ELSEVIER

European Journal of Pharmacology 411 (2001) 245–253

ejp

www.elsevier.nl/locate/ejphar

Stereochemistry and molecular pharmacology of (*S*)-thio-ATPA, a new potent and selective GluR5 agonist

Tine B. Stensbøl [a], Henrik S. Jensen [b], Birgitte Nielsen [a], Tommy N. Johansen [a], Jan Egebjerg [b], Karla Frydenvang [a], Povl Krogsgaard-Larsen [a,*]

[a] *NeuroScience PharmaBiotec Research Center, Department of Medicinal Chemistry, The Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen, Denmark*

[b] *Department for Molecular and Structural Biology, University of Aarhus, Aarhus, Denmark*

Received 7 August 2000; received in revised form 22 November 2000; accepted 28 November 2000

Abstract

(*RS*)-2-Amino-3-(5-*tert*-butyl-3-hydroxy-4-isothiazolyl)propionic acid (thio-ATPA), a 3-isothiazolol analogue of (*RS*)-2-amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid (ATPA), has previously been shown to be a relatively weak AMPA receptor agonist at native (*S*)-glutamic acid ((*S*)-Glu) receptors (EC_{50} = 14 μM), comparable in potency with ATPA (EC_{50} = 34 μM). Recent findings, that (*S*)-ATPA is a potent (EC_{50} = 0.48 μM) and selective agonist at homomerically expressed ionotropic GluR5, prompted us to resolve thio-ATPA using chiral chromatography and pharmacologically characterize the two enantiomers at native as well as cloned ionotropic glutamate receptors. The enantiomers, (*S*)- and (*R*)-thio-ATPA, were obtained in high enantiomeric excess, and their absolute stereochemistry established by an X-ray crystallographic analysis. Electrophysiologically, the two enantiomers were evaluated in the rat cortical wedge preparation, and the *S*-enantiomer was found to be an AMPA receptor agonist (EC_{50} = 8.7 μM) twice as potent as the racemate, whereas the *R*-enantiomer was devoid of activity. In accordance with this, (*S*)-thio-ATPA proved to be an agonist at homomerically expressed recombinant AMPA receptors (GluR1o, GluR3o, and GluR4o) with EC_{50} values of 5, 32 and 20 μM, respectively, producing maximal steady state currents of 78–168% of those maximally evoked by kainic acid, and 120–1600% of those maximally evoked by (*S*)-ATPA. At homomerically expressed GluR5, (*S*)-thio-ATPA was found to be a potent agonist (EC_{50} = 0.10 μM), thus being approximately five times more potent than (*S*)-ATPA. (*R*)-Thio-ATPA induced saturating currents with an estimated EC_{50} value of 10 μM, most likely due to a contamination with (*S*)-thio-ATPA. At heteromerically expressed GluR6 + KA2 receptors, (*S*)-thio-ATPA showed relatively weak agonistic properties (EC_{50} = 4.9 μM). Thus, (*S*)-thio-ATPA has been shown to be a very potent agonist at GluR5, and may be a valuable tool for the investigation of desensitization properties of AMPA receptors.

Keywords: AMPA (2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid); ATPA (2-amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid); Thio-ATPA (2-amino-3-(5-*tert*-butyl-3-hydroxy-4-isothiazolyl)propionic acid); Kainic acid; Chiral chromatography; Enantiomer; Desensitization; AMPA receptor cloned; Kainic acid receptor cloned; Cortical wedge preparation

1. Introduction

(*S*)-Glutamic acid ((*S*)-Glu, Fig. 1) is the major excitatory amino acid neurotransmitter in the central nervous system. The glutamatergic signaling pathways may be implicated in the pathological processes leading to a number of neurodegenerative illnesses such as Alzheimer's and Parkinson's disease, and are potential targets for medical intervention (Knöpfel et al., 1995; Parsons et al., 1998; Lees, 2000). Receptors activated by (*S*)-Glu form a heterogeneous group of receptors, comprising both G-protein coupled metabotropic receptors and ionotropic (*S*)-Glu receptors (Collingridge and Watkins, 1994; Wheal and Thomson, 1995; Conn and Pin, 1997; Monaghan and Wenthold, 1997; Jonas and Monyer, 1999; Braüner-Osborne et al., 2000). The ionotropic (*S*)-Glu receptors are subdivided into three pharmacologically distinct classes of receptors, namely *N*-methyl-D-aspartic acid (NMDA), (*RS*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) and kainic acid receptors (Chittajallu et al., 1999; Jonas and Monyer, 1999). The ionotropic recep-

* Corresponding author. Tel.: +45-353-06511; fax: +45-353-06040.
E-mail address: ano@dfh.dk (P. Krogsgaard-Larsen).

0014-2999/01/$ - see front matter
PII: S0014-2999(00)00916-X

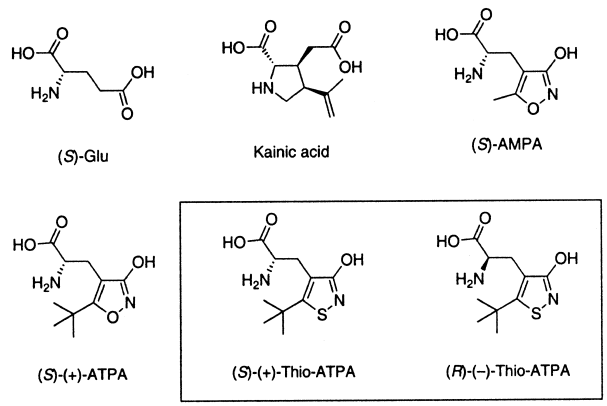

Fig. 1. Structures of (*S*)-Glu, (*S*)-AMPA, kainic acid, (*S*)-ATPA, and (*S*)- and (*R*)-thio-ATPA.

tors are homo- or heteromeric assemblies of subunits, forming an ion channel. AMPA receptors are made up of GluR1–4 subunits and kainic acid receptors contain GluR5–7 and KA1 and KA2 receptor subunits. Whereas AMPA shows low or no affinity for kainic acid receptors, kainic acid exerts a non-desensitizing response at AMPA receptors resulting in large current flows (Fletcher and Lodge, 1996). Thus, the observed effects of kainic acid at native ionotropic (*S*)-Glu receptors, in most cases, probably reflect interactions with AMPA receptors (Lees, 2000). Design of ligands that effectively discriminate between AMPA and kainic acid receptors represents a major challenge.

Initial pharmacological investigations indicated that (*RS*)-2-amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid (ATPA) (Fig. 1) was a relatively weak AMPA receptor agonist (Lauridsen et al., 1985). However, recent studies have shown that ATPA is a selective and highly potent agonist at homomerically expressed GluR5 (Clarke et al., 1997), and that [^{3}H]ATPA binds to cloned human GluR5 with a K_D of 13 nM (Hoo et al., 1999). The GluR5 agonist activity stems solely from the *S*-enantiomer, whereas (*R*)-ATPA has been characterized as a weak AMPA receptor antagonist (Stensbøl et al., 1999). (*RS*)-2-Amino-3-(5-*tert*-butyl-3-hydroxy-4-isothiazolyl)propionic acid (thio-ATPA), a 3-isothiazolol analogue of ATPA, has previously been described as an AMPA receptor agonist slightly more potent than ATPA (Matzen et al., 1997). We now describe the chiral chromatographic resolution and the pharmacological characterization of the enantiomers of thio-ATPA using native as well as cloned ionotropic (*S*)-Glu receptors.

2. Materials and methods

2.1. Materials and analytical methods

Melting points were determined in capillary tubes and are uncorrected. ^{1}H NMR spectra were recorded in D_2O on a Varian Gemini-2000 BB (300 MHz) NMR spectrometer using acetonitrile as internal standard (δ 2.06). IR spectra were recorded from KBr discs on a Perkin-Elmer 781 grating infrared spectrophotometer. Optical rotations were measured in thermostated cuvettes on a Perkin-Elmer 241 polarimeter. Circular dichroism (CD) spectra were recorded in 0.1 M HCl in 1.0 cm cuvettes at room temperature on a Jasco J-720 spectropolarimeter. Elemental analyses were performed at Microanalytical Laboratory, Department of Physical Chemistry, University of Vienna, Austria, and were within $\pm$0.4% of the calculated values.

[^{3}H]AMPA (53.1 Ci/mmol), [^{3}H]kainic acid (58.0 Ci/mmol), and [^{3}H]3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid ([^{3}H]CPP) (36.0 Ci/mmol) were all purchased from New England Nuclear (Boston, MA, USA). All other chemicals were purchased through standard commercial sources.

2.2. High performance liquid chromatography (HPLC)

The preparative chiral chromatographic resolution of thio-ATPA was performed on a Chirobiotic T column (10 × 500 mm, ASTEC) equipped with a Chirobiotic T guard column (4.6 × 50 mm, ASTEC) eluted with 1.5 ml/min of 15 mM aqueous ammonium acetate/acetic acid buffer (pH 4.0):acetonitrile (80:20). The HPLC system consisted of a Jasco M880 pump, a Rheodyne 7125 injector, a 5-ml loop, a Waters M481 spectrophotometer (240 nm), and a Hitachi D-2000 Chromato-Integrator. Reverse-phase HPLC using a Knauer LiChrosorb RP-18 column (16 × 250 mm, 5 μm) was performed on the HPLC system described above. The column was eluted at 4.0 ml/min with water:methanol (70:30). Enantiomeric excess (ee), calculated from peak areas, was determined using an analytical Chirobiotic T column (4.6 × 150 mm, ASTEC) equipped with a Chirobiotic T (4.6 × 50 mm, ASTEC) guard column. Elution was performed with 0.5 ml/min of 15 mM aqueous ammonium acetate/acetic acid buffer (pH 4.0):ethanol (50:50) and aqueous 15 mM aqueous ammonium acetate/acetic acid buffer (pH 4.0):acetonitrile (80:20) for (*S*)- and (*R*)-thio-ATPA, respectively. The HPLC system used for the analytical determinations consisted of a Waters M510 pump, a Waters U6K injector, and a Waters M991 photodiode array detector, set at 270 nm.

2.3. Resolution of (S)- and (R)-thio-ATPA

Thio-ATPA, hydrobromide (200 mg, 0.615 mmol) (Matzen et al., 1997), was dissolved in 15 mM aqueous ammonium acetate/acetic acid buffer (pH 4.0):acetonitrile (80:20) (5.0 mg/ml), filtered through a 0.45 μm membrane filter, (Millipore, type HV) and resolved in 5 mg injections. The pooled fractions of the first peak were evaporated, re-evaporated from water three times, and

dried in vacuo over P_2O_5 and KOH. In order to remove traces of ammonium acetate, the crude product was dissolved in water (10 ml) and in six injections passed through a reverse-phase column. The collected fractions were pooled, evaporated, re-evaporated three times from water, and recrystallized from water (1–2 ml) to give 38.7 mg of (*S*)-thio-ATPA (52% yield; 99.5% ee). m.p. > 250°C. ^{1}H NMR δ 3.95 (1H, dd, $J = 4.3$ and 9.1 Hz), 3.32 (1H, dd, $J = 9.1$ and 15.6 Hz), 3.32 (1H, dd, $J = 4.3$ and 15.6 Hz), 2.98 (1H, dd, $J = 9.1$ and 15.6 Hz), 1.42 (9H, s). IR 3300–2800 (br, m), 2960 (m-s), 2700–2500 (br, w), 1670–1580 (br, s), 1395 (m-s), 1360 (m-s) cm^{-1}. $[\alpha]_D^{25} = +66°$, $[\alpha]_{365}^{25} = +273°$ ($c = 0.35$, 0.1 M HCl). $\Delta\varepsilon$ (210 nm) = +0.28 m^2/mol. Anal. ($C_{10}H_{16}N_2O_3S$) C, H, N. The mother liquor furnished an additional 16.1 mg (99.7% ee) of recrystallized (*S*)-thio-ATPA (total yield 73%), having a melting point and IR spectrum identical with those of the first crop. The pooled fractions of the second peak were treated as described for the first eluting enantiomer to give 36.0 mg of (*R*)-thio-ATPA (48% yield, 96.9% ee). m.p., IR and ^{1}H NMR spectra identical with those of (*S*)-thio-ATPA. $[\alpha]_D^{25} = -66°$, $[\alpha]_{365}^{25} = -271°$ ($c =$ 037, 0.1 M HCl). $\Delta\varepsilon$ (210 nm) = −0.29 m^2/mol. Anal. ($C_{10}H_{16}N_2O_3S$) C, H, N, S. The mother liquor was evaporated and dried in vacuo over P_2O_5 and KOH to give 30.4 mg (99.0% ee) (total yield 88%). Anal. ($C_{10}H_{16}N_2O_3S$, 1/3 H_2O) C, H, N. IR spectrum and m.p. identical with those of the recrystallized product of (*R*)-thio-ATPA.

2.4. Receptor binding assays and electrophysiology at native ionotropic (S)-Glu receptors

The membrane preparations used in all receptor binding experiments were prepared according to Ransom and Stec (1988) with slight modifications as described by Stensbøl et al. (1999). Affinity for AMPA, kainic acid and NMDA receptors was determined using [^{3}H]AMPA (Honoré and Nielsen, 1985), [^{3}H]kainic acid (Braitman and Coyle, 1987), and [^{3}H]CPP (Murphy et al., 1987), respectively, with the modifications described by Stensbøl et al. (1999).

A previously described rat cortical wedge preparation (Harrison and Simmonds, 1985) in a slightly modified version (Madsen et al., 1993) was used for the evaluation of the interaction with the native ionotropic (*S*)-Glu receptors. Agonists were applied for 90 s. In experiments for the characterization of antagonists, compounds were applied for 90 s prior to a 90-s co-application of agonists and antagonists.

2.5. In vitro cRNA transcription

The in vitro run-off transcriptions were performed with ~25 μg/ml linearized templates (GluR1 flop (GluR1o), GluR3 flop (GluR3o), GluR4 flop (GluR4o), GluR5-1a, Glu R6 and KA2) in 40 mM Tris/HCl (pH 7.5), 6 mM $MgCl_2$, 5 mM NaCl, 2 mM spermidine, 10 mM DTT, 0.5 mM ATP, 0.5 mM UTP, 0.5 mM CTP, 0.1 mM GTP, 0.5 mM GpppG, 300–500 U/ml T7 polymerase (Stratagene), 400 U/ml RNase block (Promega), and 5–10 μCi/ml [^{32}P] UTP (Amersham) for quantification of the cRNAs. The transcriptions were incubated 1–2 h at 37°C.

2.6. Electrophysiology

Xenopus laevis were anesthetized in a 0.1–0.2% MS-222 (3-aminobenzoic acid ethyl ester, Sigma) solution for 15–30 min. Several ovary were removed surgically, after which the follicle layer was removed by digestion with 1 mg/ml collagenase A (Boehringer Mannheim) in OR-2 (82.5 mM NaCl, 2.0 mM KCl, 1.0 mM $MgCl_2$, 5.0 mM HEPES (pH 7.5)) at room temperature for 2–3 h. Healthy looking stage V–VI oocytes were selected after digestion and maintained in Barths' Saline (88.0 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 15.0 mM HEPES (pH 7.6), 0.30 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml gentamycin) at 18°C and injected the following day with 5–30 ng of cRNA. Currents were recorded 3–14 days after injection using a two-microelectrode clamp (OC-725C Oocyte clamp, Warner Instrument). Experiments were performed in low Ca^{2+}-Ringer (10 mM Na–HEPES pH 7.5, 1165 mM NaCl, 0.1 mM $CaCl_2$, 2.5 mM KCl, 1.8 mM $MgCl_2$) to avoid activation of the endogenous Ca^{2+} dependent Cl^- channel. The electrodes were filled with 3 M KCl and had a resistance of 0.7–2 MΩ. Oocytes were clamped at −100– − 20 mV, and recordings on homomeric GluR5 and GluR6 were performed after 10-min treatment with concanavalin A (1 mg/ml, Sigma type IV) to minimize receptor desensitization.

2.7. Data analysis

Binding data and cortical wedge data were analyzed by the nonlinear curve fitting program GRAFIT 3.0 (Leatherbarrow, 1992). Data were fitted to the following equation: $B = 100 - (100 \times [\text{Inhibitor}]^n/(\text{IC}_{50}^n + [\text{Inhibitor}]^n)$, where B is the binding as a percentage of total specific binding and n the Hill coefficient. For two-site fit the following equation was used: $B = B_{\text{Max1}} \times (1 - \text{Inhibitor}]/(\text{IC}_{50(1)} + [\text{Inhibitor}])) + (100 - B_{\text{max1}}) \times (1 - \text{Inhibitor}]/(\text{IC}_{50(2)} + [\text{Inhibitor}]))$. EC_{50} values were determined using the equation: % response = $(E_{\max} \times [\text{Agonist}]^n)/(\text{EC}_{50}^n + [\text{Agonist}]^n)$, where $E_{\max}$ is the relative maximal response and n is the Hill coefficient.

Data on the cloned receptor subtypes were acquired using Clampex 7.0 (Axon Instruments) and subsequently processed with Sigmaplot 3.0 (Jandel Scientific, SPSS). Dose–response data from individual oocytes were fitted to the equation $I = I_{\max} \times [\text{Agonist}]^n/(\text{EC}_{50}^n + [\text{Agonist}]^n)$, where I is the current, $I_{\max}$ is the maximum current in

response to the action of the agonist, and n denotes the Hill coefficient.

Relative maximal currents (Relative I_{max}) were calculated as $I_{max\ ligand}/I_{max\ kainic\ acid\ or\ AMPA}$ where $I_{max\ ligand}$ is the fitted I_{max} according to the equation (see above). $I_{max\ kainic\ acid\ or\ AMPA}$ is calculated from the current evoked by kainic acid (for GluR1o, GluR3o, GluR4o, and GluR5) or AMPA (for GluR6 + KA2) and the EC_{50} values previously defined in our laboratory for the two agonists (kainic acid EC_{50}-values/n-values for homomeric GluR1o, GluR3o, GluR4o, GluR5 have been determined to be 55.1 μM/1.28; 27.1 μM/2.30; 29.8 μM/1.80; 9.6 μM/1.38, respectively, and AMPA EC_{50} value/n value for heteromeric GluR6 + KA2 to be 110.0 μM/1.84).

The mean and S.E.M. are calculated assuming a logarithmic distribution of the EC_{50} value, (Christopoulos, 1998). Here, numbers in parenthesis (min; max) indicate ±S.E.M. according to a logarithmic distribution of EC_{50}. t-Tests were used in order to prove differences in I_{max} and pEC_{50} values.

3. Results

3.1. Chromatographic resolution of (R)-(−)-thio-ATPA and (S)-(+)-thio-ATPA and configurational assignment

The resolution of thio-ATPA was performed by chiral HPLC using the Chirobiotic T column (Armstrong et al., 1995), which contains the macrocyclic glycopeptide, Teicoplanin, as chiral selector (Fig. 2). This specialized column, which previously has been successfully used for semipreparative enantioseparations of a number of 3-isoxazolol containing α-amino acids (Johansen et al., 1997; Stensbøl et al., 1999; Vogensen et al., 2000), was shown also to be very effective for the enantioseparations of the 3-isothiazolol containing α-amino acid, thio-ATPA. The resolution was performed using a volatile mobile phase of aqueous ammonium acetate and acetonitrile. After the chromatographic enantioseparation, the enantiomeric excess of (*S*)- and (*R*)-thio-ATPA was 99.6% and 98.0%, respectively. The following recrystallizations resulted in well-defined products but with lower stereochemical purity than before the recrystallizations. This reduction in stereochemical purity was not caused by stereochemical instability of the enantiomers, since an enrichment in the stereochemical purity of the mother liquors was observed for both enantiomers. Despite this enrichment of the stereochemical purity of the mother liquor, crystalline (*S*)- and (*R*)-thio-ATPA could be isolated with stereochemical purities of 99.5% and 96.9% ee, respectively.

The absolute configuration of the two enantiomers was unequivocally established by an X-ray crystallographic analysis of (*R*)-thio-ATPA (Fig. 3). A detailed description of the X-ray structure will be published elsewhere.

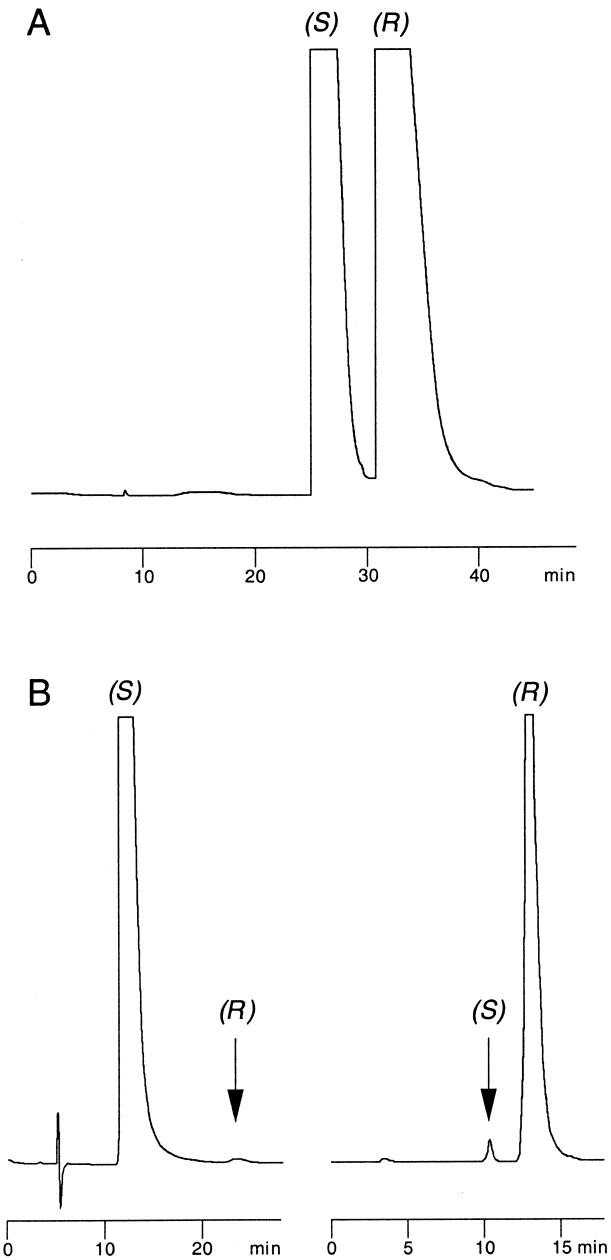

Fig. 2. Chiral HPLC chromatograms. (A) Chromatogram showing resolution of 5 mg of thio-ATPA on a Chirobiotic T column (10×500 mm). Mobile phase: ammonium acetate/acetic acid buffer (pH 4.0):acetonitrile (80:20). Flow: 1.5 ml/min. (B) Chromatograms showing injections of recrystallized (*S*)-thio-ATPA (left; ee = 99.5%) and (*R*)-thio-ATPA (right; ee = 96.9%) on a Chirobiotic T column (4.6×150 mm). Mobile phases: ammonium acetate/acetic acid buffer, pH 4.0:ethanol (50:50) and ammonium acetate/acetic acid buffer, pH 4.0:acetonitrile (80:20) for (*S*)- and (*R*)-thio-ATPA, respectively. Flow: 0.5 ml/min. Spike experiments of (*S*)- and (*R*)-thio-ATPA with thio-ATPA confirmed the identity of the observed enantiomeric impurities (not illustrated).

3.2. Homogenate receptor binding experiments

The affinities towards native AMPA, kainic acid or NMDA receptor were established using the radioligands

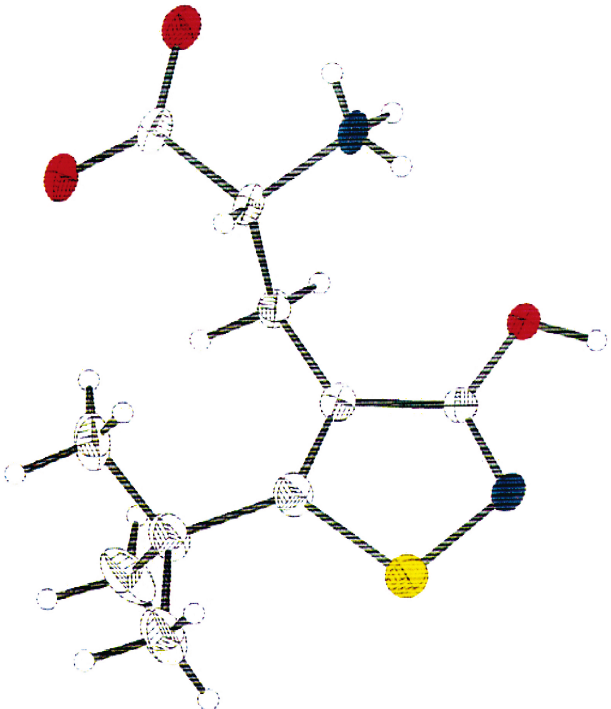

Fig. 3. Molecular structure (ORTEPII, Johnson, 1976) of (*R*)-(−)-thio-ATPA, determined by X-ray crystallography. The absolute configuration was determined to be *R* as indicated. The displacement ellipsoids of the non-hydrogen atoms are shown at the 50% probability level. The crystal structure determination revealed that both the enol and the ketone tautomers of the 3-isothiazolol moeity were present in the unit cell.

[^{3}H]AMPA, [^{3}H]kainic acid, or [^{3}H]CPP, respectively (Table 1). The affinity of the *S*-enantiomer at AMPA and kainic acid receptors was found to be twice the affinity found for the racemate, demonstrating that the *S*-enantiomer is the active enantiomer at these receptors. (*S*)-Thio-ATPA was devoid of affinity towards NMDA receptors. At [^{3}H]kainic acid labelled receptors, thio-ATPA and (*S*)-thio-ATPA were found to displace binding of the radioligand with low affinities (Table 1). Hill coefficients (n) were in both cases low ($n = 0.49$ and 0.50, respectively) indicating that thio-ATPA and (*S*)-thio-ATPA were able to discriminate [^{3}H]kainic acid labelled binding sites. Data points (1, 2, 5,... 100,000 nM) were fitted to equations assuming two independent binding sites as described in Section 2.7 and Table 1. *F*-tests ($P > 0.05$), however, did not prove that equations assuming two sites fit the data better than equation assuming only one site. (*R*)-Thio-ATPA showed neither displacement of bound [^{3}H]CPP nor [^{3}H]kainic. Low affinity was detected towards [^{3}H]AMPA labelled receptors ($IC_{50} = 31$ μM), most probably reflecting the small amount of (*S*)-thio-ATPA used.

3.3. Pharmacology at native ionotropic (S)-Glu receptors using the rat cortical wedge preparation

The functional properties of the two enantiomers at native receptors were established using the rat cortical wedge preparation. (*S*)-Thio-ATPA ($EC_{50} = 8.7$ μM) was found to be twice as potent as the racemate. This response could be antagonized by 5 μM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX), implying that depolarization was induced through interactions with AMPA receptors (Table 1 and Fig. 4). We have previously reported that depolarizations obtained by kainic acid applications results in a bi-phasic dose–response curve (Stensbøl et al., 1999), most probably reflecting the interaction of kainic acid with both kainic acid and AMPA receptors. For thio-ATPA, there was no indication of a bi-phasic dose–response. Thus, (*S*)-thio-ATPA was found to be an AMPA receptor agonist approximately three times as potent as the 3-isoxazolol analogue, (*S*)-ATPA.

(*R*)-Thio-ATPA (2 mM) induced a small depolarization, which was approximately 20% of a maximal re-

Table 1
Homogenate membrane receptor binding and rat cortical wedge electrophysiology

	IC_{50} (μM)			EC_{50} (μM)
	[^{3}H]AMPA	[^{3}H]Kainic acid	[^{3}H]CPP	Electrophysiology
AMPA[a]	0.039 (0.037; 0.041)	> 100	> 100	3.5 (3.3; 3.8)
ATPA	3.9 (3.3; 4.4)	35 (31; 40)	> 100	48[b]
(*R*)-ATPA	> 100	> 100	> 100	> 1000
(*S*)-ATPA	1.8 (1.6; 2.0)	23 (22; 24)	> 100	19 (17; 22)
Thio-ATPA	0.57 (0.54; 0.61)	22[c] (20; 25)	> 100	14 (12; 16)
(*R*)-Thio-ATPA	31 (22; 45)	> 100	> 100	> 1000
(*S*)-Thio-ATPA	0.27 (0.22; 0.34)	14[c] (11; 18)	> 100	8.7 (8.1; 9.3)

Values are expressed as the antilog to the log meanof at least three individual experiments. The numbers in parenthesis (min; max) indicate ± S.E.M. according to a logarithmic distribution. The % ee of (*R*)-ATPA, (*S*)-ATPA,(*R*)-thio-ATPA and (*S*)-thio-ATPA tested were 99.8% ee, > 99.8%, 99.0% ee and 99.2%, respectively.

[a] Vogensen et al., 2000.

[b] Ebert et al., 1992.

[c] Hill coefficients were 0.49 and 0.50 for thio-ATPA and (*S*)-thio-ATPA. Hence IC_{50} values of equations assuming two-site fit were calculated as explained in Section 2.7. For thio-ATPA, $IC_{50(1)}$, $IC_{50(2)}$, and B_{max1} were 2.0 (1.9; 2.1), > 200, 50 ± 2 μM, respectively. For (*S*)-thio-ATPA, $IC_{50(1)}$, $IC_{50(2)}$, and B_{max1} were 1.2 (0.89; 1.6), > 200, 53 ± 2 μM.

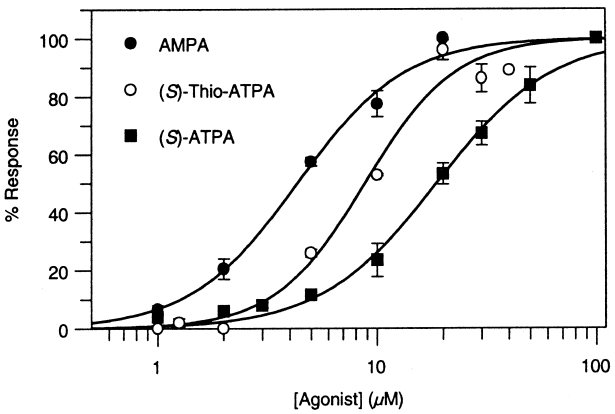

Fig. 4. Dose–response curves obtained in the rat cortical wedge preparation, showing percentage of maximal responses elicited by increasing concentrations of agonists. Each data point represents the mean value of at least three individual experiments.

sponse. Similar to the response of (*S*)-thio-ATPA, this response could be blocked by 5 μM NBQX, indicating that this response most probably originates from the small amount of (*S*)-thio-ATPA, which is present in the (*R*)-preparation.

3.4. Pharmacology at cloned ionotropic (S)-Glu receptors

Racemic thio-ATPA, (*S*)- or (*R*)-thio-ATPA were applied on two electrode voltage clamped *Xenopus* oocytes expressing homomeric AMPA receptors composed of either GluR1o, GluR3o, or GluR4o, and on homomeric kainic acid receptors GluR5o, GluR6 or heteromeric GluR6 + KA2. The large size of the oocytes prevents resolution of the peak response. Accordingly, the EC_{50} values are calculated from steady-state currents evoked by the agonists.

(*S*)-Thio-ATPA was shown to be an agonist on all of the AMPA receptor subtypes, with an EC_{50} value of 5.1 μM for GluR1o and slightly higher EC_{50} values of 32 and 20 μM for GluR3o and GluR4o, respectively (Table 2, Figs. 5A and 6). Racemic thio-ATPA exhibited approximately half the potency observed for the *S*-enantiomer (Table 2). The amplitudes of the maximal steady-state currents (I_{max}) relative to the maximal responses evoked by kainic acid were lowest for GluR1o (78%) intermediate for GluR3o (120%) and highest for GluR4o (170%). The relative maximal steady-state currents evoked by racemic thio-ATPA were in the same range (Table 2). No antagonistic effects were observed after co-application of 100 μM (*R*)-thio-ATPA and 30 μM kainic acid on AMPA receptor subtypes. (*R*)-thio-ATPA induced small non-saturating responses on AMPA receptor subtypes (data not shown). However, these effects can be explained by a contamination of 0.5% (*S*)-thio-ATPA in the (*R*)-thio-ATPA (99.0% ee).

Thio-ATPA and the *S*- and *R*-enantiomers were studied on the GluR5-1a splice variant of the kainic acid preferring GluR5 (Bettler et al., 1990; Sommer et al., 1992). Although receptor desensitization was reduced by extensive treatment with concanavalin A prior to ligand application, a significant desensitization was observed for thio-ATPA and (*S*)-thio-ATPA at concentrations > 1 μM implied by a reduction in the current amplitude of the following applications of kainic acid. A similar observation has previously been reported for ATPA and (*S*)-ATPA (Stensbøl et al., 1999). Consequently, the induced currents were normalized to subsequent applications of 25 μM kainic acid. Thio-ATPA and (*S*)-thio-ATPA were highly potent agonists on GluR5 with EC_{50} values of 0.26 and 0.10 μM, respectively (Table 2). The maximal currents induced by the racemate and the *S*-enantiomer were in the same range as the maximal current induced by kainic acid

Table 2
Apparent potencies and relative maximal steady state currents of racemic thio-ATPA, (*S*)-thio-ATPA and (*S*)-ATPA on homomeric and heteromeric ionotropic (*S*)-Glu receptors

Receptor	Thio-ATPA			(*S*)-Thio-ATPA			(*S*)-ATPA		
	EC_{50}(μM)	*n*	Relative I_{max}	EC_{50} (μM)	*n*	Relative I_{max}	EC_{50} (μM)	*n*	Relative I_{max}
GluR1o	9.1 (7.8; 11)	1.1	0.86 ± 0.050	5.2 (4.8; 5.6)[a]	0.64	0.78 ± 0.026[a]	22 (18; 27)	1.6	0.33 ± 0.11
GluR3o	50 (40; 61)	1.2	0.78 ± 0.046	32 (31; 33)[a]	0.90	1.2 ± 0.015[b]	7.9 (6.6; 9.2)	0.84	0.054 ± 0.002
GluR4o	55 (46; 68)	1.0	1.4 ± 0.20	20 (15; 27)[c]	0.80	1.7 ± 0.095[b]	7.6 (5.6; 9.6)	0.67	0.34 ± 0.051
GluR5	0.26 (0.23; 0.31)	1.1	0.78 ± 0.14	0.10 (0.09; 0.12)[a]	1.5	1.0 ± 0.027	0.48 (0.44; 0.52)	1.0	1.0 ± 0.083
GluR6	n.r.			n.r.			n.r.		
GluR6 + KA2	30 (26; 36)	1.7	0.49 ± 0.043	4.9 (4.6; 5.3)[b]	1.9	0.41 ± 0.040[a]	61 (58; 64)	2.0	0.17 ± 0.011

Values are expressed as the antilog to the log mean of at least threeindividual experiments. The numbers in parenthesis (min; max) indicate ± S.E.M. according to a logarithmic distribution. The I_{max} values are relative to maximal steady state currents evoked by kainic acid for all the receptor combinations, except GluR6 + KA2 heteromers, where the amplitudes are relative to the maximal currents evoked by AMPA. The number of observations is between 3 and 7. n.r.: no response at 300 μM. The % ee of (*S*)-ATPA, and (*S*)-thio-ATPA tested were $> 99.8\%$ and 99.2%, respectively. (*S*)-ATPA data are adapted from Stensbøl et al. (1999).

Different superscript letters a, b, c indicates that a *t*-test showed differences in pEC_{50} and I_{max} values determined for (*S*)-thio-ATPA and (*S*)-ATPA with a significance level of $P < 0.01$, $P < 0.001$ and $P < 0.05$, respectively.

(Table 2). These were, however, markedly different from those evoked by (*S*)-ATPA, as listed in Table 2.

Applications of up to 300 μM (*R*)-thio-ATPA on homomeric GluR5 evoked saturating currents with an estimated EC_{50} value of ~10 μM and an I_{max} in the same range as kainic acid. Co-application of 30 μM kainic acid and 100 μM (*R*)-thio-ATPA induces currents ~1.2-fold higher than 30 μM kainic acid alone. However, it cannot be ruled out that this activity of the *R*-enantiomer arises from an (*S*)-thio-ATPA contamination in the *R*-enantiomer as indicated above. Oocytes expressing homomeric GluR6 receptors responded to 300 μM (*S*)-thio-ATPA with less than 1% of the maximal kainic acid response after extensive treatment with concanavalin A. No currents could be induced by similar concentrations of either thio-ATPA or (*R*)-thio-ATPA.

The heteromeric GluR6 + KA2 combination was activated by thio-ATPA and (*S*)-thio-ATPA with potencies of approximately 30 and 4.9 μM and relative maximal currents of 49% and 41%, respectively (Table 2). At concentrations above 100 μM, the *R*-enantiomer evoked detectable responses with an estimated EC_{50} value more than 500-fold higher than the value found for the *S*-enantiomer (data not shown). The amplitude of the currents evoked by 1000 μM (*R*)-thio-ATPA was approximately 8% of the maximal AMPA current, equivalent to the amplitude evokable by 2.3 μM (*S*)-thio-ATPA. Hence, (*S*)-thio-ATPA contamination in the (*R*)-thio-ATPA of less than 0.5% would explain the currents observed on GluR6 + KA2. Additionally, no significant inhibition could be observed with co-applications of 50 μM AMPA and 100 μM (*R*)-thio-ATPA on the heteromeric receptor complex (data not shown).

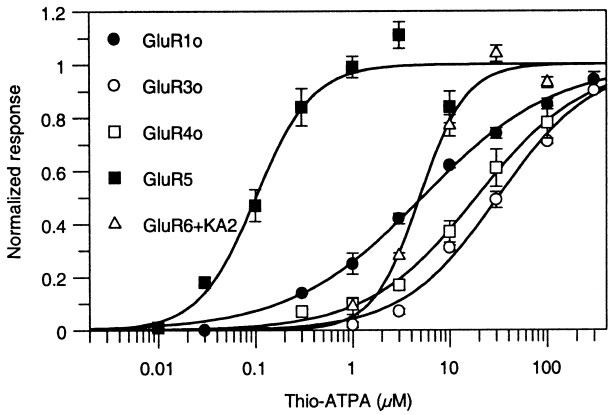

Fig. 6. Normalized dose–response of (*S*)-thio-ATPA at recombinant AMPA-preferring receptors (GluR1o, GluR3o and GluR4o) and at kainic acid preferring receptors (GluR5-1a and GluR6 + KA2) expressed in *Xenopus* oocytes.

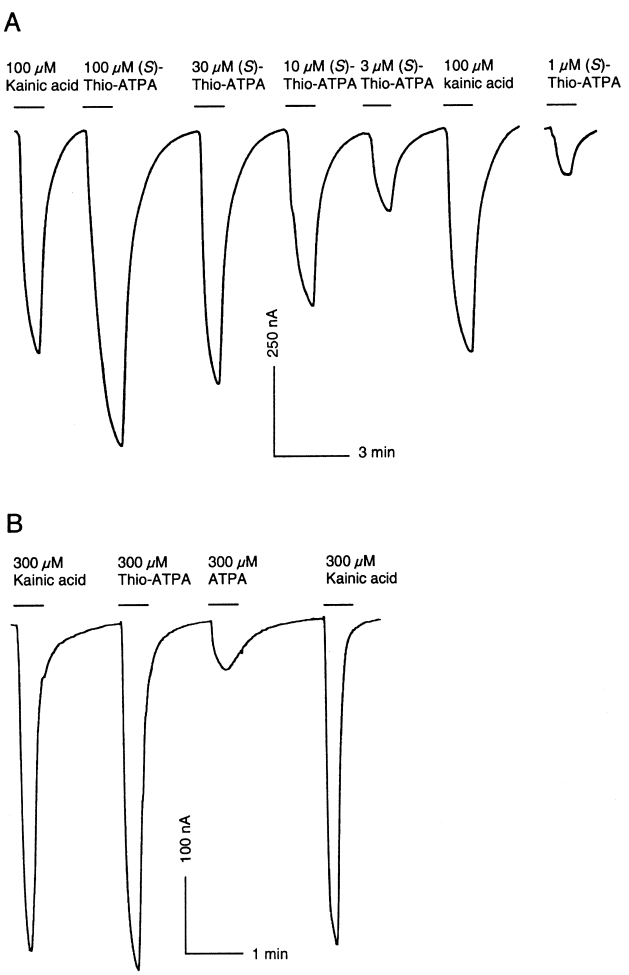

Fig. 5. Current traces of two electrode voltage clamped *Xenopus* oocytes expressing GluR4o receptors. (A) Trace showing responses after application of 100 μM kainic acid and 1–100 μM of (*S*)-thio-ATPA. (B) Trace showing responces elicited by applications of 300 μM kainic acid, 300 μM thio-ATPA and 300 μM ATPA.

4. Discussion

Over the last decade, it has become clear that AMPA and kainic acid receptors are distinct receptor populations. Amongst each of these receptor families, the diversity of receptors with different subunit compositions, and hence dissimilar pharmacology, is pronounced. The physiological relevance of these different subtypes of receptors is, however, only just beginning to be revealed. It has been suggested that presynaptic kainic acid receptors may be implicated in epilepsy through a depression of inhibitory postsynaptic potentials (IPSPs) (Clarke et al., 1997), and it has been shown that the selective GluR5 agonist ATPA is capable of inducing such depressions. Conversely, it has also been suggested that GluR5 may be involved in the regulation of excitatory synaptic transmission in the CA1 and CA3 region of the hippocampus (Vignes et al., 1998). Thus, GluR5 containing receptors might be useful targets for therapeutical intervention in epilepsy.

We have previously shown that (*S*)-ATPA activates homomerically expressed GluR5 in a potent and selective

manner. Thus, the bulky *tert*-butyl group in (*S*)-ATPA (Fig. 1) is very well accomodated by the ligand binding site at the GluR5. Although the 3-isothiazolol (pK_a 7.0) moiety of thio-ATPA is markedly less acidic than the 3-isoxazolol (pK_a 4.8) moiety in ATPA, it has earlier been proven to be an effective bioisostere of the distal carboxyl group of (*S*)-Glu (Matzen et al., 1997). In light of these findings, we decided to study the pharmacology of the two enantiomers of thio-ATPA at native as well as cloned receptors. The results presented in this paper indicate that the modes of interaction of (*S*)-thio-ATPA with ionotropic (*S*)-Glu receptors are different from those of (*S*)-ATPA.

Pharmacological characterization of the two enantiomers at native receptors showed that (*S*)-thio-ATPA was twice as potent as the racemate at all ionotropic (*S*)-Glu receptors tested, indicating that the activity stems solely from the *S*-enantiomer. Whereas depolarizations observed after applications of (*S*)-thio-ATPA in the rat cortical wedge preparation could be assigned to interactions with AMPA receptors, (*S*)-thio-ATPA showed affinity towards both [^{3}H]AMPA and [^{3}H]kainic acid labelled receptors. In contrast to (*S*)-ATPA, (*S*)-thio-ATPA inhibited the binding of [^{3}H]kainic acid labelled receptors in a bi-phasic manner. The proposed two-site fit and the affinity of approximately 1 μM towards B_{max1} of (*S*)-thio-ATPA is an indication of the interaction with kainic acid receptors, but this binding affinity is not consistent with the potency observed using recombinant receptors.

The electrophysiological characterizations of the enantiomers on recombinant receptors revealed that (*S*)-thio-ATPA is an agonist at AMPA and, in particular, at kainic acid preferring receptors with roughly 50- to 300-fold higher potency at homomeric GluR5 than at homomeric GluR1o, GluR3o, GluR4o or heteromeric GluR6 + KA2 receptors (Table 2). (*S*)-thio-ATPA shows a significantly higher selectivity ratio (200/320-fold) between GluR5 and GluR3o/GluR4o than (*S*)-ATPA (16/16-fold). The selectivity ratio between GluR5 and GluR1o is the same for the two compounds, whereas the selectivity ratio between GluR5 and GluR6 + KA2 is slightly higher for (*S*)-ATPA as compared to (*S*)-thio-ATPA. The amplitude of the currents (relative I_{max}) evoked by (*S*)-thio-ATPA at GluR1, GluR2 and GluR4 are significantly ($P = 0.0073$, 0.0001 and 0.0001, respectively) higher than those evoked by (*S*)-ATPA (Table 2). An explanation to this is likely to be found in the sterical and electrostatic properties of the sulfur atom in thio-ATPA (Fig. 1). Firstly, sulfur is more bulky than oxygen, and thus requires more space, and sulfur does not accommodate hydrogen bonding as potently as oxygen. Furthermore, the increase in potency observed for (*S*)-thio-ATPA may reflect a higher degree of solvatization of thio-ATPA as compared to ATPA, in spite of the lower acidic character of the 3-isothiazolol nucleus of thio-ATPA as compared to that of the 3-isoxazolol nucleus of ATPA, as mentioned previously. The larger currents evoked by (*S*)-thio-ATPA might reflect a smaller degree of receptor desensitization at AMPA receptors as compared to the effects of (*S*)-ATPA. Another explanation could be that ATPA with respect to thio-ATPA is acting as a partial agonist at recombinantly expressed homomeric AMPA receptors. However, it remains to be elucidated whether these hypotheses are sustainable. It is concluded that the 3-isothiazolol unit present in (*S*)-thio-ATPA enhances both potency at and selectivity towards GluR5 as compared to the 3-isoxazolol unit in (*S*)-ATPA. Furthermore, the currents induced by (*S*)-thio-ATPA are significantly larger than those evoked by the 3-isoaxzolol containing (*S*)-ATPA, suggesting that these two agonists interact with the receptor in different ways. These observations may be useful in the future design of ionotropic (*S*)-Glu receptor ligands as pharmacological tools for studies of the mechanisms underlying AMPA receptor activation and desensitization.

Acknowledgements

The technical assistance of Ms. Heidi Petersen and Mrs. Annette Kristensen, Department of Medicinal Chemistry, The Royal School of Pharmacy, and Mrs. Karen Jørgensen, Department of Chemistry, University of Copenhagen is gratefully acknowledged.

References

Armstrong, D.W., Liu, Y., Ekborgott, K.H., 1995. A covalently bonded teicoplanin chiral stationary phase for HPLC enantioseparations. Chirality 7, 474–497.

Bettler, B., Boulter, J., Hermans-Borgmeyer, I., O'Shea-Greenfield, A., Deneris, E.S., Borgmeyer, U., Hollmann, M., Heinemann, S., 1990. Cloning of a novel glutamate receptor subunit, GluR5: expression in the nervous system during development. Neuron 5, 583–595.

Braitman, D.J., Coyle, J.T., 1987. Inhibition of [^{3}H]kainic acid receptor binding by divalent cations correlates with ion affinity for the calcium channel. Neuropharmacology 26, 1247–1251.

Braüner-Osborne, H., Egebjerg, J., Nielsen, E.Ø., Madsen, U., Krogsgaard-Larsen, P., 2000. Ligands for glutamate receptors: design and therapeutic prospects. J. Med. Chem. 43, 2609–2645.

Chittajallu, R., Braithwaite, S.P., Clarke, R.J.V., Henley, J.R., 1999. Kainate receptors: subunit, synaptic localization and function. Trends. Pharmacol. Sci. 20, 26–35.

Christopoulos, A., 1998. Assessing the distribution of parameters in models of ligand-receptor interaction: to log or not to log. Trends. Pharmacol. Sci. 19, 351–357.

Clarke, V.R.J., Ballyk, B.A., Hoo, K.H., Mandelzys, A., Pellizzari, A., Bath, C.P., Thomas, J., Sharpe, E.F., Davies, C.H., Ornstein, P.L., Schoepp, D.D., Kamboj, R.K., Collingridge, G.L., Lodge, D., Bleakman, D., 1997. A hippocampal GluR5 kainate receptor regulating inhibitory synaptic transmission. Nature 389, 599–603.

Collingridge, G., Watkins, J.C., 1994. The NMDA Receptor. Oxford University Press, Oxford.

Conn, P.J., Pin, J.-P., 1997. Pharmacology and functions of metabotropic glutamate receptors. Annu. Rev. Pharmacol. Toxicol. 37, 205–237.

Ebert, B., Madsen, U., Lund, T.M., Holm, T., Krogsgaard-Larsen, P., 1992. Molecular pharmacology of cortical and spinal AMPA receptors. Mol. Neuropharmacol. 2, 47–49.

Fletcher, E.J., Lodge, D., 1996. New developments in the molecular pharmacology of α-amino-3-hydroxy-4-isoxazole propionate and kainate receptors. Phamocol. Ther. 70, 65–89.

Harrison, N.L., Simmonds, M.A., 1985. Quantitative studies on some antagonists of N-methyl-D-aspartate in slices of rat cerebral cortex. Br. J. Pharmacol. 84, 381–391.

Honoré, T., Nielsen, M., 1985. Complex structure of quisqualate-sensitive glutamate receptors in rat cortex. Neurosci. Lett. 54, 27–32.

Hoo, K., Legutko, B., Rizkalla, G., Deverill, M., Hawes, C.R., Ellis, G.J., Stensbøl, T.B., Krogsgaard-Larsen, P., Skolnick, P., Bleakman, D., 1999. [^{3}H]ATPA: a high affinity ligand for GluR5 receptor. Neuropharmacology 38, 1811–1817.

Johansen, T.N., Ebert, B., Falch, E., Krogsgaard-Larsen, P., 1997. AMPA receptor agonists: resolution, configurational assignment, and phamacology of (+)-(*S*)- and (−)-(*R*)-2-amino-3-[3-hydroxy-5-(2-pyridyl)-isoxazol-4-yl]-propionic acid (2-Py-AMPA). Chirality 9, 274–280.

Johnson, C.K., 1976. ORTEPII. Report ORNL-5138. Oak-Ridge National Laboratory, TN, USA.

Jonas, P., Monyer, H., 1999. Handbook of Experimental Pharmacology, Ionotropic Glutamate Receptors in the CNS vol. 141. Spinger-Verlag, Berlin.

Knöpfel, T., Kuhn, R., Allgeier, H., 1995. Metabotropic glutamate receptors: novel targets for drug development. J. Med. Chem. 38, 1417–1426.

Lauridsen, J., Honoré, T., Krogsgaard-Larsen, P., 1985. Ibotenic acid analogues. Synthesis, molecular flexibility, and in vitro activity of agonists and antagonists at central glutamic acid receptors. J. Med. Chem. 28, 668–672.

Leatherbarrow, R.J., 1992. GraFit version 3.0. Erithacus Software, Staines, UK.

Lees, G.J., 2000. Pharmacology of AMPA/kainate receptor ligands and their therapeutic potential in neurological and psychiatric disorders. Drugs 59, 33–78.

Madsen, U., Frølund, B., Lund, T.M., Ebert, B., Krogsgaard-Larsen, P., 1993. Design, synthesis and pharmacology of model compounds for indirect elucidation of the topography of AMPA receptor sites. Eur. J. Med. Chem. 28, 791–800.

Matzen, L., Engesgaard, A., Ebert, B., Didriksen, M., Frølund, B., Krogsgaard-Larsen, P., Jaroszewski, J.W., 1997. AMPA receptor agonists: synthesis, protolytic properties, and pharmacology of 3-isothiazolol bioisosteres of glutamic acid. J. Med. Chem. 40, 520–527.

Monaghan, D.T., Wenthold, R.J., 1997. The Ionotropic Glutamate Receptors. Humana Press, Totowa, NJ.

Murphy, D.E., Schneider, J., Boehm, C., Lehmann, J., Williams, M., 1987. Binding of [^{3}H]3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid to rat brain membranes: a selective, high-affinity ligand for *N*-methyl-D-aspartate receptors. J. Pharmacol. Exp. Ther. 240, 778–784.

Parsons, C.G., Danysz, W., Quack, G., 1998. Glutamate in CNS disorders as a target for drug development: an update. Drug News Perspect. 11, 523–569.

Ransom, R.W., Stec, N.L., 1988. Cooperative modulation of [^{3}H]MK-801 binding to the *N*-methyl-D-aspartate receptor ion channel complex by L-glutamate, glycine and polymines. J. Neurochem. 51, 830–836.

Sommer, B., Burnashev, N., Verdoorn, T.A., Kainanen, K., Sakmann, B., Seeburg, P.H., 1992. A glutamate receptor channel with high affinity for domoate and kainate. EMBO J. 11, 1651–1656.

Stensbøl, T.B., Johansen, T.N., Egebjerg, J., Ebert, B., Madsen, U., Krogsgaard-Larsen, P., 1999. Resolution, absolute stereochemistry and molecular pharmacology of the enantiomers of ATPA. Eur. J. Pharmacol. 380, 153–162.

Vignes, M., Clarke, V.R., Parry, M.J., Bleakman, D., Lodge, D., Ornstein, P.L., Collingridge, G.L., 1998. The GluR5 subtype of kainate receptor regulates excitatory synaptic transmission in areas CA1 and CA3 of the rat hippocampus. Neuropharmacology 37, 1269–1277.

Vogensen, S., Jensen, H.S., Stensbøl, T.B., Frydenvang, K., Bang-Andersen, B., Johansen, T.N., Egebjerg, J., Krogsgaard-Larsen, P., 2000. Resolution, configurational assignment and enantiopharmacology of 2-amino-3-[3-hydroxy-5-(2-methyl-2*H*-tetrazol-4-yl]propionic acid, a potent GluR3 and GluR4 preferring AMPA receptor agonist. Chirality 12, 705–713.

Wheal, H.V., Thomson, A.M., 1995. Excitatory Amino Acids and Synaptic transmission. Academic Press, London.