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Effect of the exposure to gentamicin and diltiazem on the permeability of model membranes☆

Domenico Trombetta [a], Sabrina Arena [a], Antonio Tomaino [a], Beatrice Tita [b], Giuseppe Bisignano [a], Anna De Pasquale [a], Antonella Saija [a,b,*]

[a] *Department of Farmaco-Biologico, School of Pharmacy, University of Messina, Contrada Annunziata, 98168 Messina, Italy*
[b] *Department of Pharmacology of Natural Substances & General Physiology, University of Rome "La Sapienza", Rome, Italy*

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Abstract

Preliminary observations showed that the calcium-antagonist diltiazem enhances the 'in vitro' bactericidal action of the aminoglycoside gentamicin, especially against Gram-positive bacteria. To verify if a non-specific interaction of these two drugs with biomembranes may play a role in their synergic effect on bacterial cells, we have studied the effect of exposure to gentamicin, in the absence or presence of diltiazem, on the release of carboxyfluorescein (CF) trapped in phosphatidylcholine (PC) unilamellar vesicles (LUVs) used as model membranes. A significant leakage of trapped CF from PC LUVs was registered when liposomes were treated with gentamicin and diltiazem together, employed at doses (50 and 100 μg/ml, respectively) unable to affect CF release if applied alone; the combined effect of gentamicin and diltiazem was synergic and not cumulative. The present findings demonstrate that the simultaneous exposure to gentamicin and diltiazem may induce significant alterations in the permeability of phospholipid membranes and, so, very likely, in functional properties of bacterial membranes, targets of their action. © 2001 Éditions scientifiques et médicales Elsevier SAS

Keywords: Non-antibiotics; Gentamicin; Diltiazem; Liposomes; Carboxyfluorescein

1. Introduction

A variety of compounds called 'non-antibiotics', employed in the management of pathological conditions of a non-infectious aetiology, have been shown to exhibit broad-spectrum antimicrobial activity and enhance the activity of conventional antibiotics against specific bacteria [1]. The proposed mechanism by which they exert this antimicrobial activity is thought to be via effects on the cell membrane permeability.

Particularly, diltiazem has a wide spectrum of pharmacological activities not believed to be related to the blockade of Ca^{2+} channels. In fact, some of its non-specific biological properties may be mediated, partially at least, through a perturbation effect on biological membranes [2].

Furthermore, the mechanism of action of aminoglycosides is related to their ability to inhibit protein synthesis and to produce 'in vivo' and 'in vitro' misreading. However, a non-specific interaction with biomembranes is also well documented as a part of pharmacological actions of aminoglycosides. Particularly, aminoglycosides bind tightly to acidic phospholipids (e.g. phosphatidylinositol), causing a marked decrease in the mobility of the phosphate heads in membrane bilayers [3,4].

Preliminary observations demonstrated that the calcium-antagonist diltiazem enhances the 'in vitro' bactericidal action of the aminoglycoside gentamicin, especially against Gram-positive bacteria [5]. To verify if a non-specific interaction of these two drugs with biomembranes may play a role in their synergic effect on bacterial cells, we have studied the effect of exposure to gentamicin, in the absence or presence of diltiazem, on the release of carboxyfluorescein (CF) trapped in phosphatidylcholine (PC) unilamellar vesicles (LUVs) used as model membranes. In fact, the effect of drugs

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* Corresponding author.
E-mail address: saija@pharma.unime.it (A. Saija).

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on the integrity of membranes is conveniently measured in lipid vesicles with the water-soluble fluorescent marker CF, whose fluorescence is self-quenched during containment in liposomes and leakage in the surrounding medium can be continuously and sensitively monitored [6,7].

2. Experimental

2.1. Drugs used

Crude carboxyfluorescein (Fluka Chemika-BioChemika, Buchs, Switzerland) was purified by the method described by Domingo et al. [8]; the resultant carboxyfluorescein showed no impurities by TLC. L-α-Phosphatidylcholine (from frozen egg yolk), gentamicin sulfate and diltiazem hydrochloride were purchased from Fluka Chemika-BioChemika (Buchs, Switzerland). All other chemicals were of reagent grade.

2.2. Preparation of liposomes

Multilamellar liposomes (MLVs) were obtained by freshly prepared chloroform-concentrated phospholipid solutions. The solvent was removed under nitrogen in a rotoevaporator and the resulting film was kept overnight under vacuum to remove the residual solvent. The dried film was then suspended in buffer (150 mM NaCl/5 mM Hepes, pH 7.4) containing 20 mM CF and the dispersion was vortexed intermittently for 20 min at room temperature. The resultant phospholipid concentration was about 1 mg/ml. LUVs were prepared by submitting the previously prepared MLV dispersion to extrusion through 100-nm polycarbonate membranes (Avestin Inc., Ottawa, Canada) in an extruder system (LiposoFast™ Basic, Avestin Inc.). Free CF was removed by passage of the dispersion through a 1×30 cm column of Sephadex G-50 where the vesicles eluted with the void volume. Aliquots of eluted liposomes were used to determine the amount of phospholipid by the phosphorous assay.

2.3. Carboxyfluorescein studies

The fluorescence technique described by Weinstein et al. [9] was employed to evaluate the permeability of LUVs exposed to gentamicin and diltiazem (added alone or together).

Aliquots of the liposomal stock preparations (diluted to about 20 μg/ml) were incubated with various concentrations of diltiazem or gentamicin or both at room temperature and fluorescence was recorded continuously for 10 min. At the end of each experiment, total CF was determined after lysing the liposomes with 10% Triton X-100 (150 μl); after which the mixture was heated at 100°C for 5 min and mixed vigorously for 2 min at room temperature. Fluorescence measurements were made with a Shimadzu, model RF-5301PC, spectrofluorophotometer (at an excitation and emission slit widths of 1.5 nm). CF was excited at 490 nm and emission was read at 520 nm. All experiments were done in triplicate.

The rates of CF leakage are expressed as percent of total trapped CF released

$$\%\mathrm{CF}_{\mathrm{released}} = \left(\frac{F - F_0}{F_t - F_0}\right) \times 100$$

where F is the fluorescence intensity measured at a specified time, F_0 measured at zero time, and F_t the total fluorescence measured after Triton disruption. F_t is corrected for the dilution introduced by the addition of Triton. Incubation of liposomes with higher concentrations of Triton did not affect the value of F_t, indicating that the procedure resulted in a complete release of dye from the liposomes.

3. Results and discussion

Curves reported in Fig. 1 illustrate the effect elicited by the exposure to gentamicin and diltiazem (employed alone or together) on the CF release from PC LUVs. The results expressed as percent of total trapped CF released in 10 min are shown in Table 1.

The basal rate of CF leakage (expressed as percent of total trapped CF released in a 10 min period) from PC liposomes ranged from 2 to 5%.

Gentamicin (100–750 μg/ml) caused rapid, concentration-dependent CF leakage from PC LUVs; the extent of dye release reached the peak within 4 min and slowly increased thereafter. Moreover, gentamicin was more effective than diltiazem (100–500 μg/ml), which, however, also increased the rate of dye release above the background in a concentration-dependent manner and with a peak within 6 min. A significantly more marked leakage of trapped CF from PC LUVs was registered when liposomes were treated with gentamicin and diltiazem together, employed at doses (50 and 100 μg/ml, respectively) unable to affect CF release if applied alone. As shown in Fig. 1, the combined effect of gentamicin and diltiazem was synergic and not cumulative.

The present findings demonstrate that the simultaneous exposure to gentamicin and diltiazem may induce significant alterations in the permeability of phospholipid membranes and, so, very likely, in functional properties of bacterial membranes, targets of their action.

Taking into account these results, one can speculate that the synergic antimicrobial effect of gentamicin and diltiazem observed on some bacterial strains might be

the result of two processes. It is possible that this drug combination first damages the bacterial membranes, resulting in alterations of membrane functional properties and/or in leakage of intracellular material. Secondly, this membrane damage may enhance the penetration of the antibiotic itself into the interior of the cell.

However, membrane-induced alterations induced by the exposure to the gentamicin/diltiazem association might be dependent on the composition of phospholipid bilayers. Thus, the surface charge of the target membrane might be one of the factors modulating the susceptibility of cell membranes to this drug association, so justifying the observed poor synergic effect of these two drugs against Gram-negative bacteria. Further experiments are in progress to investigate the effects of the exposure to the gentamicin/diltiazem association on the permeability of negatively charged phosphatidylcholine/phosphatidylserine vesicles. In fact, negatively charged liposomes represent a good biomimetic model to investigate the interaction of a drug with Gram-negative bacteria, given that the outer surface of Gram-negative bacteria are endowed with a strong negative charge by the presence of polyanionic lipopolysaccharide.

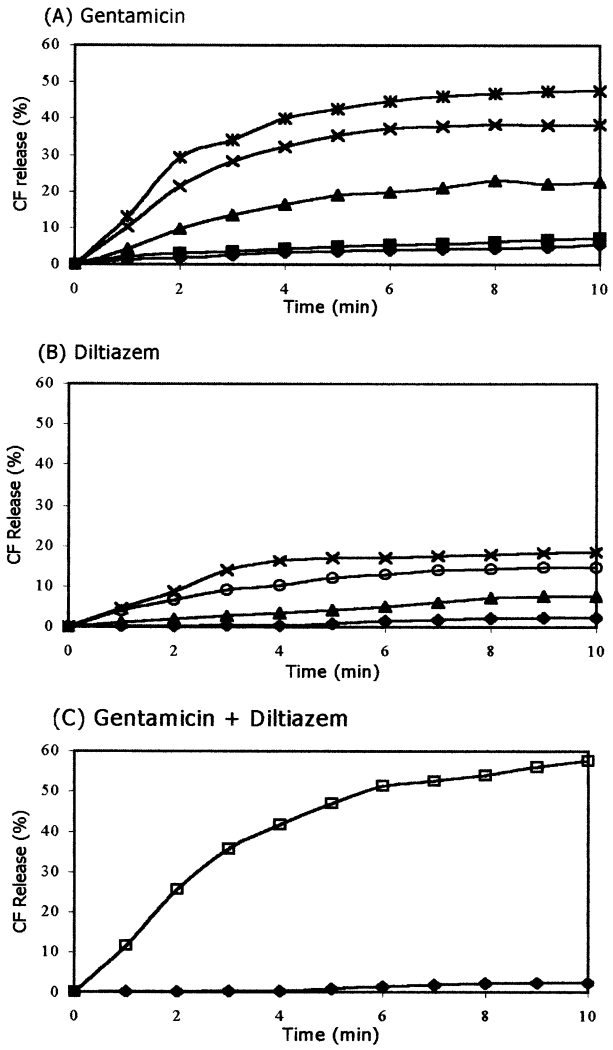

Fig. 1. Effect of the treatment with gentamicin and diltiazem, employed alone or together, on CF release from PC CF-encapsulated LUVs: ◆, control; ■, 50 μg/ml; ▲, 100 μg/ml; ○, 250 μg/ml; ×, 500 μg/ml; *, 750 μg/ml; □, gentamicin 50 μg/ml + diltiazem 100 μg/ml.

Table 1
Gentamicin-, diltiazem- or gentamicin + diltiazem-mediated CF release from CF-encapsulated LUVs (see text for more details)

Treatment	CF release in 10 min (% variation versus control)
Gentamicin (μg/ml)	
50	+1.89
100	+17.16
500	+32.70
750	+42.06
Diltiazem (μg/ml)	
100	+5.25
250	+12.28
500	+16.10
Gentamicin + Diltiazem (μg/ml)	
50 + 100	+55.36

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