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The effect of the desglycinyl metabolite of remacemide hydrochloride (FPL 12495AA) and dizocilpine (MK-801) on endogenous amino acid release from mouse cortex

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1 In this study the effect of FPL 12495AA, the desglycinyl metabolite of remacemide hydrochloride and dizocilpine (MK-801), on potassium- and veratridine-stimulated release of neurotransmitter amino acids from mouse cortical slices was investigated.

2 Veratridine (20 μM) and potassium (60 mM) produced a preferential release of glutamate and aspartate. Potassium-stimulated release was calcium-dependent, while veratridine-stimulated release was only partially affected by removal of calcium from the medium.

3 FPL 12495AA significantly inhibited veratridine- and potassium-stimulated release of glutamate and aspartate. Lower concentrations of FPL 12495AA were needed to inhibit veratridine-stimulated release of glutamate (12.5 μM) than potassium-stimulated release (100 μM).

4 Dizocilpine significantly inhibited veratridine- and potassium-stimulated release of glutamate and aspartate at concentrations of 100 μM and above.

5 FPL 12495AA and dizocilpine both have an affinity for the ion channel subsite of the N-methyl-D-aspartate (NMDA) receptor. The reduction of potassium-stimulated release of glutamate and aspartate by FPL 12495AA and dizocilpine is probably due to NMDA receptor blockade.

6 FPL 12495AA inhibited veratridine-stimulated release at a concentration of 12.5 μM while dizocilpine was effective only at a concentration of 100 μM. This difference in efficacy is probably due to the higher affinity of FPL 12495AA compared to dizocilpine at the veratridine-binding site on the sodium channel.

Keywords: Glutamate; aspartate; NMDA; remacemide; FPL 12495AA; dizocilpine (MK-801); epilepsy; cortical slices

Introduction

Remacemide hydrochloride is a novel anticonvulsant which is undergoing clinical trials in patients with partial seizures with, or without, secondary generalisation. Preclinical animal studies of remacemide hydrochloride have shown that it provides specific protection in mice against the maximal electroshock seizure (MES) test (Stagnitto *et al.*, 1990). This protection is of a potency (ED_{50} of remacemide hydrochloride given orally is 33 mg kg^{-1}) similar to that of phenytoin (11 mg kg^{-1}), carbamazepine (13 mg kg^{-1}) or phenobarbitone (20 mg kg^{-1}) and greater than valproate (631 mg kg^{-1}; Stagnitto *et al.*, 1990). The duration of protection is longer than with carbamazepine and valproate but shorter than phenytoin and phenobarbitone (Stagnitto *et al.*, 1990). Remacemide hydrochloride and its metabolites have also been shown to be neuroprotective in various animal models of hypoxia (Palmer *et al.*, 1993) and cerebral ischaemia (Bannan *et al.*, 1994).

Radioligand binding techniques using rat synaptic membrane fractions demonstrate that remacemide hydrochloride possesses relatively weak affinities for the glycine-sensitive and the non-competitive ion channel subsites of the N-methyl-D-aspartate (NMDA) receptor complex (Garske *et al.*, 1991). Metabolic transformation of remacemide hydrochloride in rats yields the more potent, and pharmacologically active, desglycine derivative, FPL 12495AA (Palmer *et al.*, 1993). After multiple doses of remacemide hydrochloride in human volunteers, at steady-state, the area under the curve of the desglycinyl metabolite is around one third that of the parent compound (Palmer *et al.*, 1993). The desglycinyl derivative is more potent as an anticonvulsant than remacemide hydrochloride against MES-induced seizures in rodents (ED_{50} = 17.1 and 21.5 mg kg^{-1}, respectively), NMDA-induced seizures (ED_{50} = 32.4 and 57.4 mg kg^{-1} respectively) and equally effective against 4-aminopyridine seizures (Cramer *et al.*, 1993). Remacemide hydrochloride and FPL 12495AA have been shown to limit sustained high-frequency repetitive firing of sodium-dependent action potentials in cultured mouse neurones, a property these compounds share with phenytoin, carbamazepine and lamotrigine (Harris *et al.*, 1992; Wamil *et al.*, 1992).

Dizocilpine has a greater affinity for the NMDA receptor channel sub-site than FPL 12495AA and remacemide. This is shown by the difference in the ability of dizocilpine, FPL 12495AA and remacemide to displace radiolabelled dizocilpine bound to the channel subsite of the NMDA receptor (IC_{50}s = 0.014, 0.48 and 68 μM respectively; Palmer *et al.*, 1992).

The endogenous amino acids γ-aminobutyric acid (GABA), (Macdonald & Meldrum, 1989), glutamate (Dingledine *et al.*, 1990) and aspartate (Ronne-Engstrom *et al.*, 1992) are involved in the pathogenesis of epilepsy and other neurological disorders. The excitatory amino acid glutamate, acting on NMDA or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors, has been implicated in the initiation and spread of epileptiform discharges and in the neurotoxicity following ischaemic injury to the brain (McNamara *et al.*, 1988; Park *et al.*, 1988; Chapman & Meldrum, 1993). In this study, we investigated the effect of the desglycinyl metabolite of remacemide hydrochloride (FPL 12495AA) and dizocilpine on both potassium- and veratridine-induced amino acid release from slices of mouse cerebral cortex.

Methods

Adult BALB/c mice of either sex from our colony at the University of Wales College of Medicine, weighing 25-30 g were used in this study. Animals were killed by cervical dislocation and the brain rapidly removed and placed in ice-cold,

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gassed (95% oxygen/5% carbon dioxide) artificial cerebrospinal fluid (aCSF). Coronal cortical slices (400 μm) were cut by a McIlwain tissue chopper and the cortical tissue separated from sub-cortical structures; the hippocampus was not used. Three to four cortical slices weighing 15–20 mg were positioned on a gauze disc and placed in a tissue bath (Barnes *et al.*, 1988) and perfused with gassed aCSF at 1 ml min^{-1} at 37°C and were allowed 60 min to equilibrate following slicing.

Three 2-min samples of perfusate were collected for the measurement of basal amino acid release. Neurotransmitter release was elicited with two 1-min pulses of veratridine hydrochloride (20 μM) at samples 4 and 14, or three 2-min pulses of potassium (60 mM) at samples 4, 14 and 24. FPL 12495AA or dizocilpine was perfused between samples 7 to 17. All samples were collected on ice and frozen immediately. Slices were weighed and amino acid release was expressed in pmol mg^{-1} 2 min^{-1}. The mean amino acid release in the two consecutive samples collected during and after the pulse of potassium or veratridine was used to obtain stimulated release.

The amino acids were assayed by high performance liquid chromatography following pre-column derivatization with *o*-pthaldialdehyde (Fluka Chemicals and Biochemicals) and the resulting fluorescence measured (LDC analytical). The mobile phase consisted of a linear gradient between sodium acetate/tetrahydrofuran and methanol (Fisons); a 25 cm long reverse-phase C_{18} column (Jones chromatography) was used to separate the amino acids. Two hundred μl of perfusate was added to 200 μl of homoserine (internal standard) and vortex mixed. Following centrifugation 200 μl were taken and mixed with 50 μl of *o*-pthaldialdehyde; 100 μl of this mixture was then injected on to the column and the resulting fluorescence measured. The amino acids aspartate, glutamate, glycine, taurine and GABA were assayed.

Composition of aCSF in mM: NaCl 124, KCl 5, NaH_2PO_4 1.25, $CaCl_2$, $MgSO_4$ 2, $NaHCO_3$ 26, D-glucose 10 and pH 7.4. For 60 mM potassium-containing aCSF a corresponding decrease in NaCl and for calcium-free aCSF an increase in NaCl was made to maintain osmolarity.

FPL 12495AA and dizocilpine were freely soluble in aCSF at the concentrations used in the study.

Drugs

The following drugs were used: FPL 12495AA (±)-1-methyl-1,2-diphenylethylamine; Fisons plc), veratridine (Sigma) and dizocilpine (Research Biochemicals International).

Statistical analysis

Results were calculated as a percentage change from the first pulse and expressed as mean ± s.e.mean. Student's *t* test for unpaired data was used to calculate the significance level for the calcium dependency. One way ANOVA followed by the Student-Neuman-Keuls test was used to calculate significance levels for drug effects.

Results

Effect of potassium (60 mM) and veratridine (20 μM) on endogenous amino acid release

Potassium (60 mM) was perfused as three 2-min pulses at samples 4, 14, 24. The first pulse stimulated a significant release of glutamate (8 fold of basal release, Figure 1a) and aspartate (4 fold, Figure 1c). The release of the other amino acids was not significantly altered. The second and third pulses of

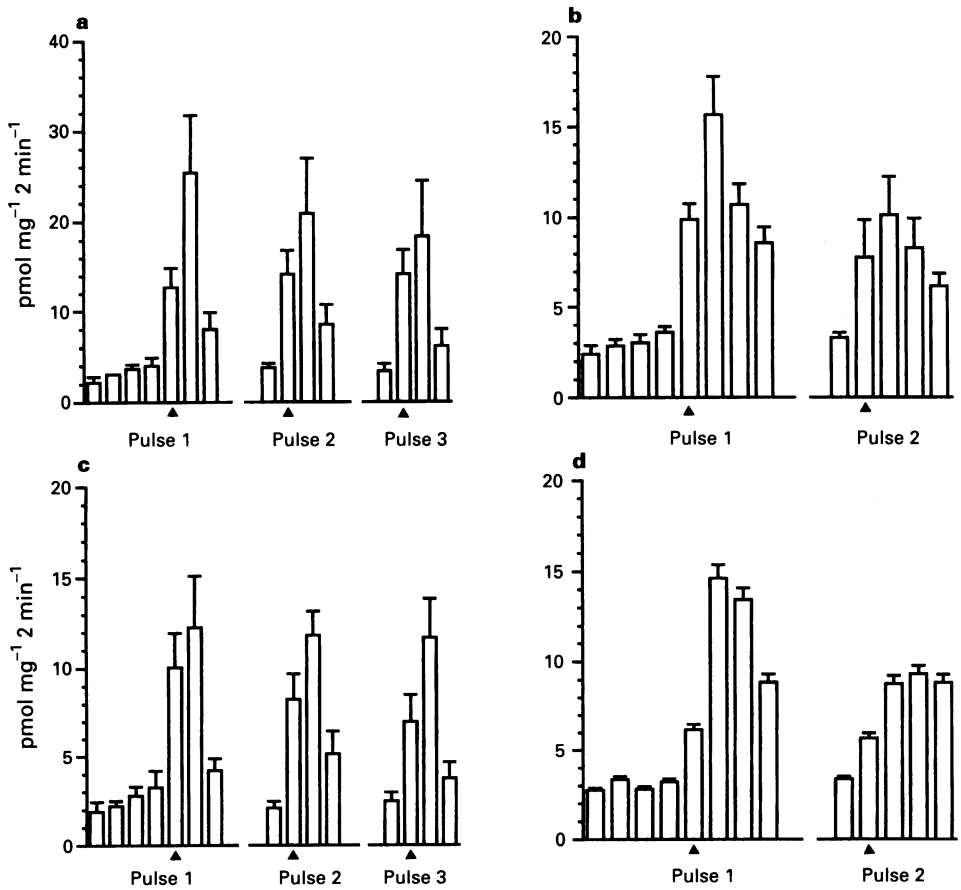

Figure 1 Release of glutamate (a and b) and aspartate (c and d) to three 2-min pulses of potassium (60 mM, a and c, ▲) and two 1-min pulses of veratridine (20 μM, b and d, ▲). The results are expressed as the mean ± s.e.mean in pmol mg^{-1} tissue 2 min^{-1}; $n = 6-9$. The interval between the potassium or the veratridine pulses was 20 min.

potassium produced an average release of amino acids of between 80 and 90% of the first pulse.

Veratridine was perfused as two 1-min pulses at samples 4 and 14. The first pulse of veratridine (20 μM) produced an increase in the release of the amino acids with a preferential release of glutamate (6 fold of basal release, Figure 1b) and aspartate (about 3 fold, Figure 1d). The release of the other amino acids was only marginally affected by veratridine. The second pulse of veratridine produced an average release of amino acids of between 60 and 90% of the first pulse.

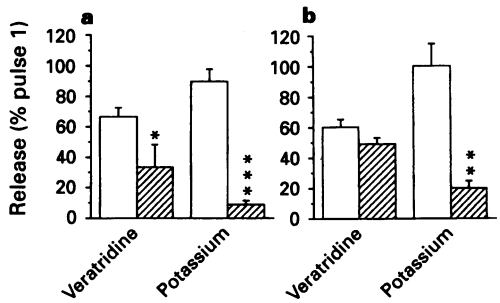

Figure 2 Veratridine (20 μM)- and potassium (60 mM)-induced release of glutamate (a) and aspartate (b) in normal (open columns) and calcium-free (hatched columns) aCSF. The results are expressed as the mean ($\pm$s.e.mean) stimulated release in the second pulse as a percentage of the first pulse; $n=4-9$. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

Potassium-stimulated release of glutamate (Figure 2a) and aspartate (Figure 2b) was calcium-dependent as shown by the decreased release in calcium-free aCSF, while veratridine-stimulated release (Figure 2a and b) was not as markedly affected by the removal of calcium from the aCSF.

Effect of FPL 12495AA and dizocilpine (MK-801) on the release of endogenous amino acids

There was no evidence that FPL 12495AA and dizocilpine in concentrations up to 200 μM had any significant effect on the basal release of amino acids.

Veratridine-stimulated release

The second pulse of veratridine produced an average release of glutamate of 67$\pm$6% of the first pulse. This release was significantly reduced by FPL 12495AA at concentrations of 12.5 μM (39.3$\pm$7.9%, $P<0.05$), 25 μM (30.9$\pm$9.4%, $P<0.01$), 50 μM (29.4$\pm$6.0%, $P<0.001$), 100 μM (26.6$\pm$6.2%, $P<0.001$) and 200 μM (23.7$\pm$6.7%, $P<0.01$; Figure 3a). Dizocilpine inhibited this release significantly at concentrations of 100 μM (36.1$\pm$2.7%, $P<0.01$) and 200 μM (36.0$\pm$7.8%, $P<0.05$, Figure 3b). The second pulse of veratridine produced an average release of aspartate of 60$\pm$5% and this release was similarly significantly reduced by FPL 12495AA at concentrations of 25 μM (30.3$\pm$4.3%, $P<0.001$), 50 μM (35.7$\pm$2.1%, $P<0.001$), 100 μM (35.6$\pm$2.8%, $P<0.001$) and 200 μM (28.5$\pm$7.1%, $P<0.001$; Figure 3c). Dizocilpine significantly inhibited aspartate release at concentrations of

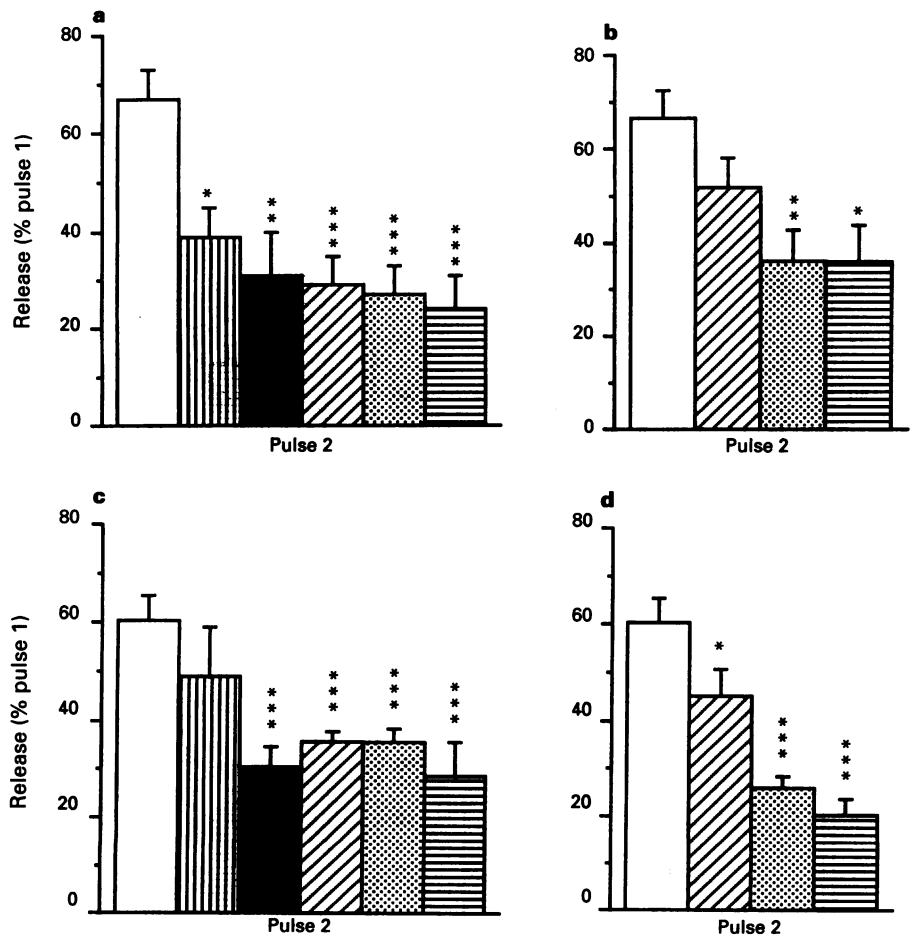

Figure 3 The effect of FPL 12495AA (a and c) and dizocilpine (b and d) on veratridine-induced (20 μM) release of glutamate (a and b) and aspartate (c and d). The results are expressed as the mean ($\pm$s.e.mean) stimulated release in the second pulse as a percentage of the first pulse. Control (open columns); effect of either FPL 12495AA or dizocilpine 12.5 μM (vertically lined columns); 25 μM (solid columns); 50 μM (hatched columns); 100 μM (stippled columns); 200 μM (horizontally lined columns). $n=4-9$; $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

50 μM (45.2±5.5%, $P<0.05$), 100 μM (25.9±2.4%, $P<0.001$) and 200 μM (20.3±3.3%, $P<0.001$, Figure 3d). The veratridine-stimulated release of the other amino acids was not significantly affected by FPL 12495AA and dizocilpine in concentrations up to 200 μM.

Potassium-stimulated release

The second and third pulses of potassium produced an average release of glutamate when compared to the first pulse of 90±7.9% and 89±7.8% respectively. This release of glutamate was significantly reduced by FPL 12495AA at concentrations of 100 μM (41.9±4%, $P<0.001$ and 55±5.1%, $P<0.01$) and 200 μM (26.7±4.8%, $P<0.001$ and 25.7±4%, $P<0.001$) respectively (Figure 4a). Dizocilpine similarly reduced the release produced by the second pulse of potassium at concentrations of 100 μM (63.4±7.9%, $P<0.05$) and 200 μM (50.2±6.5%, $P<0.01$, Figure 4b). However, unlike FPL 12495AA, dizocilpine had no significant effect on the glutamate released by the third pulse of potassium.

The second pulse of potassium produced an average release of aspartate of 100±14.4% of the first pulse. This release was significantly reduced by FPL 12495AA at 100 μM (31.7±7.7%, $P<0.001$) and 200 μM (36±9.6%, $P<0.01$) respectively (Figure 4c). The aspartate released by the second pulse of potassium was reduced by dizocilpine at concentrations of 100 μM (60.2±10.1%, $P<0.05$) and 200 μM (43.5±2.6%, $P<0.01$, Figure 4d). The aspartate released by the third pulse of potassium was not significantly affected by dizocilpine and FPL 12495AA. Potassium-stimulated release of the other amino acids was not significantly affected by FPL 12495AA and dizocilpine in concentrations up to 200 μM.

Discussion

This study showed that the desglycinyl metabolite of remacemide hydrochloride, FPL 12495AA, and dizocilpine, significantly reduced both veratridine- and potassium-stimulated release of glutamate and aspartate. FPL 12495AA reduced veratridine-induced release of glutamate at lower concentrations (12.5 μM) than those needed by dizocilpine to inhibit release (100 μM). Against potassium-stimulated release of glutamate and aspartate, both FPL 12495AA and dizocilpine were effective only in concentrations equal to, or above, 100 μM.

Radioligand binding studies, using rat synaptic membrane fractions, have shown that remacemide hydrochloride possesses relatively weak affinities for the glycine-sensitive and phencyclidine (PCP) ion channel subsites of the NMDA receptor complex (Garske *et al.*, 1991). The desglycinyl metabolite is more potent than remacemide hydrochloride in displacing dizocilpine binding from the channel subsite ($IC_{50}=0.48$ and 68 μM respectively; Palmer *et al.*, 1992). Compounds with an affinity for the channel sub-site of the NMDA receptor such as dizocilpine (Wong *et al.*, 1986; Olney *et al.*, 1987) and dextromethorphan (Wong *et al.*, 1988) have been shown to have anticonvulsant properties in animal studies. It is likely that the anticonvulsant efficacy of remacemide hydrochloride and its metabolites is secondary to non-competitive binding to the channel subsite of the NMDA receptor, blocking the channel and preventing the activation of the receptor.

The mechanism by which an increase in extracellular potassium stimulates release of excitatory amino acids is secondary to changes in the transmembrane potential resulting in the opening of voltage-sensitive calcium channels. The release of neurotransmitters in response to potassium does not involve sodium channels as tetrodotoxin, a potent sodium channel blocker, has been shown to be ineffective in inhibiting this

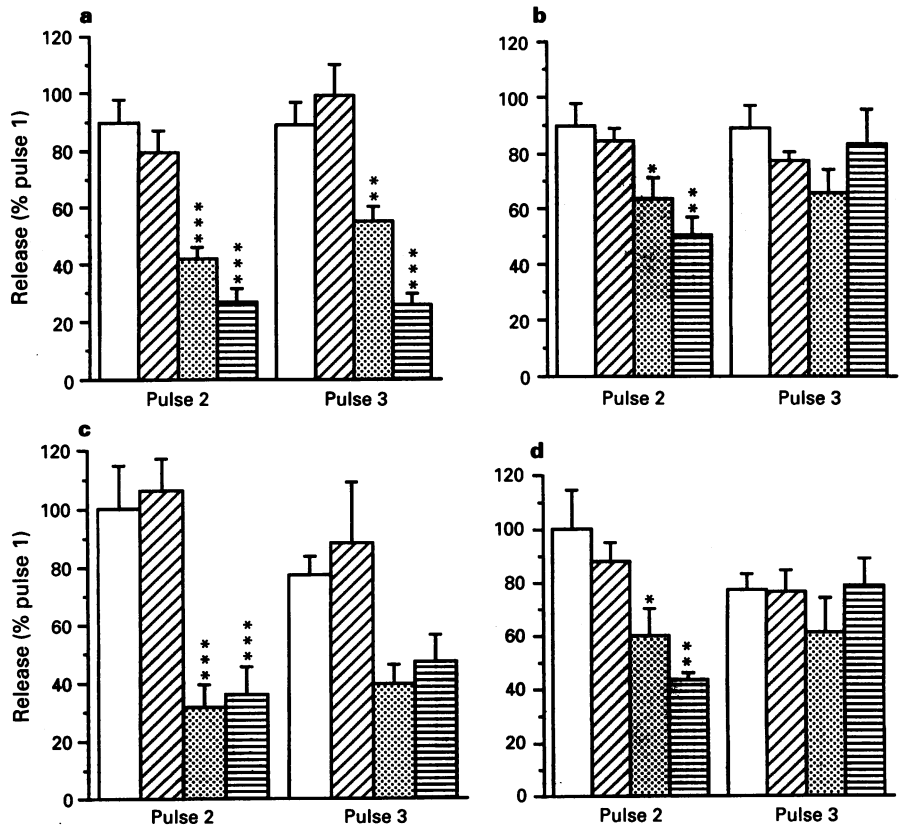

Figure 4 The effect of FPL 12495AA (a and c) and dizocilpine (b and d) on potassium-induced (60 mM) release of glutamate (a and b) and aspartate (c and d). The results are expressed as the mean (±s.e.mean) stimulated release in the second and third pulses as a percentage of the first pulse. Control (open columns); effect of FPL 12495AA or dizocilpine 50 μM (hatched columns); 100 μM (stippled columns); 200 μM (horizontally lined columns). $n=4-9$; $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

release (Dickie & Davies, 1992). The inhibition of potassium-stimulated release of glutamate and aspartate by FPL 12495AA and dizocilpine is probably as a result of blockade of the NMDA receptor.

Veratridine releases neurotransmitters by preventing the inactivation of sodium channels; tetrodotoxin, which blocks sodium channels, prevents this release (Levi *et al.*, 1980; Minchin, 1980). Drugs such as lamotrigine, which inhibit veratridine-stimulated release of excitatory amino acids but have no effect on potassium-stimulated release, are therefore thought to act by maintaining the inactivation of sodium channels (Leach *et al.*, 1986). The inhibitory effect of FPL 12495AA and dizocilpine on veratridine-stimulated release of glutamate and aspartate is probably due to both sodium channel and NMDA receptor blockade. In studies on sustained repetitive-firing in cultured neurones which involves the activation of voltage-operated sodium channels, both dizocilpine (IC_{50} 80 nM, Wamil & McClean, 1992) and FPL 12495AA (IC_{50} 600 nM, Wamil *et al.*, 1992) reduced firing. This dual action at both sodium channels and NMDA receptors may explain why FPL 12495AA was more effective in reducing veratridine-stimulated release compared to potassium-stimulated release.

Why should drugs which bind to the PCP subsite of the NMDA receptor inhibit glutamate release from the presynaptic terminal? There are two possible explanations for this inhibition. The cerebral cortex contains a large number of glutamatergic and aspartatergic neurones (which form association and commissural fibres) and these neurones probably account for the majority of the glutamate and aspartate released from the cortical slice preparation. Perfusion of cortical slices with high extracellular potassium- or veratridine-containing aCSF results in release of glutamate and aspartate from these neurones. The dendrites of these neurones possess NMDA receptors (Huntley *et al.*, 1994) and if activation of these receptors is blocked, as is probably the case by FPL 12495AA and dizocilpine, then augmentation of glutamate and aspartate release by NMDA receptor stimulation will not occur. Conversely, NMDA has been shown to stimulate glutamate release from mouse cortical slices (Rowley *et al.*, 1993). The other possibility is that activation of the NMDA receptor by the endogenous ligand initiates a positive feedback loop occurring at the synaptic level which acts on the pre-synaptic terminal increasing the release of transmitter. There are various modulators which have been shown to be involved in this positive feedback response including nitric oxide (Rowley *et al.*, 1993; Montague *et al.*, 1994) and arachidonic acid (Dickie *et al.*, 1994). Drugs which block the NMDA channel sub-site may prevent this positive feedback resulting in reduced neurotransmitter release.

The inhibitory effect of the desglycinyl metabolite on glutamate and aspartate release, demonstrated here, may be important to the anticonvulsant effect of remacemide hydrochloride.

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