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Kinetics of ultraweak light emission from human erythroleukemia K562 cells upon electroporation

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Abstract

Electroporation involves the application of an electric pulse that creates transient aqueous channels (electropores) across the lipid bilayer membranes. Here, we describe an instrument set up suitable to record ultraweak light emission from human erythroleukemia K562 cells during and immediately after delivery of electric pulses. Most of light was emitted in the first seconds after each pulse, following a complex decay which can be fitted by a double exponential equation characterized by two different time constants (T_1 and T_2), both in the order of seconds. T_1 was approximately 10-fold shorter than T_2 and both time constants were dependent on field strength of the electric pulse. The effect of various antioxidants on the amount of emitted photons and on T_1 and T_2 values was investigated, in order to shed some light on the chemical species responsible for cellular luminescence.

Keywords: Lipid peroxidation; Antioxidants; Cellular luminescence

1. Introduction

The application of an electric pulse of suitable amplitude and duration to cells leads to the reversible opening of aqueous channels (electropores) across the lipid bilayer, a process called electroporation or electropermeabilization [1]. Transport of macromolecules through electropores makes electroporation a powerful technique, used for both DNA and protein transfer to cells [2]. The process of formation and resealing of pores in cell membranes has been known for a long time [3], but its mechanism is still unclear. This is mainly due to the fact that electropores are formed in the time scale of (sub)seconds, though they can stay open up to several minutes. Therefore, experimental apparatuses able to record from the very beginning of cell electropermeabilization are needed, in order to shed light on the biochemical events leading to pore formation. To our knowledge only few studies have been focused on the events occurring within the first seconds of electroporation, measuring either fluorescence [4–6] or photooxidation kinetics [7] of cellular probes. Also electron microscopy has been used to investigate early changes in membrane structure of electropermeabilized cells [8]. Recently, we reported that ultraweak light emission is a suitable technique to follow the formation of radical species in electroporated human erythroleukemia K562 cells [9]. Indeed, measurement of cell luminescence was used to assess the involve-

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ment of lipid peroxidation in electroporation-induced membrane permeability [10]. The results reported [9,10] gave a biochemical background to previous observations, showing the generation of reactive oxygen species in mammalian cells subjected to electroporation [11]. Nevertheless, in those experiments it was possible to monitor light emission only 3 min after delivery of the electric pulse, a time point far away from the beginning of pore formation, but still indicative of significant changes in membrane properties of electroporated cells [9,10]. Here, an experimental setup was designed and built, in order to place the electroporation cuvette directly in the reflective chamber of a highly sensitive luminometer, which allowed to record luminescence during and immediately after delivery of the electric pulse. With the aim of understanding the mechanism of pore formation, the amount of light emission in the very first seconds after electroporation was analysed, as well as the possible origin of the emitted photons. Here we show that reactive oxygen species, and lipid peroxides in particular, are indeed formed mainly at the onset of electropermeabilization, which suggests that they might play a key role in electropore formation.

2. Materials and methods

2.1. Materials

Chemicals were of the purest analytical grade. 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO) was purchased from Molecular Probes (Eugene, OR, USA). Vitamin C (ascorbic acid), mannitol and vitamin E (α-tocopherol) were from Sigma Chemical (St. Louis, MO, USA), as well as the liposome kit (anionic). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was purchased from Acros Chimica (Geel, Belgium).

2.2. Cell culture and liposome preparation

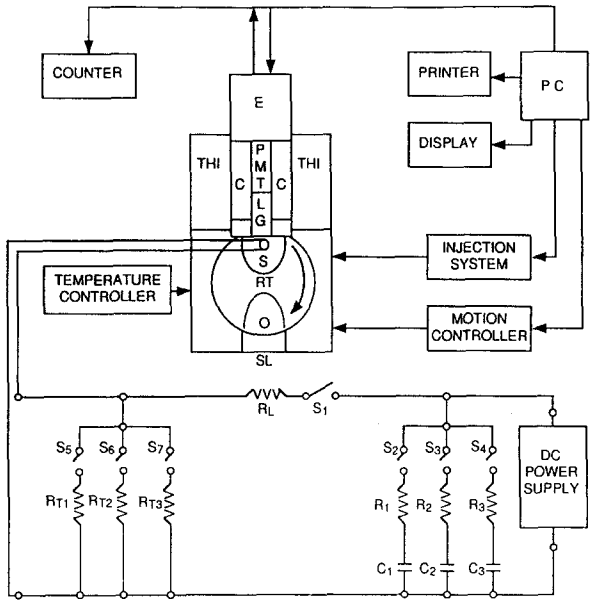
Human erythroleukemia K562 cells were cultured in RPMI 1640 medium (Gibco BRL, Renfrewshire, UK), supplemented with 10% heat-inactivated fetal calf serum (Gibco) and 2 mM L-glutamine. Mycoplasma-free cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2, then they were washed twice in Dulbecco's phosphate-buffered saline (PBS) and resuspended in PBS at a density of 1.25×10^6 cells/ml. Cell viability was determined by Trypan blue dye exclusion, as already described [12]. Egg yolk unsaturated phosphatidylcholine (PC) liposomes were prepared according to [13], using the liposome kit (Sigma). Scavengers were added to cell cultures or liposome suspensions immediately before delivering the electric pulse, and controls were treated with the same volumes of vehicle alone [9,10].

2.3. Electroporation

Electropermeabilization of K562 cells was performed with a Gene Pulser II Plus (Bio-Rad Laboratories, Hercules, CA, USA), kindly put at our disposal by Bio-Rad Italia (Milan, Italy). Exponentially decaying pulses were generated and delivered to cell suspensions in sterile disposable electroporation cuvettes of 0.4 cm path length (Bio-Rad). Aliquots of K562 cells (0.8 ml/test, i.e., 1.0×10^6 cells/test), resuspended in PBS at a density of 1.25×10^6/ml, were electroporated at a resistance of 30 Ω, in the presence or in the absence of radical scavengers. Capacitance and field strength values were chosen in a range suitable for transfection experiments [14,15], yielding electric pulses with decay time constants (τ) ranging from 0.10 ± 0.02 ms (at a capacitance of 3 μF) to 0.80 ± 0.10 ms (at a capacitance of 25 μF). Unsaturated phosphatidylcholine liposomes were electroporated under the same conditions as K562 cells, using 1.25 μmol phosphatidylcholine in 0.8 ml PBS for each experiment. The electrodes of the Gene Pulser II Plus were modified, in order to locate the electroporation cuvette in the reflective chamber of the luminometer, thus allowing measurements of light emission before, during and immediately after delivery of the electric pulse. Electrical connections between the electrodes and the electroporation cuvette were designed to avoid sparks, which might dazzle the photocathode of the luminometer. A diagram of the instrument setup is reported in Scheme 1

2.4. Luminescence measurements

Ultraweak light emission was measured in a highly



Scheme 1. Experimental setup of the LUMI-A luminometer (upper part) coupled to the Gene Pulser II Plus electroporator (lower part). PMT, photomultiplier tube; LG, light guide; E, amplifier and discriminator electronics; C, cooling channel; THI, thermal insulation; S, sample; RT, rotating turret; SL, sample loading aperture. General circuit diagram of an exponential decay wave form generator: C_{1-3} are the energy storage capacitors, which have internal resistance R_{1-3}, and can be added to the circuit by switches S_{2-4} in order to vary the total capacitance. Closing switch S_1 allows the charged capacitors to discharge to the output and into the chamber. R_L is a discharge current-limiting resistor, which is needed in some designs. R_{T1-3} are timing resistors, which can be added to the circuit by switches S_{5-7}.

sensitive LUMI-A luminometer (SEAS, Milan, Italy), described in Ref. [16]. 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 an Olivetti M24 personal computer for data analysis. Luminescence of cell suspensions was measured before, during and immediately after delivery of the electric pulse, recording photon emission every second. The instrumental dead time (approximately 1 s) was shorter than the rising time of the luminescence signal (approximately 3 s). Light emission was expressed as counts per second (cps). For calculations of peak areas, photons emitted in the first 200 s following the electric pulse were integrated in each experiment. Spectral analysis of light emitted from K562 cells upon electroporation was performed by measuring after passing luminescence through ultraviolet (RUV-370), yellow (B40-546) or red (RG-610) filters, as described [9].

2.5. Data analysis

Luminescence decay after each pulse was analyzed by means of the Sigma Plot software, using the χ^2 test for statistical evaluation. Decay curves of light emitted from K562 cells were fitted by the equation

$$L(t) = A_1\exp(-t/T_1) + A_2\exp(-t/T_2) + B$$

where $L(t)$ = light emitted at time point t, A_1+A_2 = light emitted at time point zero, T_1 and T_2 = time constants of the decay process. B values were always much smaller (approximately 2–3%) than the maximum. Data reported in this paper are the mean (± S.D.) of three independent experiments. Chi-square (χ^2) values were < 2.5. The residues were almost randomly dispersed around the fitting curve.

3. Results and discussion

Erythroleukemia K562 cells respond to external electric pulses by increasing ultraweak luminescence (Fig. 1). In previous experiments we measured cellular luminescence 3 min after the electric pulse, showing that it was enhanced up to 5-fold over the control value [9]. This finding is confirmed by the present results. However, Fig. 1 clearly shows that most of the light is emitted in the very first seconds following the electric pulse, an observation which could be made only by using an instrumentation able to record the very beginning of the process. Changing the field strength or the capacitance values of the electroporator yields more intense or longer pulses, respectively [17]. Here, both field strength and capacitance were varied within the range used for transfection experiments [14,15], which hardly affected cell viability (data not shown). Luminescence was found to depend on both parameters, showing a linear increase at increasing field strength and capacitance values (Fig. 2). These results suggest that either

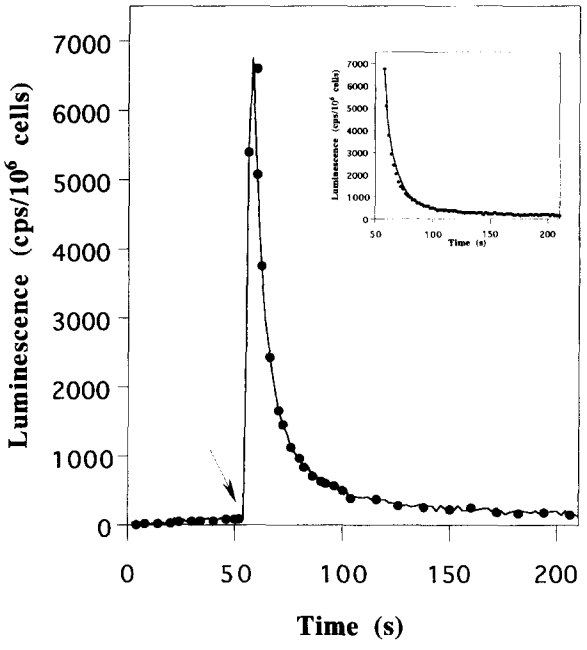
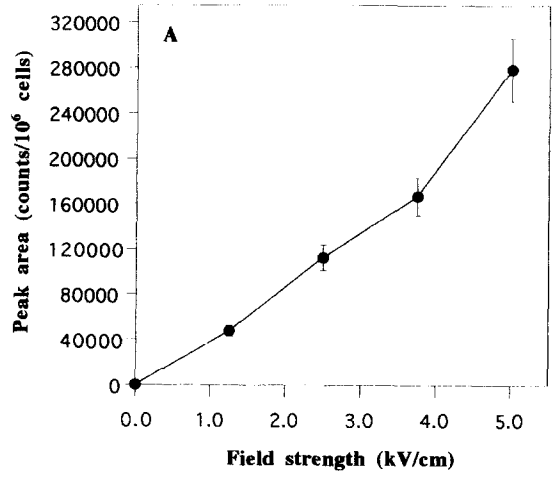


Fig. 1. Light emission from K562 cells upon electroporation. Luminescence was recorded before, during and after electroporation of K562 cells (1.0×10^6/test) at 25 μF and 2.5 kV/cm. The profile shown is representative of those obtained at different field strength and capacitance values. The arrow indicates the time point of electric pulse delivery. The inset shows the best fit of the same decay curve, obtained with the equation $L(t)=A_1\exp(-t/T_1)+A_2\exp(-t/T_2)+B$, where $A_1=5106\pm127$ cps/10^6 cells, $A_2=1400\pm102$ cps/10^6 cells, $B=186\pm6$ cps/10^6 cells, $T_1=5.5\pm0.3$ s, $T_2=29.5\pm1.5$ s and $\chi^2=2.0$. The peak area under the decay curve was $110\,000\pm10\,000$ counts.

more intense or longer pulses increase the amount of excited species responsible for light emission. It is worth recalling that electropores are reported to form and shrink to a metastable state within seconds [4–7]. This is exactly the time window during which the electroporation-induced peak of luminescence was observed (Fig. 1), suggesting that light emission may be indeed associated to pore formation. Consistently, K562 cell permeability (hence, number and/or size of pores) increases as a linear function of pulse intensity [10], as does the amount of emitted photons (Fig. 2A). A link between the processes leading to luminescence and those leading to membrane electropermeabilization was also suggested by the common shape of the decay of the two processes. In fact, luminescence decayed exponentially (Fig. 1), much alike the molecular transport through mammalian cell membranes subjected to external electric fields [4–7]. Interestingly, the experimental decay of light emission after the electric pulse could always be fitted by a double exponential equation characterized by two time constants (T_1 and T_2), whatever the capacitance and field strength applied. T_1 was approximately 10-fold shorter than T_2, indicating that light emission is made of a fast and a slow process. It is noteworthy that the fast portion of light emission (T_1) cannot be ascribed to chemicophysical processes, because broken K562 cells electroporated in the same conditions did not yield a significant level of luminescence. The cellular response to the electric shock might rather rely on enzymatic activities requiring cell integrity, most probably lipoxygenases



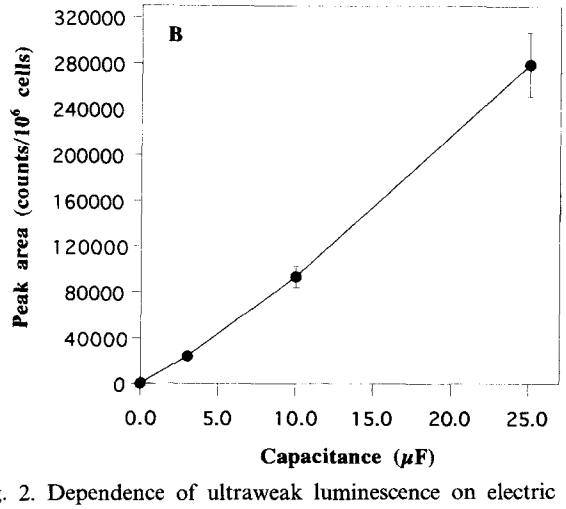


Fig. 2. Dependence of ultraweak luminescence on electric field parameters. K562 cells (1.0×10^6/test) were subjected to electroporation at either 25 μF and various field strengths (A) or 5.0 kV/cm and various capacitances (B), then peak areas of light emitted within 200 s after the electric pulse were measured.

[10]. It is tempting to suggest that T_1 and T_2 reflect two different types of radicals, able to return from the excited to the ground state at different rates. Alternatively, they might be due to the same species in different environments. Stronger electric fields (i.e., more intense pulses) dramatically shortened both T_1 and T_2 (Fig. 3A), whereas the effect of capacitance (i.e., pulse duration) on them was negligible (Fig. 3B). Therefore, light emission from K562 cells was faster at higher field strength values. This result can be interpreted by recalling that more and/or larger pores are formed on the cell membrane at increasing field strength [1]. It is conceivable that self-quenching of excited species can occur more efficiently when pore density on the cell surface is higher. Alternatively, it can be proposed that excited species can be quenched faster in larger pores, due to faster diffusion and interaction in the lipid bilayer with natural quenchers. Both hypotheses would be in agreement with the known heterogeneity of electropore number, size and morphology [1]. Light emission extrapolated at time zero ($L(0)=A_1+A_2$) showed a non-linear dependence on the field strength (Fig. 4A), though it increased linearly at increasing capacitance values (Fig. 4B). This finding strengthens the hypothesis that electropores are heterogeneous in nature and that different kinds of pores are formed at various field strengths. Indeed, an homogeneous population of pores would have shown a linear dependence on the field strength. Moreover, results reported herein are consistent with recent electro-optic data showing that opening of electropores in cell membranes can be fully described by a chemical model, based on the motion and phase transition of complex clusters of lipid molecules at the pore edges [18]. It could be suggested that a more efficient scavenging of excited species from membranes might be the molecular basis for the ability of cells to survive at increasing electric field strength. Within certain limits, the cellular machinery would be able to eliminate potentially noxious species at a rate proportional to their amount. Recently, electropermeabilization of mammalian cells has been associated to generation of reactive oxygen species, quantified by lucigenin–chemiluminescence assays [11]. The use of such an indirect method to determine free radical generation, instead of the direct assay used here, might explain some differences between the report by Gabriel and Teissié [11] and our present study. Unlike Gabriel and Teissié, we (i) did not observe a delay in light emission after the electric pulse, and (ii) could fit the experimental data with two decay time constants instead of one only. Nonetheless, it should be recalled that also differences in membrane lipid composition between human K562 cells, used by us, and Chinese hamster ovary cells, used by Gabriel and Teissié [11], might result in different kinetics of pore formation, a process which is affected by the molecular geometry (cylinders, cones, inverse cones) of membrane lipid constituents [19]. Oxygen radicals are known to be

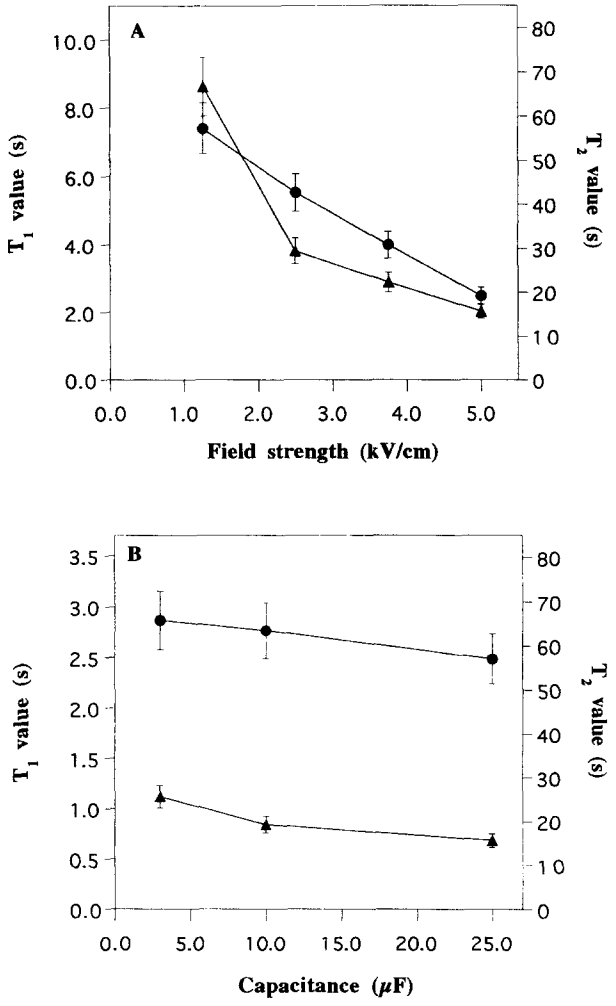


Fig. 3. Dependence of luminescence decay time constants on electrical parameters. Decay curves of light emitted from K562 cells (1.0×10^6/test) electroporated at either 25 μF and different field strengths (A) or 5.0 kV/cm and different capacitances (B) were fitted by a double exponential equation as in Fig. 1. The time constants T_1 (circles) and T_2 (triangles) were calculated and plotted versus the electrical parameters.

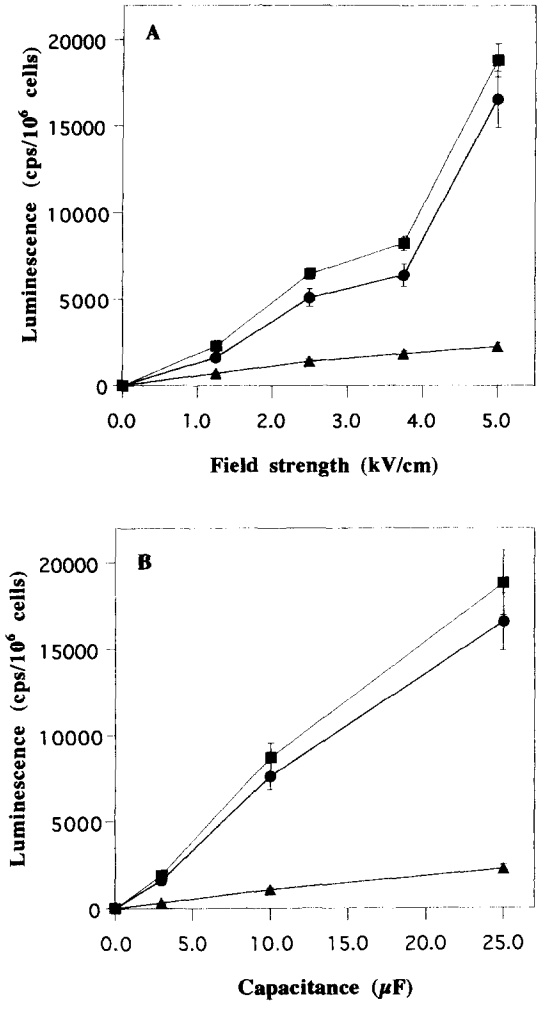


Fig. 4. Dependence of A_1 and A_2 values on electric field parameters. Decay curves of light emitted from K562 cells (1.0×10^6/test) electroporated at either 25 μF and different field strengths (A) or 5.0 kV/cm and different capacitances (B) were fitted by the double exponential equation reported in Fig. 1. The values of A_1 (circles), A_2 (triangles) and A_1+A_2 (squares) were calculated and plotted versus the electrical parameters. Values of A_1+A_2 represent light emission at time point zero, $L(0)$.

powerful oxidants of biomolecules. In particular they can start chain reactions leading to membrane lipid peroxidation [20]. That radical reactions associated to membrane lipid peroxidation may be responsible for enhanced luminescence from K562 cells is in keeping with the oxidative jump observed in electropermeabilized Chinese hamster ovary cells [11], a process which was enhanced by activators of lipid peroxidation such as nucleotide tri- and diphosphates [11]. Phospholipids have long been recognized as the primary target of electric field perturbation [21] and the role of lipid peroxidation in electroporation-induced cell permeability was further assessed [10]. Therefore, the effect of various antioxidants and radical scavengers on both the amount of emitted photons and the T_1 and T_2 values was studied, using each compound at a concentration found to be optimal in preliminary experiments. The most effective combination of field strength and capacitance in the enhancement of light emission (25 μF, 5.0 kV/cm) was used to investigate the role of reactive oxygen radicals. Trolox (a water-soluble analog of vitamin E), vitamin C and vitamin E are potent chain-breaking antioxidants able to trap hydrophilic or hydrophobic peroxyl radicals, respectively [22]. On the other hand, mannitol is a hydroxyl radical scavenger [23] whereas TEMPO is a scavenger of superoxide and hydroxyl radicals [24]. All these compounds were able to reduce the amount of light emitted from K562 cells and the T_2 value, while yielding a longer T_1 (Fig. 5A). The overall decrease of luminescence in the presence of antioxidants and scavengers extends previous work [9,11] and speaks in favor of the involvement of peroxyl and hydroxyl radicals in light emission. It suggests also that critical perturbations in the lipid bilayer, leading to light emission, should occur at the membrane–water interface rather than inside the lipid bilayer. Trolox was indeed the most effective in decreasing luminescence, whereas vitamin E was the least effective (Fig. 5A). On the other hand, the changes in T_1 and T_2 values are more difficult to explain. Lipid bilayer vesicles are widely used as a model to investigate the effects of electric fields on membranes [10,18,25]. Here, experiments performed with unsaturated (PC) liposomes, subjected to electric fields in the presence of antioxidants or radical scavengers in the same way as K562 cells, suggest that membrane proteins may play a key role in radical reactions responsible for light emission (Fig. 5B). Indeed, luminescence of PC liposomes was reduced by the various antioxidants and radical scavengers in a way fully analogous to that of K562 cells, but T_1 and T_2 values varied in a different way (compare Fig. 5A and B). Complex interactions between lipid moieties, proteins and antioxidants (scavengers) at the membrane level can lead to different lifetimes of the chemical species responsible for light emission, both in the fast and in the slow portion of the decay curve. Such a complexity was evi-

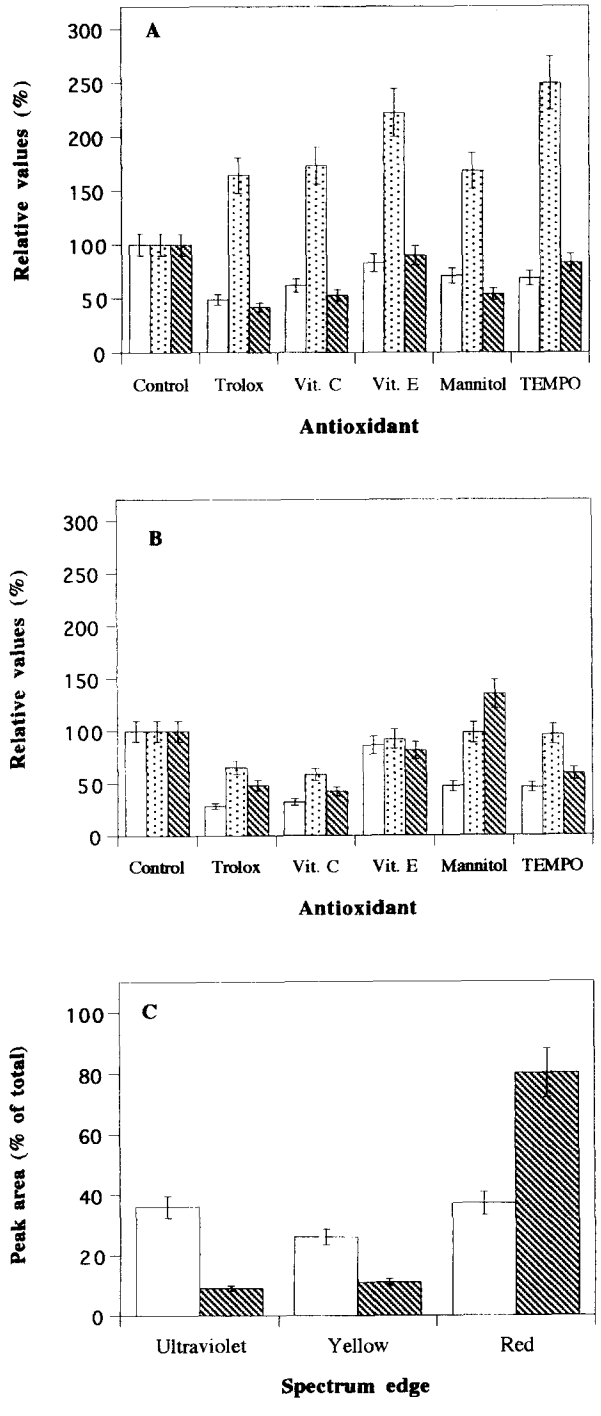


Fig. 5. Effect of antioxidants on electroporation-induced light emission. K562 cells (panel A) and unsaturated phosphatidylcholine liposomes (panel B) were electroporated at 25 μF and 5.0 kV/cm in the presence of either 1 mM trolox, 1 mM vitamin C, 1 mM vitamin E, 100 mM mannitol or 5 mM TEMPO. Peak area (empty bars), T_1 (dotted bars) and T_2 (hatched bars) values of emitted light were measured and expressed as percentage of the controls, arbitrarily set to 100 (100% corresponds to 280 000 ± 25 000 counts (peak area), 2.5 ± 0.2 s (T_1) and 15.0 ± 1.5 s (T_2), for K562 cells, and to 170 000 ± 18 000 counts (peak area), 1.8 ± 0.2 s (T_1) and 28.6 ± 2.5 s (T_2), for phosphatidylcholine liposomes). Panel C shows the spectral analysis of luminescence of K562 cells (empty bars) and phosphatidylcholine liposomes (hatched bars), subjected to electroporation at 25 μF and 5.0 kV/cm.

dent also from spectral analysis of the emitted light, showing that in mammalian cells the contribution of ultraviolet and yellow light is much higher than in lipid vesicles (Fig. 5C). Interestingly, ultraviolet light and yellow light originate from carbonyl groups, contributed by proteins and lipids, whereas red light is attributable to singlet oxygen and is related to lipid moieties only [26].

Altogether, these results show that reactive oxygen species (mainly lipid peroxides) are generated at the very beginning of cell electropermeabilization. This finding gives biochemical background to current hypotheses on the type of perturbations which occur at the membrane level upon cell exposure to electric shock [27,28]. We have already shown a linear correlation between the amount of lipid hydroperoxides formed and the cell permeability observed after the electric shock [9,10]. We also demonstrated that the luminescence was due to hydroperoxides [9,10]. Here, we have shown how the light emission is a very early event after the electric pulse. However, the very first excited species formed are not quenched by any radical scavenger used, indicating that either (a) these species do not react with them or (b) the decay is too fast to allow the encounter inside the lipid phase. In any case the instrument setup during the investigation appears to open an avenue for the analysis of the physicochemical processes leading to the formation of electropores.

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