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ELECTROPORATION ENHANCES CELL MEMBRANE PEROXIDATION AND LUMINESCENCE

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Summary: Electroporation is a most popular method of cell membrane permeabilization, by pulsed electric fields. It allows foreign molecules to enter the cell and has been used for many biotechnological applications, including transformation of mammalian cells and plant protoplasts by exogenous genetic material. However, the mechanism underlying membrane electropermeabilization is still largely unknown. Evidence is presented here that electroporation under conditions compatible with cell survival induces lipid hydroperoxide formation in the membranes of animal and plant cells. Exposure to electric fields also enhanced up to 5-fold the spontaneous emission of light from both cell types, which paralleled the amount of conjugated hydroperoxides detected in cell membranes. The emitted photons were mainly in the red edge of the spectrum, suggesting the involvement of singlet oxygen. The presence of antioxidants during electroporation did not reduce the formation of hydroperoxides nor the permeability but quenched the luminescence.

Cells can be permeabilized by exposure to electric fields, a process called electroporation (1). Under optimal conditions, electropermeabilization is compatible with cell survival, providing a direct access of external molecules to the cytosol with several biological applications already available and many more sought (reviewed in Refs 2, 3). Electroporation is a fascinating cell membrane phenomenon, whose molecular mechanism is still poorly understood (1-4). In particular, no information is available on modification of lipid bilayers upon electric shock (1-4), although electroporation-induced ethane production in plant protoplasts (5) might suggest the involvement of lipid peroxidation in cell electropermeabilization.

Spontaneous luminescence (ultraweak light emission) has been observed in almost all living organisms (6). The study of this ultraweak light emission requires single-

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photon-counting systems with cooled photomultipliers, in order to achieve a very high sensitivity (7). This luminescence has been associated with several biochemical pathways like glycolysis in animals (8) and chlororespiration in plants (9). However, the excited states produced in lipid peroxidation of animal (10) and plant (11) membranes appear to be the most common source of luminescence. Because the presence of peroxides is known to induce perturbations in the membrane structure, compatible with cell survival, the possibility that electropermeabilization of mammalian and plant cells might occur through hydroperoxide formation was investigated.

Evidence is presented that in fact electropermeabilization causes hydroperoxide formation and ultraweak light emission in cell membranes. The effect of membrane peroxidation on cell permeability is discussed as well.

MATERIALS AND METHODS

Chemicals used were of the purest analytical grade. Methanol (Merck) was of HPLC quality. Media for plant culture and protoplast isolation were from Sigma, as well as vitamin E (α-tocopherol). Cellulase, pectinase and pectolyase were purchased from Fluka. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was from Janssen.

Cell cultures

Erythroleukemia K562 cells were cultured in RPMI 1640 medium (Gibco BRL) with 10% heat-inactivated fetal bovine serum (Gibco BRL) and were maintained at 37°C and 6% carbon dioxide with biweekly media changes (12). Lentil (*Lens culinaris*) root protoplasts (LRP) were isolated from 6-day-old, dark-grown seedlings after digesting the cell wall with cellulase, pectinase and pectolyase, as reported (13). Primary cultures of walled lentil cells (LC) were produced by squeezing lentil calluses through a 50 mesh stainless steel sieve and culturing isolated cells in hormone-free Murashige-Skoog medium (14).

Electroporation

Electroporation was performed with a Gene Pulser apparatus, equipped with a capacitance extender device (Bio-Rad). Exponentially decaying pulses were generated and delivered to the different cell suspensions in sterile disposable electroporation cuvettes of 0.4 cm path length (Bio-Rad). K562 cells were washed twice in Dulbecco's phosphate-buffered saline (PBS) and were resuspended in PBS at a final concentration of 1.0×10^6/0.8 ml. Aliquots (0.8 ml/test) were electroporated at a resistance of 30 Ω and capacitance and field strength values suitable for transfection experiments (12). Freshly isolated LRP (0.5×10^6 cells/test) were electroporated at a resistance of 12 Ω and a range of capacitance and voltage values suitable for macromolecular transfer, as reported (15). Primary cultures of walled lentil cells (LC) were electroporated in Murashige-Skoog medium (1.0×10^6 cells/test) at 30 Ω and capacitance and voltage values used for transfection experiments (16). Cell permeability was determined by using Trypan blue as already described (17).

Luminescence measurements

Light emission was measured by means of a highly sensitive LUMI-A luminometer (SEAS), described in (18). Briefly, the experimental setup was formed by a reflective chamber, where the samples to be analyzed could be maintained at a constant temperature (24±0.1°C), coupled to a Hamamatsu R1104 photomultiplier. The photocathode (wavelength response 200-850 nm) was cooled down to -15±0.1°C in order to decrease the dark current. The acquisition electronics was interfaced with a M24 personal computer (Olivetti) for data analysis. Luminescence values recorded after passing through ultraviolet (R-UV 370) or red (RG 610) filters were corrected for the transmittance of each filter and the quantum efficiency of the photocathode at each wavelength. Immediately after electroporation, the luminescence of cell suspensions was measured, recording photon emission every 2 s for 4 min. Cells were then disrupted by three cycles of rapid freezing (-80°C) and thawing (+25°C) and protein

concentration of cell extracts was determined (19), using BSA as a standard. Sample luminescence was expressed as counts per s per mg protein (cps/mg P).

Analysis of membrane lipids

Cell membranes were isolated from K562 (20), LRP and LC cells (21), then the membrane lipids were extracted (22) and dissolved in methanol. The overall oxidative modification of membrane lipids was estimated by the oxidative index, *i.e.* the A_{234}/A_{205} ratio (23), recording absorbance values with a UVIKON 860 spectrophotometer (Kontron). The amount of conjugated hydroperoxides present in the samples was calculated by using the molar absorption coefficient of 25000 $M^{-1} \cdot cm^{-1}$ at 234 nm (23).

RESULTS AND DISCUSSION

Electroporation significantly (up to 5 times) increased the ultraweak light emission from both mammalian and plant cells (Fig. 1). The luminescence increase was a function of capacitance and field strength used for electroporation. Duration and intensity of the electric pulse can be varied by capacitance and field strength (1-3). More intense pulses generate a higher transmembrane potential, up to a threshold value where an irreversible breakdown of the membrane structure occurs (4). In this

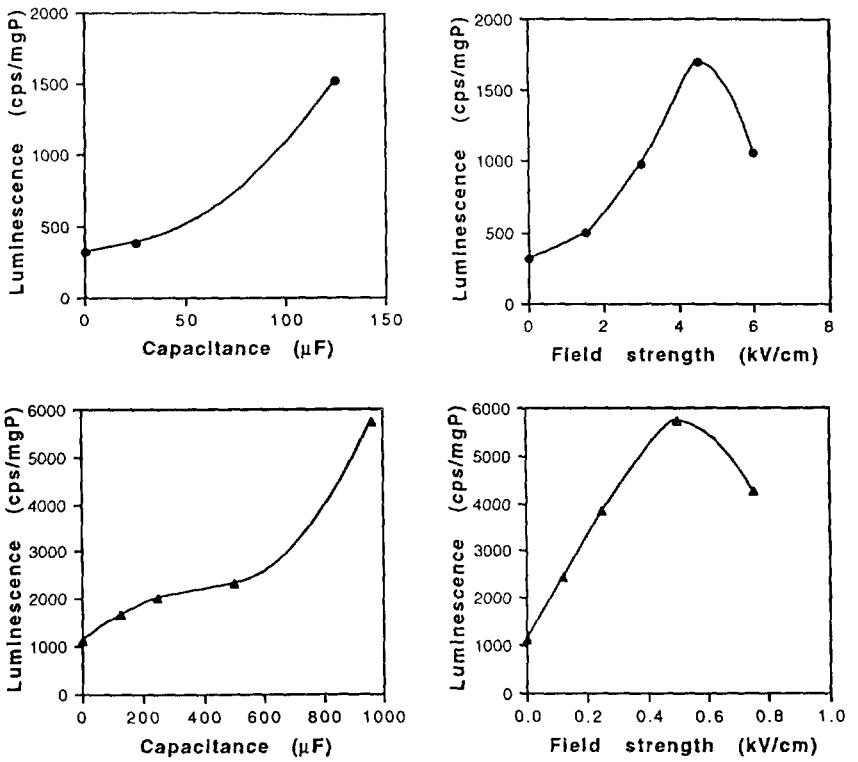

Figure 1.
Dependence of ultraweak luminescence on electric field parameters. *Upper panels*, 1.0×10^6 K562 cells were electroporated at 1.1 kV/cm and different capacitance values (left) or at 25 µF and different field strengths (right). *Lower panels*, 0.5×10^6 LRP were electroporated at 0.5 kV/cm and various capacitances (left) or at 960 µF and various voltages (right). Luminescence was measured as described in "Materials and Methods". Each data point is the mean of three independent determinations (S.D.<10%).

Table 1. Changes in hydroperoxide content and oxidative index of membrane lipids isolated from K562, LRP and LC cells

Cell type	Hydroperoxides (nmol/10^6 cells)	Oxidative index (A_{234}/A_{205})	Luminescence (cps/mg P)
K562 (-)	4.74±0.38 (100)	0.32±0.02 (100)	320±26 (100)
K562 (+)	9.04±0.72 (191)	0.57±0.04 (178)	1690±135 (528)
LRP (-)	3.23±0.26 (100)	0.32±0.03 (100)	1130±90 (100)
LRP (+)	5.94±0.47 (184)	0.58±0.05 (181)	5730±458 (507)
LC (-)	3.51±0.28 (100)	0.27±0.02 (100)	45±3.60 (100)
LC (+)	5.13±0.41 (146)	0.38±0.03 (141)	117±9.36 (260)

Membrane lipids were extracted as described in "Materials and Methods", before (-) and after (+) electroporation at 4.5 kV/cm, 25 μF (K562), 0.5 kV/cm, 960 μF (LRP) and 0.9 kV/cm, 960 μF (LC). Values are expressed ±S.D. and those in brackets represent percentages of treated (+) *vs* untreated (-) samples.

investigation, capacitance and field strength were varied within ranges widely used for cell transfection experiments (12, 15). An increase in each variable increased cell luminescence. The most effective combinations were 4.5 kV/cm, 25 μF (for mammalian cells) and 0.5 kV/cm, 960 μF (for plant protoplasts), the average time constant (τ) being 0.6±0.1 ms and 13.0±1.5 ms, respectively. In order to investigate whether lipid peroxidation could be involved in electroporation, membranes were isolated from K562 and LRP cells before and after electroporation. Table 1 shows that electroporation induced a significant increase in the amount of conjugated hydroperoxides present in the membranes of both cell types. This result was confirmed by ion-pairing HPLC determinations of malondialdehyde (24) in the samples (data not shown). The increase in hydroperoxides paralleled the increase of the oxidative index (Table 1), suggesting that lipid peroxidation was the main oxidative modification of cell membranes upon electroporation (23). Hydroperoxide content and ultraweak luminescence of K562 and LRP cells were affected in the same way by pore-forming electric fields (Table 1), although the electroporation media were very different from each other. This is indicative that the electric field affects directly the membrane lipid peroxidation and the cell luminescence, without the intermediation of the electroporation medium components. This finding extends recent observations on the electropulsation of Chinese hamster ovary cells (25). Walled lentil cells were electroporated as well, to check whether wall digestion affected the luminescence. LC emitted ultraweak light upon electroporation, with a dependence on capacitance and voltage values superimposable to that shown in Fig. 1 for LRP. The highest level of luminescence from walled cells, obtained by electroporating at 0.9 kV/cm and 960 μF

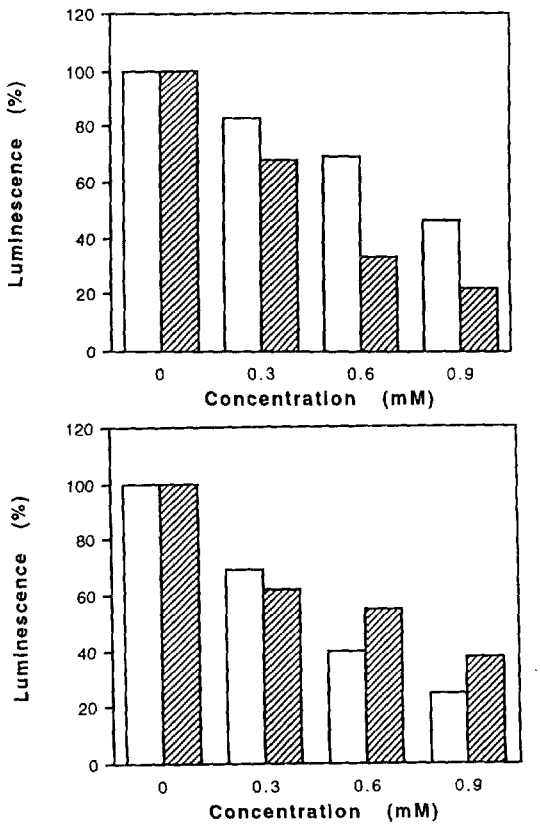

Figure 2.
Effect of inhibitors of lipid peroxidation on the electroporation-induced luminescence. 1.0×10^6 K562 cells (*upper panel*) and 0.5×10^6 LRP (*lower panel*) were electroporated in the presence of different amounts of ascorbic acid (empty bars) and trolox (dashed bars). Electric pulses were delivered at 4.5 kV/cm, 25 μF (K562) and 0.5 kV/cm, 960 μF (LRP). Each data point is the mean of three independent determinations (S.D.<10%).

(τ = 28.0±2.0 ms), was 2.6 times the control value, compared to the 5-fold increase recorded with LRP (Table 1). Analogously, the hydroperoxide content of cell membrane increased to 146% of the control in electroporated walled cells, compared to 184% in electroporated LRP (Table 1). Therefore, the presence of a wall, which protects the cell from electric shock (16), reduces the extent of both lipid peroxidation and luminescence enhancement. The good correlation between the increase in hydroperoxide content and the enhancement of luminescence of different cell types suggests that electric fields have the same effect towards mammalian and plant (walled or naked) cells. The production of hydroperoxides may perturb the membrane leading to the formation of pores. The presence of small amounts of hydroperoxides is known to cause severe structural changes in lipid bilayers, leading to a local membrane disaggregation (26).

It is well known that radical chain reactions involved in lipid peroxidation can be accompanied by light emission (10, 11). In particular, excited carbonyl groups or

Table 2. Effects of ascorbic acid, trolox and vitamin E on hydroperoxide formation, permeability and luminescence of electroporated K562 and LRP cells

Cell type	Hydroperoxides (%)	Permeability (%)	Luminescence (%)
K562	100	100	100
K562+Ascorbic acid	98±6.86	89±8.90	46±3.68
K562+Trolox	97±6.79	89±8.90	22±1.76
K562+Vitamin E	86±6.02	77±7.70	70±5.60
LRP	100	100	100
LRP+Ascorbic acid	95±6.65	87±8.70	25±2.0
LRP+Trolox	96±6.72	87±8.70	38±3.04
LRP+Vitamin E	87±6.09	79±7.90	74±5.92

K562 and LRP cells were electroporated as described in Table 1, in the presence of 0.9 mM ascorbic acid or trolox or vitamin E. Hydroperoxide content, permeability and luminescence, determined as described in "Materials and Methods", were expressed as percentages (±S.D.) of the controls.

singlet oxygen molecules may contribute to the light emission, in the ultraviolet and blue region (27) or in the red and infrared region of the spectrum (27), respectively. The luminescence of K562 and LRP cells was measured in the presence of either ultraviolet or red filters. Our results indicated that the ultraviolet emission accounted for 10% only of ultraweak luminescence from both cell types, whereas 80% of the photons were emitted in the red. Thus, the electroporation-induced luminescence was largely due to singlet oxygen, a finding well in line with the chemistry of conjugated hydroperoxides (23). The role of peroxides in electroporation was checked also by electroporating cell suspensions in the presence of vitamin E (α-tocopherol), ascorbic acid or trolox (a water-soluble analogue of vitamin E). These compounds are powerful chain-breaking antioxidants, which inhibit lipid peroxidation by trapping hydrophobic or hydrophilic peroxyl radicals, respectively (28). Figure 2 shows that the presence of the water-soluble antioxidants ascorbic acid and trolox in the electroporation mixture strongly inhibited the ultraweak light emission from the samples, in a concentration-dependent manner. On the other hand, the lipophilic vitamin E affected less severely cell luminescence, which levelled off at 70% (K562) and 74% (LRP) of the control value at the highest concentration of the antioxidant (Table 2). The effects of the different antioxidants on hydroperoxide formation and cell permeability was investigated as well. In Table 2 it is shown that the presence of ascorbic acid or trolox in the electroporation medium barely affected the formation of hydroperoxides or the permeability, whereas vitamin E significantly inhibited both processes. Taken together, these results suggest that luminescence is downstream to the hydroperoxide formation, in line with previous findings (29), whereas cell permeability is upstream. Furthermore, the different inhibitory ability of hydrophilic and hydrophobic antioxidants suggests that cell luminescence and cell permeability are controlled at different depths of the

lipid bilayer. Finally, a recent report shows that electropulsation of Chinese hamster ovary cells triggers an oxidative burst, hypothetically attributed to hydroxyl radical formation at the electropermeabilized cell level (25). Our results give biochemical support to these findings, showing that membrane lipids are the primary target of the electric field, which induces hydroperoxide formation in the lipid bilayer. The electroporation-induced lipid peroxidation can also account for the morphological changes ("blebs") observed in electrically treated cells (30).

In conclusion, we have shown for the first time that electroporation generates hydroperoxides in animal and plant cells. These hydroperoxides might represent the "excited state" needed for the formation of pores (17). They are also responsible for the increase in light emission. Therefore, ultraweak luminescence can monitor peroxide production by electric fields and hence the poration of membranes.

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