Atropine increases sevoflurane potency in cortical but not spinal networks during cholinergic overstimulation

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Abstract

In the event of mass destruction with nerve agents a number of victims can be expected to suffer from symptoms of cholinergic overstimulation due to intoxication as well as from physical trauma. Since previous studies have demonstrated that cholinesterase inhibitors may reverse general anaesthesia in humans this scenario raises the question of how these patients can be anaesthetised in order to enable surgical interventions. A likely reason for this reversal is a reduction of anaesthetic potency by acetylcholine as observed for volatile anaesthetics in vitro. In order to test whether a combination of cholinergic antagonists with general anaesthetics improves their potency, we investigated the effects of clinically relevant concentrations of atropine on sevoflurane potency in cortical and spinal slice cultures during cholinergic overstimulation. As the spinal cord and neocortex are important substrates for general anaesthetics, cultured spinal and cortical tissue slices were obtained from embryonic and newborn mice, respectively. Drug effects were assessed by extracellular voltage recordings of spontaneous action potential activity. Application of acetylcholine elevated spontaneous activity in neocortical and spinal slices. Atropine (10 nM) reduced discharge rates and reversed the increase of spontaneous activity induced by acetylcholine. In the presence of acetylcholine and atropine sevoflurane caused a concentration-dependent decrease of neuronal activity in neocortical ([EC50 = 0.35 ± 0.03 MAC]) and spinal slices ([EC50 = 0.42 ± 0.03 MAC]). Comparing our results with previous studies which investigated the effects of acetylcholine on anaesthetic potency it is concluded that small concentrations of atropine increase sevoflurane potency in cortical networks during cholinergic overstimulation. Thus, in a clinical setting, we recommend that anaesthetic drugs should be co-applied with atropine for adequate performance of general anaesthesia.

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Introduction

Organophosphorus compounds are highly toxic chemicals including many potent insecticides as well as nerve agents. The common mechanism of action for both, insecticides and nerve agents is a blockade of acetylcholinesterase resulting in an accumulation of acetylcholine in the synaptic cleft. This accumulation of acetylcholine triggers a cholinergic crisis leading to peripheral and central symptoms of poisoning like hypersalivation, bronchoconstriction, cardiovascular instability or generalized convulsions (Ben Abraham et al., 2002; Lallement et al., 1992; Tonduli et al., 1999; De Jong, 2003; Cosar and Kenar, 2006). In the event of mass destruction it seems likely that victims suffer from intoxication as well as from physical trauma (White, 2002; Ben Abraham et al., 2002). Providing medical care to these patients may require induction and maintenance of general anaesthesia in order to enable surgical interventions (Baker, 2002).

Up to now, there is little experience in how general anaesthetics act in subjects afflicted with cholinergic overstimulation. However, there is evidence from in vivo studies that application of cholinesterase blockers increases the requirement for anaesthetic drugs to perform general anaesthesia. Meuret et al. (2000) investigated the effects of the reversible acetylcholine esterase blocker physostigmine on patients anaesthetised with propofol by assessing central nervous system function by the use of Auditory Steady-State Response and Bispectral Index. They demonstrated that physostigmine restored consciousness with concomitant increases in both, Auditory Steady-State Response and Bispectral Index. Using a comparable experimental approach, Plourde et al. (2003) reported that sevoflurane anaesthesia can be antagonized by simultaneous application of physostigmine.

Abbreviations: GABA, gamma-aminobutyric acid; MAC, median alveolar concentration of an inhaled anaesthetic required to suppress movement in response to noxious stimulation in 50% of subjects.

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Transferring these results into a clinical setting implies that inhibitors of acetylcholinesterase strongly enhance anaesthetic requirement. Anaesthesiologists may be out in their estimation of anaesthetic demand since acetylcholine concentrations in the central nervous system are unknown and may undergo gross changes in patients suffering from organophosphorus intoxication. Raising impreudently the concentrations of general anaesthetics in patients suffering from organophosphorus intoxication might aggravate cardiovascular symptoms thereby increasing the probability of poor outcome.

Thus it was proposed that general anaesthetics should be used in conjunction with antagonists of muscarinic acetylcholine receptors in order to perform general anaesthesia, since acetylcholine-induced reversal of hypnosis is most likely mediated via muscarinic acetylcholine receptors (Grasshoff et al., 2007a,b). In order to test this hypothesis, in this study effects of the muscarinic acetylcholine receptor antagonist atropine on sevoflurane potency in depressing spontaneous neuronal activity in cortical and spinal networks were tested in cultured organotypic slices. Neurons in the neocortex and in the ventral horn of the spinal cord are important targets for general anaesthetics, as their depression induces fundamental components of general anaesthesia namely hypnosis, sedation and immobility (Fiset et al., 1999; Grasshoff et al., 2005; Rudolph and Antkowiak, 2004; Campagna et al., 2003; Sonner et al., 2003).

The choice of the muscarinic antagonist atropine was motivated by the fact that the drug is in clinical use for the symptomatical treatment of patients suffering from organophosphorus poisoning (Thiermann et al., 1999, 2009).

2. Materials and methods

2.1. Animals

All procedures were approved by the animal care committee (Eberhard-Karls University, Tuebingen, Germany) and were in accordance with the German law on animal experimentation. All efforts were made to minimize animal suffering and the number of animals used. Up to four 129/SvJ mice were housed in a cage with free access to food and water. The cages were stored in a temperature-controlled room with a 12-h day and night cycle.

2.2. Preparation of organotypic slice cultures

Neocortical slice cultures were prepared from 2- to 5-day-old mice as described by Gahwiler (1981). In brief, for the preparation of somatosensory cortex, animals were deeply anaesthetised with isoflurane and decapitated. Cortical hemispheres were aseptically removed and 300 μm thick coronal slices were cut. Spinal slice cultures were prepared from pregnant mice according to the method described by Braschler et al. (1989). Excised slices were placed on a coverslip and embedded in a plasma clot. The coverslips were transferred into plastic tubes containing 0.75 ml of nutrient fluid and incubated with 95% oxygen and 5% carbon dioxide at 36.0°C for 2 h. Nutrient fluid was obtained by mixing 250 ml horse serum, 250 ml Hanks’ Balanced Salt Solution, 500 ml Basal Medium Eagle, 5 ml glutamine solution (200 mM) and 10 ml glucose solution (50%). The roller tube technique was used to maintain the tissue (Gahwiler, 1981). For spinal slices, nutrient fluid included 10 mM of nerve growth factor. After 1 day in culture, antimitotics (10 μM 5-fluoro-2-deoxyuridine, 10 μM cytosine-b-d-arabinofuranoside, 10 μM uridine) were added to reduce proliferation of glial cells. All chemicals were obtained from Sigma, Taufkirchen, Germany, except the horse serum which was obtained from Invitrogen, Karlsruhe, Germany. Slices were used after 21 days in vitro for extracellular recordings.

2.3. Extracellular voltage recordings

Organotypic slices of the cortex or the spinal cord were continuously perfused with artificial cerebrospinal fluid (ACSF) consisting of 120 mM NaCl, 3.3 mM KCl, 1.13 mM NaH2PO4, 26 mM NaHCO3, 1.8 mM CaCl2, and 11 mM d-glucose. The ACSF was bubbled with 95% oxygen and 5% carbon dioxide. Bath temperature was 35°C. Glass electrodes filled with ACSF (resistance approximately 2–5 MΩ) were used for recording extracellular signals, which were bandpass filtered (200–5000 Hz) in order to isolate action potential activity. The electrodes were advanced into the tissue until extracellular single- or multi-unit spike activity (usually exceeding 100 μV in amplitude) could be clearly identified. Signals were digitized on a PC at 10 kHz via a Digidata 1200 interface and Axoscope 9 (Axon Instruments, Union City, CA, USA).

Test solutions containing sevoflurane were obtained by dissolving the liquid form of the anaesthetic in ACSF, which was equilibrated with 95% oxygen and 5% carbon dioxide. A closed, air-free system was used to prevent evaporation. Anaesthetic levels are given as multiples of median alveolar concentration of an inhaled anaesthetic required to suppress movement in response to noxious stimulation in 50% of subjects (MAC). These MAC values refer to the plasma or blood concentrations of volatile anaesthetics in mammals at 37°C. We used the EC50 values for general anaesthesia proposed by Franks and Lieb (1994). Thus we assumed that 1 MAC corresponds to an aqueous concentration of 0.35 mM sevoflurane (Grasshoff and Antkowiak, 2004).

Sevoflurane was administered via bath perfusion using gas-tight syringe pumps (ZAK, Marktredenfeld, Germany), which were connected to the experimental chamber via Teflon tubing (Lee, Frankfurt, Germany). The flow rate was approximately 1 ml/min. To ensure steady-state conditions, recordings during anaesthetic treatment were carried out 10–15 min after starting the perfusate change.

2.5. Experimental design

Data were analyzed with in-house software written in OriginPro version 7 (OriginLab Corporation, Northampton, MA, USA) and MATLAB version 7.1 (The MathWorks Inc., Natick, MA, USA).

Analysis of extracellular recordings was performed as previously described (Grasshoff and Antkowiak, 2004). After close inspection of the raw data action potentials were detected by setting a threshold well above base line noise. The mean firing rate was obtained from single- or multi-unit activity; it is defined as the number of detected action potentials divided by the recording time of 180 s. The natural firing mode of cortical neurons in culture consisted of bursts of action potentials separated by silent periods (Fig. 1A). A burst was defined as a group of action potentials with an initially high firing rate of 10 Hz. The burst rate was calculated as the number of bursts occurring during the recording period (180 s). The relative time of a culture firing bursts of action potentials (relative time in burst, also called up-state) and the mean number of events per burst were calculated based on these two basic parameters. The relative inhibition was calculated by subtracting the firing rate monitored...
in the presence of sevoflurane from the control rate (absence of the anaesthetic) for each experiment. The resulting difference was multiplied by 100 and divided by the control rate. Hence, a relative inhibition of 0% results in the case of sevoflurane lacked any inhibitory drug action and a 100% depression indicates that not a single action potential occurred in the presence of the anaesthetic.

For statistical analysis, Student’s t-test was used. Unless otherwise stated, results are given as mean ± S.E.M. Concentration–response curves were fitted by Hill equations, as previously described (Antkowiak and Helfrich-Förster, 1998). Estimated EC50 values were derived from these fits.

3. Results

3.1. Effects of acetylcholine and atropine on network activity in cortical organotypic slices

The neuronal activity patterns of cultured slices from the neocortex are characterized by spontaneous action potential firing that is organized into high frequency clusters (bursts) separated by periods of neuronal silence. A typical recording showing three bursts of action potentials is displayed in Fig. 1A. In the presence of 1 μM acetylcholine bursts of action potentials are prolonged and thereby the overall neuronal activity is elevated (Fig. 1B). This observation stands in accordance with earlier studies (Grasshoff et al., 2007a,b). When the muscarinic acetylcholine antagonist atropine (10 nM) is given in addition to acetylcholine, the activity enhancing effect of acetylcholine is abolished, as illustrated in Fig. 1C.

The basic parameter to characterize the overall neuronal activity used for the present study is the mean action potential firing rate. In the presence of 1 μM acetylcholine this parameter was increased by 33 ± 9% (n = 20, p < 0.01, compared to control condition). However, when atropine (10 nM) was added the mean firing rate was depressed by 75 ± 11% (n = 20, p < 0.001, compared to control). The burst rate in the presence of 1 μM acetylcholine was nearly unchanged (+7.6 ± 16.3%, n.s., n = 18) but greatly reduced in the additional presence of 10 nM atropine (−61.4 ± 7.4%, p < 0.001, n = 18). The relative time in burst of a cortical culture was increased by 39.0 ± 8.7% (p < 0.001, n = 18) during elevated cholinergic tone (1 μM acetylcholine) but decreased by 49.1 ± 11.3% (p < 0.001, n = 18) when atropine (10 nM) was present in addition to 1 μM acetylcholine. The mean number of action potentials per burst was elevated comparatively (58.4 ± 18.1%; p < 0.01, n = 18) in the presence of acetylcholine, and addition of atropine lowered the mean number of action potentials by 32.6 ± 13.7% (p < 0.05, n = 18).

These data demonstrate that the cortical slice cultures used for the present study have a rather high basal intrinsic cholinergic tone, as action potential firing is modestly increased by external acetylcholine, but strongly depressed by atropine. In Fig. 2 the cumulated sum of action potentials for the first 1000 ms of the mean burst is displayed to characterize the changes induced by modifications of cholinergic activity. Under control conditions (solid grey line) an almost linear rise can be observed. In the presence of 1 μM acetylcholine (dashed grey line) this is increased from the very beginning and lies completely above control values for the first 1000 ms of the mean burst. On the contrary, atropine (black line) attenuates the neuronal activity. This effect is the more pronounced with increasing the burst length.

Assuming that cortical slice cultures have an intrinsic cholinergic tone, one would expect that blocking of muscarinic acetylcholine receptors by atropine depresses spontaneous action potential firing of cortical neurons. To address this issue we tested the effects of 10, 30 and 100 nM atropine on cortical network firing in the absence of extrinsic administered acetylcholine. Expectedly, increasing atropine concentrations lead to a dose dependent depression of spontaneous activity, as displayed in Fig. 3.

3.2. Sevoflurane potency in cortical networks depends on cholinergic tone

In a previous study we found that the potency of the volatile anaesthetic sevoflurane to depress cortical network firing is decreased in the presence of acetylcholine compared to control condition (Grasshoff et al., 2007b). Here we tested whether the reduced network depressing effects of sevoflurane in the presence of acetylcholine can be restored by the addition of 10 nM atropine. The results of these experiments are shown in Fig. 4. For comparison the fits from previously published data (Grasshoff et al., 2007b) are included. When atropine was present in addition to acetylcholine (black line), the depression by sevoflurane at any given concentration was more pronounced than under control condition. Fig. 4A shows the concentration-dependent decrease of mean firing rates during three different conditions (normal, elevated and reduced cholinergic tone). The relative depression of neuronal activity by increasing concentrations of sevoflurane is...
3.3. Sevoflurane potency in spinal networks is independent of cholinergic tone

In addition to the recordings from the neocortex we investigated the effects of acetylcholine and atropine in cultured slices from the spinal cord. As in the cortex the mean action potential firing rate of spinal cord neurons was increased in the presence of 10 μM acetylcholine by 36 ± 4% (n = 256, p < 0.001, pooled data). Unlike in cortical neurons, the additional presence of 10 nM atropine restored the mean firing rate to control conditions (4 ± 14%, n = 11, n.s.), but did not further decrease action potential firing.

In contrast to effects observed in the cortex, sevoflurane potency did not depend on cholinergic status in spinal cultures. Here, sevoflurane leads to a concentration dependent depression in the presence of acetylcholine as well as in the additional presence of atropine that did not differ from controls (Fig. 5). As depicted in cortical neurons, fits from previously published data (Grasshoff et al., 2007b) were included for better comparison.

4. Discussion

4.1. Impact of organotypic cultures for studying interactions between acetylcholine, atropine and general anaesthetics

The present study was performed to characterize interactions between two drugs, sevoflurane and atropine, on network activity in different brain regions under specific conditions, namely cholinergic overstimulation. The choice of performing in vitro studies was justified by the fact that in organotypic cultures drug concentrations are under experimental control, which is much more difficult to obtain in vivo or in acutely isolated slices (Gredell et al., 2004). It has been shown that volatile anaesthetics depress spontaneous network activity of neocortical and spinal neurons in organotypic cultures at clinically relevant concentrations (Grasshoff and Antkowiak, 2004; Grasshoff et al., 2007b; Antkowiak and Helfrich-Förster, 1998). Moreover, Hentschke et al. (2005) previously demonstrated that the concentration–response relationships for volatile anaesthetics calculated from in vivo and in vitro experiments were almost identical, indicating that slice cultures are valuable tools for investigating the mechanisms of anaesthetic actions.

4.2. Relevance of drug concentrations

An important issue to be addressed in the context of in vitro studies is the question whether effects occur at clinically relevant concentrations. Sevoflurane concentrations in this study were given as multiples of median alveolar concentration (MAC). As stated in Section 2, one MAC stands for the median alveolar concentration of an inhaled anaesthetic required to suppress movement in response to noxious stimulation in 50% of subjects. Since the EC50 concentration for the inhibition of spontaneous network activity in cortical as well as in spinal cultures is around one MAC of sevoflurane, the concentration range tested in this study can be regarded as clinically relevant. Estimating the
concentration of acetylcholine which can be assumed to occur in the brain during a severe intoxication is more complex. In an in vivo study Tonduli et al. (1999) exposed freely moving rats to soman and acquired multiple sets of neurophysiologic data before and during soman intoxication. The authors measured cortical acetylcholinesterase activity and acetylcholine concentrations by microdialysis and associated both parameters with electroencephalographic recordings and power spectrum analysis of the gamma band. Acetylcholine concentrations in the cortex during intoxication can be calculated from acetylcholine levels measured in microdialysis probes during seizures. As a rough estimate the concentration range reaches from 1 to 10 μM (Grasshoff et al., 2007a,b). Concerning atropine concentrations in humans intoxicated with organophosphorus compounds, three studies were published by the group of Thiermann et al. (1997, 1999, 2009). They reported on several patients who ingested insecticides, most of them in suicidal intent. These patients had to be treated at an intensive care unit with obidoxime, a reactivator of acetylcholinesterase and atropine with both drugs being monitored. In summary, it was shown that during therapy an atropine maintenance dose above some 5 mg/h is hardly necessary. Under such a regimen, atropine plasma steady-state concentrations range between 5 and 80 nmol/L (Thiermann et al., 2009).

In conclusion the concentrations of sevoflurane, acetylcholine and atropine used in this study are in good accordance with those reported from in vivo studies with humans. Thus the interactions between sevoflurane, acetylcholine and atropine reported here can be regarded to be of clinical relevance.

4.3. Clinical relevance of the study

Previous studies on humans investigating interactions of reversible cholinesterase blockers with general anaesthetics pointed out that rising brain acetylcholine concentrations reverses general anaesthesia (Meuret et al., 2000; Plourde et al., 2003). Looking for reasons for this reversal of anaesthetic state, in vitro studies offered two putative mechanisms. First, an increase in discharge rates of cortical and spinal neurons induced by acetylcholine and, second, an impairment in anaesthetic potency in cortical networks (Grasshoff et al., 2007a,b,c). Although the decrease in anaesthetic potency was solely observed with cortical neurons, both mechanisms are likely to raise drug requirement for providing general anaesthesia: the ‘multi-site and multiple mechanisms’ concept. (Grasshoff et al., 2007a,b,c) indicating that, at least in vitro, coapplication of atropine increases drug requirement. Balancing the overstimulation by applying higher anaesthetic concentrations may be deleterious due to aggravation of cardiovascular symptoms. Here we demonstrate that, at least in vitro, coapplication of atropine increases sevoflurane potency to depress cortical network activity. An effective atropine concentration can be easily achieved by application of 1 mg atropine sulphate per hour, as previously demonstrated in patients with organophosphorus poisoning (Thiermann et al., 1997).

Conflicts of interest

The authors declare that there are no conflicts of interest.

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