Do nitrous oxide and halothane influence opioid receptor binding in SH-SY5Y human neuroblastoma cells?

D. J. CAMPBELL, D. J. ROWBOTHAM AND D. G. LAMBERT

Summary

The site of interaction of opioids and inhalation anaesthetic agents is unknown, but may be at the level of the opioid receptor. In this study we have used SH-SY5Y human neuroblastoma cells, which express both μ and δ receptors, to examine the effects of halothane on the receptor binding profiles of [3H] diprenorphine (DPN), an opioid receptor antagonist, and [3H] β-Ala², MePhe⁴, Gly(ol)⁵ enkephalin (DAMGO), a μ receptor selective agonist. Binding of [3H] DPN and [3H] DAMGO was performed at 37 °C for 60 min in the presence of air, nitrous oxide (75 %) or air containing halothane (0.5–5.0 % v/v). Compared with air controls, neither 75 % nitrous oxide nor 0.5, 1.0, 2.0 and 5.0 % halothane influenced DPN binding variables. Binding of [3H] DAMGO was unaffected by 1.0 % halothane, but 5.0 % halothane reduced the affinity, with a modest increase in Kd (1.15 (0.16) to 1.7 (0.2) nmol litre⁻¹) without effect on Bmax. Our data suggest that the site of interaction may be at a cellular level.

Key words


Clinically, opioid drugs and inhalation anaesthetic agents are known to interact, with a significantly increased potency of inhalation agents in the presence of opioids [1]. In vitro data in rats on the interaction between morphine and halothane on movement and heart rate responses to noxious stimuli indicate that the interaction is essentially additive [2]. In vitro data, although limited, suggest that the site of interaction may be at a cellular level. Halothane dramatically potentiated the inhibitory action of morphine on acetylcholine release from the guineapig ileum preparation [3], and potentiated the negative chronotropic effect of a specific kappa (κ) opioid agonist on an isolated right atrial rat heart preparation [4].

Only a few studies have examined if inhalation anaesthetic agents influence the binding of opioids to opioid receptors, and evidence to date is contradictory. Two early studies using rat brain homogenates [5, 6] found no interaction between halothane and mu (μ) opioid receptors, whereas a later study using guineapig brain [7] demonstrated that halothane inhibited both μ and δ opioid receptor binding. Nitrous oxide has been reported to inhibit μ agonist binding [8], to have specific μ agonist activity [9] or to have no effect [5] on μ opioid binding. When considering the structural similarity of all the G protein-coupled receptors [10], it is hard to reconcile marked inhibitory effects of halothane on opioid binding (as reported in [7]) with reports that adrenergic and muscarinic receptors appear relatively resistant to clinical concentrations of halothane [11].

A potentiating effect of anaesthetic agents on opioid–receptor interaction could explain the well-documented [1, 12–14] reduction in the MAC values of inhalation anaesthetics in the presence of opioids. Alternatively, the biochemical explanation of this inhalation anaesthetic–opioid interaction may lie more distally in the opioid signal transduction pathway: at the G protein, at the effector enzymes, adenylyl cyclase, phospholipase C, or both, or at ion channels. In either case, it is necessary to see if there is an effect of inhalation anaesthetics at the level of the opioid receptor before further investigation of the possible interaction of anaesthetic agents with opioid intracellular pathways.

In this study we have used SH-SY5Y human neuroblastoma cells, which express both μ and delta (δ) receptors [15], to examine the effects of halothane and nitrous oxide on the opioid receptor binding profiles of [3H][15,16(n)]diprenorphine (DPN), a non-selective opioid antagonist, and [3H][β-Ala²-MePhe⁴,Gly(ol)⁵]enkephalin (DAMGO), a μ selective agonist.

Materials and methods

CELL CULTURE AND HARVESTING

SH-SY5Y human neuroblastoma cells (passages 70–90) were cultured in minimum essential medium supplemented with l-glutamine 2 mmol litre⁻¹, penicillin 100 u. ml⁻¹, streptomycin 100 μg ml⁻¹, fungizone 2.5 μg ml⁻¹ and 10 % fetal calf serum, at 37 °C in 5 % carbon dioxide–humidified air.

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Inhalation agents and opioid binding

Table 1 Aqueous halothane concentrations estimated by gas chromatography, and equivalent % atm values (mean (SEM) n = 4). Halothane was equilibrated by surface “blowing” over 1 ml of buffer solution at 37 °C. The equivalent % atm is calculated assuming that 1.0 % atm halothane at equilibration with aqueous phase produces a concentration of 280 μmol litre⁻¹ [22].

<table>
<thead>
<tr>
<th>Vaporizer dial setting (%)</th>
<th>Aqueous halothane concn (μmol litre⁻¹)</th>
<th>Equivalent % atm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>382 (56)</td>
<td>1.35 (0.40)</td>
</tr>
<tr>
<td>2.0</td>
<td>663 (36)</td>
<td>2.35 (0.26)</td>
</tr>
<tr>
<td>5.0</td>
<td>1357 (76)</td>
<td>4.84 (0.54)</td>
</tr>
</tbody>
</table>

We used whole cell suspensions for DPN, and cell membrane suspensions for DAMGO binding experiments. Whole cell suspensions could not be used in the latter experiments, as DAMGO binding is inhibited markedly by the presence of sodium, necessitating the use of a non-physiological buffer which would cause lysis of whole cells. Confluent cells were harvested with HEPES 10 mmol litre⁻¹-buffered saline–0.02 % EDTA, pH 7.4, washed twice with, and resuspended in, Krebs–HEPES buffer, pH 7.4, of the following composition (mmol litre⁻¹): Na⁺ 143.3, K⁺ 5.9, Ca²⁺ 2.6, Mg²⁺ 1.2, Cl⁻ 153.2, HPO₄²⁻ 1.2, SO₄²⁻ 1.2, glucose 11.7 and HEPES 10. For the preparation of membranes, cells were harvested as above, then washed twice with, and resuspended in, Tris–HCl buffer 50 mmol litre⁻¹, pH 7.4. The cells were homogenised, centrifuged twice at 20 000 g for 10 min at 4 °C, and resuspended in Tris–HCl buffer. Cell or membrane suspensions were kept at 0 °C until required and used within 3 h of preparation.

MEASUREMENT OF BINDING

Binding studies and displacement studies were performed in 1-ml assay volumes of Krebs–HEPES (for whole cells) or Tris–HCl (for membranes). Binding of [³H]DPN (~ 0.03–3.0 nmol litre⁻¹) or [³H]DAMGO (~ 0.05–5.0 nmol litre⁻¹) was performed at 37 °C for 60 min. Non-specific binding (i.e. binding to structures other than opioid receptors) was defined in the presence of naloxone 10 μmol litre⁻¹, and was subtracted from the total binding to yield the specific binding (i.e. opioid receptor specific). Displacement studies used a fixed, low concentration of radioligand and increasing concentrations (~ 0.1 nmol litre⁻¹ to 10 μmol litre⁻¹) of unlabelled fentanyl or morphine. Before addition of the cells or membranes, tubes were pre-equilibrated for 15 min with the humidified test gas (~ 200 ml min⁻¹ tube⁻¹); air containing 0.5–5.0 % v/v halothane, delivered by a calibrated Fluotec 3 vaporizer; 75 % nitrous oxide in 25 % oxygen; or air alone. The concentration of anaesthetic agent delivered was checked regularly using an anaesthetic agent monitor (Capnomac) or oxygen analyser. Buffer concentrations of halothane were measured by gas chromatography [16] and showed that equilibrium was reached within 10 min. The aqueous halothane concentrations following pre-equilibration (15 min) are shown in table 1. Gas delivery was continued throughout the 60-min incubation period.

Bound and free radioactivity were separated by rapid vacuum filtration using a Brandel cell harvester onto Whatman GF/B filters and washed with 3 × 4-ml aliquots of ice-cold buffer. Protein concentrations were determined according to the method of Lowry and colleagues [17]. Radioactivity was extracted overnight and measured by liquid scintillation spectroscopy.

DATA ANALYSIS

Saturation curves were analysed according to Scatchard [18] to yield the equilibrium dissociation constant (Kd) and maximum binding capacity (Bmax). In displacement experiments, the concentration of displacer producing 50 % displacement of specific binding (IC₅₀) was obtained by computer-assisted curve-fitting using GRAPHPAD (v 2.0), and corrected for the competing mass of [³H]DPN according to Cheng and Prusoff [19] to yield the affinity constant (K). All data are mean (SEM) of 4–8 experiments. Statistical analysis of Bmax, Kd and Kᵢ values was performed using analysis of variance (ANOVA) or paired Student’s t test as appropriate, and considered significant when P < 0.05.

Results

The binding of both [³H]DPN (fig. 1) and [³H]DAMGO (fig. 2) to SH-SY5Y cells or membranes was dose related and saturable. Scatchard plots of the binding of both ligands were linear. Bmax and Kd values varied with different cell batches, therefore the values for each anaesthetic agent and concentration were compared with those of their paired air controls (tables 2, 3).

Nitrous oxide 75 % had no effect on [³H]DPN binding (table 2) in comparison with (gassed) air controls. Halothane 0.5, 1.0, 2.0 and 5.0 % similarly had no effect. Binding of [³H]DAMGO (table 3) was unaffected by 1.0 % halothane, but was inhibited by 5.0 % halothane, with a modest increase in Kᵢ from...
1.15 (0.16) to 1.70 (0.20) nmol litre\(^{-1}\) (decreased affinity), without effect on Bmax (fig. 3). Nitrous oxide was not tested for its influence on \(^{3}H\)DAMGO binding.

In the displacement experiments, specific \(^{3}H\)DPN binding was displaced in a dose-dependent manner by both morphine and fentanyl. The calculated \(K_d\) was 390 (120) nmol litre\(^{-1}\) for morphine \((n = 5)\) and 11.1 (1.6) nmol litre\(^{-1}\) for fentanyl \((n = 7)\). Halothane 5.0 % had no effect on the binding of either opioid.

**Table 2** Effect of incubation with air, nitrous oxide or halothane on \(^{3}H\)DPN binding in SH-SY5Y cells. Data are mean (SEM) of 4–8 experiments. No significant differences (ANOVA).  

<table>
<thead>
<tr>
<th>Agent</th>
<th>(K_d) (nmol litre(^{-1}))</th>
<th>Bmax (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>0.15 (0.02)</td>
<td>107 (5)</td>
</tr>
<tr>
<td>75 % Nitrous oxide</td>
<td>0.15 (0.02)</td>
<td>102 (6)</td>
</tr>
<tr>
<td>Air</td>
<td>0.16 (0.02)</td>
<td>89 (3)</td>
</tr>
<tr>
<td>0.5 % Halothane</td>
<td>0.16 (0.02)</td>
<td>87 (9)</td>
</tr>
<tr>
<td>Air</td>
<td>0.15 (0.02)</td>
<td>63 (2)</td>
</tr>
<tr>
<td>1.0 % Halothane</td>
<td>0.13 (0.02)</td>
<td>55 (1)</td>
</tr>
<tr>
<td>Air</td>
<td>0.13 (0.02)</td>
<td>78 (7)</td>
</tr>
<tr>
<td>2.0 % Halothane</td>
<td>0.21 (0.05)</td>
<td>99 (20)</td>
</tr>
<tr>
<td>Air</td>
<td>0.12 (0.03)</td>
<td>45 (6)</td>
</tr>
<tr>
<td>5.0 % Halothane</td>
<td>0.14 (0.02)</td>
<td>45 (6)</td>
</tr>
</tbody>
</table>

**Table 3** Effect of incubation with air or halothane on \(^{3}H\)DAMGO binding in SH-SY5Y membranes. Data are mean (SEM) of 5 or 6 experiments. *P \(< 0.05\) compared with paired gassed air controls (Student’s \(t\) test).  

<table>
<thead>
<tr>
<th>Agent</th>
<th>(K_d) (nmol litre(^{-1}))</th>
<th>Bmax (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>0.92 (0.09)</td>
<td>69 (11)</td>
</tr>
<tr>
<td>1.0 % Halothane</td>
<td>1.05 (0.12)</td>
<td>71 (9)</td>
</tr>
<tr>
<td>Air</td>
<td>1.15 (0.16)</td>
<td>86 (11)</td>
</tr>
<tr>
<td>5.0 % Halothane</td>
<td>1.70 (0.20)*</td>
<td>80 (17)</td>
</tr>
</tbody>
</table>

Figure 2 A representative saturation curve, with superimposed Scatchard plot, for a single \(^{3}H\)DAMGO binding experiment to SH-SY5Y membranes \((\square = \text{total binding, } \wedge = \text{non-specific binding, } \blacktriangle = \text{specific binding})\).  

**Discussion**  
We have demonstrated that opioid binding in SH-SY5Y cells and membranes was relatively unaffected by clinical concentrations of nitrous oxide and halothane. Neither nitrous oxide nor halothane had any effect on the binding of \(^{3}H\)DPN, the non-selective opioid antagonist, suggesting that there was no influence of nitrous oxide or halothane on either \(\mu\) or \(\delta\) receptor binding. The small decrease in binding affinity of the \(\mu\) selective agonist DAMGO in the presence of 5.0 % halothane was similar to, but less marked than the effects of 2.0 % halothane on agonist (dihydromorphine) binding reported previously [7]. The differential effect of 5.0 % halothane on agonist vs antagonist binding is interesting but not remarkable; frequently it is found that antagonist binding is little affected by changes in, for example, sodium or guanine nucleotide concentrations, which may markedly influence agonist binding [15]. While these findings were not unexpected, it is worth considering why our results are at variance with some of these earlier investigations [7–9].

Ori, Ford-Rice and London [7] appeared to show that 100 % nitrous oxide affects the \(K_d\) of \(\mu\) opioid binding without affecting Bmax, but this was only in comparison with non-gassed samples. Comparison instead with their samples treated with 100 % oxygen reveals that there was no difference between the effects of nitrous oxide and oxygen on \(\mu\) binding.

As halothane has the properties of an organic solvent (which can dissolve membranes), the importance of examining effects at clinically relevant concentrations cannot be overemphasized. Franks and Lieb [20] reviewed the methodological pitfalls of studies with inhalation anaesthetics. They stressed the importance of experimental temperature in determining the actual tissue concentration of anesthetic and, further, the desirability of measurement of tissue concentration of anesthetic. In the present study, anaesthetic agent was delivered continuously to the samples before and during the binding reaction. Temperature was maintained at 37 °C, and gas chromatography was used (for
halothane), both to ensure that equilibrium was reached within the 15 min pre-incubation period and to confirm that the aqueous halothane concentrations were close to those expected. In contrast, the methods of Ori, Ford-Rice and London [7] were likely to result in tissue concentrations of halothane several times higher than intended [21], because of the marked temperature dependence of water–gas partition coefficients [22]. In their study, membrane homogenate was equilibrated with halothane at 0 °C, then added to the reagents, and the mixture sealed in tubes and warmed at 25 °C. No allowance was made for the much higher solubility of halothane at lower temperatures or for the fact that the sealed tube would cause the tissue concentration at 0 °C to be maintained even when warmed to 25 °C. No attempt was made to measure aqueous concentrations, which may well have been the equivalent of 10% halothane at a physiological temperature.

In summary, we have demonstrated only minor effects of clinical concentrations of halothane and nitrous oxide on μ and δ opioid binding; these results suggest that the site of opioid and anaesthetic agent interaction is not at the level of the opioid receptor. Further studies are currently underway to determine if there is an interaction in the opioid transduction cascade distal to the receptor.

Acknowledgements

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References

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