Clinical and Experimental Pharmacology and Physiology (2004) **31**, 000–000

EFFECTS OF GLUTAMATE TRANSPORT SUBSTRATES AND GLUTAMATE RECEPTOR LIGANDS ON THE ACTIVITY OF Na^+/K^+-ATPase IN BRAIN TISSUE *IN VITRO*

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SUMMARY

1. It has been suggested that Na^+/K^+-ATPase and Na^+-dependent glutamate transport (GluT) are tightly linked in brain tissue. In the present study, we have investigated Na^+/K^+-ATPase activity using Rb^+ uptake by 'minislices' (prisms) of the cerebral cortex. This preparation preserves the morphology of neurons, synapses and astrocytes and is known to possess potent GluT that has been well characterized. Uptake of Rb^+ was determined by estimating Rb^+ in aqueous extracts of the minislices, using atomic absorption spectroscopy.

2. We determined the potencies of several known substrates/inhibitors of GluT, such as L-*trans*-pyrrolidine-2,4-dicarboxylate (L*t*PDC), DL-*threo*-3-benzyloxyaspartic acid, (2*S*,3*S*,4*R*)-2-(carboxycyclopropyl)-glycine (L-CCG III) and L-*anti,endo*-3,4-methanopyrrolidine dicarboxylic acid, as inhibitors of [^{3}H]-L-glutamate uptake by cortical prisms. In addition, we established the susceptibility of GluT, measured as [^{3}H]-L-glutamate uptake in brain cortical prisms, to the inhibition of Na^+/K^+-ATPase by ouabain. Then, we tested the hypothesis that the Na^+/K^+-ATPase (measured as Rb^+ uptake) can respond to changes in the activity of GluT produced by using GluT substrates as GluT-specific pharmacological tools.

3. The Na^+/K^+-ATPase inhibitor ouabain completely blocked Rb^+ uptake (IC_{50} = 17 μmol/L), but it also potently inhibited a fraction of GluT (approximately 50% of [^{3}H]-L-glutamate uptake was eliminated; IC_{50} < 1 μmol/L).

4. None of the most commonly used GluT substrates and inhibitors, such as L-aspartate, D-aspartate, L-CCG III and L*t*PDC (all at 500 μmol/L), produced any significant changes in Rb^+ uptake.

5. The *N*-methyl-D-aspartate (NMDA) receptor agonists (*R*,*S*)-(tetrazol-5-yl)-glycine and NMDA decreased Rb^+ uptake in a manner compatible with their known neurotoxic actions.

6. None of the agonists or antagonists for any of the other major classes of glutamate receptors caused significant changes in Rb^+ uptake.

7. We conclude that, even if a subpopulation of glutamate transporters in the rat cerebral cortex may be intimately linked to a fraction of Na^+/K^+-ATPase, it is not possible, under the present experimental conditions, to detect regulation of Na^+/K^+-ATPase by GluT.

Key words: brain energy metabolism, excitatory amino acid transporter substrates and inhibitors, Na^+ and K^+-dependent transport of L-glutamate, Na^+/K^+-ATPase, Rb^+ uptake.

INTRODUCTION

In the central nervous system (CNS), Na^+-dependent transport of L-glutamate (GluT)[1–4] plays a crucial role both in the removal of the potentially excitotoxic neurotransmitter L-glutamate from the extracellular space and in the maintenance of normal metabolic relationships between neurons and glial cells.[3,5–7] Thus, GluT is absolutely essential for homeostasis and any disturbances of GluT could be expected to have serious neurotoxic consequences.[1–4,7–12]

Normally, GluT is driven by Na^+ and K^+ transmembrane gradients generated by Na^+/K^+-ATPase (EC 3.6.1.3).[1] The enzyme Na^+/K^+-ATPase is a near-ubiquitous membrane-bound protein complex producing high ATPase activity in CNS tissue. However, the principal glutamate transporters GLT and GLAST are also among the most abundant proteins in the CNS, particularly in the cytoplasmic membranes of astrocytes.[1,13] The neurons of the CNS may be expected to have the greatest energy demands (and perhaps the highest activity of Na^+/K^+-ATPase) among brain cells because of the constant need to restore the fluctuating electrochemical gradients that are associated with normal neuronal function. Astrocytes, however, are intertwined with neurons in complex metabolic relationships,[1,3,6,14] requiring active transport systems driven by ionic gradients that also put a potentially heavy burden on energy consumption.[15] Therefore, the GLT- and GLAST-rich astrocytic membranes can be equally rich in the Na^+/K^+-ATPase.

Early experiments showed that the Na^+/K^+-ATPase inhibitor ouabain inhibited GluT in slices of rat cerebral cortex,[16] but the same result was not always obtained using other experimental models, such as cultured glial cells.[17] More recently, it has been shown that the Na^+/K^+-ATPase activity, determined by the uptake of [$^{86}Rb^+$] in cultures of glial cells, is increased in the presence of

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Received 8 June 2004; revision 17 August 2004; accepted 23 August 2004.

compounds that are known to be specific substrates of GluT.[18] Furthermore, it has been suggested that the regulation of Na^+/K^+-ATPase by GluT could be involved in the mechanism of coupling neuronal activity to energy metabolism.[18] The sensitivity of GluT to the Na^+/K^+-ATPase inhibitor ouabain,[16] together with the putative susceptibility of Na^+/K^+-ATPase to the stimulation by increased GluT,[18] could, indeed, imply a close functional and/or molecular link between the two systems. However, do such mechanisms exist in experimental models other than cell cultures, particularly in those that retain some of the morphological and metabolic complexities otherwise only found in the living CNS tissue? In the present study, we have investigated whether the hypothetical links between Na^+/K^+-ATPase and GluT occur in a preparation that preserves the main characteristics of brain tissue and can be studied under controlled conditions *in vitro*.

Approach and specific aims

We tested several typical substrates/inhibitors of GluT, as well as compounds known to be specific ligands at various classes of glutamate receptors, against the activity of Na^+/K^+-ATPase, determined by the uptake of Rb^+ in slices of brain tissue *in vitro*. We used 'minislices' (prisms) of rat cerebral cortex because GluT has been studied extensively in this particular preparation[16,19] and because it is similar to the brain slice model that has been used in detailed investigations of brain metabolism associated with glutamatergic activity.[5–7,20,21] Furthermore, using adult brain tissue rather than cultured cells should assure that the most important morphological and functional relationships between neurons and glia that are characteristic for the CNS are preserved. The principal specific aim of the present study was to test whether the activation or inhibition of GluT and/or glutamate receptors (GluR) would produce any major and rapid changes in the uptake of Rb^+ (i.e. whether the activity of Na^+/K^+-ATPase, as expressed in brain tissue, could be subject to fast regulation by glutamatergic neurotransmission in general and by the activity of GluT in particular).

METHODS

Animals and preparation of brain slices

All experiments were conducted in accordance with the guidelines laid down by the National Health and Medical Research Council of Australia and were approved by the Institutional Animal Ethics Committee. Sprague-Dawley rats, either males or females, were purchased from Laboratory Animal Services, The University of Sydney. After brief halothane anaesthesia, rats were decapitated, brains were removed rapidly from the cranial vault, freed of meningea, placed on aluminium foil kept on ice and wetted with ice-cold medium (for composition of the medium, refer to Rae *et al.*[5,6]). The cerebral cortex was dissected from the rest of the brain and from most of the underlying white matter. The isolated cortex was then blotted, weighed and sliced using a mechanical McIlwain tissue chopper (Mickle, Gomshall, UK) into prisms 0.1 mm × 0.1 mm × thickness of cortex (approximately 1–2 mm).

Uptake of Rb^+

The prisms ('minislices') were suspended at 100 mg/mL in ice-cold phosphate- and bicarbonate-buffered Krebs'–Ringer medium.[5,6] The suspension was distributed at 250 μL (25 mg tissue) per flask (9.5 mL Krebs'–Ringer medium[5,6]) kept on ice. The flasks were then transferred to a shaking water bath (90 strokes/min) and incubated at 37°C. After 15 min, Rb^+ was added at 125 μmol/L final concentration (250 μL Krebs'–Ringer medium in which 5 mmol/L KCl was replaced with 5 mmol/L RbCl) and the incubation continued, usually for a further 10 min. The incubation was terminated by rapid vacuum filtration through Whatman no. 1 filter-paper discs (2.5 cm diameter; Whatman Laboratory Division, Maidstone, England) and two rapid washings with 2.0 mL Rb^+-free medium. The negative pressure did not exceed 400 mmHg to minimize the damage to the tissue.[22] Zero-time blanks were used to correct for the residue of Rb^+ trapped by the tissue and not removed by the washing.

Estimation of Rb^+ by atomic absorption spectroscopy

The filters with tissue were placed into glass vials, 1.5 mL deionized water was added and the vials were covered and placed in a refrigerator overnight. Aliquots of the extracts were then taken and Rb^+ estimated by acetylene–flame atomic absorption spectroscopy (SpectrAA-20plus; Varian, Melbourne, Victoria, Australia) using a Photron Rb^+ lamp (λ = 780.2 nm, slit width 0.2 mm; Photron, Narre Warren, Victoria, Australia).

Uptake of [^{3}H]-L-glutamate and [^{3}H]-D-aspartate

Uptake of the radiolabelled GluT substrates L-glutamate and D-aspartate was investigated using a technique similar to that described above for Rb^+ uptake.[16] The suspension of brain slices (100 mg/mL) was centrifuged gently under conditions that are known to preserve the functional release and uptake of neurotransmitter amino acids,[23] the supernatant was removed and the slices were resuspended in an identical volume of the Krebs'–Ringer medium before being distributed at 10 mg/10 mL, into the incubation flasks. This was done to minimize the amount of endogenous L-glutamate released into the medium,[24] which could dilute the radiolabelled substrate. [^{3}H]-L-Glutamate (1 μmol/L; 7.4 kBq/10 mL) or [^{3}H]-D-aspartate (1 μmol/L; 18.5 kBq/10 mL) was used instead of Rb^+, the temperature was 25°C, the incubation time was 7 min and the volume of the medium used to wash the filters was 2 × 10 mL. In some experiments [^{3}H]-D-aspartate uptake was investigated at 37°C and compared with that at 25°C. The amount of [^{3}H] taken up by the tissue was estimated using a liquid scintillation technique (scintillation fluid: Optifluor; scintillation counter: Packard Tricarb 1900C; Canberra Packard, Mount Waverley, Victoria, Australia).

Electron microscopy

Cortical prisms were incubated as in other experiments except that the tissue concentration was increased from 25 to 100 mg/10 mL medium to ensure an adequate amount of material. Incubations were terminated by taking 1 mL aliquots of the suspension and gently pelleting the slices using 1.5 mL Eppendorf tubes in a refrigerated bench centrifuge (the average *g* force was estimated at < 500 *g* for 2 min). The prisms were fixed for 1 h in a mixture of glutaraldehyde and formaldehyde (2.5 and 1%, respectively, in 0.1 mol/L phosphate buffer at pH 7.4), washed (3 × 10 min, 0.1 mol/L phosphate buffer; pH 7.4), exposed to 1% OsO_4 in the same phosphate buffer for 1 h, dehydrated in graded ethanol solutions (30, 50 and 70% for 2 × 5 min and 95 and 100% for 3 × 10 min), infiltrated with 100% ethanol and Spurr's resin in an equal ratio and transferred to 100% Spurr's resin. The material was embedded in a BEEM capsule (Better Equipment for Electron Microscopy; ProSciTech, Kirwan, Qld, Australia) and polymerized at 60°C overnight. Serial ultrathin sections (90 nm) were cut on a Leica ultracut UCT (Leitz Austria, Vertriebs, Austria), mounted onto a piloform-coated copper grid, counterstained with uranyl acetate and lead citrate and examined in a Philips CM120 Biofilter electron microscope (Philips, Mulgrave, Victoria, Australia).

Processing of data

The data obtained in [^{3}H]-L-glutamate uptake studies and in the experiments measuring Na^+/K^+-ATPase activity using Rb^+ uptake were analysed using the Prism Software statistics package (GraphPad 3.1; San Diego,

California, USA). Lines were fitted by non-linear regression, at least two equations were compared and the simpler equation (e.g. straight line) was selected unless the *F*-test[25] indicated that a more complex equation provided a statistically better fit ($P < 0.05$; cf. legends of Figs 1–3; also the references cited therein). Newman–Keuls test was used in the case of ANOVA.

Sources of chemicals

The following fine chemicals were purchased from suppliers as indicated. (1*S*,3*R*)-1-Aminocyclopentane-1,3-dicarboxylic acid (ACPD), (*R*,*S*)-1-aminoindan-1,5-dicarboxylic acid (AIDA), (*S*)-α-amino-3-hydroxy-5-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), (*R*,*S*)-4-amino-pyrrolidine-2,4-dicarboxylate (APICA), DL-*threo*-3-benzyloxyaspartic acid (TBOA), (2*S*,3*S*,4*R*)-2-(carboxycyclopropyl)-glycine (L-CCG III), 7-chlorokynurenic acid, (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)-glycine (DCG IV), dihydrokainic acid (*S*)-3,5-dihydroxyphenylglycine (DHPG), dizocilpine (MK-801), (2*S*)-2-ethylglutamic acid (EGlu), D-*threo*-3-hydroxyaspartic acid, L-*threo*-3-hydroxyaspartic acid, L-*anti,endo*-3,4-methanopyrrolidine dicarboxylic acid (*a*,*e*-MPDC), (*R*,*S*)-*threo*-3-methylglutamic acid, (*R*,*S*)-α-methyl-4-phosphonophenylglycine (MPPG), L-*trans*-pyrrolidine-2,4-dicarboxylic acid (L*t*PDC), (*R*,*S*)-(tetrazol-5-yl)-glycine (TZG) and quinolinic acid were obtained from Tocris Cookson (Bristol, UK). *N*-Acetyl-L-aspartyl-L-glutamate (NAAG), D-aspartic acid, L-aspartic acid, creatine, cyclothiazide, glutaraldehyde, RbCl; glibenclamide, L-glutamate (monopotasium salt) and D-serine were obtained from Sigma (St Louis, MO, USA). *cis*-4-(Phosphomethyl)-2-piperidine carboxylic acid (CGS 19755) was from RBI (Nattick, MA, USA). *N*-Methyl-D-aspartate (NMDA) was synthesized and kindly given to us by Rob D Allan (Department of Pharmacology, The University of Sydney).

The following radiochemicals were purchased from Amersham Life Sciences (Amersham, UK): L-[G-^{3}H]-glutamic acid, TRK 445 B51 (1.7 TBq/mmol), D-[2,3–^{3}H]-Aspartic acid, TRK 606 B29 (1.04 TBq/mmol).

RESULTS

Uptake of Rb⁺ by prisms of cerebral cortex

At 37°C, the uptake of Rb^+ from media containing 125 μmol/L RbCl remained linear with time up to 12.5 min ($P < 0.05$, *F*-test; [25] Fig. 1). Except when testing NMDA receptor agonists and antagonists (see below), we used 15 min pre-incubation (without Rb^+) and 10 min incubations in all remaining experiments.

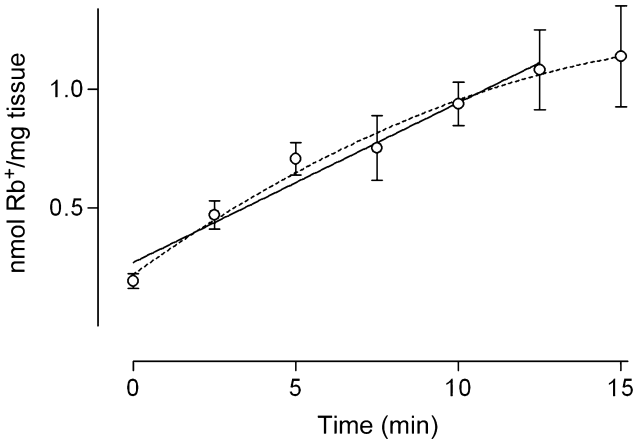

Fig. 1 Time-course of Rb^+ uptake by rat cerebral cortex prisms at 37°C. Results are expressed as nmol/mg wet weight of tissue at the end of the incubation in the presence of 0.125 mmol/L Rb^+. The 'zero' time incubation was not subtracted to illustrate the typical value of blanks that have been subtracted in other experiments. The curve was not significantly different from a straight line up to the 12.5 min time point ($P > 0.2$, *F*-test[25]).

Effect of ouabain on uptake of Rb⁺

Ouabain, an inhibitor of Na^+/K^+-ATPase, completely inhibited Rb^+ uptake. The apparent IC_{50} of ouabain inhibition was 17 μmol/L, with the value of n_H not significantly different from 1 (Fig. 2). The IC_{50} value did not change appreciably when the inhibitions smaller than 5% and greater than 95% were eliminated (data not shown), as is sometimes recommended for this type of study.[26]

Effect of GluT inhibitors on uptake of 1 μmol/L [^{3}H]-L-glutamate

Several known substrates or inhibitors for GluT have been tested using the preparation of cortical prisms. Some have been tested in the similar preparation earlier: L-aspartate, D-aspartate, L- and D-*threo*-3-hydroxyaspartate and dihydrokainate (for a review, see Balcar *et al.*[19]). The remaining compounds have been synthesized and/or identified as specific GluT substrates or inhibitors: TBOA,[27] *a*,*e*-MPDC,[28] L*t*PDC[29,30] and L-CCG III.[7,31,32] Some of the data obtained with the present experimental techniques have been shown previously as a log n_H versus log IC_{50} plot[33] to emphasize similarities in their potencies and modes of actions versus GluT in cortical prisms. Table 1 presents the results in an explicit form. The data obtained with L- and D-aspartate, L- and D-*threo*-3-hydroxy-aspartate and dihydrokainate match very closely those published earlier.[16,34,35] Statistical analysis (ANOVA followed by Newman–Keuls test) showed that, with the exception of dihydrokainate, there were no appreciable differences in the potency (IC_{50}) and in the type of inhibition (n_H) among the apparent inhibitors of

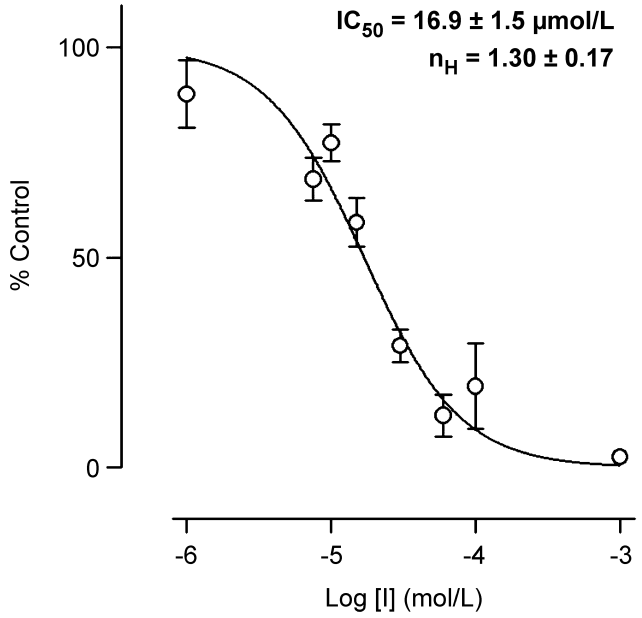

Fig. 2 Effect of ouabain on Rb^+ uptake by prisms of rat cerebral cortex. Uptake of 125 μmol/L Rb^+ is plotted against the logarithm of inhibitor (I) concentration (mol/L). Data are the mean±SEM (n = 3–7), expressed as a percentage of control. Data were analysed as explained previously.[36] Inhibition by ouabain was complete as the inhibitor concentration approached 1 mmol/L.

[^{3}H]-L-glutamate uptake (Table 1). Dihydrokainate was approximately an order of magnitude weaker inhibitor of GluT than the remaining compounds.

Effect of ouabain on uptake of 1 μmol/L [^{3}H]-L-glutamate

Ouabain was a potent inhibitor of [^{3}H]-L-glutamate uptake by prisms of cerebral cortex (Fig. 3). At submicromolar and low micromolar concentrations, there was a steep dose–response relationship that reached an apparent maximum inhibition at just above 50% of control level. Statistical analysis of the dose–response relationship using equations described previously[36,37] indicated an apparent IC_{50} = 0.39 μmol/L and maximum inhibition (I_{max}) of approximately 46% ($P < 0.5$, F-test[25,38]). [^{3}H]-L-Glutamate is metabolized rapidly by cerebral cortical prisms at 37°C, uptake of [^{3}H] stays linear with time for only a short time[16] and this is why the uptake studies with [^{3}H]-L-glutamate have usually been performed at 25°C. In order to eliminate the possibility that the type of data shown in Fig. 1 are only relevant for lower experimental temperatures, we compared the effects of ouabain on uptake of 1 μmol/L [^{3}H]-D-aspartate (not rapidly metabolized) at 25 and 37°C. The results (data not shown) confirmed the low value of IC_{50} for ouabain (3 and 1 μmol/L at 25 and 37°C, respectively)

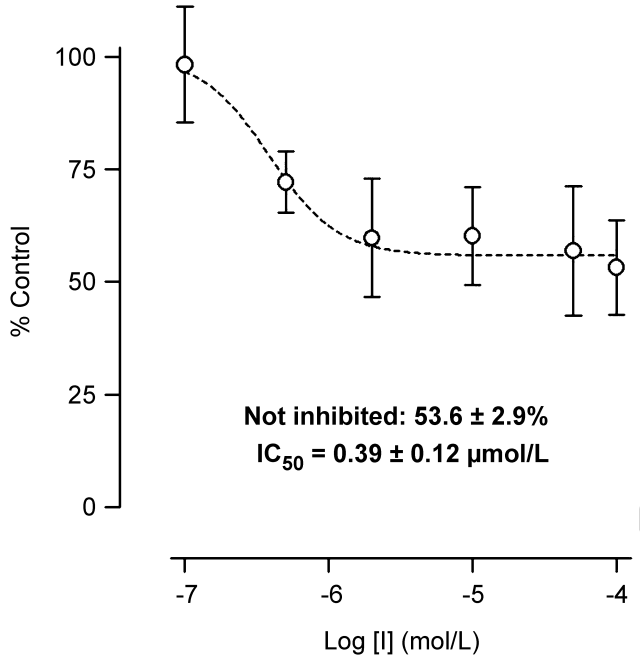

Fig. 3 Inhibition of [^{3}H]-L-glutamate uptake in rat cerebral prisms by ouabain. Uptake of 1 μmol/L [^{3}H]-L-glutamate is plotted against the logarithm of inhibitor (I) concentration (mol/L). Eight values were used for controls; uptake data are the mean±SD (n = 4–8 for each concentration). Data analysis[36] indicated that a fraction (approximately 54%) was not inhibited by ouabain up to at least 100 μmol/L inhibitor concentration.

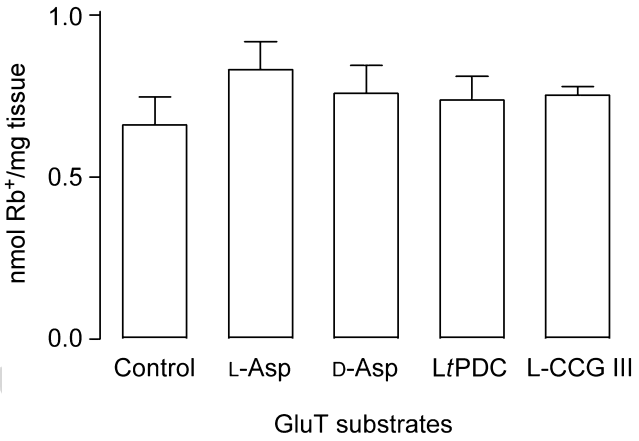

Fig. 4 Effect of glutamate transport (GluT) substrates (0.5 mmol/L) on Rb^+ uptake by rat cortex prisms. The substrates were present in the incubation medium from the beginning of the pre-incubation. Data are the mean±SD (n = 4; n = 8 for controls). There were no significant differences ($P > 0.05$) among the values (ANOVA; Newman–Keuls test) of Rb^+ uptake, expressed as nmol Rb^+/mg wet weight at the end of a 10 min incubation (corrected for zero time blank cf. Fig. 1) in the presence of 125 μmol/L Rb^+. L-Asp, D-Asp, L- and D-aspartate, respectively; L*t*PDC, L-*trans*-pyrrolidine-2,4-dicarboxylic acid; L-CCG III, (2*S*,3*S*,4*R*)-2-(carboxycyclopropyl)-glycine.

Table 1 Inhibition of radiolabelled L-glutamate uptake in brain prisms by glutamate and aspartate analogues

	IC_{50} (μmol/L)	n_H	No. values	No. concentrations	Range (μmol/L)
L-*threo*-3-Benzyloxyaspartate	5.0 ± 0.8	1.27 ± 0.25	16	8	0.93–120
L-*threo*-3-Hydroxyaspartate	5.7 ± 1.2	1.01 ± 0.17	12	8	0.93–120
a,*e*-MPDC	15.6 ± 2.0	0.93 ± 0.11	16	8	0.93–120
Dihydrokainate	256 ± 56	1.34 ± 0.39	32	9	1.87–480
L-Aspartate	21.8 ± 4.7	1.07 ± 0.24	32	8	0.93–120
D-Aspartate	8.2 ± 1.4	0.79 ± 0.11	31	8	0.93–120
L*t*PDC	16.6 ± 4.5	1.06 ± 0.21	13	8	0.93–120
L-CCG III	10.7 ± 2.0	1.14 ± 0.22	16	8	0.93–120
L-Glutamate	14.9 ± 3.0	1.13 ± 0.25	30	8	0.93–120
DL-*threo*-3-Methylglutamate	∞		16	8	0.93–120

Data for the IC_{50} and n_H are presented as the mean±SEM.

Inhibitions were calculated as shown previously.[36] Inhibitors were present in the medium from the beginning of the pre-incubation. None of the n_H values was significantly different from unity ($P > 0.05$, F-test). Statistical analysis by ANOVA (Newman–Keuls test) indicated that only the IC_{50} value for dihydrokainate was significantly different ($P < 0.01$) from the remaining IC_{50} values.

DL-*threo*-3-Methylglutamate produced no significant inhibition at a concentration of 120 μmol/L.

a,*e*-MPDC, L-*anti,endo*-3,4-methanopyrrolidine dicarboxylic acid; L*t*PDC, L-*trans*-pyrrolidine-2,4-dicarboxylic acid; L-CCG III, (2*S*,3*S*,4*R*)-2-(carboxycyclopropyl)-glycine.

and the levelling off of inhibition at ouabain concentrations < 100 μmol/L (I_{max} was estimated at 69 and 84% at 25 and 37°C, respectively).

Effects of GluT substrates and inhibitors on Rb^+ uptake

We have tested all the compounds listed in Table 1 for possible inhibition or stimulation of Na^+/K^+-ATPase activity as measured by Rb^+ uptake. None of the GluT substrates/inhibitors had any effect up to the inhibitor concentration 100 μmol/L. In some experiments, we increased the concentration of Rb^+ to 300 μmol/L and doubled the amount of tissue to 50 mg/10 mL (the uptake of Rb^+ remained linear up to 10 min under these conditions; data not shown) in order to obtain a higher signal in atomic absorption spectroscopy and increase the precision of the method but, again, we found no differences from controls in the presence of any of the GluT substrates/inhibitors. Nor did we see any effects when we increased the concentrations of GluT substrates/inhibitors up to 500 μmol/L (Fig. 4).

Effects of ionotropic glutamate receptor ligands on Rb^+ uptake

Neither AMPA nor kainate (up to 100 μmol/L) produced any significant changes in Rb^+ uptake. The AMPA-selective subtype of ionotropic glutamate receptors (iGluR) desensitizes rapidly, but this process can be attenuated by cyclothiazide.[39,40] However, neither AMPA in the presence of cyclothiazide (50 μmol/L) nor cyclothiazide alone had any effect on Rb^+ uptake.

Initially, no effects were observed when compounds related to NMDA receptors were tested, usually at 100 μmol/L. These included, for example, D-serine, the naturally occurring neurotoxin quinolinate (tested up to 2.5 mmol/L) and NMDA receptor antagonists such as 7-chlorokynurenic acid, CGS 19755 and MK-801 (tested at 10 μmol/L).

Two iGluR ligands that appeared to be effective as inhibitors of Rb^+ uptake were NMDA-type receptor ligands TZG (10 μM[41]) and NMDA (100 μmol/L), but only when pre-incubated with the tissue for 30 min, instead of the usual 15 min, in the absence of Mg^{2+}. The inhibitions were modest and apparently non-additive (< 50%; Fig. 5).

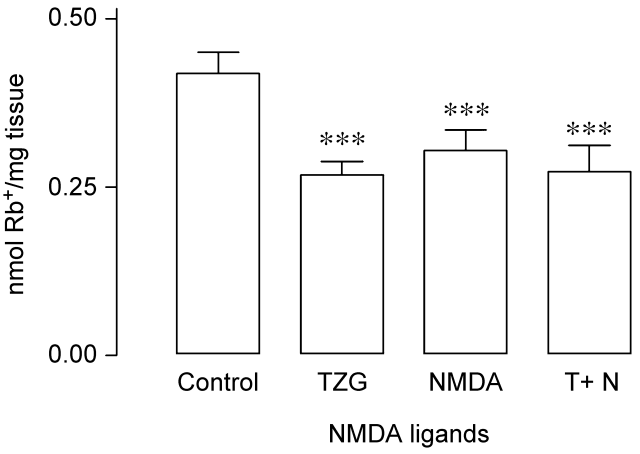

Fig. 5 Effect of *N*-methyl-D-aspartate (NMDA) agonists on Rb^+ uptake in prisms of rat cerebral cortex. Details are as per Fig. 4 except that the pre-incubation lasted for 30 min (in the presence of 10 μmol/L (*R*,*S*)-(tetrazol-5-yl)-glycine (TZG), 100 μmol/L NMDA or both (T + N)) and the incubation medium had no Mg^{2+}. ***$P < 0.001$ compared with controls.

Effects of metabotropic glutamate receptor ligands on Rb^+ uptake

We have tested several specific agonists and antagonists of classes I or II of metabotropic glutamate receptors (mGluR[42,43]): DHPG, AIDA, DCG IV, EGlu, APICA and NAAG, as well as some less selective mGluR ligands (MPPG and ACPD). None of them produced any effects on Rb^+ uptake (data not shown).

Drugs, ionophores and miscellaneous inhibitors

The sulphydryl agents *p*-chloromercuriphenyl sulphonate (*p*CS) and mercuric chloride caused 100% inhibition of Rb^+ uptake at 100 μmol/L. No effects were produced (at 100 μmol/L) by the K^+ channel blocker 4-aminopyridine, the mitochondrial ATP-dependent K^+ channel inhibitor glibenclamide, the ATP P2X receptor inhibitor Evans blue, the metabolic energy buffer creatine and its analogue cyclocreatine (data not shown).

Structural integrity of brain slices *in vitro*

Outside the superficial regions that would have been obviously damaged during the preparation of tissue prisms, electron microscopy revealed the presence of many structurally intact synapses

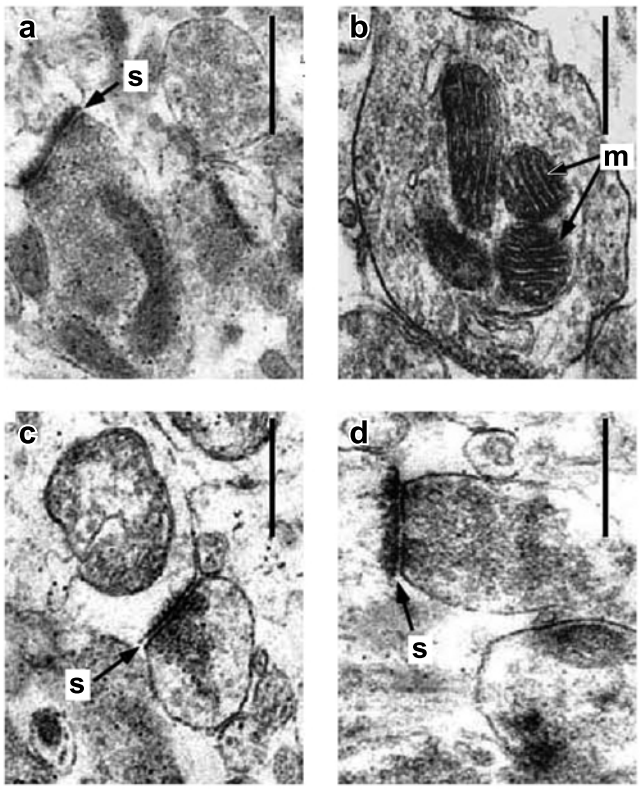

Fig. 6 Ultrastructural studies of brain slices incubated at 37°C *in vitro*. (a,b) Examples of tissue incubated for 25 min; (c,d) tissue incubated under the same conditions for 45 min. S, synapses; m, mitochondria. Bar, 0.4 μm.

both after 25 and 45 min incubations (Fig. 6). Several other structures, such as nuclei and mitochondria, were also clearly discernible.

DISCUSSION

The properties of Rb^+ in aqueous solutions appear to be so close to those of K^+ that the Na^+/K^+-ATPase can handle both ions with similar efficiencies. This characteristic of the enzyme has been used in studies of Na^+/K^+-ATPase activity in various experimental models, including cultured astrocytes.[18] However, most of the previous studies have used the radioactive isotope [^{86}Rb]. In contrast, the technique used in the present experiments is based on the use of non-radioactive Rb^+. Such methodology would make the studies of Na^+/K^+-ATPase activity accessible to laboratories not equipped for handling radiotoxic isotopes such as [^{86}Rb].

Non-radioactive Rb^+ was rapidly taken up by 'minislices' (prisms) of rat brain cortex *in vitro* and, at 37°C, the uptake remained linear with time for long enough to obtain a sufficient amount of Rb^+ in the tissue to be reproducibly estimated by acetylene–flame atomic absorption spectroscopy. Moreover, the uptake of Rb^+ was completely abolished in the presence of the typical Na^+/K^+-ATPase inhibitor ouabain. Thus, we have accepted the Rb^+ uptake, as studied under the present conditions, as a satisfactory quantitative measure of Na^+/K^+-ATPase activity in brain tissue *in vitro*.

Na^+/K^+-ATPase exists in the form of a complex consisting of α and β protein subunits.[44,45] The α-subunits (α_1, α_2, α_3 and α_4) posses the substrate (ATP)-binding site and are responsible for the translocation of Na^+ and K^+ they also vary in their sensitivity to ouabain.[46] In brain tissue, the α_1-subunit is expressed both in neurons and glia, whereas the α_2-subunit is found predominantly in glia and the α_3-subunit is found in neurons.[47,48]

The principal glutamate transporters in rat cerebral cortex are GLT and GLAST.[1,13] Their expression can be as high as 1–2% of total tissue protein and both are located predominantly in astrocytes.[1,13,48] This probably explains very high capacities of some astrocyte culture systems to accumulate L-glutamate (for a review and comparison of kinetic constants, such as V_{max} of GluT in various culture systems, see Balcar *et al.*[49]). Transporting large amounts of L-glutamate in a Na^+- and K^+-dependent manner would result in extra demands on Na^+/K^+-ATPase and could explain the sensitivity of GluT to ouabain.[50] Indeed, activation of GluT by adding high concentrations of GluT substrates, such as L-aspartate and L-glutamate, to cultured astrocytes can increase Na^+/K^+-ATPase activity by as much as 40 or 50%[18,51] and this additional activity appears to be exerted by a Na^+/K^+-ATPase subunit (α_2) highly sensitive to the inhibition by ouabain.[18] Furthermore, colocalization of GLAST and the α_2-subunits of Na^+/K^+-ATPase has been described in the rodent CNS.[52] In addition, a study *in vivo* has shown that interference with GluT will abolish the characteristic pattern of [^{14}C]-2-deoxyglucose ([^{14}C]-2-DOG) uptake imparted onto somatosensory cortex by specific types of stimuli.[53] Could such a change in energy consumption (as indicated by altered [^{14}C]-2-DOG uptake *in vivo*) be caused by 'uncoupling' of GluT from the GluT (and ouabain)-sensitive fraction of Na^+/K^+-ATPase?

Even though we have observed that a fraction of GluT, as measured by [^{3}H]-L-glutamate uptake, is potently inhibited by ouabain ($IC_{50} < 1$ μmol/L), manipulating GluT, either by compounds that are poorly transported and may act as GluT inhibitors (TBOA and *a*,*e*-MPDC) or by compounds that are good substrates for GluT (L-aspartate, D-aspartate and also L-CCG III, which seems to be very avidly transported by cortical slices *in vitro* against a steep concentration gradient[7]) had no effect on the overall rate of Rb^+ uptake. The stoichiometry of GluT with respect to L-glutamate, Na^+ and K^+ can be very complex.[1,54] Consequently, the activity of some glutamate transporters may, indeed, depend critically on large Na^+ and K^+ gradients generated by Na^+/K^+-ATPase molecules in their immediate surroundings. It is therefore possible that a significant fraction of GluT (approximately half) indeed depends on α_2-containing Na^+/K^+-ATPase (highly 'ouabain sensitive' $IC_{50} < 1$ μmol/L) but, at least in the present studies, this accounts for only a small proportion of the total Na^+/K^+-ATPase activity that seems to be driven by less ouabain-sensitive α_1-containing Na^+/K^+-ATPase ($IC_{50} > 10$ μmol/L).

It is obvious that using a McIlwain tissue chopper for the preparation of tissue 'prisms' from rat cerebral cortex will result in damage to both neurons and astrocytes. However, uptake of L-glutamate and Rb^+, as well as the preserved synaptic contacts and intracellular organelles revealed by electron microscopy, strongly indicate that the preparation retains a high degree of functional and structural integrity, even after a lengthy incubation *in vitro*. Thus, the lack of effect of GluT substrates on Rb^+ uptake cannot be easily explained by a loss of normal metabolic and structural relationships caused by possible superficial disruption of the tissue and/or by experimental conditions.

Furthermore, results from additional preliminary experiments have indicated that the uptake of [^{14}C]-2-DOG, measured in the cerebral cortex prisms under conditions similar to those used when studying uptake of Rb^+ and [^{3}H]-L-glutamate, was not affected by 100 μmol/L concentrations of L-aspartate, D-aspartate and TBOA (i.e. by two transportable and one non (or poorly)-transportable GluT substrate). In these studies, GluT substrates were put into the incubation medium at the beginning of the 15 min pre-incubation and, following the addition of [^{14}C]-2-DOG, the incubation was continued for a further 15 min. Under such conditions, control uptake of [^{14}C]-2-DOG was found to remain linear with time for at least 40 min and was inhibited by approximately 50% by 1 mmol/L ouabain (data not shown).

In conclusion, it is possible to use the uptake of non-radioactive Rb^+, estimated by atomic absorption spectroscopy, to study reproducibly the activity of Na^+/K^+-ATPase in small amounts of brain tissue *in vitro*. Our findings indicate that even if a fraction of Na^+/K^+-ATPase activity may be tightly linked to glutamate transport, it would not represent a large enough proportion of the total Na^+/K^+-ATPase to be detected by Rb^+ uptake under the present experimental conditions. If such a relationship holds for brain tissue *in vivo*, GluT-linked perturbations in Na^+/K^+-ATPase activity would not produce a strong enough signal to provide a rapid communication channel between changes in glutamatergic (excitatory) activity and local uptake of glucose from the blood stream. Rather, more specific signalling mechanisms, perhaps involving glutamate receptors or transporters positioned directly on brain microvessels, may have to be considered.[55,56]

ACKNOWLEDGEMENTS

Thanks are expressed to Mr William Lowe for expert technical assistance with atomic absorption spectroscopy and to Dr Anne Simpson from the Electron Microscopy Unit, The University of Sydney, for help with the ultrastructural analysis of the tissue. CR was supported by a National Health and Medical Research Council of Australia Fellowship. The work was supported, in part, by a grant from the Rebecca L Cooper Foundation, by a Sydney University Sesquicentennial Research Grant and by a grant from the Australian Health Management Fund to VJB.

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