Pore-forming properties of elicitors of plant defense reactions and cellulolytic enzymes

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Abstract Using the planar lipid bilayer technique, it is shown that a yeast elicitor as well as several cellulolytic enzymes used in protoplasting plant cells contain components which strongly interact with the bilayers. This results in the appearance of transmembrane ion fluxes which may pass through membrane defect structures and even large conductance pores with unitary conductances above 400 pS. Since membrane depolarization is an immediate response in the process of defense elicitation in plant cells, elicitors may act directly with the lipid phase of cell membranes, causing depolarizations and thus initiating the process of elicitation. When using enzymatically prepared protoplasts in electrophysiological work, contributions to electrical activity by membrane active constituents originating from the enzymes used must be expected.

Key words: Cellulase; Ion channel; Pectolyase; Planar lipid bilayer; Yeast elicitor

1. Introduction

Elicitors are compounds that induce defense reactions in plants. A wide range of abiotic and biotic elicitors, including glycoproteins, peptides, fungal steroids and oligoglycans, detergents and heavy metals, have become known [1–3]. Studies of the modes and mechanisms of action of elicitors are actively pursued. While some appear to be relatively host-selective, many elicitors act on diverse species, e.g. the abiotic elicitors, but also biotic elicitors such as yeast elicitor which is used widely to study the process of elicitation in plant cell cultures [4,5].

The earliest detectable response of a plant cell challenged with an elicitor is induced ion currents, mainly an efflux of K^+ and Cl^-, an influx of Ca^{2+}, cellular acidification and apoplastic alkalinization [6–9]. Membrane-depolarizing agents have been shown to elicit strongly, and elicitors to evoke the efflux of Cl^- and other anions from plant cells [4,10–12].

The primary sites of elicitor action are mostly unknown. Elicitor responses are assumed to be mediated by membrane receptors [2,3,9,13], especially in the light of the stringent structure-activity relationships shown to exist for some elicitors [14,15], and high-affinity elicitor-binding sites have been identified in some cases [14–20], but only one elicitor-binding protein, the β-glucan elicitor-binding protein from soybean, has been purified [21,22] and recently cloned [23]. It is not yet known, however, if this binding protein is the elicitor receptor.

While receptor-mediated activation of ion channels appears to be one mechanism of elicitor action [9], it may not be the only one and alternatives, especially to understand the activity of the numerous non-host-specific elicitors, have to be considered. In this paper, we show that elicitors may directly interact with lipid bilayers, forming pores or bilayer discontinuities that are highly conductive for ions. We further demonstrate that cellulolytic enzyme preparations from fungal sources, some of which have earlier been shown to elicit plant defense [18,24], contain components able to induce a broad variety of ion conduction structures in lipid bilayers. Elicitors thus may not require receptor-based mechanisms in order to be active. Our findings have implications for electrophysiological work on protoplasts prepared using cellulolytic enzymes.

2. Materials and methods

Cell suspension cultures of *Eschscholtzia californica*, a kind gift of Prof. Dr. M.H. Zenk, Munich, were grown in LS medium [25] on a gyratory shaker (100 rpm) at 24°C in continuous dim light (3.8 μmol photons/m^2 s) in a 7 day growth cycle. Cell cultures 2 days after inoculation at the beginning of the logarithmic growth phase were used for all experiments.

Yeast elicitor was prepared according to [4]. Briefly, 1 kg of commercial baker's yeast (*Saccharomyces cerevisiae*) was dissolved in 1.5 l of sodium citrate buffer (20 mM, pH 7.0) and autoclaved at 121°C and 1.1 bar for 60 min. The autoclaved suspension was centrifuged at $10\,000 \times g$ for 20 min. The resulting supernatant was mixed with 1 volume of ethanol and stirred gently overnight. The resulting precipitate was then centrifuged at $10\,000 \times g$ for 20 min. The supernatant of this centrifugation step was subjected to another ethanol precipitation overnight. The precipitate was lyophilized and stored at −20°C until use as an elicitor. Commercial sources of protoplasting enzymes were hemicellulase from *Aspergillus niger* (Sigma, Deisenhofen, Germany), pectolyase Y-23 from *Aspergillus japonicus* (ICN Biochemicals Inc., Aurora, OH, USA) and cellulase Onozuka RS from *Trichoderma viride* (Yakult Honsha Co., Tokyo, Japan).

Cell cultures of *E. californica* were incubated with different test substances under sterile conditions for 1 day under the growth conditions described above. After this incubation period, alkaloids were extracted as described [8]. Briefly, 1 ml of the cell suspension was mixed with 1 ml ethanol containing 1% (v/v) HCl and extracted for 30 min at 40°C. The samples were then centrifuged for 10 min at 5000 rpm and 1 ml aliquots of the extracts were used for the following determinations. Total extracted aromatics were determined photometrically at a wavelength of 280 nm [4]. Benzophenanthridine alkaloids were determined by fluorescence measurements (λ_{ex} 460 nm, λ_{em} 570 nm) [8] using an AB-2 Luminescence spectrometer (SLM Aminco, Rochester, NY, USA).

Electrophysiological experiments were carried out exactly as described previously [26–28]. Planar lipid bilayers were prepared from a solution of 80 parts (w/w) 1-palmitoyl-2-oleoyl-glycero-3-phosphatidylcholine and 20 parts (w/w) 1,2-dioleoyl-glycero-3-phosphatidylethanolamine (Avanti Polar Lipids Inc., Alabaster, AL, USA) dissolved in *n*-decane (15 mg/ml). We used self-made Perspex cuvettes, the hole on which the bilayer was painted being 0.1 mm in diameter. We define the *trans*-compartment of the cuvette to be at ground potential. The sign of the membrane voltage therefore refers to the *cis*-

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compartment, and a positive current (upward deflection in the figures) corresponds to a cation transfer from the *cis*- to the *trans*-compartment or an anion transfer from the *trans*- to the *cis*-compartment. Test substances were always added to the *cis*-compartment. We used a BLM-120 membrane amplifier (Biologic, Echirolles, France) with a low-pass, linearized, five-pole Tchebicheff filter in all our experiments. Current signals were filtered at corner frequencies between 3 and 10 kHz and recorded continuously on a digital tape recorder (Model DTR-1204, Biologic). For computer analysis on a Power Macintosh 4400/200, the recorded signals were digitized with a sample rate of 10 kHz using an ITC-16 (Instrutech Inc., New York, USA) computer interface and Pulse software (Heka Electronic, Lambrecht/Pfalz, Germany).

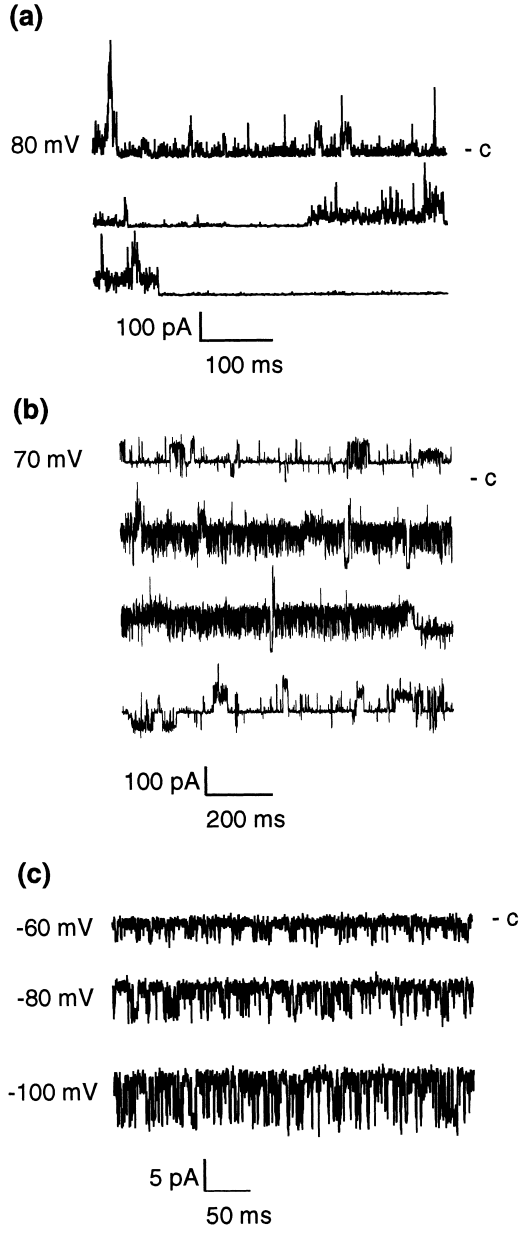

Fig. 1. Effect of yeast elicitor (10 μg/ml) on planar lipid bilayers. Parts a, b and c are characteristic for the variety of effects observed after the addition of yeast elicitor to the aqueous solution of the *cis*-compartment. Shown are the results of three different experiments. Experimental conditions: electrolyte solution 1 M KCl, 10 mM HEPES, pH 7.0. c, closed level (deflections from this level in either direction represent currents passing across the membrane).

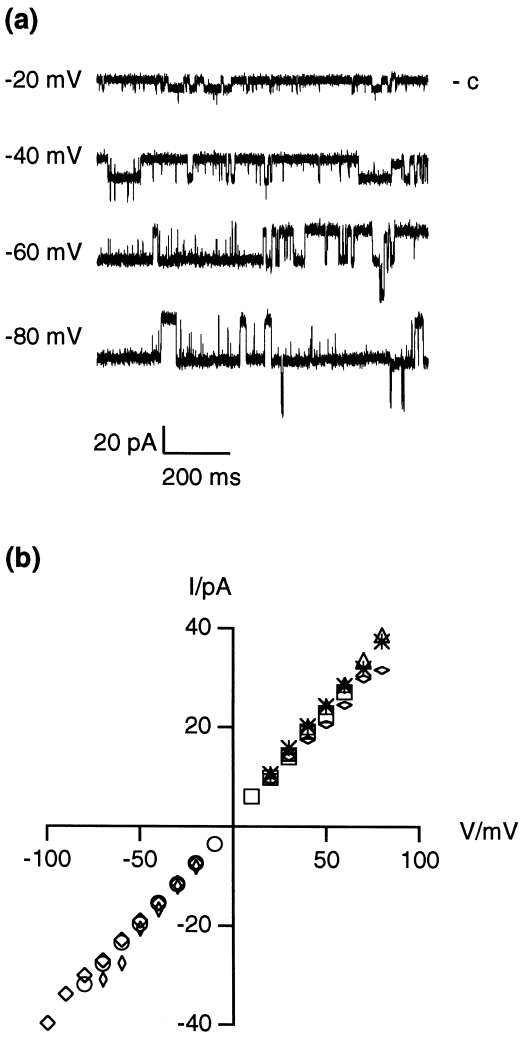

Fig. 2. Single-channel current fluctuations (a) and current-voltage relationship (b) of a large-conductance ion channel formed in the bilayers after the addition of yeast elicitor (10 μg/ml). The I/V curve (b) depicts the data of seven independent experiments. From the fitting of the data points, a single-channel conductance of $\Lambda = 418$ pS was determined. Experimental conditions are the same as in Fig. 1.

3. Results and discussion

It has been demonstrated several times that plant cells are depolarized in reaction to elicitor treatment, this being attributed to an activation of endogenous ion channels by the elicitor [9,12,24]. However, by testing several elicitors in the protein-free lipid bilayer system, we could show that some elicitors by themselves have pore-forming properties, therefore allowing ions to permeate the membrane without the necessity of receptor binding and ion channel activation. Figs. 1 and 2 give examples of current fluctuation patterns, observed after the addition of low concentrations of yeast elicitor [4,5] to planar lipid bilayers. The elicitor concentrations used (10 μg/ml) were those reported to result in elicitation in a wide range of plant tissues [4,5] and allowed to discriminate discrete current fluctuations in the bilayer preparations. Only effects that were seen regularly (i.e. in at least 50% of all bilayers) are shown. With increasing doses of elicitor, the frequency and complexity of the observed events increased,

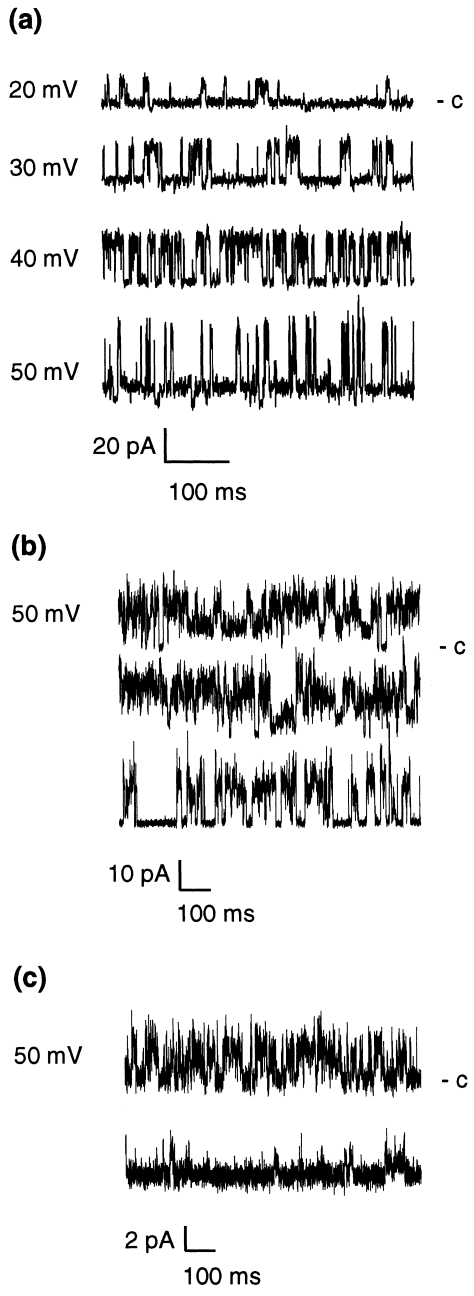

Fig. 3. Membrane activity of protoplasting enzyme solutions. Shown are the effects of (a) hemicellulase from *Aspergillus niger*, (b) cellulase from *Trichoderma viride* and (c) pectin lyase from *Aspergillus japonicus*, on planar lipid bilayers. Experimental conditions: a: electrolyte solution: 1 M KCl, 10 mM HEPES, pH 7.0, protein concentration: 15 μg/ml; b,c: electrolyte solution: 100 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.0, protein concentration: 15 μg/ml.

leading eventually to bilayer instabilities. Leak currents of the lipid bilayers were negligible. The level marked 'c' (closed) in all figures represents zero current at the sensitivity level of our analysis.

We observed a variety of different effects, some typical situations being represented by the current traces shown (Figs. 1a–c and 2a). These ranged from bursts of flickers (Fig. 1a) to long continuous open states superimposed by rapid flicker (Fig. 1b), continuous series of rapid successions of opening and closing events (Fig. 1c) to clear, channel-like, switching between open and closed states (Fig. 2a). In most of the cases, quantification was precluded by the variability of the events observed, which we trace back to several different, membrane-active and presumably interacting, components present in the elicitor preparation. Some of the currents seen may arise from membrane defect structures caused by components in the elicitor preparation, while pore formation is suggested in events as shown in Fig. 2a. In this case, the current-voltage relationship (Fig. 2b) reveals an ion-conducting pore that does not rectify (i.e. conducts ions in both directions) and exhibits a very large unitary conductance of 418 pS. At present, it is not known if the pore conducts cations and/or anions. In any case, such pores, when formed in the plant plasma membrane, would rapidly depolarize the membrane potential and thus could elicit the cell's defense without having to interact with cellular proteins. This might explain the broad activity of the yeast elicitor in very many diverse plant species [29].

As it has been reported that protoplasting enzymes derived from fungi, like pectin lyase from *Aspergillus japonicus*, elicit

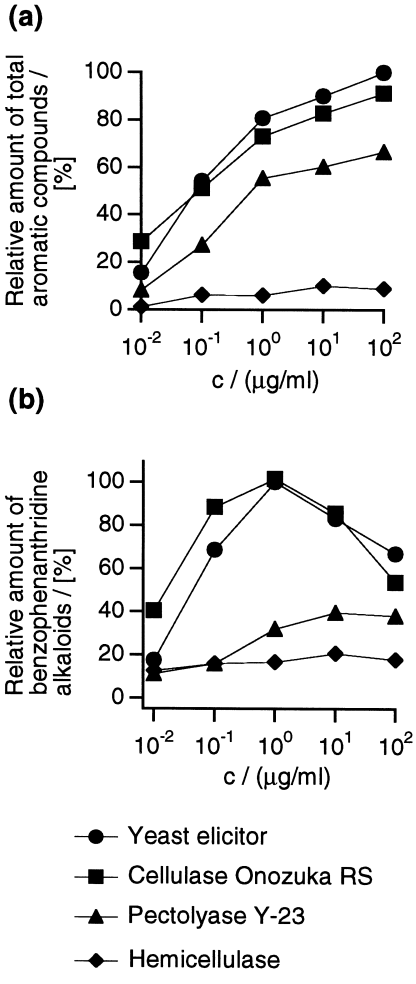

Fig. 4. Accumulation of total aromatic compounds (a) and of benzophenanthridine alkaloids (b) in *E. californica* cell cultures treated with different concentrations of yeast elicitor or protoplasting enzymes. a: Absorption at $\lambda = 280$ nm. b: Fluorescence emission at $\lambda_{em} = 570$ nm ($\lambda_{ex} = 460$ nm). Shown are the mean values of $n = 7$ experiments (a) or $n = 3$ experiments (b), normalized for the maximum effect observed for yeast elicitor (set at 100%).

and depolarize plant cell cultures [12], we next tested several protoplasting enzymes on planar lipid bilayers. Representative examples of these experiments are given in Fig. 3. We again observed a variety of effects, suggesting a number of different factors to be involved in each preparation. However, it is obvious that hemicellulase from *Aspergillus niger*, cellulase from *Trichoderma viride* and pectin lyase from *Aspergillus japonicus* all produce strong current fluctuations in planar lipid bilayers, again either by pore formation and/or by causing membrane defect structures. A large number of fungi are known for the production of pore-forming peptides, for example alamethicin, a model pore in electrophysiology [30,31], which is produced by the ascomycete *T. viride* [32]. However, the current fluctuations we recorded when using the *T. viride* cellulase do not resemble those produced by pure alamethicin. Some, but not all the enzyme preparations, which induce current fluctuations in artificial lipid membranes, also acted as elicitors in a plant cell culture of *E. californica*. This cell suspension culture accumulates benzophenanthridine alkaloids in response to a range of different elicitors [4]. As a second parameter, the accumulation of aromatic compounds – usually associated with strong elicitation [33] – was monitored. Fig. 4 shows the kinetics of accumulation of total extractable aromatic compounds and of benzophenanthridine alkaloids in *E. californica* cell cultures treated with yeast elicitor and protoplasting enzymes at very low concentrations. Whereas cellulase from *T. viride* and pectin lyase from *A. japonicus* showed a strong elicitor activity, the hemicellulase preparation from *A. niger* did not elicit at all. Whether in this case the pore-forming substance(s) cannot permeate the cell wall or whether pore formation alone is not sufficient to induce elicitation remains to be elucidated. Our results are in agreement with earlier observations that cyclic peptide antibiotics such as amphothericin B and nystatin are strong elicitors [4]. These peptides are well-known for forming large-conductance pores in cellular membranes [34].

4. Conclusions

In addition to receptor-mediated processes, direct interactions of elicitors of plant defenses with the cellular membranes, leading to depolarizing transmembrane ion currents, must be taken into consideration as primary mechanisms of elicitor action. This hypothesis appears especially attractive in order to understand the action of broad-range elicitors, but it may also further our understanding of abiotic elicitor action, e.g. the elicitor activity of detergents or heavy metals. More detailed experiments are needed in this field, and the planar lipid bilayer technique may prove helpful to guide the isolation of the active principles from the preparations studied here. The technique will also be of great value when studying membrane activities of pure protein and peptide elicitors, as it allows electrophysiological assays to be conducted in the absence of any cellular proteins. In the long run, this technique will prove germane to assay the interactions of the host-specific elicitors with their protein targets, especially if they are ion channels.

Finally, our results show that one has to be cautious in interpreting current fluctuations seen after elicitor treatment of plant cells as solely representing endogenous channels. There is also a risk of contamination with fungal pore-forming components in patch-clamp experiments, when enzymatically produced protoplasts are used.

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