Charged anaesthetics alter LM-fibroblast plasma-membrane enzymes by selective fluidization of inner or outer membrane leaflets

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The functional consequences of the differences in lipid composition and structure between the two leaflets of the plasma membrane were investigated. Fluorescence of 1,6-diphenylhexa-1,3,5-triene (DPH), quenching, and differential polarized phase fluorimetry demonstrated selective fluidization by local anaesthetics of individual leaflets in isolated LM-cell plasma membranes. As measured by decreased limiting anisotropy of DPH fluorescence, cationic (prilocaine) and anionic (phenobarbital and pentobarbital) amphipaths preferentially fluidized the cytofacial and exofacial leaflets respectively. Unlike prilocaine, procaine, also a cation, fluidized both leaflets of these membranes equally. Pentobarbital stimulated 5'-nucleotidase between 0.1 and 5 mM and inhibited at higher concentrations, whereas phenobarbital only inhibited, at higher concentrations. Cationic drugs were ineffective. Two maxima of (Na⁺ + K⁺)-ATPase activation were obtained with both anionic drugs. Only one activation maximum was obtained with both cationic drugs. The maximum in activity below 1 mM for all four drugs clustered about a single limiting anisotropy value in the cytofacial leaflet, whereas there was no correlation between activity and limiting anisotropy in the exofacial leaflets. Therefore, although phenobarbital and pentobarbital below 1 mM fluidized the exofacial leaflet more than the cytofacial leaflet, the smaller fluidization in the cytofacial leaflet was functionally significant for (Na⁺ + K⁺)-ATPase. Mg²⁺-ATPase was stimulated at 1 mM-phenobarbital, unaffected by pentobarbital and slightly stimulated by both cationic drugs at concentrations fluidizing both leaflets. Thus the activity of (Na⁺ + K⁺)-ATPase was highly sensitive to selective fluidization of the leaflet containing its active site, whereas the other enzymes examined were little affected by fluidization of either leaflet.

INTRODUCTION

The relationships that exist between the activities of membrane-associated enzymes, and the composition and physical properties of membrane lipids, have attracted considerable attention (Gordon & Mobley, 1985). Integral membrane proteins, i.e. those which are firmly embedded in the bilayer, may display an absolute asymmetric transbilayer orientation by facing either the cytoplasm (cytofacial) or the extracellular space (exofacial). Alternately, such proteins may appear on both sides of the membrane (penetrant). Membrane-penetrant enzymes may have different activities or allosteric sites on the two sides of the membrane. The activity of several membrane-bound enzymes is affected by their lipid environments, e.g. 5'-nucleotidase (exofacial), adenylate cyclase (cytofacial) and (Na⁺ + K⁺)-ATPase (penetrant). Previously such correlations have generally been made with 'bulk' or overall membrane lipid composition. However, more recently it was recognized that the lipids of the cell surface membrane are not symmetrically distributed across the membrane bilayer (Zwaal *et al.*, 1973; Op den Kamp, 1979). With few exceptions (Sessions & Horwitz, 1981), the anionic lipids are enriched in the cytoplasmic face of eukaryotic-cell plasma membranes. Cholesterol also appears enriched in

the exofacial leaflet of myelin and red blood cells; in contrast, it is enriched in the cytofacial leaflet of LM fibroblasts, sperm tail and insect K_c-cell plasma membranes (reviewed by Schroeder, 1984). Since the two leaflets of the plasma membrane are not coupled, they are therefore also asymmetric with respect to structure and fluidity (reviewed by Schroeder, 1984, 1985). Because of this asymmetric distribution of charged lipids, it has been hypothesized that charged amphipathic molecules, often anaesthetics, preferentially insert into one leaflet and thereby fluidize that leaflet (Sheetz & Singer, 1974).

The above hypothesis has been tested indirectly by measurement of the effects of charged anaesthetics on the activity of plasma-membrane enzymes that were cytofacial, exofacial or penetrant (Dipple *et al.*, 1982; Houslay *et al.*, 1980b, 1981; Houslay & Palmer, 1978; Salesse *et al.*, 1982a,b; Sinensky *et al.*, 1979). However, a unifying principle is not evident, owing to some uncertainty in the interpretation of the results (Gordon *et al.*, 1980; Dipple *et al.*, 1982; Houslay & Palmer, 1978; Sinensky *et al.*, 1979; Houslay *et al.*, 1980a, 1981; Houslay & Gordon, 1983; Whetton *et al.*, 1983). For example, specificity of charged amphipaths demonstrated by inflections in Arrhenius plots of 5'-nucleotidase activity were not demonstrated in the same way in rat and in hamster liver plasma membranes. The present study tests the functional

Abbreviations used: DPH, 1,6-diphenylhexa-1,3,5-triene; (Na⁺ + K⁺)-ATPase, (Na⁺ + K⁺)-stimulated ATPase; Mg²⁺-ATPase, Mg²⁺-dependent ATPase.

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consequences of alterations in fluidity in each leaflet of the LM-cell plasma membrane. The effects of anaesthetics on 5'-nucleotidase, $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ and $\text{Mg}^{2+}\text{-ATPase}$ activity of the isolated plasma membrane are examined and related to the preferential fluidization hypothesis. A preliminary account of a portion of this work was presented as an abstract (Sweet & Schroeder, 1985).

EXPERIMENTAL

Chemicals

Prilocaine was generously given by Astra Pharmaceuticals (Södertälje, Sweden, and Worcester, MA, U.S.A.). Lubrol 12A9 was kindly given by Imperial Chemical Industries Ltd. (Bury, Lancashire, U.K.). Pentobarbital, phenobarbital, procaine, 5'-AMP, ATP (vanadium-free) and ouabain were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DPH was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Bovine serum albumin was purchased from Miles Laboratories (Elkhart, IN, U.S.A.). All other reagents were of highest commercially available quality.

LM-cell plasma-membrane isolation

LM cells, a strain of transformed mouse fibroblasts (American Type Culture Collection, CCL 1.2), were grown in suspension as described by Schroeder *et al.* (1976). Periodic testing assured freedom from mycoplasma, bacterial and fungal contamination. Plasma membranes were obtained by a modification (Schroeder & Gardiner, 1984) of a method described elsewhere (Schroeder *et al.*, 1976). The membranes were 7–8-fold enriched in $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ over crude cell homogenate. The LM plasma membranes are right-side-out vesicles of uniform lipid composition (Schroeder *et al.*, 1982). This plasma-membrane preparation is much more homogeneous than are plasma membranes isolated from liver. Not only is liver heterogeneous in cell type, but the three functional domains of the hepatocyte (the blood-sinusoidal, contiguous and bile-canicular areas) differ in lipid and protein composition (Kremmer *et al.*, 1976).

Rat liver plasma-membrane isolation

Sprague-Dawley rats (220 g body wt.), obtained from Charles River Breeding Laboratory (Wilmington, MA, U.S.A.), were given water and Purina Laboratory Rodent Chow *ad lib*. After they were killed by decapitation, the liver was removed and perfused with 0.9% NaCl, pH 7.4, for 5–10 min. The membranes were prepared by the methods described previously (Schroeder, 1983; Pohl, 1976) and stored at -80°C . The membranes were 18–22-fold enriched in 5'-nucleotidase and $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ over the crude homogenate.

Lubrol solubilization of 5'-nucleotidase

5'-Nucleotidase was solubilized from rat liver and LM-cell plasma membranes with Lubrol 12A9 essentially as described by Swislocki & Tierney (1975). Briefly, a 2% Lubrol 12A9 solution was added to membranes (1.5–2 mg of protein/ml) to give a final concentration of 4 mg of Lubrol 12A9/mg of protein. After gentle vortex-mixing and incubation at 0°C for 10–15 min, the mixture was centrifuged at 108 000 *g* for 1 h. The supernatant was taken as solubilized 5'-nucleotidase.

5'-Nucleotidase specific activity in LM-cell homogenates was stimulated 11.5% by 4 μg of Lubrol 12A9/ μg of protein, with no further change up to a 16:1 (w/w) ratio of Lubrol to protein. A 4:1 ratio was used for the solubilized enzyme in the membrane-perturbation experiments.

Enzyme determinations

The specific activity of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ was determined by a modification (Schroeder & Gardiner, 1984) of a published method (Schimmel *et al.*, 1973). The minimum concentration of ouabain that fully inhibited the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ was 1 mM, a concentration below the critical micellar concentration of ouabain. This concentration was used throughout. The pH optimum of LM plasma-membrane $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ was very broad, between 6.0 and 8.2, thus ensuring that minor changes in pH on addition of alkaline barbiturate solutions to the buffered reaction mixture could not account for changes in observed activity. The ATPase activity remaining in the presence of ouabain was designated as $\text{Mg}^{2+}\text{-ATPase}$.

The specific activity of 5'-nucleotidase was determined by a modification (Schroeder & Gardiner, 1984) of a described method (Schimmel *et al.*, 1973). As a further modification, the reaction mixture was buffered to pH 7.0. A broad pH optimum between 4.0 and 7.5 ensured a lack of effect of adding alkaline barbiturate solutions.

Protein was determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Trinitrophenylation and isolation of plasma membranes

To determine fluorescence parameters to probe molecules in each membrane leaflet, techniques previously established in our laboratory were used (Schroeder, 1978, 1980; Schroeder & Kinden, 1980; Hale & Schroeder, 1982). Briefly, membranes were prepared either without covalently linked fluorescence-quenching agent or with quencher linked to the outer leaflet of the plasma membranes as follows. One-half of each culture was treated with trinitrobenzenesulphonic acid under non-penetrating conditions (4°C , 4 mM) as described by Fontaine & Schroeder (1979). Excess reagent was removed and plasma membranes were isolated by a modification (Schroeder & Gardiner, 1984) of a published procedure (Schroeder *et al.*, 1976). Microsomal fractions and mitochondria were also routinely isolated in order to determine if any of the trinitrobenzenesulphonic acid penetrated the cell (Fontaine & Schroeder, 1979).

Fluorescence spectroscopy

Absorption-corrected fluorescence of DPH in LM plasma membranes was determined with a computer-centered spectrofluorimeter (Holland *et al.*, 1973) as described previously (Hale & Schroeder, 1982; Schroeder *et al.*, 1984). All fluorescence measurements were made at 37°C . Fluorescence lifetime, τ , and steady-state fluorescence polarization, P , were obtained with a T-format SLM4800 sub-nanosecond spectrofluorimeter (SLM Instruments Inc., Champaign-Urbana, IL, U.S.A.) essentially as described by Hale & Schroeder (1982). Fluorescence lifetimes were simultaneously measured relative to a reference solution of dimethyl-*p*-bis(phenyloxazol-2-yl)benzene in ethanol (Lakowicz

Fig. 1. Action of charged anaesthetics on membrane-bound and detergent-solubilized 5'-nucleotidase activity from LM-cell plasma membranes

In all panels, the activity of 5'-nucleotidase was measured in native membranes (●) or in Lubrol-12A9-solubilized preparations (▲). (a) Effects of phenobarbital; (b) effects of pentobarbital; (c) effects of procaine; (d) effects of prilocaine. Assays were performed at 37 °C with time and protein concentration adjusted to keep linearity. Activity is calculated as $100\% \times (\text{specific activity with anaesthetic})/(\text{specific activity without anaesthetic})$. Data are expressed as means $\pm$ s.e.m. with $n = 3$, except $n = 1$ where error bars are omitted.

et al., 1981). The data were fitted to a multiple-lifetime analysis by the method of Weber (1981). Diphenylhexatriene displayed a single lifetime at 9.71 ± 0.13 ns in LM plasma membranes. Differential polarized phase fluorimetry, as described by Weber (1978) and extended by Lakowicz *et al.* (1979), was used to obtain the rotational rate, R (rad/s), and the limiting anisotropy, r_{∞} , of membrane-bound DPH. The rotational relaxation time in ns equals $(6R)^{-1}$. DPH was incorporated into plasma membranes at a concentration of $0.2 \mu\text{g}/100 \mu\text{g}$ of membrane protein as described by Schroeder *et al.* (1984). The excitation wavelength for DPH in LM plasma membranes was 362 nm, and fluorescence emission was monitored at 424 nm.

Drug treatment

Anaesthetics were prepared fresh daily in deionized water with pH adjusted to 10 with NaOH and, as necessary, with HCl. The drugs were added directly to membranes resuspended ($50 \mu\text{g}$ of protein/ml) in phosphate-buffered saline (Schroeder *et al.*, 1976) in the fluorimeter cuvette. The pH of the buffered sample was not changed significantly by addition of the anaesthetic solution. Measurements commenced usually within 1 min after addition of drug. No effect of longer incubation times was noted.

RESULTS

Effect of charged anaesthetics on LM-fibroblast plasma-membrane 5'-nucleotidase

In LM plasma membranes, phenobarbital failed to activate the ectoenzyme 5'-nucleotidase and was moder-

Fig. 2. Effect of charge anaesthetics on the absorbance-corrected fluorescence of DPH

The absorbance-corrected fluorescence of DPH in ethanol ($0.05 \mu\text{g}$ of DPH/ml) was determined as described in the Experimental section for phenobarbital (○), pentobarbital (●), procaine (■), prilocaine (▲) and chlorpromazine (×). Each point is the result of a single determination.

Fig. 3. Effect of charged anaesthetics on rotational rate and limiting anisotropy of DPH in Lubrol and Lubrol-lipid micelles

Fluorescence parameters were determined as described in the Experimental section. The concentration of Lubrol 12A9 of 5–6 mg/ml of membrane buffer was the same as used to extract the membranes. For Lubrol 12A9–lipid micelles, lipid extracted from membranes containing 1 mg of protein was added to 4 mg of Lubrol 12A9. The concentration of Lubrol 12A9 was 5 mg/ml. Lipid was extracted from LM plasma membranes by the Bligh & Dyer (1959) method. The concentration of DPH was 10 μ g/ml of Lubrol 12A9 (or Lubrol 12A9 plus lipid). The effects of phenobarbital (●) and prilocaine (▲) are shown for rotational relaxation time (ns) and limiting anisotropy. (a) Lubrol 12A9 micelles; (b) micelles of Lubrol 12A9 plus lipid extracted from LM-cell plasma membrane. For limiting anisotropy: (c) Lubrol 12A9 micelles; (d) micelles of Lubrol 12A9 plus lipid. Each point is the result of a single determination.

ately inhibitory at concentrations greater than 1 mM (Fig. 1a). This inhibition could arise either through fluidization of the membrane or by phenobarbital directly affecting the enzymic activity. Therefore the 5'-nucleotidase was solubilized with Lubrol. Phenobarbital above 1 mM strongly inhibited the Lubrol-solubilized 5'-nucleotidase (Fig. 1a), suggesting direct inhibition. Qualitatively similar results were obtained with pentobarbital (Fig. 1b) added either to intact plasma membrane or to Lubrol-solubilized 5'-nucleotidase. The intact membranes exhibited a 21% stimulation of 5'-nucleotidase at concentrations below 5 mM-pentobarbital. Enzyme activity was inhibited at higher drug concentrations. As with phenobarbital, pentobarbital inhibited the activity of the Lubrol-solubilized 5'-nucleotidase. Procaine was without effect on 5'-nucleotidase in intact membranes and had a modest non-dose-dependent inhibitory effect on Lubrol-solubilized enzyme (Fig. 1c). Prilocaine was without effect on either the membrane-bound or the solubilized 5'-nucleotidase (Fig. 1d). These data contrasted with those for rat liver plasma membranes (results not shown), where phenobarbital stimulates and prilocaine has no effect on 5'-nucleotidase activity. This observation confirms that of earlier investigators (Dipple *et al.*, 1982).

Effect of charged anaesthetics on rotational relaxation time and limiting anisotropy of DPH in Lubrol and Lubrol-lipid micelles

In the preceding section an indirect effect of the anaesthetics to fluidize the Lubrol micelle, thereby decreasing 5'-nucleotidase activity, was not ruled out. Therefore the fluorescent probe DPH was used to measure the effect of the anaesthetics on fluidity of the Lubrol micelles. Before this determination, the absence of a direct interaction of the anaesthetics with the fluorescent molecule must be established. Phenobarbital, pentobarbital, procaine and prilocaine were without effect on absorbance-corrected fluorescence of DPH in ethanol (Fig. 2). These anaesthetics also had no effect on polarization of DPH in ethanol (results not shown). Furthermore, increasing concentrations of these anaesthetics did not change the fluorescence lifetime of DPH in LM plasma membranes (results not shown). Thus there was no fluorescence quenching by direct interaction of these anaesthetics with DPH. In contrast, chlorpromazine, a tranquillizer/anaesthetic believed to interact preferentially with the inner leaflet of plasma membranes, quenched the absorbance-corrected fluorescence of DPH

Fig. 4. Action of charged anaesthetics on $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity in LM-cell plasma membranes

(a) Effect of phenobarbital; (b) effect of pentobarbital; (c) effect of procaine; (d) effect of prilocaine. The specific activity was calculated as the activity in the absence of ouabain minus the activity in the presence of 1 mM-ouabain. Assays were performed at 37 °C with time and protein concentration adjusted to maintain linearity. Activity is expressed as $100\% \times (\text{specific activity in the presence of anaesthetic})/(\text{specific activity in the absence of anaesthetic})$. Data are expressed as means $\pm$ S.E.M. ($n = 3$). An asterisk indicates $P < 0.05$ versus no drug (Student's t test).

in ethanol (Fig. 2). In addition, absorbance-corrected fluorescence and fluorescence lifetime of DPH in LM plasma membranes were decreased by chlorpromazine, also indicative of quenching (results not shown). Consequently, chlorpromazine was not used in these experiments. The above data indicate that DPH may be used to measure the effect of phenobarbital, pentobarbital, procaine and prilocaine on the physical properties of Lubrol micelles and LM plasma membranes.

The effects of phenobarbital and prilocaine on the fluidity of Lubrol and Lubrol-membrane-lipid micelles were determined. The rotational relaxation time of DPH in Lubrol micelles, 0.8 ns (approx. 70% of that observed in intact LM plasma membranes), was unaltered by addition of phenobarbital or prilocaine (Fig. 3a). When lipids extracted from LM plasma membranes were solubilized by Lubrol to form mixed micelles, the rotational relaxation time of DPH incorporated therein remained at 0.8 ns. Addition of either phenobarbital or prilocaine between 0.3 and 10 mM increased the rotational relaxation time to 0.9–1.0 ns (Fig. 3b). The limiting anisotropy of diphenylhexatriene in Lubrol micelles was very low, approx. 0.0016 (Fig. 3c), indicating little or no restriction to motion. This value was very much smaller (more 'fluid') than the value of 0.14 in the intact LM plasma membrane. Addition of phenobarbital or prilocaine increased the limiting anisotropy (more 'rigid') in Lubrol micelles to 0.005–0.008 in a non-dose-dependent manner, and in Lubrol-lipid micelles in a dose-dependent fashion up to 0.012, respectively (Figs. 3c and 3d). Even at the maximum increase, the limiting anisotropy was about one-tenth of the minimum limiting anisotropy of

0.10 seen for the exofacial leaflet of LM plasma membranes in the presence of 10 mM-pentobarbital and -prilocaine (results not shown). Hence, the motional restrictions on DPH solubilized into Lubrol micelles were very much less than even in membranes fluidized by high concentrations of anaesthetics. Thus changes in the fluidity of the Lubrol micelle cannot account for the observed inhibition of Lubrol-solubilized 5'-nucleotidase by these anaesthetics.

Modulation of LM plasma-membrane $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ by charged anaesthetics

By contrast with 5'-nucleotidase, an ectoenzyme, $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ is a penetrant enzyme that has an exofacial ouabain-binding site and a cytofacial ATP-hydrolysis site. In intact LM plasma membranes, the activity of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ exhibited a biphasic stimulation with increasing phenobarbital concentration (Fig. 4a). There was an initial stimulation, with a maximum near 0.5 mM, followed by a minimum near 2.5 mM (Fig. 4a). This minimum was followed by a small stimulation with a maximum near 5 mM and a subsequent decline in activity, which continued to the highest dose used (Fig. 4a). A similar curve was obtained with pentobarbital, except that the maxima were shifted slightly, to 0.25 and 2.5 mM, and the minimum was shifted to 0.8 mM (Fig. 4b). The activity of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ was stimulated by approx. 20% at 0.5 mM-phenobarbital and 0.25 mM-pentobarbital.

A simpler pattern of changes in $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity was observed with the cationic drugs. Procaine moderately inhibited the enzyme at low concentrations

Fig. 5. Action of charged anaesthetics on Mg^{2+} -ATPase activity in LM-cell plasma membranes

(a) Effect of phenobarbital; (b) effect of pentobarbital; (c) effect of procaine; (d) effect of prilocaine. Activity was determined in the presence of 1 mM-ouabain. For other details, see legend to Fig. 4. An asterisk indicates $P < 0.05$ versus no drug (Student's t test) ($n = 3$).

(0.01 mM), with a stimulation up to control values near 0.5 mM, followed by inhibition at higher drug concentrations (Fig. 4c). A qualitatively similar curve was observed with prilocaine (Fig. 4d). However, inhibition was observed only at and above 5 mM-prilocaine. The peak stimulation (30% stimulation over the no-drug value) occurred near 0.4 mM, a concentration slightly lower than that at which procaine stimulated the enzyme.

Effects of charged anaesthetics on Mg^{2+} -ATPase of LM plasma membranes

The activity of Mg^{2+} -ATPase was stimulated by 1 mM-phenobarbital, with a gradual decline at higher concentrations (Fig. 5a). In contrast, pentobarbital did not affect the activity (Fig. 5b). Very small changes in enzyme activity were observed with procaine and prilocaine. There was no effect below 1 mM with either drug, and a broad peak of 10% and 8% maximal stimulation, respectively, was observed between 1 and 10 mM (Figs. 5c and 5d respectively).

Individual monolayer structure of LM plasma membranes

The structures of the intact LM plasma membrane (inner and outer monolayer), the exofacial leaflet (extracellular monolayer) and the cytofacial leaflet (cytoplasmic monolayer) were examined with DPH as fluorescence reporter molecule and trinitrophenyl groups as quenching agents. The trinitrobenzenesulphonic acid labelling reaction was carefully monitored by three methods to ensure that the reagent did not penetrate into the cells and label both sides of the plasma membrane. (1) As an 'internal control', mitochondria and microsomal fractions were isolated from the same cells from which the trinitrophenylated plasma membranes were isolated. Appearance of trinitrophenyl groups in mitochondria or microsomal fractions requires not only flip-flop or penetration of the cell surface membrane, but also diffusion through the cytoplasm. Only 1.7 ± 0.2 and $1.9 \pm 0.4\%$ of microsomal and mitochondrial phospho-

tidylethanolamine were trinitrophenylated, respectively. When isolated microsomal fractions or mitochondria were treated with trinitrobenzenesulphonic acid at 4°C , 53 and 63% of the phosphatidylethanolamine was trinitrophenylated respectively. Trans-bilayer flip-flop of trinitrophenylated phosphatidylethanolamine was insignificant under the conditions used for fluorescence measurement herein; additional plasma-membrane phosphatidylethanolamine does not become available for trinitrophenylation. (2) Approximately half of the DPH fluorescence/mg of membrane protein was quenched in the trinitrophenylated membrane. This percentage quenching was maximal, since addition of trinitrophenylglycine, a water-soluble non-penetrant quencher, does not further decrease the DPH fluorescence (results not shown). (3) The fluorescence lifetime of DPH in non-trinitrophenylated LM-cell plasma membranes was best described as having a single component near 9.7 ± 0.13 ns. In contrast, the fluorescence lifetime of DPH in plasma membranes in which the outer monolayer was trinitrophenylated was 7.9 ± 0.1 ns. When analysed for multiple fluorescence lifetimes by the method of Weber (1981), the latter lifetime was resolved into two fluorescence lifetime components, one near 9.8 ± 0.3 ns (corresponding to unquenched DPH in the inner monolayer) and another near 2.2 ± 0.4 ns (corresponding to DPH quenched by trinitrophenyl groups in the outer monolayer). When fractional fluorescence values, also obtained by heterogeneity analysis, were converted into mole fractions, 49 ± 1 mol% of DPH was localized in the outer monolayer and 51 ± 1 mol% was localized in the inner monolayer of the LM plasma membrane. Quantum yield is proportional to fluorescence lifetime. Therefore the quantum yield of the quenched fluorescence in the outer monolayer was only one-fifth that in the inner monolayer. Consequently, more than 90% of the measured fluorescence signal of DPH in plasma membranes trinitrophenylated in the outer monolayer was due to DPH located in the inner

Table 1. Effect of local anaesthetic concentration on plasma-membrane individual-leaflet limiting anisotropy of DPH

LM fibroblasts were treated with or without trinitrobenzenesulphonic acid under non-penetrating conditions as described in the Experimental section. Plasma membranes were isolated, DPH was incorporated (1:100 probe/lipid molar ratio), and limiting anisotropy was determined at 37 °C also as described in the Experimental section. Values from cells treated with trinitrobenzenesulphonic acid represent the inner leaflet, and values from intact cells represent the inner+outer leaflet. Values for the outer monolayer were calculated from a fluorescence-intensity-normalized average of the inner and outer monolayer such that [limiting anisotropy in the inner+outer leaflet] equals the [(limiting anisotropy in the inner leaflet) × (fraction of total fluorescence in the inner leaflet)] plus [(limiting anisotropy in the outer leaflet) × (fraction of total fluorescence in the outer leaflet)]. Individual values are means ± S.E.M. ($n = 3$); * $P < 0.05$ compared with no anaesthetic added, by Student's t test.

Anaesthetic	Concn. (mM)	Limiting anisotropy	
		Outer leaflet	Inner leaflet
None	0	0.119 ± 0.002	0.155 ± 0.002
Phenobarbital	0.1	0.111 ± 0.001*	0.155 ± 0.002
	10.0	0.108 ± 0.002*	0.143 ± 0.003*
Pentobarbital	0.5	0.110 ± 0.002*	0.150 ± 0.002
	10.0	0.102 ± 0.003*	0.138 ± 0.004*
Prilocaine	0.5	0.115 ± 0.002	0.147 ± 0.002*
	10.0	0.101 ± 0.004*	0.129 ± 0.002
Procaine	0.5	0.114 ± 0.003	0.154 ± 0.003
	10.0	0.108 ± 0.002*	0.147 ± 0.002*

monolayer. When both monolayers were trinitrophenylated (e.g. at 37 °C), then only the short-lifetime component appeared. If as little as 10% of the inner-monolayer fluorescence probe molecules were quenched by trinitrobenzenesulphonic acid that had penetrated to the inner monolayer, the fluorescence lifetime in that monolayer would be significantly decreased. The data show that this does not occur.

The limiting anisotropy and order parameter of diphenylhexatriene in the inner monolayer (0.155 ± 0.002 and 0.630 ± 0.006 respectively) were significantly higher than in the outer monolayer (0.119 ± 0.002 and 0.550 ± 0.006 respectively). Concomitantly, the rotational relaxation time of DPH was also longer (1.28 ± 0.03 ns) in the inner monolayer than in the outer monolayer (1.12 ± 0.03 ns).

Correlation between DPH limiting anisotropy and ($\text{Na}^+ + \text{K}^+$)-ATPase activity

Since the major focus of this paper is based on the premise that local anaesthetics are capable of selectively fluidizing a specific leaflet, the effects of the four local anaesthetics on the individual leaflet limiting anisotropy of DPH fluorescence were determined (Table 1). At low concentrations (0.1–0.5 mM) phenobarbital and pentobarbital selectively fluidized the outer leaflet of the LM plasma membrane, whereas prilocaine selectively fluidized the inner leaflet. Procaine, however, had no effect at

0.5 mM concentration. It is also clear from Table 1 that some spill-over to the non-specific monolayer occurred at low concentrations, whereas at high concentration (10 mM) all four drugs fluidized both leaflets, indicating no specificity. If the percentage activity at a given concentration is plotted against the exofacial-leaflet DPH limiting anisotropy, a parameter that reflects restriction to motion ('fluidity'), for that anaesthetic concentration, no clustering of activity maxima for the four drugs at any value of limiting anisotropy was noted (Fig. 6a). This weighs against exofacial fluidity changes effecting phosphatase activity changes. If the same plot was made for cytofacial limiting anisotropy and percentage specific activity, the maximal percentage specific activity for all four drugs occurs in the limiting anisotropy range of 0.1535–0.1500 (Fig. 6b). This suggests that small changes in cytofacial-leaflet fluidity (< 3.25%) by any of the four drugs tested lead to large changes (20–30%) in ATP hydrolysis. Clearly, the selectivity of any of these drugs for one or the other leaflet is not absolute, and small changes in fluidity in the non-preferred leaflet can, in this and perhaps other sensitive enzymes, lead to activity changes.

DISCUSSION

Sheetz & Singer (1974) originally proposed that cationic penetrant amphipathic drugs preferentially distributed into the cytofacial monolayer, whereas anionic penetrant and ionic impenetrant amphipathic drugs preferentially distributed into the exofacial monolayer of the erythrocyte plasma membrane. Regardless of the mechanism of selective fluidization of one or the other monolayer, with one exception (Conrad & Singer, 1979, 1981) most evidence is consistent with selectivity for individual monolayers by charged amphipaths (Sheetz & Singer, 1974; Bondy & Remien, 1981; Gaffney *et al.*, 1983; Dipple *et al.*, 1982; Houslay *et al.*, 1977, 1980b, 1981). Herein, the testing of the hypothesis was extended to LM-cell plasma membranes, which have a transbilayer gradient of cholesterol and fluidity opposite to that found in erythrocytes and other normal (i.e. not transformed) cells tested (Schroeder, 1980, 1984, 1985; Hale & Schroeder, 1982). In the outer leaflet of the LM plasma membrane, DPH had a lower limiting anisotropy than in the inner leaflet. The significance of the magnitude of difference in DPH limiting anisotropy between monolayers, namely 0.036, can be illustrated by comparison with the effect of temperature on this parameter. The limiting anisotropy of diphenylhexatriene in LM plasma membranes was 0.181 ± 0.002 ($n = 3$) and 0.137 ± 0.002 ($n = 6$) at 24° and 37 °C respectively. Thus the difference in limiting anisotropy between the inner and outer monolayer was as large as that produced by a 10.6 °C change in temperature. Taken together, these data indicate that the motion of DPH in LM plasma membranes was less rapid and considerably more restricted in the inner than in the outer monolayer.

As predicted, 5'-nucleotidase was not affected by the cytofacial drug prilocaine or the non-selective drug procaine in the rat liver plasma membrane (results not shown), confirming previous work (Dipple *et al.*, 1982), or in the LM plasma membranes (Figs. 1c and 1d). Results similar to those reported for rat liver plasma-membrane 5'-nucleotidase (Dipple *et al.*, 1982) were also reported for the effects of charged drugs on the

Fig. 6. Relationship between limiting anisotropy and % specific activity of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$

The results presented in Fig. 4 for % specific activity versus concentration of anaesthetic were transformed by using limiting-anisotropy data, which were obtained as described in the Experimental section. The curves relating limiting anisotropy to concentration of anaesthetic and concentration of anaesthetic to $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity were used to construct these curves: actual data points are not shown. The limiting anisotropy at a given concentration was estimated from the curve and this value was plotted against the % specific activity at that concentration as estimated from the curve; actual data points from the Figures were not used. The limiting anisotropy of the exofacial leaflet is presented in (a) and of the cytofacial leaflet in (b); $\times$ represents the limiting anisotropy in the absence of any anaesthetic for each leaflet. Results are shown for phenobarbital (—), pentobarbital (---), procaine (-.-.) and prilocaine (....). The curve for prilocaine in the cytofacial leaflet (b) is truncated; very small changes occur in the % specific activity in the omitted portion.

synaptosome-associated acetylcholinesterase activity (Deliconstantinos & Tsakiris, 1985). The exofacial-preferential drugs, phenobarbital and pentobarbital, had little or no influence on the LM plasma-membrane-bound enzyme until high concentrations of anaesthetic were reached. Transformation itself may induce such changes. LM cells are a highly malignant transformed cell line (Kier & Schroeder, 1982). 5'-Nucleotidase is shed from tumour cells into the blood or culture medium in quantities sufficient to suggest its use as a diagnostic test for many neoplasms (Kim *et al.*, 1977; Schroeder & Gardiner, 1984). The isolated LM plasma membrane is 8-fold enriched in $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$, whereas 5'-nucleotidase is only 6-fold enriched over the crude homogenate, indicating that the fraction of 5'-nucleotidase lost from the membrane may be substantial. Differences in the extent of association of 5'-nucleotidase to liver membranes from different species have also been inferred previously. In rat liver plasma membranes, there is a characteristic temperature of 28 °C in Arrhenius plots of e.s.r. order parameter (Gordon *et al.*, 1980) and also in Arrhenius plots of rat liver 5'-nucleotidase activity (Dipple *et al.*, 1982). The 28 °C inflection in the e.s.r. order parameter was assigned to the exofacial leaflet, and the activity of 5'-nucleotidase was responsive only to agents that perturbed that e.s.r. inflection (Dipple *et al.*, 1982). The 5'-nucleotidase from hamster liver behaved differently. The two characteristic temperatures in Arrhenius plots of hamster liver 5'-nucleotidase activity corresponded to the two characteristic temperatures of 4-anilinonaphthalene-1-sulphonic acid fluorescence

(Houslay & Palmer, 1978). One characteristic temperature of fluorescence was assigned to each of the leaflets on the basis of characteristic temperatures of seven other enzymes of known membrane asymmetric distribution. Since 5'-nucleotidase activity in hamster liver membranes sensed transitions in both membrane leaflets, but rat liver 5'-nucleotidase sensed transitions only in the exofacial leaflet, the hamster 5'-nucleotidase may be more deeply buried in the membrane.

We are not aware of any previous work demonstrating an effect of altered individual leaflet fluidity on $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. In a study of the effects of chlorpromazine and four of its metabolites (all cationic) on the activity of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ and on the polarization of DPH fluorescence, no consistent relationship between the signs of changes in polarization and enzyme activity was observed (Keeffe *et al.*, 1980). However, chlorpromazine quenched the fluorescence of DPH (Fig. 2). Since at least some metabolites of chlorpromazine might reasonably be expected to retain the molecular structure necessary for quenching, and because quenching and motion (fluidity) affect polarization independently (Knox, 1968; Kelly & Patterson, 1971), these data are very difficult to interpret. Furthermore, only the average polarization of the whole membrane (inner plus outer leaflet) was determined. It is clear from the present work that exofacial-leaflet-preferential drugs (phenobarbital and pentobarbital) affect the cytofacial ATP-hydrolysis site of LM plasma-membrane $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$.

Although alterations of the lipid environment can

affect ouabain binding (Abeyawardena & Charnock, 1983), the effects of these anaesthetics on $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ are not explained by altered ouabain binding. First, since a ouabain concentration giving maximal inhibition was used, enhancement of binding would lead to no additional effect. Second, since $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity is a derived quantity, decreased binding would result in an apparent decrease in activity, exactly opposite to the observed results. This argument holds also if either one or both of the two molecular forms of the α -subunit of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$, which differ in their affinity for ouabain (Sweadner, 1979; Lytton *et al.*, 1985), are present. Thus altered ouabain binding, although not rigorously excluded by the available data, cannot explain the observed barbiturate effects.

The $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity maxima occurring at the higher concentrations of barbiturates were in the range of limiting anisotropy of 0.146–0.143 in the cytofacial leaflet, but were dissociated in the exofacial leaflet. This suggests a second mechanism of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activation by further fluidization of the cytofacial leaflet. Procaine did not decrease the limiting anisotropy to this range, and thus provided no evidence on this proposal. However, activation was not noted with fluidization by prilocaine in this range of limiting anisotropy, thus excluding a second, fluidity-associated, mechanism. An alternative explanation was suggested by the fact that both anionic, but neither cationic, drugs evoked the second peak. It was demonstrated that, for $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$, ionic conditions at the surface, particularly regarding formation of an electrical double layer, influence activity (Ahrens, 1981). Furthermore, ionic conditions (regardless of the specific properties of the ions) also influenced the binding constants of the activating Na^+ and K^+ ions, thereby altering $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity (Ahrens, 1983). The combination of these effects leads to a (three-dimensional) 'activity surface' for concentrations of Na^+ and K^+ and activity (Ahrens, 1983). Although the ionic composition of the assay was constant in our experiments, the addition of anions to the membrane surface does alter the surface charge potential (Schlieper & Steiner, 1983). At certain concentrations, a stimulation of activity followed by an inhibition would be seen as the conditions passed through the maximum on the 'activity surface'. It is possible that the required concentrations may occur at the second maximum. This effect would be, of course, superimposed on direct fluidization effects and indirect effects on fluidity caused by interaction of ions with the charged membrane surface, which can alter motional parameters of the membrane lipids (Träuble & Eibl, 1974). However, the available data cannot discriminate among these proposals.

In contrast with $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$, which is sensitive to small changes in the fluidity of the cytofacial leaflet, the activity of $\text{Mg}^{2+}\text{-ATPase}$ was insensitive to large changes in the fluidity of either leaflet. Opposite to the small effects of fluidization on $\text{Mg}^{2+}\text{-ATPase}$ activity seen in this study with anaesthetics, substantial decreases in activity of $\text{Mg}^{2+}\text{-ATPase}$ were associated with decreased polarization of DPH fluorescence induced by phospholipase A_2 or concanavalin A treatment of rat liver plasma membranes (net fluidization of the whole membrane) (Riordan, 1980). As with rat liver $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$, there was no correlation between the polarization and rat liver $\text{Mg}^{2+}\text{-ATPase}$ activity changes produced by

chlorpromazine and its metabolites (Keeffe *et al.*, 1980). As discussed above, the quenching of DPH fluorescence by chlorpromazine makes the interpretation of such results very difficult.

In summary, $5'$ -nucleotidase in LM plasma membranes is little influenced by changes in fluidity caused by charged anaesthetics, unlike $5'$ -nucleotidase in rat liver plasma membranes, which is activated at certain concentrations of barbiturates. Fluidization by some charged anaesthetics seems to activate LM-cell plasma-membrane $\text{Mg}^{2+}\text{-ATPase}$ slightly. In contrast, the activity of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ in LM plasma membranes is stimulated by anionic and cationic anaesthetics. Activation occurs within a narrow range of limiting anisotropy in the cytofacial leaflet regardless of whether the fluidization is achieved by cytofacial-preferential drugs or exofacial-preferential drugs 'spilling over' to the cytofacial leaflet. Only the (anionic) barbiturates produce a second stimulation peak at high concentrations, which suggests that ionic changes in the membrane surface may cause the second peak. These results demonstrate that the activity of plasma-membrane-bound enzymes may or may not be affected by changes in fluidity of one or the other membrane leaflet, depending on the enzyme and the membrane. This study also warns that the relatively low selectivity ratio of these drugs may lead to false conclusions about which leaflet influences the activity of an enzyme, unless the fluidity changes are simultaneously measured. However, these drugs can be a useful tool in delineating the effect of the fluidity of individual membrane leaflets on the activity of membrane-bound enzymes.

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