Journal of Physiology (1991), **432**, *pp.* 483–508
With 8 figures
Printed in Great Britain

ACTIONS OF KETAMINE, PHENCYCLIDINE AND MK-801 ON NMDA RECEPTOR CURRENTS IN CULTURED MOUSE HIPPOCAMPAL NEURONES

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(*Received 19 April 1990*)

SUMMARY

1. Stable *N*-methyl-D-aspartic acid (NMDA) receptor-mediated currents in cultured mouse hippocampal neurones were evoked by 20 ms pressure pulse applications of L-aspartate, repeatedly applied at 30 or 40 s intervals, to the cell body region of the neurone. We have characterized the voltage- and use-dependent blockade of the currents by three dissociative anaesthetics: ketamine, phencyclidine (PCP) and MK-801 in mouse hippocampal neurones grown in dissociated tissue culture.

2. We have used a simple model of the blockade, based on the 'guarded receptor hypothesis' to interpret our data. The model assumes that receptors are maximally activated at the peak of the response with an open probability (P_o) approaching 1, that there is no desensitization and that the blocking drug only associates with, or dissociates from, receptor channels which have been activated by agonist (e.g. open channels).

3. The model allows us to estimate forward and reverse rate constants for binding of the blockers to open channels from measurements of the steady-state level of blockade and the rate of change of the current amplitude per pulse during onset and offset of blockade. As predicted by the model, the estimated reverse rate was independent of blocker concentration while the forward rate increased with concentration. Changing the level of positively charged ketamine (pK_a 7·5) tenfold by changing pH from 6·5 to 8·5 caused a corresponding change in the forward rate while having no effect on the reverse rate. Most of the voltage dependence of the blockade could be accounted for by reduction of the reverse rate by depolarization.

4. Estimated forward rate constants for ketamine, PCP and MK-801 were similar to one another when measured under similar conditions and were 3×10^4–3×10^5 M^{-1} s^{-1}. Most of the differences in potency of the three blockers could be accounted for by differences in the reverse rate constants which were approximately 0·2, 0·03 and 0·003 s^{-1} for ketamine, PCP and MK-801, respectively. The estimated rate constants actually are the product of the rate constants and $1/P_o$.

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Suggestions that maximum P_o is much less than 1 for NMDA channels imply that both forward and reverse rate constants of blockade may in fact be larger than we have calculated. However, their magnitudes, relative to one another, are unaffected by this consideration.

5. The reverse rate constant of blockade increased at positive potentials. This increase was prevented when the neurone was loaded with *N*-methyl-D-glucamine, an impermeant cation which prevented outward currents. This observation suggests that the voltage-dependent blockade by dissociative anaesthetics is in fact current dependent and reflects displacement of anaesthetic molecules, bound to the vicinity of the outer mouth of the channel, by intracellular cations that move out of the cell via the channel at positive potentials. This suggestion is supported by the observation that the voltage dependence of the blockade by the neutral PCP analogue, 1-(1-(2 thienyl)-cyclohexyl)morpholine (TCM) is similar to that of the blockade by PCP.

6. The blockade by dissociative anaesthetics was reduced by 0·5 mM-Mg^{2+} which itself caused blockade of the NMDA currents. Occupancy of the receptor binding site by the competitive NMDA antagonist DL-2-amino-5-phosphonovaleric acid (APV) had no direct effect on the action of the dissociative anaesthetics other than that expected from preventing activation of the receptor.

INTRODUCTION

The NMDA subtype of excitatory amino acid receptor (Monaghan, Bridges & Cotman, 1989) is selectively blocked by a variety of dissociative anaesthetics, such as ketamine, phencyclidine (PCP) and MK-801 (Anis, Berry, Burton & Lodge, 1983; MacDonald, Miljkovic & Pennefather, 1987; Wong, Kemp, Priestley, Knight, Woodruff & Iverson, 1987; Kushner, Lerma, Zukin & Bennett, 1988). Honey, Miljkovic & MacDonald (1985) demonstrated that the blockade is voltage dependent, with a much greater blockade observed at hyperpolarized than depolarized potentials. MacDonald *et al.* (1987) showed subsequently that in order to reach or recover from a steady-state block at a given potential it was necessary to repeatedly apply the agonist; the blockade was use dependent. The blockade thus, appeared to be dependent upon the presence of the agonist, implying that the state of the NMDA receptor determined the degree of antagonism (MacDonald *et al.* 1987).

State-dependent changes in the rates of onset and offset of blockade of ionic channels have been extensively described for voltage-dependent channels (Armstrong, Swenson & Taylor, 1982; Hille, 1984; Catterall, 1987; Carmeliet, Nilius & Vereecke, 1989; Swandulla & Armstrong, 1989) as well as for ligand-gated channels (Lingle, 1983; Gurney & Rang 1984). Trapping of the antagonists on closed channels is a prominent feature of state-dependent blockade including that of NMDA receptor channels by ketamine, PCP and MK-801 (MacDonald *et al.* 1987; Wong *et al.* 1987; Huettner & Bean, 1988; Kushner *et al.* 1988). A variety of binding studies have confirmed that it is the agonist-activated form of the NMDA receptor which interacts most readily with dissociative anaesthetics (Loo, Braunwalder, Lehmann & Williams, 1986; Foster & Wong, 1987; Reynolds, Murphy & Miller, 1987; Snell, Morter & Johnson, 1987; Kloog, Haring & Sokolovsky, 1988). There also is evidence at the single channel level that dissociative anaesthetics cause a selective block of

open NMDA receptor channels (Huettner & Bean, 1988; Nowak & Wright, 1990; Wright & Nowak, 1990).

A simple explanation for the voltage dependence of the action of dissociative anaesthetics is that as positively charged cations they experience a portion of the membrane electric field as they approach a binding site within the open channel. This mechanism would be analogous to that proposed by Woodhull (1973) to account for the blockade of Na^+ channels by H^+ ions. A similar mechanism has been proposed for the blockade of NMDA receptor channels by divalent cations such as Mg^{2+}, (Nowak, Bregestovski, Ascher, Herbert & Prochiantz, 1984; Ascher & Nowak, 1988). Although this model seems appropriate for small, permeant cations it becomes much more difficult to reconcile the bulky molecular dimensions of dissociative anaesthetics with a model which requires that they move significant distances into the open pore of NMDA receptor channels.

An alternative model for voltage-dependent channel blockade has been presented recently by MacKinnon & Miller (1988) for the blockade of calcium-activated K^+ channels by charybdotoxin. Charybdotoxin is a polycationic molecule and these authors have provided evidence that rather than toxin molecules entering the channel and crossing a portion of the transmembrane field to reach a binding site, the toxin binds to a region of negative charge located at the mouth or vestibule of the K^+ channel. The rate of association with this site was found to be independent of voltage while the rate of dissociation appeared to be enhanced by depolarization. It was suggested that at depolarized potentials outwardly permeating K^+ ions might sterically interact with the bound toxin molecules speeding their dissociation from the binding site.

We compare here, in a semi-quantitative way, the blockade of NMDA receptor-activated currents by three dissociative anaesthetics and provide evidence that this blockade is not consistent with the Woodhull model (1973). Instead we report that the block of NMDA currents by dissociative anaesthetics more closely resembles the current-dependent charybdotoxin block of calcium-activated K^+ channels.

METHODS

Whole-cell patch-clamp recordings were made from hippocampal neurones grown in dissociated tissue culture using techniques previously described (MacDonald *et al.* 1987; MacDonald, Mody & Salter, 1989). Briefly, hippocampii were dissected from fetal (E18) Swiss White or CB7 Evans mice which were killed by cervical dislocation and cells were mechanically dissociated. The dissection and dissociation were performed in cooled Hank's solution and containers were kept on ice until the cells were plated. Cells were plated at densities below 1 million ml^{-1} in 35 mm, collagen-coated culture dishes. Monolayer cultures were then employed for electrophysiological recordings following approximately two weeks in culture.

Immediately prior to beginning each experiment the culture dish was thoroughly washed with an extracellular solution containing (in mM): 140 NaCl, 1·3 $CaCl_2$, 5·4 KCl, 25 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 33 glucose. The pH was adjusted to 7·4 and the solution contained tetrodotoxin (Sigma) at a concentration of 1–3 μM and 1 μM-glycine (Sigma). L-Aspartate (250 μM) or in a few cases NMDA (500 μM) were dissolved in the extracellular solution and applied by pressure (Picospritzer II, 10–150 kPa) from glass micropipettes with tips located approximately 10–20 μm from the soma under study as previously described (MacDonald *et al.* 1989). Pressure applications of 20 ms duration routinely evoked highly reproducible L-aspartate currents that peaked within 50–200 ms and recovered fully by 1–2 s provided a low frequency (either 0·034 or 0·025 Hz) of application was employed in order to minimize desensitization.

Each dish could be continuously perfused with the extracellular solution. However, this was not

usually done because the non-laminar flow of solution greatly reduced the reproducibility of amino acid responses and thus in most cases recordings were made in a static bath. Ketamine (1–20 μM: Parke Davis), 1-(1-phenylcyclohexyl)piperidine (PCP; 250 nM to 5 μM), (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]-cyclohepten-5,10-imine maleate (MK-801; 120–1000 nM; Merck Sharpe), DL-2-amino-5-phosphonovaleric acid (APV) (50 μM; Cambridge Research Biochemicals) and Mg^{2+} (100–500 μM), were dissolved in the extracellular solution and the entire volume of the culture dish was exchanged at least five times to ensure equilibration of each new solution. In the absence of drugs, such an exchange of the bathing solution had no influence on the size of the recorded currents. In some experiments a rapid laminar flow system was used to change the extracellular solution.

Patch electrodes were constructed from borosilicate thin-walled glass (o.d. 1·5 mm, TW150F-4, WPI) which contained a filament to facilitate filling. A Narashige PP88 vertical puller was used to form electrodes of an approximately uniform tip diameter (1·5–2 μm) and each electrode was fire-polished. The intracellular solution contained (in mM): 140 CsCl or KCl, 10 HEPES, 11 ethyleneglycol-*bis*-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), 35 CsOH or KOH used to dissolve the EGTA, 1 mM $CaCl_2$. In some experiments 140 mM-*N*-methyl-glucamine titrated with HCl was used to replace CsCl or KCl. In all cases an ATP-regenerating system containing (in mM) 2 TEA, 2 $MgCl_2$, 4 Tris-ATP, 20 phosphocreatine and 50 U ml^{-1} creatine phosphokinase (all from Sigma) was also included. This solution greatly retards the wash-out of NMDA receptor-activated currents observed in these cells (MacDonald *et al.* 1989). All intracellular solutions had a pH of 7·3. To minimize degradation of labile components in the solutions they were either made fresh just prior to the beginning of the experiment or were aliquoted into smaller volumes and frozen until they were used. After 4–6 h the solution was then discarded. Recordings were all done at room temperature (21–23 °C).

The majority of recordings were made using a single-electrode voltage-clamp switching between hold and current injection at frequencies of 20–28 kHz (Axoclamp 2A) and therefore series resistance compensation was not required. Care was taken to ensure adequate capacitive settling of the clamp during the response to excitatory amino acids. In the other experiments a non-switching patch clamp with series resistance compensation (Axopatch 1A) was employed but the voltage-clamping technique had no effect on the outcome of the experiments.

The recording of current and voltage as well as the application of excitatory amino acids was controlled via an acquisition program (EXPS; NIH, Laboratory of Neurophysiology) running on a LSI-11 23 computer or on an IBM compatible computer using P-clamp (Axon Instruments). Data were recorded continuously on a Gould Brush pen recorder and also were routinely sampled (2·5 ms per point) and stored on hard disc for analysis.

THEORY

Modified guarded receptor hypothesis

We have proposed that the 'guarded receptor' model can be used to explain how dissociative anaesthetics block NMDA receptor channels (MacDonald *et al.* 1987). This model was proposed by Starmer and his associates (Starmer, Grant & Strauss, 1984; Starmer, Yeh & Tanguy, 1986) to account for the use-dependent blockade of the voltage-activated Na^+ channel by charged local anaesthetics. The model states that Na^+ channel gates provide a barrier for access to and from a channel binding site, which has unchanging affinity for local anaesthetics. Accordingly, the block depends strongly upon the duration and frequency of channel gating. The voltage dependence of the blockade is attributed to the voltage dependence of both activation and inactivation gates, to shifts in that voltage dependence due to formation of the drug–channel complex and to interaction of drug charge with the electrical field in the membrane.

For the blockade of NMDA channels by dissociative anaesthetics, the model can be simplified considerably because of differences between NMDA-gated and voltage-gated channels. For example, gating of channels coupled to NMDA receptors is

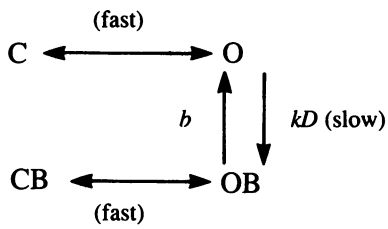
primarily agonist dependent and not greatly affected by voltage (but see Discussion). Furthermore, we have found that within our experimental constraints the development of the block is not dependent upon the frequency of agonist application (MacDonald *et al.* 1987). Coupled with this, is the observation that once the NMDA agonist dissociates from its binding site and the open-blocked channel becomes a closed-blocked channel the dissociative anaesthetic is apparently trapped within or on the channel and dissociates only very slowly. As a consequence it seems unlikely that blocker molecules can gain access to their binding site at any appreciable rate unless the channels are in the open agonist-associated state.

Finally, studies involving the binding of radiolabelled dissociative anaesthetic analogues indicate the NMDA receptor agonists have little effect on the equilibrium affinity of the dissociative anaesthetic blockers for the NMDA receptor channel (Kloog *et al.* 1988). As predicted by the guarded receptor hypothesis, opening of the channel simply increases the rates of association and dissociation of the blockers. Since the presence of agonist does not affect the equilibrium affinity of the blocker, the blocker cannot affect the equilibrium affinity of the agonist.

We show below that after introduction of a dissociative anaesthetic into the medium bathing a neurone, the magnitude (X_i) of currents evoked by a given pulse in a train of pulses of an NMDA agonist, decreases exponentially with increasing pulse number towards a steady-state value (X_n). Therefore, a semilogarithmic plot of ($X_i - X_n$) *versus* pulse number gives a straight line. In order to derive from this line the forward and reverse binding rate constants, that define the interaction between dissociative anaesthetic and channels coupled to NMDA receptors, we make the following assumptions.

First we assume that the response to a brief pulse of agonist is due to a rapid rise in the open probability of all channels in the neurone somata to a peak value, P_o, that then declines with a time constant, τ_p, due to a combination of diffusion of agonist away from the cell and dissociation of the agonist from its receptor. We further assume that each of the responses is equivalent to one caused by an instantaneous change in open probability to P_o, that remains constant for a time equal to τ_p, and then returns to zero. In both cases the number of channels opened will equal $n\tau_p P_o/\tau_c$ where n is the number of channels that can experience the pulse of agonist and τ_c is the mean open time of the channel.

Second, we assume that in the absence of blocker the channel exists in one of two states, opened (O) or closed (C). The blocker induces additional states, open-blocked (OB) and closed-blocked (CB) but, can only associate or dissociate from the open form of the channel. This situation is described by the following kinetic scheme:



where k is the forward rate constant of blockade, D is the concentration of blocker

and b is the reverse rate constant of blockade. If we assume further that blockade has no effect on the binding of agonist or on the agonist induced transition between open and closed states, then at the peak of the response

$$O/(C+O) = OB/(CB+OB) = P_o, \tag{1}$$

$$\text{also since} \quad O+OB = P_o(C+O)+P_o(CB+OB),$$

$$(O+OB)/(C+CB+O+OB) = P_o. \tag{1A}$$

Thus, P_o refers to the probability that channels are in the open state and not only that they are in a conducting state.

With such a scheme, in the presence of a dissociative anaesthetic, a step change in open state probability to a value P_o would be associated with a current that declined exponentially to a new steady-state value with a time constant of blockade, τ_b, such that

$$1/\tau_b = P_o(b+kD). \tag{2}$$

Since we have assumed that our responses to pulses of agonist are equivalent to constant responses of duration τ_p, the rate of development of blockade per pulse, λ, is such that

$$\lambda = \tau_p P_o(b+kD) = \Delta \ln (X_i - X_n)/\Delta i, \tag{3}$$

where i is the pulse number, X_i is the magnitude of the response to the ith pulse of agonist and X_n is the steady-state value of that response.

At equilibrium,

$$\text{fraction blocked} = \frac{CB+OB}{CB+C+OB+O} = \frac{OB/P_o}{(OB+O)/P_o},$$

$$= \frac{OB}{OB+O} = \frac{kD}{b+kD} = \left(1-\frac{X_n}{X_0}\right), \tag{4}$$

where X_0 is the magnitude of the control response. Combining eqns (3) and (4) we obtain

$$\text{forward rate} = kD = (1-X_n/X_0)\lambda/\tau_p P_o, \tag{5}$$

$$\text{reverse rate} = b = (X_n/X_0)\lambda/\tau_p P_o. \tag{6}$$

In this paper we will make the simplifying assumption that $P_o = 1$. That is, we assume that each pulse of agonist can open all of the NMDA receptor channels in the cell soma.

After the dissociative anaesthetic is washed from the bathing medium NMDA receptor-activated currents recover exponentially and plotting $\ln(X_i - X_n)$ *versus* pulse number gives a straight line with slope λ. In this case the concentration of the dissociative anaesthetic is assumed to be zero and therefore the forward rate is also zero. This allows an alternative calculation of the reverse rate constant which we denote as the wash reverse rate, b', where from eqn (3),

$$\text{wash reverse rate} = b' = \lambda/\tau_p P_o. \tag{7}$$

RESULTS

Agonist applications

In most experiments we have employed L-aspartate to activate NMDA receptors. This agonist shows high specificity for NMDA receptors and does not activate other

subtypes of excitatory amino acid receptors at the concentrations employed (MacDonald *et al.* 1987). Responses to L-aspartate are entirely blocked by appropriate concentrations of Mg^{2+}, ketamine or APV. Furthermore, results were qualitatively similar when NMDA was used instead of L-aspartate (MacDonald *et al.* 1987). A high

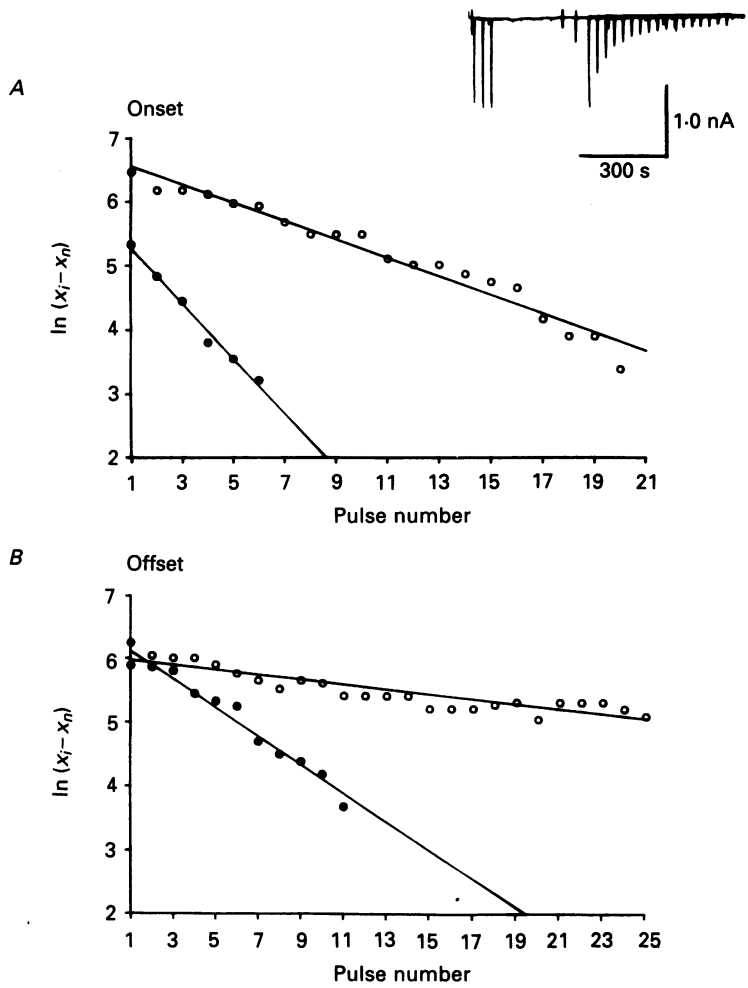


Fig. 1. A comparison of the onset (*A*) and the recovery (*B*) of blockade by the same concentrations (5 μM) of ketamine (●) and PCP (○) at −60 mV. The magnitude of the *i*th pulse in pA (X_i) is substracted from the steady-state value (X_n) and the difference is plotted semilogarithmically *vs.* pulse number. The slope of the line (λ) and the steady-state fractional inhibition (X_n/X_0) can be used to calculate forward and reverse rate constants of blockade (see text). The inset shows onset of the block of currents by PCP. Both the onset and offset of the block by ketamine is faster than that by PCP even though PCP causes a greater steady-state level of blockade. Calculated rate constants for a group of cells are given in the text.

concentration of agonist (250 μM) was used in order to cause nearly maximum activation of receptors close to the application site. Also, the applications were brief (20 ms) in order to limit the region of the cell membrane activated. The latter feature

would maximize the spatial adequacy of the voltage clamp. Furthermore, the brevity of agonist application would tend to minimize the degree of desensitization occurring during each response thereby facilitating complete recovery from desensitization between responses.

Voltage and use dependence of blockade of macroscopic currents by dissociative anaesthetics

Figure 1 illustrates that the blockade of L-aspartate responses by ketamine required fewer pulses of agonist to approach an equilibrium than did the blockade by PCP. For the same concentration of blocker both onset and offset rates were consistently greater for ketamine. In a series of cells, apparent forward and reverse rate constants were calculated in order to compare ketamine and PCP. The calculated forward rate constants for this group of cells were about the same for the two blockers (ketamine: $n = 5$, $k = 1{\cdot}7 \pm 1{\cdot}3 \times 10^4$ M^{-1} s^{-1}; PCP; $n = 10$, $k = 3{\cdot}3 \pm 1{\cdot}5 \times 10^4$ M^{-1} s^{-1}) but the reverse rate constant calculated for PCP was five times lower than that for ketamine (ketamine: $b = 0{\cdot}180 \pm 0{\cdot}06$ s^{-1}; PCP: $b = 0{\cdot}037 \pm 0{\cdot}02$ s^{-1}). In agreement with the model, reverse rate constants calculated from the rate plots during onset of the blockade were very similar to those calculated from the rate plots during recovery following removal of blocker from the bath (ketamine: $b' = 0{\cdot}23 \pm 0{\cdot}01$ s^{-1}, $n = 5$; PCP; $b' = 0{\cdot}038 \pm 0{\cdot}02$ s^{-1}, $n = 2$).

Under optimal conditions, the first response to a pulse of agonist after adding the use-dependent blocker to the bathing medium was of the same amplitude as control responses (see Fig. 1). However, this was not always the case. The first L-aspartate response after washing in the dissociative anaesthetic was often depressed to some degree and with high concentrations of dissociative anaesthetics this initial blockade could be substantial. In some cases, the initial blockade clearly could be attributed to leakage of agonist from the pressure pipette. It also is possible that a portion of the initial blockade was due to spontaneous release of excitatory amino acids from the tissue itself; some low level of synaptic activity was occasionally observed even in the presence of tetrodotoxin.

The concentration dependence of the blockade by PCP

Our ability to demonstrate state-dependent phenomenon as well as the voltage dependence of the blockade was related to the concentrations of dissociative anaesthetics employed. When we first initiated these experiments (Honey *et al.* 1985) little data was available to indicate appropriate concentrations of these compounds for interaction with the PCP site of the NMDA channel. Currently, there are a number of such studies available and it is possible to use K_i values for unlabelled ketamine, PCP and MK-801 for displacement of binding of a labelled high-affinity ligand such as [^{3}H]MK-801 or [^{3}H]TCP. For example, values of about 1 μM for ketamine, 45–80 nM for PCP and about 2–15 nM for MK-801 seem typical (Keana, Scherz, Quarum, Sonders & Weber, 1988). Initially we used, as have others (Huettner & Bean, 1988; Karschin, Aizenman & Lipton, 1988; Halliwell, Peters & Lambert, 1989), concentrations of ketamine and PCP (Honey *et al.* 1985) well in excess of these K_i values. However, it was clear that when high concentrations were

used it was extremely difficult to reverse the block the washing out the dissociative anaesthetics and a number of distinctive features of the block of NMDA currents were obscured. In particular, the apparent forward rates of the blockade and the potency appeared to be increased in earlier experiments where we employed higher concentrations.

Because of cell-to-cell variations and the technical difficulties of repeatedly evoking stable responses during the slow blockade by dissociative anaesthetics we examined a number of the features of blockade in some of our best recordings. For example, we examined effects of two different concentrations of PCP in the same cell with the objective of determining whether or not only the forward rate was sensitive to antagonist concentration. In Fig. 2*A*, a cell was held at −60 mV and PCP was applied twice at two different concentrations, with recovery permitted between applications. This figure illustrates that the rate of onset increased with increasing blocker concentrations. Calculation of the apparent forward and reverse rate constants demonstrated that the forward rate constants, were similar ($k = 1{\cdot}8 \times 10^5$ M^{-1} s^{-1}, 250 nM and $k = 1{\cdot}4 \times 10^5$ M^{-1} s^{-1}, 1000 nM) as expected if the forward rate increased with agonist concentration. As predicted for a simple bimolecular reaction, the reverse rate constants were also similar for both concentrations of PCP ($b = 0{\cdot}038$ s^{-1}, 250 nM and $0{\cdot}040$ s^{-1}, 1000 nM).

The importance of reaching steady-state blockade and approaching equilibrium

Our comparison of the forward and reverse rates of blockade by ketamine and PCP suggests that it is a major difference in the rate of dissociation from the channels which accounts for their difference in potency. Therefore, dissociative anaesthetics such as MK-801, which is at least tenfold more potent than PCP, would be anticipated to dissociate at an even lower rate. As a consequence, we would predict that the number of pulses required to reach a given steady-state level of blockade would increase in proportion to the increase in potency. Our calculation of the fraction of blocked channels would be extremely sensitive to a failure to achieve a steady-state level of blockade and a substantial error could be introduced by not continuing the agonist applications until this was achieved. Also, the level of blockade is best defined with concentrations of dissociative anaesthetics that induce intermediate levels of blockade. In the case of ketamine the rate of dissociation was sufficiently rapid that it was relatively easy to achieve an intermediate level of steady-state blockade. However, in the case of PCP we may have failed in some of our initial measurements thus reducing the accuracy of estimates of rate constants based on those data. In the case of MK-801 the rate of onset was exceptionally slow (Fig. 2*B*). Using our protocol it was necessary to apply hundreds of pulses in order to approach the steady state. The forward rate constants calculated for MK-801 were similar to those for PCP and ketamine but the reverse rate constants were at least ten times slower than those for PCP (20 nM-MK-801; $k = 3{\cdot}1 \times 10^5$ M^{-1} s^{-1}, $b = 0{\cdot}003$ s^{-1}). This again suggests that the relative potency of the three blockers is related to the rate at which they dissociate from the binding site. The rate of onset of block by MK-801 was also found to be concentration dependent and the results were consistent with only the forward rate being dependent on blocker concentration.

While the MK-801 blockade clearly was use dependent, it has been reported by

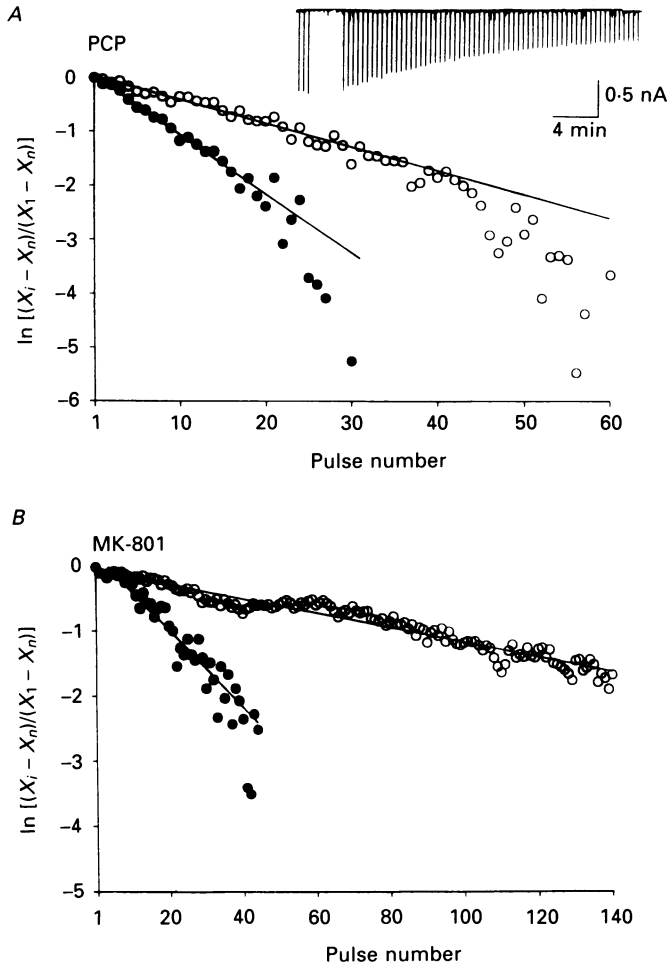


Fig. 2. Rate of onset of the blockade of L-aspartate currents by PCP and MK-801 at two different concentrations. See Fig. 1 for explanation of axes. *A*, the neurone was held at −60 mV and PCP was applied at a concentration of 250 nM (○) or 1000 nM (●). Recovery was achieved between applications of PCP. Note that the rate of onset was greater at the higher concentrations. There was incomplete recovery between applications of the two concentrations of PCP hence values have been normalized. The inset shows three control currents followed by the development of blockade in the presence of 250 nM-PCP. *B*, The rate of onset of blockade at 100 nM-MK-801 (●) was substantially greater that at a concentration of 20 nM (○). Only partial recovery from the blockade was achieved between applications of MK-801 as the rate of recovery following wash-out of this compound was exceptionally slow. Hence, the current amplitudes have been normalized.

Halliwell *et al.* (1989) and by Karschin *et al.* (1988) that the steady-state block is not voltage dependent. The former investigators recorded NMDA currents in cultured hippocampal neurones under conditions very similar to our own. However, they employed concentrations of MK-801 (300 nM) approximately 150 times higher than the K_i value. If excessively high concentrations of blocker are used (hundreds or

thousands of times greater than the K_i value) then the block will be substantial at all values of membrane potential and it becomes extremely difficult to measure changes in the relative fraction of blocked channels due to changes in membrane potential.

We therefore tested for voltage dependence using concentrations of MK-801 much closer to the K_i value (tenfold higher). In addition, because so many pulses were required to approach the steady-state level of blockade we introduced some high-frequency trains of pulses between series of test pulses. In Fig. 3 these trains are seen to reduce the amount of time required to develop the block by increasing the time of agonist exposure. Once a substantial level of block had been established (but not steady-state block) the membrane potential was changed to +40 mV. The application of a train of pulses in the continued presence of MK-801 caused the amplitude of the responses to increase, demonstrating a partial degree of relief of the block at this depolarized holding potential. That the block was relieved by holding at +40 mV was further illustrated when the holding potential was returned to −60 mV. The inward currents were substantially larger than they had been when the membrane potential had been previously held at this hyperpolarized value. Subsequently, repeated pulses caused a use-dependent re-establishment of the blockade.

The decline of the responses during the train was seen even in the absence of MK-801 and reflects a form of desensitization. MacDonald *et al.* (1987) showed that when pulses of agonist, similar to those used here, were delivered at a rate greater than 0·05 Hz, the responses declined during the train. This desensitization was much less pronounced at +40 mV (see Fig. 6*E*). Recovery from desensitization can occur within a minute at −60 mV (MacDonald *et al.* 1987) and can account for the rapid recovery of response amplitude after the trains in Fig. 6*A*, *B*, *C* and *E*.

The voltage dependence of the apparent rate constants

It was critical in these experiments to make highly stable and long-term recordings. While this could be done with difficulty at hyperpolarized membrane potentials it was considerably more difficult to do so at depolarized potentials. Holding at depolarized potentials appears to be deleterious to the seal of the patch electrode. Therefore, the numbers of experiments performed at depolarized potentials was much more restricted than at hyperpolarized potentials.

When membrane potential was stepped rapidly to positive potentials we observed a slow inward current relaxation in many cells. Returning to hyperpolarized potentials was also associated with a slow outward current relaxation. L-Aspartate currents evoked at depolarized potentials and during the slow inward relaxation were depressed while those to kainate were uninfluenced (MacDonald *et al.* 1987). Although we did not attempt to determine the origin of the current relaxation, it probably represents the activity of a variety of slowly deactivating outward currents a component of which may be a calcium-activated chloride current. NMDA receptor currents may be inhibited by increases in $[Ca^{2+}]_i$ and thus this selective inhibition of NMDA receptor currents during the relaxation may reflect the underlying recovery of the calcium transient. Alternatively, the change in amplitude of the L-aspartate currents may reflect voltage dependence of the agonist–receptor interaction (Wright

& Nowak, 1990). Regardless of the origin of the effect we always waited several minutes following a step change in membrane potential before measuring the response to the pulses of agonist.

In several cells we examined whether or not we could measure the voltage dependence of the apparent rates of association and dissociation for PCP. Cells were

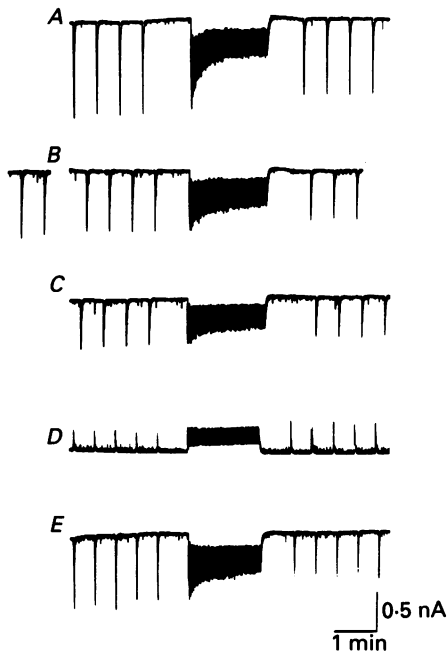


Fig. 3. Voltage dependence of the block of L-aspartate currents by MK-801 (20 nM). Consecutive portions of the pen recorder tracings are shown from *A* to *E*. Pulses of agonist were applied every 30 s. Onset of blockade was accelerated by a high-frequency train of applications (0·5 Hz) given for a period of 100 s every 5 min. In *A*, responses to single pulses and a train of pulses of L-aspartate are shown immediately following exposure to MK-801. Traces *B* and *C* show the fourth and sixth trains 20 and 30 min later, respectively. The two currents shown at the beginning of trace *B* were applied just before a 5 min cessation of agonist application. The next currents were evoked just after this period. Since there is no difference in amplitude, it is unlikely that desensitization was accumulating as a consequence of the agonist applications. Five minutes before trace *D* the holding potential was changed to +40 mV where test pulses and two trains of stimulations 5 min apart were applied. The third train is shown in *D*, a significant recovery of the test response was apparent following the first two trains and again following the third train. When membrane potential was returned to −60 mV (*E*) this recovery was retained and another train of pulses was required to re-establish at least part of the blockade.

held at +40 mV and PCP was applied at a relatively high concentration (2 μM). At this concentration substantial blockade could be produced at +40 mV. Following a wash-out of the drug, 2 μM-PCP was reapplied at −60 mV (Fig. 4). The rate of decline per pulse was similar at the depolarized and hyperpolarized potentials but the steady-state level of blockade was much greater at −60 mV (89% *vs.* 50%). The

apparent reverse rate constant of blockade was voltage dependent and increased at depolarized membrane potentials, the reverse rate constant increased from 0·03 s^{-1} at −60 mV to 0·38 s^{-1} at +40 mV. The forward rate constant did not decrease at depolarized potentials but instead demonstrated a slight increase ($1{\cdot}2 \times 10^5$ M^{-1} s^{-1}

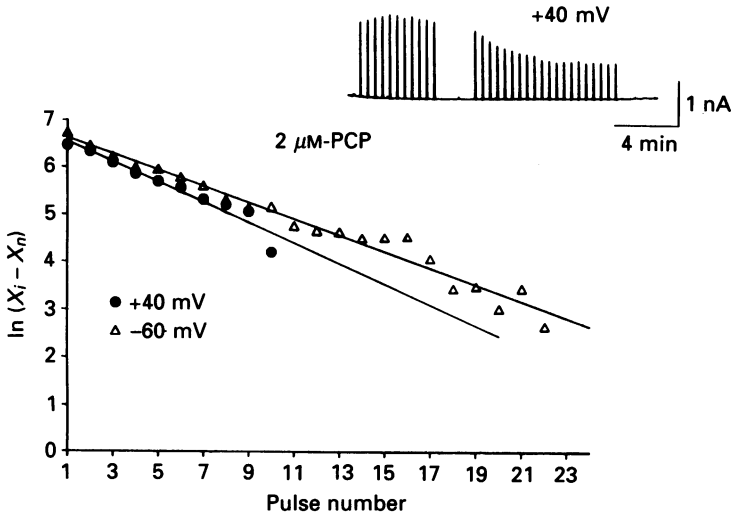


Fig. 4. The onset of blockade by 2 μM-PCP was examined at two different voltages, +40 mV and −60 mV. See Fig. 1 for explanation of axes. Inset shows onset at +40 mV during the first application of PCP. The rate of onset of blockade at the two voltages was similar; however, the steady-state level of blockade was greater at −60 mV (89% *vs.* 50%). Calculation of rate constants indicated that the forward rate of blockade was increased slightly and the reverse rate of blockade was greatly increased by changing the voltage from −60 to +40 mV (see text).

−60 mV; $1{\cdot}8 \times 10^5$ M^{-1} s^{-1} at +40 mV). The apparent EC_{50} of PCP was 2 μM at +40 mV *versus* 250 nM at −60 mV. These results are in direct contrast to the voltage dependence of the onset of blockade of NMDA receptor channels by divalent cations such as Mg^{2+} (Ascher & Nowak, 1988) where the forward rate of blockade is decreased by depolarization.

The charge of ketamine determines its potency

We decided to see if the charge or dipole on dissociative anaesthetic molecules could influence their ability to block NMDA receptor currents. The pK_a of ketamine is 7·5 (Volle, Alkadhi, Branisteanu, Reynolds, Epstein, Smilowitz, Lambert & Henderson, 1982). With 10 μM-ketamine therefore, at pH 8·5 only 0·9 μM is in the charged form *versus* 9·9 μM at pH 6·5. Acidification in itself significantly reduced NMDA receptor-activated currents by approximately 30% (Fig. 5). Despite this direct effect of acidification it was clear that the degree of blockade by ketamine was highly dependent upon extracellular pH. When apparent rate constants were calculated using 10 μM-ketamine as the concentration of blocker the reverse rate was

not substantially altered by changing pH ($n = 6$ for each group; pH 6·5: $b = 0{\cdot}18 \pm 0{\cdot}5\ s^{-1}, b' = 0{\cdot}34 \pm 0{\cdot}47\ s^{-1}$; pH 8·5: $b = 0{\cdot}22 \pm 0{\cdot}15\ s^{-1}, b' = 0{\cdot}22 \pm 0{\cdot}02\ s^{-1}$). In contrast, the onset rates differed by a factor of about 10 (pH 6·5: $k = 4{\cdot}9 \pm 2{\cdot}7 \times 10^4\ M^{-1}\ s^{-1}$; pH 8·5: $k = 0{\cdot}55 \pm 0{\cdot}32 \times 10^4\ M^{-1}\ s^{-1}$). Thus, the relative

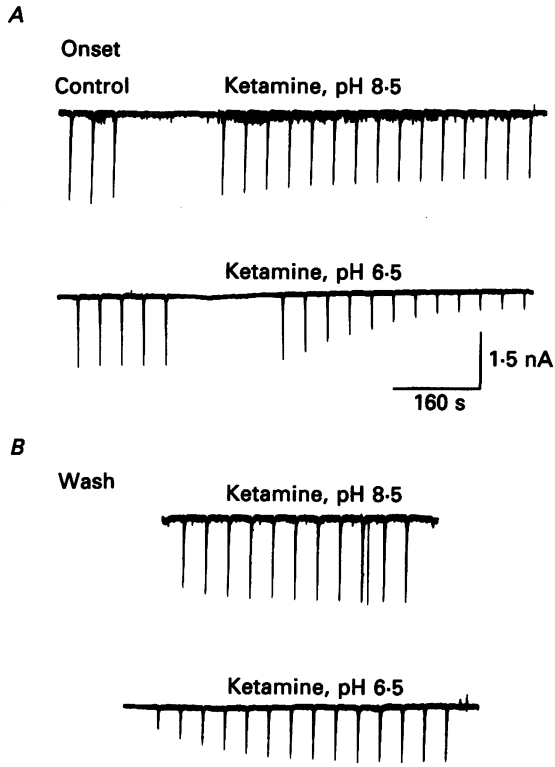


Fig. 5. Altering the extracellular pH changes the potency of ketamine and suggests that it is the charged form of ketamine which blocks NMDA receptors. Current responses to L-aspartate were recorded at a holding potential of −60 mV. In *A*, the onset of the blockade by ketamine (10 μM) is shown either at pH 6·5 or pH 8·5. Full recovery was achieved between applications and this recording lasted for approximately two hours. Note the reduction in the control currents at pH 6·5. The effectiveness of the ketamine blockade is considerably greater at the more acidic pH. In *B*, the recovery of the currents at each pH is shown. Calculations of the forward and reverse rate constants demonstrated the reverse rate constants were not greatly altered by such a difference in pH. However, the forward rate constant was changed tenfold as predicted from the change in concentration of the charged form of ketamine. Note, that the L-aspartate solutions used to evoke responses were at pH 7·4 in the pressure pipette; however, we expect that the pH of the aspartate solution would rapidly equilibrate with that of the bathing solution after ejection.

difference in potency of ketamine at the two different pH values could be accounted for entirely on the basis that it is the charged form which blocks NMDA receptor-activated currents.

We did not attempt to examine the voltage dependence of the blockade at the two different pH values because of the difficulty of making recordings at depolarized

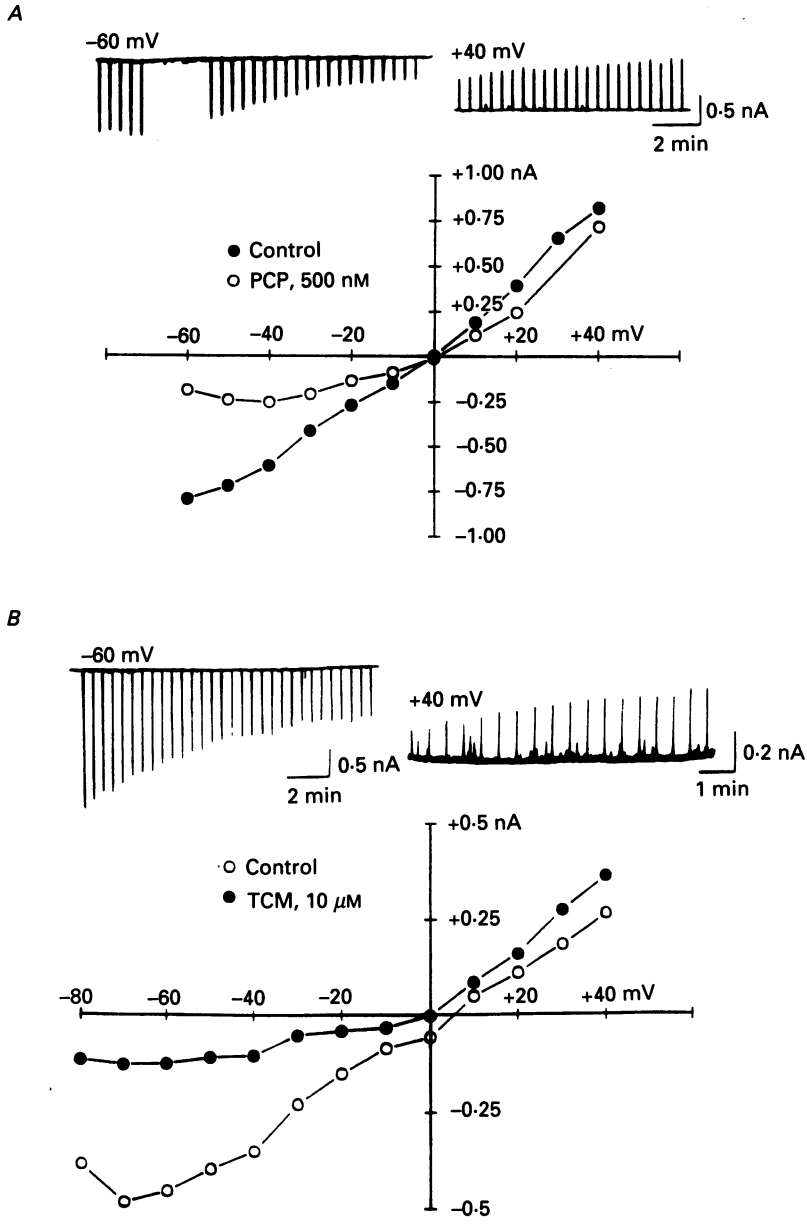


Fig. 6. PCP and TCM cause a use- and voltage-dependent block of L-aspartate currents in a cultured hippocampal neurone. *A*, PCP blockade, inset shows onset of blockade at −60 mV in the continued presence of PCP. Changing the membrane potential to +40 mV led to a use-dependent partial recovery from the blockade established at −60 mV. The graph plots the current–voltage relations of peak currents evoked by L-aspartate at different holding potentials. In the absence of PCP (●) the current–voltage relationship is fairly linear. ○, the magnitude of the currents after a steady-state level of block by PCP (500 nM) was achieved at a given holding potential. *B*, TCM blockade. TCM is an analogue of TCP which possesses a much lower dipole charge than either TCP or PCP. The addition of an oxygen in the primary ring of the TCP molecule greatly reduces the potential

potentials with many changes of bathing solution. As an alternative, however, we used an analogue of PCP, 1-(1-(2-thienyl)-cyclohexyl)morpholine (TCM), which has only a very weak dipole as a consequence of replacing the piperidine ring of TCP by a morpholine ring. Aguayo & Albuquerque (1986) have demonstrated TCM blocks endplate currents in a voltage-independent fashion even though the parent compounds demonstrated a clear voltage dependence. In addition, the potency of TCM was about ten times less than that of PCP in blocking endplate currents and we anticipated that this might also be true for the block of NMDA receptor-activated currents. We therefore applied the analogue TCM at a concentration of 10 μM (Fig. 6*B*). At this concentration and a holding potential of −60 mV, TCM caused a block of the NMDA receptor-activated currents that was approximately similar in magnitude to that produced by 500 nM-PCP (Fig. 6*A*). The block by TCM was characteristically use dependent. Unlike the case for ACh receptors, however, the blockade of NMDA receptor-activated currents by 10 μM-TCM was voltage dependent, and the degree of voltage dependence was similar to that observed with 500 nM-PCP.

Suppression of outward current flow through NMDA channels prevents recovery

Our results are *not* consistent with the hypothesis that it is the charge on the dissociative anaesthetic molecules which determines the voltage dependence of the block by these agents. Thus, in our experiments an outward flux of Cs^+ ions might be driving the blocker from NMDA receptor channels thereby accelerating the reverse rate of blockade. In this regard, we examined whether or not the voltage dependence of the block could be reduced by replacing permeant cations with an impermeant one. We substituted Cs^+ with *N*-methyl-D-glucamine and examined the voltage dependence of the block by ketamine and PCP. Because outward currents are not generated or are very small with this recording solution it is not possible to examine the dissociation rates at depolarized potentials. However, the voltage dependence of the re-establishment of the blockade following applications at depolarized potentials could be examined. Therefore, the block was established at −60 mV followed by a sufficient number of agonist pulses to re-equilibrate the block at +40 mV. Upon returning the membrane potential to −60 mV the response to the first pulse of agonist was compared to that expected for a steady-state level of blockade. In the absence of ions to permeate in an outward direction, recovery from blockade at +40 mV was substantially reduced (Fig. 7). This suggests that permeation of the channels by cations is indeed an important contributor to the voltage dependence of the dissociation rate.

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positive charge on TCM. Despite the lack of polarity, TCM caused a use- and voltage-dependent block of L-aspartate currents. Inset shows L-aspartate currents during the onset of the block by 10 μM-TCM, at −60 mV holding potential followed by partial recovery from blockade at +40 mV. Calculation of the rate constants gave values of $k = 2{\cdot}0 \times 10^4$ M^{-1} s^{-1} and $b = 0{\cdot}046$ s^{-1} at −60 mV. The graph plots the current–voltage relations of peak currents evoked by L-aspartate at different holding potential after a steady-state blockade has been established. Note the strong depression of L-aspartate currents at hyperpolarized potentials (−60 mV, 73% depression). In contrast, at +40 mV no depression was observed but rather a slight and unexplained enhancement of outward currents was observed.

Interaction with the open channel blocker Mg^{2+}

The forward rate and voltage dependence of the blockade of NMDA receptor-activated channels by Mg^{2+} are consistent with its interaction with a site located deep within the channel pore (Ascher & Nowak, 1988). If dissociative anaesthetics

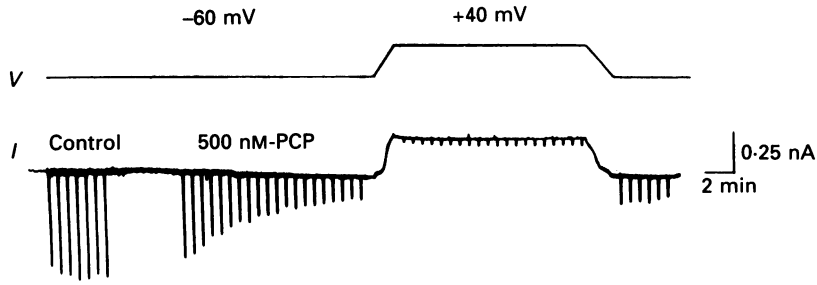

Fig. 7. Replacement of internal Cs^+ with the impermeant *N*-methyl-D-glucamine greatly diminishes the voltage dependent recovery of the blockade by PCP. Control L-aspartate currents are stable in amplitude prior to application of PCP. In the presence of 500 nM-PCP and a holding potential of −60 mV, a use-dependent blockade was observed. Further applications of this agonist at +40 mV evoked inward currents as a consequence of a shift in the reversal potential anticipated following the replacement of permeant intracellular cations with an impermeable one. Nevertheless, once the potential was returned to −60 mV the inward currents in response to L-aspartate failed to demonstrate an appreciable voltage-dependent recovery had occurred at +40 mV. It is of note that the first few responses at +40 mV appear smaller than the last few. This does not reflect recovery from blockade since there is no evidence of recovery upon return to −60 mV. Rather, the difference probably reflects the transient depression of L-aspartate currents observed during inward current relaxations found in some of these cultured neurones upon rapid depolarization (see MacDonald *et al.* 1987).

were to bind to the channel then we might expect that Mg^{2+} would inhibit access to the channel and therefore slow the onset of blockade. Divalent cations may offer further protection by reducing the probability of channel opening (Nowak *et al.* 1984; Ascher & Nowak, 1988; Christine & Choi, 1990). Protection by 10 mM-Mg^{2+} was reported by Huettner & Bean (1988) for the blockade by MK-801 and by MacDonald, Miljkovic & Schneiderman (1986) for ketamine. In the present study we have found that this protection can be provided by substantially lower concentrations of Mg^{2+} (Fig. 8*A*). At a concentration of 500 μM-Mg^{2+}, which produced an approximately 80% blockade of NMDA receptor-activated currents by itself, steady-state blockade by PCP was reduced. There was no obvious action of Mg^{2+} upon the apparent rate of dissociation of ketamine suggesting that a channel blocked by ketamine cannot bind Mg^{2+}.

Competitive antagonist site

One alternative explanation for the blockade by dissociative anaesthetics is that simple occupation of the agonist site influences the affinity or access of the blockers for their site, rather than the conformational change in the receptor complex from a closed to open channel state. We examined this possibility by testing to see if the use-

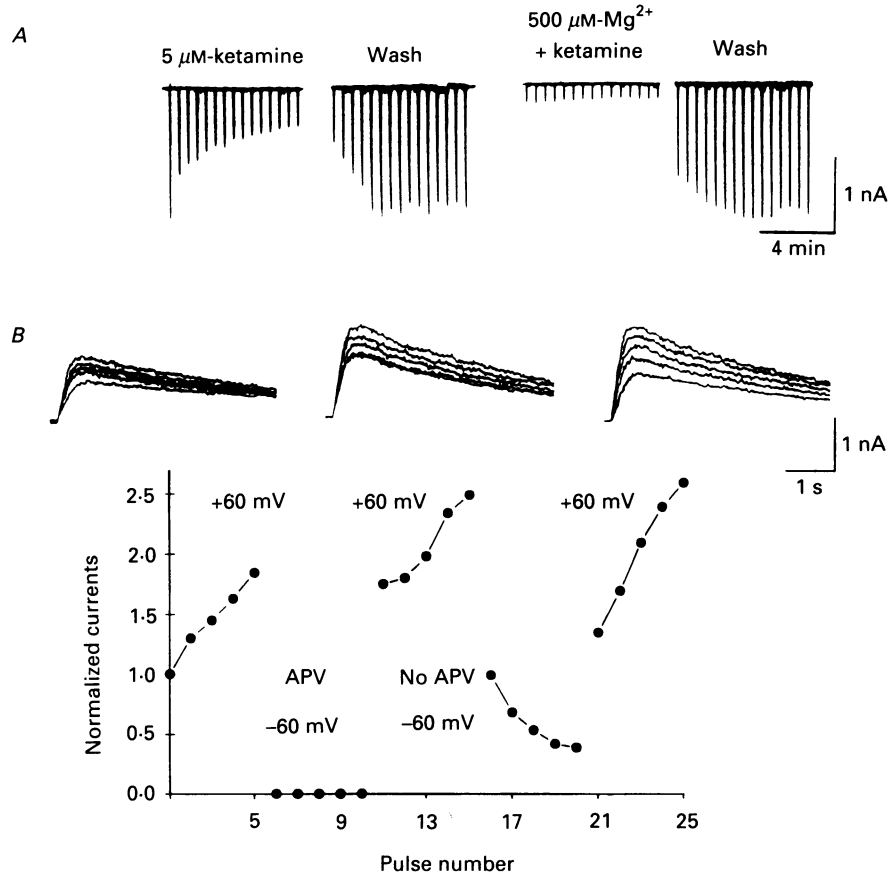

Fig. 8. *A*, the block of L-aspartate currents by ketamine can be prevented by exposure to relatively low concentration of Mg^{2+}. The onset of the ketamine block at a holding potential of −60 mV is shown, as is the use-dependent recovery of the currents after ketamine had been washed out of the bath. Note that after washing out ketamine the first response remained blocked by 60% and then there was a use-dependent recovery. Following this recovery a solution containing ketamine and Mg^{2+} (500 μM) was placed in the bathing chamber. L-Aspartate currents were substantially reduced by Mg^{2+} leaving small residual current responses which showed use-dependent blockade. However, after removal of both ketamine and Mg^{2+} the initial current was depressed by only 35% demonstrating that even though the NMDA receptors were exposed to the same applications of agonist a much lesser degree of blockade to ketamine developed in the presence of Mg^{2+}. *B*, exposure to the competitive antagonist APV does not enhance the onset nor recovery of the use-dependent block by ketamine. This neurone was continuously exposed to 5 μM-ketamine at all times and repeated exposures to L-aspartate were made to develop a steady-state level of blockade −60 mV (not shown). The holding potential was then changed sequentially as shown in the plot. Individual current amplitudes were normalized to the initial current recorded at +60 mV. Actual outward currents recorded at +60 mV and superimposed upon one another are shown above the appropriate regions of the plot. At the beginning of the plot the cell was held at +60 mV and five applications of L-aspartate were made (pulses 1–5). The amplitude of the outward currents demonstrated a use-dependent partial recovery. The membrane potential was then returned to −60 mV and APV (100 μM), a concentration sufficient to block the inward L-aspartate currents, was added to the bathing solution in the continued presence of ketamine (pulses 6–10). Previous applications of APV to this neurone permitted a

dependent block by ketamine was influenced by occupation of the recognition site by a competitive antagonist. We observed that concentrations of APV which totally blocked NMDA receptor-activated currents neither promoted onset nor recovery from blockade (Fig. 8*B*).

DISCUSSION

Our hypothesis is that dissociative anaesthetics bind in the vicinity of the extracellular mouth of the NMDA channel pore. Our observations that the blockade of NMDA receptor-activated currents by dissociative anaesthetics is state dependent implies that NMDA channels must be gated open before the binding site for dissociative anaesthetics will be exposed. The interaction with Mg^{2+} suggests that both Mg^{2+} and the dissociative anaesthetics are mutually exclusive in their binding. Since there is good evidence that Mg^{2+} binds within the channel, it is likely that dissociative anaesthetics bind close to the channel. Finally, the fact that movement of ions through the channel at positive potentials influences the dissociation rate also implies that the binding site is close to the channel mouth.

We have used a simple model to compare the blockade of NMDA receptor-mediated current by three dissociative anaesthetics. This model is a highly simplified version of the 'guarded receptor hypothesis' (Starmer *et al.* 1984, 1986) and allows us to estimate roughly the rate constants of binding and dissociation of the anaesthetics. We have assumed that the kinetics of association and dissociation of the anaesthetic with the closed state of the receptor are sufficiently slow relative to the time course of the experiment that for our purposes they can be discounted. We have assumed also that once associated with the channels the anaesthetic molecules do not prevent dissociation of the excitatory amino acid agonists and channel closure. Moreover, if the channel closes while associated with a dissociative anaesthetic molecule, that molecule becomes trapped on or within the closed channel.

The kinetics of the blockade therefore depend strongly upon the gating properties of the channels themselves. For our purposes we have explicitly assumed that the probability of channel opening during a pulse of agonist is close to one and that

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determination of how long it was necessary to wait for full recovery of the L-aspartate currents from blockade by this competitive antagonist. Therefore, once the APV was removed (but not ketamine) we returned the holding potential to +60 mV and made five more applications of L-aspartate (pulses 11–15). The initial outward current (pulse 11) was similar in amplitude to that evoked by pulse 5 demonstrating that little if any re-establishment of the block by ketamine had occurred during the applications at −60 mV in the presence of APV. Thus the ketamine blockade did not develop at −60 mV even though the neurone had been exposed both to the agonist and the competitive antagonist. As a control, the membrane potential was changed once again to −60 mV and the same number of applications of L-aspartate were made in the absence of APV (pulses 16–20). A use-dependent block of the inward currents developed but the number of applications was insufficient to return to the steady-state level of block. Finally, once the holding potential was again changed to +60 mV the initial outward current (pulse 21) was much smaller than the previous response at this potential (pulse 15), indicating that the block by ketamine at −60 mV had developed and had been retained. With subsequent applications of L-aspartate (pulses 22–25) a use-dependent and partial recovery of the block occurred again. Note that the response to pulse 21 is only slightly larger than the first pulse of the series.

desensitization of the receptors is relatively constant between agonist pulses. However, there is evidence from single-channel experiments which suggests that these two assumptions may be incorrect. For example, Huettner & Bean (1988) reported the maximum probability of NMDA-gated channel opening appears to be unexpectedly low. This low probability of opening would lead us to underestimate the forward and reverse rate constants of blockade. If they are right, our forward and reverse rate constants should be divided by 0·002, the maximum open channel probability reported by Huettner & Bean (1988). When this is done the forward rate for MK-801 is about the same magnitude, 10^6–10^7 M^{-1} s^{-1}, as that estimated from the shortening of single-channel open times (Huettner & Bean, 1988). The corrected forward and reverse rate constants for blockade of NMDA channels also approach those estimated for local anaesthetic interactions with nicotinic ACh channels (Neher & Steinbach, 1978). This suggests that dissociative anaesthetics could fall into the broad category of open channel blockers which interact rapidly with the open channel. It should be noted, however, that the correction would not affect the *relative* magnitude of the rate constants nor the steady-state affinities estimated from the ratio of forward and reverse rate constants.

One possible explanation for an unexpectedly low probability of channel opening is the observation of a rapid component of desensitization of NMDA channels which has been reported in outside-out patch recordings (Sather, Johnson, Henderson & Ascher, 1989). Desensitization under these conditions is accentuated as the concentration of agonist is increased, often to a degree such that the steady-state current is at most 20% of the initial level. The time constant of the onset of this desensitization is in the range of 100–400 ms and well within the time course of the time to peak of our individual current responses to L-aspartate. This suggests that, even though we have made an attempt to apply the agonist rapidly and have minimized any build up of slow desensitization during the train of pulses, the majority of receptors may rapidly become desensitized. The amplitude of our current response may be related, at least in part, to NMDA receptors cycling between a low or non-conducting desensitized state and an open state which can then be blocked by the dissociative anaesthetics.

One of our assumptions is that the forward and reverse rates of binding of dissociative anaesthetics with closed NMDA receptor channels are sufficiently low that they can be ignored. Kloog *et al.* (1988) have estimated that in the absence of agonist, [^{3}H]TCP dissociates with a rate constant of about 10^{-4} s^{-1} or 2·8 h^{-1}. Although the actual affinity of TCP for the NMDA receptor in the absence of agonist was estimated to be close to that of the agonist-associated receptor, the rates of interaction with the activated receptor were much faster than with non-activated receptors. In our experiments, it is difficult to determine to what degree closed NMDA receptor channels interact with dissociative anaesthetics primarily because it is not possible to ensure that there is no spontaneous release of excitatory amino acids from cells in the cultures. Furthermore, it also is possible that spontaneous openings of NMDA channels occurs in the absence of agonist (or in the presence of antagonist) analogous to that reported from ACh receptors (Jackson, 1984). Thus, there is considerable potential for opening of the NMDA channels independent of our applications of L-aspartate. Nevertheless, opening may not be necessary for channel

blockade. It is possible that the lipid-soluble character of the dissociative anaesthetic can allow them to cross, albeit slowly, the state-dependent barrier that regulates access to their binding site.

The lipid solubility of dissociative anaesthetics also suggests that they might be accumulated in the membranes of the various cells found in our dissociated tissue cultures, particularly when high concentrations are employed. Therefore, the membranes might provide a concentration sink of dissociative anaesthetics. This would make it difficult to ensure that the blockers are entirely washed out of the preparation and might account for at least some of the difficulty in reversing the block of L-aspartate currents following exposures to high concentrations of dissociative anaesthetics. A similar lack of reversibility was reported for the chlorisondamine block of ACh currents by Neely & Lingle (1986). A sink of lipid-bound blocker also seems likely to account for our previous observations that following two consecutive periods of washing out ketamine from the bath the block can be re-established (see Fig. 7 of MacDonald *et al.* 1986).

In spite of these difficulties and concerns, we have used our model to obtain rough estimates of the kinetic rate constants governing the binding of ketamine, PCP and MK-801 to the NMDA receptor channel. In the case of MK-801, the dissociation rate was so slow that it was not practical to make such measurements routinely. Nevertheless, it was clear that the apparent forward rate constants for ketamine, PCP and MK-801 at −60 mV were of the same order of magnitude, but that the apparent reverse rates differed substantially. These results suggest that the difference in affinity (K_d) of NMDA channels for dissociative anaesthetics is determined primarily by the rate at which they dissociate from open NMDA channels. Of course, alterations in NMDA gating of channels induced by binding of the dissociative anaesthetics also might play a role. Our estimated values of effective K_d values at −60 mV calculated from the ratios of apparent rate constants were approximately 10 nM for MK-801, 100–250 nM for PCP and 1–10 μM for ketamine. These values are of the same order as those deduced from binding studies (see Keana *et al.* 1988).

The apparent forward rates need to be corrected for probability of channel opening. If the value of 0·002, suggested by Huettner & Bean 1989, is used the rates approach the appropriate order of magnitude for fast channel blockade. The rates of dissociation from the channel should also be influenced by the probability of channel opening and by any voltage dependence of the probability of the NMDA channels being in the open state. Thus, our calculated forward and reverse rate constants may be much lower than the molecular rate constants which must be determined more directly from single-channel experiments.

Our estimates suggest that the forward rate depends upon the concentration of the anaesthetic while the reverse rate is concentration independent. This observation is consistent with the bimolecular aspect of our simple model. However, our method of estimating the rate constants is critically dependent upon attaining a steady-state and intermediate level of blockade. This was extremely difficult to achieve with low concentrations of MK-801 and would also be likely to introduce errors to some extent into our measurements for ketamine and PCP. The slow onset of the blockade means that the period of agonist exposure required to reach equilibrium is critical. The use of high concentrations of MK-801 and PCP can speed the onset of the block but also

introduces other difficulties because the accuracy of estimates of the level of steady state of blockade is compromised (Karschin *et al.* 1988; Halliwell *et al.* 1989).

We have proposed that dissociative anaesthetics cause a use- and voltage-dependent blockade of open NMDA receptor channels with trapping of the blocker on the closed channels. As each of these dissociative anaesthetics is positively charged the voltage dependence could arise by assuming that each molecule of blocker experiences the transmembrane field as it accesses a binding site in the channel pore. However, none of our present evidence supports this mechanism of dissociative anaesthetic block. In particular, the forward rate constant is not appropriately dependent upon membrane potential. The rate of association is not reduced by depolarization as predicted from the Woodhull model (1973) but rather it is enhanced. Nowak & Wright (1990) have recently reported that the microscopic forward rate constant of blockade by tiletamine estimated from single-channel data is also voltage independent. This contrasts with the case of the Mg^{2+} blockade of NMDA receptor channels where the forward rate constant is highly voltage dependent (Nowak *et al.* 1984; Ascher & Nowak, 1988).

Recent single NMDA receptor channel experiments by Wright & Nowak (1990) have shown that the probability of NMDA receptor channel opening (frequency and mean open time) is also increased by membrane depolarization. This would facilitate the open channel blockade by dissociative anaesthetics at depolarized potentials and may account for slight enhancement of the onset of blockade by PCP that we observed at depolarized potentials. The forward rate of blockade of calcium-activated K^+ channels by charybdotoxin also exhibits a slight voltage dependence that disappears when channel open probability is taken into account (MacKinnon & Miller, 1988).

The voltage dependence of the charybdotoxin blockade is strongly dependent upon the generation of an outward current through the K^+ channels (MacKinnon & Miller, 1988). Thus, substitution of impermeant ions on the intracellular side of the channels eliminated the voltage dependence of the reverse rate. McKinnon & Miller (1988) have suggested a model to explain this result whereby permeating cations would accelerate the dissociation of the blocker from its binding site. We have shown that the voltage-dependent recovery of the dissociative anaesthetic block at positive potentials is also highly dependent upon the induction of outward current flow through NMDA channels implying that a similar mechanism may account for our observed voltage dependence of the 'off' rates of the blockade by dissociative anaesthetics. A reduction in the reverse rate in the absence of outward current also occurs in the case of MK-801 (J. E. Huettner, personal communication).

Evidence from single-channel studies has suggested that the NMDA channel pore mouth or vestibule may be ringed with negative charges (Ascher, 1988). These negative-charged groups might provide substantial electrostatic interaction with positively charged dissociative anaesthetics and provide a partial polar binding site, as suggested by Manallak, Wong, Costa, Andrews & Beart (1988) for the PCP receptor. In addition, the lipophilic ring structures of the dissociative anaesthetics would still be capable of interacting with hydrophobic regions of the binding site. Thus, changing the concentration of positively charged ketamine would reduce the potency of this dissociative anaesthetic by reducing any potential electrostatic

interactions with the channel pore. Similarly the lower potency of the neutrally charged dissociative anaesthetic TCM could be accounted for by the same reduction in this electrostatic interaction. A number of compounds that are unrelated to dissociative anaesthetics have also been reported to cause a voltage-dependent and/or use-dependent block of NMDA currents (Bertolino & Vicini, 1988; O'Dell & Christensen, 1988; Keana, McBurney, Scherz, Fischer, Hamilton, Smith, Server, Finkbeiner, Stevens, Jahr & Weber, 1989; Priestley, Woodruff & Kemp, 1989; Sernagor, Kuhn, Vyklický & Mayer, 1989). This wide variety of agents acting via similar mechanisms suggests that they might also interact electrostatically with the vestibule region of the channel pore.

Manallack *et al.* (1988) have proposed a three-site model whereby PCP and MK-801 would bind to two hydrophobic and one additional polar site where a hydrogen bond is made between the nitrogen atom of the anaesthetic and the PCP receptor. Electrophysiological evidence suggests that these sites would be closely associated with the open state of NMDA channel and that the pore mouth or vestibule may represent a significant site of electrostatic interaction with dissociative anaesthetics. Once the NMDA channels close, dissociative anaesthetics become trapped on or in the channel complex. It may be that a portion of the dissociative anaesthetic molecule enters part way into the channel pore as suggested for the trapping of chlorsondamine in ACh channels (Neely & Lingle, 1986) or perhaps they can interact with a hydrophobic pocket located within the NMDA channel pore itself (Neher & Steinbach, 1978). Although dissociative anaesthetics would associate at a rapid rate with open channels the low rate of channel opening would greatly impede the onset of the blockade (Huettner & Bean, 1988). Furthermore, the relatively slow dissociation of blockers from open channels (consistent with a 'slow channel block' of NMDA channels) or the low probability of channel opening in combination with the trapping of drug molecules within the closed state would lead to a particularly slow rate of recovery from the block by dissociative anaesthetics.

Antagonists of NMDA receptors would be anticipated to block components of excitatory postsynaptic potentials which are generated by activation of NMDA receptors (Sutor & Hablitz, 1989). In the case of a competitive antagonist the kinetics of blockade would be largely dependent upon the pharmacokinetics of delivery of the antagonist to the vicinity of these receptors. However, the dissociative anaesthetics exhibit use dependence and trapping. Thus, it may be necessary for NMDA-mediated EPSPs to be evoked repeatedly in order to attain or recover from a steady-state level of blockade (Schneiderman & MacDonald, 1989). In the case of some uncompetitive nicotinic antagonists, synaptic activity will amplify their blocking potency (Gurney & Rang, 1984). The kinetics of this blockade, both onset and recovery, are apparently further slowed by the low probability of the NMDA channels being in the open state (Huettner & Bean, 1988; Wright & Nowak, 1990). The blocking kinetics of the dissociative anaesthetics with respect to trains of EPSPs may provide information about the maximum open probabilities of synaptic NMDA channels. In summary, the blockade by dissociative anaesthetics provides a unique mechanism of modulating excitatory activity in the central nervous system, one which depends upon not only the state of the NMDA receptors but also upon their previous activity.

This work was supported by a grant to J. F. MacDonald from the Medical Research Council of Canada. P. Pahapill is a Career Scientist of the Ontario Ministry of Health.

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