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INTERACTION OF THE CALCIUM ANTAGONIST FANTOFARONE WITH PHOSPHOLIPIDS: ELECTROSTATIC EFFECTS

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Abstract—The binding of fantofarone, a novel calcium channel antagonist, to cytoplasmic membranes and lipid vesicles has been studied by means of its fluorescence. The binding characteristics (dissociation constant K_d and total number of binding sites B_{max}) were determined using saturation isotherms. In brain synaptic and cardiac sarcolemmal membranes, fantofarone binds to a single site with a K_d value of $\approx 1.4 \times 10^{-6}$ M and a B_{max} value of ≈13 fantofarone molecules bound per 100 lipid molecules. Using vesicles made from egg phosphatidylcholine (PC), fantofarone was shown to possess a $K_d \approx 7.5 \times 10^{-6}$ M and a $B_{max} \approx 15$. When other classes of naturally-found lipids were incorporated into PC vesicles, a decrease in K_d with no modification in B_{max} was observed for all acidic lipids studied. The decrease in K_d was inhibited by sodium and calcium. None of the experimental conditions modified the spectral properties or lifetimes of fantofarone. We conclude that an electrostatic interaction between fantofarone and negatively charged lipids takes place at the surface of the membrane and this interaction explains the decreased K_d value (increase in affinity) observed in cytoplasmic membranes.

Key words: fantofarone; electrostatic interaction; lipid; calcium channel antagonists

Drugs acting on calcium channels have been studied extensively over the last two decades. Although normally there are thought to be three principal classes of drug binding sites at the L-type calcium channel, there is now increasing evidence to suggest that there is a larger number of binding sites [1]. This has been suggested on the basis of the results obtained with new ligands chemically unrelated to each other [1]. Among these new compounds, the indolizine sulfone, fantofarone†, has been shown to be a potent antagonist of the L-type calcium channel [2–6]. This compound is a potent antagonist of potassium-induced contractions in rat aorta with an IC_{50} value of approximately 6 nM [2–3] and binds with a high affinity to the α_1-subunit of the L-type calcium channel at a specific binding site (K_d values between 0.08 and 0.037 nM) [2, 3, 5, 6]. In addition, fantofarone is the sole antagonist of the L-type calcium channel able to discriminate between the two activities supported by the α_1-subunit, namely the voltage-sensor and the calcium pore [7].

Fantofarone is positively ionized at biological pHs (its pK_a is 8.7 [8]) and hydrophobic. The binding of the molecule on plasma proteins [9] and lipids [10] has been studied by means of its fluorescent properties. In the latter studies, a clear increase in affinity of fantofarone for lipids extracted from erythrocytes over phosphatidylcholines has been observed. This observation prompted us to study further the interactions of fantofarone with lipids. This was done by varying the composition of lipid vesicles. We found that the increased affinity of natural lipid mixtures is due to an electrostatic interaction between fantofarone and negatively charged lipids. This electrostatic interaction between fantofarone and negatively charged lipids is of importance as this may promote the binding of fantofarone to the cytoplasmic membrane and consequently to the L-type calcium channel [11].

MATERIALS AND METHODS

Phospholipids (PC, PS, PE, PA, PI, CL, SM), lipids (OL) and CH were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Fantofarone (SR 33557) was obtained from the Sanofi Chemical Department. All other chemicals were analytical reagent grade from commercial sources.

Vesicle preparation. MLVs were prepared by vortexing a film of dry lipids deposited on the wall of a glass vessel in the presence of the appropriate buffer (Tris–HCl, 20 mM, pH 7.4) at room temperature. The resulting MLV were then freeze-thawed five times employing alternative liquid nitrogen and warm water cycles [9]. The MLV were subsequently transferred in a device (Lipex Biomembranes Inc., Vancouver, Canada) which allowed the extrusion by eight successive passes through 0.1 μM pore size polycarbonate filters (Nucleopore) [12, 13].

Membrane preparation. Highly purified heart sarcolemma was prepared from adult rat heart,

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† Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; fantofarone, 2-isopropyl-1-((4-(3-(*N*-methyl-*N*-(3,4-dimethoxy-β-phenethyl)amino)propyloxy)-benzenesulphonyl))indolizine; PC, egg phosphatidyl-choline; PS, L-α-phosphatidyl-L-serine; PE, L-α-phosphatidyl-ethanolamine; PA, L-α-phosphatidic acid; PI, phosphatidylinositol; CL, cardiolipin; SM, sphingomyelin; OL, oleic acid; CH, cholesterol; MLV; multilamellar vesicles.

following the method previously described [14]. In essence, the purification procedure consisted of collagenase digestion, treatment with KCl-pyrophosphate, differential centrifugations, and fractionation using a sucrose density gradient. Synaptosomes were prepared from adult rat brain as described by Abita *et al.* [15]. The purification procedure consisted of several steps of differential centrifugations followed by fractionation using a sucrose density gradient. The sarcolemma and synaptosomes were frozen in liquid nitrogen and stored at −80° in a 5×10^{-3} M Tris–HCl buffer (pH 7.4) containing 250×10^{-3} M sucrose and 1×10^{-4} M dithioerythritol. Phospholipid content of the sarcolemma, the synaptosomes and the vesicles was determined [16] in order to adjust the final concentration to the required value.

Measurement of fluorescence properties. All fluorescence measurements were obtained using either a scanning spectrofluorometer or a sub-nanosecond phase fluoremeter, both from SLM Instruments Inc. (Urbana, IL, U.S.A.) as described previously [10]. These measurements were carried out using monochromators in both the emission as well as the excitation channels. Fluorescence lifetimes (τ) were calculated by the phase shift and modulation methods using modulation frequencies of 6, 18 and 30 MHz [17]. A solution of 2,2′*p*-phenylene*bis*(5-phenyl)oxazole in absolute ethanol ($\tau = 1.35$ nsec) or DPH in heptane ($\tau = 6.72$ nsec) was used as reference. As substrates such as fantofarone fluoresce much more intensively when bound than when free [9, 10], the concentration of bound fantofarone at each point was calculated using the following equation [18]:

$$\text{Fantofarone}_b = \frac{F_o}{F_m} \times 30,$$

where fantofarone$_b$ is the concentration of bound fantofarone, F_o is the observed fluorescence and F_m is the maximum fluorescence of a solution containing 30 μM fantofarone. The maximum fluorescence of the solution of fantofarone was determined by titration with the lipid vesicles at a wide range of concentrations (0.05–1.5 mg lipid/mL). Using [^{3}H]-fantofarone, it was determined that the passive adsorption of fantofarone did not exceed 2% of the total molecule concentration. Saturation isotherms were analysed by a computer-assisted method of non-linear regression, based on the Clark equation [19]:

$$[\text{B}] = \frac{B_{\max}[F]}{K_d + [\text{F}]}.$$

The density of binding sites ($B_{\max}$ expressed as the number of bound fantofarone molecules per 100 lipid molecules) and the equilibrium dissociation constant (K_d expressed in molar terms) were calculated, taking into account the experimental values of B (specifically bound ligand) and of F (free ligand).

RESULTS

In a preliminary study, the original observation, namely the increased affinity of fantofarone for the lipid compound of the erythrocyte, was verified and amplified. Adsorption isotherms were performed using rat heart sarcolemma membranes and guinea pig brain synaptic membranes. Analysis of the isotherms indicated that fantofarone binds to a single site with the following characteristics $K_d = 1.3 \pm 0.4 \times 10^{-6}$ M, $B_{\max} = 13.8 \pm 2.0$ for the sarcolemma and $K_d = 1.5 \pm 0.5 \times 10^{-6}$ M, $B_{\max} = 11.2 \pm 1.8$ for the synaptic membranes (mean ± SD, N = 3). The total number of binding sites is expressed as the number of fantofarone molecules bound per 100 lipid molecules. These data are comparable to the values obtained using erythrocytes [10].

Table 1. Effect of lipid composition on the characteristics of fantofarone binding*

Vesicle composition (M/M)		N	Binding Characteristics K_d†	$B_{\max}$‡
PC		5	7.5 ± 0.6	15.3 ± 1.1
PC/PS	(85/15)	4	1.1 ± 0.6	14.6 ± 1.7
PC/PE	(85/15)	3	3.1 ± 0.5	14.0 ± 1.5
PC/SM	(85/15)	4	10.0 ± 0.8	16.1 ± 0.9
PC/CH	(60/40)	4	15.0 ± 1.4	17.5 ± 2.5
PC/PS/CH	(53/17/30)	4	3.5 ± 0.8	15.5 ± 0.9
PC/PA	(85/15)	3	2.0 ± 0.6	15.7 ± 0.6
PC/PI	(85/15)	3	3.5 ± 0.7	15.4 ± 1.2
PC/CL	(85/15)	3	2.0 ± 0.5	18.1 ± 1.6
PC/OI	(80/20)	3	3.5 ± 0.8	15.9 ± 1.8

* Results are shown as the mean ± SD of N experiments.
† $K_d \times 10^{-6}$ M.
‡ $B_{\max}$: number of fantofarone molecules bound per 100 lipid molecules.

In a second study, the effects of main lipid classes (PS, PE, SM, CH, PA, PI) present in the cytoplasmic membrane on the binding characteristics of fantofarone were studied by incorporation of a fixed amount of these lipids in vesicles made of PC. Adsorption isotherms were constructed and analysed to calculate the equilibrium dissociation constant (K_d) and the number of fantofarone binding sites expressed as the number of molecules bound per 100 lipid molecules ($B_{\max}$). The results shown in Table 1 indicate that none of the added lipids modified the number of fantofarone bound molecules but that the equilibrium dissociation constant K_d was affected in general. With the exception of SM, all other lipids investigated modified the K_d values: CH was the only lipid to increase the K_d value while all others decreased the K_d value with respect to the K_d value for the binding of fantofarone to PC. In ternary mixtures comprised of PC, CH and PS, the K_d value is decreased as compared to the K_d for PC alone (Table 1).

Representative Scatchard plots are shown in Fig. 1. These indicate that fantofarone binds to one single type of site. This has been verified for all lipids studied (data not shown). Also the shape and the position of the maxima of both excitation and emission spectra are not modified when compared to the reference spectra taken in PC vesicles. For further comparison, excitation and emission

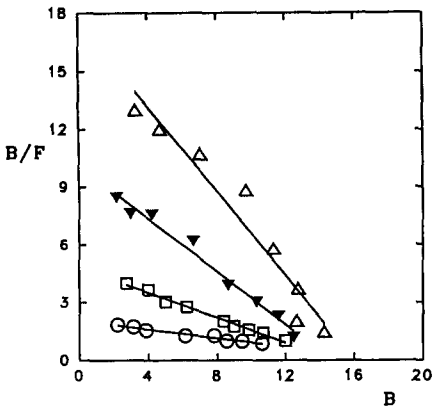

Fig. 1. Effect of phospholipid composition on the binding of fantofarone to vesicles. Vesicle composition (M/M): PC(100, ○), PC/PA (85/15, ▼), PC/PE (85/15, □), PC/PS (85/15, △). Experimental conditions: fantofarone 1×10^{-6}–3×10^{-5} M, phospholipids: 1.3×10^{-4} M, buffer, Tris–HCl, 20 mM, pH 7.4. The data are from one typical experiment.

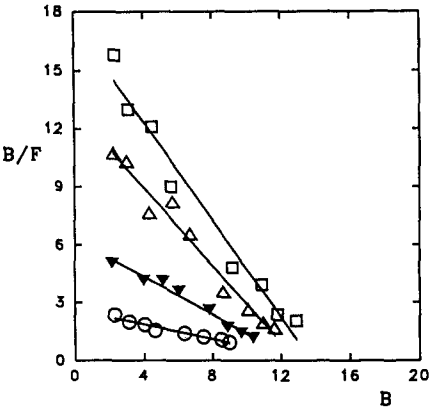

Fig. 3. Effect of increasing amount of phosphatidylserine on the binding of fantofarone to vesicle. Vesicle composition (M/M) PC (100, ○), PC/PS (92.5/7.5, ▼, 85/15, △, 77.5/22.5, □). Experimental conditions: fantofarone 1×10^{-6}–3×10^{-5} M, phospholipids: 1.3×10^{-4} M, buffer, Tris–HCl, 20 mM, pH 7.4. The data are from one typical experiment.

fluorescence spectra of fantofarone in methanol were also included in Fig. 2. With the exception of the amplitude, there is no modification of excitation and emission spectra of fantofarone in lipid vesicles (either PC or PC/PS) as compared to the organic solvent.

In a third series of studies, one selected phospholipid (PS) was incorporated at various concentrations in the PC matrix in order to study the effects of this parameter on the characteristics of fantofarone binding: within the range of PS (0 to 22.5%) incorporated in the PC vesicles, there was

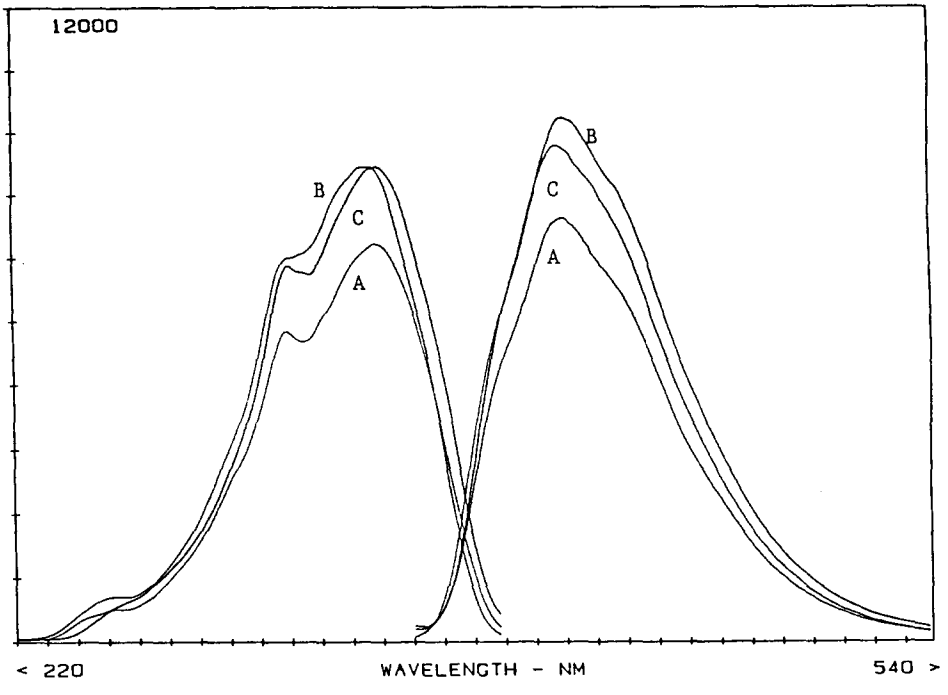

Fig. 2. Excitation (left) and emission (right) fluorescence spectra of fantofarone in the presence of PC (A) and PC/PS (85/15) (B) vesicles and in methanol (C). The spectra were recorded under identical conditions using 10^{-5} M fantofarone solutions. The total phospholipid concentration was 1.3×10^{-4} M in Tris–HCl 20 mM, pH 7.4 buffer.

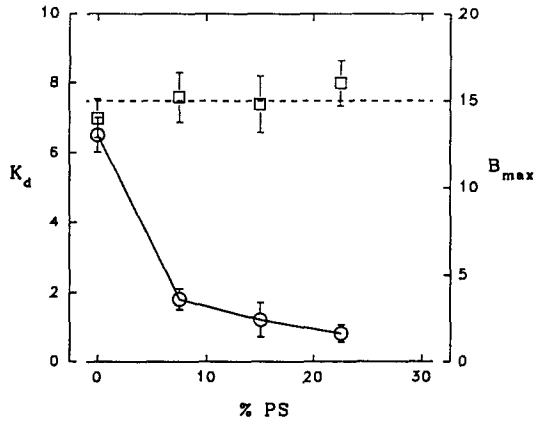

Fig. 4. Binding characteristics of fantofarone to PC/PS vesicles as a function of PS concentration. Right ordinate (○): $K_d \times 10^{-6}$ M, left ordinate (□): B_{max}, number of fantofarone molecular bound per 100 lipid molecules. The data are the mean ± SD of 3–4 determinations.

Table 2. Effect of lipid composition and cations on the characteristics of fantofarone binding*

Vesicle composition (M/M)	+ions†	N	Binding characteristics K_d‡	B_{max}§
PC	—	4	7.3 ± 0.3	14.9 ± 2.9
	+Na^+ 134	3	12.0 ± 0.5	16.9 ± 2.3
	+Ca^{2+} 10	3	21.0 ± 1.0	19.0 ± 2.2
PC/PS	—	3	0.9 ± 0.3	14.2 ± 1.8
(85/15)	+Na^+ 134	3	2.5 ± 0.6	14.2 ± 2.0
	+Ca^{2+} 10	3	11.0 ± 0.9	16.0 ± 2.1

* The data are the mean ± SD of N experiments.
† Ion concentration × 10^{-3} M.
‡ $K_d \times 10^{-6}$ M.
§ B_{max}: number of fantofarone molecular bound per 100 lipid molecules.

no variation in the total number of binding sites (B_{max} = 14–16) while there was a gradual decrease in K_d (PS, 0%, $K_d = 6.5 \pm 0.5 \times 10^{-6}$ M; PS, 7.5%, $K_d = 1.8 \pm 0.3 \times 10^{-6}$ M; PS, 15%, $K_d = 1.2 \pm 0.5 \times 10^{-6}$ M; PS, 22.5%, $K_d = 0.8 \pm 0.2 \times 10^{-6}$ M; mean ± SD, N = 3) (Figs 3 and 4). A similar experiment was performed with either PA or OI incorporated to the same range of concentration; the results were comparable to those obtained with PS. The fluorescence lifetimes of fantofarone bound to lipid vesicles made of either PC or PC/PS (85/15) has been determined. In the two vesicles, the results obtained are comparable. The fluorescence lifetime of fantofarone has two-well defined components. The predominant fluorescence lifetime component (τ_1) of 13.3 ± 1.4 nsec for PC and 12.7 ± 1.4 nsec for PC /PS represents 80–90% of the time resolved decay (mean ± SD, N = 3–4). The second lifetime component (τ_2) of 6.6 ± 1.0 nsec for PC and 6.1 ± 1.5 nsec for PC/PS represents 20–10% of the time resolved decay (mean ± SD, N = 3–4).

In a fourth series of studies, the effects of two cations (Na^+ and Ca^{2+}) on the binding characteristics of fantofarone to vesicles made of either PC or PC/PS were determined (Table 2). Incubation with either Na^+ (134 mM) or Ca^{2+} (10 mM) had no effect on the number of bound fantofarone molecules (Table 2). By contrast, the cations increased the K_d values in the two vesicle systems with Ca^{2+} having a more pronounced effect than Na^+. The fluorescence lifetimes of fantofarone bound to lipid vesicles made of either PC or PC/PS in the presence of either Na^+ or Ca^{2+} has been determined. In all the experimental conditions studied, the results are comparable. The fluorescence lifetime of fantofarone was best characterized by two components: τ_1 of 13–15 nsec representing approximately 85% to the time resolved decay and τ_2 of 4–6 nsec representing approximately 15% of the latter.

DISCUSSION

The results presented in this study demonstrate the effects of the lipid bilayer composition on the binding properties of the new calcium antagonist fantofarone. Neither excitation and emission fluorescence spectra, nor fluorescence lifetimes, were affected by any of the various experimental conditions used in this study, indicating that the changes in fluorescence intensity only reflect changes in the binding characteristics of fantofarone to the lipid vesicles.

In a previous article [10], we showed that for a series of PC's, the binding characteristics of fantofarone are insensitive to the length of the hydrocarbon chain and to their degree of saturation. In this paper, the incorporation of lipids other than PC in the PC bilayer had pronounced effects on K_d values and no effect on the number of binding sites. A series of lipids including PE, PS, PA, PI, CL and OI decreased the value of K_d. All these lipids have one characteristic in common: they are negatively charged at neutral pH [20]. Thus an electrostatic interaction between the negatively charged lipid and the positively charged fantofarone takes place at the surface of the membrane; this interaction increases the affinity of fantofarone for the lipid matrix. The effect is unspecific in that it is observed in the case of a series of negatively charged lipids bearing a large variety of polar head structures. Also, the number of binding sites is not modified by the negatively charged lipid species nor by its concentration, which would be expected in the case of a specific lipid–fantofarone interaction. Thus the charge present at the surface of the membrane is more important than the structure of the polar head.

This conclusion is reinforced by two observations. Firstly the decrease in K_d is related to the amount of negative charge. The K_d value is close to its minimal value when there is 15% of negatively charged lipids in the PC matrix. This corresponds to the optimal random dispersion of a negatively charged lipid within a neutral PC matrix (the lamellar liquid crystalline phase) [21]. This corresponds also to the maximal number of fantofarone molecules bound to the vesicles. Secondly, cations are able to increase the K_d values (a decrease in affinity). The

cations were used at concentrations well below their K_d for phospholipids [22]; thus they are unlikely to compete with fantofarone for a specific binding site. Rather their general effect is to screen out the electrostatic charge. Electrostatic phenomena associated with a charged membrane are described by the Gouy-Chapman equation:

$$\sigma y^2 = 2000\ \varepsilon_o \varepsilon_r RT \Sigma_i C_{i,\,eq.} [\exp - z_i F_o \Psi_o / RT - 1],$$

which relates the charge density, σ, to the surface potential Ψ_o, where $C_{i,eq.}$ is the concentration of species i (i.e. Na^+) in the bulk aqueous phase, z_i is the signed valency of species i and F_o the Faraday constant. As expected from the presence of the signed valency z_i in the Gouy-Chapman equation, divalent cations are much more potent than monovalent ions (Ca^{2+} vs Na^+ in this manuscript) in decreasing the K_d values.

Among the lipids tested, CH was the only lipid to have an opposite effect on K_d: CH decreased the K_d of fantofarone for the lipid matrix. We have no explanation for this observation. Among the lipids tested, CH is the only lipid to lack positive or negative charge; an electrostatic interaction cannot explain the effect of CH. Rather, it could be hypothetized that CH impairs the affinity of fantofarone for the lipid membrane by a general effect on lipid fluidity or by the localization of the hydroxyl moiety at the level of the supposed binding site of fantofarone [10]. It is striking to note that, in ternary mixtures made of PC, CH and PS, the electrostatic effect is dominant since the K_d of fantofarone is decreased as observed in the absence of CH.

A membrane pathway for the binding of dihydropyridine calcium channel antagonists to the L-type calcium channel has been proposed by Rhodes and coworkers [23]. This two-step pathway postulates that specific binding of the dihydropyridines to a site on the calcium channel is preceded by partitioning into and diffusion through the plasma membrane bilayer of the target cell. This proposal has received several lines of experimental support (see 23 for a review). Among these studies, some have indicated that the molecular interaction of dihydropyridines with the membrane bilayer can be correlated with certain functional parameters. It is known that fantofarone also interacts with the L-type calcium channel [2–8]. The compound is lipophilic, penetrating into the lipophilic lipid bilayer [10]. By analogy with the dihydropyridines, a membrane pathway could be hypothetized for fantofarone. In this respect, an electrostatic interaction between the positively charged drug and acidic lipids which increases affinity will promote such a pathway. In addition, electrostatic interaction could provide a mode of selection for the target membrane, affinity for charged membranes like the cytoplasmic membrane [11] and/or the mitochondria being increased with respect to uncharged or less charged membranes.

In conclusion, an electrostatic interaction between negatively charged lipids and fantofarone has been found. This interaction leads to an increase in affinity of fantofarone for the membranous lipids with no modification of the maximal number of bound molecules.

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