Isoflurane increases the uptake of glutamate in synaptosomes from rat cerebral cortex

M. Larsen, E. Hegstad, J. Berg-Johnsen and I. A. Langmoen

Summary
We have studied the effect of increasing concentrations of isoflurane on high- and low-affinity uptake of L-glutamate using synaptosomes from rat cerebral cortex. In the high-affinity uptake range, 0.5% isoflurane had no effect on uptake velocity, while 1.5% and 3.0% isoflurane caused an increase in mean $V_{\text{max}}$ to 131 (SEM 54) and 210 (103) % of control, respectively. There was no significant change in the $K_m$ value. $V_{\text{max}}$ and $K_m$ values for low-affinity uptake of L-glutamate were unchanged by 1.5% isoflurane. These results provide evidence for an isoflurane-induced increase in high-affinity uptake of glutamate into presynaptic terminals. This effect may contribute to a reduction of transmitter in the synaptic cleft and thereby decreased excitatory synaptic transmission. (Br. J. Anaesth. 1997; 78: 55–59)

Key words

As early as 1906, Sherrington reported that chloroform blocks reflex activity in the spinal cord at lower concentrations than those needed to inhibit impulse propagation along nerve fibres. Later studies have shown that other inhalation agents depress excitatory synaptic transmission in the superior cervical ganglion and in various parts of the central nervous system, including the spinal cord, cuneate nucleus, olfactory bulb, olfactory cortex and dentate area. Isoflurane, a halogenated methyl-ethyl-ether, is used widely because of its advantages compared with older volatile anaesthetics. In the rat hippocampus, isoflurane inhibits excitatory signal transduction by depression of the activity in thin unmyelinated, afferent fibres, reduction of excitatory synaptic transmission and hyperpolarization of the postsynaptic neurone.

The effect on excitatory synaptic transmission seems quantitatively to be the most important. Synaptic transmission may be depressed by drug-induced reduction in the release of the transmitter from the presynaptic terminal, altered reuptake or reduced sensitivity of the postsynaptic receptor. Intracellular recordings from lumbosacral motor neurones in cats have shown that diethyl ether depresses synaptic potentials, thus decreasing the amount of excitatory transmitter released while leaving the chemosensitivity of the postsynaptic membrane unchanged. We have shown recently that isoflurane reduced both synaptic (calcium-dependent) and non-synaptic (calcium-independent) potassium-stimulated release of glutamate from rat cerebral cortex, without affecting the response on the postsynaptic membrane. The effect on the presynaptic terminal may be caused by alteration in the uptake mechanism in addition to reduction in the release of transmitter.

To study further how isoflurane acts presynaptically, we have investigated the effect on neuronal reuptake mechanisms of glutamate using synaptosomes prepared from rat cerebral cortex.

Materials and methods

PREPARATION OF SYNAPTOSOMES
Synaptosomes were prepared, as described by McMahon and colleagues. Briefly, Wistar rats, treated according to approved rules, were killed and the brain carefully removed. The cortex from each hemisphere was isolated and homogenized in 15 ml of sucrose 0.32 mol litre$^{-1}$ (1–4°C) using a glass homogenizer with a Teflon pestle (clearance 0.25 mm). The homogenate was centrifuged at 3000 $g$ for 3 min and the supernatant spun at 15 000 $g$ for 30 min. The pellet formed consisted of two layers. The superficial layer containing the majority of the synaptosomes was resuspended selectively in 6 ml of sucrose 0.32 mol litre$^{-1}$, stored at 1–4°C and used within 6 h from preparation. The pellet was diluted before use in HEPES-buffered medium (HBM) containing (mmol litre$^{-1}$): NaCl 117.5, KCl 3.75, MgCl$_2$ 1, CaCl$_2$ 1.3, KH$_2$PO$_4$ 1.25, NaHCO$_3$ 20, glucose 10, HEPES 20, NaOH 6 (pH 7.4). Fresh HBM was prepared daily.

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L-GLUTAMATE UPTAKE

Synaptosomes (2–4 mg protein ml⁻¹) were incubated at 37°C in a series of different concentrations of l-glutamate corresponding to the high-(5.5 x 10⁻⁵ to 2.5 x 10⁻⁴ mol litre⁻¹) and low-(1 x 10⁻⁴ to 1 x 10⁻⁵ mol litre⁻¹) affinity uptake systems, respectively. A fixed concentration (2.2 x 10⁻⁵ mol litre⁻¹) of radiolabelled glutamate (46 Ci mmol⁻¹) (Amersham Laboratories, Buckinghamshire, UK) was added to each l-glutamate concentration. The chambers, each containing 1.5 ml of incubation medium, were sealed and saturated with 95% oxygen–5% carbon dioxide. Isoflurane 0.5, 1.5 or 3.0% (Abbot Laboratories Ltd., Kent, UK) was added using a vaporizer (Fortec, Ohmeda 5250 RGM, Englewood, USA) and the medium shaken gently every 30 s. Uptake was terminated at 4 min as the synaptosomes were placed on a Millipore filter and the medium shaken for 1 min. Uptake was then removed from the filter before scintillation fluid (Pico-Fluor 15, Packard, Groningen, The Netherlands) was added. The labelling was measured by counting disintegrations per minute in a β-scintillation counter (LKB Wallac, San Francisco, CA, USA) with a counting efficiency of 25–30%. Protein content was measured using the method of Lowry and colleagues.

In each setting, a control experiment and experiments using 0.5, 1.5 and 3.0% isoflurane were performed using one batch of synaptosomes from one brain. The number of experiments (n = 9) thus reflects the number of rats used.

Results are presented as mean (SEM). Differences between the two groups were tested using the Wilcoxon signed rank sum test and for three or more groups with the Kruskal–Wallis test. Differences between the two groups were tested using the Wilcoxon signed rank sum test and for three or more groups with the Kruskal–Wallis test. Differences were considered significant when P < 0.05.

Results

In the first set of experiments, concentrations of l-glutamate were increased from 0.55 x 10⁻⁵ to 2.5 x 10⁻⁴ mol litre⁻¹ corresponding to the high-affinity uptake system with a reported K_m value of 0.2–5.0 x 10⁻⁵ mol litre⁻¹. Uptake velocity for l-glutamate was measured in the control situation and under the influence of isoflurane in increasing concentrations (0.5, 1.5 and 3.0%). The results are summarized in table 1. By plotting the mean values from table 1 in a double reciprocal plot (Lineweaver–Burk plot) as shown in figure 1, straight lines with regression coefficients (r²) of 0.905 to 0.992 were drawn. V_max and K_m were calculated from the intercepts of the lines on the Y and X axis, respectively. The lowest concentration, 0.5% isoflurane, had no effect on uptake velocity as the control and 0.5% isoflurane plots were parallel. However, 1.5 and 3.0% isoflurane produced an increase in uptake velocity, as seen from the reduced steepness of the plot in figure 1. As all lines cross the X axis at approximately the same point, the high-affinity transporter to glutamate was presumably unaffected by isoflurane.

Calculated K_m and V_max values for the high-affinity uptake system are presented in table 2. V_max in the control situation was 1.47 (1.25) µmol g⁻¹ min⁻¹, and there was no significant change in response to 0.5% isoflurane. However, 1.5 and 3.0% isoflurane increased V_max to 1.97 (0.80) and 3.09 (1.52) µmol g⁻¹ min⁻¹, respectively (P < 0.05), corresponding to 131% and 210% increases, on average, K_m for the high-affinity transporter was 52.9 (50.2) µmol litre⁻¹. Isoflurane 0.5, 1.5 and 3.0% had no effect on K_m.

In the second set of experiments the effect of isoflurane on the low-affinity uptake system was investigated, using glutamate concentrations from 1 x 10⁻⁴ to 1 x 10⁻³ mol litre⁻¹. The double reciprocal plot in

Table 1  Uptake velocity (µmol g⁻¹ min⁻¹) at different concentrations of l-glutamate in the high affinity range in the synaptosomal fraction from rat cerebral cortex. The effects of 0.5, 1.5 and 3.0% isoflurane were tested (mean (SEM), n = 9).* P < 0.05

<table>
<thead>
<tr>
<th>Test group</th>
<th>L-glutamate concentration (x 10⁻⁵ mol litre⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.55  0.62  0.70  1.00  1.60  2.50</td>
</tr>
<tr>
<td>Isoflurane 0.5%</td>
<td>0.55 (0.11) 0.53 (0.09) 0.65 (0.11) 1.27 (0.18) 1.25 (0.30) 1.54 (0.60)</td>
</tr>
<tr>
<td>Isoflurane 1.5%</td>
<td>0.52 (0.17) 0.63 (0.23) 0.71 (0.23) 1.08 (0.36) 1.37 (0.44) 1.67 (0.49)</td>
</tr>
<tr>
<td>Isoflurane 3.0%</td>
<td>0.79 (0.18) 0.93 (0.21) 0.85 (0.22) 1.27 (0.39) 2.22 (0.62) 2.50 (0.91)</td>
</tr>
</tbody>
</table>

*P < 0.05

Figure 1 Double-reciprocal plots of the effect of 0.5, 1.5 and 3.0% isoflurane on high-affinity glutamate transport in cortical synaptosomes. Regression lines are drawn. (Control (△), r²=0.905; 0.5% isoflurane (○), r²=0.952; 1.5% isoflurane (□), r²=0.958; and 3.0% isoflurane (△), r²=0.992.) Each point

from 1 x 10⁻⁴ to 1 x 10⁻³ mol litre⁻¹. The double reciprocal plot in
**Discussion**

Volatile anaesthetics seem to exert their effects in the central nervous system by affecting several different sites, including both excitatory and inhibitory pathways; for example isoflurane affects conduction in unmynelinated fibres, excitatory synaptic transmission, membrane properties of the postsynaptic neurone and inhibitory synaptic transmission.\(^9\)\(^11\)\(^12\)\(^21\) However, the quantitatively most important effect is probably decreased excitatory synaptic transmission\(^10\) which can be caused by decreased amount of transmitter released from the presynaptic terminal, more efficient reuptake from the synaptic cleft or reduced sensitivity of the postsynaptic membrane. This study showed an anaesthetic-induced increase in the uptake velocity of transmitter glutamate presynaptically. Taken together with the previously demonstrated decrease in transmitter release,\(^16\) increased uptake further attenuates the excitatory synaptic transmission by faster termination of transmitter action.

The synaptosomal preparation used in this study is a mixture of pinched off nerve endings and to a large extent free from glial cells and cell debris.\(^22\) The preparation consists of sealed plasma membrane vesicles with preserved release and uptake mechanisms.\(^23\) Synaptosomes from rat cerebral cortex exhibit both high- and low-affinity uptake mechanisms for glutamate\(^18\) and a high-affinity uptake system for \(\gamma\)-aminobutyric acid.\(^23\) Because of well functioning secretory and uptake mechanisms, the method is regarded as well suited for studying neuronal release and uptake mechanisms of transmitters.\(^20\)

Both neurones and glial cells have uptake systems important in terminating the effect of transmitter glutamate in the synaptic cleft.\(^20\) We have demonstrated uptake systems both in low- and high-affinity areas with \(K_m\) values of approximately 1 mmol litre\(^{-1}\) and 50 \(\mu\)mol litre\(^{-1}\), respectively. These are in agreement with previous reports of two different uptake systems in rat cortical synaptosomes, one in the low-affinity area with a \(K_m\) value of approximately 1 mmol litre\(^{-1}\) and the other in the high-affinity area with a \(K_m\) value of 2–50 \(\mu\)mol litre\(^{-1}\).\(^18\)\(^24\) The physiological role of the low-affinity transporter is still unclear although it has been suggested that it prevents peak concentrations of extracellular glutamate.\(^23\) The high-affinity uptake system, which is active in glial cells and in glutamatergic neurones, is important in terminating transmitter action.\(^25\)

The mechanisms that regulate these transporter proteins are not fully understood. Four different glutamate transporters have recently been identified and cloned in human brain.\(^25\)\(^26\) EAAT 1 (excitatory amino acid transporter) is localized only in the plasma membrane of glial cells, EAAT 2 in neurones and glial cells, EAAT 3 in neuronal plasma membranes\(^27\) and EAAT 4 predominantly in cerebellum.\(^26\) EAAT 1 is activated as protein kinase C (PKC) catalyses phosphorylation of the transporter protein.\(^28\) In cultures of glial cells, stimulation of PKC activity with phorbol esters increases the high-affinity transport of l-glutamate seen as increased \(V_{\text{max}}\) whereas \(K_m\) is unaltered.\(^29\) PKC stimulation also enhanced \(V_{\text{max}}\), but not \(K_m\), of a cloned \(\gamma\)-aminobutyric acid transporter in *Xenopus* oocytes through translocation of the transporter from a cytoplasmic compartment to the plasma membrane.\(^30\) Several volatile anaesthetics, including halothane and to some extent isoflurane, increase PKC-mediated phosphorylation of several rat brain cytoplasmic proteins in a calcium- and phospholipid-dependent manner.\(^31\) One of these proteins, a major substrate for PKC, might have a regulatory role in neurotransmission.\(^31\) As halothane and enflurane appear to inhibit PKC itself, potential targets for anaesthetic action in intact neurones could be complex processes leading to release of intracellular calcium or production of diacylglycerol, which are both required for PKC activation. Furthermore, experimental observations have indicated that neurones

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**Table 2** Estimated values for \(K_m\) and \(V_{\text{max}}\) for the high-affinity uptake system for glutamate in synaptosomes and the effects of 0.5, 1.5 and 3.0% isoflurane (mean (SEM), \(n=9\)). \(^*P<0.05\)

<table>
<thead>
<tr>
<th>Test group</th>
<th>(K_m) ((\times 10^{-4} \text{ mol litre}^{-1}))</th>
<th>(V_{\text{max}}) ((\mu\text{mol} \text{ g}^{-1} \text{ min}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>52.9 (50.2)</td>
<td>1.47 (1.25)</td>
</tr>
<tr>
<td>Isoflurane 0.5%</td>
<td>52.0 (21.0)</td>
<td>1.45 (0.49)</td>
</tr>
<tr>
<td>Isoflurane 1.5%</td>
<td>46.5 (19.1)</td>
<td>1.97 (0.80)*</td>
</tr>
<tr>
<td>Isoflurane 3.0%</td>
<td>61.7 (30.4)</td>
<td>3.09 (1.52)*</td>
</tr>
</tbody>
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**Figure 2** Double-reciprocal plot of the effect of 1.5% isoflurane on the low-affinity uptake system for l-glutamate in synaptosomes. Regression lines are drawn. (Control (□), \(r^2=0.909\); 1.5 % isoflurane (○), \(r^2=0.911\)). Each point represents the mean of seven experiments.
may release factors that influence the activity of glutamate and GABA transporters in glial cells and thus suggest an alternative mechanism of action for anaesthetic agents. 33

The potency of inhaled anaesthetics is described as the minimum alveolar concentration (MAC) of the gas producing surgical anaesthesia in 50% of subjects. The MAC value for isoflurane in middle-aged humans is 1.15% and for rats, 1.38%, and does not alter with increasing duration of anaesthesia. Concentrations exceeding 1 MAC are needed to


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