Carbon dioxide negatively modulates $N$-methyl-$d$-aspartate receptors

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Background. Carbon dioxide (CO$_2$) dose-dependently decreases minimum alveolar concentration (MAC) of anaesthetics in rats. CO$_2$ also dose-dependently decreases cerebrospinal fluid pH. N-methyl-$d$-aspartate (NMDA) channels exhibit pH sensitivity and are putative targets for inhaled anaesthetics. We hypothesized that CO$_2$ dose-dependently decreases rat NMDA channel current via an acidifying effect at concentrations relevant to CO$_2$ MAC.

Methods. To test this hypothesis, we studied rat NR1/NR2A glutamate receptors expressed in voltage-clamped *Xenopus* oocytes. To measure pH effects, we used perfusates adjusted between 7.3 and 5.3 with HCl. To measure CO$_2$ effects, we used equimolar sodium perfusates containing either 0 or 24 mM NaHCO$_3$ and CO$_2$ between 0% and 87% atm. Solution compositions were measured using a blood gas analyser with values corrected using a calibrated pH meter and gas chromatograph with solutions at 37°C.

Results. We found that decreasing pH decreased NMDA current. Moreover, pH effects produced by adding CO$_2$ to NaHCO$_3$-containing perfusates were identical to those produced by adding HCl to normal perfusates. The pH inhibiting 50% of NMDA current was 6.52. The CO$_2$ concentration inhibiting 50% of rat NMDA current was 63% for solutions with 24 mM NaHCO$_3$. CO$_2$ exhibited a linear dose-dependent NMDA response analogous to that observed for in vivo CO$_2$ anaesthetic potency in rats.

Conclusions. CO$_2$ and hydrogen ions act via the same mechanism to inhibit NMDA receptors. Moreover, CO$_2$ inhibits rat NMDA receptors in a manner that is consistent with CO$_2$ MAC-sparing effects in rats.

Keywords: anaesthesia; anaesthetic gases; carbon dioxide; hypercapnia; ion channels; $N$-methyl-$d$-aspartate; pH; receptor pharmacology

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Since at least the time of the Egyptian pharaohs, carbon dioxide (CO$_2$) has been used as an anaesthetic.$^1$ CO$_2$ is also a general anaesthetic, producing immobility to noxious stimuli at 1/3 atm in dogs$^2$ and at 1/2 atm in rats.$^3$ However, the mechanism for CO$_2$ general anaesthesia, as true for the mechanism for all inhaled anaesthetics, remains unknown.$^4$

The potency of conventional inhaled anaesthetics increases as a function of their lipid solubility.$^5$ However, the anaesthetic potency of CO$_2$ is much greater than would be predicted by its solubility in oil.$^6$ Unlike conventional inhaled anaesthetics, CO$_2$ decreases solution pH and decreasing cerebrospinal fluid (CSF) buffering capacity enhances CO$_2$ anaesthetic potency.$^2$ Thus, the narcotic effects of CO$_2$ may not be due to the CO$_2$ molecule itself. Rather, CSF acidification alone could be the mechanism for CO$_2$ anaesthesia.

The $N$-methyl-$d$-aspartate (NMDA) receptor is a glutamatergic heterotetrameric cation channel with high calcium conductance composed of NR1 and NR2 subunits.$^7$ NMDA receptors are inhibited by acidosis$^8$ and NMDA-evoked neurone potentials are decreased by hypercapnia.$^9$ NMDA antagonism is thought to contribute to the immobilizing action of volatile anaesthetics$^{10}$ and the gaseous anaesthetics nitrous oxide and xenon.$^{11}$ Likewise, NMDA antagonism could be important for CO$_2$ anaesthetic actions.

We hypothesized that CO$_2$ is an NMDA antagonist and that this antagonism, at least in part, explains CO$_2$ narcosis. To be correct, several criteria must be fulfilled.
CO₂ should significantly and reversibly inhibit NMDA currents in vitro at concentrations that significantly decrease anaesthetic requirements for other inhaled agents in vivo. CO₂ should also inhibit NMDA currents as a function of extracellular pH; therefore, more alkaline solutions should blunt CO₂ antagonism. Finally, since P<sub>CO₂</sub> produces a linear dose-dependent decrease in inhaled anaesthetic requirement in rats, P<sub>CO₂</sub> should also produce a linear dose-dependent decrease in rat NMDA current.

**Methods**

**Oocyte preparation and NMDA expression**

Adult female South African clawed toed frogs (Xenopus laevis) were anesthetized in a 0.3% tricaine bath and unilaterally ovarioctomized using a protocol approved by the Animal Use and Care Committee at the University of California, Davis. Eggs were defolliculated using 0.2% collagenase Type I ( Worthington Biochemical, Lakewood, NJ, USA) and stored in modified Barth solution [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 20 mM HEPES, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 5 mM sodium pyruvate, gentamicin, penicillin, streptomycin, filtered, pH 7.4]. All salts were of analytical grade and otherwise not possible using headspace gas chromatography and a standard benchtop pH meter. Oxygen was added as needed to the syringe headspace to ensure an O₂ tension between 12 and 33 kPa (90 and 250 mm Hg). Further CO₂ or air headspace additions and equilibrations were used to achieve solutions pairs having the following P<sub>CO₂</sub> (in % atm): 0, 10, 20, 30, 40, and 50. P<sub>CO₂</sub> of each solution was measured again at the end of each experiment.

For P<sub>CO₂</sub> plus bicarbonate test solutions, 24 mEq NaHCO₃ replaced an equimolar quantity of NaCl to make bicarbonate-BaFR (BaFR: 91 mM NaCl, 24 mM NaHCO₃, 2.5 mM KCl, 1.8 mM BaCl₂, 10 mM HEPES, 0.1 mM EGTA, filtered, pH 7.4) and bicarbonate-BaFREG (bicarbonate-BaFR plus 0.1 mM glutamate and 0.01 mM glycine). CO₂ was added to pairs of solutions and measured, as described above, to achieve the following P<sub>CO₂</sub> (in % atm): 0, 10, 20, 30, 40, and 85. Solutions in syringes containing 85% atm CO₂ were first saturated with 100% O₂ before CO₂ additions, and the syringe gas cap was never exposed to room air. P<sub>CO₂</sub> of each solution was measured again at the end of each experiment.
For pH test solutions, 1 M HCl was added to 13 pairs of BaFR and BaFREG solutions to generate a range of pH values between 5.3 and 7.4. The pH was measured at room temperature using a calibrated benchtop pH analyser (Accumet XL20, Fischer Scientific).

**Study design**

Oocytes were studied in a linear-flow perfusion chamber that had a 250 μl channel volume. All solutions were administered by a syringe pump (Pump 33, Harvard Apparatus, Holliston, MA, USA) at 1.5 ml min⁻¹ using glass syringes and PTFE tubing during which a −80 mV membrane potential was maintained using a standard two-electrode voltage clamp technique (GeneClamp 500B, Axon Instruments, Union City, CA, USA). After a 5 min baseline measurement during perfusion with BaFR, the perfusate was switched to BaFREG for 30 s followed by a 5 min washout with BaFR. This was repeated three to four times to verify constancy of the control agonist response (<10% change in peak current).

The perfusate was then switched to the test solution in BaFR for a 5 min washin, followed by a 30 s exposure to the counterpart test solution in BaFREG. Test solution washout with BaFR ensued for 5 min followed by another 30 s exposure to BaFREG. Data were only used for analysis if NMDA current responses after washout differed by ≤10% from responses before washin of the test solution. Data were recorded by commercially available data acquisition software (Chart, version 5, AD Instruments, Colorado Springs, CO, USA).

**Equipment calibration**

The automated blood gas analyser was used to measure pH and PCO₂ of test solutions, since placing solutions in open air vessels required for benchtop pH meter analysis and headspace gas chromatography would have resulted in CO₂ loss and consequently an increase in measured pH. However, the blood gas analyser is not normally calibrated over the range of PCO₂ and pH values used in this study; moreover, gas partial pressures are measured at 37°C which results in higher PCO₂ and lower pH values compared with measurements at ambient temperature (23°C).

A CO₂ standard curve was required to correct blood gas analyser values. Known CO₂ standard concentrations spanning the range of experimental values were generated using Dalton’s Law and a digital barometer (Fisherbrand Traceable, Fischer Scientific) and used to calibrate a gas chromatograph (Clarus 500, Perkin Elmer, Waltham, MA, USA). Chromatography was performed by gas injections directly on to a 1.8 m CTR-I concentrically packed column (Grace, Deerfield, IL, USA) with a 0.25 ml sample loop, 20 ml min⁻¹ carrier gas flow rate, 100°C oven temperature, and 120°C detector temperature. This produced a 1.4 min CO₂ retention time and excellent separation from other air component peaks. Gas caps of different air and CO₂ mixtures were added to gastight glass syringes containing 50 ml BaFR. After vigorous shaking, syringes were placed in a temperature-calibrated rotating hybridization incubator (Model 400, SciGene, Sunnyvale, CA, USA) at 37°C for 1 h. CO₂ concentration in the syringe headspace was then immediately measured by gas chromatography, and the BaFR P̄CO₂ was measured by the blood gas analyser. The resulting standard curve was linear only for ≤70% atm CO₂. Consequently, the P̄CO₂ of test solutions containing 87% CO₂ was calculated by subtracting the P̄O₂, which equalled 13.4 kPa (101 mm Hg) and fell within the instrument’s automated calibration range, from the total barometric pressure.

The pH values for all samples were corrected to 37°C. This was accomplished by constructing a standard curve for the benchtop pH meter using identical samples encompassing the entire study range measured at 25°C and 37°C. Because the pH of many test solutions were outside the automated calibration range, a standard curve was also constructed for the blood gas analyser and benchtop pH meter using samples measured at 37°C.

**Statistical analysis**

Data were described by mean (SEM). The NMDA current–pH response curve was fit to the Hill equation from which the median effective pH (pH₅₀) and Hill coefficient (n₅₀) were estimated using a pharmacology modelling programme (WinNonlin, Pharsight, Cary, NC, USA). Differences between the pH response curve and those for CO₂ or CO₂+HCO₃ were analysed using t-tests with Dunn-Sidak corrections for multiple comparisons. Buffering capacity of BaFR and BaFR+HCO₃ solutions was analysed by linear regression (SPSS v.11) of pH and log₁₀(CO₂) followed by a t-test of the slopes. Linear portions of the NMDA current–CO₂ response curves were analysed using linear regression, and the x-intercept was used to predict CO₂ concentration producing maximal NMDA channel inhibition. A P-value of <0.05 was considered significant.

**Results**

Baseline NMDA currents in the presence of maximal agonist (BaFREG) ranged from 0.8–8.5 μA, indicating good receptor expression in oocytes. In contrast, sham-injected oocytes did not exhibit any current change when exposed to BaFREG solution. A sample NMDA current tracing is shown in Figure 1. CO₂ and H⁺ both caused a concentration-dependent decrease in baseline NMDA current. After washout with BaFR solution, NMDA current responses to agonist were indistinguishable from baseline responses, indicating that effects of CO₂ and H⁺ on NMDA receptors are reversible (Fig. 1).

As expected, pH decreased as a function of increasing CO₂. The log-log regressions of H⁺ and CO₂ had an
R²>98% for solutions with and without bicarbonate (Fig. 2); the slope of each line equals the buffering capacity of the corresponding solution. Differences between the buffering capacities for solutions with and without bicarbonate were modest, but statistically significant. The ΔpH/Δlog(%CO₂) was -1.39 (0.10) and -0.87 (0.02) for BaFR with and without bicarbonate, respectively.

The sigmoid pH–current response curve (Fig. 3) was described by a Hill equation having an absolute maximum NMDA current change (ΔIₘₐₓ) equal to -98 (1)%; a current change without pH inhibition (ΔI₀) equal to 9 (3)%; a pH₉₀ equal to 6.52 (0.02); and an n_H equal to 23 (1). Since ΔIₘₐₓ - ΔI₀ = 107 and (pH₉₀)ₚH₃O⁺ = 6.31 x 10¹⁸, this relationship mathematically reduces to:

\[ ΔI = -98 + \frac{107 \times pH^{23}}{pH^{23} + 6.31 \times 10^{18}} \]

where ΔI is the per cent change in NMDA current. Although control measurements were performed at pH=7.4 at 23°C, the pH after temperature correction to 37°C was 7.24. If the Hill relationship is extrapolated back to pH=7.4, ΔI increases approximately 4%.

When plotted as a function of pH, the effects of CO₂+HCO₃ on NMDA current were indistinguishable from the effects of pH alone (Fig. 3). However, the addition of CO₂ alone to BaFR caused a left shift of the dose–response curve. Negative NMDA current modulation for the three highest values of the CO₂ curve—corresponding to the three lowest CO₂ concentrations in BaFR—was significantly less than for the comparable pH changes produced by HCl.

Equimolar NaCl replacement by NaHCO₃ reduced CO₂ potency as an NMDA antagonist (Fig. 4). Addition of 10% CO₂ caused a much more precipitous decrease in NMDA current for BaFR solutions without NaHCO₃, corresponding to a much greater decrease in solution pH. However, for larger partial pressures, absolute changes in NMDA inhibition for a given change in CO₂ were identical. Least squares slope estimates were -0.94 (0.06) and -0.95 (0.05).
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Discussion

CO₂ causes reversible and concentration-dependent inhibition of NMDA receptor current. Moreover, the magnitude, pH dependence, and rectilinear NMDA current responses all support the relevancy of this receptor to the mechanism of action for CO₂ anaesthesia.

High \( P_{CO₂} \) produces general anaesthesia. The minimum alveolar concentration (MAC), equal to the end-tidal anaesthetic concentration that prevents movement in response to a maximal noxious stimulus in 50% of individuals,\(^{14}\) is 32.6 kPa (245 mm Hg) in dogs\(^{2}\) and 53.6 kPa (403 mm Hg) in rats.\(^3\) Assuming an \( in vivo \) NMDA response similar to that for bicarbonate-buffered solutions, CO₂ at MAC should inhibit 20% of NMDA current in dogs and 40% in rats. Contemporary volatile anaesthetics at MAC inhibit NMDA current by 22–34%,\(^{15}\) and spinal NMDA receptors contribute to the immobilizing action of these agents.\(^{10}\) NMDA receptor inhibition likely also contributes to the mechanism of action of CO₂ anaesthesia. Since selective NMDA antagonists cannot by themselves cause complete anaesthesia in rats,\(^{10}\) CO₂ must act on other anaesthetic- or pH-sensitive targets also.

Carbon dioxide exerts its effects on NMDA receptors principally through the acidification of extracellular fluid rather than by a direct interaction between receptors and...
the CO₂ molecule itself. Increased H⁺ concentration has previously been shown to act on the NR1 subunit surface loop encoded by exon 5 to inhibit NMDA receptor function.¹⁶ When plotted as a function of pH, NMDA antagonism caused by HCl and CO₂ in bicarbonate-buffered solutions is identical. This suggests that H⁺ ions generated by carbonation likewise inhibit NMDA via an identical extracellular receptor effect on the exon 5 surface loop. In dogs, CO₂ potency is enhanced when CSF bicarbonate—and hence buffering capacity—is decreased. Moreover, halothane MAC-sparing effects are more closely correlated with CSF pH than PCO₂, with CSF pH approximately equal to 6.7 at 1 MAC of CO₂ in dogs.² The CSF pH in rats at 1 MAC of CO₂ is probably even more acidic, since the ED₅₀ of CO₂ is higher in rats than in dogs, resulting in an even greater degree of NMDA receptor negative modulation. Relevance of NMDA to CO₂ narcosis in vivo is thus supported by the pH-mediated NMDA antagonism of CO₂ in vitro.

Rat NMDA receptors exhibit rectilinear inhibition by CO₂ in bicarbonate-buffered solutions; that is to say, there is no threshold response observed. Similarly, CO₂ decreases volatile anaesthetic MAC rectilinearly in rats,³ except that the slope of the NMDA current response is only 40% of the slope of the MAC response. In dogs, however, there is a CO₂ threshold response to decreasing halothane MAC.² Yet, the CSF pH threshold for MAC reduction in hypercapnic dogs still approximates the pH at which significant rat NMDA inhibition was observed. Therefore, different CO₂ MAC responses between dogs and rats may not arise from differences in NMDA or other receptor pH sensitivity, but rather from differences in CSF bicarbonate or CSF buffering capacity in the animals studied. Formulation of BaFR perfusates with a higher either bicarbonate concentration or buffering capacity than in BaFR solutions for a given pH e. Thus, marked intracellular acidosis in BaFR+HCO₃ solutions with high PCO₂ may alter the structural conformation of transmembrane or intracellular portions of NMDA subunits, consequently nullifying the binding or efficacy of CO₂ at this second site of action.

The NMDA current pH₅₀ in this study was 6.52. However, in rat cerebellar granule cells, NMDA currents are 50% of maximum at pH=7.3.⁵ This discrepancy could result from differences between the NMDA subunits expressed or between the cellular expression models. Additionally, neuronal NMDA measurements were reported for room temperature. If corrected to 37°C using the BaFR standard curve from this study, the NMDA pH₅₀ in cerebellum neurones is approximately 7.1. Correcting pH and gas partial pressures to normal body temperature allows comparison of in vivo and in vitro physiologic responses for a comparable per cent of ionized imidazole moieties on the receptor protein.²⁰ Moreover, if identical systems at different temperatures are compared at the same Pco₂, then the cooler system will have a lower CO₂ activity and a higher solubility coefficient and aqueous CO₂ content. Hence, CO₂ partial pressure would appear a more potent NMDA inhibitor at lower temperatures because there is more dissolved CO₂. This is analogous to ED₅₀—temperature relationships observed with alcohols and inhaled anaesthetics, for which potency is also a function of aqueous—and not gas phase—concentration.²¹ ²² Nonetheless, the open probability and conductance through open NMDA receptors—and also agonist and antagonist association time constants with activated receptors—decrease as temperature decreases,²³ ²⁴ and hence the temperature used for measurement may still cloud to some degree in vivo and in vitro comparisons.

NMDA inhibition by CO₂ implies that hypventilation might exhibit the pharmacologic effects common to other NMDA antagonists, such as amnesia and analgesia. In evidence, the long-term potentiation, an NMDA-mediated enhancement of synaptic connections important for learning and memory, is inhibited in rat hippocampus slices by 20% CO₂.²⁵ Remarkably, humans breathing only 6.5% CO₂ perform markedly slower on reasoning tests and have a tendency, albeit not statistically significant, to forget new facts.²⁶ Furthermore, rats that inhale 70/30% CO₂/O₂ for 30 s exhibit antinociception to hot plate, tail flick, and tail pinch tests for at least 1 h after exposure.²⁷ If these phenomena are due in part to NMDA antagonism, then the present study would suggest that even small current decreases caused by CO₂ could have important and long-lasting effects, perhaps most importantly during and after...
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anaesthesia and surgery. Thus, the central nervous system effects of hypoventilation may be as widespread as the NMDA receptor itself.

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