

# The Respiratory Burst and Electrolyte Leakage Induced by Sulfhydryl Blockers in *Egeria densa* Leaves Are Associated with H<sub>2</sub>O<sub>2</sub> Production and Are Dependent on Ca<sup>2+</sup> Influx<sup>1</sup>

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In leaves of *Egeria densa* Planchon, *N*-ethylmaleimide (NEM) and other sulfhydryl-binding reagents induce a temporary increase in nonmitochondrial respiration ( $\Delta QO_2$ ) that is inhibited by diphenylene iodonium and quinacrine, two known inhibitors of the plasma membrane NADPH oxidase, and are associated with a relevant increase in electrolyte leakage (M. Bellando, S. Sacco, F. Albergoni, P. Rocco, M.T. Marrè [1997] *Bot Acta* 110: 388–394). In this paper we report data indicating further analogies between the oxidative burst induced by sulfhydryl blockers in *E. densa* and that induced by pathogen-derived elicitors in animal and plant cells: (a) NEM- and Ag<sup>+</sup>-induced  $\Delta QO_2$  was associated with H<sub>2</sub>O<sub>2</sub> production and both effects depended on the presence of external Ca<sup>2+</sup>; (b) Ca<sup>2+</sup> influx was markedly increased by treatment with NEM; (c) the Ca<sup>2+</sup> channel blocker LaCl<sub>3</sub> inhibited  $\Delta QO_2$ , electrolyte release, and membrane depolarization induced by the sulfhydryl reagents; and (d) LaCl<sub>3</sub> also inhibited electrolyte leakage induced by the direct infiltration of the leaves with H<sub>2</sub>O<sub>2</sub>. These results suggest a model in which the interaction of sulfhydryl blockers with sulfhydryl groups of cell components would primarily induce an increase in the Ca<sup>2+</sup> cytosolic concentration, followed by membrane depolarization and activation of a plasma membrane NADPH oxidase. This latter effect, producing active oxygen species, might further influence plasma membrane permeability, leading to the massive release of electrolytes from the tissue.

The ability of NEM and a number of other sulfhydryl group blockers to induce  $\Delta QO_2$  in *Egeria densa* Planchon leaves has been reported previously (Albergoni et al., 1996; Bellando et al., 1997; Marrè and Albergoni, 1998).  $\Delta QO_2$  induced by these reagents is completely insensitive to mitochondrial inhibitors such as cyanide, salicylhydroxamic acid, and propyl gallate and to the uncoupler carbonylcyanide-*m*-chlorophenylhydrazon, whereas it is suppressed by quinacrine and diphenylene iodonium, two inhibitors of plasma membrane NADPH oxidase, an enzyme responsible for the oxidative burst induced by pathogenic factors in granulocytes and in plants (Babior, 1992; Doussière and Vignais, 1992; Auh and Murphy, 1995; Desikan et al., 1996; Murphy and Auh, 1996; Van

Gestelen et al., 1997). The respiratory burst induced by sulfhydryl blockers is also associated with an early depolarization of the transmembrane electrical potential difference ( $E_m$ ), with a rapid increase in electrolyte leakage (mainly K<sup>+</sup>, but also HPO<sub>4</sub><sup>2-</sup> and Cl<sup>-</sup>), and with an increase in H<sup>+</sup> influx (Bellando et al., 1997; Marrè and Albergoni, 1998). All of these features are similar to the effects of pathogenic elicitors in higher plants in which the hypersensitive reaction has been investigated. This suggests that a common general mechanism is involved in the two orders of phenomena.

In addition to its intrinsic value, the elucidation of the mode of action of sulfhydryl reagents on O<sub>2</sub> uptake and plasma membrane permeability might contribute to the understanding of the mechanism of the pathogen-induced oxidative burst and the increase in membrane permeability that are characteristic of the hypersensitive response (Apostol et al., 1989; Conrath et al., 1991; Goodman and Novacky, 1994). Two important observations concerning the pathogen-derived, elicitor-induced oxidative burst are: (a) the production of active oxygen species, mainly O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> (Doke, 1983; Sutherland, 1991; Levine et al., 1994; Mehdy, 1994; Doke and Miura, 1995; Lamb and Dixon, 1997; Xing et al., 1997), which are potentially responsible for changes at the plasma membrane; and (b) the demonstration of a strict Ca<sup>2+</sup> dependence of the effects of treatments with a variety of pathogenic elicitors, leading to the conclusion that an increase in the cytosolic Ca<sup>2+</sup> level is a very early step in the sequence of events involved in the phenomenon (Bach et al., 1993; Tavernier et al., 1995; Levine et al., 1996; Gelli et al., 1997; Jabs et al., 1997; Pugin et al., 1997).

The present investigation was undertaken to determine whether the effects of sulfhydryl blockers in *E. densa* are associated with the production of active oxygen species, and whether in this case, as well as in the induction of the respiratory burst, membrane depolarization and electrolyte leakage depend on the presence of Ca<sup>2+</sup> in the medium and on its influx into the tissue. We also investigated the possibility that the production of H<sub>2</sub>O<sub>2</sub>, as a product of the

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Abbreviations: BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane;  $E_m$ , transmembrane electric potential difference; NEM, *N*-ethylmaleimide;  $QO_2$ , oxygen uptake;  $\Delta QO_2$ , increase in nonmitochondrial respiration; SOD, superoxide dismutase.

oxidative burst, could contribute to the changes in membrane permeability associated with electrolyte leakage.

## MATERIALS AND METHODS

### Plant Material

*Egeria densa* Planchon plants were grown in flowing tap water in a greenhouse. Young, fully expanded leaves were cut about 2 to 4 cm from the branch apex, randomized, and then grouped into samples. Before the experiments, the samples were washed twice for 15 min with 20 mL of 0.5 mM CaSO<sub>4</sub> and preincubated for 30 min in 20 mL of 0.5 mM CaSO<sub>4</sub> and 5 μM DCMU, with continuous shaking at 20°C in the dark. DCMU, a specific inhibitor of photosynthetic O<sub>2</sub> evolution and C fixation, was added to prevent the eventual effects of photosynthesis caused by occasional exposure to light (Marrè et al., 1989; Albergoni et al., 1996).

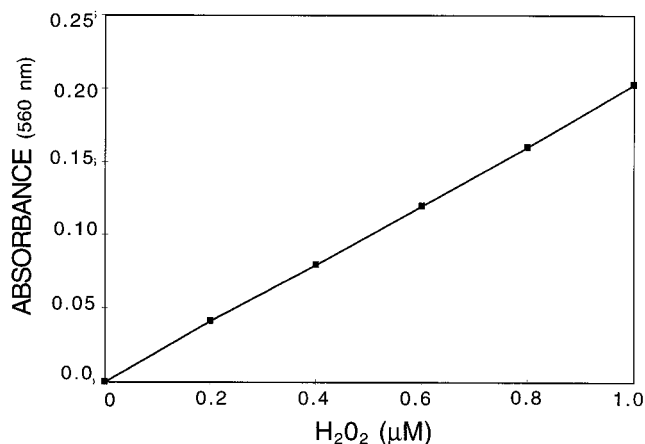
### QO<sub>2</sub> Measurements

QO<sub>2</sub> was measured on four leaves (about 30 mg fresh weight) in 2 mL of medium using a Clark-type O<sub>2</sub> electrode (unit KW2, control box CB1, Hansatech, Norfolk, UK). Measurements were carried out continuously, as described by Marrè and Albergoni (1998), and medium was renewed every 8 min to restore maximal concentration of dissolved O<sub>2</sub>. The basal medium contained 5 μM DCMU, 10 mM Mes buffer (pH 6.0), with BTP. CaSO<sub>4</sub> and other reagents were added at the concentrations specified in the legends to the figures.

### H<sub>2</sub>O<sub>2</sub> Production

The low values of H<sub>2</sub>O<sub>2</sub> accumulation in the samples revealed that there was a problem with the measurement procedure. The widely used luminol method presented the disadvantages of a relatively low sensitivity, together with a large variability. After several trials using this method (as described by Viard et al., 1994), we decided to follow the method described by Jiang et al. (1990) using the peroxide-mediated oxidation of Fe<sup>2+</sup>, followed by the reaction of Fe<sup>3+</sup> with xylenol orange. This method produced reproducible results in the 0.1 to 1 μM H<sub>2</sub>O<sub>2</sub> concentration range (Fig. 1).

The possibility of interference by other chemicals present in the various assay media used in our experiments was excluded by a series of tests, and the specificity for H<sub>2</sub>O<sub>2</sub> was tested by determining the effects of H<sub>2</sub>O<sub>2</sub> elimination by catalase. For the determination of H<sub>2</sub>O<sub>2</sub> with this method 500-μL aliquots of the incubation medium, drawn from the O<sub>2</sub> electrode chamber every 8 min, were added to 500 μL of the reaction mixture containing 500 μM ammonium ferrous sulfate, 50 mM sulfuric acid, 200 μM xylenol orange, and 200 mM sorbitol. After 45 min at room temperature the changes in A<sub>560</sub> were evaluated with a Biochrom Ultraspec 2 spectrophotometer (LKB, Bromma, Sweden). The addition of catalase (1600 units) to the sample aliquots for 5 min decreased the absorbance changes to less



**Figure 1.** Standard curve of the H<sub>2</sub>O<sub>2</sub> concentration determined by the xylenol method in the range of 0.1 to 1 μM. H<sub>2</sub>O<sub>2</sub> was measured with 100 μM xylenol orange, 250 μM Fe<sup>2+</sup>, and 100 μM sorbitol in 25 mM H<sub>2</sub>SO<sub>4</sub>. A<sub>560</sub> was read after 45 min at room temperature. Data are the means of triplicate determinations. SD did not exceed ±3%.

than 10%. The H<sub>2</sub>O<sub>2</sub> values reported in Figures 1 and 6 are those left after subtraction of the small values found after treatment with catalase.

### Electrophysiological Measurements

A single leaf held in a 5-mL plexiglass chamber was irrigated with a continuously flowing (approximately 15 mL min<sup>-1</sup>) aerated medium thermoregulated at 20°C. The basal medium was the same as that described above, except that 0.5 mM CaSO<sub>4</sub> was added to the solution. Other reagents were added at the concentrations reported in the legends to the figures. Micropipettes (tip resistance, 10 MΩ) filled with 1 M KCl were used as microsalt bridges of Ag/AgCl electrodes and were inserted vertically into the tissue by means of a micromanipulator (Leitz, Wetzlar, Germany). E<sub>m</sub> was measured with a high-impedance electrometer amplifier (model WPI K5-700, World Precision Instruments, New Haven, CT) connected to a chart recorder. The data presented represent one of at least three experiments for each set of experimental conditions.

### Measurement of Electrolyte Leakage

After pretreatment, samples of 10 leaves (approximately 100 mg fresh weight) were incubated in 10 mL of a stirred solution thermoregulated at 20°C. Basal medium contained 5 μM DCMU and 10 mM Mes buffer (pH 6.0), with BTP. Other reagents were added at the concentrations specified in the legends to the figures. The conductivity of the medium was measured continuously by a conductivity meter (model MO101, Analytical Control, Milan, Italy) connected to a chart recorder for the required time. The data represent the changes in medium conductivity during the treatments, calculated per gram fresh weight, taking 0 as the value of conductivity measured at the end of the equilibration period before starting the treatment.

### Ca<sup>2+</sup> Uptake and Net Flux Measurements

Ca<sup>2+</sup> influx was measured as <sup>45</sup>Ca<sup>2+</sup> uptake, and the radioactivity was provided as <sup>45</sup>CaCl<sub>2</sub> (1.48 GBq mol<sup>-1</sup>). After pretreatment, samples of leaves (100 mg fresh weight) were incubated in 20 mL of basal medium containing 0.5 mM CaSO<sub>4</sub>, 10 mM Mes-BTP (pH 6.0), and 5 μM DCMU, with or without 0.2 mM NEM. To remove by exchange the <sup>45</sup>Ca<sup>2+</sup> contained in the free space and in the cell wall, after 30 and 90 min of treatment the samples were briefly rinsed twice in 20 mL of an unlabeled ice-cold solution containing 10 mM CaSO<sub>4</sub>, 10 mM Mes-BTP (pH 6.0), and 5 μM DCMU. The leaves were then transferred twice (for 2.5 and 18 min, respectively) in 20 mL of the same unlabeled solution and finally collected, blotted on filter paper, and placed into scintillation vials. Digestion and bleaching of the tissue were performed as described by Romani and Beffagna (1991). The radioactivity was measured by liquid-scintillation counting in a TriCarb counter (Packard Instruments, Meriden, CT) after addition of 10 mL of scintillation liquid (Hionic Fluor, Packard Instruments).

Net Ca<sup>2+</sup> fluxes were measured in samples of leaves incubated in the same experimental conditions described above, without <sup>45</sup>Ca<sup>2+</sup>. The changes in Ca<sup>2+</sup> concentrations in the assay media were measured after 90 min of incubation using a Ca<sup>2+</sup>-selective electrode (MI-600, Microelectrodes, Bedford, NH).

### SOD Assay

*E. densa* leaves (130 mg/mL) were homogenized in 50 mM potassium phosphate (pH 7.8) containing 0.1 mM EDTA and 0.3% (w/v) Triton X-100 at 4°C. The homogenate was filtered through four layers of cheesecloth and promptly used for the SOD assay. SOD activity was determined spectrophotometrically as the inhibition of xanthine-xanthine oxidase-mediated reduction of Cyt *c* (McCord and Fridovich, 1969). The assay was performed at 25°C in a 3-mL cuvette containing 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 50 μM Cyt *c*, and 1 mM xanthine. The reduction of Cyt *c* was followed by a double-beam spectrophotometer (ZWS II, Sigma). The data are means of three determinations on two different leaf extracts.

## RESULTS

### H<sub>2</sub>O<sub>2</sub> Production in NEM and the Ag<sup>+</sup>-Induced Oxidative Burst

Previously, it was shown that the ΔQO<sub>2</sub> induced by NEM or by Ag<sup>+</sup> was insensitive to cyanide and salicylhydroxamic acid but completely inhibited by diphenyleneiodonium and quinacrine. This suggested that it was mediated by a type of NADPH oxidase operating in the oxidative burst induced by pathogen-derived elicitors, both in granulocytes and in plants, and responsible for the production of O<sub>2</sub><sup>-</sup> and (through SOD) H<sub>2</sub>O<sub>2</sub> (Mehdy, 1994; Doke and Miura, 1995; Desikan et al., 1996). To confirm this

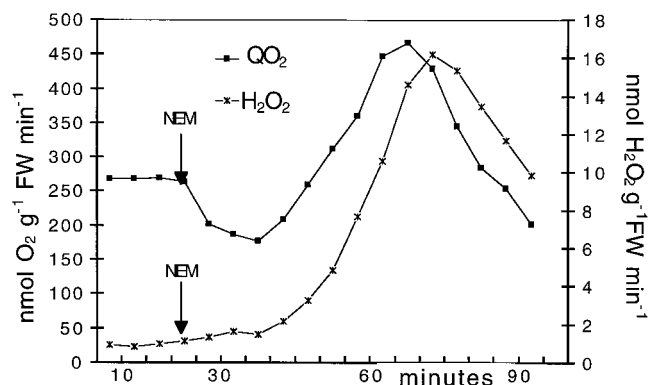
conclusion we investigated whether H<sub>2</sub>O<sub>2</sub> was the product of the oxidative reaction, as in the case of treatment with the sulfhydryl blockers. Figure 2 shows that NEM-induced ΔQO<sub>2</sub> was indeed closely associated with and parallel to the production of H<sub>2</sub>O<sub>2</sub>. Similar results were obtained on leaves in which the oxidative burst had been induced by treatment with 5 μM Ag<sup>+</sup>. Attempts to demonstrate the appearance of O<sub>2</sub><sup>-</sup> in the incubation medium failed to give significant and reproducible results. This might be a consequence of SOD rapidly metabolizing the products of NADPH oxidase activity. Measurements of SOD activity in *E. densa* leaves gave a very high value of this enzyme (1.3 ± 0.15 μmol min<sup>-1</sup> g<sup>-1</sup> fresh weight; see "Materials and Methods").

### Ca<sup>2+</sup> Dependence of Sulfhydryl Reagent-Induced ΔQO<sub>2</sub>, Membrane Depolarization, and Electrolyte Leakage

#### Effects of Ca<sup>2+</sup> Concentration in the Medium

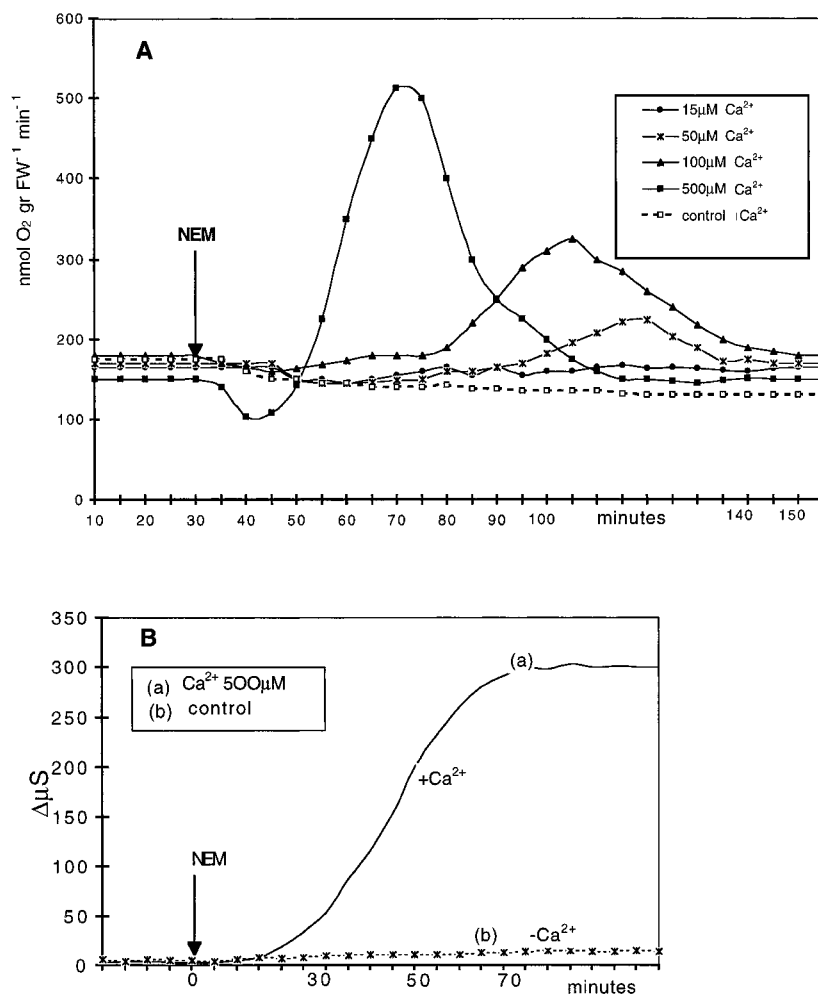
In our previous studies on the oxidative burst induced by the sulfhydryl blockers in aquatic plants, 0.5 mM Ca<sup>2+</sup> was always present in the medium (Albergoni et al., 1996; Bellando et al., 1997; Marrè and Albergoni, 1998). In this study the relevance of extracellular Ca<sup>2+</sup> concentration on the NEM- and Ag<sup>+</sup>-induced increase in O<sub>2</sub> consumption was investigated at Ca<sup>2+</sup> concentrations ranging from 0 to 0.5 mM. The results shown in Figure 3A indicate that the ability of NEM to induce the respiratory burst was completely abolished by the absence of extracellular Ca<sup>2+</sup> (control) or by its presence at 15 μM, whereas it was barely appreciable at 50 μM, and steadily increased from 0.1 to 0.5 mM Ca<sup>2+</sup>. Figure 3B shows that the absence of Ca<sup>2+</sup> in the medium completely suppressed the NEM-induced electrolyte leakage, an aspect of sulfhydryl-blocker action similar to that reported for the pathogenic elicitor-induced respiratory burst (Atkinson et al., 1990; Bach et al., 1993; Goodman and Novacky, 1994; Jabs et al., 1997).

The effect of Ca<sup>2+</sup> in conditioning the NEM-induced oxidative burst and electrolyte leakage might have de-



**Figure 2.** Effects of 0.2 mM NEM on the rate of O<sub>2</sub> uptake and of H<sub>2</sub>O<sub>2</sub> production by *E. densa* leaves. The incubation medium contained 10 mM Mes buffer (pH 6.0) with BTP, 5 μM DCMU, and 0.5 mM CaSO<sub>4</sub>. Aliquots for ΔQO<sub>2</sub> measurements and H<sub>2</sub>O<sub>2</sub> determination came from the same incubation medium. Data are from one representative experiment out of five. FW, Fresh weight.

**Figure 3.** Effects of increasing concentrations of  $\text{Ca}^{2+}$  on 0.2 mM NEM-induced respiratory burst (A) and  $\text{Ca}^{2+}$  dependence of NEM-induced electrolyte leakage (B). Experimental conditions were as in Figure 2.  $\Delta\text{QO}_2$  data (A) are from one representative experiment out of three for each  $\text{Ca}^{2+}$  concentration tested. Conductivity change data (B) are the means of three experiments. SE did not exceed  $\pm 8\%$ . gr  $\text{FW}^{-1}$ , Per gram fresh weight.



depended on an increase of its influx in the cells. To test this hypothesis, we measured the effects of NEM on  $\text{Ca}^{2+}$  influx in a medium containing  $^{45}\text{Ca}^{2+}$  (Fig. 4) after 30 and 90 min of incubation. The results shown in Figure 4A demonstrate a very large increase of  $\text{Ca}^{2+}$  influx in the NEM-treated leaves. This finding was confirmed by measurements of  $\text{Ca}^{2+}$  concentration changes in the incubation medium, which showed a decrease of  $20 \mu\text{mol g}^{-1}$  fresh weight (Fig. 4B). Although changes in the  $\text{Ca}^{2+}$  content in the cell walls contributed somewhat to these results, these data are interpreted as indicating a marked stimulation of  $\text{Ca}^{2+}$  influx in the NEM-treated leaves.

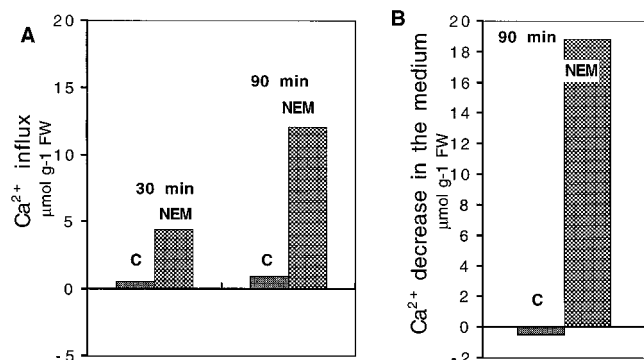
#### Effects of $\text{La}^{3+}$ on $\Delta\text{QO}_2$ and $\text{H}_2\text{O}_2$ Production

In several plant systems the ability to respond to a stress stimulus (i.e. temperature shock, drought, salinity, or pathogen-derived elicitors) requires the presence of extracellular  $\text{Ca}^{2+}$ . The inhibition by  $\text{Ca}^{2+}$ -channel blockers such as  $\text{La}^{3+}$  suggests that  $\text{Ca}^{2+}$  influx increases the cytosolic  $\text{Ca}^{2+}$  concentration (Atkinson et al., 1990; Bach et al., 1993; Price et al., 1994; Tavernier et al., 1995; Gelli et al., 1997; Pugin et al., 1997; Takahashi et al., 1997). The results shown in Figures 5 and 6 indicate that in our system the

induction of  $\Delta\text{QO}_2$  and the production of  $\text{H}_2\text{O}_2$  by NEM or  $\text{Ag}^+$  were completely inhibited by the presence of 0.25 mM  $\text{La}^{3+}$ . After treatment with NEM, the onset of the oxidative burst was seen after a lag of about 20 min, and the addition of  $\text{La}^{3+}$  was still able to counteract the appearance of  $\Delta\text{QO}_2$ . As shown in Figure 7, the development of the oxidative burst was completely prevented when  $\text{La}^{3+}$  was added 15 min after NEM, and was interrupted almost immediately when  $\text{La}^{3+}$  was added at the  $\Delta\text{QO}_2$  already initiated.

#### Effect of $\text{La}^{3+}$ on the $E_m$ and on Electrolyte Leakage

Previous experiments have shown that sulfhydryl blockers induced a respiratory burst and an increase in electrolyte leakage that were preceded by an early, strong depolarization of the plasma membrane (Bellando et al., 1997; Marrè and Albergoni, 1998). As shown in Figure 8, the addition of 0.25 mM  $\text{LaCl}_3$  to the flowing solution before the addition of either NEM or  $\text{Ag}^+$  completely suppressed the depolarizing effect of the two drugs (Fig. 8B). In other experiments  $\text{La}^{3+}$  was added 20 min after NEM, i.e. toward the end of the lag period between the addition of NEM and the onset of the respiratory burst, but when the depolar-



**Figure 4.**  $^{45}\text{Ca}^{2+}$  influx in *E. densa* leaves (A) and changes in external  $\text{Ca}^{2+}$  concentration (B) induced by 0.2 mM NEM. The  $^{45}\text{Ca}^{2+}$  uptake was determined after 30 and 90 min of incubation, as described in "Materials and Methods." Changes in external  $\text{Ca}^{2+}$  concentration were measured in the medium with a  $\text{Ca}^{2+}$ -selective electrode after 90 min of incubation. Samples were incubated under the same conditions described for the influx, except for the absence of  $^{45}\text{Ca}^{2+}$ . The data represent the means of two experiments run in triplicate. SE did not exceed  $\pm 10\%$ . FW, Fresh weight.

ization of NEM had fully developed and electrolyte leakage had already started. In this case  $\text{La}^{3+}$  completely reversed the NEM-induced depolarization (Fig. 8C) (for a similar effect of  $\text{La}^{3+}$  on elicitor-induced depolarization in tobacco cells, compare Pugin et al., 1997).  $\text{La}^{3+}$  alone did not significantly change the basal  $E_m$  value of *E. densa* cells, indicating a weak ability of this cation to enter the cells. The  $\text{La}^{3+}$ -induced inhibition of the membrane depolarization caused by NEM or  $\text{Ag}^+$  was associated with an equally complete suppression of the increase in electrolyte leakage (Fig. 9).

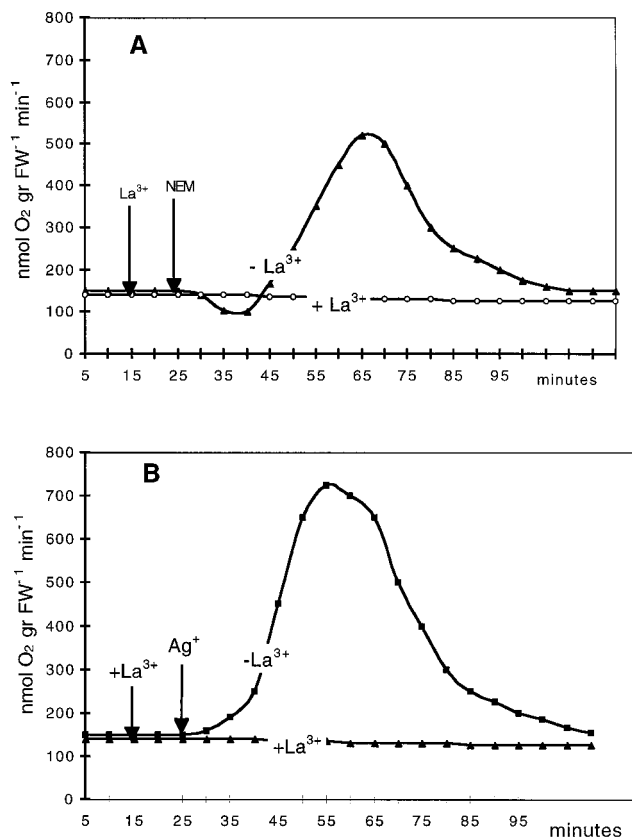
#### Effect of $\text{La}^{3+}$ on $\text{H}_2\text{O}_2$ -Induced Electrolyte Leakage

A role has been proposed for  $\text{H}_2\text{O}_2$  in the changes in the plasma membrane leading to electrolyte leakage in the oxidative burst induced by pathogen-derived elicitors (Levine et al., 1994; Mehdy, 1994; Lamb and Dixon, 1997). This also appears to be true in the case of sulfhydryl-blocker-induced  $\text{H}_2\text{O}_2$  production in *E. densa*. Figure 10 shows that infiltration of *E. densa* leaves with 1 and 10 mM  $\text{H}_2\text{O}_2$  induced a rapid increase in electrolyte leakage.  $\text{H}_2\text{O}_2$  concentrations less than 0.5 mM did not induce any detectable response.

The ability of  $\text{H}_2\text{O}_2$  to induce electrolyte leakage might depend on the activation of  $\text{Ca}^{2+}$  influx (Price et al., 1994; Levine et al., 1996). Thus, one might expect an effect of  $\text{La}^{3+}$  on the effect of  $\text{H}_2\text{O}_2$  on membrane permeability and electrolyte leakage. In our experiments the presence of  $\text{La}^{3+}$  completely inhibited the  $\text{H}_2\text{O}_2$  effect on electrolyte leakage, suggesting the involvement of some step influenced by  $\text{Ca}^{2+}$  extracellular concentration (for a similar conclusion in soybean cells, see Levine et al., 1996).

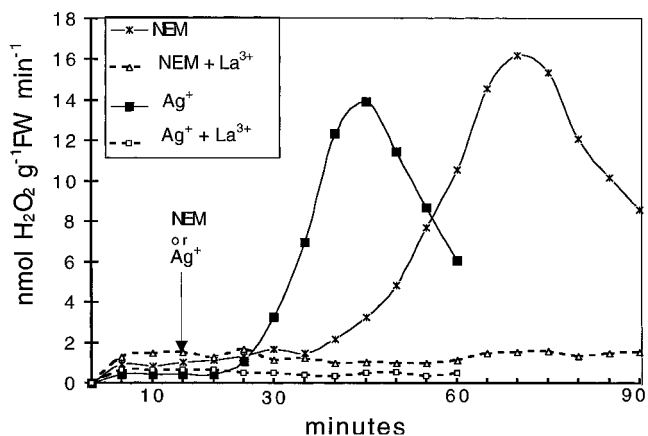
#### DISCUSSION

The results presented in this paper integrate the previously reported evidence concerning the effects of sulfhy-

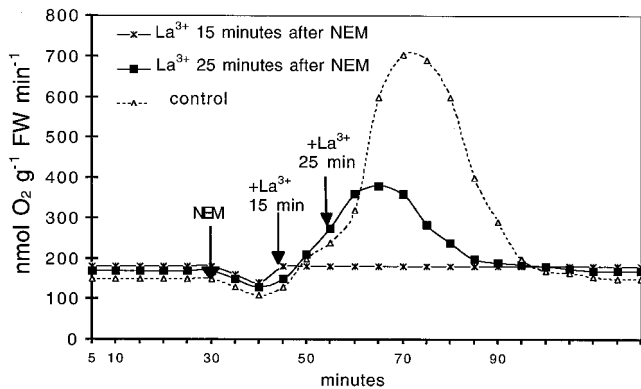


**Figure 5.** Effects of 0.25 mM  $\text{La}^{3+}$  on 0.2 mM NEM-induced (A) or 5  $\mu\text{M Ag}^+$ -induced (B) respiratory burst. Experimental conditions were as in Figure 2. Data are from one representative experiment out of three.  $\text{gr FW}^{-1}$ , Per gram fresh weight.

dryl reagents on respiration and ion transport in *E. densa* leaves. These new data demonstrate that the respiratory burst induced by sulfhydryl blockers (NEM or  $\text{Ag}^+$ ) in *E. densa* leaves was associated with a parallel production of  $\text{H}_2\text{O}_2$ . The presence of  $\text{Ca}^{2+}$  in the incubation medium was strictly required for the effects of NEM and  $\text{Ag}^+$  on trans-



**Figure 6.** Effects of the presence of 0.25 mM  $\text{La}^{3+}$  (added at time 0) on the rate of  $\text{H}_2\text{O}_2$  production induced by 0.2 mM NEM and 5  $\mu\text{M Ag}^+$ . Experimental conditions were as in Figure 2. Data are from one representative experiment out of three. FW, Fresh weight.



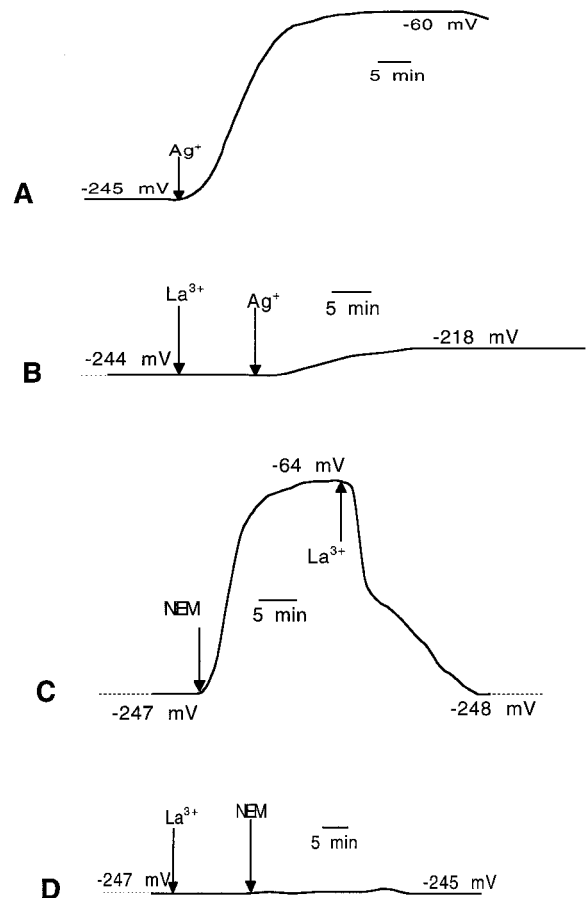
**Figure 7.** Effects of the addition of  $\text{La}^{3+}$  15 and 25 min after the addition of NEM on the NEM-induced respiratory burst. Experimental conditions were as in Figure 2. Data are from one representative experiment out of three. FW, Fresh weight.

membrane potential, electrolyte leakage,  $\text{O}_2$  uptake, and  $\text{H}_2\text{O}_2$  production, and  $\text{Ca}^{2+}$  influx was markedly stimulated by NEM. With 0.5 mM  $\text{Ca}^{2+}$  present, the addition of the  $\text{Ca}^{2+}$ -channel blocker  $\text{La}^{3+}$  completely inhibited all of these effects of NEM or  $\text{Ag}^+$ , suggesting that  $\text{Ca}^{2+}$  action depends on its influx. The addition of  $\text{H}_2\text{O}_2$  to the medium induced an electrolyte leakage of the same order as that induced by NEM or  $\text{Ag}^+$ , and this effect was completely inhibited by  $\text{La}^{3+}$ .

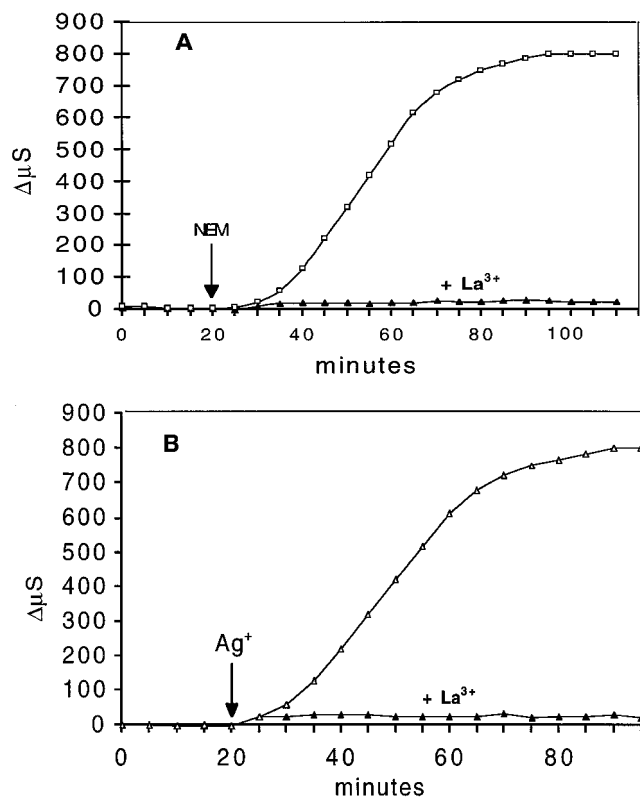
The integration of these results with those of previous studies allows a coherent interpretation of the mechanism of action of the sulfhydryl blockers on respiration and membrane activities in *E. densa* leaves. According to a model covering the available evidence to date, the primary interaction of the sulfhydryl blockers with the sulfhydryl groups of some cell component would lead to a rapid increase in  $\text{Ca}^{2+}$  influx. The consequent increase in cytosolic  $\text{Ca}^{2+}$  concentration would influence ion-transport systems at the plasma membrane (for a similar interpretation of the effects of  $\text{Ca}^{2+}$ , see Schroeder and Hagiwara, 1989; Price et al., 1994; Tavernier et al., 1995; Pugin et al., 1997), and would therefore explain the observed rapid membrane depolarization and increased release of electrolytes. According to our previous data, the main effects on ionic fluxes induced by sulfhydryl reagents are the effluxes of  $\text{K}^+$ ,  $\text{Cl}^-$ , and  $\text{H}_2\text{PO}_4^-$  and the influx of  $\text{H}^+$  and  $\text{Ca}^{2+}$  (according to the present results). In guard cells an increase in cytosolic  $\text{Ca}^{2+}$  concentration has been reported to favor the opening of anion ( $\text{Cl}^-$  and  $\text{H}_2\text{PO}_4^-$ ) channels and the closure of inwardly rectified  $\text{K}^+$  channels (Schroeder and Hagiwara, 1989), which would explain the changes in anion fluxes in our system. Membrane depolarization would depend at least in part on anion efflux and might be responsible for the opening of outward  $\text{K}^+$  channels and for  $\text{K}^+$  efflux. Thus, one might propose that all changes in the fluxes of these ions and in the  $E_m$  are consequences of  $\text{Ca}^{2+}$  entry into the cell, whereas the increase of  $\text{H}^+$  net uptake (this is potentially important because of its influence on cytosolic pH and the membrane potential) would depend on a  $\text{Ca}^{2+}$ -induced increase in passive membrane permeability to  $\text{H}^+$  and perhaps also on the inhibition of

the  $\text{H}^+$ -ATPase by the sulfhydryl blockers (Brooker and Slayman, 1982).

The identification of  $\text{H}_2\text{O}_2$  production during the  $\text{QO}_2$  burst confirms the previous conclusion that an  $\text{O}_2^-$ -producing NADPH oxidase of the type speculated to be involved in the hypersensitive reaction mediates the oxidative burst induced by the sulfhydryl blockers (Doussièrè and Vignais, 1992; Felix et al., 1994; Auh and Murphy, 1995; Van Gestelen et al., 1997; Xing et al., 1997), a conclusion previously drawn from the inhibition of the  $\Delta\text{QO}_2$  effect by diphenylene iodonium and quinacrine (Bellando et al., 1997). The activation mechanism of the NADPH oxidase by the sulfhydryl blockers remains open to investigation and probably involves various steps, as proposed for NADPH oxidase activation by pathogen-derived elicitors (Legendre et al., 1992; Morrè et al., 1993; Mehdy, 1994; Vera-Estrella et al., 1994). The conclusion that the responses of membrane potential and electrolyte leakage are largely independent of the activation of the NADPH oxidase is suggested by the



**Figure 8.** Effects of the presence of 0.25 mM  $\text{La}^{3+}$  on 5  $\mu\text{M}$   $\text{Ag}^+$ -induced (A and B) and 0.2 mM NEM-induced (C and D) membrane depolarization. Note that in C,  $\text{La}^{3+}$  was added after NEM addition, so that NEM-induced depolarization had already reached its maximum (about 15 min) but  $\Delta\text{QO}_2$  had not yet started (compare Fig. 7). Data are from one representative experiment out of three. A, Depolarization by 5  $\mu\text{M}$   $\text{Ag}^+$ ; B, its inhibition by  $\text{La}^{3+}$ ; C, depolarization by 0.2 mM NEM and its reversal by  $\text{La}^{3+}$ ; and D, inhibition by the presence of  $\text{La}^{3+}$  of the NEM-induced depolarization.

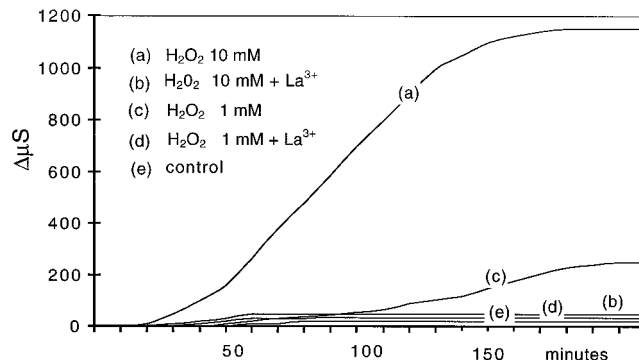


**Figure 9.** Effects of 0.25 mM  $\text{La}^{3+}$  on 0.2 mM NEM-induced (A) and 5  $\mu\text{M}$   $\text{Ag}^+$ -induced (B) electrolyte leakage. Basal medium was as in Figure 2 with 0.5 mM  $\text{CaSO}_4$  always present; 0.25 mM  $\text{La}^{3+}$  was added at time 0. Data are the means of three experiments. SE did not exceed  $\pm 10\%$ .  $\Delta\mu\text{S}$ , Change in medium conductivity.

findings that: (a) after treatment with NEM, the two responses are clearly separated in time; (b) NADPH oxidase inhibitors completely suppress the oxidative burst, whereas they inhibit electrolyte leakage only partially (Bellando et al., 1997; Marrè and Albergoni, 1998); and (c) the addition of  $\text{La}^{3+}$  15 to 20 min after that of NEM completely prevents the oxidative burst, although membrane depolarization has already reached its maximum and electrolyte leakage has already started. The findings that the addition of  $\text{La}^{3+}$  completely suppresses NEM-induced  $\Delta\text{QO}_2$  and  $\text{H}_2\text{O}_2$  production and that it reverses the previous effects of NEM on membrane potential suggests that all of the leaf responses to the sulfhydryl blockers require the continuous influx of  $\text{Ca}^{2+}$  into the cells.

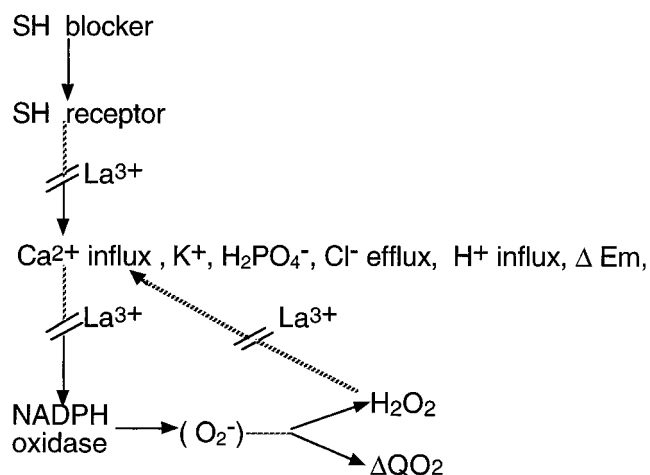
These experiments demonstrate that  $\text{Ca}^{2+}$  influx is strictly required for sulfhydryl reagent-induced changes in both ion transport and oxidative metabolism. However, an unresolved question is whether the influx of  $\text{Ca}^{2+}$  by itself is sufficient to induce the oxidative burst. The necessary involvement of ion fluxes other than that of  $\text{Ca}^{2+}$  in the induction of the oxidative burst has been suggested by Jabs et al. (1997). Further data are requested to define the situation in our system.

The model shown in Figure 11 summarizes the main lines of our interpretation of the action of the sulfhydryl blockers in *E. densa* leaves. A strict similarity is evident



**Figure 10.** Effects of the presence of 1 and 10 mM  $\text{H}_2\text{O}_2$  in the incubation medium on electrolyte leakage in the presence and absence of 0.25 mM  $\text{La}^{3+}$ . To facilitate  $\text{H}_2\text{O}_2$  entry, before the experiment was started leaves were rapidly infiltrated with the same medium used in the treatment. Experimental conditions were as in Figure 2. Data are from one representative experiment out of four.  $\Delta\mu\text{S}$ , Change in medium conductivity.

between this model and that proposed for the action of pathogen-derived elicitors in the induction of the oxidative burst (for review, see Lamb and Dixon, 1997). Our model is similar to that presented by Pugin et al. (1997) in a recent and very complete investigation regarding the action of cryptogein in tobacco cells, in which the effects of this elicitor on membrane depolarization, ion transport, and oxidative metabolism appeared to be very similar to those induced by the sulfhydryl blockers in *E. densa* leaves. According to our model, the  $\text{H}_2\text{O}_2$  produced by NADPH oxidase might converge with the primary effect of the sulfhydryl reagent increasing the passive permeability of the plasma membrane to ions. Such an effect of active



**Figure 11.** Proposed model for the action of the sulfhydryl (SH) blockers and  $\text{La}^{3+}$  on membrane potential, electrolyte leakage,  $\text{H}^+$  influx,  $\text{O}_2$  uptake, and  $\text{H}_2\text{O}_2$  production burst in *E. densa* leaves. According to this scheme,  $\text{Ca}^{2+}$  influx would influence the effects on NADPH oxidase independently of its action on ion transport and transmembrane potential. However, the present evidence is compatible with the alternative interpretation, that the activation of NADPH oxidase is a consequence of the changes at the ion-transport level (see Jabs et al., 1997).

oxygen species produced by pathogen-derived elicitors in various plant materials has been proposed by various authors (Keppler and Novacky, 1987; Levine et al., 1994; Mehdy, 1994; Lamb and Dixon, 1997). In our case an interesting feature of the activation of electrolyte leakage by  $\text{H}_2\text{O}_2$  was its inhibition by  $\text{La}^{3+}$ , suggesting that  $\text{Ca}^{2+}$  influx was involved. A stimulation of  $\text{Ca}^{2+}$  influx by exogenous  $\text{H}_2\text{O}_2$  has been reported by Price et al. (1994) in tobacco cells, and a stimulating effect of  $\text{Ca}^{2+}$  on anion efflux (and thus plasma membrane depolarization) has been reported by Schroeder and Hagiwara (1989). Thus, it seems possible that the effect of  $\text{H}_2\text{O}_2$  depends on its nature as a sulfhydryl-oxidizing agent, endowed with a mode of action on  $\text{Ca}^{2+}$  influx similar to that of  $\text{Ag}^+$  or NEM, rather than on its direct interaction with the plasma membrane.

The concentrations of  $\text{H}_2\text{O}_2$  in the medium required to induce a consistent electrolyte leakage are much higher than those that accumulated in the medium after treatment with NEM. This might be attributable to the fact that in the first case,  $\text{H}_2\text{O}_2$  reaches the cell from the outside, whereas in the second case, it is produced inside of the cell. A high  $\text{H}_2\text{O}_2$  concentration has been used in studies of the possible relevance of  $\text{H}_2\text{O}_2$  in pathogen-derived effects on ion transport (Apostol et al., 1989; Levine et al., 1994, 1996; Price et al., 1994). However, the very high  $\text{H}_2\text{O}_2$  concentration required to induce leakage raises some doubt about its possible role in NEM action.

Taken together, these new results emphasize the similarity between the effect of the sulfhydryl blockers in *E. densa* and the effects of pathogen-derived elicitors on plasma membrane activities and oxidative metabolism in a number of plant materials. This underscores the need for a comparative analysis of the possibly common steps in the mechanism of action of the sulfhydryl reagents and the pathogen-derived elicitors. Useful information in this regard should come from the investigation of the effects of nonspecific pathogen-derived elicitors in the *E. densa* system, an approach that we plan to develop in the near future. Progress in the study of the responses to the sulfhydryl blockers is of interest not only for the understanding of the mode of action of the sulfhydryl reagents, the nature of plasma-membrane redox systems, and the role of  $\text{Ca}^{2+}$  in the regulation of other ion fluxes, but also for the elucidation of the mechanism of defense reactions in plants.

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