MEMBRANE TRANSPORT WITHOUT RECEPTORS?

THE ROLE OF CYCLOSPORINES AND SILYMARINES STRUCTURES FOR THEIR INTERACTIONS WITH LIPIDS OF HEPATOCYTE PLASMA MEMBRANE

Jiří Šebestian[1,2], Štěpánka B. Šebestianová[3], Vladimíra Moulisová[1,2], and Alexandr Jegorov[4]

[1]Institute of Physical Biology, Univ. South Bohemia, Nový Zámek 136, CZ-373 33 Nové Hrady
[2]Laboratory of Biomembranes, Fac. Biol., Univ. South Bohemia, Branišovská 31, CZ-370 05 České Budějovice
[3]Dept. Genetics, Fac. Biol., Univ. South Bohemia, Branišovská 31, CZ-370 05 České Budějovice,
[4]IVAX CR, Research Unit, Branišovská 31, CZ-370 05 České Budějovice

Address for correspondence: E-mail: sebest@jcu.cz

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Abstract
The paper summarizes experimental results on interaction of cyclosporines and silymarines with different models of hepatocyte plasma membranes (from different phospholipid vesicles through plasma membrane vesicles up to hepatocytes).

Introduction

Cyclosporines (Cs) are non-ribosomatically synthesized toxic cyclic undecapeptides that include some non-coded aminoacids. Well known and the most common of them is cyclosporine A (CsA) which is widely used in medicine as a powerful immunosuppressant in organ transplantations and for suppression of multidrug resistance in tumor chemotherapy. The cyclopeptidic structure features have some other important natural toxins (i.e. alga toxin microcystine, mushroom toxins amanitin and phalloidin etc.). Flavanolignans (called silymarines) from Milk Thistle (Silybum marianum) are known to help against hepatotoxic effects of cyclic peptides. Mechanisms of actions in the liver cell are well known for both cyclosporines and silymarines. But much less is known about their transport through the cell plasma membrane. Because of their hydrophobic nature they are believed (at least partly) to enter the cell by passive diffusion through the lipidic part of hepatocyte plasma membrane.

We investigated the interaction of these compounds with different models of hepatocyte plasma membranes (from different phospholipid vesicles through plasma membrane vesicles up to hepatocytes). The interactions were monitored by changes in membrane lipid fluidity after cyclosporine or silymarine addition. The membrane fluidity was observed by measuring of the steady-state fluorescence anisotropy of diphenylhexatriene (DPH) and its polar derivative, TMA-DPH.

Materials and Methods

DPH and TMA-DPH were purchased from Molecular Probes (USA). All the other chemicals used were of analytical grade from Sigma-Aldrich (Czech Republic).

1. Animals

Two months old male Wistar rats weighing 200-250 g were used for hepatocyte isolation, liver cytoplasmic membrane preparation and liver phospholipid isolation. Animals were kept in standard laboratory conditions with free access to water and standard pellet laboratory diet (KrmiMo Mohelski, Brno, Czech Republic). All procedures with animals were approved by the Ethics Commettee, Ministery of Education, Czech Republic.

2. Hepatocytes preparation

Rat hepatocytes were isolated by modified two-step collagenase perfusion (Moldeus et al., 1978). The hepatocytes were collected in PBS, filtered on gauze and washed three times by centrifugation (50 g). Cells were resuspended in William's medium E and washed once more by centrifugation (50 g). Cells were counted using trypan blue exclusion test and viability was typically 90 %. Freshly prepared hepatocytes were resuspended in William's E medium supplemented by 1 % bovine serum to the final concentration 6´105 cells/ml.

3. Cytoplasmic membrane vesicles isolation

Cytoplasmic membranes from hepatocytes were isolated by differential centrifugation and isopycnic centrifugation using discontinual sucrose gradient according to Scott et al. (1993).

4. Phospholipid isolation from hepatocytes

Phospholipids were isolated by classical chloroform/methanol extraction with modification according to Cartwright (1993) from isolated hepatocyte plasma membranes. Isolated phospholipids were stored dissolved in chloroform under nitrogen in -70 °C before using.

5. Phospholipid vesicles preparations

Monolayer phospholipid vesicles were prepared from commercial phosphatidylcholin from egg yolk and phosphatidyl ethanolamine from sheep brain (Sigma-Aldrich, Prague, Czech republic) or from isolated hepatocyte plasma mem-

brane phospholipids by very gentle sonication for 1 minute in 4 °C under nitrogen atmosphere.

6. Labelling of hepatocytes, plasma membrane vesicles and phospholipid vesicles

DPH in final concentration 5´10-7 M was directly added to the stirred sample and let to incorporate for 30 minutes at 37 °C. Incorporation of TMA-DPH was very quick (less than 1 minute) thus TMA-DPH was added directly to the sample in the fluorescence cuvette to the final concentration 5´10-7 M. Final volume in the cuvette was 2 ml. Final concentrations of ingredients were: hepatocytes 3´105 cells/ml (=75 mg of protein/ml), membrane vesicles 75 mg of protein/ml, phospholipid vesicles 0.3 mg/ml, investigated compound (silymarine or cyclosporine) 10 mM. Fluorescence anisotropy was measured as soon as possible after the investigated compound addition (30 seconds) and each 5 minute after that up to 30 minutes. Fluorescence probes and investigated compound were dissolved before addition in spectroscopic pure ethanol and final concentration of ethanol was 0.5 % (v/v).

7. Fluorescence anisotropy measurement

Steady-state anisotropy measurements were performed with a SLM 4800S fluorometer (SLM Instruments, Urbana, IL), equipped with the standard polarization accessory. The excitation wavelength was set to 360 nm. Fluorescence was detected through a cut-off emission filter (Schott, 50% transmittance at 405 nm). And the emission monochromator was set to 430 nm. The correction for the fluorescence intensity of non-labelled hepatocytes (usually 10 % intensity of the TMA-DPH labelled cells) was calculated according to Kuhry et al., (1985). The background fluorescence of non-labelled vesicles of all types did not exceed 1% of the experimental values. The steady-state anisotropy was calculated according to Lakowitz (1983). The steady-state anisotropy values presented in this work correspond to the average of at least three determinations performed with the independent vesicle preparations.

8. Other methods.

Protein concentrations were determined by the Lowry et al. (1951) assay in the presence of 0.07 % (w/v) sodium deoxycholate in alkali pH using BSA as the standard.

Results and discussion

The experimental results are summarized in Figure 1. Because the standard deviations of measurements were much higher at 37 °C than that found in case of 20 °C and the pattern of fluorescent anisotropy values was the same at both temperatures (see the third graph) we decided to measure all other graphs at 20 °C.

We found that the side chain of the second aminoacid is very important for interactions of cyclosporines with lipids. The membrane fluidity increases in the raw: CsC << CsA < CsD < CsG, where the second aminoacid in CsC is threonin, in CsA: a-aminobutyric acid, in CsD: valin and in CsG: norvalin. The same membrane fluidity as in case of CsA was found for interactions of cyclosporines with changes in other than second aminoacid; including CsH, which 3D structure completely differs from other cyclosporines (position 11 D-N-methylvalin instead of L-MeVal). The role of the second amino acid is supported by the fact that this side chain is in 3D structure of cyclosporines the most exposed residue to the space above the plane of cyclopeptide ring.

On the other hand the change in the first amino acid which is the most exposed residue under the cyclopeptide chain, has no effect (i.e. fluidity measured for AcetylCsA was the same as for CsA). These results and the same patterns were found for each type of vesicles and for both probes. The changes were in order of 10-3 anisotropy units.

Different results and patterns were found in case of hepatocytes. Changes in membrane fluidity were found only in case of CsG but in the order of 10-2 anisotropy units!

No changes were found in case of silymarines.

Conclusions

We concluded that:

1) The most important for hydrophobic interaction of cyclosporines with our model membranes is the second amino acid residue.

2) The intensity of the interaction increases with hydrophobicity of the second amino acid residue.

3) Hydrophobic interactions of cyclosporines in vivo can play a role only in case of CsG.

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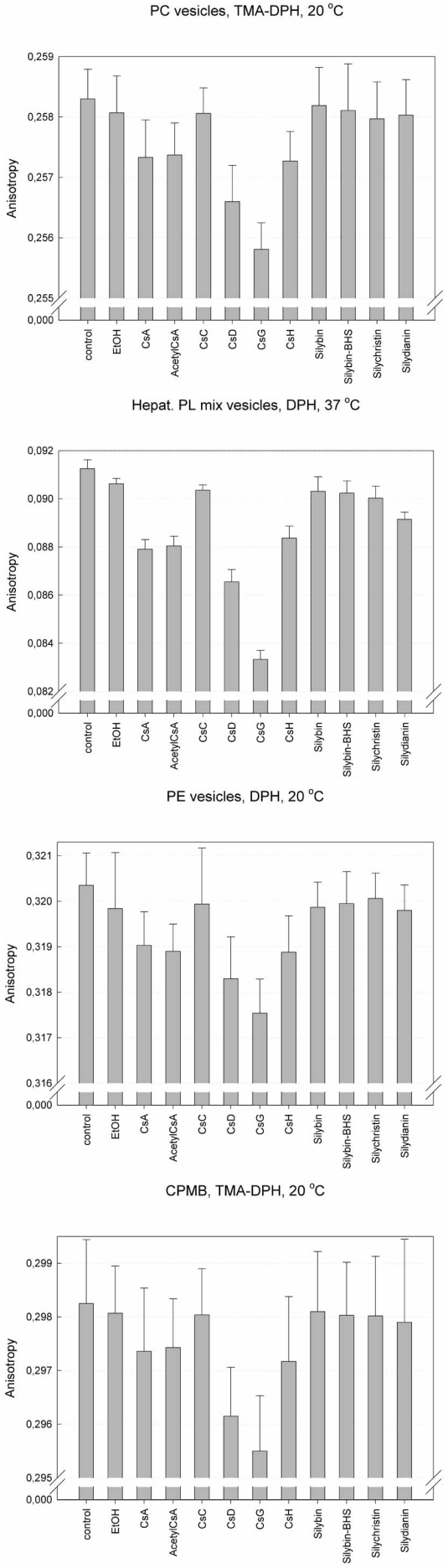
References

1. Moldeus et al., *Methods Enzymol.* **52** (1978) 62-71.
2. Scott et al., *Methods Mol. Biol.* **19** (1993) 59-69.
3. Cartwright, *Methods Mol. Biol.* **19** (1993) 153-161.
4. Kuhry et al., *Biochim. Biophys. Acta* **845** (1985) 60-67.
5. Lakowitz, Principles of Fluorescence Spectroscopy, Plenum Press, New York, 1983.
6. Lowry et al., *J. Biol. Chem.* **193** (1951) 263-272.



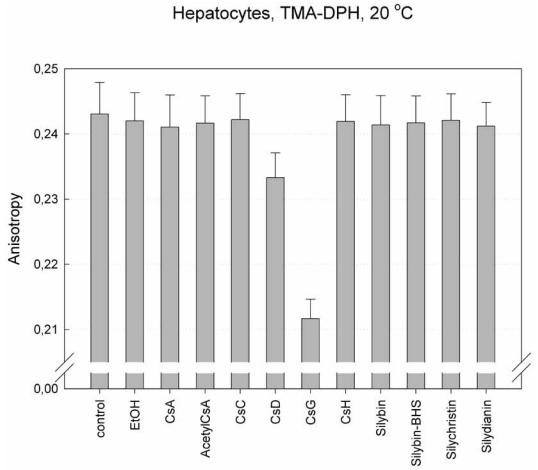


Fig. 1. Membrane lipid fluidity after addition of cyclosporines or silymarines measured by fluorescence anisotropy

Abbreviations:

TMA-DPH: Amphiphilic fluorescence probe 1-[4-(trimethylamino)phenyl]-6-phenyl-hexa-1,3,5-triene.

DPH: Hydrophobic fluorescence probe 1,6-diphenyl-hexa-1,3,5-triene

PC: Vesicles prepared from pure phosphatidylcholine - the main phospholipid of hepatocyte membrane that dominates in the inner half-layer of hepatocyte membrane.

PE: Vesicles prepared from pure phosphatidylethano lamine- the second main phospholipid of hepatocyte membrane that dominates in the outer half-layerof hepatocyte membrane.

Hepat. PL: Vesicles prepared from the mixture of phospholipids isolated by chlorofom-methanol extraction of hepatocyte plasma membranes

CPMB: Vesicles prepared from cytoplasmic membranes of hepatocytes (including proteins)