Inhalation anaesthetic competition at high-affinity cocaine binding sites in rat brain synaptosomes

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Summary
We have shown previously that inhalation anaesthetics inhibit dopamine transport in rat brain synaptosomes. In order to determine if this inhibition is associated with occupancy of the cocaine site, we examined binding of [3H](2β-carbomethoxy-3β-(4-fluorophenyl)-tropane) ([3H]-CFT) in the presence of halothane or isoflurane 0.01-6 mmol litre^{-1} in rat brain synaptosomes. Both anaesthetics inhibited [3H]-CFT binding (mean K_b 0.61 (SEM 0.12) and 0.75 (0.21) mmol litre^{-1}, respectively), by increasing K_a (13.8 (0.6) and 29.8 (12.8) mmol litre^{-1}, respectively) compared with control (8.02 (0.5) nmol litre^{-1}) (P < 0.01). Halothane did not change B_{max}, but isoflurane increased it significantly. Cocaine protected CFT sites from N-ethylmaleimide alkylation, but neither anaesthetic did. Photoaffinity labelling with halothane significantly inhibited [3H]-CFT binding compared with UV-exposed controls. We conclude that clinically relevant concentrations of both anaesthetics inhibit high-affinity CFT binding, and the data suggest overlapping sites for halothane and CFT. (Br. J. Anaesth. 1994; 73: 820-825)

Key words

The inhalation anaesthetics halothane and isoflurane have been shown to non-competitively inhibit dopamine transport into rat brain synaptosomes [1], suggesting allosteric inhibition of the dopamine transporter. This in vitro result may explain in vivo microdialysis studies which show an increase in extracellular dopamine concentration with induction of halothane anaesthesia [2-4]. Furthermore, because systemic administration of the dopamine precursor, L-dopa, has been shown to reduce the ED_{50} (minimum alveolar concentration, MAC) of halothane by approximately 50% in mice [5], elevated synaptic dopamine concentrations may be a component of the mechanism of halothane anaesthesia. Because cocaine and its analogues are also non-competitive antagonists of the dopamine transporter [6], we hypothesized that the inhalation anaesthetics produce inhibition of the transporter by interaction at this same site. We explored this question in rat brain synaptosomes and found evidence for a competitive interaction between cocaine and halothane, but not isoflurane, which may explain the previously reported inhibition of the dopamine transporter by these two inhalation anaesthetics.

Materials and methods

CHEMICALS
[3H]-2β-Carbomethoxy-3β-(4-fluorophenyl)-tropane ([3H]-CFT), a potent cocaine analogue [7] with a specific activity of 83.4 Ci mmol^{-1}, was purchased from Dupont-NEN (Boston, MA, USA). Cocaine HCl was obtained from Astra Pharmaceutical Products (Westborough, MA, USA), halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) was obtained from Halocarbon Laboratories (Hackensack, NJ, USA) and the preservative thymol was removed using an activated alumina column [8]. Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) was obtained from Anaquest Corp (Murray Hill, NJ, USA). Other chemicals were of analytical grade and were purchased from Sigma (St Louis, MO, USA).

TISSUE PREPARATION

Animal studies were approved by the Institutional Animal Care and Use Committee. Whole brains (excluding the cerebellum and midbrain, approximately 2 g) of male adult Sprague-Dawley rats were processed as follows: brains were removed rapidly from animals anaesthetized with pentobarbitone 50 mg kg^{-1} i.p., washed, minced and homogenized in 20 volumes of ice cold sucrose solution 0.32 mol litre^{-1} using a Potter-Elevehjem glass homogenizer with a Teflon pestle. The crude homogenate was centrifuged at 1000 \times g for 15 min, the pellet resuspended and homogenized again in sucrose 0.32 mol litre^{-1} and then centrifuged at 1000 g for 15 min. The combined supernatants were recentrifuged at 30000 g for 30 min. The final pellet (P2 fraction) containing synaptosomal membranes was resuspended in 5 volumes of 2°C Tris 10 mmol litre^{-1}-NaCl buffer 100 mmol litre^{-1}, pH 7.3, and used immediately. Protein concentration was determined by Coomassie blue assay [9]. Preliminary studies indicated that the low remaining concentration (<1 \mu mol litre^{-1}) of barbiturate had no effect on [3H]-CFT binding.

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Binding experiments were performed in Teflon-sealed microvials (600-μl capacity) containing various concentrations of \(^3\)H-CFT (see below) in Tris 10 mmol litre\(^{-1}\)–NaCl buffer 100 mmol litre\(^{-1}\), pH 7.3, and about 300 μg of synaptosomal protein. Included in half of the vials was cocaine 100 μmol litre\(^{-1}\) to determine non-specific binding. Vials were incubated at 2 °C for 90–120 min (preliminary experiments showed stable binding between 30 and 120 min). Bound and free label were separated by vacuum filtration using Whatman GF/B filters pre-soaked in 0.05% poly(ethyleneimine). Filters were washed with 9 ml of 2 °C saline 154 mmol litre\(^{-1}\) and incubated for at least 12 h with scintillation fluid in vials before counting in an LKB Wallac 1209 counter for 3 min.

**COMPETITION EXPERIMENTS**

The inhalation anaesthetics halothane and isoflurane were added to the microvials as possible competitors for \(^3\)H-CFT binding. At fixed concentrations of \(^3\)H-CFT, increasing concentrations of halothane and isoflurane (0.01 to approximately 5 mmol litre\(^{-1}\)) were produced by injecting appropriate volumes of higher concentrations dissolved in buffer using Hamilton (Reno, NV, USA) gastight syringes into the capped Teflon-sealed microvials. This final addition of anaesthetic solution completely filled the microvials, which were then vortexed, incubated at 2 °C for 2 h, filtered and counted as described above. In order to verify anaesthetic concentrations, parallel microvials containing only buffer and experimental concentrations of anaesthetic were also incubated for 2 h and subjected to gas chromatographic analysis after hexane extraction. Competition data were transformed to percent of control (0 mmol litre\(^{-1}\) anaesthetic) and fitted with sigmoid curves with negative Hill coefficients using non-linear least squares regression. Maximum binding (100%) was constrained. The inhibition constant (\(K_d\)) was calculated from the IC\(_{50}\) using the predetermined \(K_d\) for CFT and the known \(^3\)H-CFT concentration in the experiments.

**BINDING ISOTHERMS**

In order to determine the anaesthetic influence on variables of the CFT saturable binding component, fixed concentrations of anaesthetic (halothane 1 mmol litre\(^{-1}\) or isoflurane 1.7 mmol litre\(^{-1}\), final concentration) were produced in the presence of increasing concentrations of \(^3\)H-CFT (0.75 mmol litre\(^{-1}\), final concentration). Control experiments (0 mmol litre\(^{-1}\) anaesthetic) were also performed. Both sets of experiments were conducted at 2 °C with a 2-h incubation period. Scatchard analysis was used to determine \(K_d\) and \(B_{max}\) (EBDA/LIGAND software) [10]. The low CFT concentration range used did not allow examination of low-affinity sites reported previously [6, 7], and thus the data were fitted to single site models.

**PROTECTION ASSAYS**

To assess if the anaesthetics may protect the cocaine site from alkylation, as with cocaine analogues [11], we preincubated synaptosomal membranes in \(N\)-ethylmaleimide (NEM) in four groups. These groups were incubated for 30 min at 2 °C with cocaine 100 μmol litre\(^{-1}\), isoflurane 8 mmol litre\(^{-1}\), halothane 5 mmol litre\(^{-1}\) or no addition (NEM control) and then an additional 30 min after adding sufficient NEM to achieve final concentrations of 0.1, 1.0 and 10.0 mmol litre\(^{-1}\). Control samples were processed identically except that nothing was added during each incubation period. After the second incubation, all groups were diluted with Tris 5 mmol litre\(^{-1}\)–NaCl buffer 100 mmol litre\(^{-1}\), pH = 8, and centrifuged at 30000 × g for 30 min at 4 °C. Pellets were resuspended and washed by centrifugation twice. The final pellets were resuspended in 5.0 ml of buffer and used in binding assays immediately, as described above. Pilot studies showed complete removal of the anaesthetics from the membranes by this washing procedure, as indicated by recovery of control levels of CFT binding.

**PHOTOAFFINITY COMPETITION EXPERIMENTS**

We have shown previously that halothane may be used as a photoaffinity ligand [12, 13]. With exposure to UV light, debromination of halothane yields a chlorotrifluoroethyl (CFTE) radical [14] which binds covalently near to its site of generation [15]. Because we were concerned that the binding kinetics of these small molecules to protein sites may be too fast to allow detection with the above NEM protection experiments (see Discussion), we performed photoaffinity competition experiments in which CFT binding was evaluated after covalent attachment of halothane to synaptosomes with UV light. Synaptosomes were prepared as above and separated into three fractions. Two fractions were exposed for 50 s to 254-nm light (Oriel low pressure Hg calibration lamp, distance 6 mm), in 5-mm path-length quartz cuvettes with continuous stirring; one of these fractions contained halothane 1 mmol litre\(^{-1}\) (final concentration). The last fraction was a non-exposed control. The fractions were diluted and washed three times as in the NEM experiments, and used immediately in \(^3\)H-CFT binding assays, as described above.

**Results**

The cocaine analogue CFT bound specifically and with high affinity to synaptosomal membranes prepared from rat brain. Preliminary experiments (data not shown) demonstrated the well established sodium dependence of CFT binding [6]. Because we also found that \(^3\)H-CFT binding was inhibited by Tris concentrations greater than 50 mmol litre\(^{-1}\), we used routinely Tris 10 mmol litre\(^{-1}\) in these experiments. The binding isotherms in the absence of anaesthetics demonstrated saturation with an apparent mean \(K_d\) of 8.0 (SEM 0.8) nmol litre\(^{-1}\) and a \(B_{max}\) of 229 (50) fmol/mg protein, in agreement.
Table 1  Binding variables for $^3$H-CFT to synaptosomes (mean (SEM) of three experiments, each in triplicate). $^*P < 0.05$, $^{**}P < 0.001$ compared with controls.

<table>
<thead>
<tr>
<th>Group</th>
<th>$K_d$ (nmol litre$^{-1}$)</th>
<th>$B_{max}$ (fmol/mg protein)</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.0 (0.8)</td>
<td>229 (50)</td>
<td>0.97 (0.1)</td>
</tr>
<tr>
<td>Halothane</td>
<td>13.8 (1.0) $^*$</td>
<td>219 (61)</td>
<td>0.96 (0.1)</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>29.8 (12.8) $^{**}$</td>
<td>387 (45)</td>
<td>0.98 (0.1)</td>
</tr>
</tbody>
</table>

Figure 1  Representative Scatchard plots for binding isotherm experiments: control (○), halothane 1 mmol litre$^{-1}$ (△) and isoflurane 1.7 mmol litre$^{-1}$ (□). The lines represent least squares linear regression of the data.

Figure 2  Competition experiments for halothane (△) and isoflurane (□), expressed as percent of control $^3$H-CFT binding (0 anaesthetic) (mean (SEM)). Data were fitted by non-linear least squares to sigmoid plots of approximately −1 Hill coefficient and fixed upper limit (100%). $K_i$ values are given in the text.

with published data for this cocaine analogue in synaptosomal preparations [7]. The results were different for the two anaesthetics studied. Halothane significantly decreased only the apparent $K_d$, while isoflurane altered both $K_d$ and $B_{max}$ (table 1, fig. 1). Competition experiments with the anaesthetics demonstrated near complete inhibition of CFT (~1 nmol litre$^{-1}$) binding and an apparent $K_i$ of 0.61 (0.12) nmol litre$^{-1}$ for halothane and 0.75 (0.12) nmol litre$^{-1}$ for isoflurane (fig. 2). Competition experiments with higher concentrations of CFT (6 nmol litre$^{-1}$) showed essentially an identical $K_i$ value for halothane (0.64 (0.10) nmol litre$^{-1}$), whereas that for isoflurane was slightly higher (1.03 (0.66) nmol litre$^{-1}$) (ns). The larger variation with isoflurane was caused by its consistent ability to increase $^3$H-CFT binding at low isoflurane concentrations and the difficulty of fitting sigmoid curves to this pattern.

The NEM protection assays demonstrated that although cocaine 100 µmol litre$^{-1}$ during NEM exposure (0.1, 1.0 or 10.0 mmol litre$^{-1}$) significantly protected CFT binding sites (fig. 3) from alkylation, neither halothane 5 mmol litre$^{-1}$ nor isoflurane 8 mmol litre$^{-1}$ offered significant protection compared with no addition.

The photoaffinity competition experiments demonstrated that although UV exposure alone reduced specific CFT binding to 55.2 (3.2)% of control (non-UV exposed) values, there was a significant further reduction to 29.8 (1.8)% when the UV exposure was conducted in the presence of halothane 1 mmol litre$^{-1}$ ($P < 0.01$ compared with UV-only values) (fig. 4). Because this further reduction in CFT binding by halothane and UV light could also be caused by CTFE free radical-induced damage, independently of covalent occupation of the CFT site, we compared the influence of generation of free radicals by UV photolysis of other photolabile molecules. Benzoquinone (BQ) and 5-diazouracil (5-DU) form free radicals on exposure to UV light. UV absorbance at 254 nm was used to match the concentration of these compounds to halothane under the assumption that concentrations that produce similar absorbance at this wavelength also produce similar free radical flux. These concentrations (BQ 5 µmol litre$^{-1}$ and 5-DU 10 µmol litre$^{-1}$) were then tested for inhibition of $^3$H-
CFT binding without photolysis, to assure lack of competition with CFT. No significant inhibition of CFT binding occurred at these concentrations. After photolysis in the presence of these compounds, no further reduction in $^3$H-CFT binding occurred compared with UV-exposed controls. In fact, a non-significant trend towards protection from UV-induced damage was observed (110 (7.0) % and 104 (5) % for 5-DU and BQ, respectively, compared with UV only).

Discussion

The precise mechanism by which cocaine and its analogues cause inhibition of the dopamine transporter is not known. Although some kinetic studies demonstrated competitive inhibition [16, 17], others suggested non-competitive kinetics [18]. Also, site alkylation by NEM [11] and site-directed mutagenesis [19] studies provide more direct evidence that the cocaine binding site is allosteric with respect to the substrate (dopamine) site. Recent cloning and expression of both rat [20] and human [21] dopamine transporters should help to resolve this issue. It is notable that 92 % homology exists between dopamine transporters from the two species, permitting reasonable extrapolation of these results to the human protein.

The data presented here demonstrated that the inhalation anaesthetic halothane inhibited $^3$H-CFT binding with an apparent $K_i$ very similar to the $IC_{50}$ for inhibition of dopamine [1] and serotonin transport [22]. The inhibition of $^3$H-CFT binding by halothane appeared to be competitive by Scatchard analysis of binding isotherms and the results of the photoaffinity competition experiments were consistent, suggesting overlapping sites for halothane and CFT. The inhibition of $^3$H-CFT binding by isoflurane is more complex, having mixed kinetic characteristics. In addition, the $K_i$ for isoflurane inhibition of dopamine and serotonin transport, about 2.3 mmol litre$^{-1}$ [1, 23] was substantially higher than that for inhibition of $^3$H-CFT binding, suggesting that the two may not be related, at least in the case of isoflurane. Unfortunately, the site of isoflurane binding could not be evaluated with photoaffinity competition methods because of its photostable structure. Similarly, the ability of cocaine to protect the site from halothane photo-labelling would be difficult to interpret because of the photolabile nature of cocaine. The complex inhibition of CFT binding by isoflurane may be related to the biphastic nature of inhibition of dopamine transport noted previously [1]. An increase in CFT binding at low isoflurane concentrations (ns) was suggested in these studies and is consistent with the increase in $B_{\text{max}}$ noted in the presence of this anaesthetic. A biphastic binding influence of isoflurane has also been observed in other proteins, such as the GABA$_A$ complex [24]. The mechanism or implication of this complex, presumably allosteric influence of isoflurane, is not yet clear, but it emphasizes that substantial differences can exist between the effects of these small molecules and argues against the notion of a unitary mechanism of action of inhalation anaesthetics.

It should be noted that the functional dopamine uptake experiments were performed at higher temperatures (22–37 °C) than our binding studies (2 °C). Although it is well known that gas–buffer and gas–membrane partition coefficients (binding) change dramatically with temperature [25], our method of direct injection and dilution of aqueous anaesthetic stock solutions, combined with evidence suggesting that buffer-membrane partitioning is stable over a wide temperature range (4–40 °C) [25], implies that binding variables are temperature independent. We evaluated this in a small series of experiments with two concentrations of each anaesthetic (halothane 0.11 and 0.83 mmol litre$^{-1}$; isoflurane 0.66 and 2.0 mmol litre$^{-1}$) in binding experiments at 37 °C and found the percent inhibition of CFT binding to be well within the standard error limits (slightly to the left of the mean) of the 2 °C curve. Therefore, the apparent $K_i$ does not appear to have a major temperature dependence, as expected.

Previous studies using NEM alkylation suggested that certain sulphhydryl groups are essential for cocaine binding [11]. Importantly, dopamine was unable to protect the cocaine site, demonstrating its allosteric nature. Our experiments demonstrated that the CFT site was not protected by either halothane or isoflurane, implying that the anaesthetic and CFT sites do not overlap, in contrast with the photoaffinity competition experiments and the Scatchard analysis (for halothane). It is not clear, however, if ligands with substantially faster dissociation rates than the high-affinity "natural" ligand (e.g. cocaine) would be able to afford site protection in this non-equilibrium alkylation assay. The dissociation rate for cocaine is six or seven orders of magnitude slower than that of halothane, as
estimated by halothane dissociation rates from serum albumin by NMR studies [26], and published dissociation times for cocaine [6]. This was the rationale for conducting the photoaffinity competition experiments, as the creation of a covalent bond prevents dissociation of halothane from its binding site.

Results of the photoaffinity competition experiments suggested that halothane and CFT sites overlap because covalent linkage of the halothane photolysis product near the equilibrium binding site for halothane significantly reduced CFT binding. Quantitation is difficult, however, because photolysis of halothane is inefficient and is not complete in only 50 s of UV exposure. Further, because UV light causes site destruction independently, longer UV exposures would be difficult to interpret.

Alternative interpretations of the photoaffinity competition results are possible. The data could be consistent with non-overlapping sites. The dopamine transporter conformational state which binds CFT at lower affinity, possibly because of an allosteric binding by the anaesthetic, may now become a “fixed” conformational state because of covalent occupancy of that site. However, it seems unlikely that the covalently bound CTFE group, the most likely adduct formed by photolysis of halothane [14], which lacks the important and bulky bromine atom, would induce the same conformational change as halothane itself.

The in vivo relevance of anaesthetic competition at the cocaine binding site is not clear. The high-affinity cocaine (CFT) binding sites investigated in this study are thought to be located on catecholamine transporters, transmembrane proteins which are responsible for termination of catecholamine transmission by reuptake into presynaptic terminals. Inhibition of such transporters by cocaine and its analogues increases the synaptic concentration of catecholamines and presumably enhances such transmission. This is thought to be the mechanism of cocaine-induced euphoria [6], especially as it relates to dopaminergic transmission. It is difficult to know if anaesthetics cause euphoria, as at these concentrations (2–3 times MAC) they also produce unconsciousness, presumably because of actions at other neuronal targets. However, it is attractive to speculate that an important side effect of halothane, its cardiac arrhythmogenic potential [27], is caused by this cocaine-like influence on catecholamine transport in the heart, especially as the $K_I$ for CFT binding is similar to the $ED_{50}$ for halothane (for movement), approximately 0.3 mmol litre$^{-1}$. Also, it is well known that isoflurane has a significantly lower potential to produce arrhythmia and it is a less potent inhibitor of dopamine [1] or serotonin [23] transport and CFT binding (this study). Further studies of noradrenaline transport are required to examine this question. Finally, the suggestion of overlapping sites for halothane and CFT, and the different effects of the two agents, support the notion that at least some effects of inhalation anaesthetics are mediated by direct interaction with protein and that this influence is different for different inhalation anaesthetics.

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