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Lack of selectivity between anesthetic stereoisomers for an inhibitory site which is located on a membrane protein

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Lack of selectivity towards anesthetic stereoisomers is one of the few criteria available for the identification of an anesthetic target site. Until now this criterion has not been tested for anesthetics that directly interact with sensitive membrane proteins which are considered the targets of anesthetic action. In this communication we show that stereoisomers of 2-butanol and 2,4-pentanediol did not differ in their inhibitory potency for a site located on the $Na^+/K^+/Cl^-$ co-transporter protein. This suggests that an inhibition site on a membrane protein can fulfill the criterion of lack of stereoisomer selectivity.

Introduction

Lack of selectivity towards stereoisomers of anesthetics has been proposed as one of the characteristics of the anesthetic target site [1,2] Such a lack in stereoselectivity has been demonstrated for enantiomers of secondary aliphatic alcohols in tadpoles [2] and for enantiomers of the volatile anesthetic halothane in two model systems [3,4] However, significant differences between the anesthetic potency of enantiomers of hexobarbital and *N*-methylcyclobarbital in rats [5] and between enantiomers of ketamine in mice [6] have been observed The stereospecific actions of these compounds were not merely a consequence of differences in biodegradation, but were most likely a result of the interaction with specific receptors [7–9] The barbiturates and ketamine should therefore be excluded from studies on the effect of enantiomers of anesthetics on possible anesthetic target sites

Non-selectivity in enantiomer binding seems to be a strong argument for the lipid bilayer as the target of the anesthetic molecules However, membrane proteins also contain hydrophobic areas with which the anesthetics can interact [10] Whether this interaction is non-selective with respect to enantiomers of anesthetics has not been investigated It has only been observed that enantiomers of halothane induce a conformational change in hemoglobin equally [11]

Recently we have characterized a membrane protein in primary cultures of rat [12] and human astrocytes [13] which simultaneously transports 1 Na^+ ion, 1 K^+ ion and 2 Cl^- ions into the cell The effect of general anesthetics on this membrane function was studied [14] The IC_{50} values for co-transporter inhibition by halothane and the *n*-alkanols (C_1–C_5) were almost identical with the ED_{50} (effective dose 50) values for anesthetizing tadpoles Larger sized anesthetics, however, did not inhibit this function according to their anesthetic potency It seems therefore unlikely that the $Na^+/K^+/Cl^-$ co-transporter is involved in general anesthesia

A further interesting aspect was the mechanism of inhibition of the co-transporter by the two groups of anesthetics The smaller sized anesthetics inhibited by competing with the chloride ion for binding to the co-transporter protein The larger sized anesthetics showed a non-competitive interaction with respect to chloride binding [14] These data suggested that the smaller sized anesthetics directly interact with a hydrophobic area of the co-transport protein The inhibition site on the co-transporter therefore only has a model character for substances that fit this proposed hydrophobic area such as halothane and the lower *n*-alkanols (C_1–C_5) This implies for example that the co-transport system cannot be used to study the absolute cut-off in potency for *n*-alkanols larger than *n*-dodecanol Within the group of substances that fit the proposed hydrophobic area on the co-transport protein we have tested enantiomers of 2-butanol and 2,4-pentanediol in order to find out whether these enantiomers differ in potency

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with respect to $Na^+/K^+/Cl^-$ co-transport inhibition

Materials and Methods

Materials

Rubidium-86 (spec activity 1 96 mCi/mg) was obtained from New England Nuclear Bumetanide was a gift from Leo Pharmaceuticals (Ballerup, Denmark) The stereoisomers of 2-butanol and 2,4-pentanediol were from Aldrich (Steinheim, F R G) All other chemicals were from Merck (Darmstadt, F R G) Cell culture Petri dishes were from Nunc (Roskilde, Denmark)

Cell culture

C6 rat glioma cells (ATTC CCL 107) [15] were maintained in plastic tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 10% fetal calf serum (GIBCO) in a 5% CO_2/95% humidified air atmosphere and were passaged by trypsinization (0 25% trypsin)

Measurement of co-transport activity

Permanent cultures of rat glioma C6 cells on 3 cm Petri dishes were preincubated for 10 min at 36 °C in 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0 4 mM $MgSO_4$, 25 mM glucose and 25 mM Hepes-Tris (pH 7 3), followed by a 4 min incubation in hyperosmolar medium (same medium with 250 mM sorbitol) containing 0 5 μCi/ml $^{86}Rb^+$ The reaction was stopped by rinsing the dishes three times with 2 ml ice-cold PBS The cells were then denatured with 1 ml of cold 5% trichloroacetic acid and centrifuged, and the amount of radioactivity in the supernatant was determined by Cerenkov counting The protein content of each dish was determined using the method of Lowry et al [16] Co-transport activity was measured as the bumetanide (10 μM) sensitive component of the total $^{86}Rb^+$ (as substitute for potassium) uptake The loop diuretic bumetanide is a specific inhibitor of $Na^+/K^+/Cl^-$ co-transport activity [17]

Results and Discussion

Mechanism of the inhibition of the $Na^+/K^+/Cl^-$ co-transporter by 2-butanol and 2,4-pentanediol

The $Na^+/K^+/Cl^-$ co-transporter in rat glioma C6 cells simultaneously transports 1 Na^+ ion, 1 K^+ ion and 2 Cl^- ions from the outside to the inside of the cells [14] Omittance of Na^+ (replaced by cholinium) or Cl^- (replaced by gluconate) from the incubation medium inactivates the co-transport activity [12,13] Since the co-transporter is not very active under steady-state conditions in the C6 glioma cells, we stimulated its activity by performing the experiments in hyperosmolar medium (addition of 250 mM sorbitol [14])

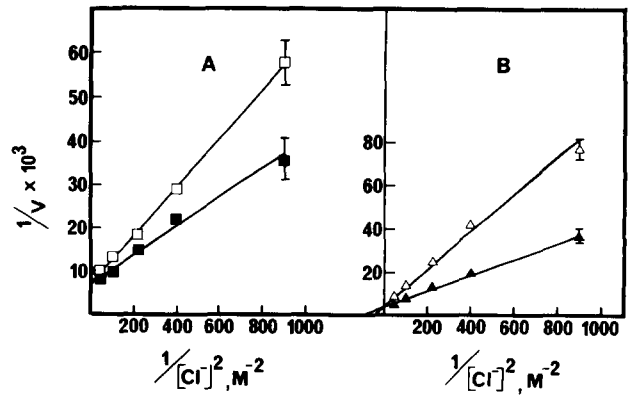

Fig 1 Competitive inhibition of the $Na^+/K^+/Cl^-$ co-transporter by 2,4-pentanediol (A) and 2-butanol (B) All $^{86}Rb^+$ uptake measurements in the presence and absence of anesthetic were performed with and without 10 μM bumetanide to obtain the bumetanide sensitive part of the total $^{86}Rb^+$ uptake Incubation was performed as described in Materials and Methods The chloride ion was replaced by gluconate to obtain incubation media with various Cl^- concentrations keeping the concentration of the other ions constant To decide whether the binding is competitive we constructed a modified Lineweaver-Burk plot for enzyme functions having two substrate sites [17,18] Plotted is the reciprocal of the $^{86}Rb^+$ transport activity ($1/v$) (units for v are nmol/mg protein per ml) against $1/[Cl^-]^2$ in the presence and absence of anesthetics The experiments were performed four times with basically the same result Data are means ± deviation of duplicate measurements of a representative experiment (■, ▲), control, (□), 100 mM 2,4-pentanediol, (△) 20 mM 2-butanol

In order to study the stereoselectivity of the enantiomers of 2-butanol and 2,4-pentanediol we had to verify that these substances interact with the same site of the co-transporter as described for halothane and the lower *n*-alkanols (from methanol through pentanol) [14] Since these latter compounds were shown to inhibit the co-transporter by competing with the chloride ion for binding to the co-transporter we checked whether 2-butanol and 2,4-pentanediol inhibit the co-transporter in a similar manner The results of this experiment are presented in Figs 1A and 1B A modified Lineweaver-Burk presentation of the data was needed because of the presence of two binding sites for the chloride ion [18,19] The reciprocal of the $^{86}Rb^+$ transport activity ($1/v$) is plotted against $1/[Cl^-]^2$ in the presence and absence of the alcohols The intercept with the ordinate gives the V_{max} of the co-transport activity The V_{max} is not affected by 2,4-pentanediol or 2-butanol suggesting a competitive inhibition (Figs 1A and 1B) It seems therefore very likely that these two substances interact with the same area of the co-transport protein as the lower *n*-alkanols and the volatile anesthetic halothane

Effect of stereoisomers of 2-butanol and 2,4-pentanediol on the activity of the $Na^+/K^+/Cl^-$ co-transporter

Stereoselective action of enantiomers of anesthetics on the $Na^+/K^+/Cl^-$ co-transporter would imply that the anesthetics interact at three points with a chiral center in its target molecule Non-selectivity will be

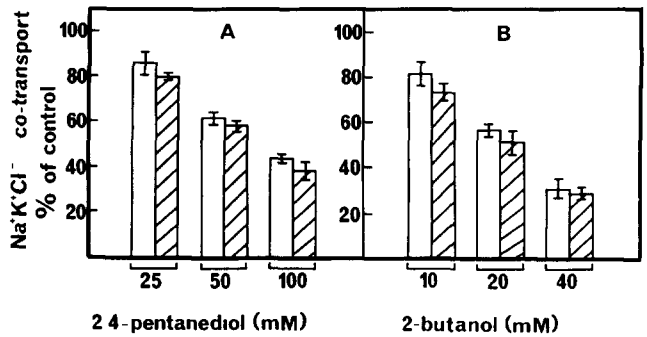

Fig 2 Inhibition of the $Na^+/K^+/Cl^-$ co-transporter by enantiomers of 2,4-pentanediol (A) and by enantiomers of 2-butanol (B) Incubations were performed as described in Materials and Methods The activity of the $Na^+/K^+/Cl^-$ co-transporter in the absence of alkanol was taken as 100% and the activity in the presence of alkanol was calculated as percent of the uninhibited activity Data are means ± S E of four experiments performed in triplicate □, (−)-enantiomer, ▨, (+)-enantiomer

observed if the binding site does not have a chiral center, or if binding involves only one or two points of attachment Since the proposed hydrophobic pocket on the $Na^+/K^+/Cl^-$ co-transport protein is of limited size (pentanol is the largest molecule that fits [14]), only stereoisomers of small anesthetic molecules were tested Fig 2 shows the inhibitory effect of enantiomers of 2-butanol and 2,4-pentanediol on the $Na^+/K^+/Cl^-$ co-transporter For 2-butanol an IC_{50} value of 23 mM was found which is close to the reported ED_{50} value of 17 mM for tadpoles [2] For 2,4-pentanediol we observed an IC_{50} value of approx 75 mM for co-transporter inhibition Unfortunately, no anesthetic potency data are available for this substance On the basis of the membrane buffer partition coefficient [20] one would expect an anesthetic potency for tadpoles of 150–200 mM Fig 2 further shows that the enantiomers of 2-butanol and 2,4-pentanediol did not significantly differ in their inhibition of the $Na^+/K^+/Cl^-$ co-transporter This is to our knowledge the first demonstration that enantiomers of anesthetics are equally potent for an inhibitory site which is located on a membrane protein The results presented here and previously [14] show that the $Na^+/K^+/Cl^-$ co-transporter has model character for the proposed anesthetic target site(s) The lower *n*-alkanols and halothane inhibit the co-transporter according to their lipid solubility [14] showing that a membrane protein can provide an lipid-like environment for the anesthetics The lack of stereoselectivity is further an indication that the anesthetics interact in a rather unspecific manner with the co-transporter The results presented here and previously [14] suggest that general anesthesia can be caused by direct interaction of general anesthetics with hydrophobic areas of membrane proteins

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