used as the main osmotic component of the external bathing solution and perfusion medium (PM). The external medium and PM were made, approximately, isotonic to solutions with 0.2 and 0.3 mol/l sucrose, respectively. The difference in osmotic pressure stabilized the plasmalemma in the course of intracellular perfusion. The free Ca^{2+} concentration in PM was controlled with EGTA or EDTA (Ca^{2+} — chelating agents) buffers. The perfusion media containing EGTA (EG-PM) are listed in Table 1. To activate the Cl channels the same media used with EGTA replaced by 1 mmol/l $CaCl_2$ (Ca-PM). The concentration of free Ca^{2+} ($10^{-6}-10^{-4}$ mol/l) was obtained using no buffer or according to the method of Portzehl et al. (1964) using the buffer system Ca-Mg-EDTA (or EGTA). The composition of this buffer system is given in Table 2. Also this system was a component of the perfusion medium which contained (in mmol/l): KCl 13; sucrose 240; HEPES 20; pH 7.1. For additional checking of free Ca^{2+} a calcium electrode (Orion, USA) was used. Binding of Ca^{2+} to methyl sulphate anions was calculated according to Pollard et al. (1977).

The tonoplast was removed from the cells by washing with saline PM containing 3 mmol/l EGTA, as described by other authors (Williamson 1975; Tazawa et al. 1976; Shimmen and Tazawa 1977; 1980; Sanders 1980; Smith and Walker 1981). The free Ca^{2+} level was less than 10^{-7} mol/l. When PM contains a concentration of free Ca^{2+} similar to that in vacuolar sap (≈ 10 mmol/l), the tonoplast cannot be removed even by long-term perfusion (Tazawa 1964; Berestovsky et al. 1973 a, b). It seems likely that Ca^{2+} stabilizes both the tonoplast itself and its adhesion contact with the plasmalemma occurring at two narrow (5—10 μm) zones (ZC) running like a ribbon along the entire length of the cell (Fig. 1).

The tonoplast removal procedure was as follows: an isolated cell was slightly blotted to decrease its turgor, placed into the chamber grooves (Fig. 2) and sealed with vaseline. Compartment B was then filled with the external medium. When the cell had almost completely lost its turgor, both ends were cut off and compartments A and D (each 2 cm^3 in volume) were filled in succession with the perfusion media.

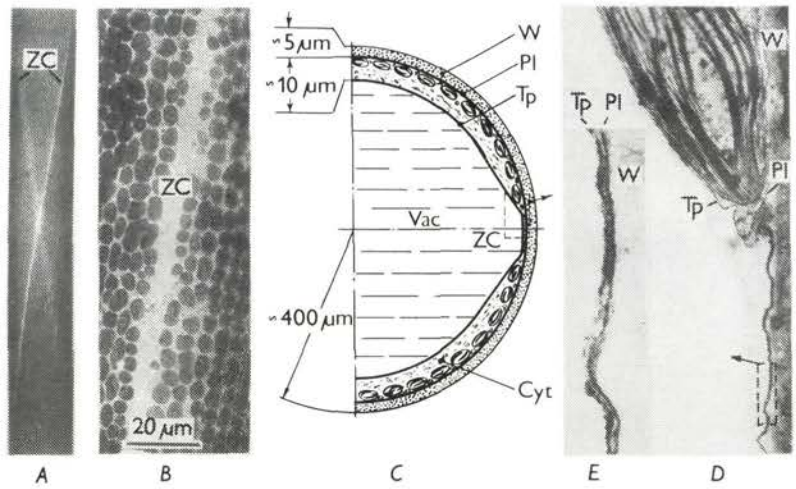

Fig. 1. General view of a segment (*A*) and a patch of the surface (*B*) of a *Nitellopsis obtusa* internodal cell and its cross section (*C*, *D*, *E*). The cell has two diametrically opposite chloroplast-free zones of adhesion contact (ZC) between the tonoplast (Tp) and the plasmalemma (Pl) running like a screw ribbon 5—10 μm in width (*A*, *B*) along the entire cell length; *D*, *E* — ultrastructure of ZC. W, Vac, Cyt — wall, vacuole and cytoplasm, respectively.

The liquid level difference in these two compartments determined the direction of the perfusion. During the first 10—15 min (incubation period) the perfusion of the vacuole with EGTA solution was slow with a subsequent slight increase in the perfusion rate. The removal of the tonoplast without plasmalemma damage was achieved at a liquid level difference of less than 1 mm (*Chara* cells will withstand differences in level up to 6—7 mm (Williamson 1975; Sanders 1980)). The process of tonoplast and cytosol removal was checked visually using a stereomicroscope, and it was assumed to be complete when intensive extrusion of membrane vesicles and the bulk of cytoplasmic granules from a cell ceased. Single granules were observed in perfusate outflow throughout the experiment and this was used to check the perfusion rate and direction.

The perfusion solution in the working section of a cell was exchanged in the course of an working section of a cell was exchanged in the course of an experiment as follows. Initially, compartments A and D were filled with different solutions and replacement of media inside the cell was carried out by changing the direction of the perfusate flow. To maintain a constant rate of perfusion (50—200 μm/s), 10—30 μl doses of solution were systematically added to one of the compartments A or D, or in the case of overfilling, appropriate doses of liquid were withdrawn from the corresponding compartment.

A constant perfusate flow was required to maintain a prefixed concentration of ions at the working section of the cell membrane. During prolonged current flow the ionic concentrations near the membrane surface would change significantly (see Appendix). At the outer membrane surface similar effects may develop but to a lesser extent due to the cylindrical shape of cells and to the large volume of the external solution. To minimize these concentration alternations, the external solution was exchanged by perfusion when large currents were initiated.

The membrane was voltage-clamped using 4 electrodes (Smith and Walker 1981) and an intracellular preamp-clamp (Model 8500, Dagan, USA). Membrane potential (V_m) was recorded using

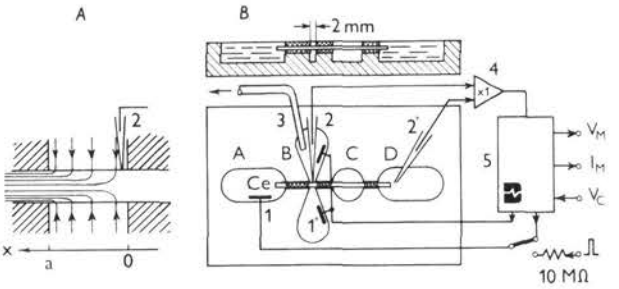

Fig. 2. The electric lay-out and the experimental chamber used for cell perfusion under voltage clamp conditions. Compartments A and D were filled with the perfusion medium (PM), *B* — with bathing solution, and compartment C contained air. 1,1′ — Ag/AgCl current electrodes, 2,2′ — voltage electrodes (glass micropipettes), 3 — outlet tube for the bathing solution, 5 — voltage-clamp circuit equipped with probe 4. *A* — scheme for current flow through a cell, Ce, in the working compartment B.

micropipettes tip diameter 5—10 μm filled with agar containing 100 mmol/l KCl (Fig. 2). The tip of electrode 2 was placed near the cell surface on the right side of the cell working section (close to compartment C). The portion of the cell sited between compartments D and B served as prolonged electrode 2′. Compartment C with air insulation was inserted between compartments D and B to prevent electrical coupling along the cell wall. Ag/AgCl current electrodes were placed in compartments A and B, respectively. This arrangement of current and voltage electrodes and the rather narrow (2 mm) working section of the cell isolated with vaseline gaps in compartment B provided a quite satisfactory temporal and spatial control of membrane voltage (Lunevsky et al. 1983). It follows from general considerations that the higher the conductance of the intracellular medium and the lower the conductance of both the cell membrane and the external solution, the less the relative voltage difference $(V_m(a) - V_m(o))/V_m(o)$ between two boundary points of working section of the cell (Fig. 2*A*). With respect to this, we usually used external solutions of low ionic strength. To decrease membrane current density, a relatively impermeant anion, methyl sulphate, as used.

To measure the membrane conductance and current reversal potentials, rectangular and ramp voltage pulses of 1 s duration were applied. When the membrane conductance changed during the command pulse, the duration of the voltage pulse was decreased to 20—40 ms. A pen recorder and a storage oscilloscope were used to record the voltage.

All experiments were conducted at room temperature (20—22 °C).

Generally the membrane and Cl channel selectivity were characterized by a permeability ratio as calculated by the Goldman equation for electrolytes with monovalent cations and mono- or bivalent anions.

Results

Perfusion of a cell with EGTA-containing solution resulted in a changed membrane voltage (Fig. 3). Initially, the membrane was depolarized, followed by hyperpolarization. Finally, the membrane voltage stabilized at a constant level $-(100 \div 120$ mV). Spontaneous spike generation was often present during the depolarization phase. As a rule the resistance of the membrane gradually increased

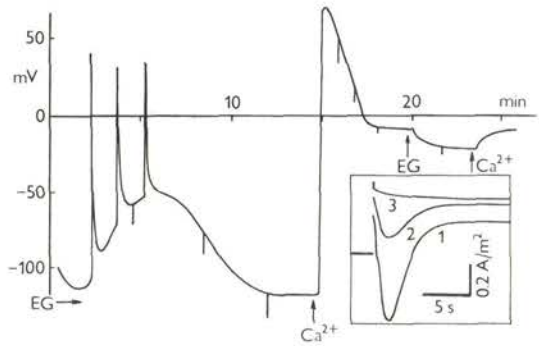

Fig. 3. Alterations in membrane potential, V_m, in the course of the tonoplast removal with EGTA perfusion medium (EG) (in mmol/l): EGTA 3; KCl 150; Tris-HCl 5; pH 7.4) and after its replacement by the same solution containing 1 mmol/l Ca^{2+} instead of EGTA. The external medium contained (in mmol/l): LiCl 1.0; $CaCl_2$ 0.25; KCl 0.1; sucrose 180; pH 7.2. Rectangular (0.5 s) current pulses were applied to the cell prior to (amplitude 0.1 μA) and after Ca^{2+} introduction (amplitude 1 μA). Inset: oscillograms of the transient current under voltage clamp conditions (holding potential -120 mV, voltage command step $+70$ mV) show the disappearance of electrical excitability ($1 \rightarrow 3$) along with the tonoplast removal. Curve 3 corresponds to the phase when the plasmalemma voltage stabilizes at a steady-state level (the interval between 12 and 15 min).

and reached 0.8—1.2 Ohm.m^2 (8—12 kOhm.cm^2) by the end of the hyperpolarization phase, similarly as in *Chara corallina* (Smith and Walker 1981). The membrane hyperpolarization coincided in time with the start of intensive extrusion of membrane vesicles and cytoplasmic granules from the cell which ceased at the midpoint of the hyperpolarization phase; also transient membrane currents produced by voltage clamp disappeared (Fig. 3 inset). A comprehensive analysis has shown that both the slow (chloride) component of the transient current and the fast cation component (with activation and inactivation times of 0.1 and 0.5 s, respectively) generated by voltage-dependent calcium channels (Lunevsky et al. 1980, 1983) have disappeared during the perfusion with EGTA. The disappearance of the transient current upon perfusion was therefore used as a criterion of tonoplast removal from the cell. The loss of cell excitability upon perfusion with an EGTA solution in the absence of ATP and Mg^{2+} was also observed by Shimmen and Tazawa (1977, 1980).

The replacement in PM of EGTA by 1 mmol/l Ca^{2+} led to a fast depolarization of the plasmalemma up to $+70$ mV followed by repolarization and a drop in its resistance by more than an order of magnitude (in the presence of 150 mmol/l KCl in PM with Ca^{2+}, the resistance often fell to 0.01 Ohm.m^2) (Fig. 3, right panel). The average peak value of membrane depolarization under the conditions shown in Fig. 3, was 68 ± 2 mV. The calculated value of the equilibrium potential

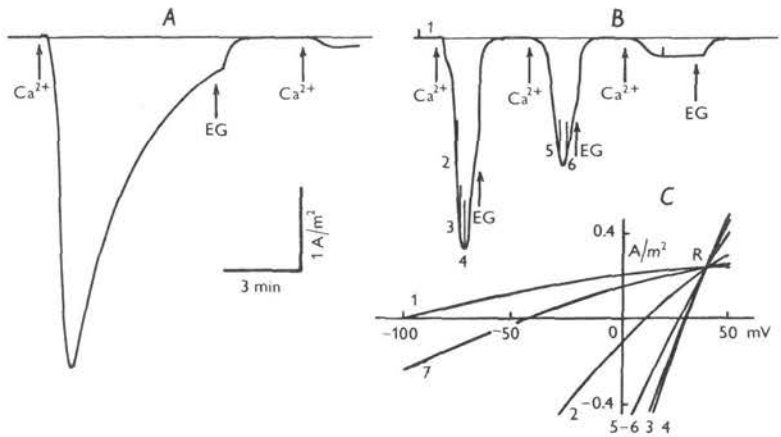

Fig. 4. *Activation of plasmalemma chloride conductance by intracellular Ca^{2+} ions after tonoplast removal. A, B* — records of transient currents (in two different cells) under voltage clamp at −120 mV (*A*) and −100 mV (*B*). *Vertical bars (numbered in B) show the moments of application of ramp-wave* voltage pulses (150 mV, 30 ms) to register the instantaneous voltage-current curves (IVC). *C* — a set of *instantaneous IVC corresponding to the currents recorded in B. Curve 1 was obtained prior to the* application of Ca^{2+} solution. Point R corresponds to the reversal potential (V_r) of the chloride current. *The internal perfusion medium (PM) contained (in mmol/l):* KCl 15; *sucrose* 270; HEPES 1.0; *and* $CaCl_2$ 1.0, pH 7.1 (Ca-PM), or EGTA 3.0; pH 7.5 (EG-PM). Bathing solution: NaCl 0.5; KCl 0.08; *sucrose* 180; HEPES 1.0, *pH* 7.0. *Calibration bars refer to A and B.*

for Cl^- ions, $E_{Cl} = 110$ mV (with Cl^- activities taken into account). The selectivity of the membrane (the chloride channels activated by Ca^{2+} plus the potassium channels) under the same conditions was high as shown by the permeability ratio: $P_{Cl}/P_K = 17$. However, the selectivity is substantially dependent on the concentration of penetrating ions (see below).

A voltage change similar to that shown in Fig. 3 was observed upon the perfusion of the cell with low Cl and MS solutions; however, both the voltage and resistance values in extreme points were different.

Most of our experiments were conducted under voltage clamp after the tonoplast had been removed and V_m had stabilized at a steady-state level. A typical example of current alterations induced by solution exchange (similar to that shown in Fig. 3) is given in Fig. 4*A*. The only anion present was Cl^- and its concentration (17 mmol/l) in PM was similar to that in native cell cytoplasm (see Table 1 in Lunevsky et al. 1983). Introduction of Ca^{2+}-containing solution induced an inward transient current with distinct activation-inactivation kinetics (Fig. 4*A*). Exchange of the solution for EGTA caused the current to drop practically to zero. If the exposures to the Ca^{2+} solution were relatively short, a series of transient currents

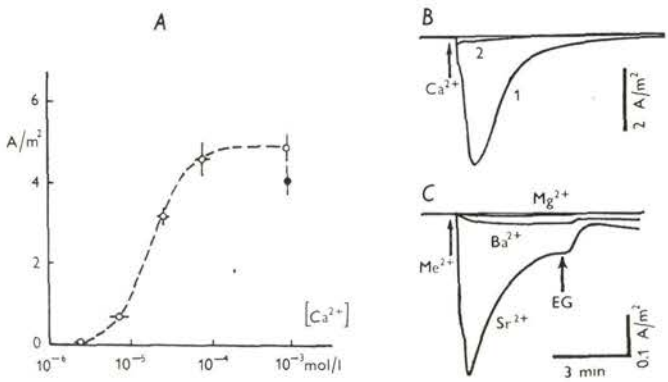

Fig. 5. *A* — Dependence of transient current peak value on Ca^{2+} concentration in the perfusion medium (PM). (o) — PM_{Cl} (in mmol/l): KCl 13; sucrose 240; HEPES 20 + Ca-Mg-EDTA buffer (Table 2), pH 7.1. External medium: NaCl 0.5, sucrose 160; HEPES 1.0; pH 7.1. (●) — PM_{MS}: KCH_3SO_4 150; $Ca(OH)_2$ 1.0; HEPES 1.0; pH 7.0. External medium: $NaCH_3SO_4$ 1.5; KCH_3SO_4 0.1; sucrose 180; pH 7.0. The holding potential, $V_m = -100$ mV. Each point on the graph was obtained for no less than 5 cells. *B* — Inhibitory effect of ethacrynic acid (EA) on transient current induced by 1 mmol/l Ca^{2+}. 1 — control; 2 — after incubation with EA (another cell). PM and outward medium correspond to those in legend to Fig. 4, the only difference being that 20 mmol/l HEPES and 10^{-4} mmol/l EA with NaOH (pH 7.1) were added to PM and outward medium, respectively. Cell 2 was treated with EA during 12 min prior to Ca-PM application, $V_m = -80$ mV. *C* — The current induced by Me^{2+} – PM. Sr^{2+} curve: Low Cl EG-PM was replaced by the same solution containing 0.5 mmol/l EGTA + 10 mmol/l $SrCl_2$, pH 7.1. Mg^{2+}, Ba^{2+} curves: Cl EG-PM was replaced by the same medium but with 0.5 mmol/l EGTA + 10 mmol/l $MgCl_2$ or $BaCl_2$, pH 7.2. External medium for all cases was (in mmol/l): KCl 0.1; NaCl 1.0; EGTA 0.2; sucrose 180; Tris-HCl 10; pH 7.2. $V_m = -(100 \div 120)$ mV.

decreasing in amplitude were evoked (Fig. 4*B*). This suggests that Cl channels become inactivated only in the presence of Ca^{2+} ions. The channels inactivated irreversibly, and no restoration could be observed at least during subsequent 20—30 min. The inactivation time constant varied from 1 to 3 min, and the activation time was perfusion rate-dependent. Response of Cl channels to the addition of Ca^{2+} into PM did not depend on the duration (up to 20 min) of the cell preperfusion (during which the tonoplast had been removed) with EGTA solution through all water-soluble endogenous components including ATP become lost by the cell.

During the exchange of EG-PM for CA-PM solution a pronounced acidification should have occurred at the border of these solutions. This acidic zone may have affected both the activation and inactivation of Cl channels. To test this assumption, experiments were performed with low Cl solutions containing 20 mmol/l HEPES (instead of 1 mmol/l). In this case, mixing of equal EG-PM volumes (3 mmol/l EGTA) and CaPM (1 mmol/l $CaCl_2$) changed pH by as little as 0.2. Perfusion of cells with highly buffered PM induced transient currents (e.g. Fig.

5*B*) similar to those (within the limits of normal dispersion) shown in Fig. 4. Due to this PM listed in Table 1 were used in most of our experiments.

Instantaneous current-voltage curves (IVC) recorded prior to the appearance of the transient current (curve 1) and during its development are shown in Fig. 4*C*. Note that all instantaneous IVCs of the membrane with activated channels intersect at a single point R lying on curve 1.

Based on the above data, following conclusions may be drawn. (1) At all stages of its development, the transient current is produced by a single channel population with a constant ionic selectivity; this fact is demonstrated by the intersection of all IVCs at the same point determining the reversal potential (V_r) of the transient current. (2) Ca^{2+} has no effect on potassium channels in the resting membrane since the point R lies on the initial I—V curve 1 for these channels. (3) At the resting potential ($V_m = -120$ mV) which corresponds to the reversal potential for the potassium current (zero-current potential), the transient current passed only through Ca^{2+}-activated chloride channels. This current was carried only by anions since in this case, the holding membrane voltage was close to the equilibrium potential for cations.

Experiments with ethacrynic acid also suggested that the transient current was mediated by Ca^{2+} activation of Cl channels. Earlier it was shown in native algal cells (Lunevsky et al. 1980, 1983) that ethacrynic acid is an effective inhibitor of chloride channels. In experiments with perfused cells ethacrynic acid (10^{-4} mmol/l) was added to the outer solution in the working section B (Fig. 2*B*). After 10—12 min cell incubation in the above solution, replacement of EG-PM Ca-PM induced a transient current which was by one order of magnitude smaller as compared to that in control (Fig. 5*B*). The inhibitory effects of ethacrynic acid were similar for both perfusion media, low Cl and MS (Table 1).

The differential membrane resistance, r_m, as measured at the peaks of the currents at the holding potential, was 250 and 400 Ohm.cm^2 (0.025 and 0.040 Ohm.m^2) for A and B in Fig. 4, respectively. The majority of cells in similar solutions had an intermediate value of r_m. In these solutions, the reversal potential, $V_r \approx 40$ mV, and the corresponding selectivity of chloride channels, i. e. $P_{Cl}/P_K = 5.3$, was 3 times lower than that for the membrane in PM with 150 mmol/l KCl (see above). On the other hand, when 0.5 mmol/l NaCl in the external medium was replaced by 3 mmol/l ($MgCl_2$, the P_{Cl}/P_K ratio for chloride channels increased up to 7. This increase in selectivity for anions in the presence of bivalent cations in the external medium, which was also found with other solutions may have been due to a decrease in the negative membrane surface charge induced by these cations (Sokolik and Yurin 1981). The least permeant anions through the chloride channels were $CH_3SO_4^-$ and SO_4^{2-} with membrane concentration gratients ($C_{in} : C_{out}$, mmol/l) of 150 : 1.5 and 25 : 1, respectively. The selectivity sequence was: $Cl^- \gg CH_3SO_4^- \approx K^+ \gg SO_4^{2-}$ ($P_K/P_{SO_4} \sim 10$), which is similar to that for the

anion-selective channel reconstituted in planar lipid bilayer membrane from *Paramecium* mitochondria (Schein et al. 1976).

The dependence of the peak value of the anion current on Ca^{2+} concentration showed saturation (Fig. 5*A*). The half-value of the peak current was reached at $(17 \pm 4) \times 10^{-6}$ mol/l Ca^{2+}. Our previous estimates (Lunevsky et al. 1983) have shown that Ca^{2+} concentration in the cell cytoplasm reaches approximately the same value (2×10^{-5} mol/l) during the action potential, when Ca^{2+} enters the cell through the plasmalemmal Ca channels activated during the depolarization. This may indicate that, during the action potential, approximately one half of the chloride channels became activated. In this case the peak current densities at $V_m = -(80 + 100)$ mV, usually did not exceed $2 \div 4$ A/m^2. Also these values nearly correspond to half magnitude of currents induced by direct intracellular application of Ca^{2+} ($\geqslant 10^{-5}$ mol/l) in PM containing 15 mmol/l Cl^-.

To determine the ionic specificity of the chloride channel activation mechanism, Mg^{2+}, Ba^{2+} and Sr^{2+} were tested. When the cell was in a Ca-free external medium with extra 50 μmol/l EGTA, neither Mg^{2+}, nor Ba^{2+} in PM were able to activate the chloride channels, even if given in a concentration of 10 mmol/l (Fig. 5*C*). Millimolar concentrations of Sr^{2+} ions induced transient currents similar in their kinetics to those activated by Ca^{2+} (Fig. 5*B*), but of an essentually lower amplitude. In the presence of Ca^{2+} in the external medium, intracellular perfusion with Mg^{2+} or Ba^{2+} solutions (millimolar concentrations) initiated transient currents, but the results were poorly reproducible. Mg^{2+} and Ba^{2+} seem to activate some cationic channels through which Ca^{2+} enters the cell interior and activates the chloride channels. A similar result has been reported for activation of tonoplast chloride channels by Mg^{2+} and Ba^{2+} in the extracellular medium (Vostrikov 1976; Lunevsky et al. 1983). These ions enter the cytoplasm through preliminary depolarized plasmalemma (up to $-30 \div -80$ mV) by an elevation of the external K^+ concentration and, they likely make the tonoplast permeable to Ca^{2+} ions. The latter enter the cytoplasm from the vacuole, where $[Ca^{2+}] \sim 10$ mmol/l, and activate the tonoplast chloride channels. The results obtained in the present study agree well with earlier findings of Findlay and Hope (1964) reported for algal cells *in vivo*. These authors showed that activation of the plasmalemma transient current requires external Ca^{2+} or Sr^{2+}, and that neither Mg^{2+} nor Ba^{2+} are effective.

Discussion

After the tonoplast removal by a continuous perfusion with an EGTA-containing saline solution, only the plasmalemma and the cortical layer of the cytoplasm (cytogel) with chloroplasts and mitochondria remain. The layer is 5 μm thick. It is natural to suppose that perfusion with EGTA solution for 10—20 min removes the bulk of water-soluble substances (including Ca^{2+}, ATP, and substrates of enzymatic

reactions) from the thin cortical layer. As a result, Ca^{2+} channels lose their electroexcitability (the fast component of the transient current disappears). However, chloride channels are still able to be activated by Ca^{2+} ions. It should also be noted that the cationic (calcium) component of the transient current disappears before V_m has reached its steady-state level (Fig. 3), i.e. within several minutes after the removal of the tonoplast. The similarities of the Ca^{2+} channels in algal cells and neurone somata (Lunevsky et al. 1980; 1983) suggest that cyclic nucleotides (Doroshenko et al. 1982) may control Ca^{2+} currents through algal membranes as well. The level of cyclic nucleotides in the cortical layer decreases sharply in the course of cell perfusion with EGTA solution, thus preventing the transition of Ca^{2+} channels into the open state.

Ca^{2+} ions play a dual role in controlling the chloride channels: they trigger activation and, later on, cause irreversible inactivation of the channels (Fig. 4*A*, *B*). The latter arises from the fact that EGTA substitution for Ca^{2+} in PM prevents irreversible inactivation. In principle, three ways of Ca^{2+}-dependent control of chloride channels are possible: (1) direct action on the channel-forming molecule; (2) Ca-induced release of some mediator from organelles (chloroplasts, mitochondria); (3) action on cytoskeletal components (microfilaments, microtubules) localized in the cortical layer, which might control mechanically switching (activation) of the channels. The possibility of combined effects of Ca^{2+} may also be suggested.

As for the first variant, we can say nothing definite at present. There is a serious objection against the second possibility because activation of plasmalemma chloride conductance under electrical excitation is rather fast (0.1—0.3 s), and it tracks the influx of Ca^{2+} ions through voltage-activated Ca^{2+} channels. This period of time is too short to account for any appreciable Ca-induced release of a hypothetical mediator. Moreover, in the algal rhizoids lacking chloroplasts the same transient chloride currents are generated as in green cells (unpublished data). Also it is very unlikely that Ca^{2+}-stimulated release of some mediators from organelles might cause slow inactivation of the channels, since the mediator concentration could not reach any appreciable value due to diffusion into the stream of PM.

The most promising working hypothesis, to our mind, is that cytoskeleton elements and, probably, some other macromolecules of cortical cytogel control the functioning of ionic channels in general (Matsumoto and Sakai 1979a; Berestovsky and Aleksandrov 1983) and chloride channels, in particular. A number of facts and considerations support this hypothesis.

All cells contain a submembrane cortical gel-like layer, which includes cytoskeletal elements, mainly bundles of microfilaments (Krasovskaya and Pavlenko 1977; Vasiliev and Gelfand 1981). Numerous microfilaments are attached to membrane integral proteins. Ionic channels, in turn, are also formed by integral

proteins. Microfilaments are arranged into bundles and ionic channels are also able to form clusters (Kazachenko and Geletyuk 1984). Thus, ionic channels may be formed just by proteins bound to some microfilaments from bundles. It is also well known, that many membrane receptor proteins in different cells are anchored. In addition to microfilaments formed by actin or actin-like contractile proteins, the cortical layer may contain myosin (Poglazov and Levitsky 1982) as well as microtubules with tubulin. These elements of the cortical layer are all extremely sensitive to Ca^{2+}, Mg^{2+} and ATP. Moreover, glycerinated models of both nonmuscular and muscular cells are capable to contract in the presence of these agents (Krasovskaya and Pavlenko 1977). The muscular models even contract in the presence of Sr^{2+} (Meisescu and Thieleczek 1979). Therefore, an elevation of the Ca^{2+} (or Sr^{2+}) concentration *in vivo* may lead to mechanical tension and a relative shift of both the cytoskeletal elements and cytogel as a whole. Such local shifts, may apparently change the intramembrane position of those integral proteins which are bound to cytogel molecules. Thus, if the channels are linked with cytogel elements, the latter may control channel functioning.

The hypothesis is still very preliminary, and all the facts are still of a desultory nature. It is supported by data obtained in cultures of mammal neurones (Fukuda et al. 1981) suggesting a possible involvement of microfilaments and microtubules in controlling the sodium and calcium channels, respectively. The authors found a decrease in the rate of rise of Na^+ and Ca^{2+} spikes induced by cytochalasin B and colchicin, respectively. Brehm and Eckert (1978) reported that the influx of Ca^{2+} ions (and, to a lesser extent, that of Sr^{2+}) leads to inactivation of calcium channels in *Paramecium*, and Ba^{2+} was shown to be ineffective in this respect. This correlates with Ca^{2+}- and Sr^{2+}-induced activation of the cytogel contractile system and the chloride channels of algal cell plasmalemma. The lack of an inactivation mechanism in different channels reconstituted in pure lipid membranes may also serve as an indirect indication of the participation of cortical elements in the channel function.

The slow irreversible inactivation of chloride channels in the presence of Ca^{2+} (Fig. 4*A*, *B*) agrees well with the phenomenon of Ca^{2+}-induced depolymerization of microtubules *in vitro* (Sakai and Matsumoto 1978) and in the cortical layer of perfused squid giant axons which lose their electrical excitability in these conditions (Matsumoto et al. 1979; Matsumoto and Sakai 1979 a, b). These latter authors restored membrane excitability by the addition of some proteins and tubulin-tyrosine ligase into the PM providing the formation of microtubules. Introduction of proteins in obligatory due to the fact that proteins from depolymerized microtubules are washed out by PM. It seems reasonable to suggest that, in our case, the same situation caused an irreversible inactivation of chloride channels.

An additional evidence that microtubules participate in control of the algal Cl channels was obtained in our previous experiments with colchicin introduced into

the PM. After 30—45 min treatment of the cell with colchicin (0.15 mmol/l) the peak value of the Ca^{2+}-induced Cl^- current was by 3—5 times lower than that in control and when colchicin concentration was 2.5 mmol/l a 10-fold decrease was observed.

On the other hand, in electrically excited cells *in vivo* Ca^{2+} concentration in cytoplasm increases only for a short period of time (2—3 s). Thus the depolymerization of microtubules should be very weak and it should make no essential contribution to the inactivation of chloride channels. Therefore, as suggested earlier (Lunevsky et al. 1983), in an intact cell inactivation of Cl channels is mediated by a decrease in the free Ca^{2+} concentration in the cytoplasm. Thus, the kinetics of inactivation reflects that of the decrease in the free Ca^{2+} concentration.

The hypothesis on the participation of cytoskeletal elements in the control of ionic channels appears to be rather attractive, realistic and constructive. It points to an additional way (in parallel with the biochemical one) by which intracellular processes and structures may participate in the control of membrane transport. The hypothesis is based on a highly attractive idea on the existence of a universal molecular mechanism (or a limited number of mechanisms) providing all kinds of motility from the submolecular (channels) to the cellular level.

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Appendix

Let us try to estimate change in ionic concentration near the membrane under prolonged flow of an anionic current for the most unfavourable case, when the density of the inward chloride current (I) reaches 10 A/m^2, (1 mA/cm^2). Let us suppose that only anionic current flows across the membrane, and that anions and cations carry currents equally in the external solution. This means that anions leave (through the membrane of the working section) the intracellular space with rate a I (per mol/m^2.s) and enter the space along the cell with a rate $I/2$. The other half of I corresponds to the outflux of cations from this space. Thus, if diffusion of ions from the lateral ends to the working section is neglected, the amount of ions in this intracellular space would decrease with the rate $I/2$ and the average ionic concentration $\bar{c}$ would decrease with the rate $d\bar{c}/dt = Is/2v = I/r$, where r, s and v are radius, membrane area and volume of the cell in the working section, respectively. For the cell with an $r = 0.4$ mm and $I = 10$ A/m$^2 \approx 1 \times 10^{-4}$ mol/m^2.s, $d\bar{c}/dt$ would be 0.25 mmol/l.s = 15 mmol/l.min.

The above words for the case of the equality of cation and anion mobilities: $u_+ = u_-$. When $u_+ \gg u_-$ (e.g. for KCH_3SO_4) the current I along the cell is carried

mainly by cations and the anion component will be $\ll I/2$. That is why $d\bar{c}/dt$ in this case will be twice that at $u_+ = u_-$.

Now let us estimate the ratio of the diffusion ion flux (i_D) from lateral ends of the cell working section and the ion flux carried by electric current, $i_E = I \cdot s/2$(at $u_+ = u_-$).

$$i_D = 2\pi r^2 D \left|\frac{\Delta_c}{\Delta_x}\right|, \quad i_E = \pi r a I,$$

where D is the ionic diffusion coefficient; $\Delta c/\Delta x$ is the gradient of ion concentration at $x = 0$ and $x = a$ (Fig. 2*A*); a is the length of the cell working section. Introducing actual values: $a = 2$ mm (Fig. 2*B*), $D = 2 \times 10^{-9}$ m^2/s (for KCl) and r and I, mentioned above, we obtain $i_D/i_E \approx 10\ |\Delta c/\Delta x|$, where $\Delta c/\Delta x$ is in mol/l.mm. This suggests that i_D is comparable with i_E at $|\Delta c/\Delta x| \sim 0.1$ mol/l.mm. When $\Delta x \sim 1$ mm ($\sim a/2$) which corresponds to partial filling of the working section with diffusing ions, we obtain $\Delta c \sim 0.1$ mol/l. The latter means that lateral diffusion of ions at a small disbalance of concentrations ($\Delta c < 10^{-2}$ mol/l) can be neglected.

In the case of continuous perfusion, there exists a non-stirring layer with a thickness $\delta \ll r$ near the intracellular membrane surface. Then the anionic concentration near the membrane would be lower than that in the perfusate by $\Delta c = \delta \cdot dc/dr = \delta \cdot I/2D$. Assuming that $D = 2 \times 10^{-9}$ m^2/s and $\delta = 10$ μm, we obtain $\Delta c = 0.5$ mmol/l.

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