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Effects of lonidamine on the membrane electrical properties of Ehrlich ascites tumor cells

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The effects of lonidamine on membrane electrical properties of Ehrlich ascites tumor cells are investigated Using a dielectric relaxation technique based on the Maxwell-Wagner effect and elaborated by a 'single-shell' fitting procedure, the data indicate that both membrane conductivity and membrane permittivity increase after treatment of these cells with lonidamine while the conductivity of the cytosol remains unchanged Changes in membrane proteins and/or lipids are suggested which lead to altered membrane structure and/or function

Lonidamine, Maxwell-Wagner effect, Membrane property, Ehrlich ascites tumor cell

1 INTRODUCTION

The anti-neoplastic drug, lonidamine, a dichlorinated derivative of indazole-3-carboxylic acid, has been shown to exert a variety of effects on both normal and tumor cells These effects include the impairment of energy metabolism [1,2] and the induction of biochemical and ultrastructural changes in mitochondrial and plasma membranes [3,4]. This last result is of particular importance since the disruption of the cell membrane may also perturb the normal ionic equilibrium of cells and thus their function

The electrical properties of cell membranes can be obtained using a variety of electrophysiological techniques such as the micropipette method or the patch-clamp (using an internal electrode) technique These methods, although widely used, are invasive and are sometimes inappropriate in detecting small variations between untreated controls and their experimental counterparts In addition, these techniques can provide information on only a small number of cells and not on large populations which, in certain cases, need to be studied For these reasons, non-invasive dielectric relaxation studies in the radiowave frequency range can be a useful tool in examining the electrical characteristics of the biological membranes of large populations of cells (10^7 to 10^{10} cells) This technique is based upon the fact that cell membranes separate media of different dielectric properties (for instance, the cytosol from the external medium) which also results in charge accumulation at these interfaces. Thus, the total cell suspension conductivity which shows a marked frequency dispersion (β- dispersion) between 10^4 and 10^8 Hz known as the Maxwell-Wagner effect [5], can be used to determine the characteristics of the membrane by the use of a proper curve-fitting procedure. The electrical properties of the cell membrane (membrane conductivity and membrane permittivity) as well as the conductivity of the cytosol can be extracted from the conductivity measurement of the cell suspension

The aim of the present study was to determine whether lonidamine affects membrane electrical properties of Ehrlich ascites tumor cells by adding this drug to log phase (6 days) and plateau phase (11 days) cells The results obtained using the dielectric method described show that lonidamine does alter membrane electrical properties and that this alteration is more pronounced during plateau phase.

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2 MATERIALS AND METHODS

2 1 *Cells*

Ehrlich ascites tumor cells were grown in 2-month-old male Swiss mice and harvested at the 6th and 11th day after inoculation The cells were collected from the sacrificed animals and suspended in a medium containing a final concentration of 105 mM NaCl, 5 mM KCl, 50 mM TES (pH 7 4) (NKT) The cells were centrifuged at 300 × *g* for 5 min at room temperature and washed three times with NKT medium The packed cells were counted (Coulter Counter ZM, Coulter Electronics, Luton, UK) and resuspended in the same medium at a concentration

of 2×10^8 cells/ml Contamination with other cells, such as leukocytes, did not exceed 0 6%, according to the differential counting of smears stained by the May-Grunwald method The viability of the cells was about 95–98% as indicated by phase-contrast microscopy in the presence of Trypan blue

2 2 *Lonidamine incubation*

The incubations were carried out in 25 ml Erlenmeyer flasks at 30°C in a Dubnoff metabolic shaker The complete reaction medium contained NKT medium and 0 2 ml of cellular suspension (4×10^7 cells) After 10 min of preincubation, lonidamine (dissolved in 0 7% dimethylsulfoxide) to a final concentration of 0 2 mM was added to the cells The controls received the same amount of solvent free of the drug The final volume was always kept at 3 0 ml The incubation lasted 1 h, at the end of which the cells were collected by centrifugation at $500 \times g$ for 5 min at room temperature, washed 3 times with NKT and prepared for either conductivity measurements or cell diameter determination At least 5 separate experiments were conducted

2 3 *Conductivity measurements*

Cells (30×10^7) were resuspended in 1 0 ml NKT buffer (pH 7 4) Conductivity measurements were carried out in the frequency range from 10 kHz to 100 MHz by means of two impedance analyzers Hewlett-Packard model 4192A (frequency range 10 kHz to 10 MHz) and model 4193A (frequency range 0 5 MHz to 100 MHz) The conductivity cell is described in detail elsewhere [6] It consisted of a section of cylindrical waveguide excited well beyond its cut off frequency mode Cell constants were determined by calibration with standard liquids of known conductivity according to Bottomley [7] The measurements were carried out at a temperature of 20°C maintained within 0 1°C Errors in conductivity were estimated within 1% across the frequency range

2 4 *Cell diameter determination*

A Coulter Channelyzer 256 (Coulter Electronics, Luton, UK) was used to evaluate the cell diameter distribution for each experiment of both control and lonidamine-treated cells All Coulter readings on the same sample were repeated at least 5 times The following diameter values were determined $6\,2 \times 10^{-4}$ cm for 6 days control, $6\,4 \times 10^{-4}$ cm for 11 days control, $7\,0 \times 10^{-4}$ cm for 6 days treated and $8\,2 \times 10^{-4}$ cm for 11 days treated The standard deviations were about 3% of the mean values

2 5 *Dielectric model*

The electrical properties of the cell membrane (membrane conductivity σ_S and membrane permittivity ε_S) as well as the conductivity of the cytoplasm σ_P can be extracted from the conductivity of the whole cell suspension as a function of frequency The analysis, similar to the one described by Asami et al. [8], is based on a single-shell model in which the cell is represented as a sphere covered by a shell (the cell membrane) of conductivity $\sigma_S^* = \sigma_S + i\omega\varepsilon_0\varepsilon_S$, a cell interior (cytosol) of conductivity $\sigma_P^* = \sigma_P + i\omega\varepsilon_0\varepsilon_P$, randomly distributed in a continuous medium of complex conductivity described by $\sigma_m^* = \sigma_m + i\omega\varepsilon_0\varepsilon_m$ In these expressions, ε_0 is the permittivity of free space and ω is the angular frequency of the applied field The mean diameter values were determined by Coulter analysis as described previously and used in the fitting procedure The thickness of the cell membrane was taken as 7 5 nm

3. RESULTS AND DISCUSSION

Fig 1 shows electrical conductivity as a function of frequency of suspensions of control Ehrlich ascites tumor cells at 6 days (curve a) and 11 days (curve b) of growth and ascites cells treated with lonidamine at 6 days (curve c) and 11 days (curve d) of growth. The conductivity of the buffer solution is also shown (curve

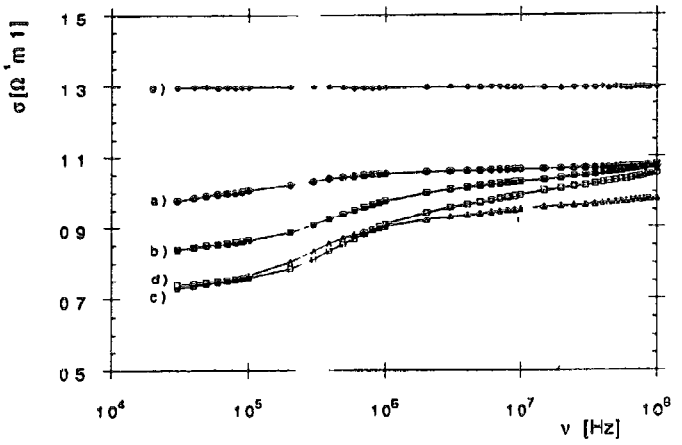

Fig 1 Conductivity of suspensions of Ehrlich ascites tumor cells at 20°C as a function of frequency (a) control cells collected at 6 days, (b) control cells at 11 days, (c) cells treated with lonidamine at 6 days, (d) lonidamine-treated cells at 11 days, and (e) NKT buffer (pH 7 4) The solid lines represent the calculated values derived from the fitting procedure

e) Curve (e) shows that the conductivity of the buffer solution is constant from 10^5 to 10^8 Hz This may be expected since there is no interfacial polarization in this salt solution. In contrast, curves (a)–(d) show the marked conductivity dispersion data typical of interfacial polarization in heterogeneous systems The conductivity increment (from low to high frequencies) was less pronounced in both 6- and 11-day controls (curves a and b) than in cells treated at these two growth times (curves c and d) These results seem to indicate that lonidamine induces variations in membrane properties such as in membrane structure and/or function. This preliminary observation prompted further examination of the differences observed.

Fig 2a gives the means and standard deviations of membrane conductivity σ_S of 6- and 11-day lonidamine-treated Ehrlich ascites tumor cells after the data from Fig 1 had been analyzed by the fitting procedure based upon the 'single-shell' model described previously and normalized with respect to their respective controls (zero line) The zero line (control value) is the mean value of control measurements of cells collected at both 6 and 11 days. As can be seen, both treatment at 6 and 11 days resulted in an increase in membrane conductivity with respect to controls. However, 11-day treatment seems to induce a greater variation in this membrane parameter than lonidamine administration at 6 days (35% vs 67%, respectively) Membrane conductivity is a measure of the overall ionic transport across the cell membrane through ion channels Therefore, conductivity may be viewed as a measure of the dynamic processes dependent upon net ionic fluxes Thus, the higher conductivity value observed after lonidamine treatment seems to indicate an increase in ionic flux across the cell membrane, perhaps by formation and/or activation of membrane channels and/or pumps. It has

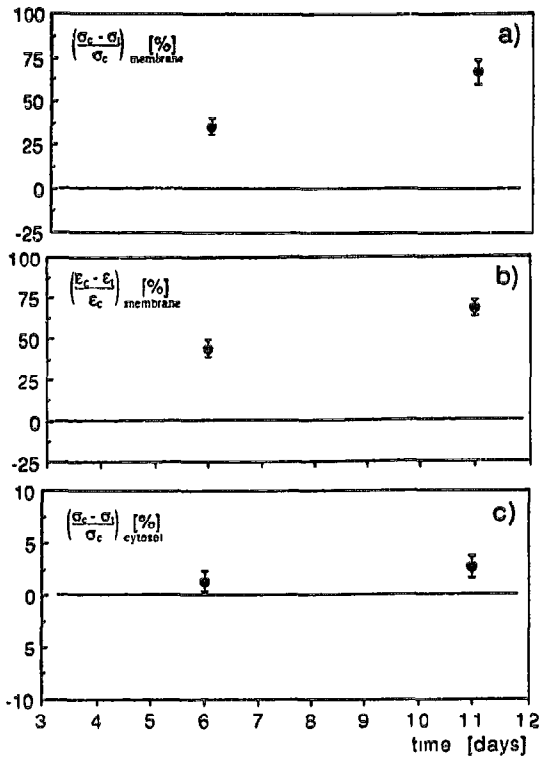

Fig 2 Per cent variation in membrane conductivity (a), membrane permittivity (b) and the conductivity of the cytosol (c) as a function of the day Ehrlich ascites tumor cells were collected (6 and 11 days) are shown All values represent the means and standard deviations of at least 5 separate experiments each normalized with its respective control Note the increased variation of these three membrane parameters in cells collected at 11 days with respect to those collected at 6 days

been previously demonstrated using the same dielectric relaxation method described in this work that variations in membrane conductivity in chick embryo myoblasts may be due to variations in ion channels and/or pumps, particularly in an alteration in K^+ channels and, consequently, the Na^+/K^+ equilibrium [9] More direct evidence for a perturbation of specific ionic pumps (the Na^+,K^+-ATPase) in Ehrlich ascites tumor cells by lonidamine comes from our preliminary measurements of membrane electrical parameters in the presence of ouabain, an inhibitor of Na^+,K^+-ATPase This analysis, although preliminary, does demonstrate a modification of membrane properties upon addition of ouabain. In addition, evidence for a perturbation of the ionic equilibrium itself of Ehrlich ascites tumor cells by lonidamine comes from a recent study in which both cell volume and intracellular Na^+ and K^+ concentration were examined [10]. In this study, it was found that after treatment with this drug, there was an increase in cell volume in these Ehrlich cells due to an augmented uptake of Na^+ and an increased outflow of K^+ These alterations in Na^+/K^+ exchange regulatory mechanisms may explain, at least in part, the variations in membrane conductivity observed in the present study.

A similar behavior in membrane permittivity ε_S also occurs in these cells (Fig 2b) Again, treated cells at both 6 and 11 days have higher normalized permittivity values than controls (zero line). In addition, it is apparent once more that 11-day treatment induces the greatest change in permittivity (43% versus 68%, respectively) Membrane permittivity is a measure of the distribution of charges and/or polar groups across cell membranes Thus, this membrane parameter is indicative of the static charges present in the membrane such as proteins and lipids which appear to be altered by lonidamine. In fact, variations in both of these cell constituents induced by lonidamine have been observed in Ehrlich ascites tumor cells as well as T lymphocytes and human erythrocytes [3,4]. Specifically, freeze-fracture studies demonstrated that lonidamine alters the distribution of intramembrane particles [3] and also the membrane lipid distribution [4] A selective transfer of phosphatidylcholine and cholesterol from the membrane to the incubation medium and the consequent enrichment in phosphatidylethanolamine in the membrane itself was observed [4] Thus, the variations in membrane permittivity induced by lonidamine are supported by observed effects of this drug on cell membrane morphology

Membrane conductivity and membiane permittivity are strongly interrelated and their effects cannot be easily separated For instance, changes in ionic pumps may not only affect conductivity, but may also influence membrane permittivity since any changes in structure of these pumps may also cause variations in fixed membrane charges In addition, phospholipid type and/or charge can interfere with active tiansport processes directly. In fact, the type of phospholipid environment in which ion channels are embedded can vary not only permittivity, but also the transport properties of the channels [11,12] Conductivity is much higher in negatively charged membranes than in neutral ones [11,13].

Finally, in Fig 2c are shown values of the conductivity of the cytosol σ_P of 6- and 11-day treated Ehrlich ascites tumor cells normalized with respect to controls (zero line). As can be seen, there is no variation in this parameter at either 6 or 11 days of lonidamine treatment Apparently, this drug seems not to affect the cytosol of Ehrlich cells at this concentration. However, since the morphological effects discussed are known to be concentration-dependent [3], it cannot be excluded that greater concentrations of this drug wil not affect Ehrlich cell cytosol conductivity Consequently, more exhaustive experiments are in progress in order to determine the role of lonidamine concentration on the cell cytosol as well as on both membrane conductivity and membrane permittivity variations. More detailed experiments are also in progress in order to evaluate the time of the cell cycle when lonidamine administration causes the greatest variations in cell electrical properties.

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