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Calcium release from $InsP_3$-sensitive internal stores initiates action potential in *Chara*

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Abstract Neomycin and U73122 are known to suppress inositol 1,4,5-trisphosphate ($InsP_3$) production by inhibition of phospholipase C. We studied the effects of these inhibitors on the excitatory currents, I_{ex}, in *Chara corallina* under voltage-clamp conditions. Computer simulations of the experimental effects by a minimum model for the excitatory reaction pathway allow the assignment of the inhibitory effects to one specific reaction step, i.e. the release of Ca^{2+} from $InsP_3$-sensitive internal stores. In contrast, the inhibitory effect of La^{3+} on I_{ex} suggests inactivation of Cl^- channels. Furthermore, ryanodine-sensitive Ca^{2+} stores seem to be irrelevant for electrical excitation in *Chara*.

Key words: *Chara* action potential; Internal calcium store; Excitatory reaction pathway; Computer simulation; Neomycin; U73122

1. Introduction

The plasma membrane of characean algae is electrically excitable (for review see [1]). It is generally accepted that depolarization beyond a critical threshold voltage causes an increase in the concentration of free cytoplasmic Ca^{2+}, $[Ca^{2+}]_{cyt}$ [2,3]. This elevation is thought to activate Ca^{2+}-dependent Cl^- channels giving rise to further depolarization of the membrane voltage (V_m) (for review see [4]). Repolarization is probably in part due to removal of Ca^{2+} from the cytoplasm and consequent inactivation of the Cl^- channels (for review see [5]).

Some authors believe that this temporal rise in $[Ca^{2+}]_{cyt}$ directly originates from outside through Ca^{2+}-conducting channels in the plasma membrane (e.g. [6]). On the other hand, this rise in $[Ca^{2+}]_{cyt}$ has been suggested to be due to release of Ca^{2+} from internal stores ([5,7,8] and references therein). The mechanism by which Ca^{2+} could be mobilized from internal stores is still poorly understood. It was found that elevation of the second messenger inositol 1,4,5-trisphosphate ($InsP_3$) initiated action potentials in *Chara* [9]. In analogy to animal cells, this indicated a possible mechanism of ligand-mediated mobilization of Ca^{2+} from internal stores in these cells [9]. It did not, however, prove necessarily the role of $InsP_3$ in releasing Ca^{2+} in the course of electrically triggered action potentials

Inhibitors have been described which interfere with ligand-activated Ca^{2+} release from internal stores in animal cells. The aminoglycoside neomycin inhibits phospholipase C (PI-PLC), an enzyme responsible for mobilizing $InsP_3$ from its membrane-bound precursor phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) [10,11]. The aminosteroid 1-(6-[17β-3-methoxyestra-1,3,5-(10)trien-17-yl]amino/hexyl)-1*H*-pyrrol-2,5-dione (U73122) also inhibits PI-PLC in animals [12–15] and plants [16]. Ryanodine, a plant alkaloid, inhibits (>100 μM) or stimulates (<10 μM) Ca^{2+} release from internal stores distinct from the $InsP_3$-sensitive ones [17,18]. Recent application of neomycin and ryanodine on plant cells revealed that these inhibitors are, with probably the same mode of action, also effective in plant cells to interfere with Ca^{2+} mobilization from internal stores [18–20].

Here, the question was addressed whether $InsP_3$- or ryanodine-sensitive release of Ca^{2+} from internal stores contributes to the action potential in *Chara corallina*. Therefore, inhibitors of the internal Ca^{2+} metabolism were applied and their effects on ionic currents of excited cells quantified.

2. Materials and methods

2.1. Cells and solutions

Chara corallina Klein ex Willd. was cultured as reported previously [21]. For experiments, the second or third internodal cell from the apex of a plant was dissected and stored in artificial pond water (APW; 0.5 mM KCl, 1 mM NaCl, 0.5 mM $CaCl_2$, 2 mM *N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid] (HEPES)/NaOH) at least 24 h prior to experiments.

Standard bath solution was stagnant APW. Inhibitors were applied by exchanging the standard bath solution by APW with dissolved inhibitors neomycin sulfate, ryanodine, or $LaCl_3$. U73122 and U73343 were pre-dissolved in dimethyl sulfoxide (DMSO, final concentration of DMSO in APW: 0.5% w/v). No effects of 0.5% DMSO alone were observed in control experiments. Neomycin, ryanodine, U733122, and U73343 were purchased from Calbiochem/Novabiochem (La Jolla, CA, USA).

2.2. Experimental setup

Voltage clamp experiments were carried out according to [22]. In short, 1.4 mm sections of internodal cells were brought under voltage control using a voltage clamp amplifier (μP, Wye Science, Wye, UK). A personal computer with pClamp hard- and software (V 5.5.1, Axon Instruments, Foster City, CA, USA) was used for issuing clamp protocols and current recording. For electrical excitation, the following clamp protocol was used: the membrane was clamped first for 60 ms to a voltage close to the free running voltage V_f. Then V_m was clamped for 3.2 s to a test voltage (V_c) positive of V_f. The clamp protocol was repeatedly applied at 1 min intervals. This time was found to be sufficiently long with respect to the refractory period of the cells. Among 40 different cells considered in the analysis, V_c varied between -50 mV and 20 mV due to different excitability of individual cells. In fresh preparations, the current responses frequently decayed upon repetitive stimulation towards a steady response. Therefore, the cells were pre-stimulated until the amplitude of subsequent control responses (without inhibitor) no longer differed by $>10\%$. Correspondingly, an experiment (series of excitations after addition of inhibitor) was usually terminated when the amplitude of subsequent I_{ex} values no longer differed significantly. In addition, the cytoplasmic

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streaming was observed microscopically. Short cessation of cytoplasmic streaming upon clamping to positive voltages was used as an indication for excitation [23].

2.3. Data analysis

Data were analyzed using the computer programs CLAMPFIT (Axon Instruments, Foster City, CA, USA) and xmgr, Version 4.00 (P.J. Turner, pturner@teleport.com). The excitation currents I_{ex}, as defined in Fig. 1A, were standardized to the origin $I_{ex,\,t=0}=0$, by subtracting a steady offset current, because the absolute position of I_{ex} was not important here. Time courses of I_{ex} were approximated by a the sum of two exponential components:

$$I_{ex}\,(t) = a_1\exp(-\lambda_1 t) + a_2\exp(-\lambda_2 t) \quad (1)$$

with amplitudes a_1 and a_2, and time relaxation coefficients λ_1 and λ_2 (see Fig. 1B). A third, delaying component ,frequently occurring at the beginning of the current response, was ignored for numerical analysis with Eq. 1 by extrapolation of the maximal (negative) slope back to zero current. This procedure yields a corrected time origin for the apparent event following Eq. 1. Similarly, capacitative current transients (<1 ms) upon voltage steps were ignored as well.

2.4. Modelling

Fig. 2 illustrates a simplified model for Ca^{2+} mobilization during action potentials in *Chara*. The formalism will be used as a working hypothesis. An aliquot of Ca^{2+} ($Q_{tot}=1$) is assumed to be translocated during an action potential. Before a stimulus ($t<0$), Q_{tot} resides in stores: $Q_{s,\,t<0}=Q_{tot}=1$. Upon stimulation ($t\geq 0$), this quantity is released from stores into the cytoplasm. There it causes a transient rise of released quantity, Q_r, before it is taken up as the portion Q_b (buffered quantity) into the entire Ca^{2+} buffering system comprising various compartments and mechanisms.

This reaction scheme can be expressed by the differential equations

$$dQ_r/dt = -k_r Q_s \quad (2a)$$

$$dQ_r/dt = k_r Q_s - k_b Q_r \quad (2b)$$

$$dQ_b/dt = k_b Q_r \quad (2c)$$

which can be used to calculate the time course of Q_r, i.e. of the transient increase of $[Ca^{2+}]_{cyt}$:

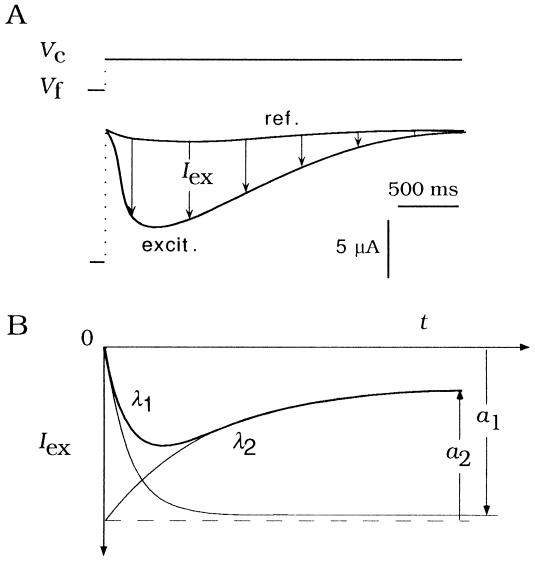

Fig. 1. Definitions. A: Examples of excitatory and refractory response of the clamp current upon positive voltage steps from −150 to +10 mV; excit and ref: stimulation 60 and 3 s after last excitation respectively; definition: $I_{ex}=I_{excit.}-I_{ref.}$. B: Symbols used for quantitative characterization of I_{ex} by the sum of two exponentials, Eq. 1, with the amplitudes $a_{1,2}$ and the relaxation coefficients $\lambda_{1,2}(=1/\tau_{1,2})$.

$$Q_r(t) = \frac{k_r}{k_r-k_b}(\exp(-k_b t)-\exp(-k_r t)) \quad (3)$$

Upon a change in Q_r, the activity of the Cl^- channels relaxes with $\exp(-(k_i+k_a)t)$ towards a new equilibrium conductance $G_{Cl,\,max}/(1+k_i/k_a)$, where k_i and $k_a=k_a^0 Q_r$ are the rate constants for inactivation and activation of the Cl^- conductance respectively. For the explicit calculation of the time course of I_{ex} (with arbitrary amplitude scaling), we chose $k_i=k_a^0=2\ s^{-1}$, and calculated the time course of the Cl^- conductance iteratively as $G_{Cl}=G_{Cl,\,max}/(1+k_i/k_a)$ with $k_{a,j}=k_{a,j-1}+k_a^0 Q_{r,j}\,\Delta t$ which, in turn, yields the desired time course of

$$I_{ex} = (V_m-E_{Cl})\cdot G_{Cl,\,max}/(1+\ k_i/k_a) \quad (4)$$

as the product of the constant driving force, V_m-E_{Cl}, at V_t and the Cl^- conductance.

3. Results

3.1. Excitatory and refractory currents

Fig. 1A shows an example for the excitatory/refractory characteristics of the current response in *C. corallina* upon a larger voltage step from the free running voltage V_f to $V_c \gg V_f$. When >60 s have elapsed after the past stimulus, the clamp current falls in a sigmoidal time course to the (negative) peak. If such a sigmoidal current response is observed, we consider the event an excitatory one, no matter how big the actual amplitude of the current response. The corresponding trace in Fig. 1A is marked by 'excit'. On the other hand, when only a short time period has elapsed since the past excitation (i.e. 3 s in the example in Fig. 1A), the current response upon a new V-step already starts with its maximum (negative) slope, i.e. it does not accelerate up to an inclination point but decays right away toward zero at the (negative) peak. We consider such a response refractory. Correspondingly, this tracing in Fig. 1A is marked with 'ref'. In control experiments (not shown), these refractory current responses did not change significantly with respect to the presence of the inhibitors used here. Therefore, the differences between the non-refractorial current responses and the refractorial responses have been focussed upon as the excitatory currents,

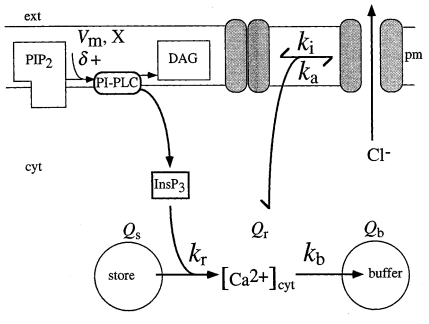

Fig. 2. Working hypothesis for Ca^{2+} mobilization from internal stores and activation of Cl^- channels during action potential. Depolarization (δ+ of V_m) activates PI-PLC by a yet unknown mechanism (X). PI-PLC mobilizes $InsP_3$ from PIP_2 in the plasma membrane (pm). This $InsP_3$ causes release of an aliquot Q_s of Ca^{2+} from internal stores with the rate constant k_r. The following elevation of $[Ca^{2+}]_{cyt}$, Q_r, stimulates the chloride conductance, G_{Cl} (Cl^- channels), in the pm via the rate constant $k_a=k_a^0 Q_r$ for activation of G_{Cl}, where k_a^0 is k_a at a reference amount ($\equiv 1$) Q_r; k_i is the rate constant for inactivation of the Cl^- channels. Q_r will be taken up by processes globally described by k_r, as the portion Q_b of the total Ca^{2+} buffer.

$I_{ex} = I_{excit.} - I_{ref.}$. These excitatory currents, I_{ex}, have been analyzed according to the formalism in Fig. 1B.

3.2. Neomycin

Fig. 3 shows examples of the effect of neomycin on I_{ex} of a *Chara* cell. Individual tracings were recorded every 2 min after addition of neomycin. Explicit examples of the effect of 200 μM neomycin exposure on I_{ex} are given in Fig. 3A. Qualitatively, in neomycin the amplitude of I_{ex} decreased and the response slowed down. Eq. 1 has been fitted to each of the tracings. The resulting, neomycin-induced changes of the two amplitudes, a_1 and a_2, and of the two relaxation coefficients, λ_1 and λ_2, are plotted in Fig. 3B,C, respectively.

The two amplitudes, a_1 and a_2, in Fig. 3B show a parallel and approximately exponential decay to zero with a time constant of about 10 min incubation. Similar experimental results have been recorded from five different cells. These bulk results show that in neomycin, the amounts of a_1 and a_2 are similar, i.e. that the current transients return to about their starting level.

As for the relaxation coefficients λ, (Fig. 3C), the greater one, λ_1 – for the increase of the (negative) current transient – decayed within the first 2 min to about 70% of its control value and decayed further to about 50% with a time constant of roughly 10 min. The smaller relaxation coefficient, λ_2 – for the decrease of the (negative) current transient – falls in a similar way as λ_1 but not as much.

Qualitatively similar but slower changes of parameters were obtained with 100 μM ($n = 2$) and 20 μM ($n = 4$) neomycin (data not shown).

In 10 experiments with 10–200 μM neomycin, the inhibitor was washed out after excitability had vanished completely. Twenty minutes after washing, only one of these cells became excitable again.

3.3. U73122

In order to examine the conclusion that the observed effect of neomycin reflects inhibition of PI-PLC, the effect of U73122 (another inhibitor of PI-PLC) on I_{ex} was investigated as well. Representative results from one cell are shown in Fig. 4. Some individual tracings of the effect of U73122 on I_{ex} are given in Fig. 4A. Fig. 4B,C shows the average changes of the parameters a_1 and a_2, λ_1 and λ_2, as fitted by Eq. 1 to each individual tracing of I_{ex} in progressive periods of incubation in 10 μM U73122.

The global effect of U73122 on I_{ex} consists of a decrease of the amplitude, just as with neomycin; similar is also the relatively large effect on the amplitudes during the first 2 min of incubation, followed by minor decreases. Kinetic differences of the effects of U73122 compared to those of neomycin consist of smaller changes of a_1, compared to a_2, and the lack of the initial decrease of λ_1 during the first 2 min of incubation. The former finding corresponds to the observation that in the presence of U73122, I_{ex} does not return to the starting level within seconds. This U73122-induced, apparently residual level in I_{ex} ($|a_1| > |a_2|$ in Fig. 4B) is well represented by the example tracings in Fig. 4A. It is, however, not a genuine steady-state current; it rather reflects an about 10-fold prolonged time for return to the starting current. This corresponds to recordings of the free running voltage in the pres-

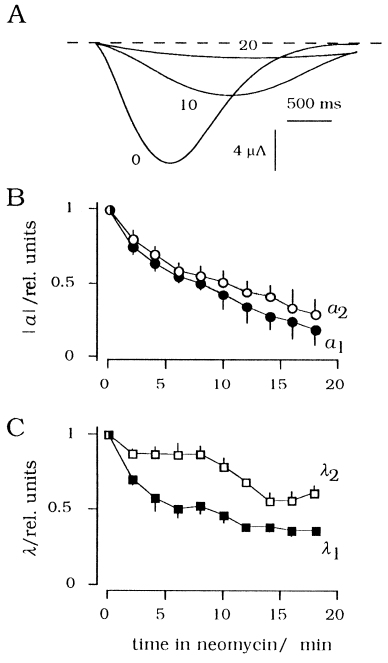

Fig. 3. Progressive effect of incubation with 200 μM neomycin on I_{ex} in *Chara*. Results from one cell. A: Individual recordings, before (0) and 10 (or 20) min after exposure to neomycin. B: Normalized time courses of amplitudes (virtually equivalent reference values for a_1 and a_2 at time zero: −39 and +41 μA respectively); means ± S.D. from five cells. C: Normalized time courses of relaxation coefficients (reference values for λ_1 and λ_2 at time zero: 1.8 and 1.5 s^{-1} respectively; means ± S.D. from five cells.

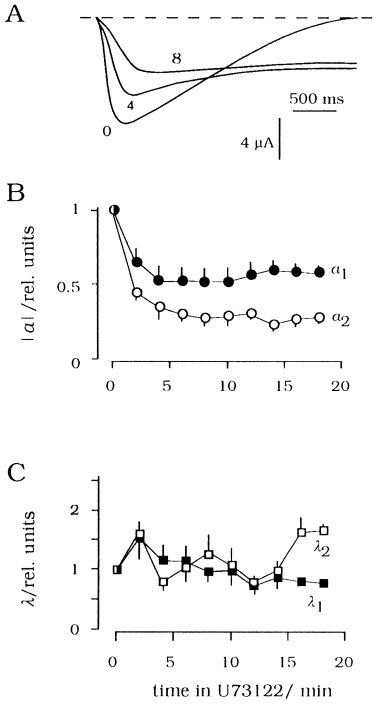

Fig. 4. Progressive effect of incubation with 10 μM U73122 on I_{ex} in *Chara*. Results from one cell. A: Individual recordings, before (0) and 4 (or 8) min after exposure to U73122. B: Normalized time courses of amplitudes (virtually equivalent reference values for a_1 and a_2 at time zero: −38 and +40 μA respectively); means ± S.D. from four cells. B: Normalized time courses of relaxation coefficients (reference values for λ_1 and λ_2 at time zero: 2.6 and 0.75 s^{-1} respectively; means ± S.D. from four cells.

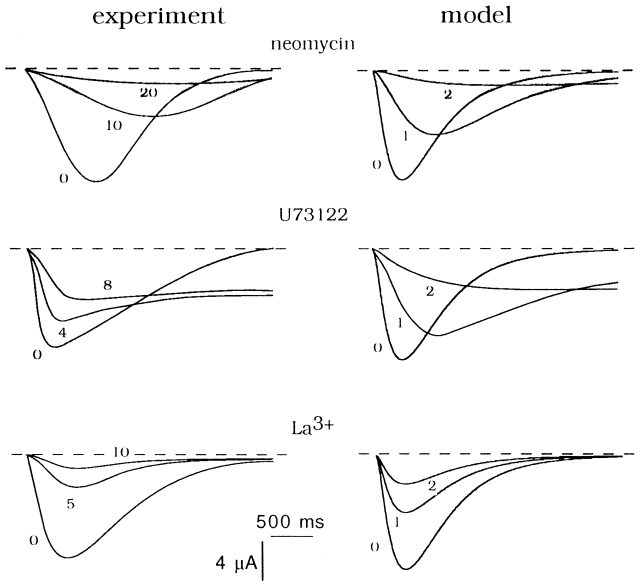

Fig. 5. Comparison of experimental (left column) and simulated (right column) data on the (progressive) effects of 200 μM neomycin, 10 μM U73122, and 200 μM La^{3+} on I_{ex} in *Chara*. Experimental data for neomycin and U73122 identical with top panels A in Figs. 3 and 4 respectively; experimental data for (200 μM) La^{3+}: effects of 0, 5 and 10 min incubation. Simulations by the model in Fig. 2 with the following parameters; common to all model tracings: k_i and k_a (at $Q_r = 1$) = 2 s^{-1}; $k_b = 5$ s^{-1}; $G_{Cl,\,max}{\cdot}(V-E_{Cl})$: 50 μA, $Q_{s,\,t<0} = 1$, $Q_{r,\,t<0} = 0$, $Q_{b,\,t<0} = 0$, $k_{r,\,t<0} = 0$; for neomycin $k_{r,\,t\geq 0} = 16{\cdot}decr$ s^{-1} with $decr = 1$, 0.1, or 0.01; for U73122: as for neomycin plus $\Delta Q_{t=\infty} = 0.05$ and $1/\tau_{\Delta Q} = k_{r,\,t=0}$, for La^{3+}: $k_{r,\,t\geq 0} = 16$ s^{-1} throughout, and $G_{Cl} = G_{Cl,\,max}{\cdot}decr$ with $decr = 1$, 0.5, or 0.25.

ence of 10 μM U73122 which showed delayed repolarization of action potentials.

All cells which lost excitability by incubation in U73122 regained excitability by washing for 15 min with U73122-free medium. This good reversibility is another instance where U73122 behaves differently from neomycin.

Experiments with smaller concentrations of U73122 (eight cells with 5 μM and three cells with 1 μM) resulted in qualitatively similar but somehow slower responses (not shown) compared to the results in Fig. 4.

U73343 is a structural analogue of U73122 but has no effect on PI-PLC (e.g. [13]). When *Chara* cells ($n = 4$) were treated in the same way as shown in Fig. 4 with 10 μM U73343, I_{ex} was not affected over >12 min of exposure (data not shown).

3.4. Ryanodine

Since in animal cells, <10 μM ryanodine stimulates Ca^{2+} release from internal stores but >100 μM inhibits release [17], ryanodine was tested here over a concentration range from 1 μM to 1 mM for any stimulating or inhibiting effect on I_{ex} via Ca^{2+} release from internal stores. However, 45 min exposure to 1 μM, 200 μM, or 1 mM ryanodine had no notable effect either on I_{ex} or on V_f (three cells tested for each concentration; data not shown).

3.5. Lanthanum

Since La^{3+} has been suggested to inhibit Ca^{2+} channels in *Chara* [24–27], the effect of La^{3+} on I_{ex} of *Chara* was tested here as well. Incubation with 200 μM La^{3+} caused a simple downscaling of I_{ex} rather than a change of the kinetics as with neomycin or U73122. Therefore, the effect of La^{3+} on I_{ex} is not presented here in as much detail as for neomycin and for U73122. Only some original tracings are given in Fig. 5 (left bottom panel).

4. Discussion

Before discussing the individual results with respect to our working hypothesis (Fig. 2), the following should be recalled briefly. Depolarization leads – by a mechanism discussed below – to cleavage of $InsP_3$ from $PtdInsP_2$ by PI-PLC. The following cascade comprises $InsP_3$-induced release of Ca^{2+} aliquots from internal Ca^{2+} stores, a transient increase of $[Ca^{2+}]_{cyt}$-activated Cl^- conductance, and rebuffering of the surplus Ca^{2+}.

4.1. Comparison of model and experimental data

The aim of this section is to interpret the experimental data in terms of the hypothetical reaction scheme in Fig. 2, rather than to verify this simple model quantitatively. This would not leave any room for alternative models and/or for additional insights. Fig. 5 shows typical experimental data and simulated time courses of the effects of neomycin, U73122, and La^{3+} on I_{ex}, during progressive times of incubation, i.e. control tracings before incubation (labelled 0) and at two different times during incubation. Equidistant incubation times are given in minutes for the experimental data, and in arbitrary time intervals for the model tracings.

The central part of the model are transient increases in $[Ca^{2+}]_{cyt}$ according to Eq. 1, with $Q_s \equiv 1$, $Q_r \equiv 0$, and $k_r \equiv 0$ before the start when k_r steps from zero to a finite value. Under these circumstances, Eq. 1 predicts an initially linear rise of Q_r out of the origin. The measured current change of I_{ex}, however, starts not immediately with the maximum (negative) slope but in a sigmoidal way. There may be several reasons for this delay. One likely possibility is that several reaction steps are involved between the applied voltage step and the actual rise of k_r. Another reason for the observed delay could be the gating kinetics of the Cl^- conductance. This possibility is chosen here, because it keeps the formal treatment of the Ca^{2+} metabolism simple, and because it accounts for the known time constants (range 100 ms) for gating of Cl^- channels in plant cells. In this case, the delay would increase with the time constant τ for $[Ca^{2+}]_{cyt}$-induced gating of the Cl^- conductance is $\tau = 1/(k_i + k_a)$, where k_i is the rate constant for inactivation and $k_a = k_a^0{\cdot}[Ca^{2+}]_{cyt}$ the rate constant for $[Ca^{2+}]_{cyt}$-induced activation with k_a^0 being k_a at a reference value of $[Ca^{2+}]_{cyt}$. In fact, in the presence of the inhibitors neomycin and U73122 this delay becomes progressively larger in the experiments, and can be simulated by diminished $[Ca^{2+}]_{cyt}$ peaks.

Simulation of the effects of inhibitors on I_{ex} has been most successful in the case of neomycin, where a neomycin-induced decrease in k_r was sufficient to describe the obvious effects of the inhibitor on I_{ex}: the mentioned delay of the maximum slope, a pronounced decrease of the amplitude, and a delay of the (negative) current peak. A corollary of this model is a complete return of the observed excitation current to its start value.

The effect of U73122 of I_{ex} could not be simulated by the same, simple model as for neomycin, because of the appar-

ently residual component in I_{ex} (Fig. 4). In order to account for this observation, an additional, U73122-induced elevation of $[Ca^{2+}]_{cyt}$ by the amount ΔQ has been postulated which does not readily vanish in the range of seconds, but more slowly. The time course $\Delta Q(t)$ of this additional, U73122-induced $[Ca^{2+}]_{cyt}$ increase may be described by

$$\Delta Q_r(t) = \Delta Q_{r,\,t=\infty}(1-\exp(-t/\tau_{\Delta Q})) \quad (5)$$

using standard nomenclature. For the model curves (middle right panel), somehow arbitrary values of $\Delta Q_{r,\,t=\infty} = 5\%$ of $Q_{s,\,t<0}$ and $\tau\Delta Q = 1/k_{r,\,t=0}$ were chosen. Comparable, U73122-induced elevations of $[Ca^{2+}]_{cyt}$ have already been reported from animal cells [28,29]. This corresponds to the finding that U73122 concentrations which inhibit PI-PLC can also elevate $[Ca^{2+}]_{cyt}$ by inhibition of Ca^{2+} pumps [16].

The effect of La^{3+} on I_{ex} (experimental data in Fig. 5, left bottom panel) differs qualitatively from those of neomycin and U73122, in so far as La^{3+} does not seem to have any effect on the temporal characteristics (sigmoidal onset and time of maximum amplitude) of I_{ex}; it rather seems to cause a mere downscaling of the amplitude. This behavior can hardly be explained by an effect of La^{3+} on the PI-PLC $\rightarrow$ $InsP_3$ $\rightarrow$ Ca^{2+} signalling pathway but easily by a direct inhibition of G_{Cl} by La^{3+}. The simple model predictions of the latter interpretation are illustrated in the bottom right panel of Fig. 5. Our conclusion that (200 μM) La^{3+} strongly inhibits the transient G_{Cl} during I_{ex} in *Chara* is novel but not incompatible with previous reports from *Chara* about La^{3+} inhibiting Ca^{2+} conducting cation channels [7,25,26], volume-sensitive Cl^- channels [30] and hyperpolarization-activated Cl^- channels [31] which depend only weakly on $[Ca^{2+}]_{cyt}$ [32].

4.2. General aspects

The effects of the PI-PLC inhibitors neomycin and U73122 on I_{ex} can be successfully simulated by a model in which membrane excitation is initiated by mobilization of $InsP_3$ from $PtdInsP_2$ and subsequent $InsP_3$-generated release of Ca^{2+} from internal stores. The effect of neomycin on I_{ex} can be ascribed solely to the inhibition of PI-PLC. For the effect of U73122 at least one additional reaction must be anticipated. Ca^{2+} mobilization from ryanodine-sensitive stores does not contribute to excitation.

The interpretation of excitation in the context of $InsP_3$-mediated Ca^{2+} release is consistent with other findings, that action potentials can be elicited in *Chara* by injection of $InsP_3$ [9], that Ca^{2+} is released from internal stores during an action potential [7,8]) and that $InsP_3$-mediated Ca^{2+} release from internal stores in animal cells as well as activation of Ca^{2+}-dependent Cl^- channels in *Chara* occur in discrete portions [33,34].

The model does not as yet explain the postulated dependence of PI-PLC activity on membrane voltage. A potential explanation for this could be that in plant cells, PI-PLC is activated by low concentrations of cytosolic Ca^{2+} [35–37]. Therefore, it could be speculated that a small voltage-dependent Ca^{2+} influx starts the cascade of events by activating PI-PLC and consequently $InsP_3$ mobilization.

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