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Excitatory amino acid antagonists and endogenous aspartate and glutamate release from rat hippocampal slices

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1 The effect of excitatory amino acid agonists and antagonists on the efflux of endogenous aspartate and glutamate from the rat hippocampus *in vitro* was studied.

2 None of the compounds tested had any effect on the basal efflux of endogenous aspartate and glutamate.

3 2-Amino-5-phosphonovaleric acid (APV), 2-amino-7-phosphonoheptanoic acid (APH) and MK-801 all reduced the potassium-evoked efflux of aspartate and glutamate by between 14.9% and 34.3% ($P < 0.05$).

4 The depression of efflux brought about by APV was still observed in the presence of tetrodotoxin.

5 Neither N-methyl-D,L-aspartate nor quinolinic acid had any effect on the potassium-evoked efflux of aspartate and glutamate.

6 These results imply the existence of presynaptic amino acid receptors that are capable of modulating the efflux of endogenous aspartate and glutamate.

Introduction

Some compounds with antagonist activity at the N-methylaspartate (NMA) class of excitatory amino acid receptor are potent inhibitors of epileptiform phenomena and seizures in a number of animal models (Meldrum, 1987). Although this anticonvulsant activity is usually explained by the antagonism of postsynaptic NMA receptors, few attempts have been made to assess the possible contribution of receptors located on presynaptic terminals. McBean & Roberts (1981) have previously described the modulation of [^{3}H]-D-aspartate release from a rat hippocampal prism preparation. Although a range of glutamate analogues were active in inhibiting the potassium-evoked release of [^{3}H]-D-aspartate in this system, NMA was devoid of activity. However, the NMA specific antagonist 2-amino-5-phosphonovaleric acid (APV) produced a marked inhibition of the potassium-evoked [^{3}H]-D-aspartate release. These effects were tetrodotoxin (TTX)-insensitive. Subsequently Bowker *et al.* (1986) confirmed this observation and suggested an involvement of presynaptic NMA receptors.

In a study of rat olfactory cortex, Collins *et al.* (1983) examined the release of endogenous aspartate and glutamate. N-methyl-D-aspartate (NMDA) was able to reduce significantly the potassium-evoked release of aspartate, without affecting the release of glutamate. These authors also suggested that this effect was mediated by the activation of presynaptic NMDA receptors, but this is clearly difficult to reconcile with the observations just quoted that APV also inhibited [^{3}H]-D-aspartate release. Since the use of radiolabelled probes has been found to produce results different from those in studies of endogenous compounds in a number of recent investigations of transmitter release (Ferkany & Coyle, 1983; Herdon *et al.*, 1985), we here examine the effects of APV and other amino acid antagonists on the net efflux of endogenous aspartate and glutamate.

Methods

Male Wistar rats were decapitated and the brain quickly removed into ice cold Krebs-bicarbonate solution (composition, mM): KH_2PO_4 2.2, $MgSO_4$ 1.2, KCl 2.0, glucose 10.0, $NaHCO_3$ 25.0, NaCl 115.0, $CaCl_2$ 2.5.

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The cerebellum was separated by means of a coronal cut at the rostral end of the cerebellum. Next, a sagittal cut was made to separate the two hemispheres. The cortex of each hemisphere was reflected by means of two fine spatulae, and the hippocampus dissected out.

The hippocampus was then transferred to a McIlwain tissue chopper and placed on a filter paper moistened with Krebs-bicarbonate medium. Transverse slices were then prepared (400 μm) and the slices incubated for 30 min in 100 ml of medium gassed with 95% O_2/5% CO_2 at 37°C in order to allow recovery and to achieve a stable level of amino acid efflux. Release of aspartate and glutamate was determined by placing single slices in vials containing 1 ml oxygenated buffer for 15 min at 37°C. This system was preferred to a perfusion system because it yielded sufficient concentrations of aspartate and glutamate for immediate analysis by high performance liquid chromatography (h.p.l.c.) without further concentration. In addition it provides a model of the events following the focal administration of drugs into brain tissue, since many of these compounds are taken up only poorly by nervous tissue, and may therefore exert both a powerful and prolonged releasing action and an inhibition of uptake of neuroactive substances. Thus this system monitors the net efflux of aspartate and glutamate following such a stimulus.

Depolarization was produced by incubation with the compound of interest or with a submaximal stimulation by 44 mM KCl or a combination of both. Krebs-bicarbonate buffer containing elevated potassium had an equivalent reduction of sodium to maintain isotonicity.

The calcium dependency of aspartate and glutamate efflux was investigated by incubating slices as above, the calcium ions having been replaced by 1 mM ethyleneglycol-*bis*(β-amino ethyl ether)N,N'-tetraacetic acid (EGTA). The effect of magnesium ions on the stimulated release was investigated by omission of this ion from the medium. Aliquots of medium were removed after 15 min and frozen at −20°C. Tissue slices were homogenized and the protein concentration measured by the method of Lowry *et al.* (1951).

Analysis

Aliquots (100 μl) of each sample were rapidly mixed with 10 μl of an *o*-phthalaldehyde/2-mercaptoethanol reagent and allowed to stand for 30 s before injection onto the chromatographic column. A high performance liquid chromatograph (h.p.l.c.) (Gilson), consisting of two model 302 pumps, a model 702 gradient programmer and a Rheodyne model 7125 injector (20 μl loop) was used. A Spectra-Glo fluorimeter (Gilson) was used to detect components in the column eluate; excitation wavelength, 390 nm; emission cut off filter, 475 nm, at maximum sensitivity. Separation was performed on a reverse phase 'μ-Bondapak' C_{18} analytical column (Waters) fitted with a C_{18} 'guard pack' precolumn (Waters).

Acetonitrile and methanol were h.p.l.c. grade solvents (Rathburn Chemicals Ltd.). Phosphoric acid and anhydrous disodium hydrogen phosphate were analar grade (BDH Chemicals).

Amino acid standard and *o*-phthalaldehyde solutions were purchased from Sigma, (5 μl of 2-mercaptoethanol was added to the *o*-phthalaldehyde reagent every three days after opening).

Solvent A: anhydrous disodium hydrogen phosphate; 3.55 g was dissolved in 400 ml of reagent grade water and the pH adjusted to 7.2 with concentrated H_3PO_4. The volume was brought to 500 ml to give a 0.05 M solution.

Each day 125 ml of this solution was diluted to 460 ml with water, made up to 500 ml with acetonitrile and then filtered through a 0.45 μm durapore filter (Millipore) under vacuum. The resulting solution therefore contained 0.0125 M Na_2HPO_4 and 8% acetonitrile.

Solvent B: water (400 ml), acetonitrile (300 ml) and methanol (300 ml) were premixed and filtered through a 0.45 μm durapore filter (Millipore) under vacuum.

Amino acid derivatives were identified by their retention times relative to reference injections of standard aspartate and glutamate injected every 10 samples. The amino acid concentrations were quantified by comparing the peak heights to those obtained in the reference injections. This was found to be a reliable method for the quantification of the amino acids (Connick, 1987).

The gradient programme, expressed as time in min from injection (% solvent B) was; 0(0), 10(0), 30(100). The flow rate was 1.5 ml per min at room temperature.

Statistics

Throughout this study statistical difference has been assessed relative to control conditions by use of a paired Student's *t* test. Levels of significance are indicated by the following: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Results

The system allowed the resolution of aspartate, glutamate, serine, glutamine, glycine, alanine, GABA and taurine. As we have shown previously, the release of glutamate and aspartate by 44 mM K^+, 0.5

Table 1 Summary of effects of excitatory amino acid agonists and antagonists on basal efflux of endogenous aspartate and glutamate *in vitro*

	Control		*Experimental*		
Compound	*Aspartate*	*Glutamate*	*Aspartate*	*Glutamate*	n
Quinolinic acid:					
5 mM ($-Mg^{2+}$)	100.0 ± 10.9	100.0 ± 5.9	112.3 ± 12.3	106.1 ± 4.0	6
5 mM (+44 mM KCl)	100.0 ± 10.8	100.0 ± 15.8	96.3 ± 11.2	93.5 ± 8.2	6
NMDLA:					
1 mM ($-Mg^{2+}$)	100.0 ± 8.7	100.0 ± 5.4	96.4 ± 12.3	115.0 ± 6.5	10
1 mM (+44 mM KCl)	100.0 ± 11.0	100.0 ± 9.6	95.9 ± 3.4	98.7 ± 11.5	10
Kynurenic acid:					
1 mM			87.8 ± 10.3	N.D.	8
APV:					
200 μM			109.0 ± 8.3	112.9 ± 7.3	8
APH:	100.0 ± 6.1	100.0 ± 7.0			8
200 μM			115.2 ± 10.5	118.8 ± 12.8	8
MK801:					
10 μM			103.0 ± 5.8	114.1 ± 11.3	8
100 μM			106.1 ± 5.7	116.5 ± 16.1	8

Amino acid efflux was determined as described in the methods. Results are as mean % of the control efflux (either basal or potassium stimulated) ± s.e. mean from (n) experiments conducted in triplicate. Typical levels of aspartate and glutamate released during resting conditions were 33.0 ± 4.6 (pmol mg^{-1} protein $15 min^{-1}$) ($n = 6$) and 138.0 ± 15.8 (pmol mg^{-1} protein $15 min^{-1}$) ($n = 6$) respectively.
NMDLA = N-methyl-D,L-aspartate; APV = 2-amino-5-phosphonovaleric acid; APH = 2-amino-7-phosphonoheptanoic acid.

or 5 mM kainate was dependent on the presence of calcium ions in the medium. Basal efflux was unchanged in the absence of calcium but K^+-evoked release of glutamate was only 46.6% ± 4.6% ($n = 6$) ($P < 0.05$) that of the release when Ca^{2+} was present, and the release of aspartate only 62.2% ± 10.8% ($n = 6$) ($P < 0.05$) that of release in the presence of calcium (data not shown). Neither quinolinic acid nor NMDLA was able to modify either the basal or potassium-evoked release of endogenous glutamate or aspartate, even in the absence of magnesium ions (Table 1) which are known to attenuate NMDLA receptor mediated mechanisms (Evans *et al.*, 1977; Lehmann *et al.*, 1983).

Whilst agonists acting via the NMDLA receptor class had no effect on amino acid release *in vitro* (Table 1), NMDLA antagonists caused a small, though significant reduction of the potassium-evoked release of aspartate and glutamate (Figure 1).

None of the NMDLA antagonists tested had any effect on the basal efflux of aspartate or glutamate (Table 1).

MK801 10 μM ((+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate) failed to reduce significantly the potassium-evoked efflux of either aspartate or glutamate, whilst 100 μM MK801 inhibited the efflux of aspartate and glutamate by 25.7% ± 10.9% and 34.3% ± 16.0% respectively (Figure 1).

2-Amino-7-phosphonoheptanoic acid (APH) at 200 μM again reduced the potassium stimulated efflux of aspartate and glutamate by 14.9% ± 4.7% and 19.1% ± 5.7% and 200 μM APV produced a similar inhibition; by 16.3% ± 6.4% and 21.1% ± 5.8% respectively.

The nonspecific amino acid antagonist, kynurenic acid, failed to affect either the basal or potassium-evoked efflux of aspartate. Unfortunately the release of glutamate could not be determined due to the simultaneous elution of glutamate and kynurenic acid itself from the h.p.l.c. column.

The reduction of evoked release produced by APV could still be demonstrated in the presence of the sodium channel blocker TTX. TTX itself reduced the 44 mM KCl-evoked efflux of aspartate and glutamate (Figure 2). The inhibition of the evoked efflux of aspartate and glutamate by APV was 16.1% and 21.1% respectively of control in the absence of TTX and 17.7% ± 3.1% and 20.3% ± 5.3% respectively of the TTX containing solution.

Discussion

In this study the depressant action of APV previously described against the evoked release of [^{3}H]-D-aspartate (McBean & Roberts, 1981; Bowker *et al.*, 1986) has been confirmed against the evoked efflux of endogenous aspartate and glutamate. In

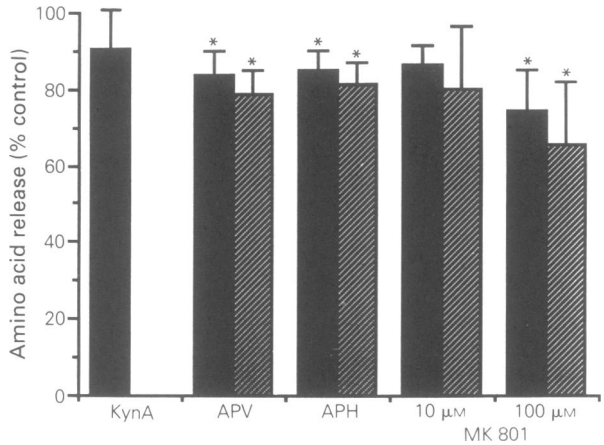

Figure 1 The effect of amino acid antagonists on the evoked efflux of endogenous amino acids from rat hippocampal slices. Amino acid efflux was determined as described in the text, both in 44 mM KCl Krebs-bicarbonate medium containing 1 mM kynurenic acid (Kyn A), 200 μM(±)-D,L,2-amino-5-phosphonovaleric acid (APV), 200 μM(±)-D,L,2-amino-7-phosphonoheptanoic acid (APH), 10 μM MK801 and 100 μM MK801. Results are mean % evoked efflux of aspartate (solid column) and glutamate (hatched columns) from between 8 and 10 experiments conducted in triplicate; s.e. mean shown by vertical lines.

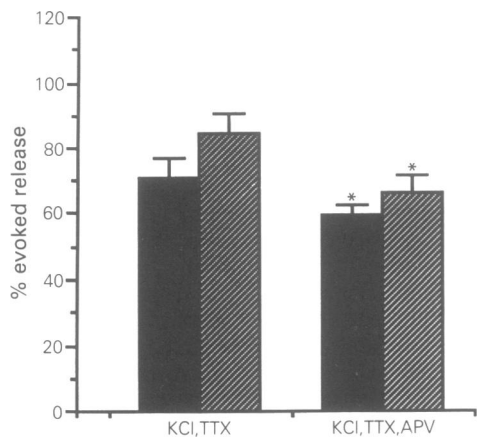

Figure 2 The effect of tetrodotoxin (TTX) on the reduction of the evoked efflux of endogenous aspartate and glutamate produced by 2-amino-5-phosphonovaleric acid (APV) in rat hippocampal slices. Amino acid efflux was determined as described in the text both in 44 mM KCl Krebs-bicarbonate medium and 44 mM KCl Krebs-bicarbonate medium containing 0.5 μM TTX and 44 mM KCl Krebs-bicarbonate medium containing 0.5 μM TTX in addition to 200 μM(±)-D,L,2-amino-5-phosphonovaleric acid. Results are mean % evoked efflux of aspartate and glutamate from 8 experiments conducted in triplicate. Statistical difference between the release in the presence of K^+, TTX and APV compared with that which occurred in the presence of K^+ and TTX: * $P < 0.05$.

addition, this effect has been demonstrated in the presence of the sodium channel blocker TTX, indicating that a presynaptic receptor, or at least a process not dependent upon axonal conduction, is involved in this modulation of release (this term is used reservedly, since there are no data regarding the effects of APV, APH etc. on the uptake systems for acidic amino acids). In view of the specific NMDLA antagonist activity of APV, APH and MK801 (Stone & Burton, 1988), these results imply that a presynaptic NMDLA receptor is responsible for the mediation of the depression of evoked amino acid efflux. However, in view of the high concentrations of the antagonists required to produce these effects, when compared with electrophysiological studies, the possibility exists that these compounds are acting non-specifically at both NMDLA and other receptors. Indeed it is possible that at such concentrations, the NMDLA antagonists such as APV, which normally have little efficacy against glutamate responses act to prevent the excitant effect of the endogenous glutamate and aspartate released by the potassium depolarization. It is difficult to reconcile the effect of NMDLA antagonists on amino acid release with the result of Collins *et al.* (1983), who reported that NMDA itself could depress the evoked release of endogenous aspartate in slices of rat olfactory cortex. Neither NMDLA nor quinolinate had any effect on the potassium-evoked amino acid efflux in this study, and similarly NMDA was ineffective in modulating the potassium-evoked release of [^{3}H]-D-

aspartate from the hippocampal prism preparation (McBean & Roberts, 1981). It is therefore clear that responses found in the olfactory cortex are very different from those of the hippocampus, whilst the latter tissue shows similar effects with a variety of approaches.

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References

BOWKER, H.M., CHAPMAN, A.G. & MELDRUM, B.A. (1986). The action of ω-phosphono-2-amino carboxylic acids on release of [^{3}H]-D-aspartate from hippocampal mini-slices. *Br. J. Pharmacol.*, **88**, 328P.

COLLINS, G.G.S., ANSON, J. & SURTEES, L. (1983). Presynaptic kainate and N-methyl-D-aspartate receptors regulate excitatory amino acid release in the olfactory cortex. *Brain Res.*, **265**, 157–159.

CONNICK, J.H. (1987). The neurochemistry of quinolinic acid and related compounds in the central nervous system. *University of London Ph.D. Thesis.*

CONNICK, J.H. & STONE, T.W. (1986). The effect of kainic, quinolinic and beta-kainic acids on the release of endogenous aspartate and glutamate from rat brain slices. *Biochem. Pharmacol.*, **35**, 3631–3635.

EVANS, R.H., FRANCIS, A.A. & WATKINS, J.C. (1977). Selective antagonism by Mg^{2+} of amino acid-induced depolarisation of spinal motorneurones. *Experientia*, **33**, 489–491.

FERKANY, J.W. & COYLE, J.T. (1983). Evoked release of aspartate and glutamate: disparities between prelabelling and direct measurement. *Brain Res.*, **278**, 279–282.

HERDON, H., STUMPISH, J. & NAHORSKI, S.R. (1985). Differences between the release of radiolabelled and endogenous dopamine from superfused rat brain slices: effect of depolarisation stimulation, amphetamine and synthesis inhibitors. *Brain Res.*, **348**, 309–320.

LEHMANN, J., SCHAFFER, P., FERKANY, J.W. & COYLE, J.T. (1983). Quinolinic acid evoked [^{3}H]acetylcholine release in striatal slices: mediation by NMDA-type excitatory amino acid receptors, *Eur. J. Pharmacol.*, **96**, 111–115.

LOWRY, O.H., ROSENBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin-phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

McBEAN, G. & ROBERTS, P.J. (1981). Glutamate preferring receptors regulate the release of D-[^{3}H]-aspartate from rat hippocampal slices. *Nature*, **291**, 593–594.

MELDRUM, B.S. (1987). Excitatory aspartate and glutamate and epilepsy. In *Excitatory Amino Acid Transmission.* ed. Hicks, T.P., Lodge, D. & McLennan, H. Alan R. pp. 189–197. New York: Liss Inc.

STONE, T.W. & BURTON, N.R. (1988). NMDA receptors and endogenous ligands in the vertebrate CNS. *Progr. Neurobiol.* **30**, 333–368.

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