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THE EFFECTS OF MEMBRANE LIPID ORDER AND CHOLESTEROL ON THE INTERNAL AND EXTERNAL CATIONIC SITES OF THE Na^+-K^+ PUMP IN ERYTHROCYTES

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Cholesterol depletion alters the apparent affinity of the internal cationic sites and the maximal translocation rate but not the affinity of the external cationic sites of the Na^+-K^+ pump in human erythrocytes. To test whether these effects were mediated by a direct cholesterol-internal site interaction or by a change in membrane lipid order, the effects of five fluidizing amphiphiles (chlorpromazine, imipramine, benzyl alcohol, sodium oleate and sodium benzenesulphonate) on the kinetic parameters of the Na^+-K^+ pump were determined. The cholesterol removal and all the agents used induced dose-response decreases in membrane lipid order as measured by fluorescence polarization or ESR. Positive and neutral amphiphiles mimicked the effects of cholesterol removal on the affinity of the internal sites of the pump and to a lesser extent on the maximal translocation rate. Anionic amphiphiles had no effect on internal sites, probably because they distributed preferentially within the outer leaflet on the membrane. These results indicate that cholesterol controls the affinity of the internal sites of the Na^+-K^+ pump by altering the membrane lipid order. In contrast, neither cholesterol depletion nor the agents used altered the affinity of the external sites of the Na^+-K^+ pump. This difference in sensitivity to membrane lipid order suggests that internal and external cationic sites, although borne by the same protein, are in different lipid environments.

Introduction

The Na^+-K^+ pump and its enzymatic equivalent, the [Na^+-K^+] ATPase (EC 3.6.1.3) is one of the most thoroughly studied transport systems. This intrinsic protein is a minor constituent of the erythrocyte membrane, spanning the membrane, and responsible for the energy-dependent transport of Na^+ and K^+. On the cytosolic face of the membrane, it exhibits cationic sites with a high affinity for Na^+ and on the external face, sites with a high affinity for K^+. When the pump is energized by intracellular ATP it transports the K^+ inwardly and the Na^+ outwardly, thus maintaining the ionic gradients between aqueous intra- and extracellular phases [1,2]. In a previous study [3] we have shown that cholesterol depletion causes substantial modifications of the kinetic parameters of the Na^+-K^+ pump: the apparent affinity for internal Na^+ is decreased and the maximal translocation rate is increased, whereas the affinity for external K^+ remains unchanged. It is not known whether these effects are a reflection of a direct control exerted by cholesterol molecules or are indirectly mediated by a change in the ordering of phospholipids. This second hypothesis is based on the observa-

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Abbreviation: DPH, 1,6-diphenyl-1,3,5-hexatriene.

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tion that cholesterol controls the membrane lipid order [4,5], which may be a factor in the activation of membrane transport systems [6–8].

It was therefore of particular interest to examine the effects of change in membrane lipid order on the activity of the Na^+-K^+ pump independently of the cholesterol content. We have determined the kinetic parameters of the pump in erythrocytes with a normal cholesterol to phospholipid molar ratio in the presence of various amphiphilic molecules which fluidize the membrane. The molecular formula and the electrical charge of the drugs were as different as possible in order to avoid any nonspecific interaction with the pump. Results suggest that cholesterol affects the affinity of internal cationic sites, and to a lesser extent the maximal translocation rate of the Na^+-K^+ pump, by changing the membrane lipid order. In contrast, external cationic sites, although borne by the same protein, appear to be independent of the physical state of the erythrocyte lipid membrane.

A preliminary account of this work has been published elsewhere [9].

Materials and Methods

Erythrocyte preparation and cholesterol depletion

Freshly drawn heparinized blood from healthy volunteer adults was centrifuged for 10 min at $2000 \times g$ at 4°C, and the plasma and buffy were removed by aspiration. The packed erythrocytes were washed three times with 8 vol. ice-cold 150 mM choline chloride solution and resuspended in the same solution to give a final haematocrit of about 40%. Unilamellar egg phosphatidylcholine vesicles were prepared by sonication according to the method of Bruckdorfer et al [10]. Briefly, ethanolic solutions of phosphatidylcholine (50–100 mg), glycerol tri[9,10-(n)-^{3}H]oleate or [^{14}C]dipalmitoyl-phosphatidylcholine (nonexchangeable markers) and, occasionally, cholesterol (22–44 mg) were evaporated under N_2. The thin film of lipid was resuspended in 10 ml solution A ((mM) NaCl, 140; KCl, 10; $MgCl_2$, 1; Na_2HPO_4, 2; NaH_2PO_4, 0.5 (pH 7.4); glucose, 10; penicillin 5000 UI/ml) using a vortex mixer and sonicated for 10 min with a probe sonifier under N_2 in an ice-water bath. The dispersions were centrifuged at $100000 \times g$ for 1 h to remove titanium particles and undispersed lipids. Clear supernatants were used for incubations with erythrocyte suspensions. They were carried out for 15 h at 37°C under agitation and at a haematocrit of about 10%. The mixture contained 3–10 mg vesicle phosphatidylcholine/ml erythrocyte. Control incubations were run in solution A alone or in the presence of cholesterol-phosphatidylcholine vesicles (molar ratio, 0.85). At the end of the incubation, the erythrocytes were washed three times with 150 mM choline chloride and membranes were prepared according to the method of Dodge et al. [11]. Vesicle contamination expressed as percent vesicle phosphatidylcholine relative to cell membrane phospholipids never exceed 1–3%.

Steady-state fluorescence depolarization

This was measured at 37°C, with DPH as a probe, on an Elscint (MVIa) microviscosimeter according to the method of Shinitzky and Barenholz [12], either on cholesterol-depleted membranes or on control membranes in the presence of the amphiphilic agents. Membranes (40 μg membrane protein/ml) were loaded with DPH ($0.5 \cdot 10^{-6}$ M in 140 mM NaCl/10 mM Tris-HCl pH 7.4) by 1 h incubation at 37°C. Amphiphiles were then added during different periods and at different concentrations before the polarization measurements. Recent theoretical and experimental studies [13–17] have shown that steady-state fluorescence anisotropy cannot be interpreted in terms of the so-called microviscosity by applying the Perrin equation. It furnishes information on the structural order in membranes. Thus. we will use the parameter P, degree of fluorescence polarization, defined by:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

in which $I_{\parallel}$ and $I_{\perp}$ are, respectively, the intensities of the parallel and perpendicular polarized light beams, as an index for the change in order parameter of the membrane lipids induced by cholesterol depletion or by the different drugs.

ESR studies

The measurements were done on a Varian E-9 spectrometer equipped with a variable temperature control unit. The spectra were recorded with field modulation of 100 kHz and microwave power at 10

mV. The scanning time was 16 min and the temperature was 37°C. The spin labels, 12-nitroxymethylstearate and 5-nitroxypalmitate, were dried from a solution in ethanol and incubated with 0.5 ml washed erythrocytes (haematocrit 45–50%) for 15 min at room temperature with agitation. The molar ratio of probe to membrane lipid molecules did not exceed 1/50. No evidence of spin exchange was indicated from the spectra. The cells were then diluted to 10 ml with solution of the amphipaths in 150 mM NaCl/5 mM Tris-HCl, pH 7.4, and allowed to stand for 5 min. The cells were centrifuged and the supernatant removed. 0.2-ml aliquots of the drug solution were added to the packed cells to resuspend. The suspensions were put into a quartz flat cell for introduction into the spectrometer.

When vesicles were used instead of intact cells they were prepared after lipid extraction of erythrocytes in chloroform/methanol (2/1, v/v) and evaporation of the extract under N_2. The extract was then sonicated in 140 mM NaCl/10 mM Tris-HCl, pH 7.4, using a Rapidis Sonic Power Instrument fitted with a sonicator bath, 180 kW. Probe incorporation, drug addition and spectrometry were performed as described above.

The motion parameter (τ_0) was calculated from the formula of Henry and Keith [18] where W_0 is the mid-field line width, h_0 the mid-field and h_{-1} the high-field height:

$$\tau_0 = K \cdot W_0 \left[\left(\frac{h_0}{h_{-1}} \right)^{1/2} - 1 \right]$$

The constant $K = 6.5 \cdot 10^{-10}$ s (see Henry and Keith, Ref. 18). The term τ_0 can be used as parameter for the nitroxy probe anisotropic mobility for comparison purposes and not as the correlation time (τ_c).

Finally, the empirical parameter h_{-1}/h_0 was also used as an indication of membrane fluidity.

Cation fluxes

Ouabain-sensitive Na efflux. Washed erythrocytes were prepared to contain different concentrations of internal Na^+ ($[Na]_i$) and a constant concentration of internal K^+ ($[K]_i$) by the *p*-chloromercuribenzene sulphonate cysteine method and loaded with ^{22}Na [3]. ^{22}Na efflux from these cells was measured after 15 and 30 min, at 37°C, and haematocrit of 1–2%, in solution A (in which NaCl was only 40 mM and the isotonicity maintained with 100 mM choline chloride, to prevent any increase in $[Na]_i$ of drug-treated cells during the incubation) without or with ouabain ($5 \cdot 10^{-4}$ M), in the absence (control) or in the presence of the amphiphiles. At times required, cell suspensions (900 μl) were centrifuged at 12 000 × *g* for 30 s through a layer (200 μl) of dibutyl phthalate (density 1.042–1.045), pre-cooled at 0–4°C. This method permits a rapid inactivation of ion transport across the cell membrane because of physical isolation of cells and medium [19]. Supernatants (700 μl) were collected for counting. Aliquots of total cell suspension were counted after precipitation with trichloroacetic acid (see below).

Ouabain-sensitive K influx. Influx was measured on washed erythrocytes after 20 min incubation, at 37°C and haematocrit 8%, in the absence (control) or in the presence of the amphiphiles, with or without ouabain, in solution A containing ^{42}K and in which KCl was varied from 0.1 to 3 mM at the expense of choline chloride. At that time, cell suspensions (100 μl) were centrifuged at 12 000 × *g* for 30 s in a pre-cooled medium containing dibutyl phthalate (200 μl) and 150 mM choline chloride + [^{3}H]inulin (900 μl). The supernatant and the oil were carefully removed by aspiration. The cell pellets were lysed with water and deproteinized with trichloroacetic acid (final concentration, 5%) with vigorous vortex mixing before and after acid addition [19]. The tubes were centrifuged for 1 min, and aliquots of the supernatant were counted directly for ^{42}K by Cerenkov radiation and by liquid scintillation after an interval of 7 days, during which most of ^{42}K had decayed. ^3H counts were used to calculate the contamination of the pellet by extracellular medium (5–10%) and to correct the ^{42}K uptake thereafter. This procedure offers the advantage of obtaining cell samples with low extracellular space by one short centrifugation.

Analytical procedures

Erythrocyte membrane lipids were extracted using the procedure of Rose and Oklander [20]. Cholesterol and phospholipid contents of the lipid extract were measured as described by Rudel and Morris [21] and Bartlett [22], respectively. Membrane protein was estimated by the method of Lowry et al. [27] using bovine serum albumin as standard. Na^+ and K^+ were measured by flame photometry and

haemoglobin by absorption photometry at 541 nm. ^{22}Na, glycerol tri[^{3}H]oleate, [^{3}H]inulin, [^{14}C]dipalmitoylphosphatidylcholine were counted by liquid scintillation and ^{42}K by Cerenkov radiation all in a liquid scintillation counter.

Chemicals

Sodium benzenesulphonate and benzyl alcohol were obtained from Fluka Ltd. (Basel, Switzerland); chlorpromazine, imipramine, sodium oleate, *p*-chloromercuribenzene sulphonate, cysteine, ouabain, egg phosphatidylcholine (fraction V E) and cholesterol (recrystallized) from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DPH was purchased from Aldrich Chemical Co, Inc. (Milwaukee, WI, U.S.A.) and 12-nitroxymethylstearate from Syva Associates (Palo Alto, CA, U.S.A.). 5-Nitroxypalmitate was a gift from Dr. P. Devaux. Dibutyl phthalate was from Koch-light lab. Ltd. (Colnbrook Bucks, U.K.). Glycerol tri[^{3}H]-oleate, [^{3}H]inulin, [^{14}C]dipalmitoylphosphatidylcholine were obtained from Amersham (France) and ^{22}Na and ^{42}K from C.E.A. (France).

Results

Cholesterol- and drug-induced changes in membrane lipid order as measured by steady-state fluorescence polarization: time course and dose-response curves

Incubation of erythrocytes with phospholipid vesicles containing no cholesterol caused a progressive reduction in the cholesterol to phospholipid molar ratio of the cell membrane whereas control cells incubated with cholesterol-phospholipid vesicles in a molar ratio 0.85 exhibited no change in their cholesterol to phospholipid molar ratio. Under both experimental conditions there was no significant uptake or exchange of phospholipids as tested by [^{14}C]dipalmitoylphosphatidylcholine or glycerol tri[^{3}H]oleate when compared with cholesterol movements [24]. The reduction of the cholesterol to phospholipid molar ratio of the erythrocyte membrane from 0.85 to 0.6 caused the fluorescence polarization of ghosts to decrease linearly from 0.290 to 0.235, not shown. This is in agreement with previous results of Cooper et al. [8]. Under our experimental conditions, a 20–25% depletion in membrane cholesterol resulted in a decrease of about 10% in the fluorescence polarization.

Addition of amphiphiles to erythrocyte membranes also caused the fluorescence polarization to decrease. Fig. 1 shows the time course of this decrease elicited by 40 mM sodium benzenesulphonate. At this dose the drug reduced the fluorescence polarization in less than 10 min to a value which remained unchanged for more than 30 min, a period long enough to allow flux measurements. Similar time courses were observed for sodium oleate, imipramine and benzyl alcohol. The effect of chorpromazine, the self-absorption of which interfered with the fluorescence emission of DPH, was not tested by this method. Fig. 2 shows that after 10 min incubation of the membranes with the amphiphiles, the fluorescence polarization was dose-dependent and that similar changes were obtained with quite different concentrations of drugs. This rather wide range in the concentrations probably reflected the difference in the ability of the drugs to partition in the membrane [25–27].

Drug-induced changes in membrane lipid order as measured by ESR

This second technique was initially designed to measure the effect of chlorpromazine on the membrane lipid order. The effects of the other amphiphiles were also tested. Intact erythrocytes labelled with two different probes (12-nitroxymethylstearate

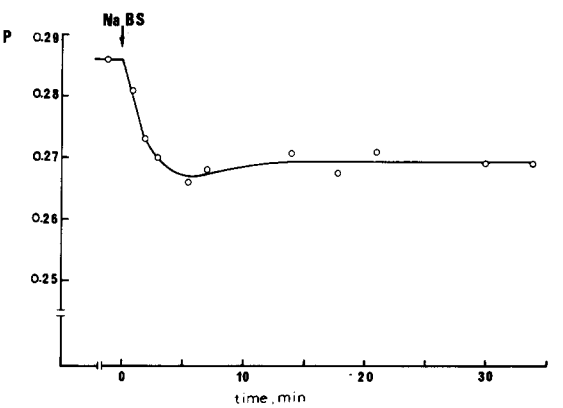

Fig. 1. Time dependence of the fluorescence polarization *P* of DPH in erythrocyte membranes after addition of 40 mM sodium benzenesulphonate. Time of addition is indicated by the arrow. Membrane protein concentration was 40 $\mu g \cdot ml^{-1}$, i.e., approx. 1% haematocrit, and temperature was 37°C (see experimental section).

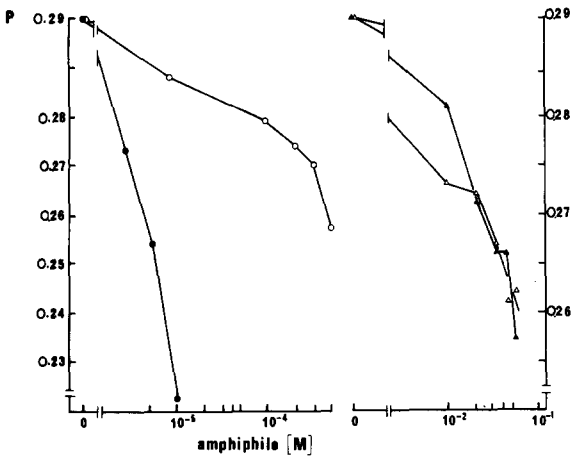

Fig. 2. Drug concentration dependence of the fluorescence polarisation P of DHP in erythrocyte membranes. Drugs were added 10 min before the measurements. (●———●, sodium oleate; ○———○, imipramine; ▲———▲, sodium benzenesulphonate; △———△, benzyl alcohol). Membrane protein concentration was 40 $\mu g \cdot ml^{-1}$, i.e., approx. 1% haematocrit, and temperature was 37°C (see experimental section).

or 5-nitroxypalmitate) and also vesicles prepared from a lipid extract of erythrocytes and labelled with 12-nitroxymethylstearate were used in these experiments. The amphiphiles were added 10 min before the measurement at the concentrations which produced a 10% decrease in the fluorescence polarization (see Fig. 2). Table I shows that the motion parameter (τ_0) describing the probe rotation mobility and, to a lesser extent, the empirical parameter h_{-1}/h_0, the ratio of the height of the upper field to that of the medium field, were changed in the presence of all the amphiphiles tested. This occurred in cells as well as in lipid vesicles, indicating that the drugs altered the membrane physical state by interacting with the membrane lipids.

It may be noted that for the same concentration of a given drug the amplitude of the apparent viscotropic effects depended somewhat on the method used and on the associated physical parameters. This may result from the fact that these parameters differ in their physical meaning and also because the nitroxy groups located at the 5th or 12th position explored different regions across the membrane bilayer. As a whole these results confirmed those obtained by fluorescence polarization and show that amphiphiles altered the lipid order of the erythrocyte membrane.

Cholesterol and drug-induced changes in Na^+-K^+ pump activity

In these experiments the concentrations of the amphiphiles in the external medium were adjusted in order to cause a fluidizing effect similar to that produced by a 20% cholesterol depletion, as measured by fluorescence depolarization (see Fig. 2). For chlorpromazine a concentration of 100 μM was selected, from the data of Seeman et al. on the protective anti-haemolytic effects of this drug [28]. Our ESR results show that at this concentration the effects of chlorpromazine were of the same order of magnitude as those induced by the other amphiphiles (Table I). The effects of the amphiphiles on the apparent affinities and maximal translocation rate of the Na^+-K^+ pump were determined as in Refs. 3, 29 and 30, i.e., by measuring the ouabain-sensitive cation fluxes as a function of the concentration of the cation concerned, keeping constant all other ionic concentrations on both sides of the membrane.

Internal cationic sites

The ouabain-sensitive $^{22}Na^+$ efflux was measured as a function of the internal Na^+ content of erythrocytes incubated in the presence or in the absence of amphiphiles. The apparent dissociation constant of the Na-site complex (K'_{Na}) and the maximal translocation rate (M'_{max}) were calculated graphically from the equation given in the legend of Fig. 3. This figure shows that both K'_{Na} intercept of the curve with the abscissa and M'_{max} were increased by the addition of 40 mM benzyl alcohol. Similar results were obtained with 100 μM chlorpromazine and 500 μM imipramine (Table II), thought the effects on M'_{max} were less pronounced. In contrast, 40 mM sodium benzenesulphonate (Fig. 4) and 5 μM sodium oleate had no effect on either parameter (Table II). For comparison, the effects of 20% cholesterol depletion on K'_{Na} and M'_{max} [3] are also reported in Table II. It thus appears that cationic and neutral amphiphiles were able to mimic the results obtained with cholesterol depletion, whereas anionic amphiphiles, though increasing the membrane lipid order, were unable to alter the pump activity.

TABLE I

EFFECT OF AMPHIPHILE AGENTS ON MEMBRANE LIPID ORDER MEASURED EITHER BY STEADY STATE FLUORESCENCE DEPOLARIZATION OR ESR

P, fluorescence polarization ratio of Fig. 2. τ_0, motion parameter of 12-nitroxymethylstearate and 5-nitroxypalmitate in erythrocytes or in sonicated lipid extracts of the cell membranes. h_0/h_{-1}, mid-field to high-field height ratio obtained from the spectra of the same probes. All measurements were carried out at 37°C after incubation with drugs for 10 min and haematocrit of 1–2%. Values are means of 3–27 polarization experiments or of 2–4 ESR experiments. The probes used are in parenthesis.

Physical parameters	Preparation						
	Erythrocyte membranes (DPH)	Sonicated lipid extracts (12-nitroxymethylstearate)		Erythrocytes (12-nitroxymethylstearate)		Erythrocytes (5-nitroxypalmitate)	
	P	τ_0 ($s \times 10^{10}$)	h_{-1}/h_0	τ_0 ($s \times 10^{10}$)	h_{-1}/h_0	τ_0 ($s \times 10^{10}$)	h_{-1}/h_0
Control	0.290	23.9	0.76	49.7	0.50	45.5	0.30
Benzyl alcohol, 40 mM	0.263	8.6	0.85	22.1	0.76	36.1	0.33
Imipramine, 500 μM	0.268	17.3	0.83	41.0	0.59	35.2	0.33
Chlorpromazine, 100 μM	–	18.3	0.78	26.6	0.68	38.1	0.33
Sodium benzenesulphonate, 40 mM	0.266	17.7	0.76	42.6	0.59	34.1	0.35
Sodium oleate, 5 μM	0.267	17.5	0.80	36.9	0.60	32.5	0.37

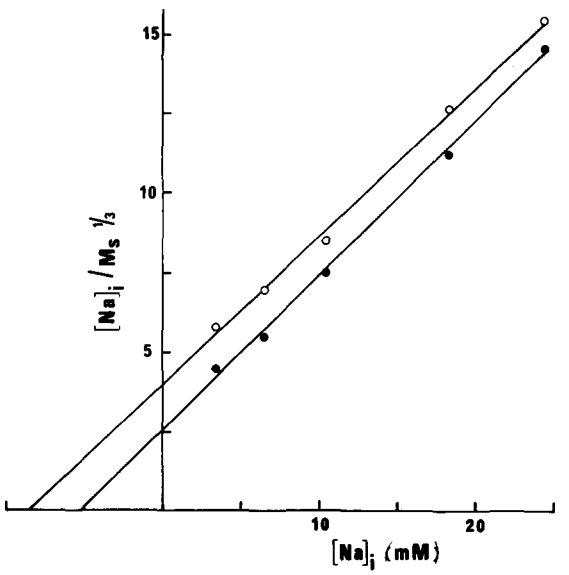

Fig. 3. Effect of internal Na^+ concentration ($[Na]_i$) on the ouabain-sensitive Na^+ efflux (M_s) from erythrocytes. Control medium (•———•); benzyl alcohol-containing (40 mM) medium (○———○). Results are plotted according to the equation: $[Na]_i/M_s^{1/3} = K'_{Na}/M'_{max}{}^{1/3} + [Na]_i/M'_{max}{}^{1/3}$ (see Ref. 29). Measurements were carried out at 37°C at a haematocrit of 1–2%.

TABLE II

EFFECT OF CHOLESTEROL DEPLETION AND AMPHIPHILIC AGENTS ON KINETIC PARAMETERS OF THE SODIUM PUMP

K'_{Na} apparent dissociation constant of the internal sodium-site complex, and M'_{max}, maximal translocation rate of the sodium pump were obtained by methods described in the experimental section. All measurements were carried out at 37°C at a haematocrit of 1–2%. Values of K'_{Na} and M'_{max} after cholesterol depletion were taken from the results of Claret et al. [3]. Values are means ± S.E., with the number of experiments shown in parenthesis. Values that are significantly different from the control using a paired *t*-test are shown: * $P < 0.05$.

Treatment	K'_{Na} (mM)	M'_{max} (mmol/l cell per h)
Control (8)	4.2 ± 0.2	5.4 ± 0.3
Cholesterol depletion, 20–25% (4)	6.5 ± 0.5 *	13.9 ± 1.2 *
Benzyl alcohol, 40 mM (5)	8.1 ± 0.6 *	7.6 ± 0.9 *
Imipramine, 500 μM (5)	8.4 ± 1.0 *	6.0 ± 0.5
Chlorpromazine, 100 μM (4)	6.9 ± 0.4 *	7.2 ± 0.7 *
Sodium benzenesulphonate, 40 mM (4)	4.2 ± 0.2	5.4 ± 0.3
Sodium oleate, 5 μM (4)	4.2 ± 0.2	5.4 ± 0.5

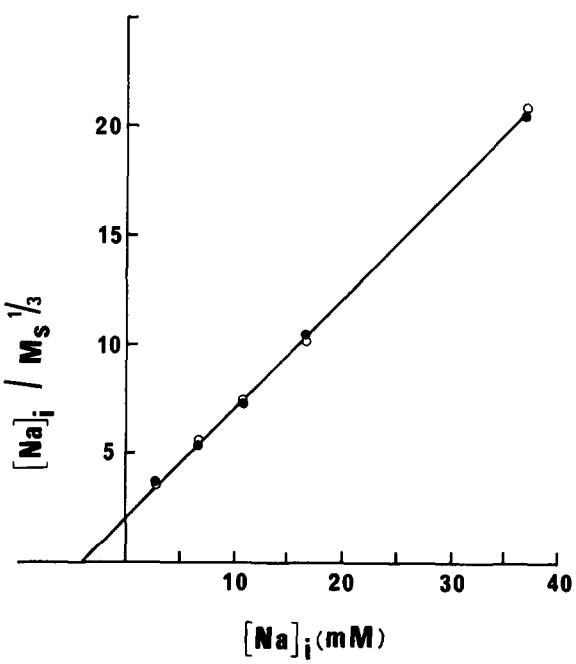

Fig. 4. Effect of internal Na^+ concentration ($[Na]_i$) on the ouabain-sensitive Na^+ efflux (M_s) from erythrocytes. Control medium (•———•); sodium benzenesulphonate-containing (40 mM) medium (○———○). Results are plotted according to the equation: $[Na]_i/M_s^{1/3} = K'_{Na}/M'_{max}{}^{1/3} + [Na]_i/M'_{max}{}^{1/3}$ (see Ref. 29). Measurements were carried out at 37°C at a haematocrit of 1–2%.

External cationic sites

The uptake of ^{42}K was measured as a function of the external K^+ concentration at constant internal K^+ and Na^+ contents. The cells were equilibrated with the amphiphiles for 10 min and for each external K^+ concentration, the value of K^+ influx was plotted against the values of K^+ influx in a paired control (Fig. 5). It has been shown that this representation is the most straightforward way of comparing K^+ influx curves [30]. Fig. 5 shows that straight lines with zero intercepts were obtained for sodium benzenesulphonate (Fig. 5A) and for benzyl alcohol (Fig. 5B). The same results were observed for the other amphiphiles and also in cholesterol-depleted erythrocytes (not

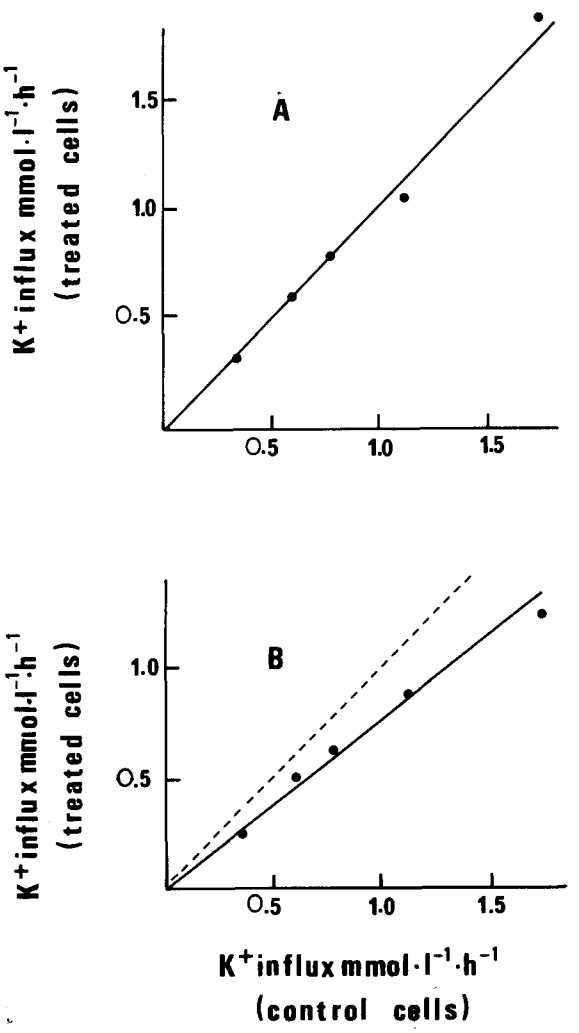

Fig. 5. Plots of the ouabain-sensitive K^+ influx into cells suspended in drug-containing media against the ouabain-sensitive K^+ influx into cells suspended in control medium. External K^+ concentration ranged from 0.1 to 3 mM. Cells contained 9 mM Na^+ and 135 mM K^+. Measurements were carried out at 37°C at a haematocrit of 8%. A, sodium benzenesulphonate (40 mM); B, benzyl alcohol (40 mM). The dotted line represents the line with slope 1 (see text).

shown). This means that addition of amphiphiles to cells or cholesterol depletion do not affect the apparent affinity of external sites for K^+. For chlorpromazine, imipramine, benzyl alcohol and cholesterol depletion the slopes of the lines were different from unit, indicating that the K^+ influx in treated cells differed only by a constant factor from that in control cells. This was due to the fact that these drugs affected the affinity of the internal cationic sites and the maximal translocation rate as did cholesterol depletion. In agreement with this conclusion the slopes of the lines for sodium oleate and sodium benzenesulphonate, which had no effect on these parameters, were not significantly different from unity.

Discussion

Cholesterol is known to affect the packing of hydrocarbon chains of phospholipid in both artificial and natural membranes [4,5]. Its partial removal from cell membranes, such as that reported here, causes a decrease in lipid ordering or an increase in membrane fluidity. These results confirm those obtained by others showing that the effect is proportional to the change in the cholesterol to phospholipid molar ratio [8]. Cholesterol is present in both leaflets of the bilayer [31–34] and studies on exchange kinetics between human erythrocytes and various external receptors (lipoproteins, artificial lipid vesicles) have shown that all the molecules are available for exchange or depletion and behave as a single homogeneous pool [24,35]. This means that the depletion and resulting decrease in membrane lipid order is likely to occur in the internal as well as in the external leaflet of the erythrocyte membrane.

All the amphiphilic agents used in the present study decrease the lipid ordering of the cell membrane as measured by fluorescence polarization or by ESR. In erythrocyte membranes containing a high cholesterol to phospholipid molar ratio, these agents have a disordering effect because they disrupt the molecular interactions which allow cholesterol to exert its rigidifying effect on phospholipids [36]. The fluidizing effects of the amphiphiles were rapid and dose-dependent (see Figs. 1 and 2). The concentrations required to decrease the fluorescence polarization by about 10% (a value obtained in 20% cholesterol-depleted membranes) were quite different for each agent, probably due to the difference in their partition coefficient between the membrane and the medium. Accordingly, oleate or chlorpromazine may be mainly incorporated within the membrane, whereas the major fraction (>85%) of benzyl alcohol or benzenesulphonate remained in the aqueous phase

[25–27]. These results agree with a number of studies on the effects of local anaesthetics, tranquilizers or antidepressants including benzyl alcohol, imipramine and chlorpromazine on natural or model membranes [25–27]. To our knowledge, the effects of sodium oleate and sodium benzenesulphonate on membrane lipid order have never been reported in the literature.

It is likely that the amphiphiles do not distribute evenly in both leaflets of the cell membrane. Recent reports [37,38] have provided experimental evidence for an asymmetric distribution of compounds depending on their charge at neutral pH. Tenforde et al. [37] calculated that only about 2.5% of chlorpromazine present in the erythrocyte membranes was located in the outer leaflet. Matayoshi [38] determined that more than 70% of the anionic amphiphile pyrene derivative was present in the outer leaflet, whereas the cationic derivative concentrated in the inner half of the membrane after a while due to low permeability. The authors proposed that, according to the Sheetz and Singer 'bilayer couple hypothesis', the cationic amphiphiles might preferentially partition in the inner leaflet because of the negative field which arises from phosphatidylserine. Anionic amphiphiles would concentrate in the outer leaflet which contains neutral phospholipids, due to repulsion from the inner half of the membrane. If the same holds for the amphiphiles used in the present study, it could be concluded that the disordering effects reported for benzyl alcohol and cholesterol depletion affect both leaflets of the membrane, whereas anionic (oleate and benzenesulphonate) and cationic molecules (chlorpromazine and imipramine) disturb preferentially the outer and the inner part of the membrane, respectively.

Cholesterol depletion and addition of cationic or neutral amphiphiles (chlorpromazine, imipramine, benzyl alcohol) caused the same changes in the apparent affinity for internal Na. This suggests that the effect of cholesterol depletion is mediated through changes in the order of the lipids surrounding the Na^+ sites and thus that cholesterol does not directly control these sites. This is in agreement with the fact that the activity of many enzymes or transport systems is dependent on the physical state of the membrane lipids [6–8]. The disordering effects resulting from cholesterol removal or amphiphile addition could also cause a redistribution of the phospholipids located in the microenvironment of the protein and which have been shown to play a role in the activation of the [Na^+-K^+] ATPase [6,7]. The fact that anionic amphiphiles disorder the erythrocyte membrane (as measured by fluorescence polarization or by ESR) without altering the affinity of the internal cationic sites does not contradict this conclusion. This finding is probably relevant to the data discussed above on the distribution of amphiphiles in erythrocyte membranes depending on their charge. According to Refs. 37–39 it is likely that anionic sodium salts used in this study did not perturb the cytosolic sites of the Na^+-K^+ pump because they were not able to distribute substantially into the inner leaflet of the membrane. As compared with the relative similarity of the changes in the apparent affinity of the internal sites, the modifications of the maximal translocation rate due to cationic and neutral amphiphiles were far less pronounced than those caused by cholesterol depletion (see Table II). This may indicate that cholesterol has two effects on the maximal translocation rate: a minor one mediated through a change in membrane lipid order of the inner leaflet of the membrane, which may be mimicked by intercalation of the neutral or cationic amphiphiles, and a major one exerted through a specific interaction with the Na^+-K^+ pump which cannot be observed in the presence of any of the fluidizing agents.

In contrast to internal sites, external sites appear unaltered by cholesterol depletion or by any of the amphiphiles used. Thus an interesting observation of this study is that sites borne by the same intrinsic protein are differently affected by the lipid order of the leaflet in which they are located. On the cytosolic face, Na^+ sites appear to be controlled by the order or the arrangement of phospholipids themselves modulated by the level of cholesterol. On the external face K^+ sites are independent of the presence of cholesterol and of the lipid order of the membrane. This difference of lipid-protein interactions on both sides of the erythrocyte membrane could suggest that the sites are located in different lipid environments.

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