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SOLUBILITY OF PHENOTHIAZINES IN RED BLOOD CELL MEMBRANES AS EVIDENCED BY PHOTOAFFINITY LABELING

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Radioactively labeled 7-azido-fluphenazine and 7-azido-triflupromazine methiodide have been synthesized and their binding to membranes of intact red blood cells and to ghosts was compared after irradiation. The results indicated that tertiary phenothiazines react with integral membrane components. We conclude from the results that amphiphilic substances solubilize in biological membranes. This is in contradiction to the proposal that these compounds are excluded from the hydrophobic core of biological membranes (Conrad & Singer (1979) Proc.Natl.Acad.Sci.U.S.A. 76, 5202-5206 and (1981) Biochemistry 20, 808-818).

It has been generally accepted that amphiphilic molecules solubilize in biological membranes causing changes in organisation and in the physical state of the membrane (1). This assumption was based on binding studies separating free from bound substances by equilibrium dialysis or centrifugation (1-3) as well as on ESR, NMR or fluorescence studies (4-10). Intact red blood cells and isolated membranes have been used for these investigations.

Recently, however, solubilization of amphiphilic molecules in biological membranes has been questioned by Conrad and Singer (11,12). These investigators concluded on the basis of data which had been obtained by "hygroscopic desorption" of amphiphilic substances - among them the phenothiazine derivative chlor-

Abbreviations used: Periodic acid-Schiff reagent (PAS), sodium dodecyl sulfate (SDS), SDS-polyacrylamide-gel-electrophoresis (SDS PAGE), thin layer chromatography (TLC).

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promazine - from blood cells, ghosts and phospholipid vesicles, that amphiphilic substances are excluded from the hydrophobic core of biological membranes.

Since interactions of amphiphilic drugs like the phenothiazines with membranes are thought to be important for their action as anaesthetics and tranquilizers (1) we investigated their binding sites in red blood cell membranes employing photoaffinity labeling with phenothiazine derivatives. Using this approach we were able to detect binding to integral membrane components. Our findings lead to the conclusion that tertiary phenothiazines do solubilize in the hydrophobic core of the membrane.

METHODS

Synthesis

7-Nitro-2-trifluoromethyl derivatives were synthesized according to Florey and Restivo (13) and converted to the corresponding 7-azido-derivatives by reduction, diazotisation and displacement of the diazonium group by the azido group.

Tritiated 7-azido-fluphenazine in a specific activity of 100 mCi/mmole was obtained by reduction of 4-[3-[2-(trifluoromethyl)-7-nitro-10H-phenothiazine-10-yl]propyl]-1-piperazine-1,1-diethoxyethane with tritiated sodium borohydride, followed by reduction of the nitro group and conversion into the azide as described above. The quaternary derivative was synthesized from 7-azidotriflupromazine using ^{14}C-labeled methyliodide (spec.activity 11.6 mCi/mmole). The synthesis will be described in detail elsewhere.

Photolysis

Washed human red blood cells were suspended in 150 mM NaCl, 5 mM phosphate pH 7.4 to a haematocrit of 10% and treated with the phenothiazines in concentrations as indicated. The mixture was incubated at room temperature in the dark for a few minutes.

Unsealed ghosts were prepared according to Steck (14). The membranes (10^9 ghosts/ml) were suspended in 5 mM phosphate buffer pH 7.4 and treated with the phenothiazines in the indicated concentration.

A high pressure mercury vapor lamp (Phillips HPX 125 W/L) was used for photolysis and set up in a distance of 5 cm from the samples. The light was filtered by a 2 cm water layer and a 5 mm thick pyrex glass. Samples were kept in an ice bath with gentle stirring and purged with nitrogen for 15 min before photolysis.

Photolyzed intact red cells were washed three times by centrifugation and resuspension in the incubation buffer containing 1% bovine albumin. After three additional washes with

albumin free incubation buffer, ghosts were prepared according to Steck (14).

Photolyzed ghosts were washed three times by suspension in the haemolysis buffer containing 1% bovine albumin and centrifugation at 22,000 x g_{max} for 15 min. This was followed by three additional washes with buffer without albumin.

Radioactivity was determined in the lysate before and after isolation of membranes and in the ghost preparations.

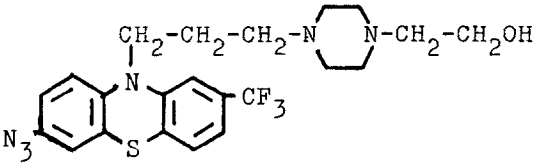
SDS PAGE

Samples (ghosts) were solubilized in a buffer containing 0.0265 M tris HCl (pH 6.8), 2% SDS, 10% glycerol and 5% ß-mercaptoethanol. The solution was boiled for 2 min and subjected to SDS PAGE on slab gels according to Laemmli (15).

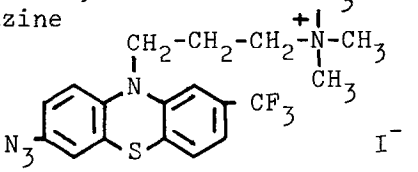
For determination of radioactivity, the Coomassie Blue or PAS stained gels were sliced into 3 mm pieces which were digested as described by Inough and Beckwith (16).

RESULTS

Two radioactively labeled phenothiazine derivatives containing azido groups as photoaffinity labels were synthesized.



4-[3-[2-(Trifluoromethyl)-7-azido-10H-phenothiazine-10-yl]propyl]-1-piperazine ethanol; 7-Azidofluphenazine



N,N,N-Trimethyl-2-(trifluoromethyl)-7-azido-10H-phenothiazine-10-propanaminium iodide; 7-Azidotriflupromazine methiodide

The compounds protected erythrocytes against osmotic haemolysis and caused shape changes similar to the phenothiazines used therapeutically (data not shown). The concentrations used for the experiments described in the following were about 10 times lower than the concentration which caused maximal protection against haemolysis, in order to avoid possible membrane damaging effects. When these compounds were incubated with washed human red blood cells, irradiated with light above 300 nm and washed extensively with albumin containing buffer to remove the nonreacted material,

7.4% incorporation of the tertiary compound and 7.8% of the quaternary compound could be detected in the membrane (table 1). Incorporation in the nonphotolyzed control was negligible (table 1). In contrast to the compound with quaternary nitrogen, the tertiary compound was found also in the haemolysate (8.5%). When isolated ghosts were used, more incorporation (15% and 19%, table 1) was obtained and photolysis time was shorter (5 min).

SDS PAGE was carried out with solubilized membranes derived from photolysed red blood cells and ghosts. Analysis of the gels for incorporation of radioactivity revealed the following:

1. Membranes of intact red blood cells

7-Azidofluphenazine reacted with the integral membrane proteins band 3 and glycophorin (PAS I) as well as with the peripheral protein spectrin and with phospholipids (fig. 1A). Reaction with 7-azido-triflupromazine methiodide could be detected only for the phospholipids (fig. 2A), which were identified by

Table 1.

Incorporation of radioactively labeled 7-azido-phenothiazines in red blood cells and ghosts before and after photolysis.
a: [^{3}H]7-azido-fluphenazine, 4 x 10^{-6}M, spec.act. 100 mCi/mmole
b: [^{14}C]7-azido-triflupromazine methiodide, 2 x 10^{-5}M, spec.act. 11.6 mCi/mmole

		Incorporation (% of total concentration)		
		tertiary compound (a)	quaternary compound (b)	photolysis time (min)
intact cells (10% haematocrit)	membranes	0.12	0.3	0
		7.4	7.8	10
	cytoplasm	0.7	0.2	0
		8.5	0.2	10
ghosts (10^{9} cells/ml)		0.4	0.7	0
		15.0	19.0	5

Red blood cells or ghosts were incubated with a or b, photolyzed, washed, and further processed as described in Methods.

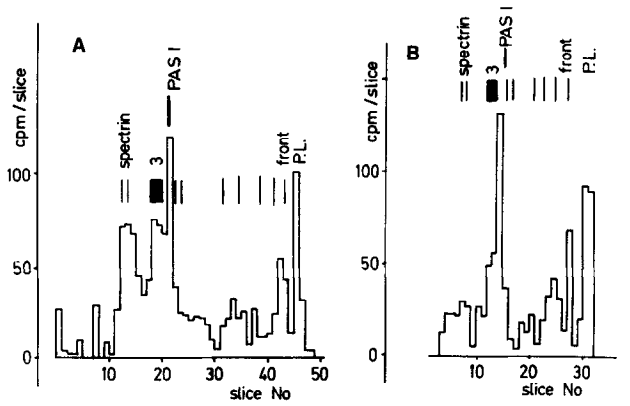


Fig.1 Labeling of red blood cells by [^{3}H]7-azidofluphenazine. The membranes from red blood cells (A) and ghosts (B) were incubated with [^{3}H] 7-azidofluphenazine (4 x 10^{-6}M, spec.act. 100 mCi/mmole) and subjected to photolysis as in the Methods. The membranes were isolated as described and subjected to analysis by SDS PAGE (7.5% acrylamide). The gel was cut into 3 mm slices and radioactivity determined as described in Methods. The protein bands which are visible after Coomassie Blue or PAS staining were drawn schematically on top of the gel.

TLC after lipid extraction and by PAS staining after SDS-PAGE (not shown).

2. Isolated ghosts

7-Azidofluphenazine was found to label mainly integral membrane proteins and phospholipids (fig. 1B), whereas 7-azidoflupromazine methiodide labeled the peripheral protein spectrin in addition (fig. 2B).

DISCUSSION

The present study was undertaken in order to find out about binding of phenothiazines in or to erythrocyte membranes. This should be particularly interesting, since erythrocytes are commonly used to study membrane perturbations by anaesthetics and to elucidate the molecular basis of anaesthetic action.

The photoaffinity labeling approach described in this work, using a compound with tertiary nitrogen which can be uncharged and a compound with quaternary nitrogen having a strong permanent charge, allows some conclusions about possible binding

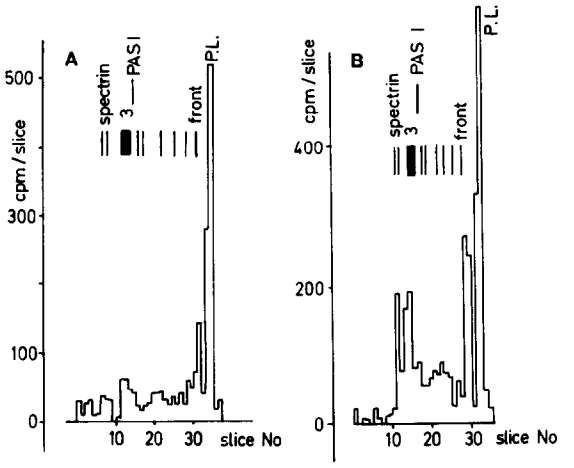


Fig.2 Labeling of red blood cells by [^{14}C] 7-azidotriflupromazine methiodide. The membranes from red blood cells (A) and ghosts (B) were incubated with [^{14}C] 7-azidotriflupromazine methiodide (2 x 10^{-5}M, spec.act. 11.6 mCi/mmole) and subjected to photolysis as in the Methods. The membranes were isolated as described and subjected to analysis by SDS PAGE (7.5% acrylamide). For further details see legend to fig.1.

sites in the membrane. Although phenothiazines are photosensitive by themselves (17,18) forming free radicals upon UV-irradiation, a nitrene generating azido group was introduced in order to achieve shorter life time and greater reactivity of the light-activated molecule.

The finding that the tertiary N-derivative labels integral proteins in intact cells, whereas the quaternary N-compound reacts with phospholipids only, is a strong indication for solubilization of the tertiary compound in the membrane. Moreover, the ability of the tertiary phenothiazine to label cytoplasmic proteins released by haemolysis indicates the compound can cross the membrane (table 1), whereas this does not occur in the quaternary case. This is clearly in contrast to Conrad and Singer's finding, that amphiphilic molecules like the phenothiazines, do not solubilize in biological membranes (11,12). These authors assume that the compounds form some type of micelles with components of the membrane surface. These aggregates can be removed

under the conditions of "hygroscopic desorption", but stick with the cells when the free molecules are separated from the bound by centrifugation of the cells. This type of binding might apply for quaternary compounds, where a strong permanent charge prevents the molecule from permeation into the bilayer. Our results show labeling of phospholipids only for these compounds. However, we think that the binding is more likely caused by immersion of the hydrophobic part of the phenothiazine into the bilayer - causing the observed labeling of phospholipids - while the hydrophilic charged part of the molecule remains on the surface of the cell.

In the case of unsealed ghosts, the peripheral protein on the inside and the cytoplasmic domains of the integral proteins were accessible for the quaternary compound and thus became labeled. In contrast, no reaction with peripheral proteins was found for the tertiary compound. This provides further evidence for preferential localization of the tertiary phenothiazines in the hydrophobic core of the bilayer.

Whereas there is enough evidence for solubility in the membrane, we cannot conclude from our experiments that the hydrophobic sites of membrane proteins are specific binding sites for the drug. The relatively long life-time and certain electrophilic properties of aromatic nitrenes might cause some preferential labeling of compounds with reactive groups like proteins suggesting a specific binding site.

Formation of free radicals and their reaction with oxygen to more hydrophilic sulfoxides, which is known to be catalyzed by hemoglobin (19) might be the reason that in case of intact erythrocytes the peripheral membrane protein spectrin is labeled by the tertiary compound and that a considerable amount of the drug is found in the cytoplasm.

Several papers appeared, which describe results in contradiction to Conrad and Singer's findings: Bondy and Remien (20) found considerable partitioning of chlorpromazine in blood cells at lower concentrations, using the hygroscopic desorption method of Conrad and Singer (11,12); Gains and Dawson (9) obtained a similar binding affinity of 8-anilinonaphtalene-1-sulfonate to Triton X 100 micelles and to submitochondrial particle calculated from fluorescence measurements; Moules et al. (8) could not detect micelle formation on the surface of sarcoplasmic reticulum membranes by a ESR study with spin labeled fatty acids and by fluorescence studies with dansyl propanol; finally, using EPR techniques, Gaffney et al. (10) obtained data on partitioning of spin labeled amphiphiles between membranes with and without proteins. Very little difference could be detected.

In contrast to these studies, our photochemical approach enabled us to analyse binding sites of amphiphilic substances in biological membranes directly. We conclude on the basis of these results that small amphiphilic molecules do solubilize in biological membranes.

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