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Effects of Nitrous Oxide and Halothane on μ and κ Opioid Receptors in Guinea-pig Brain

Carlo Ori, M.D., Felicia Ford-Rice, B.S.,† Edythe D. London, Ph.D.‡*

The effects of two general anesthetics, nitrous oxide and halothane, and oxygen on μ and κ opioid receptor subtypes from guinea-pig brain were investigated. μ receptor binding was defined using [^{3}H]dihydromorphine as the ligand. Nitrous oxide (100%) and halothane (2%) decreased the [^{3}H]dihydromorphine binding affinity (Kd_{air} = 0.87 nM, Kd_{N_2O} = 1.45 nM, $Kd_{halothane}$ = 2.30 nM) without affecting the density of binding sites. A decrease in the [^{3}H]dihydromorphine binding affinity without influence on the density of binding sites was also observed in the presence of 100% oxygen (Kd_{O_2} = 1.40 nM). κ receptor binding was defined using [^{3}H](-)ethylketocyclazocine as the ligand, in the presence of 100 nM D-ala^2-D-leu^5-enkephalin and 30 nM morphine. While 100% nitrous oxide caused a slight decrease in [^{3}H](-)ethylketocyclazocine binding affinity (Kd_{air} = 0.24 nM, Kd_{N_2O} = 0.31 nM) and a substantial decrease in the density of binding sites ($Bmax_{air}$ = 115 fmol/mg protein, $Bmax_{N_2O}$ = 84 fmol/mg protein), halothane dramatically affected both the affinity ($Kd_{halothane}$ = 0.70 nM) and density ($Bmax_{halothane}$ = 38 fmol/mg protein). Oxygen (100%) reduced [^{3}H]dihydromorphine binding affinity. Differential effects of two anesthetics on the same receptor or distinct actions of the same anesthetic on different receptors could indicate the presence of specific targets for anesthetics at the membrane level. Conversely, effects of volatile anesthetics on opioid receptors could reflect a nonspecific perturbation of the lipidic and proteinaceous components of the membranes. (Key words: Anesthetics, gases: nitrous oxide. Anesthetics, volatile: halothane. Receptors, opioid: μ and κ. Theories of anesthetic action.)

NO AGREEMENT, at present, exists as to the molecular mechanisms underlying the production of an anesthetic state. It is conceivable that different anesthetics might produce a common endpoint by different mechanisms.[1] One site of action suggested for volatile and gaseous anesthetics has been the opioid receptors.[2,3] Opioid receptor involvement in nitrous oxide analgesia has been suggested by both *in vivo*[3,4] and *in vitro*[5,6] studies. Nitrous oxide has been described as a specific agonist at μ opioid receptors *in vitro*[7,8] with no effect at κ receptors.[9] In contrast, an *in vitro* study has indicated that halothane does not interact with μ opioid receptors.[10]

* Visiting Scientist from Istituto di Anestesiologia e Rianimazione, Universitã degli Studi di Padova, 35100 Padova, Italy.

† Predoctoral Student, University of Maryland, School of Pharmacy, Department of Pharmacology and Toxicology, Baltimore, Maryland.

‡ Chief, Neuropharmacology Laboratory, NIDA Addiction Research Center.

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Address reprint requests to Dr. London: Chief, Neuropharmacology Laboratory, NIDA Addiction Research Center, P.O. Box 5180, Baltimore, Maryland 21224.

The present study was designed to compare the action of two inhalational anesthetics, nitrous oxide and halothane, and oxygen on *in vitro* binding at μ and κ opioid receptor subtypes.

Materials and Methods

Adult male Hartley guinea-pigs were killed by CO_2 asphyxiation and were decapitated. The brains were removed rapidly and stored at −70° C. Methodology in the experimental protocol was approved by an intramural research animal welfare committee that follows NIH guidelines for compliance with the United States Department of Agriculture regulations, established under the Animal Welfare Act. Crude membranes were prepared fresh daily. The whole brain including cerebellum was weighed, thawed, and homogenized in 10 volumes (w/v) of Tris-HCl buffer, 50 mM, pH 7.4 at 0° C (Tris buffer) with a Brinkmann polytron homogenizer (setting 5, 20 s). After centrifugation at 40,000 × g for 10 min, the resulting pellet was resuspended in 10 volumes (w/v) of Tris buffer containing 100 mM NaCl and 50 μM GTP, and incubated at 37° C for 45 min to remove endogenous peptides. The suspension was centrifuged at 40,000 × g for 10 min, and the pellet was resuspended in 90 volumes (w/v) of Tris buffer. The suspension was stirred until used in the assays. Protein concentration was determined according to Lowry *et al.*[11]

The effects of nitrous oxide and halothane on opioid receptor binding were tested in membranes that were treated by bubbling 100% nitrous oxide or 2% halothane through oxygen (delivered *via* a calibrated Fluotec MK III vaporizer; Cyprane) into the membrane homogenates at a flow rate of 2 $l \cdot min^{-1}$ for 1 h at 0° C. Additional membrane preparations were tested similarly with 100% oxygen to determine if oxygen in the halothane treatment contributed to potential changes in binding parameters. A volume of 3.8 ml of treated or untreated (control/no gas bubbled) membranes was added to borosilicate tubes containing labelled and unlabelled drugs to initiate the reaction. The tubes were then immediately sealed and transferred to a water bath at 25° C for binding assays. μ and κ receptor binding were defined using [N-methyl-^{3}H]-dihydromorphine ([^{3}H]DHM) and (-)-[9-^{3}H(N)]-ethylketocyclazocine ([^{3}H](-)EKC) as the radioligands, respectively.[12] Samples used for μ receptor binding contained [^{3}H]DHM (specific activity 78.9 Ci/mmol; New England Nuclear Corp.), at concentrations ranging from

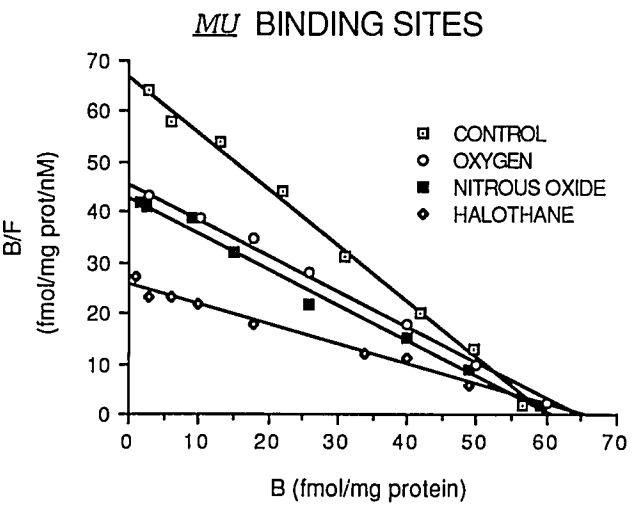

FIG. 1. Scatchard plot of [^{3}H]dihydromorphine binding to cerebral μ receptors. The concentration range was 0.05–10 nM. Each point represents the mean ± SEM of four separate experiments performed in triplicate. Lines were drawn from all points by best-fit linear regression analysis.

0.05 to 10 nM. Levorphanol (10 μM) was used to determine nonspecific binding. All the experiments involving [^{3}H]DHM were conducted in subdued lighting.

Samples used for assay of κ receptor binding contained (-)-[9-^{3}H(N)]-ethylketocyclazocine, [^{3}H](-)EKC (specific activity 30 Ci/mmol; New England Nuclear Corp.), at concentrations varying from 0.01 to 5 nM, and 100 nM DADL plus 30 nM morphine to prevent radioligand binding to δ and μ receptors, respectively. Levorphanol, 10 μM, was also used to assess nonspecific binding. Samples, prepared in triplicate, were incubated for 1 h (μ receptor) or 40 min (κ receptor). Free ligand was separated from bound radioactivity by reduced pressure filtration through Whatman GF/C glass fiber filters, using a Brandel cell harvester. The filters were rinsed with three 4-ml aliquots of ice-cold Tris buffer and placed in vials with 5 ml of Formula 963 scintillation cocktail (New England Nuclear Corp.). Radioactivity was measured by liquid scintillation spectrometry the following day using the Beckman Model LS 2800 counter.

TABLE 1. μ Receptor Binding

	Kd (nM)	Bmax (fmol/mg protein)
Control (no bubbling)	0.87 ± 0.05	59 ± 2.9
Nitrous oxide (100%)	1.45 ± 0.08*	62 ± 2.6
Halothane (2% through 100% O_2)	2.30 ± 0.19*†	63 ± 5.2
Oxygen (100%)	1.40 ± 0.08*	65 ± 2.7

Data are taken from figure 1. Each value represents the mean ± SEM for Kd and Bmax data generated from four separate experiments performed in triplicate. Both O_2 and N_2O produced slight increases in Kds for μ binding with no changes in Bmax. Halothane greatly decreased the affinity without affecting Bmax.

* Significantly different from control by Bonferroni test, $P < 0.05$.

† Significantly different from oxygen by Bonferroni test, $P < 0.05$.

Scatchard analysis was performed on saturation binding data obtained with the ligands [^{3}H](-)ethylketocyclazocine and [^{3}H]dihydromorphine, respectively, to determine if anesthetics altered the apparent maximum number (Bmax) of κ and μ receptors or the affinities (Kds) of the receptors for the radioligands. From the Law of Mass Action, $B = Bmax \cdot F/Kd + F$, where B is the amount of ligand bound (estimate of ligand-receptor complex) at any free ligand concentration (F), and Kd is the concentration of ligand at which binding is at half maximal saturation. Scatchard analysis is a linear transformation of B *versus* F (sigmoidal curve) into parameters of B/F *versus* B (linear function) at equilibrium.[13]

Scatchard analyses were performed using a Hewlett-Packard 9815A computer and linear least-squares regression analysis, giving estimates of goodness of fit by experimental data to linear regressions (r^2) and the 95% confidence limits of slopes (Kd) and x-axis intercepts (Bmax). Data were then analyzed by one-way analysis of variance. When a statistical treatment effect was detected, individual means were compared by Bonferroni *t* statistics. The criterion for statistical significance was $P \leq 0.05$.

Results

Scatchard plots of the binding of [^{3}H]DHM were linear (fig. 1). Therefore, only one class of binding site appeared to be labelled by this ligand throughout the range of concentrations used. The apparent Kd and Bmax for [^{3}H]DHM in the untreated membranes were 0.87 nM and 59 fmol/mg protein, respectively (table 1). Oxygen and nitrous oxide produced a statistically significant reduction in binding affinity of [^{3}H]DHM in an identical manner (Kd_{O_2}) = 1.40 nM, Kd_{N_2O} = 1.45 nM) but did not affect the density of binding sites ($Bmax_{O_2}$ = 65 fmol/mg protein, $Bmax_{N_2O}$ = 62 fmol/mg protein). Halothane treatment profoundly reduced the affinity of μ receptors ($Kd_{halothane}$ = 2.30 nM) but had no effect on receptor density ($Bmax_{halothane}$ = 63 fmol/mg protein).

Under the experimental conditions used in the assays, [^{3}H](-)EKC bound a single population of binding sites, with an apparent Kd of 0.25 nM and Bmax of 115 fmol/mg protein in the untreated membranes (fig. 2). Both oxygen and nitrous oxide produced slight but statistically significant decreases in the binding affinity of [^{3}H](-)EKC at κ receptors (Kd_{O_2} = 0.29 nM, Kd_{N_2O} = 0.31 nM), but only nitrous oxide significantly reduced the density of binding sites ($Bmax_{O_2}$ = 113 fmol/mg protein, $Bmax_{N_2O}$ = 84 fmol/mg protein (table 2). Halothane decreased both the Kd (0.70 nM) and Bmax (38 fmol/mg protein) for [^{3}H](-)EKC binding. The magnitude of these effects exceeded those of oxygen and nitrous oxide.

Discussion

The present study demonstrated that the inhalational anesthetics, nitrous oxide and halothane, can perturb specific binding of [^{3}H]DHM and [^{3}H](-)EKC to μ and κ opioid receptors in membrane preparations from guinea-pig brain. It was noteworthy that μ and κ receptors were affected differently by the anesthetics. Nitrous oxide decreased the affinity, but not the density, of μ sites, indicating a competitive inhibition at the receptor. However, the relatively large concentration of nitrous oxide used (estimated at approximately 30 mM in another study using experimental conditions similar to ours[6]) indicated that a nonspecific interaction at the target site could be involved.

Nitrous oxide reduced the apparent density of κ receptors, suggesting a noncompetitive inhibition. In a previous study on [^{3}H]naloxone binding in membranes of rat forebrain, Daras *et al.*[5] found that nitrous oxide decreased the affinity of high affinity sites and increased the affinity of low affinity sites, and revealed a "superhigh" affinity site. However, studies with [^{3}H]naloxone do not elucidate interactions with specific opioid receptor subtypes. Prior studies on the interaction of nitrous oxide at μ opioid receptors have produced conflicting results. No effect,[14] an inhibition of [^{3}H]DHM binding,[6] and the appearance of a superhigh affinity binding site[5] have all been reported. The relevance of the findings from *in vitro* receptor binding assays to physiological effects of nitrous oxide are unclear. However, an antagonistic action of nitrous oxide at κ receptors (*e.g.*, noncompetitive inhibition, as observed in the present study) agrees with data from a study of 15 human volunteers, 11 of whom showed naloxone-induced facilitation of nitrous oxide analgesia.[15] Thus, it is possible that part of the analgesic action of nitrous oxide may result from opioid antagonism.

Halothane produced a pattern of effect similar to that of nitrous oxide, but the magnitude of the halothane effect was markedly greater. Nonetheless, the underlying mechanism could be the same. This difference may be related to the greater potency of 2% halothane, compared with that of 100% nitrous oxide.[16] Halothane behaved in a competitive manner at the μ receptor, while it substantially influenced all the binding parameters at the κ receptor.

While the effect of halothane on κ receptors seemed unrelated to the fact that the halothane was delivered in oxygen, the decrease in μ receptor affinity could be due in part to oxygen. It seems possible that halothane may interact with oxygen in an additive or synergistic manner. We are aware of only two previous studies that addressed the interaction of halothane with opioid receptors, revealing no influence on either μ[10,14] or κ[10] receptor subtypes. Substantial dissimilarities between the methods used in those studies and in the present experiments might be the reason for the opposite results. Lawrence and

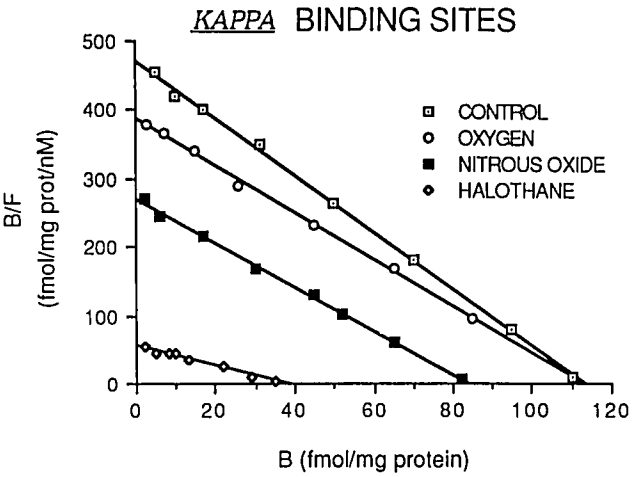

FIG. 2. Scatchard plot of [^{3}H](l)ethylketocyclazocine binding to cerebral κ receptors. The concentration range was 0.01–10 nM. Each point represents the mean ± SEM of four separate experiments performed in triplicate. Lines were drawn from all points by best-fit linear regression analysis.

Livingston[14] applied halothane solubilized in olive oil to the membranes, Inoki *et al.*[10] used aliquots of a solution saturated with the anesthetic, while we directly bubbled halothane with oxygen in the membranes preparation until saturation. Furthermore, Inoki *et al.*[10] used [^{3}H](±EKC) to label κ receptors, although it is not a reliable radioligand for this receptor subtype when employed in the absence of competing ligands for μ and δ receptors.[17] In contrast, we used [^{3}H](-)EKC, in the presence of adequate concentrations of morphine and D-ala^2-D-leu^5-enkephalin to block μ and δ opioid receptor binding. Finally, the different proportion of μ and κ receptors in rat and guinea-pig brain[18] might be another factor leading to discrepancies.

Oxygen promoted a statistically significant decrease in μ, but not κ, receptor affinity without an effect on the densities of the binding sites in the present study. The similarity of the effects of oxygen and nitrous oxide on μ

TABLE 2. κ Receptor Binding

	Kd (nM)	Bmax (fmol/mg protein)
Control (no bubbling)	0.25 ± 0.01	115 ± 2.8
Nitrous oxide (100%)	0.31 ± 0.02	84 ± 3.8*†
Halothane (2% through 100% O_2)	0.70 ± 0.04*†	38 ± 1.6*†
Oxygen (100%)	0.29 ± 0.01	113 ± 3.1

Data are taken from figure 2. Each value represents the mean ± SEM of Kd and Bmax data generated from four separate experiments each performed in triplicate. The values of Kd for κ binding sites were slightly but significantly increased both by O_2 and N_2O, but only N_2O altered Bmax. Halothane greatly decreased both affinity and density of κ binding sites.

* Significantly different from control by Bonferroni test, $P < 0.05$.

† Significantly different from oxygen by Bonferroni test, $P < 0.05$.

receptor binding in our study could be explained by an influence on opioid receptors through an oxidative mechanism.[8,19] An interaction of oxygen with μ receptors has been reported previously.[5] In that study, Daras *et al.*[5] found effects of oxygen at high affinity [^{3}H]naloxone binding sites in membrane preparations of rat forebrain that were similar to the effects on μ receptor binding in the present study.

The fact that oxygen, a nonanesthetic substance, increased the Kd of μ opioid receptors indicates that increases in Kd of μ receptors induced by nitrous oxide and halothane may be unrelated to the anesthetic or analgesic actions of these drugs. It is even possible that this effect may be a consequence of bubbling gas through the homogenate for 1 h. Nonetheless, the increase in Kd for κ receptors induced by halothane and decrease of Bmax for κ receptors seen with both anesthetics were not observed in response to bubbling with oxygen. Therefore, the effects on κ receptors may be more related to anesthetic or analgesic effects than the observed findings on μ receptors.

The effects of halothane on κ receptor binding suggest a mixed (competitive and noncompetitive) inhibition of κ receptor binding. The receptor binding data alone do not reveal whether the complex interaction of halothane with the κ receptor is agonistic or antagonistic. Furthermore, *in vivo* studies of halothane morphine interactions have suggested antagonism and agonistic activity of halothane in opioid systems, depending on the parameters measured and the doses of the drugs used. For example, halothane antagonized the effect of morphine on the reaction threshold to pressure and the cardiac acceleration response to tail clamp, but facilitated with motor response to tail clamp in rats.[20,21] Thus, the biological correlate of halothane interaction with opioid receptors is complicated. Furthermore, studies of physiological and behavioral interactions between halothane and opioids in intact animals may be complicated by indirect interactions of halothane with opioid systems.

It is well known that inhalational anesthetics affect the physical properties of lipids and proteins, and they produce various effects on complex biological models, such as membranes, enzymes, or receptors.[5,10,22] Although many examples of interactions between inhalational anesthetics and biological models are known, none of them clearly elucidates the mechanism of action of anesthetics. Opioid receptors represent a possible target influenced by general anesthetics, but are not necessarily involved in the production of the anesthetic state. The differential specificity of anesthetics on receptor subtypes in our experiments may depend on the physico-chemical properties of anesthetic molecules and different conformation of the receptor subtypes.

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