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Dielectric Properties of the Plasma Membrane of Cultured Murine Fibroblasts Treated with a Nonterpenoid Extract of *Azadirachta indica* Seeds

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Abstract Neem oil is a natural product obtained from the seeds of the tree *Azadirachta indica*. In this report, we investigate the alterations of the biophysical properties of the plasma membrane caused by treatment with the nonterpenoid fraction of neem oil that we defined as methanolic extract (MEX). The dose-response effect was evaluated and a MEX-dependent cytoxicity evidenced. The effect of MEX on the plasma membrane was studied by a well-established dielectric spectroscopy technique: electrorotation, which allows single-cell analysis. Our results show a structural/functional alteration of the plasma membrane with an evident increase of specific capacitance and conductance. The biological implications of this effect are discussed.

Keywords Neem oil · Dose-response effect · Electrorotation · Membrane structure/function

Introduction

Neem oil exhibits strong cytotoxic activity as well as antibiotic and antiviral properties. Moreover, a possible use as an antitumor drug has been envisaged (Schmutterer 1990; Connell 1991; Mulla and Su 1999; Brahmachari 2004). This oil is constituted by a complex mixture of substances obtained from the seeds of *Azadirachta indica* (A. Juss, neem tree). Ayurvedic medicine uses decoctions and infusions of seeds, leaves and roots which show antipyretic, antiseptic, antiviral and putative antitumor properties. The natural insecticidal properties of neem extracts are currently exploited in agriculture for pest control since these extracts affect feeding, growth, reproduction and metamorphosis of many insect species (Cohen, Quistad and Casida 1996; Di Ilio et al. 1999).

Azadirachtin, a tetranor-triterpenoid molecule, is the best-characterized component of neem extracts and a potent inhibitor of insect cell proliferation. Its effect on mammalian cells is a matter of dispute. Other terpenoids, similar in structure to azadirachtin, are able to inhibit the growth of mammalian cells to a lesser degree (Rembold and Annadurai 1993; Akudugu, Gäde and Bohm 2001; Salehzadeh et al. 2002). Some authors attribute the overall biological effects to azadirachtin; but the high variability of content and the complex formulation of neem commercial preparations do not allow definitive conclusions, and results are conflicting (Sidhu, Kumar and Behl 2003).

In our laboratory, we have studied the biological activity of different natural compounds (Iacoangeli, Melucci-Vigo and Risuleo 2000; Campanella et al. 2002) and, in a recent work, the effects of these neem oil components in cultured mouse fibroblasts (Di Ilio et al. 2006). We observed a cytotoxic effect induced by application of a methanolic oil extract (MEX). Our results suggest that MEX might cause cell death via the apoptotic pathway, though the mode of apoptosis is yet to be elucidated.

This report is focused on the structural/functional alterations of the plasma membrane consequent to treatment with

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MEX and studied by the spectroscopic technique known as electrorotation (Arnold and Zimmermann 1982; Mischel, Voss and Pohl 1982; Gimsa et al. 1991; Gimsa 2001). This powerful technique allows single-cell examination, leading to evaluation of the dielectric parameters of the plasma membrane: specific capacitance (C) and specific conductance (G). The alteration of these parameters is suggestive of a change in the plasma membrane structure and/or function.

Materials and Methods

Cell Cultures, Treatments with MEX and Cytotoxicity Assays

Cultures of murine fibroblasts (3T6 cell line) were routinely maintained at 37°C in 5% carbon dioxide atmosphere in Dulbecco's modified Eagle medium supplemented with 10% newborn serum, glutamine 50 mM final concentration (f.C.) and penicillin-streptomycin (10^3 U/ml each).

MEX (*see below*) was assayed by administration (5 mg/ml) to cells plated 24 h earlier. Treatments were continued for 24 h in the presence of 2% ethanol (f.c.) that was added also to controls. Each treatment group was in triplicate.

Aflatoxin-free neem oil and pure azadirachtin (99.8% purity grade) were kindly provided by Trifolio-M (Lahnau, Germany). Each batch of oil (10 ml) was extracted six times with a double volume of methanol (120 ml total extraction volume). The methanol-extracted fraction was dried at low temperature under vacuum and produced 1.2 mg of solid sediment (average yield 12%). The pellet was redissolved in ethanol at a stock concentration of 100 mg/ml. The stock solution of MEX was treated, prior to use, at 90°C for 24 h to destroy thermally unstable terpenoids. The residuum was redissolved as above to obtain a stock solution at the same concentration. In cytotoxicity experiments, MEX was further diluted at test concentrations of 40, 20, 10 and 5 mg/ml.

Prior to each measurement, cells were trypsinized, washed twice in phosphate-buffered saline without calcium and magnesium (PBSA) and finally resuspended in an osmolar solution (0.3 M sucrose). The conductivity of the solvent was varied by adding NaCl at different concentrations (0.5, 1.0 and 1.5 mM).

To assess the cytotoxic activity of MEX, 3T6 fibroblasts (5×10^4) were seeded in a 24-well plate. After 24 h, cells were treated to final concentrations of MEX of 0.8, 0.4, 0.2 and 0.1 mg/ml. Controls contained 2% ethanol. Treatment continued for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983) was performed to establish the viability of cells. Pure azadirachtin was administered to 3T6 fibroblasts at concentrations of 0.05 and 0.1 mg/ml. Cell viability was estimated after 24 and 48 h by vital staining with trypan blue. The azadirachtin concentration was chosen considering that in the methanolic extracts of neem oil the presence of this limonoid varies from 0.1 to 0.3 μg/ml (A. S. van der Esch, personal communication).

Electrorotation Apparatus

We used a standard apparatus for electrorotation, working in the frequency range of 100 Hz–500 kHz. In this interval it is possible to monitor only the relaxation at the solvent/plasma membrane interface.

The rotating electrical field was generated by superimposing four square waves out of phase by 90° with respect to each other. The square pulses were applied to the copper miniplate electrodes of the measuring cell, which form a central circular cavity with a volume of about 10 μl (Fig. 1). The whole setup was glued onto a microscope slide. A 33x objective (focal length 1 cm) was used, and the microscope was connected to a charge-coupled device camera, producing a further magnification (20×). The rotating cells were observed via a monitor where they appeared with a diameter of about 1 cm. At each session, the rotation period of 20 cells was considered.

The whole setup was constructed according to the project developed by Jan Gimsa (University of Rostock, Rostock, Germany).

Results and Discussion

Cytotoxicity Assay

The bioactivity of MEX is strongly concentration-dependent (Fig. 2), and a significant difference between control and treated samples was detected. The ethanol present in all samples was nontoxic in our experimental conditions (*not shown;* Di Ilio et al. 2006). Concentrations lower than 0.1 mg/ml had no effect on cell viability. These results suggest an activity threshold between 0.1 and 0.2 mg/ml. The calculated 50% inhibitory concentration (IC_{50}) was

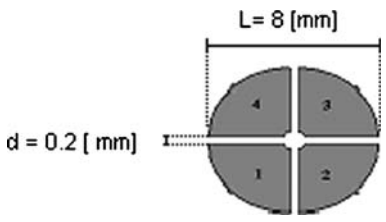

Fig. 1 Schematic diagram of the four-electrode measuring cell. Square waves out of phase by 90° generate the rotating electrical field. The copper miniplate electrodes form a central circular cavity of about 10 μl, and the whole setup is glued onto a microscope slide

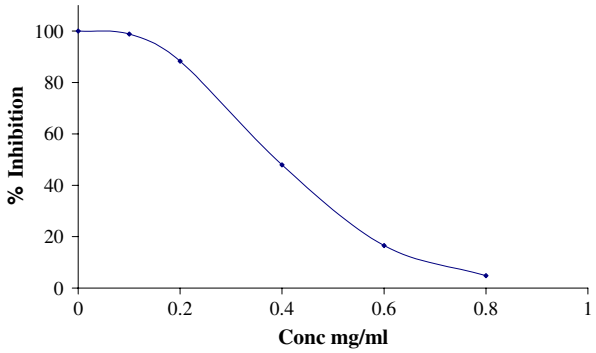

Fig. 2 Decrease of cell survival as a function of increasing concentrations of MEX. On the *y* axis is reported the actual number viable cells

Table 1 Cell viability after treatment with two concentrations of azadirachtin

	Cell viability (%)	
	24 h ± SD	48 h ± SD
Control (EtOH only)	99 ± 5	100 ± 7
AZA 50 μg/ml	95 ± 9	87 ± 8
AZA 100 μg/ml	102 ± 7	108 ± 6

SD, standard deviation; EtOH, ethanol; AZA, azadirachtin

0.39 after treatment with MEX. Pure azadirachtin did not affect cell viability in our experimental conditions (Table 1). This result demonstrates that the cytotoxic effect is actually due to the terpenoid-free MEX and not to putative terpenoid contaminants such as the limonoid azadirachtin. This is not in contrast with data showing that this compound is toxic to cultured insect cells, but mammalian cells exhibit an intrinsically higher resistance to azadirachtin (Salehzadeh et al. 2002).

Electrorotation: Theory

The application of an electric field to a polydispersed cell suspension induces a dipole moment due to the accumulation of charges at the plasma membrane/solvent interface. When the mechanism of interfacial polarization relaxes, a phase shift between the electric field and the induced dipole moment occurs. As a consequence, a torque moment is generated. The rotation period of the cell depends upon the frequency of the applied field according to the following formula (considering the single shell model):

$$T\ (f) = T_{\min} \frac{1 + (\frac{f}{f*})^2}{2(\frac{f}{f*})} \tag{1}$$

where f is the frequency of the applied field, f^* is the relaxation frequency and $T_{\min}$ is the value of the rotation period at the relaxation frequency. The value of f^* depends on the solvent conductivity according to the following formula:

$$f* = \frac{1}{2\pi rC}\left[\frac{1}{\frac{1}{\sigma_e} + \frac{1}{\sigma_i}} + rG\right] \tag{2}$$

where C and G are, respectively, the specific capacitance and conductance of the cell plasma membrane with a radius r, σ_e is the solvent conductivity and σ_i is the conductivity of the cytoplasm considered as homogeneous. Since $\sigma_e << \sigma_i$, equation 2 becomes

$$f* = \left(\frac{1}{\pi r \quad C}\right)\sigma_e + \left(\frac{1}{2\pi \quad C}\right)G \tag{3}$$

An exhaustive overview of the electrorotation theory can be found in the literature (Arnold and Zimmermann 1982; Mischel et al. 1982; Gimsa et al. 1991; Gimsa 2001).

Electrorotation: Experimental

Figure 3A shows the average rotation period plotted *vs.* different rotating field frequencies in untreated control

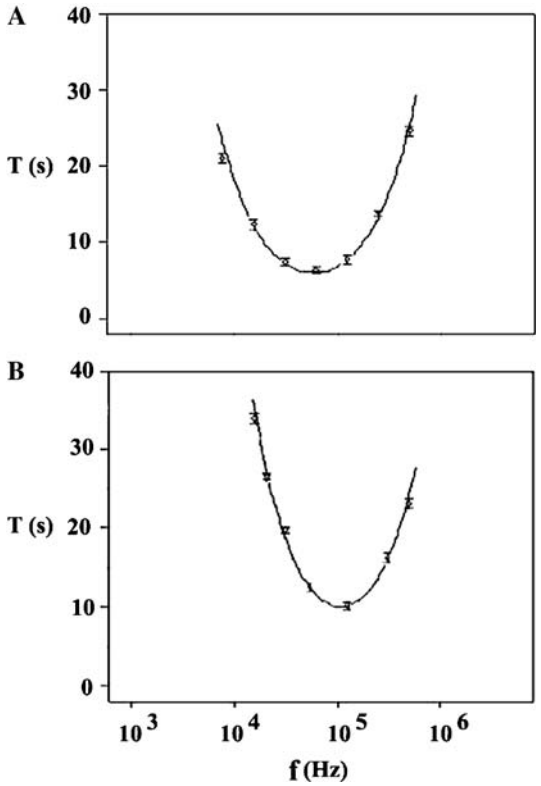

Fig. 3 Average rotation period as a function of the rotating field frequency in untreated control cells (*A*) and cells treated with MEX (*B*). The solvent was salt-free 0.3 M sucrose. *Continuous curves* are the result of the best fit according to equation 1 in the text

cells. These measurements were performed in a salt-free 0.3 M sucrose solution. The experimental data were fitted according to equation 1 to determine the relaxation frequency. Measurements were repeated at different conductivities of the solvent (0.5, 1.0 and 1.5 mM NaCl); the related plots, which are very similar to one another, are not shown for the sake of concision. The relaxation frequencies f^* are reported as a function of the solvent conductivity and, as expected from equation 3, form a straight line (Fig. 4). The average cell radius was estimated on a statistically significant number of cells (above 15 items), and its value was 8.2 ± 0.4 μ. From the size of the cell radius and the linear fit shown in Figure 4, the values of C and G, referred to the control samples, were calculated (Table 2). While the C value was in good agreement with the literature data, an evident discrepancy in the G value exists (Gimsa et al. 1994; Cen et al. 2004). One way to rationalize this difference is that fibroblast cells are measured in a condition of high viability and metabolic activity: this may cause a higher intramembrane ion transport.

Cells treated with MEX at the concentration of 5 mg/ml were investigated by the same experimental approach. The results referring to this set of measurements are reported in Figures 3B and 4. It should be pointed out that cells exposed to MEX showed a decrease of size. The average radius, estimated as above, was 7.3 ± 0.4 μ. The values of C and G calculated for the treated samples are reported in Table 2. It is evident that the treatment caused a significant increase of both parameters, with C and G being, respectively, three- and fivefold higher than in control samples. The higher C value denotes a higher polarizability of the membrane, while the dramatic increase of G strongly suggests a highly enhanced ion permeability. These data are in surprising agreement with previous results, obtained in our laboratory, showing that both infection of these cells with the murine polyomavirus and serum deprivation also stimulated intramembrane ion transport after 24 h of treatment (Bonincontro, Iacoangeli and Risuleo 1996; Bonincontro et al. 1997). Therefore, the alteration of these two parameters may represent a generalized response to cell stress.

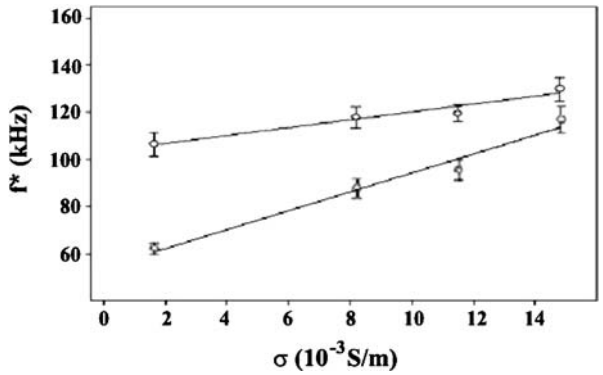

Fig. 4 Relaxation frequency (f^*) as a function of solvent conductivity. *Straight lines* result from the best fit according to equation 3 in the text. *Lower line*, control cells; *upper line*, MEX treated cells

Table 2 C and G values calculated for control and MEX-treated samples (averaged on 20 different cells)

3T6 cells	Specific capacitance (C)	Specific conductance (G)	Average cell radius
Control	0.9 ± 0.2 μF/cm^2	0.3 ± 0.1 S/cm^2	8.2 ± 0.4 μ
MEX-treated (24 h, 5 mg/ml)	2.6 ± 0.6 μF/cm^2	1.7 ± 0.5 S/cm^2	7.3 ± 0.4 μ

Conclusions

Our results show that treatment with MEX is toxic to cells and that the toxicity is due to active principles present in the extract and not to possible terpenoids such as azadirachtin. As a matter of fact, administration of this pure compound has no effect on cell viability. Apoptosis may be the result of cell suffering. This possibility is also suggested by former results from our laboratory and confirmed by the observed cell size reduction, monitored in this work. Size reduction is typical of this cell death mode. The biophysical action of MEX causes a higher membrane polarizability and a highly enhanced ion permeability. The overall conclusion drawn from the dielectric data is that treatment with this derivative of neem oil increases the fluidity of the membrane.

This natural mixture has raised increasing interest for its potential biomedical applications. Results from our laboratory strongly suggest that a partially purified fraction of neem oil plays a key role in the control of cell proliferation. The data presented here are very encouraging and stimulate us to continue studies on this natural plant oil. The final goal is to purify and characterize the component(s) endowed with biomolecular activity.

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