Characteristics of non-specific permeability and H\(^+\)-ATPase inhibition induced in the plasma membrane of *Nitella flexilis* by excessive Cu\(^{2+}\)

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**Abstract.** Effects of Cu\(^{2+}\) on a non-specific conductance and H\(^+\)-ATPase activity in the plasma membrane of the freshwater alga *Nitella flexilis*. Agardh was studied using a conventional microelectrode voltage-clamp technique. We show that a Cu\(^{2+}\)-induced increase in the non-specific conductance is related to the formation of pores in the plasma membrane. Pore formation is the result of unidentified chemical reactions, since the Q\(_{10}\) for the rate of increase of conductance over time was about 3. Various oxidants and antioxidants (10 mmol/l H\(_2\)O\(_2\), 10 mmol/l ascorbate, 100 μg/ml superoxide dismutase, and 100 μg/ml catalase) did not alter Cu\(^{2+}\)-induced changes in the plasma membrane conductance, suggesting that the effect of Cu\(^{2+}\) was unrelated to peroxidation of plasma-membrane lipids. In contrast, organic and inorganic Ca\(^{2+}\)-channel antagonists (nifedipine, Zn\(^{2+}\), Cd\(^{2+}\), Fe\(^{2+}\), Ni\(^{2+}\)) inhibited the Cu\(^{2+}\)-induced non-specific conductance increase. This suggests that changes in Ca\(^{2+}\) influx underlie this effect of Cu\(^{2+}\). Decreasing the pH or the ionic strength of external solutions also inhibited the Cu\(^{2+}\)-induced plasma-membrane conductance increase. Copper was also found to inhibit plasma-membrane H\(^+\)-ATPase activity with half-maximal inhibition occurring at about 5–20 μmol/l and full inhibition at about 100–300 μmol/l. The Hill coefficient of Cu\(^{2+}\) inhibition of the H\(^+\)-ATPase was close to two.

**Key words:** Calcium metabolism – Copper toxicity – Conductance (non-specific) – H\(^+\)-ATPase inhibition – *Nitella* (Cu\(^{2+}\) toxicity) – Plasma membrane (Cu\(^{2+}\) toxicity)

**Introduction**

Damage to the plasma membrane (PM) is one of the primary events in the chain of Cu\(^{2+}\)-induced toxic effects in plant cells (De Vos and Schat 1991; Strange and Macnair 1991; Demidchik et al. 1997). Copper concentrations above 3–5 μmol/l increase non-specific PM permeability, inhibit Cl\(^-\) channels, and suppress electrogenic H\(^+\)-ATPase activity (Demidchik et al. 1997). The increase in non-specific conductance and inhibition of the H\(^+\)-ATPase are probably most damaging because they lead rapidly to PM depolarisation, disruption of ionic homeostasis and subsequent perturbation of enzymatic reactions.

To date, there is little knowledge of the mechanism underlying the Cu\(^{2+}\)-induced increase in non-specific conductance. Presently there are three main ideas on possible causes: (i) Ca\(^{2+}\) displacement from the PM surface (Bergmann 1992); (ii) membrane lipid oxidation caused by free-radical formation (De Vos and Schat 1991; Gutteridge 1998), and (iii) disruption of intracellular Ca\(^{2+}\) homeostasis (Stohs and Bagchi 1995). The fact that substitution of Ca\(^{2+}\) for Al\(^{3+}\) does not damage the PM (Kinraide 1994) likely rules out the displacement of surface Ca\(^{2+}\) as a primary mechanism of Cu\(^{2+}\)-induced membrane breakdown. Thus, other mechanisms remain to be examined. Taking into account the capability of Cu\(^{2+}\) to catalyse production of highly toxic hydroxyl radicals by reactions of the Haber-Weiss cycle (De Vos and Schat 1991), membrane lipid peroxidation is a plausible explanation for PM destruction. However, to date there are only a few reports dealing with Cu\(^{2+}\)-induced oxidative stress in plant cells in vivo. Therefore, we tested the involvement of lipid peroxidation in the Cu\(^{2+}\) effects on PM conductance. It is known that Cu\(^{2+}\) toxicity in animal cells involves disruption of intracellular Ca\(^{2+}\) homeostasis (Stohs and Bagchi 1995). It may be hypothesised that a similar mechanism takes place in plant cells. Here we report some results supporting this view.

Mechanisms of Cu\(^{2+}\)-induced H\(^+\)-ATPase suppression have been investigated in vitro (Serrano 1990);
however, the effects have not been studied in vivo. We have also attempted to address this question.

In this work, we have used the giant-celled green alga, *Nitella flexilis*. The advantages of electrophysiological research using charophyte algae are well known (Hope and Walker 1975; Sokolik and Yurin 1981, 1986). Charophyte cells make it possible to examine the passive transport systems such as K⁺ channels, Cl⁻ channels and non-specific PM leakage separately as well as the active transport (H⁺-ATPase electrogenic pump) of ions across the plant PM (Spanswick 1981; Sokolik and Yurin 1986; Tester 1990; Yurin et al. 1989, 1991; Demidchik et al. 1997). It therefore provides an ideal model system in which to study analytically the various possible effects of Cu²⁺ on the PM of a plant cell.

**Materials and methods**

**Materials**

Giant internodal cells of the freshwater alga *Nitella flexilis* L. Agardh were used. Cells for *N. flexilis* culture were collected from Braslav’s Lakes (Belarus) and grown in our laboratory in a bathing medium of APW (artificial pond water) with micronutrients (Coleman and Findlay 1985). The temperature was 20 ± 1.5 °C, and the cells were subjected to a daily cycle of 12 h of light and 12 h of darkness, light being provided by fluorescent lamps. The second or third internodal cells were selected, these being 4-6 cm long and 0.4-0.6 mm in diameter. To inhibit the light-stimulated electrogenic H⁺-ATPase, cells were kept in darkness for 2 or 3 d (dark-adapted cells; Sokolik and Yurin 1981, 1986; Demidchik et al. 1997). At the initial stage of the experiment, a cell was placed in APW, a microelectrode was inserted into the cell and then an assay solution was let into the chamber.

**Solutions**

Artificial pond water (0.1 mmol/l KCl, 0.1 mmol/l CaCl₂, 1 mmol/l NaCl) was used as the basic solution. To this basic APW were added sulphates (ion/salt: Cu²⁺/CuSO₄ × 5H₂O, Fe²⁺/FeSO₄ × 7H₂O, Zn²⁺/ZnSO₄ × 7H₂O) or chlorides (ion/salt: Cd²⁺/CdCl₂ × 2.5H₂O, Ni²⁺/NiCl₂ × 2H₂O, Ca²⁺/CaCl₂, Na⁺/NaCl, K⁺/KCl, Cs⁺/CsCl, Li⁺/LiCl). Since divalent cations form insoluble precipitates at pH > 6.0–7.0 it was necessary to lower the medium pH to 5.6 (Kiss et al. 1991). To this end, we used Mes (up to 1 mmol/l). Nifedipine, Cu and Zn-containing superoxide dismutase (SOD; source: bovine erythrocytes) and catalase (source: bovine liver) were from Sigma-Aldrich Corporation (USA).

**Experimental procedures**

Conventional microelectrode voltage-clamp techniques were used for recording the electrical characteristics of the PM, as described elsewhere (Sokolik and Yurin 1981, 1986). The tip of a microelectrode was filled with 3 mol/l KCl and it was inserted into the cell cytoplasm to measure the electrical properties of the PM. The fact that the microelectrode tip was placed in the cytoplasm was determined by cell response to a short-term illumination, the type of action potential and resting potential difference (RPD; Yurin et al. 1991). Experiments were only carried out if the RPD across the PM in the APW solution was close to the K⁺ equilibrium potential (∼140 to ∼160 mV).

The PM voltage was clamped at the RPD level and then was slowly hyperpolarized at a rate of about 0.2–0.5 mV/s until the desired conditioning voltage (CV) was attained (∼140 to ∼170 mV).

![Fig. 1. A Typical example of the effect of hyperpolarising pulses from −150 mV to −160 mV on PM current during illumination (the initiation of illumination is indicated by an arrow). B Time course of PM conductance (G) changes during illumination; G values were calculated using magnitudes of PM current (I) in response to hyperpolarising pulses (G = I/E = 10.01 V). A pulse amplitude of 10 mV was taken because it did not significantly change PM potential-dependent characteristics, as evidenced by linearity of instantaneous and steady-state voltage-current characteristics over −150 mV to −160 mV (Sokolik and Yurin 1986)](image)

To investigate long-term transitional processes (activation of light-stimulated H⁺-ATPase, Cu²⁺-induced increase in PM conductance), short (duration = 10 s; separated by 10 s) hyperpolarising pulses of 10–20 mV amplitude were used (Fig. 1A). The size of currents induced by hyperpolarising voltage pulses allowed the calculation of the PM conductance (G) (Fig. 1B).

To study the kinetics of light-induced changes in G (that are due to activation of the H⁺-ATPase: Spanswick 1981; Yurin et al. 1991) (see Fig. 1), light from an incandescent electric lamp at 12 W/m² was used. To measure the non-specific PM conductance (Fig. 2), K⁺ channels were inhibited by 5 mmol/l Cs⁺, and the H⁺-ATPase was inhibited by keeping cells in darkness for 2–3 d (Sokolik and Yurin 1986; Yurin et al. 1991). In this case, inward

![Fig. 2. The effect of different assay solutions on PM conductance (G values were calculated according to the experimental scheme described in Fig. 1. Assay solutions were changed in the following sequence: APW → APW + 5 mmol/l Cs⁺ → APW + 5 mmol/l Cs⁺ + 5 mmol/l Na⁺ → APW + 5 mmol/l Cs⁺ + 5 mmol/l K⁺. G′, K⁺-channel conductance, as blocked by 5 mmol/l Cs⁺; G′′, the non-selective conductance increment evoked by addition of monovalent cations to solution. Values are the average of seven measurements ± SD)](image)