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Selective block of *N*-methyl-D-aspartic acid (NMDA)-evoked whole-cell currents in mouse cultured spinal neurones by CGP 40116

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1 CGP 40116 is the active (**R**)-enantiomer of the most potent *N*-methyl-D-aspartic acid (NMDA) receptor antagonist presently available: 2-amino-4-methyl-5-phosphono-3-pentenoic acid (CGP 37849). In this study, we describe the effect of CGP 40116 on whole-cell currents induced by excitatory amino acids in cultured mouse spinal cord cells by use of the whole-cell patch-clamp technique.

2 We found that application of CGP 40116 in the nM range, concentration-dependently inhibited whole-cell current evoked by 20 μM NMDA in mouse cultured spinal neurones ($IC_{50} \pm$s.e.mean 48 ± 8 nM CGP 40116).

3 The compound appeared to be highly selective for the NMDA current. At concentrations as high 1 μM, currents evoked by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or kainic acid were not affected by CGP 40116. The threshold concentration for antagonism of NMDA-induced responses was 10 nM suggesting a selectivity ratio of $\geqslant 100$ fold for NMDA receptors versus AMPA or kainate receptors.

4 CGP 40116 produced a parallel rightward displacement of the NMDA log concentration-current curve indicating competitive antagonism at the transmitter recognition site of the NMDA receptor complex. An apparent dissociation constant for the antagonist was calculated from the displacement of the agonist concentration-current curve: 117 ± 53 nM CGP 40116 (estimated $K_d \pm$s.e.). Like other competitive NMDA antagonists, CGP 40116 blocked NMDA-evoked current in a voltage-independent manner.

Keywords: CGP 40116; competitive NMDA receptor antagonists; whole-cell current; patch-clamp; spinal cord; cell culture

Introduction

N-methyl-D-aspartic acid (NMDA) receptor antagonists have received much attention as possible therapeutic agents against neurological disorders associated with excessive activation of these receptors. Such disorders include epilepsy, stroke and neurodegenerative diseases like amyotrophic lateral sclerosis, Huntington's or Parkinson's disease (Meldrum & Chapman, 1994). The NMDA-type glutamic acid receptor possesses a number of pharmacologically distinct antagonist binding sites (Wong & Kemp, 1991; Watkins, 1994). (**RS**)-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid (CGP 37849) is the most potent antagonist presently available, acting selectively at the transmitter recognition site of the NMDA receptor and displaying little or no activity at other receptor binding sites (Fagg *et al*., 1990). The compound inhibits the binding of the NMDA antagonist [^{3}H]-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid ([^{3}H]-CPP) with a $K_i \sim 35$ nM, and it has been shown to possess anticonvulsant properties after oral administration (Fagg *et al*., 1990; Schmutz *et al*., 1990). *In vitro*, CGP 37849 blocked NMDA-induced neuronal firing in hemisected spinal cord, and hippocampal and nigral brain slices from rat, without affecting quisqualic or kainic acid responses (Pozza *et al*., 1990). Similar selectivity was found in the ability of the compound to block neuronal firing induced by iontophoretically applied NMDA in rat hippocampus *in vivo* (Fagg *et al*., 1990; Pozza *et al*., 1990).

In the mean while, the active enantiomer of this competitive NMDA antagonist has become available. In the present study, we describe the effect of this (**R**)-enantiomer, CGP 40116, on whole-cell currents induced by excitatory amino acids on cultured mouse spinal cord cells, by use of the whole-cell patch-clamp technique. A preliminary study by Schmutz *et al*. (1991) described selective inhibition of [^{3}H]-CPP binding ($K_i \sim 19$ nM), potent oral anticonvulsant activity and selective antagonism of NMDA-evoked responses in the cortical wedge preparation by this compound. The present paper provides the first detailed description of the functional effects of CGP 40116 on NMDA-evoked current.

Methods

Whole-cell currents were recorded from mouse spinal cord neurones in primary dissociated cell cultures. Spinal cord cells were obtained from albino Swiss mouse foetuses (12–14 days of gestation). Neurones were grown and maintained *in vitro* as described in detail previously (De Deyn & Macdonald, 1988). Cultures were 4–6 weeks old when used in tight-seal whole-cell recording experiments.

Spinal neurones were visualized by an inverted microscope (Zeiss IM35). Patch pipettes were pulled from glass capillaries (Jencons H10/15) with a Flaming-Brown P87 Micropipette Puller (resistance 3–5 MΩ). After heat-polishing, the pipettes were positioned with a Narashige Micromanipulator, and after seal formation (>3 GΩ), whole-cell configuration was obtained by disruption of the patch membrane (Hamill *et al*., 1981). All cells were clamped at a membrane potential of -60 mV except where indicated differently. Membrane currents were measured with an Axon Instruments Axoclamp-2A in discontinuous single-electrode voltage-clamp mode with optimal sampling rate of 2–3 kHz confirmed on a second oscilloscope. Currents were stored via a TL-1-125 Labmaster interface on IBM-compatible computer, and analysed by pClamp6 and conventional software. Curve fitting and error calculations were done with SigmaPlot and SigmaStat software.

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Pipette solution contained (in mM): KCl 30, K-aspartate 110, HEPES 10, EGATA 2, Na-ATP 5, $MgCl_2$ 2, $CaCl_2$ 1, adenosine 3–5′-cyclic monophosphate (cyclic AMP) 0.1 (315–320 mOsm with sucrose; pH 7.4 with KOH). Cells were superfused with bath solution containing (in mM): NaCl 140, KCl 3.5, $CaCl_2$ 1, HEPES 10, glucose 5, glycine 1 and tetrodotoxin 0.5×10^3 (315–320 mOsm with sucrose; pH 7.4 with NaOH). A fast-perfusion system was used to apply bath and drug solution to the cells, allowing a solution switch within 500 ms (flow rate $\sim$700 μl min^{-1}). The flow outlet of the perfusion system (diameter $\sim$1 mm) was placed within 1 mm of a selected spinal neurone. NMDA and kainic acid were purchased from Sigma Chemical Co. (St. Louis, USA). (**RS**)- α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) was purchased from Tocris Neuramin (Bristol, U.K.). CGP 40116 was a gift from Ciba Pharmaceuticals. Other chemicals were from Merck and of analytical grade.

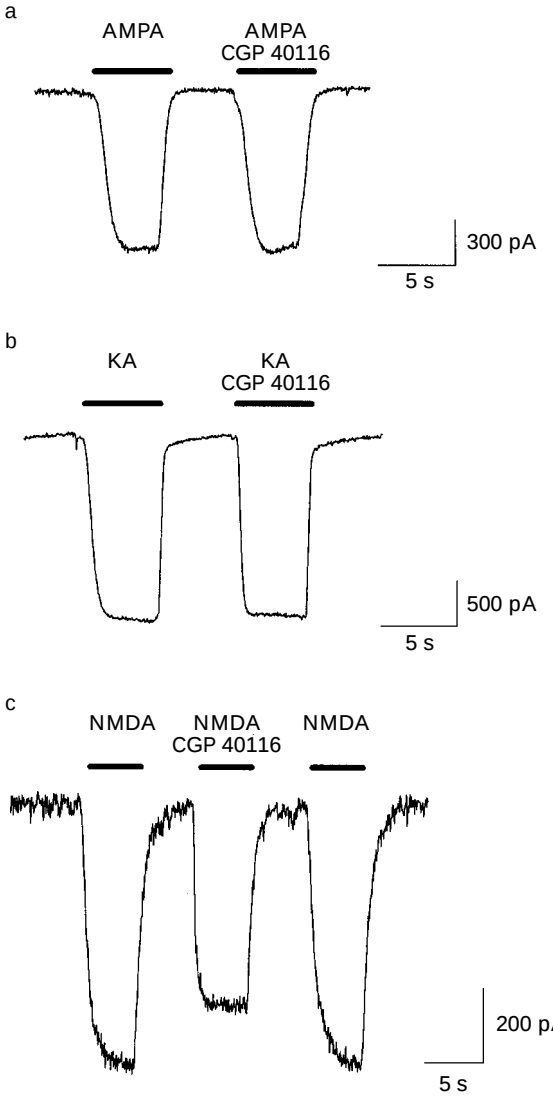

Figure 1 Typical examples of the effect of CGP 40116 on inward whole-cell currents evoked by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainic acid (KA) and *N*-methyl-D-aspartic acid (NMDA). Mouse spinal neurones in primary dissociated cell culture were clamped at -60 mV and perfused with solutions containing 20 μM AMPA (a), 20 μM KA (b) or 20 μM NMDA (c) with or without CGP 40116 during the period indicated by the horizontal bars above each current tracing. Cells are from different cultures. In the case of AMPA and KA (a and b), a concentration of 1 μM CGP 40116 was co-applied with agonists; in the case of NMDA (c), 10 nM CGP 40116 was co-applied with the 20 μM NMDA, followed by a second application of 20 μM NMDA without CGP 40116.

Results

The effect of CGP 40116 was examined on whole-cell currents evoked by the glutamate receptor agonists, α-amino-3-hydroxy-5-methyl-4-methyl-4-isoxazole propionic acid (AMPA), kainic acid (KA) and NMDA (Figure 1). On current evoked by 20 μM AMPA (Figure 1a) or 20 μM KA (Figure 1b), a concentration as high as 1 μM CGP 40116 had no effect (2 cells). Application of 20 μM NMDA to cells clamped at -60 mV evoked an inward whole-cell current of 440 ± 240 pA (mean $\pm$ s.d.; 7 cells). CGP 40116 blocked 10 nM reversibly inhibited this NMDA-evoked current (Figure 1c); 1 μM CGP 40116 blocked $95.7 \pm 4.2\%$ of the NMDA-evoked current (mean $\pm$ s.d.; 5 cells; not not shown in figure). Even though this latter concentration almost completely blocked the NMDA-evoked current, the block was easily reversible, and a 5 s washout period proved to be more than sufficient for complete return of NMDA-evoked current to its initial value.

Application of CGP 40116 concentration-dependently blocked NMDA-evoked current over the broad concentration range of 1 nM to 10 μM (Figure 2a). Figure 2b shows the log concentration-response curve for CGP 40116 antagonism of the inward whole-cell current evoked by 20 μM NMDA. The IC_{50} was calculated from:

$$(I - k)/I_{max} = C^{n_H}/[C^{n_H} + (IC_{50})^{n_H}]$$

In this Hill equation, I represents the NMDA-evoked current in the presence of CGP 40116; C, the concentration of

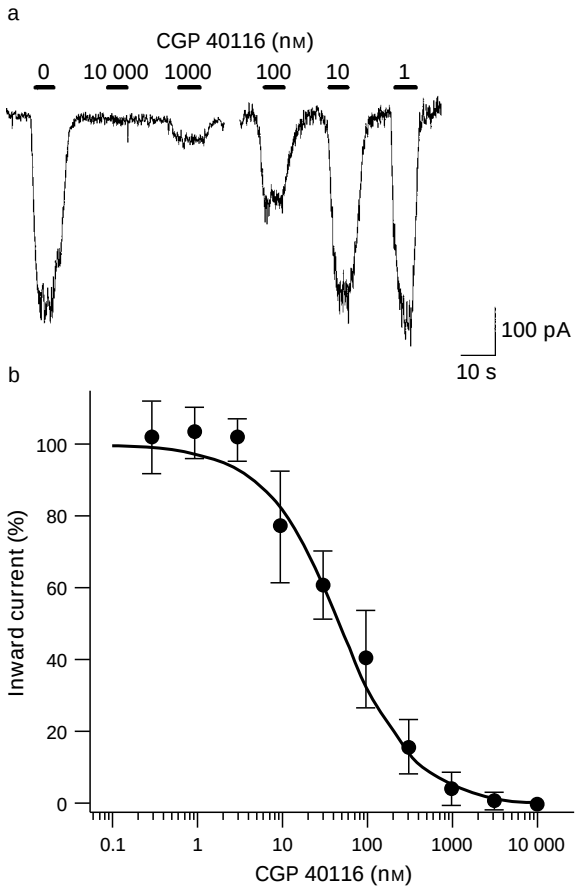

Figure 2 Concentration-dependent inhibition of NMDA-evoked by CGP 40116. Block of current evoked by 20 μM NMDA (horizontal bars) in the presence of the indicated concentration of CGP 40116 (a). The log concentration-response relation of CGP 40116 antagonism of NMDA-evoked current was fitted to a Hill equation with $IC_{50} = 48 \pm 8$ nM CGP 40116 (b). Each data point is the mean of 5–7 cells expressed as % of response to 20 μM NMDA; vertical lines show s.d.

CGP 40116; IC_{50}, the concentration of CGP 40116 for half-maximal inhibition of NMDA-evoked current; I_{max}, current evoked in the absence of antagonist; n_H the Hill coefficient; and k, the Y asymptote. The curve in Figure 2b was obtained with the following values: $IC_{50} = 48 \pm 8$ nM CGP 40116 (coefficient $\pm$s.e.; $P = 0.001$); $I_{max} = 100\%$ $n_H = -0.95$; and $k = 1.8\%$.

Figure 3 shows the effect of 100 nM CGP 40116 on whole-cell current evoked by 100 μM NMDA in a cell clamped at potentials ranging from -60 to $+40$ mV. Application of 100 μM NMDA evoked an outward current at positive clamp potentials ($+20$ and $+40$ mV). It was demonstrated that CGP 40116 blocked the inward as well as the outward NMDA-evoked current. In fact, the proportion of current blocked by CGP 40116 appeared to be unaffected by clamp potential.

Finally, Figure 4 shows the effect of 100 and 1000 nM CGP 40116 on the log concentration-response curve of NMDA-evoked inward current in cells clamped at -60 mV. The NMDA-evoked currents in the absence of the antagonist were fitted to a Hill equation with the following values (EC_{50}, the concentration of NMDA for half-maximal current): $(EC_{50})_o = 80 \pm 9$ μM NMDA (coefficient $\pm$s.e. $P = 0.003$); $I_{max} = 17\%$; $n_H = 0.82$; and $k = -4.3\%$. Application of CGP 40116 with NMDA shifted the NMDA log concentration-response curve to the right. The NMDA-evoked currents in the presence of 100 nM CGP 40116 were fitted to a Hill equation with the following values: $(EC_{50})_{100\text{ nM CGP }40116} = 148 \pm 26$ μM NMDA (coefficient $\pm$s.e.; $P = 0.005$); $I_{max} = 111\%$; $n_H = 0.82$ and k -4.3%. Thus, the application of 100 nM CGP 40116 with NMDA resulted in a 1.9 fold increase in the EC_{50} of NMDA, whereas I_{max} was not changed by application of CGP 40116. An apparent dissociation constant for CGP 40116 (K_d) can be calculated from the rightward displacement of the NMDA log concentration-response plots as

$$K_d = C/(x - 1)$$

with C, the concentration of CGP 40116, and x, the dose-ratio of NMDA in the presence of CGP 40116:

$$x = (EC_{50})_{100\text{ nM CGP }40116} / (EC_{50})_0 = 1.86$$

which yielded an apparent dissociation constant of 117 ± 53 nM CGP 40116 (apparent $K_d \pm$s.e.). Notably, a K_d of 60 nM CGP 40116 was estimated from the effect of 1 μM CGP 40116 on the NMDA log concentration-response curve of one cell (Figure 4 (▲)), which falls within the 95% confidence limits of the 100 nM CGP 40116 calculation.

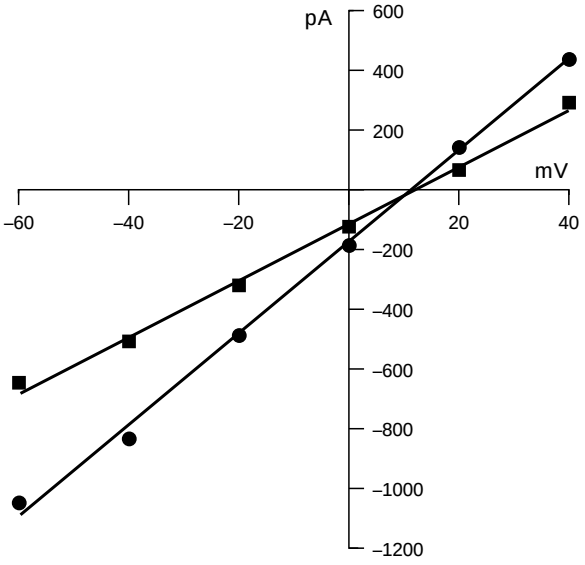

Figure 3 The effect of CGP 40116 on whole-cell current evoked by NMDA in a cell clamped at potentials ranging from -60 to $+40$ mV. At negative potentials, 100 μM NMDA evoked an inward current, whereas at positive potentials ($+20$ and $+40$ mV), an outward current was evoked (●); CGP 40116 (100 nM) blocked the inward and outward NMDA-evoked current (■). The proportion of current blocked by CGP 40116 appeared to be unaffected by clamp potential.

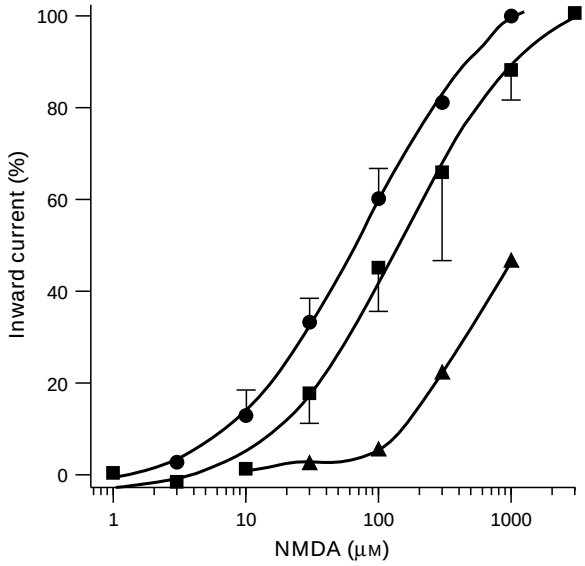

Figure 4 The effect of 100 nM and 1 μM CGP 40116 on the log concentration-response curve of NMDA-evoked inward current in mouse cultured spinal neurones. The NMDA-evoked current (●) was fitted to a Hill equation with $EC_{50} \approx 80$ μM NMDA. Application of 100 nM (■) or 1 μM (▲) CGP 40116 shifted the NMDA log concentration-response curve to the right. The NMDA and NMDA + 100 nM CGP 40116 data points (expressed as % of current evoked by 1 mM NMDA) are means $\pm$ s.d.(vertical lines) of 5–7 cells; the effect of 1 μM CGP 40116 was demonstrated on one cell only.

Discussion

The first antagonists of NMDA-type glutamic acid receptor were compounds acting at the transmitter recognition site (see Collingridge & Lester, 1989; Wong & Kemp, 1991; Jane *et al*., 1994). Evans *et al*. (1982) showed that a series of ω-phosphonic α-carboxylic amino acids antagonize NMDA responses in frog isolated spinal cord with the (**R**)-enantiomer of 2-amino-5-phosphonovaleric acid (APV) as the most potent agent ($K_i \sim 1.4 \mu$M). Ascher *et al*. (1988) demonstrated that APV concentration-dependently inhibits whole-cell current evoked by NMDA in mouse central neurones in primary dissociated cell culture. The introduction of a double bond and a methylgroup into the carbon chain of APV has produced the most potent NMDA antagonist presently available. The racemate of this compound (CGP 37849) was introduced in 1990 as the first orally active anticonvulsant NMDA antagonist (Fagg *et al*., 1990; Schmutz *et al*., 1990). It was shown to bind selectively at the transmitter recognition site of the NMDA receptor complex, to have no effect on neuronal release or reuptake of glutamic acid, and to have little or no activity at 18 other receptor binding sites examined (Fagg *et al*., 1990).

The compound investigated in this study, CGP 40116, is the active (**R**)-enantiomer of CGP 37849. Like its racemate, CGP 40116 inhibits [^{3}H]-CPP binding ($K_i \sim 19$ nM), and has potent oral anticonvulsant activity (Schmutz *et al*., 1991). CGP 40116 was shown to protect against NMDA-induced death of mouse cortical neurones in primary cell cultures,

concentration-dependently, with an ED_{50} of 3.2 μM (Maier *et al*., 1995). In rats and rabbits, CGP 40116 has been found to protect against experimentally-induced focal cerebral ischaemic damage (Sauer *et al*., 1993; 1994; Maier *et al*., 1995). The compound has also been shown to protect against neuronal damage caused by severe insulin-induced hypoglycaemia (Nellgård & Wieloch, 1992) or by status epilepticus (Fujikawa *et al*., 1994). Here we found that application of CGP 40116 in the nM range, concentration-dependently inhibited NMDA-evoked whole-cell current in mouse cultured spinal neurones ($IC_{50} \sim 48$ nM). The compound appeared to be highly selective for the NMDA current. At concentrations up to 1 μM, AMPA- or kainic acid-evoked currents were not affected by the application of CGP 40116. The threshold concentration for antagonism of NMDA-induced responses was 10 nM suggesting a selectivity ratio of $\geqslant$100 fold for NMDA-induced receptors versus AMPA or kainate receptors. According to Schmutz *et al*. (1991), CGP 40116 also antagonizes NMDA-evoked neuronal firing in the rat cortical wedge preparation without effecting quisqualic or kainic acid responses.

The effect of the antagonist on the NMDA log concentration-current relation indicates competitive antagonism at the transmitter recognition site of the NMDA receptor complex. Like other competitive NMDA antagonists, CGP 40116 blocked NMDA-evoked current in a voltage-independent manner. Antagonists like phencyclidine, ketamine or dizocilpine, on the other hand, which act at the ion-channel site of the NMDA receptor, typically produce a voltage-dependent block of NMDA receptor currents (Honey *et al*., 1985; MacDonald *et al*., 1987; Huettner & Bean, 1988). MacDonald *et al*. (1987) contrasted the effect of the competitive antagonist APV with that produced by the non-competitive antagonist ketamine on the current-voltage relation of (D)-aspartic acid-evoked whole-cell current in cultured hippocampal neurones. They found that 50 μM APV was as efficacious against outward as inward current, whereas 50 μM ketamine blocked inward current at hyperpolarizing potentials but had little effect on outward current.

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