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Excitation of *Characeae* Cell Membranes as a Result of Activation of Calcium and Chloride Channels

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Summary. Ionic channels responsible for excitation of plasmalemma and tonoplast of fresh-water *Nitellopsis obtusa* were studied using the voltage-clamp technique. Voltage was clamped on each separate membrane. Chloride channels were inhibited with ethacrynic acid. 1. Along with anion (chloride) channels the cation channels have been revealed in the membranes. The corresponding channels are similar in both types of membranes. 2. The cation channels are controlled by membrane voltage being activated under membrane depolarization. The channels possess activation-inactivation kinetics. For *N. obtusa* characteristic times of activation and inactivation are ~0.1 and 0.5 sec, respectively. 3. Conductance of cation channels depends on the type of cation and the orders of conductivity decrease are the following: $Rb^+ > K^+ > Cs^+ > Na^+ > Li^+$ and $Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$. Ratio of conductance for bivalent ions to that of monovalent ones decreases with the increase of normal concentrations. When the external medium contains both mono- and bivalent cations in comparable concentrations, the current is mainly determined by the latter. In natural environment of the algae such conditions are realized for Ca^{2+} ions which create the bulk of the inward current through cation channels under cell excitation. That is why we term these channels "calcium" ones. 4. Ca^{2+} ions entering the cytoplasm through the calcium channels located in both membranes activate the chloride channels. Ba^{2+} and Mg^{2+} also activate the chloride channels but to a lesser extent than Ca^{2+} does. Characteristic inactivation time of these channels in *N. obtusa* is about 1 to 2 sec.

Key words *Characeae* · voltage clamp · Ca^{2+} channel · Cl^- channel · ion selectivity · ethacrynic acid

Introduction

In order to characterize the molecular mechanism of membrane ionic channel functioning we need comprehensive information on channel arrangement and on chemical content and structure of the molecules forming the channel. This information can be obtained from the studies of channel-forming substances after their isolation from the cells. In our opinion, the most promising test for identification of channel-forming substances in the process of their isolation is reconstruction of the channels with predicted properties in bilayer lipid membranes (BLM). BLM may serve not only as a specific "electrode" for these substances, but also as a model of cellular membrane which allows for the study of the structure and functional properties of cellular channels in more detail. However, this can be achieved only in the case when the properties of reconstructed channels are highly similar to those of intact ones, so our knowledge on the properties and structure of intact channels must be as wide as possible. Similarity between the reconstructed and intact channels attests that the function of isolated channel-forming substances is preserved.

The approach mentioned has been successfully applied in our study of *Characean* algae. This became possible due to the discovery of water-soluble channel-forming molecular complexes in algal cytoplasm [2]. Some of these complexes were shown to be capable of forming the potential-dependent cation channels in BLM. Their selectivity to mono- and bivalent cations as well as the character of voltage control are similar to those of cellular calcium channel; only inactivation phenomenon is lacking [22, 23]. A typical "single" channel in BLM comprises an aggregation of tens of elementary channels switching (between two states – open and closed) simultaneously. The conductance of an elementary channel in 0.1 M KCl solution is about 2.5 pS [33]. It represents a water pore more than 8 Å in diameter with an anionic site [23]. Reconstructed channels are formed by protein molecules with molecular weight no more than 20 kD (according to some data ~5 kD) [22, 33].

In the present paper we tried to summarize the data obtained from comprehensive studies of intact ionic channels in algal cell membranes. By the time our work began (1971) it was well-established that

* Deceased.

in the course of generation of algal action potentials, Cl^- ion outflux into external medium is enhanced [17]. This fact along with some other data led to the suggestion that Cl^- is the main current-carrying ion [11, 16, 25] and that Ca^{2+} ions control the value of transient chloride current [14]. It was also established that both membranes, plasmalemma and tonoplast, are excitable, and that excitation in the membranes proceeds simultaneously accompanying each other. When the tonoplast is excited, its conductance for Cl^- ions increases [13]. We succeeded in extending this evidence. Using the voltage-clamp technique, the chloride transient current has been shown to be accompanied by the calcium one; we have studied the selective properties of the calcium channels and kinetics of their functioning as well as the character of interaction between the channels of both types and interrelation of both cell membranes. These results and those on reconstructed calcium channels were published earlier mainly in Russian [1, 2, 4–6, 22, 23, 33].

Materials and Methods

The experiments were carried out mainly at internodal cells of fresh-water *Characean* alga *Nitellopsis obtusa*. The cells were 10 to 20 cm in length and 0.6 to 0.8 mm in diameter. Cytoplasm motility, which stops usually at excitation, served as an indicator of normal functional state of a cell. Algae were collected from Lithuanian lakes in September. Isolated cells survived in artificial pond water (APW, 0.1 mM KCl, 1.0 mM NaCl, 0.5 mM $CaCl_2$, pH 7.3) at room temperature till the middle of April. In some experiments the cells of *Nitella flexilis* grown in aquaria were used. The analysis of vacuolar sap was carried out using flame photometry (Table 1). There exists no direct evidence on ionic concentrations in *Nitellopsis obtusa* cytoplasm. So to estimate them approximately, in Table 1 we give the ionic concentrations for different types of algae which possess similar contents of vacuolar sap. It is seen that the scatter in the data for different algal types does not exceed that between specimen of the same species, so this approach of estimating the ionic concentrations in *Nitellopsis* cytoplasm seems to be quite reasonable. Concentration of free Ca^{2+} in cytoplasm was taken to be $<10^{-6}$ M according to the data for algal [29] and animal cells. It should be noted that temporal characteristics and amplitudes of transient currents in *Nitellopsis* cell membranes vary from year to year and depend on the "shelf life" in artificial media. All experiments were conducted at room temperature (19 to 21 °C).

Voltage clamping on any membrane was performed on the portion of an algal cell (2 to 3 mm in length) situated in the central compartment of the experimental chamber isolated with two vaseline gaps (each 10 mm in width) from the lateral compartments (Fig. 1*a*). For better space voltage clamping the Ag/AgCl current electrodes were placed in both lateral compartments. Membrane voltage was measured by glass microelectrodes (filled with 2.5 M KCl) inserted into the vacuole and cytoplasm in the center of the working area. The end of an external microelectrode was placed at the cell wall. Localization of a microelectrode in cytoplasm or vacuole was judged by the appearance of a transient current curve under alteration of holding potential level and by the value of current reversal potential. Microelectrodes were connected with high-resistance input preamplifiers [4]. It was possible to control the membrane potential with the accuracy of 1%; at least, the time constant of the voltage-clamp system [6] was 1 to 2 msec. To measure the "instantaneous" differential membrane resistance short rectangular voltage pulses (20 to 100 msec) were superimposed on the voltage-step command. Current curves were displayed with the help of storage oscilloscope CI-37 [7] or three-channel loop oscillograph to provide simultaneous recording of current and voltage. Families of current curves were obtained by the sequence of single pulses of command voltage applied at 3 to 6-min intervals. This time is quite sufficient for the membrane to restore its electrical properties.

At voltage clamping on plasmalemma the cell wall is incorporated in series with it between two microelectrodes, so the quality of voltage clamp on plasmalemma depends on the wall resistance R_w. Since the wall represents a fibrous cationic exchanger with concentration of fixed anions of the order of hundreds of millimoles [9, 10, 31], it could be expected that its resistance would be much less than that of excited plasmalemma. Actually, the specific resistance of the wall along the cell axis, even at low ionic strength of external solution (~2 mM) is ~500 Ω cm [31]. Taking into account the character of ultrastructural organization of a cell wall, it seems reasonable to suppose that its transverse specific resistance would be of the same order of magnitude. Then the wall, 10 μm in thickness, would possess the R_w of ~1 Ω cm^2. On the other hand, according to our data, the amplitude of the inward chloride current under voltage clamp on the system involving the wall and plas-

Table 1. Concentration of ions in vacuole and cytoplasm of algal cells

Species of algae	Concentration of ions (mM), vacuole/cytoplasm K^+	Na^+	Cl^-	Ca^{2+}	Refs.
Nitellopsis obtusa	124/–	27/–	–/15	14/–	our data
	150/–	0,65(?)/–	167/–	10/–	[16]
	74–159/–	26–77/–	143–249/–	–	[24][a]
Nitella flexilis	80/125	27/5	135/36	–	[19]
	73/78	4/2	179/27	–	[30]
Chara corallina	48–112	27–49	100–161	–	[35][b]
	112–128	3–10	10–21		

[a] Species from brackish water.
[b] Generalized data of different authors.

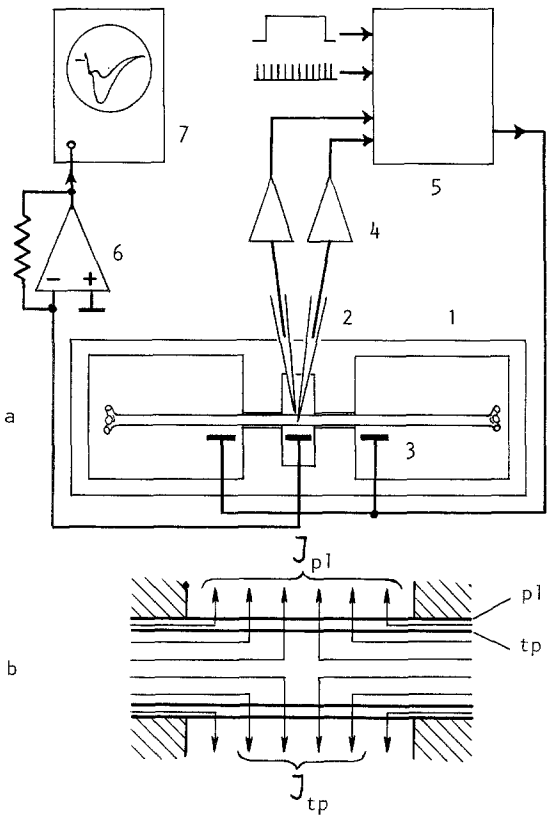

Fig. 1. *a.* Schematic diagram of the experimental arrangement with the voltage-clamp set-up. 1 – Plexiglass chamber with three compartments; central compartment is 3 mm in width; 2 – Glass microelectrodes filled with 2.5 M KCl; 3 – Ag/AgCl current-recording electrodes in each compartment; 4 – Probe voltage preamplifiers; 5 – Voltage-clamp set-up; 6 – Current monitor; 7 – Storage oscilloscope CI-37 (USSR) or three-channel loop oscillograph. *b.* Scheme of the current passing through the working area of the cell. $I_{pl} > I_{tp}$ by the value of the current flowing at isolated regions of the cell along the cytoplasm layer

malemma reaches 1 mA/cm^2 at electrochemical membrane potential difference for Cl^- ions being of ≈ 100 mV. This corresponds to the value of a total resistance of the wall and membrane, $\sim 100\ \Omega\ cm^2$. Thus, R_w represents only a few percent of the plasmalemma resistance, so its value can be neglected.[1]

In the voltage-clamp system used, the current to be registered almost totally flows in the working area through both membranes no matter which membrane is clamped. Difference of the currents through plasmalemma (I_{pl}) and tonoplast (I_{tp}) is determined, as shown schematically in Fig. 1*b*, by fraction of the total current along the cytoplasm layer at isolated areas of the cell. Let us estimate the contribution of this current. According to microscopic measurements the cytoplasm layer is 10 to 15 μm in thickness and the cells are 0.6 to 0.8 mm in diameter. This suggests that cytoplasm cross-section accounts for 5 to 10% of that of vacuole. Ionic concentration of vacuole sap and its specific conductance are about 1.5-fold higher than those in cytoplasm (Table 1) and, thus, the density of longitudinal current is also higher by the same value. Hence, the value of $(I_{pl}-I_{tp})/I_{pl}$ does not exceed 3 to 6% and can be neglected assuming that I_{tp} equals the current registered. However, here and later we shall use the same indexes to denote the current in order to make clear which membrane is clamped.

The quality of space voltage clamp of the membrane was estimated in the experiments with the use of the third microelectrode. It was found that the voltage alternates from the periphery of the working area to its center directly with the value of current flow. Under most unfavorable conditions, when the current was ~ 0.7 mA/cm^2 (7 A/m^2), the difference between voltages at these two points reached 15 mV, so at high transient currents their true time course is somehow distorted. In our case it was of main interest to estimate the accuracy of determination of the voltage at which some component of a transient current changes its direction (reversal potential, V_r). As a rule, this phenomenon occurs at low current values; under such conditions the voltages at different sites of the working area coincide within 1 to 2 mV. Hence, in the scheme proposed the value of V_r can be estimated quite perfectly.

To identify the kind of ions transferring one or another transient current component we used the method based upon the dependence of reversal potential of the current component on ionic content of the medium. In the case of a single type of penetrating ion V_r corresponds to the equality of the electrochemical potentials for that type of ion on both sides of the membrane. V_r depends neither on the number of channels, nor on kinetics and mechanism of their functioning since at this potential the current through the channel is equal to zero (on the assumption that channel selectivity remains constant with time).

Results

Components of Plasmalemma Ionic Currents

Figure 2 represents typical families of transient current curves registered at the *Nirellopsis* and *Nitella* cells under stepped depolarizing changes in the holding potential on plasmalemma (V_{pl}). The character of time- and voltage-dependence of current allows to conclude that the ionic current comprises several components.

The first (quick) component of the transient current (i_{t1}) changes its direction from inward (in Fig. 2 and everywhere except Fig. 8–10 inward direction is downward) to outward with depolarization onset. For different cells in APW the value of reversal potential of this component (V_{r1}) ranges from -60 to -20 mV (in Fig. 2*a* $V_{r1} \approx -45$ mV) and duration of rising phase of this component does not exceed several hundred milliseconds.[2]

The second (slow) transient current component (i_{t2}) possesses a characteristic activation-inactivation kinetics. Reversal potential of this component (V_{r2}) lies within more positive range of voltage

[1] The values of specific transverse resistance of a wall $\sim 10^5\ \Omega$ cm [35] and R_w $\sim 5 \times 10^3\ \Omega\ cm^2$ [27, 34] appear to be highly overestimated.

[2] When membrane voltage exceeds V_{r1} by 10 to 30 mV at the rising phase of the inward current the teeth are often observed (Fig. 3*a*). This phenomenon is followed by a sharp delay of the current upstroke and noise appearance. Under repeated depolarizations the current amplitudes decrease (i.e. the cell "dies"). The origin of the current teeth remains unclear.

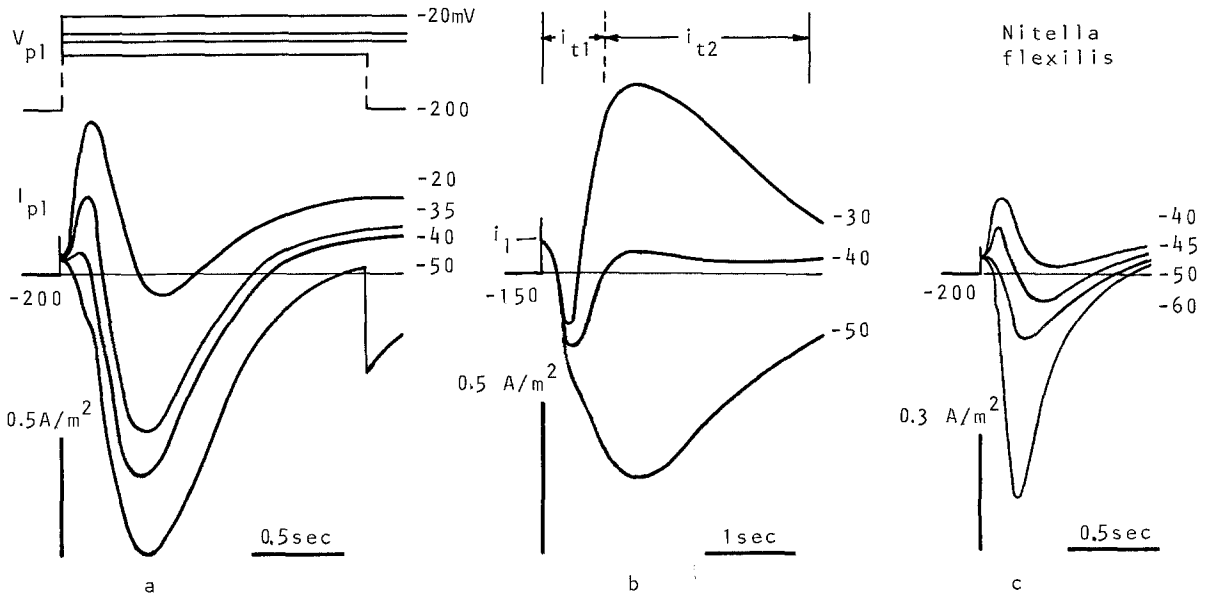

Fig. 2. Transient plasmalemma ionic currents in the cells of *Nitellopsis obtusa* (*a*, *b*) and *Nitella flexilis* (*c*) under step change in the level of holding voltage on plasmalemma (V_{pl}). Numbers to the right of each current curve indicate V_{pl} (in mV). Extracellular medium contains (in mM): *a*, *c* – APW (KCl 0.1, NaCl 1.0, $CaCl_2$ 0.5); *b* – KCl 10, NaCl 40, $CaCl_2$ 0.1. All the curves exhibit two components of transient current: quick (i_{t1}) and slow (i_{t2}) and leakage current (i_l). Besides, one can see the reversal of the current components at some value of V_{pl} (reversal potential – V_r), i.e. the quick component changes its sign at $V_{r1} = -45$ mV (*a*, *c*), the slow one at $V_{r2} = -40$ mV(*b*). Here and everywhere (except Figs. 8–10) the downward direction corresponds to the inward current

(Fig. 2*a*) than that of the first component in the case of solutions with low chloride concentrations, and within a more negative one (Fig. 2*b*) for the solutions with high chloride concentrations. Threshold voltage for the cell in APW, at which transient current arises, is −90 to −120 mV. Maximal peak inward current (i_{t2}) is observed, as a rule, at voltages exceeding threshold by 10 to 20 mV; its value depends on the functional state of the cell and varies in the range of 4 to 7 A/m^2 (400 to 700 μA/cm^2).

In addition, the leakage current (i_l) which has a value equal to the product of the values of a potential jump and resting membrane conductance, arises immediately after the potential jump. This is the potassium current, since the permeability of plasmalemma at resting state for K^+ is by order of magnitude higher than that for other ions [35]. There are some indications that steady-state current under excitation is also of potassium nature [20]. Its value, as a rule, is not equal to the initial value of i_l; this indicates that the leakage current also changes with time.

Thus, transient ionic current through plasmalemma involves at least two clearly distinct components. Significant difference in the values of their reversal potentials points to different ionic nature of these two components. Hence, these facts allow us to suggest that plasmalemma contains two distinct populations of ionic channels activating under membrane depolarization.

Chloride Nature of the Slow Component of the Transient Current

At depolarization of plasmalemma of a cell placed into APW the current i_{t2} has inward direction and changes it only within the range of positive membrane potentials. Hence, the current can be transferred either by external cations or cytoplasmic anions. From comparison of the ionic contents of APW and cytoplasm (Table 1) it transpires that the equilibrium potentials only for Ca^{2+} and Cl^- ions are capable of providing the inward membrane current. Only for these ions the equilibrium potential equals zero in the range of positive voltages on the membrane since the concentration of free Cl^- in APW is much lower and that of Ca^{2+} – much higher than in cytoplasm ($[Ca^{2+}]_{cyt} < 10^{-6}$ M). To identify the ionic nature of the current, we took into account the fact that changes in external activity (concentration) of current-forming ions had to shift the reversal potential V_{r2} by the value predicted by the Nernst equation (in the case of constant concentration of those ions in cytoplasm).

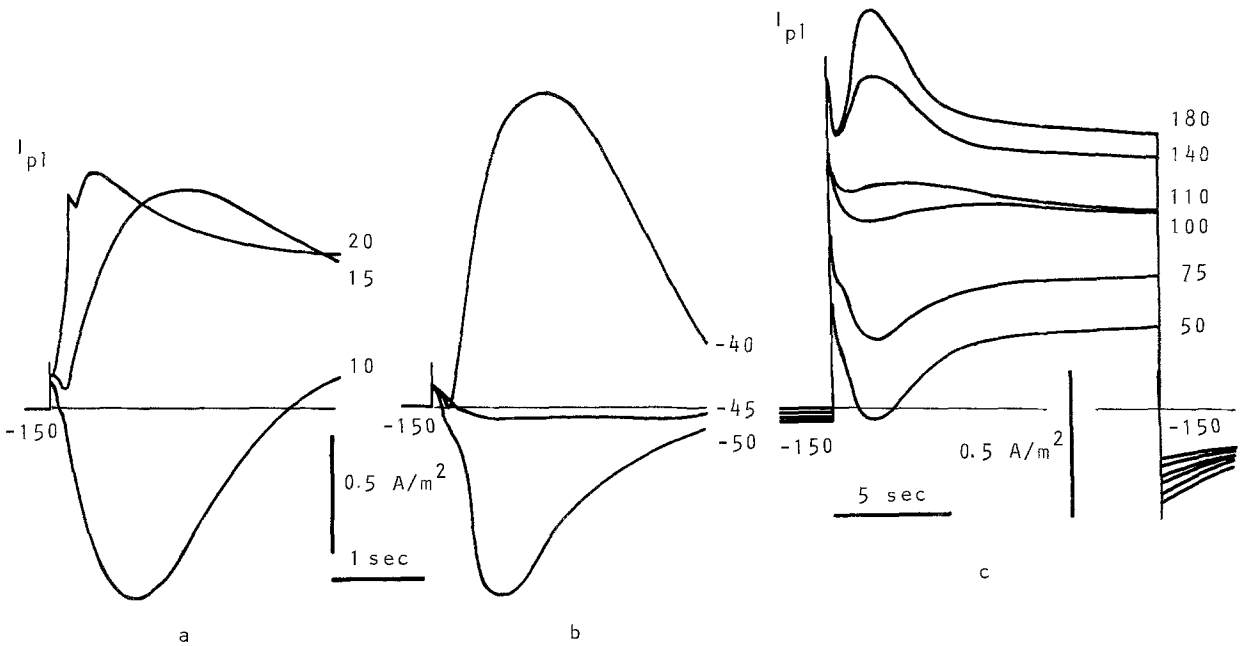

Fig. 3. Shift of reversal potential of the slow component of plasmalemma transient current (i_{t2}) from $V_{r2} \approx -12$ to -45 mV under variation of outside $CaCl_2$ concentration from 8 mM (*a*) to 100 mM (*b*). (*c*) An example of reverse of slow current component ($V_{r2} = 105$ mV) at voltage clamping between the vacuole and external medium (APW) under continuous vacuole perfusion (cytoplasm "perfusion", *see* text) with 100 mM KCl, 50 mM NaCl, 10 mM $CaCl_2$, pH 6.0. Here and everywhere we present experimental results for *Nitellopsis obtusa* only

Table 2. Shift of reversal potential (ΔV_{r2}) of a slow component of the plasmalemma current (i_{t2}) in *Nitellopsis obtusa* as a function of Cl^- activity in the external solution (A_e)

External solution $CaCl_2$ (mM)	A_e (mM)	ΔV_{r2}^{exp} (mV)	ΔV_{r2}^{cal} (mV)
1. 25	32		
100	104	$-29 \pm 3(7)$[a]	-30
2. 8	12		
100	104	$-60 \pm 6(2)$	-55
3. 30	38		
30 + 70 CaG[b]		$6 \pm 1(3)$	

[a] Number of experiments are shown in parentheses.
[b] CaG – calcium gluconate.

For the sake of convenience i_{t2} was studied in conditions when $V_{r2} < V_{r1}$. This does occur if the concentrations of $CaCl_2$ or NaCl in the external solution are no less than 15 to 20 mM. Examples of reversal of i_{t2} in the solutions with increased ionic concentrations are presented in Fig. 2*b*. Activities of Cl^- and Ca^{2+} ions were changed by simultaneously altering the concentrations of $CaCl_2$ four- to 10-fold. Oscillograms for the currents in such an experiment are shown in Fig. 3 (*a* and *b*). The data on V_{r2} shifts at variations of external activity (A_e) of Cl^- ions are listed in Table 2. It is seen that V_{r2} shifts to the direction of electrochemical equilibrium of Cl^- ions and experimental values of ΔV_{r2} are very similar to those calculated from the formula: $\Delta V_{r2} = 58 \log (A_{e2}/A_{e1})$, where A_{e1} and A_{e2} are the initial and final activities of Cl^-, respectively. It should be noted that gluconate anions make no contribution to i_{t2} (*see* Table 2). Slight shift of V_{r2} in response to Ca-gluconate addition can be related to the decrease in activity of Cl^- ions due to increased ionic strength of the solution. Thus, ionic channels through which the current i_{t2} is transferred are highly selective for Cl^- ions as compared with cations of the solution.

Earlier [6] we made the same conclusion on the basis of similar studies on *Nitellopsis* cells under continuous perfusion of the cell vacuole and voltage clamp at the system of membranes: tonoplast and plasmalemma. When perfusion of vacuole was carried out at elevated rate or when the perfusate was pumped forward and backward, in some cases the tonoplast seems to be damaged. Then, in the course of time, the reversal potential V_{r2} reaches a new equilibrium value different from the initial one. This new value coincides well with calculated value of the equilibrium potential for Cl^- ions. Fig. 3*c* gives an example of such an experiment. Chloride origin of the current i_{t2} is also supported by the experiments with ethacrynic acid

Table 3. Shift of reversal potential (ΔV_{r1}) of the fast component of plasmalemma current (i_{t1}) at concentration changes of cations (Na^+, K^+, H^+, Ca^{2+}) in the external solution

Nitellopsis obtusa				*Nitella flexilis*			
External solution (mM)	A_e[a] (mM)	ΔV_{r1} (mV)	α	External solution (mM)	A_e (mM)	ΔV_{r1} (mV)	α
I. Concentration changes of monovalent cations in the presence of 0.5 mM Ca^{2+} (APW)							
1. APW[b]	1			1. APW	1		
APW + 10 NaCl	9	0(2)[c]	0	APW + 10 NaCl	9	0(3)	0
2. APW	1			2. APW	0.1		
APW + 10 NaP[d]	9	0(2)	0	APW + 10 KCl	9	0(3)	0
3. APW	1			3. APW + 5 NaCl	5.5		
APW + 25 NaCl	22	10 ± 2(2)	0.13	APW + 50 NaCl	42	13(1)	0.26
4. APW	1			4. APW pH 7			
APW + 50 NaP	41	13 ± 3(4)	0.14	APW pH 4.5		3 ± 2(3)	0
5. APW pH 7				5. APW pH 7			
APW pH 5		0(2)	0	APW pH 10		−10(2)	0.05
6. APW pH 7		0(1)	0				
APW pH 9							
II. Ca^{2+} concentration changes							
1. APW	0.45			1. APW	0.45		
APW + 3.5 $CaCl_2$	3.2	16 ± 4(14)	0.65	APW + 4.5 $CaCl_2$	4	19 ± 3(13)	0.8
2. APW	0.45	20 ± 1(3)	0.85	2. APW	0.45	20 ± 1(2)	0.85
APW + 5 CaG	3.2			APW + 5 CaG	3.2		
3. APW	0.45	20 ± 3(3)	0.7	3. 0.6 $CaCl_2$	0.55	15 ± 4(5)	0.8
APW + 10 CaG	4.5			3.0 $CaCl_2$	2.5		
4. APW + 3.5 $CaCl_2$	3.2	27 ± 4(17)	1.05				
APW + 40 $CaCl_2$	24.5						
III. Concentration changes of monovalent cations in the Ca^{2+}-free solution							
1. 0.1 KCl + 1 NaCl		26 ± 7(5)	0.6	1. 5 NaCl	19 ± 3(2)		0.5
0.1 KCl + 6 NaCl				25 NaCl			
2. 0.1 KCl + 10 NaCl				2. 1 NaF + 4 NaCl	26 ± 4(4)		0.65
0.1 KCl + 10 KCl		0(1)	0	1 NaF + 24 NaCl			
				3. 5 Na_2SO_4	25 ± 5(2)		0.65
				25 Na_2SO_4			
				4. 10 NaCl	0(2)		0
				10 KCl			

[a] A_e-activity of ion which concentration is changed in the solution. Ca^{2+} activity in the CaG solutions was calculated using the association constant of 65 [8].
[b] In APW $V_{r1} = -35 \pm 8(20)$ and $-38 \pm 9(16)$ for *Nitellopsis* and *N. flexilis*, respectively; in the solution of 0.1 mM KCl + 1 mM NaCl $V_{r1} = -66 \pm 12(7)$ for *Nitellopsis*.
[c] Number of experiments is shown in parentheses.
[d] P – propionate, G – gluconate, $\alpha = z\Delta V_{r1}/58 \log(A_{e2}/A_{e1})$.

(Fig. 5) which is well-known to inhibit the chloride fluxes in erythrocytes [26].

Since i_{t2} is the chloride current, the activity of Cl^- ions in cytoplasm (A_i) can be calculated from the Nernst equation knowing the values of V_{r2} and Cl^- activity in the external solution (A_e). As a result we obtain: $A_i = 15 \pm 6$ mM.

Calcium Forms the First Component of Transient Current

The ions transferring the quick component of a transient current were identified in earlier experiments [2] by a procedure similar to that described above. To obtain more accurate values of V_{r1} the ionic concentrations were chosen at which reversal of i_{t1} occurs at more negative voltage on plasmalemma than those for the current i_{t2}. The results are listed in Table 3. Parameter "α" is used to characterize the degree of coincidence between experimental values of ΔV_{r1} and those at thermodynamic equilibrium. When these values are equal, $\alpha = 1$.

At increasing Ca^{2+} concentration in APW (Fig. 4, Table 3) the reversal potential shifts substantially. At 10-fold change in Ca^{2+} activity in

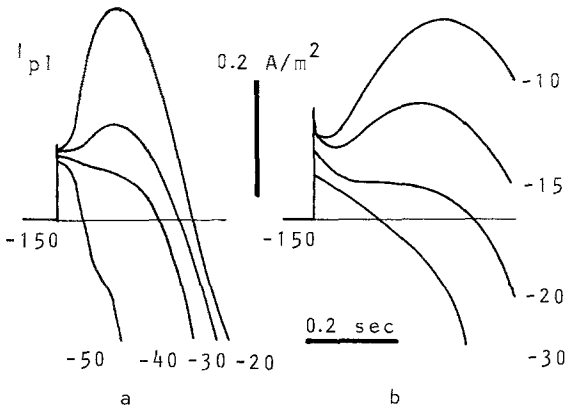

Fig. 4. Shift of reversal potential of the quick component of plasmalemma transient current (i_{t1}) in response to alteration of Ca^{2+} concentration in the external medium from 0.5 mM (APW, *a*) to 4.5 mM (*b*). $V_{r1} \approx -40$ mV (*a*) and -20 mV (*b*). This Figure shows i_{t1} and only the initial stage of i_{t2}

the solution with low ionic strength, ΔV_{r1} is less than 29 mV and slightly exceeds 29 mV in the solutions with high ionic strength. Absence of thermodynamically equilibrated shifts of V_{r1} (especially in the solutions with low ionic strength) can be in principle due to both the interference of the current i_{t2} while determining the reversal of i_{t1}, and participation of some other (except for Ca^{2+}) ions in the current transfer. However, it is clearly seen from Table 3, that alterations in Ca^{2+} activity due to the changes in $CaCl_2$ or calcium gluconate concentrations are practically the same. At the same time, the actions of these solutions on the values of i_{t2} are different since, as shown above, gluconate ions do not pass through the chloride channels. These results point to the absence of any pronounced influence of both i_{t2} and outside Cl^- concentration on V_{r1}.

Participation of monovalent cations in the current i_{t1} was tested by variation of Na^+ and K^+ concentrations in APW. As seen from Table 3, these cations affect V_{r1} only in concentrations exceeding 25 mM. Thus, when APW contains 0.5 mM Ca^{2+} the effect of monovalent cation on the reversal potential of the current i_{t1} is very weak. The effect of monovalent cations on V_{r1} can be accounted for competition between these cations and Ca^{2+} ions for the passage through the channels, in which the latter win. In fact, in a special set of experiments with calcium-free solutions (Table 3) we observed a sharp increase in the effect of Na^+ and K^+ on V_{r1}; on the average it was 0.6, the thermodynamically equilibrium value of ΔV_{r1}, and in some experiments almost equal to it.

Thus, Ca^{2+}, Na^+ and K^+ pass through the channels responsible for i_{t1} current transfer, the value of V_{r1} is determined by equality of the efflux of monovalent ions from the cell and the influx of external cations into the cell. Under physiological conditions (fresh water) during the action potential only Ca^{2+} ions enter the cytoplasm through these channels (no shift of V_{r1} is seen at variation of monovalent cation concentration in APW).[3] Ca^{2+} ions are known to control a number of intracellular processes, in particular, cytoplasm flow which stops at action potential generation [29] as well as opening of chloride channels (*see below*). This suggests that the main functional role of cationic channels is to provide the entrance of Ca^{2+} into the cell during excitation. That is why we term these channels "calcium" ones [23] but not "cationic" or "calcium-sodium" as in our previous papers [2, 5]. It should be noted that in neurones Ca^{2+} channels gained their name also due to their functional property but not due to selective ones, since they are more permeable for Ba^{2+} and Sr^{2+} than for Ca^{2+}, and they are also permeable for Na^+ [21].

It is well known that Mn^{2+}, Co^{2+}, Ni^{2+}, La^{3+} and verapamil [8] inhibit the calcium-sodium channels in excitable membranes of animal cells. We used these substances in the following concentrations: $MnCl_2$ – 0.5 to 20 mM; $NiCl_2$ – 4.5 mM; $La(CH_3COO)_3$ and verapamil – saturated solutions. Lanthanum and manganese inhibit both current components by three- to 10-fold with practically no influence on the value of V_{r1}. Nickel slows the kinetics of current development significantly and affects V_{r1}, but weaker than calcium. Verapamil has no appreciable effect on ionic currents. The fact that Mn^{2+} and La^{3+} inhibit both components of transient currents, points to possible existence of functional interrelation between calcium and chloride channels and this suggestion has been proved by further experiments (*see below*).

Selective Filter of Calcium Channel

For a detailed study of calcium channel selectivity it was necessary to block the current through the Cl^- channels. In earlier experiments this was achieved by brilliant yellow dye that is known to be a blocker of anion permeability in erythrocytes

[3] Let us estimate the increase of Ca^{2+} concentration in cytoplasm (cytoplasm layer is 10^{-5} m in thickness) in the course of action potential (AP). Assuming that the bulk of Ca^{2+} enters the cytoplasm during the rising phase of AP ($\sim$0.2 sec) and, thereby, $i_{Ca} \sim 0.1$ A/m^2 ($\sim 3 \times 10^{17}$ ion/sec m^2), $\Delta C_{Ca} \sim (3 \times 10^{17} \times 0.2)/10^{-5} = 6 \times 10^{21}$ ion/m$^3 = 10^{-5}$ M, this value is just sufficient to stop the cytoplasm flow [29]. At rest, as mentioned above, the concentration of free Ca^{2+} in cytoplasm is $< 10^{-6}$ M.

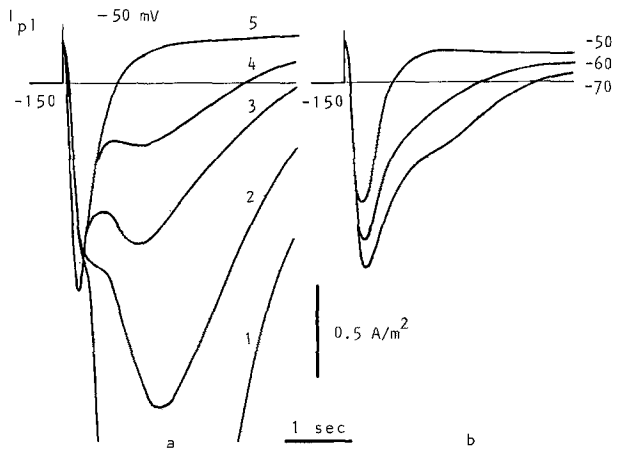

Fig. 5. Inhibition of chloride component of plasmalemma transient current induced by ethacrynic acid (EA). *a*. 1 – Initial current (prior to EA application) at plasmalemma depolarization up to −50 mV, peak current value is ~4 A/m². Oscillograms 1–5 were taken at 10-min intervals. Curve 5 reflects cation current through calcium channels. *b*. Dependence of cation current amplitude and contribution of chloride component on the level of plasmalemma depolarization near the reversal potential, $V_{r2} \approx -55$ mV, 40 min after EA application. External medium: APW+60 mM NaCl+0.15 mM EA, pH 7.3. In *a* and *b* different cells are shown

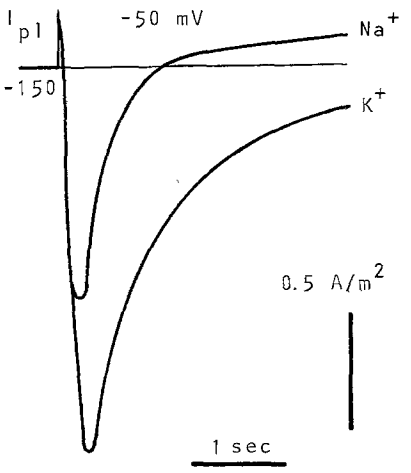

Fig. 6. Dependence of the amplitude of plasmalemma transient current on the kind of cation at $V_{pl} = -50$ mV (chloride channels are blocked by EA). External solution contains 60 mM KCl or NaCl

[7]. Brilliant yellow (1×10^{-5} to 5×10^{-4} M) inhibits the current i_{t2} and has no effect on i_{t1}. Efficiency of inhibition depends on flow direction of the current: the outward current (i.e. influx of Cl^- ions into the cell) was inhibited much more effectively. The inward current was inhibited only by two- to threefold; this was insufficient to reveal the real time course of i_{t1}. Besides, brilliant yellow has an irreversible toxic effect on the cell which appears 30 to 40 min after the drug application as an increase in the leakage current and as a slowing down of the rising phase of the current i_{t1}.

Much better results were obtained when ethacrinic acid (Egyt, Hungary) was used as an inhibitor of chloride channels [23]. It appeared to be effective in concentrations of $\sim 10^{-4}$ M and its effect was completely reversible. Inhibition, as a rule, takes 30 to 40 min. The evolution of the curves of transient currents in the course of inhibition is best shown in the solutions with high ionic strength, when i_{t1} is of great value (Fig. 5*a*). The chloride channels, however, are not completely inhibited. Fig. 5*b* shows that at voltages different from the reversal potential V_{r2} (≈ -55 mV (*b*) and > -50 mV (*a*)) the contribution of the chloride current component is still present. The current through calcium channels is registered adequately enough only within the range of V_{r2} which changes from −30 to −55 mV for different cells at 60 mM Cl^- in the external medium. As seen from Figs. 5–7, the calcium channels first activate – the rising phase duration is 100 to 250 msec – and then inactivate with a characteristic time of about 0.5 to 1 sec.

To determine the properties of calcium channel selective filter we studied the amplitudes of the fluxes of mono- and bivalent ions at high ionic strength of the solution, and when chloride current is inhibited with ethacrynic acid (EA). High ionic concentrations (including Cl^-) are required to provide a significant inward current through calcium channels at $\approx V_{r2}$ (the range of minimal contribution of chloride current) due to positive shift of V_{r1} and negative shift of V_{r2}, as well as to minimize the effect of surface potential of the membrane (and, possibly, of the wall). The amplitudes of inward current, i_{t1}, in the region of $V_{pl} = -50$ mV were compared in the experiments with replacement of one cation for another (the cells were selected with $V_{r2} \approx -50$ mV). Figure 6 shows an example of substitution of 60 mM NaCl for 60 mM KCl. The results of similar experiments with replacement of 60 mM KCl by 60 mM solutions of chloride salts of other alkali metals are summarized in Table 4. It is seen that current amplitudes differ significantly. At the same time it was revealed that the ions (Cs^+, K^+ and Na^+) substantially different in size but taken in equimolar concentrations give the same value of V_{r1}.

In the experiments with bivalent ions, the initial solution with 60 mM NaCl was replaced by 10 mM solutions of $CaCl_2$, $BaCl_2$, $SrCl_2$, or $MgCl_2$. In these cases some difficulties arose due to the shifts of channel activation curve to depolarizing direc-

Table 4. Comparison of currents (I_i) carried by different ion species (i) through intact (cell)[a] and reconstructed (BLM)[b] calcium channels. The amplitude ratios of I_i to I_K are given

Ion	Cell		BLM		Ionic radius (according to Pouling) Å
	C_i mM	$I_i:I_K$	C_i mM	$I_i:I_K$	
Cs^+	60	0.75±0.12(4)[c]	100	0.75±0.01	1.65
Rb^+		1.25±0.05(6)		1.13±0.1	1.48
K^+		1.00		1.00	1.33
Na^+		0.55±0.08(9)		0.63±0.08	0.95
Li^+		0.40±0.08(4)		0.40±0.05	0.6
Ba^{2+}	10	>0.1	25	0.52±0.02	1.35
Sr^{2+}		>0.1		0.40±0.02	1.13
Ca^{2+}		0.17±0.02(7)		0.28±0.02	0.99
Mg^{2+}		>0.1		0.12±0.01	0.65
$M^+ + M^{2+}$	60 Na^+ +0.5 Ca^{2+}	0.35±0.05(6)	100 K^+ +0.5 Ba^{2+}	0.75±0.02	

[a] Currents measured through plasmalemma clamped at −50 mV.
[b] Experimental conditions were the following [23]. BLM separated two compartments I and II. Compartment I was filled with 100 mM solution of KCl imitating the cytoplasm. Channel-forming substance was added into this solution. Compartment II was filled with solution containing the ions under study (C_i). Amplitudes of single-channel currents were registered.
[c] Number of experiments is shown in parentheses.

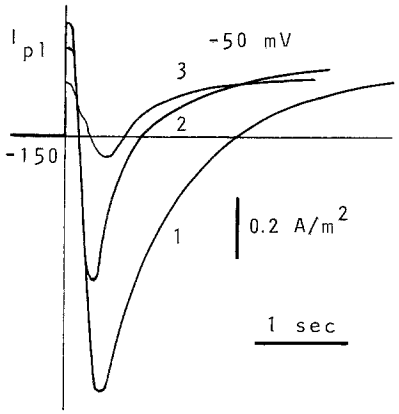

Fig. 7. Transient cation currents in plasmalemma at different contents of extracellular medium, $V_{pl} = -50$ mV. 1 – 60 mM NaCl; 2 – 60 mM NaCl + 0.5 mM $CaCl_2$; 3 – 10 mM $CaCl_2$. Comparison of curves 1 and 2 demonstrates inhibitory effect of Ca^{2+} ions on sodium current through the calcium channels

tion induced by bivalent cations. In some experiments, when holding potential was shifted from −60 to −40 mV, i.e. when voltage value was shifted to $V_{r1}(>0)$, the amplitude of the inward current still increased due to growing numbers of activated calcium channels. That is why we considered only the results obtained in the experiments where the current amplitude regularly decreased. Figure 7 demonstrates the experiments with addition of 0.5 mM $CaCl_2$ to the initial solution containing 60 mM NaCl and subsequent replacement of this mixture by 10 mM $CaCl_2$. In the cases of Ba^{2+}, Sr^{2+} and Mg^{2+} ions only the lower limit of current amplitudes can be estimated since at −50 mV noncomplete activation of channels occurs and the currents have reduced amplitudes. Table 4 and Fig. 7 show that the amplitudes of currents for bivalent ions are much lower than those for monovalent ions (at concentrations mentioned), and that the former ions inhibit the current of monovalent ones. Due to this competition, V_{r1} is more affected by bivalent ions (Table 3). Physical aspects of this phenomenon were considered elsewhere [23]. Similar data for single calcium channels reconstructed in BLM are given in Table 4 for comparison [23]. In this case the channel conductance for bivalent ions was studied in more detail. Conductance for mono- and bivalent ions increases with crystalline radii of ions, apart from Cs^+ ions.

Ionic Currents in Tonoplast

Tonoplast voltage, V_{tp} (determined as the difference of potentials between cytoplasm and vacuole, $V_{cyt} - V_{vac}$), is about −10 mV at resting state. When the voltage clamped on the tonoplast is shifted to positive level, there appears a transient current from cytoplast to vacuole (Fig. 8*a*; in Figs. 8–10 $i_{tp} > 0$ corresponds to the same direction). The value of threshold potential initiating the tonoplast excitation varies between 10 and 30 mV. This tran-

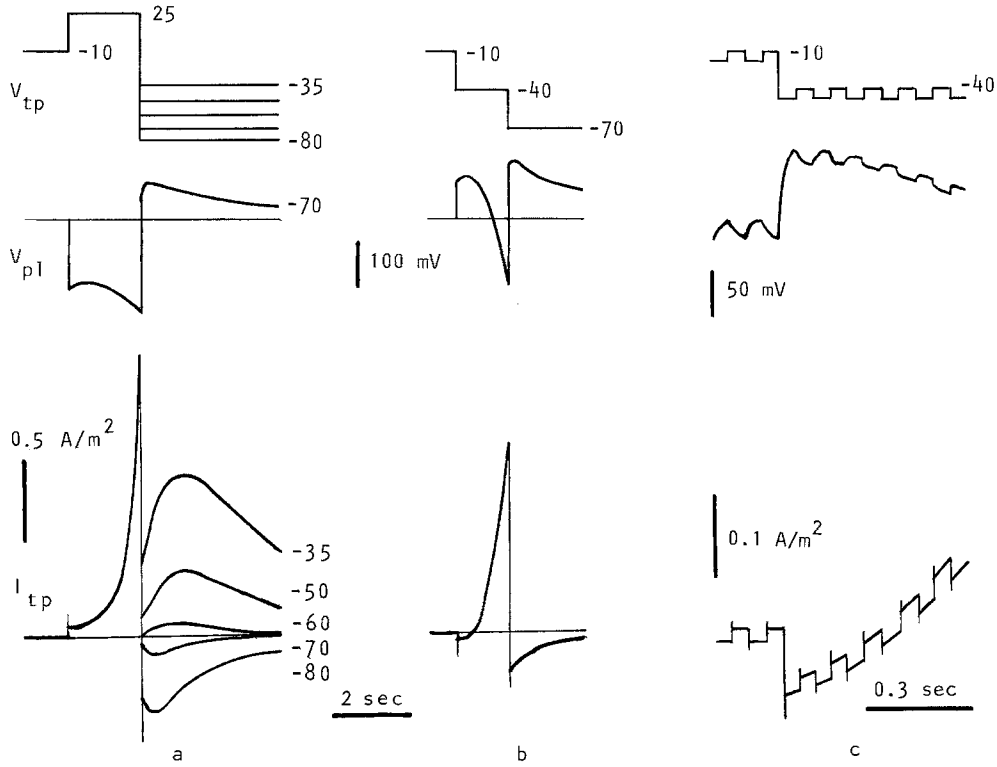

Fig. 8. Activation of tonoplast ionic current (I_{tp}) by positive (*a*) and negative (*b*) shifts of voltage (V_{tp}) from the resting potential level (≈ -10 mV) and (*a*) estimation of reversal potential of tonoplast transient current after preliminary excitation. The difference of electric potentials between cytoplasm and vacuole is taken to be V_{tp}. Here and in Figs. 9 and 10 positive values of I_{tp} correspond to the flow from cytoplasm to vacuole, i.e. inward current. In the central part (*b*) the voltage curves on plasmalemma registered simultaneously with the current are shown. Extracellular solution: APW. Records in *a*, *b* and *c* are made for different cells. *a*. Family of transient current curves taken for determination of reversal potential (≈ -65 mV). The record of V_{pl} was obtained at V_{tp} stepped down to -70 mV. *c*. V_{tp} with testing pulses equal to 6 mV. It is seen that immediately after V_{tp} jump from -10 to -40 mV the tonoplast resistance does not change but that of plasmalemma sharply decreases, i.e. plasmalemma calcium channels are activated

sient current (increasing with time) flows through plasmalemma and hyperpolarizes it up to the levels resulting in a series of electric break-downs which sometimes lead to cell damage. To avoid this, membrane voltage V_{tp} was stepped to various negative levels when the current i_{tp} reached the value of 1 to 1.5 A/m^2 (100–150 μA/cm^2). Immediately after the voltage jump the ionic channels are still in the open state for some period of time, thus providing the current flow decaying with time, and its reversal potential can be determined. In fact, instead of monotonous decay the current reaches its maximum, especially pronounced at voltages higher than the reversal potential, as seen in the oscillograms (Fig. 8*a*). This phenomenon will be considered below. In the region of reversal potential the current equals zero from the very beginning, indicating preferable activation of the only type of tonoplast channels at this stage. The reversal potential of this current in most cases lies between -50 and -70 mV.

Activation of tonoplast conductance could be induced, as unexpectedly appeared, not only by the shift of membrane voltage V_{tp} to positive direction, but to negative range as well (Fig. 8*b*). In the latter case the threshold of excitation varies between -20 and -40 mV. The shape of the rising phase of the current is similar to that shown in Fig. 8*a*. When after activation of i_{tp} the voltage V_{tp} is stepped to more negative levels, the transient decaying current appears (Fig. 8*b*) which has the same reversal potential as in the case shown in Fig. 8*a*. This indicates that in both cases the same ionic channels are activated. It should be noted, however, that at excitation of tonoplast by negative step of voltage (Fig. 8*b*), plasmalemma is depolarized and (as seen from Fig. 8*c*) its resistance decreases (it excites) just before the tonoplast current increases. This fact will be considered later.

According to our data from the analysis of reversal potentials of plasmalemma chloride channels, the activities of Cl^- ions in cytoplasm and

vacuole range between 9 and 21 mM and 100 and 160 mM, respectively (*see* Table 1). So the equilibrium potential for Cl^- ions (from -40 to -70 mV) is in the range of reversal potentials observed for the tonoplast currents; as for cations the equilibrium potential is of positive value. Thus, we may naturally believe that the shifts of tonoplast voltage in both positive and negative directions lead to activation of the chloride channels.

However, if the tonoplast contains the chloride channels one should expect, by analogy with plasmalemma, that it also involves calcium channels. Moreover, the molecules forming the calcium channels are present in cell cytoplasm as shown earlier [2]; the cytoplasm may serve as a donor for incorporation of Ca channels into the tonoplast. To check this assumption such experimental conditions are required which provide the predicted quick but weak cation component of the current being of opposite direction to the large chloride current; in such a case the cation component would be easier revealed.

The range of voltages where the current components would be opposite, lies between their reversal potentials. Reversal potential of chloride current, as determined above, equals from -50 to -70 mV. To estimate the reversal potential of cation current we measured it in a single calcium channel reconstituted in a bilayer lipid membrane (BLM) [2] at ionic concentrations on both sides of BLM similar to those in cytoplasm and vacuole.[4] It was found to be equal to 5 to 10 mV. Thus, the voltage range of interest (V_{tp}) lies between -70 mV and 10 mV. On the other hand, as shown above, the threshold of tonoplast excitation exceeds 10 mV (10 to 30 mV), i.e. it does not lie within the above range. So we used a procedure similar to that shown in Fig. 8*a*. Conductivity of tonoplast clamped at $V_{tp}<0$ was activated by a short (20-msec) positive impulse. If, as assumed, in this case the calcium channels become activated, then after impulse we should observe a quick negative current flowing through the not-yet-inactivated calcium channels, and more smoothly increasing positive (higher than reversal potential) chloride current. As shown in Fig. 9, this assumption was true: there exists an earlier negative component of the current (from vacuole into cytoplasm), its amplitude being more increased, the more negative the V_{tp}.

Thus, we may conclude that tonoplast current,

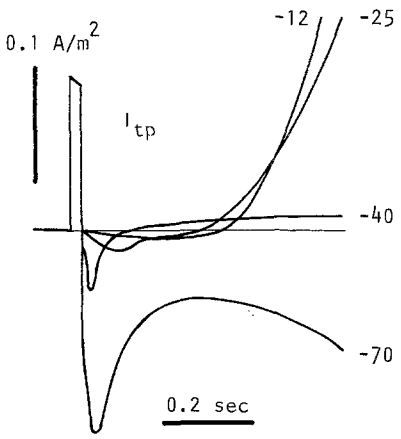

Fig. 9. Determination of the quick component of tonoplast transient current (I_{tp}) by means of excitation with a short (20-msec) depolarizing pulse (+90 mV) from the steady-state level $V_{tp}<0$. Figures near the curves denote the V_{tp} values (mV). External medium: 10 mM KCl, required to reduce the plasmalemma resistance and to weaken its excitability. It is seen that the peak cationic current is followed by decaying chloride component, the reversal potential of which is somehow less than -40 mV

as well as plasmalemma transient current, consists of two components: cationic (earlier) and chloride (late) ones.

Calcium Ions Activate the Chloride Channels

As mentioned above, the chloride channels in tonoplast are activated by shifts of voltage to both positive and negative directions. It seems to be highly unlikely that in both cases channel activation is induced by direct effect of electric field; at least in one direction the mechanism of activation could be of chemical nature, i.e. with participation of some mediator.

As the above analysis showed (*see* Fig. 8*b, c*) at the voltage step, V_{tp} from -10 to -40 mV depolarization of plasmalemma and decrease in its resistance precede the development of tonoplast current. This indicates that at first plasmalemma calcium channels are activated and Ca^{2+} ions enter the cytoplasm and only after that chloride currents appear in tonoplast. Hence, it is natural to suppose that Ca^{2+} ions may activate chloride channels.

To check this supposition it was necessary to control the Ca^{2+} influx into cytoplasm. So in the working compartment a steady flow of Ca^{2+}-free solution containing 10 to 20 mM KCl was applied transversely to the cell axis (elevated K^+ concentration is necessary for activation of Ca channels in plasmalemma due to decreasing potential to -50 to -70 mV). Then 0.5 ml of 20 mM $CaCl_2$ was added into the flow. Under the voltage clamp

[4] Channel-forming substance was added to 120 mM KCl (analog of cytoplasm) and on the opposite side of BLM the bathing solution contained (in mM): 120 KCl, 20 NaCl, 10 $CaCl_2$.

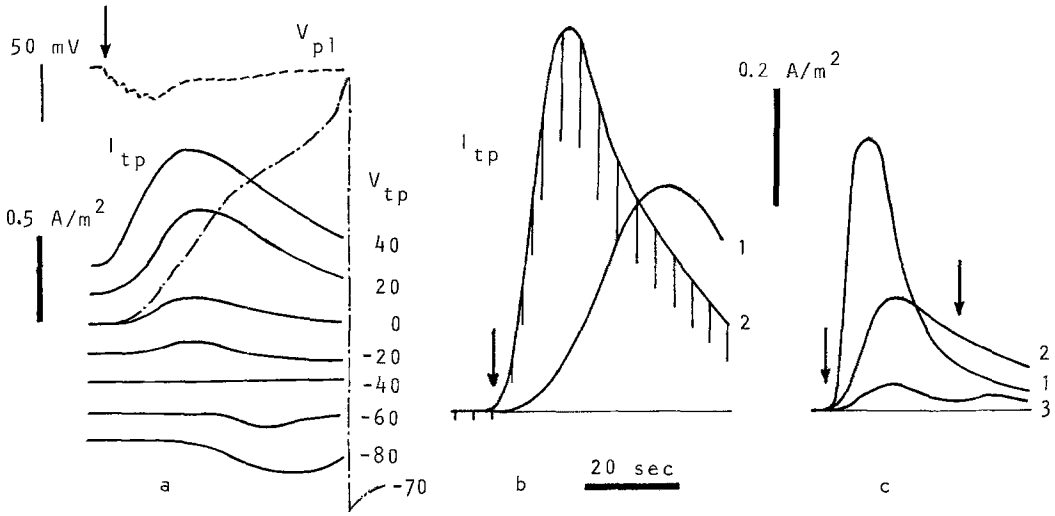

Fig. 10. Activation of tonoplast chloride channels by bivalent cations applied to the flow of external solution bathing the working area of the cell. Arrow indicates the moment of addition of 0.5-ml dose of the solution containing bivalent cations. Tonoplast voltage is held at some constant level. *a.* V_{tp} is shown to the right of the current curves the beginning of which are vertically shifted in respect to each other; upper trace shows plasmalemma voltage. To the external solution (containing 20 mM KCl) 0.5-ml doses of 20 mM $CaCl_2$ solution are added. Under continuous application of 20 mM $CaCl_2$ the current I_{tp} monotonously increases (dash-and-dot line, V_{tp} jumps from 0 to −70 mV). It is seen from the transient current curves that its reversal potential equals −40 mV. *b.* $V_{tp}=0$; external solution: 50 mM KCl+3 mM EDTA. 0.5-ml doses of 10 mM (1) or 40 mM (2) solution of $CaCl_2$ are applied. In the latter case 5 mV testing voltage (V_{tp}) pulses are applied. *c.* $V_{tp}=-5$ mV. External solution: 40 mM KCl+0.15 mM EDTA. Doses of 40 mM $CaCl_2$ (1), $BaCl_2$ (2) and $MgCl_2$ (3) solutions are applied (first arrow). At the moment indicated by the second arrow, a 40-mM dose of $CaCl_2$ solution was added in all three cases

(V_{tp}) condition this induces transient tonoplast current (Fig. 10*a*). In this case the value of reversal potential was close to that of the tonoplast chloride current under electric excitation. The amplitude of transient current increased with elevation of external Ca^{2+} concentration from 10 to 40 mM (Fig. 10*b*); at prolonged exposure of the cell to 20 mM Ca^{2+} solution permanent increase of the current was observed (Fig. 10*a*, a dash-and-dot line). Ionic channels of tonoplast were also activated by Ba^{2+} and Mg^{2+} ions applied externally (Fig. 10*c*). Their effect, however, was weaker than that of Ca^{2+} ions. Additions of 40 mM NaCl or KCl had no effect on the tonoplast current. The delay (2 to 5 sec) between the moments of addition of Ca^{2+} into extracellular solution and excitation of tonoplast corresponded to the time periods required for diffusion of Ca^{2+} ions into the cytoplasm through an unstirred layer (~100 μm) near the plasmalemma. Therefore, experimental results indicate that Ca^{2+} ions entering the cytoplasm really activate the tonoplast chloride channels. If this is the case, Ca^{2+} will activate the chloride channels in plasmalemma as well.

In the first set of experiments we tried to activate the chloride channels of clamped plasmalemma by short injections of Ca^{2+} ions into the outside perfusion medium. Necessary partial activation of calcium channels was achieved by depolarization of plasmalemma up to from −70 to −50 mV at 10 mM KCl or 40 mM NaCl in the outside solution. Under such conditions sometimes we succeeded to observe activation of chloride channels in plasmalemma (Fig. 11*a*). However, 30 to 50 min later the membrane lost both electrical and chemical excitability.

The second set of experiments was similar to that described above for tonoplast excitation with negative step V_{tp} (Fig. 8*b*). But in this case the plasmalemma was hyperpolarized in the regime of voltage clamp, so the tonoplast voltage was first depolarized ($\Delta V_{tp}>0$) due to the inward current (flowing through both membranes). In the case, when ΔV_{tp} exceeds the threshold of excitation, calcium channels of tonoplast activate and Ca^{2+} ions enter the cytoplasm from vacuole, and, according to our supposition, activate the chloride channels in plasmalemma. In fact under such conditions at sufficient hyperpolarization of plasmalemma we observe the onset of inward current (Fig. 11*b*). To be certain of chloride origin of this current we determined the reversal potential of transient current after depolarizing step of V_{pl} using the method shown in Fig. 8*a* (in Fig. 11*b* this current is registered after the reversal of V_{pl} from −350 to −150 mV). It appears that the reversal potential of this current coincides with that of the chloride component in the usual method of plasmalemma

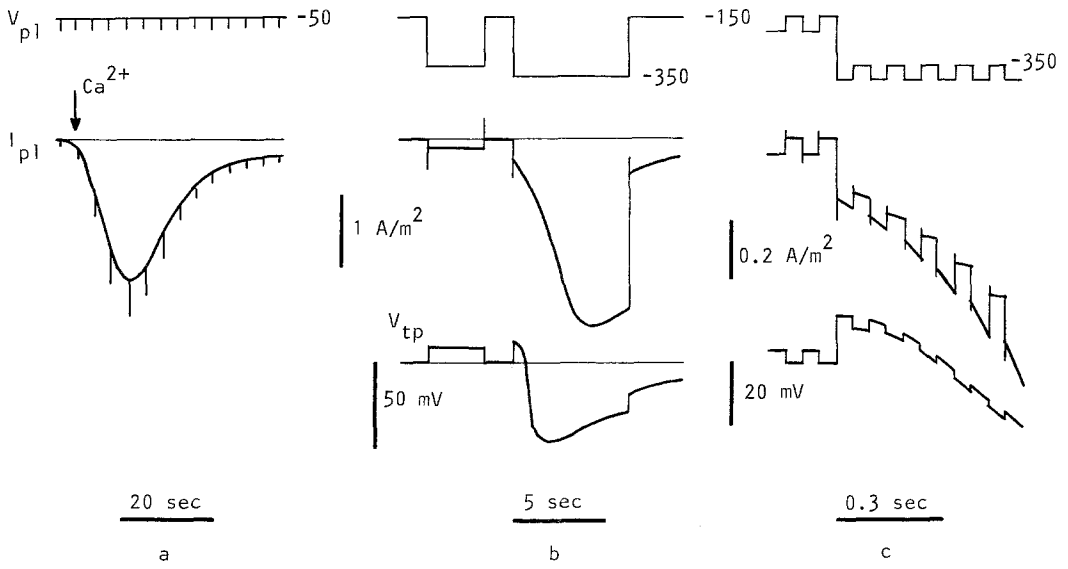

Fig. 11. Activation of chloride channels of plasmalemma: *a.* induced by Ca^{2+} ions (0.5 ml of 20 mM $CaCl_2$) added to the bathing solution of 40 mM NaCl. $V_{pl} = -50$ mV (resting potential). Regular testing voltage pulses (5 mV, 0.1 sec) are applied. Calibrations in *a* and $b = 1$ A/m^2; *b.* induced by hyperpolarization of plasmalemma up to -350 mV. The first impulse (≈ -310 mV) is subthreshold. At excitation the tonoplast voltage first increases by a jump and then transforms into negative action potential. External solution: APW; *c.* the same as in *b*, but the beginning of excitation is shown with superimposed testing pulses (50 mV) on V_{pl}. It is seen that primarily tonoplast resistance sharply falls, and then the amplitude of the current impulses, I_{pl}, slowly increases

excitation by a depolarizing step. Superimposition of testing voltage pulses shows, as expected, that plasmalemma hyperpolarization is followed first by decrease in tonoplast resistance (it excites) and then the plasmalemma resistance smoothly falls (Fig. 11 *c*), i.e. plasmalemma chloride channels are activated. Thus, experimental results obtained prove that Ca^{2+} plays the role of activator of chloride channels.

Discussion

As a whole the results of the studies on cell excitation are summarized in the scheme shown in Fig. 12. Both plasmalemma and tonoplast contain similar ionic channels, i.e. calcium and chloride channels. Shifting of voltage on any membrane in depolarizing direction opens potential-dependent calcium channels and calcium ions enter the cytoplasm. Ca^{2+} ions interacting with chloride channels of both membranes activate them and Cl^- ions flow through the membranes. After that, first the calcium channels are inactivated (close) and then the chloride channels. Under depolarization of plasmalemma the outward K^+ flux occurs through its noninactivating leakage channels. Presence of the inward calcium and chloride currents (with inactivation kinetics) and outward potassium current is sufficient to provide generation of action potential in plasmalemma.

Since the chloride channels are activated by free calcium ions, inactivation of these channels is determined to a large extent, if not exclusively, by asymptotic decrease in Ca^{2+} concentration due to adsorption of these ions by cytoplasm molecules and cellular organelles, in particular, by mitochondria, and due to active transport to vacuole, where Ca^{2+} concentration is about 10 mM. In fact, as seen from Fig. 10 *a* (dash-and-dot line), under prolonged perfusion of a depolarized cell with $CaCl_2$, when Ca^{2+} ions continuously enter the cytoplasm, the chloride current of the tonoplast monotonously increases. This phenomenon justifies the absence of apparent inactivation of chloride channels in this case. Not so with the mechanism of inactivation of potential-dependent calcium channels. Taking into account qualitative kinetic similarities of the current through potential-dependent calcium channels and sodium channels in nerve fibers, one could assume a common mechanism of their inactivation.

Existence of calcium and chloride channels in plasmalemma was demonstrated above while analyzing two components of transient current developed at membrane depolarization under voltage-clamp conditions. These results coincide well with earlier data: increase in Cl^- flux in the course of action potential [15] and dependence of plasmalemma current on Cl^- and Ca^{2+} concentrations as shown by Findlay and Hope [11, 14]. Two com-

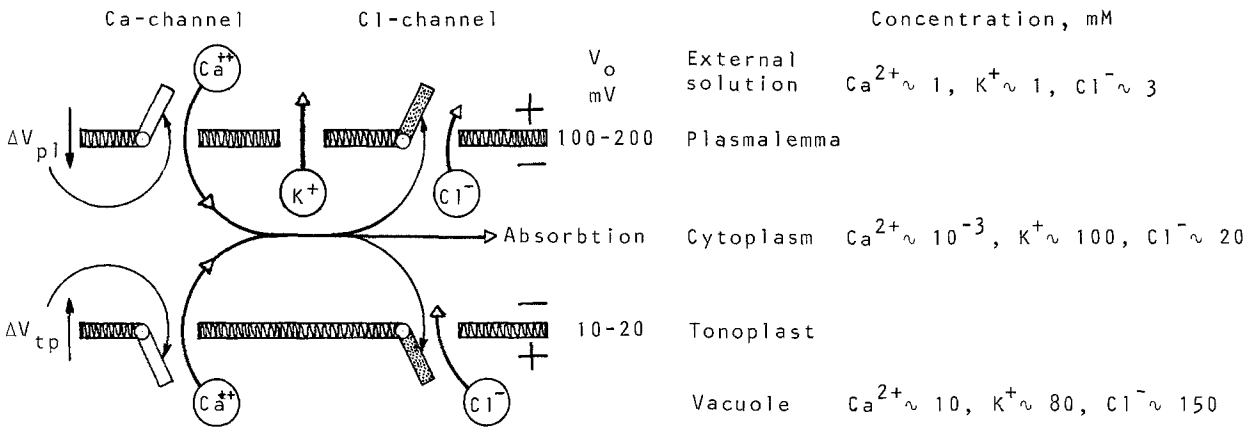

Fig. 12. Schematic presentation of excitable system of plasmalemma and tonoplast. Depolarization ($\Delta V>0$) of any membrane causes temporary opening of Ca channels which in turn allows Ca^{2+} ions to enter the cell cytoplasm and to activate Cl channels in both membranes. Then inactivation of Ca channels occurs and mitochondria and other cytoplasmic elements absorb free Ca^{2+} ions. As a result, the chloride channels close and the system returns to its initial state due to outward K^+ current

ponents of plasmalemma transient current were also described in [32] and [3], and it should be noted that Beilby and Coster [3] while studying the *Chara corallina* cells identified clearly the second component as the chloride one, according to the reversal potential, and proved calcium origin of the first component.

Existence of chloride channels in tonoplast is obvious from the comparison of reversal potential of the tonoplast transient current with equilibrium potentials for the Cl^-. Earlier in [13] and then in [18] similar conclusions were made from the studies of action potentials in tonoplast. Recently [28], the method of continuous perfusion of *Nitella* cell vacuole was applied which provided maintenance of intrinsic hydrostatic pressure inside a cell. This method allowed for the introduction of a microelectrode into cell cytoplasm and the replacement of the solutions inside the vacuole and voltage clamp of the same tonoplast simultaneously. Under substitution of experimental solution ΔV_r of the tonoplast transient current was shown to coincide well with the shift of equilibrium potential for chloride ions. The data considered hold no doubt about the existence of chloride channels in the tonoplast.

A number of comparatively indirect results supports the existence of calcium channels in the tonoplast: (i) presence of two components in the tonoplast current, when membrane potential is shortly depolarized and then hyperpolarized to the level at which opposite directions of currents through the chloride and calcium channels can be expected (Fig. 9); (ii) activation of plasmalemma chloride channels under the voltage shift to hyperpolarizing direction (Fig. 11*b*). In this case the channels in tonoplast are first to be activated due to its depolarization. Since activation of the chloride channels is mediated by Ca^{2+} ions this means that in tonoplast the calcium channels transporting Ca^{2+} ions from vacuole to cytoplasm are activated.

It remains uncertain, however, how the tonoplast calcium channels maintain their capability of functioning being at the resting state when the voltage is close to zero (the region of high depolarization for plasmalemma calcium channels). It may be accounted for by the existence of "adaptation" mechanism in algal cell membranes. In fact, when depolarization of plasmalemma is maintained for a long period of time and then the voltage is shifted to even higher depolarization level, one can again register transient currents (G.A. Volkov, *personal communication*). Another evidence was obtained on reconstituted calcium channels [2]: the channel silent (no transmission between open and closed states) at some "depolarizing" voltage can be forced to operate again at the same voltage after a short shift of the latter to even more positive values.

Activation of the chloride channels in tonoplast and plasmalemma by calcium ions from the cytoplasm was demonstrated by direct experiments (Figs. 10 and 11*a*). Moreover, a mediatory role of Ca^{2+} was indirectly justified by the experiments showing mutual influence of tonoplast and plasmalemma membranes at different conditions of excitation as well as by the studies of the effects of inhibitors of calcium channels (*see above*) on the chloride component of plasmalemma current. In fact, calcium channels are inhibited. This leads to a sharp decrease of calcium influx to cytoplasm,

and, hence, less number of chloride channels become activated. At the same time, in these experiments the chloride component was not inhibited when excitation was achieved by shifting the voltage in plasmalemma to hyperpolarization (as shown in Fig. 10*b*). In this case chloride channels in plasmalemma are activated by calcium ions entering the cytoplasm through the noninhibited calcium channels in tonoplast, which excites due to the fact that $\Delta V_{tp} > 0$.

An increase in tonoplast conductivity for chloride ions observed immediately after stepped changes of tonoplast voltage to negative level can be also attributed to the mediatory role of Ca^{2+} ions (Fig. 8*a*). This fact indicates that after the voltage step the Ca^{2+} concentration in cytoplasm continues to grow for some time due to Ca^{2+} influx from vacuole since inactivation of the tonoplast calcium channels requires a certain period of time.

The idea about stimulatory effect of Ca^{2+} ions on the fluxes of chloride ions through plasmalemma was put forward earlier by Findlay and Hope [14] on the basis of the amplitude analysis of transient currents. Later, studying the relation between excitation of tonoplast and plasmalemma, Findlay suggested that tonoplast is activated by some ion entering through the plasmalemma channels [12]. Our results support and detail these ideas. So the relation between excitations in plasmalemma and tonoplast observed in all experiments becomes perspicuous [13, 18].

Table 4 provides only rough representation of selective properties of calcium channels, since, as shown with reconstituted Ca channel [23], at increasing cationic concentrations (C_i) the current through the channel tends asymptotically to some limit (saturation current) depending on the type of cation and its valency. In the case of the same valency there exists direct proportion between the value of the saturation current and concentrations at which it can be achieved. The concentrations of monovalent cations range approximately from 0.1 M (Li^+) to 3 M(Rb^+) and those of bivalent cations from 1.5 mM(Mg^{2+}) to 5 mM(Ba^{2+}). Thus, Table 4 contains saturation current of bivalent cations compared with monovalent cationic currents that have values far from the saturation ones (apart from Li^+). So, the above-mentioned ratios I_i/I_K for monovalent ions are characteristic for $C_i \sim 100$ mM. While comparing the currents of mono- and bivalent ions at similarly low concentrations, e.g., at 5 mM, the currents of bivalent ions would be several times higher than those of monovalent ions ($I_{Ba}:I_K \sim 5$), since in this case the currents of monovalent ions are proportional to C_i. Hence, while comparing selective properties of calcium channels in algal cells with those on other objects, the concentration dependence should be taken into account.

In plant electrophysiology *Characea* cells are approximately of the same significance as squid giant axons in electrophysiology of animals; however, the ionic channels of the former are much less well studied, to a great extent due to the lack of specific blockers of these channels. So we hope that the revealed effect of ethacrynic acid to block the chloride channels in algal cells (as it does in animal cells) will provide more detailed characterization of both calcium and chloride algal channels. On the other hand, the capability of ethacrynic acid to block chloride channels in both animal (such as erythrocytes, kidney epithelium) and algal cells justifies a certain structural similarity of corresponding channels. Moreover, comparative analysis of the properties of selective filters of calcium channels in algal cells, mollusc neurones and myocardium cells also proves their similarity [21, 23]. Thus, it seems highly probable that algal cell channels are the evolutionary precursors of those in animal cells and, therefore, the studies on algal cell channels can result in gaining some important information on structure and function of such types of channels in general.

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