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Short communication

A new structural class of subtype-selective inhibitor of cloned excitatory amino acid transporter, EAAT2

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Abstract

We have studied the pharmacological effects of (*RS*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) and the enantiomers of (*RS*)-2-amino-3-(3-hydroxy-1,2,5-thiadiazol-4-yl)propionic acid (TDPA) on cloned human excitatory amino acid transporter subtypes 1, 2 and 3 (EAAT1–3) expressed in Cos-7 cells. Whereas AMPA and (*R*)-TDPA were both inactive as inhibitors of [^{3}H]-(*R*)-aspartic acid uptake on all three EAAT subtypes, (*S*)-TDPA was shown to selectively inhibit uptake by EAAT2 with a potency equal to that of the endogenous ligand (*S*)-glutamic acid. (*S*)-TDPA thus represents a new structural class of EAAT2 inhibitor that will serve as a lead for the design of EAAT selective inhibitors.

Keywords: EAAT; (*RS*)-2-Amino-3-(3-hydroxy-1,2,5-thiadiazol-4-yl)propionic acid; TDPA; EAAT2 inhibitor, selective

1. Introduction

(*S*)-Glutamic acid is the major excitatory amino acid in the central nervous system (CNS) and is involved in many important processes such as learning, memory and plasticity; glutamate receptors and transporters are thus considered as important drug targets (Bräuner-Osborne et al., 2000; Seal and Amara, 1999; Vandenberg, 1998). Upon release into the synaptic cleft, (*S*)-glutamic acid activates an array of pre- and postsynaptically located receptors which either belong to the ligand-gated ion channels (ionotropic glutamate receptors) or the G-protein coupled receptors (metabotropic glutamate receptors) (Bräuner-Osborne et al., 2000). In order to terminate the synaptic transmission, (*S*)-glutamic acid is transported into neuronal or glial cells by excitatory amino acid transporters (EAATs). At present, five different human EAAT subtypes have been cloned (EAAT1–5), of which EAAT1–3 have been suggested to be the subtypes responsible for the major part of the uptake process based on their expression patterns and the observation that EAAT4,5 predominantly act as ligand-gated chloride channels (Vandenberg, 1998; Seal and Amara, 1999). Given that several pathological conditions are thought to be caused by either too low or too high levels of (*S*)-glutamic acid in the CNS, regulation of EAAT subtypes has been suggested as pertinent drug targets (Vandenberg, 1998; Seal and Amara, 1999). However, only a few compounds have been shown to interact with EAATs and a very limited number of compounds inhibit EAATs in a subtype selective fashion (Vandenberg, 1998; Seal and Amara, 1999). Important examples of the latter are kainic acid, dihydrokainic acid and L-*trans*-2,3-pyrrolidine dicarboxylate, which have all been shown to be selective nonsubstrate inhibitors of the EAAT2 subtype (Arriza et al., 1994; Koch et al., 1999). However, there is a great need for the development of new subtype-selective inhibitors which could serve as pharmacological tools to further elucidate the physiological function and therapeutic relevance of individual EAAT subtypes. We report here the pharmacology of a novel selective EAAT2 inhibitor

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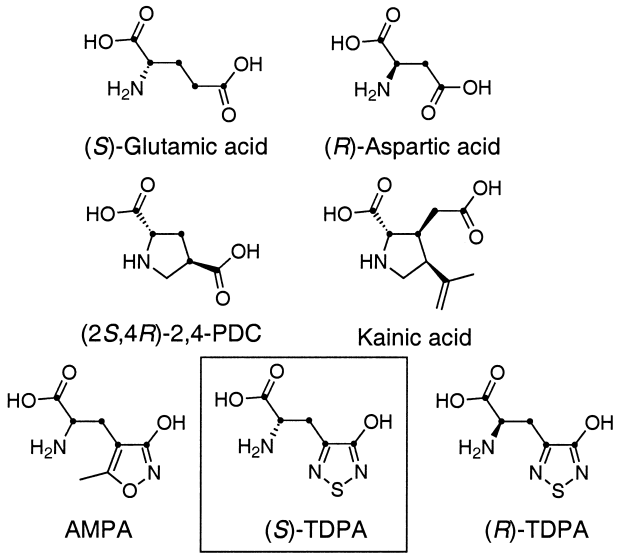

Fig. 1. Structures of inhibitors tested in this study.

containing the 3-hydroxy-1,2,5-thiadiazol nucleus as a carboxyl group bioisostere (Fig. 1).

2. Materials and methods

2.1. Generation of expression plasmids

The cloning of the three human EAAT1–3 has previously been described (Arriza et al., 1994). The coding regions were transferred from pBluescript cloning vector (Stratagene, La Jolla, CA) to the pcDNA3 expression vector (Invitrogen, La Jolla, CA) using the following procedures. The open reading frames of EAAT1 and EAAT3 were amplified by the polymerase chain reaction (PCR) using the following primers: EAAT1-U (5′-CGCG CTCGAG AAT ATG ACT AAA AGC AAT G-3′), EAAT1-D (5′-CGCG TCTAGA CTA CAT CTT GGT TTC ACT G-3′), EAAT3-U (5′-CGCG GAATTC GCC ATG GGG AAA CCG GCG-3′) and EAAT3-D (5′-CGCG CTCGAG CTA GAA CTG TGA GGT CTG). PCR was performed using *Pfu* polymerase (Stratagene) and a PTC-100 thermal cycler (MJ Research, Waltham, MA). The reactions were cycled 30 times at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 2 min. The PCR product was run on a 1% agarose gel containing 0.1 μg/ml ethidium bromide and single band of expected sizes were purified using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). The purified products were digested with the appropriate restriction enzymes and ligated into pcDNA3. Finally, the amplified regions were sequenced on an ABI 310 using Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer, UK) to verify that the PCR reaction had not introduced any unwanted mutations. EAAT2 was cut out of the pBluescript vector using the unique restriction sites *Eag*I and *Sal*I which were upstream and downstream of the open reading frame, respectively, and the insert was ligated into *Not*I/*Xho*I digested pcDNA3 vector.

2.2. Cell maintenance and uptake assay

The cell maintenance and uptake assay is based on the previously published protocol by Arriza et al. (1994). Cos-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (both Gibco, Paisley, Scotland) in a humidified incubator containing 5% CO_2 and 95% atmosphere. One million cells were split into a 10-cm tissue culture dish and the following day, cells were transfected with 5 μg plasmid DNA using SuperFect (Qiagen) as a DNA carrier. The day after transfection, cells from one 10-cm dish were transferred to a 48-well tissue culture plate and 2 days post-transfection, uptake assays were performed.

Each well was washed three times with 150 μl room temperature modified phosphate buffered saline (mPBS, 137 mM NaCl, 8.1 mM Na_2PO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 5.6 mM D-glucose, all Sigma, St. Louis, MO) over a period of 10 min. Each well was then incubated for 10 min in mPBS containing 100 nM [^{3}H](*R*)-aspartic acid (Amersham, Buckinghamshire, UK), 900 nM cold (*R*)-aspartic acid (Sigma) and test ligand. Washing the wells three times with 150 μl ice-cold mPBS terminated uptake, and finally cells were lysed by addition of 200 μl 0.1% sodium dodecyl sulphate (SDS, Sigma). The lysate was transferred to scintillation vials containing 2 ml OptiFluor scintillation fluid (Packard, Groningen, Netherlands) and counted on a scintillation counter. It was determined that using this protocol, there was a linear relationship between incubation time and uptake for at least 60 min.

2.3. Data analysis

Counting values from cells transfected with transporter were subtracted values from mock transfected cells (empty pcDNA3) performed in parallel experiments. Inhibition data were then fitted to the Michaelis–Menten equation using the programme KaleidaGraph (Synergy Software, Reading, PA) and the IC_{50} values were determined. K_i values were calculated from the Cheng–Prusoff equation (Cheng and Prusoff, 1973). Each data point was determined in triplicate and each experiment was performed three to four times. Inhibition constants are shown as mean pK_i values $\pm$S.E.M.

2.4. Pharmacological agents

(*S*)-Glutamic acid, (*R*)-aspartic acid and kainic acid were obtained from Sigma. (*S*)-*trans*-pyrrolidine-2,4-dicarboxylic acid [L-*trans*-2,4-PDC, (2*S*,4*R*)-2,4-PDC] was obtained from Tocris Cookson (Bristol, UK). (*S*)- and (*R*)-2-Amino-3-(3-hydroxy-1,2,5-thiadiazol-4-yl)propionic acid [(*S*)- and (*R*)-TDPA] were synthesised in our laboratory in enantiomerically pure forms (> 99.9% e.e. Johansen et al., in preparation). (*RS*)-2-Amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) was synthesised in our laboratory according to a previously published protocol (Begtrup and Sløk, 1993).

3. Results

In the present study, we have used inhibition of the transport of [^{3}H]-(*R*)-aspartic acid into Cos-7 cells transiently transfected with EAAT1–3 to determine the pharmacological effects of transport inhibitors. We decided to use radiolabelled (*R*)-aspartic acid as a tracer substrate rather than (*S*)-glutamic acid that has previously been used to characterise cloned EAATs (Arriza et al., 1994). This choice was based on a significantly lower background signal observed with the nonmetabolizable [^{3}H]-(*R*)-aspartic acid compared to [^{3}H]-(*S*)-glutamic acid (data not shown). The inhibition constants of the tested compounds are shown in Table 1.

Table 1
Affinity constants of inhibitors on cloned excitatory amino acid transporters

Compound	pK_i		
	EAAT1	EAAT2	EAAT3
(*S*)-Glutamic acid	4.4 ± 0.0	4.0 ± 0.1	3.9 ± 0.0
(*R*)-Aspartic acid	4.3 ± 0.0	4.2 ± 0.0	4.3 ± 0.1
(2*S*,4*R*)-2,4-PDC	4.0 ± 0.1	4.5 ± 0.1	3.7 ± 0.0
Kainic acid	< 3.0	3.9 ± 0.0	< 3.0
AMPA	< 3.0	< 3.0	< 3.0
(*S*)-TDPA	< 3.0	4.0 ± 0.1	< 3.0
(*R*)-TDPA	< 3.0	< 3.0	< 3.0

Values are expressed as means ± S.E.M. of three to four independent experiments.

4. Discussion

We have studied the inhibition of [^{3}H]-(*R*)-aspartic acid uptake by human EAATs of already known inhibitors and two newly synthesised glutamic acid bioisosteres (Fig. 1). Our results with the known uptake inhibitors agree nicely with the results of previously published studies using either electrophysiology on *Xenopus* oocytes or radiolabelled ligand uptake into mammalian cells. Thus, as shown in Table 1, we observed that (*S*)-glutamic acid and (*R*)-aspartic acid act as nonselective inhibitors on all three transporter subtypes with K_i values in the range of 37–120 μM. Previously published K_m values from mammalian cells and *Xenopus* oocytes were in the range of 47–97 μM (Arriza et al., 1994) and 7–28 μM (Vandenberg, 1998). Both (2*S*,4*R*)-2,4-PDC and kainic acid (Fig. 1) selectively inhibit uptake by the EAAT2 subtype. In our study, the former compound displayed fourfold and sixfold selectivity for EAAT2 compared to EAAT1 and EAAT3, respectively, whereas the latter compound displayed more than 10-fold selectivity for EAAT2. Albeit both compounds were shown to act slightly less potently on EAAT2 than previously reported, 32 μM and 120 μM, respectively, the selectivity profiles were identical to those observed in previous studies using mammalian cells (Arriza et al., 1994; Shimamoto et al., 1998) and *Xenopus* oocytes (Shimamoto et al., 1998).

We have previously shown that replacement of the distal carboxyl group of glutamic acid with heterocyclic bioisosteric groups such as the 3-hydroxyisoxazole unit affords compounds with pronounced differences in selectivity profiles for (*S*)-glutamic acid receptors (Bräuner-Osborne et al., 2000). In order to further investigate the effect of different bioisoteric groups on glutamate pharmacology, we have synthesised (*S*)- and (*R*)-TDPA. As shown in Table 1, AMPA and (*R*)-TDPA are both inactive as inhibitors of all three transporter subtypes, whereas (*S*)-TDPA displays selective inhibition of EAAT2 with a potency equal to that of (*S*)-glutamic acid. This profile is very similar to that of kainic acid (Table 1). Like kainic acid, (*S*)-TDPA also potently activates glutamate receptors (Johansen et al., in preparation). This reduces the potential use of (*S*)-TDPA as a pharmacological tool. However, given the very short list of compounds acting as selective inhibitors of EAAT subtypes, it does serve as an important lead for the design of new EAAT inhibitors. Furthermore, it is interesting to note the dramatic difference on EAAT2 inhibition between (*S*)-TDPA and AMPA, which contain the 3-hydroxy-1,2,5-thiadiazole and 3-hydroxyisoxazole units, respectively, as the distal acidic groups (Fig. 1). It is suggested that the lack of inhibitory effect of AMPA on EAAT2 is caused by the presence of the 5-methyl sub-

stituent of AMPA that cannot be accommodated by the binding site of EAAT2. However, other explanations such as differences in electronic distribution in the heterocyclic rings of (*S*)-TDPA and AMPA cannot be ruled out. In light of the urgent need for new selective EAAT inhibitors, we are currently designing and testing ligands which will further elucidate the mechanism behind the differences between EAAT2 inhibition by AMPA and TDPA.

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