



Budipine is a low affinity, *N*-methyl-D-aspartate receptor antagonist: patch clamp studies in cultured striatal, hippocampal, cortical and superior colliculus neurones

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Abstract

The NMDA receptor antagonistic effects of budipine were assessed using concentration- and patch-clamp techniques on cultured striatal, hippocampal, cortical and superior colliculus neurones. Inward current responses of striatal neurones to NMDA (200 μM) at -70 mV were antagonized by budipine in a concentration-dependent manner (50% inhibitory concentration (IC_{50}) 59.4 ± 10.7 μM , $n = 17$) with 24 times lower potency than memantine but similar potency to amantadine. In striatal neurones, budipine blocked outward currents at $+70$ mV with an IC_{50} of 827 μM , suggesting that the binding site is less deep in the channel ($\delta = 0.45$) than for memantine. However, more detailed analysis of the fractional block by budipine 300 μM in hippocampal neurones gave a δ -value of 0.90, but revealed that 28% block is mediated at a voltage-independent site. This voltage-insensitive site was accessible in the absence of agonist. Budipine exhibited concentration-dependent open channel blocking kinetics ($\kappa_{\text{on}} = 0.71 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) whereas the fast offset rate was concentration-independent ($\kappa_{\text{off}} = 0.63 \text{ s}^{-1}$). Calculation of the ratio $\kappa_{\text{off}}/\kappa_{\text{on}}$ revealed an apparent K_d value of 88.7 μM . Budipine, memantine and amantadine had similar effects against NMDA-induced currents in cultured hippocampal, cortical and superior colliculus neurones, although amantadine was somewhat more potent in cultured striatal neurones. The relevance of NMDA receptor antagonism to the anti-Parkinsonian effects of budipine remains to be established. © 1998 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Budipine (1-tert-butyl-4,4-diphenylpiperidine) is a relatively old agent with established clinical efficacy in the symptomatological treatment of Parkinson's disease (e.g. Menge and Brand, 1985; Jellinger and Bliesath, 1987; see Kornhuber et al., 1995a for further references). Until recently, the mechanism of action of this compound was unclear. Thus, whilst high concentrations block muscarinic receptors, the potency of budipine in this regard is much lower than that of the well characterized antimuscarinic drug biperidine (Menge and Brand, 1985). Similarly, the reported effects of budipine on monoamine oxidase B and dopamine transporters are relatively weak (Menge and Brand,

1985; Jackisch et al., 1993). It now seems apparent that budipine could also act as a relatively low affinity uncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist as it displaces the binding of tritiated ligands to the channel site (Klockgether et al., 1993, 1996; Kornhuber et al., 1995a; Porter and Greenamyre, 1995), blocks NMDA-induced acetylcholine release in striatal slices in a use-dependent manner (Jackisch et al., 1994), and increases the threshold for NMDA-induced convulsions in mice (Klockgether et al., 1993). This raises the possibility that at least part of the anti-Parkinsonian effects of budipine is mediated by blockade of hyperactive glutamatergic pathways in the basal ganglia (see Carlsson and Carlsson, 1990; Schmidt et al., 1992; Klockgether and Turski, 1993; Danysz et al., 1994, 1995, 1997). Moreover, the relatively low affinity of budipine in this regard may be partially responsible for its good clinical tolerability

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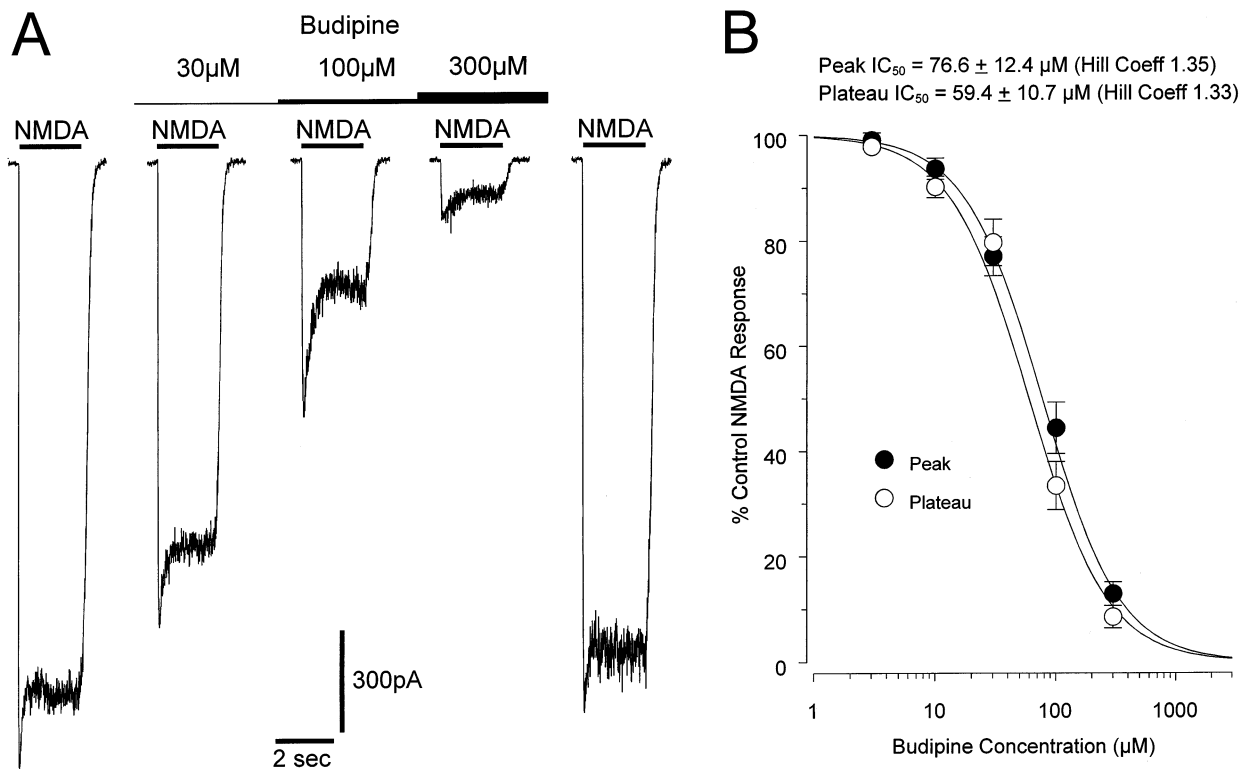


Fig. 1. Concentration dependence of the blockade of NMDA receptors by budipine in cultured striatal neurones. NMDA (200 μM) was applied for 2.5 s every 30 s in the continuous presence of glycine (1 μM) and various concentrations of budipine (3–300 μM). (A) Original data for a single striatal neurone—NMDA was applied as indicated by the bars. The left and right panels show control and recovery responses, respectively. The middle three panels show equilibrium responses in the continuous presence of budipine 30, 100 and 300 μM , respectively. (B) Peak and steady-state (plateau) NMDA current responses were normalized to control levels and plotted as means (\pm S.E.M.) against budipine concentration ($n = 17$). Estimation of IC_{50} values and curve fitting were made according to the four-parameter logistic equation (GraFit, Erithacus Software).

(Chen et al., 1992; Parsons et al., 1993; Rogawski, 1993). Thus, the anti-Parkinsonian drugs amantadine (1-amino-adamantane) and orphenadrine [*N,N*-dimethyl-2-(*O*-methyl-*a*-phenylbenzyloxy)ethylamine] are also low-affinity NMDA receptor antagonists (Kornhuber et al., 1991, 1995b; Danysz et al., 1994, 1995, 1997; Parsons et al., 1995, 1996; Blanpied et al., 1997). The good clinical tolerability of amantadine has been attributed, at least in part, to its strong voltage dependency, low potency and associated fast open channel unblocking kinetics (Parsons et al., 1995, 1996; Blanpied et al., 1997; Danysz et al., 1997). Previous data give no hints as to the kinetics or voltage dependency of the uncompetitive NMDA antagonistic effects of budipine. The aim of the present study was to assess such parameters using patch clamp recordings from cultured striatal, hippocampal, cortical and superior colliculus neurones.

2. Methods

Patch clamp recordings from cultured neurones were performed as described previously (Parsons et al., 1993). In brief, the relevant CNS regions were obtained

from rat embryos (E20–E21) and were then transferred to calcium- and magnesium-free Hank's buffered salt solution (Gibco) on ice. Cells were mechanically dissociated in 0.05% DNAase/0.3% ovomucoid (Sigma) following an 8 min pre-incubation with 0.66% trypsin/0.1% DNAase (Sigma). The dissociated cells were then centrifuged at $18 \times g$ for 10 min, re-suspended in minimum essential medium (Gibco) and plated at a density of 150000 cells cm^{-2} onto poly-D,L-ornithine (Sigma)/laminin (Gibco) -precoated plastic petri dishes (Falcon). The cells were nourished with NaHCO_3 /HEPES-buffered minimum essential medium supplemented with 5% foetal calf serum and 5% horse serum (Gibco) and incubated at 37°C with 5% CO_2 (95% humidity). The medium was exchanged completely following inhibition of further glial mitosis with cytosine- β -D-arabinofuranoside (5 μM ; Sigma) after about 6–7 days in vitro. Thereafter the medium was exchanged partially twice weekly.

Patch clamp recordings were made from these cultured neurones, after 12–15 days in vitro, with polished glass electrodes (3.5–4.5 M Ω) in the whole cell mode at room temperature (20–22°C) with the aid of an EPC-7 amplifier (List). Care was taken to record from small neurones (soma 15–20 μm \varnothing) to minimize problems of

Table 1
Pooled data on the antagonism of NMDA-induced steady-state currents by amantadine, memantine and budipine in cultured striatal, hippocampal, cortical and superior colliculus neurones

	Striatum			Hippocampus			Cortex			Superior colliculus		
	IC ₅₀ (μM)	Hill	n	IC ₅₀ (μM)	Hill	n	IC ₅₀ (μM)	Hill	n	IC ₅₀ (μM)	Hill	n
Amantadine	56.7 ± 8.4	1.10 ± 0.05	5	80.8 ± 10.4	1.19 ± 0.05	9	104.4 ± 19.5	0.94 ± 0.05	9	71.0 ± 11.1	1.12 ± 0.10	10
Memantine	2.51 ± 0.50	1.01 ± 0.02	9	2.87 ± 0.11	0.99 ± 0.04	9	2.70 ± 0.75	1.00 ± 0.08	4	2.3 ± 0.3	1.11 ± 0.05	25
Budipine	59.4 ± 10.7	1.33 ± 0.05	17	59.8 ± 8.8	1.34 ± 0.01	6	80.9 ± 11.7	1.39 ± 0.01	4	52.3 ± 4.8	1.29 ± 0.10	14

Inward currents were evoked by NMDA (200 μM) in the continuous presence of glycine (1 μM) at -70 mV. IC₅₀ values and Hill coefficients are presented as means ± S.E.M. NB: Data with memantine and amantadine in superior colliculus have been published previously.

space clamp and buffered diffusion for concentration clamp experiments. Test substances were applied by switching channels of a custom made fast superfusion system with a common outflow (15–20 ms exchange times). Both the application of solutions and the storage of digitized data to the hard disk of an IBM Pentium PC were controlled by the acquisition program TIDA for Windows (HEKA Electronic, Lambrecht, Germany).

The contents of the intracellular solution were as follows (mM): CsCl (120), TEACl (20), EGTA (10), MgCl₂ (1), CaCl₂ (0.2), glucose (10), ATP (2), cAMP (0.25); pH was adjusted to 7.3 with CsOH or HCl. The extracellular solutions had the following basic composition (mM): NaCl (140), KCl (3), CaCl₂ (0.2), glucose (10), HEPES (10), sucrose (4.5), tetrodotoxin (3 × 10⁻⁴), glycine (1 × 10⁻³) (pH 7.3). Only results from stable cells were accepted for inclusion in the final analysis, i.e. following recovery of responses to NMDA by at least 75% of their depression by the antagonists tested. All compounds were obtained from Sigma with the exception of budipine, which was a kind gift of Dr Kolassa at Byk-Gulden, Konstanz, and memantine, which was synthesized at Merz.

3. Results

In cultured striatal neurones, budipine antagonized inward current responses to NMDA (200 μM) at -70 mV in a concentration-dependent manner (50% inhibitory concentration (IC₅₀) on steady-state currents of 59.4 ± 10.7 μM, n = 17) with 24 times lower potency than memantine but similar potency to amantadine (Fig. 1, Table 1). The Hill coefficient of 1.33 ± 0.05 was significantly greater than unity, indicating the possibility of interactions of budipine with multiple sites on the NMDA receptor complex.

The kinetics of this blockade were studied by applying budipine 30–300 μM for 5 s during 18 s applications of NMDA 200 μM at alternating potentials of -70 and +70 mV (Fig. 2). Sometimes, kinetics were better fit by a double exponential, but this was not

apparent on all cells or at all concentrations (Fig. 2). Where present, double exponential fits were weighted and averaged for simplicity. These studies indicated that budipine exhibited concentration-dependent open channel blocking kinetics ($\kappa_{\text{on}} = 0.71 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) whereas the fast offset rate was concentration-independent ($\kappa_{\text{off}} = 0.63 \text{ s}^{-1}$). Calculation of the ratio $\kappa_{\text{off}}/\kappa_{\text{on}}$ revealed an apparent K_d value of 88.7 μM, which agrees quite well with the IC₅₀ calculated at equilibrium. For comparison, memantine and amantadine have κ_{on} values of 7.52 × 10⁴ and 5.97 × 10⁴ M⁻¹ s⁻¹, and κ_{off} values of 0.21 and 4.59 s⁻¹, respectively, on cultured hippocampal neurones (Parsons et al., in press).

The effects of budipine in a subset of striatal neurones were voltage-dependent (Fig. 2), i.e. it antagonized NMDA-induced currents at -70 mV with an IC₅₀ of 67.9 ± 12.5 μM (n = 5) but was considerably less potent at +70 mV (estimated IC₅₀ 827 ± 223 μM). Moreover, offset kinetics were faster at more depolarized potentials (Fig. 2). However, calculations on the basis of these IC₅₀ values using the Boltzmann equation indicated that budipine is not as voltage-dependent as memantine, despite its lower affinity. Thus, δ was 0.45 for budipine compared to $\delta = 0.71$ –0.83 for memantine (Bresink et al., 1996; Frankiewicz et al., 1996; Blanpied et al., 1997; Chen and Lipton, 1997). There are two possible interpretations of this finding. It is possible that the channel site is genuinely more superficial than for memantine. Alternatively, budipine might interact with two sites, one voltage-dependent and the other non-voltage-dependent. This point was initially addressed on a subset of striatal neurones by applying budipine either: (1) only between NMDA applications, (2) only with NMDA, or (3) both between and with NMDA applications (Fig. 3). Budipine antagonized steady-state currents to 70.9 ± 5.7, 21.6 ± 3.9 and 8.1 ± 1.6% of control, respectively (n = 5). These data indicate that although the main effect of budipine is mediated via open channel blockade, a component is mediated at a voltage-insensitive site, accessible in the absence of agonist.

Further experiments were made with budipine memantine and amantadine on cultured hippocampal,

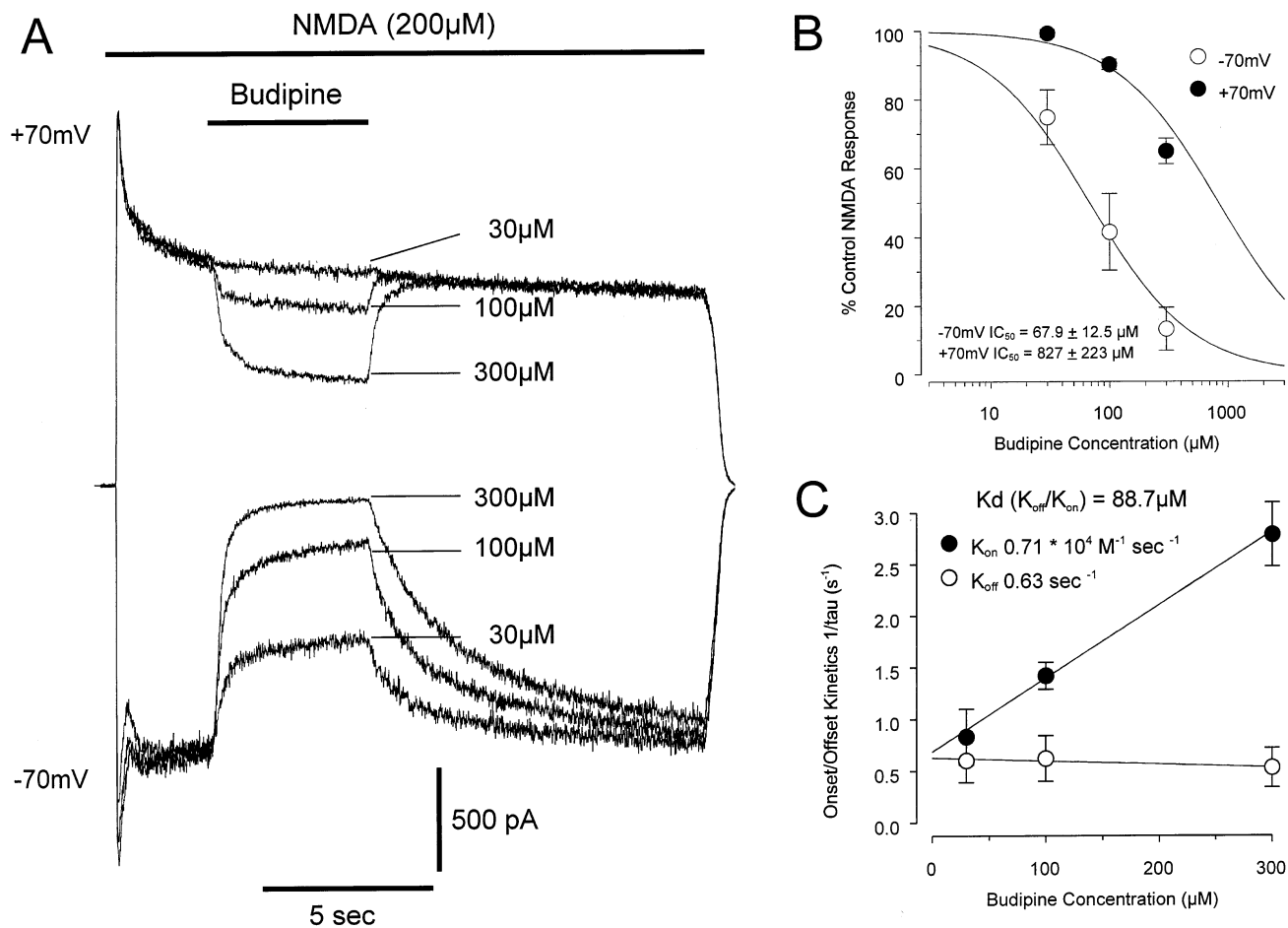


Fig. 2. Kinetics and voltage dependence of the blockade of NMDA receptors by budipine on cultured striatal neurones. (A) Original data for a single striatal neurone. NMDA (200 μM) was applied for 18 s every 60 s at alternating potentials of -70 and +70 mV. Budipine was applied at 30, 100 and 300 μM for 5 s as indicated by the bar. The traces are averages from 60 recordings, 10 at each concentration of budipine and both holding potentials. Between recorded traces, NMDA was applied for 5 s at +70 mV in the absence of budipine to ensure recovery from residual blockade at negative potentials. (B) Pooled data on the voltage dependency of budipine on striatal neurones. Steady-state blockade of NMDA current responses was assessed as a percentage of both control and recovery currents and plotted as means (± S.E.M.) against budipine concentration at -70 and +70 mV. It should be noted that the IC₅₀ at +70 mV can only be regarded as an estimate, as the highest concentration of budipine only blocked responses to 64.9 ± 3.7% of control. (C) Kinetics of NMDA channel block by budipine at -70 mV. Budipine exhibited concentration-dependent open channel blocking kinetics ($\kappa_{on} = 0.71 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) whereas the fast offset rate was concentration-independent ($\kappa_{off} = 0.63 \text{ s}^{-1}$). Calculation of the ratio κ_{off}/κ_{on} revealed an apparent K_d value of 88.7 μM.

cortical and superior colliculus neurones, and the data are summarized in Table 1. Budipine had similar effects on all four neuronal types. Although budipine appeared to be somewhat less potent against cultured cortical neurones, it should be noted that only four cortical neurones were tested.

Hippocampal neurones were used to make a more detailed analysis of the fractional block by budipine 300 μM at different membrane potentials (Fig. 4; see Subramaniam et al., 1994). These studies indicate that the apparently weaker voltage dependency of budipine was not due to actions at a more superficial channel site. The data were very well fit by the following equation:

$$[1 + [\text{budipine}]/\text{IC}_{50(0)} \exp(-z\delta FV/RT)]^{-1}$$

where β is the fraction of voltage-independent sites, $\text{IC}_{50(0)}$ is the affinity at 0 mV, δ is the fraction of the electric field sensed by the voltage-dependent site, z is the charge of budipine, V is the membrane potential, and F , R and T have their usual meaning.

This analysis indicated that the channel site for budipine experiences 90% of the membrane electric field ($\delta = 0.897$) but confirmed that 28% block ($\beta = 0.284$) is mediated at a voltage-independent site. Note that offset kinetics were also progressively faster at more depolarized potentials although this effect was not quantified. Similar analysis was not performed on striatal neurones.

Memantine had similar effects on all neuronal types and the effects of memantine on cultured hippocampal

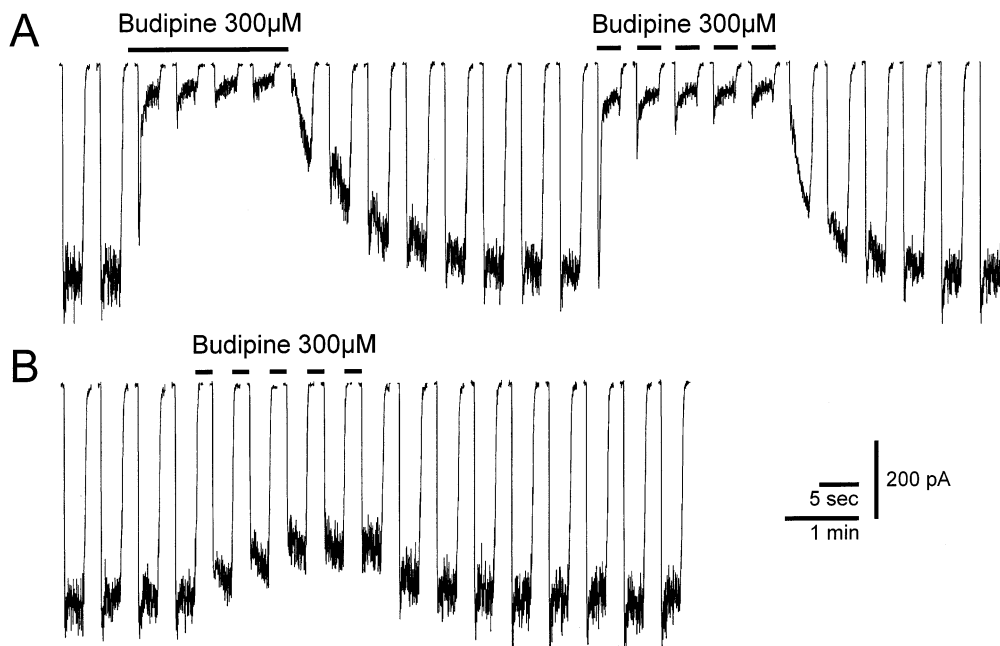


Fig. 3. Open and closed channel blockade by budipine 300 μM on a single striatal neurone. NMDA (200 μM) was applied for 2.5 s every 30 s in the continuous presence of glycine (1 μM) and at a constant membrane potential of -70 mV. This inter-response interval has been omitted for clarity (two time-scales). (A) Left: Budipine was continuously present for 2 min as indicated by the bar. Note that the first peak response following a 15–20 s pre-incubation was less affected. Right: Budipine 300 μM was co-applied with NMDA. Note that the first peak response following a 15–20 s pre-incubation was even less affected than in (A). (B) Budipine was present only between five successive NMDA applications.

neurones are illustrated for comparison in Fig. 5. The effects of memantine were strongly voltage-dependent and lower concentrations even showed a tendency to potentiate outward currents at $+70$ mV (Fig. 5(C)). Amantadine was somewhat more potent against NMDA-induced currents in cultured striatal neurones than in the other structures tested.

4. Discussion

The results of the present study support previous data (Klockgether et al., 1993, 1996; Jackisch et al., 1994; Kornhuber et al., 1995a; Porter and Greenamyre, 1995) indicating that budipine is an uncompetitive NMDA receptor antagonist. In line with its relatively low affinity, the open NMDA receptor channel blockade by budipine showed fast offset kinetics and allowed full expression of its strong voltage dependency (Parsons et al., 1995). The fact that the potency of budipine in blocking NMDA-induced [^3H]acetylcholine release in striatal slices was 10-fold higher than in displacing [^3H]MK-801 binding in the same structure has been interpreted to imply that the effects of budipine on NMDA receptors are mediated at a different site to those of MK-801 (Jackisch et al., 1994). This assumption is supported to some degree by the present data, as: (1) high concentrations of budipine were able to partially block NMDA responses even when present

only between successive NMDA applications; and (2) analysis of fractional block by budipine 300 μM at different holding potentials in hippocampal neurones indicates that 28% block is mediated at a voltage-independent site.

Although the degree of closed channel blockade was similar to that predicted by the fractional block experiments at different holding potential, it should be noted that the former could also be explained by access to the channel site via a lipophilic route (Blanpied et al., 1997). Moreover, the interpretation of such experiments is confounded by technical aspects when studying the effects of low-affinity antagonists with fast kinetics. For example, blockade in the continuous presence of budipine could be more pronounced than when applied together with NMDA, because after removal of both budipine and NMDA, this antagonist could leave some channels due to the relatively slow offset kinetics of NMDA. Similarly, budipine could gain access to some channels only when present between NMDA applications during the slow offset phase of NMDA responses, thereby producing apparent closed channel blockade.

However, the fractional block experiments clearly indicate that budipine also has voltage-independent actions at a second site, and that the voltage-dependent component experiences a similar degree of the transmembrane field to memantine (Bresink et al., 1996; Frankiewicz et al., 1996; Blanpied et al., 1997; Chen and Lipton, 1997) and amantadine (Parsons et al., 1995; Blanpied et al., 1997).

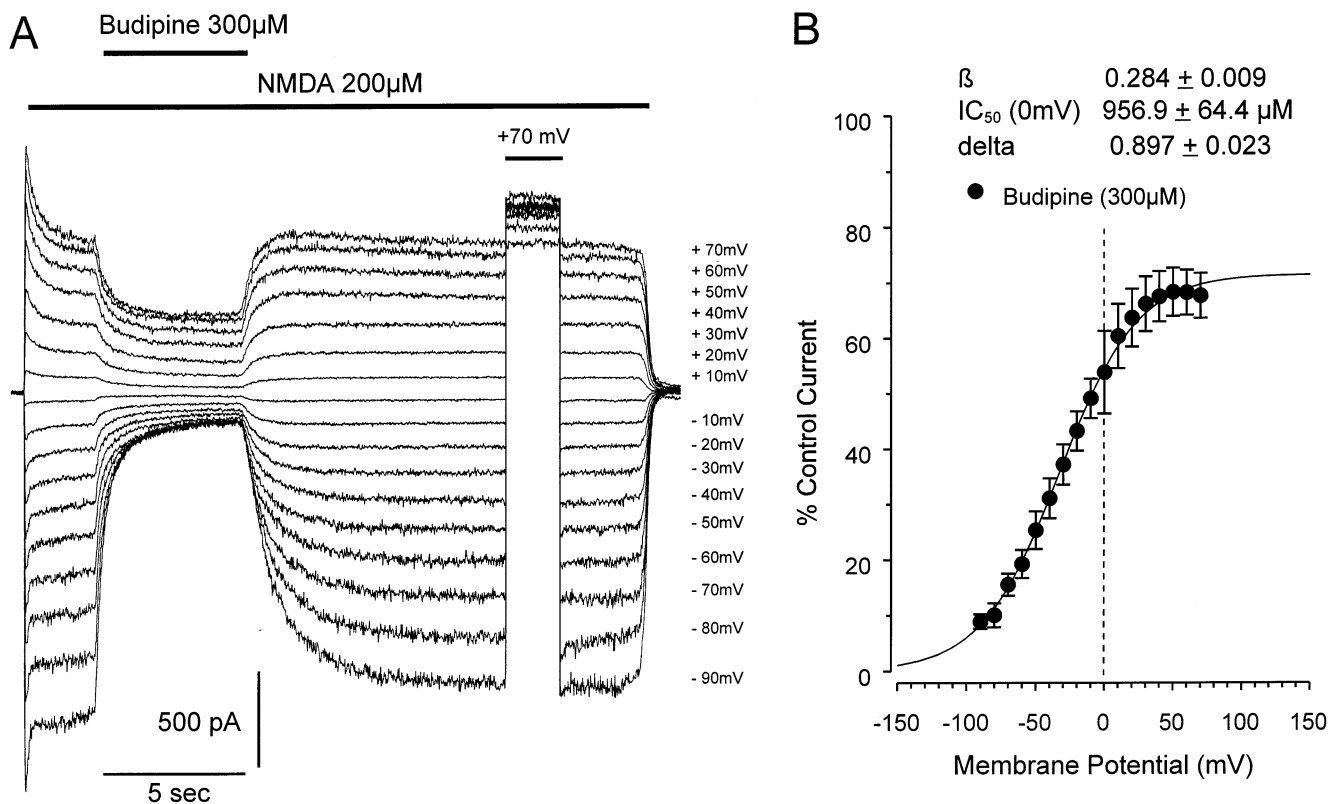


Fig. 4. Fractional block by budipine 300 μM at various holding potentials in hippocampal neurones. (A) Original data for a single hippocampal neurone. NMDA (200 μM) was applied for 22 s every 60 s at different holding potentials from -90 to $+70$ mV in 10 mV increments. Budipine 300 μM was applied for 5 s as indicated by the bar. Then 9 s following removal of budipine, neurones were clamped to $+70$ mV for 2 s in the continuing presence of NMDA to ensure complete relief of blockade. The traces are averages from 170 recordings, 10 at each holding potential. Note that offset kinetics were faster at more depolarized potentials. (B) Pooled data from seven neurones were well fit by the following equation: Fractional current = $(1 - \beta)[1 + [\text{budipine}]/IC_{50(0)} \exp(-z\delta FV/RT)]^{-1}$. The fraction of voltage-independent sites (β) was 0.284, i.e. 28%, the fraction of the electric field sensed by the voltage-dependent site (δ) was 0.897 and the $IC_{50(0)}$ was 957 μM . Other parameters have their normal meaning.

Data from Kornhuber et al. (1995a) indicate that budipine is 6 times more potent at the σ_1 binding site. There is considerable evidence that some sigma ligands can modulate NMDA receptor activity at a site other than the MK-801 channel domain, possibly via interactions with the strychnine-insensitive glycine modulatory site (e.g. Monnet et al., 1992; Fletcher et al., 1995). Moreover, some of the anti-Parkinsonian effects of amantadine may be mediated at this site (Kornhuber et al., 1993; see Danysz et al., 1997). It was not possible to assess the potency of budipine at the voltage-independent site as higher concentrations of budipine would act at both sites, even at $+70$ mV. However, it seems unlikely that budipine is more potent at this site, as 30 μM was completely without effect at $+70$ mV.

The moderate potentiation of NMDA-induced outward currents at positive potentials seen with memantine in hippocampal neurones could be related to the finding that Mg^{2+} and ketamine increase NMDA receptor-mediated currents in cultured mouse hippocampal neurones and HEK-293 cells expressing NMDA $\zeta 1/\epsilon 2$ receptors by increasing the affinity of the glycine_B

site (Wang et al., 1994; Wang and Macdonald, 1995). Such a facilitation would be predicted to be more pronounced with lower concentrations of glycine, although this point was not addressed in the present study. This could have important functional implications, as the differentiation between block of NMDA receptors at negative potentials and less block at depolarized potentials would be enhanced by such a mechanism, and would facilitate the ability of memantine to differentiate between mild/chronic pathological and physiological activation of NMDA receptors (Parsons et al., 1995; Frankiewicz et al., 1996). A similar potentiation was not seen with budipine, although it is possible that an opposite effect at the same site could underlie the second, voltage-independent component of the blocking actions of budipine.

It should be noted that the potency of budipine in the present study on cultured striatal neurones was about 10-fold lower than that reported for the inhibition of NMDA-induced [3H]acetylcholine release in striatal slices (Jackisch et al., 1994). It is possible that this difference is due to the differential expression of

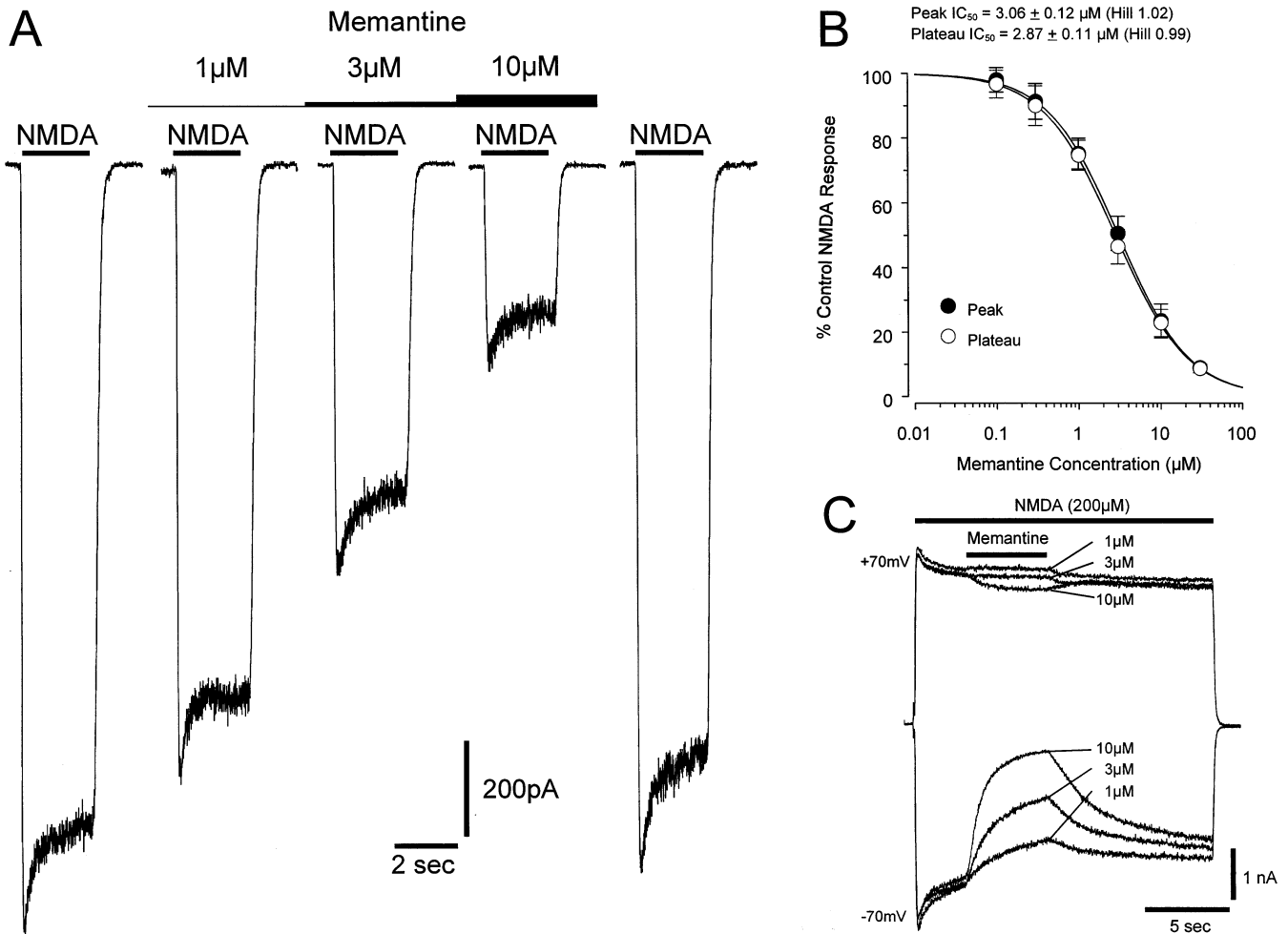


Fig. 5. Concentration dependence, kinetics and voltage dependence of the blockade of NMDA receptors by memantine in cultured hippocampal neurones. (A) Original data for a single hippocampal neurone—NMDA was applied as indicated by the bars. The left and right panels show control and recovery responses, respectively. The middle three panels show equilibrium responses in the continuous presence of memantine 1, 3 and 10 μM , respectively. (B) Peak and steady-state (plateau) NMDA current responses were normalized to control levels and plotted as means (\pm S.E.M.) against memantine concentration ($n = 9$ per concentration). (C) NMDA (200 μM) was applied for 18 s every 60 s at alternating potentials of -70 and $+70$ mV. Memantine was applied at 1, 3 and 10 μM for 5 s as indicated by the bar. Between recorded traces, NMDA was applied for 5 s at $+70$ mV in the absence of memantine to facilitate recovery from residual blockade at negative potentials.

NMDA receptor subtypes in slices and cultured neurones (Monyer et al., 1992; e.g. see Ujihara and Albuquerque, 1992; Williams et al., 1993; Priestley et al., 1996). Thus, although we previously reported that the anti-Parkinsonian drug amantadine was more potent against NMDA-induced currents in freshly dissociated striatal neurones than in hippocampal neurones (Parsons et al., 1996), this difference was less clear in the present study on cultured neurones. Similarly, the 3-fold greater potency of memantine on freshly dissociated hippocampal than striatal neurones (Parsons et al., 1996) was not seen in the present study. This difference between cultured cells and freshly isolated tissues is further supported by comparing the present patch-clamp data and previous biochemical studies. Whilst memantine was 23-fold more potent than amantadine on cultures of striatal neurones, this difference was only

3–4-fold against NMDA-induced Ach release in striatal slices (Lupp et al., 1992; Stoof et al., 1992). On the other hand, the fact that memantine is 2–3-fold more potent against NMDA 2C and 2D receptors than NMDA 2A and 2B receptors (Bresink et al., 1996; Parsons et al., in press) is reflected in relatively greater potency in the cerebellum, both in freshly isolated tissue (Porter and Greenamyre, 1995) and in cultures (Weller et al., 1993). We do not have any patch-clamp data on the effects of memantine in cultured cerebellar neurones.

The relevance of these NMDA antagonistic effects of bupropion to its anti-Parkinsonian properties remain uncertain. Thus, it is not clear what striatal or CSF levels are reached in the therapy of Parkinson's disease. Data from Zech et al. (1985) indicate that serum levels reach around 0.3 μM following a single oral administration of

a therapeutically relevant dose. In the rat, brain levels of budipine are around 2–10 times higher than in the blood (Zech et al., 1985), indicating that low μM concentrations of budipine may also be achieved in human brain under therapeutic conditions. However, brain concentrations do not necessarily reflect free concentrations at receptors in the CNS, as other uncompetitive NMDA receptor antagonists such as memantine and amantadine show pronounced lysosomal accumulation (Honegger et al., 1993), with the result that free CSF concentrations are some 10–20-fold lower than total brain levels (see Danysz et al., 1997).

In conclusion, budipine is a low affinity, voltage-dependent NMDA receptor antagonist with fast, channel blocking kinetics—at least in cultured neurones. Although the main effect of budipine was via open channel blockade, a component was clearly mediated at a voltage-insensitive site. The relevance of such effects to its anti-Parkinsonian properties remains to be established. It would seem important to test the possibility that budipine is more potent on NMDA receptors in freshly dissociated neurones from various basal ganglia structures with similar patch-clamp experiments.

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