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CERCOSPORA BETICOLA TOXINS. USE OF FLUORESCENT CYANINE DYE TO STUDY THEIR EFFECTS ON TOBACCO CELL SUSPENSIONS*

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Key Word Index—*Cercospora beticola*; fungus; beticolin; cyanine dye; transmembrane potential; ATPase, tobacco cell.

Abstract—The fluorescent dye 3,3′-diethylthiadicarbocyanine iodide [diS-C_2-(5)] was used to observe plasmalemma transmembrane potential variations of tobacco cells treated with uncoupler (FCCP), respiratory inhibitors (azide and cyanide), and H^+-ATPase inhibitors (DCCD and a carbanilate derivative). These chemicals induced an increase in fluorescence, indicating a dissipation of the transmembrane potential. The [diS-C_2-(5)] was also used to study the effects of two *Cercospora beticola* toxins on tobacco cells. Changes in fluorescence of [diS-C_2-(5)] suggested that these two toxins caused a dissipation of the transmembrane potential with a different magnitude whereas kinetics of their association with membranes were comparable. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The transmembrane potential in plant cells is the result of charge transport between the cells and the external medium. Measurement of its variation could be used in order to study membrane sensitivity to effectors such as hormones or toxins.

Besides electrophysiology, which is the conventional method used for the cell membrane potential determination [1], other techniques have been developed. For example, it was shown that lipophilic ions, for which equilibrium concentration between extracellular medium and cellular compartment depends on the transmembrane potential, have been used to assess the transmembrane potential in plant cells [2–4]. This kind of measurement can also be done with fluorescent dyes as reported in ref. [5], since correlations between fluorescence intensities of the dyes and measured membrane potentials have been found [6, 7]. Dialkylthiacarbocyanine optical probes have been widely used to monitor potential variations in animal cells, bacteria, yeast, organelles and vesicles (for review see ref. [8]), whereas, as far as we know, reports concerning plant cells are sparse [9].

In the present paper, we evaluated the conditions for using the fluorescent dye 3,3′-diethylthiadicarbocyanine [diS-C_2-(5)] in order to analyse plasmalemma transmembrane potential variations in tobacco cells. A correlation between the fluorescence changes of positively ([diS-C_2-(5)]) or negatively (oxonol) charged probes and plasma membrane potential measured with a microelectrode or cations such as tetraphenylphosphonium has been demonstrated [4, 10]. It seems that the plasma membrane potential is the main factor which determines the distribution of the hydrophobic ion between the medium and the cells, whereas the mitochondrial and vacuolar membrane potential did not seem to be significantly involved.

The validity of the method was assayed using ionophores such as FCCP (carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone) and valinomycin, or respiratory inhiitors such as NaN_3 and NaCN. Then the method was applied to the study of the effect of ATPase inhibitors such as DCCD (*N,N′*-dicyclohexylcarbodiimide), SW26 (2,2,2-trichloroethyl 3,4-dichlorophenylcarbamate) and beticolin-1 and -2, secondary metabolites produced by *Cercospora beticola* [11–14]. Beticolin-1 (previously known as CBT) was reported to inhibit ATP dependent proton transport across plasmalemma [15]. Recently we have shown that purified plasmalemma ATPase was a direct target for beticolin-1 [16].

RESULTS AND DISCUSSION

As demonstrated in animal cells [7], the addition of the carbocyanine dye [diS-C_2-(5)] to a tobacco cell

*Part XIV in the series '*Cercospora beticola* Toxins'. For part XIII see *Phytochemistry* (1996) **42**, 979.

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suspension caused an initial rapid increase in fluorescence followed by a slow decline in light emission (during 4–5 min) down to a steady level F_0 (Fig. 1). As reported in ref. [5], the fluorescence of the carbocyanine dye is quenched when this compound is added to polarized cells having negative inside potential difference. This could be explained by the mechanism of action of carbocyanine dye as described in ref. [17]: carbocyanine dyes with their delocalized positive charge, penetrate lipid membranes and distribute within the lipid boundary in response to the membrane potential. Hyperpolarization causes intracellular dye accumulation resulting in a formation of less fluorescent dye aggregates.

In contrast, cell depolarization results in dye accumulation in the extracellular medium, the predominating dye monomers being responsible for the increase in fluorescence intensity as shown by addition of 5 μM FCCP (Fig. 1, level F). Using 5 μM FCCP, the dose-response curve of [diS-C_2-(5)], expressed as $\Delta F = F - F_0$ is presented in Fig. 1, insert 1. ΔF increased linearly with dye concentration from 0 to 2 μM and then reached a plateau. Thus, optimal conditions for cyanine dye fluorescence measurements were obtained with a dye concentration of 2 μM which is a concentration generally used in fluorescence measurement involved in the study of membrane potential variations [3].

In the same way, the dose-response curve of FCCP (Fig. 1, insert 2) showed that ΔF increased for FCCP concentrations up to 0.25 μM and then remained constant (ΔF_M). Addition of valinomycin (2.5 μM) did not change the ΔF_M value obtained with FCCP. This increase in fluorescence, induced by FCCP, can be correlated with the variations of transmembrane potential. It was surprising that F_M was obtained with 0.25 μM FCCP whereas 10 μM FCCP was generally used to uncouple the plasma membrane or tonoplast membrane [2, 3, 18]. The fact that a lower FCCP concentration was needed, in our experimental conditions, could be explained by its uncoupler effect on mitochondria which deprives cells of ATP and decreases all ATPase activities. The membrane potential of mitochondria is much more sensitive to FCCP than that of the plasma membrane [19].

Then, two compounds known as respiratory inhibitors, NaN_3 and NaCN, were used to assess the validity of the method. Both are inhibitors of mitochondrial cytochrome oxidase but to different degrees. NaCN is a powerful inhibitor of this enzyme, whereas NaN_3 leads to incomplete inhibition [20]. Moreover, NaN_3 is a selective inhibitor of the pH 9.0 ATPase activity in mitochondria [21].

The addition of NaN_3 to the cells (Fig. 2) caused an important increase in $\Delta F\%$ which reached a plateau at a value of about 80% for 600 μM NaN_3, whereas $\Delta F\%$ for NaCN (Fig. 2) reached only 30% for 500 μM NaCN. The effect of NaCN was enhanced by addition of salicylhydroxamic acid (SHAM) an inhibitor of the mitochondrial alternate oxidase [22]. Added after NaCN, SHAM (10 mM) enhanced the $\Delta F\%$ value up

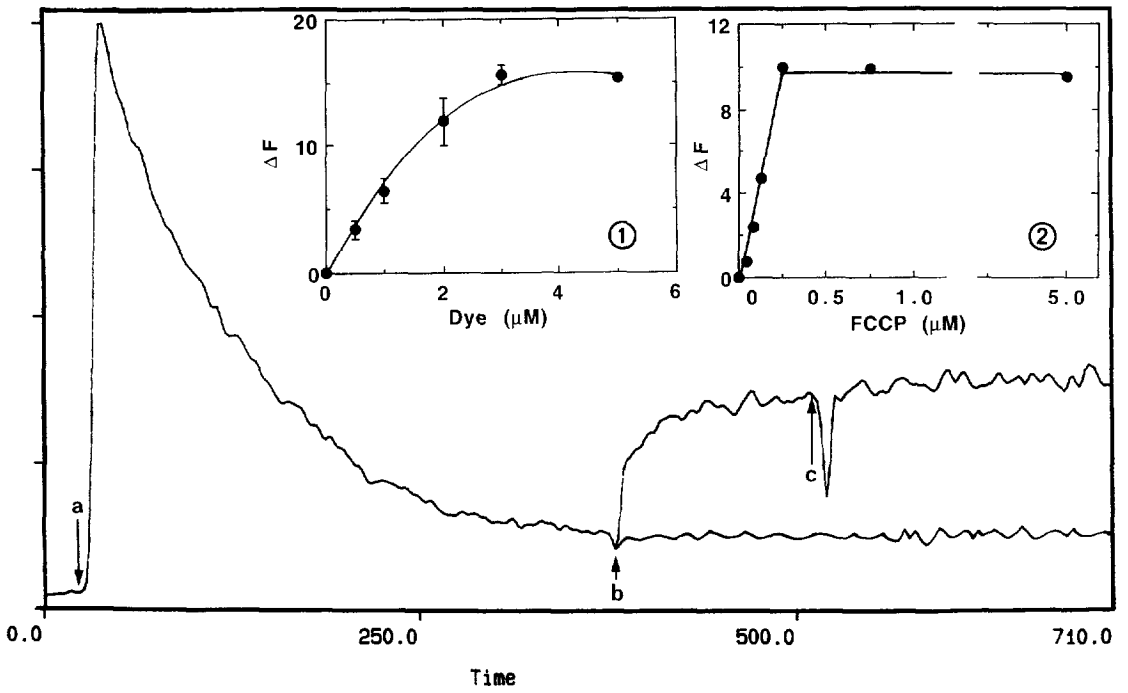

Fig. 1. Time course of [diS-C_2(5)] fluorescence after addition to tobacco cells. Cells were prepared as described in the Experimental, [diS-C_2(5)] 2 μM, FCCP 5 μM and valinomycin 2.5 μM were added as shown by arrows a, b and c, respectively. Fluorescence in arbitrary unit, time in s. Insert 1: effect of diS-C2(5) concentration on ΔF. Results represent the mean value of three independent experiments and are expressed as $\Delta F = F - F_0$. Insert 2: effect of FCCP concentration on ΔF. Results represent the mean value of three independent experiments and are expressed as in insert 1.

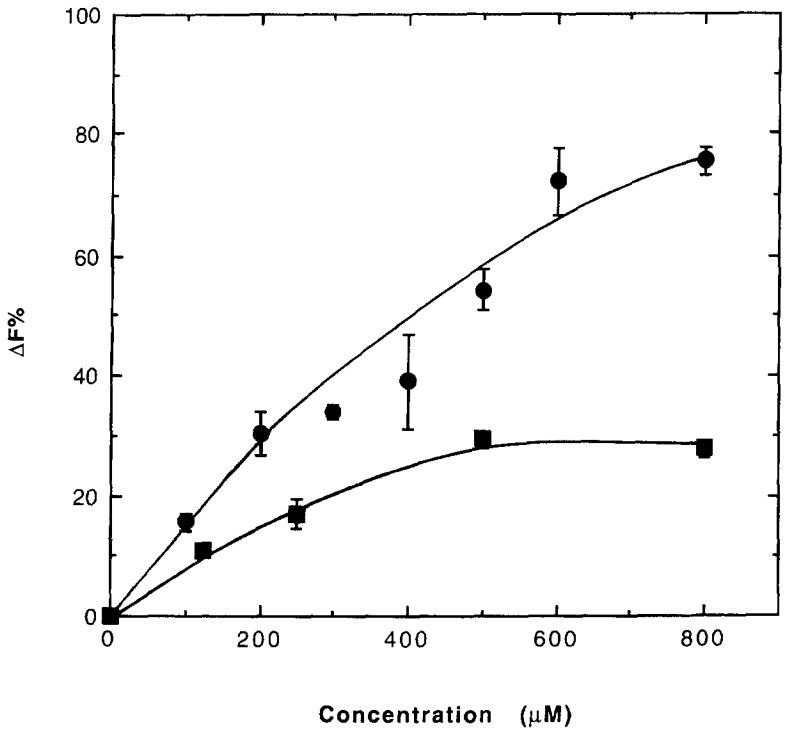

Fig. 2. Effects of NaCN (■) or NaN_3 (●) on $\Delta F\%$. Results represent the mean value of three independent experiments and are expressed as $\Delta F\% = (\Delta F/\Delta F_M) \times 100$. Determinations were performed at the steady level reached at the equilibrium of the dye (2 μM) into cells and after addition of variable NaCN or NaN_3 concentrations (ΔF) or obtained after the addition of 5 μM FCCP (ΔF_M).

to 70% (Fig. 3, upper trace) whereas, when it was used alone, it had no effect (Fig. 3, lower trace). The addition of NaN_3 should deprive the cells of ATP and thus plasmalemma H^+-ATPase activities should decrease. The magnitude of the fluorescence increase obtained with NaN_3 (80%) was greater than that obtained with NaCN (30%) (Fig. 2). This result is consistent with the importance of mitochondrial metabolism for the maintenance of the transmembrane potential and may explain the smaller amount of uncoupler required in our experiments. Moreover, the effect of NaCN is emphasised by the addition of SHAM (Fig. 3), suggesting that the alternative oxidase pathway is involved in the NaCN insensitive potential component.

The effects of DCCD and SW26 have also been studied. The first is known to block the H^+-channel of H^+-ATPases [21], and the second, which is a carbanilate derivative, has been reported as a specific inhibitor of plasma membrane H^+-ATPase [23, 24]. We previously verified that H^+-ATPase activity of the plasma membrane, prepared from tobacco leaves, was not significantly inhibited by cyanine dye added in the assay medium (95±2% of the control at 8 μM).

DCCD led to the same increase in fluorescence as FCCP ($\Delta F\%$ of about 100%, at 200 μM DCCD, Fig. 4) which indicates a complete dissipation of the transmembrane potential of tobacco cells. SW26, the other H^+-ATPase inhibitor assayed, abolished the transmembrane potential only partially ($\Delta F\%$ of about 70%, at 50 μM SW26, Fig. 4). These results suggest that the effect of these two plasma membrane H^+-ATPase inhibitors on tobacco cells is different. DCCD inhibits the plasma membrane H^+-ATPase and the synthesis of ATP in mitochondria so that all ATP-dependent processes are restrained. SW26 is a specific inhibitor of the plasma membrane ATPase and it does not act on the mitochondrial ATPase [23]. In this case, the activity of all ATP-dependent processes except the plasma membrane H^+-ATPase could be maintained.

This method was then extended to the analysis of the effects of beticolin-1 and -2, two fungal toxins produced by *C. beticola*. Their interaction with tobacco cells was studied, following their fluorescence in the presence of cells (Fig. 5, insert). The slow increase in fluorescence was not significantly different for the two beticolins, indicating that their interactions with cells were similar. It has been verified that the intrinsic fluorescence of beticolins did not interfere with the dye fluorescence measurements (data not shown). Modifications in fluorescence of the cyanine dye induced by cell-beticolins interactions have also been performed (Fig. 5). The dose-dependent fluorescence curve showed that $\Delta F\%$ did not exceed 20%, even at high concentration (7 μM) for beticolin-1, whereas it reached 50% with 3 μM beticolin-2.

These results suggest that beticolin-1 and -2 partially abolished the transmembrane potential, the efficacy on its dissipation by beticolin-2 and by SW26 being

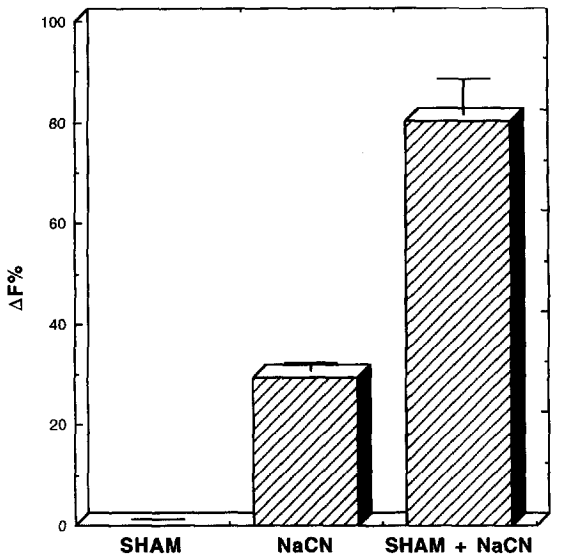

Fig. 3. Effect of SHAM on $\Delta F\%$. Experimental conditions were described in Fig. 2. NaCN 1.5 mM (three repetitions), SHAM 10 mM (two repetitions), NaCN 1.5 mM + SHAM 10 mM (two repetitions), FCCP 5 μM.

similar, and better than that of beticolin-1. This is consistent with the recent demonstration of the inhibition of plasma membrane H^+-ATPase by these toxins [15] and which demonstrated that beticolin-2 was more active than beticolin-1 (I_{50} were 0.6 μM and 6.7 μM, respectively).

Finally, since the kinetics of association of beticolin-1 and -2 with the cells were very similar, it is probable that the partial depolarization of the plasma membrane after the addition of beticolins is rather due to the inhibition of plasma membrane H^+-ATPase than to a perturbation of the plasma membrane itself. The inhibition of ATPases and the resulting decrease in the plasma membrane potential caused by beticolins could explain the toxicity of these molecules.

The plasma membrane potential is a result of the proton translocation catalysed by the ATPase and of the other energy-dependent transport processes across this membrane. The fluorescence changes of [diS-C_2-(5)] after the addition of respiratory inhibitors, ATPase inhibitors or uncouplers show that this method could be used to measure the potential changes in plasma membrane of tobacco cells. The effectors lowering the pool of ATP in cells as well as ionophores, which depolarize membranes, reduced the magnitude of the transmembrane potential measured with [diS-C_2-(5)].

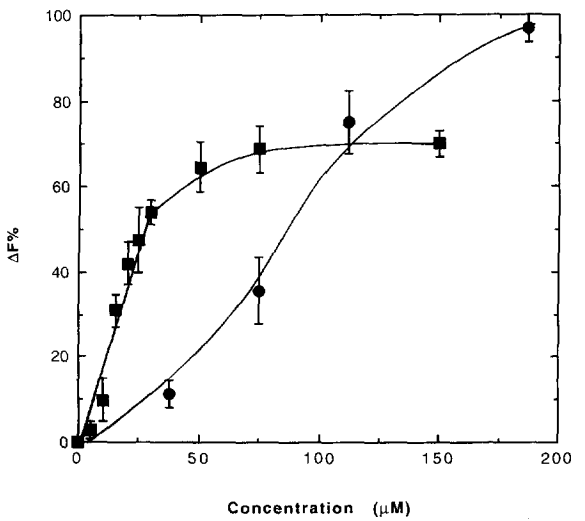

Fig. 4. Effect of DCCD (●) and SW26 (■) on $\Delta F\%$. Results represent the mean value of three independent experiments and are expressed as $\Delta F\% = (\Delta F/\Delta F_M) \times 100$. Determinations were performed at the steady level reached at the equilibrium of the dye (2 μM) into cells and after addition of variable DCCD or SW26 concentrations (ΔF) or obtained after the addition of 5 μM FCCP (ΔF_M).

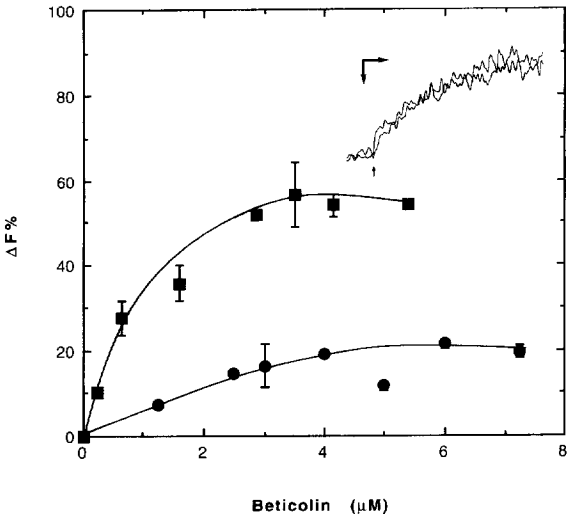

Fig. 5. Effect of beticolin-1 (●) and beticolin-2 (■) on $\Delta F\%$. Results represent the mean value of three independent experiments and are expressed as $\Delta F\% = (\Delta F/\Delta F_M) \times 100$. Determinations were performed at the steady level reached at the equilibrium of the dye (2 μM) into cells and after addition of variable beticolin concentrations (ΔF) or obtained after the addition of 5 μM FCCP (ΔF_M). Insert: time course of beticolin-1 and -2 fluorescence in the presence of tobacco cells, Ex 345 nm, Em 500 nm. Arrow indicates the addition of beticolin. Fluorescence in arbitrary units and time in min.

Therefore, this method seems to be efficient to determine the effect of fungal toxin on plant cell membranes, as shown with beticolins, and could provide a useful tool in plant–pathogen interaction studies.

EXPERIMENTAL

Chemicals. SW26 was a gift from Pr. J.-P. Calmon (ENSA Toulouse, France). The [diS-C_2-(5)], SHAM and FCCP were purchased from Sigma. DCCD and valinomycin were obtained from Aldrich, NaCN and NaN_3 from Merck. All chemicals were dissolved in EtOH except SW26 which was dissolved in DMSO, and NaCN and NaN_3 in H_2O. Beticolin-1 and beticolin-2 were isolated from the mycelium of a *C. beticola* strain (CM) as described in ref. [11].

Cell suspension cultures. Chlorophyll-free tobacco cell suspensions (*Nicotiana tabacum*, var. Xanthi) were grown in Chandler's medium [25] on a rotatory shaker (125 rpm, 25° in darkness).

Measurements of fluorescence. Cells in exponential growth phase were filtered and washed 3× with a buffer (10 ml) containing 0.14 M mannitol, 20 mM Mes/Tris pH 6.5, 0.5 mM K_2SO_4, 0.5 mM $CaCl_2$, 0.5 mM NaCl. Cells resuspended in this buffer (50 mg fresh wt ml^{-1} were placed on a shaker (150 rpm) and preincubated for 30 min at 25°. Just before fluorescence measurement, 2 ml aliquots of suspension cells were put into a stirred fluorometric cuvette of a Shimadzu RF 5001 PC spectrofluorimeter at 25°. The fluorescence probe [diS-C_2-(5)] was then added, its fluorescence being measured with an excitation wavelength set at 650 nm and an emission wavelength set at 670 nm. In order to assess interaction of beticolins with cells, their fluorescence variations in the presence of cells were observed according to [26]. All results represent the mean value of three independent experiments and are expressed as $\Delta F\% = (\Delta F/\Delta F_M) \times 100$. Determinations were performed at the steady level reached at the equilibrium of the dye into cells and after addition of the chemical assayed (ΔF) or obtained after the addition of FCCP (ΔF_M).

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