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INHIBITION OF ANION AND GLUCOSE PERMEABILITIES BY ANESTHETICS IN ERYTHROCYTES

THE MECHANISMS OF ACTION OF POSITIVELY AND NEGATIVELY CHARGED DRUGS

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Summary

(1) The mode of action of anesthetics as inhibitors of Cl^- and glucose transports in human red cells was studied. The term anesthetic is taken in its broad meaning as defined by Seeman (Seeman, P. (1972) Pharmacol. Rev. 24, 583—655) and covers anionic and cationic liposoluble compounds which reversibly block the rising phase of the action potential, without effect on the resting membrane potential.

(2) Phenothiazine derivatives were chosen as prototypes of anesthetics because they represent a set of compounds having the same basic chemical structure, the phenothiazine ring, but with either a positive or a negative charge.

(3) The Cl^- self-exchange is inhibited by both cationic and anionic derivatives. However, to obtain the same level of inhibition, it is necessary to use a concentration 10—100 times higher with cationic than with anionic drugs.

(4) At a concentration which inhibits Cl^- permeability, cationic derivatives induce a very strong morphological change (cup-shaped cells: stomatocytes or spherostomatocytes) and protect erythrocytes against osmotic hemolysis, signifying that the membrane is fully expanded. Conversely, with anionic derivatives, inhibition occurs at a concentration which does not induce any apparent shape change or protect against osmotic hemolysis: there is no significant membrane expansion.

(5) Glucose permeability, measured by glucose exit, is inhibited by cationic and anionic phenothiazine, but always at a concentration which fully expands the membrane as indicated by morphological changes and anti-hemolytic

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effects. It is interesting to point out that whilst glucose exit shows inhibition by cationic derivatives, glucose exchange flux is scarcely altered.

(6) It is concluded that cationic and anionic anesthetics are general inhibitors of transmembrane solute movements involving a facilitated-diffusion process. However, the mechanism of inhibition is not identical for all: inhibition of glucose permeability by anionic and cationic anesthetics, as well as inhibition of Cl^- permeability by cationic anesthetics may be of a non-specific nature and result from their interaction with the bilayer (this indirect effect is discussed); on the other hand, inhibition of Cl^- permeability by anionic anesthetics may result from a specific perturbation of the transport mechanism according to recent evidence in some cases (Cousin, J.L. and Motais, R. (1979) J. Membrane Biol. 46, 125—153; Zaki, L., Ruffing, W., Gärtner, E.M., Fasold, H., Motais, R. and Passow, H. (1977) 11th FEBS Meeting, Copenhagen, A4 17-671.

Introduction

The usual definition of an anesthetic is that of a drug which, when applied to the muscle or nerve cell, reversibly blocks the rising phase of the action potential (i.e., the rapid entry of Na^+ into the cell through the so-called Na^+ channel) without appreciable effect on the resting membrane potential. According to this definition, a great number of anionic or cationic lipid-soluble compounds are anesthetics [1].

Although much attention has been focused on the mode of action of anesthetics, the manner in which the Na^+ conductance channel is inhibited by the drug is still unknown. Recent theories emphasized either a direct interaction between anesthetic and ion channel protein structure [2,3] or an anesthetic-induced alteration in the physical state of the lipid milieu which secondarily modifies the functional properties of the channel protein [4,5].

Since these proposed mechanisms involve anesthetic interaction either with membrane lipid or with the hydrophobic regions likely to be common to most intrinsic membrane proteins, it is expected that anesthetics can inhibit translocation across membranes of different solutes (and not only Na^+) as long as they flow through integral proteins. Indeed, facilitated diffusion of anions in the red blood cell, which is considered to occur through a specific intrinsic membrane protein, the band 3 protein, is inhibited by a large variety of anionic and cationic lipid-soluble compounds which belong to the class of anesthetics as defined above [6—18]. These inhibitors include: fatty acids, phenol derivatives, pharmacological drugs (local anesthetics, diuretics, anti-inflammatory agents, uricosuric agents, analgesics, vasodilators, sedatives), etc.

Recently in several papers on the effect of anionic liposoluble anesthetics on Cl^- transport in erythrocytes, we showed the importance of certain physicochemical properties of the molecules in their inhibitory potency [13—16, 18] and produced strong evidence that they interact with the band 3 protein [19, 20]. The cationic liposoluble anesthetics also inhibit Cl^- transport but at higher concentrations [6,11,12] suggesting a different and more indirect mode of action. In an attempt to provide evidence for different sites of action of anionic

and cationic inhibitors, we have studied the influence on Cl^- transport of several compounds having the same basic chemical structure (i.e., the phenothiazine ring), but with either a positive or a negative charge, and tried to correlate it with shape changes and internal concentration. In addition we have made comparable studies on glucose transport in erythrocytes.

Materials and Methods

Preparation of cells

Freshly collected human blood was obtained from the blood bank and stored at 4°C. Before use the red cells were separated by centrifugation, and plasma and buffy coat removed by aspiration. They were then washed three times in appropriate buffer and used as described below.

Flux measurements

^{36}Cl effluxes. The Cl^- self-exchange fluxes were measured in intact cells at Donnan equilibrium (pH 7.4 and 0°C). Labelling of cells with ^{36}Cl, isolation of labelled cells, determination of radioactivity in cell-free medium and automatic technique of flux measurement have previously been described [17]. However, in the present work the hematrocrit was 0.5%. The composition of the solution used was: 150 mM NaCl, 10 mM KCl, 20 mM Tris and 10 mM glucose. In experiments with inhibitors the cells were not incubated with the inhibitors prior to the flux measurements: the drugs were only present in the experimental media at suitable concentrations.

[^{14}C]Glucose effluxes. The glucose exchange fluxes were performed under the same conditions as the Cl^- effluxes in order to compare the effects of drugs on both transport systems.

Human red cells were washed several times in saline buffer of the following composition: 140 mM NaCl, 20 mM Tris, 80 mM glucose at pH 7.4. Between each centrifugation, the suspension was kept at room temperature for a few minutes to allow equilibration of glucose between medium and cell water: the subsequent incubation was performed at 0°C, pH 7.4. The cells were then packed to a hematocrit of 80%. [^{14}C]Glucose (Centre d'Etudes Nucléaires de Saclay, France) was added to the cell suspension and allowed to equilibrate for more than 10 half-times of the subsequent efflux at 0°C. At the start of efflux measurement 300-μl of labelled cell suspension were injected into 50 ml of vigorously stirred cold saline-glucose medium (final hematocrit 0.50%; pH 7.4, 0°C). The efflux of [^{14}C]glucose from cells to the medium was measured by serially isolating cell-free medium from the cell suspension by rapid filtration as described by Dalmark and Wieth [21], and the radioactivity of a constant volume of filtrate was measured.

The kinetics for glucose, as for Cl^- efflux, were well described by a two-compartment model with constant volume. The equation describing the time-dependence of the specific activity in a cell-free medium is:

$$Q_t = Q_\infty (1 - e^{-(k_o + k_i)t})$$

where Q_t and Q_∞ are the specific activities at time, t, and isotopic equilibrium,

respectively, and the exponents k_o and k_i are the rate coefficients for isotope efflux and influx, respectively.

In our experiments k_i could be neglected because the hematocrit was low (0.5%). The rate constant was calculated from the relationship between $\ln(1 - Q_t/Q_\infty)$ and the time t by linear regression analysis. The slope of the graph was assumed to be equal to $-k_o$.

Glucose exit. The exit of glucose from preloaded human erythrocytes was determined from the loss of water volume measured by 90° light scattering [22]. After the cells were washed, a suspension was made by adding red cells to a medium containing 150 mM NaCl, 10 mM Tris, 300 mM glucose (hematocrit 10%, pH 7.4). The cells were kept at 37°C for at least 30 min to allow for the equilibration of glucose between medium and cell water. The suspension was then adapted to 25°C. For measurement, a 0.1 ml sample of the 10% cell suspension was added to 14.9 ml of isotonic NaCl (300 mM NaCl, 10 mM Tris) in measuring cuvettes at 25°C. The change in light scattering with time was measured until equilibrium was attained. The 'exit time', which is inversely proportional to the initial velocity of sugar efflux [23], was obtained by extrapolating the initial slope of the tracing to the equilibrium value. In experiments with inhibitors the cells were not incubated with the inhibitors prior to the net flux measurements: the drugs were only present in the experimental media at suitable concentrations.

Determination of the anti-hemolytic effect

Washed red cells were suspended in 160 mM NaCl, 20 mM Tris-HCl, pH 7.4, at 0°C at a hematocrit of 40%. The cells were then subjected to hypotonic hemolysis by adding 50-μl of the stock cell suspension to 4-ml of cold hypotonic milieu (final hematocrit 0.5%) containing different concentrations of phenothiazine derivatives. After 5 min of incubation at 0°C, the suspensions were centrifuged and the hemoglobin concentration in the supernatant determined from the absorbance at 413 nm. Hemolysis at different drug concentrations is expressed relative to the hemolysis observed in the absence of any drug. The concentration of the hypotonic milieu was chosen to obtain an absolute hemolysis of 25% in the absence of any drug. The above experimental conditions (0°C, hematocrit 0.5%; no preincubation with the drug) are the same as those used for isotopic flux measurements.

Scanning electron microscopy

Washed red cells were suspended at 0.5% hematocrit in a cold isotonic mixture, pH 7.4, containing glutaraldehyde (1%), sodium phosphate (120 mM) and the indicated concentrations of drugs. The cell suspension was incubated for 1 h at 4°C, then washed with 120 mM sodium phosphate, pH 7.4 (three times), fixed with osmic acid (1%) in sodium phosphate, and washed with distilled water (four times). The fixed cells were dehydrated sequentially with ethanol and propylene oxide, a droplet of the suspension was placed on a cover-glass and allowed to dry in air. The dried samples were coated with AuPd and examined in a CAMECA MEB 07 scanning electron microscope.

Materials

All the phenothiazine derivatives were kindly provided by Rhône Poulenc.

Results

Effect of phenothiazine derivatives on Cl^- transport

Phenothiazine has a three-ringed structure in which two benzene rings are linked by a sulfur atom and a nitrogen atom (Fig. 1). The derivatives considered in this study differ in the nature of the substituents at position 2 or 10.

The cationic phenothiazines, of which chlorpromazine (No. 1, Fig. 1) may be taken as a prototype, are psychotropic drugs. The anionic phenothiazines do not possess such an activity but have anti-inflammatory properties.

Fig. 2 shows the effect on Cl^- fluxes of varying the concentration of an anionic derivative (drug No. 12) and of a cationic derivative (chlorpromazine) in the medium. It appears from this figure that I_{50}, the concentration of the drug required in the external medium to produce 50% inhibition (0°C, pH 7.4), is two orders of magnitude higher for the cationic than for the anionic derivative (I_{50}, respectively, $1.5 \cdot 10^{-4}$ and $2 \cdot 10^{-6}$ M).

The experiments reported in Fig. 3 show the rapidity with which the drugs affect the Cl^- self-exchange. When untreated erythrocytes (i.e., no previously incubated in the presence of the drugs) are suspended in a medium containing $1 \cdot 10^{-4}$ M chlorpromazine or $2.5 \cdot 10^{-6}$ M drug No. 12, the Cl^- effluxes are inhibited and follow first-order kinetics. This indicates that the level of inhibition was obtained within the 2 s preceding the first sample.

The data obtained with the phenothiazine derivatives are summarized in Table I and show that:

(1) All the tested derivatives are inhibitors of Cl^- permeability.

(2) There is clearly a strong difference in the capacity to inhibit Cl^- permeability between cationic and anionic agents: to obtain the same level of inhibi-

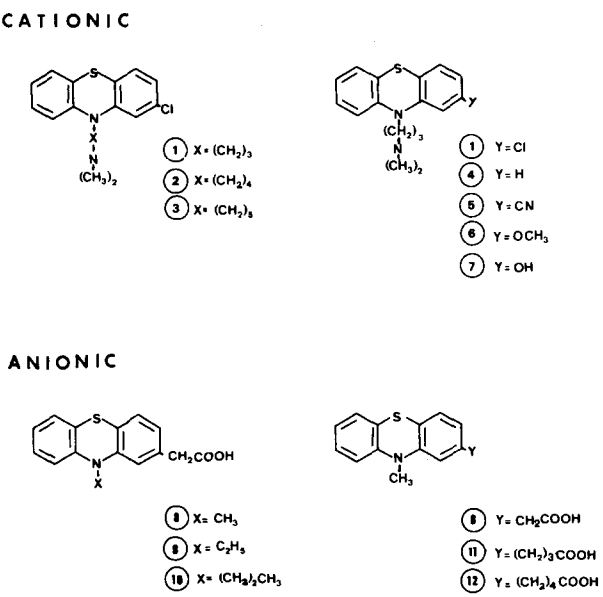


Fig. 1. Structure of phenothiazine derivatives used in this study (Nos. 1—12). Drug No. 1 is chlorpromazine. Y refers to a substituent at position 2. X refers to a substituent at position 10.

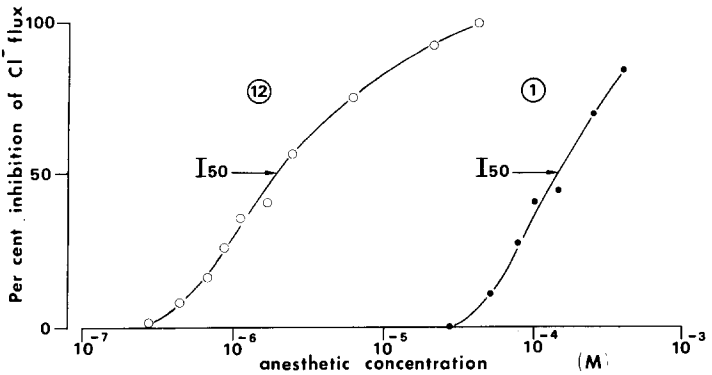


Fig. 2. Inhibition of Cl^- flux in human red cells as a function of the concentration of a cationic (No. 1) or an anionic (No. 12) derivative. The cells were not preincubated with the drugs prior the experiments: the drugs were only present in the experimental media, and thus got into contact with cells at time zero. Hematocrit 0.5%.

tion it is necessary to use a concentration 10—100 times higher with cationic than with anionic drugs.

(3) At least for cationic compounds, comparison of the effects of drugs Nos. 1—3, shows that the more lipophilic the substituent groups are, the greater the activity they confer on the parent molecule. This suggests that the level of membrane concentration could be an important parameter involved in the inhibition.

It should be noted that the inhibitory capacity of cationic phenothiazine is independent of the presence of Ca^{2+} in the external solution.

Effect of phenothiazine on glucose transport

Effect on glucose exit. Exit of glucose from glucose-preloaded erythrocytes was progressively inhibited by increasing concentrations of phenothiazine derivatives in the media. The concentration of a phenothiazine derivative

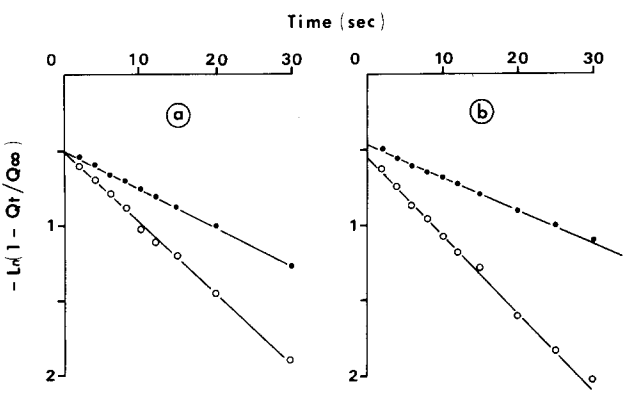


Fig. 3. Effect of: (a) chlorpromazine ($1 \cdot 10^{-4}$ M) and (b) No. 12 ($2.5 \cdot 10^{-6}$ M) on Cl^- self-exchange in human red cells. Ordinate, $\ln(1 - Q_t/Q_\infty)$, Q_t and Q_∞ are the concentrations of $^{36}Cl^-$ in external medium at time t and at isotopic equilibrium, respectively; abscissa, time in s; (○) control cells, (●) cells injected at time 0 in medium containing chlorpromazine or drug No. 12.

TABLE I

EFFECT OF ANIONIC AND CATIONIC DERIVATIVES ON Cl^- AND GLUCOSE PERMEABILITIES

	Reference number of drug (see Fig. 1)	Effect on Cl^- permeability (I_{50} in M)	Effect on glucose permeability	
			Exit (K_i in M)	Exchange (I_{50} in M)
Cationic phenothiazines	1	$1.5 \cdot 10^{-4}$	$8.0 \cdot 10^{-5}$	
	2	$6.0 \cdot 10^{-5}$	$3.4 \cdot 10^{-5}$	
	3	$4.0 \cdot 10^{-5}$	$2.0 \cdot 10^{-5}$	
	4	$4.0 \cdot 10^{-4}$	$2.1 \cdot 10^{-4}$	
	5	$4.0 \cdot 10^{-4}$	$1.8 \cdot 10^{-4}$	
	6	$2.5 \cdot 10^{-4}$	$1.2 \cdot 10^{-4}$	
	7	$1.6 \cdot 10^{-4}$	$1.2 \cdot 10^{-4}$	
Anionic phenothiazines	8	$6.5 \cdot 10^{-6}$	$4.0 \cdot 10^{-4}$	$8.0 \cdot 10^{-5}$
	9	$9.0 \cdot 10^{-6}$	$1.8 \cdot 10^{-4}$	$6.0 \cdot 10^{-4}$
	10	$2.0 \cdot 10^{-6}$	$2.0 \cdot 10^{-4}$	$2.0 \cdot 10^{-4}$
	11	$3.5 \cdot 10^{-6}$	$1.8 \cdot 10^{-4}$	$4.0 \cdot 10^{-4}$
	12	$2.0 \cdot 10^{-6}$	$7.0 \cdot 10^{-5}$	$7.0 \cdot 10^{-5}$

necessary to double the exit time (k_i) was determined from plots of exit time vs. phenothiazine concentration [23], as illustrated in Fig. 4 for an anionic and a cationic derivative (drug No. 12 and chlorpromazine, respectively).

It can be observed that the anionic and cationic drugs have the same capacity to inhibit glucose, a situation very different to that concerning Cl^- permeability (Fig. 2). Under our experimental conditions we did not observe with chlorpromazine the complex pattern of inhibition (biphasic effect) shown by Baker and Rogers [24].

The k_i values so obtained for all phenothiazine derivatives are summarized in Table I.

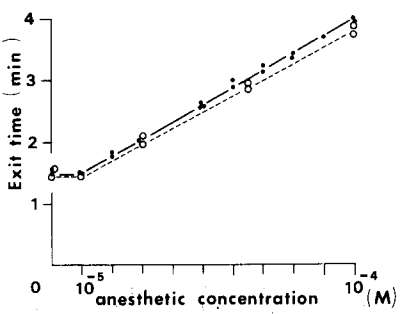
All of the twelve derivatives tested were found to be inhibitors of glucose exit from human red cells and the anionic and cationic agents have a similar inhibitory capacity (the K_i values for the compounds lie within the range $2 \cdot 10^{-5}$—$2 \cdot 10^{-4}$ M). Moreover, by comparison of compounds having homologous substituents (Nos. 1, 2 and 3, or 8, 9 and 10, or 8, 11 and 12) it appears that the relative inhibitory activity of either cationic or anionic drugs is correlated with their liposolubility.

The inhibitory effects on the exit of glucose were found to be completely reversible upon washing the cells at all sub-lytic concentrations of the drug and were independent of the presence of Ca^{2+} in the external solution (not shown).

Effect on glucose exchange. The behavior of glucose-exchange flux in the presence of anionic phenothiazine is similar to that described for glucose net flux: the drug concentration required to produce 50% inhibition is close to that which produces a 2-fold inhibition of exit rate (Table I).

Conversely, in the presence of cationic phenothiazine, the exchange flux is practically unaffected over the concentration range which produces a 2-fold inhibition of exit rate, and at a higher concentration some decrease in the rate of exchange is seen but this does not develop as rapidly as for the exit.

These data on cationic derivatives confirm the results obtained by Baker and Rogers [24] showing that chlorpromazine inhibits glucose exit whereas the



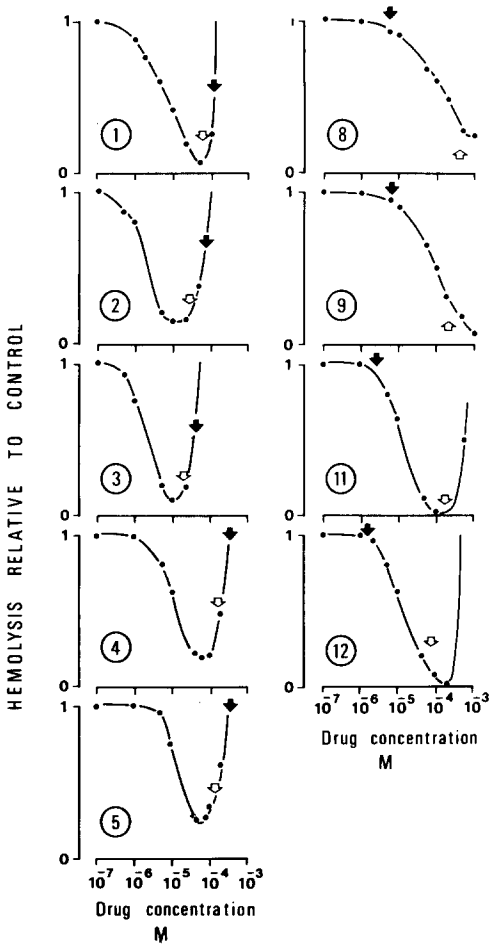


Fig. 4. The effect of an anionic derivative, No. 12 (○) and a cationic derivative No. 1 (chlorpromazine) (●) on the exit time of 300 mM glucose from human red cells at 27°C determined by the optical technique. External glucose concentration due to addition of extracellular space was 2 mM. The cells were not preincubated with the drugs prior the experiments: the drugs were only present in the experimental media. Hematocrit 0.066%.

Fig. 5. Protection against hemolysis as a function of drug concentration. Left, cationic drugs; right, anionic drugs. Solid arrows indicate the concentration of the drug necessary to inhibit 50% of Cl^- transport (I_{50}), open arrows the concentration necessary to double the exit time of glucose (k_i). The number on the curves corresponds to the number of the drug in Fig. 1.

glucose-exchange flux was scarcely altered. As quoted by Baker and Rogers [24] this "effect ... discloses a fundamental difference between exchange and net flux".

Expansion of the membrane by phenothiazine derivatives

Under the conditions in which the above experiments were carried out, phenothiazine derivatives alter the shape of the cells (Fig. 7). As the concentration of anionic derivatives increases, the cell changes from a discocyte to a discoechinocyte and then to a spheroechinocyte. In the presence of cationic

derivatives the cell becomes cup-shaped. Sheetz and Singer [25] have proposed that these shape changes can result from the fact that charged drugs bind differentially to the two membrane surfaces because of the asymmetry of membrane lipid distribution, thereby inducing asymmetrical expansion of the membrane. Such an asymmetrical expansion of the lipid bilayer may disrupt the required structural organization of the transporting channels and thus may inhibit transport. To test whether such an explanation can account for the above results on Cl^- and/or glucose permeabilities for cationic and/or anionic compounds, we need to obtain quantitative data for each drug comparing the permeability effects with the degree of membrane expansion.

Membrane expansion can be quantified by measuring the degree of protection from hypotonic hemolysis in the presence of different concentrations of anesthetic; the hemolytic protection is interpreted as being due to expansion of the membrane allowing greater increases in cell volume before lysis [1]. As shown in Fig. 5, increasing drug concentrations protect against hemolysis with maximum protection in the range $1 \cdot 10^{-5}$—$1 \cdot 10^{-4}$ M for cationic and $1 \cdot 10^{-4}$—$1 \cdot 10^{-3}$ M for anionic compounds. At higher drug concentrations hemolysis protection decreases and with a sufficient amount of drug, spontaneous hemolysis occurs. With the cationic compounds (left-hand curves) inhibition of Cl^- transport (solid arrows) appears only for a prehemolytic concentration of the drug, i.e., when the membrane is fully expanded. With the

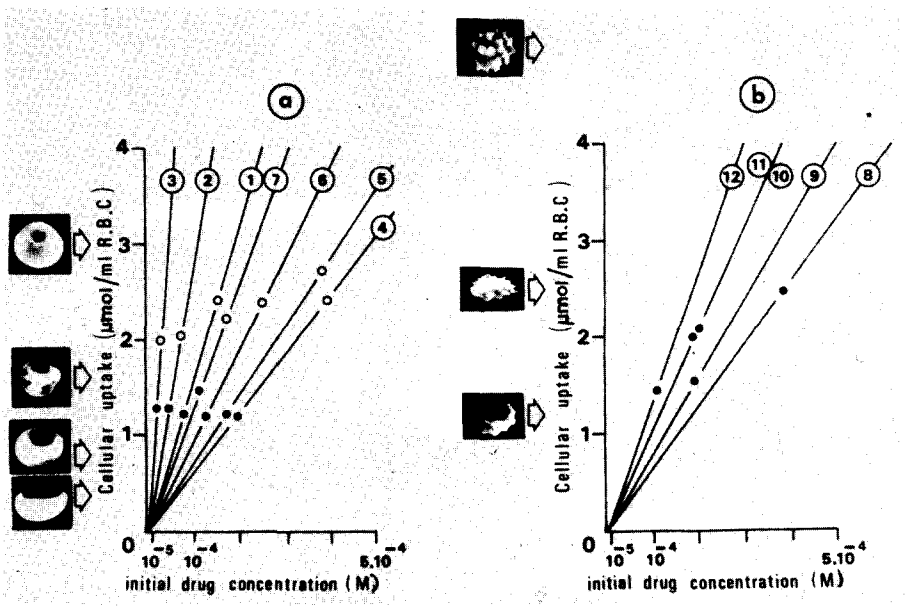


Fig. 6. Amount of drug adsorbed by erythrocytes as a function of the concentration of drug in the external medium at the suspension time. A given amount of drug absorbed by the cell corresponds to a given morphological state of the cell as indicated by the arrow: (a) cationic phenothiazines; (b) anionic phenothiazines. The number on the curves corresponds to the number of the drug in Fig. 1. On this rearranged representation of the data of Mohandas and Feo [26], we have plotted the I_{50} values for Cl^- (○) and the k_i values for glucose exit (●). With the anionic derivatives the values for I_{50} were so small (approx. $5 \cdot 10^{-6}$ M) that it was impossible to plot them on the curves. R.B.C., red blood cells.

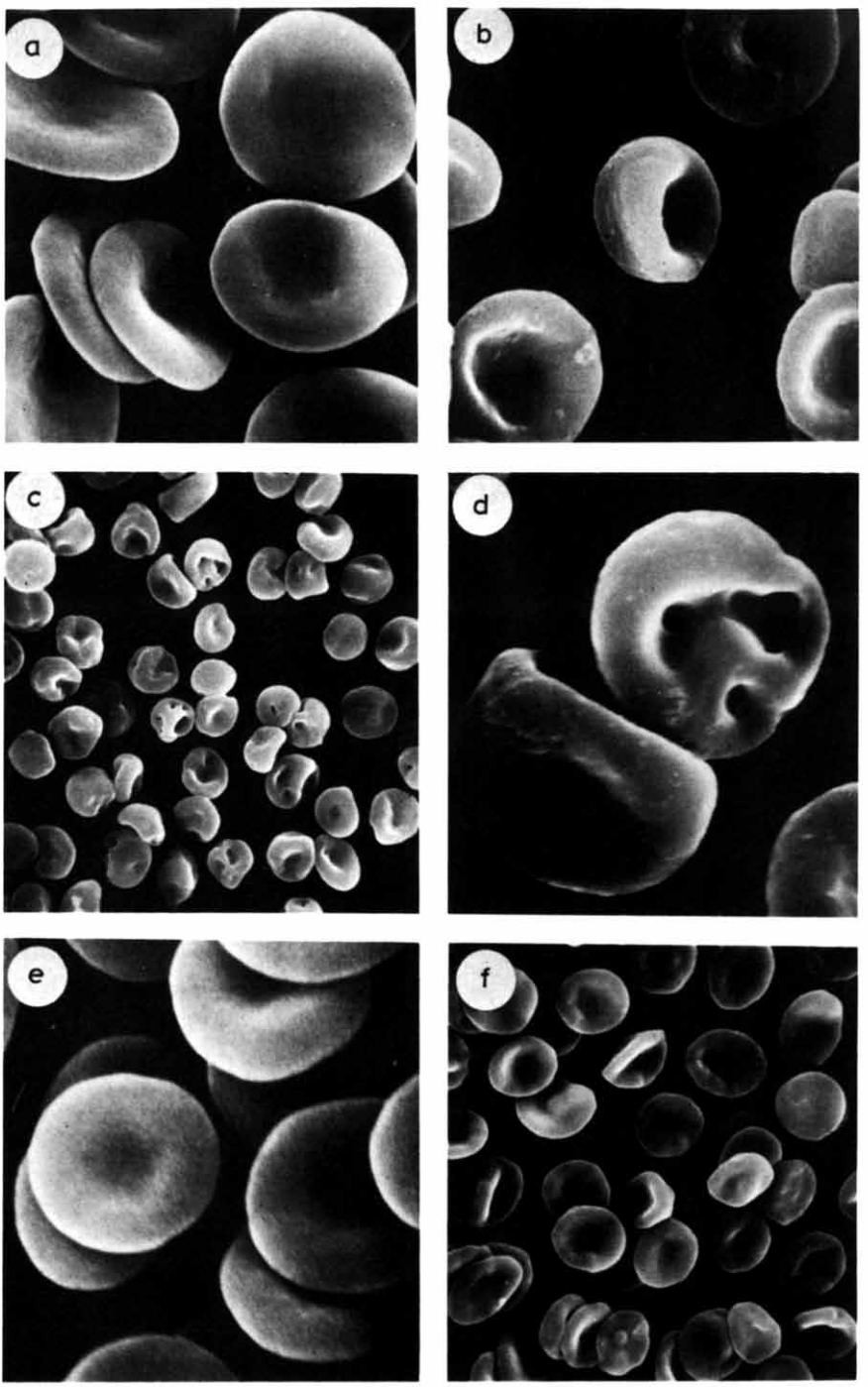


Fig. 7. Perturbation of red cell shape by phenothiazines derivatives: (A) control cells; (B) chlorpromazine ($8 \cdot 10^{-5}$ M); (C) and (D) chlorpromazine ($2.5 \cdot 10^{-4}$ M); (E) anionic drug No. 12 ($3 \cdot 10^{-6}$ M); (F) anionic drug No. 12 ($2 \cdot 10^{-4}$ M).

anionic compounds (right-hand curves), on the contrary, inhibition of Cl^- transport occurs for drug concentrations which do not protect against hemolysis, i.e., when the membrane is not expanded. It is worth noticing that for glucose transport (open arrows) inhibition by cationic, but also by anionic compounds, occurs at concentrations which stabilize the erythrocyte membrane, i.e., which fully expand the membrane.

Mohandas and Feo [26], working with the same phenothiazine derivatives as we used in this study, have simultaneously determined the amount of compound absorbed by intact erythrocytes when they are suspended in a given concentration of compound (hematocrit 1%) and the degree of shape change. They demonstrated that, under their experimental conditions, the amount of absorbed drug is proportional to that of free drug and that the red cell morphology so obtained depends only upon cellular concentration and not upon the chemical structure of the compound. Our experimental conditions being similar (hematocrit 0.5 or 0.06%), it is thus possible, by using their data, to correlate for each compound the inhibitions of transport with the amount of drug absorbed by the cells. Fig. 6 is a rearranged representation of their data for cationic derivatives on which we plotted I_{50} values for Cl^- and k_i values for glucose exit. It is clear that the cationic drugs inhibit Cl^- permeability when the internal concentration lies within the range 2—3 μmol/ml of red blood cells, corresponding to a very strong morphological change (Fig. 7c and d). For glucose exit, inhibition is also obtained when a large amount of drug is absorbed by the erythrocyte (1.2—1.6 μmol/ml of red blood cells) inducing an important modification of red cell morphology (Fig. 7b).

Fig. 6b is a similar representation for anionic phenothiazine. For Cl^- data, it appears that all the half-inhibition concentrations (approx. $5 \cdot 10^{-6}$ M) correspond to such a small amount of drug absorbed by erythrocytes that it was not possible to plot it (less than 0.1 μmol/ml of red blood cells). This amount of drug does not induce any apparent shape change (Fig. 7e). Considering now the contrasting glucose data, it is evident that inhibition for exit and for exchange fluxes occurs only when the shape of the erythrocyte changes to a crenated disk after absorption of an approx. 15- to 30-fold higher concentration than in the Cl^- experiments (Fig. 7f).

Discussion

Inhibition of Cl^- permeability by anesthetics

As discussed previously, the transfer of anions across the red cell membrane is reversibly inhibited by a large variety of anionic and cationic compounds. For all compounds tested thus far, the inhibition is non-competitive [9,11,19] even for anionic agents which might be assumed to compete with penetrating anions. The question arises as to whether or not these chemically different compounds block the Cl^- channel in the same way.

These agents possess a common denominator: they have an amphiphilic structure (i.e., a hydrophobic moiety and a hydrophilic end) and therefore they tend to adsorb to interfaces.

An initial hypothesis to explain the inhibiting effect on Cl^- permeability is to assume that hydrophobic adsorption of anionic drugs to the lipids would

produce a modification of the surface potential, thus modifying the relative concentration of anions and cations at the membrane interface [27,28]. Such a purely electrostatic mechanism predicts a decrease in Cl^- permeability, but an increase in cation transport. It has been shown that 8-anilino-1-naphthalenesulfonic acid and niflumic acid, anionic drugs which reversibly bind to the erythrocyte membrane, effectively decrease the anion exchange but instead of increasing the cation movements decrease them [19,28,29] *. Thus, such a hypothesis cannot be considered without introducing serious limitations such as a compulsory spatial separation between cation transport sites and inhibitor binding sites, etc. It is also worth noticing that the inhibitory action of cationic agents (e.g., local anesthetics) cannot be explained by such changes of the surface potential which would increase and not inhibit anion permeability. Moreover, Gunn and Cooper [11] have provided evidence that the protonated and the unprotonated forms of a local anesthetic are inhibitory.

It is more reasonable to attribute the effects of amphiphilic compounds on Cl^- permeability to some alteration of the protein constituting the anion channel. This is supported by recent experiments [12] showing that local anesthetics inhibit SO_4^{2-} transport both in intact erythrocytes and in reconstituted liposome membranes containing band 3 protein whereas they increase SO_4^{2-} permeability in purely lipid membranes. It remains to be seen whether amphiphilic compounds affect the protein directly or indirectly through a modification of the lipid phase.

Essentially, two possible modifications of the lipid phase must be considered: perturbation of the fluidity and membrane expansion.

As absorption of anesthetic induces a disordering effect on the surrounding lipid structure, it has been proposed that such an effect could prevent conformational changes of the Na^+ channel which allows Na^+ flux [4,5]. Feinstein et al. [12] put forward arguments to discard this possibility concerning inhibition of SO_4^{2-} transport by local anesthetics. Nor can such an interpretation account for inhibition of Cl^- or oxalate transport by anionic drugs: anion permeability is decreased both by anionic drugs and by a decrease in temperature, of which the effects on membrane fluidity are the opposite, and inhibition by anionic compounds is even more pronounced at 0 than at 37°C (Brahms, Sola, Cousin and Motais, unpublished data).

The inhibiting effect of amphiphilic compounds on Cl^- transport could result from drug-induced membrane expansion indirectly modifying the configuration of the Cl^- channel. All inhibitors of anion permeability effectively cause shape changes of red cells, in accordance with the concept put forward by Sheetz and Singer [25]. It must be pointed out that the shape changes induced by these agents are instantaneous as is the inhibiting effect. This hypothesis is attractive because it is a single explanation of the inhibiting effect for both cationic and anionic agents.

Deuticke and Gerlach [30] evaluated this hypothesis by simultaneously measuring phosphate permeability and morphological changes for a series of amphiphilic compounds and concluded that 'the processes inducing shape

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* The increase in cation leaks observed at high 8-anilino-1-naphthalenesulfonic acid concentrations is related to prehemolytic membrane disruption.

transformation cannot be the reason for the decrease of permeability since a number of substances was found to induce shape changes without any effect on phosphate transfer'. On the basis of this statement this interpretation of the inhibitory action of anesthetics has been systematically discarded [12,19,29]. In fact, of the six compounds considered by Deuticke and Gerlach as ineffective, we tested three and found, as illustrated in Fig. 8, that all of them efficiently inhibit Cl^- permeability. Thus, this hypothesis must be reconsidered by comparing the degree of membrane shape change with permeability effects.

From the quantitative data reported above (Figs. 5 and 6a) for cationic derivatives, showing that inhibition always occurs at concentrations which stabilize the erythrocyte membrane and induce a major morphologic change (stomatocyte III in Bessis classification (Fig. 7c, d) it seems reasonable to consider that such an unspecific mechanism could explain the inhibition of Cl^- transport by the cationic phenothiazine derivatives. This conclusion can certainly be extended to all the other cationic anesthetics, since a good correlation also exist between inhibition of anion transport [6,11,12] and protection of erythrocytes against hypotonic hemolysis [1] by local anesthetics such as dibucaine and tetracaine.

The inhibition of Cl^- transport by anionic phenothiazine derivatives, on the other hand, cannot be explained by such a mechanism since we found that inhibition occurs before any morphological change (Figs. 6b and 7e) and membrane expansion (Fig. 5) could be detected. This suggests a more specific site of action for anionic phenothiazines. Could this inhibition be attributed with reasonable certainty to a direct effect on Cl^- channel protein structure? The arguments outlined below strongly support this view.

The first is the evidence recently put forward for such an interaction between band 3 protein and several anesthetics. In one study [20] it was found

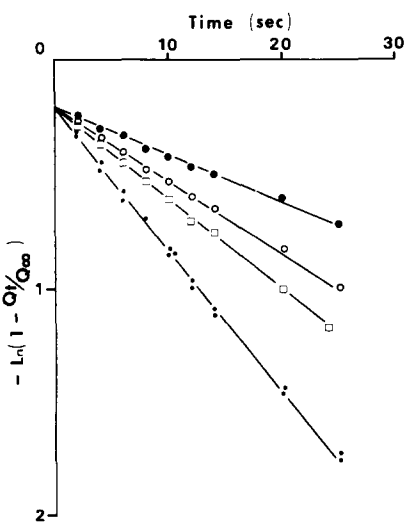

Fig. 8. Inhibitory effect of $5 \cdot 10^{-6}$ M hexadecylpyridinium (•); Triton X-100, 70 mg/ml (○); and lysolecithin 5 mg/ml (□) on Cl^- self-exchange in beef red cells. Control cells (•). Ordinate and abscissa as in Fig. 3.

that a variety of chemically unrelated anionic inhibitors of Cl^- transport such as salicylate, tetrathionate, furosemide, piretanide, ethacrynic acid, etc., reduce the rate of dinitrophenylation of the band 3 protein by 1-fluoro-2,4-dinitrobenzene indicating band 3 as a site of action of these anionic anesthetics. In another study [19] the interaction of an anionic anesthetic, niflumic acid, with band 3 protein has been even more directly demonstrated. Indeed: (1) niflumic acid inhibits the binding reaction of 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), a specific inhibitor of Cl^- transport, with band 3 protein; (2) conversely, the binding of niflumic acid, at a low concentration of the drug, is abolished when Cl^- transport is fully inhibited by covalently bound SITS; (3) the number of these SITS-protectable niflumate binding sites corresponds to the number of band 3 copies per cell.

The second supporting evidence arises from an evaluation of the relationship between chemical structure and Cl^--inhibiting activity which has been made for a great variety of anesthetics. From these studies [13—16,18] it appears that substitution in aromatic rings with lipophilic groups results in a significant increase in the inhibitory potency, and similarly that the presence of an electrophilic group (e.g., nitro) confers an enhanced anion inhibitory capacity. On the contrary, addition of a nucleophilic group (e.g., amino) on the aromatic ring produces a reduction in the inhibitory power. It is interesting to note that it is exactly the same physico-chemical parameters which are involved in the interaction between the inhibitory site and the sulfonic derivatives of stilbene (SITS analogs) which are thought to be specific inhibitors of Cl^- transport and bind to band 3 protein [31]. In fact, the site with which SITS analogs interact is outlined as a nucleophilic target located either in a hydrophobic pocket or adjacent to it [31]; to establish such a structure-activity relationship, a range of structural changes inducing wide variation in electronic distribution and lipid solubility must be found in the set congeners. Thus, a similar analysis could not be made with phenothiazine derivatives. However, accounting from the fact that the same relationships have been obtained with all the cationic anesthetics tested thus far, it is probable that phenothiazines follow the same rule.

In view of the above data, it seems reasonable to consider that all the anionic anesthetics are potentially able to react with the protein involved in anion transport, the capacity to interact depending upon certain physico-chemical properties of the molecules as defined above.

Inhibition of glucose permeability by anesthetics

Band 3 protein does not seem to be homogeneous. Proteins that migrate at the same rate in polyacrylamide gel electrophoresis probably do not mediate sugar transport, since there is recent evidence which supports the role of band 4,5 as the glucose transport system in the erythrocyte [32]. The fact that many anionic anesthetics, which have been considered in the above section as interacting specifically with the anion protein channel, interfere with many other transport systems including that of glucose raises the alternative possibility that: either they act differently on glucose transport and Cl^- transport or our conclusion was wrong and the effects on Cl^- transport are of a nonspecific nature.

The results obtained with anionic phenothiazines (Figs. 5, 6b and 7f) clearly

show that glucose inhibition occurs when the amount of drug absorbed by erythrocytes is at least one order of magnitude higher than that which produces Cl^- inhibition. Moreover, this amount of drug induces an important shape change in the red blood cell and fully protects it against hypotonic hemolysis. Thus, the present results indicate that anionic phenothiazines inhibit glucose exit and Cl^- permeability in different ways, the former presumably through a conformational change brought about by the membrane-expanding anesthetics.

An analogous interpretation can be given concerning the mode of action of cationic anesthetics on glucose exit (Figs. 5, 6a and 7b).

These data are in agreement with the results of Lacko et al. showing that membrane lipids are important for the inhibition of glucose transport by alcohols [33], steroids [34] and local anesthetics [35].

In conclusion, these observations suggest that the perturbation of glucose permeability by anionic and cationic anesthetics, as well as the perturbation of Cl^- permeability by cationic anesthetics, may have a common factor: because they are amphiphatic molecules they may bind preferentially to one of the halves of the bilayer and as a consequence asymetrically expand the membrane and indirectly alter protein channels. Inhibition of Cl^- permeability by anionic anesthetics, on the other hand, would be by specific perturbation of the transport mechanism.

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