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Volatile anesthetics inhibit the ion flux through Ca^{2+}-activated K^+ channels of rat glioma C6 cells

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Ca^{2+}-activated K^+ channels in rat glioma C6 cells were investigated using monolayers of these cells in petri dishes. The ion flux through the channels was studied with $^{86}Rb^+$ after addition of a Ca^{2+}-ionophore to the incubation medium. Both the influx and efflux of $^{86}Rb^+$ through these Ca^{2+}-activated K^+ channels were inhibited by the general anesthetic halothane (at clinical concentrations). Other volatile anesthetics such as isoflurane, enflurane and methoxyflurane also inhibited the Ca^{2+}-activated K^+ channels at clinical concentrations. Inhibition of these channels by general anesthetics could have profound effects on signal transmission in the brain.

Introduction

Glial cells appear to play a key role in regulation of the K^+ concentrations in the extracellular space surrounding the neurons [1–3]. It is suggested that glial cells cope with local increases in K^+ concentration via a removal mechanism which has been termed spatial buffering [4–6]. The prerequisites for spatial buffering are a high glial membrane selectivity and permeability for K^+ ions and an electrical coupling between glial cells. K^+ depolarization of such glial cells due to active neurons will lead to a local inflow of K^+ ions at the site of depolarization and an outflow of K^+ ions at membrane sites with more negative potential. Spatial buffering therefore redistributes local increases in potassium using the specialized properties of the glial syncytium. Recent evidence [7–9] has suggested that spatial buffering is triggered by depolarization of glial cells which leads to an opening of voltage-dependent Ca^{2+} channels and a subsequent rise of the Ca^{2+} concentration which in turn activates a special class of K^+ channels (Ca^{2+}-activated K^+ channels).

Since the maintenance of potassium concentrations within physiological levels in the brain is important for proper signal transmission, we have investigated whether volatile anesthetics interfere with spatial buffering by inhibition of the ion flux though Ca^{2+}-activated K^+ channels.. The results of this study show that volatile anesthetics in clinical concentrations interfere with the ion flux through these channels.

Materials and Methods

Materials

Halothane was obtained from Hoechst (Frankfurt, F.R.G.). All other anesthetics were from Abbott (Wiesbaden, F.R.G.). Rubidium-86 (spec. act. 1.37 mCi/mg) was obtained from New England Nuclear, *Leiurus quinquestriatus* scorpion venom and A23187 were from Sigma (St. Louis, MO, U.S.A.) and ionomycin and fura-2-acetoxymethyl ester (fura-2/AM) were from Calbiochem (Frankfurt, 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, F.R.G.).

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 10% CO_2/90% humidified air atmosphere and were passaged by trypsinization (0.25% trypsin).

$^{86}Rb^+$ influx and efflux experiments

Permanent cultures of rat glioma C6 cells on 3 cm petri dishes were preincubated for 10 min at 36 °C in

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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 an incubation in buffer A containing 0.25 μCi $^{86}Rb^+$ and 2 μM of Ca^{2+}-ionophore for the indicated period. 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].

For efflux measurements rat glioma C6 cells were pre-incubated for 1 h in buffer A containing 0.25 μCi $^{86}Rb^+$. The cells were then quickly rinsed four times with 2 ml buffer A. Efflux was then measured for 1 min in 1 ml buffer A containing 2 μM of Ca^{2+} ionophore in the presence or absence of 50 μg/ml scorpion venom. The $^{86}Rb^+$ efflux data were corrected for differences in $^{86}Rb^+$ loading of each petri dish.

$^{86}Rb^+$ influx measurements in the presence of volatile anesthetics

For these experiments the culture dishes were placed in a plastic gas flow chamber at 36°C under a flow of air to which the desired anesthetic concentration was administered with special vaporizers (Drägerwerk, Lübeck). Since volatile anesthetics diffuse very slowly into H_2O containing media, we pre-equilibrated the uptake buffer for 20 min with an air stream containing the desired anesthetic concentration. The diffusion of the anesthetic in the uptake buffer was monitored with a ga chromatograph as described previously [12].

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

Rat glioma C6 cells were washed by centrifugation and resuspended in buffer A ($1 \cdot 10^7$ 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. Measurements of Ca^{2+} was as described previously [13].

Results

Characteristics of the $^{86}Rb^+$ ion flux through Ca^{2+} activated K^+ channels

Monolayers of rat glioma C6 cells on 3 cm petri dishes were treated with Ca^{2+}-ionophores (A23187 or ionomycin) to raise the intracellular Ca^{2+} concentration and to activate Ca^{2+}-activated K^+ channels. The Ca^{2+}-activated $^{86}Rb^+$ (used here as a substitute for potassium) uptake was not affected by apamin up to a concentration of 2 μM, however, the ion flux was inhibited by crude *Leiurus quinquestriatus* scorpion venom (IC_{50} = 2.5 μg/ml) (Table I). Complete inhibition was obtained by a concentration of 50 μg/ml of the latter toxin. The active component in the *Leiurus* venom is probably charybdotoxin since the Ca^{2+}-activated K^+ channel wa also inhibited by a purified preparation of this toxin (Table I). The IC_{50} of the inhibition by charybdotoxin was 1.6 nM (not shown). The scorpion toxin sensitive part of the total $^{86}Rb^+$ uptake was considered to represent the specific $^{86}Rb^+$ flux through Ca^{2+}-activated K^+ channels. Fig. 1A shows the activation of the Ca^{2+}-activated K^+ channels by the addition of ionophore to the incubation medium. Half maximal activation was obtained with 0.4 μM A23187. For the Ca^{2+}-ionophore ionomycin, half maximal activation was at 0.05 μM (not shown). Using fura-2 loaded rat glioma cells, we estimated that half maximal activation of the Ca^{2+}-activated K^+ channels was obtained at a cytoplasmic Ca^{2+} concentration of 400 nM (not shown). Fig. 1B shows the time-course of $^{86}Rb^+$ uptake into the glioma cells via Ca^{2+}-activated K^+ channels. $^{86}Rb^+$ uptake is approximately linear for 1 min and then levels off.

Effect of volatile anesthetics on the $^{86}Rb^+$ ion flux through Ca^{2+}-activated K^+ channels

Fig. 2A shows the effect of increasing concentrations of the general anesthetic halothane on the $^{86}Rb^+$ influx through Ca^{2+}-activated K^+ channels. Clinical concentrations of the anesthetic (0.5–2 vol% in air corresponding to 0.26–1.04 mM of the anesthetic in buffer

TABLE I

Effect of various toxins on the $^{86}Rb^+$ uptake into rat glioma C6 cells in the presence or absence of 2 μM A23187

Monolayers of glioma cells on 3 cm petri dishes were preincubated for 10 min in buffer A (see Materials and Methods). After preincubation the cells were changed to buffer A containing 0.25 μCi/ml $^{86}Rb^+$ in the presence or absence of 2 μM A23187 and various toxins as indicated in the table. Incubation was for 4 min at 36°C. The incubations were stopped by rinsing the cells with ice-cold PBS. The measurement of the $^{86}Rb^+$ uptake into the cells was as described in Methods. Data are means ± S.D. of quadruplicate determinations of a representative experiment. The average scorpion toxin-sensitive $^{86}Rb^+$ uptake was 195.4 ± 19.3 nmol/mg protein per 4 min (n = 15).

Additions	$^{86}Rb^+$ uptake (nmol/mg protein per 4 min)	
	− 2 μM A23187	+ 2 μM A23187
None	70.4 ± 14.1	287 ± 57.4
50 μg/ml scorpion venom	57.3 ± 5.2	87.8 ± 17.6
25 nM charybdotoxin	57.0 ± 8.8	106.5 ± 21.3
2.5 μM apamin	93.9 ± 8.9	265.8 ± 53.2

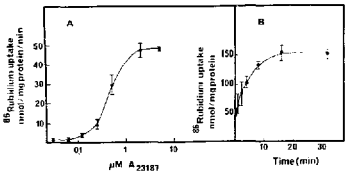

Fig. 1. Effect of the ionophore A23187 on the $^{86}Rb^+$ uptake through Ca^{2+}-activated K^+ channels (A) and the time-dependent uptake of $^{86}Rb^+$ after addition of 2 μM A23187 (B). After a preincubation of 10 min in buffer A the cells were changed to buffer A containing the indicated concentrations of ionophore, 0.25 μCi/ml $^{86}Rb^+$ with or without 50 μg/ml *Leiurus quinquestriatus* scorpion venom. For the experiment in A the rat glioma cells were incubated with A23187 for 4 min at 36 °C and in B for the indicated times. The incubation was stopped by rinsing with ice-cold phosphate-buffered saline as described in Materials and Methods. The scorpion toxin-sensitive part of the total $^{86}Rb^+$ uptake is plotted in the figures. Data are means ± S.D. of triplicate determinations of a representative experiment, for Fig. 1A and are means ± S.E. for Fig. 1B ($n = 3$).

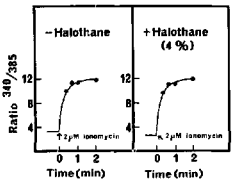

Fig. 3. Influence of 4% halothane on the 2 μM ionomycin-induced rise of the cytoplasmic Ca^{2+} concentration of rat glioma C6 cells. C6 cells at a concentration of 10^7 cells/ml in DMEM (-serum) were mixed with 0.5% volume of 1 mM fura-2/AM in DMSO. Measurement of Ca^{2+} after addition of 2 μM ionomycin was carried out at 36 °C with a modified 4-8202 Aminco Bowman spectrofluorimeter (Silver Spring, MD, U.S.A.) fitted with a magnetic stirrer and a thermostated cuvette holder. Intracellular fura-2 fluorescence was measured at two quickly alternating excitation wavelengths (340/385 nm) and continuously recorded at 500 nm. Ca^{2+} values were calculated using the 340/385 nm ratio of fluorescence intensities as described previously [13]. Shown in the figure is a representative set of data out of five independent experiments.

A) significantly inhibited the $^{86}Rb^+$ influx. At the halothane concentration which anesthetizes 50% of rats (1.05 vol%), we observed a 50% inhibition of the $^{86}Rb^+$ influx. Almost identical results (48 ± 3% inhibition by 1% halothane ($n = 4$)) were obtained in the presence of 50 nM charybdotoxin. Replacement of the halothane containing incubation medium by a medium without halothane resulted in an almost complete return of the original channel transport activity (not shown). Fig. 2B shows that the efflux through the K^+ channels is also inhibited in a dose-dependent manner by the anesthetic.

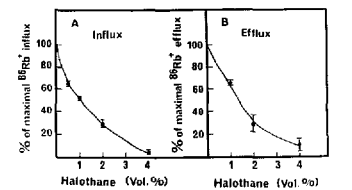

Fig. 2. Effect of the general anesthetic halothane on $^{86}Rb^+$ influx (A) and efflux (B) through Ca^{2+}-activated K^+ channels of rat glioma C6 cells. Influx and efflux were measured for 1 min at 36 °C in the presence or absence of the volatile anesthetic. Measurements were performed in the presence or absence of 50 μg/ml crude *Leiurus quinquestriatus* venom in order to determine the specific flux through Ca^{2+} activated K^+ channels. The uptake measurements in the presence of halothane were performed as described in Materials and Methods. Data are means ± S.E. of three separate experiments performed in quadruplicate. The average scorpion toxin sensitive $^{86}Rb^+$ influx in the absence of halothane was 57.3 ± 7.8 nmol/mg protein per min ($n = 3$).

Comparison of Figs. 2A and 2B show that influx and efflux through Ca^{2+}-activated K^+ channels are about equally sensitive to halothane.

To exclude the possibility that ionophore-induced Ca^{2+} transport was affected by the anesthetic we studied the rise in the Ca^{2+} concentration with the Ca^{2+}-indicator fura-2 in the presence and absence of 4% halothane. Measurement was only for 2 min in order to prevent photobleaching of fura-2. The rise in the 340/385 ratio (Fig. 3) was not affected by the anesthetic, indicating that halothane does not interfere with the ionophore-induced Ca^{2+} rise.

We further investigated whether other volatile anesthetics (enflurane, isoflurane and methoxyflurane) also inhibit the Ca^{2+}-activated K^+ channels in these glioma cells. All three volatile anesthetics inhibited these channels in a dose dependent manner (Fig. 4). Similar results were obtained in the presence of 50 nM charybdotoxin (37 ± 4.1% inhibition by 2% isoflurane ($n = 4$)). The inhibitory potency (IC_{50}) of the anesthetics for the Ca^{2+}-activated K^+ channels also correlated very well ($r = 0.99$) with the concentrations of the anesthetics that anesthetize 50% of test animals (Fig. 5). These observations suggest that charybdotoxin-sensitive Ca^{2+}-activated K^+ channels in glial cells are a target site for volatile anesthetics. Whether inhibition of these K^+ channels by volatile anesthetics plays a role in anesthesia remains to be investigated.

Discussion

The data in this study provide the first evidence for the presence of charybdotoxin sensitive Ca^{2+}-activated

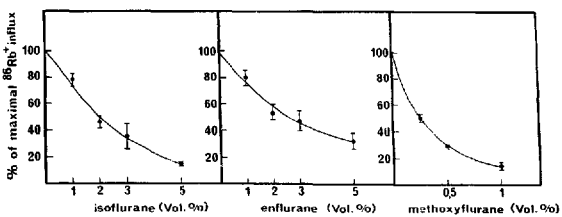

Fig. 4. Inhibition of Ca^{2+}-activated K^+ channels in rat glioma C6 cells by the volatile anesthetics isoflurane, enflurane and methoxyflurane. Influx was measured for 1 min at 36 °C in the presence and absence of the volatile anesthetics. The scorpion toxin-sensitive part of the total $^{86}Rb^+$ uptake was considered as specific influx through the Ca^{2+}-activated K^+ channels. The specific influx in the absence of anesthetic was taken as 100% and the other fluxes were calculated as % of this maximum influx. All other procedures were as described in Materials and Methods. Data are means ± S.E. of three separate experiments performed in triplicate. The average scorpion toxin sensitive $^{86}Rb^+$ influx in the absence of isoflurane was 67.3 ± 20.1, in the absence of enflurane 60.3 ± 6.3 and in the absence of methoxyflurane 69.1 ± 9.8.

K^+ channels in glial cells. To date, charybdotoxin-sensitive K^+ channels have been implicated in action potential repolarization in nerve [14], fluid secretion in exocrine cells [15,16] epithelial salt transport [17] and neurosecretion in pituitary cells [18]. It is tempting to speculate that these charybdotoxin sensitive channels in glial cells are involved in spatial buffering. The observation that both influx and efflux through these channels can occur, makes it a potential candidate for such a function.

The effects of volatile anesthetics on the charybdotoxin-sensitive K^+ channels in rat glioma C6 cells differ from the effects of general anesthetics on Ca^{2+}-activated K^+ channels in human erythrocytes [19]. In the latter, general anesthetics stimulated rather than inhibited the Ca^{2+}-dependent efflux of K^+ when used at clinical concentrations. Since at least three different Ca^{2+}-activated K^+ channels have been described [20], the above discrepancy may be due to the detection of different types of Ca^{2+}-activated K^+ channels in the two systems.

In order to assess the relevance of the inhibition of Ca^{2+}-activated K^+ channels in glial cells for anesthesia it is necessary to study (a) whether glial cells in vivo also express these channels and (b) whether these channels are involved in spatial buffering.

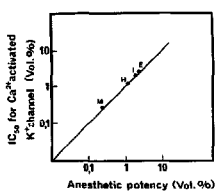

Fig. 5. Relationship between general anesthetic concentrations needed to anesthesize 50% of test animals and the concentrations necessary for 50% inhibition of Ca^{2+}-activated K^+ channel in rat glioma C6 cells. The line through the origin represents the theoretical situation where the anesthetic concentration is identical to the concentration that inhibits the Ca^{2+}-activated K^+ channel by 50% (IC_{50}). The IC_{50} values for Ca^{2+}-activated K^+ channel inhibition of the volatile anesthetics were extrapolated from Fig. 2A and Fig. 4. The animal potency data of the anesthetics for rats were from Ref. 21. Since the anesthetic potency of methoxyflurane for rats is not available in the literature we have used the anesthetic potency for mice (Ref. 22). (H, halothane; I, isoflurane; E, enflurane and M, methoxyflurane).

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