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Effects of high pressure on the channel gated by the quisqualate-sensitive glutamate receptor of locust muscle and its blockade by ketamine; a single-channel analysis

Alister G. Macdonald [a], Robert L. Ramsey [b], John Drewry [a] and Peter N.R. Usherwood [b]

[a] *Department of Biomedical Sciences, Marischal College, University of Aberdeen, Aberdeen (UK) and* [b] *Department of Life Science Nottingham University, University Park, Nottingham (UK)*

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The effects of high pressure on the channel gating kinetics of the quisqualate-sensitive L-glutamate receptor (qGluR) of locust muscle have been investigated using a megaohm seal patch-clamp technique. Pressure was applied with helium gas and recordings were carried out at 20.5°C with Rb^+ as the main charge-carrying cation in the patch pipette. The mean open time of the qGluR channel was unaffected by 10 and 30 MPa, but it was significantly reduced at 50 MPa. A high proportion of brief openings (mean 0.808 ms) was seen at 50 MPa but not at lesser pressures. Also, in contrast to lesser pressures, 50 MPa prolonged the mean closed time and reduced both the frequency and probability of channel opening. 10^{-6}M ketamine significantly reduced the mean channel open time, as previously reported. A pressure of 10 MPa which alone had no effect on the qGluR channel, restored the mean open time in the presence of 10^{-6}M ketamine to the value obtained in the absence of the anaesthetic. This implies the shortening of qGluR channel open time by ketamine involves a large $+\Delta V$ and, therefore, probably conformational changes in the channel. However 10 MPa did not restore the distribution of open times to normal.

Introduction

Ketamine, a general anaesthetic, causes open-channel block of postjunctional nicotinic acetylcholine receptors (nACh-R) [1–3] and non-competitively antagonises *N*-methyl-D-aspartate receptors [4]. These effects are seen at doses which are similar to those associated with clinical anaesthesia, e.g., < 20 μM [5]. Channel blockers are conveniently classified as either steric or allosteric [6,7]. In the former case the blocking molecule is thought to bind to the channel, thereby occluding it and reducing its conductance to near zero. Allosteric blockers are thought to elicit conformational changes in a channel and thus indirectly abolish conductance. The celebrated 'flickering' blockade of nACh-R channels by local anaesthetics exemplifies blockers with binding times which are short enough to interrupt the current flowing through an open channel many times before the channel closes [8]. Ketamine blockade of the channel gated by the quisqualate-sensitive L-glutamate receptor (qGluR) of locust muscle probably involves a much slower dissociation from the channel [9].

One interesting distinction between steric and allosteric block, which has received little attention, is in the thermodynamics of the antagonist-binding reaction. From the above definition, steric blockade involves simple binding and negligible disturbance to the channel protein, whilst allosteric blockade implies the converse. Thus, thermodynamic analysis of steric blockade should reveal relatively small changes in enthalpy (ΔH) and entropy (ΔS) whilst large values would be expected of allosteric blockade. McLaren and Quastel [10] adopted this approach in their analysis of miniature endplate currents recorded from the nAch-R channel in the presence of various blockers. They found, for example, that the channel-binding reaction involved a larger ΔS in the case of octanol than for the charged local anaesthetic procaine. In the present work we have extended the rationale to a single channel analysis of ketamine blockade, and to the thermodynamic parameter ΔV, the molar volume change. The equation for the change in free energy, $\Delta G = \Delta H - T\Delta S = (\Delta E + P\Delta V) - T\Delta S$ shows that ΔV and the in-

Correspondence to: A.G. Macdonald, Department of Biomedical Sciences, Marischal College, University of Aberdeen, Aberdeen AB9 1AS, UK.

ternal energy ΔE make up the enthalpy change ΔH. At constant temperature the ΔV of an association-dissociation equilibrium can be revealed by the application of pressure to the system. Thus, from $d \ln K/dp = -\Delta V/RT$ it may be shown that K, the equilibrium constant of such an equilibrium is increased 4% by 10 MPa when $\Delta V = -10$ ml/mol, but more than two fold when ΔV is -200 ml/mol. Conversely, K is reduced when the sign of ΔV is positive. The formation of simple ionic bonds or hydrophobic interactions generally involves values of ΔV no bigger than 20 ml/mol [11], whereas multiple, thermodynamically coupled events associated with conformational changes in proteins (and conceivably the allosteric blockade of ion channels) can involve values at least one order of magnitude greater [12–14]. Thus, steric blockade should be much less sensitive to high pressure than allosteric blockade.

In addition to these general considerations there is an additional, specific reason for studying the effect of pressure on anaesthetic-ion channel interactions. In a number of cases anaesthetised animals are 'awakened' by the application of a moderate hydrostatic pressure (i.e., 10 MPa), and rats and mice anaesthetised with ketamine are no exception [15,16]. Various explanations for the pressure reversal of anaesthetic effects have been debated, including the direct effect of pressure on the anaesthetic binding reaction and indirect, physiological mechanisms of reversal [17].

We have recently shown that 10^{-6}M ketamine blocks the open state of the qGluR channel in locust skeletal muscle [9]. The kinetics of the qGluR channel have been extensively studied using a combined two-electrode voltage clamp and a megaohm seal-patch technique [18,19]. Data derived from these studies and analysed according to Kerry et al. [18], yielded a model comprising at least 4 open and 4 closed states. This model has been subsequently confirmed and extended by Bates et al. [20]. The locust muscle qGluR channel was originally chosen for our pressure experiments because studies of this channel are less subject to the constraints imposed on patch-clamp recording at high pressure. We decided to use a medium-sized pressure vessel fitted with a high-pressure window for microscopy, multiple electrical connections and an internal micromanipulator, all of which provide for a variety of electrophysiological recordings. In contrast, Conti and his colleagues extended their study of the effects of pressure on voltage gated channels in the squid axon to the nAch-R channel by means of the 'flying patch' clamp, which incorporated a gigaohm seal patch recording apparatus inside a small pressure vessel [21–23]. In our approach, successive and sometimes prolonged megaohm seal recordings of a qGluR channel under high pressure are carried out with a single pipette at the muscle resting potential. This method exploits two distinctive properties of the channel, the elimination of desensitization by prior treatment with concanavalin A and its high conductance (115–150 pS) [24].

The purpose of this paper is to report the effects of high pressure on the kinetics of the locust qGluR channel and on the open-channel block caused by ketamine.

Materials and Methods

The experiments were undertaken on the metathoracic extensor tibiae muscle of adult, female locusts (*Schistocerca gregaria*), bathed in standard locust saline (mM: 180 NaCl; 10 KCl, 2 $CaCl_2$, 3 Hepes (pH 6.8)) as before [9]. To control for the possible effects of a reduction in saline pH which 50 MPa may cause, [13,22], some experiments were undertaken at atmospheric pressure using saline (in both the electrode and the bath) adjusted to pH 6.4. The qGluR channel properties were unaffected by the reduction in pH. Prior to use, the muscles were routinely treated with 1–2 μM concanavalin A in the saline for 30 min to block desensitization. A megaohm seal patch clamp technique was used to record single qGluR-channel activity [25]. The patch pipette normally contained locust saline in which RbCl was substituted for NaCl to enhance the channel conductance [26,27], 10^{-4}M L-glutamate as agonist and 10^{-6}M ketamine as appropriate [9]. Substituting Rb^+ for Na^+ in the patch pipette improves the signal-to-noise ratio by 25%. Although this also prolongs the channel mean closed time and reduces the probability of opening, it has no effect on the mean open time, which is the main focus of the ketamine effect [26,27]. All recordings were undertaken at the muscles' resting potential, which is approx. -65 mV in normal conditions [26].

The muscle preparation was mounted in a dish containing 4 ml of locust saline and, when appropriate, it was equilibrated with 10^{-6}M ketamine hydrochloride (free of stabiliser; Parke Davis, UK) by perfusing the dish at 1 ml/min for 40 min. Patch pipettes contained the same ketamine concentration. Recordings were carried out at high pressure using an apparatus modified after Macdonald et al. [28]. The pressure vessel was a 135-mm bore steel cylinder, vertically mounted. A high-pressure window was mounted in the top-end plug, whilst electrical and pressure connections and a light guide passed through the lower-end plug. The muscle preparation was positioned beneath the inner face of the pressure window and within range of a binocular microscope (Bausch & Lomb Stereozoom) mounted outside. The muscle was illuminated from below by a light guide. An electrically-driven micromanipulator (Microinstruments, Oxford, UK) within the vessel enabled the patch pipette to be gently

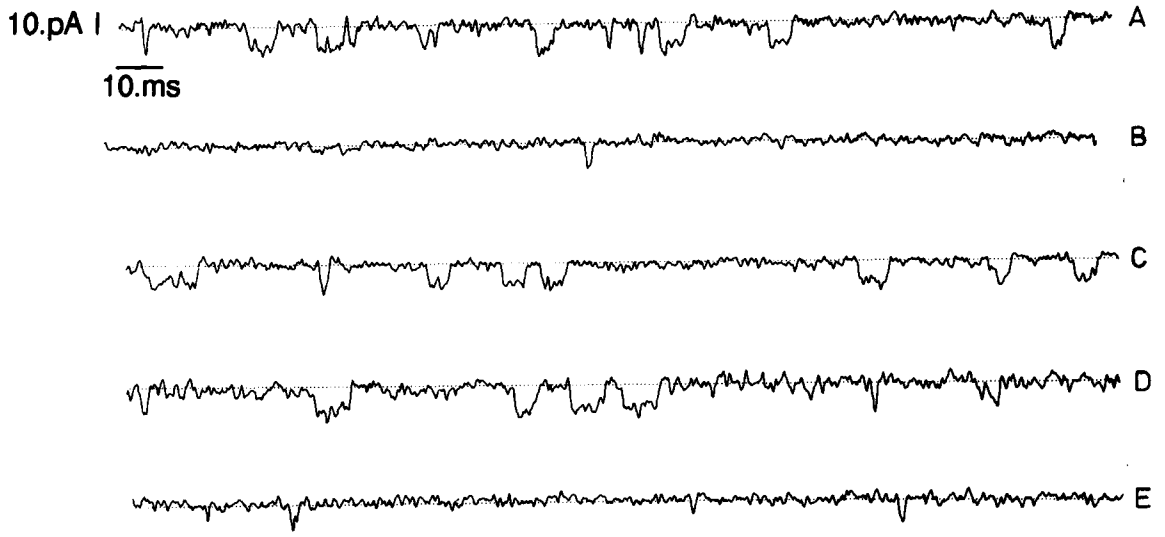

Fig 1. Single-channel recordings from different qGluR channels, using 10^{-4} M L-glutamate and 180 mM Rb^+ (pH 6.8 at atmospheric pressure) in the patch pipette. (A) control conditions; (B) equilibrated with 10^{-6}M ketamine; (C) equilibrated with 10^{-6}M ketamine and pressurised with helium to 10 MPa; (D) ketamine absent, pressurised to 10 MPa and (E) ketamine absent, pressurised to 50 MPa. The muscle fibres were at resting potential and the recordings low-pass filtered at 1 kHz.

pressed onto the extrajunctional muscle membrane, which contains qGluR at low population density, to form megaohm seals. The electrode headstage was contained within a miniature pressure-resistant casing (100 × 25 mm diameter) also mounted on the micromanipulator.

The vessel was pressurised with helium gas (British Oxygen Company) which was added to the air-filled vessel at a rate of 0.16 MPa/min for the first 5 MPa and somewhat faster thereafter, the ambient pO_2 remaining normal. The heat of compression was monitored by a thermistor located in the gas near the muscle. The maximum transient heat rise was less than 3 C°, but recordings were delayed for 30 min after the completion of compression to ensure that the temperature of the muscle was 20.5 ± 0.5°C. Channel activity was thus recorded after a period at high pressure of 0.5–4 h, during which time there was no sign of any deterioration in the condition of the muscle.

Single-channel recordings were low-pass filtered at 1 kHz prior to being analyzed off line using the single threshold crossing program of Dempster [29]. This also provides for the detailed inspection of the analogue recording for multiple, i.e., superimposed channel currents, and all such cases were discarded. All data analyzed conformed to the criteria of Kerry et al. [18].

TABLE I

Effect of high pressure on the qGluR channel; probability of opening, mean open and closed times and frequency of opening

Median values are given for individual recording sites, with the number of sites/number of animals in brackets.

	Probability of opening	Mean open time (ms)	Mean closed time (ms)	Frequency of opening (s^{-1})	
Rb^+ in patch pipette					
Atmospheric pressure (pH 6.8)	0.011	1.26	116.55	7.9	(10/7)
10 MPa	0.016	1.20	76.71	12.8	(13/3)
30 MPa	0.018	1.04	44.96	21.8	(6/2)
50 MPa	0.004 [a,c]	0.88 [a]	233.91 [a,c]	4.2 [b,c]	(12/4)
Atmospheric pressure (pH 6.4)	0.013	1.11	77.92	12.6	(10/4)
Na^+ in patch pipette					
Atmospheric pressure (pH 6.8)	0.057 [d]	1.46	21.19 [d]	45.0 [d]	(6/5)

Mann-Whitney test of significance: [a] Significantly different from atmospheric pressure (pH 6.8 and 6.4), $p < 0.01$; [b] significantly different from atmospheric pressure (pH 6.8), $p < 0.01$ and from atmospheric pressure (pH 6.4), $p < 0.02$; [c] significantly different from 30 MPa, $p < 0.01$; [d] significantly different from Rb^+ control data, $p < 0.01$.

TABLE II

Effects of high pressure on the qGluR channel. A, open time and B, closed time distributions

The exponentials giving the best fits to pooled data are shown in time categories and the proportion of events is given in brackets. The number of recording sites/events is shown in the right hand column in A.

II-A. Open times

	< 1 ms		> 1 ms	
Control (pH 6.8)	0.349 (0.359)	1.21 (0.539)	3.16 (0.106)	10/18837
10 MPa	0.338 (0.397)	1.10 (0.488)	3.05 (0.115)	13/50840
30 MPa	0.414 (0.361)	1.42 (0.495)	4.67 (0.144)	6/45681
50 MPa	0.808 (0.916)	1.42 (0.084)	–	12/14698

II-B. Closed times (the number of recording sites / events as in A)

	< 10 ms		< 100 ms		> 100 ms
Control (pH 6.8)	2.59 (0.310)	19.92 (0.351)		102.8 (0.295)	359.0 (0.0444)
10 MPa	2.26 (0.317)	32.33 (0.407)		112.9 (0.257)	657.2 (0.0183)
30 MPa	4.514 (0.412)	23.08 (0.483)	82.67 (0.091)	319.2 (0.0137)	–
50 MPa	2.26 (0.361)	61.81 (0.202)		243.1 (0.412)	1276.0 (0.0248)

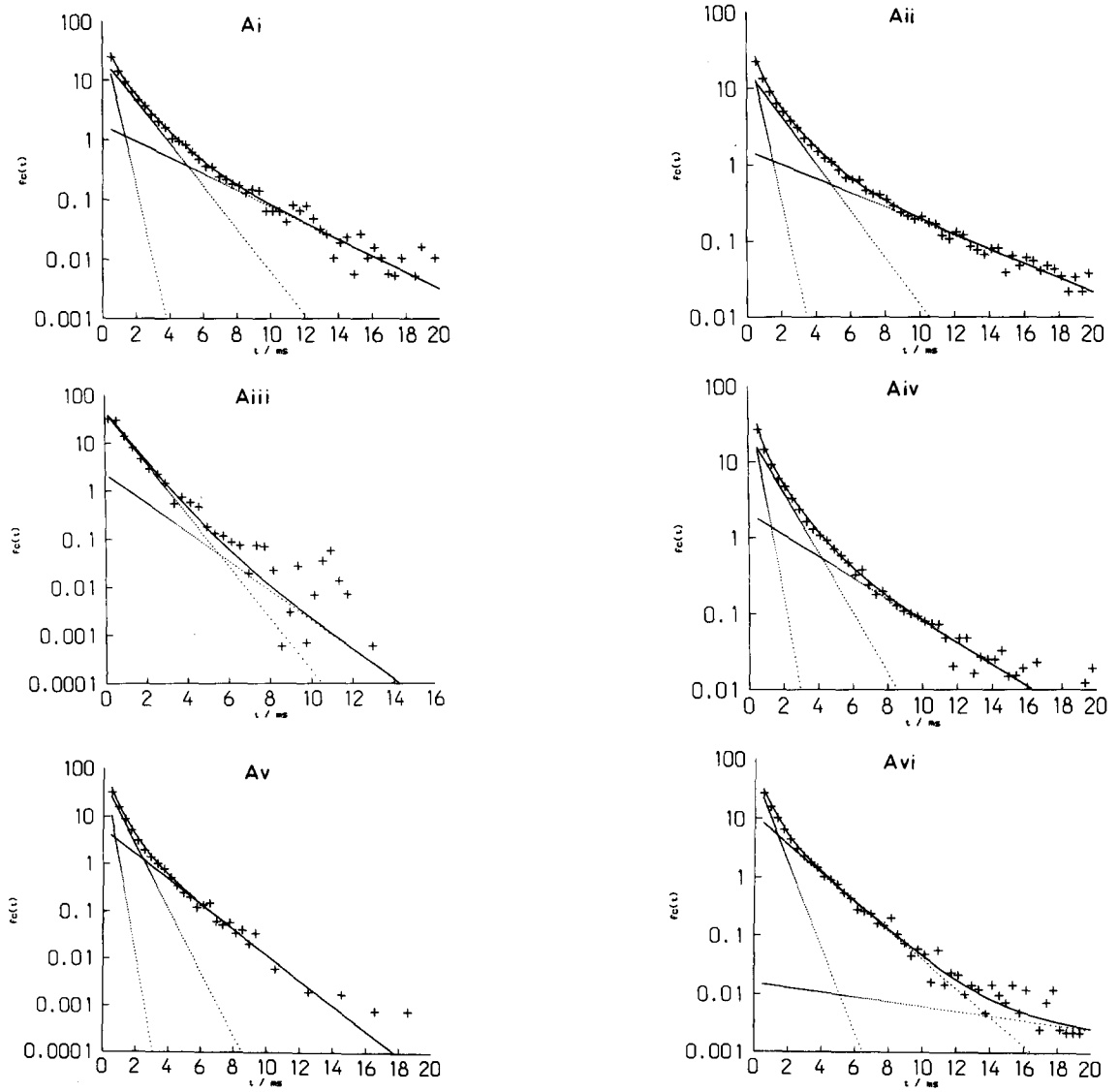

Fig 2. qGluR channel open and closed time distributions, shown as log probability density function as in Ref. 7 against dwell time. (A) Open times; (i) control; (ii) 30 MPa; (iii) 50 MPa; (iv) 10 MPa; (v) Ketamine, 10^{-6}M, normal pressure and (vi) Ketamine, 10^{-6}M at 10 MPa. (B) Closed times; (i) control; (ii) 10 MPa; (iii) 30 MPa and (iv) 50 MPa. The crosses represent the experimental data, the dotted lines the individual exponential components and the continuous line the multiexponential fit. See also Tables II and IV.

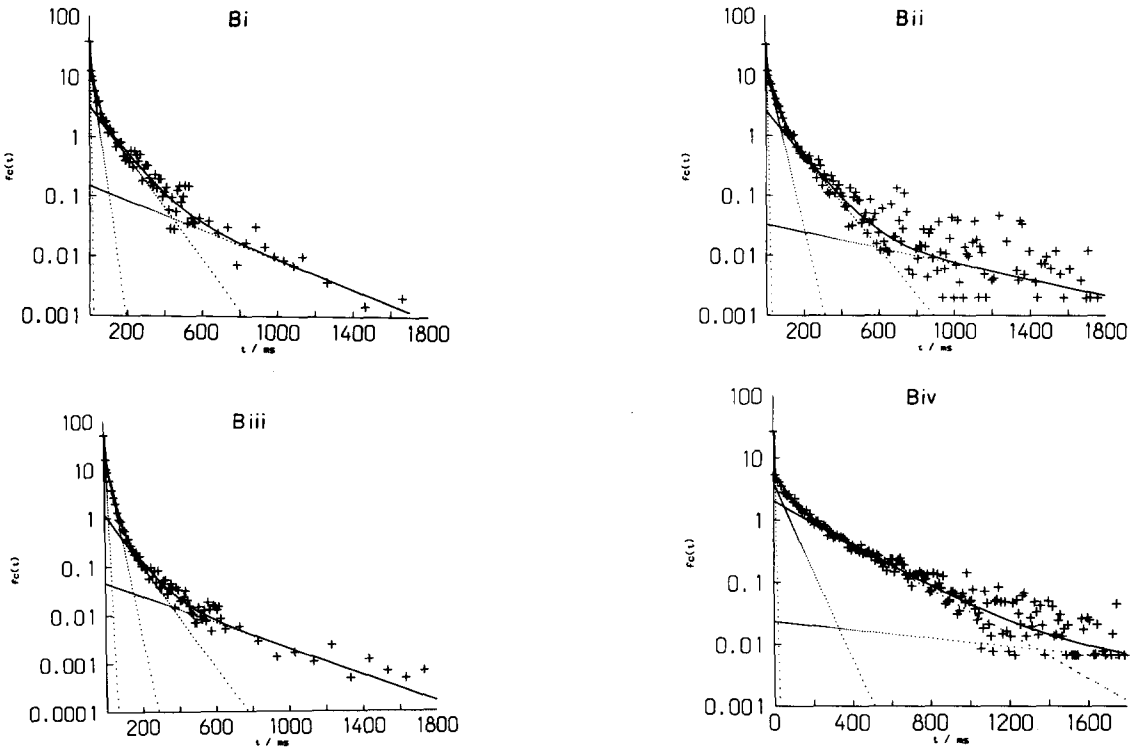

Fig. 2 (continued).

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) were determined for individual recording sites. Dwell time histograms were constructed from data pooled from individual sites using the above program and exponentials fitted using the procedure of Kerry et al. [18]. The minimum bin size used for fitting open time distributions was 0.4 ms [30]. The pooling procedure assumes that the qGluR channels of locust muscle are essentially identical.

Results

Pressure appeared to have no obvious effect on the single-channel conductance, (Fig. 1). Ketamine also had no obvious effect on the conductance of the qGluR channel when Na^+ was the main charge-carrying ion [9], and the same was true in the present experiments when Rb^+ was the main ion. The control data obtained in the present study with Rb^+ in the patch pipette, were M_o 1.26 ms, M_c 116 ms, P_o 0.011 and F_o 7.9 s^{-1}. With Na^+ as the main cation in the patch pipette $M_o = 1.46$ ms, $M_c = 21.2$ ms, $P_o = 0.057$ and $F_o = 45$ s^{-1} (Table I). These differences between the Na^+ and Rb^+ data are consistent with those reported by Collins et al. [27], and the results with Na^+ as the main cation are similar to previously published data, analysed after low-pass filtering at 3 kHz [9].

Pressure

Table I summarises the effects of pressure on the channel parameters. Significant changes in channel-

TABLE III

Effect of ketamine and high pressure on the qGluR channel

Median values are given for individual recording sites, with the number of sites/number of animals in brackets.

	Probability of opening	Mean open time (ms)	Mean closed time (ms)	Frequency of opening (s^{-1})
Atmospheric pressure				
Ketamine-free	0.0111	1.266	116.55	7.9 (Table I)
10^{-6} M Ketamine	0.0071 [b]	0.760 [a]	121.95	8.2 (10/4)
10 MPa				
10^{-6} M Ketamine	0.013	1.12 [c]	103.38	9.5 (14/3)
Ketamine-free	0.016	1.20	76.71	12.8 (Table I)

Mann Whitney test of significance: [a,b] Significantly different from atmospheric pressure control (pH 6.8), $p < 0.01$ and $p < 0.05$, respectively; [c] significantly different from ketamine-treated, atmospheric pressure, $p < 0.02$.

TABLE IV

Effect of ketamine and high pressure on the open times of the qGluR channel

The exponentials giving the best fits to pooled data are shown in two time categories, the proportion of events is given in brackets and the number of recording sites/events is shown in the right-hand column.

	Open times				
	< 1 ms		> 1 ms		
Control atmospheric pressure	0.349 (0.359)		1.21 (0.539)	3.16 (0.106)	10/18837
10^{-6} M Ketamine atmospheric pressure	0.221 (0.333)	0.645 (0.533)	1.62 (0.135)	–	10/21496
10 MPa, Ketamine-free	0.338 (0.397)		1.10 (0.488)	3.05 (0.115)	13/50840
10 MPa, 10^{-6} M Ketamine		0.596 (0.607)	1.77 (0.390)	10.94 (0.0033)	14/42619

gating properties were only obtained with the highest pressure, i.e., 50 MPa, which approximately halved P_o, M_o and F_o and doubled M_c.

The results of the analysis of channel-dwell times are shown in Table II and Figs. 2 and 3. The proportion of brief openings was almost trebled at 50 MPa and dominated by a population with a mean open time of 0.808 ms. Changes in M_c were associated with the appearance of additional long closings at 50 MPa.

Ketamine

At atmospheric pressure 10^{-6}M ketamine approximately halved P_o and M_o (Table III), changes which are consistent with those in our earlier report despite the different recording conditions. Table IV and Fig. 3 show that there were more brief open times when ketamine is present, which is also consistent with our previous report [9]. Half of the openings were in a category with a mean of 0.645 ms.

Ketamine and pressure

The effect of 10^{-6}M ketamine on M_o was much reduced by 10 MPa, (Table III, Fig. 3). The distribution of open times was not restored to that seen in the controls however (Table IV, Fig. 3). The effect of 10 MPa on P_o in the presence of 10^{-6}M ketamine fell short of statistical significance and clearly M_c and the frequency of opening were unaffected (Table III).

Discussion

Channel kinetics

The partial pressure of helium may have exerted an influence on qGluR channel properties distinct from

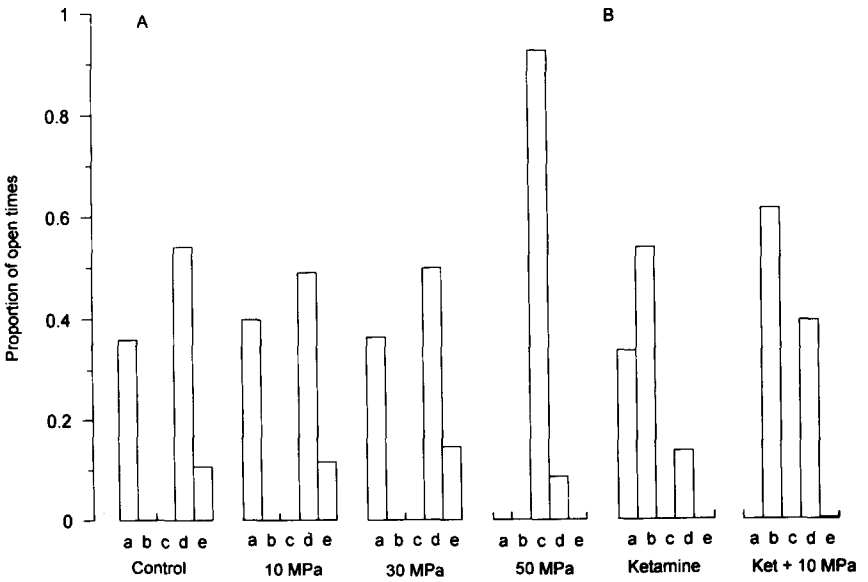

Fig 3. The qGluR channel open times (A) as affected by pressure and (B) by 10^{-6}M ketamine, alone and in combination with 10 MPa pressure. Each column refers to a time constant given in Table II or IV and is classified as follows; (a) 0.221–0.414 ms; (b) 0.596–0.645 ms; (c) 0.808 ms; (d) 1.10–1.77 ms; (e) 3.05–10.94 ms.

that exerted by purely hydrostatic pressure, but the evidence for such a phenomenon is inconclusive in the present context [31,32]. Here we adopt the orthodox view that any helium effect is likely to be very small and that the effect of hydrostatic pressure dominates. Four conclusions may be drawn from the results of these experiments. First, the qGluR channel differs from the nAChR channel in its kinetic response to high pressure. For instance, according to Heinemann et al. [23], both M_o and M_c of the nAChR channel were linearly increased by pressure up to 40 MPa. Second, the qGluR channel at 50 MPa and 20°C is functioning in a lipid bilayer whose degree of order is comparable to that of a bilayer at 11°C, atmospheric pressure. Pressure reduces the fluidity of lipid bilayers in both natural and model membranes by an amount which may be offset by an increase in temperature, the coefficient being 0.18 C°/MPa [33]. If steady-state channel kinetics are determined by bilayer order, i.e., the structural component of fluidity [34], then the present results should match those obtained from cooling the channel. In fact pressure reduced M_o, whereas Gration [35] reported that cooling increased M_o and decreased the conductance of qGluR. It would be interesting to extend the present pressure study to cover a wide range of temperatures, particularly to see how cooling modifies the effect of pressure on channel kinetics. Third, as in the case of the nAChR channel, there is no obvious reason for supposing that the agonist-channel interactions are pressure-labile. In the conditions used in the experiments reported here, increasing the concentration of glutamate linearly increases M_o and P_o, and vice versa [19]. This is a pattern not seen with increase in pressure, which presumably is neither enhancing nor reducing glutamate binding to the receptor. It seems reasonable to conclude, therefore, that pressure acts on the qGluR channel by affecting the activation steps in its closing and opening reactions.

50 MPa clearly favours the closing reactions, increasing M_c and reducing M_o. 30 MPa appears to exert an opposite effect in reducing M_c but it does not achieve statistical significance. Whilst pressures up to and including 30 MPa have little effect on the open time distributions it is interesting that 50 MPa abolishes the longest of the open states and strongly favours a population of channels with a distinctive mean open time of 0.808 ms (Table II). In the case of the opening reactions pressures up to 30 MPa appear to be neutral whilst 50 MPa retards them, prolonging three of four categories of closed times (Table II). Ultimately, the effects of pressure on channels are to be interpreted in the same way as for other proteins and there are numerous examples of protein dynamics being affected by the moderate hydrostatic pressures used here [12,13,36]. It remains to be seen which is the most important; sub-unit interactions, small ligand binding or intramolecular effects [37,14].

Channel blockade

M_o, which is reduced by 10^{-6} M ketamine, is restored to normal by 10 MPa (Table III), and the high proportion of short openings in the presence of ketamine is reduced by 10 MPa (Fig. 3). In detail, a significant population of channels with a mean open time of 0.645 ms appears in the presence of ketamine. The application of 10 MPa, which alone has no effect on the dwell time distributions, causes the population of channels with the shortest open time (0.221 ms) to disappear. It has no effect on the main population (mean open time 0.645 ms at atmospheric pressure and 0.596 m at 10 MPa) and it trebles the proportion of channels with a mean open time of 1.62 ms at atmospheric pressure (1.77 ms, 10 MPa). Thus, although M_o is restored to normal, the dwell time distribution is not.

Qualitatively the restoration of the M_o to normal by 10 MPa implies that ketamine binds to the channel (thereby reducing M_o) with a $+\Delta V$ and that pressure dissociates it from its initial binding site. From the dose-response relationship for the ketamine effect on the channel [9], it appears that 10 MPa reduces the potency of ketamine 100-fold, and hence its binding constant is similarly affected. This suggests that the $+\Delta V$ of ketamine binding is large. From $\mathrm{d}\ln K/\mathrm{d}p = -\Delta V/RT$, assuming K varies 100-fold, linearly, over the 0.1–10 MPa range and one ketamine binds per channel, it follows that the ΔV of the intereaction is approx. 1 l/mol. Whilst a $+\Delta V$ of binding is not unexpected, the estimated ΔV is paradoxically large for the simple binding of a positively-charged near-spherical molecule such as ketamine (molecular weight 238). The ΔV for enflurane – bacteriorhodopsin binding is +16.1 ml/mol at 20°C [38], and for the partitioning of enflurane from an aqueous phase into a phospholipid bilayer it is 18.2 ml/mol [39]. The binding of methoxyflurane to poly(L-lysine HBr) entails an even smaller $+\Delta V$ [40]. Interestingly, the diethyl ether–serum albumin binding reaction entails a $+\Delta V$ of 295 ml/mol which may include conformational changes in the protein [41]. Although the $+\Delta V$ estimated above is improbably large, due to the simplistic nature of the calculation, we conclude that the marked sensitivity of the ketamine-channel interaction to 10 MPa is evidence for conformational changes which are likely to contribute to ketamine's open-channel blockade. This conclusion can be tested by measuring M_o as a function of pressure with a time resolution superior to that possible in the present study. The stoichiometry of the ketamine-channel interaction and the multiple open states of the channel would also have to be taken into account.

A general conclusion emanating from this study is

that pressure could reverse ketamine anaesthesia in animals by dissociating it from binding sites similar to those studied here.

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