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Inhibition of NMDA-gated ion channels by the P2 purinoceptor antagonists suramin and reactive blue 2 in mouse hippocampal neurones

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1 The action of suramin and reactive blue 2 on *N*-methyl-D-aspartate (NMDA)-activated ion current was studied in mouse hippocampal neurones in culture by use of whole-cell patch-clamp recording.

2 Suramin and reactive blue 2 inhibited steady-state current activated by 25 μM NMDA with IC_{50} values of 68 and 11 μM, respectively.

3 Reactive blue 2 produced a gradual decline of NMDA-activated current to a steady-state, but this slow onset was not an indication of use-dependence, as it could be eliminated by exposure to reactive blue 2 before NMDA application. In addition, NMDA-activated current recovered completely from inhibition by reactive blue 2 in the absence of agonist.

4 The slow onset of inhibition by reactive blue 2 was not apparently due to an action at an intracellular site, as inclusion of 250 μM reactive blue 2 in the recording pipette did not alter inhibition by 25 μM reactive blue 2 applied externally.

5 Reactive blue 2 and suramin inhibited NMDA-gated channels in a voltage-independent manner.

6 Reactive blue 2, 25 μM, decreased the maximal response to NMDA from 1441 to 598 pA without changing its EC_{50}. In contrast, 75 μM suramin increased the EC_{50} for NMDA from 13 to 35 μM, and decreased the maximal response to NMDA from 1822 to 1498 pA. Schild analysis of suramin inhibition of NMDA-activated current yielded a nonlinear plot.

7 Both agents decreased the maximal response to glycine without altering its EC_{50}.

8 Suramin and reactive blue 2 appear to inhibit NMDA receptor-channels in a manner that is noncompetitive with respect to both NMDA and glycine. However, inhibition by suramin differed from that by reactive blue 2, in that suramin significantly increased the EC_{50} of NMDA.

Keywords: NMDA; suramin; reactive blue 2; purinoceptor; glycine; hippocampus

Introduction

N-methyl-D-aspartate (NMDA) receptors are cation channels activated by glutamate, which is now recognized as the predominant excitatory neurotransmitter in the central nervous system. NMDA receptor-channels are widely distributed in the brain and are implicated in multiple physiological phenomena, such as motor coordination (Willetts *et al*., 1990), neurotoxicity and neurodegenerative disorders (Lipton & Rosenberg, 1994), and certain types of neuronal plasticity believed to underlie some forms of learning and memory (Bliss & Collingridge, 1993). NMDA receptor function is also affected by a number of drugs, most notably dissociative anaesthetics (Lodge & Anis, 1982) and ethanol (Lovinger *et al*., 1989).

Another neurotransmitter that has recently received much attention is extracellular adenosine 5′-triphosphate (ATP). Among other actions, ATP can mediate fast excitatory synaptic transmission in the brain (Edwards *et al*., 1992) and in autonomic ganglia (Evans *et al*., 1992; Silinsky *et al*., 1992). Fast excitatory neurotransmission mediated by ATP results from its activation of P2X purinoceptors, a class of ligand-gated cation channels (Bean, 1992). Although a number of P2X receptor subunits have recently been cloned (Buell *et al*., 1996), little is known regarding the physiological functions of this class of receptor-channels. The potential importance of these receptor-channels in neuronal function is evidenced not only by their role in mediating excitatory postsynaptic potentials in the brain and periphery (Evans *et al*., 1992; Silinsky *et al*., 1992; Edwards *et al*., 1992), but also by their widespread distribution throughout the central and peripheral nervous systems (Collo *et al*., 1996).

Despite considerable structural differences between NMDA and P2X receptors (e.g., a proposed topology of three membrane-spanning regions and one re-entrant loop in NMDA receptors vs two transmembrane domains in P2X purinoceptors (Brake *et al*., 1994; Kuner *et al*., 1996)), these receptor-channels appear to be similar to each other in certain respects. For example, both NMDA and P2X receptor-channels conduct Ca^{2+} (Rogers & Dani, 1995), and thus both types of receptor-channels can directly elevate intracellular Ca^{2+} concentration. NMDA and P2X receptor-channels are also highly sensitive to modulation by a number of metal ions, such as Mg^{2+} (Nowak *et al*., 1984; Li *et al*., 1997b) and Zn^{2+} (Westbrook & Mayer, 1987; Cloues *et al*., 1993; Li *et al*., 1993b; 1997a), as well as protons (Traynelis & Cull-Candy, 1990; King *et al*., 1996; Li *et al*., 1996). Like NMDA receptor-channels, at least some types of P2X purinoceptors are inhibited by ethanol (Li *et al*., 1993a). Interestingly, results of recent studies have shown that the P2 purinoceptor antagonists suramin and reactive blue 2 inhibit radioligand

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binding to the NMDA receptor (Balcar *et al*., 1995), and NMDA-activated current in CNS neurones (Nakazawa *et al*., 1995; Motin & Bennett, 1995). However, the mechanisms of action of each of these agents on the NMDA receptor have not been determined. The aim of this study was to determine whether NMDA receptor inhibition is a common property of P2 purinoceptor antagonists and to determine the mechanisms by which these agents inhibit NMDA receptor-channels.

Methods

Cultures of hippocampal neurones on glial feeder layers were prepared from 15- to 17-day foetal mice. Hippocampi were dissected in Hank's buffered salt solution (HBSS) containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), DNase type I (Boehringer-Mannheim, Indianapolis, IN, U.S.A.), and 1 mM sodium pyruvate (pH 7.4); incubated in 0.25% trypsin (Gibco BRL, Grand Island, NY, U.S.A.) in HBSS at 37°C for 15 min; washed three times in HBSS at room temperature; and triturated 30–40 times with a fire-polished Pasteur pipette. Neurones were plated on confluent layers of hippocampal glia in minimum essential medium (MEM) containing 10% heat-inactivated equine serum (HyClone, Logan, UT, U.S.A.) and 1 mM sodium pyruvate. After 4 h, half of this medium was replaced with a maintenance medium consisting of MEM, 1 mM sodium pyruvate, and N2 serum supplement (Gibco BRL); this medium was subsequently given half-changes weekly. Neurones were cultured for 1 to 4 weeks before use in experiments. The care and use of animals in this study was approved by the Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism in accordance with National Institutes of Health guidelines (protocol number: LMCN-SP-01).

Patch-clamp recording of whole-cell currents was performed at room temperature with an Axopatch-1D or Axopatch 200 (Axon Instruments Inc., Foster City, CA, U.S.A.) patch-clamp amplifier. Gigaohm seals were formed by use of electrodes with tip resistances of 2–5 MΩ, and series resistances of 4–15 MΩ were compensated by 80–90%. Membrane potential was held at −50 mV, unless noted otherwise. In most cases, data were filtered at 2 kHz (8-pole Bessel) and acquired on computer (5 kHz sampling frequency) during experiments with a Labmaster TL-1 or DigiData 1200A interface and AxoTape or pCLAMP software (Axon Instruments). Data were also recorded on videotape with a VR-10B digital data recorder (Instrutech Corp., Great Neck, NY) connected to a videocassette recorder (Sony SLV-440). In some cases, data were filtered at 4 kHz (8-pole Bessel) and recorded on videotape, and were later replayed through a low-pass 8-pole Bessel filter (2 kHz corner frequency) and acquired at a sampling frequency of 5 kHz on a computer.

Neurones were superfused at 1–2 ml min^{-1} in an extracellular medium containing (in mM): NaCl 150, KCl 5, $CaCl_2$ 0.2, HEPES 10, glucose 10, tetrodotoxin 0.0002 and strychnine 0.001 (to block glycine receptor-mediated chloride currents); pH was adjusted to 7.4 with NaOH and osmolality to 340 mosmol kg^{-1} with sucrose. Low Ca^{2+} was used to minimize NMDA receptor inactivation (Zilberter *et al*., 1991). Unless noted otherwise, the recording pipette solution contained (in mM): CsCl 95, Mg_4ATP 4, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) 10, HEPES 10, creatine phosphate 20, creatine phosphokinase 50 u ml^{-1}; pH was adjusted to 7.4 with CsOH and osmolality to 310 mosmol kg^{-1} with sucrose.

Solutions of agonists and drugs were prepared in extracellular medium and were applied to neurones by use of one of two systems. In most experiments, solutions were applied by gravity flow with a linear multi-barrel array of fused silica tubing (300 μm i.d.) placed within 100 μm of the cell body. Cells were constantly bathed in extracellular medium flowing from one barrel (flow rate ~ 3 μl s^{-1}), and treatment solutions were applied by opening a valve and moving the barrel array so that the desired solution bathed the cell. In some experiments, a rapid solution exchange system was used. This system consisted of two 300 μm i.d. fused silica exit tubes set at ~30° angles to each other. Each exit tube was connected to the outflow of a manifold composed of four barrels of 300 μm i.d. fused silica tubing, glued together in a cylindrical pattern and inserted into a length (~5 mm) of silicone rubber tubing (500 μm i.d.). Each manifold barrel was connected to a reservoir via a solenoid valve (The Lee Co., Westbrook, CT, U.S.A.). Solution flowed continuously from one of the two exit tubes, and solutions were rapidly changed by closing and opening valves to switch the flow between the two exit tubes. Solenoid valves were controlled by computer by means of locally-written software (VDriver; R.W. Peoples) to allow for rapid and precise opening and closing. With this sytem, the 10–90% rise time of the junction potential at an open pipette tip was ~2 ms. In all experiments, solutions containing excitatory amino acids were applied at intervals of at least 90 s.

Statistical analysis of concentration-response data was performed by the nonlinear curve-fitting programme ALLFIT (DeLean *et al*., 1978), which uses an analysis of variance (ANOVA) procedure. Values for concentration yielding 50% of maximal effect (IC_{50} for inhibition, EC_{50} for activation) and slope factor (n) are those obtained by fitting the data to the logistic equation:

$$y = \frac{E_{max} - E_{min}}{1 + (x/IC_{50})^{-n}} + E_{min}$$

where x and y are concentration and response (i.e. % inhibition), respectively, and E_{max} and E_{min} are the maximal and minimal responses, respectively. In some cases, when E_{min} did not differ significantly from zero, this variable was constrained to zero in the curve fitting procedure. Statistical evaluation of differences between means was determined by ANOVA or Student's *t* tests by use of the programme InStat (GraphPad Software, San Diego, CA). All values are presented as the mean ± s.e.mean.

Suramin was obtained from FBA Pharmaceuticals (West Haven, CT, U.S.A.) and all other drugs, including pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and reactive blue 2 (Basilen Blue), were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Effects of suramin, reactive blue 2 and PPADS on NMDA-activated current

Application of 25 μM NMDA and 1 μM glycine to cultured hippocampal neurones activated currents exhibiting slight to moderate desensitization. Suramin, at a concentration of 100 μM, inhibited peak and steady-state NMDA-activated current to a similar extent (Figure 1a). Recovery from inhibition by suramin was complete after 90 s and did not require the presence of agonist. Reactive blue 2, 25 μM, produced a moderate inhibition of peak NMDA-activated current, followed by a gradual decline of the current to a

steady-state (Figure 1b). The time constant (τ) for the decline of 25 μM NMDA-activated current in the presence of 25 μM reactive blue 2 was 495 ± 54 ms ($n=7$). Recovery from inhibition by reactive blue 2 occurred in 90 s or less, and did not require the presence of agonist. In contrast, PPADS at a concentration of 100 μM had little or no effect on NMDA-activated current (Figure 1c).

Concentration-response analysis revealed that suramin inhibited current activated by 25 μM NMDA in a concentration-dependent manner, and that it affected peak and steady-state current similarly (Figure 2a). Suramin inhibited NMDA-activated current with IC_{50} values of 63 ± 16 and 68 ± 14 μM for peak and steady-state current, respectively; these values did not differ significantly (ANOVA, $P>0.05$). The slope factors of the curves for peak and steady-state current were also similar (1.4 ± 0.34 and 1.2 ± 0.31, respectively; ANOVA, $P>0.05$). Suramin, at high concentrations, was able to inhibit current activated by 25 μM NMDA essentially completely, as evidenced by E_{max} values of 100% inhibition for peak and steady-state current.

Reactive blue 2 also inhibited current activated by 25 μM NMDA in a concentration-dependent manner (Figure 2b), although it inhibited steady-state current more potently than peak current (IC_{50} values of 11 ± 0.78 vs 53 ± 3.7 μM, respectively; ANOVA, $P<0.05$). The slope factors of the curves for peak and steady-state current did not differ significantly (0.69 ± 0.034 and 0.66 ± 0.031, respectively; ANOVA, $P>0.05$). Reactive blue 2 was able to inhibit NMDA-activated current essentially completely, although higher concentrations were required to inhibit peak current than were required to inhibit steady-state current.

Investigation of the gradual onset of reactive blue 2 inhibition of NMDA-activated current

The observation that reactive blue 2 inhibition of NMDA-activated current was gradual in onset raised the possibility that its action might require the presence of agonist, as would occur if reactive blue 2 acted by enhancing desensitization or by blocking the open channel. To test this possibility, we compared the effect on peak NMDA-activated current of reactive blue 2 applied for 5 s before

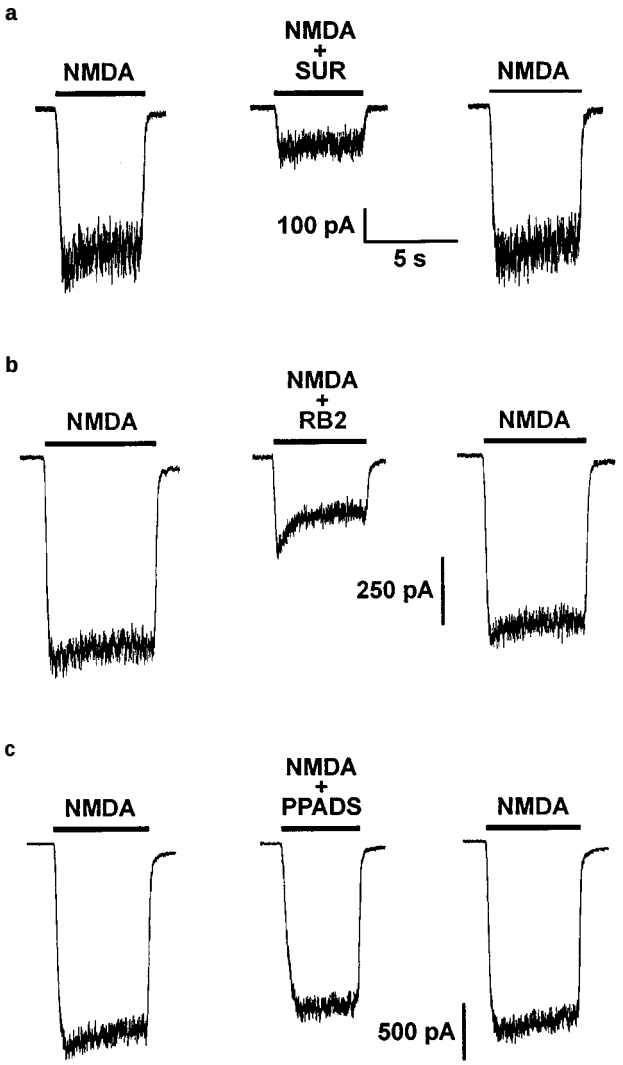

Figure 1 Suramin and reactive blue 2, but not PPADS, inhibited NMDA-activated current. Traces are currents activated by 25 μM NMDA and 1 μM glycine and their modulation by (a) 75 μM suramin (SUR), (b) 25 μM reactive blue 2 (RB2) and (c) 100 μM PPADS. NMDA (+ glycine) or NMDA (+ glycine) and antagonist application is indicated by bars, as labelled. Note the gradually decaying component of NMDA-activated current in the presence of reactive blue 2. Time scale in (a) also applies to (b) and (c).

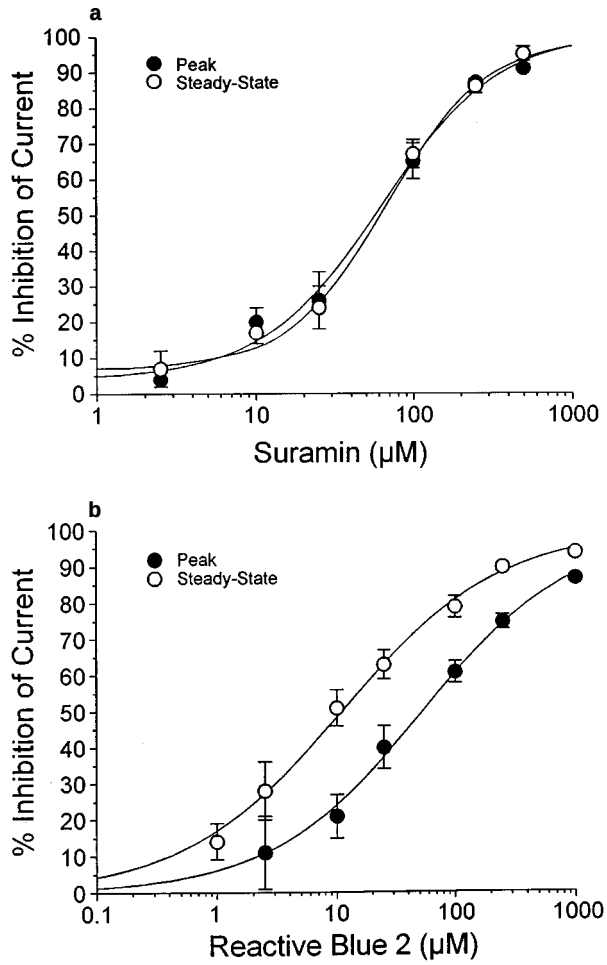

Figure 2 (a) Concentration-response curve for suramin inhibition of peak and steady-state current activated by 25 μM NMDA and 1 μM glycine. Each data point is the mean of 3–6 neurones; vertical lines indicate s.e.mean. (b) Concentration-response curve for reactive blue 2 inhibition of peak and steady-state current activated by 25 μM NMDA and 1 μM glycine. NMDA (+ glycine) and antagonists were applied simultaneously, as in Figure 1. Each data point is the mean of 4–7 neurones. Error bars not visible are smaller than the size of the symbols.

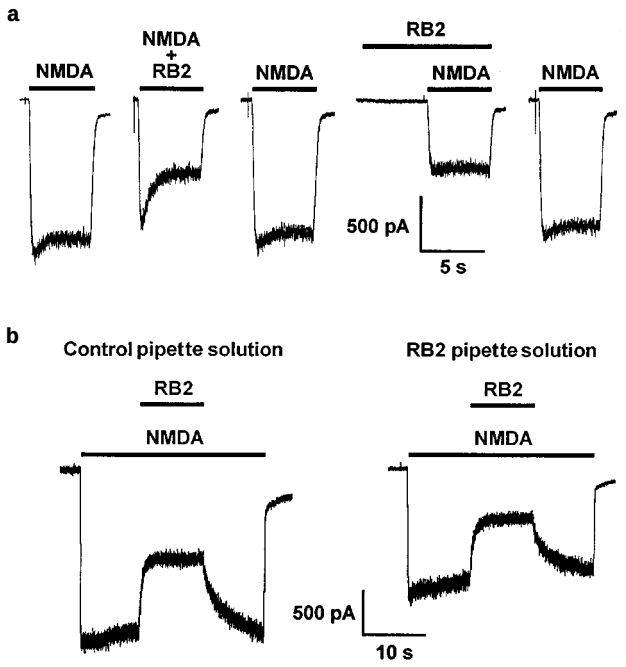

Figure 3 Gradual onset of reactive blue 2 inhibition of NMDA-activated current was not due to use-dependence or to an intracellular site of action. (a) Traces are currents activated by 25 μM NMDA and 1 μM glycine and their inhibition by 25 μM reactive blue 2 (RB2). Note that the gradual onset of inhibition produced by reactive blue 2 applied simultaneously with NMDA was eliminated by 5 s pre-application of reactive blue 2. (b) Traces are currents activated by 25 μM NMDA and 1 μM glycine and their inhibition by 25 μM reactive blue 2 (RB2) obtained from one neurone with normal recording pipette solution, and from one neurone with pipette solution containing 250 μM reactive blue 2.

application of NMDA and glycine with that applied simultaneously with NMDA and glycine. As shown in Figure 3a, in an individual cell the simultaneous application of NMDA and reactive blue 2 resulted in a peak:steady-state current amplitude ratio of 1.93, whereas when reactive blue 2 alone was applied for 5 s before NMDA, this ratio was 1.00. The average values of peak:steady-state ratio were 1.62 ± 0.12 for 25 μM reactive blue 2 applied simultaneously with NMDA, and 1.08 ± 0.048 for reactive blue 2 applied for 5 s before NMDA; these values differed significantly (paired Student's t test; $P < 0.05$, $n = 4$).

An alternative explanation for the gradual onset of reactive blue 2 inhibition of NMDA-activated current is that it might reflect the time required for reactive blue 2 to cross the cell membrane and reach an intracellular site of action. We evaluated this by determining whether a high concentration of reactive blue 2 in the recording pipette solution could alter the inhibition of NMDA-activated current by a 10 fold lower concentration of reactive blue 2 applied externally. Figure 3b shows records of NMDA-activated current and its inhibition by 25 μM reactive blue 2 in one neurone under control conditions, and one neurone in which the recording pipette solution contained 250 μM reactive blue 2. As can be seen, the inhibition of NMDA-activated current by reactive blue 2 applied externally was similar in both cells. The average inhibition of NMDA-activated current by 25 μM reactive blue 2 was not altered by inclusion of 250 μM reactive blue 2 in the recording pipette solution (46 ± 2 vs 49 ± 6% inhibition for control and reactive blue 2 pipette solution, respectively; Student's t test; $P > 0.05$, $n = 4$).

Effect of membrane potential on inhibition of NMDA-activated current by suramin and reactive blue 2

To assess whether inhibition of NMDA-gated channels by reactive blue 2 or suramin was voltage-dependent, the amplitude of NMDA-activated current was measured at holding potentials between -60 and $+60$ mV. The current traces in Figure 4a show that, in an individual neurone, a voltage ramp stimulus applied during exposure to NMDA and glycine produced an approximately linear and symmetrical current in the absence or the presence of 75 μM suramin or 25 μM reactive blue 2. The current-voltage plot in Figure 4b shows that, in this neurone, suramin or reactive blue 2 inhibition of NMDA-activated current was not dependent upon membrane potential between -60 and $+60$ mV. Similar

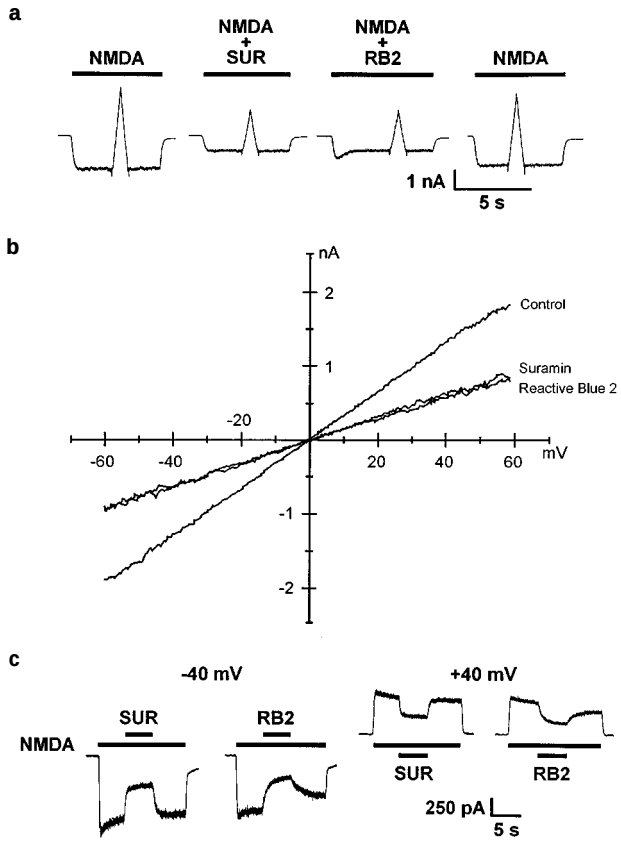

Figure 4 Suramin and reactive blue 2 inhibition of NMDA-activated current is not voltage-dependent. (a) Traces of currents activated by 25 μM NMDA and 1 μM glycine in the absence and the presence of 75 μM suramin (SUR) or 25 μM reactive blue 2 (RB2). Application of a voltage ramp stimulus (described below) produced the large deflections evident in the current traces. Membrane potential was held at -50 mV, except during the ramp stimulus. (b) Plot of NMDA-activated current as a function of membrane potential under control conditions and in the presence of 75 μM suramin or 25 μM reactive blue 2. Membrane potential was rapidly changed with the following protocol: step from -50 to -60 mV, ramp to $+60$ mV, ramp to -60 mV, step to -50 mV (slew rate of ramp segments: 240 mV s^{-1}). Data used for analysis were acquired during the negative-going phase of the voltage ramp stimulus to minimize effects of voltage-activated ion channels. Current obtained with the same stimulus in the absence of NMDA was subtracted from currents obtained in the presence of NMDA. Data in (b) are from the records shown in (a); similar results were obtained in 5 other neurones. (c) Traces of current activated by 25 μM NMDA and 1 μM glycine at membrane potentials of -40 and $+40$ mV and its inhibition by 75 μM suramin (SUR) or 25 μM reactive blue 2 (RB2). Neurones were held at each membrane potential for at least 60 s before application of NMDA, glycine and antagonists.

results were obtained in 5 other neurones tested. Suramin or reactive blue 2 also did not change the reversal potential of NMDA-activated current (2 ± 1 and 1 ± 1 mV, respectively, vs a control value of 0 ± 1 mV; ANOVA; $P>0.05$, $n=6$). To ensure that suramin or reactive blue 2 did not produce a voltage-dependent inhibition that equilibrated too slowly to be observed with the ramp stimulus, we also compared the inhibitory effects of these agents in neurones held at membrane potentials of -40 and $+40$ mV. As shown in Figure 4c, changing the membrane holding potential from -40 to $+40$ mV did not affect the inhibition of NMDA-activated current by reactive blue 2 (58 ± 11 *vs* $64\pm8\%$ inhibition at -40 and $+40$ mV, respectively; paired Student's *t* test; $P>0.05$, $n=4$) and suramin (50 ± 3 *vs* $53\pm2\%$ inhibition at -40 and $+40$ mV, respectively; paired Student's *t* test; $P>0.05$, $n=4$).

Effect of agonist concentration on suramin and reactive blue 2 inhibition of NMDA-activated current

To distinguish among several possible mechanisms of inhibition, we determined the effect of NMDA concentration on inhibition by suramin and reactive blue 2. Reactive blue 2 inhibition was not reversed by increasing the concentration of NMDA from 10 to 1000 μM (Figure 5a, upper traces). Concentration-response analysis (Figure 5b) revealed that reactive blue 2, 25 μM, reduced the E_{max} of NMDA from 1441 ± 62 pA to 598 ± 57 pA ($P<0.05$, $n=6$), but did not alter the EC_{50} of NMDA (14 ± 4.8 μM vs a control value of 19 ± 2.8 μM; $P>0.05$, $n=6$) or the slope of the curve (1.4 ± 0.64 vs a control value of 1.3 ± 0.26; $P>0.05$, $n=6$).

In contrast to the results obtained with reactive blue 2, inhibition by 75 μM suramin was highly dependent upon NMDA concentration. The current traces in Figure 5a (lower traces) illustrate that as the NMDA concentration was increased from 10 μM to 1 mM in a typical neurone, the inhibition by 75 μM suramin decreased markedly. A concentration-response plot for NMDA in the absence and the presence of 75 μM suramin (Figure 5c) revealed that suramin shifted the curve to the right, increasing the NMDA EC_{50} from 13 ± 0.82 to 34 ± 5.3 μM ($P<0.05$, $n=6$), and decreased the E_{max} of the curve from 1822 ± 33 to 1453 ± 67 pA ($P<0.05$, $n=6$). Figure 5d shows the marked dependence of suramin inhibition on NMDA concentration.

Schild analysis of suramin inhibition of NMDA-activated current

To assess whether the rightward shift in the NMDA concentration-response curve produced by suramin resulted

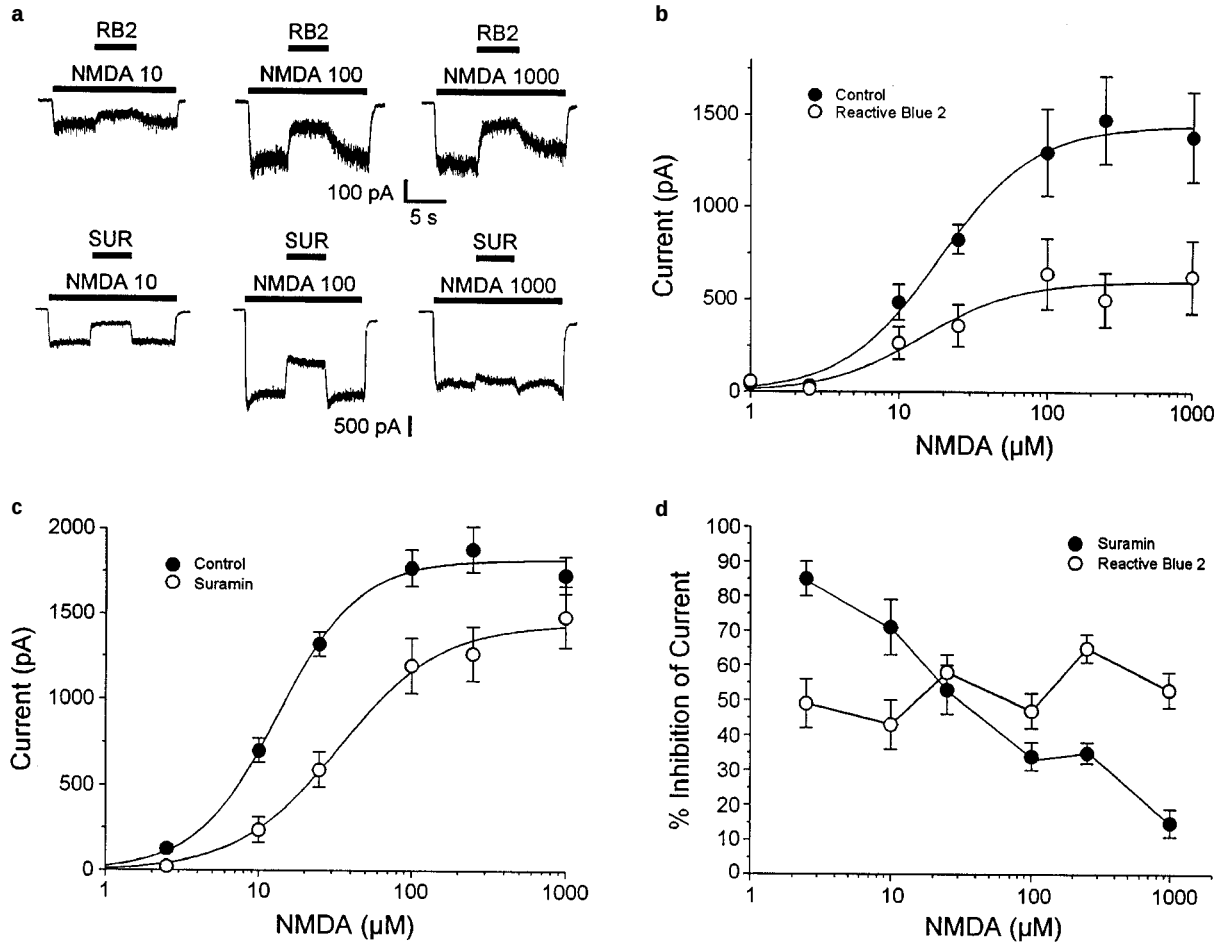

Figure 5 Effect of NMDA concentration on inhibition of NMDA-activated current by reactive blue 2 and suramin. (a) Traces of currents activated by various concentrations (in μM) of NMDA and 1 μM glycine and their inhibition by 25 μM reactive blue 2 (RB2) or 75 μM suramin (SUR). (b) Concentration-response curve for NMDA in the absence and the presence of 25 μM reactive blue 2. Each data point is the mean of 4–9 neurones. (c) Concentration-response curve for NMDA in the absence and the presence of 75 μM suramin. Each data point is the mean of 6 neurones. (d) % inhibition by 75 μM suramin and 25 μM reactive blue 2 plotted against NMDA concentration. Each data point is the mean of 4–9 neurones. In (b), (c) and (d) vertical lines show s.e.mean and error bars not visible are smaller than the size of the symbols.

from competitive inhibition, we determined the effect of multiple concentrations of suramin on the NMDA concentration-response curve in order to perform Schild analysis (Arunlakshana & Schild, 1959). Increasing the concentration of suramin from 25 to 250 μM progressively shifted the NMDA concentration-response curve to the right, increasing the EC_{50} for NMDA from 18 to 105 μM, without changing the slope of the curve (Figure 6a). Although increasing the concentration of suramin also decreased the E_{max} of NMDA, this effect appeared to reach a maximum at 75 μM suramin. The effect of suramin on the EC_{50} of NMDA appeared to be maximal at 250 μM suramin, as the EC_{50} for NMDA in the presence of 500 μM suramin did not differ from that in the presence of 250 μM suramin (105 ± 30 *vs* 105 ± 29, respectively; $P > 0.05$). A Schild plot of the data (Figure 6b) showed a marked deviation from linearity, which precluded determination of a pA_2 value for suramin.

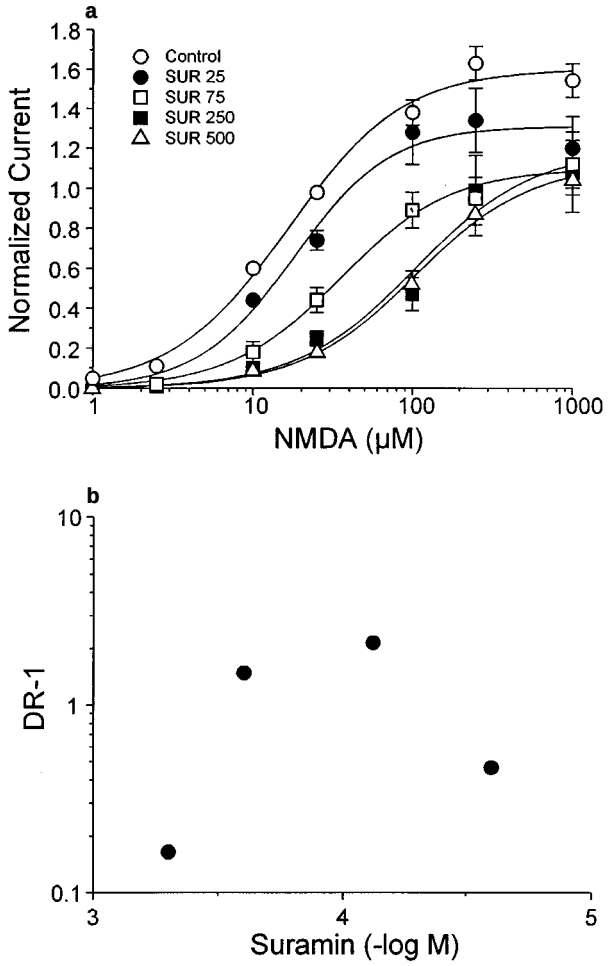

Figure 6 Effect of various concentrations of suramin on the NMDA concentration-response. (a) Concentration-response curve for current activated by NMDA (normalized to that activated by test pulses of 25 μM NMDA) in the absence and the presence of suramin, 25 μM (SUR 25), 75 μM (SUR 75), 250 μM (SUR 250) and 500 μM (SUR 500). Each data point is the mean of 4–21 neurones; vertical lines show s.e.mean. Respective E_{max}, slope factor and EC_{50} values (in μM) were: 1.6 ± 0.061, 1.2 ± 0.18 and 17 ± 2.3 for the control curve; 1.3 ± 0.056, 1.4 ± 0.33 and 18 ± 2.6 for 25 μM suramin; 1.1 ± 0.077, 1.3 ± 0.28 and 35 ± 8.3 for 75 μM suramin; 1.2 ± 0.12, 1.2 ± 0.26 and 105 ± 29 for 250 μM suramin; and 1.1 ± 0.12, 1.2 ± 0.29 and 105 ± 30 for 500 μM suramin. Error bars not visible are smaller than the size of the symbols. (b) Graph plots dose-ratio -1 (DR-1) *vs* suramin concentration for the data in (a). It was not possible to determine a pA_2 value for suramin due to the marked nonlinearity of the plot.

Effect of glycine concentration on inhibition of NMDA-activated current by reactive blue 2 and suramin

The observation that the effects of reactive blue 2 and suramin on NMDA-activated current were not due to an interaction with the agonist binding site of the receptor-channel led us to question whether either of these agents interacted with the glycine coagonist site of the receptor-channel (Johnson & Ascher, 1987). Figure 7a (upper traces) shows records of current activated by 25 μM NMDA and its inhibition by 25 μM reactive blue 2 in 0.1, 1 and 10 μM glycine in an individual hippocampal neurone. As is evident, inhibition of NMDA-activated current by reactive blue 2 in this neurone was not influenced by glycine concentration. Concentration-response analysis revealed that reactive blue 2 decreased the E_{max} of glycine ($P < 0.005$, $n = 6$) without altering its EC_{50} ($P > 0.05$), which is consistent with a mechanism of inhibition that is noncompetitive with respect to glycine (Figure 7b). Similarly, inhibition of NMDA-activated current by suramin, 75 μM, did not appear to depend upon glycine concentration. Figure 7a (lower traces) shows that in an individual neurone, increasing the concentration of glycine did not reverse the inhibitory effect of suramin. The effect of suramin on the concentration-response curve for glycine was also consistent with a mechanism of inhibition that is noncompetitive with respect to glycine (Figure 7c). Suramin, 75 μM, decreased the E_{max} of the glycine concentration-response curve ($P < 0.05$, $n = 4-5$) without significantly affecting the EC_{50} of glycine ($P > 0.05$). The lack of dependence of the inhibitory effects of suramin and reactive blue 2 on the concentration of glycine are also apparent in the plot of % inhibition *vs* glycine concentration shown in Figure 7d.

Discussion

The results of the present study demonstrate that the purinoceptor antagonists suramin and reactive blue 2 inhibit NMDA-gated ion channels at concentrations comparable to those required to inhibit ATP responses in P2X receptors (Nakazawa *et al.*, 1991; Garcia-Guzman *et al.*, 1997). This effect appears to result from a direct interaction of suramin and reactive blue 2 with NMDA receptor-channels, rather than from an inhibition of purinoceptors, as the purinoceptor antagonist PPADS had little or no effect on NMDA-activated current and as the characteristics of inhibition by suramin and reactive blue 2 differed from each other.

In initial experiments, suramin appeared to inhibit NMDA receptors in a primarily competitive manner, as 75 μM suramin shifted the NMDA concentration-response curve to the right and produced progressively less inhibition as the concentration of NMDA increased. However, further experiments revealed that increasing concentrations of suramin did not produce parallel rightward shifts in the NMDA concentration-response curve. Increasing the concentration of suramin from 250 μM to 500 μM did not produce a further increase in the EC_{50} of NMDA, suggesting that the site of action of suramin is saturated at a concentration of 250 μM. Furthermore, a Schild plot of the data from these experiments deviated markedly from linearity. Thus, despite the large increases in the EC_{50} of NMDA produced by moderate to high concentrations of suramin, suramin appears to act in a manner that is noncompetitive with respect to NMDA, perhaps by an allosteric action to decrease agonist affinity.

The increase by suramin in the EC_{50} of NMDA observed in the present study agrees with the findings of Balcar *et al.*

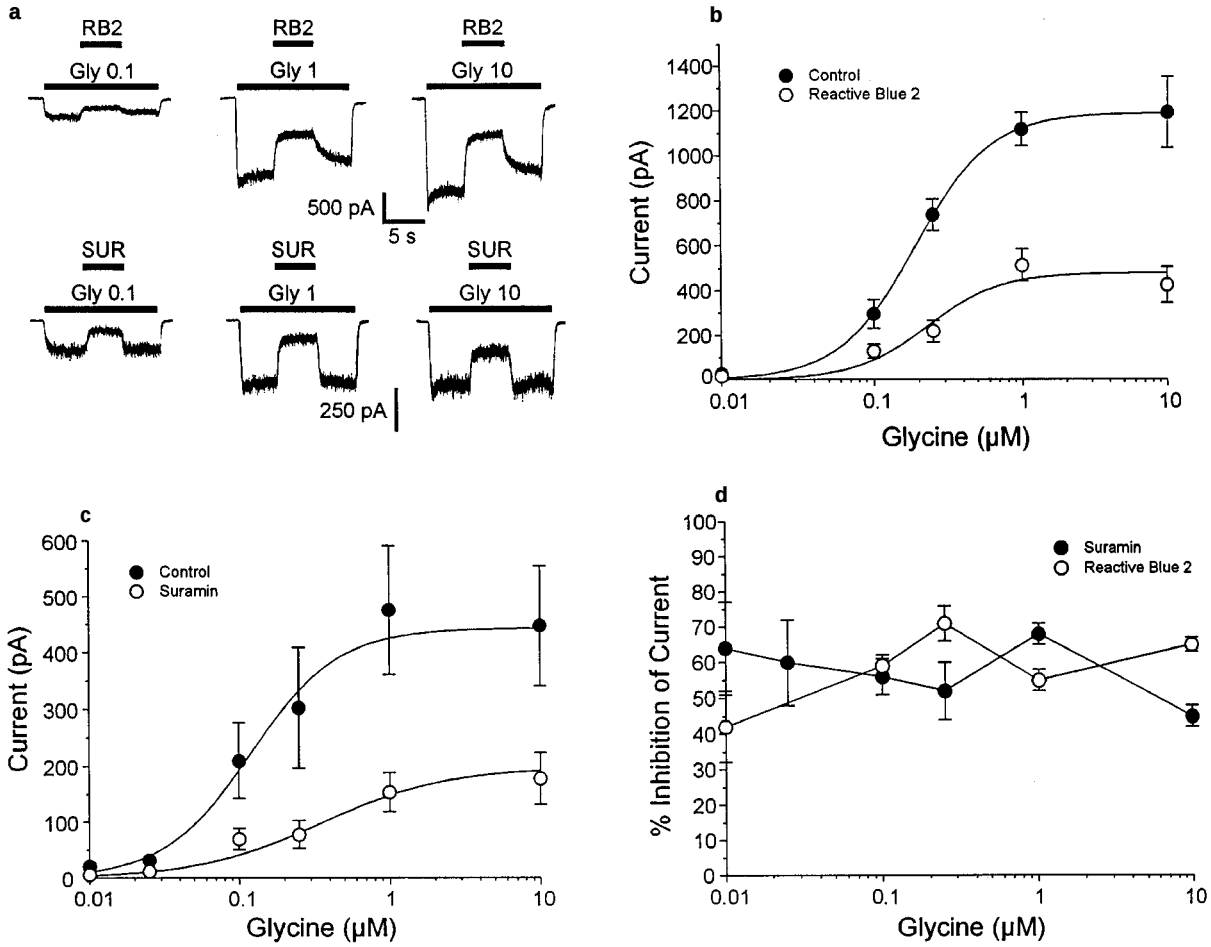

Figure 7 Effect of glycine concentration on inhibition of NMDA-activated current by reactive blue 2 and suramin. (a) Traces of currents activated by 25 μM NMDA at various concentrations (in μM) of glycine and their inhibition by 25 μM reactive blue 2 (RB2) or 75 μM suramin (SUR). (b) Concentration-response curve for glycine enhancement of current activated by 25 μM NMDA in the absence and presence of 25 μM reactive blue 2. Each data point is the mean of 5 neurones. (c) Concentration-response curve for glycine enhancement of current activated by 25 μM NMDA in the absence and the presence of 75 μM suramin. Each data point is the mean of 4 neurones. (d) % inhibition by 75 μM suramin and 25 μM reactive blue 2 plotted against glycine concentration. Each data point is the mean of 4–5 neurones. In (b), (c) and (d), vertical lines show s.e.mean and error bars not visible are smaller than the size of the symbols.

(1995), who showed that suramin displaced binding of the competitive NMDA antagonist [^{3}H]-CGP 39653 in slices of neocortex and hippocampus. In contrast, the observations of the present study do not appear to agree with those obtained by Nakazawa *et al.* (1995), who observed that suramin produced similar inhibition of current activated by 100 or 500 μM NMDA. This apparent discrepancy most probably results from the range of NMDA concentrations tested. Nakazawa *et al.* tested the effect of suramin at only two NMDA concentrations, both of which were close to the maximally-effective concentration, rather than over the full NMDA concentration range, as in the present study. Our results in this study indicate that there would be little difference in the inhibitory effect of suramin at 100 and 500 μM NMDA, but a marked difference in the effect of suramin at low and high NMDA concentrations.

In the present study, both suramin and reactive blue 2 inhibited NMDA-activated current in a voltage-independent manner. Our observation that suramin inhibition was not voltage-dependent between -60 and $+60$ mV is in contrast to a previous study (Nakazawa *et al.*, 1995), in which suramin inhibited NMDA-activated current to a greater extent at -90 mV than at -30 mV. While the reasons for this difference are not clear, the authors of the previous study tested the effect of suramin at only two membrane potentials, which does not allow for adequate analysis of the current-voltage relationship. In the present study, we tested the effect of suramin using a voltage ramp stimulus over the range $+60$ to -60 mV. Within this range, the current-voltage relationship was approximately linear, and the degree of inhibition produced by both suramin and reactive blue 2 did not depend on the membrane holding potential. We also verified that the inhibition by suramin and reactive blue 2 did not have a voltage-dependent component that equilibrated too slowly to be observed with the ramp stimulus.

Suramin inhibition of NMDA receptor-channels did not appear to involve open-channel block, as inhibition by suramin exhibited no use-dependence or voltage-dependence. Not all agents that produce open-channel block do so in a voltage-dependent manner (MacKinnon & Miller, 1988; Marszalec & Narahashi, 1993), due to a low proportion of charged blocker molecules at physiological pH (e.g., pentobarbitone, which blocks non-NMDA glutamate receptor-channels (Weight *et al.*, 1991; Marszalec & Narahashi, 1993), is 75% uncharged at pH 7.4), or to a site of action beyond the influence of the membrane electrical field (MacKinnon & Miller, 1988). However, evidence of agonist-dependence is generally required to establish a channel-block mechanism of

inhibition by use of whole-cell recording and, in the present study, suramin inhibition of NMDA receptor-channels did not exhibit such use-dependence. An action of suramin to increase receptor desensitization could also be largely excluded, based upon the observation that suramin produced the greatest inhibition at low agonist concentrations, at which desensitization is lowest, and the least inhibition at high agonist concentrations, at which desensitization was greatest. In addition, the inhibition by suramin was not due to an interaction with, or effect upon, the glycine coagonist site, as suramin decreased the E_{max} of the glycine concentration-response curve without altering the EC_{50} of glycine. Thus suramin appears to interact with an allosteric site on the NMDA receptor-channel, perhaps one of the several known modulatory sites.

Reactive blue 2 inhibited NMDA receptors in a clearly noncompetitive manner, as it decreased the E_{max} of the NMDA concentration-response curve without affecting the EC_{50} of NMDA, and produced a similar degree of inhibition at all NMDA concentrations that were tested. This finding is in contrast to the observation of Nakazawa *et al.* (1995) that reactive blue 2 inhibited current activated by 100 μM NMDA to a much greater extent than current activated by 500 μM NMDA. The reason for the discrepancies between the results of Nakazawa *et al.* and those of the present study is not clear. The drug referred to as reactive blue 2 in the previous study was Cibachron blue F3G-A, which differs from the Basilen blue E-3G that was used in the present study in having the sulphate group on the A ring in the *ortho* position rather than the *meta* or *para* positions. Further work is needed to determine if this difference in structure can account for the difference in the mechanism of action.

Although it is clear from the results of the present study that reactive blue 2 inhibits NMDA receptor-channels in a noncompetitive manner, the precise site and molecular mechanism of its action could not be determined in the present study. The onset of inhibition by reactive blue 2 was relatively slow, which could be an indication of use-dependence. However, when this possibility was evaluated by pre-application of reactive blue 2 in the absence of NMDA, inhibition of peak and steady-state current did not differ. In addition, reactive blue 2 inhibition of NMDA-activated current did not persist in the absence of agonist, as occurs when a use-dependent blocking agent is trapped within an ion channel upon channel closing (MacDonald *et al.*, 1987). Inhibition by reactive blue 2 was also independent of membrane voltage. Thus reactive blue 2 does not appear to act by blocking the open NMDA-gated ion channel. Reactive blue 2 also did not appear to act by increasing receptor desensitization, as it did not affect the observed desensitization rate, and it produced relatively constant inhibition across the range of agonist concentrations tested. In addition, inhibition by reactive blue 2 was noncompetitive with respect to glycine, indicating that it did not bind to the glycine coagonist site, or influence the binding of glycine to this site. Reactive blue 2 thus appears to inhibit the NMDA receptor-channel by interacting with an allosteric site, although the identity of the site is, at present, unclear. An indication of the location of this site may be provided by the slow onset and offset of action of reactive blue 2, which appears to reflect the difficulty that the drug encounters in reaching its site of action. The intracellular domain of the NMDA receptor-channel can be largely excluded as the location of this site, as reactive blue 2 in the recording pipette did not alter the effect of reactive blue 2 applied externally. The site may thus be located in the extracellular domain in a region between subunits or in the interior of an individual subunit.

The IC_{50} values for reactive blue 2 and suramin inhibition of NMDA-activated current obtained in the present study were 11 and 63 μM, respectively. These IC_{50} values are consistent with results obtained by other investigators for displacement by suramin of specific binding of [^{3}H]-CGP 39653 in slices of neocortex and hippocampus (Balcar *et al.*, 1995), suramin inhibition of NMDA-evoked firing of locus coeruleus neurones (Fröhlich *et al.*, 1996), and inhibition by suramin and reactive blue 2 of NMDA-activated current in rat hippocampal neurones (Nakazawa *et al.*, 1995). The potencies of suramin and reactive blue 2 for inhibition of P2X receptors vary depending upon the experimental preparation used, but are generally in the range 5–180 μM for suramin (Nakazawa *et al.*, 1991; Khakh *et al.*, 1995; Garcia-Guzman *et al.*, 1997) and 1–40 μM for reactive blue 2 (Nakazawa *et al.*, 1991; Fieber & Adams, 1991; Garcia-Guzman *et al.*, 1997). The observations of the present study, that both suramin and reactive blue 2 inhibit NMDA receptor-channels at concentrations similar to those required for P2X purinoceptor inhibition, indicate a need for caution in interpreting results of physiological studies in which these agents are used as selective purinoceptor antagonists.

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