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pH-DEPENDENT INFLUENCE OF MEMBRANE-INCORPORATED FLUNARIZINE ON Ca-BINDING TO PHOSPHATIDYLSERINE MONOLAYER MEMBRANES

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Abstract—The pH-dependent ^{45}Ca binding to phosphatidylserine monolayers was investigated. Ca binding increased with increasing pH. Between pH 10 and pH 11 a steep increase of Ca binding could be observed. This increase was interpreted to be due to complex Ca binding opposed to ionic binding at low pH. Flunarizine added to the spreading solution of the monolayer dose dependently displaced up to 100% Ca at pH 5 independently of phospholipid packing. At pH 11 less than 20% of Ca could be displaced by flunarizine. Intermediate results were found at pH 7. Flunarizine displaced less Ca from dense than from loosely packed monolayers at pH 7. The results suggest two binding states of flunarizine: ionic binding at low pH and apolar binding at high pH. The latter is much less effective in displacing Ca from phosphatidylserine monolayers. The Ca displacing properties of charged flunarizine may prevent a deleterious phospholipid reorientation within the membrane induced by the intracellular Ca rise during ischemia.

Flunarizine is used as an agent protecting the brain from the deleterious effects of ischemia. Besides its effect on cerebral hemodynamics the drug is discussed to directly improve the survival of cerebral cells during an ischemic insult. It has been proposed that the often observed massive rise of intracellular free Ca concentrations during severe ischemia triggers a reorganisation of membrane ultrastructure [1]. This membrane alteration is thought to be a major factor determining irreversibility of ischemia. Ca binding to negatively charged phospholipids is considered to play a key role in this process [2]. The objective of this study was to characterize the interaction of flunarizine with phosphatidylserine (PS) in terms of Ca binding and charge of flunarizine.

Flunarizine is known to displace Ca from acidic phospholipid model membranes [3] like other positively charged amphiphilic drugs [4–7]. The pK_a of 7.7 (Janssen Pharmaceutica, Analytical Department, 1980) suggests the coexistence of uncharged and positively charged flunarizine incorporated into artificial and biological membranes. Therefore, Ca displacement from phosphatidylserine (PS) monolayers by flunarizine was investigated pH dependently. The drug was spread simultaneously with the phospholipid, because flunarizine is nearly insoluble in the aqueous subphase at high pH.

MATERIALS AND METHODS

^{45}Ca is a weak β-emitter. Radioactivity is heavily quenched by fluids or solids but less by air. This enables one to measure upper layers of a ^{45}Ca solution by a Geiger–Müller counter located directly above the surface. The difference of the radioactivity of an aqueous ^{45}Ca-solution before and after spreading an insoluble monolayer represents the radioactivity bound to the monolayer.

The subphase consisted of 2 mmol/l TES (*N*-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid)-histidine and 5 mmol/l NaOH. pH was adjusted to the desired range by addition of HCl. The Ca concentration amounted to 1×10^{-5} mol/l. The free Ca concentration did not change between pH 5 and pH 11 as measured by an ionsensitive electrode (Radiometer, Copenhagen). ^{45}Ca was added to result in a specific activity of 0.6 Ci/mol. Phosphatidyl-serine (PS; Sigma Chemical Company, Munich, F.R.G.) was dissolved in chloroform containing 0.5% methanol. The spreading solution for the monolayer amounted to 1 mg lipid/ml and was stored under nitrogen at −70°. Flunarizine (Flu) was directly added to the spreading solution. Film density was adjusted to 80, 100 and 120 Å^2/PS.

Thirty millilitres of the subphase buffer were filled into a circular Teflon dish with 100 mm inner diameter. Radioactivity was determined before and 10 min after spreading of the monolayer by a methane flow tube with an endwindow directly above a sampler changer (Berthold, Wildbad, F.R.G.). Readings were taken after 10 min, when rapid changes had ceased. Ca binding did not change for the next hour for more than 10%.

The temperature was kept constant at 23°. For the determination of the absolute amount of Ca bound to the monolayer, the system was standardized by samples of known specific activity [8]. Therefore, molar Ca/PS ratios could be calculated. Surface tension of monolayers at constant area was determined by a tensiometer (Meßgeräte-Werk Lauda, Lauda-Königshofen, F.R.G.) equipped with a platinum Wilhelmy plate. Rapid changes in readings ceased at least 15 min after formation of the monolayer

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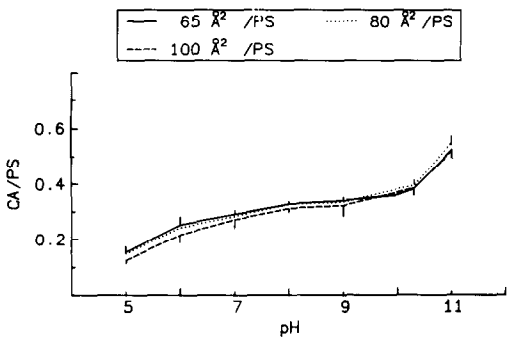

Fig. 1. Ca-binding to phosphatidylserine (PS) monolayers as a function of pH. ^{45}Ca binding is given as molar ratio. Film density ranged from 65 to 100 Å^2/molecule PS. Each point represents the mean of 5–10 experiments.

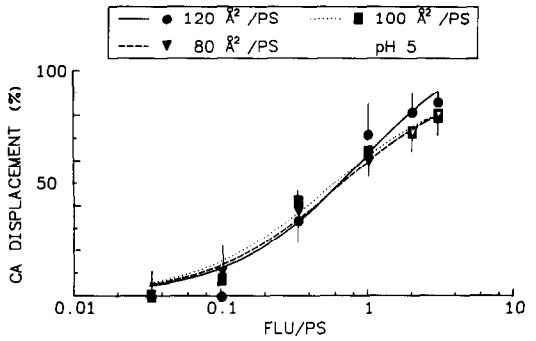

Fig. 3. % Ca-displacement from phosphatidylserine (PS) monolayers at pH 5 upon increasing amounts of flunarizine (Flu) incorporated into the film. Drug content is given as molar ratio Flu/PS upon spreading. Correction due to desorption of drug from the film was not applied to the abscissa. For further details see text. Monolayers were spread at 80, 100 or 120 Å^2/molecule PS. Symbols represent mean ± SD (N = 5–6). Lines are the optimal fit of the data to a hyperbolic dose–response curve.

irrespective of film composition. Surface tension did not decrease for more than 8% for the next hour.

For the determination of flunarizine loss from the drug containing monolayer (Flu/PS = 1) ^{3}H-flunarizine (56 Ci/mol) was added to the spreading solution. Samples (500 μl) were drawn from the subphase with a stationary cannula at the bottom of the Teflon dish. Radioactivity was determined by liquid scintillation counting (Beckmann Instruments). The sampling syringe was rinsed with methanol and the radioactivity in the washing fluid was added to the sample counts.

Data were fitted to a hyperbolic dose response curve by a non-linear least square computer program [9].

RESULTS

pH-dependent binding of Ca to phosphatidylserine (PS) monolayer membranes is given in Fig. 1. Ionic binding increases over the entire pH range. Above pH 10 a steep increase in Ca binding could be observed. Only minor influence on the Ca/PS ratios was found upon decreasing film density from 65 Å^2/molecule PS to 100 Å^2/molecule PS.

The loss of ^{3}H-flunarizine from the film is given in Fig. 2. A maximal desorption of drug could be observed after 45–60 min. There is evidence for a delayed equilibrium at pH 11 (Fig. 2). The losses at 60 min were not significantly different ($P < 0.05$) from each other. At 10 min, when all Ca binding or Ca displacement data were obtained, the loss of flunarizine amounted to 20% or less. This value gives an estimate of the error in the abscissa for Figs 3–5.

At pH 5 flunarizine completely displaces Ca from PS monolayer films independent of film packing (Fig. 3). Fitting the data to a hyperbolic dose–response curve revealed that the half-maximal Ca displacement is not significantly ($P > 0.05$) influenced by monolayer density. At pH 7, flunarizine is not able to displace Ca completely from a film with 80 Å^2/molecule PS (fitted maximal Ca displacement: 66.8%) or 100 Å^2/molecule PS (fitted maximal Ca displacement: 85.6%). At 120 Å^2/molecule PS Ca can be completely displaced (fitted maximum: 104.8%). Fitting the data to a dose–response curve reveals that the half-maximal Flu/PS ratio is not significantly ($P > 0.05$) influenced by film density (Fig. 4). At pH 11 flunarizine displaces less than 20% of bound Ca (Fig. 5). The data cannot be properly described by a hyperbolic dose–response curve. This

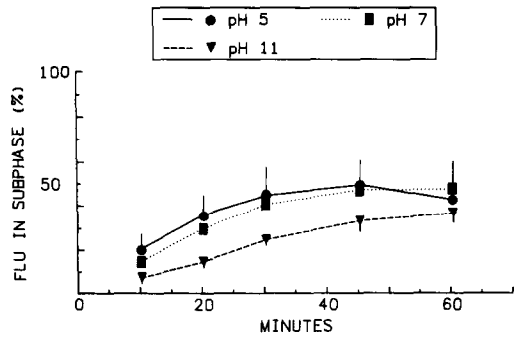

Fig. 2. Time-dependent % total loss of ^{3}H-flunarizine from a phosphatidylserine (PS) monolayer at 100 Å^2/PS containing ^{3}H-flunarizine (Flu) in a 1 : 1 molar ratio. For further information see text. Monolayers were spread at pH 5, pH 7 and pH 11. Symbols represent mean ± SD (N = 5–6).

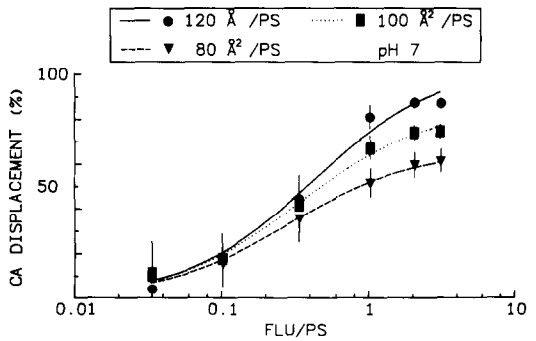

Fig. 4. Ca-displacement from phosphatidylserine monolayers by flunarizine at pH 7. For further details see Fig. 3.

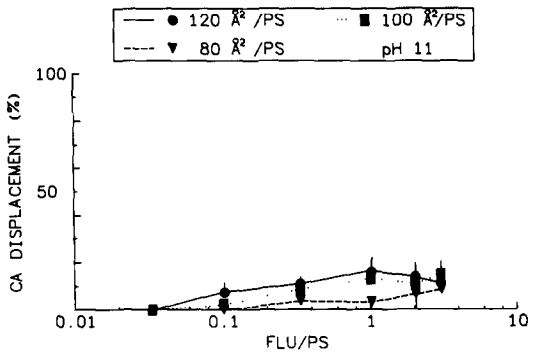

Fig. 5. Ca-displacement from phosphatidylserine monolayers due to flunarizine at pH 11. For further details see Fig. 3.

may be due to the experimental error resulting from low maximal Ca displacement under this condition.

Incorporation of Flu into PS films consistently shows higher surface pressures for mixed films than for pure films (Fig. 6). The extent of the increase in surface pressure is equal for the two highest Flu/PS ratios employed in the whole study. The increase in surface pressure due to the drug has about the same order of magnitude for all three pH employed (Fig. 6).

DISCUSSION

The pH-dependent Ca binding curve is in excellent agreement with the binding curve reported by Lüllmann *et al.* [6], which was performed under very similar conditions. However, the curve established by Lüllman *et al.* [6], ends at pH 10. Above pH 10 a steep increase in Ca binding was observed under the present conditions. This is thought to be due to unmasking of a complex binding site by high pH. The pK_a of the amino group involved in complex Ca binding [10, 11] is a function of the ionic composition of the subphase (for discussion see Ref. 12). Therefore, the inflection point for the appearance of the complex Ca binding site differs somewhat with experimental conditions. The data presented here are in line with results reported by Rojas and Tobias [13] and Seimiya and Ohki [8].

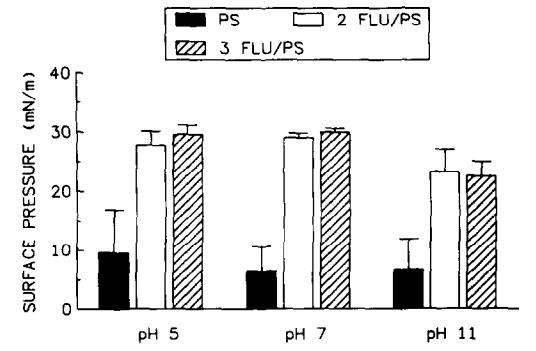

Fig. 6. Surface pressure of pure PS films and mixed Flu/PS films at different pH. PS density was in all experiments 100 Å²/molecule. Given are means ± SD.

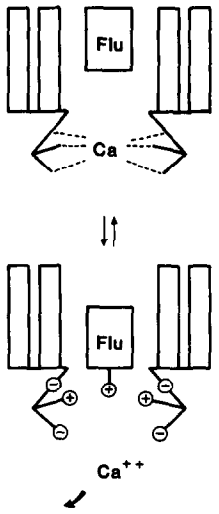

Fig. 7. Model demonstrating apolar binding and Ca displacement of charged flunarizine (Flu) in phosphatidylserine (PS) monolayers. The hydrocarbon part of PS is given by bars. The charges of the phosphate, the amino and the carboxylic moiety at pH 7 are shown additionally.

A possible explanation for the effective Ca displacement (i.e. disappearance from the surface) might be that the Flu/PS complex solubilizes at low pH and mimicks Ca displacement. However, this is contradicted by the observation that the surface pressure of mixed films is much higher than of pure PS films, i.e. mixed films are packed more densely. Furthermore, the about equal increase in surface pressure at pH 5, pH 7 and pH 11 suggests a very similar monolayer composition. Although desorption of the drug from the film is somewhat higher at low pH, about 80% of the drug is retained at pH 5 vs 93% at pH 11 (the amount of desorption is given for the time of measurement for Ca displacement). Considering the dose–response curve this difference cannot explain the poor Ca displacing activity of flunarizine. It is concluded that solubilisation of a flunarizine–PS complex cannot account for the observed differences.

At the condition involving primarily an ionic binding site (pH 5) the data can be described by a dose–response curve, suggesting competition of the positively charged flunarizine with Ca binding sites (e.g. see Ref. 14). All of the Ca could be displaced independently of the amount of PS packing. At the condition primarily involving a complex binding site (pH 11) flunarizine was poorly effective in displacing Ca from the PS monolayer. Flunarizine may be expected to be uncharged at pH 11 ($pK_a = 7.7$; Janssen Pharmaceutica, Analytical Department, 1980) so that in contrast to pH 5 a hydrophobic interaction with the membrane prevails. At an intermediate pH of 7 flunarizine could not displace all Ca from the monolayer under any conditions, suggesting the coexistence of the active charged and the hardly active uncharged form of flunarizine. Hydrophobic bonds are more effective in a dense monolayer and may favour the uncharged drug. A schematic presentation is given in Fig. 7. Additionally, Ca dis-

placement may be inhibited by more firm Ca binding at 80 Å^2/molecule than at 120 Å^2/molecule as described for the Ca binding to phosphatidylinositol [5]. The latter two effects may both explain the low efficacy of flunarizine to displace Ca from closely packed monolayers at pH 7.

The hypothesis of Verkleij and Post [2] suggests that Ca induces a lipid reorientation within the membrane, which subsequently loses its barrier function. The results reported here may indicate that charged flunarizine prevents Ca binding to negatively charged phospholipids. This may protect the membrane from lipid reorganisation and may explain antiischemic properties of flunarizine.

In conclusion, the results reported here propose that flunarizine can bind and interact with phosphatidylserine monolayer membranes in two ways: the ionic binding state (at low bulk pH) can completely displace Ca from the monolayer whereas the uncharged binding state (high bulk pH) is poorly effective in this respect. Alternatively, the observation could also be explained as a result of an insensitivity of the Ca–PS complex to flunarizine. In the light of the hypothesis of Verkleij and Post [2], only charged drug may possess antiischemic properties.

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