0273-2289/00/87/0219/$13.50

Toxicity Assessment of Tamoxifen by Means of a Bacterial Model

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Received May 1, 1999; Revised September 1, 1999; Accepted October 1, 1999

Abstract

A strain of *Bacillus stearothermophilus* was used as a model to study physical perturbations induced in the membrane by the cytostatic tamoxifen (TAM). This study was carried out using two lines of criteria: (1) bacterial growth, and temperature growth range, with determination of growth parameters as a function of TAM concentration; and (2) biophysical studies by differential scanning calorimetry (DSC) and by means of two fluorescent probes to evaluate perturbations promoted by the drug on the structural order of bacterial lipid membranes. The inhibition of growth induced by TAM, the structural bilayer disordering, and the shift in the phase transition temperature to a lower range were also determined in the presence of Ca^{2+}, i.e., a natural membrane stabilizer, to elucidate further perturbing effects of TAM on membranes with putative implications in cell toxicity. Growth inhibition promoted by TAM is potentiated by an increase in growth temperature above the optimal range, but attenuated or relieved by the addition of 2.5 m*M* Ca^{2+} to the culture medium. Consistently, fluorescence polarization and DSC studies showed that Ca^{2+} ions (2.5 m*M*) effectively compensated for the destabilizing effects promoted by TAM in bacterial lipid membranes.

Index Entries: *Bacillus stearothermophilus*; calcium; tamoxifen; bacterial growth; membrane physical effects.

Introduction

In the past decade, the triphenylethylene compound tamoxifen (TAM) has been the subject of intensive research because of its therapeutic interest

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in all stages of breast cancer *(1)* and its potential use as a tumor-preventing agent in women at high risk for breast cancer *(2,3)*. It is becoming evident that TAM and other estrogen antagonists can influence cell proliferation by mechanisms not restricted to the classical estrogen receptor system *(4)*. On the other hand, cellular membranes are important target sites for several anticancer drugs, being interactive with membranes likely involved either in the molecular mechanisms of action or in the cytotoxic side effects of the drugs *(5–8)*. Owing to its high partitioning *(9,10)*, a strong interaction of TAM with membranes may in principle be predicted. The physical integrity and structure of membranes greatly depend on fluidity, which has been associated with modulation of the activity of important functions of proteins in biological membranes *(11)*. The effects of TAM on the physical state of membrane phospholipids have been investigated by means of fluorescent probes of fluidity in several models, either lipid vesicles or native membranes *(10,12)*.

To elucidate the molecular mechanisms of TAM at the membrane level and putative cytotoxic effects, a microorganism has been used as a model *(10)*. Microorganisms are often good experimental tools to probe membrane-mediated toxic effects, because impairment of cell growth usually occurs *(13–16)*, being the growth inhibition dependent on drug concentration and, in most cases, directly related to the partitioning of drugs in membranes *(17)*.

The present study follows the work of previous studies on the effects of TAM on the growth of *Bacillus stearothermophilus (10)*. This microorganism has been selected owing to the strict dependence of growth on the physical state of membrane lipids, as affected by temperature, drugs, and divalent cations *(10,18,19)*.

Divalent cations induce alterations in the physical state of lipids resulting in perturbations of membrane functions *(20)*. However, data concerning the effect of divalent cations on the toxicity of membrane-active drugs are scarce. To understand further the physicochemical basis of TAM-mediated cytotoxicity, TAM perturbations were evaluated on growth and on the physical properties of bacterial phospholipid membranes in the presence of Ca^{2+}, a natural membrane stabilizer *(18,21,22)*.

Materials and Methods

Chemicals

2-[4-(1,2-Diphenyl-1-butenyl) phenoxyl]-*N*,*N*-dimethylethanamine (TAM) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Sigma (St. Louis, MO). 3-[p-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid (DPH-PA) was purchased from Molecular Probes.

Organism and Culture Conditions

The strain of *B. stearothermophilus* used was isolated from discs impregnated with spores supplied by Mast, United Kingdom (lot no. 8879).

Stock cultures were maintained at –80°C. Growth conditions have been previously described *(23)*. Liquid cultures were started with an early stationary inoculum from a medium (dilute L-Broth) with a residual Ca^{2+} concentration (115 μ*M*) and were grown in 1-L Erlenmeyer flasks containing 200 mL of the same medium supplemented or not with Ca^{2+} to a final concentration of 2.5 m*M*. The flasks were shaken at 100 strokes/min in a New Brunswick water bath shaker. TAM aliquots from a concentrated ethanolic solution were added to the growth medium, to yield concentrations ranging from 1 to 10 μ*M*. The bacterial in medium containing TAM had to be grown in silanized Erlenmeyer flasks, because this drug strongly binds to glass material *(9)*. Growth was measured by turbidimetry at 610 nm in a Spectronic 201 spectrophotometer.

Lipid Analysis

Growth was stopped at the beginning of the stationary phase and the cells were harvested by centrifugation. Cells were then washed three times with buffer (10 m*M* Tris-Cl, pH 7.0). The lipids were extracted by the method of Bligh and Dyer *(24)* and quantified by measuring the amount of inorganic phosphate *(25)* after hydrolysis of the extracts at 180°C in 70% $HClO_4$ *(26)*. The polar lipids were isolated by preparative thin-layer chromatography on 2-mm-thick silica gel plates (Merck) developed in acetone and extracted with the mixture of solvents $CHCl_3$:CH_3OH:H_2O (45:45:10 [v/v/v]) followed by a phase separation by mixing the lipid extract with an equal volume of $CHCl_3$:H_2O (1:1 [v/v]). The lower phase was collected and evaporated to dryness on a rotary evaporator, and the lipid residue was dissolved in chloroform (a few milliliters) and stored under nitrogen atmosphere at –20°C.

Liposomes

Aliquots from lipid solutions in $CHCl_3$ (polar lipid extract) containing 1.34 mg of lipid for fluorescence experiments and 4 mg of lipid for differential scanning calorimetry (DSC) experiments were evaporated to dryness on a rotary evaporator and then kept for a few hours under high vacuum to remove traces of solvent. The dry residues were hydrated at 50°C under N_2 atmosphere, by gently shaking with 5 mL of 50 m*M* KCl, 10 m*M* Tris-maleate (pH 7.0), supplemented with 2.5 m*M* $CaCl_2$ (Ca^{2+} liposomes) or without Ca^{2+} supplement (control liposomes). Then, the suspensions were vortexed for 1 min and stabilized overnight.

Fluorimetric Measurements

Two fluidity probes were used: DPH and DPH-PA. DPH in tetrahydrofuran and DPH-PA in dimethylformamide were incorporated into liposome suspensions (345 μ*M* in phospholipid), as previously described *(27)*, to give a lipid:probe molar ratio of about 400. After incubation at 55°C in the

dark for 18–20 h, TAM was added from an ethanolic stock solution, and the mixtures were allowed to equilibrate for 20 min at 37°C, before fluorescence measurements. The fluorimetric measurements were performed with a Perkin-Elmer LS 50 computer-controlled spectrofluorometer. The excitation was set at 336 nm and the emission at 450 nm (3- and 4-nm band pass). All the fluorescence measurements were corrected for the contribution of light scattering by using appropriate blanks without added probes. However, these corrections were generally negligible.

The degree of fluorescence polarization (P) was calculated according to Shinitzky and Barenholz *(28)* from the following equation:

$$P = [(I_{||} - G \cdot I_{\perp})/(I_{||} + G \cdot I_{\perp})]$$

in which $I_{||}$ and $I_{\perp}$ are the intensities of the light emitted with its polarization plan parallel ($||$) and perpendicular ($\perp$) to that of the exciting beam; and G is the correction factor for instrument polarization, given by the ratio of vertically to horizontally polarized emission components when the excitation light is polarized in the horizontal direction.

A high degree of fluorescence polarization, which is a measure of the probe rotational diffusion, means a high structural order or a low membrane fluidity of the probe environment. Note that fluidity is used here in an operational sense and is defined as being inversely proportional to polarization of DPH probes. This fluidity is related but not identical to the physical definition of fluidity in isotropic media.

Differential Scanning Calorimetry

After stabilization, TAM was added from an ethanolic stock solution and the mixtures were allowed to equilibrate for 20 min at 37°C. The lipid dispersions were centrifuged at 450,000*g* for 45 min at 4°C, and the pellets were hermetically sealed in aluminum pans. The DSC measurements were made in a Perkin-Elmer Pyris 1 differential scanning calorimeter at a scan rate of 5°C/min over the temperature range from 10 to 50°C. Data acquisition and analysis were performed using the software provided by Perkin-Elmer. Three distinct temperatures were defined in thermotropic profiles: the temperature of the onset (T_o), the temperature corresponding to the completion of the phase transition (T_f), and the temperature at the endothermic peak (T_m). The onset (T_o) and completion of the phase transition (T_f) were determined from the intersections of the peak slopes with the baseline of the thermograms *(29)*. For each sample, three heating scans were recorded and the mean transition temperatures calculated.

After the experiments, the aluminum pans were opened and the samples dissolved in chloroform:methanol (5:1) mixtures. Phospholipids were quantitated by measuring inorganic phosphate *(25)* released after hydrolysis of the extracts at 180°C in 70% $HClO_4$ *(26)*.

Results and Discussion

Ca^{2+} Relieves Inhibition of Growth Induced by TAM in Cultures of B. stearothermophilus

Populations of *B. stearothermophilus* were used as a tool model to study drug toxicity mediated by physical perturbations at the membrane level, because growth of this bacterium is strongly affected by environmental agents (drugs, ions, and temperature) that perturb the physical properties of membrane lipids *(10,18,19)*. This strain of *B. stearothermophilus* grows in the temperature range of 40 to 70°C (optimally between 55 and 65°C) in a complex medium *(23)*. The addition of 2.5 m*M* Ca^{2+} stimulates growth at supraoptimal temperatures, extending the range of the maximal temperature for growth above 71°C *(23)*.

Table 1 shows the influence of temperature and Ca^{2+} on bacterial growth, under the effect of TAM. The effects on growth were quantitatively evaluated by determination of growth parameters, such as, the lag phase, the specific growth rate, and the cell density reached by the culture at the beginning of the stationary phase. The length of the lag period depends on several conditions, namely the age of the inoculum. However, by using the same inoculum, the comparison of lag times under different growth conditions provides valuable information about growth behavior. Because the variability of this growth parameter in different growth experiments is appreciable, means and standard deviations (SDs) were not determined. According to previous studies, the addition of TAM in a concentration range from 2.5 to 10 μ*M* inhibits bacterial growth as a function of concentration. Growth inhibition with increasing drug concentrations was characterized by a progressive extension of the lag period, a lower specific growth rate, and a lower bacterial yield, as documented in Table 1. These trends could be relieved by the addition of Ca^{2+} (Table 1 and Fig. 1).

Without Ca^{2+} supplementation, 2.5 μ*M* TAM promoted a limited inhibition of growth at 65°C, but induced a significant decrease in the specific growth rate and in the cell density at 69°C and impaired growth at 71°C.

However, growth in a Ca^{2+}-supplemented medium was not sensitive to 2.5 μ*M* TAM at 65 and 69°C; at 71°C, the extension of growth was still significant, and similar to the control in the basal medium without drug. The addition of 5.0 μ*M* TAM to cultures grown at 65°C in the basal medium elicited significant alterations in growth parameters, triplicating the lag time and decreasing the specific growth rate and the cell density to 73.4 and 60% of controls (cultures grown without drug), respectively; at supraoptimal temperatures, growth was completely inhibited. However, Ca^{2+} supplementation of growth medium containing 5.0 μ*M* TAM allowed significant growth at 69°C and limited growth at 71°C. The addition of increasing TAM concentrations (7.5 and 10 μ*M*) to the basal medium impaired growth at all assayed temperatures, but in a Ca^{2+}-supplemented medium, significant growth still occurred at 65°C with 7.5 μ*M* TAM. Although lim-

Table 1
Specific Growth Rates (h^{-1}), Final Cultures Densities (maximum OD), and Lag Times of Cultures of *B. stearothermophilus* Grown in Media Supplemented (+) or Not (–) with 2.5 m*M* Ca^{2+} and with Different Concentrations of TAM at Optimal (65°C) and Supraoptimal (69 and 71°C) Growth Temperatures

Additives to growth medium		Growth temperature						
		65°C			69°C		71°C	
TAM (μ*M*)	Ca^{2+} (2.5 m*M*)	Specific growth rate (h^{-1})[a]	Lag time (min)[b]	Maximum OD[c]	Specific growth rate (h^{-1})[a]	Maximum OD[c]	Specific growth rate (h^{-1})[a]	Maximum OD[c]
0	–	2.303 ± 0.060 (5)	60	0.243 ± 0.013 (2)	1.758 ± 0.210 (2)	0.190 ± 0.020 (2)	1.290 ± 0.150 (2)	0.153 ± 0.003 (2)
	+	2.430 ± 0.176 (7)	40	0.275 ± 0.005 (2)	1.948 ± 0.117 (2)	0.225 ± 0.005 (2)	1.435 ± 0.115 (2)	0.178 ± 0.033 (2)
2.5	–	2.050 ± 0.058 (4)	80	0.225 ± 0.005 (2)	1.379 ± 0.006 (2)	0.138 ± 0.003 (2)	0.0 (2)	—
	+	2.290 ± 0.026 (2)	40	0.255 ± 0.005 (2)	1.794 ± 0.100 (2)	0.215 ± 0.005 (2)	1.200 ± 0.160 (2)	0.153 ± 0.003 (2)
5.0	–	1.690 ± 0.045 (4)	170	0.145 ± 0.005 (2)	0.0 (2)	—	0.0 (2)	—
	+	2.100 ± 0.107 (4)	60	0.235 ± 0.005 (2)	1.318 ± 0.055 (2)	0.175 ± 0.015 (2)	0.869 ± 0.088 (2)	0.118 ± 0.033 (2)
7.5	–	0.0 (2)	—	—	0.0 (2)	—	0.0 (2)	—
	+	2.000 ± 0.145 (5)	90	0.216 ± 0.015 (2)	0.765 (1)	0.15 (1)	0.0 (2)	—
10.0	–	ND[d]	ND[d]	ND[d]	ND[d]	ND[d]	ND[d]	ND[d]
	+	1.280 ± 0.090 (4)	110	0.2 (2)	0.0 (2)	—	0.0 (2)	—

[a]Mean ± SD, with the number of experiments in parentheses. The specific growth rate was determined from a logarithmic plot of the culture pseudoabsorbance (610 mm) against the time in hours. A statistic evaluation of least squares was used to calculate the specific growth rate (h^{-1}) from the slope of the best linear (linear regression) fit to the data points of the exponential growth phase. A specific growth rate of 0.0 means that there was no growth.

[b]The values of lag time refer to a single experiment taken as a typical one, in which different growth conditions were assayed simultaneously. Lag times were obtained by extrapolation of the exponential portion of the growth curves; the point of intersection of the extrapolated exponential phase with the time axis when the OD equals the original inoculum was referred to as the lag time.

[c]The values of maximal ODs correspond to the bacterial concentrations at the end of the exponential phase and were determined by the intersection point of the tangent to the exponential and to the stationary portions of the growth curves.

[d]Not determined.

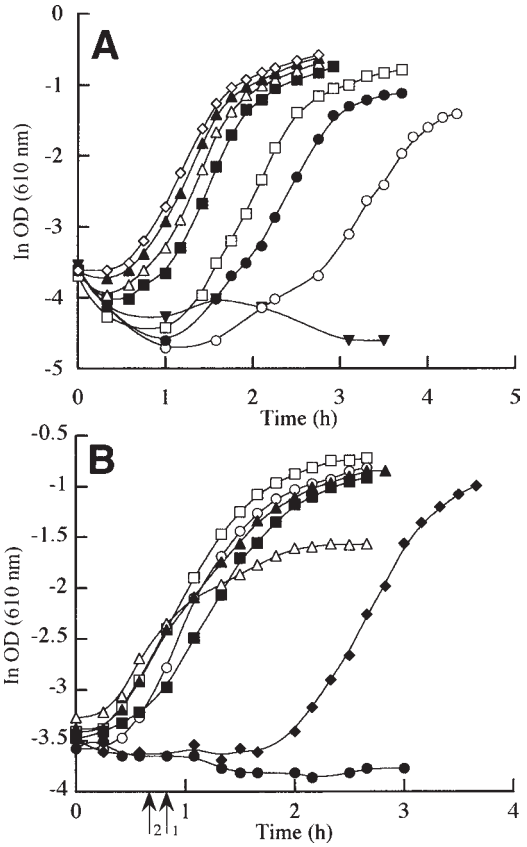

Fig. 1. Effect of Ca^{2+} addition to cultures of *B. stearothermophilus* inhibited by 7.5 μ*M* TAM at 65°C. **(A)** Cells were grown in the presence of 7.5 μ*M* TAM added to the basal medium (▼) or to a medium containing Ca^{2+} at a final concentration of 0.25 m*M* (○), 0.5 m*M* (●), 1.0 m*M* (□), 2.5 m*M* (■), 5.0 m*M* (△), 10 m*M* (▲), and 15 m*M* (◇). **(B)** Cells were grown in the basal medium, without drug added (○), in the basal medium containing 7.5 μ*M* TAM (●), in the basal medium to which 7.5 μ*M* TAM was added (arrow 1) 55 min after inoculation (△), in the basal medium to which 7.5 μ*M* TAM plus 2.5 m*M* Ca^{2+} were added (arrow 1) 55 min after inoculation (▲), in the basal medium containing 7.5 μ*M* TAM to which 2.5 m*M* Ca^{2+} was added (arrow 2) 40 min after inoculation (◆), in a medium supplemented with 2.5 m*M* Ca^{2+} without drug added (□), and in a medium supplemented with 2.5 m*M* Ca^{2+} containing 7.5 μ*M* TAM (■). The results shown are typical of three separate experiments.

ited, growth also occurred in the Ca^{2+} medium at 65°C with 10 μ*M* TAM and at 69°C with 7.5 μ*M* TAM.

The concentration dependence of Ca^{2+} effects on relieving the growth inhibition promoted by TAM was studied at 65°C (Fig. 1A). At this temperature, growth in the basal medium (without Ca^{2+} supplementation) was completely inhibited by 7.5 μ*M* TAM, but the addition of Ca^{2+} relieved the inhibition. The effect of Ca^{2+} was concentration dependent and particularly significant between 0.25 and 2.5 m*M*, eliciting progressively shorter lag

periods, higher specific growth rates, and higher bacterial yields (Fig. 1A). Thus, Ca^{2+} opposed the effect of increasing TAM concentrations. Concentrations of Ca^{2+} above 2.5 m*M* (up to 15 m*M*) had an additional but limited effect on growth: the lag time was slightly reduced and the maximal cell densities and the specific growth rates were slightly increased (Fig. 1A).

The opposite effects of Ca^{2+} and TAM on bacterial growth are comparatively documented in Fig. 1B. The addition of 7.5 μ*M* TAM to a culture in exponential phase (55 min after inoculation) induced an abrupt cessation of growth, being the stationary phase reached for a lower OD as compared to the control culture (grown in a medium without drug). However, when 2.5 m*M* Ca^{2+} was simultaneously added with the drug, growth was only slightly affected, reaching a cell density similar to that of the control.

On the other hand, the inhibition of growth induced by 7.5 μ*M* TAM added to the growth medium on inoculation was reversed by 2.5 m*M* Ca^{2+} added to the culture 40 min after, resulting in a similar growth rate as observed in the control culture with 2.5 m*M* Ca^{2+}. Finally, the relieving effect of Ca^{2+} on growth of a culture inhibited by 7.5 μ*M* TAM could be reversed on chelation with EGTA (data not shown).

Ca^{2+} Effects on Physical State of Bilayers of Bacterial Polar Lipids Perturbed by TAM

The thermotropic behavior of the membrane lipids phase was studied by means of two fluorescent probes of fluidity: DPH and its propionic acid derivative (DPH-PA) and by DSC.

Fluorescence polarization provides a measure of the probe rotational diffusion strongly dependent on fluidity *(28,30,31)*. DPH, a probe buried in the bilayer core *(28)*, and DPH-PA, a probe anchored close to the interfacial region by its charged propionic group *(32)*, were used in this study to compare the physical alterations induced by TAM and Ca^{2+} at different depths across the bilayer thickness.

According to previous findings *(10)*, the addition of 50 μ*M* TAM to bacterial lipid dispersions induces a decrease in fluorescence polarization in the gel phase and along the phase transition temperature range (Fig. 2). This effect was detected by both fluidity probes, indicating a disorder effect induced by TAM across the bilayer thickness. However, in the fluid phase, small disordering effects were noticed in the center of the bilayer, as monitored by DPH (Fig. 2A), whereas a slight condensation of the lipid packing in the bilayer outer regions was detected by DPH-PA (Fig. 2B). TAM disordering effects were similarly induced in bacterial lipid dispersions prepared with or without Ca^{2+}, i.e., in Ca^{2+} and control liposomes. However, the effect of TAM condensation in the fluid phase, detected by DPH-PA, only occurred in control liposomes. The thermotropic profiles of Ca^{2+} liposomes differed significantly from control liposomes, as detected with DPH and DPH-PA. In agreement with previous findings *(18)*, Ca^{2+}-supplemented liposomes exhibited a significant order of increase along the transition temperature range and in the fluid phase (temperatures above the transition)

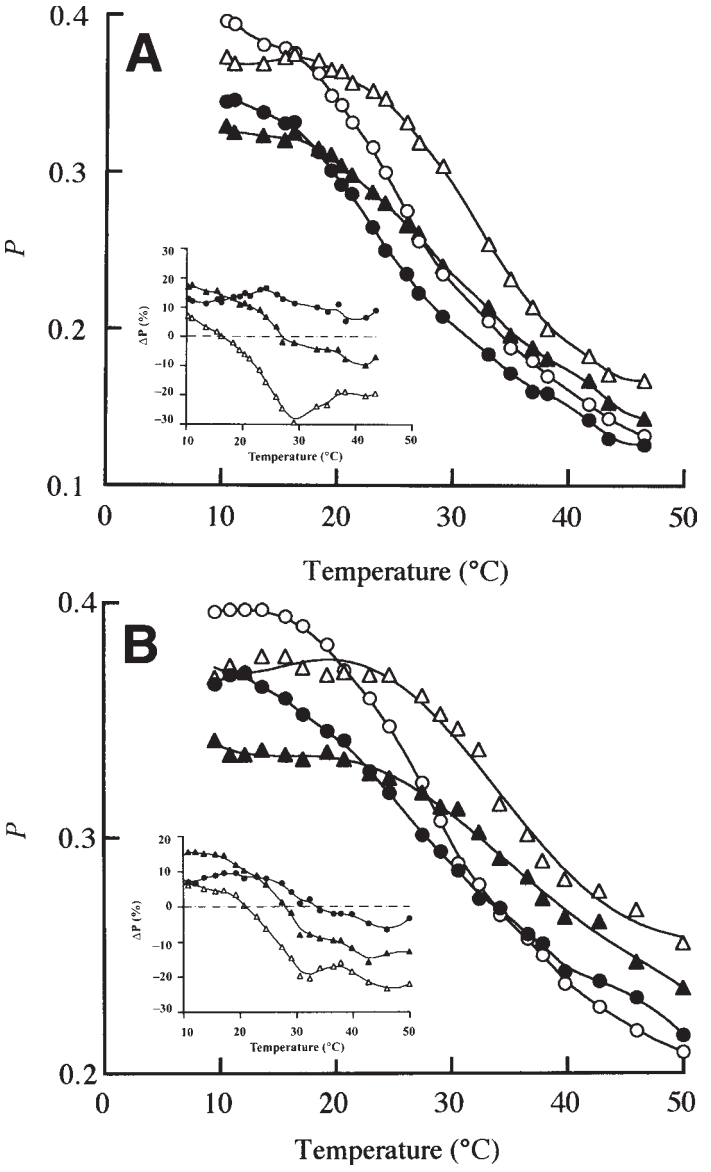

Fig. 2. Thermograms of fluorescence polarization (*P*) of DPH **(A)** and DPH-PA **(B)** in liposomes prepared with the polar lipids of cells grown in the basal medium at 65°C. Control liposomes (○, ●) were prepared in a buffer without Ca^{2+}, and Ca^{2+} liposomes (△, ▲), in a buffer with 2.5 m*M* Ca^{2+}. Control and Ca^{2+} liposomes were incubated without TAM (○, △) or with 50 μ*M* TAM (●, ▲). The thermotropic profiles are typical of three independent experiments. Polarization data are the average of three readings, and error bars are not represented because, for most points, they are encompassed by the size of the symbols. The insets represent the differential polarization (Δ*P*) of DPH (A) and DPH-PA (B) as a function of temperature, for control liposomes incubated with 50 μ*M* TAM (●) and Ca^{2+} liposomes incubated with 50 μ*M* TAM (▲) or without TAM (△). Δ*P*(%) was determined by subtracting the polarization values obtained for the liposome preparations referred to above from those obtained for the control liposomes without drug supplement.

Table 2
Characterization of Phase Transitions Detected by DSC in Control Liposomes and Ca^{2+} Liposomes Incubated with or without 50 μ*M* TAM

Sample	T_m (°C)	T_o (°C)	T_f (°C)
Control liposomes	31.9	22.7	37.1
Control liposomes + 50 μ*M* TAM	30.3	21	35.8
Ca^{2+} liposomes	36	24	40.6
Ca^{2+} liposomes + 50 μ*M* TAM	35.3	23.5	39.7

and a slight disordering in the gel phase (temperatures below the transition). Although the main effect of TAM was the disordering of the bilayer structure of Ca^{2+} liposomes and control liposomes, the general order of Ca^{2+} liposomes remained higher than that of control liposomes in the presence of TAM, over all of the transition temperature range and in the fluid phase. This event reported by DPH and DPH-PA suggests an effective counteraction of the disordering effects of TAM by Ca^{2+}. Accordingly, differential plots (Fig. 2, insets) showed that the addition of TAM to control membranes (without Ca^{2+} supplementation) induced positive Δ*P* values, over all of the temperature range as reported by DPH (Fig. 2A) and for the temperature range of 10–33°C, as reported by DPH-PA (Fig. 2B). Conversely, for liposomes prepared in a Ca^{2+}-supplemented buffer, negative Δ*P* values were observed over the transition temperature range and in the fluid phase, indicating opposite effects of TAM and Ca^{2+} in the membrane physical order. Consistently, in the same temperature range, the dispersions of bacterial lipids in the presence of Ca^{2+} and TAM showed an intermediate behavior, as compared to those prepared in the presence of Ca^{2+}, without TAM, and incubated with TAM in the absence of Ca^{2+}.

DSC studies were performed with the bacterial polar lipids to characterize further the gel-to-liquid-crystalline phase transition. According to previous studies *(18)*, the addition of Ca^{2+} to the preparation medium of liposomes (Ca^{2+} liposomes) induces a pronounced shift in the endothermic peak to higher temperatures (Table 2). By contrast, the incubation of liposomes (control and Ca^{2+} liposomes) with TAM (50 μ*M*) produced a shift of the endotherm to lower temperatures (Table 2). Because the deviation of the phase transition temperature range induced by TAM was smaller as compared to the deviation induced by Ca^{2+}, the phase transition of Ca^{2+} liposomes incubated with drug remained at higher temperatures than that of control liposomes incubated with the same concentration of drug. These results are consistent with those obtained by fluorimetric techniques showing opposite effects of Ca^{2+} and TAM on the structural order of liposome bilayers.

Opposed effects exerted by Ca^{2+} and membrane-active agents that increase lipid fluidity (e.g., benzyl alcohol) on membrane-associated functions (e.g., the activity of adenylate cyclase of hepatocyte membranes) were interpreted as evidence of a Ca^{2+}-induced compensatory effect on lipid structural order *(22)*. Ca^{2+} has a direct and rapid effect on lipid packing of rat hepatocyte plasma membranes, decreasing the freedom of motion of lipid molecules as a consequence of binding to anionic sites of the lipid bilayer, probably the polar head groups of acidic phospholipids. This effect, varying linearly in the range of 0–4 m*M*, is reversed by the subsequent removal of Ca^{2+} by EDTA *(22)*. Similarly, we observed that relief of growth inhibition in TAM cultures could be readily suppressed by the addition of EGTA (data not shown), indicating that removal of calcium by chelation is sufficient to cancel its protective effect. This fact, associated with the need for millimolar concentrations (≥2.5 m*M*) to obtain the maximal Ca^{2+} effects on growth improvement, strongly suggests that Ca^{2+} action involves a reversible alteration in the physical state of the membrane. If intracellular events were involved, a low Ca^{2+} concentration would be required because it is kept very low within cells *(33)*. A similar requirement for high Ca^{2+} concentrations (above 2.5 m*M*) was observed for the improvement in growth at supraoptimal temperatures *(23)*.

On the other hand, the extension of growth improvement by Ca^{2+} in TAM-stressed cultures depends on temperature (Table 1). Thus, 2.5 m*M* Ca^{2+} relieved growth inhibition induced by 7.5 μ*M* TAM at 65°C, but, under the same conditions, only limited cell growth was induced by Ca^{2+} at 69°C and no relieving effect was observed at 71°C. Because Ca^{2+} and temperature have direct and opposed effects on the physical state of lipids *(18)* and on bacterial growth *(23)*, it is conceivable that opposite effects of Ca^{2+} and TAM on growth are also the consequence of Ca^{2+} counteraction of the physical perturbations induced by TAM in bilayer lipids.

Although the liposome model is useful in the interpretation of drug perturbations in membranes, direct extrapolation of results to complex native membranes must be done cautiously. Interaction of TAM and membrane lipids has been extensively demonstrated with different lipid systems and native membranes *(12,34,35)*, indicating that the lipid bilayer is a putative locus to account for TAM cytotoxicity, and, essentially, our data are in agreement with these previous conclusions. An important physical event often associated with the presence of divalent cations, namely Ca^{2+}, is the induction of isothermic lateral phase separations in membrane systems containing charged lipids *(36–39)*. The coexistence of solid and fluid domains, induced by interactions of Ca^{2+} with acidic phospholipids, changes the percolative membrane structure and the mobility of certain membrane components within the connected percolated phase, which may have functional significance *(40)*. According to the hydrophobic-matching principle, which establishes the need to match the hydrophobic regions of integral membrane proteins and their host bilayer lipids, Ca^{2+}-induced alterations of the lipid bilayer structure in the vicinity of an integral mem-

brane-bound protein is a putative physical mechanism for controlling protein function *(40)*.

On the other hand, induction of lipid-protein mismatch and subsequent inhibition of membrane functions was proposed as a hypothetical mode of action of certain lipophilic xenobiotics *(41)*. Therefore, Ca^{2+}/TAM antagonistic effects on the bacterial growth may also be related to a Ca^{2+} effect on the lipid-protein interfaces, via lipid structural changes, which eventually compensates for the TAM-induced lipid-protein mismatch. It is significant that Ca^{2+} can induce important modifications of lipid shape geometry, namely of diphosphatidylglycerol *(42)*, which accounts for 26 mol% of the total phospholipid of the membranes of our bacterial model *(18)*. These changes in geometry (from cylindrical to conical shape) could be important to the improvement of packing and lining up of polar and nonpolar groups of lipid and proteins, minimizing lipid-protein mismatch. However, this hypothesis predicts TAM-localized effects taking place on limited membrane domains specifically involved in essential membrane and cell functions. Therefore, TAM concentrations that impaired cell growth could be well below those affecting the physical properties of the bulk lipid, which are reported by fluidity fluorescent probes and DSC. This would explain the difference in concentrations (by a factor of 10) proven effective in growth inhibition (2.5–10 μM) and in fluidity of bacterial lipid membranes (25–100 μM).

Conclusion

The bacterial growth impairment induced by TAM is presumably related to membrane disordering effects promoted by the cytostatic. Consistently, TAM inhibitory effects on growth were potentiated by the deviation of growth temperature from the optimal temperature range and were attenuated by the addition of Ca^{2+} to the growth medium, up to 2.5 m*M*. An increase in growth temperature above the optimal temperature range had a destabilizing effect on the membrane lipid bilayer in addition to the perturbing effect of TAM, explaining the stronger inhibition of growth induced by TAM at supraoptimal temperatures. By contrast, the addition of Ca^{2+} stabilized the membrane, counteracting inhibition of bacterial growth induced by TAM.

Although our observations strongly suggest a direct Ca^{2+} effect mediated by an increase in the structural order of the membrane, other mechanisms including the induction of lateral phase separations cannot be excluded. The coexistence of an indirect Ca^{2+} effect, involving biochemical alterations of the membrane lipid composition, is currently under study.

Acknowledgment

This work was supported by Praxis XXI grants Praxis/2/2.1/BIO/1156/94, Praxis/2/2.1/SAU/1400/95, and Praxis JNICT (PBIC/C/BIO/1162/92).

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