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Effect of Elicitors on the Plasmamembrane of *Petunia hybrida* Cell Suspensions[1]

Role of ΔpH in Signal Transduction

Marc J. M. Hagendoorn, Arne M. Poortinga, Harro W. Wong Fong Sang, Linus H. W. van der Plas[2], and Hendrika S. van Walraven*

Biological Laboratory, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

ABSTRACT

Primary processes during elicitation of the phenylpropanoid pathway (PPP) were studied in *Petunia hybrida* cell suspensions. We tested the hypothesis that decrease of the proton gradient across the plasma membrane activates the PPP. Induction of the PPP was determined by measuring phenylalanine ammonia lyase activity. A variety of ATPase inhibitors and ionophores were tested for the ability to elicit the PPP. The ATPase inhibitors orthovanadate and *N,N'*-dicyclohexylcarbodiimide and the ionophores carbonyl cyanide-4-trifluoromethoxyphenylhydrazone and nigericin were all effective elicitors. Carbonyl cyanide-4-trifluoromethoxyphenylhydrazone and nigericin elicit also when used in combination with *N,N'*-dicyclohexylcarbodiimide. Valinomycin had little effect on phenylalanine ammonia lyase activity. Treatment with orthovanadate or nigericin led to the formation of lignin. Alkalinization of the external medium by *N,N'*-dicyclohexylcarbodiimide, carbonyl cyanide-4-trifluoromethoxyphenylhydrazone, and nigericin was observed directly with the use of a sensitive pH electrode and internal acidification was deduced from the changes in emission intensity of the fluorescent probe bis[3-propyl-5-oxoisoxazol-4-yl] pentamethineoxonol. These data indicate that changes in the activity of the plasmamembrane H^+-ATPase, and subsequent decrease of the proton gradient (particularly of the pH gradient) by itself are sufficient to influence phenylalanine ammonia lyase activity of *P. hybrida* cells and are therefore important intermediates in signal transduction.

In plant cell and tissue cultures, generally no significant production of secondary metabolites is observed. To initiate this production, many strategies have been followed, such as treatment with so-called elicitors (see ref. 8 and references therein). For example, biotic elicitors, isolated from plant pathogens, induced the production of isoflavonoid phytoalexins in soybean (7). Also abiotic elicitors, such as high medium osmolarity (15), are reported to induce the production of secondary metabolites.

Previously, we (10) examined the possibility of inducing PPP[3] (an example of a secondary biosynthesis pathway) in *Petunia hybrida* cell suspensions. This has been done by addition of nigeran, a polyglucan isolated from *Aspergillus niger*, and orthovanadate, an abiotic elicitor (see also ref. 12). Changes in the activity of the PPP were followed by measuring the activity of the enzyme PAL, a key enzyme of the general part of this pathway. Together with an increased PAL activity, addition of orthovanadate caused considerable lignin production (10).

The primary reactions upon elicitation with a biotic elicitor are thought to be composed of the following processes (6). The elicitor is recognized by and bound to a specific receptor protein on the plasma membrane (23). Then it has been suggested that inhibition of the plasma membrane ATPase is the next step in elicitation (12) because orthovanadate, an inhibitor of plasma membrane ATPases (3), can act as an elicitor as well (10, 12). Inhibition of the plasma membrane ATPase by orthovanadate would reduce the proton electrochemical gradient across this membrane. This idea has been corroborated by the finding that an external pH shift can give rise to elicitation also (10, 12). The proton gradient consists of a ΔpH (acidic outside) and a $\Delta\psi$ (positive outside). The collapse of both components upon addition of a biotic elicitor in various plant cells has been described in the literature (20).

To test this hypothesis, we used two different approaches. The first consists of the treatment of *P. hybrida* cells with DCCD, an inhibitor of proton channels such as H^+-ATPases (3), and various ionophores. These ionophores influence the relative contribution of transmembrane ΔpH and/or $\Delta\psi$. We investigated which treatments resulted in elicitation, as indicated by an increase in PAL activity. The second approach is a direct observation of the changes in membrane energization with the use of a pH electrode and a fluorescent probe.

In this way, we have been able to study the role in elicitation of changes in the ΔpH and the $\Delta\psi$ of the plasma membrane.

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[2] Present address: Department of Plant Physiology, Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands.

[3] Abbreviations: PPP, phenyl propanoid pathway; DCCD, *N, N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide-4-trifluoromethoxyphenylhydrazone; PAL, phenylalanine ammonia lyase; oxonol VI, bis[3-propyl-5-oxoisoxazol-4-yl] pentamethineoxonol; ΔpH, pH gradient; $\Delta\psi$, membrane potential.

MATERIALS AND METHODS

Chemicals

DCCD was purchased from Merck (Darmstadt, Federal Republic of Germany), valinomycin from Boehringer (Mannheim, Federal Republic of Germany), FCCP from Fluka (Buchs, Switzerland), and nigericin from Calbiochem (La Jolla, CA). Oxonol VI was synthesized and kindly donated by Dr. W. Hanstein (Ruhr-Universität Bochum, Federal Republic of Germany). All other chemicals were of analytical grade. Stock solutions of DCCD, ionophores, and oxonol VI were prepared in ethanol in a concentration of, respectively, 10, 1, and 1 mM. Sodium orthovanadate was dissolved in water at pH 5.5 ± 0.2 with HCl.

Plant Material and Tissue Culture

The V30 cell line was initiated from *Petunia hybrida* Violet 30 hypocotyls and was maintained on Murashige-Skoog medium (21) containing 30 g glucose and 1 mg naphthalene acetic acid per liter. The Murashige-Skoog medium contained 20 mM K^+, and the initial pH of the medium was 5.4. The batch cultures were maintained in 250-mL Erlenmeyer flasks containing 70 mL of culture medium, covered with aluminum foil. The cells were subcultured every 14 d. At the start of an experiment, 10 mL of a 14-d-old cell suspension was added to 25-mL fresh medium in a 100-mL flask. The cells were grown in the dark at 28°C, on a gyratory shaker at 125 rpm. In all experiments, cells were used 2 to 3 d after inoculation.

PAL Activity

About 0.5 g fresh weight of cells was ground in a mortar and pestle in liquid nitrogen and, after thawing, in 2.0 mL 0.1 M sodium borate, pH 8.8, containing 0.1 g quartz. To remove phenolics, 100 mg Dowex-1, chloride form, was added. The extract was centrifuged in a Sigma 201-M for 10 min at 24,000*g*. The PAL activity in the supernatant was determined as the change in extinction at 290 nm in a cuvette containing 0.5 mL extract and 60 mM phenylalanine in 0.1 M sodium borate, pH 8.8, according to the method of Zucker (30). An LKB Ultrospec K. 4053 was used. The protein content of the extracts was determined according to the method of Bradford (2) using BSA as a standard. The PAL activity is expressed as μkat/kg protein, 1 μkat being 1 μmol product formed/s.

Lignin Determination

The semiquantitative method is described by Hagendoorn *et al.* (10). Cells (5 g fresh weight) were washed in boiling water and filtered through a Büchner funnel. The residue was extracted with 96% ethanol. The extractive free weight obtained by this step was dried (60 min, 90°C), and 50 to 100 mg of extractive free weight was used for an acid ethanolysis (25 mL 4% HCl in ethanol) at 60°C. After filtration through filter paper, 25 mg of phloroglucinol was added to the filtrate and the A_{540} was determined after exactly 3 min.

Fresh Weight/Dry Weight and *in Vivo* Respiration

Fresh weight was determined after harvesting the cells on a Büchner funnel applied with a paper filter. For determination of dry weight, the cells were dried at 60°C for 24 h. *In vivo* respiration was determined according to the method of Van der Plas *et al.* (29).

pH Measurements and Fluorescence Assays

External pH was measured with a sensitive micro-electrode (Ingold) while continuously stirred in a thermostatically controlled reaction vessel. Fluorescence spectra were recorded on an SLM-Aminco fluorimeter equipped with a thermostated multi-purpose cuvette (16). Fluorescence intensities at set wavelength were followed with an Oriel 3090 fluorimeter using a similar cuvette.

pH determinations and fluorescence assays were carried out at 28°C using cultured cells or culture medium only. It was important to take the cells out of the cultivation incubator immediately before measurement, to prevent pH changes occurring in the very weakly buffered suspensions. For further details, see legends to the Figures.

RESULTS AND DISCUSSION

Effect of ATPase Inhibitors and Ionophores on the PPP

Figure 1 shows the PAL activity 24 h after treatment with various concentrations of DCCD and ionophores. A significant increase in PAL was obtained by addition of less than 1 μM DCCD, FCCP, and nigericin, whereas addition of valinomycin had no significant effect on the PAL activity.

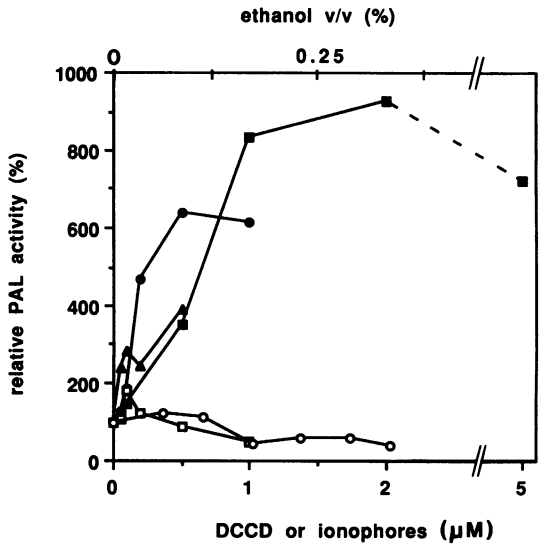

Figure 1. Dependence of the PAL activity of *P. hybrida* cells on the concentration of DCCD and various ionophores. Ethanol is used to prepare stock solutions of the added compounds and is therefore also tested. The PAL activity is given as percent of the activity in control cells (untreated); 100% corresponds to 19 ± 6 μkat/kg protein. Additions are: ■, DCCD; ●, nigericin; ▲, FCCP; □, valinomycin; ○, ethanol.

Table I. *PAL Activities of P. hybrida Cell Suspensions before and after Treatment with the ATPase Inhibitors Orthovanadate and DCCD, and the Ionophores FCCP, Nigericin, and Valinomycin*

The used concentrations of the additions give maximal PAL activities (see Fig. 1 and ref. 10 for orthovanadate). The conditions of the pH-shift experiment are described in ref. 10. The results of representative experiments (performed in duplicate) are shown.

Treatment	PAL Activity	
	Control	Treated
	μKat/kg protein	
Orthovanadate (50 μM)	11	70
pH-shift (from 5.6 to 6.7)	8	70
DCCD (2 μM)		
Experiment 1	<1	92
Experiment 2	10	32
Experiment 3	<1	62
FCCP (0.5 μM)	16	65
FCCP (0.5 μM) + DCCD (2 μM)	<1	40
Nigericin (0.5 μM)	18	114
Nigericin (0.5 μM) + DCCD (2 μM)	14	127
Valinomycin (0.2 μM)	21	26
Valinomycin (0.2 μM) + DCCD (2 μM)	<1	16
Valinomycin (0.2 μM) + orthovanadate (25 μM)	2.5	33

Table I summarizes the maximal elicitation caused by different (combinations of) treatments. A comparison is made with orthovanadate and pH-shift that have proved to be effective eliciting treatments of *Petunia* cells in previous studies (10). The control PAL activities may differ due to differences in growth phase of the cells between the experimental series. Dilution stress (9), occurring shortly after subculturing, may also contribute to these differences. Variation in PAL activity after treatment (see Table I, DCCD treatment) may occur due to the same factors. Treatment by a combination of FCCP or nigericin with DCCD gave results similar to those of the compound by itself. Valinomycin alone did not cause elicitation (see also Fig. 1). In the presence of valinomycin, elicitation by DCCD and orthovanadate still occurred, but to a lower extent.

Orthovanadate inhibits different types of plasma membrane ATPases (3). The results of the DCCD experiments suggest that inhibition of an H^+-translocating ATPase is an intermediate in elicitation. The effects of FCCP, which collapses both $\Delta\psi$ and ΔpH by H^+ transport, show that a proton gradient is important. There is a K^+ gradient across the plasma membrane ($K^+_{in} > K^+_{out}$) (28), and also a large Δ pH (pH_{in} 7–7.5; pH_{out} 5.4) (17). Nigericin promotes the electroneutral exchange of H^+/K^+, thus dissipating ΔpH. Addition of nigericin led to very high PAL activities (see Table I), indicating that changes in ΔpH alone are able to elicit PAL. This is in accordance with the observation that elicitation occurs after alkalinization of the medium.

The lack of influence of valinomycin may in theory be due to two factors. First, it is possible that the potassium gradient across the plasma membrane is in equilibrium with the $\Delta\psi$. In that case, no effect of valinomycin can be expected. However, if we consider values from the literature for $\Delta\psi$ and internal $[K^+]$, this situation is quite unlikely. Values for $\Delta\psi$ in suspension cultures are usually lower than −100 mV, and $[K^+]_{in}$ is around 100 mM (28). Since $[K^+]_{out}$ in our experiments is 20 mM, valinomycin addition will drastically increase the $\Delta\psi$ to a value of approximately −40 mV.

The second possibility, therefore, is that depolarization of the $\Delta\psi$ alone does not lead to elicitation of PAL. Moreover, the existence of an intact $\Delta\psi$ might be necessary for proper elicitation. The lower degree of elicitation by DCCD or orthovanadate in the presence of valinomycin supports the latter idea.

A time course of PAL activity after addition of orthovanadate or nigericin is shown in Figure 2. Orthovanadate is an inhibitor of the plasma membrane ATPase and nigericin is an affector of the ΔpH solely. Within 12 h, a significant increase of the PAL activity was observed in both cases. Nigericin showed the same kinetics as orthovanadate in increasing PAL activity. The effect of these elicitors is slower than that of biotic elicitors, which is in accordance with earlier work (10, 12, 26). Increase in PAL activity is generally due to *de novo* synthesis of the enzyme (19).

We also examined the product formed after nigericin treatment. Earlier studies have shown that orthovanadate addition and alkalinization of the medium led to a rise in the production of lignin (10). Six days after nigericin addition, an increase in lignin production of approximately 4 times was found, compared with control cells (results not shown). This indicates that elicitors, which affect the plasma membrane H^+-ATPase or ΔpH, lead to the same end product (lignin).

Effect of ATPase Inhibitors and Ionophores on Growth and Respiration

We compared the increase in dry weight of treated cells after 24 h, with that of control cells. While cells treated with valinomycin showed no growth inhibition, cells treated with nigericin showed only 40% of the increase in dry weight observed with untreated cells. Cells treated with FCCP and DCCD showed intermediate results with 70% of the dry weight increase of control cells. This indicates that an increase

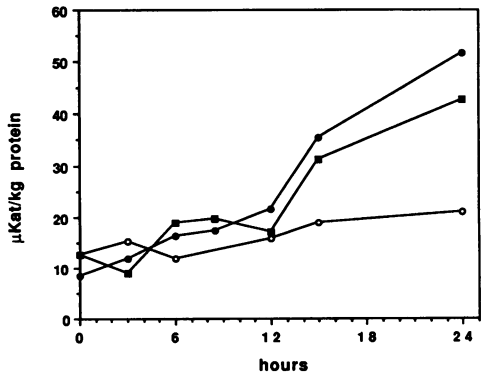

Figure 2. Time course of PAL activity of *P. hybrida* cells after treatment with orthovanadate (50 μM, ■) and nigericin (0.5 μM, ●) as compared with untreated cells (○). Points are means of two measurements. PAL activity is expressed as μkat/kg protein.

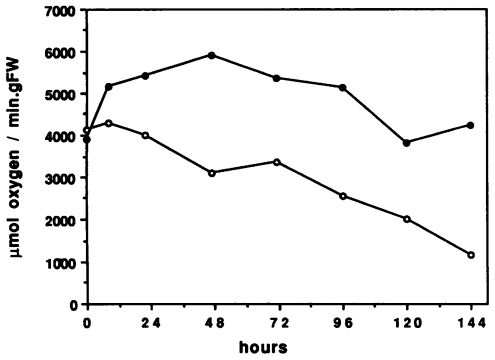

Figure 3. Respiration rate of *P. hybrida* cells after treatment with orthovanadate. Total oxygen uptake of the cells/min/mg fresh weight (corrected for residual respiration) is depicted. Orthovanadate was added (t = 0) 5 d after inoculation. Points represent mean values of eight determinations from two different experimental series. ○, untreated cells; ●, cells treated with orthovanadate (50 µM).

in PAL activity is correlated with a decrease in growth rate, as has been reported earlier for orthovanadate, where the dry weight did not increase at all (10).

To investigate whether cells could survive the eliciting treatments, we followed the cell respiration after addition of orthovanadate, the strongest growth inhibitor. Figure 3 shows that the cell respiration increased within 12 h and remained higher than the respiration of control cells for at least 6 d. Together with the lignin production, 6 d after orthovanadate addition (10), this shows that cells can survive this kind of treatment. Probably, the energy flow is primarily directed to the synthesis of new enzymes and products upon addition of an elicitor. If treated cells are transferred to fresh medium, they grow at the same rate as control cells (9).

Orthovanadate, DCCD, and the ionophores can be expected to affect also the mitochondrial membrane. Therefore, we also investigated the respiratory rate immediately upon addition of these compounds. In the concentrations used for elicitation, all additions except orthovanadate led to an instantaneous increase in respiration (about 20%). This effect was due to the ethanol in which all stock solutions were prepared. Orthovanadate, which was dissolved in water, had no instantaneous effect on cell respiration. FCCP in a concentration above 1 µM, which was just slightly higher than used for elicitation, gave rise to uncoupling of the mitochondrial membrane and a concomitant increase in respiration up to a factor 1.7.

pH Measurements and Oxonol VI Fluorescence Assays

If elicitation of plant cells acts via inhibition of the plasma membrane H^+-ATPase and subsequent collapse of membrane energization, it should be possible to observe changes in ΔpH and/or $\Delta\psi$ directly. Low and Heinstein (20) have reported a simultaneous collapse of both ΔpH and $\Delta\psi$ upon addition of fungal elicitor with the use of the fluorescent probes pyranine and oxonol VI, respectively. The collapse in ΔpH and $\Delta\psi$ followed a lag phase that depended on the concentration of elicitor.

In *Petunia* cells, external pH changes could be observed with a pH electrode due to the weak buffering capacity of the cultivation medium (Fig. 4). As expected, addition of DCCD, FCCP, and nigericin led to alkalinization. The final pH level was reached slowly, in more than 10 min, and a lag in response was observed. The changes in pH were only small: maximally 0.1 pH unit. The control experiment with ethanol showed no pH effect, nor did addition of valinomycin.

Oxonol VI can be used to monitor both positive and negative $\Delta\psi$ in a fluorescence assay (24) and has been used to determine $\Delta\psi$ in plant cells before (28). We have measured $\Delta\psi$ by oxonol VI fluorescence under the conditions where it functions as a $\Delta\psi$ probe (pH of external medium exceeding 6, see ref. 24). High concentrations of DCCD (about 50 µM) led to a decrease in $\Delta\psi$ by about 10 to 20%. A similar decrease was observed with low concentrations of FCCP and valinomycin (0.1–0.5 µM). However, no membrane depolarization was observed with 1 to 5 µM DCCD or 25 to 50 µM orthovan-

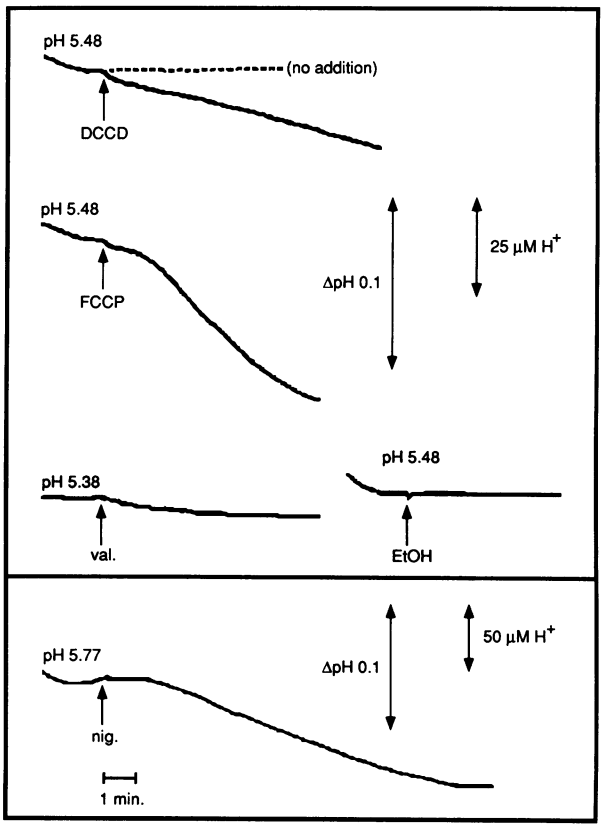

Figure 4. DCCD- and ionophore-induced changes in external pH of *P. hybrida* cell suspensions. Ethanol is used to prepare stock solutions of the added compounds and is therefore also tested. The pH value before addition is given at the start of each trace. A decrease in signal indicates external alkalinization. All traces were obtained with cells 2 d after inoculation, with the exception of the nigericin experiment, which was performed with cells 3 d after inoculation. The concentrations of the additions are: DCCD, 10 µM; FCCP, 0.5 µM; nigericin, 0.5 µM; valinomycin, 0.5 µM; ethanol, 0.1% v/v. Standard amounts of oxalic acid were used to quantitate the changes in proton concentration in the external medium. A downward trace indicates alkalinization.

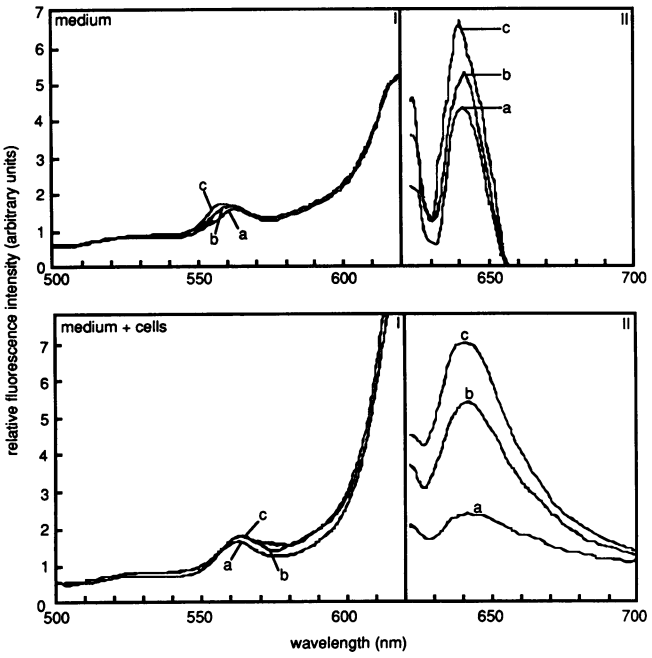

Figure 5. Effect of pH on excitation (I) and emission (II) spectra of oxonol VI in cultivation medium (upper panel) and *P. hybrida* cell suspensions (lower panel). Oxonol VI was present in a concentration of 2.5 μM. The excitation spectrum was determined with the emission wavelength, set at 645 nm, and the emission spectrum with the excitation wavelength, set at 609 nm. The sensitivity of the instrument was 5 times increased when recording the emission spectrum in cultivation medium (upper panel II). The pH values in the upper panel were: a, 5.20; b, 6.42; c, 8.68; and in the lower panel: a, 4.95; b, 5.62; c, 9.25. The pH values were determined simultaneously with a pH electrode and refer to external pH. The pH was manipulated by addition of HCl or NaOH.

adate, the concentrations effective in elicitation (see Fig. 1). Under these conditions, we have never observed complete collapse of fluorescence, as reported previously (20), upon addition of an elicitor.

These observations correspond well with the results of previous studies (4, 5), in which much higher concentrations of vanadate and DCCD were required for slight membrane depolarization than for actual inhibition of the plasma membrane H^+-ATPase. The changes in plasma membrane $\Delta\psi$ of plant cells are so small because they are probably compensated for by K^+ fluxes by another channel (see refs. 3 and 28 for reviews).

Smith *et al.* (24) demonstrated that the level of fluorescence of oxonol VI was highly dependent on the pH with a critical range below pH 6. At a low pH value, oxonol VI shifts from the anionic form, which is responsible for the fluorescence, to the poorly soluble neutral form. Since the pH value of cell suspensions is low and varies around pH 5.5 (see Fig. 4), there are problems using oxonol VI for studies of $\Delta\psi$.

The effect of pH on the fluorescence excitation and emission spectrum is given in Figure 5. The effect of the presence of cells is a slight shift in excitation and emission maximum to higher wavelengths and, more important, an enormous increase in fluorescence yield (approximately 8–10 times). The pH effect is therefore very striking in the presence of cells. According to Bashford *et al.* (1), the mentioned changes in fluorescence properties are associated with binding of the dye to the membrane. The pH at which the largest change occurs is around 5.5, which corresponds to the pH of the cell suspensions.

This phenomenon is also apparent in Figure 6, in which the fluorescence was measured at set wavelengths. An excitation wavelength of 556 nm was used rather than 609 nm (13, 20) because the latter overlaps with the emission maximum (see also Fig. 5). In traces 1 and 2 of Fig. 6, it is shown that a low fluorescence level at pH 5.3, and a high level at pH 5.6

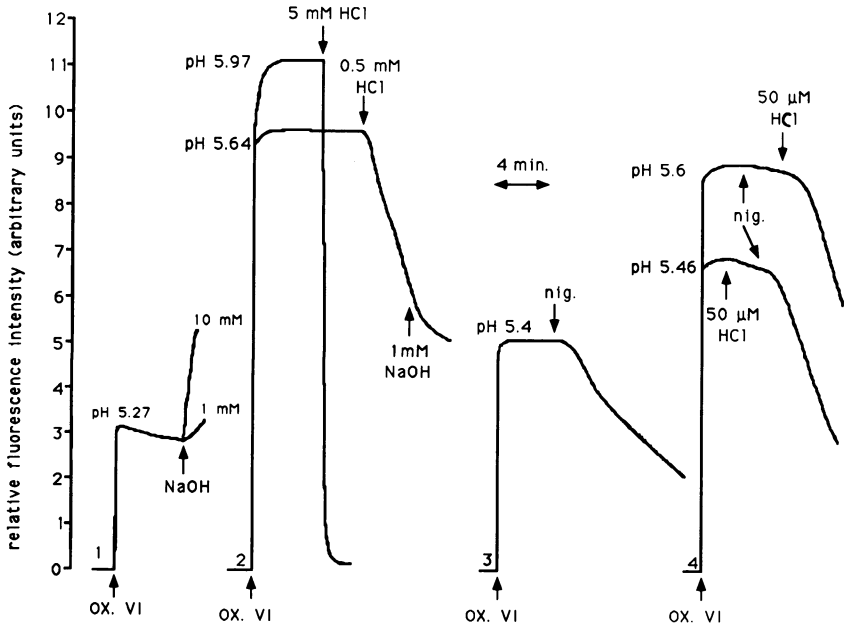

Figure 6. Effect of acid, base, and nigericin additions at various pH on the fluorescence level of oxonol VI in a suspension of cultured *P. hybrida* cells. See also legend to Figure 5. Oxonol VI was present in a concentration of 0.5 μM. The excitation and emission wavelengths were set at 556 nm and 654 nm, respectively. The initial pH values in the presence of oxonol VI are given at the start of each trace. Nigericin was added in a concentration of 0.5 to 1 μM. The concentrations of acid and base in the Figure are the final concentrations (mol/L).

to 6, can be influenced by base and acid addition, respectively. Low amounts of NaOH and HCl led to a slow response including a lag phase. This suggests that oxonol VI responds to internal events. Further evidence for the latter is obtained from comparative experiments using pyranine, a hydrophilic fluorescent pH indicator (13, 20) remaining in the external medium. Addition of small amount of base or acid led to an immediate change in fluorescence (results not shown). These observations are in accordance with the idea that oxonol VI accumulates at the inside of the membrane (1), thus reflecting internal pH in our studies. In Figure 6, trace 3, the effect of nigericin in the "critical" pH range is demonstrated. A collapse in fluorescence was observed after addition of nigericin. Above the "critical" pH range, nigericin had almost no effect on fluorescence. A collapse of fluorescence upon nigericin addition could only be obtained after lowering the pH by addition of minute amounts of acid, which by itself had little effect (Fig. 6, trace 4). The observed effects are not the result of surface charge or ionic strength-dependent changes in oxonol VI binding to the plasma membrane or cell wall (results not shown).

The nigericin experiment suggests that internal acidification occurs during elicitation and is probably responsible for the observed changes in oxonol VI fluorescence. This corroborates the observation (Fig. 1 and Table I) that alteration of the ΔpH across the plasma membrane is an important factor in the type of elicitation we studied. Low and Heinstein (20) described the same kind of oxonol VI signal as we showed in Figure 6, trace 3. They interpreted it as a change in $\Delta\psi$. However, a change in $\Delta\psi$ does not occur in the nigericin experiments of Figure 6. Since the pH of the cultured cells in the oxonol VI experiments performed by Low and Heinstein (20) is between 5 and 5.7 (11), internal pH changes may also contribute to their oxonol VI fluorescence changes.

Two other reports in the literature describe the importance of changes in cytoplasmic pH upon elicitation. In *Phaseolus vulgaris* cell suspensions (22), a decrease in cytoplasmatic pH is also observed upon elicitation. In parsley cell suspensions, the cytoplasmic pH dropped by approximately 0.25 pH unit upon addition of a biotic elicitor (14).

The knowledge of the further intracellular signal transduction processes after elicitation is still sparse, and sometimes contradictory. There are indications that Ca^{2+} fluxes are involved (25). Also the phosphatidyl inositol-cascade can be a part of this mechanism, but evidence on this point is contradictory (18, 27).

CONCLUSION

These data indicate that changes in the activity of the plasma membrane H^+-ATPase and subsequently of the ΔpH are sufficient to increase PAL activity and product formation in *P. hybrida* cells, and are therefore important intermediates in signal transduction. An interesting observation in this respect is that a decrease in the concentration of auxin (naphthaleneacetic acid) is able to increase the product formation in *P. hybrida* cells upon elicitation (9). As auxins have been shown to enhance H^+ extrusion by stimulating the plasma membrane H^+-ATPase (our unpublished results and ref. 17), the antagonistic action of elicitors and auxin gives further support for this hypothesis.

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