Effects of halothane on transport of 5-hydroxytryptamine by platelet membranes

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Na⁺-dependent uptake of 5-HT (5-hydroxytryptamine) into plasma membrane vesicles derived from bovine blood platelets and ATP-dependent 5-HT uptake into storage vesicles in platelet lysates were measured. Na⁺-dependent uptake was temperature-dependent, inhibited by imipramine and exhibited Michaelis–Menten kinetics (apparent K_m , $0.12 \pm 0.02 \mu\text{M}$; $V_{\max}$, 559 ± 54 pmol/min per mg of protein). Halothane had no effect on Na⁺-dependent transport of 5-HT in plasma-membrane vesicles. ATP-dependent 5-HT transport into storage granules also exhibited Michaelis–Menten kinetics (apparent K_m , $0.34 \pm 0.03 \mu\text{M}$; $V_{\max}$, 34.3 ± 1.7 pmol/min per mg of protein) and was inhibited by noradrenaline (norepinephrine), but not by imipramine. Exposure of the granules to halothane resulted in a progressive decrease in $V_{\max}$. The results demonstrate a possible site for disruption of platelet function by anaesthetics.

INTRODUCTION

5-HT is involved in a number of important physiological and pathophysiological processes. These include neurotransmission (Paasonen, 1968; Sneddon, 1973), platelet function (Born *et al.*, 1972), and regulation of blood pressure (Block & Stalcup, 1982). In addition, there is evidence that 5-HT can moderate the level of anaesthesia produced by the general anaesthetic halothane (2-bromo-2-chloro-1,1,1-trifluoroethane). Depletion of 5-HT in neurons of the raphe nuclei decreased the MAC for halothane, whereas increased levels of 5-HT increased MAC (Roizen *et al.*, 1978).

In aminergic neurons, high concentrations of 5-HT are accumulated and stored in electron-dense granules (Blackburn *et al.*, 1967). An analogous process occurs in platelets. Transport of 5-HT into these intracellular storage organelles requires either ATP or a pH gradient across the granule membrane (Rudnick, 1977; Rudnick *et al.*, 1980). The characteristics of the storage system in platelets are similar to those of storage systems for biogenic amines found in chromaffin granules of the adrenal medulla (Smith, 1968; Kirshner, 1974), in mast cells (Mota *et al.*, 1954) and in synaptic vesicles of neurons (Whittaker, 1971). Halothane has been shown to inhibit uptake of catecholamines into chromaffin granules isolated from bovine adrenal medulla (Sumikawa *et al.*, 1980). In the same study, release of catecholamine from the granules was also observed, but only at high concentrations of halothane. A similar effect of the anaesthetic on amine uptake into platelets could affect the function of platelets in haemostasis.

Plasma 5-HT is maintained at low levels not only by storage in platelets, but also by rapid uptake and inactivation by vascular endothelial cells. Uptake is mediated by a specific transport mechanism and is followed by deamination by monoamine oxidase to a non-vasoactive metabolite. We demonstrated previously

halothane's effect of inhibiting transport of 5-HT into endothelial cells of lungs perfused *in situ* and into isolated lung cells in culture (Watkins *et al.*, 1983).

Because of the similarities of uptake, storage and release of biogenic amines between the variety of tissues mentioned above, platelets have been used by others as a model for studies of neuronal uptake of monoamines. Platelets can be easily obtained in large quantities and at high purity. Because platelets exhibit an uptake system in the plasma membrane that has characteristics similar to that observed in endothelial cells, as well as an uptake system in storage granules, we have used subcellular fractions of platelets to define further the effects of halothane on a membrane transport process in both types of membrane.

EXPERIMENTAL

Preparation of plasma-membrane vesicles and storage granules

Fresh bovine blood was obtained from a local slaughterhouse. Platelets were isolated, lysed and fractionated as described by Barber & Jamieson (1970) as modified by Rudnick & Nelson (1978). All vessels and pipettes with which platelets came in contact were plastic. Only one band containing membrane vesicles was separated by density-gradient centrifugation. Vesicles were stored at -70°C at 1.5–3 mg of protein/ml in 10 mM-Tris/HCl, pH 7.5, containing 0.25 M-sucrose. ATP-dependent transport of 5-HT into intracellular storage organelles was measured in unfractionated lysates prepared as described by Rudnick *et al.* (1980), and stored (-70°C) at 10–15 mg of protein/ml.

Transport assays

5-HT uptake by plasma-membrane vesicles was assayed essentially as described by Rudnick (1977).

Abbreviations used: 5-HT, 5-hydroxytryptamine; MAC, mean alveolar concentration of anaesthetic required to produce anaesthesia in 50% of subjects; C_e , external concentration; C_i , internal concentration.

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Unless stated otherwise, all transport assays on both plasma-membrane vesicles and storage granules were performed at 37 °C. Vesicles were equilibrated for 5 min with 0.1 M-potassium phosphate, pH 6.7, containing 1 mM-MgSO₄. Uptake experiments were begun by adding 0.02 ml of membrane suspension (28–50 µg of protein) to 0.2 ml of either 0.1 M-NaCl or 0.1 M-LiCl, containing 1 mM-MgSO₄ and [1,2-³H]5-HT (New England Nuclear Corp.) at concentrations indicated in the Figures. The reactions were terminated by dilution with 0.15 M-NaCl (4 °C), and the suspensions were filtered (Millipore; 0.45 µm pore size; HAWP) and washed twice with 5 ml of 0.15 M-NaCl (4 °C). The filters were dried, placed in scintillation vials, and dissolved in 1 ml of 2-methoxy-ethanol ('ethylene glycol monomethyl ether'). Scintillation cocktail (ACS II, Amersham; 10 ml) was added to the vials and radioactivity was assayed using a Beckman LS 250 liquid-scintillation spectrometer.

Unfractionated platelet lysates were equilibrated with 10 mM-Hepes, pH 7.4, containing 0.3 M-sucrose. Transport was initiated by dilution of lysate (0.02 ml) into 0.2 ml of 10 mM-Hepes, pH 8.5, containing 0.3 M-sucrose, 2.5 mM-MgSO₄, 5 mM-KCl, 5 mM-KCl, 5 mM-ATP (except where indicated) and 0.015–1.5 µM-[1,2-³H]5-HT. Reactions were terminated by dilution with cold 0.15 M-NaCl. 5-HT uptake was estimated by the same filtration assay described above for plasma-membrane vesicles. Calculations of 5-HT uptake were based on the specific radioactivity of [³H]5-HT (5000 d.p.m./pmol) added to the reaction mixtures.

Inhibitors, when present, were added to the buffers before addition of the membrane suspensions. Halothane (Ayerst Laboratories) was added to the reaction buffers from a stock of the same buffer saturated with the anaesthetic and the tubes were capped immediately. Similar results were obtained when the reaction mixtures were equilibrated with water-saturated halothane

vapour. The relative ease of the former method of administration of halothane dictated its use in the experiments described here. The concentration of halothane in the reaction mixtures at the end of incubation was determined using g.l.c. as described previously (Watkins *et al.*, 1983). Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Kinetic constants (K_m and V_{max}) were estimated from a linear transformation of the Michaelis-Menten equation (Hofstee, 1952).

Statistics

Statistical significance of differences between means was determined by Student's *t* test for unpaired data. Significance of differences was established at the $P < 0.05$ level.

RESULTS

Uptake of 5-HT by plasma-membrane vesicles and storage granules

In order to establish the usefulness of subcellular membrane fractions prepared from bovine platelets as a model for studies of 5-HT transport, it was necessary to test their responses to known inhibitors of Na⁺-dependent 5-HT transport and to compare these responses with those observed in platelet membrane preparations from other species. In the presence of an initial Na⁺ gradient of 0.1 M (C_e , 0.1 M; C_i , 0) and K⁺ gradient of 0.1 M (C_e , 0; C_i , 0.1 M), plasma-membrane vesicles from bovine blood platelets sequestered 5-HT to a maximum level of 180–200 pmol/mg of protein when 5-HT was present in the reaction mixture at 0.1 µM (Fig. 1). Maximal uptake was reached by 30 s at 37 °C (Fig. 1a) and by 2 min at

Fig. 1. 5-HT transport into isolated platelet plasma-membrane vesicles

Transport was measured as described in the Experimental section. All additions to the incubation mixture were equilibrated to 37 °C or 18 °C as indicated in the Figure. 5-HT was present at 0.1 µM. ■, $C_e = 0.1$ M-Na⁺, $C_i = 0$; □, $C_e = 0.1$ M-Li⁺, $C_i = 0$; ○, same as closed squares + 1 µM-imipramine.

Fig. 2. ATP-dependent uptake of 5-HT in platelet lysates

Transport was measured as described in the Experimental section, with the additions indicated in the Figure. 5-HT was present at 0.15 μM ; 5 mM-ATP was included in the incubation mixtures containing inhibitors. Points represent means $\pm$ S.E.M. (as indicated by the bars) of 3–11 observations. Where bars are not shown, they did not extend beyond the symbols. ■, + 5 mM-ATP; □, – ATP; ●, + 1 μM -imipramine; ○, + 50 μM -noradrenaline.

18 °C (Fig. 1*b*), followed in both cases by a decline in vesicular content of [^3H]5-HT. No uptake was observed at 0 °C (results not shown). When Li^+ was substituted for Na^+ in the external environment of the vesicles, 5-HT transport was reduced to 10% of maximal uptake. In the presence of the tricyclic antidepressant imipramine, uptake of 5-HT was decreased substantially. Imipramine is well known to inhibit Na^+ -dependent transport of 5-HT into neurons, intact platelets, platelet plasma-membrane vesicles and endothelial cells (Carlsson *et al.*, 1969; Ross & Renyi, 1969; Rudnick, 1977; Watkins *et al.*, 1983).

In Fig. 2, uptake of [^3H]5-HT (0.15 μM) into storage granules was shown to be dependent on the presence of ATP in the medium. Imipramine did not inhibit 5-HT transport into the granules. An excess of the competitive substrate, noradrenaline, prevented 5-HT uptake by storage granules, as expected. Fig. 2 also shows that maximal uptake of 5-HT was probably not reached by 10 min of incubation, at which time the granules had accumulated 70 pmol of 5-HT/mg of lysate protein.

Although there are temporal differences in the characteristics of 5-HT uptake between subcellular fractions of bovine blood platelets and those from other species, rates of transport and responses to inhibitors are similar to those reported by Rudnick (1977) for human platelet plasma-membrane vesicles, by Rudnick *et al.* (1980) for porcine platelet lysates and by Sneddon (1973) and Brase & Loh (1976) for intact platelets.

Fig. 3. Effect of halothane on uptake of 5-HT by plasma-membrane vesicles

In (a) the 5-HT content of vesicles in 0.1 M-LiCl was subtracted from the 5-HT content of vesicles in 0.1 M-NaCl. 5-HT was present at 0.1 μM . In (b), rates of uptake determined during 15 s of incubation are presented as a function of substrate concentration. Halothane was present (○) where indicated at 2 mM (■, control). Points represent the means for 3–11 observations. For the purpose of clarity, standard errors are not shown, but did not exceed 14% of the mean. Kinetic constants were determined as described by Hofstee (1952):

Treatment	K_m (μM)	V_{max} (pmol/min per mg)
Control	0.12 ± 0.02	559 ± 54
Halothane	0.14 ± 0.01	583 ± 30

Fig. 4. Dependence of 5-HT uptake into storage granules on ATP and protein concentration

The concentration of ATP (a) and lysate protein (b) added to the incubation mixture was varied. Standard incubation mixtures included 5 mM-ATP and 0.2–0.3 mg of lysate protein/ml. 5-HT was present in these experiments at $0.15 \mu\text{M}$. Points represent means $\pm$ S.E.M. for four observations.

Fig. 5. Inhibition of ATP-dependent 5-HT uptake by halothane

Halothane concentration was varied between 0.04 and 2.7 mM in the incubation mixture. ATP-dependent rates of uptake were determined between 1 and 5 min of incubation. 5-HT was present at $0.15 \mu\text{M}$. The open symbol represents the rate of uptake in the presence of 0.48 M-thymol, the level of preservative found in 1 mM-halothane. The inhibition by halothane is described by the equation:

$$y = 30x + 10$$

where y = percentage inhibition and x = the concentration of halothane (mM). Points represent means $\pm$ S.E.M. for 3–21 observations.

Effect of halothane on membrane transport of 5-HT

The rate of Na^+ -dependent transport of 5-HT by plasma-membrane vesicles was linear for at least 20 s (Fig. 3a), and was directly dependent on the concentration of protein between 0.09 and 0.27 mg/ml of incubation mixture (results not shown). An incubation time of

Fig. 6 Effects of halothane on kinetic parameters of ATP-dependent 5-HT uptake in platelet lysates

The concentration of 5-HT in the incubation mixture was varied in the presence of increasing levels of halothane, and kinetic constants were determined as described by Hofstee (1952):

Symbol	Treatment	K_m (μM)	V_{max} (pmol/min per mg)
■	Control	0.34 ± 0.03	34.3 ± 1.7
□	0.4 mM-Halothane	$0.50 \pm 0.04^*$	36.7 ± 1.9
○	1.0 mM-Halothane	0.36 ± 0.05	$15.9 \pm 1.4^*$
●	2.0 mM-Halothane	0.30 ± 0.07	$9.4 \pm 1.2^*$

* $P < 0.01$ (halothane-treated versus control).

Points represent means $\pm$ S.E.M. (represented by bars) of three to nine observations. Where bars are not shown, they fall within the symbols.

15 s was used in studies designed to determine the saturability of the uptake process by substrate. The effect of 5-HT concentration on initial velocity of transport is shown in Fig. 3(b). Transformation of the data by the method of Hofstee (1952) provided estimates of the kinetic constants of the reaction (Fig. 3b). These values are similar to those reported by Rudnick (1977) for plasma-membrane vesicles from humans (K_m $0.6 \mu\text{M}$; V_{max} 750 pmol/min per mg of protein) and for intact platelets (K_m 0.1–1.0 μM) reported by various investigators (Born & Gillson, 1959; Sneddon, 1973; Lingjaerde, 1970; Brase & Loh, 1976). Halothane, even at a concentration as high as 2 mM, had no effect on the kinetics of 5-HT uptake into the plasma-membrane vesicles (Fig. 3b).

The rate of uptake of 5-HT into platelet lysate was linear for at least 5 min. For estimation of kinetic parameters, the rate of ATP-dependent uptake between 1 and 5 min was used. In other experiments, the concentrations of ATP and protein in the reaction mixtures were varied (Fig. 4). At 2 mM-ATP and above, the initial rate of transport was directly proportional to protein concentration up to 0.3 mg/ml. Determinations

of initial rates of transport were made by using 5 mM-ATP and 0.2–0.3 mg of lysate protein/ml.

Halothane effected a dose-related decrease in ATP-dependent 5-HT uptake (Fig. 5). Significant inhibition was first observed at 0.2 mM-halothane; nearly complete inhibition occurred at 2.7 mM. Thymol (0.48 μ M), a preservative found in commercial preparations of halothane, had no effect on the rate of transport of 5-HT (Fig. 5). The inhibition by halothane represents a decrease in transport of 12% for each 1% (v/v) increment of the volatile anaesthetic in the gas phase above the incubation mixture.

Uptake of the amine into storage vesicles was also characterized by Michaelis–Menten kinetics (Fig. 6). A low concentration of halothane (0.4 mM) in the reaction mixture raised the apparent K_m slightly. Higher concentrations of the anaesthetic resulted in a progressive decrease in the V_{max} to 27% of control. When the tubes containing the anaesthetic-treated reaction mixtures (at 2 mM-halothane) were uncapped, the concentration of halothane decreased exponentially ($y = -3.87 \times 10^{-5} x^3 + 4.46 \times 10^{-3} x^2 - 0.161 x + 1.968$; $y = \text{mM}$, $x = \text{s}$ after uncapping; calculated on a DEC Rainbow 100+ personal computer). After 30 min at 37 °C, the halothane concentration decreased from 2.0 to 0.10 mM. During this period, inhibition of 5-HT transport by halothane decreased from 66% ($P = 0.01$; $n = 4$) to 24% ($P > 0.05$; results not shown). Thus the effect of the anaesthetic on uptake of monoamine into storage granules was at least partially reversible upon removal of the effector. This suggests that the inhibition observed in these preparations was not due to irreversible damage to the granule membranes.

DISCUSSION

Halothane has been shown to affect a number of plasma-membrane-mediated events in a variety of cell types. Malinconico *et al.* (1983) reported that halothane decreased fast Ca^{2+} uptake in beating-heart cells in culture. Increasing the levels of Ca^{2+} in the culture medium reversed the inhibition, suggesting competitive inhibition of binding or uptake. Halothane's ability to stimulate adenylate cyclase in human platelets and to inhibit platelet aggregation was reported by Walter *et al.* (1980). Platelet phosphodiesterase was not affected. Thus the interplay between halothane and 5-HT may be involved in the increased bleeding times associated with anaesthesia (Daalsgard-Nielsen *et al.*, 1981). In another study using platelets, halothane was reported to inhibit platelet aggregation and to decrease the rate of uptake of 5-HT into intact platelets in a non-competitive manner (Daalsgard-Nielsen & Gormsen, 1980). In those studies, however, uptake values used to calculate kinetic constants did not appear to represent initial rates. The total amount of 5-HT taken up by the platelets during 8 min of incubation was not decreased by the anaesthetic.

In studies using intact lungs, halothane inhibited transport of noradrenaline (Naito & Gillis, 1973; Bahkle & Block, 1976) and 5-HT (Watkins *et al.*, 1983). The anaesthetic also inhibited 5-HT metabolism by lung cells in culture (Watkins *et al.*, 1983). Transport of both monoamines by pulmonary endothelial cells is dependent on temperature and extracellular Na^+ , and exhibits Michaelis–Menten kinetics with an apparent K_m of

0.5–6 μ M (Iwasawa *et al.*, 1973; Rickaby *et al.*, 1982; Catravas & Gillis, 1983; Watkins *et al.*, 1983). These characteristics of monoamine uptake are similar to those reported for transport of 5-HT in platelet membrane preparations (the present study and Rudnick, 1977) and in intact platelets (Born & Gillson, 1959; Lingjaerde, 1970; Sneddon, 1973; Brase & Loh, 1976). In intact lungs, halothane was shown to inhibit specifically 5-HT transport by endothelial plasmalemma in a competitive manner (Watkins *et al.*, 1983). Inhibition of transport was not observed, however, in plasma-membrane vesicles derived from bovine platelets. Whether this is due to a difference in the components of the transport systems or to loss of a control mechanism during the procedure for preparation of the vesicles requires further investigation using plasma-membrane vesicles derived directly from pulmonary endothelial cells.

There is evidence, however, that the uptake system for monoamines in storage granules exhibits universal similarities (Kanner *et al.*, 1979; Rudnick *et al.*, 1980). In at least two tissues, halothane inhibited monoamine transport by storage granules. In addition, Sumikawa *et al.* (1980) reported that noradrenaline uptake by electron-dense granules from adrenal chromaffin cells was decreased by the anaesthetic. In the present study, the inhibition of 5-HT uptake into storage granules was dependent on the concentration of anaesthetic and, at halothane concentrations of 1 mM and above, was non-competitive. At 0.4 mM-halothane, the slight, but statistically significant, increase in the apparent K_m may reflect biphasic alterations in the fluidity of the granular membrane, as was observed by Rosenberg (1979) for synaptic plasma membranes.

It is difficult to extrapolate from the concentrations of halothane used in these experiments to those associated with clinical anaesthesia. Anaesthetic levels of halothane range between 0.5 and 5% of the inspired air in a variety of animals (e.g. MAC = 0.8% in humans; 2.0% in rats). Aqueous solutions in equilibrium with 4% (v/v) halothane contain the anaesthetic at 1.09 mM. The actual concentration in the reaction mixture or in blood depends on the lipid content. Clinical concentrations of volatile anaesthetics in the blood and tissues are approx. 1 mM (Larson *et al.*, 1962). The concentrations of halothane used in these studies correspond to a range from 0.2 to 18%. In these experiments, inhibition of 5-HT uptake was observed at levels of the anaesthetic equivalent to 1.5% and above.

Whereas the transport of 5-HT across plasma membranes is driven by gradients of Na^+ and K^+ , the driving force for movement of the amine across granular membranes appears to be a combination of the pH and electrical potential gradients. Whether halothane inhibits 5-HT transport by disrupting these gradients or by a direct effect on the membrane (Trudell & Cohen, 1975) or on the proteins involved in transport (Eyring *et al.*, 1973) requires further investigation. In addition, studies designed to examine similarities and differences between the transport proteins and membrane environments of the two 5-HT transport systems are required. Characterization of the role of anaesthetics in effecting changes in specific membrane-associated processes may aid in the elucidation of the mechanism of action of anaesthesia and will further our understanding of cellular membrane function.

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