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Interaction of gentamicin polycation with model and cell membranes

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ABSTRACT

The interaction of positively-charged antibiotic gentamicin with cell membranes was studied to determine if any changes in membrane organization were induced by the drug. Opossum kidney epithelia (OK) cells were used as models of eukaryotic cells.
Two methods were used: laurdan fluorescence spectroscopy and fluorescence anisotropy recordings on 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH) labeled cell suspensions. Both methods showed an altered membrane hydration and fluidity of gentamicin treated cells. Liposomes prepared from dimyristoyl-phosphatidylcholine (DMPC) mixed with cardiolipin, which mimics the heterogeneous charge composition of the natural cell membrane, were used to determine the effect of gentamicin on artificial bilayers. The membrane lipid packing as revealed by generalized polarization (GP) and fluorescence anizotropy variation with increasing temperature was studied. It was found that the generalized polarization of liposomal membranes containing a negatively charged lipid (cardiolipin) is higher in the presence of gentamicin; in the membrane of living cell (OK), gentamicin induces, on the contrary, a decrease of general polarization. Considering the role of membrane organization in the function of transmembrane channels and receptors, our findings suggest hypotheses that may explain the permeation of gentamicin through the living cell membrane by using these channels.

1. Introduction

Aminoglycosides are hydrophilic polycationic compounds carrying 3 to 5 amino groups which are widely used as antibiotic drugs in the treatment of infections caused by aerobic Gram-negative bacilli. Their polycationic character is mainly responsible for their antibacterial activity [1].

Although the antibacterial activities of aminoglycosides are well understood, their toxicity in eukaryotic cells is not. The prolonged clinical use of aminoglycosides leads to serious complications, mainly due to their nephrotoxicity and ototoxicity [2–6].

While their nephro- and ototoxicity seem to depend on intracellular accumulation of these antibiotics [7–12], numerous studies have also demonstrated the ability of these polycationic drugs to acutely depress synaptic transmission at the neuromuscular junction, presumably by blocking presynaptic voltage-gated Ca^{2+} channels [13,14]. Aminoglycosides block a wide variety of ion channels, such as mechanosensitive ion channels [15–17], purinergic ionotropic channels [18] and nicotinic acetyl-choline receptors [19,20]. The molecular mechanisms by which aminoglycosides block these different ion channels in the cell plasma membrane still remain poorly understood [21,22].

In comparison with other aminoglycosides, gentamicin has lower bacterial resistance [23] and is used as an antibiotic for general purposes; its toxicity is related to its ability to electrostatically bind with negatively charged phospholipids' head groups and, once it has penetrated inside the cell, to interfere with protein synthesis [24]. The ability to interact with protein synthesis by binding to the eukaryotic mitochondrial RNA [25] opens up the perspective of using aminoglycosides in curing severe genetic diseases such as Duchenne muscular dystrophy [26], A hemophilia [27] or in limiting HIV infections [28,29].

As the prospect of treating genetic diseases by intervening in the ribosome machinery of protein synthesis implies using a chronic drug treatment, the effects of gentamicin on various cell properties have to be known and controlled.

The cell membrane is the first biological structure encountered by the drug; the aim of our paper is to identify any changes in the cell membrane induced by gentamicin. To determine this, changes in fluorescence anisotropy and generalized polarization were measured in gentamicin treated culture cells and membrane models (liposomes).

Abbreviations: TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate; DMPC, dimyristoyl-phosphatidylcholine; OHC, outer hair cell; OK, opossum proximal tubule kidney cell; CL, cardiolipin; GP, generalized polarization; *r*, fluorescence anisotropy; PIP2, phosphatidylinositol; AG, aminoglycoside(s); MET, mechanoelectric transducer.

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Small changes in lipid packing and bilayer organization may prove critical for key cell functions such as transport and signaling. Gentamicin also has an impact on the conformation and function of membrane channels and receptor molecules.

2. Materials and methods

Monitoring the thermal behavior of cell membranes was used to provide information about the molecular packing of lipids within the bilayer, and membrane fluidity and integrity in the presence of gentamicin. Using liposomes as simplified membrane models allowed us to reveal particular aspects of drug interaction with the phospholipidic bilayer.

2.1. Cell cultures

OK opossum proximal tubule kidney cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured in antibiotic-free Dulbecco's Modified Eagle's Medium with 2 mM L-glutamine, with 4.5 g/L glucose (PAA, Austria) supplemented with 10% fetal bovine serum (Gibco, Scotland, UK) at 37 °C, 5%CO_2. Subconfluent cells were harvested by trypsination (0.05% w/v Trypsin-4Na^+EDTA, Biochrom AG, Germany).

We used concentrations of gentamicin (Fluka) comparable with those used for in vivo testing of gentamicin's ability to modulate protein synthesis on transgenic animal models suffering from Duchenne muscular dystrophy (up to 500 μg/mL) [26,30,31].

In the case of liposomes, the minimal gentamicin concentration which did not cause the liposome suspension aggregation [32,33] was established in preliminary experiments.

2.2. Laurdan fluorescence spectroscopy of OK epithelial cells

Subconfluent OK cultured cells were washed with PBS, trypsinized and centrifuged at 250×*g* for 10 min; the resulting pellet was resuspended in PBS at a cell density equivalent to an optical density of 0.3 at 450 nm. 2 mL of cell suspension was then incubated with 1 μM (final concentration) laurdan for 5 min and subsequently, with 500 μg/mL (final concentration) gentamicin for 5 min. Laurdan internalization into intracellular membranes was avoided by using this short incubation time [33]. The cell suspension was placed into a fluorometric cuvette with 10 mm optical path length. A Horiba Jobin-Yvon Fluorolog 3 spectrofluorometer with Peltier Quantum thermostated sample holder (TLC50, Quantum Northwest Inc, USA) was used for fluorescence measurements. All measurements were done under magnetic stirring.

Laurdan emission spectra were recorded in the temperature range of 10–40 °C using $\lambda_{ex} = 364$ nm and $\lambda_{em} = 400$–550 nm.

Laurdan is known to be a fluorophore sensitive to the polarity of the environment [34]. In nonpolar media, its emission spectrum is characterized by an emission peak at around 430–440 nm (called "emission in blue", I_B), while in polar media, the emission spectrum presents a maximum at 480–500 nm ("emission in red", I_R). In intermediate situations, the overall recorded spectra will be a mixture of the two components. The so called "generalized polarization" (GP) is defined as:

$$GP = \frac{I_B - I_R}{I_B + I_R} \tag{1}$$

where I_B and I_R are the fluorescence intensities emitted by laurdan at the specific wavelengths for the gel phase (less polar) and liquid crystalline phase of the membrane respectively; the liquid crystalline phase is more polar due mainly to water penetration in the polar part of the lipid bilayer. According to this definition, a higher GP value corresponds to the prevalence of the gel phase, while a lower GP corresponds to the predominantly liquid crystalline phase. GP may vary between +1 and −1.

Main phase transition temperature was considered the inflection point of the sigmoidal fit of the GP vs. temperature curves.

2.3. Fluorescence anisotropy measurements on OK cells

Fluorescence anisotropy of lipophilic probes dissolved in a membrane is directly related to the microfluidity of the lipid environment. The more fluid there is the environment, the smaller is the value of the fluorescence anisotropy.

Fluorescence anisotropy (r) is defined as:

$$r = \frac{I_{VV} - G_{VH}}{I_{VV} + 2GI_{VH}} \quad \text{with} \quad G = \frac{I_{HV}}{I_{HH}} \tag{2}$$

where the fluorescence intensities (I) are recorded in polarized light at $\lambda_{ex} = 385$ nm and $\lambda_{em} = 434$ nm, with polarizers oriented as specified by the indices (V = vertical, H = horizontal); G is a correction factor related to the specific sensitivity for polarized light of the fluorometer.

The fluorescence anisotropy may vary between 0 (for a "perfect fluid" environment) and 1 (for a "perfect rigid" environment). However, some considerations related to the statistics of the excitation and emission processes, limit the anisotropy to lower values ($r_{max} \leq 0.4$).

After preparing the OK cells in the same way as for laurdan fluorescence spectroscopy measurements, they were incubated 2 min with 1 μM TMA-DPH per 2 mL of cell suspension and subsequently, with 500 μg/mL (final concentration) gentamicin for 5 min.

I_{VV} and I_{VH} were measured simultaneously for each sample in the 10–40 °C temperature range.

Both in laurdan fluorescence and anisotropy measurements, the cell viability was checked by a Trypan Blue assay at the end of each experiment and was found to be about 90% compared to the viability before the experiment.

2.4. Liposome preparation and labeling

The liposome preparation procedure was the following: a well defined amount of the appropriate mixture of lipids was dissolved in chloroform (5 mg lipids/mL chloroform) and dried in a round bottomed flask, under a nitrogen stream, at 40 °C and with continuous rotation, to form a uniform film of lipids on the walls of the flask. The lipids were then resuspended in a previously de-aerated PBS at a final lipid concentration of around 10 mM. The milky suspension was sonicated until it became clear and subsequently centrifuged to eliminate possible titanium particles. The procedure led to a very homogenous population of liposomes, with an average hydrodynamic radius of 110 nm (as measured by dynamic light scattering — results not shown).

The concentration of the liposome suspension was adjusted to have an optical density of less than 0.3 at 450 nm in order to minimize the light scattering. The liposomes were stable for a few days if kept at 4 °C, under nitrogen.

Labeling with laurdan (0.1 mM stock solution in DMSO) was done 5 min before each experiment, at 1 μM final dye concentration.

2.5. Statistics

A statistical analysis of experimental data based on two tailed unpaired t-Student test was used.

3. Results

3.1. Laurdan emission spectroscopy

3.1.1. Recordings on liposomes

In order to check if gentamicin interacts differently with charged and neutral membranes, liposomes with two different compositions were prepared: i) using a neutral phospholipid alone (DMPC) and

ii) using a DMPC mixed with CL (a negatively charged lipid), at a molar ratio of 9:1. In Fig. 1 typical laurdan emission spectra of both types of liposomes are shown.

Based on the above spectra, generalized polarization values were calculated, according to Eq. (1). In Fig. 2, the thermal evolution of generalized polarization GP was represented as the difference between GP value at a given temperature [GP(t)] and its value at 10 °C, [GP(10 °C)].

In the case of DMPC liposomes, the presence of gentamicin polycation does not affect the phase transition at all (the experimentally determined phase transition temperature is 25.5 °C, in the presence as well as in the absence of gentamicin); in the case of DMPC + CL liposomes, the phase transition temperature is lower (23.9 °C) and the presence of gentamicin completely suppresses the phase transition (Fig. 2). A statistically significant effect of gentamicin on the GP values ($p<0.05$) is observed only in cardiolipin containing liposomes at temperatures above the characteristic phase transition temperature of DMPC + CL (23.9 °C).

This result suggests an electrostatic interaction between gentamicin and liposome membranes. It shows also an increased resistance to thermal disorganization of the negatively charged liposomes, induced by gentamicin.

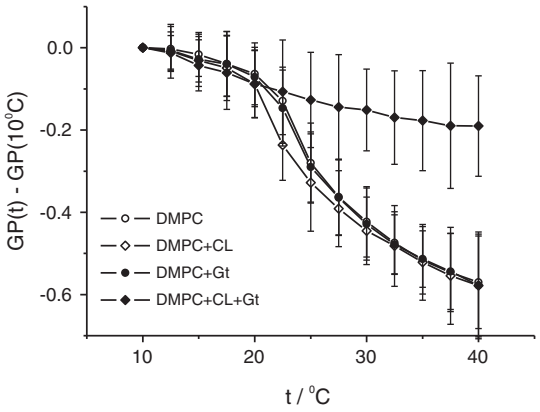

Fig. 2. Temperature dependence of generalized polarization (GP) for liposomes of different composition, with and without gentamicin. Error bars represent the standard deviations calculated for 10 experiments.

3.1.2. Recordings on cultured cells

OK epithelial cells were chosen in our study since these cells are known to be especially sensitive to the aminoglycosides toxicity [1]. Cultured immortal kidney cells serve also as models for auditory hair cells since kidney epithelia share many characteristics with inner ear sensory hair cells, such as the pharmacological sensitivity to aminoglycoside antibiotics and mechanosensitivity [35,36].

Fluorescence emission spectra of laurdan incubated OK cells' suspensions were recorded at temperatures between 10 and 40 °C.

Experiments were performed on 8 different batches of cultured OK cells and typically, there was a difference between the emission spectra of gentamicin treated and control cells as shown below.

In control tests, the emission of laurdan shows a decline of the fluorescence quantum yield with increasing temperature (Fig. 3a).

In gentamicin tests (Fig. 3b), the decrease of the fluorescence quantum yield is less significant and the emission in red (I_R) is situated at higher wavelengths (500–520 nm) as compared to laurdan emission in pure lipid membranes (liposomes). As the temperature increases, there is an increase of the "red maximum", reflecting the membrane thermal disorganization, polar species penetration and relaxation of the excited laurdan molecules due to the created polar environment within the membrane. This effect is much stronger in the presence of gentamicin than in controls and becomes statistically significant at temperatures above 25 °C

There is a difference in the response to gentamicin of artificial and cell membranes: while GP of gentamicin treated DMPC-CL membrane does not change much with increasing temperature (pointing to membrane

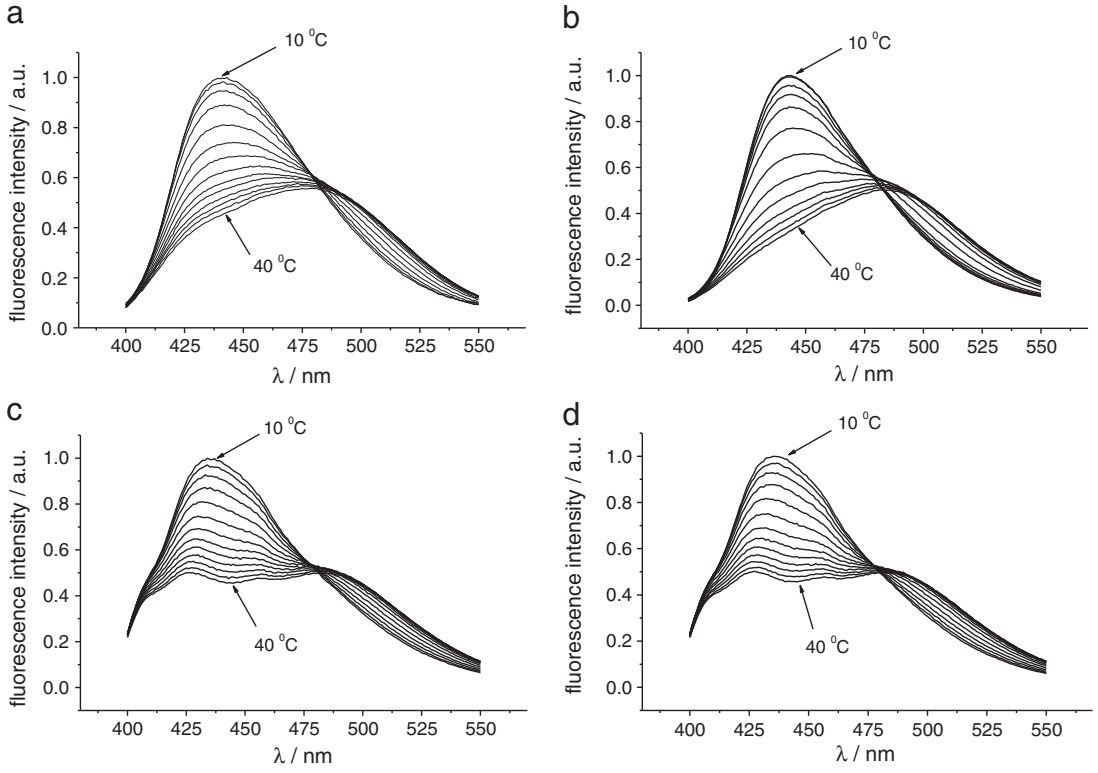

Fig. 1. Normalized emission spectra of laurdan containing liposomes of different composition recorded at different temperatures between 10 and 40 °C. a) DMPC liposomes; b) DMPC liposomes incubated with 500 μg/mL gentamicin; c) DMPC + CL (9:1 molar ratio) liposomes; and d) DMPC + CL (9:1 molar ratio) liposomes incubated with 500 μg/mL gentamicin.

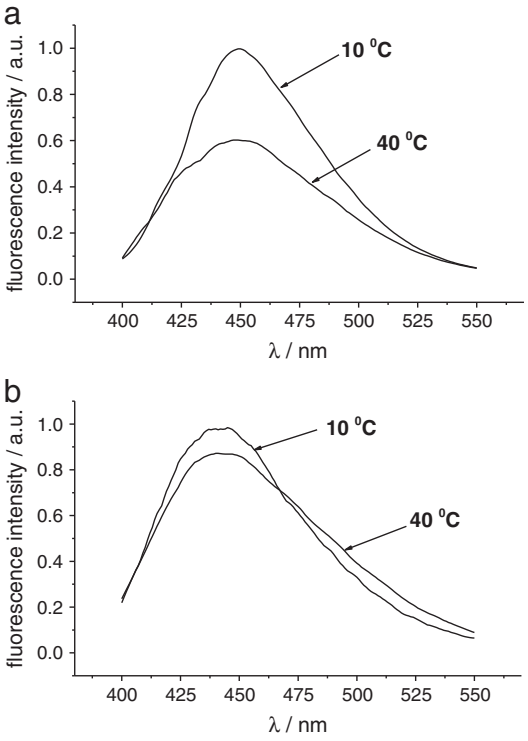

Fig. 3. Emission spectra of laurdan in OK cells. a) Control cells, and b) cells incubated with 500 μg/mL gentamicin. Data are shown only for two extreme temperatures (10 °C and 40 °C) for clearer viewing.

stabilization in the presence of the drug), the opposite trend is observed in membranes of OK cells. This opposite response may be explained by the different bilayer composition and charge distribution of polar heads, the presence of glycocalix and big transmembrane proteins as well as the mechanisms of gentamicin uptake by the living cell.

3.1.3. Fluorescence anisotropy

Fluorescence anisotropy (r) of gentamicin treated and control OK cell suspensions was recorded at temperatures between 10 and 40 °C. Temperature dependence of fluorescence anisotropy was represented as a difference between **r** values at each temperature and r at 10 °C (Fig. 5).

Compared to the controls, gentamicin treated cells were shown to be more rigid at all explored temperatures; this trend becomes statistically significant ($p<0.05$) only close to physiological temperatures (higher than 30 °C) (Fig. 5). As one might expect, in the case of natural cellular membrane, there is no clear phase transition temperature (no inflection point in the **r** dependence on the temperature), due to the chemical complexity of the membrane. The effect of gentamicin (filled circles in Fig. 5) on the fluorescence anisotropy in this case is similar to that exerted on the generalized polarization of CL containing liposomes: suppression of the temperature dependence (Fig. 2, filled diamonds).

The molecular action of the drug we observed (membrane hydration and fluidity changes), may be explained as resulting from neutralization of the negative charges present at the cell surface by polycationic gentamicin. When the positively charged molecules of gentamicin interact electrostatically with the membrane, they compete with water molecules for the negatively charged sites (mainly negative phosphate groups) at the membrane surface. Because the gentamicin molecule is bigger than water molecules and carries more positive charges, its binding energy to the membrane is anticipated to be higher than that of water molecules. As temperature rises, more and more negative sites on the membrane are exposed (due to the increased thermal movement of phospholipids) and, correspondingly, more and more gentamicin molecules attach. This leads to the observed decrease of the generalized polarization of the cytoplasmic membrane.

In controls (where only water molecules penetrate the membrane), the phenomenon is less important, because water molecules are much smaller than gentamicin and their electrostatic interaction with the membrane is weaker. Once they have penetrated the membrane, due to their large volume, the gentamicin molecules hinder the thermal movement of phospholipids and of the fluorescent probe; this is reflected in the observed insensitivity of the fluorescence anisotropy to the sample heating (Fig. 5).

This electrostatic interaction, evoked in many previous investigations of gentamicin treated lipid bilayers (e.g. [32,33,37]), thus seems a good explanation of our observations. It is also supported by earlier observation that Ca^{2+} ions as well as poly-L-aspartic acid (a polyanionic peptide) act as protectors of renal tubules and cochlear hair cells against aminoglycoside toxicity, because Ca^{2+} competes for negatively charged phospholipids groups as poly-L-aspartic acid does for antibiotic's positive charges [37,38]; in addition, Ca^{2+} was shown to annihilate the gentamicin effect on thin lipid films [23].

4. Discussion and conclusions

We studied the effect of gentamicin on the organization of artificial and natural membrane bilayers by observing their thermal behavior in the presence of the drug.

Neutral lipid (DMPC) liposomes and negatively charged vesicles obtained by adding CL to DMPC were used in order to check for the nature of the vesicles' interaction with the drug molecule. As revealed by the comparative behavior of charged and neutral liposomes, the interaction of gentamicin with vesicles containing CL is mainly electrostatical. The thermal behavior of these liposomes was altered by gentamicin, showing a higher GP values at all explored temperatures, while there was no effect on neutral liposomes (Figs. 1 and 2).

The attachment of gentamicin to the CL-DMPC liposomes resulted in the annihilation of the typical thermal behavior of the phospholipid bilayer: in control liposomes, the temperature dependence of GP showed a standard S-shaped curve, while in gentamicin treated vesicles, GP was almost insensitive to temperature (Fig. 2, filled diamonds). The electrostatic interaction of gentamicin polycation with negatively charged phospholipids stabilizes the membrane structure, making it less sensitive to the temperature increase. This may be explained by the well known fact that no sharp phase transition is observed in membranes having a complex chemical composition (the same phenomenon is reported in membranes of any living cell, including OK cells — Fig. 4, open circles).

Although intracellular mechanisms were proposed and demonstrated for the *cellular action* of the gentamicin (accumulation in lysosomes, phospholipidosis, ribosome binding and modulation of protein synthesis), little is known about the specific effect of this drug on the *cell membrane*.

One important feature of the living cell is the presence of membrane potential. Negative inside, it provides a driving force for the drug polycation to challenge the cell entry. It was shown in earlier works on *Staphylococcus aureus* [39,40] that aminoglycoside uptake and its subsequent lethal effect are proportional to the magnitude of the membrane potential. The same was later shown by Marcotti et al. [41] on mouse outer hair cells (OHC); the authors also suggest that the aminoglycoside molecule they used (dihydrostreptomycin) blocked the Ca triggered K^+ channel by entering the cell through this channel.

Our results show that overall, close to physiological temperature (over 30 °C), the presence of gentamicin induces a greater susceptibility of the cell membrane to disorganization and penetration of polar species (water etc.) (Fig. 4); it also makes the membrane less fluid near to the exoplasmic region of the bilayer as shown by TMA-DPH fluorescence anisotropy recordings (Fig. 5). A similar effect of gentamicin was earlier found by ^{31}P-NMR studies on lipid vesicles [32] and on cultured pig proximal tubule-derived cells [42]; the latter used the fluorescence

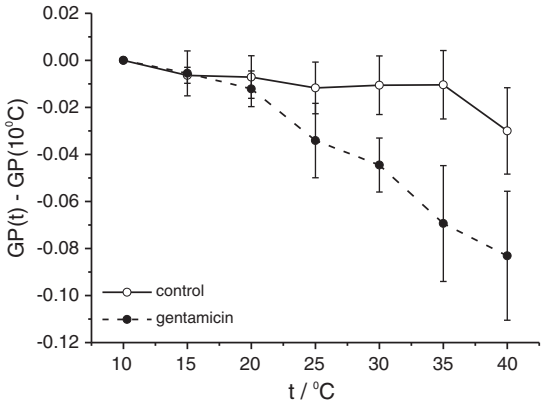

Fig. 4. Generalized polarization of OK cell membranes vs. temperature in control and gentamicin treated samples: (GP(t) — generalized polarization at temperature t, GP(10 °C) — generalized polarization at 10 °C).

recovery after photobleaching technique to compute the fluorophore diffusion coefficient in gentamicin incubated cells.

These observations are in line with experimental studies showing that the opening of TPRV1 channels (present in kidney epithelia, in OHC and vestibular cells as well as in a variety of neurosensory cells), mediates the direct entry of aminoglycosides into cytosol. This fast uptake is *cell selective* and leads to direct chemical interaction with PIP2 (phosphatidylinositol), resulting in the generation of endogenous agonists for new aminoglycoside permissive channels as well as to interaction with mitochondria and ribosomes; as previously shown in the case of liposomes of different anionic lipid composition and in experiments on isolated OHC, gentamicin is specifically binding with high affinity to PIP2 [43–46]. In the living cell, this binding impedes PIP2 hydrolysis and thus the exertion of its physiological role, namely the formation of the second messenger, phosphoinositol triphoshate; the phosoinositide cascade is thus compromised with drastic consequences to the cell.

TPRV1 are non selective cationic channels which may be activated by a wide variety of exogenous and endogenous chemical and physical factors. They were reported to conduct the direct entry of gentamicin by electrophoretic passage of the molecule through cation channels towards the electrically-negative interior of the cell [22]. More recently, the entry of gentamicin through mechanotransducing channels of the outer hair cells was reported [47].

The mechanism by which gentamicin makes these channels to open for the drug passage are not clear. Knowing the mechanism of drug permeation through the cell membrane is important for imagining and designing methods of its limitation. The changes in membrane organization we observed through fluorescence methods are good candidates

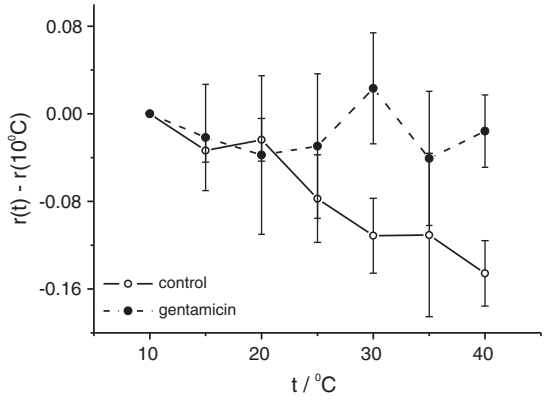

Fig. 5. The influence of gentamicin on OK cell fluorescence anisotropy at different temperatures. Averages at each temperature were calculated from normalized anisotropy values measured in 11 independent experiments.

for the mechanism by which these channels are activated, letting the drug molecules' passage. Humans take antibiotics to kill germs and have to protect as much as possible their body cells, especially if these are extrasensitive to these drugs.

It may be concluded that, at least for the type of cell we have investigated (OK cultured cells), the gentamicin presence induces, most probably, the shielding of the superficial membrane negative charges; this in turn determines a reorganization of the bilayer into a predominantly liquid crystalline phase, more susceptible to the intrusion of polar species. It is important to emphasize that this trend is more significant at physiological temperature, involving, apparently, the active mechanisms of the living cell. The drug induced reorganization of the lipid bilayer, *which we observed*, is likely to trigger the accelerated uptake of gentamicin by TPRV1 and mechanoelectric transducer channels, as a result of the channel protein switching to a favorable conformation, which allows the passage of the drug molecules into the cell; this would be the most important *biomedical* consequence of the drug induced membrane organization changes we report in this paper.

In order to decide on this mechanism, cell viability should be checked in gentamicin exposed and control cells which express TPRV1 and MET receptors comparatively to cells which do not normally express these channels. Research pending in our laboratory is focused presently on these tests as well as on the membrane potential changes induced by gentamicin.

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