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Participation of NMDA and non-NMDA excitatory amino acid receptors in the mediation of spinal reflex potentials in rats: an *in vivo* study

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1 The effect of various intravenously administered excitatory amino acid (EAA) antagonists on the dorsal root stimulation-evoked, short latency (up to 10 ms) spinal root reflex potentials of chloralose-urethane anaesthetized C_1 spinal rats was studied, in order to gain information on the involvement of non-NMDA (AMPA/kainate; AMPA = α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate) and NMDA (N-methyl-D-aspartate) receptors in their mediation. The competitive non-NMDA antagonist, 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline (NBQX; 1–32 mg kg^{-1}), the non-competitive non-NMDA antagonist, 1-(amino)phenyl-4-methyl-7,8-methylendioxy-5H-2,3-benzodiazepine (GYKI 52466; 0.5–8 mg kg^{-1}), the competitive NMDA antagonist 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP, 2–8 mg kg^{-1}) and two non-competitive NMDA antagonists: MK-801 (0.5–2 mg kg^{-1}) and ketamine (2–32 mg kg^{-1}) were used as pharmacological tools.

2 Validating the applied pharmacological tools regarding selectivity at the applied doses, their effects were tested on direct (electrical) as well as on synaptic excitability of motoneurones evoked by intraspinal stimulation. Furthermore, their effect was investigated on the responses elicited by microiontophoretic application of EAA agonists (AMPA, kainate and NMDA) into the motoneurone pool, where the extracellular field potential evoked by antidromic stimulation of the ventral root was recorded to detect the effects of EAA agonists.

3 NBQX and GYKI 52466 were able to abolish completely the mono-, di- and polysynaptic ventral root reflexes (MSR, DSR, PSR) and the synaptic excitability of motoneurones, while hardly influencing direct excitability of motoneurones. They markedly attenuated AMPA and kainate responses whilst having little or no effect on NMDA responses.

4 Apparently 'supramaximal' doses of CPP and MK-801 slightly inhibited MSR (by about 10%) moderately reduced DSR and PSR (by about 20–30%) and did not influence excitability of motoneurones. They selectively blocked responses to NMDA.

5 Ketamine dose-dependently inhibited MSR, DSR and PSR. Nevertheless, diminution of none of the responses exceeded 50%. It reduced both direct and synaptic excitability of motoneurones, thus displaying a local anaesthetic-like effect, which may contribute to its reflex inhibitory action. It depressed responses to NMDA whilst having negligible effects on responses to AMPA and kainate.

6 We conclude that non-NMDA receptors play a substantial role in the mediation of MSR, DSR and PSR, while NMDA receptors contribute little to this. Neither MSR nor PSR is mediated exclusively by non-NMDA or NMDA receptors, respectively.

7 The drugs investigated in this study, with the exception of ketamine, proved to be useful tools for elucidation of the involvement of EAA receptors in various processes *in vivo*

Keywords: Glutamate receptors; AMPA; kainate; NMDA; NBQX; GYKI 52466; CPP; MK-801; spinal reflex; spinal cord

Introduction

There is abundant evidence that endogenous L-glutamate is released in the spinal cord by dorsal root stimulation (Kawagoe *et al.*, 1986) and glutamate receptors (excitatory amino acid, EAA, receptors) are involved in mediating synaptic transmission in the spinal cord (for review see: Mayer & Westbrook, 1987; Evans, 1989).

Ionotropic EAA receptors have been classified into three groups based on their agonist selectivity: N-methyl-D-aspartate-(NMDA), kainate- and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate- (AMPA) receptors (Collingridge & Lester, 1989; Watkins *et al.*, 1990). Pharmacological and electrophysiological distinction between AMPA and kainate receptors, however, is a matter of controversy (see e.g. Zeman & Lodge, 1992), and molecular biological studies have also failed to demonstrate clear selectivity of these agonists (Sommer & Seeburg, 1992). Consequently the terms non-NMDA or AMPA/kainate receptors are also in use.

Along with the development of EAA antagonists numerous studies have been performed to reveal the involvment of EAA receptors in the mediation of reflex transmission in the mammalian spinal cord. Since at first only selective NMDA antagonists (e.g. D-amino-5-phosphonovalerate, AP5; D-2-amino-7-phosphonoheptanoate, AP7) were available, early studies had to draw conclusions from the effect of NMDA antagonists alone or from a comparison with the effect of non-selective antagonists (such as *cis*-2,3-piperidine dicarboxylate, PDA, or kynurenate). The conclusion of Evans *et al.* (1981), using PDA and AP5 in immature rat spinal cord, that NMDA receptors mediate polysynaptic, while non-NMDA receptors mediate mono-

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synaptic excitation, was further supported by Ganong *et al.* (1983), by intracellular investigations of Jahr & Yoshioka (1986) using kynurenate in the same *in vitro* preparation or by iontophoretic application of antagonists on dorsal horn neurones of cats *in vivo* (Davies & Watkins, 1983). Similar conclusions were drawn by Polc (1985) studying the effect of AP7 and of the apparent kainate antagonist nuciferine, an apomorphine related alkaloid (Polc & Haefely, 1977) on feline spinal ventral root reflex potentials *in vivo*.

Nevertheless, it is practically impossible to draw clear and reliable conclusions especially on the involvement of non-NMDA receptors in polysynaptic excitation with non-selective antagonists. Therefore, the discovery of more potent and selective non-NMDA antagonist, quinoxalinediones (Honoré *et al.*, 1988) had a great impact on further development. Turski *et al.* (1990) comparing the effect of the potent NMDA antagonist 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) and that of 6-cyano-7-nitroquinoxaline (CNQX, administered intrathecally) on the monosynaptic Hoffmann (H)-reflex and on the polysynaptic flexor reflex also came to the above conclusion. On the other hand, Long *et al.* (1990) comparing the effect of CNQX and AP5 on the ventral root reflex in mature rat spinal cord *in vitro* have shown considerable depression of the polysynaptic reflex response by 3 μM CNQX, a concentration which did not inhibit the NMDA response.

Recently new selective non-NMDA antagonists have been reported which are also active by systemic administration *in vivo*: the competitive antagonist 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline (NBQX; Sheardown *et al.*, 1990) and the non-competitive antagonist, 1-(amino)phenyl-4-methyl-7,8-methylendioxy-5*H*-2,3-benzodiazepine (GYKI 52466; Tarnawa *et al.*, 1990; Donevan & Rogawski, 1993). Penetration of these antagonists through the blood brain barrier allows reliable antagonist studies to be performed *in vivo*.

Spinal root reflexes in the rat and cat spinal cord *in vivo* have often been used in studies of the modulatory effects of catecholamine-, 5-hydroxytryptamine-, GABA-related and centrally acting muscle relaxant drugs (e.g Barasi & Roberts, 1974; Ono, 1982; Ryan & Boisse, 1984; Kaneko *et al.*, 1987; Yamazaki *et al*, 1992). However, the participation of EAA receptors, especially that of non-NMDA receptors in the mediation of these reflex potentials has not yet been systematically studied.

In order to draw conclusions on the EAA receptor involvement in the mediation of spinal reflexes, we have investigated the effects of EAA antagonists, which are active by systemic administration, on ventral and dorsal root potentials of the rat spinal cord evoked by dorsal root stimulation *in vivo*. To obtain clear results we intended to use the antagonists in a wide dose-range and/or to apply a 'supramaximal' dose as far as possible. To avoid misinterpretation due to non-selectivity of the pharmacological tools used, several antagonists of different types were employed: the competitive non-NMDA antagonist NBQX, the non-competitive non-NMDA antagonist GYKI 52466, the competitive NMDA antagonist, CPP (Davies *et al.*, 1986) and the non-competitive NMDA antagonists, MK-801 (Wong *et al.*, 1986) and ketamine (Anis *et al.*, 1983). The effects of these drugs were also investigated on the motoneurone excitability to exclude a possible non-specific 'membrane stabilizing' action which may influence spinal reflexes (Ono *et al.*, 1984). Furthermore, we investigated the effects of these drugs against responses of AMPA, kainate and NMDA applied iontophoretically on the motoneurones, in order to gain information on their selectivity and reliability at the given site *in vivo*.

Methods

In all experiments male Wistar rats weighing 270–400 g were anaesthetized with chloralose (25 mg kg^{-1}, i.p.) and urethane (1 g kg^{-1}, i.p.). The vagus nerves were severed and the common carotid arteries ligated bilaterally at the cervical region. Blood pressure was monitored via a cannula in the carotid artery. The femoral vein was also cannulated to allow intravenous injections. A tracheal cannula was inserted and the animals were artificially ventilated throughout the experiment. The spinal cord was infiltrated with lignocaine (1%, 50 μl) and transected at the C_1 level. Animals were fixed in a spinal stereotaxic frame and a dorsal laminectomy was performed on vertebrae L_1–L_6. Ventral and dorsal roots below L_4 were cut bilaterally, dorsal and ventral roots of the segments L_4 and L_5 were isolated. A pool was formed from the skin of the back and filled with warm paraffin oil. Dorsal and ventral roots used for stimulation and recording were placed on bipolar silver wire electrodes. Rectal and oil pool temperatures were maintained at 36 ± 0.6°C with a heating pad and a heating lamp, respectively. In the majority of the experiments a 1:1 mixture of Ringer solution and glucose solution (6%) was infused (10–20 ml kg^{-1} h^{-1}) in order to keep the mean arterial blood pressure of spinal animals above 60 mmHg. This was essential for the recording of stable reflexes (particularly of the sensitive polysynaptic reflex) over a long period and for avoiding secondary effects to drug administration.

Spinal reflex study

The L_5 dorsal root was stimulated by single impulses (stimulus strength: supramaximal voltage; pulse width: 0.05 ms; frequency: 10 min^{-1}). The ventral root reflexes (VRR) from the L_5 ventral root and dorsal root potential (DRP) from the L_4 dorsal root were recorded ipsilaterally using differential preamplifiers. For perfect monophasic recording of VRR the nerve was crushed between the two poles of the electrode. For recording of the DRP one pole of the recording electrode was placed as close as possible to the entry of the dorsal root without touching the spinal cord. The other pole was in contact with the distal end of the same dorsal root. Responses were band-pass filtered (0.5 Hz–5 kHz), averaged (averager: Nihonkohden DAT-1100) and displayed on a digital storage oscilloscope (Nihonkohden VC-10). An average of 8 responses was plotted every minute on a two channel oscillographic thermorecorder. Moreover, different components of the ventral root reflex response were separated based on their post-stimulus latency and duration using a time-window signal selector, and electronically integrated (see Figure 1a) after appropriate amplification. Five integrated responses were summed and the integrator reset every 30 s. Integrated responses were recorded on a multi-pen ink-writing recorder (Nihonkohden RM-45). Pen deflection was exactly proportional to the area under the curve of the selected response. (For more details on this signal processing see: Farkas *et al.*, 1989.) Quantitative evaluation of VRR components was based on the integrated records, while the amplitude of DRP was evaluated from records of the averaged responses.

Excitability test

Excitability of the motoneurone soma and of the primary afferent fibres were measured according to the technique described earlier (Ono *et al.*, 1979). Anaesthetized animals were paralyzed with (+)-tubocurarine (1 mg kg^{-1} starting dose plus 1 mg kg^{-1} h^{-1} in infusion). A tungsten microelectrode, insulated except its tip, was inserted into the motoneurone pool, which was stimulated by negative pulses (stimulus strength: 0.2–0.5 mA; pulse width: 0.05 ms; frequency: 10 min^{-1}). The compound action potential evoked by direct stimulation of motoneurones (MN, first peak), and the one caused by (mono)synaptic activation of motoneurones (MS, second peak), were recorded from the L_5 ventral root. The antidromic action potential which reflects excitability of the primary afferent fibres (PAF) was recorded

from the L_5 dorsal root. Exact tip position and stimulus strength were adjusted to yield similar amplitude of MN and MS. The signal processing and recording system were the same as that described above for the spinal reflex study.

Microiontophoretic study

Anaesthetized animals were paralyzed with (+)-tubocurarine. The L_5 ventral root was stimulated (stimulus strength: 2–5 V; pulse width: 0.5 ms; interval: 500 or 600 ms). The field potential evoked by antidromic stimulation (antidromic field potential, AFP) was recorded via a seven barrelled glass microelectrode, having a tip diameter of 5–7 μm, inserted into the motoneurone pool. Outer barrels were filled with solutions of AMPA (0.01 M, pH 7.5), NMDA (0.15 M, pH 8), kainate (0.02 M, pH 8) and 3 M NaCl (compensating current barrel) solutions. The central recording barrel was also filled with 3 M NaCl yielding a tip resistance of 3–8 MΩ *in situ*. Excitatory amino acid agonists were regularly (intervals: 3–6 min) applied microiontophoretically into the motoneurone pool, ejection current being 15–45 nA. Usually it was necessary to use a retaining current of 1–5 nA to avoid leakage.

In some iontophoretic experiments the above described analog signal processing and recording system was used. In the majority of the iontophoretic experiments, however, the amplified responses (band-pass filtering: 50 Hz–5 kHz) were fed into an IBM PC AT compatible computer via an analog-to-digital converter (Scientific Solutions, Lab Master TM-100; conversion rate: 20 kHz). Every AFP response was again integrated. The integrated responses and their amplitudes were recorded on-line via a digital-to-analog converter and a chart recorder (Graphtec, Mark VII). The averaged shape of each ten AFP responses from 15 s before to 1 min after the iontophoretic ejection was automatically stored. The average values of every ten integrated responses and of every ten amplitude values arising throughout the experiment were also stored for later evaluation. Stimulations, recordings and iontophoretic applications were all controlled by the computer programme: 'Stimulat 1.0' (by P. Molnár). The integrated value represented the area bounded by the negative wave of AFP and the zero line. Amplitude was defined as the difference between the negative peak and the zero line. Zero line was determined from the pretrigger segment.

Drugs

The anaesthetic solution contained 0.25% α-chloralose (TCI) and 10% urethane (Aldrich) dissolved in distilled water. The cannula for blood pressure monitoring was filled with saline containing 200 iu ml^{-1} heparin. NBQX (gift from Novo Nordisk) 2 mg ml^{-1} was freshly dissolved in 6% glucose solution containing 0.01 N NaOH. GYKI 52466 (gift from I. Tarnawa) 2 mg ml^{-1} was freshly dissolved in 6% glucose solution. ± MK-801 (dizocilpine maleate) 0.5 mg ml^{-1} (synthesized at Richter), ± CPP (RBI) 2 mg ml^{-1} and ketamine HCl (Ketalar-50, Parke Davis) 2 or 8 mg ml^{-1}, were dissolved in saline. Lignocaine (Xylocaine, 1%, Fujisawa), (+)-tubocurarine chloride (TCI), NMDA (RBI), AMPA (RBI) and kainate (kainic acid, Sigma) were also used.

Results

Spinal reflex study

The ventral root reflex (VRR) potential evoked by dorsal root stimulation typically consists of three components (see Figure 1a, Figure 2f, Figure 3d; sell also e.g. figures of Barasi & Roberts, 1974; or Ono, 1982). A first spike (monosynaptic reflex, MSR) is followed by a second smaller spike, apparently reflecting still rather synchronized discharge which, based on its latency, may be regarded to represent a disynaptic reflex (DSR; Fukuda *et al.*, 1977). The time from stimulation to the peak of MSR was about 1.8–2 ms, while the time between the first and second peak was 0.8–1.2 ms (cf. 'disynaptic excitatory path from group Ib afferents' in Eccles, 1957). The second spike (which in other studies has often been termed polysynaptic reflex) is followed by a smaller gradually declining potential (termed polysynaptic reflex, PSR in the present study) lasting for about 10 ms from the stimulation. It has usually been ignored in other studies of the rat spinal reflex, since it has a magnitude of 0.1–0.2 mV and is less synchronized than the MSR of 1–2 mV. It is clearly observed, however in perfect monophasic recording (see also Methods) by increasing the amplification. During the stabilisation period of experiments the three components (MSR, DSR, PSR) seemed to alter independently, i.e. both concurrent and opposite spontaneous changes could be observed. PSR seemed to be the most labile and sensitive to experimental conditions, injections of placebo (Figure 1d) and changes in the blood pressure. Therefore, it seemed reasonable to differentiate between these three components. These components were separated for integration based on their different post-stimulus time of appearance as shown in Figure 1a. This separation is acceptable if latencies of various components do not undergo changes during the experiment. Latencies of components proved to be surprisingly stable. The only error in this separation was occasional partial shifting of the late MSR part into the window of DSR when MSR was almost completely blocked by NBQX or GYKI 52466. This error in either integrated response can be estimated to be less than 5% in those experiments where it occurred, and hence it may be disregarded. A typical time-window setting was: 1.25–2.5 ms for MSR, 2.6–3.4 ms for DSR and 3.4–10 ms for PSR. Although time-windows were individually adjusted from experiment to experiment only minor deviations (0.1–0.2 ms), if any, from the above values had to be set.

All drug effects referred in this section were obtained from previously drug-free animals except for the initial dose of anaesthetic, for cumulative administrations of the same drug or for repeated administrations of increasing doses of GYKI 52466 after appropriate time for recovery.

The effect of NBQX was investigated in 4 rats. The cumulative dosing protocol, records from an experiment and average data are presented in Figure 1. NBQX (1–32 mg kg^{-1}, i.v.) dose-dependently attenuated MSR, DSR and PSR. The sensitivity order was DSR > MSR > PSR. While 1 mg kg^{-1} NBQX considerably depressed the DSR, appreciable decrease in PSR was only achieved with 4 mg kg^{-1}. MSR and DSR but not PSR were completely abolished by 32 mg kg^{-1} NBQX. However, if it was attempted (2 cases) additional doses (16 or 32 mg kg^{-1}) completely abolished PSR as well. Dorsal root potential (DRP) and reflex (DRR) were much more resistant to NBQX but high doses also caused significant (24%) inhibition. Because of the poor water solubility of NBQX, a large amount of NaOH solution had to be injected. Therefore, solvent control experiments ($n = 6$) were performed with the solvent for NBQX (Figure 1d), most of them before investigation of the effect of NBQX. The solvent caused no statistically significant change except for a transient 5% increase in PSR which proved to be statistically significant only at 5 min ($P < 0.05$, paired t test). This effect may be a secondary one via marked elevation of the blood pressure or via the effect of the high pH.

The effects of various single doses of GYKI 52466 (0.5–8 mg kg^{-1}, i.v.) were investigated (each dose in 4–5 experiments; see Figure 2). This compound also dose-dependently inhibited all the three VRR components. The highest dose could also produce complete inhibition. The sensitivity order, as for NBQX, was DSR > MSR > PSR, and DRP was considerably (by 27%) inhibited only by the high doses causing complete inhibition of VRR components. The dose-response relationship for GYKI 52466 was steeper and

this compound was about 4 fold more potent but had a much shorter duration of action than NBQX. It is noteworthy that relative depressions of various spinal root potentials (MSR, PSR and DRP) in rats by GYKI 52466 found in this study were surprisingly similar to those described in our previous study in cats (Tarnawa *et al.*, 1989).

The effect of CPP (2 mg kg^{-1}, i.v.) was studied in 5 rats (see Figure 3a, b and d). The onset of the effect was very slow, the maximum effect being achieved in 30–60 min. CPP caused moderate inhibition of DSR and PSR (34 and 33% in average, respectively), and slight (10%) but statistically significant depression of MSR ($P<0.01$ at 30 min; paired t test). The inhibition of MSR was distinct, since the MSR records were very stable and a clear-cut breakpoint on the integrated record (as in Figure 3a) could always be observed after administration of the drug. Administration of an additional 2 mg kg^{-1} ($n=3$) or even later of an additional 4 mg kg ($n=1$) apparently did not cause any further inhibi-

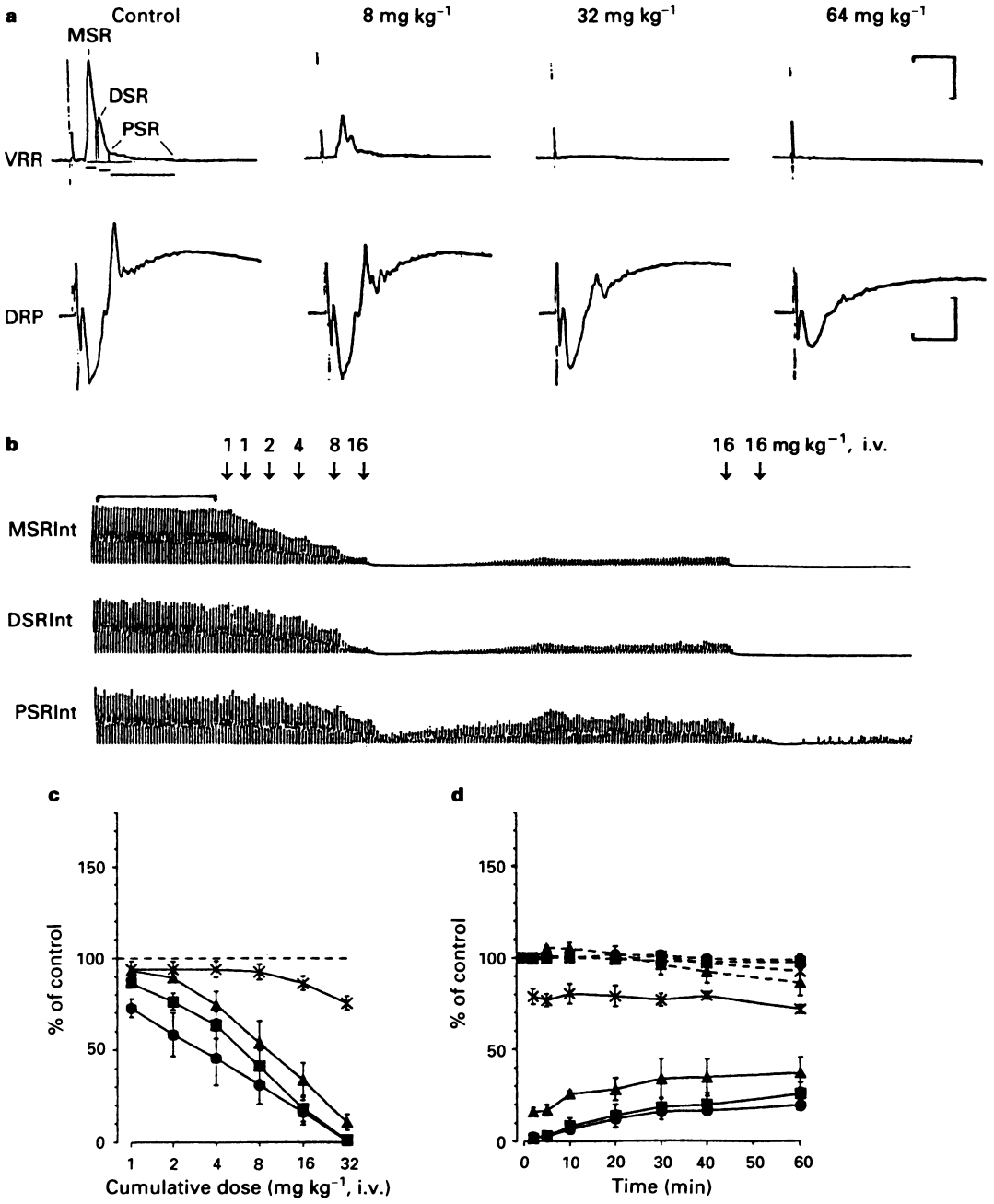

Figure 1 Effect of NBQX on the spinal root potentials evoked by dorsal root (L_5) stimulation studied by cumulative i.v. administration of doses 1–32 mg kg^{-1}. (a)–(b) Records from an experiment where 1 h after the last administration, additional 16 mg kg^{-1} was administered twice. (a) Averaged reflex potentials (average of 8) from an experiment. Upper trace: ventral root reflex (VRR, calibration: 0.5 mV, 4 ms); lower trace: ascending part of the dorsal root potential (DRP) with the dorsal root reflex superimposed (calibration: 0.2 mV, 4 ms), from left to right: control; after 8 mg kg^{-1} after 32 mg kg^{-1}; after 64 mg kg^{-1} (cumulative doses), respectively. Note that all components of the ventral root reflex could be abolished completely. Horizontal bars under the control ventral root reflex show the time-segments selected for integration of the corresponding response. Vertical lines and zero line close the areas under the curve which are represented by the integrated records. (b) Integrated reflex (Int) records of the monosynaptic- (MSR), disynaptic- (DSR), and polysynaptic reflex (PSR); (calibration: 20 min). (c) Dose-response relationship for the different potentials ($n=4$): (■) MSR Int; (●) DSR Int; (▲) PSR Int; (×) DRP Ampl. (d) Time-course of the effect after cumulative administration of 32 mg kg^{-1} ($n=3$). Abscissa scale: time from the last administration. (■——■) MSR Int; (●——●) DSR Int; (◀——▶) PSR Int; (X——X) DRP Ampl. Data obtained with the solvent for NBQX (4 ml kg^{-1}) (the same symbols but with broken line). Data are presented as mean ± s.e.mean.

tion of either reflex response suggesting that the effect of 2 mg kg^{-1} was supramaximal. Amplitude of DRP was unaffected by CPP.

The effect of MK-801 (0.5 mg kg^{-1}, i.v.) was studied in 4 animals (see Figure 3c). The onset of the effect was also rather slow, maximum effect being usually attained 20–30 min after the administration. DSR, PSR and MSR (in order of sensitivity) were decreased slightly (by 20, 15 and 9%, respectively), the latter two changes were not statistically significant. Despite the lack of statistical significance the clear-cut effect of MK-801 was apparent in most experiments. Moreover, repetition of the dose of 0.5 mg kg^{-1} yielded further significant depression of DSR and PSR in 2 out of 3 trials. Calculated average maximum depressions in these 3 experiments were: MSR: 15%, PSR: 21% and DSR: 31%. A further dose of 1 mg kg^{-1}, however, did not cause any further inhibition (3 trials). The amplitude of DRP was unaffected by MK-801.

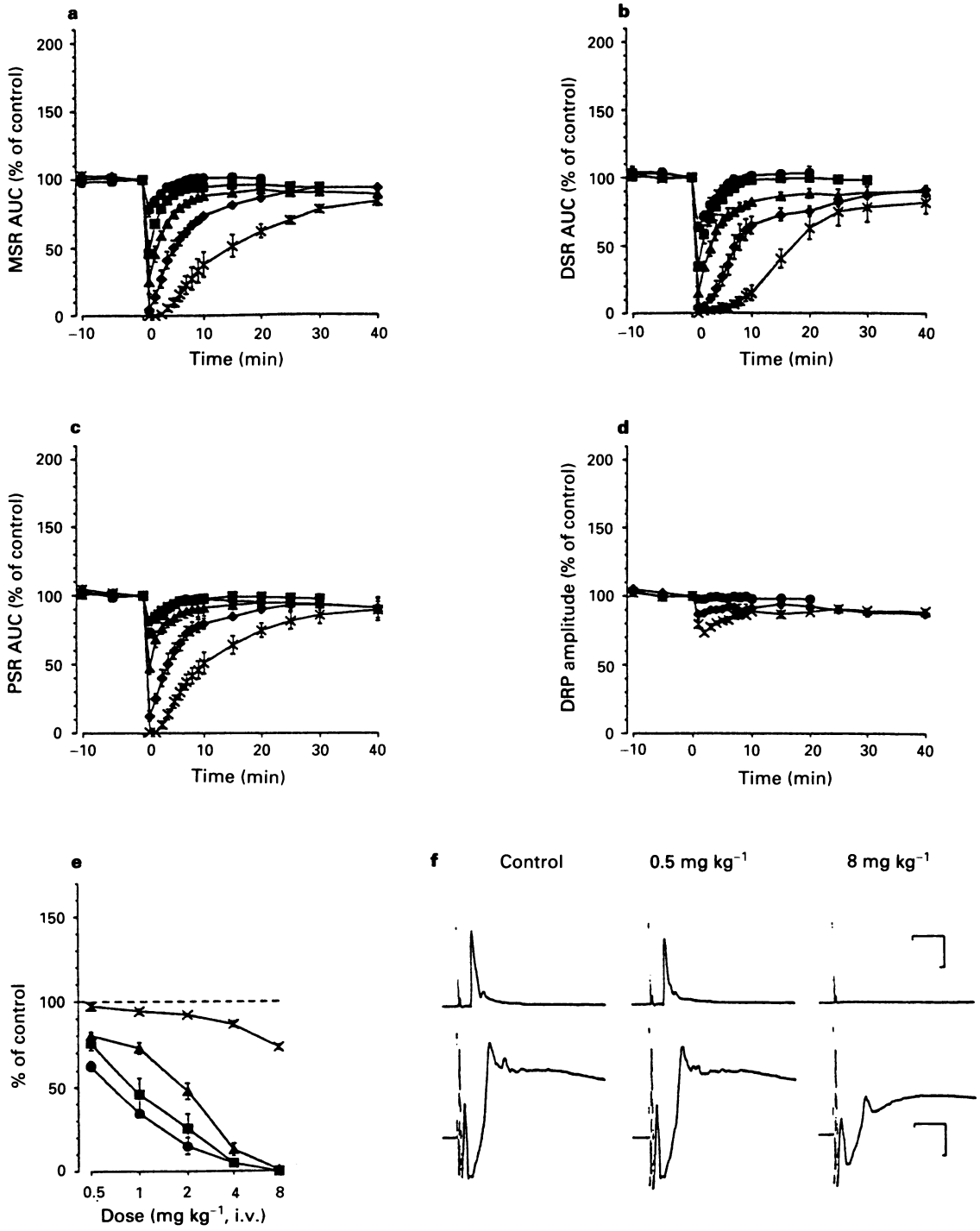

Figure 2 Effect of various i.v. doses of GYKI 52466 on the spinal root potentials. (a)–(c) Time-effect curves of integrated ventral root responses: (a) MSR, (b) DSR, (c) PSR. AUC = area under curve. (d) Time-effect curve of the amplitude of DRP. In (a), (b), (c) and (d) doses are (●) 0.5 mg kg^{-1}; (■) 1 mg kg^{-1}; (▲) 2 mg kg^{-1}; (◆) 4 mg kg^{-1}; (×) 8 mg kg^{-1}. (e) Dose-response relationship for the different potentials. (■) MSR Int; (●) DSR Int; (▲) PSR Int; (×) DRP Ampl. Data are presented as mean ± s.e.mean in 4–5 experiments. (f) Averaged reflex potentials from an experiment. Upper trace: ventral root reflex (VRR, calibration: 1 mV, 4 ms); lower trace: ascending part of the dorsal root potential (DRP) with the dorsal root reflex superimposed (calibration: 0.2 mV, 4 ms). From left to right: control, 2 min after injection of 0.5 mg kg^{-1}, 2 min after injection of 8 mg kg^{-1}. Note that all components of the ventral root reflex were abolished completely.

Ketamine, which had a rapid onset of action, was investigated in 4 animals according to a cumulative dosing protocol similar to that for NBQX. Results are presented in Figure 3e. Unlike CPP and MK-801, ketamine did not reach a plateau on the dose-response curve and at the highest applied dose produced more marked inhibition (32%, 48% and 30% for MSR, DSR and PSR, respectively) of the first two VRR components than did the other two NMDA antagonists. At a dose of 32 mg kg^{-1} its effect proved to be rather durable (Figure 3f). The amplitude of the DRP was not influenced by ketamine.

Excitability test

Neither success rate nor long-term stability of these experiments were as good as those of the reflex study. Therefore, in these experiments, if the administered drug had no effect or apparent recovery was achieved, further drug

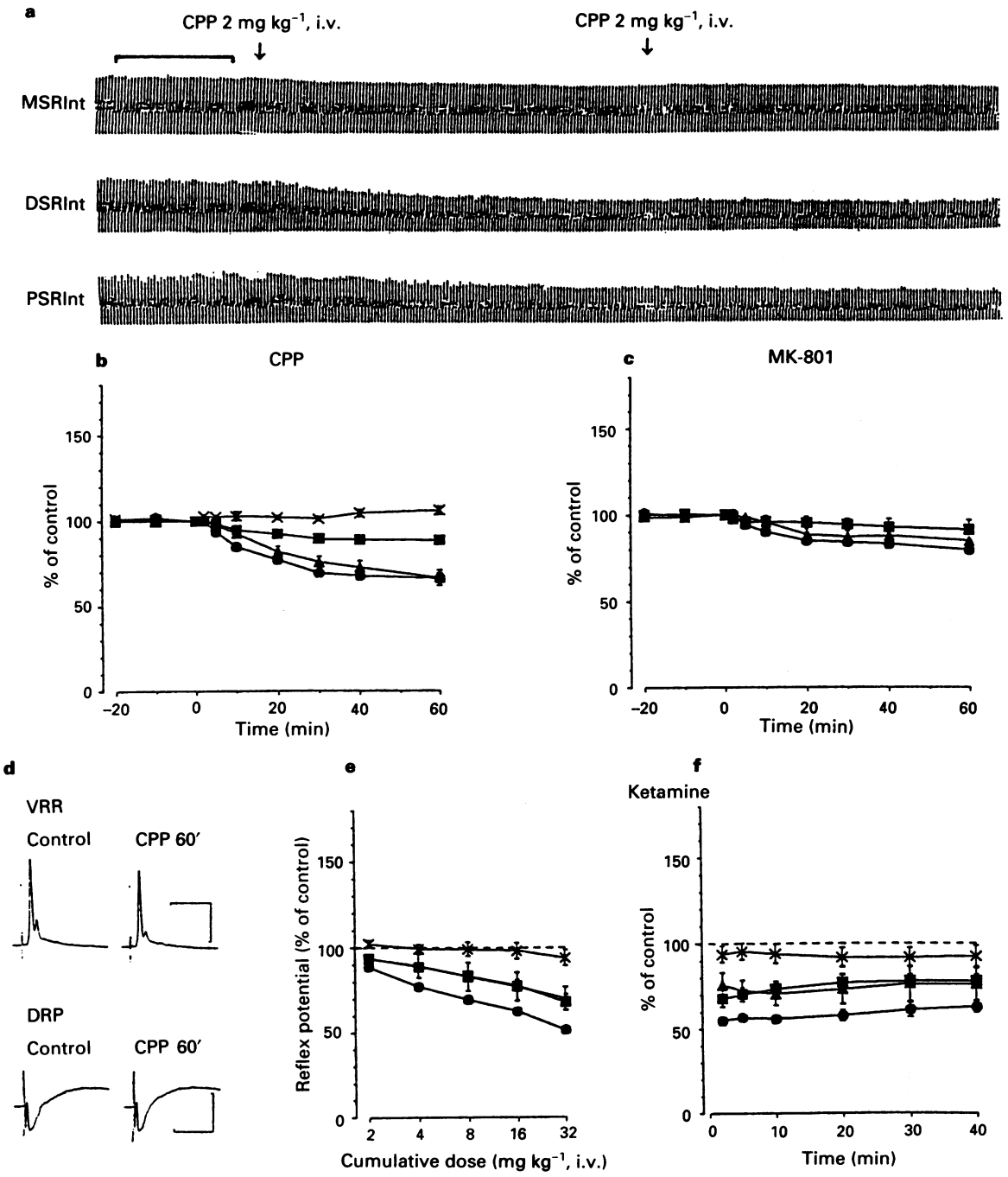

Figure 3 Effect of i.v. administered NMDA antagonists on the spinal root potentials. (a) Records from an experiment where 1 h after the administration of 2 mg kg^{-1} of CPP, additional 2 mg kg^{-1} was administered (calibration: 20 min). Note that no remarkable further reflex depression could be achieved. (b) Effect of CPP (2 mg kg^{-1}, i.v. at time: 0 min) on the time-course of integrated ventral root responses (MSR, DSR, PSR) and of amplitude of DRP ($n = 5$). (c) Effect of MK-801 (0.5 mg kg^{-1}, i.v., at time: 0 min) on the time-course of integrated ventral root responses (MSR, DSR, PSR). For the purpose of clarity the curve indicating no change in amplitude of DRP, was omitted from this figure ($n = 4$). (d) Averaged reflex potentials from the experiment shown in (a). Upper trace: ventral root reflex (calibration: 1 mV, 8 ms); lower trace: ascending phase of DRP (calibration: 0.4 mV, 8 ms) left: control; right: 60 min after administration of CPP (first dose). (e) Cumulative dose-response curves for ketamine ($n = 4$). (f) Time-course of the effect of ketamine after cumulative administration of 32 mg kg^{-1} ($n = 4$). In (b), (c), (e) and (f) (■) MSR Int; (●) DSR Int; (▲) PSR Int and (×) DRP Ampl. Abscissa scale: time from the last administration. In all graphs data are presented as mean ± s.e.mean but symbols often mask error bars.

effects were studied. Nevertheless, all drugs were investigated in 'drug-free' (except for the anaesthetic) animals as well, to confirm the findings. Representative records for each drug involved are presented in Figure 4. Ventral horn stimulation elicited two peaks of compound action potentials (MN and MS; see Methods) with different latencies recorded from the ventral root. Sometimes further activity of lower amplitude could be recorded following the monosynaptic (MS) component, which presumably reflected polysynaptic excitement of motoneurones (see for example Figure 4a and e).

GYKI 52466 (doses ranging 2–8 mg kg^{-1}, studied in 7 animals) reliably and selectively blocked all synaptic components of the ventral root response (see Figure 4a). Apart from a small (less than 10%) reduction observed in some experiments, GYKI 52466 exerted no effect on the MN component. Such a slight reduction seemed to appear if the drug was administered relatively long after the spinal transection. In Figure 4a, 8 mg kg^{-1} GYKI 52466 had no effect on MN. In a later phase of the same experiment, 2 mg kg^{-1} GYKI 52466 (not shown) and 8–32 mg kg^{-1} NBQX (see Figure 4b) had an apparent slight depressant effect. Excitability of primary afferents was not influenced by GYKI 52466.

Very similar results, i.e. selective abolition of MS, very slight or no inhibition of MN and no effect on PAF (see Figure 4b), were obtained with NBQX (studied in 4 animals). The time course of the effect of the two drugs was different and conform with that detected in the reflex study.

CPP and MK-801 ($n = 4$ and 3, respectively) apparently did not have any effect on either response. No inhibition of MS, like that of MSR in the reflex study, could be detected but taking into consideration the insufficient long-term stability, detection of an effect of 10% with such a slow onset may not be expected with this method.

On the contrary, ketamine (doses ranging 8–32 mg kg^{-1}, $n = 5$) considerably depressed MN and MS to a comparable

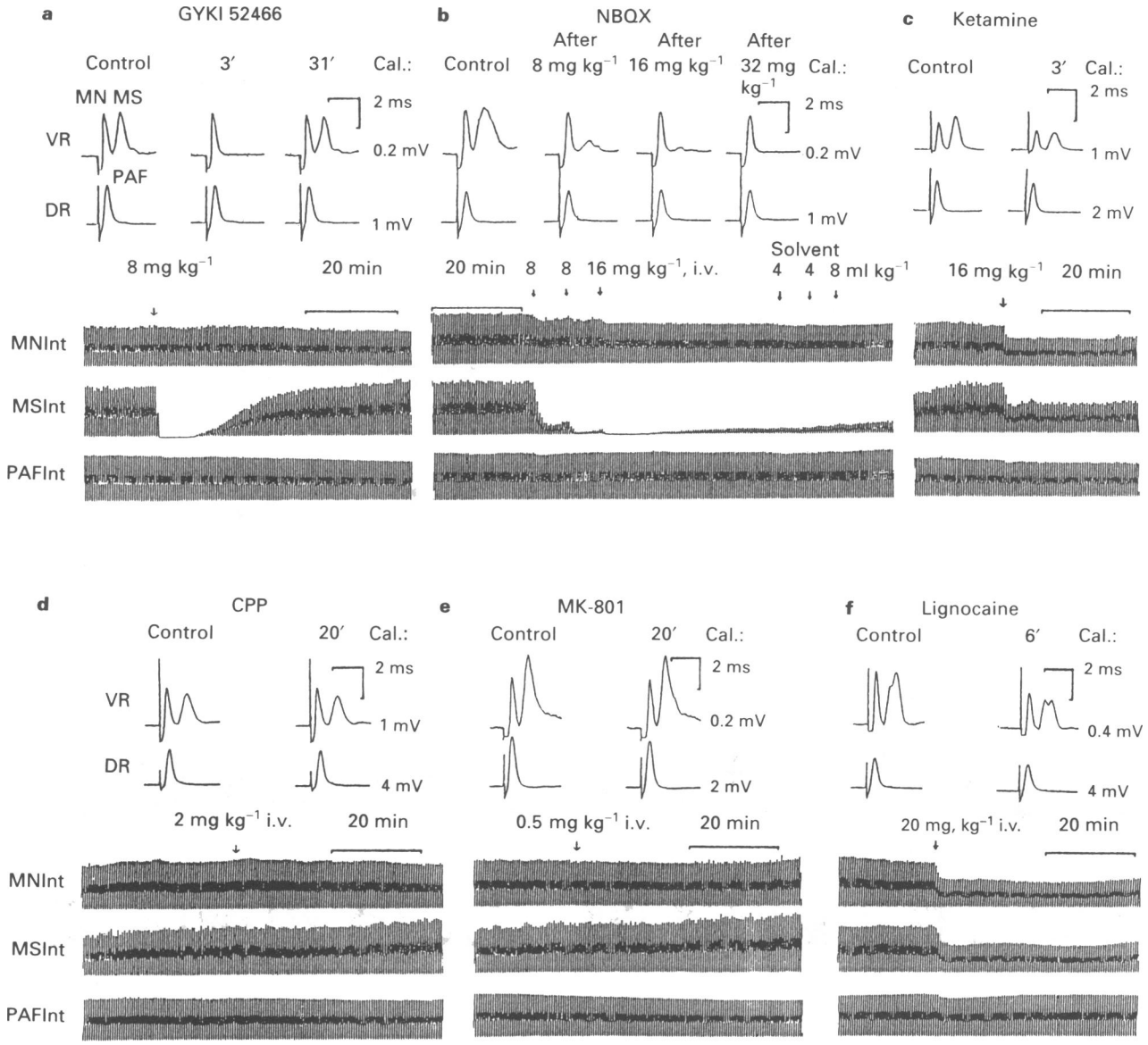

Figure 4 Effect of various excitatory amino acid antagonists and lignocaine administered i.v. in the excitability test. Panels (a) and (b) present records from the same experiment. GYKI 52466 (first drug in the experiment) was injected 4 h 33 min, while NBQX 8h 9 min after spinal transection. Tip position of the stimulating tungsten microelectrode was moved 40 μm more ventral between the two drugs. All other panels (c)–(f) show first drug administrations and records from different experiments. In each panel VR: averaged potential from the L_5 ventral root; DR: averaged potential recorded from the L_5 dorsal root; MN: potential representing direct stimulation of motoneurones; MS: potential representing monosynaptic activation of motoneurones; PAF: antidromically conducted compound action potential generated by direct stimulation of primary afferents; MNInt, MSInt, PAFInt: integrated records of the corresponding potentials. Treatments and calibrations are presented in the figure.

extent (Figure 4c). The excitability of primary afferents was also slightly decreased. The profile of ketamine was similar to that of the local anaesthetic, lignocaine (Figure 4f).

Microiontophoretic study

Iontophoretic administration of EAA agonists produced characteristic alterations in the antidromic field potential (AFP) of the motoneurone pool. Typical AFP, and its reliable response to agonists in the L_5 segment could be recorded from electrode tip positions of 0.7–1.1 mm lateral from the midline (lateral from the dorsal root entry zone) and 1.7–2.2 mm depth (ventral) from the dorsal spinal cord surface. Figure 5 shows records from an experiment in which almost all characteristic phenomena could be observed. The AFP starts with a positive wave followed by a negative one (P_1 and N_1, respectively, terms used by Engberg *et al.*, 1979).

Iontophoretic application of increasing 'doses' (i.e. ejection currents) of AMPA yielded the following alterations. First, some increase in the amplitude accompanied by shortening of the duration of the N_1 wave appeared; however, the enhancement of the amplitude often failed to appear. This was followed by appearance of a second positive wave (P_2). Then the N_1 wave decreased, disappeared and usually a large positive wave appeared. Altogether these phenomena caused dual changes in the amplitude i.e. both increase and decrease. However, the integrated record mostly showed a net decrease. The 'resting' AFP recovered usually within 2 min after cessation of the iontophoresis.

The effects of kainate were quite similar to those of AMPA, except that after cessation of the iontophoretic application, a gradual enhancement of AFP ('afterfacilitation') developed and lasted for several minutes. Even if the inhibitory response, which usually appeared promptly, was missing, the 'afterfacilitation' developed slowly starting with some delay, suggesting that it may be the result of diffusion of kainate to a relatively large area.

In contrast, NMDA always increased both the amplitude and the integrated response. Depression of the N_1 wave or appearance of a positive wave could only be observed after using very large ejection currents exceeding 50 nA. However, when we tried to apply NMDA with such high currents repeatedly, it led to apparent destruction of the otherwise very stable AFP response. In the recovery from the enhancing effects of either kainate or NMDA stepwise changes or units behaving in an 'all-or-nothing' manner could often be observed.

In some cases following iontophoretic application of the EAA agonists we could observe, on a free running oscilloscope, neurones (probably motoneurones) firing with extracellular action potentials of 1–2 mV. After administration of AMPA or kainate, parallel with the above described change in the AFP, the negative action potentials became

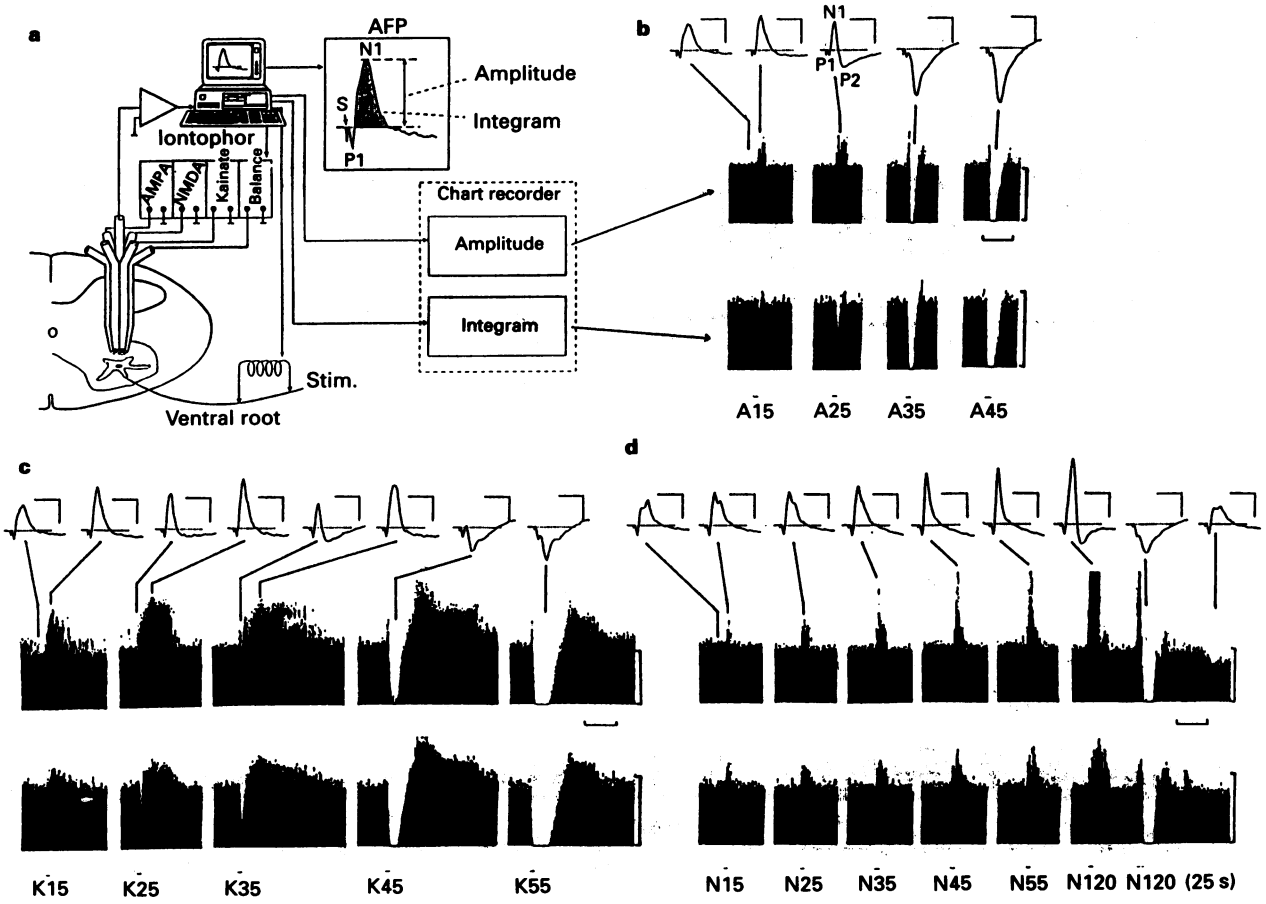

Figure 5 Schematic drawing of the experimental arrangement in the iontophoretic study and a typical antidromic field potential (AFP) recorded from the motoneurone pool, consisting of a positive wave (P_1) followed by a negative one (N_1) with the stimulus artefact (S) (a); and records from an experiment showing typical alterations in the AFP, induced by iontophoretic application of EAA agonists AMPA (A), kainate (K) and NMDA (N) by different ejection currents (in nA, numbers following the letters) (b–d). In each panel: averaged AFP of 10 responses (upper row; negativity is upwards; calibration: 1 mV, 2 ms); chart record segments of the amplitude record (middle row), each response is represented by a pen deflection every 500 ms (calibration: 1 mV, 2 min); simultaneous chart record segments of the integrated response record (bottom row), each response is represented by a pen deflection proportional to the area under the N_1 wave (calibration: 1 μVs, 2 min). Horizontal bars below the integrated records show iontophoretic applications lasting for 15 s (except the last application of 25 s). Lines connect the averaged potentials with corresponding parts of the amplitude record. Amplitude records at last four NMDA applications were truncated by the recorder.

biphasic (negative-positive), then often reversed to be only positive before they disappeared (depolarization blockade). A transient firing could also be observed in the recovery phase judged from the integrated AFP record. Ejection of NMDA also elicited firing; however, reversal of the extracellular action potentials or depolarization blockade did not occur.

Based on the above findings, the depressant effects of AMPA and kainate (between 50–100%) as well as the enhancing effects of NMDA on the integrated response

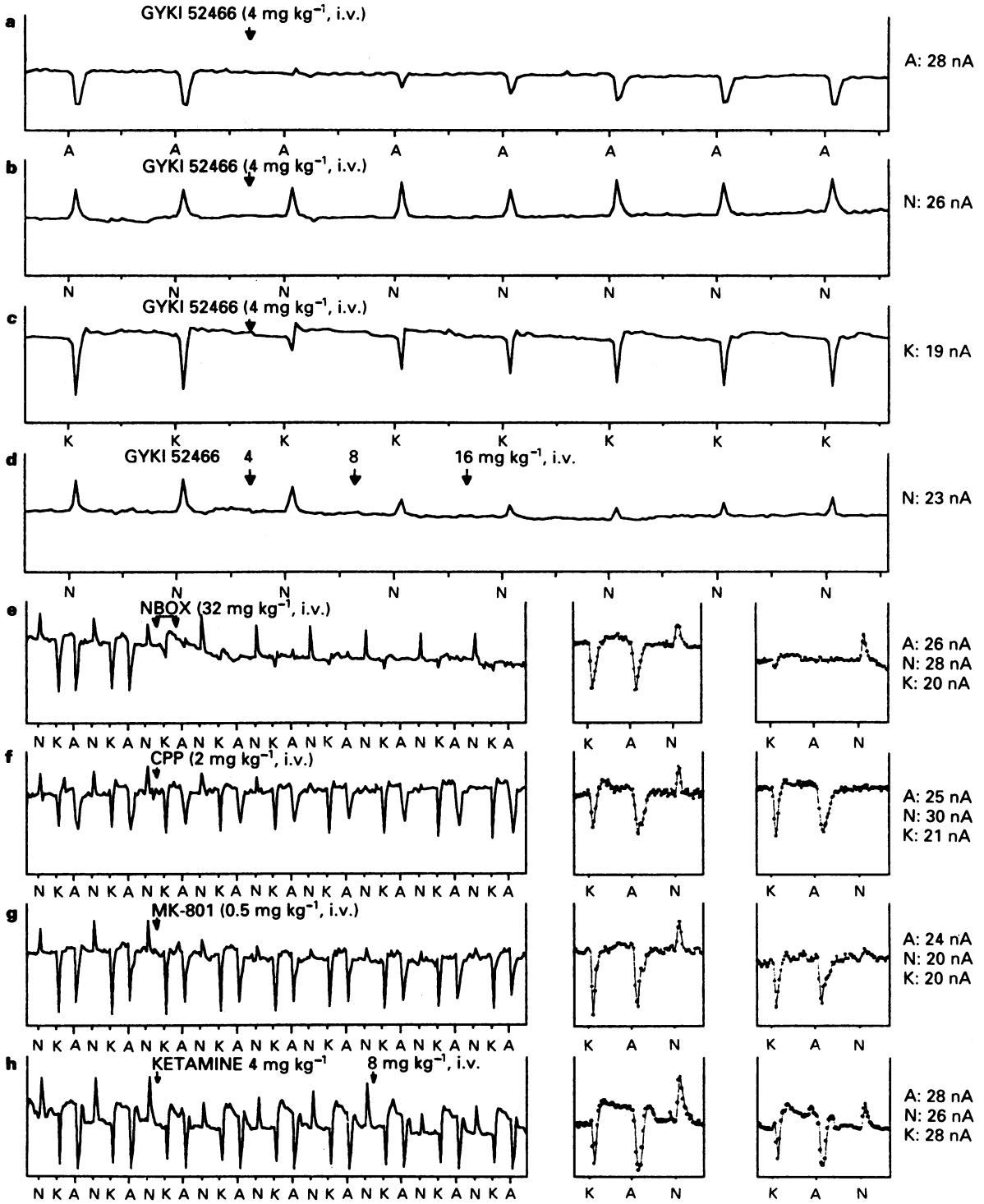

Figure 6 Effect of various excitatory amino acid antagonists on the alteration induced in the antidromic field potential of the motoneurone pool by regular alternating iontophoretic application of AMPA (A), NMDA (N) and kainate (K). Tickmarks above letters in each chart indicate start of the iontophoretic ejection current lasting for 15 s. Ordinates: integrated field potential (uncalibrated). Abscissae: time. Distance between two tickmarks represents 3 min. Each line-graph connects points (10 or 12 points min^{-1}) representing the average integrated value of 10 responses. (a)–(c) Successive records from the same experiment. The gap in the time between these graphs was that used for the determination of the appropriate ejection current and for the stabilization of responses to the next agonist. (d) Record from another experiment, in which the effects of high doses of GYKI 52466 on the NMDA responses were studied. (e–h) The rows present records from different animals obtained with administrations of various antagonists. Left graph: compressed time-scale, continuous record. Right two graphs: segments from the same record with a more extended time-scale. Left panel: control responses; right panel starts 46 min (e–g) or immediately (h) after the (first) drug administration. Applied iontophoretic currents are presented at the right side.

seemed to be the best methods for detection of the effects of EAA antagonists.

The effect of GYKI 52466 on EAA agonist-induced responses was investigated in 11 rats. Four of these experiments were performed according to the same protocol as that shown in Figure 6a–c. After stabilization of responses to an EAA agonist, 4 mg kg^{-1} GYKI 52466 was injected 2 min before the iontophoresis. Following the recovery from the effect of GYKI 52466, recurrent ejection of the next agonist was started and in this manner the effect of GYKI 52466 against all the three agonists was tested. In the other 7 experiments effects of higher doses or effects against only one or two agonists were tested similarly. GYKI 52466 typically abolished the responses to AMPA or caused reversal of the apparently 'depressant' responses to a very slight facilitation (Figure 6a). Responses to NMDA were typically not affected by 4 mg kg^{-1} GYKI 52466 (see Figure 6b); however, 8 mg kg^{-1} clearly reduced NMDA responses in 2 out of 3 trials. Administration of higher doses (semicumulative administration up to 28 mg kg^{-1}, Figure 6d) yielded marked depression but did not cause complete abolition of NMDA responses. Repeated ejection of kainate every 6 min usually resulted in some long-term 'enhancement' in the 'resting' AFP, i.e. the interval between two ejections was not sufficient for complete recovery. The 'depressant' response to kainate was markedly reduced but never abolished completely (see Figure 6c). The 'afterfacilitation' was inconsistently affected: slightly enhanced or reduced or not affected by GYKI 52466. In some cases the 'resting' AFP was also slightly diminished.

The antagonists having longer action were tested against alternating ejection of all the three agonists (Figure 6e–h). It must be noted that some maintained potentiating effect of kainate due to an insufficient recovery was often present in these experiments. NBQX (32 mg kg^{-1}, $n = 4$) markedly reduced or abolished responses to AMPA, or caused reversal to a slight facilitation. Responses to NMDA were apparently not affected, while those to kainate were markedly reduced but not completely abolished (see Figure 6e). NBQX consistently reduced 'resting' AFP but the 'afterfacilitation' was inconsistently affected like that described at GYKI 52466.

CPP (2 mg kg^{-1}) completely (in 2 out of 4 trials) or almost completely abolished the effect of NMDA, while responses to AMPA or kainate were practically unaffected (see Figure 6f). The maximum effect was achieved in 45–60 min.

MK-801 (0.5 mg kg^{-1}, $n = 4$) markedly reduced the NMDA response but complete abolition was achieved only in one case. It also exerted practically no effect on AMPA or kainate-induced alterations. The very slight gradual decrease in the 'resting' AFP and in the effect of kainate seen in Figure 6g were not consistent findings and the latter one may probably be attributed to a spontaneous drifting. The effect of MK-801 reached a maximum in about 30 min.

The effect of ketamine (4 mg kg^{-1}) was investigated in 6 rats, 3 of them according to the protocol used for GYKI 52466, and the others (e.g. Figure 6h) according to that for NBQX, CPP and MK-801. Ketamine markedly reduced or in 2 cases transiently abolished the effect of NMDA. Effects of AMPA or kainate were minimally reduced or unaffected. In most cases, some reduction in the 'resting' AFP was observed.

Discussion

Before drawing conclusions from the reflex studies on the involvement of EAA receptors in the reflex transmission, the adequacy of the methods and suitability of the antagonists used should be considered.

As far as we know, our data have for the first time demonstrated a complete and selective blockade of the MS response in the excitability test by some pharmacological agents (i.e. GYKI 52466 and NBQX). Selective abolition of MS by antagonists of the supposed transmitter of excitatory synapses on motoneurones gives further support to the view that the MS spike clearly represents synaptic excitation, while MN may represent direct electrical excitability of motoneurones (Ono *et al.*, 1979). The inconsistent very slight depressant effect of the two non-NMDA antagonists on the MN may indicate that a slight tonic facilitatory influence modulating electrical excitability of motoneurones may have been present in our spinal rats, perhaps depending on the time allowed for recovery from the 'spinal shock'. Of course, a very slight local-anaesthetic like effect (not comparable to their reflex inhibitory action) cannot be fully excluded. The local anaesthetic-like effect of ketamine, clearly seen in the higher dose-range (e.g. at 8 mg kg^{-1}) may explain its relatively greater effect on MSR (in comparison with the other two NMDA antagonists) and the lack of reaching a plateau on the dose-response curves in the reflex study. This finding suggests ketamine is not a valuable tool for answering the basic questions of the present study.

Interpretation of iontophoretically applied drug-induced changes in the AFP is difficult (for more details see Engberg *et al.*, 1979). Enhancing effects (Barasi & Roberts, 1977; Roberts *et al.*, 1988; in rats) as well as depressant effects by depolarizing agents (Barasi & Roberts, 1974; Engberg *et al.*, 1979) have already been described. Based on the above studies and on our experience possible conflicting factors influencing the AFP can be summarized as follows: (a) Antidromically evoked action potentials often fail to invade the motoneurone soma or dendritic regions (see also Renshaw, 1942; Coombs *et al.*, 1955). Slight depolarization or enhanced membrane resistance can increase the number of motoneurones invaded, leading to an increase in the AFP. (b) Depolarization may decrease the amplitude of the action potential in those motoneurones which have already been invaded, resulting in a decrease in the extracellularly recorded AFP. (c) Profound depolarization can lead to depolarization blockade. This can easily be achieved with kainate but hardly with NMDA (Engberg *et al.*, 1978). Although both compounds depolarize motoneurones, kainate increases, while NMDA gives an apparent decrease in the membrane conductance (Engberg *et al.*, 1978). (d) Compensatory outward currents for the action potential (inward current) generated at remote membrane parts of the motoneurones act as current 'source' at the electrode tip yielding a positive wave superimposed on the negative wave caused by the action potential of close segments. A marked increase in the membrane conductance in the vicinity of the electrode tip can enhance the current density of this 'source', yielding biphasic or inverted (positive) extracellular action potential recording, narrow N_1 wave, appearance of a P_2 wave (perhaps representing invasion of remote dendritic regions), or of a large positive wave on the AFP, phenomena typically observed after ejection of AMPA or kainate.

In summary, AFP does not simply reflect depolarization of motoneurones but also conductance mechanisms activated by the depolarizing agent. While NMDA presumably acted via the first and possibly only insignificantly via the second mechanism, in the action of kainate and AMPA third and fourth items of the above mechanisms may have dominated in determining the net effect on the AFP leading to an opposite alteration.

Whatever the underlying mechanism, the described iontophoretic method seemed to be suitable for testing interactions between agonists and antagonists on motoneurones *in vivo*. All the EAA antagonists (NMDA or non-NMDA) used in this study proved to be selective in attenuating responses of the corresponding agonists at the same doses as those applied in the reflex study. Although both NBQX and GYKI 52466 had apparently somewhat greater effect on AMPA- than on kainate-induced changes, responses to both agonists were markedly reduced; thus we could not demonstrate clear-cut selectivity of either GYKI 52466 or NBQX towards AMPA vs. kainate responses on spinal motoneurones. This is

in agreement with results obtained with GYKI 52466 on cat spinal motoneurones (Engberg *et al.*, 1993), but in contrast with results of Ouardouz & Durand (1991), who reported GYKI 52466 to antagonize glutamate but not kainate responses on rat abducens motoneurones.

In summary, according to the results of the excitability test and of the iontophoretic study, both CPP and MK-801 but not ketamine proved to be suitable tools for investigating involvement of NMDA receptors, while NBQX and GYKI proved to be suitable for testing that of non-NMDA receptors in the reflex transmission.

The effect of 2 mg kg^{-1} CPP seemed to be 'supramaximal' in depressing the spinal reflex, while 0.5 mg kg^{-1} MK-801 (in contrast with our preliminary finding on which this selection was based) proved 'submaximal'. Therefore, data obtained with 2 mg kg^{-1} CPP (see Figure 3) demonstrate most clearly the contribution of NMDA receptors (about 10% to MSR and 20–30% to PSR and DSR). The fact that both non-NMDA antagonists could completely abolish not only MSR but also DSR and PSR, indicates that non-NMDA receptors play a substantial role in the mediation of all short latency reflexes (up to 10 ms). The lack of a non-NMDA antagonist resistant part, despite the indication of contribution of NMDA receptors to PSR, is consistent with the view that NMDA receptors *in vivo* are under a considerable voltage-sensitive Mg^{2+} blockade (Nowak *et al.*, 1984) and the synaptically released transmitter is unable to depolarize motoneurones to a suprathreshold level via NMDA receptors alone.

Our results conflict with the general view that non-NMDA and NMDA receptors mediate MSR and PSR, respectively. Not even MSR was fully resistant to NMDA antagonists. This is in good agreement with the slight depression of MSR and of the peak of monosynaptic excitatory postsynaptic potential (e.p.s.p.) of motoneurones by AP5 in rat spinal cord *in vitro* reported recently by King *et al.* (1992). The term 'monosynaptic' or 'polysynaptic' in the field of electrophysiology has been based on the estimation of synaptic delay(s) and on the assumption of a single transmitter substance acting on a single receptor. However, data are accumulating for long lasting effects mediated via receptors activated by a presumably monosynaptically released transmitter. A significant depression of the descending phase and a slight depression of the peak of clearly monosynaptic e.p.s.ps by NMDA antagonists have been demonstrated (for review see Headley & Grillner, 1990). In the case of dorsal root stimulation (not functionally separated input) disynaptic and polysynaptic e.p.s.ps are presumably summed with the descending phase of the monosynaptic one. The higher but not exclusive effect of NMDA antagonists on DSR and PSR vs. MSR may result from the NMDA receptor-mediated 'late' effect of the monosynaptically released transmitter, too. Since in complex systems, such as the mammalian spinal cord, the differentiation is difficult even with intracellular recording, the terms 'monosynaptic' or 'polysynaptic' (which for simplicity we have also been using) may be misleading regarding the underlying mechanisms, and the use of 'early-' or 'late-' or given latency components would be more correct.

The fact that MS (i.e. the synaptically evoked) peak was fully antagonized in the excitability test by non-NMDA antagonists does not support the hypothesis that, in contrast with primary afferents, interneuronal 'secondary afferents' may use an NMDA-like transmitter (see Davies & Watkins, 1983), since 'secondary afferents' may also have been excited by the intraspinal stimulation in our experiments.

It must be emphasized that the present study extended only for short latency (up to 10 ms) polysynaptic components that are evoked by *single* shock stimulation and are dependent on activation of A fibres only (concluded from input-output curve, i.e. responses reached a maximum below 5T). Turski *et al.* (1992) using EMG recording reported no effect of NBQX on the 'polysynaptic' flexor reflex (time window: $\approx$10–100 ms) evoked by high intensity *train* stimulation in mice. On the contrary, apart from the present data, we have found GYKI 52466 to abolish the flexor reflex (time window: 10–50 ms) in cats also recorded by EMG but evoked by high intensity *single* shocks (Farkas *et al.*, 1992). The difference in the latency is not likely to be sufficient to explain fully this discrepancy. Marked depression of long lasting (up to 800 ms) PSR components (Long *et al.*, 1990) and of late e.p.s.p. components (King *et al.*, 1992) by CNQX has been reported using single shock stimulation of the dorsal root in *in vitro* rat spinal cord preparations. AMPA receptors become desensitized very quickly (time const.: <10 ms; Mayer *et al.*, 1991) and complete recovery from desensitization after short pulse ejection of glutamate onto motoneurones, comparable to the synaptic release, takes more than 1 s. (Onodera & Takeuchi, 1991). On the other hand, the onset (Onodera & Takeuchi, 1991) and the desensitization kinetics of NMDA receptors is slower and lesser in degree (time const.: $\approx$300 ms, Mayer *et al.*, 1991). These features may predestine *AMPA receptors* for preferential mediation of *single volleys*, while participation of *NMDA receptors* may become dominant in mediation of *impulse trains*, which may liberate larger amounts of the transmitter being present in (or around) the synaptic cleft for a much longer period. In the generation of long latency components, even if they were evoked by a single afferent volley, presumably train like firing of interneurones is involved giving more importance to the NMDA receptors than that demonstrated in the present study for short latency PSR components. Moreover, it must be noted that the use of a competitive antagonist (e.g. CNQX or NBQX) may yield underestimation of the involvement of non-NMDA receptors if train like firing liberates excess amounts of the transmitter resulting in insufficient antagonism. The glycine receptor mediated NMDA antagonist feature of CNQX at 10 μM (Long *et al.*, 1990) limits usefulness of this compound due to the inability to produce a 'supramaximal' but still selective effect.

DRP which represents depolarization of primary afferents evoked by a GABA-ergic interneurone is thought to be associated with the presynaptic inhibition (for review see Levy, 1980). Since the first synapse of this pathway is that formed by primary afferents, involvement of some EAA receptors may be assumed. Our results do not indicate contribution of NMDA receptors to the mediation of DRP (but only its early part was studied). From the partial (about 25%) inhibition of DRP by both non-NMDA antagonists it cannot be decided whether non-NMDA receptors are only partly involved, or the high safety factor in this pathway and thus the lack of a supramaximal effect is responsible for the only partial inhibition. The fact that the dose-response curves of both non-NMDA antagonist started to get steeper suggests the latter conclusion. Higher doses should have been used to gain a clear conclusion but this was limited by poor solubility of NBQX, while GYKI 52466 above the dose of 4 mg kg^{-1} seemed to lose its selectivity towards AMPA/kainate vs. NMDA responses in this complex *in vivo* system. Whether this apparent non-selectivity is due to the relative re-establishment of the voltage-sensitive Mg-blockade of NMDA receptors by removal of all excitatory influences via selective blockade of non-NMDA receptors or whether other mechanisms are involved (true non-selectivity) remains open.

In conclusion, our results demonstrate that non-NMDA EAA receptors play a substantial role in the mediation of short latency mono-, di- and polysynaptic spinal reflexes, while NMDA receptors contribute to a smaller extent. We suggest that differential function of non-NMDA and NMDA receptors is not specifically distributed between mono- and polysynaptic pathways but rather determined by the firing patterns involved. To get a clear picture of the specific involvement of non-NMDA receptors in the mediation of longer latency reflex components and train- or physiological stimulus evoked reflexes further studies are necessary with

supramaximal doses of highly selective antagonists. GYKI 52466 and NBQX may be suitable tools for such investigations *in vivo* and probably *in vitro* as well; however, as was pointed out in the discussion of DRP, they also have their limitations.

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