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Effects of kainic acid analogues on the high affinity uptake of D-[^{3}H] aspartate into rat cerebellar homogenates

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Glutamate and aspartate are considered to serve as excitatory neurotransmitters in the mammalian central nervous system [1]. In common with many transmitters, their physiological actions are thought to be terminated by transport into presynaptic nerve terminals and glia, by a Na^+-dependent high affinity uptake mechanism [2]. Interference with this inactivation process will potentiate the depolarizing actions of these substances [3], and also will reveal their potential excitotoxic properties [4].

A number of closely related excitatory analogues of glutamate and aspartate are effective inhibitors of this uptake system [5]. D-Aspartate for example, is transported equally well as L-glutamate or L-aspartate, and provides a useful non-metabolizable alternative substrate for investigating uptake mechanisms [6].

The availability of glutamate/aspartate uptake inhibitors (lacking overt direct depolarizing actions) is important for investigating the physiological role of excitatory amino acid transport in nervous tissue. The potent excitant and excitotoxin α-kainic acid is a weak inhibitor of high affinity L-glutamate uptake; however, much more potent in this respect is its reduced congener dihydrokainate, which lacks direct excitatory actions at post-synaptic receptors [7].

As part of a study of potential excitatory amino acid receptor antagonists, we have synthesized and examined a number of novel kainic acid analogues [8, 9]. We report here on the ability of these substances to influence the high affinity uptake of D-[^{3}H]aspartate into rat cerebellar crude synaptosomal preparations.

Materials and methods

Preparation of crude cerebellar synaptosomes. Male adult Wistar rats (300 g) were killed by decapitation, and their cerebella excised rapidly. Following removal of the pial meninges, the tissues were homogenized in 10 vol. (w/v) 0.32 M sucrose, with a Teflon-glass homogenizer (0.25 mm clearance), and then diluted to 50 vol. with 0.32 M sucrose, to dilute endogenous amino acids. Following centrifugation at 1000 *g* for 10 min, the supernatant was removed and centrifuged at 17,000 *g* for a further 20 min. The synaptosome-enriched P_2 pellet obtained was resuspended in 10 vol. of 0.32 M sucrose for the assay.

Uptake assay. Assays were performed in 5 ml Teflon centrifuge tubes containing 2 ml Krebs bicarbonate buffer pH 7.4 (NaCl 114 mM; KCl 5 mM; KH_2PO_4 1.2 mM; $MgSO_4$ 1.2 mM; $CaCl_2$ 2.6 mM; $NaHCO_3$ 25 mM; glucose 11.7 mM) together with 20 μl D-[^{3}H]aspartate (10 nM final concentration, sp. act. 22 Ci/mmol, Amersham International, U.K.), gassed with 95% O_2–5% CO_2 and preincubated at 37° for 2 min. Non-specific uptake was defined in a parallel set of tubes where sodium was replaced by an equimolar concentration of choline. The incubations were initiated by addition of 20 μl of the P_2 preparation (equivalent to approximately 50–60 μg protein per assay tube), continued for 3 min at 37° and were terminated by transference onto ice. The tubes were then spun at 16,000 *g* for 10 min, the pellets washed several times with warm buffer (37°) and the tissue was solubilized with 300 μl of 2% SDS overnight. Following the addition of 3 ml scintillant (LKB optiphase 'RIA'), radioactivity was determined by liquid scintillation counting. High affinity uptake was determined by subtraction of the values obtained in the absence of Na^+ from those obtained in the presence of Na^+.

Protein was determined by the method of Lowry *et al.* [10] using bovine serum albumin (Sigma) as standard.

All common analytical reagents were obtained from Sigma UK or from B.D.H. Chemicals, Poole, Dorset, U.K.

Results and discussion

High affinity D-[^{3}H]aspartate uptake into adult rat cerebellar synaptosomes was rapid and showed an absolute dependence on Na^+ (data not shown). Uptake was saturable (Fig. 1a) with a K_m of 10 ± 1.5 μM and a V_{max} of 6.25 ± 0.94 nmol/mg protein/3 min (Fig 1b).

A number of kainic acid analogues were tested for their ability to influence this high affinity uptake (Fig. 2). α-Dihydrokainate was the most potent of the analogues tested with an IC_{50} of approximately 110 μM, whilst α-kainate was slightly weaker with an IC_{50} of about 350 μM (Table 1). The other kainate analogues tested were substantially less potent, and several, *viz.* α-homokainate, β-homokainate, α-alloketokainate, β-alloketokainate, α-ketokainate, β-ketokainate, α-kainyl glycine, β-kainyl glycine, α-kainyl-aminomethylphosphonate, β-kainyl aminomethylphosphonate and α-carboxykainate were all totally ineffective in influencing high affinity D-[^{3}H]aspartate uptake into adult rat cerebellar synaptosomes at concentrations up to 1.5 mM.

As reported previously [7], both α-kainate and, more potently, α-dihydrokainate were found to inhibit the high affinity uptake of D-[^{3}H]aspartate into rat brain tissues.

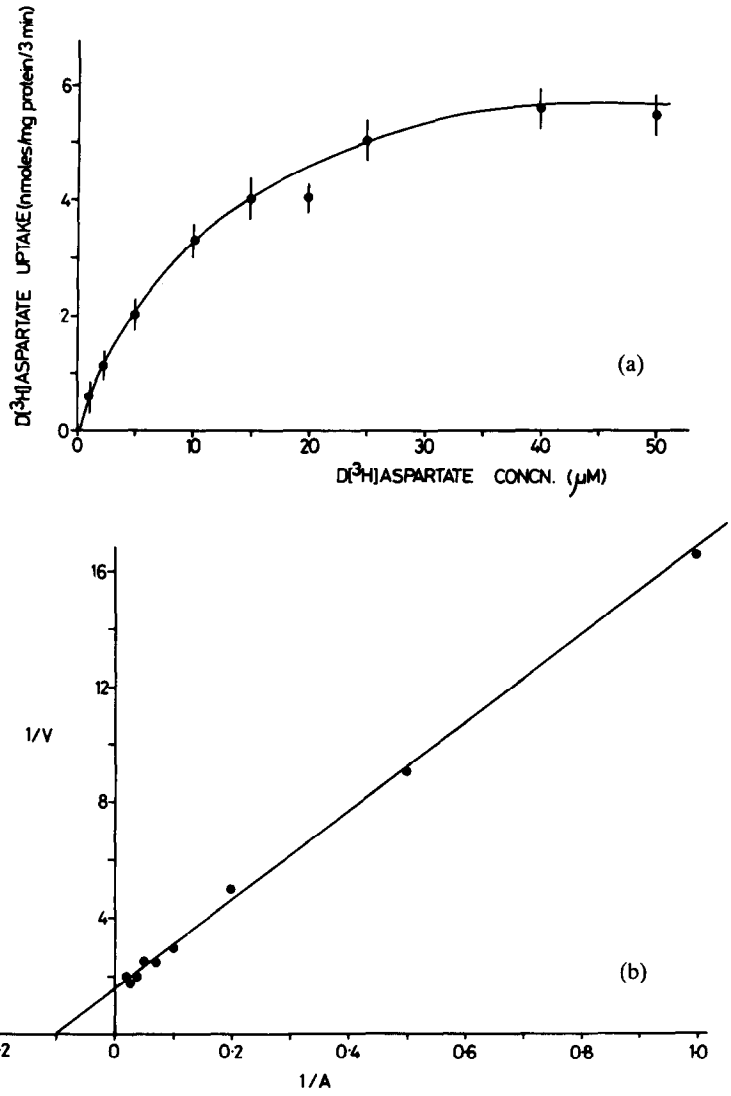

Fig. 1. (a) Saturability of the high affinity uptake of D-[^{3}H]aspartate into adult rat crude cerebellar synaptosomes. Each assay tube was initially 'spiked' with 10 nM D-[^{3}H]aspartate and then diluted with increasing amounts of unlabelled D-aspartate to bring it up to their respective final required concentration. Assays were carried out as described in Materials and Methods with the incubation initiated by addition of P_2 synaptosomes. Each point is the mean ± S.E.M. of three independent experiments assayed in quadruplicate. (b) Lineweaver–Burk analysis of the data obtained from the saturation plot of Fig. 1a. The reciprocal of the initial velocity of D-[^{3}H]aspartate uptake (V) in pmol/mg protein/3 min is plotted against the reciprocal of the D-[^{3}H]aspartate concentration (A) in μM. Each point is the mean of three independent experiments assayed in quadruplicate with S.E.M. of about 10–15%. The linear plot was obtained using a computer-fitted linear regression least square analysis programme.

None of the new kainate analogues tested exhibited equivalent inhibitory potencies and, indeed, the majority were devoid of activity.

We have demonstrated previously that those kainate analogues which retained an isopropylene side chain at the C-3 position on the pyrrolidine ring with no long chain substitutions on either of the carboxyl groups were inhibitors of [^{3}H]kainic acid binding [8] and appeared to act as agonists or partial agonists, in being able to increase cerebellar slice cyclic GMP levels [9]. Those analogues which did not retain the isopropylene side chain and possessed long chain substitutions on the carboxyl groups were much less potent in inhibiting [^{3}H]kainic acid binding, but did exhibit some ability to antagonize the elevation of cerebellar cyclic GMP levels by kainic acid.

None of the compounds examined in this study can therefore be regarded as potentially useful glutamate/aspartate uptake inhibitors, and threo-3-hydroxy-aspartate and α-dihydrokainate remain the most effective, albeit rather weak agents presently available.

Table 1. Kainic acid analogues as inhibitors of high affinity uptake of D-[^{3}H]aspartate into adult rat crude cerebellar synaptosomes

Inhibitor	IC_{50} (μM)
α-Dihydrokainate	110 ± 16.5
α-Kainic acid	350 ± 52.5
β-Dihydrokainate	825 ± 124
2-Carboxy-4-ethyl-3-pyrrolidine methanol	930 ± 140
2-Carboxy-4-propanol-3-pyrrolidine acetic acid	970 ± 144
α-Allokainic acid	1050 ± 158
β-Kainic acid	1325 ± 200

Adult rat cerebellar synaptosomes were incubated with each inhibitor over a concentration range of 100 μM to 1.5 mM for 3 min at 37°, in the presence of 10 nM D[^{3}H]aspartate. The IC_{50} values were determined from the plots shown in Fig. 2. Results are means ± S.E.M. from three independent inhibition curves. Inactive compounds tested: α-homokainate, β-homokainate, α-alloketokainate, β-alloketokainate, α-ketokainate, β-ketokainate, α-kainylglycine, β-kainylglycine, α-kainylaminomethylphosphonate, β-kainylaminomethylphosphonate and α-carboxykainate.

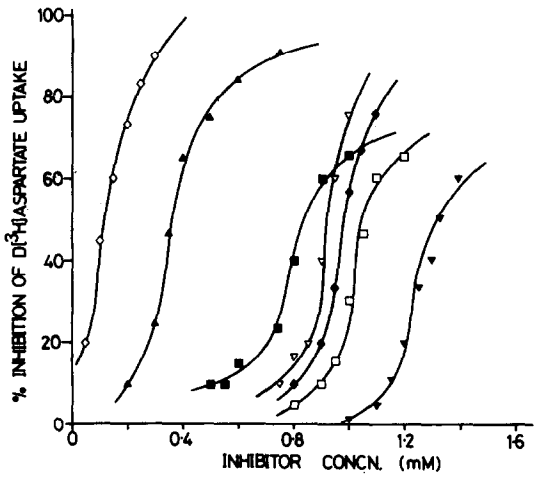

Fig. 2. Dose v/s % inhibition plots for D-[^{3}H]aspartate uptake in adult rat crude cerebellar synaptosomes incubated with α-dihydrokainate (◇), α-kainic acid (▲), β-dihydrokainate (■), 2-carboxy-4-ethyl-3-pyrrolidine methanol (▽), 2-carboxy-4-propanol-3-pyrrolidine acetic acid (◆), α-allokainic acid (□) and β-kainic acid (▼), for 3 min at 37° in the presence of 10 nM D-[^{3}H]aspartate. Each point is the mean of three independent experiments assayed in quadruplicate with S.E. of approximately 10–15%.

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Enzymatic cleavage of 5-substituted-2′-deoxyuridines by pyrimidine nucleoside phosphorylases

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Some 5-substituted derivatives of 2′-deoxyuridine (5-ethyl-, 5-propyl-, 5-isopropyl-, (E)-5-(2-bromovinyl)-2′-deoxyuridine) have antiviral activity [1–6]. The corresponding pyrimidine bases have only very weak antiviral activity compared with nucleosides [7]. It has also been reported that 5-hexyl-2′-deoxyuridine is active towards Ehrlich and