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SULOCTIDIL–PHOSPHOLIPID INTERACTION IN BILAYER MEMBRANES: EFFECTS ON LIPID DYNAMICS

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Abstract—The effects of suloctidil on the dynamic properties of dipalmitoyl-DL-α-phosphatidylcholine and dipalmitoyl-DL-α-phosphatidylcholine–phosphatidylserine in bilayer systems have been studied by fluorescence polarization using 1,6-diphenyl-1,3,5-hexatriene as a fluorophore. A downward shift of the transition temperature of DPPC† in the presence of suloctidil and a general decrease in the fluorescence depolarization values suggest that suloctidil destabilizes the bilayer. These effects show a linear relationship with the amount of suloctidil present in the bilayer. Changes in the structure of the drug, pH of the buffer, lipid composition together with the measurement of the amount of suloctidil in the bilayer allow evaluation of the partition coefficient of the drug and suggests an electrostatic interaction between the drug and the acid phospholipid. The probable localization of suloctidil in the bilayer is discussed. These observations confirm the proposed mode of action of suloctidil deduced from its effect on Na^+/K^+ ATPase activity and on membrane fluidity of rat brain synaptosomes.

The efficacy of suloctidil in the treatment of cerebral and peripheral insufficiences and their pathologies [1, 2] is probably related to an effect on the membranes. This was a conclusion of the mode of action of suloctidil on erythrocyte membranes [3], platelets [4] and on synaptosomes [5].

A comparison of the effect of suloctidil on Na^+/K^+ ATPase activity and on lipid fluidity, indicated that suloctidil inhibits synaptosomial ATPase by affecting the stability of the lipid bilayer. However, there was no direct correlation between the inhibition of Na^+/K^+ ATPase and the increase in membrane fluidity induced by uncharged analogues of suloctidil. Therefore, the existence of an electrostatic interaction with acidic phospholipids necessary for Na^+/K^+ ATPase activity was proposed, this interaction occurring after solubilization in the lipid matrix [5].

The study of such an electrostatic interaction is the aim of the present study. A simple membrane model was chosen to study the interactions between suloctidil and phospholipids: multilamellar vesicles (MLV) or sonicated unilamellar vesicles (SUV) consisted of phosphatidylcholine or mixtures of phosphatidylcholine–phosphatidylserine. The effect of suloctidil and related compounds was evidenced by fluorescence polarization of a hydrocarbon probe (1,6-diphenyl-hexatriene, DPH) buried in the apolar region of the bilayer [6]. All observations are related to the concentration of suloctidil in the membrane. The use of tritiated suloctidil allowed quantitative radioactive measurements of its penetration within the membrane.

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† Abbreviations: DPH: 1,6-diphenyl hexatriene; DPPC: dipalmitoyl-DL-α-phosphatidylcholine; MLV: multilamellar vesicles; PS: phosphatidylserine; SUV: small unilamellar vesicles.

MATERIALS AND METHODS

Materials

Dipalmitoyl-DL-α-phosphatidylcholine (99%) (DPPC), L-α-phosphatidylcholine from egg yolk (type VII-E) (Egg PC) and phosphatidylserine (PS) have been purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and were used without further purification.

Suloctidil, CP 556 S (erythro-*p*-(isopropylthio)-α-[1-(octylamino)-ethyl] benzyl alcohol), CP 1136 S (erythro-*p*-(isopropylthio)-α-(1-aminoethyl)benzyl alcohol) and CP 894 S (erythro-*p*-(isopropylthio)-α-[1-(octylthio)-ethyl]benzyl alcohol) are Continental Pharma products. The chemical structures of these compounds are given in Fig. 1. Tritiated suloctidil is a generous gift of Continental Pharma.

Methods

Preparation of the vesicles. Vesicles were prepared from individual phospholipids as DPPC or Egg PC or from mixtures containing DPPC–PS in a 95–5 or 85–15 ratio. A chloroform solution containing the required lipid composition was evaporated to dryness under a stream of N_2. Trace amount of solvent were removed under reduced pressure overnight. The thin film which formed on the walls of the flask was dispersed in a Tris–HCl buffer (pH: 7,4; NaCl 0.15 M) by mechanical agitation (Vortex for 30 sec) at a temperature above the phase transition temperature of the phospholipid. The resulting multilamellar vesicles (MLV) were analysed as such or after sonication (Branson Sonifier B 12, power input 70 W) for one hour at a temperature above the phase transition temperature of the phospholipid (T_m). Under those circumstances unilamellar vesicles (SUV) with a molecular weight of $\pm 2 \cdot 10^6$ and an average diameter of 250 Å are formed [7].

Fluorescence polarization measurements. The flu-

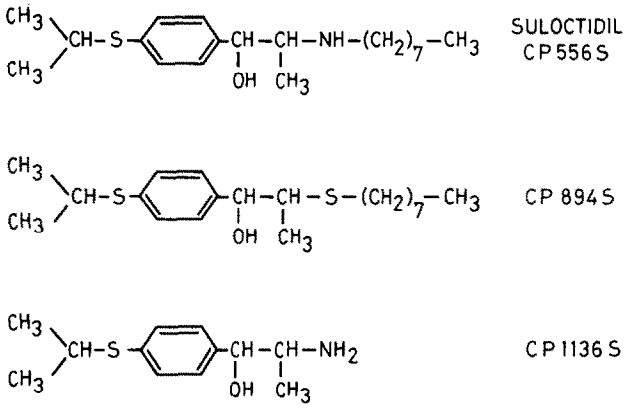

Fig. 1. Chemical structure of the compounds.

orescent hydrocarbon 1,6-diphenyl-1,3,5-hexatriene (DPH — Aldrich) was used as a probe for monitoring the degree of fluidity of the hydrocarbon region of the lipid bilayer. The vesicles were labelled by the incorporation of DPH in the lipid organic solution at a ratio 1:500 prior to evaporation. Fluorescence polarization was measured in an Elscint Microviscometer, Model MV 1 (Elscint LTH, Haifa, Israel). The excitation light was provided by a mercury lamp equipped with a cut-off filter (cut-off 365 nm) and a polarizer. The emitted light is detected in two independent cross-polarized channels after passing through a filter which cuts off wavelength below 418 nm. Fluorescence polarization (P) is defined as follows: (equation 1)

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \qquad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of light emitted with its polarization plane parallel ($\parallel$) and perpendicular ($\perp$) to that of the exciting beam. The accuracy of the measure is better than 5 per cent and temperature of the samples are kept within 0.5°. Corrections for light scattering are made by use of reference samples without DPH [6].

Fresh stock solutions of the drugs in methanol are prepared daily. An appropriate amount of these stock solution is added to the reaction mixture. The methanol concentration in the mixture never exceeds 0.5 per cent. Preliminary experiments showed that methanol below a concentration of one per cent has no effect on P measurements. Some experiments have been performed by incorporation of the drugs at a known ration in the lipid organic solution prior to evaporation and to the formation of the vesicles.

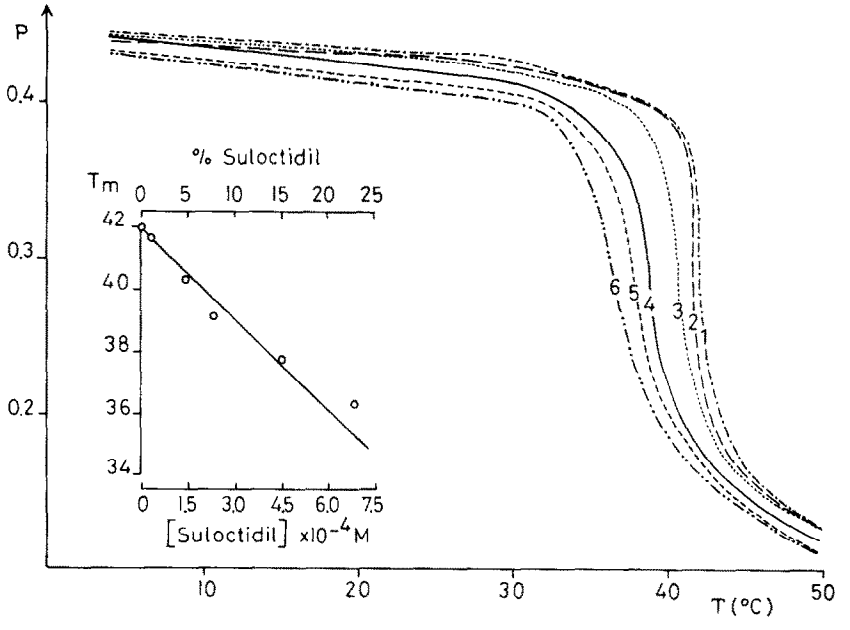

Fig. 2. Fluorescence depolarization (P) versus temperature (°C) scan of DPH in DPPC MLV in absence (1) or in presence of increasing quantity of suloctidil (2: 1.5%, 3: 3%, 4: 6%, 5: 12%, 6: 24%). The quantity of suloctidil is expressed in per cent of the quantity of DPPC. Insert: change of the main transition mid point temperature (T_m) as a function of suloctidil concentration.

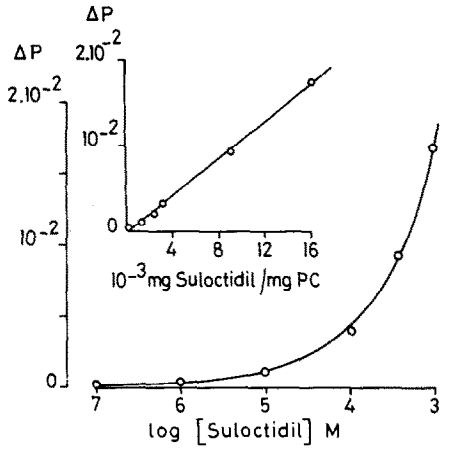

Fig. 3. DPH mobility ΔP as a function of suloctidil concentration, at 25°. Insert: the increase in DPH mobility ΔP as a function of the suloctidil membranous concentration.

After 15 min incubation above T_t, the samples were cooled and heating scans are recorded.

Radioactivity measurements. Drug concentrations in the vesicles are determined as follow: the required amount of [^{3}H]suloctidil is added as described above to an aqueous dispersion of SUV (1 mg/ml). After an incubation time of 20 min at 25°, free suloctidil is separated from the adsorbed suloctidil by rapid chromatography (2-min) on a small Sephadex PD 10 column. Control experiments with SUV alone and suloctidil alone show a complete separation. After separation, an aliquot (100 μl) of the fraction containing SUV is transferred in a vial containing 8 ml of Insta-Gel® (Packard Instrument Company). The amount of phospholipids in each fraction is monitored with trace amount of [^{14}C]cholesterol. All samples were counted to at least 2000 counts in a Packard Model 2450 scintillation spectrometer.

RESULTS

Figure 2 shows a typical depolarization of fluorescence versus temperature scan of a MLV suspension prepared from DPPC without (upper curve) and with increasing amount of suloctidil. Incorporation of suloctidil into the lipids leads to the disappearance of the lower transition temperature, to a dose-dependent downward shift of the main transition temperature which indicates a fluidification of the bilayer and to a general decrease of P suggesting the DPH moves more freely in the hydrocarbon matrix over the entire range of temperature. The last two effects of suloctidil are proportional to the amount of drug present in the system as shown by the inserts of Figs 2 and 3.

According to the Perrin equation [8]:

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(1 + \frac{\tau RT}{V\eta}\right) \qquad (2)$$

where P is the measured fluorescence depolarization,
P_o is the 'limiting value' of P,
τ is the fluorescence life time,
V is the effective molar volume of the probe,
η is the viscosity of the medium.

The movements of the probe within the medium depend on both τ and η. It is generally assumed that τ is constant at one temperature and thus P is directly related to η. However, the presence of a third compound can modify τ if this compound acts as a quencher. A direct measurement of τ in the absence or presence of quencher checks this possibility. An estimate can be obtained by measuring the intensity of total fluorescence F since

$$\frac{\tau}{\tau_{\text{quencher}}} = \frac{F}{F_{\text{quencher}}}$$

where τ and τ_{quencher} are the fluorescence life times measured in the absence and in the presence of the quencher.

F and F_{quencher} are the fluorescence intensities measured in the absence and the presence of the quencher and are equal to $F = I_{\parallel} + 2\,I_{\perp}$, where $I_{\parallel}$ and $I_{\perp}$ are defined as in equation (1). The identical fluorescence intensities measured in absence and in presence of suloctidil, independent of its concentration, allows us to conclude that suloctidil has no effect on τ and that consequently the observed variations in P values do represent fluidification of the lipid bilayer.

A more classical approach to study the suloctidil–phospholipid interactions was then used and suloctidil was introduced at a known concentration in the aqueous dispersion and incubated with the vesicles. P was recorded at 25° in the absence of suloctidil and after 20 min incubation when equilibrium is

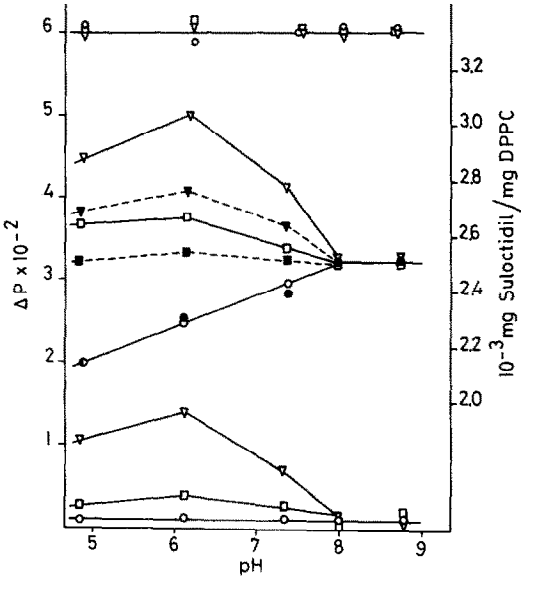

Fig. 4. Left ordinate: the increase in DPH mobility (ΔP) as a function of the pH and vesicles composition (o: DPPC; □: DPPC–PS 95–5; ▽: DPPC–PS 85–15) for suloctidil (center curves), CP 894 S (upper line) and CP 1136 S (lower curves). Drug concentration: 10^{-4} M/l temperature 25°. Right ordinate: suloctidil concentration within the membrane in the same experimental conditions. (●: DPPC; ■: DPPC–PS 95–5; ▼: DPPC–PS 85–15)

reached. In all cases, we observe a fall in P corresponding to the increase of the fluidity of the bilayer. The variation of fluorescence polarization (ΔP) is defined as the difference of fluorescence polarization in absence and in presence of the drug. The increase of fluidity of DPPC SUV is given in Fig. 3 as a function of the suloctidil aqueous concentration. The suloctidil concentration in the membranes has been measured in the same conditions. A plot of the suloctidil concentration in the membrane is given in Fig. 3 (insert) as a function of ΔP. The amount of suloctidil present in the bilayer is related to the aqueous concentration of suloctidil; an increase of the latter corresponds to an increase of the membranous concentration of suloctidil. But Fig. 3 shows also that ΔP is proportional to the suloctidil membranous concentration.

From this data, the membrane/buffer partition coefficient of suloctidil can be evaluated as:

$$K = \frac{C_m}{C_{aq}} \cdot \frac{V_{aq}}{V_m}$$

where C_m and C_{aq} are the suloctidil concentrations in the membrane and in the aqueous phase respectively and V_{aq} and V_m are the volumes of the buffer and of the membrane, respectively. In our system, V_{aq}/V_m is 10^3 if we assume that partial molar volumes are constant on mixing and that water and phospholipids have equal densities [9]. In these conditions, an average value log $K = 2$ has been found. This value is close to the value of the octanol/buffer partition coefficient (log $K_{oct/buffer} = 1.87$). This suggests a good correlation between the partition coefficients in both DPPC/buffer and octanol/buffer systems. Therefore no specific interactions between suloctidil and DPPC are to be expected. The partition of the drug is entirely controlled by its hydrophobic content.

Fluorescent technic yields more information about the suloctidil–phospholipid interactions using analogues of suloctidil and by modifying the conditions of interactions. These conditions were varied by the incorporation of different concentrations of an acidic phospholipid (PS) in the SUV and by changing the pH of the aqueous phase. PS has been chosen since it is one of the negatively charged phospholipids which is essential for Na^+/K^+ ATPase activity [10, 11]. The effect of these different parameters on the dynamics of the phospholipids are summarized in Fig. 4. At the concentration of 10^{-4} M/l, the three compounds fluidify the bilayer but to different extents depending on their structure, the presence of charged phospholipids in the SUV and the pH. Above pH 8, suloctidil has a maximal and constant effect on the DPPC SUV fluidity. Decreasing the pH decreases the perturbation induced by suloctidil. Incorporation of 5% or 15% PS in the bilayer does not modify the effect of suloctidil above pH 8; below this value, the presence of PS produces an increase in the fluidity as compared to DPPC SUV. This behaviour can be interpreted in function of the ionization of suloctidil which possess an amino group and consequently as an electrostatic interaction with the phospholipids. The derivative CP 1136 S differs from suloctidil by the absence on the *n*-octyl chain (Fig. 1). It is thus less lipid soluble and produces indeed at the same aqueous concentration a smaller effect than suloctidil. However, a quantitatively similar behaviour is observed particularly in the presence of the mixed PS–DPPC vesicles (Fig. 4). In the third derivative (CP 894 S), the NH group has been replaced by a sulphur atom. This compound has exactly the same effect on the bilayer fluidity whatever the pH of the aqueous phase or the composition of the SUV. Its effect is amplified as compared to suloctidil and CP 1136 S but completely insensitive to ionic interactions (Fig. 4).

Figure 4 shows the concentration of suloctidil in the bilayer (right ordinate). The change in suloctidil concentration supports qualitatively the observed differences in ΔP. This suggests that ΔP values are related to the amount of the drug in the membrane and that suloctidil-phospholipid interactions are sensitive to the degree of ionization of the drug and to the presence of acidic phospholipids.

DISCUSSION

The data suggest that suloctidil and related compounds interact with phospholipids. Indeed, a reduction of the phase transition temperature and a general increase of the hydrocarbon fluidity is observed in the presence of these compounds, whatever the physical state of the bilayer. In the case of suloctidil, these two effects are linear with respect to the drug concentration within the membrane. These non-specific effects could be related to the hydrophobic moiety of the drug.

The introduction of charged phospholipids or the modification of the pH of the buffer modifies the behaviour of suloctidil towards the lipid matrix as shown by the variations of fluorescence depolarization and of the drug concentrations within the membrane. This change can be interpreted as a consequence of electrostatic interactions between suloctidil-ion and the phospholipids. Above pH 8, the drug is uncharged and is therefore insensitive to electrostatic interactions. Below this pH value, the ionized suloctidil will be more soluble in water which explains the decrease of its effect on the bilayer fluidity and will interact with PS through electrostatic attraction.

At the pH value of 7.4, the presence of an acidic phospholipid produces an increase of the effect of suloctidil and an increase of its concentration. However, the increase in fluidity as compared to the study of uncharged vesicles (Fig. 3) is no longer proportional to the suloctidil concentration in the membrane. We suggest that the charge interaction between the positively charged suloctidil and PS produces a complex which modifies the phospholipid dynamics in a different way than in the case of an unspecific adsorption and penetration of the drug. This could be due simply to a different position or localization of suloctidil within the bilayer. From a detailed analysis of the effect of a large number of lipid-soluble compounds on the calorimetric behaviour of DPPC bilayer, Jain and co-workers [12–14] have proposed that "qualitative and quantitative differences in the phase transition characteristics are determined by the position or the localization of solutes in different regions in the bilayer along its

thickness". In the case of amphiphatic compounds, the interaction depends on the polar and on the hydrocarbon regions of the bilayer, a key region being the C_1–C_8 region of the acyl chains [13–15].

Changes of pH modify also the suloctidil–PC interactions. This could correspond to changes in drug levels since electrical behaviour of PC is constant over the studied pH range [16]. For DPPC SUV, the biphasic behaviour observed by fluorescence polarization measurements and radioactivity measurements can be viewed as a result of the protonation of the amino group. Once the drug is ionized, its effect on the bilayer fluidity decrease linearly with the pH. This decrease can be the consequence of an increase of the water solubility of suloctidil. However, the decrease in P is no longer proportional to the suloctidil concentration in the membrane if the values of P and suloctidil membranes are compared to the values of Fig. 3 (insert).

The modification of ΔP which is abnormally high in comparison to the decrease of the suloctidil concentration within the membrane could also be attributed to a modification of the localization of the drug in the bilayer; the more hydrophobic the drug, the more important its effect because it is more deeply buried within the bilayer.

For mixed DPPC–PS systems, the change in the DPPC–suloctidil interaction in function of the pH could explain the pH dependent decreasing effect of suloctidil although the initial adsorption due to charge–charge interaction is pH-independent. The importance of the initial adsorption is clearly shown in Fig. 4 where both ΔP and the suloctidil concentration within the membrane are higher in mixed DPPC–PS than in DPPC vesicles.

Further evidence for electrostatic interaction are given by an examination of the behaviour of parent compounds. CP 1136 S is an amino derivative and has a behaviour which is qualitatively equivalent to suloctidil. CP 894 S is totally insensitive to the pH change and to the lipid composition due to the absence of an ionizable function.

CP 894 S produces a larger increase of the fluidity of the lipid bilayer than suloctidil at the same aqueous concentration. This must be attributed to the replacement of the NH group of suloctidil by a sulphur atom: the only chemical difference between the compounds. This can however produce several effects. First of all, this may modify the partition coefficient of CP 894 S, induce an increase of the drug concentration within the membrane and consequently affect the lipid dynamics. Secondly, this could modify the spatial conformation of the drug within the membrane. There is indeed a small angular difference between the chemical bonds in the secondary amine and sulfide. The *n*-octyl chain will be overstated differently in the two drugs and provide different shapes to suloctidil and CP 894 S. The absence of electrostatic interaction will modify the position and/or localization of CP 894 S in the bilayer and produce greater effect than suloctidil.

The effect of suloctidil and related compounds has been tested on rat brain synaptosomes with respect to the fluidity of the lipid component and Na^+/K^+ ATPase activity [5]. It has been shown that suloctidil fluidified the synaptosomal lipid matrix and inhibited the Na^+/K^+ ATPase. These two parameters are strongly correlated for suloctidil and other positively charged drugs like chlorpromazine. In absence of positive charge (derivative CP 894 S), no inhibition of the Na^+/K^+ ATPase activity was observed in spite of a large effect on the synaptosomal fluidity. It was therefore proposed that suloctidil inhibited the Na^+/K^+ ATPase activity by an electrostatic interaction with some acidic phospholipids which have been shown to be essential for the Na^+/K^+ ATPase activity [10, 11] after its solubilization in the lipid bilayer. The present work, performed in the same range of concentration with a physico-chemical model of membrane, confirms the effect of suloctidil on the lipid dynamics and demonstrates the existence and the importance of an electrostatic interaction between the drug and an acidic phospholipid. This conclusion is reinforced by the behaviour of compound CP 894 S. Indeed, this compound displays no electrostatic interactions with PS nor does it inhibit the Na^+/K^+ ATPase [5] although it fluidifies the bilayer to a large extent.

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