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Communication

Effect of D-*myo*-Inositol 1,4,5-Trisphosphate on the Electrical Properties of the Red Beet Vacuole Membrane

Joël Alexandre and Jean-Paul Lassalles*

Laboratoire "Echanges Cellulaires," URA CNRS 203, Faculté des Sciences, BP 118, 76134 Mont-Saint-Aignan Cedex France

ABSTRACT

The effect of channel opening in the tonoplast by D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] has been examined on red beet (*Beta vulgaris*) vacuoles. Patch-clamp measurements of the vacuolar potential and current were performed on vacuoles isolated in 0.1 micromolar free Ca^{2+} medium. With vacuoles clamped at +30 millivolts, the Ins(1,4,5)P_3 induced changes in current were depending on the Ca^{2+} buffer strength in the external medium. The spontaneous depolarization of vacuoles in which H^+-pumps were activated by 5 millimolar MgATP was increased from +6 to +18 millivolts by 1 micromolar Ins(1,4,5)P_3. We have interpreted our data by assuming that even with 2 millimolar EGTA to buffer Ca^{2+} at 0.1 micromolar in the external medium, Ins(1,4,5)P_3 released enough Ca^{2+} from the vacuole to produce an accumulation of this ion near the tonoplast. Apart from their dependency with free Ca^{2+} in the cytoplasm, the electrical properties of the tonoplast could be depending on the Ins(1,4,5)P_3 and Ca^{2+} buffer values in the cytoplasm.

A great variety of Ca^{2+}-mediated effects in plants has already been described (for a review, see *e.g.* 14). They range from the molecular level, where Ca^{2+} dependent molecules like calmodulin are found (4), up to the cellular level, where Ca^{2+} induces rotational streaming of the cytoplasm in leaf cells (18) or modifies callose deposition onto cell walls (14). More recently, by using patch clamp techniques (8), it was possible to detect the effect of Ca^{2+} on a cellular organelle, the isolated vacuole (12). In different vacuole types (12, 13), there are Ca^{2+} dependent channels which carry cations as well as anions. These nonspecific channels are closed by an increase in $(Ca^{2+})_o$[1] which, as a consequence, allows H^+-pumps of the tonoplast (6, 9–11) to depolarize the membrane.

The importance of interactions between Ca^{2+} and vacuole has still been emphasized when it was found that Ins(1,4,5)P_3 can release calcium from the vacuolar compartment of plants (15, 17) and a fungus (5). Ins(1,4,5)P_3 is a well known inositide encountered in cell signaling processes in animal cells where it produces a Ca^{2+} release from nonmitochondrial cellular stores (3). It is not known if, under the *in vivo* conditions, it is also used in plant cells to increase the free cytoplasmic Ca^{2+} and eventually induce a physiological response by releasing part of the high Ca^{2+} content of the vacuolar reservoir (10^{-4}–10^{-2} M) (4). However, we have found that less than micromolar concentrations of Ins(1,4,5)P_3 were sufficient to open Ca^{2+} channels in the tonoplast (2). The effect of Ins(1,4,5)P_3 was reversible and quite specific when compared with other inositides. These studies were performed on vacuoles isolated in nonphysiological high (10^{-3} M) Ca^{2+} medium, in order to facilitate study of the Ins(1,4,5)P_3 opened channels [in the range 0 to +100 mV the background current due to NSC is negligible when compared to the Ins(1,4,5)P_3 induced current].

Here we present our results on the effect of Ins(1,4,5)P_3 on vacuoles under the more biological condition of low $(Ca^{2+})_o$ and we compare the effect of Ins(1,4,5)P_3 with an increase in $(Ca^{2+})_o$ on the vacuolar potential of vacuoles depolarized by 5×10^{-3} M MgATP.

MATERIALS AND METHODS

The red beet (*Beta vulgaris*) root vacuoles used in our experiments were prepared by mechanical slicing of the tissue. Individual vacuoles (45 ± 5 μm) were selected from the preparation medium and transferred to the recording chamber filled with an external medium by aspirating into a micromanipulated pipette (de Fonbrune micromanipulators, Cit-Alcatel).

Patch-clamp measurements of the vacuolar current, *I*, and potential, V, were performed in the 'whole vacuole' configuration.

The patch pipettes were pulled from soft glass (microhematocrit capillary 'blue tip,' Lancer Sherwood Medical) by a two stage puller (PP83 Narishige) to a resistance of 5 to 10 MΩ in 0.1 M KCl. After filling the pipette with the selected pipette medium, its tip was dipped for a few seconds in a 30% silane, 70% carbon tetrachloride solution. This was found to improve the signal to noise ratio, most probably by forming a hydrophobic surface at the pipette shank. Giga seals (10–30 GΩ) were usually obtained without fire polishing the tip of the pipette. A short voltage pulse (1 V–100 ms) was sometimes necessary to disrupt the membrane under the patch pipette, allowing transition between the 'vacuole attached' and the 'whole vacuole' configurations.

[1] Abbreviations: $(Ca^{2+})_o$ extravacuolar calcium concentration; NSC, nonspecific channels; Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; *I*, vacuolar current; *V*, vacuolar potential.

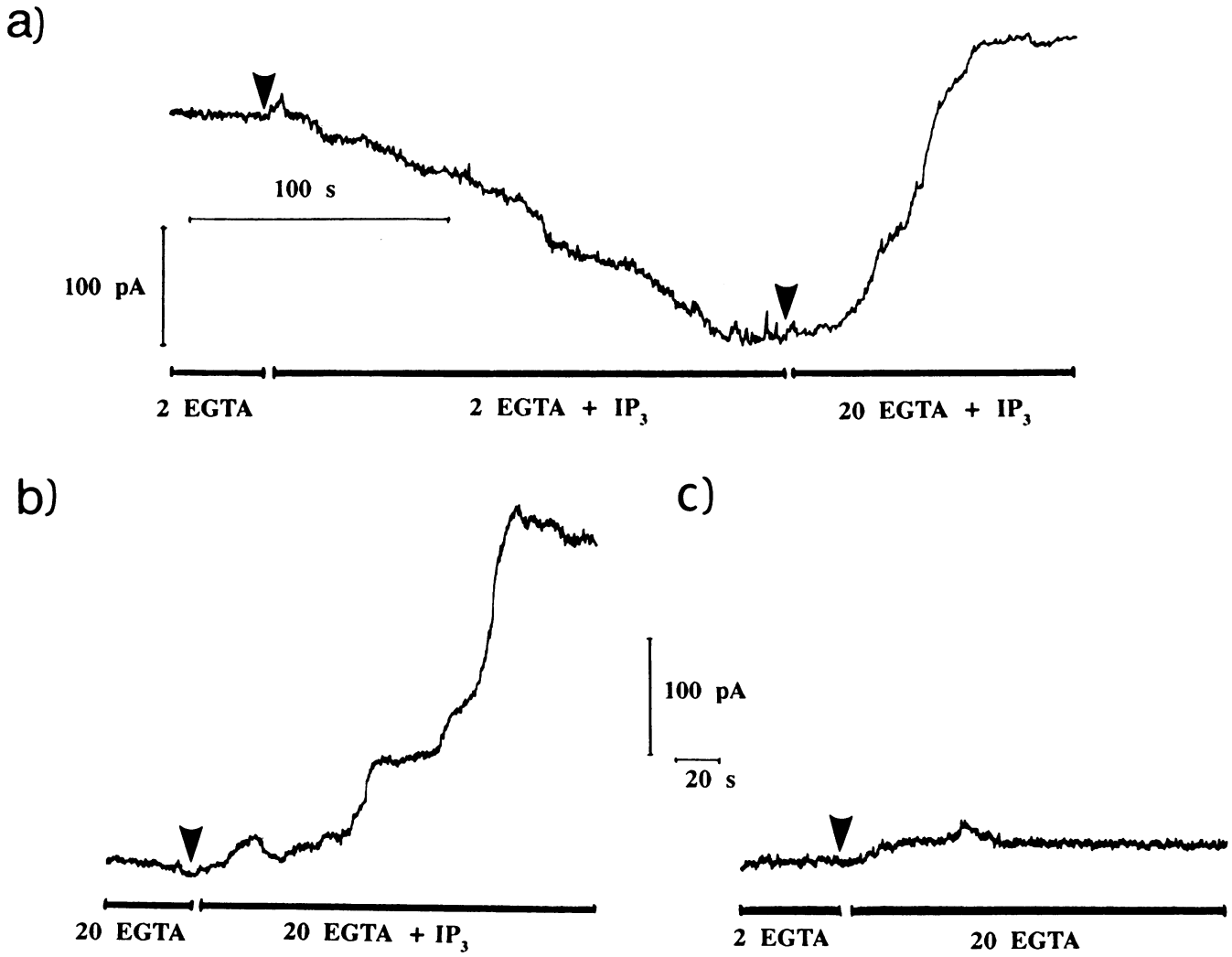

Figure 1. Effects of Ins(1,4,5)P_3 and Ca^{2+} buffer strength on vacuolar current. In each experiment shown, the recorded current was positive and corresponded to an outward current from the vacuole into the external medium. External medium and pipette solutions contained 0.6 M sorbitol, 0.1 M KCl, 2×10^{-3} M $MgCl_2$. For each experiment shown, EGTA, calcium and Ins(1,4,5)P_3 were different in the external medium (as indicated) but $CaCl_2$ was kept at 5×10^{-3} M in the pipette. Tris, 5×10^{-3} M, was added to the bath solution and pH was adjusted to 7.5 with Mes; 5×10^{-3} M Mes was added to the pipette solution and pH was adjusted to 5.5 with Tris. Changes in the external medium were initiated at the times indicated by the arrows in each current trace. *V* was clamped to +30 mV in all experiments. The free Ca^{2+} concentration outside was buffered to 10^{-7} M, either with 2×10^{-3} M EGTA or with 20×10^{-3} M EGTA. In each record, baseline for the current is arbitrary. (a) Effect of a 10-fold increase in EGTA concentration on Ins(1,4,5)P_3 induced nonspecific channel closure. Ins(1,4,5)P_3 was applied at the first arrow and EGTA concentration changed at the second arrow. (b) Outward current induced by Ins(1,4,5)P_3 when applied in the presence of high EGTA concentration. (c) Effect of change in EGTA alone.

Current Clamp Measurements

Once in the 'whole vacuole configuration,' *I* was clamped to zero. V reached a constant value (refered to as the spontaneous value of the vacuolar potential) after 5 to 10 min, a time delay assumed to be due to replacement of the vacuolar solution by the pipette medium.

Voltage Clamp Measurements

Recording of *I* in different media was as follows. Once the spontaneous value for *V* had been measured for a vacuole in a first external medium, *V* was clamped at + 30 mV (positive inside the vacuole) for several minutes. After a constant value for *I* had been obtained, the chamber was perfused with another external medium. The time evolution of *I* toward a new constant value was followed. When new wash of the vacuole with the first medium did not reverse the current to its initial value, the experiment was discarded, all the changes in external medium including those due to Ins(1,4,5)P_3, being reversible (2).

The perfusion rate into the chamber (150 μL) was 100 to 250 μL/min. Current and potential were measured at room temperature with an RK300 (Biologic Meylan, France) patch-clamp amplifier and low-pass filtered at 300 Hz with an 8-

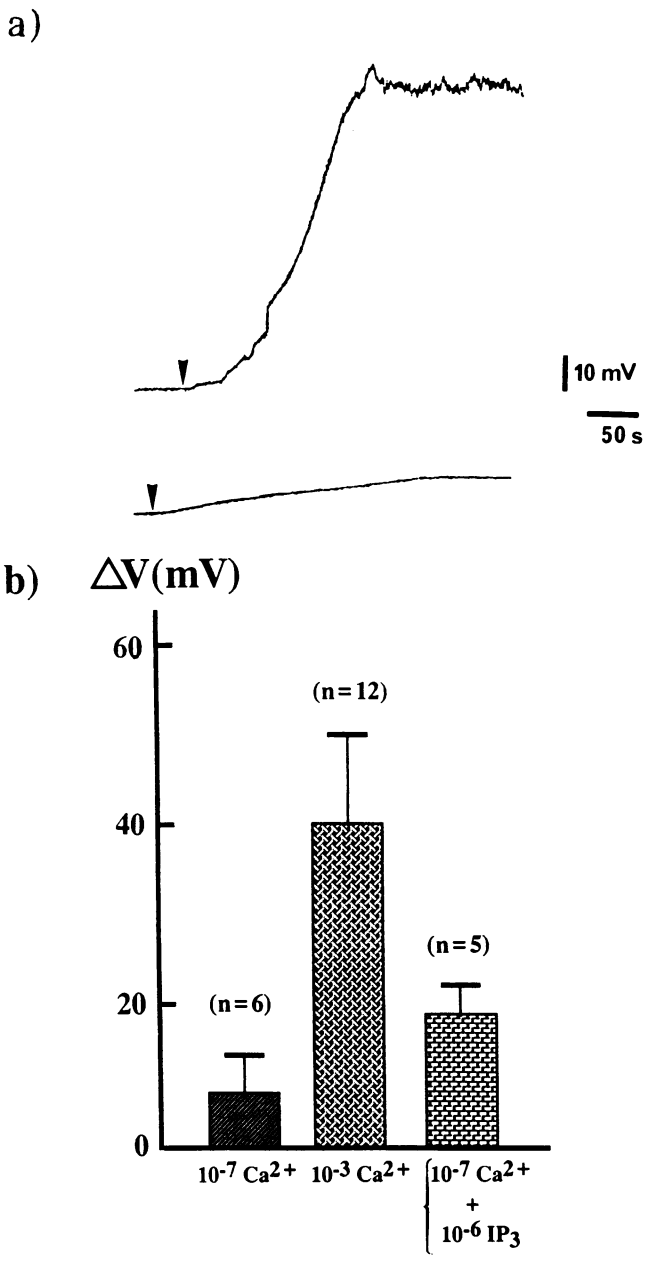

Figure 2. Effect of extravacuolar Ca^{2+} and Ins(1,4,5)P_3 on vacuolar potential V. (a) Variations of spontaneous potential for two vacuoles after addition of 5×10^{-3} M MgATP in the external medium (arrows). The medium also contains 10^{-3} M Ca^{2+} (upper record) or 10^{-7} M Ca^{2+} (lower record). (b) Steady state values of spontaneous vacuolar potential were recorded as shown in (a), before and after addition of 5×10^{-3} M MgATP in several external media. For each vacuole the increase in potential, *V*, was measured. Histogram represents values for *V* in each medium (mean + SD). (*n*): Number of experiments. For each experiment, pipette medium: 0.6 M sorbitol, 0.1 M KCl, 2×10^{-3} M $MgCl_2$, 5×10^{-3} M $CaCl_2$, 5×10^{-3} M Mes, pH solution adjusted to 5.5 with Tris; external medium: 0.6 M sorbitol, 0.1 M KCl, 2×10^{-3} M $MgCl_2$, 5×10^{-3} M Tris. Ins(1,4,5)P_3, free Ca^{2+} and MgATP: as indicated in each case. pH solution adjusted to 7.5 with Mes.

pole butterworth filter (Kemo, Ltd, U.K.). Data were stored on a video cassette recorder (Sony SL T50) and digitized at 900 Hz for computer analysis (HP 98580).

Chemicals

Ins(1,4,5)P_3 and MgATP were purchased from Sigma. The free 10^{-7} M Ca^{2+} concentration was buffered with 1.5×10^{-3} M $Ca^{2+}/2 \times 10^{-3}$ M EGTA or with 15×10^{-3} M $Ca^{2+}/20 \times 10^{-3}$ M EGTA assuming a value of 2.6×10^{-8} M for the apparent dissociation constant of Ca^{2+} and EGTA at pH 7.5 (16). The 10^{-3} M Ca^{2+} concentration was unbuffered.

RESULTS AND DISCUSSION

Effects of Ins(1,4,5)P_3 on Isolated Vacuoles: Ca^{2+} Buffer Dependency

Nine vacuoles were studied in 10^{-7} M free Ca^{2+} external medium, buffered with 2×10^{-3} M EGTA. Vacuoles were held at +30 mV and *I* was recorded. The application of Ins(1,4,5)P_3 produced a slow decrease in *I*. When the current was nearly stabilized at its minimum value, the medium was switched to one with the 10^{-7} M free Ca^{2+} medium buffered with 20×10^{-3} M EGTA. The current increased at a greater rate than that during the decrease and stabilized at a value greater than its magnitude before the addition of Ins(1,4,5)P_3 (Fig. 1a). In one kind of control experiment three vacuoles were held at +30mV in a 10^{-7} M free Ca^{2+}, 20×10^{-3} M buffered medium. Before the addition of Ins(1,4,5)P_3 the currents were about 300 pA. With the addition of Ins(1,4,5)P_3, the currents rapidly increased by about 500 pA. This effect is illustrated for one vacuole in Figure 1b. A second control experiment simply switched from 2×10^{-3} M buffer to the 20×10^{-3} M buffer without the addition of Ins(1,4,5)P_3. This change resulted in a small increase (about 20 pA) in the vacuolar current showing that the previous results were not an EGTA effect. This result is illustrated in Figure 1c. In a previous work (2), it was shown that Ins(1,4,5)P_3 has no direct effect on NSC and we already explained preliminary results on Ca^{2+} buffer effects (1) by assuming that Ins(1,4,5)P_3 released Ca^{2+} may interact with the NSC, depending on the Ca^{2+} buffer strength of the external medium. The results presented here confirm this hypothesis. If the Ins(1,4,5)P_3 released Ca^{2+} could not influence the NSC, only a net increase in current should result with the addition of Ins(1,4,5)P_3 by opening more channels in the tonoplast. The experiments illustrated in Figure 1a show that the released Ca^{2+} could close the NSC when EGTA outside was 2×10^{-3} M but not 20×10^{-3} M. The Ca^{2+} buffer capacity of the cell cytoplasm in the *in vivo* situation is expected to be smaller than that of 2×10^{-3} M EGTA medium (7). Under this assumption, the experiments described in Figure 1 show that an early effect of Ins(1,4,5)P_3 in plant cells could be a modification of the NSC conductance of the tonoplast.

Effect of $(Ca^{2+})_o$ and Ins(1,4,5)P_3 on MgATP Depolarized Vacuoles

The effect of Ca^{2+} on the spontaneous potential was first recorded in whole vacuole experiments, current being

clamped to zero. For each vacuole tested, we used 5×10^{-3} M MgATP to depolarize the vacuole, MgATP being more efficient than pyrophosphate (10, 11) in this case. The recorded values for V (Fig. 2a) were small (+6 mV) in 10^{-7} M $(Ca^{2+})_o$. They were larger (+40 mV) in 10^{-3} M $(Ca^{2+})_o$. When vacuoles were voltage clamped to 0 mV under symmetrical KCl conditions, the current I corresponding to the pump current (6, 10, 11) was found to be $(Ca^{2+})_o$ independent: 60 ± 14 pA ($n = 6$) in 10^{-7} M $(Ca^{2+})_o$ and 65 ± 8 pA ($n = 11$) in 10^{-3} M $(Ca^{2+})_o$. Variations of V with $(Ca^{2+})_o$ were interpreted by assuming that Ca^{2+} ions had no effect on the H^+-ATPase and that an increase in $(Ca^{2+})_o$ resulted in a closure of the NSC (12) and depolarization of the tonoplast.

In another set of experiments, the effect of Ins(1,4,5)P_3 was detected on the vacuolar potential (current clamped to zero) measured on vacuoles under symmetrical KCl conditions, with 10^{-7} M free Ca^{2+} buffered with 2×10^{-3} M EGTA in the external medium. The depolarization of the tonoplast was increased from its low value (+6 mV) in 10^{-7} M $(Ca^{2+})_o$ to a mean value of +18 mV when 10^{-6} M Ins(1,4,5)P_3 was added in the external medium (Fig. 2b). The Ins(1,4,5)P_3 induced depolarization was less important than that resulting from an increase in $(Ca^{2+})_o$ from 10^{-7} to 10^{-3} M, but it was obtained with $(Ca^{2+})_o$ being kept at the more physiological level of 10^{-7} M.

Here again, it is possible to explain this increase in V by assuming that Ins(1,4,5)P_3 released Ca^{2+} interacts with the NSC despite the presence of a Ca^{2+} buffer in the extravacuolar medium.

Our experiments on isolated vacuoles indicate that besides $(Ca^{2+})_o$, Ins(1,4,5)P_3 and $(Ca^{2+})_o$ buffer strength control the electrical properties of the tonoplast. In the *in vivo* situation, we do not know if a Ca^{2+} efflux from the vacuole can overbalance the capacity of Ca^{2+} buffers or Ca^{2+} pumps to remove free Ca^{2+} from the cytoplasm. For example, we ignore how fast are the Ca^{2+} buffers in the cytoplasm or what level can be reached by Ins(1,4,5)P_3 in this compartment. However, it can be expected that the values for these cellular parameters sometimes allow Ins(1,4,5)P_3 to rapidly induce important changes in the vacuolar potential and ion conductance of the tonoplast without inducing any significant change in the mean cytoplasmic Ca^{2+} concentration.

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LITERATURE CITED

1. **Alexandre J, Lassalles JP, Kado RT** (1989) IP_3 opens calcium channels of the red beet vacuole tonoplast. *In* J Dainty et al, ed, Plant Membrane Transport. Elsevier Science Publishers B. V. (Biomedical Division), The Netherlands, pp 249–254
2. **Alexandre J, Lassalles JP, Kado RT** (1990) Opening of Ca^{2+} channels in isolated red beet vacuole membrane by inositol 1,4,5-trisphosphate. Nature **343:** 567–570
3. **Berridge MJ, Irvine RF** (1989) Inositol phosphate and cell signalling. Nature **341:** 197–205
4. **Blowers D, Collinge M, Gibroy S, Harvey H, Trewavas A** (1987) Regulation of and by calcium in plants with particular respect to membranes. A report on progress. *In* C Leaver, H Sze, eds, Plant Membranes: Structure, Function, Biogenesis. Alan R Liss, Inc, New York, pp 371–381
5. **Cornelius G, Gebauer G, Techel D** (1989) Inositol tris-phosphate induces calcium release from *Neurospora crassa* vacuoles. Biochem Biophys Res Commun **162:** 852–856
6. **Coyaud L, Kurkdjian A, Kado RT, Hedrich R** (1987) Ion channels and ATP-driven pumps involved in ion transport across the tonoplast of sugar beet vacuoles. Biochim Biophys Acta **902:** 263–268
7. **Fabiato A** (1985) Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac purkinje cell. J Gen Physiol **85:** 247–289
8. **Hamil OP, Marty A, Neher E, Sackmann B, Sigworth FJ** (1981) Improved patch-clamp techniques for high-resolution current recordings from cells and cell-free membrane patches. Pflügers Arch Eur J Physiol **391:** 85–100
9. **Hedrich R, Flügge UI, Fernandez JM** (1986) Patch-clamp studies of ion transport in isolated plant vacuoles. FEBS Lett **204:** 228–232
10. **Hedrich R, Kurkdjian A** (1988) Characterization of an anion-permeable channel from sugar beet vacuoles: effect of inhibitors. EMBO J **7:** 3661–3666
11. **Hedrich R, Kurkdjian A, Guern J, Flügge UI** (1989) Comparative studies on the electrical properties of the H^+ translocating ATPase and pyrophosphatase of the vacuolar-lysosomal compartment. EMBO J **8:** 2835–2841
12. **Hedrich R, Neher E** (1987) Cytoplasmic calcium regulates voltage-dependent ion channels in plant vacuoles. Nature **329:** 833–836
13. **Hedrich R, Schroeder JI** (1989) The physiology of ion channels and electrogenic pumps in higher plants. Annu Rev Plant Physiol **40:** 539–569
14. **Kauss H** (1987) Some aspects of calcium-dependent regulation in plant metabolism. Annu Rev Plant Physiol **38:** 47–72
15. **Ranjeva R, Carrasco A, Boudet A** (1988) Inositol tris-phosphate stimulates the release of calcium from intact vacuoles isolated from *Acer* cells. FEBS Lett **230:** 137–141
16. **Ringbom A** (1976) Les complexes en Chimie Analytique. DUNOD, Paris
17. **Schumaker KS, Sze H** (1987) Inositol 1,4,5-trisphosphate releases Ca^{2+} from vacuolar membrane vesicles of oat roots. J Biol Chem **262:** 3944–3946
18. **Takagi S, Nagai R** (1988) Light-affected Ca^{2+} fluxes in protoplasts from *Vallisneria* mesophyll cells. Plant Physiol **88:** 228–232