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Dual inhibitory action of enadoline (CI977) on release of amino acids in the rat hippocampus

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Abstract

The effect of the κ-opioid receptor agonist enadoline (CI977, (5*R*)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrilidinyl)-1-oxaspiro[4,5]dec-8-yl-4-benzofuranacetamide monohydrochloride), on the release of amino acids was studied in the hippocampus of freely moving rats. K^+, 100 mM, or veratrine, 100 μM, were applied for 10 min via the dialysis probe, either alone (control groups) or together with CI977 (after a 10 min pretreatment with CI977 in the perfusion medium). To test the specificity of the response to CI977, nor-binaltorphimine, a selective κ-opioid receptor antagonist, was delivered together with CI977 in two groups of animals. To test the effect of systemic injection, CI977 was given subcutaneously 30 min prior to either stimulus. K^+-induced release of glutamate and aspartate was significantly reduced by CI977, 2.5 mM; release of γ-aminobutyric acid (GABA) was reduced by 250 μM CI977 in the probe. The effect of CI977 on release of glutamate and aspartate, but not of GABA, was reversed by nor-binaltorphimine (45 μM). Systemic treatment with CI977, 1 or 10 mg/kg, did not reduce K^+-induced release of glutamate. Veratrine-induced release of aspartate and glutamate was significantly inhibited by 25 μM and release of GABA by 250 μM CI977 in the probe, and this effect was not modified by nor-binaltorphimine (58 μM). Systemic injection of CI977 1 mg/kg significantly reduced veratrine-induced release of glutamate. These results indicate that CI977 regulates release of amino acids by two independent mechanisms. The weaker inhibition of the stimulated release involved activation of the κ-opioid receptor; the more potent inhibition appears to be connected with Na^+ channels.

Keywords: κ-Opioid receptor; CI977; Excitatory amino acid; Epilepsy; Na^+ channel

1. Introduction

κ-Opioid receptor agonists have anticonvulsant activity in animal models of epilepsy. CI977 ((5*R*)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrilidinyl)-1-oxaspiro-[4,5]dec-8-yl-4-benzofuranacetamide monohydrochloride) a new, potent, selective, analgesic κ-opioid receptor agonist (Halfpenny et al., 1990; Hunter et al., 1990) has potent anticonvulsant activity against *N*-methyl-DL-aspartate-induced seizures in mice (Singh et al., 1990). Another κ-opioid receptor agonist, U-50488H ((±)-*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide methane sulfonate), and the structurally related benzamide, U-54494A (*cis*-3,4-dichloro-*N*-2-[(1-pyrrolidyl)-cycloexyl]benzamide), are protective against seizures in rodents induced by excitatory amino acid agonists and other chemoconvulsants, as well as against maximal electroshock and sound-induced seizures in DBA/2 mice (Von Voigtlander et al., 1987). CI977 and other κ-opioid receptor agonists are also neuroprotective in middle cerebral artery occlusion and other models of cerebral ischaemia (Boxer et al., 1991; Kusumoto et al., 1992; Mackay et al., 1993, Birch et al., 1991; Hayward et al., 1993b).

Since both epilepsy and ischaemia are believed to be associated with enhanced release of excitatory amino acids, the influence of neuroprotective κ-opioid receptor agonists on glutamate release has been studied. It has been shown that both CI977 and U-54494A inhibit high K^+-induced release of glutamate, in vitro (Conner-Kerr and Terrian, 1993; Lambert et al., 1991).

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U-54494A also inhibits kainate-induced glutamate release in the hippocampus of anaesthetized rats (Payson et al., 1990).

In this study we investigate the effects of the κ-opioid receptor agonist CI977, either delivered via the dialysis probe or given systemically, on the release of amino acids induced by high K^+ or by veratrine in the hippocampus of freely moving rats.

2. Material and methods

2.1. Microdialysis

Male Sprague-Dawley rats (250–270 g) were used in the study. Concentric, vertical dialysis probes with 2–2.5 mm tip membrane (Hospal) were stereotactically implanted into the dorsal hippocampus (A: 3.8, V: 5.3 mm both from intraaural line, L: 2.8 mm from the sagittal suture) under ether anaesthesia. After a 90–120 min recovery period the probes were flushed with perfusion medium (NaCl 125 mM, KCl 3.3 mM, $CaCl_2$ 1.85 mM, $MgSO_4$ 2.4 mM, KH_2PO_4 1.25 mM). Baseline level samples were collected for 40 min (10 min samples, 2 μl/min flow rate). In the control group, medium containing 100 mM KCl or 100 μM veratrine hydrochloride was perfused through the probe for 10 min. In the experimental groups, the following compounds were included in the perfusion medium for 10 min before and during the K^+ stimulus: (1) 0.25 or 2.5 mM CI977, (2) 2.5 mM CI977 + 45 μM nor-binaltorphimine. 45 μM nor-binaltorphimine alone was included for 20 min in the perfusion medium. Two additional 10 min samples were collected after the application of the compounds. Separate groups of animals received 0.12 ml of saline or 1 or 10 mg/kg of CI977 subcutaneously 30 min prior to the K^+ stimulus.

In the experimental groups where a veratrine stimulus was used, the following concentrations of drugs were included in the perfusion medium: (1) 2.5 or 25 μM CI977, (2) 25 μM CI977 + 58 μM nor-binaltorphimine. In separate groups of animals CI977, 1 or 10 mg/kg, was injected subcutaneously (s.c.) 30 min prior to the veratrine stimulus.

At the end of the experiment rats were overdosed with barbiturate, their brains removed, frozen and cut in a cryostat in order to assess position of the probe tip.

2.2. High performance liquid chromatography analysis

The 20 μl dialysate samples were analyzed by high performance liquid chromatography (HPLC) after orthophthaldialdehyde derivatization of amino acids. The amino acid derivatives were separated on a Spherisorb ODS2 5 μm column (25 cm × 4.6 nm) using a 20–56% methanol gradient in 0.1 M sodium acetate, 2.5% tetrahydrofuran buffer, pH 5.75. The HPLC system consisted of a Spectraphysics SP8800 ternary HPLC pump, a Kratos FS 950 Fluorimeter and a Spectraphysics Chromjet Integrator. Samples were injected automatically by Carnegie Medicin CMA/200 refrigerated Mi-

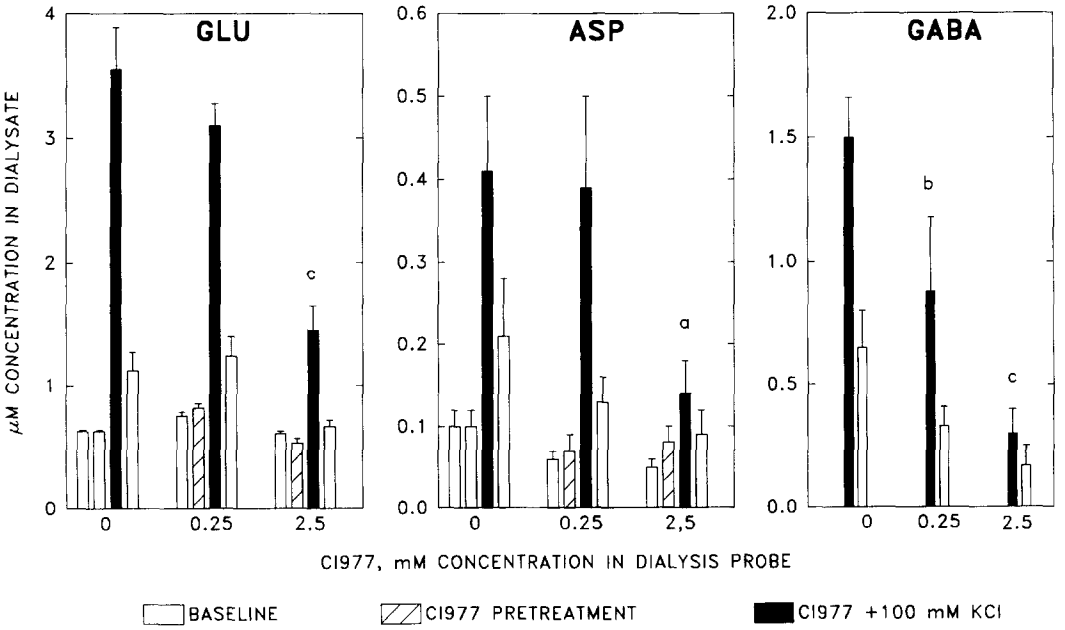

Fig. 1. The influence of 0.25 and 2.5 mM CI977 on basal levels and on K^+-enhanced levels of glutamate, aspartate and GABA in hippocampal dialysate. Each column represents the mean value of six to eight experiments (μM in dialysate), with S.E.M. indicated by error-bars. The letters a, b and c indicate values that were significantly different from corresponding K^+-enhanced values in the control groups (= 0 μM CI977 in dialysate). Statistical analysis was carried out using Student's t-test; [a] $P \leq 0.05$, [b] $P \leq 0.01$, [c] $P \leq 0.001$.

Table 1
Effect of CI977 on K^+-evoked release of glutamine and taurine

Experimental group	Glutamine, μM		Taurine, μM	
	Basal	Post-stimulus	Basal	Post-stimulus
Control (100 mM KCl)	13.06 ± 0.74	10.45 ± 0.30 [a]	5.81 ± 0.71	17.14 ± 1.30 [a]
100 mM KCl + 0.25 mM CI977	12.72 ± 0.52	10.22 ± 0.34 [a]	7.18 ± 0.75	19.17 ± 2.35 [a]
100 mM KCl + 2.5 mM CI977	12.45 ± 0.65	10.58 ± 0.13 [a]	4.93 ± 0.57	16.02 ± 1.18 [a]

Values are expressed as μM concentrations (± S.E.M.) in hippocampal dialysate of rats. Significant K^+-induced changes from basal levels were analyzed using Student's t-test ([a] $P \leq 0.001$); these changes were not significantly affected by CI977.

crosampler. Levels of the following amino acids were determined from the chromatograms: aspartate, glutamate, serine, glutamine, glycine, alanine, taurine and GABA. The baseline concentrations (μM in the dialysate) of the amino acids were calculated using the pooled pre-stimulus fractions. The K^+-evoked changes in concentration of aspartate, glutamate and GABA of the control group were compared with those of experimental groups. K^+-induced changes in the levels of glutamine and taurine were expressed as per cent decrease or increase below/above the baseline levels. The per cent changes in glutamine and taurine of the control group were statistically compared with those of experimental groups using Student's t-test.

2.3. Materials

We are grateful to Dr G. Woodruff (Parke Davis, Cambridge) for the donation of CI977 (enadoline) and to Dr A. Lipkowski, Polish Academy of Sciences, Warsaw, for the donation of nor-binaltorphimine. Amino acid standards, ortho-phthaldialdehyde and inorganic salts were purchased from Sigma (Poole, UK). HPLC columns, solvents and diethyl ether were purchased from Fisons (Loughborough UK).

3. Results

Histological examination of the brains confirmed that all probe tips were located in the dorsal hippocampus, vertically crossing CA1, CA4 and dentate granule neuronal layers.

Basal levels (μM in hippocampal dialysate) of the amino acids were as follows: glutamate 0.69 ± 0.07, aspartate 0.05 ± 0.03, glutamine 13.06 ± 0.74, taurine 5.81 ± 0.71, serine 1.25 ± 0.36, glycine 5.47 ± 1.27 and alanine 4.13 ± 1.0. In some groups GABA levels were below detection limit during basal conditions therefore

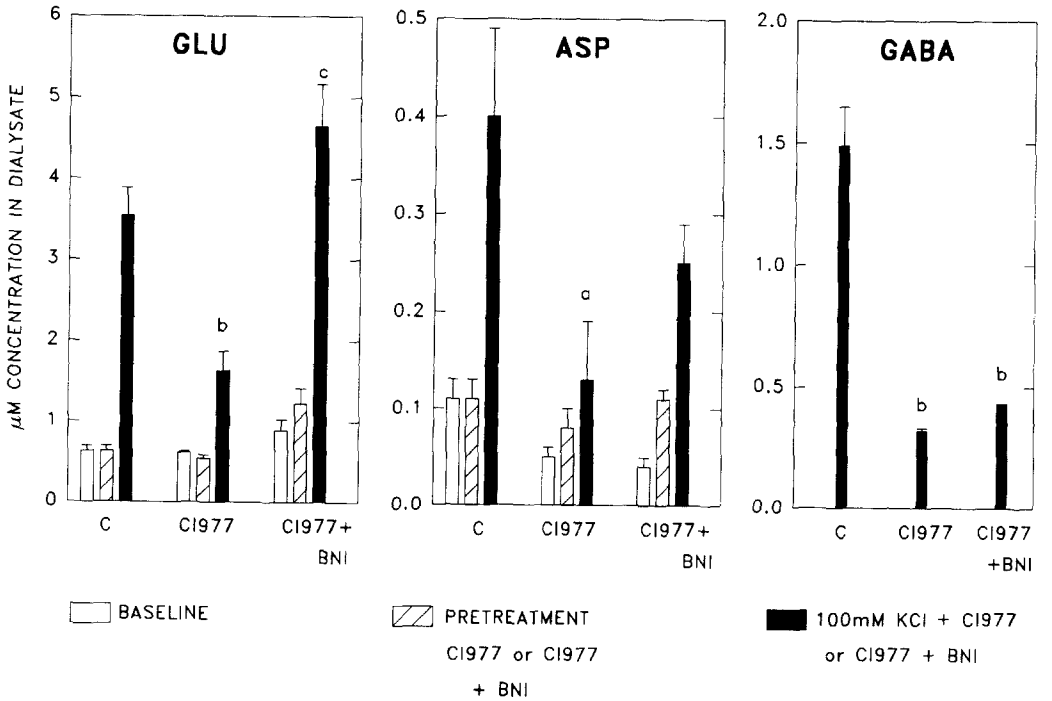

Fig. 2. The influence of nor-binaltorphimine on the effect of CI977 on high K^+ increase in the levels of aspartate, glutamate and GABA. Each column represents the mean value of six to eight experiments (μM in dialysate), with S.E.M. indicated by error-bars. The letters a and b indicate values that were significantly lower than corresponding K^+-enhanced values in the control group (C); [a] $P \leq 0.05$, [b] $P \leq 0.001$ or significantly higher as compared to these reduced by 2.5 mM CI977 [c] $P \leq 0.01$. Statistical analysis was carried out using Student's t-test. BNI: nor-binaltorphimine.

stimulus-induced changes are not expressed as percentage of basal level for this amino acid.

3.1. K^+-evoked amino acid release

In the presence of 100 mM KCl in the perfusion medium, significant increases in the extracellular concentration of aspartate (+285%, $P \leq 0.001$) and glutamate (+463%, $P \leq 0.001$) occurred (Fig. 1). There was a concomitant K^+-evoked rise in extracellular GABA level from below detection limit to 1.50 ± 0.16 μM. In the sample following the K^+ stimulus the taurine level was also significantly increased (+195%, $P \leq 0.001$); glutamine level in the same sample was significantly decreased (−20%, $P \leq 0.001$) (Table 1). In the experimental groups 0.25 or 2.5 mM CI977, applied during a 10 min period prior to the K^+ stimulus, had no significant effect on the basal levels of the amino acids, except for causing a 10% decrease in extracellular taurine level ($P \leq 0.02$) at 2.5 mM (data not shown). The K^+-induced elevations of extracellular aspartate, glutamate and GABA levels, however, were significantly reduced by 2.5 mM CI977, and that of GABA also by 0.25 mM CI977 (Fig. 1). K^+-evoked changes in the levels of taurine and glutamine were not affected by either dose of CI977 (Table 1).

Nor-binaltorphimine (45 μM) delivered together with 2.5 mM CI977 via the probe reversed completely the inhibitory effect of CI977 on K^+-evoked release of glutamate. The decrease in the release of aspartate was partially (non-significantly) reversed, while inhibition of GABA release was not affected by nor-binaltorphimone (Fig. 2). Changes in taurine and glutamine were also unaffected.

Nor-binaltorphimine (45 μM) delivered alone via the probe had no effect on the basal levels of the investigated amino acids (glutamate: 1.34 ± 0.38 vs. 2.25 ± 0.22 μM; aspartate 0.04 ± 0.01 vs. 0.07 ± 0.03 μM).

Systemic injection of CI977, 1 or 10 mg/kg, resulted in the rapid development of severe ataxia lasting throughout the experiments. However, the K^+-induced increases in the concentration of glutamate and aspartate were not changed (5.1 ± 0.64 vs. 5.74 ± 1.59 μM and 0.35 ± 0.17 vs. 0.25 ± 0.01 μM, respectively) by systemic CI977.

3.2. Veratrine-evoked amino acid release

Veratrine hydrochloride (100 μM) delivered via the probe elevated glutamate and aspartate levels to $402 \pm 48\%$ and $676 \pm 119\%$ of the baseline respectively (Fig. 3). The peak GABA level following the stimulus was 0.56 ± 0.08 μM. The concentration of glutamine following the veratrine stimulus was significantly reduced

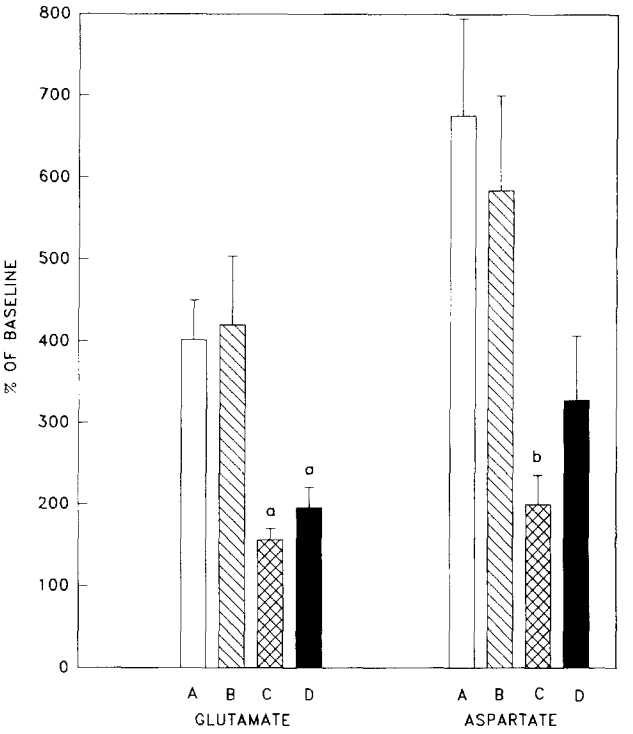

Fig. 3. The influence of CI977 and nor-binaltorphimine on veratrine-evoked release of aspartate and glutamate in rat hippocampus. Each column represents the mean value of 6–16 experiments (% of the baseline level of the sample showing the highest amino acid content post stimulus. (A) 100 μM veratrine; (B) 100 μM veratrine +2.5 μM CI977; (C) 100 μM veratrine +25 μM CI977; (D) 100 μM veratrine +25 μM CI977+58 μM nor-binaltorphimine. $^aP \leq 0.01$; $^bP \leq 0.05$.

(from 14.78 ± 0.33 μM to 12.67 ± 0.81 μM $P \leq 0.01$, Student's paired t-test, not shown).

CI977 (2.5 μM) delivered via the probe for 10 min prior to and during the stimulus did not modify the changes in amino acid levels due to veratrine. CI977 (25 μM) resulted in a significant suppression of the veratrine-evoked increase in the extracellular levels of glutamate and aspartate (to $156 \pm 14\%$, $P \leq 0.01$ and $200 \pm 36\%$, $P \leq 0.05$ of baseline levels respectively). Nor-binaltorphimine (58 μM) delivered together with CI977 did not reverse its inhibitory effect on veratrine-induced amino acid release (Fig. 3). The peak GABA level was reduced significantly by CI977 250 μM from 0.56 ± 0.08 μM to 0.17 ± 0.03 μM ($P \leq 0.01$) and this effect was not reversed by nor-binaltorphimine (the peak level = 0.27 ± 0.08 μM, not shown).

Systemic injection of CI977, 1 mg/kg, 30 min before the veratrine stimulus, significantly lowered veratrine-evoked release of glutamate (to $247 \pm 19\%$ of the baseline, $P \leq 0.05$). The evoked release of aspartate and GABA was not significantly affected by this injection. CI977, 10 mg/kg, produced a further reduction in the evoked glutamate release (to $215 \pm 24\%$ of the baseline, $P \leq 0.05$). The veratrine-evoked GABA release

was also reduced by this dose of CI977 (from 0.56 ± 0.08 μM in the control group to 0.3 ± 0.05 μM, $P \leq 0.05$).

4. Discussion

The main finding of this study is that CI977, a κ-opioid receptor agonist, inhibits the release of glutamate by two different mechanisms. The mechanism connected with activation of the κ-opioid receptor requires a very high concentration of CI977 and is reversible by the selective antagonist nor-binaltorphimine (Portoghese et al., 1987). There is however a more potent mechanism not connected with its action on the κ-opioid receptor. Veratrine-induced release of neurotransmitter amino acids is significantly reduced by a low (25 μM) concentration of the compound in the perfusion medium (dialysis membrane allows approximately 10% of this concentration to reach the tissue). This effect is not reversed by nor-binaltorphimine, a specific antagonist of the κ-opioid receptor (Fig. 3). The systemic injection of CI977 reduces veratrine-evoked release of glutamate, with 10 mg/kg being only marginally more effective than 1 mg/kg. The residual effect of veratrine on glutamate release may be related to its very high concentration around the dialysis probe.

Inhibition of the K^+- and electrically evoked release of glutamate by low nanomolar concentrations of κ-opioid receptor agonists has been shown in several in vitro studies (Lambert et al., 1991; Conner-Kerr and Terrian, 1993; Pinnock, 1992).

Systemic administration of CI977, 1 or 10 mg/kg, failed to inhibit K^+-induced release of glutamate (Fig. 4). A similar negative result was reported in another hippocampal microdialysis study with the anticonvulsant benzamide, U-54494A, 50 mg/kg, a compound which is closely related to the κ-opioid receptor agonist U-50488H (Payson et al., 1990). Kainate-induced release of glutamate, on the other hand, was reduced by the systemic injection of U-54494A, 1 to 15 mg/kg (Payson et al., 1990). The effect of the κ-opioid receptor agonists on K^+-induced release of glutamate appears to be less significant in vivo than in vitro.

This study demonstrates that CI977 shows two independent inhibitory mechanisms of amino acids release. Inhibition of K^+-evoked release is connected with the activation of the κ-opioid receptor as this effect is reversible by the antagonist of the receptor, nor-binaltorphimine. It has been shown by Pinnock (1992) using in vitro preparation of locus coeruleus that electrical stimulation of the afferents induces postsynaptic potentials inhibited by CI977 and this effect is reversed by nor-binaltorphimine or glutamate receptor antagonists.

These results indicate that the pre-synaptic κ-opioid receptors activated by CI977 inhibit release of excitatory amino acids. This mechanism of action may not however be relevant for the neuroprotective action of the compound. As shown in this study, CI977 at doses of 1 or 10 mg/kg, has no effect on K^+-induced release of glutamate.

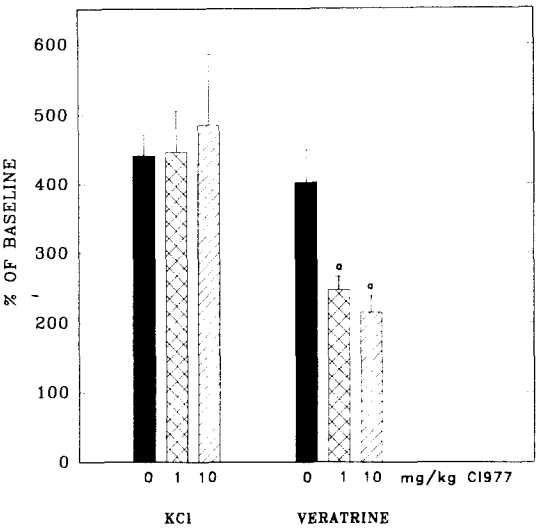

Fig. 4. The influence of the s.c. injection of CI977 on K^+- and veratrine-induced release of glutamate in rat hippocampus. Each bar represents the mean value of 6–16 experiments (% of the baseline level of the sample showing the highest glutamate content during/post stimulus). [a] $P \leq 0.05$.

In contrast, veratrine-induced release of glutamate was reduced by a 100-fold lower concentration of CI977 delivered locally, and by 1 mg/kg s.c., corresponding to CI977 doses that are effective against ischaemic glutamate release (Hayward et al., 1993a) and neuronal damage (see Table 2).

Veratrine induces release of amino acids by opening Na^+ channels. Compounds that inactivate voltage-dependent Na^+ channels prevent veratrine-induced glutamate release in vitro and in vivo (Lees and Leach,

Table 2
Cerebroprotective and anticonvulsant efficacies of CI977 in rodents

Anticonvulsant activity	ED_{50} (mg/kg)	Reference
NMDA-seizures, clonic (i.v., −30 min, mice)	0.17 (0.04–0.65)	Singh et al. (1990)
Sound-induced clonic seizures (i.p., −30 min, DBA/2 mice)	9.6 (6.5–14.5)	*
Cerebroprotective activity	**Protective dose (mg/kg)**	**Reference**
MCA occ., rats; i.v., 30 min	0.5	Boxer et al. (1991)
MCA occ., rats; s.c., −30 min	0.3	Kusomoto et al. (1992)
MCA occ., rats; s.c., −30 min	1.0	Hayward et al. (1993b)

* Chapman, Millan and Meldrum, unpublished results. MCA occ.: middle cerebral artery occlusion.

1993; Leach et al., 1986). BW1003C87 (5-[2,3,5-trichlorophenyl]pyrimidine-2,4-diamine ethane sulfonate), which prolongs inactivation of Na^+ channels, produces a similar degree of protection against ischaemic cortical neuronal damage (but with higher doses, 10–20 mg/kg) in the middle cerebral artery occlusion and the transient forebrain ischaemia models (Meldrum et al., 1992, 1994; Lekieffre and Meldrum, 1993). The more potent neuroprotective effect of CI977 may be due either to a more potent action on the Na^+ channel or to a combination of more than one mechanism of action. It has been reported that protective effects of the κ-receptor agonists may also be due to inhibition of Ca^{2+} entry (Conner-Kerr and Terrian, 1993; DeCoster et al., 1991; Von Voigtlander et al., 1987). Na^+ channel inactivation per se powerfully inhibits net Ca^{2+} entry during ischaemia (because the Na^+/Ca^{2+} exchanger, which is the principal mechanism for removing intracellular Ca^{2+}, depends on the normal plasmalemmal Na^+ electrochemical gradient (Meldrum et al., 1994). It has been shown that riluzole, another drug protective against ischaemic, *N*-methyl-D-aspartate-induced, and veratrine-induced neuronal damage (Wahl et al., 1993; Malgouris et al., 1994) also acts as an inhibitor of extracellular Ca^{2+} accumulation by two independent mechanisms. One of them is an inactivation of Na^+ channels (Herbert et al., 1994; Hubert et al., 1994).

Our results thus suggest that CI977 inhibits release of glutamate, aspartate and GABA by inactivation of Na^+ channels independently of its action on the opioid receptor and that this effect is relevant to its neuroprotective action.

The anticonvulsant potency of CI977 in DBA/2 mice (ED_{50} value, 9.6 mg/kg, i.p.) is much lower than that reported (Singh et al., 1990) for CI977 against *N*-methyl-DL-aspartate-induced seizures in mice (ED_{50} value of 0.17 mg/kg, i.v.), but comparable to the anticonvulsant efficacies of U-50488H and U-54494A (ED_{50} values of 10–50 mg/kg, s.c.) against a wide range of seizures in mice (Von Voigtlander et al., 1987; Fisher et al., 1993). As reported by Singh et al. (1990), the anticonvulsant effect of CI977 on *N*-methyl-DL-aspartate-induced seizures was reversed by D-serine, an agonist at the glycine site of the *N*-methyl-D-aspartate receptor. It suggests that CI977 may also act at this site, making it particularly effective against *N*-methyl-DL-aspartate-induced seizures. This mechanism of action was not however confirmed by binding studies (Singh et al., 1990).

In conclusion, this study shows that CI977, a κ-opioid receptor agonist, weakly inhibits K^+-evoked release of glutamate and aspartate via activation of this receptor, and more potently inhibits veratrine-induced release of these amino acids via a mechanism that is independent of κ-opioid receptor activation. This second mechanism of action apparently involves an effect on Na^+ channels and is probably relevant to the neuroprotective action of CI977.

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