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Research paper

Biophysical characterization of the drug–membrane interactions: The case of propranolol and acebutolol

Catarina Pereira-Leite [a,1], Cláudia Carneiro [a,1], José X. Soares [b], Carlos Afonso [b], Cláudia Nunes [a,*], Marlene Lúcio [a], Salette Reis [a]

[a] REQUIMTE, Laboratório de Química Aplicada, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal
[b] CEQUIMED-UP, Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal

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ABSTRACT

The interaction of propranolol and acebutolol with biological membranes was assessed in the present work by using a range of biophysical techniques and liposomes, as membrane mimetic models. Liposomes were made of zwitterionic phosphatidylcholines and experiments were performed at physiologic pH and at various membrane physical states (gel, ripple and fluid phases). Fluorescence techniques were used to study the partition coefficient of β-blockers, the influence of drugs on membrane fluidity and the drugs–membrane binding. Moreover, small and wide angle X-ray scattering techniques were used to evaluate the β-blockers effect on long range bilayer order and hydrocarbon chain packing. The gathered results highlighted the importance of electrostatic interactions between propranolol and acebutolol with membranes. Furthermore, both β-blockers exhibited a membrane-fluidizing effect and the capacity to disturb the membrane organization. In general, propranolol unveiled a more pronounced effect on membrane fluidity and structure than acebutolol. In the current study, the obtained results were also correlated with the cardioprotective properties of the β-blockers studied.

1. Introduction

β-blockers are competitive antagonists of catecholamines at β-adrenoceptors and are used in the treatment of several conditions, such as hypertension, angina pectoris, cardiac arrhythmias, myocardial infarction, anxiety disorders and migraine. Propranolol is a non-cardioselective β-blocker, which has membrane-stabilizing properties but does not possess intrinsic sympathomimetic activity. Contrastingly, acebutolol is a β_1-selective-blocking agent, with both intrinsic sympathomimetic activity and membrane stabilizing properties [1,2].

The interaction of drugs with membranes plays an essential role in their biological activity, since drugs must interact with membranes through their entire *in vivo* course, and their therapeutic target is often embedded within membranes [3]. Moreover, numerous cardiovascular diseases appear to be related with modifications of membrane lipid composition and structure [4,5]. Therefore, the study of the influence of these drugs on membrane organization could shed light on cardioprotective properties that are not related with β-adrenoceptors antagonism.

In this regard, the present study aims to systematically characterize the effects of propranolol and acebutolol on the biophysical properties of biological membranes and further correlate these effects with some of their pharmacological properties. Since biological membranes are too complex to accurately assess their biophysical properties, liposomes, as membrane model systems, made of egg phosphatidylcholine (EPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were used to mimic the constitution and dimensionality of biomembranes. EPC and DPPC were chosen because natural plasma membranes contain a high amount of phosphatidylcholines [6,7], and these phospholipids are commonly used to analyze the biophysical properties of cellular membranes [8–11]. DPPC was also chosen for its suitability to mimic the lipid polymorphism found at the membrane level. In fact, the use of DPPC beyond EPC allowed us to perform experiments at lipid gel

Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; EPC, egg phosphatidylcholine; K_p, partition coefficient; LUVs, large unilamellar vesicles; L_α, lipid fluid phase; $L_{\beta'}$, lipid gel phase; MLVs, multilamellar vesicles; $P_{\beta'}$, lipid ripple gel phase; SAXS, small angle X-ray scattering; T_m, main phase transition temperature; TMA-DPH, 1-(4-trimethylammoniophenyl)-6-phenyl-1,3,5-hexatriene; WAXS, wide angle X-ray scattering.

* Corresponding author. Laboratório de Química Aplicada, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal. Tel.: +351 220428672; fax: +351 226093483.
E-mail address: cdnunes@ff.up.pt (C. Nunes).
[1] These authors have contributed equally.

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phase ($L_{\beta'}$), ripple gel phase ($P_{\beta'}$) and fluid phase (L_α). To further mimic the *in vivo* conditions, studies were performed at physiologic pH.

The use of complementary biophysical techniques is important to obtain an ample characterization of drug–membrane interactions. Therefore, the partition coefficients of propranolol and acebutolol were determined by fluorescence spectroscopy; the drugs' effect on membrane fluidity was assessed by fluorescence anisotropy measurements; the β-blockers-membrane binding was studied by fluorescence measurements and the effect of both drugs on membrane structure was evaluated by small and wide angle X-ray scattering techniques (SAXS and WAXS, respectively).

Although there are some studies reporting the propranolol–membrane interactions [12–14], to our knowledge, no study has been reported regarding the interaction of acebutolol with biological membranes. Furthermore, this research may provide significant insights for a better understanding of these β-blockers pharmacological effects and thus for predicting or modulating the *in vivo* action of other cardioprotective drugs.

2. Materials and methods

2.1. Reagents

Acebutolol, propranolol and egg phosphatidylcholine (EPC) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was supplied by Avanti Polar Lipids, Inc. (Alabama, USA). The probes: 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4-trimethylammoniophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) and 8-Anilino-1-naphthalenesulfonic acid (ANS) were purchased from Molecular Probes (Invitrogen Corporation, Carlsbad, California, USA). All other chemicals were purchased from Merck (Darmstadt, Germany). All reagents were used without further purification.

Drug solutions were prepared with hepes buffer (pH 7.4). This buffer was prepared with double-deionized water (conductivity less than 0.1 μS cm^{-1}) from a Millipore system, and the ionic strength (I = 0.1 M) was adjusted with NaCl.

2.2. Preparation of liposomes

Liposomes were obtained according to the thin film hydration method [15–17]. Concisely, the lipid, EPC or DPPC, was dissolved in a chloroform/methanol (3:2, v/v) mixture. The organic solvents were then evaporated under a nitrogen stream to yield a dried lipid film. The resultant lipid film was hydrated with hepes buffer and vortexed at room temperature for EPC or at 45 °C for DPPC (temperature above the main phase transition temperature) to obtain multilamellar vesicles (MLVs). Large unilamellar vesicles (LUVs) were prepared by extrusion of MLVs suspension through polycarbonate filters with a pore size of 100 nm at room temperature for EPC or at 45 °C for DPPC.

Labeled liposomes were prepared by co-dissolving the lipid and the probe (DPH or TMA-DPH) in the organic solvents mixture to give a probe/lipid molar ratio of 1:300. Afterward, labeled LUVs were similarly obtained by evaporation of the organic solvents, by dissolution of the dried lipid film and by extrusion of the MLVs suspension.

2.3. Determination of partition coefficients by spectrofluorimetry

The partition coefficient (K_p) of propranolol and acebutolol between liposomes and an aqueous phase was determined by spectrofluorimetry. Hepes buffered LUVs suspensions contained a fixed concentration of propranolol (10.4 μM) or acebutolol (40 μM) and increasing concentrations of EPC (in the range 0–1000 μM). The corresponding reference solutions were identically prepared in the absence of drug. All suspensions were incubated at room temperature in the dark for 2 h. The emission spectra were recorded in a Perkin Elmer LS-50B steady-state fluorescence spectrometer between 300–800 nm and 350–600 nm for propranolol and acebutolol, respectively. The excitation wavelengths were set to 286 and 320 nm for propranolol and acebutolol, respectively. The results were mathematically treated to eliminate the spectral interferences due to the light scattered by lipid vesicles. Therefore, the corrected emission spectra were obtained by subtracting each reference spectrum from the correspondent sample spectrum, and if necessary, the first derivative spectra were determined using Microcal Origin 6.1™. The partition coefficient was then calculated from the corrected emission spectra or the first derivative spectra at a wavelength where the scattering was eliminated by fitting Eqs. (1) or (2) (respectively) to the experimental data using a non-linear regression method, where the adjustable parameter is the partition constant, K_p:

$$I_T = I_w + \frac{(I_w - I_a)K_p[L]V_m}{1 + K_p[L]V_m} \quad (1)$$

$$D_T = D_w + \frac{(I_m - I_w)K_p[L]V_m}{1 + K_p[L]V_m} \quad (2)$$

In these equations, I is the corrected fluorescence intensity of the total amount of drug (I_T), drug distributed on the lipid membrane phase (I_m) and drug distributed in the aqueous phase (I_w). D is the first derivative intensity ($D = (d^nI)/d\lambda^n$) obtained from the fluorescence intensity values of the total amount of drug (D_T), drug distributed on the lipid membrane phase (D_m) and drug distributed in the aqueous phase (I_w). *[L]* represents the lipid concentration (in M), and V_m is the lipid molar volume. For EPC, V_m is 0.688 L mol^{-1} [18].

2.4. Microviscosity, order and cooperativity studies by anisotropy measurements

The effect of acebutolol and propranolol on the membrane main phase transition temperature, order and phase transition cooperativity was studied by fluorescence anisotropy. A fixed concentration of labeled liposomes of DPPC (500 μM) with DPH or TMA-DPH was used. The drug incorporation into the labeled liposomes was performed by incubation and inclusion procedures. In incubation procedure, an aliquot of drug solution was added to the previously obtained labeled LUVs suspension, followed by 30 min of incubation period at 45 °C. The fixed concentrations of acebutolol and propranolol obtained by this preparation procedure were 50 μM and 10 μM, respectively. In order to achieve higher concentrations of the drugs studied, the inclusion procedure was used by co-dissolving and co-drying the drugs with the lipid and the probe during LUVs preparation. The experiments were performed in a Perkin Elmer LS-50B steady-state fluorescence spectrometer equipped with a constant-temperature cell holder. The excitation wavelength was set to 360 nm for both probes, and the emission wavelengths were set to 426 and 440 nm for DPH and TMA-DPH, respectively. The anisotropy was recorded at several temperatures between 25 and 60 °C, with an accuracy of 0.1 °C. The results obtained with labeled liposomes in the presence and absence of the drugs studied were collected as anisotropy versus temperature and data was fitted using:

$$r_s = r_{s1} + p_1T + \frac{r_{s2} - r_{s1} + p_2T - p_1T}{1 + 10^{B(1/T - 1/T_m)}} \quad (3)$$

where r_s is the fluorescence anisotropy, T is the temperature (°C), p_1 and p_2 are the slopes of the linear fits to the data before and after