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Permeability changes of connexin32 hemi channels reconstituted in liposomes induced by extremely low frequency, low amplitude magnetic fields

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Abstract

The effect of extremely low frequency and low amplitude magnetic fields on gap junctional permeability was investigated by using reconstituted connexin32 hemi channel in liposomes. Cytochrome *c* was loaded inside these proteoliposomes and its reduction upon addition of ascorbate in the bulk aqueous phase was adopted as the index of hemi channel permeability. The permeability rate of the hemi channels, expressed as ΔA/min, was dependent on the incubation temperature of proteoliposomes. The effect of exposures to magnetic fields at different frequencies (7, 13 and 18 Hz) and amplitudes (50, 50 and 70 μT, respectively), and at different temperatures (16, 18 and 24 °C) was studied. Only the exposure of proteoliposomes to 18-Hz (B_{acpeak} and B_{dc}=70 μT) magnetic field for 60 min at 16±0.4 °C resulted in a significant enhancement of the hemi channel permeability from ΔA/min=0.0007±0.0002 to ΔA/min=0.0010±0.0001 (P=0.030). This enhancement was not found for magnetic field exposures of liposomes kept at the higher temperatures tested. Temperature appears to influence lipid bilayer arrangement in such a way as being capable to mask possible effects induced by the magnetic field. Although the observed effect was very low, it seems to confirm the applicability of our model previously proposed for the interaction of low frequency electromagnetic fields with lipid membrane.

Keywords: Gap junction; Connexin32 hemi channel; Channel reconstitution; Permeability change; Liposome; Magnetic field

1. Introduction

There is increasing evidence that extremely low frequency electromagnetic fields (ELF-EMFs) can influence gap junction intercellular communication (GJIC) [1–6]. The rationale of these studies arises from two main reasons. One is the public concern about the potential health risks due to magnetic field exposure from power lines and electrical appliances [7,8]. Another important reason is that the alteration of GJIC is considered an important event during the promotion stage of carcinogenesis process [9]. However, many such effects have been reported with contradictory results, difficulties in replication [10] and no clear biophysical mechanisms have emerged.

Gap junctions (GJ) are composed of channels that permit the passage of ions and low molecular weight metabolites between adjacent cells, without exposure to the extracellular environment [11,12]. These pathways are formed by the interaction between two hemi channels on the surface of opposing cells. These hemi channels are formed by the association of six identical subunits, named connexins (Cx), which are integral membrane proteins [13]. Cell coupling via GJ is dependent on the specific pattern of Cx gene expression. In addition, gating mechanisms regulate GJ cellular communication.

Detailed studies on gating and permeability of GJ channels using cellular systems present experimental

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difficulties since, unlike other channels, the gap junction channel in situ spans two plasma membranes [14,15], and both ends of the pore, and its modulatory sites, are in cytoplasm. Because the access to the pore is via cytoplasm, for selectivity studies one cannot alter permeant species with impunity, and for modulatory studies is difficult to distinguish direct from indirect effects [16]. These factors could partially justify the different and irreproducible results reported in literature for the effect of ELF-EMF exposures.

In this study, we used purified connexin32 from rat liver reconstituted into lipid bilayers to analyze the influence of ELF-EMFs on hemi channel permeability. Indeed, the elucidation of the gating, permeability, and modulation of connexin channels has been greatly aided by the reconstitution of purified connexins into artificial membranes [17–23].

In addition, we selected a priori the components of the magnetic fields for the exposures of the proteoliposomes according to the model previously proposed for the interaction between ELF-EMFs and membrane components [24]. Observations reported by our group have indicated that combined 7-Hz sinusoidal 50-μT peak and quasi-parallel 50-μT static magnetic fields can induce a significant enhancement of lipid bilayer permeability in cationic liposomes. This effect was associated to the presence of a charged lipid (e.g., a dipole) on the membrane [25].

2. Materials and methods

2.1. Chemicals

Soybean phosphatidylcholine (PC), *n*-octyl-β-glucopyranoside, Triton X-100, sodium ascorbate and cytochrome *c* (from horse heart cat. no. C3934) were purchased from Sigma (St. Louis, MO, USA). Saccharose, potassium chloride, sodium bicarbonate, Tris (hydroxymethyl) amino methane, and HEPES-Na were purchased from Serva and Carlo Erba.

2.2. Reconstitution of gap-junction connexin32 into liposomes

To prepare the reconstituted gap-junction proteins in liposomes, we followed the protocol of Diez and Villalobo [20]. Briefly, crude rat liver plasma membrane fractions were isolated by a discontinuous sucrose gradient method as extensively described elsewhere [26]. A typical membrane preparation contained 23±7 mg of total protein and exhibited a 5′ -nucleotidase activity of 948±35 nmol/min/mg protein (data from 30 preparations). The activity of this plasma membrane enzymatic marker was enriched with respect to the crude homogenate by 17±2-fold. Then, gap-junction plaques from plasma membrane (usually 160 mg of protein) were isolated by the alkali-precipitation method. In this study, gap-junction protein (70–80 μg of connexin32) was suspended in 1-ml medium containing 20 mM Tris–HCl (pH 7.4)–50 mM KCl, 20 mg/ml *n*-octyl-β-glucopyranoside, and 10 mg/ml PC. The protein–lipid–detergent mixture was sonicated on ice 25 times for 15 s each using a 3-mm microprobe at setting 15. Then, 4 mg/ml cytochrome *c* was added, and dialysis was carried out against 1 l of a dialysis-buffer containing 20 mM Tris–HCl (pH 7.4)–50 mM KCl for 24 h at 4 °C with five changes of buffer. The gap-junctions reconstituted in liposomes, hereinafter referred as proteoliposomes, were applied to a 0.9×20-cm Sephadex G-200 column to separate the cytochrome *c*-loaded proteoliposomes from non-entrapped cytochrome *c* molecules. The proteoliposomes were collected in the void volume (1.2 ml) by measuring their turbidity at 630 nm. Plain liposomes without gap junctions were prepared in parallel under the same conditions. To insure removal of trace calcium, column and dialysis membrane were pre-washed with EDTA 0.01%. All reagents were prepared in Ca-free, deionized water, and disposable plastic tubes and pipettes were utilized.

Overall exposure experiments were carried out usually within 2 h from the end of liposome preparations.

2.3. Transmission electron microscopy (TEM) analysis

For negative staining of gap junction plaques and liposome preparations, 50 μl of each sample was placed on a 200-mesh carbon-formvar coated copper grid and stained with 2% (w/v) uranyl acetate. Samples were examined using a Philips CM12 transmission electron microscope (Philips Instruments, Eindhoven, The Netherlands) operating at 80 kV. The mean diameter of both proteoliposomes (40±5 nm) and plain liposomes (25±4 nm) was measured on TEM images using the Java image-processing program Image J 1.26. The immunolocalization of gap junction on the surface of proteoliposomes was done according to the following procedure. After washing with Tris-buffer and before negative staining, liposomes were incubated with rabbit anti-connexin32 immune serum (named GJr-904) or with preimmune serum (kindly donated by Dr. Antonio Villalobo of CISC, University of Madrid) as control at a working dilution of 1:1000 for 1 h at room temperature. After washing in PBS/BSA 1%, primary antibody detection was obtained by reaction with goat anti-rabbit IgG coupled to 5-nm gold particles (British BioCell Int., Cardiff, UK).

2.4. Hemi channel activity measurements

The procedure for assaying the connexon hemi channels activity involved intraliposomal cytochrome *c* reduction measurements, using sodium ascorbate as substrate (Fig. 1). We followed the appearance of the reduced form of cytochrome *c* at its peak absorbance (λ=417 nm) on a Cary 50 spectrophotometer via an optical probe. The change in absorbance of liposomes (0.15 ml) was monitored at 417 nm for 0.5 min. Ascorbate (0.3 mM) was then added, and

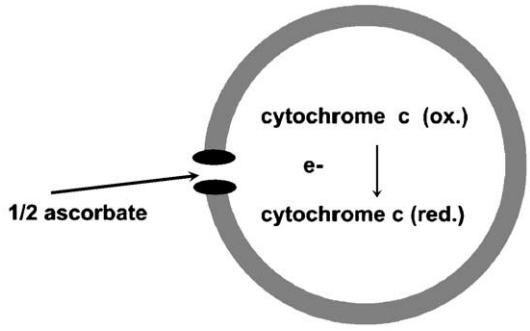

Fig. 1. Assay system for reconstituted gap junction channel activity. The drawing depicts ascorbate passing through a connexon channel reconstituted into a liposome and the reduction of the entrapped cytochrome *c*. The oxidized and reduced forms of cytochrome *c* are represented by (ox) and (red), respectively. The donation of one electron is represented by e^-.

changes in absorbance due to reduction of entrapped cytochrome *c* were monitored for a further 3 min (or 15 min when indicated). The permeability rate of the hemi channel, expressed as ΔA/min, was calculated from the values of absorbance at 417 nm against time (min), $\Delta A = A_t - A_0$, where A_0 and A_t are the absorbance unit at 417 nm for reaction mixture after addition of the ascorbate at time 0 and t. The total amount of cytochrome *c* present in the lumen of liposomes was determined after their rupture by adding Triton X-100 (2% w/v) in the reaction mixture.

Each spectrophotometric measurement was carried out at a constant temperature as indicated. Between kinetic measurements, liposome suspensions were placed in an ice bath.

2.5. ELF-EMFs exposure

We conducted our experiments by using a triaxial exposure system (Fig. 2) providing high precision control of all the fields components. Each magnetic field component was generated by two parallel coils [25], i.e., each set of coils controls the component direct along its axis. In the center of the apparatus we obtained a large isofield region (8×8×8 cm) with a field uniformity of 1%. By means of a fluxgate triaxial sensor (MGA-03MC, Bartington) with an accuracy of ±0.5 μT, a feedback system generates a magnetic field controlling both static and dynamic components. The ELF system was located in a temperature-controlled room, which governed the exposure temperature. A temperature range of 16–24 °C was possible. The indicated temperature was continuously monitored by using a sensor (with ±0.05 °C of accuracy) placed in the center of the exposure apparatus, and recorded by a computer.

2.6. Experimental procedure

Aliquots (0.15 ml) of sham or proteoliposomes in a final volume of 1.33-ml Tris–KCl buffer were placed in a 3-cm^3 silica cuvette positioned, at 10-min intervals, in the region of maximum uniformity of the field in the exposure apparatus. Typically three replicate plain liposomes or proteoliposomes samples were exposed to magnetic fields, with static component of the quasi-parallel to dynamic one and perpendicular to the cuvette axis, for 60 min. The experimental conditions were as follows: B_{ac} frequencies of 7, 13 and 18 Hz, with differing B_{acpeak} and B_{dc} amplitudes of 50, 50 and 70 μT.

Placing the cuvette in the exposure system in local geomagnetic field conditions performed exposure of sham samples.

After 60 min of exposure, a non-perturbative optical probe (indicated with arrow in Fig. 2) directly connected to the spectrophotometer was placed in the cuvette, and the kinetic measurements carried out as described in Section 2.4. The use of the optical probe made it possible to follow the kinetic measurements in the sample while continuing its exposure to the applied magnetic fields.

2.7. Statistical analysis

Data from exposed and sham samples were compared at each frequency. The data were subjected to unpaired two-tailed Student's tests at 95% confidence interval using the program 'Graph Pad Prism' [27].

3. Results

3.1. Functionality of connexon hemi channels reconstituted in liposomes

The gap-junction plaques showed a high degree of purification; the ordered array of connexons is visible (Fig. 3, panel a). The proteoliposomes and plain liposomes were almost 100% unilamellar and homogeneous (Fig. 3, panels b and c), while the reconstitution of connexons in the

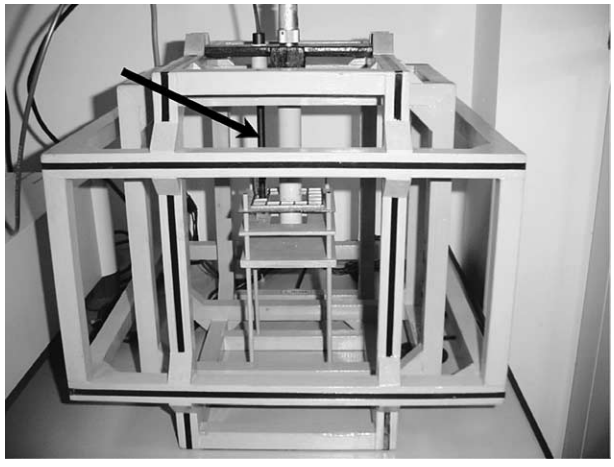

Fig. 2. Exposure system. The cuvettes are placed in the center of apparatus where a uniform magnetic field of 1% is established. Real-time monitoring of absorbance changes is possible during application of EM fields by means of the optical probe indicated by the arrow.

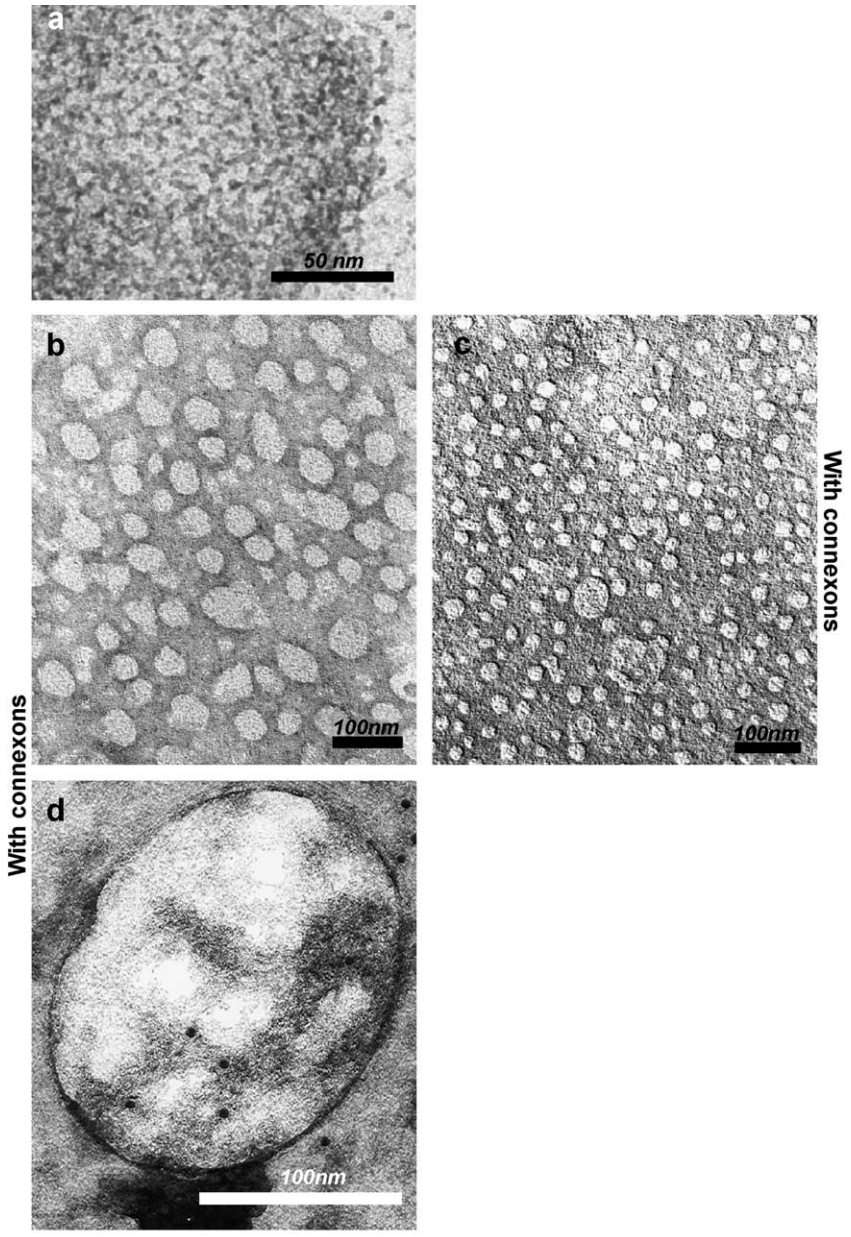

Fig. 3. TEM of samples negatively stained with 2% (wt./vol.) uranyl acetate. (a) Isolated rat liver gap junction plaque. (b, c) Liposome preparations formed with and without connexons after incubation for 24 h at 5 °C. The mean diameters of vesicles were 40±5 and 25±4 nm, respectively. (d) Detail of a liposome formed with connexons after immunogold labeling using the rabbit anti-connexin32 immune serum (GJr-904): a 5-nm gold particle staining of connexons on the liposome surface is visible.

lipid bilayer significantly increased (ca 60%) the mean vesicles diameter of the proteoliposomes (Fig. 3, panel b). Furthermore, the immunolocalization of connexons on the liposome surface (Fig. 3, panel d) clearly indicates that connexon hemi channels have been successfully reconstituted in the lipid bilayer.

In order to demonstrate that the connexon hemi channels reconstituted in the proteoliposomes were functional, we measured the reduction of entrapped cytochrome *c* into the plain liposomes and proteoliposomes. In Fig. 4 is shown a typical curve of changes of absorbance at 417 nm against time (min). The addition of ascorbate to plain liposomes (trace A) failed to reduce the entrapped cytochrome *c*, indicating no simple diffusion of ascorbate across the bilayer. The later addition of Triton X-100, which permeabilizes the liposomes, provoked a fast reduction of the total cytochrome *c* loaded into plain liposomes. In contrast, the addition of ascorbate to proteoliposomes (trace B), with connexons reconstituted, resulted in a fast reduction of entrapped cytochrome *c* (ΔA/min=0.0048±0.0006, n=3). The later addition of Triton X-100 further reduced the cytochrome *c*, indicating that functional connexon hemi channels were reconstituted in proteoliposomes, as already reported elsewhere [20].

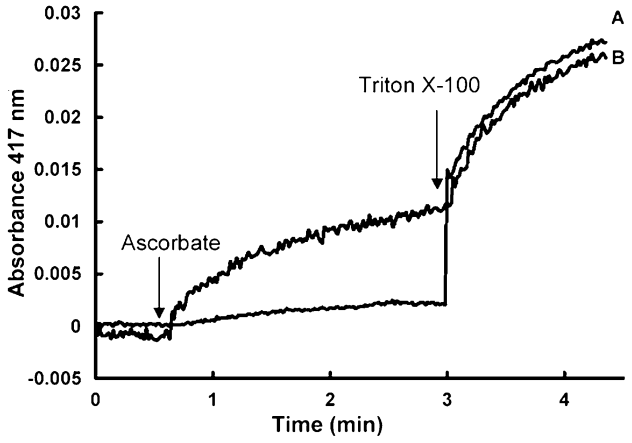

Fig. 4. Reduction of intraliposomal cytochrome *c* in plain liposomes without reconstituted gap junctions (trace A) and proteoliposomes with reconstituted gap junctions (trace B) are presented. The liposomes were incubated at 24±0.5 °C for 60 min before spectrophotometric measurements. When indicated 0.3 mM ascorbate and 0.2% (w/v) Triton X-100 were added. For clarity the plot of absorbance at 417 nm was arbitrarily reset to 0.

3.2. Magnetic field exposures

The authors have previously reported that exposure to magnetic fields (7 and 13 Hz) at specific amplitudes of B_{acpeak} and B_{dc} of 50 μT can induce changes on the permeability of cationic liposomes [25]. This effect has been explained by a theoretical model that studied the transfer of power from a magnetic field to an electric dipole (i.e., polar head group of DPPC) on the lipid membrane (i.e., cationic liposomes) [24]. This analysis indicated that the transfer of power was possible only at specific frequencies [25].

The charged group dynamics can be described by the following Langevin-type equation:

$$\frac{\mathrm{d}\vec{L}}{\mathrm{d}t} = -\gamma\vec{B}\times\vec{L} - v\vec{L}$$

where $\vec{L}$ is the angular momentum, $\vec{B} = \vec{B}_0 + \vec{B}_1\cos(\omega t)$ is the applied magnetic field ($\vec{B}_0$ is the static component whereas $\vec{B}_1$ is the alternating one), $\gamma = q/2m$ is the gyro magnetic factor and v is the collision frequency (s^{-1}) representing the viscosity.

It should be noted that, as a first approximation, in the above equation the random component (modeling the thermal bath) has been neglected. Starting from this equation it is possible to evaluate the effect of the application of an external magnetic field both on the trajectory of the motion and on the energetic transfer to the biological structure. It can be shown that the power exchanged between the magnetic field and the charged molecule is

$$P(t) = -\gamma\left|\frac{\partial\vec{B}}{\partial t}\cdot\vec{L} + \frac{\partial\vec{L}}{\partial t}\cdot\vec{B}\right|$$

However, in absence of viscosity the power transferred to the molecule depends only on the derivative of the alternating magnetic field. Thus, we would be able to calculate the evolution of the molecule trajectory and the power transferred by the field to the structure in motion. The principal equation represents a linear system with periodic coefficients that can be in general solved only by a numerical integration. This has been done by implementing a fourth-order Runge–Kutta solver.

The numerical solution allows us to study the exposure conditions (e.g., polarization of the fields, applied frequency, initial orientation of the trajectory) in order to evaluate those which will assure the best transfer of energy or the maximum displacement from the original position. It can be shown that by applying a rotating magnetic field with a rotation frequency close to the Larmor frequency:

$$f_{\mathrm{s}} = \frac{\gamma}{2\pi}\left|\vec{B}_0\right|$$

A re-orientation of the angular momentum can be observed. This condition is referred to as resonance. The angular momentum tends to spiral outwards until it completes an entire revolution along the *z*-axis. It should be noted that with off-resonance frequencies the resonant behavior is lost and orbit plane oscillates only due to the Larmor precession.

In order to confirm these experimental and theoretical observations, in this study we carried out the exposure experiments of proteoliposomes at the following field parameters: 7, 13 and 18 Hz magnetic fields at amplitudes of $B_{\mathrm{acpeak}}=B_{\mathrm{dc}}$ of 50, 50 and 70 μT, respectively.

In the first experiments, we performed cytochrome *c* reduction measurements of proteoliposomes after 60 min of exposure at 24±0.5 °C. In all cases the permeability rate of connexon hemi channel was not changed by the ELF magnetic field exposure (data not shown).

3.3. Effects of temperature

Because the channel activity of reconstituted connexons has been reported to be dependent on the incubation temperature of the proteoliposomes [28], we carried out subsequent experiments at lower temperatures of 18±0.5 and 16±0.4 °C. As expected, by decreasing the incubation temperature of the sham proteoliposomes from 24 to 16 °C, a slower cytochrome *c* reduction rate was observed, confirming similar observations [20]. At 24 °C we obtained a permeability rate of connexon hemi channel (ΔA/min=0.0048±0.0006, n=6) that was significantly higher (P=0.0003, df=4) than at 16 °C (ΔA/min=0.0007±0.0002, n=3). Thus, permeability changes induced by magnetic field exposure could be masked eventually by the channel reaching its maximum opening under our experimental conditions (24±0.5 °C). Furthermore, to improve the analysis of the kinetic curve, in these latter experiments, we prolonged the monitoring time of the change in the absorbance at 417 nm, after addition of ascorbate, from 2.5 to 15 min.

Interestingly, when proteoliposomes were exposed to the magnetic fields for 60 min while keeping the incubation

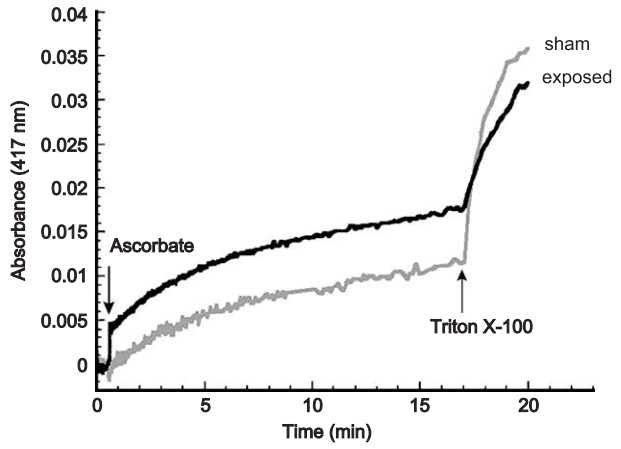

Fig. 5. ELF effects on the connexon channel permeability. Real-time measurements of entrapped cytochrome *c* reduction in proteoliposomes during their exposure to 18 Hz/B_{acpeak} and B_{dc}=70 μT. Earlier exposures of the samples to the field for 60 min at 16±0.4 °C were carried out before starting spectrophotometric measurements. When indicated 0.3 mM ascorbate and 0.2% (w/v) Triton X-100 were added. For clarity the plot of absorbance at 417 nm was arbitrarily reset to 0.

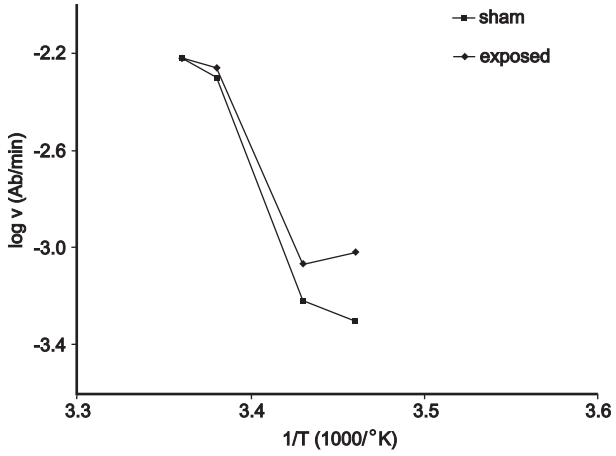

Fig. 7. Effects of 18-Hz magnetic fields at 70 μT on permeability of reconstituted connexons in liposomes. Plots of the permeability rates (log Δ*A*/min) of cytochrome *c* against indicated temperatures (1/*T*) (1000/°C) are presented. Values at 22±0.5 C and 18±0.5 °C are obtained from two experiments. Values at 24±0.5 and 16±0.4 °C are obtained from three experiments as reported in Section 3.

temperature at 16±0.4 °C, an enhancement of the hemi channel permeability resulted, but only for exposure to 18 Hz magnetic field at B_{acpeak}=B_{dc} of 70 μT. A typical curve of changes of absorbance at 417 nm against time (min) is shown in Fig. 5. The addition of Triton X-100 further reduced the entrapped cytochrome *c*, indicating that sham and exposed proteoliposomes were not leaky and functional hemi channels were present.

We also analyzed the raw data by fitting the curve using MathCAD software (Fig. 6). A significant (*P*=0.030) enhancement of the permeability rate from Δ*A*/min=0.0007±0.0002 to Δ*A*/min=0.0010±0.0001 was calculated on three different experiments. Instead no significant differences on the hemi channel permeability resulted in proteoliposomes exposed to 7 and 13 Hz at B_{acpeak}=B_{dc} of 50 μT (data not shown).

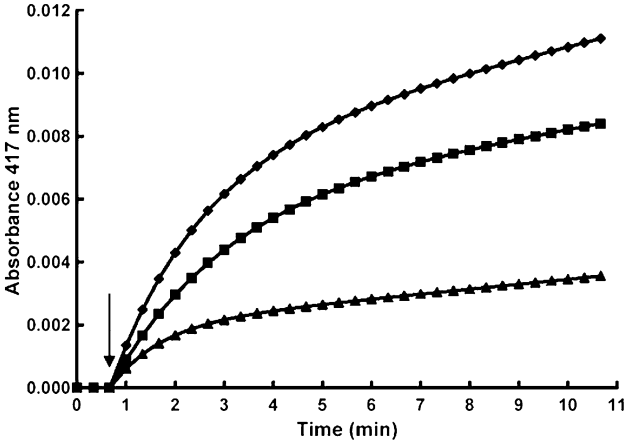

Fig. 6. Effects of 18-Hz magnetic fields at 70 μT on permeability of reconstituted connexons in liposomes at 16±0.4 °C. Plots of absorbance at 417 nm against time (min) of proteoliposomes exposed (–♦) and sham (–■) are shown. Plain liposomes (–▲) are also shown. The arrow indicates the time at which ascorbate was added. The change in absorbance (Δ*A*/min) represents the permeability rate of liposomes to ascorbate. The graph is representative of three experiments. The plots shown are the results of curve fitting the raw data using MathCAD software. The fitting equation was $y = a\left(1 - e^{\frac{-t}{b}}\right) + ct$.

To clarify the role of the incubation temperature in eliciting the above field effect, we exposed proteoliposomes to 18-Hz magnetic fields also at 18±0.5 and 22±0.5 °C. The results are shown in Fig. 7. In exposed proteoliposomes kept at 16±0.4 °C, a significant enhancement of the hemi channel permeability resulted, whereas no significant increment of hemi channel activity was observed in exposed proteoliposomes kept at 22±0.5 or 18±0.5 °C.

Additionally, plain liposomes without reconstituted connexon hemi channels showed no effects induced by magnetic field exposures in all cases (data not shown).

4. Discussion

The results show that exposure to 18-Hz magnetic fields at B_{acpeak}=B_{dc} of 70 μT may enhance the permeability rate of rat liver gap junction channels reconstituted in proteoliposomes.

Plain liposomes without connexon channel reconstituted show no reduction of entrapped cytochrome *c* by externally added ascorbate, whereas proteoliposomes with reconstituted connexon channels show a large reduction of entrapped cytochrome *c* after addition of ascorbate in the bulk aqueous phase. These findings demonstrate that connexin32 formed functional hemi channels in our proteoliposomes, in agreement with previous reports [20,21,23]. However, in this study, slower permeability rates resulted in sham proteoliposomes incubated at room temperature, possibly due to the use of smaller aliquots

(0.15 vs. 0.5 ml) of proteoliposomes in the assay. Alternatively, a different average number of connexin structures per liposome might be obtained in our reconstituted system in comparison to the ones in literature [20,21,23] leading to a slower reduction of the cytochrome *c* present in our proteoliposomes.

Because this reconstituted system makes it possible to study the effect of exposure to magnetic fields on hemi channel function in a more simplified environment than in whole cells, we have set out to test the applicability of our proposed mechanism of interaction between extremely low magnetic fields and membrane [24]. This mechanism studied the transfer of power from a magnetic field to a dipolar structure.

In the reconstituted system used here, a plausible target for the interaction with the field should be charged molecules such as the amine- ($-NH_3^+$) or carboxy- (COO^-) terminals of connexin32, normally localized to the cytoplasmic surface of cell membrane. However, we do not know if the connexons (i.e., six subunits of connexin32) reconstituted in proteoliposomes are an inside-in or an inside-out conformation, thus it is difficult to consider that the abovementioned molecules are involved in the interaction with the field. For this reason, we have firstly considered here that the polar head group $(CH_3)_3$-N^+ of phosphatidylcholine is the dipolar structure involved in the transfer of power by the magnetic fields at the specific frequencies of 7, 13 and 18 Hz, as previously reported [25]. Furthermore, it has been hypothesized that the power transferred can provoke a local motion of this dipole, which in turn can induce a lipid bilayer rearrangement, leading to a final impairment of the liposome permeability. In a similar way, in this study, if a lipid bilayer rearrangement of proteoliposomes is induced by exposure to magnetic fields, consequently a possible impairment of connexon hemi channel permeability could result.

In contrast to the expectation, we did not obtain any effect on the connexon hemi channel permeability when proteoliposomes were exposed at 24 ± 0.5 °C to the magnetic fields tested.

An earlier study [20], using a similar reconstituted system, showed that the activity of the connexons channel exhibits a transition temperature. The authors calculated a transition temperature of 28.2 °C from the Arrhenius plot of reduction rates of entrapped cytochrome *c*, and they suggested that the increase of fluidity of phospholipids in the liposomal membrane might affect the rearrangement of connexin molecules leading to the opening of the hemi channels.

At this time, an important question arises: Can temperature affect the opening of hemi channels in such way as to mask the physical phenomenon induced by the magnetic field on the lipid bilayer? To answer this, we looked for experimental conditions capable of revealing minor changes in the hemi channel permeability, for instance, by decreasing the temperature so that the transport conditions in the test system were not optimal.

In fact, by decreasing the temperature we observed that exposure to magnetic fields can affect gap junction hemi channel permeability. Thus, the lack of 18-Hz magnetic field effect on the channel activity at the higher temperatures could be accounted for by the occurrence of a maximum opening of the channel itself, masking the field effects on the lipid bilayer. Therefore, we cannot exclude the critical role played by the dipole of phosphatidylcholine in relation to the permeability changes observed and the applicability of our model as well [24].

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