Biochimica et Biophysica Acta, 1023 (1990) 436–440
Elsevier

BBAMEM 74808

Lipid solubility is not the sole criterion for the inhibition of a Ca^{2+}-activated K^+ channel by alcohols

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(Received 27 October 1989)

Key words: Calcium ion activated potassium ion channel; Potassium ion channel; *n*-Alkanol; Glioma cell; Charybdotoxin; Calcium ionophore; Ionophore A23187

The effect of a homologous series of *n*-alkanols (C_2–C_8) on the $^{86}Rb^+$ influx through charybdotoxin sensitive Ca^{2+}-activated K^+ channels of rat glioma C6 cells was investigated. The lipid solubility of the *n*-alkanols was not the sole determinant of the inhibitory potency of these substances for ion flux inhibition. 1-Hexanol for example was about 8-times less potent than one would expect on the basis of its lipid solubility. The introduction of a second OH-group in this molecule (giving 1,6-hexanediol) or a structural shift in the OH-group of 1-hexanol from position 1 to 3 strongly increased the potency of the alcohol. The above data cannot be explained by a pure lipid site of action of the alcohols. Therefore it seems likely that direct effects on protein are involved in the inhibitory action of some of the alcohols.

Introduction

The mechanism by which general anesthetics cause anesthesia is largely unknown. Due to the excellent correlation between lipid solubility and anesthetic potency research has mainly been focused on the disturbance of the physical properties of the lipid bilayer by the anesthetics [1–3]. Recent evidence, however, suggests that general anesthetics directly interfere with hydrophobic regions of proteins [4–6]. Especially membrane proteins which span the lipid bilayer are known to contain hydrophobic domains [7] with which anesthetics can interact. Unfortunately the activity of such proteins critically depends on their lipid environment. However, a detailed study of the effect of anesthetics on such functions can provide indirect evidence for an interaction of anesthetics with hydrophobic regions of such proteins [5].

Recently we have characterized a Ca^{2+}-activated K^+ channel in rat glioma C6 cells [8]. The exact function of this channel is still unknown, but its properties suggest that it could be involved in the regulation of the potassium concentration in the central nervous system [8]. The ion flux through this K^+ channel was inhibited by clinical concentrations of the volatile anesthetics halothane, enflurane, isoflurane and methoxyflurane [9]. Interestingly the IC_{50} values for channel activity were identical with the ED_{50} values for anesthesia. This prompted us to study this K^+ channel in more detail.

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Materials and Methods

Materials

Rubidium 86 (spec. act. 1.37 mCi/mg) was obtained from New England Nuclear, *Leiurus quinquestriatus* scorpion venom, ionophore A23187 and the *n*-alkanols were from Sigma (St. Louis MO, U.S.A.). Ionomycin was from Calbiochem (Frankfurt, F.R.G.). The alkanediols were from Aldrich Chemie (Steinheim, F.R.G.). Charybdotoxin was a gift of Prof. Chris Miller, Brandeis University, Waltham, MA (U.S.A.). All other chemicals were from Merck (Darmstadt).

Cell culture

C6 rat glioma cells (ATTC CCL 107) [10] were maintained in plastic tissue culture flasks (Nunc, Roskilde, Denmark) in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) with 10% (v/v) fetal calf serum (GIBCO) in a 5% CO_2/95% humidified air atmosphere and were passaged by trypsinization (0.25% trypsin).

$^{86}Rb^+$ influx experiments

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) (buffer A) followed by a 5 min incubation in buffer A containing 0.25 μCi $^{86}Rb^+$ and 2 μM of Ca^{2+}-ionophore. To determine the specific $^{86}Rb^+$ flux through the Ca^{2+}-activated K^+ channels, experiments were performed in the presence and absence of 50 μg/ml of crude *Leiurus quinquestriatus* venom and the toxin sensitive part of the total $^{86}Rb^+$ influx was taken as specific flux through the K^+ channels. After incubation the cells were quickly rinsed three times with ice-cold phosphate-buffered saline. After the last wash the cells were scraped off in the presence of 1 ml 5% trichloroacetic acid. After a low-speed centrifugation the radioactivity in the supernatant was measured by Cerenkov radiation in a scintillation counter. The sediment was dissolved in 1 M NaOH and the protein content was determined by the method of Lowry et al. [11].

Fura-2 loading and Ca^{2+} measurements

Rat glioma C6 cells were washed by centrifugation and resuspended in buffer A (10^7 cells/ml) mixed with 0.5% volume of 1 mM fura-2/AM in dimethylsulfoxide. After incubation at 15 °C for 1 h, the cells were washed twice and resuspended in buffer A. Ca^{2+} concentrations were measured as described previously [12].

Results

Measurement of the ion flux through Ca^{2+}-activated K^+ channels

Experiments were performed with monolayers of rat glioma C6 cells on Petri dishes. Ca^{2+}-activated K^+ channels were stimulated by the addition of a Ca^{2+}-ionophore (A23187 or ionomycin) to the incubation medium [8]. Activated channels mediate both an influx and efflux of $^{86}Rb^+$ (as substitute for potassium). Both fluxes appeared to have the same sensitivity towards the volatile anesthetic halothane [9]. For reasons of convenience we have chosen the $^{86}Rb^+$ influx which is linear for about 4 min [8] to study the effect of a homologous series of *n*-alkanols on the Ca^{2+}-activated K^+ channel.

Fig. 1A shows the result of a typical experiment in which the effect of ethanol on the $^{86}Rb^+$ influx was studied. The scorpion toxin sensitive component of the total $^{86}Rb^+$ influx was considered as the specific influx of $^{86}Rb^+$ through the Ca^{2+}-activated K^+ channels. Similar results were obtained when a purified preparation of charybdotoxin was used. The scorpion toxin sensitive $^{86}Rb^+$ fluxes in the presence of ethanol were calculated as % of the control influx. From such experiments an IC_{50} for channel inhibition was calculated (Fig. 1B, Table I).

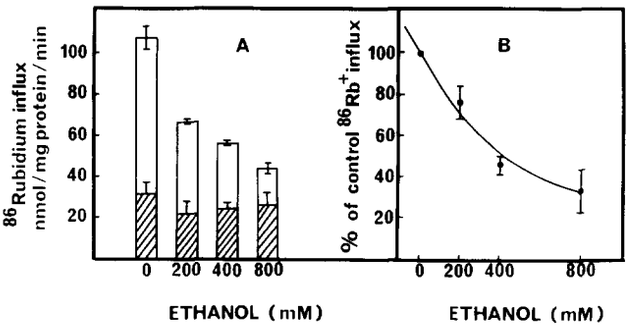

Fig. 1. Effect of ethanol on the $^{86}Rb^+$ influx into rat glioma C6 cells after addition of 2 μM A23187 to the incubation medium. (A) Monolayers of the cells on 3 cm Petri dishes were preincubated for 10 min in Buffer A (see Methods). After preincubation the cells were changed to buffer A containing 2 μM A23187 and 0.25 μCi/ml $^{86}Rb^+$. One set of dishes was incubated for 1 min at 36 °C in the presence and the other set of dishes in the absence of 50 μg/ml scorpion venom. The incubations were stopped by rinsing the dishes three times with ice-cold PBS. □, without scorpion venom; ▨, with 50 μg/ml *Leiurus quinquestriatus* scorpion venom. (B) The scorpion toxin sensitive part of the total $^{86}Rb^+$ uptake in the absence of ethanol was taken as 100% and the other fluxes calculated as % of this uninhibited influx. Data are means ± S.E. of three experiments performed in duplicate.

Inhibition of the Ca^{2+}-activated K^+ channels by 1-alkanols

By testing a homologous series of inhibitory substances one can obtain more information about the inhibitory site than by testing unrelated inhibitors. For each additional C-atom in the *n*-alkanol series lipid solubility and anesthetic potency increase with approximately a factor 3. This allows us to study how good the inhibitory potencies of the *n*-alkanols for the K^+ channel correlate with their lipid solubility. Any anomalities in the correlation are most likely related to special features of the inhibitory site. A leveling off of the inhibitory potency of the *n*-alkanols for the function under investigation can give an approximation of the size of the inhibitory site as we have shown previously [5].

We first ruled out the possibility that the *n*-alkanols interfered with the ionophore induced rise in the cytoplasmic Ca^{2+} concentration. Using fura-2 loaded rat glioma C6 cells we did not find an impairment of the ionomycin induced Ca^{2+} rise in the presence of the alkanols (not shown). Furthermore, the Ca^{2+}-activated K^+ channel in our glioma cells was not significantly affected by increases in cAMP, cGMP or protein kinase C activity (data not shown). We can therefore exclude the possibility that the alkanols have stimulatory or inhibitory effects on the channel via these second messenger systems. In a manner similar to the one presented in Fig. 1 we have tested other *n*-alkanols. Methanol was omitted from this series since at high concentrations of methanol, needed to estimate an IC_{50}, the cells did not stay attached to the Petri dishes. The

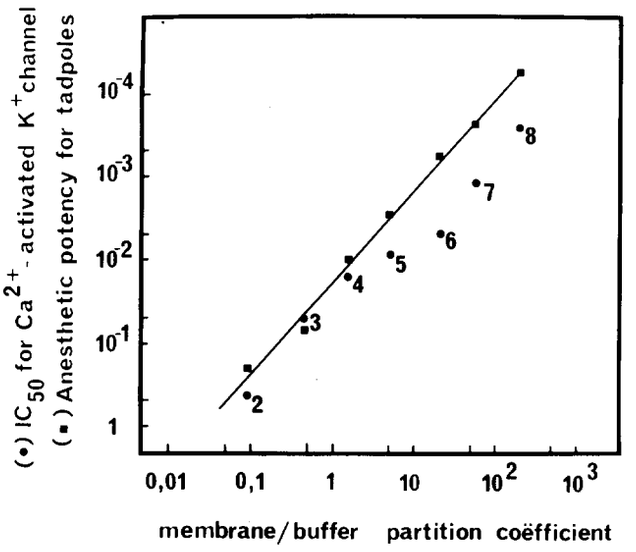

Fig. 2. Relation between the IC_{50} values for the Ca^{2+}-activated K^+ channel and the ED_{50} values for anesthetizing tadpoles for a homologous series of *n*-alkanols. The IC_{50} values for Ca^{2+}-activated K^+ channel inhibition were obtained in experiments analogous to the one presented in Fig. 1A. The ED_{50} values (loss of righting reflex in 50% of the tadpoles) were from Ref. 13. The membrane/buffer partition coefficients were obtained by dividing the octanol-water partition coefficients by a factor 5 (Ref. 14). The *n*-alkanols are indicated by their number of C-atoms (2 = ethanol, 8 = octanol). The values on the ordinate are in mol/liter.

IC_{50} of methanol exceeded at least 5 M.

In order to correlate the inhibitory potency of the *n*-alkanols with lipid solubility we plotted the IC_{50} for Ca^{2+}-activated K^+ channel inhibition of the individual *n*-alkanols against their membrane/buffer partition coefficient (which is a measure of their lipid solubility) (Fig. 2)). In addition we plotted in Fig. 2 the anesthetic potency of the *n*-alkanols for tadpoles (Effective $Dose_{50}$, ED_{50}) against their membrane/buffer partition coefficients (data derived from Alifimoff et al. [13]). The latter points are all on or very close to a straight line, indicating the excellent correlation of anesthetic potency with lipid solubility. Using this line as reference we can now conveniently compare for each of the *n*-alkanols the IC_{50} for Ca^{2+}-activated K^+ channel inhibition with the reported anesthetic potency for tadpoles. It is clear from Fig. 2 that the IC_{50}'s for Ca^{2+}-activated K^+ channel inhibition of ethanol, propanol and butanol are rather close to the reported anesthetic potency of these substances. From pentanol upwards the IC_{50} for Ca^{2+}-activated K^+ channel inhibition is much higher than the reported anesthetic potency for tadpoles. For 1-hexanol this difference is approximately a factor 8 and for 1-heptanol a factor 5. This suggests that other factors beside lipid solubility determine the inhibitory potency of these substances for the Ca^{2+}-activated K^+ channel.

Structure activity relationship for inhibition of Ca^{2+}-activated K^+ channels by alcohols

In view of the above data it was of interest to study modified alcohols and compare their inhibitory activity with those of the corresponding *n*-alkanols.

Table I shows the IC_{50} values of some alkanediols for Ca^{2+}-activated K^+ channel inhibition. Since no anesthetic potency data of the alkanediols for tadpoles are available in the literature we calculated the membrane concentration of the anesthetics in order to compare the potency of the different modified alcohols with their parent (*n*-alkanol) compound. This procedure is based on the assumption that the membrane concentration of the anesthetics is the parameter which is directly related with Ca^{2+}-activated K^+ channel inhibition. The concentration of the anesthetics in the membrane compartment can be calculated from the IC_{50} of the alcohols using the membrane buffer partition coefficients from the literature (Table I). Correction for the quantity of anesthetic dissolved in the membrane compartment was not necessary due to the small volume of this compartment and the low membrane buffer partition coefficients of the alcohols. Using the membrane concentration of the alcohols we can now directly compare the effect of structural changes of the alcohol molecule on its membranous IC_{50}. The introduction of an additional OH-group in *n*-hexanol (giving 1,6-hexanediol) reduces

TABLE I

Calculation of the anesthetic concentration in the membrane compartment of C6 cells at the alcohol concentration which inhibit the Ca^{2+}-activated K^+ channel by 50%

The IC_{50} values of the individual alcohols for the Ca^{2+}-activated K^+ channel were obtained in experiments analogous to the one in Fig. 1. The membrane concentration of the individual alcohols (IC_{50} membranous) was calculated by multiplying the membrane/buffer partition coefficient (*P* m/b) (Ref. 14) with the aqueous IC_{50}. The membrane buffer partition coefficient for 1,4-butanediol was calculated using the formula and data of Ref. 24.

	IC_{50} aqueous (mM)	*P* (m/b)	IC_{50} membranous (mM)
1,4-Butanediol	550	0.104	57.2
1,5-Pentanediol	230	0.303	69.7
1,6-Hexanediol	32	0.957	30.6
1,7-Heptanediol	30	3.03	90.9
Ethanol	410	0.096	39.4
n-Propanol	56	0.438	24.5
n-Butanol	17	1.52	25.8
n-Pentanol	9	5.02	45.2
n-Hexanol	5	21.4	107.0
n-Heptanol	1.2	51.4	61.7
2-Propanol	80	0.264	21.1
2-Butanol	15	0.815	12.2
3-Pentanol	12	4.69	56.3
3-Hexanol	3.2	8.73	27.9

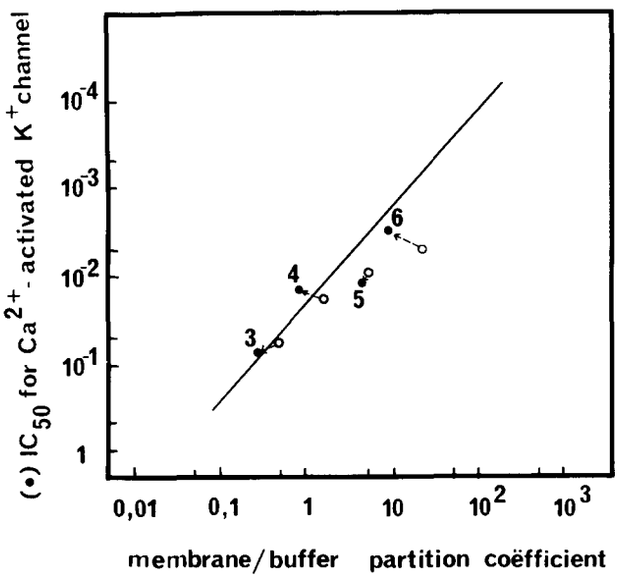

Fig. 3. Effect of changing the OH-group of some *n*-alkanols from the terminal to a 2- or 3-position on the inhibitory potency of the alcohols for the Ca^{2+}-activated K^+ channel. The solid line indicates the approximate concentrations of the substances that induce a loss of righting reflex in 50% of the tadpoles (ED_{50}). The alcohols are indicated by numbers (3 = 2-propanol; 4 = 2-butanol; 5 = 3-pentanol and 6 = 3-hexanol). The arrows indicate the shifts from the *n*-alkanol position to the modified alcohol position. The values on the ordinate are in mol/liter.

the membranous IC_{50} about 3.5-times which means that 1,6-hexanediol is 3.5-times more potent than *n*-hexanol. On the other hand 1,7-heptanediol and 1,5-pentanediol are less potent than the parent molecules (Table I).

We further tested the effect of changing the OH-group of some *n*-alkanols to a 2- or 3-position inside the molecule. Fig. 3 shows the result of such changes for propanol, butanol, pentanol and hexanol. For propanol (OH from 1 → 2) and pentanol (OH from 1 → 3) the changes in position do not greatly affect the potency for channel inhibition (see Table I). However, similar changes in butanol (OH from 1 → 2) and hexanol (OH from 1 → 3) increase the potency of the alcohol with a factor 2. For hexanol (OH from 1 → 3) the increase in potency is almost a factor 4. These data are again an indication that for some of the tested alcohols other factors beside lipid solubility determine the inhibitory potency for the Ca^{2+}-activated K^+ channel.

Discussion

In this communication we have shown that lipid solubility of a homologous series of *n*-alkanols is not the sole determinant of the potency of these substances for Ca^{2+}-activated K^+ channel inhibition (Figs. 2 and 3). By introducing an additional OH-group (Table I) or by changing the position of the OH-group from the 1- to other positions within the molecule (Fig. 3) a drastic increase in the potency of the alcohols was obtained. These results indicate that other factors beside lipid solubility determine the inhibitory potency of at least some of the alcohols for the Ca^{2+}-activated K^+ channel.

The data in this communication do not reveal whether the observed inhibition of the ion flux is the result of the interaction of the alcohols with a single site or whether it is a combination of different effects. Although we have ruled out that the cAMP, cGMP and protein kinase C second messenger systems affect the ion flux through the channel, other possibilities of regulation such as effects of the alcohols on membrane potential or Ca^{2+}-calmodulin activated protein kinase are not excluded. The data further do not reveal how the alcohols affect the ion flux through the channels. The inhibition could be the result of a decrease in the driving force (due to membrane depolarization), a decrease in the channel open probability, a decrease in the number of active channels or a decrease in single-channel conductance.

A possible explanation for the aberration of the inhibitory potency from lipid solubility could be that the alcohols directly interact with protein. Structural features of the inhibitory site would in this case modulate the inhibitory potency of the alcohols. Such a structural selectivity in inhibitory potency has been predicted by Richards et al. [15] and Janoff et al. [16] for anesthetics that interact with protein.

In favor of such a interpretation are also data from the literature. General anesthetics directly interact with the luciferase protein and the inhibitory potency of the anesthetics for enzyme inhibition correlates very well with the anesthetic potency of the substances [17]. However, close inspection of the *n*-alkanol series shows that hexanol and heptanol have about equal potency with respect to the luciferase inhibition [18]. Their anesthetic potencies, however, differ by a factor 2.5 [13]. This looks quite similar to what we have observed in this report.

The modulation of the inhibitory potency is even more stringent when the inhibitory site is of limited size so that only small anesthetic molecules can bind. Such a situation exists for the inhibition of β-lactoglobulin by *n*-alkanes [18] and the inhibition of the $Na^+/K^+/Cl^-$ co-transporter in glial cells by *n*-alkanols [5]. The observed inhibitory data cannot be simply explained by a change in the physical properties of the lipid bilayer which in turn affects the activity of the Ca^{2+}-activated K^+ channel. Other possibilities such as effects of the alcohols on both lipid bilayer and protein can, however, not be excluded.

The fact that general anesthetics can inhibit the function of proteins by competing with the binding of endogenous ligands was first shown for the water-soluble luciferase enzyme [4] where the anesthetics compete

with luciferin binding. A similar observation has recently been made for an integral membrane protein, the $Na^+/K^+/Cl^-$ co-transporter in glial cells [5]. Anesthetic concentrations of halothane and the lower *n*-alkanols inhibit this membrane function by competing with the chloride ion for binding to the protein. This competitive inhibition also suggests a direct interaction of the anesthetics with protein.

Other experimental evidence also supports a protein site of action of general anesthetics. The free energy change for transferring a methylene group of the *n*-alcohol to the more hydrophobic environment of cytochrome oxidase was consistent with the partitioning of the *n*-alcohols in an octanol-like environment on the enzyme or the protein/phospholipid interface [19].

The picture which is emerging is that clinical concentrations of general anesthetics affect many sensitive functions [20–22]. Therefore, the anesthetic target site might consist of more than one sensitive membrane protein and anesthesia might be caused by an impairment of a number of sensitive membrane functions. The inhibited functions may vary between different groups of anesthetics or the same function may be inhibited at different sites by different groups of anesthetics [21,23]. The final result of these various perturbations will be the anesthetic state.

Acknowledgements

We thank Mrs. Helga Sennefelder and Ms. Ulli Wagner for skillful assistance and Mrs. Christel Stoppe for typing. We further thank Prof. Chris Miller for his kind gift of charybdotoxin. This project was supported by a grant from the Wilhelm Sander-Stiftung, Neustadt/Donau, F.R.G.

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