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Halothane inhibits the neurotoxin stimulated [^{14}C]guanidinium influx through 'silent' sodium channels in rat glioma C_6 cells

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We have investigated the effect of pharmacological agents on [^{14}C]guanidinium ion influx through sodium channels in C_6 rat glioma and N18 mouse neuroblastoma cells. The sodium channels of the N18 cells can be activated by aconitine alone, indicating that they are voltage-dependent channels. In contrast, sodium channels in the C_6 cells require the synergistic action of aconitine and scorpion toxin for activation and are therefore characterized as so-called silent channels. The general anesthetic halothane used at clinical concentrations, specifically inhibited the ion flux through the silent sodium channel of C_6 rat glioma cells. The voltage-dependent channels of the N18 cells were insensitive to halothane at the concentrations tested.

Halothane *Sodium channel* *[^{14}C]Guanidinium* *Rat glioma C_6 cell* *Neuroblastoma N18 cell*

1. INTRODUCTION

Ion flux experiments provide an easy way to study the characteristics of sodium channels in neuronal cell lines [1,2]. Several neurotoxins interact with sodium channels and are therefore used as a tool to characterize properties of such channels. On the basis of their effect on the channel they are divided in 4 groups (review, [3]): (a) tetrodotoxin and saxitoxin which specifically block the Na^+ channel [4,5]; (b) some alkaloids from plants such as veratridine and aconitine and alkaloid toxins from animals including batrachotoxin and grayanotoxin cause persistent activation [6–9]; (c) peptide toxins such as scorpion α-toxin and sea anemone toxin, which slow down channel inactivation [10–13]; (d) American scorpion β-toxins, which enhance activation [14]. The ion flux through the sodium channel can be studied with $^{22}Na^+$ [1,15–17] or [^{14}C]guanidinium ions [16–19]. We have used the latter method to study the sodium channels in C_6 rat glioma and N18 mouse neuroblastoma cells.

C_6 cells have electrically silent Na^+ channels in the plasma membrane. This was shown by different authors using electrophysiological measurements and $^{22}Na^+$ flux measurements through sodium channels opened by the combined action of veratridine or aconitine with scorpion α-toxin [1,18,20]. In contrast, electrically excitable mouse neuroblastoma N18 cells have voltage-dependent Na^+ channels which can be opened by veratridine or aconitine alone [1,18,20]. This behaviour is in very good agreement with the electrophysiological behaviour of these channels [1,20]. Both silent and voltage-dependent Na^+ channels of C_6 and N18 cells are sensitive to tetrodotoxin and saxitoxin, respectivley. These toxins block the channels. The voltage-dependent or silent character of sodium channels in these two cell types were verified by us using toxins as described by different authors cited above. Further, we determined the sensitivity of the two channel types towards the general anesthetic halothane and show here that the silent sodium channel is more sensitive towards the anesthetic than the voltage-dependent sodium channel.

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2. MATERIALS AND METHODS

2.1. *Materials*

Tetrodotoxin and scorpion toxin (*Androctonus australis*) were from Sigma (Taufkirchen, FRG), aconitine from Roth (Karlsruhe) and halothane from Hoechst (Frankfurt). [^{14}C]Guanidinium chloride (specific activity 35–55 mCi/mM) from the Centre de l'Energie Atomique (Saclay, France). Chemicals were from Merck (Darmstadt).

2.2. *Cell cultures*

C_6 rat glioma cells (ATTC CCL107) [22] were cultured in plastic tissue culture flasks in Dulbecco's modified Eagle's meidum (DMEM) with 10% fetal calf serum in a 10% CO_2/90% humidified air atmosphere and were passaged by trypsinization (0.25% trypsin). The N18 mouse-neuroblastoma cells [23] were cultured as described above for the C_6 cells except that they were passaged with 0.005% trypsin. All plastic tissue culture dishes were from Nunc (Roskilde, Denmark).

2.3. *[^{14}C]Guanidinium uptake measurements*

[^{14}C]Guanidinium uptake was measured essentially as previously described [16]. Experiments were performed at 36°C. Cells in 3 cm dishes were preincubated for 5 min in a 25 mM Hepes-Tris buffer (pH 7.4) containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. The preincubation medium was then removed and 1 ml ion uptake buffer was added (25 mM Hepes-Tris buffer (pH 7.4) containing 1 mM NaCl, 10 mM guanidinium chloride, 129 mM cholinium chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose and 0.17 μCi/ml [^{14}C]guanidinium chloride). Ion uptake measurements were terminated by aspirating off the radioactive assay medium and washing the culture dishes 3 times with 2 ml wash buffer (25 mM Hepes-Tris buffer (pH 7.4), containing 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose and 140 mM choline chloride). The washing procedure was performed in less than 15 s. The cells were then suspended in 1 ml cold 5% trichloroacetic acid and the radioactivity measured in a liquid scintillation counter (Packard 460 Cl using Bray's solution as scintillator). Cell protein was measured by the method of Lowry et al. [24].

2.4. *[^{14}C]Guanidinium ion uptake in the presence of halothane*

Preincubation and uptake buffer were equilibrated for 15 min with the desired halothane concentration by bubbling the solutions with 95% air/5% CO_2 containing either 1, 2 or 4% halothane. The halothane concentration was administered to the air using a 'Halothan Vapor 19' vaporizer (Drägerwerk, Lübeck) and was checked for accuracy with an Engström EMMA analyzer (Elektromedizin, München).

The culture dishes with the equilibrated media were placed in a plastic gas flow chamber at 36°C under a flow of 1, 2 and 4% halothane (flow rate 3 l/min). The concentration of the anesthetic in the preincubation and uptake buffer was checked with a gas chromatograph (Packard, model 430 – using a 2 m glass column filled with chromosorb WHP 100/120 containing 2% OV101 – oven temperature 30°C). All other treatments were as described above.

3. RESULTS AND DISCUSSION

3.1. *Effects of neurotoxins on the sodium channels in C_6 rat glioma and N18 mouse neuroblastoma cells*

We have studied the ion influx of [^{14}C]guanidinium ions through sodium channels of C_6 rat

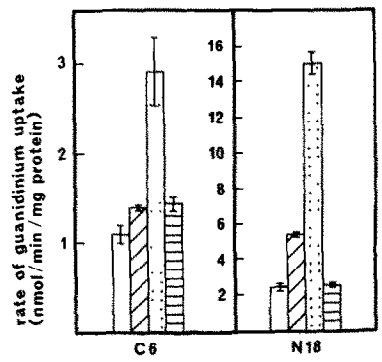

Fig.1. [^{14}C]Guanidinium uptake by rat glioma C_6 cells and mouse neuroblastoma N18 cells measured during 30 min: □, without toxin addition; ▨, with 10^{-4} M aconitine; ⊞, with 10^{-4} M aconitine plus 10^{-5} M scorpion toxin; ▤, 10^{-4} M aconitine plus 10^{-5} M scorpion toxin plus 10^{-6} M tetrodotoxin. The protein content of each dish was determined using the method of Lowry et al. [24]. Data are means ± SD of triplicate determinations.

glioma and N18 mouse neuroblastoma cells. The sodium channels of the non-excitable C_6 rat glioma cell line have been characterized as so-called silent sodium channels [20,21]. This type of channel is opened by the combined action of aconitine and scorpion toxin or veratridine and scorpion toxin and is inhibited by 1 μM tetrodotoxin as shown in fig.1.

In contrast, the voltage-dependent sodium channels in neuronal cells can be activated by the alkaloids veratridine, aconitine or batrachotoxin alone without scorpion toxin [1,2]. As shown further in fig.1, aconitine stimulated the basal influx in N18 cells by a factor of 2.5. But in the presence of the alkaloids and the scorpion toxin together we find a 7-fold stimulation of the ion influx. This ion influx was completely inhibited by 1 μM tetrodotoxin, indicating specific ion movement through the sodium channels. In the conditions used to find the pharmacological characteristics of the ion channels of our cells we used an incubation time of 30 min. This incubation time was suitable because the neurotoxin-stimulated [^{14}C]guanidinium ion uptake increased up to about 30 min as shown in fig.2. At 30 min we found a 6–7-fold higher level of uptake in the neurotoxin-stimulated cells than in the cell culture without toxins. Similar kinetics but with lower neurotoxin stimulation were obtained with C_6 cells (not shown). Ouabain at a concentration of 1 mM did not affect influx measurements, indicating that the Na^+/K^+ ATPase did not pump [^{14}C]guanidinium ions out of the cells (not shown).

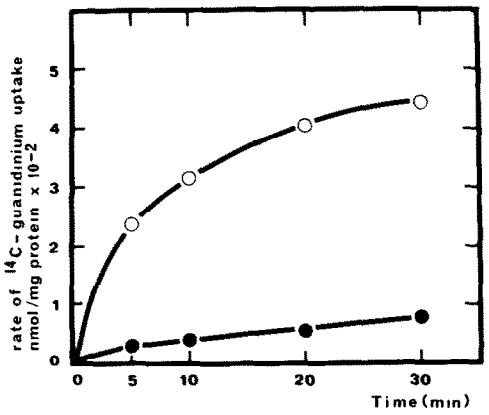

Fig.2. Stimulation of [^{14}C]guanidinium uptake by the synergistic action of aconitine and scorpion toxin. N18 neuroblastoma cells were incubated at 36°C for 30 min in uptake buffer containing 0.17 μCi/ml of [^{14}C]guanidinium (see section 2) and assayed for the indicated times with (○) or without (●) 10^{-4} M aconitine and 10^{-5} M scorpion toxin. The protein concentration of each petri dish was determined using the method of Lowry et al. [24]. Data are means ± SD of triplicate determinations.

3.2. *Effect of halothane on the sodium channels in C_6 and N18 cells*

We further characterized the sodium channels in the two neural cell lines with respect to their sensitivity towards the general anesthetic halothane. Clinical concentrations of the anesthetic significantly affected the [^{14}C]guanidinium ion movement through the silent sodium channels of rat glioma C_6 cells (fig.3A). Under similar conditions halothane did not have a significant inhibitory effect on the ion flux through the voltage-dependent sodium channels of N18 cells (fig.3B). This observation is in agreement with published data of electrophysiological experiments [25]. About 5–10 times the clinical concentration of anesthetic is

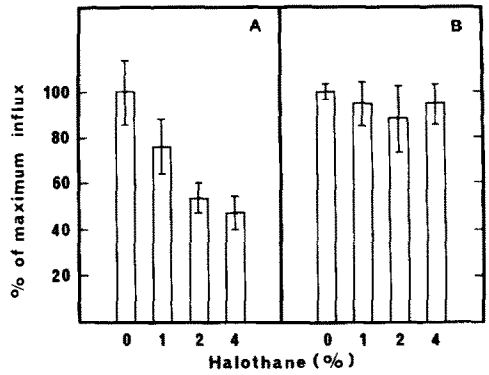

Fig.3. Effect of halothane on [^{14}C]guanidinium influx through scorpion toxin (10^{-5} M) and aconitine (10^{-4} M) activated ion channels in C_6 rat glioma (A) and N18 mouse neuroblastoma cells (B). Preincubation and uptake buffer (see section 2) were equilibrated with either 1, 2 or 4% halothane. The cells on 3 cm petri dishes were then allowed to take up the [^{14}C]guanidinium ion under the corresponding halothane atmosphere for 30 min. The [^{14}C]guanidinium influx in the presence of the two neurotoxins was taken as 100% and the fluxes in the presence of halothane were calculated as % of this maximum influx. Experiment was performed 3 times. Data are means ± SD of triplicate determinations of a representative experiment.

needed to suppress the sodium current through excitable channels. The function of the silent sodium channel is still unclear. If this channel has some important transport function our finding might have significance for the development of the anesthetic state. On the other hand, it would be interesting to study silent sodium channels in other neural [19,20] and non-neural cells [17,26,27] to find out if the sensitivity towards halothane is a general property of silent sodium channels. Local anesthetics inhibit voltage-dependent and silent sodium channels to the same degree [18]. As we have shown here, halothane specifically inhibits silent sodium channels. Therefore, it is tempting to speculate that the binding site of halothane on the silent sodium channel differs from the binding sites of local anesthetics.

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