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The effects of nitrous oxide on a glutamate-gated ion channel and their reversal by high pressure; a single channel analysis

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Abstract

Nitrous oxide reversibly affects the kinetics, but not the conductance, of the qGluR channel of locust muscle. 0.5 atm N_2O at 20.5° C was without effect but both 1.5 and 2.7 atm significantly reduced the probability of the channel opening, the frequency of opening and the mean open time, and prolonged the mean closed time. 100 atm helium was without effect on these parameters, but when 98.5 atm He was combined with 1.5 atm N_2O they, and the associated dwell time distributions, were restored to normal. 100 atm similarly combined with 2.7 atm N_2O exerted a comparable trend which fell short of significance. The results are consistent with nitrous oxide binding to the channel with a significant molar volume increase, which pressure opposes. This suggests that nitrous oxide may cause conformational changes in the channel, and that the pressure reversal of nitrous oxide anaesthesia in animals could be caused by molecular antagonism.

Keywords: L-Glutamate; Nitrous oxide; High pressure; Ion channel; Anesthesia

1. Introduction

The molecular basis of anaesthesia has intrigued chemists and physiologists for decades. The clinical potency of general anaesthetics, of which nitrous oxide is a classical example, is closely correlated with their solubility in reference solvents, such as octanol. Their chemical structure is relatively unimportant [1]. Furthermore in a number of behavioural experiments with vertebrates it has been shown that anaesthetic potency, including that of nitrous oxide, is reduced by high hydrostatic pressure [2]. The underlying mechanism for this is not clear. On the one hand pressure may act through an indirect physiological process or, on the other, it may act directly and thermodynamically, altering the putative anaesthetic–target interaction [3].

The complexity of the state of the general anaesthesia is such that various cellular and subcellular sites of action are likely to be involved, and it is not surprising that ion channels, particularly ligand gated channels, are now widely regarded as important [4]. In several cases general anaesthetics have been shown to block the channels in excitatory synapses (e.g., [5]) and enhance channel activity in inhibitory GABA-ergic synapses [6].

This paper is concerned with the quisqualate-sensitive glutamate-gated channel of locust muscle (qGlu-R), which has a number of features which make it relatively convenient for the hyperbaric experiments required with nitrous oxide, see Materials and methods and [7]. The partial pressure of nitrous oxide which causes anaesthesia in humans and rats is 1.01 and 1.5 atm, respectively [8,9]. The ED_{50} for the loss of the righting reflex in mice is 1.2–1.5 atm [10] and for newts at 20° C it is 0.7 atm [11]. Furthermore the experiments reported here use high hydrostatic pressure to study the nitrous oxide-channel interaction. The paper shows that near clinical doses of nitrous oxide affect the channel's kinetics and that 100 atm pressure reverses the effects.

2. Materials and methods

The qGlu-R channel in the metathoracic extensor tibiae muscle of adult female locusts (*Schistocerca gregaria*) was used. This cation selective channel has a conductance of 130 pS [12]. Here it was gated by 10^{-4} M L-glutamate in the patch pipette and single channel recordings were

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Table 1
Effect of nitrous oxide, 100 atm He and nitrous oxide in the presence of 100 atm on the qGlu-R channel; probability of opening, mean open and closed times and frequency of opening

	Probability of opening	Mean open time (ms)	Mean closed time (ms)	Frequency of opening (s^{-1})	
Control	0.031	1.25	36.30	27.16	10/4
0.5 atm N_2O	0.034	0.91	32.99	29.39	9/3
1.5 atm N_2O [a]	0.003	0.83	247.60	4.00	19/4
2.7 atm N_2O [b]	0.007	0.61	98.19	13.09	19/3
100 atm He	0.023	0.95	30.94	30.41	11/4
1.5 atm N_2O [c] at 100 atm	0.025	1.13	32.04	30.40	14/3
2.7 atm N_2O at 100 atm	0.010	0.72	83.68	15.25	10/4

Median values are given for individual recording sites. The number of sites/number of animals is shown in the right hand column.
Mann-Whitney tests of significance.
[a] Significantly different from control ($P < 0.002$) except for M_c ($P < 0.02$).
[b] Significantly different from control ($P < 0.05$) except for M_o ($P < 0.002$) and P_o ($P < 0.02$)
[c] Significantly different from 1.5 atm N_2O ($P < 0.002$) except for M_o ($P < 0.02$).

obtained using a mega-ohm seal patch electrode technique, with prior treatment of the muscle with 1–2 μM concanavalin A blocking desensitization [13]. These features enable recordings of single channel activity to be made in a high pressure vessel using an electrically driven micromanipulator to place the patch pipette onto the muscle membrane repeatedly, under pressure [7].

Standard locust saline was used to bathe the muscle (mM: 180 NaCl; 10 KCl; 2 $CaCl_2$; 10 Hepes (pH 6.8). The patch pipette contained the same solution except that RbCl was substituted for NaCl to enhance channel currents at the muscle's resting potential [14], plus 10^{-4} M glutamate. All recordings were carried out at 20.5 $\pm$ 0.5° C.

The preparation was equilibrated with a selected partial pressure of nitrous oxide, previously mixed with 1 atm air in a separate gas cylinder (both BOC, medical grade). The mixture was first flushed through the patch clamp pressure vessel to remove the contained air, and then the pressure was increased to give the selected pN_2O (0.5, 1.5 or 2.7 atm) additional to 1 atm of air. The preparation was also subjected to a selected pN_2O at a high pressure of helium (BOC) by preparing a nitrous oxide-air-helium mixture in a separate cylinder, such that on pumping the mixture into the patch clamp pressure vessel the selected pN_2O, was obtained, plus 1 atm of air and a pHe of 100 − pN_2O atm. The transfer of gases to the patch clamp vessel took approximately half an hour, after which a further 3 h was allowed for the nitrous oxide to equilibrate before recording began. The muscle membrane was immersed by 2–3 mm of saline, and equilibration proceeded by diffusion and convection, the latter arising from the temperature fluctuations ($\pm$5° C) which occurred during compression. The tip of the patch electrode was simultaneously immersed in the surface layer of the muscle bath and hence the solution within, close to the tip, also equilibrated with nitrous oxide. Three types of measurement demonstrated that the muscle membrane equilibrated within the 3 h period. First, an unstirred oxygen electrode mounted in a mock-up of the muscle bath showed that step changes in ambient, gaseous PO_2 equilibrated at a depth of 3 mm of saline in approx. 90 min, the rate of equilibration being much influenced by incidental convection within the bath. Second, comparable measurements using mass spectrometry to detect the influx of oxygen and nitrogen in a similar set up yielded an equilibration time of 60 min [15]. Third, the results of the present experiments, collected over a period of 3–5 h after the selected pressure was applied, showed no time dependence.

Single channel currents were filtered at 1 kHz prior to off line analysis using a single threshold crossing program and all data analyzed conformed to the criteria in [13], including a detailed inspection for, and rejection of, multiple superimposed channel currents. Individual recording sites yielded the probability of the channel being open (P_o), mean open time (M_o), mean closed time (M_c), and the frequency of channel opening (F_o). Dwell time histograms were prepared from data pooled from individual recording sites and exponentials were fitted [13].

3. Results

No effects on channel conductance were apparent, but a number of experiments qualitatively demonstrated the reversible effect of nitrous oxide on the opening/closing transitions. For example, 4 atm, and separately 8 atm N_2O abolished detectable channel activity which was restored within a few minutes of decompression. The results are

Fig. 1. The qGlu-R channel open and closed time distributions, shown as log probability density function [13] against dwell time in ms. (A) Open times, (B) closed times. (i) Control; (ii) 0.5 atm N_2O; (iii) 1.5 atm N_2O; (iv) 2.7 atm N_2O; (v) 100 atm He; (vi) 1.5 atm N_2O at 100 atm; (vii) 2.7 atm N_2O at 100 atm. Crosses represent experimental data, the dotted lines the exponential components and the continuous line the multi-exponential fit. See also Table 2.

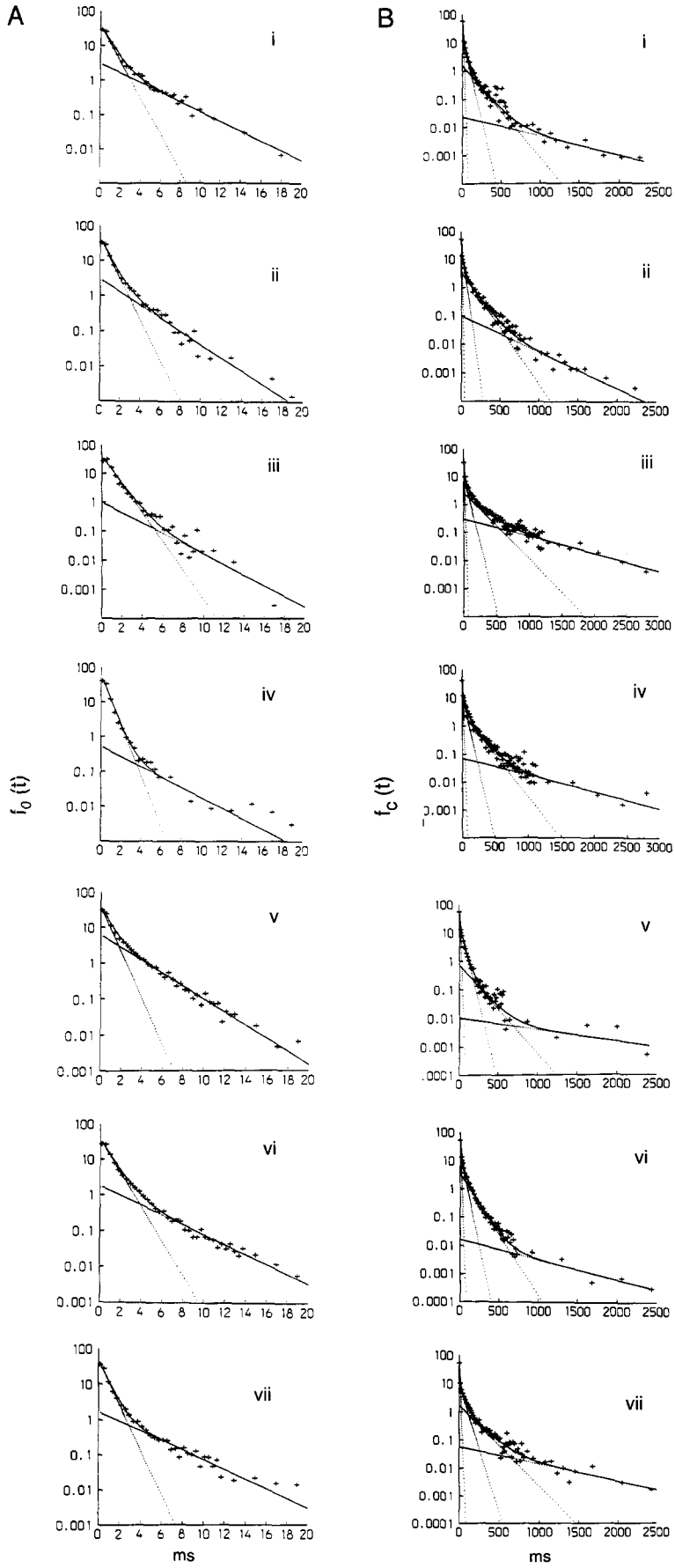
A
B
i
ii
iii
iv
v
vi
vii
f_0 (t)
f_c (t)
ms
ms

Table 2
Effect of nitrous oxide, 100 atm He and nitrous oxide in the presence of 100 atm on the qGlu-R channel

(A) Open times	r_1	Proportion of events	r_2	Proportion of events	
Control	0.825	(0.76)	3.065	(0.24)	10/20526
0.5 atm N_2O	0.750	(0.82)	2.337	(0.18)	9/14206
1.5 atm N_2O	0.872	(0.94)	2.413	(0.06)	19/23610
2.7 atm N_2O	0.594	(0.96)	2.929	(0.04)	19/31662
100 atm He	0.674	(0.63)	2.400	(0.37)	11/23752
1.5 atm N_2O at 100 atm	0.911	(0.85)	3.178	(0.15)	14/53997
2.7 atm N_2O at 100 atm	0.675	(0.86)	3.125	(0.14)	10/13210

(B) Closed times	r_1	Proportion of events	r_2	Proportion of events	r_3	Proportion of events	r_4	Proportion of events
Controls	4.780	(0.575)	36.30	(0.302)	131.5	(0.114)	618.0	(0.008)
0.5 atm N_2O	2.821	(0.528)	22.71	(0.288)	110.5	(0.175)	342.6	(0.015)
1.5 atm N_2O	5.447	(0.299)	48.20	(0.305)	196.1	(0.274)	575.7	(0.121)
2.7 atm N_2O	5.631	(0.369)	41.51	(0.336)	145.0	(0.263)	724.0	(0.031)
100 atm He	5.343	(0.521)	39.75	(0.414)	140.1	(0.058)	1032	(0.006)
1.5 atm N_2O at 100 atm	5.426	(0.457)	33.49	(0.331)	101.4	(0.205)	610.5	(0.006)
2.7 atm N_2O at 100 atm	5.662	(0.547)	47.18	(0.277)	149.8	(0.153)	692.9	(0.023)

(A) Open time distributions and (B) closed time distributions. The time constants giving the best fits to pooled data are shown, two for the open states and four for the closed states, and the proportions which each contributes to the total dwell times (Fig. 1). The number of recording sites/events is shown in the right hand column in A.

therefore concerned with reversible effects on channel kinetics, and are summarised in Table 1. P_o is significantly reduced by 1.5 and 2.7 atm N_2O but not by 0.5 atm. M_o, M_c and F_o are likewise unaffected by 0.5 atm N_2O but are affected by the higher partial pressures. 100 atm He has no significant effect on channel kinetics, consistent with previous work [7] but here referring to prolonged exposure, matching the time to equilibrate the nitrous oxide. The combination of 2.7 atm N_2O and 100 atm produces no significant change from the data obtained from 2.7 atm N_2O alone. However, the effect of 1.5 atm N_2O at 100 atm is significantly different from that of 1.5 atm N_2O. All four parameters, P_o, M_o, M_c and F_o, are restored to their control values at 100 atm (i.e., in the presence of 98.5 atm He), Table 1.

The results of a more detailed examination of the data using dwell time distributions is given in Table 2 and Fig. 1. In all experimental conditions the open time distributions are consistent with the channel adopting two open states with short and long time constants. In control conditions the former, ($r = 0.825$ ms) comprise 76% of openings and the latter, ($r = 3.065$ ms), 24%. In 100 atm He the open time distribution is broadly similar to the control pattern. The open states are also little affected by 0.5 atm N_2O but higher partial pressures of nitrous oxide, which reduced M_o, clearly increase the proportion of short lived states and simultaneously reduce the proportion of long openings (Table 2 and Fig. 2). The effect of 1.5 atm N_2O at a total pressure of 100 atm is to produce a population of open states whose two components are intermediate between those of the control (and 100 atm He) and 1.5 atm N_2O. Similarly, 2.7 atm N_2O at 100 atm also produces an intermediate population of open states (Table 2 and Fig. 2). These will be considered in detail in Discussion.

The closed time distributions are consistent with the channel adopting four closed states in all experiments, each with a characteristic time constant, but their magnitude and proportions vary according to conditions. In 100 atm He the closed time distribution is very similar to that seen in control conditions (Fig. 2). The lowest dose of N_2O, 0.5 atm, reduces the duration of each of the four time constants slightly (Table 2) but, overall, not significantly. Higher doses which significantly prolong M_c decrease the proportion of the shortest time constant and increase the proportions of the longest two. Dwell times in 1.5 and 2.7 atm N_2O each at a total pressure of 100 atm show a marked difference from those seen in N_2O alone, with the high proportion of long lived closings restored to near control values at high pressure (Table 2). These will be considered in detail, below.

4. Discussion

4.1. Nitrous oxide

The results reveal the steady state kinetics of the qGlu-R channel in equilibrium with nitrous oxide. In view of the prompt reappearance of channel activity when the muscle was decompressed from high partial pressures of nitrous oxide, it is concluded that nitrous oxide's effect is freely reversible and that enzymic or metabolically mediated steps are not involved in its action [16]. The effective partial pressures of nitrous oxide are similar to anaesthetic

doses, see Introduction, much lower than the 4.9 atm required to halve the amplitude of the compound action potential in frog nerve, at 20° C [17].

The lowest dose of nitrous oxide studied here, 0.5 atm, has no significant effect on mean parameters (Table 1) but 1.5 atm N_2O has a marked effect, reducing P_o by an order

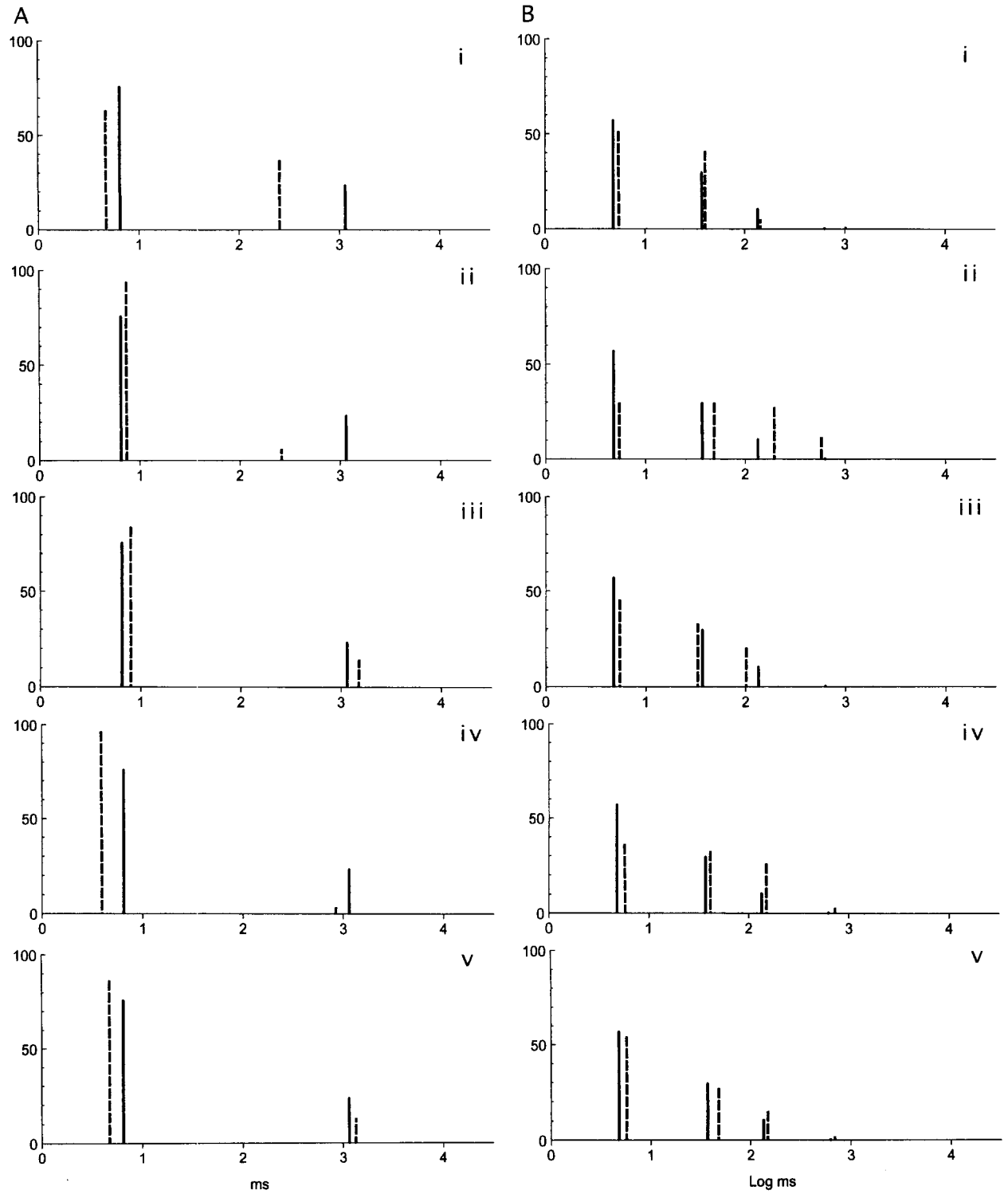

Fig. 2. Dwell time distributions, from Table 2. Time constants are plotted on the horizontal scale and the proportion which each class of dwell time contributes to the total is plotted on the vertical scale. (A) Open times. Control data are shown by the continuous line in all cases; the dashed lines refer to (i) 100 atm He; (ii) 1.5 atm N_2O; (iii) 1.5 atm N_2O and 100 atm (98.5 atm He) (iv) 2.7 atm N_2O (v) 2.7 atm N_2O and 100 atm (97.3 atm He). (B) Closed times. Control data are shown by the continuous line in all cases; the dashed lines refer to (i) 100 atm He; (ii) 1.5 atm N_2O; (iii) 1.5 atm N_2O and 100 atm (98.5 atm He); (iv) 2.7 atm N_2O; (v) 2.7 atm N_2O and 100 atm (97.3 atm He). Data for 0.5 atm N_2O are not shown in A or B.

of magnitude, shortening M_o and prolonging M_c. A steep dose–response curve is characteristic of general anaesthetics. Presumably nitrous oxide binds, reversibly, to the channel in such a way as to stabilise the closed state and favour the return to it from the open state. The nature of the binding is to some extent revealed by the high pressure experiments.

4.2. Pressure

100 atm He alone has no significant effect on mean channel parameters. Previous work showed that 300 atm He was also without effect whilst 500 atm He reduced P_o and M_o and increased M_c [7]. It was concluded the effect was mainly one of hydrostatic pressure and not of helium. Here the effect of 100 atm pressure on the dwell time distributions has to be considered. It has a negligible effect on the duration and proportions of the four closed states (Fig. 2B-i) but it does change the open time distributions a little (Fig. 2A-i). It reduces the duration of the longer time constant but increases its proportions and it reduces the proportion of the openings with the shorter time constant.

4.3. Nitrous oxide and pressure

It is clear from the Table 1 that 100 atm pressure combined with 1.5 atm N_2O, restores the M_o and M_c to normal, whereas when combined with 2.7 atm N_2O it does not.

Consider the closed time distributions first. A comparison of the distribution of closed times in 2.7 atm N_2O with those in 2.7 atm N_2O at 100 atm pressure shows a tendency for pressure to restore the distribution to normal. The proportion of the closings with the shortest time constant is increased at high pressure and that of the two intermediate classes is decreased, producing a pattern of distribution more similar to the control distribution than that seen in 2.7 atm N_2O alone (Fig. 2B-iv and -v). The combination of 1.5 atm N_2O with 100 atm pressure shows a similar but stronger trend (Fig. 2B-ii and -iii). In this case, as we have noted, the global mean closed time for 1.5 atm N_2O at 100 atm pressure is restored to normal, and is significantly different from that at 1.5 atm alone (Table 1). A reasonable interpretation of these results is that 100 atm pressure dissociates the N_2O from the channel, completely in the case of 1.5 atm N_2O, and less so in 2.7 atm N_2O.

Can this effect of pressure be seen in the open time distributions? The issue is somewhat complicated by the small effect which 100 atm has on the open times. The effect of both 1.5 and 2.7 atm N_2O on the open times is to increase the proportion of the shorter and hence decrease the proportion of the longer time constants (Fig. 2A-ii and -iv). The combination of 2.7 atm N_2O and 100 atm shifts the pattern of open times towards the control condition (Fig. 2A-v). Similarly the combination of 1.5 atm and 100 atm also shifts the pattern of open time to the control condition (Fig. 2A-iii), only in this case the global M_o is significantly different from that in 1.5 atm N_2O alone. Thus, the tendency for pressure to oppose the binding of N_2O is also apparent in the channel openings.

It follows that the interaction of N_2O with the channel (i.e., channel protein with associated lipid) which affects the kinetics, involves a positive molar volume change (ΔV). From first principles ($\mathrm{d}\ln K/\mathrm{d}P = -\Delta V/RT$) in which K is a binding constant and the other terms have their usual meaning, it may be shown that for 100 atm to dissociate N_2O from the channel, ΔV must be large, hence the binding process complicated. For example, the partitioning of volatile general anesthetics such as enflurane and chloroform into a lipid bilayer entails a ΔV of 20.6 and 11.1 ml mol^{-1}, respectively [18], suggesting that if nitrous oxide (with a smaller molecular weight of 44) were to bind 'simply' to the channel, the ΔV would be less than 20 ml/mol. However for 100 atm pressure to effectively reduce 1.5 atm to 0.5 atm N_2O or less, i.e., reduce the putative binding constant proportionately, ΔV would have to be at least 100 ml mol^{-1}. This large volume change seems more likely to arise from conformational changes in the channel than from multiple, thermodynamically coupled binding of the anaesthetic. An example is provided by diethyl ether (molecular weight 74.1) which binds to delipidated bovine serum albumin with $\Delta V = 295$ ml/mol [19].

It is interesting to compare the effect of nitrous oxide on the qGlu-R channel reported here with that of ketamine [7,20]. Ketamine's octanol: water partition coefficient is higher than that of nitrous oxide [21] and it is a larger molecule (molecular weight 238). At a concentration of 10^{-6} M at 20° C, which is somewhat lower than a clinical anaesthetic dose at body temperature, ketamine reduces the M_o of the qGlu-R-channel but has no effect on M_c. 100 atm restores M_o to normal, presumably by acting on the large ΔV in the ketamine-channel binding reaction [20].

Finally, it is worth noting that if the anaesthetic-channel interactions which occur in the CNS are similar to those described here then the pressure-reversal of anaesthesia could be caused by a direct thermodynamic, rather than an indirect physiological, process.

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