Biochimica et Biophysica Acta, 688 (1982) 163–168
Elsevier Biomedical Press

BBA 71194

THE EFFECTS OF GENERAL ANAESTHETICS ON GLUCOSE AND PHOSPHATE TRANSPORT ACROSS THE MEMBRANE OF THE HUMAN ERYTHROCYTE

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(Received February 1st, 1982)

Key words: Anesthetic; Glucose transport; Phosphate transport; (Human erythrocyte membrane)

A study has been carried out into the effects of clinically important general anaesthetics, althesin, thiopentone and propanidid, on the transport of glucose and phosphate across the membrane of the human erythrocyte. In general these three substances all inhibit both transport processes but with characteristic inhibition profiles and varying degrees of efficacy. Glucose transport was more sensitive to the hydrophobic steroids and phosphate transport to propanidid. Some hydrophobic agents, e.g., iodobenzene and its azide, were not inhibitory. Removal of cholesterol to some extent augmented the inhibitory effects of most of these compounds (not propanidid). It is argued that these effects are due to the penetration of the anaesthetics into the lipid bilayer and either subsequent disruption of the lipid annuli surrounding the integral membrane proteins and/or direct anaesthetic-protein interaction.

Introduction

The transport of hydrophilic substances across a lipid bilayer is believed to be mediated by protein molecules which are in general stereospecific, inhibitable and exhibit saturation kinetics. These proteins appear to traverse the bilayer and thus part of their structure is assumed to be in intimate contact with the hydrophobic milieu of the membrane. The demonstration of a less mobile component of the lipid fraction and of defined lipid requirements for some membrane proteins has given rise to the notion of annular lipids associated with proteins, whose characteristics differ from the bulk lipid. It is a reasonable extrapolation of this concept, therefore, that substances which alter this hydrophobic environment may affect protein-mediated transport activity.

Certain anaesthetics belong to the group of molecules which may be able to pass into the cell membrane due to their hydrophobic nature. Because of the general correlation between solubility in hydrophobic media and anaesthetic potency [1–3], it has been postulated that the cell membrane is the site of their action. The mechanism of this effect, however, is obscure. Changes in lipid fluidity [4–11], membrane thickness [12–15], and membrane volume [16,17] have been suggested but interactions of several kinds may be responsible for the effect, including the displacement of calcium ions [18].

This research was undertaken in order to establish whether certain clinically important general anaesthetics, of differing structure but all of which are neutral and may penetrate the lipid bilayer, can alter transport activity, most particularly in the human erythrocyte but with obvious implications for other cell types. At the same time, it was

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Abbreviation: SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid.

hoped that the characteristics of their effects may give a clue to their primary target and provide some evidence of the way in which integral membrane proteins respond to changes in their general hydrophobic environment.

Experimental

Materials

Type A Rh^+ human red blood cells were obtained from the Regional Blood Transfusion Centre, Seacroft Hospital, Bridlepath Lane, Leeds. Althesin, propanidid and thiopentone were obtained from Glaxo Ltd., Bayer UK Ltd., and May and Baker Ltd., respectively, and pregnenolone from Sigma. Propanidid (pure, not the formulation for clinical usage) and pregnenolone were dissolved in ethanol and used such that the final ethanol concentration in both experimental and control systems were less than 1% (v/v). Cholesterol Grade 1, α-L-phosphatidylcholine type III-E from egg yolk and bovine serum albumin were supplied by Sigma. All other reagents were Analar grade or better, and were obtained from BDH Chemicals.

Methods

Transport experiments. The transport of phosphate was monitored as described earlier [19] except that the pH was 7.2. Cells were preincubated for 30 min at 25°C with 50 mM KH_2PO_4, 0.3 M triethanolamine/citric acid, pH 7.2, and this buffer then removed. Anaesthetics were subsequently added, as either their soluble formulations for clinical usage or dissolved in ethanol, to washed cells suspended in 0.3 M triethanolamine/citric acid, pH 7.2. The mixtures were incubated for 10 min at 25°C before [^{32}P]phosphate was added. Washed erythrocytes were not preincubated with KH_2PO_4 prior to experiments on glucose transport which was measured as before, again at pH 7.2 [19]. Sulphate transport was carried out as for phosphate transport except that K_2SO_4 was used instead of KH_2PO_4 and [^{32}P]phosphate was replaced by [^{35}S]sulphate. All concentrations of anaesthetics shown were examined in duplicate in one experiment. This experiment was repeated at least on five different occasions. The points represent the average of these five studies.

Glucose transport in whole cells. Erythrocytes were prepared as for phosphate transport, and diluted with buffer to a 50% hematocrit value. Then 0.2 ml of the cell suspension was placed in a centrifuge tube and 1 ml of 0.3 M triethanolamine/citric acid, pH 7.2, containing 0.5 mM D-glucose, 1.0 mM D-galactose and 0.5 μCi of D-[^{3}H]glucose was added with thorough mixing. At the specified time, 6 ml of ice-cold 0.3 M triethanolamine/citric acid/3 mM $HgCl_2$ was added and the tube kept on ice until the end of the experiment. All the samples were then centrifuged for 5 min and the supernatants removed. The pellet was lysed with 0.1 ml of water and the protein precipitated with 5% trichloroacetic acid. Samples were counted for radioactivity as for phosphate transport.

Cholesterol depletion. Cells were depleted of cholesterol according to the method of Cooper et al. [20]. Liposomes were prepared by sonication of phospholipid/cholesterol mixtures of molar ratios between 10:1 and 15:1, suspended in 0.3 M triethanolamine/citric acid, pH 7.2. Bovine serum albumin in the same buffer was added to the liposome preparation to give a final protein concentration of 5 mg/ml. Washed erythrocytes were incubated with gentle shaking in the above mixture (control cells in buffer alone) for 15 h at 37°C. The treated cells were recovered by gentle centrifugation (3000 × *g* for 10 min) and washing in 0.3 M triethanolamine/citric acid, pH 7.2. They were used in the transport experiments after the preincubations described above. Cholesterol removed from the cells [21] was estimated from the membrane content before and after depletion, as assayed by the method Leffler and McDougal [22].

Results

Effects on phosphate transport

Despite their different structures, all three anaesthetics, and the steroid derivative, pregnenolone, (results not shown), had similar effects on phosphate exchange across the membranes of human erythrocytes.

The profile of inhibition was characteristic, with an initial rapid dose-dependent loss in activity quickly reaching a plateau which varied between 25% and 55% activity, below which no further

inhibition was obtained. The anaesthetic concentration at which this plateau was reached was generally below 1 mM. Neither the hydrophobic probe 1-iodo-4-azidobenzene which binds to the intramembraneous regions of the anion exchange protein when activated by light [23], nor ethanol inhibited the transport activity over this concentration range (Fig. 1). Ouabain, a much more water-soluble steroid than either althesin or pregnenolone, also did not appear to inhibit phosphate exchange. The inhibition was relatively pH independent in the range examined (6.5–7.4).

When after preincubation with anaesthetic, the erythrocytes were washed once with a 50-fold volume of buffer, no inhibition of phosphate transport was obtained, indicating a complete reversal of the anaesthetic effect presumably due to its removal from the cell membrane. Althesin required a second wash for complete restoration of transport activity. Whatever form of membrane-interaction occurs, therefore, it is freely and readily reversed. Simultaneous incubation of erythrocytes with both althesin and propanidid gave additive effects but only up to the plateau region which was altered only slightly by the combination of anaesthetics (Fig. 2).

Inhibition of phosphate transport by SITS was not affected by the presence of any of the anaesthetics. The inhibition of sulphate exchange gave the same characteristics as those seen for phosphate.

Effects on glucose transport. Under the same conditions as those for phosphate exchange, the transport of glucose responded differently to the various anaesthetics (Fig. 3). Althesin at 0.4 mM, completely abolished transport activity whilst propanidid produced the previous plateau profile re-

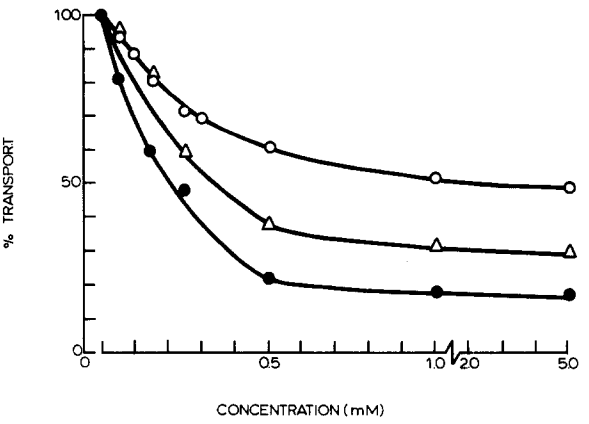

Fig. 2. The effect of althesin plus propanidid on phosphate exchange. Washed human erythrocytes were preincubated with althesin (○——○) alone, propanidid (△——△) alone or both (●——●), and phosphate uptake monitored as above.

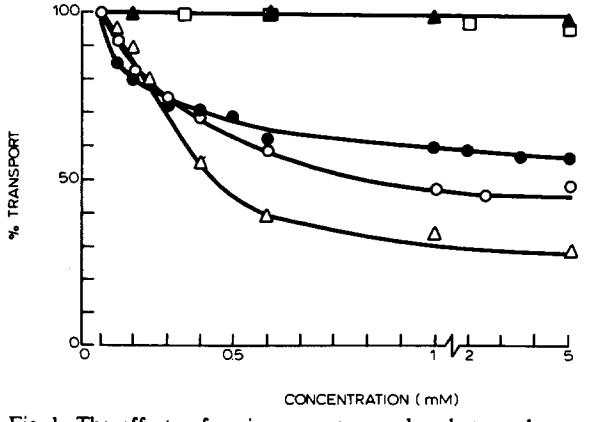

Fig. 1. The effects of various agents on phosphate exchange. Washed human erythrocytes were preincubated with varying amounts of althesin (○——○), propanidid (△——△), thiopentone (●——●), 1-iodo-4-azidobenzene (□——□), and ethanol (▲——▲). The rate of [^{32}P]phosphate uptake was then monitored for 60 min and the rate of the linear initial transport calculated.

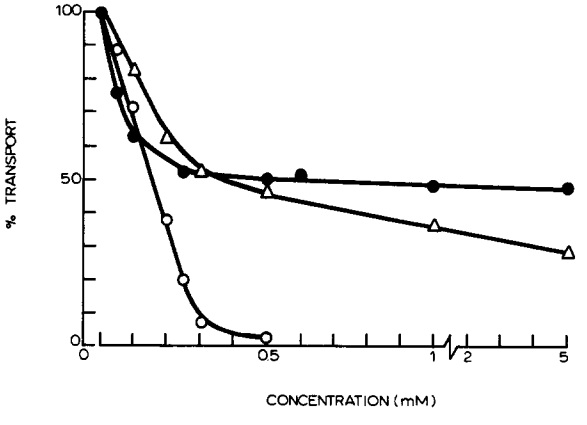

Fig. 3. The effect of anaesthetics on glucose uptake. Washed human erythrocytes were preincubated with varying amounts of althesin (○——○), propanidid (△——△) and thiopentone (●——●). The accumulation of [^{3}H]glucose in the erythrocytes was monitored.

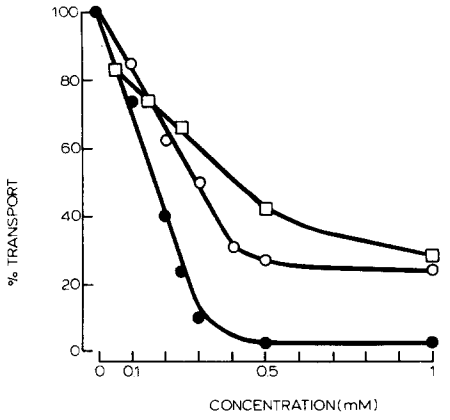

Fig. 4. The effect of althesin plus propanidid on glucose uptake. Washed human erythrocytes were preincubated with althesin alone (●——●), propanidid (○——○) or both (□——□). Glucose uptake was monitored as before.

aching a maximum of approx. 70% inhibition above 0.4 mM, a pattern not significantly different from its effects on anion transport. Pregnenolone and thiopentone produced effects similar to but less pronounced than althesin and propanidid, respectively.

The wash out experiments yielded the same results for glucose as for phosphate transport but

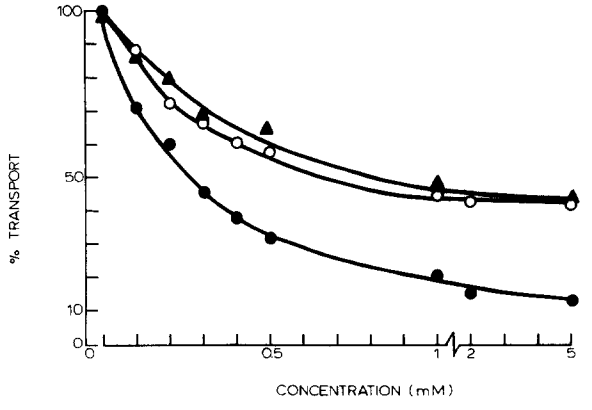

Fig. 5. The influence of altered cholesterol content on althesin inhibition of phosphate exchange. Cholesterol was removed from or added to erythrocyte membranes by incubation of cells with phosphatidylcholine/cholesterol liposomes at 37°C for 15 h [20]. Treated erythrocytes were then preincubated with and without althesin prior to monitoring phosphate exchange. ○——○, Untreated cells; ▲——▲, cholesterol augmented; ●——●, cholesterol depleted.

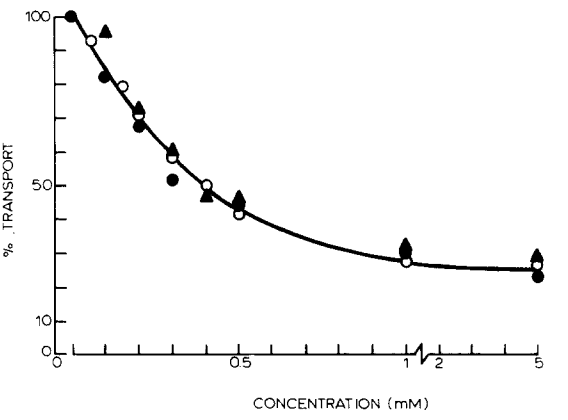

Fig. 6. The effect of altered cholesterol content on propanidid inhibition of phosphate exchange. Experiment as in fig. 5.

the effects of the simultaneous addition of althesin and propanidid were unexpected (Fig. 4). At the higher but still non-saturating levels of anaesthetic, inhibition of transport was apparently less than with either agent alone, in marked contrast to their effects on the activity of the anion exchange protein.

No attempt was made to separate the two components in althesin (alphaxalone and its acetate) in order to examine whether both components were equally effective.

Effects on cholesterol depleted/augmented cells. The procedure employed to remove cholesterol

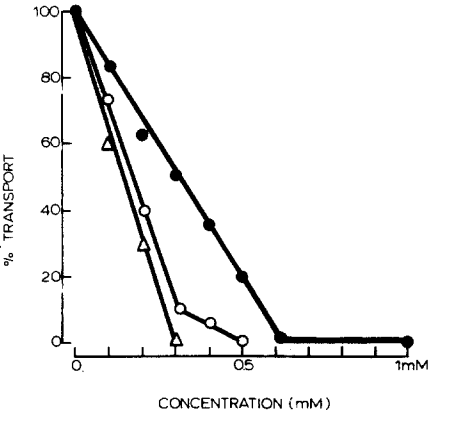

Fig. 7. The effect of altered cholesterol content on althesin inhibition of glucose uptake. △——△, Cholesterol depleted; ○——○, untreated cells; ●——●, cholesterol augmented.

from membranes of red blood cells was very effective giving an average depletion of 39%. The transport activity in these depleted cells for both glucose and phosphate was reduced by about 12%. Under these conditions, althesin inhibition of phosphate transport was markedly increased whereas the propanidid effects were largely unaffected (Figs. 5 and 6). In the case of glucose transport the influence of both agents were only slightly augmented (Fig. 7).

Attempts to increase the level of cholesterol in red blood cells by incubating with cholesterol/lipid mixtures of up to 5:1 (molar ratio) give at best only a 10% increase in the cholesterol content. Under these conditions the inhibitory effects of the anaesthetics on both transport processes were marginally reduced.

Discussion

The results show that the transport of both glucose and phosphate is inhibited by all three anaesthetics. The order of effectiveness and the characteristics of inhibition, however, vary, althesin being the most potent inhibitor of glucose transport and propanidid of phosphate exchange. This feature illustrates the specific nature of the two transport processes and may indicate direct anaesthetic-protein interaction (discussed later), particularly in the althesin-glucose system. Only in the case of althesin inhibition of glucose transport was complete inhibition observed (also widely observed for other steroids [24]); in the remaining systems, a characteristic plateau was reached.

In some instances the position of this plateau was altered by the removal of cholesterol from the membrane suggesting that at least part of the effect may be due either to the inability of the membrane to absorb more anaesthetic or to the increased relative amount of anaesthetic in the bilayer. On the other hand the limited effect of cholesterol depletion on inactivation by propanidid and the additive effect of anaesthetic combinations indicate that these cannot be complete explanations. One can rule out any action at the active site itself since the anaesthetics do not interfere with the inhibitory action of SITS on phosphate exchange. In general, the relatively high level of anaesthetic required for inhibition (cf. the action of niflumic acid [25]) mitigates against interaction at very specific sites associated with transport activity [26].

The mechanism by which these anaesthetics cause inhibition, however, is not at all clear [27]. As judged by criteria of molecular weights, and the different effect of proteases and inhibitors (-SH reagents, SITS, etc.) on transport activity, it would appear that the two protein mediators are structurally quite dissimilar. This would tend to suggest that their exposed surfaces are quite different and hence that direct interaction between the anaesthetics and these regions of the proteins is unlikely. Althesin inhibition of glucose transport may be different since the total character of its effect contrasts with the other agents. It can be postulated, therefore, that in general the membrane itself is altered in some way and that at least some of the associated proteins are responding to varying extents to these changes.

One such change might involve the nature of water associated with the membrane. This feature is not at all well understood, although it is known that hydrophobic substances may alter the molecular properties of structured water [28–31]. This in turn may influence protein conformation and/or activity. This rather nebulous proposal must remain a less likely alternative, however, for it fails to explain the quite different effects of the steroids, althesin, cholesterol and ouabain on the phosphate exchange.

The most popular theory of anaesthetic action postulates that these agents are able to penetrate the lipid bilayer and exert their effects from within the hydrophobic phase. Although there is no direct evidence in this case that such penetration does occur, other hydrophobic molecules do possess this property and hence, by extrapolation, anaesthetics are thought to be no different in this respect. The different effects of ouabain which is water soluble, from pregnenolone and althesin which are not, support this assumption. It ought to be borne in mind, however, that there is a body of opinion which suggests that amphipathic molecules, e.g. chlorpromazine, do not penetrate the bilayer [32].

One of the most pronounced consequences of such entry into the hydrophobic phase is seen in altered fluidity characteristics of the lipid bilayer

[4–11]. The concentrations required, however, for measurable changes and the directions of those changes do not correlate well with the observed effects [9]. In the experiments reported here the removal of cholesterol or the inclusion of 1-iodo-4-azidobenzene might both be expected to induce alterations in fluidity but in neither case is transport significantly affected.

One is left, therefore, with the possibility that it is the less mobile lipid components more adjacent to the protein surface that are being influenced, or that direct interactions between the anaesthetic molecules and hydrophobic regions of the polypeptide chains are occurring. In both instances the freedom of the proteins to undergo conformational rearrangements might be restricted and hence cause this characteristic inhibition pattern. Although these last are our favoured explanations, we have no evidence to distinguish between them and they may indeed not be mutually exclusive.

Finally, this ability of general anaesthetics to inhibit transport systems in the membrane of the red blood cell suggests that similar effects may be expected to occur in many other cell types including nervous tissue. Anaesthesia, therefore, may result from the particular sensitivity of nerve cell membranes or the resultant alterations in their intercellular communications.

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