Biochemical Pharmacology, Vol. 25, pp. 1783–1785. Pergamon Press, 1976. Printed in Great Britain.

THE ACTION OF ANESTHETICS ON THE PHOSPHATIDE PATTERN OF RED BLOOD CELL AND YEAST MEMBRANES

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(*Received* 25 *August* 1975; *accepted* 19 *January* 1976)

Abstract—A number of local anesthetics, tranquilizers, detergents and alcohols cause, at high concentrations, an increase in the level of diphosphatidylglycerol and a decrease in phosphatidylglycerol in erythrocytes and yeast cells. This change in phospholipid pattern is inhibited by the addition of cytidine-5'-monophosphate.

A variety of lipid-soluble compounds, including local anesthetics, tranquilizers and detergents at low concentrations will protect cell membranes from osmotic lysis [1]. At higher concentrations (usually above 10^{-3} M) these anesthetics are directly lytic to the erythrocyte membrane. This action of anesthetics is well established. Anesthetics also elicit erythrocyte membrane expansion. Van Steveninck *et al.* [2, 3] reported that 10^{-4} M chlorpromazine caused a 10% increase in the critical haemolytic volume. Anesthetic amines displace membrane-bound Ca^{++} [4]. In this paper we present a new common action of anesthetics on biological membranes. Our experiments indicate that different anesthetics, at high concentrations, cause a change in the phosphatide pattern of the membrane.

MATERIAL AND METHODS

Erythrocytes. Bovine blood with heparin as anticoagulant was centrifuged; plasma, buffy coat and the upper layer of the red cells were discarded. The packed red cells were washed three times with an isotonic citrate–phosphate–glucose solution (30 g trisodium citrate, 150 mg NaH_2PO_4 and 1 g glucose/l) according to Mollison *et al.* [5].

The phospholipids were labeled with ^{32}P, because the changes were too small to be detected by a colorimetric method. The incorporation of ^{32}P into the phosphatides of the red cell was much more rapid in the citrate–phosphate–glucose medium than in the isotonic NaCl[5]. The phosphatides were labeled by incubating an erythrocyte suspension in isotonic citrate–phosphate–glucose (1 ml packed cells + 1 ml medium) with sodium-^{32}P-orthophosphate (50 μCi ^{32}P/ml packed cells) for 17 hr at 25°. Under these conditions the uptake of ^{32}P was about 0.25 μCi/μmole phospholipid.

Possible bacterial growth was checked by inoculation of the red cell suspension onto agar (3%) containing 1.5% Merck standard II-nutritive bouillon and 1% glucose, with subsequent incubation at 37° for 48 hr. Bacterial growth after 17 hr of labeling with ^{32}P did not exceed 1 bacterium per 2×10^4 erythrocytes.

The presence of leucocytes in the erythrocyte layer was checked by leucocyte counting in a Bürker chamber. After the described washing procedure and the repeated discarding of the top-layer of red cells between each washing, the leucocyte:erythrocyte ratio did not exceed $1:10^5$.

Yeast. Baker's yeast, Strain Delft-1, was cultured aerobically at 27° on a liquid medium described by Van Steveninck *et al.* [6]. The cultured yeast was washed three times in about 30 vol of distilled water. Subsequently, the yeast was starved aerobically overnight in distilled water and washed again twice. The phosphatides were labeled with ^{32}P by incubating a 10% aqueous suspension of 30 g yeast with 2 mCi sodium-^{32}P-orthophosphate at 25°, for 2 hr, with aeration. After this time the pattern of the incorporation of ^{32}P into the yeast phospholipids was comparable with that of bovine erythrocytes labeled as described above. Incorporation of ^{32}P was about 0.25 μCi/μmole phospholipid.

Extraction and detection of phospholipids. Lipids were extracted from red cells according to the method of Folch *et al.* [7]. Lipid extraction from yeast cells was described previously [8].

The paper chromatographic separation and identification of phosphatides was accomplished according to a procedure of Letters [9]. In this two-dimensional procedure a strip-transfer method was used in such a way that phosphatides were separated in the first direction on formaldehyde impregnated paper [10], and in the second direction on Whatman SG 81 silicic acid impregnated paper. The mobile phase in the first direction was the upper phase of the butan-1-ol–acetic acid–water solvent system equilibrated with ether [10]. The mobile phase in the second direction was the di-isobutylketone–acetic acid–water system described by Marinetti [11].

The relative positions of the phosphatides is shown in Fig. 1.

The quantitative estimation of phosphatides and radioactive phosphorus was described previously [8].

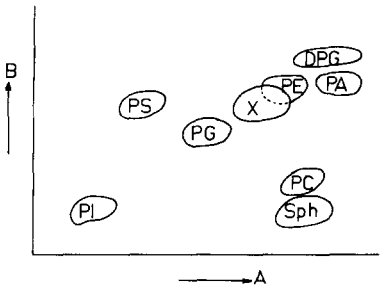

Fig. 1. Two-dimensional paper chromatography of phosphatides. A, first direction; B, second direction. Abbreviations: DPG, diphosphatidylglycerol; PA, phosphatidic acid; PE, phosphatidylethanolamine; X, unidentified; PS, phosphatidylserine; PG, phosphatidylglycerol; PC, phosphatidylcholine; Sph, sphingomyelin; PI, phosphatidylinositol.

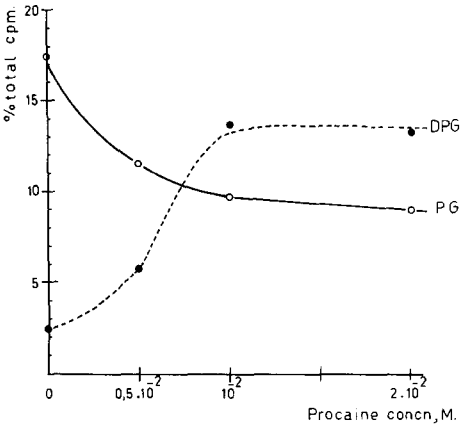

Fig. 2. The change in % total ^{32}P cpm in phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) in relation to procaine concentration.

Overlapping spots (X, PE) could be distinguished if their specific radioactivity was markedly different. The relative positions of these spots was established by comparison of the normal phospholipid staining methods with the blackening of X-ray film.

RESULTS

The action of anesthetics on the phosphatide pattern of bovine erythrocytes. The red cell suspension, after labeling with ^{32}P was diluted to 10% with isotonic citrate–phosphate–glucose. Procaine was added to a final concentration of 10^{-2} M. After incubating for 1 hr at 25° the cells were packed by centrifugation, the supernatant discarded and the lipids of the erythrocytes extracted and analyzed as described in the preceding section.

After the same incubation period in citrate–phosphate–glucose medium the control, without procaine, showed the following pattern of radioactivity. The figures are the mean per cent ^{32}P radioactivity (per cent of the total counts ^{32}P per minute in all phospholipid) ± S.D.: PA, 1.0 ± 0.3; PC, 0.9 ± 0.3; PE + X, 71.7 ± 3.1; DPG, 3.0 ± 0.6; PG, 18.3 ± 2.0; PS, 2.2 ± 0.5; Sph, 0.9 ± 0.3; I, 2.0 ± 0.4.

Similar experiments were performed with 2×10^{-4} M promethazine (phenergan), 2×10^{-4} M chlorpromazine, 2×10^{-4} M cetylpyridinium chloride (CPCl), 8×10^{-4} M cetyltrimethylammonium bromide (CTAB), 1 M methanol, 0.5 M ethanol and 0.015 M *n*-pentanol.

Each anesthetic action causes an increase in diphosphatidylglycerol (DPG) and a decrease in phosphatidylglycerol (PG). Table 1 shows the changes in per cent ^{32}P radioactivity. In the other phospholipids the changes in per cent radioactivity did not exceed the experimental error.

The effect of procaine concentration is shown in Fig. 2.

Procaine concentrations larger than 2×10^{-2} M caused considerable hemolysis. Because there was a certain degree of hemolysis in all experiments it was desirable to check whether the change in the phos-

Table 1. Effects of anesthetics on % total ^{32}P cpm in diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG) of bovine red blood cells

Anesthetic	Procaine		Chlorpromazine		Promethazine		Cetylpyridinium chloride	
Concn. (M)	0	10^{-2}	0	2×10^{-4}	0	2×10^{-4}	0	2×10^{-4}
DPG, % total cpm	2.6	13.2	3.0	11.8	3.0	11.3	3.3	14.1
PG, % total cpm	17.4	9.0	20.3	12.2	16.8	9.3	18.2	8.5

Anesthetic	Methanol		Ethanol		*n*-Pentanol		Cetyltrimethyl-ammonium bromide	
Concn. (M)	0	1	0	0.5	0	0.015	0	8×10^{-4}
DPG % total cpm	2.8	8.6	3.1	12.5	3.2	10.8	3.4	8.3
PG % total cpm	16.6	9.6	18.8	8.3	17.3	9.9	18.3	12.1

Table 2. The action of chlorpromazine on % total cpm ^{32}P in DPG and PG of yeast cells

	Blank	Chlorpromazine 2×10^{-4} M
DPG % total cpm	2.4	9.5
PG % total cpm	18.8	9.6

Table 3. Inhibition of the anesthetic induced change in the phosphatide pattern of yeast cells by the addition of cytidine-5′-monophosphate

Chlorpromazine concn. (M)	—	2×10^{-4}	2×10^{-4}	2×10^{-4}	—
CMP concn. (M)	-	-	5×10^{-3}	5×10^{-2}	5×10^{-2}
DPG % total cpm	2.4	9.8	4.7	2.5	2.8
PG % total cpm	19.5	10.1	16.0	20.0	19.1

phatide pattern was caused by hemolysis. Osmotic hemolysis up to 100% in hypotonic NaCl solutions did not change the phosphatide pattern at all.

The action of anesthetics on the phosphatide pattern of yeast cells. Chlorpromazine (2×10^{-4} M) added to the medium during the last 30 min of the labeling of the phosphatides of yeast with radioactive phosphorus, caused a change in the phosphatide pattern similar to that described for bovine erythrocytes (Table 2).

Procaine (10^{-2} M) and promethazine (2×10^{-4} M) caused similar changes. Other anesthetics were not tested in the experiments with yeast cells.

Inhibition of the anesthetic induced change in the phosphatide pattern by cytidine-5′-monophosphate. According to the current scheme of phospholipid biosynthesis based on the results of Kennedy and coworkers [12–15], PG, a cytosine liponucleotide and DPG are interconnected as follows:

$$\text{PA} \underset{\text{CTP PP}}{\rightleftharpoons} \text{CDP-diglyceride} \underset{\text{GP CMP}}{\rightleftharpoons} \text{PGP} \rightleftharpoons$$

$$\text{PG} \underset{\text{CDP-digl. CMP}}{\rightleftharpoons} \text{DPG}$$

(CTP = cytidine-5′-triphosphate, PPi = pyrophosphate, CDP-diglyceride = cytidine diphosphate diglyceride, GP = glycerol-3-phosphate, CMP = cytidine-5′-monophosphate PGP = phosphatidylglycerolphosphate).

According to this scheme the change in the phosphatide pattern from PG to DPG, as shown in Tables 1 and 2, can be inhibited by the addition of CMP. This is confirmed in experiments with yeast Strain Delft-1.

When 2×10^{-4} M chlorpromazine together with 5×10^{-3} M CMP is added to a 10% aqueous suspension of yeast cells, in the final stage of the incorporation of ^{32}P, the change in the phosphatide pattern is inhibited to about one third of the change with chlorpromazine alone; 5×10^{-2} M CMP causes a complete inhibition (Table 3).

CONCLUSION

In addition to the effects of anesthetics on biomembranes reviewed by Seeman [1] we have shown that several anesthetics, in high concentrations, cause a change in the phosphatide pattern of biomembranes. The change is in that specific part of the Kennedy scheme of phospholipid biosynthesis that requires the action of a phosphatidyltransferase system viz. CDP-diglyceride–phosphatidylglycerol phosphatidyltransferase (cardiolipin synthetase). The inhibition of the change by CMP emphasizes the fact that this part of the synthesis scheme is susceptible to anesthetic action.

The question whether the different anesthetics can stimulate this particular transferase activity is the object of further study.

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