

The Antiepileptic Drug Diphenylhydantoin Affects the Structure of the Human Erythrocyte Membrane

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Z. Naturforsch. **59c**, 427–431 (2004); December 3, 2003/January 19, 2004

Phenytoin (diphenylhydantoin) is an antiepileptic agent effective against all types of partial and tonic-clonic seizures. Phenytoin limits the repetitive firing of action potentials evoked by a sustained depolarization of mouse spinal cord neurons maintained *in vitro*. This effect is mediated by a slowing of the rate of recovery of voltage activated Na⁺ channels from inactivation. For this reasons it was thought of interest to study the binding affinities of phenytoin with cell membranes and their perturbing effects upon membrane structures. The effects of phenytoin on the human erythrocyte membrane and molecular models have been investigated in the present work. This report presents the following evidence that phenytoin interacts with cell membranes: a) X-ray diffraction and fluorescence spectroscopy of phospholipid bilayers showed that phenytoin perturbed a class of lipids found in the outer moiety of cell membranes; b) in isolated unsealed human erythrocyte membranes (IUM) the drug induced a disordering effect on the polar head groups and acyl chains of the erythrocyte membrane lipid bilayer; c) in scanning electron microscopy (SEM) studies on human erythrocytes the formation of echinocytes was observed, due to the insertion of phenytoin in the outer monolayer of the red cell membrane. This is the first time that an effect of phenytoin on the red cell shape is described. However, the effects of the drug were observed at concentrations higher than those currently found in plasma when phenytoin is therapeutically administered.

Key words: Phenytoin, Antiepileptic Drug, Erythrocyte Membrane

Introduction

Phenytoin (diphenylhydantoin) is an antiepileptic agent effective against all types of partial and tonic-clonic seizures. The epilepsies are devastating disorders and therapy is symptomatic in that antiepileptics inhibit seizures but a cure is not yet available. One mechanism of action of drugs such as phenytoin, efficient against the common forms of epileptic seizures, is to limit the sustained repetitive firing of a neuron by promoting the inactivated state of voltage-activated Na⁺ channels (McNamara, 2001; Farber *et al.*, 2002). This family

of channels are integral proteins responsible for the initiation of action potentials in many excitable tissues. For this reasons we thought it of interest to study the binding affinities of phenytoin with cell membranes and their perturbing effects upon membrane structures. This article describes the interaction of phenytoin with well-established molecular models of biomembranes. Such models are human erythrocytes and lipid bilayers. The latter consisted of multibilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes located in the outer and inner monolayers of the human erythrocyte membrane, respectively (Boon and Smith, 2002). The capacity of phenytoin to perturb their structures was determined by X-ray diffraction. The antiepileptic ability to perturb the structure of DMPC large unilamellar vesicles (LUV) was examined by fluorescence spectroscopy. Intact human erythrocytes

Abbreviations: IUM, isolated unsealed human erythrocyte membranes; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; LUV, large unilamellar vesicles; SEM, scanning electron microscopy; DPH, 1,6-diphenyl-1,3,5-hexatriene; laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; r, anisotropy; GP, general polarization.

incubated with phenytoin were observed by scanning electron microscopy (SEM) while their membranes, consisting of resealed ghosts (IRM) were studied by fluorescence spectroscopy. Erythrocytes were chosen because although less specialized than many other cell membranes, they carry enough functions in common with them such as active and passive transport and the production of ionic and electric gradients to be considered representative of the plasma membrane in general.

Materials and Methods

Scanning electron microscopy (SEM) studies on human erythrocytes

In vitro interaction with erythrocytes was attained by incubating red blood cells suspensions derived from healthy human male donors not receiving any pharmacological treatment. Blood samples were obtained by puncture of the ear lobe and by aspiration into a tuberculin syringe without a needle containing 50 units/ml heparin in saline solution (0.9% NaCl). Red blood cells were then centrifuged, washed twice in saline solution, resuspended in saline solution containing phenytoin at a final 10 mM concentration, and incubated for 1 h at 37 °C. Controls were cells resuspended in saline solution without phenytoin. Specimens were then fixed overnight at 5 °C by adding one drop of each sample to plastic tubes containing 1 ml of 2.5% glutaraldehyde, washed twice in distilled water, placed on siliconized Al stubs and air-dried at 37 °C for 30 min. Al stubs were then gold coated for 3 min at 13.3 pascal in a sputter device (Edwards S 150, Sussex, England). Specimens were examined in a Etec Autoscan SEM (Etec Corp., Hayward, CA, USA).

Fluorescence measurements of isolated unsealed human erythrocyte membranes (IUM) and DMPC large unilamellar vesicles (LUV)

The influence of phenytoin on the physical properties of IUM and DMPC LUV was examined by fluorescence spectroscopy using DPH and laurdan (Molecular Probe, Eugene, OR, USA) fluorescent probes. DPH is widely used as a probe for the hydrophobic regions of the phospholipid bilayers because of its favorable spectral properties. Its steady-state fluorescence anisotropy measurements were used to investigate the structural properties of IUM and DMPC LUV as it provides a measure of the rotational diffusion of the fluoro-

phor, restricted within a certain region such as a cone, due to the lipid acyl chain packing order. Laurdan, an amphiphilic probe, has a high sensitivity of excitation and emission spectra to the physical state of membranes. With the fluorescent moiety within a shallow position in the bilayer, laurdan provides information of the dynamic properties at the level of the phospholipid glycerol backbone. The quantification of the laurdan fluorescence shift was effected using the general polarization (GP) concept (Parasassi and Gratton, 1995), which is related to the lipid polar head organization in the zone of the erythrocyte membrane and DMPC LUV.

Erythrocytes were separated from heparinized venous blood samples obtained from normal casual donors by centrifugation and washing procedures. IUM were prepared by lysis according to Dodge *et al.* (1963). DMPC LUV suspended in water were prepared by extrusion of frozen and thawed multilamellar liposome suspensions (final lipid concentration 0.3 mM) through two stacked polycarbonate filters of 400 nm pore size (Nucleopore, Corning Costar Corp., MA, USA) under nitrogen pressure at 10 °C above the lipid phase transition temperature. DPH and laurdan were incorporated into IUM and LUV by addition of small aliquots of concentrated solutions of the probe in dimethylformamide and ethanol, respectively, and incubated at 37 °C for 45 min. Fluorescence spectra and anisotropy measurements were performed on a Spex Fluorolog (Spex Industries Inc., Edison, NJ, USA) and in a phase shift and modulation Gregg-200 steady-state and time-resolved spectrofluorometer (ISS Inc., Champaign, IL, USA), respectively, both interfaced to computers. Software from ISS was used for data collection and analysis.

Measurements of LUV suspensions were made at 18 °C and 37 °C and measurements of IUM were made at 37 °C using 10 mm path-length square quartz cuvettes. Sample temperature was controlled by an external bath circulator (Cole-Parmer, Chicago, IL, USA) and monitored before and after each measurement using an Omega digital thermometer (Omega Engineering Inc., Stamford, CT, USA). Anisotropy measurements were made in the L configuration using Glan Thompson prism polarizers (I. S. S.) in both exciting and emitting beams. The emission was measured using a WG-420 Schott high-pass filter (Schott WG-420, Mainz, Germany) with negligible fluorescence.

DPH fluorescence anisotropy (r) was calculated according to the definition: $r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$, where I_{\parallel} and I_{\perp} are the corresponding parallel and perpendicular emission fluorescence intensities with respect to the vertically polarized excitation light (Lakowicz, 1983). Laurdan fluorescence spectral shifts were quantitatively evaluated using the GP concept (see above) which is defined by the expression $GP = (I_b - I_r) / (I_b + I_r)$, where I_b and I_r are the emission intensities at the blue and red edges of the emission spectrum, respectively. These intensities have been measured at the emission wavelengths of 440 and 490 nm, which correspond to the emission maxima of laurdan in the gel and liquid crystalline phases, respectively (Parasassi *et al.*, 1990). Phenytoin was incorporated in IUM and LUV suspensions by addition of small aliquots of a concentrated solution and incubated at 18 °C or 37 °C, depending on the work temperature, for ca. 15 min. Blank subtraction was performed in all measurements using labelled samples without probes.

X-ray diffraction studies of phospholipid multilayers

Synthetic DMPC (lot 80H8371, A grade, M_r 677.9), DMPE (lot 13H83681, A grade, M_r 635.9) and phenytoin (lot 39H0752, 99% purity, M_r 252.3) from Sigma (MO, USA) were used without further purification. About 3.5 mg of each phospholipid were mixed in 2.0 mm-dia glass capillaries (Glas-Technik & Konstruktion, Berlin, Germany) with 200 μ l of aqueous phenytoin solutions (concentration range 1 mM to 10 mM). They were investigated by X-ray diffraction using flat-plate cameras with 0.25 mm diameter glass collimators provided with rotating devices. The blanks consisted of pure samples of each phospholipid with excess water. Specimen-to-film distances were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. Ni-filtered CuK_{α} radiation from a Philips PW 1140 X-ray generator was used. The relative reflection intensities on film were measured by peak integration using a Bio-Rad GS-700 densitometer (Hercules, CA, USA) and Molecular Analyst/PC image software; no correction factors were applied. The experiments were performed at 17 ± 2 °C, which is below the main transition temperatures of both DMPC and DMPE. Higher temperatures would have induced transitions to more

fluid phases making the detection of structural changes harder.

Results and Discussion

Scanning electron microscopy (SEM) studies on human erythrocytes

SEM observations of human erythrocytes incubated with phenytoin suspensions equivalent to 10 mM revealed that the drug induced changes in the normal shape of the cells. In fact, about half the erythrocytes underwent an echinocytic type of alteration, *i.e.*, developed a form characterized by blebs or protuberances over the cell membrane.

Fluorescence measurements of isolated unsealed human erythrocyte membranes (IUM) and DMPC large unilamellar vesicles (LUV)

Phenytoin concentration-dependent effects on IUM and DMPC LUV were explored at two different depths of the lipid bilayer: at the hydrophilic/hydrophobic interface level, estimated from the laurdan fluorescence spectral shift through the GP parameter, and in the deep hydrophobic core, determined by the DPH steady-state fluorescence anisotropy (r). Fig. 1A and 1B show that increasing concentrations of phenytoin (0 to 2.0 mM) decreased both the r and GP values of IUM at 37 °C. These results imply that the drug induced structural perturbations in both the acyl chain and polar group packing arrangement of the erythrocyte membrane lipid bilayer. Fig. 1C and 1D show that the incorporation of phenytoin to DMPC LUV in the same range of increasing concentrations also decreased the r and GP values at 18 °C. Similar measurements performed at 37 °C showed that while the r value remained practically constant in the 0 to 2.0 mM concentration range, there was a mild increase in GP, implying an ordering of the polar headgroups of DMPC. It should be taken into account that at 37 °C this lipid is in a much more fluid state than at 18 °C.

X-ray diffraction studies of phospholipid multilayers

The molecular interactions of phenytoin with multilayers of the phospholipids DMPC and DMPE in an aqueous medium were determined by X-ray diffraction. Fig. 2A shows a comparison of the diffraction patterns of DMPC alone and of DMPC incubated with phenytoin in the range of

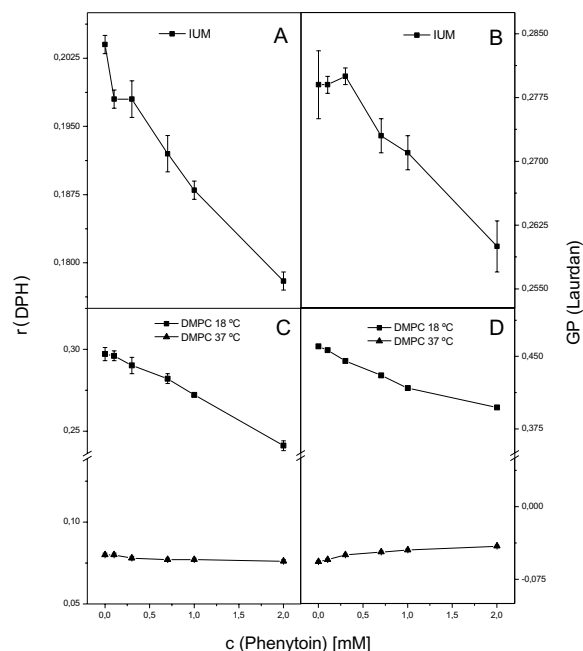


Fig. 1. Effect of phenytoin on (A) the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH), (B) the general polarization (GP) of laurdan embedded in isolated human erythrocyte membranes (IUM) at 37 °C, (C) the anisotropy (r) of DPH, and (D) the general polarization (GP) of laurdan embedded in large unilamellar vesicles (LUV) of dimyristoylphosphatidylcholine (DMPC) at 18 °C (■) and 37 °C (▲).

1 mM up to 10 mM. As expected, water altered the structure of DMPC: its bilayer width increased from about 5.5 nm in its dry crystalline form (Suwalsky, 1996) to 6.45 nm when immersed in water, and its reflections were reduced to only the first three orders of the bilayer width. On the other hand, a new and strong reflection of 0.42 nm showed up, whose appearance was indicative of the fluid state reached by DMPC bilayers and corresponded to the average distance between its fully extended acyl chains organized with rotational disorder in hexagonal packing. Addition of 1 mM phenytoin caused only a very slight decrease in the phospholipid reflection intensities. However, phenytoin 3.5 mM and 5 mM caused a marked decrease of these intensities, whereas 10 mM induced the complete disappearance of the low angle reflections [indicated as (a) in the figure], which were replaced by a diffuse halo, and a considerable weakening of the 0.42 nm reflection. This result implies that the drug induced serious

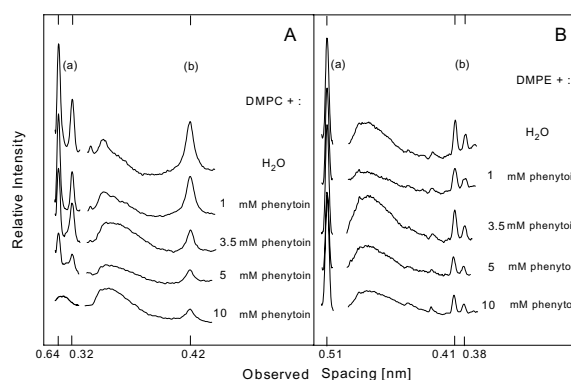


Fig. 2. Microdensitograms from X-ray diffraction diagrams of dimyristoylphosphatidylcholine (DMPC) (A) and dimyristoylphosphatidylethanolamine (DMPE) (B) in water and aqueous solutions of phenytoin; (a) low-angle and (b) high-angle reflections.

molecular disorder in the DMPC bilayer, especially in the region of the polar head groups. Fig. 2B shows the results of the interaction of phenytoin with DMPE. The perturbing effect of this compound upon the structure of DMPE bilayers was practically negligible in the low-angle region even at a 10 mM concentration, although phenytoin 5 mM and 10 mM somewhat reduced the phospholipid high angle reflection intensities [indicated as (b)] in the figure. As a matter of fact, these two phospholipids differ only in their terminal amino groups, these being $^+N(CH_3)_3$ in DMPC and $^+NH_3$ in DMPE. Moreover, both molecular conformations are very similar in their nonaqueous crystalline phases (Suwalsky, 1996) with the hydrocarbon chains mostly parallel and extended, and the polar groups lying perpendicularly to them. However, the gradual hydration of DMPC results in water filling the highly polar interbilayer spaces. Thus, its bilayer width increases from 5.45 nm when dry up to about 6.4 nm when it is fully hydrated. This phenomenon allows the incorporation of phenytoin into DMPC bilayers producing its structural perturbation and almost complete destruction at a 10 mM concentration. On the other hand, DMPE molecules pack tighter than those of DMPC due to their smaller polar group and higher effective charge, resulting in a very stable bilayer system that is not significantly affected by water (Suwalsky, 1996) nor by a number of drugs (Suwalsky *et al.*, 1994, 2002) including phenytoin.

In conclusion, this report presents the following evidence that phenytoin interacts with membrane

phospholipids: a) X-ray diffraction and fluorescence spectroscopy showed that phenytoin interacted with DMPC, a class of lipids found in outer monolayers of cell membranes; b) in IUM, the drug induced a disordering effect on the polar head groups and acyl chains of the erythrocyte membrane lipid bilayers; c) SEM observations of human erythrocytes showed the formation of echinocytes, an effect due to the insertion of phenytoin in the outer monolayer of the red cell membrane (Sheetz and Singer, 1974).

Plasma therapeutic concentrations of phenytoin are in the 0.04–0.08 mM range and toxic effects, which show marked differences among individuals, are encountered at concentrations as high as

0.4 mM (Lebdeh *et al.*, 2001). The effects of the drug detected in the present work, including alterations in erythrocyte structure, were observed at concentrations much higher than those currently set up in plasma when phenytoin is therapeutically administered. This finding is certainly of therapeutic interest as it indicates that phenytoin cytotoxicity at the cell membrane level is not significant.

Acknowledgements

The authors thank José Morales and Fernando Neira for their valuable technical assistance. This work was supported by a grant from FONDECYT (1020476).

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