External Electric Fields Stimulate the Electrogenic Calcium/Sodium Exchange in Plant Protoplasts†

Annick Graziana,‡ Raoul Ranjeva,*,‡ and Justin Teissié§

Centre de Physiologie Végétale de l'Université Paul Sabatier, URA 241, and Centre de Recherche de Biochimie et Génétique Cellulaires du CNRS, UPR 8201, 118 Route de Narbonne, F. 31062 Toulouse Cédex, France

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ABSTRACT: External electric fields of low intensity stimulated calcium influx in protoplasts isolated from carrot cell suspension cultures in field intensity dependent and frequency-dependent ways. The field-induced calcium uptake involved a temperature-dependent system that was saturable by external calcium. The induction process appeared mainly cumulative as long as the morphology of the protoplasts did not change (up to 10 min). The stimulation elicited by the electric fields was effective even after switching the field off; the influx increased for 5 min and then slowed down to its initial value 15 min later. During electrostimulation, an additional amount of ATP was accumulated; on removal of the stimulatory field, the extra amount of ATP was consumed, whereas the plasma membrane was hyperpolarized and sodium ions were expelled from the protoplasts. Inhibition of either ATP accumulation or consumption results in the inhibition of both calcium influx and sodium efflux, demonstrating that these processes are coupled. From the data obtained in this work, it may be concluded that the electric field stimulates an ATP synthase like activity; the consumption of the ATP thus formed elicits an electric potential (probably due to the efflux of cations and more specifically sodium) that drives the influx of calcium.

Calcium controls a number of physiological processes in higher plants from the molecular to the multicellular level (Hepler & Wayne, 1985) and is considered as the main second messenger involved in the transduction of external stimuli including light, fungal elicitors, plant growth substances, and different types of stress (Hepler & Wayne, 1985; Kauss, 1987). Such a conclusion stems from the characterization of calcium-dependent enzyme activities and by a more indirect approach using cells and protoplasts. Thus, lowering the exchangeable amounts of calcium in culture media with EGTA[1] or increasing the $[Ca]_i$ with ionophores mimics/inhibits the effects elicited by some of the above-mentioned stimuli. Moreover, addition of calcium channel blockers or agonists makes it possible to regulate the initiation and developement of gametophore buds in the moss *Funaria* (Conrad & Hepler, 1988), which suggests that calcium entry through specific channels is a critical event in the differentiation process. Moreover, membranes isolated from plant tissues or cell suspension cultures bind calcium channel blockers in a specific manner (Andrejauskas et al., 1985; Graziana et al., 1988; Harvey et al., 1989), and the site occupancy leads to the inhibition of calcium uptake in protoplasts (Graziana et al. 1988). Similarly, the phytotoxin zinniol competes with channel antagonists and stimulates the calcium influx up to a lethal dose (Thuleau et al., 1988).

It is therefore highly probable that functional calcium channels exist in plants as is the case in animals; however, it is still unclear how far they are structurally and functionally related to their animal counterpart. Thus, there is no cross-reactivity between either polyclonal or monoclonal antibodies raised against muscle T-tubule calcium channels and plant membranes (Graziana et al., 1988). More importantly, the voltage dependence of calcium entry into plant cells is implicitly accepted but has never been demonstrated. Protoplast electropulsation offers an approach to a controlled alteration of membrane potential.

External electric fields are known to dramatically change the permeability of biological membranes and to elicit important effects such as cell fusion (Neumann et al., 1989), but electropulsation can activate membrane-bound ATPase of red blood cells, *Escherichia coli*, or submitochondrial particles (Teissié & Tsong,, 1981; Teissié et al., 1981; Serpersu & Tsong, 1983, 1984; Teissié, 1986). By using low-intensity external fields, one should be able to specifically modulate the potential of the plasma membrane but not of the tonoplast, the plasma membrane playing the role of a Faraday cage. As a consequence, the plasma membrane potential would be shifted by only a few tens of millivolts and did not induce physiological damage. We used such a strategy to study the mechanism of calcium uptake in plant protoplasts, and we report, in this paper, on the stimulation of a voltage-sensitive calcium influx into carrot protoplasts. The present study provides evidence that the process is triggered only when the membrane potential reaches a critical threshold, lasts a while after electrostimulation, and involves the activation of cell metabolism.

Experimental Procedures

Chemicals. $^{45}CaCl_2$ (25.92 mCi/mg) was from Du Pont (Wilmington, DE). [^{14}C]Dextran and (−)-D888 were from Amersham (London, U.K.). Caylase 345 (a protease-poor cellulase) was from Societé Cayla (Toulouse, France). Pectolyase Y23 was from Seishim Pharmaceuticals Co. (Tokyo,

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* To whom correspondence should be addressed.

‡ Centre de Physiologie Végétale de l'Université Paul Sabatier, URA 241.

§ Centre de Recherche de Biochimie et Genetique Cellulaires du CNRS, UPR 8201.

[1] Abbreviations: (−)-D888, (−)-desmethoxyverapamil; DCCD, *N,N'*-dicyclohexylcarbodiimide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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Japan). Carbocyanine was from Molecular Probes (Junction City, OR). Evans blue was from Sigma (St. Louis, MO). Other analytical grade products were from standard sources.

Cell Cultures and Protoplast Preparation. Carrot cell suspension cultures were grown as already described (Graziana et al., 1988). Protoplasts were prepared aseptically from 8- to 10-day-old cultures (midexponential phase) by a method adapted from Graziana et al. (1988). The original procedure was modified as follows: (i) The concentrations of the digestion enzymes were respectively lowered to 0.8% instead of 2% for Caylase 345 and to 0.02% instead of 0.1% for pectolyase Y23. The mixture was stirred for 1 h in 25 mM Mes–Tris buffer at pH 5.5 containing 700 mM mannitol and clarified by centrifugation (10 min at 2000*g*) and the resulting supernatant sterilized by filtration. (ii) One gram of cells was transferred into a 8-cm diameter Petri dish containing 20 mL of the enzyme mixture and incubated overnight at 20 °C with gentle stirring (44 rpm). Thereafter, the protoplasts were processed and purified exactly as described by Graziana et al. (1988).

Protoplast Electropulsation. Basically, the procedure previously described for red blood cells was improved and adapted to the plant material (Teissié & Tsong, 1981). The electric field was generated by means of three parallel stainless steel electrodes (9.8 mm wide, 30 mm long) 4 mm apart. The two external electrodes were connected together, and alternating square-wave voltage was applied between the central and outer electrodes, generating a field in between. The amplitude, symmetry, and frequency of the stimulating voltage were predetermined (Enertec generator 4430, Saint-Etienne, France) and monitored (Tektronix oscilloscope 7313, Beaverton, OR). The electrodes wee dipped in a disposable spectrophotometer cuvette containing 1.5 mL of protoplast suspension and thermostated at the temperatures specified (Figure 1).

Calcium Uptake. Unless stated otherwise, protoplasts (1.5×10^6/mL) were suspended in 1.5 mL of buffer containing 600 mM mannitol in 10 mM Hepes–KOH at pH 6.7 and electrostimulated for 5 min. After electrostimulation, calcium uptake was initiated by adding 1 μCi of $^{45}CaCl_2$ and $CaCl_2$ to give a final concentration of 0.1 mM. At the indicated times, 300-μL aliquots were filtered through HAWP Millipore filters (Millipore, Saint Quentin, France) and washed (three times) with 2 mL of ice-cold 20 mM Tris-HCl buffer at pH 7.5 containing 500 mM mannitol and 100 mM $MgCl_2$. The radioactivity remaining on the filters was counted in a liquid scintillation spectrometer (Packard 460 C). Experiments were done in triplicate with three independent repetitions, as described (Graziana et al., 1988). Controls with unstimulated protoplasts were run in parallel.

Sodium Determination. Protoplasts were electrostimulated for 5 min, then, at indicated times, protoplast suspensions were centrifuged (3 min at 300*g*), and the sodium content of the supernatants was measured by atomic absorption spectrophotometry (Varian AA 275 series). Controls was unstimulated protoplasts were run in parallel.

Plasma Membrane Integrity. The integrity of the plasma membrane was checked by exclusion of nonpermeant molecules, i.e., Evans blue or [^{14}C]dextran. Whatever the intensity of the electric field used (at most 25 V/cm) none of these compounds accumulated in the protoplasts. As an example, only 10^3 dpm remained out of 4×10^5 dpm of labeled dextran added to 3×10^5 protoplasts.

Fluorescence Measurements. The changes in membrane potential and ATP content have been determined by using procedures adapted to plant materials. Thus, fluorescence was measured with a JY3 spectrofluorimeter (Jobin-Yvon, Paris, France) (λ_{ex} 551 nm; λ_{em} 568 nm) using 20 μM carbocyanine and 3×10^5 protoplasts in a final volume of 1.5 mL (Low & Heinstein, 1986). ATP was estimated by the luciferin/luciferase assay following Pradet (1967).

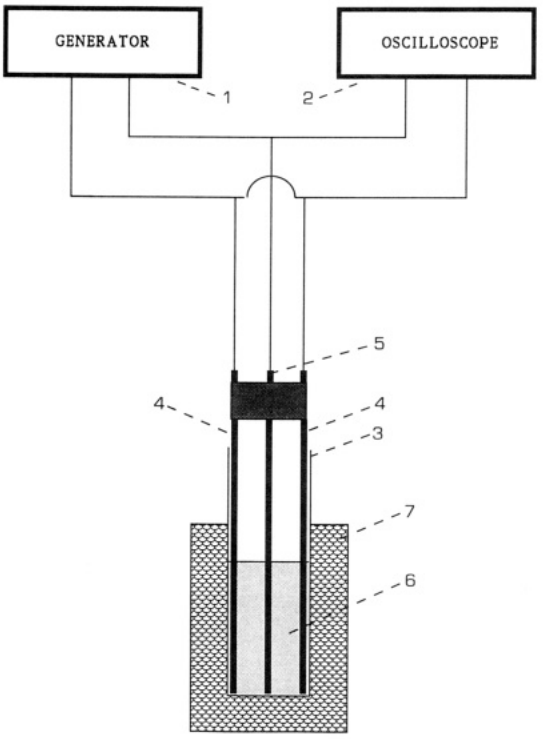

FIGURE 1: Protoplast electropulsator. The carrot protoplasts (6) are in a disposable cuvette (3) where they are submitted to the electric field generated between the lateral (4) and central (5) flat electrodes. The temperature of the sample is controlled by means of a thermostating block (7). The ac field is generated by the voltage delivered by the generator (1) and monitored on the oscilloscope (2).

RESULTS

External Electric Fields Stimulate Calcium Influx in a Voltage- and Frequency-Dependent Way. Figure 2a depicts the effects of increasing electric field intensities (at the constant frequency of 100 Hz) on the uptake of calcium by carrot protoplasts. It clearly appears that the influx was stimulated only when the field reached 12.5 V/cm. Beyond this critical threshold value, the process was stimulated by more than 2-fold at 25 V/cm. Higher fields were not used due to practical constraints. Addition of 2 μM calcimycin, a calcium ionophore, released calcium from the protoplasts, demonstrating that the electrostimulation led to an actual calcium influx and not a simple adsorption onto plasma membrane (see Figure 4a).

Electric field induced effects on cells are known to depend upon the frequency of the external stimuli (Teissié & Tsong, 1981; Serpersu & Tsong, 1984). Therefore, the protoplasts were maintained at a constant external field (25 V/cm) while the frequency was raised. The data reported in Figure 2b show that the influx increased with frequencies up to 100 Hz, reached a plateau at 1 kHz, and returned back to zero at 100 kHz.

Voltage-Induced Calcium Uptake Depends on Temperature and External Calcium Concentrations. Figure 3a depicts the effects of temperature on the calcium uptake by protoplasts in various experimental conditions. In the absence of electric fields, the influx of calcium increased by 30% when the temperature was raised from 0 to 30 °C. In contrast, the voltage-dependent uptake behaved in a very different manner.

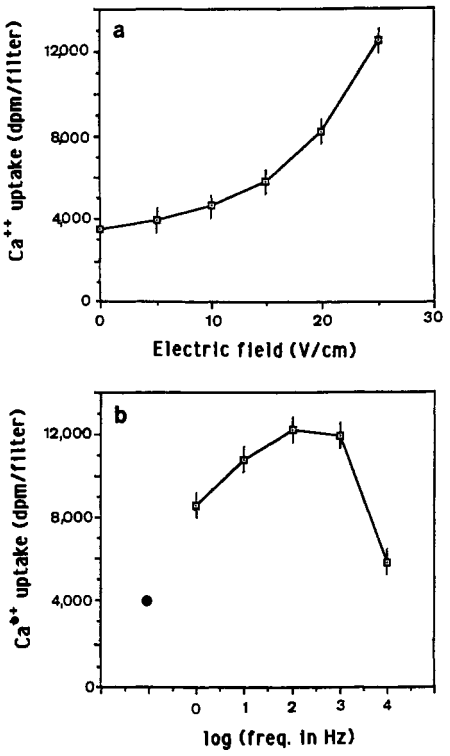

FIGURE 2: Effects of electric fields on calcium uptake by carrot protoplasts. The protoplast preparations were treated for 5 min at 20 °C by electric fields, and then labeled calcium was added and the influx measured after 1 min (300 000 protoplasts/assay). (a) Effects of increasing fields at constant frequency (100 Hz). (b) Effects of increasing frequency at constant field (25 V/cm) (●, control without electric treatment).

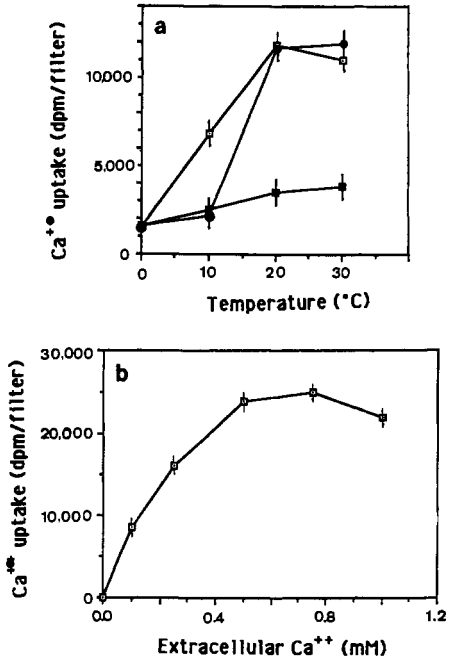

FIGURE 3: Effects of temperature and external calcium on voltage-induced calcium uptake. The protoplasts were treated for 5 min at 25 V/cm and 100 Hz as specified, and the influx was measured as in Figure 2. (a) Effects of temperature: (■) without electric fields (control); (●) treatment and uptake at constant temperature; (⊡) treatment at indicated temperature and uptake at 20 °C. (b) Effects of calcium concentrations.

When temperature was maintained constant during the electric treatments and the influx measurements, it turned out that the calcium uptake was stimulated by 5-fold between 0 and 20–30 °C. Moreover, when the electric treatments were operated at different temperatures and kinetics were run at 20 °C, the process stimulated by electric fields was not effective at 0 °C but was significantly increased at 10 °C, suggesting that electropulsation was modulating a temperature-dependent system. As shown in Figure 3b, the external calcium concentration influenced the calcium uptake in a dose-dependent manner, the process being saturable.

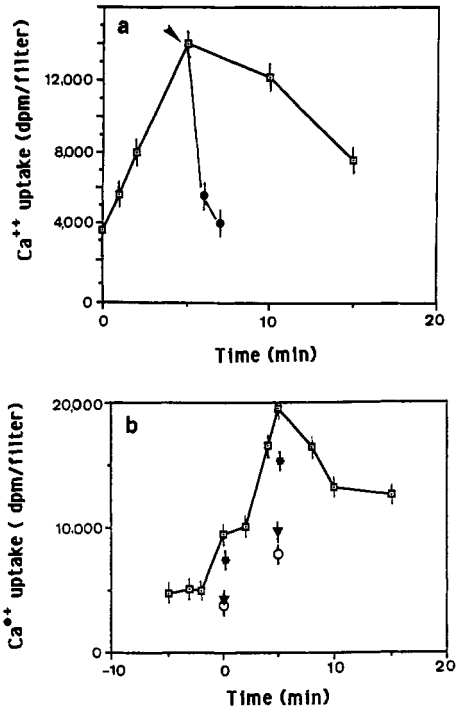

FIGURE 4: Time course of voltage-dependent calcium uptake by carrot protoplasts. (a) The electric field (25 V/cm) was applied for indicated times at 20 °C; then labeled calcium was added and the calcium influx measured (⊡) as in Figure 2. In a parallel experiment 2 μM calcimycin was added (arrowhead) and the calcium remaining in protoplasts measured (●). (b) The electric field was applied for 5 min (time −5 to 0), and then the stimulatory electrode was removed (time 0). Labeled calcium was added at the indicated times and the uptake measured for 1 min. (*) 5×10^{-5} M (−)-D888, (○) 20×10^{-5} M amiloride, or (▼) 10^{-5} M bepridil was added at time −5 min.

The Time Course of the Voltage-Dependent Uptake of Calcium Proceeds in a Particular Manner. Two aspects of the calcium uptake time course were studied, namely, (i) the duration of the treatment with electric fields and (ii) and relaxation time of the system where the treated protoplasts return back to the resting state.

First, the induction process appeared mainly cumulative in that the influx increased with treatment duration up to 5 min (Figure 4a). Then, a maximum was reached and the influx decreased slowly to almost its initial value after 15 min. Microscope observations of the protoplasts showed that they were not disturbed by the short duration treatments whereas a large number of them were morphologically changed after longer durations and were permeable to Evans blue.

Second, the stimulation elicited by the electric field was still effective after switching the field off (Figure 4b). The poststimulation process was very striking because the rate of influx increased dramatically for 5 min (almost twice as high as time 0) and then slowed down but did not return to the 0 time value even 15 min later. In addition, it turned out that the voltage-dependent component of the calcium influx was essentially insensitive to the calcium channel blocker D888 but was severely inhibited by the amiloride derivative 5-*N*-(4-chlorobenzyl)-2′,4′-dimethylbenzamil, which is known to inhibit calcium/sodium exchange (Slaughter et al., 1988), and

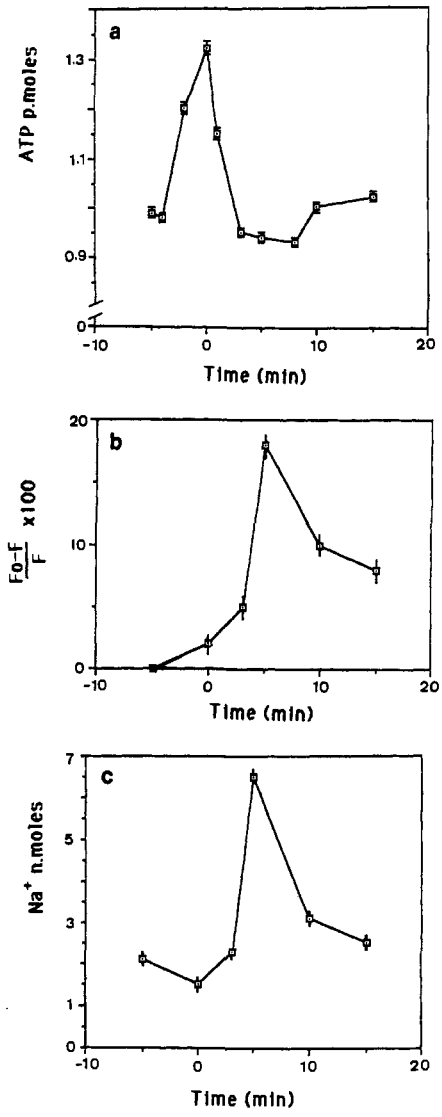

FIGURE 5: Effects of electric fields on membrane polarization, ATP synthesis, and sodium efflux: (a) protoplast ATP content; (b) change in relative fluorescence of the cationic dye carbocyanine; (c) sodium efflux.

by bepridil, which acts on calcium channels and calcium/sodium exchanges (Garcia et al., 1988) in animal systems.

Voltage-Dependent Calcium Uptake Is Correlated to ATP Consumption, Hyperpolarization, and Sodium Efflux. Considering the unexpected time-dependent behavior of the uptake system, we investigated the metabolic consequences of the electrostimulation and the activation of the process by temperature (postfield effect). We determined the changes in membrane potential and in ATP content. During electrostimulation, the ATP content of the protoplasts increased by 50% (Figure 5a). On removal of the stimulatory electrode, the extra amount of ATP was almost completely consumed within 5 min corresponding to the maximum influx of calcium. As shown in Figure 5b, the fluorescence of the cationic dye carbocyanine decreased dramatically on electrostimulation of protoplasts. The changes in fluorescence, indicative of membrane hyperpolarization (inside more negative), exactly followed the rate of calcium influx during and after the electric treatment which is a back effect of the field. Such behavior suggests that the calcium uptake system is associated to electrogenic processes.

Due to the sensitivity of the process to an amiloride derivative which has been reported to interfere with the movements of sodium (Kaczorowski el al., 1989) the amount of sodium ion released by the protoplasts was measured. Since the medium used during electrostimulation was sodium-free, the protoplasts were the only source of sodium. Moreover, the medium was buffered by 10 mM Hepes–KOH; consequently, potassium ions basically did not interfere with the process; in contrast, substitution of KOH by NaOH as the buffer component resulted in the inhibition of the calcium influx (not shown).

From the qualitative point of view, sodium was expelled from the protoplasts during the period of ATP consumption and reentered the cellular milieu when the ATP level and the membrane potential returned close to their resting values (Figure 5c).

From the quantitative point of view, the calculations were made as follows. In the case of calcium, the specific radioactivity was 14 333 dpm/nmol and the amount of labeled calcium associated to an aliquot of 0.3 mL of protoplasts was 12 000 dpm (i.e., 0.82 nmol) after a 1-min uptake. In the case of sodium, the amount released by the same number of protoplasts was 2.3 nmol. From these values, it is concluded that approximately 1 calcium ion enters the protoplasts whereas 3 sodium ions move out.

Addition of either an uncoupler (50 μM DCCD) or an inhibitor of ATPase (200 μM vanadate) during electrostimulation (time −5 to 0) led to the total or severe inhibition of the induced calcium uptake (Figure 6a). In the case of vanadate, higher concentrations of the compound led to an electrolysis-like effect so that it was not possible to check if vanadate was able to fully inhibit the process. However, it is clear that addition of DCCD suppressed all the electric field induced effects, i.e., increase in ATP (Figure 6b), change in membrane potential, as shown by the fluorescence signal (Figure 6c), and calcium inflow. Addition of vanadate to nonelectrostimulated protoplasts did not stimulate the calcium influx; therefore, the inhibition of a proton pump is not enough to trigger the process.

Discussion

Manipulation of calcium movements (Conrad & Hepler, 1988) and electrostimulation (Goldsworthy, 1986) have been shown to elicit pleiotropic effects on plants including cell division and proliferation (Goldsworthy, 1986); however, no clear relationship between these processes has been established to date. The results reported in this paper establish a direct link between electrogenic activities induced by the electric field and uptake of calcium by protoplasts.

External electric fields are known to exert two major effects on cell suspensions: Joule heating and modulation of the membrane potential (Neumann et al., 1989). Joule heating is not dependent on the frequency of the stimulus whereas the uptake of calcium by protoplasts is (Figure 2b). Therefore, as established for red blood cells (Teissiē & Tsong, 1981; Serpersu & Tsong, 1983, 1984), the observed electrogenic process that is mediated by external ac fields cannot be explained by thermal effects. Membrane permeabilization (electropermeabilization) is also known to be induced by an electric field and is routinely used for direct gene transfer (Neumann et al., 1989). In the present work the range of field intensities is lower by 1 order of magnitude than necessary for electropermeabilization (20 V/cm instead of 200 V/cm) (Neumann et al., 1989). Moreover, permeabilization would allow ATP to leak out of the cell but not to accumulate as observed in this work (Figure 5a).

Consequently, electropermeabilization does not occur during calcium uptake. These conclusions suggest that uptake is related to a mechanism involving the triggering of electrogenic

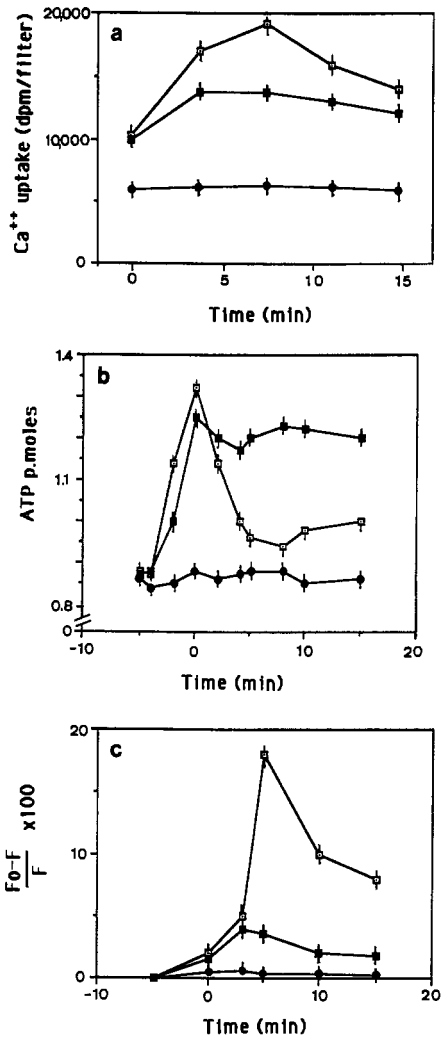

FIGURE 6: Effects of dicyclohexylcarbodiimide (●) and vanadate (■) (□ no addition of inhibitors) (a) on calcium uptake, (b) on ATP content of the protoplasts, and (c) on the change of relative fluorescence of the cationic dye carbocyanine. Experimental conditions as in Figure 4b.

activities. Calcium uptake proceeds only when the field is larger than a given threshold and depends on the frequency. Uptake is dependent upon the field intensity above the threshold.

Calcium accumulates with the duration of the ac stimulation up to 5 min as does rubidium on pumping in red blood cells (Serpersu & Tsong, 1983). The observed decrease for longer times is related to morphological alterations of the protoplasts and probably associated to a loss of functionality and even viability.

The uptake is clearly temperature-dependent and saturable by an excess of extracellular calcium. Therefore, the system involved is probably associated with an active process rather than with the gating of channels, which is basically temperature-independent. The inability of calcium channel blockers to inhibit the calcium influx strengthens this hypothesis (Figure 4b).

However, a direct effect of the external field on calcium uptake is not the major process, if present, because the accumulation of ATP is associated with the ac stimulation and precedes the pumping. The external field induces a net synthesis of ATP, and this extra amount of ATP is then used by ATPases. This multistep process elicits various membrane activities, among which is a calcium/sodium exchange. The process is inhibited by compounds known to interfere with ATP

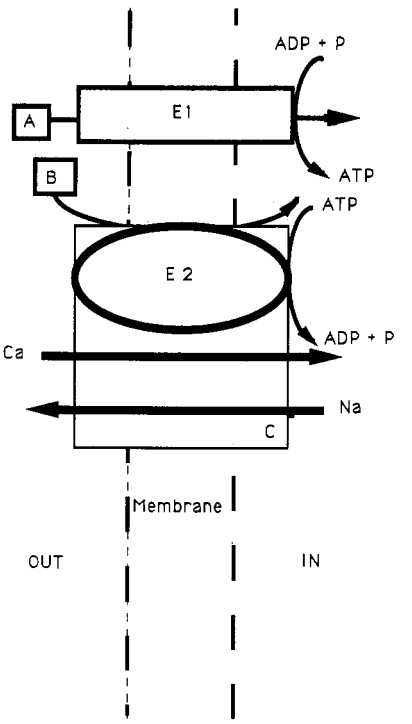

FIGURE 7: Proposed energy conversion pathways in electropulsed protoplasts. The applied field induced potential A which triggered ATP synthesis by E1 which is DCCD-sensitive. The extra amount of ATP is consumed by the C complex. C can in fact be considered as a mutienzyme system where E2, a vanadate-sensitive ATPase, increases potential B at the expense of the cytoplasmic ATP, this increase in potential being used by a Ca/Na antiport, which is sensitive to the amiloride derivative.

production and consumption and differently sensitive to drugs acting on calcium movements. Thus, the voltage-dependent calcium uptake is insensitive to desmethoxyverapamil (a calcium channel bloker) but is inhibited by an amiloride derivative [5-*N*-(-chlorobenzyl)-2′,4′-dimethylbenzamil] and bepridil. The amiloride derivative has been reported to have an important activity against Na^+/Ca^{2+} exchanger and a low activity against Na^+ channel (Kleyman & Cragoe, 1988); moreover, bepridil is known to act also on the exchanger by a "mechanism-based" inhibition (Kaczorowski et al., 1989). The data obtained by this indirect approach using inhibitors are strengthened by the fact that a flux of calcium (measured by the amount of labeled calcium trapped by the protoplasts) is correlated to an opposite movement of sodium. Therefore, it may be concluded that a sodium/calcium exchange is one of the main calcium uptake systems activated by electrostimulation. The ATP consumption fuels the efflux of sodium, and the electric potential induced drives the influx of calcium. The association of the two events of supported by the inhibition of both fluxes by amiloride (Figure 4b).

As depicted in Figure 7, the process may involve two steps, a direct (step A) and a back effect (step B) of the electrostimulation on protoplasts.

The first one (A) is the ac electric field stimulation of ATP synthase like activity that is known to be triggered by electric fields as described for submitochondrial particles and bacteria (Garcia et al., 1988; Goldsworthy, 1986). The external field is then amplified at the membrane level and, when large enough, triggers various activities. Once the critical value is passed, the activity increases with the increased area of the cell surface where the membrane field is beyond the activating threshold. From the data of Figure 2a, an increase in potential of 10–15 mV is needed to stimulate the ATP synthase like activity. The dependence upon the frequency was described

as a consequence of a time limitation in the conformation change of ATP synthase (Tsong & Astumian, 1986; Westerhoff et al., 1986; Tsong, 1989). Since the optimum frequency is about 100 Hz (Figure 2b) the time necessary for the conformational change of ATP synthase induced by membrane electric field is between 1 and 5 ms.

The second step (B) is a back effect of step A that occurs when the field has been switched off (Figure 4b). Several processes take place simultaneously where ATP consumption induces an increase in membrane potential and a calcium/sodium exchange. Since the ion transfer is clearly electrogenic, it is associated with the change in membrane potential.

The interaction of sodium and calcium is considered to be important in plant physiology. Thus, calcium is a critical factor in the resistance of plants to salinity (i.e., NaCl), and external NaCl has been recently shown to disturb the intracellular calcium, most probably by stimulating calcium efflux (Lynch & Läuchli, 1988). The data of Lynch and Läuchli (1988) may be consistent with the occurrence of a sodium/calcium exchanger as suggested in the present paper.

Patch-clamp experiments done with membranes from animal cells have shown that calcium/sodium exchange generates electric current, is linked to hyperpolarization due to the unbalanced charge movements, and needs ATP (Kimura et al., 1986). In the plant system, calcium ATPase and proton/calcium antiport have been characterized on both microsomes and isolated vacuoles (Schumaker & Sze, 1986). However, these systems are supposed to be mainly effective for protection of the cytoplasm against lethal calcium concentrations. From the data described in this paper, it appears that low-voltage stimulation may be used to load the plant cell with calcium in a controlled manner. As such, electrostimulation may induce changes in cytoplasmic calcium, which is known to be an important second messenger. This may explain the biochemical effects of electrostimulation on plant metabolism and development (Rathore et al., 1988).

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