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The Selective Activation of the Glutamate Receptor GluR5 by ATPA Is Controlled by Serine 741

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ABSTRACT

Only a few agonists exhibit selectivity between the AMPA and the kainate subtypes of the glutamate receptor. The most commonly used kainate receptor preferring agonist, (*S*)-2-amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid [(*S*)-ATPA], is an (*R*,*S*)-2-amino-3-(5-methyl-3-hydroxy-4-isoxazolyl)propionic acid (AMPA) derivative in which the methyl group at the 5-position of the isoxazole ring has been replaced by a *tert*-butyl group. When characterized by the two-electrode voltage clamp method in *Xenopus laevis* oocytes, ATPA exhibits at least 50-fold higher potency on the kainate receptor subtype, GluR5, compared with the AMPA receptors. Through mutagenesis studies of GluR5 and the AMPA receptor subtype, GluR1, we demonstrate that this pronounced selectivity for ATPA can be ascribed to Ser741 in GluR5 and Met722 in GluR1. Examination of other aliphatic substitutions at the 5-position of the isoxazole ring revealed that (*R*,*S*)-2-amino-3-(5-isopropyl-3-hydroxy-4-isoxazolyl)propionic acid (isopropyl-AMPA) displayed a 6-fold higher potency for GluR5 than for GluR1, whereas the analogs, propyl-AMPA and isobutyl-AMPA, did not exhibit significantly different potencies. Our study suggests that the GluR5 selectivity was a result not only of steric interference between the bulky *tert*-butyl group in ATPA and the methionine (Met722) in GluR1 but also a serine-dependent stabilization of the active conformation of GluR5 induced by ATPA. The stabilization was agonist-dependent and observed only for ATPA and isopropyl-AMPA, not for other AMPA analogs with bulky substitutions at the 5-position of the isoxazole ring.

Glutamate receptors are the most abundant excitatory receptors in the central nervous system. Activation and modulation of the glutamatergic system play a crucial role in our understanding of the neuronal activity in the healthy brain as well as for the mechanisms underlying various neurological and psychiatric disorders. The contributions of the different glutamate receptor subtypes to neuronal activity are to a large extent identified using subtype-selective compounds or, more recently, by studies of genetically modified animals (Bräuner-Osborne et al., 2000; Doherty and Collingridge, 2001).

The traditional pharmacological division of the glutamate receptors into AMPA, kainate, and *N*-methyl-D-aspartic acid receptors based on the potencies of the respective agonists is reflected at the level of sequence identity between of the receptor subtypes forming the receptor complex. Thus, the subunits GluR1 to GluR4 form the AMPA receptors and the subunits GluR5 to GluR7 and KA1 and KA2 form the kainate receptors (Hollmann and Heinemann, 1994).

The increasing understanding of the molecular diversity underlying the ionotropic glutamate receptor system has challenged the development of subtype-selective ligands (Dingledine et al., 1999; Bräuner-Osborne et al., 2000). AMPA activates the AMPA receptors expressed in oocytes with an EC_{50} in the range of 1.3 to 3.5 μM (Vogensen et al., 2000), whereas kainate receptors formed from the GluR5 to GluR7 subunits are either activated with $EC_{50} > 1$ mM or not at all (Egebjerg et al., 1991; Sommer et al., 1992; Schiffer et al., 1997). Surprisingly, (*S*)-ATPA, an AMPA analog in which the methyl group at the 5-position in the isoxazole ring is replaced by a *tert*-butyl group (Lauridsen et al., 1985), exhibits a strong preference for GluR5 compared with the AMPA receptors (Clarke et al., 1997; Stensbøl et al., 1999). Analogs of AMPA with different 5-substitutions of the isoxazole ring have been studied extensively (Krogsgaard-Larsen et al., 1996). These derivatives seem to contribute to the selectivity and potency both within the AMPA receptor family and be-

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ABBREVIATIONS: AMPA, (*R*,*S*)-2-amino-3-(5-methyl-3-hydroxy-4-isoxazolyl)propionic acid; ATPA, (*R*,*S*)-2-amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid; TM, transmembrane; GluR, glutamate receptor; isopropyl-AMPA, (*R*,*S*)-2-amino-3-(5-isopropyl-3-hydroxy-4-isoxazolyl)-propionic acid; propyl-AMPA, (*R*,*S*)-2-amino-3-(5-propyl-3-hydroxy-4-isoxazolyl)propionic acid; isobutyl-AMPA, (*R*,*S*)-2-amino-3-(5-isobutyl-3-hydroxy-4-isoxazolyl)propionic acid; (*S*)-2-Me-Tet-AMPA, (*S*)-2-amino-3-(5-(2-methyltetrazolyl)-3-hydroxy-4-isoxazolyl)propionic acid; LCR, low Ca^{2+} Ringer; conA, concanavalin A.

tween the AMPA and kainate receptors. In particular, the AMPA analog with a 2-methyltetrazolyl substituent at the 5-position increases the potency and activates GluR4 and GluR1 with EC_{50} values of 9 and 160 nM, respectively, but remains AMPA receptor-selective by activating the kainate receptor GluR5 with an EC_{50} of 9 μM (Vogensen et al., 2000). In contrast, the *tert*-butyl substitution in ATPA resulted in 100-fold higher potency at GluR5 (0.66 μM) compared with GluR1 (62 μM) when expressed in *Xenopus laevis* oocytes (Stensbøl et al., 1999). The selectivity is even more pronounced (1000-fold) compared with the peak current in the AMPA receptors observed after fast application (Clarke et al., 1997). Studies performed on cortical wedges, which mainly reflect activation of the AMPA receptors, showed that the 5-ethyl analog of AMPA was more potent than AMPA, whereas the propyl and butyl analogs showed decreased potencies (Sløk et al., 1997). These observations and studies of willardiine analogs (Wong et al., 1994; Jane et al., 1997; Swanson et al., 1998) have resulted in a hypothesis proposing that the AMPA and kainate receptors might contain a hydrophobic cavity that can accommodate hydrophobic substituents to a certain size at the 5-position of the isoxazole ring of the AMPA molecule (Krogsgaard-Larsen et al., 1996).

The current structural model of the ionotropic glutamate receptors suggests a tetrameric complex formed by two dimers (Armstrong and Gouaux, 2000; Robert et al., 2001). The membrane topology of each subunit is three transmembrane- (TM) spanning segments, where the pore is formed by the two N-terminal TM-spanning segments and a re-entrant loop located between these TM segments. The ligand-binding domain is composed of two lobes formed from the part preceding the first TM and the extracellular region between the second and third TM (for review see Dingledine et al., 1999; Bräuner-Osborne et al., 2000). A soluble form of the ligand-binding domain of the GluR2 subunit has been expressed and crystallized in the apo form and also cocrystallized with a number of ligands, including the agonists kainate, glutamate, AMPA, and the antagonist 6,7-dinitro-2,3-quinoxalinedione (Armstrong and Gouaux, 2000; Armstrong et al., 1998). The crystal structure data suggest that the agonist-induced closure of the binding domain gives rise to the opening of the channel and, furthermore, the degree of closure correlates with the agonist specific properties of the channel, including the degree of desensitization.

In the current study, we attempt, based on mutagenesis, molecular modeling, and the use of AMPA analogs, to identify the amino acid(s) determining the difference in potency for ATPA on the AMPA receptor GluR1 and the kainate receptor GluR5.

Materials and Methods

Glutamate Receptor Ligands and Reagents. The AMPA analogs (*S*)-ATPA (Lauridsen et al., 1985; Stensbøl et al., 1999), isopropyl-AMPA, (*R*,*S*)-2-amino-3-(5-propyl-3-hydroxy-4-isoxazolyl)propionic acid (propyl-AMPA), (*R*,*S*)-2-amino-3-(5-isobutyl-3-hydroxy-4-isoxazolyl)propionic acid (isobutyl-AMPA) (Sløk et al., 1997), and (*S*)-2-amino-3-(5-(2-methyltetrazolyl)-3-hydroxy-4-isoxazolyl)propionic acid [(*S*)-2-Me-Tet-AMPA] (Vogensen et al., 2000) were synthesized as described previously. All other pharmacological tools and reagents were purchased from regular commercial sources.

Mutagenesis. The mutations were introduced by the standard overlap polymerase chain reaction method, using Pfu polymerase. The mutated polymerase chain reaction fragments were inserted between the *Bsp*EI and *Mlu*NI in GluR1flop and *Bln*I and *Eco*RI, *Bln*I, and *Xba*I or *Sal*I and *Xba*I in GluR51a. The inserted fragments were sequenced. All constructs were inserted in the pGEMHE (Liman et al., 1992) oocyte expression vector.

In Vitro cRNA Transcription. DNA (3 μg) was linearized using the appropriate enzymes. Run-off transcription was performed for 2 h at 37°C in 100 μl using the following concentrations; 7 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 40 mM Tris-HCl, pH 8.0, 37.5 mM dithiothreitol, 0.5 mM ATP, 0.5 mM UTP, 0.5 mM CTP, 0.1 mM GTP, 0.5 mM CAP (GpppGTP), 400 U/ml RNase block, and 300 to 500 U/ml T7 RNA polymerase. Trace amounts of [α-^{32}P]UTP were included to allow quantification of the transcribed cRNA.

Electrophysiology. A female *Xenopus laevis* frog was anesthetized and three to five ovarian lobes were surgically removed. The follicle layer was removed by washing twice in Barth's solution (88.0 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 15.0 mM HEPES pH 7.6, 0.30 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 10 μg/ml penicillin, and 10 μg/ml streptomycin), once in OR-2 (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4) followed by treatment with collagenase A (1 mg/ml in OR-2) for 3 h at RT. Oocytes at stages 4 to 5 were isolated and injected the following day with 50 nl (5–50 ng) of cRNA. The oocytes were kept at 18°C in Barth's medium before recordings were performed 3 to 12 days after injection, using a two-electrode voltage clamp (Warner OC-725C; Warner Instruments, Inc., Hamden, CT).

The recording solution was low Ca^{2+} Ringer (LCR; 10 mM HEPES-NaOH, pH 7.4, 115 mM NaCl, 0.1 mM $CaCl_2$, 2.5 mM KCl, and 1.8 mM $MgCl_2$). The LCR buffer was chosen to prevent activation of the endogenous Ca^{2+}-activated Cl^- channel. The oocytes were clamped at −70 to −20 mV. Electrodes (borosilicate glass capillaries, outer diameter, 1.5 mm; inner diameter, 1.17 mm; with inner filament; Harvard apparatus LTD, Kent, UK) were filled by 3 M KCl and exhibited resistances around 0.7 to 2 MΩ. Oocytes expressing GluR5 were treated with 1 mg/ml concanavalin A (type IV; Sigma Chemical, St. Louis, MO), in normal frog Ringer solution (10 mM HEPES-NaOH, pH 7.4, 115 mM NaCl, 1.8 mM $CaCl_2$, 2.5 mM KCl, and 0.1 mM $MgCl_2$) for 5 min, before recording. Stock solutions of the drugs were made in LCR at a concentration of 2.5 mM, pH adjusted to 7.4 with NaOH.

Data Analysis. The data were acquired using Clampex 7.0 or 8.0 (Axon Instruments, Inc., Union City, CA). Data analysis was done with SigmaPlot 3.0 (SPSS Science Inc., Chicago, IL). All responses were normalized to the average of a kainate response applied before and after the agonist application. After normalizing to the kainate responses, the data were fitted to the equation: $I = I_{max} \times ([L]^n{}_H / [(EC_{50})^n{}_H + [L]^n{}_H])$, where I_{max} is the estimated maximal current and I is the current measured after application of a given concentration of the ligand L ([L]). n_H is the Hill coefficient. Mean and S.E.M. values were calculated assuming a logarithmic distribution of EC_{50} and n_H values. To estimate the relative maximal current, the estimated $I_{maxligand}$ was normalized to I_{maxKA} corresponding to a 300 μM kainate response.

Molecular Modeling. The model of (*S*)-ATPA in the binding site of GluR2 was obtained using the crystallographic structure of the AMPA-GluR2 complex (pdb-code 1FTM). To perform the energy minimizations, the subset of amino acids was expanded to contain amino acids within a sphere of 12 Å from the (*S*)-AMPA molecule using the Insight II software (Accelrys, Inc., Princeton, NJ). The methyl group in (*S*)-AMPA was replaced by a *tert*-butyl group to convert it into (*S*)-ATPA and hydrogens were added to the complex. Energy minimization was performed using the MMFF force field as implemented in the MacroModel Software version 6.5 (Schrödinger, Inc., Portland, OR). The protein part of the complex was kept fixed, whereas the (*S*)-ATPA molecule and the water molecules were subjected to unconstrained energy minimization. To avoid large geometrical distortions of the (*S*)-ATPA molecule, it was necessary to remove the water molecule mediating the interaction between (*S*)-AMPA and the back-

bone of Ser654 and Thr655 in the AMPA-GluR2 complex. The models for (*S*)-ATPA-GluR1 and (*S*)-ATPA-GluR5 shown in Fig. 3 were obtained by replacing amino acids differing from GluR2.

Results

The amino acids lining the agonist binding cavity of GluR2 were identified from the crystal structure of the co-complex between kainate and the GluR2 ligand-binding domain (Armstrong et al., 1998). To pinpoint amino acids that might interact directly with ATPA, residues located closer than 6 Å from the kainate molecule were selected and amino acids at equivalent positions in GluR1 and GluR5 were determined from sequence alignment of GluR1, GluR2, and GluR5 (Table 1). Only three of the 13 identified residues, Leu650$_{R2}$, Thr686$_{R2}$, and Met708$_{R2}$ [the subscript indicates that the numbering refers to the equivalent position in GluR2 (Armstrong et al., 1998)] were different between GluR1 and GluR5. A subsequent docking of (*S*)-ATPA into the GluR2 crystal structure (see *Materials and Methods*) indicated that the *tert*-butyl group at the 5-position of ATPA might interact with the residues Thr686$_{R2}$ and Met708$_{R2}$.

To examine whether the difference in ATPA potency between GluR1 and GluR5 could be ascribed to the amino acid differences at the positions 686$_{R2}$ and 708$_{R2}$, mutant forms of GluR1 containing the GluR5 sequence at these positions, and vice versa, were generated. The receptors were analyzed by two-electrode voltage clamp using the *Xenopus laevis* oocytes expression system. The experiments on GluR5 wild type and mutants were performed after concanavalin A (conA) treatment to eliminate transition into the desensitized state. Mutations at the positions equivalent to Thr686$_{R2}$ in GluR1(T700S) and GluR5(S721T) reduced the potencies of ATPA by 1.5- and 2-fold, respectively (Table 2). However, mutating the position equivalent to 708$_{R2}$ in GluR1(M722S) increased the potency of ATPA by more than 10-fold (EC_{50} from 62 to 4.6 μM), whereas the reciprocal mutation in GluR5(S741M) resulted in a 15-fold decrease in the potency of ATPA (EC_{50} from 0.66 to 9.5 μM). Introduction of both mutations in GluR1(T700S, M722S) resulted in a receptor with an EC_{50} for ATPA between the wild type and the single mutant at the position equivalent to 708$_{R2}$, whereas ATPA exhibited reduced potency on GluR5(S721T, S741M) compared with the single mutant GluR5(S741M) (Table 2).

The larger size of the methionine in GluR1 compared with the serine in GluR5 suggested that the lower potency of ATPA at GluR1 might result from steric interference between Met722 in GluR1 and the *tert*-butyl group at the 5-position of ATPA. This was examined by substituting the methionine with an alanine in GluR1. Surprisingly, GluR1(M722A) resulted in a decrease in potency of ATPA (EC_{50} of 97 μM), suggesting that steric hindrance alone could not account for the lower potency on GluR1 compared with GluR5. A similar analysis was performed on GluR5, where Ser741 was mutated to residues of different sizes. Substituting with the smaller alanine, GluR5(S741A), resulted in a 48-fold reduction in ATPA potency, and substitution of Ser741 with leucine or valine also resulted in reduced potencies (20-fold decrease) to levels similar to GluR5(S741M) (Table 2).

The maximal responses activated by ATPA were compared with the responses elicited by 300 μM kainate. The mutant forms of GluR1 showed a significantly ($P < 0.005$) higher ratio than the wild-type GluR1 (0.2 ± 0.02) (Fig. 1C). Despite the conA treatment of the GluR5-expressing oocytes, GluR5(S721T) exhibited significantly ($p < 0.02$) higher relative maximal current. However, this effect was abolished in the GluR5(S721T, S741M) mutant.

In addition to the mutagenesis approach to characterize the interaction between the ligand and the residue at the 708$_{R2}$ position, we determined the potencies of other AMPA analogs with different aliphatic substituents at the 5-position (Sløk et al., 1997). Extending the side chain from a methyl (in AMPA) to a propyl group reduced the potency on GluR1 from 3.4 (Vogensen et al., 2000) to 7.9 μM, and introduction of larger substituents reduced the potency even further, with ATPA as the least potent (Table 3). At GluR5, the propyl and isobutyl analogs exhibited more than 40-fold reductions in potencies compared with ATPA, whereas the isopropyl substituent only reduced the potency 5-fold compared with

TABLE 1

Amino acids lining the ligand binding site in GluR1-GluR5

Amino acids located adjacent (< 6 Å) to kainate in the kainate-GluR2 cocrystal aligned with equivalent residues in GluR5.

	GluR1–4	GluR5
Lobe A		
402$_{R2}$	Glu	Glu
450$_{R2}$	Tyr	Tyr
478$_{R2}$[a]	Pro	Pro
479$_{R2}$	Leu	Leu
480$_{R2}$[a]	Thr[b]	Thr
485$_{R2}$[a]	Arg	Arg
Lobe B		
650$_{R2}$	Leu[b]	Val[b]
654$_{R2}$[a]	Ser	Ser
655$_{R2}$[a]	Thr	Thr
686$_{R2}$	Thr[b]	Ser[b]
705$_{R2}$[a]	Glu	Glu
708$_{R2}$	Met[b]	Ser[b]
732$_{R2}$	Tyr	Tyr

[a] Residues with direct interaction with kainate.
[b] Residues are different between GluR1–R4 and GluR5.

TABLE 2

Potency of (*R*,*S*)-ATPA on mutant and wild-type forms of GluR1 and GluR5

EC_{50} and n_H coefficients for GluR1 and GluR5 wild-type and mutant receptors expressed in *X. laevis* oocytes and analyzed by two-electrode voltage clamp. Oocytes expressing GluR5 were pretreated with conA to remove desensitization. Min/max are ± 1 × S.E.M., assuming a logarithmic distribution of the potency. Values represent data from at least three individual oocytes. There are no significant differences in the Hill coefficients between the GluR1 mutants or between the GluR5 mutants when activated by ATPA.

	ATPA			
Receptor	EC_{50}	Min/Max	n_H	Min/Max
	μM			
GluR1wt[a]	62	57/68	0.61	0.5/0.7
GluR5wt[a]	0.66	0.61/0.72	1.1	1.0/1.2
GluR1(M722S)	4.6	4.0/5.3	0.74	0.71/0.77
GluR5(S741M)	9.5	8.7/10	1.1	1.1/1.2
GluR1(T700S)	97	80/120	0.73	0.69/0.77
GluR5(S721T)	1.4	1.2/1.6	0.84	0.80/0.88
GluR1(T700S, M722S)	14	13/16	0.75	0.72/0.78
GluR5(S721T, S741M)	23	22/24	0.94	0.91/0.97
GluR1(M722A)	97	85/110	0.7	0.68/0.73
GluR5(S741A)	32	28/35	1.0	0.94/1.1
GluR5(S741L)	18	13/25	0.98	0.94/1.0
GluR5(S741V)	14	12/16	0.95	0.89/0.99

wt, wild-type.
[a] Stensbøl et al. (1999).

Fig. 1. Electrophysiological characterization of GluR1 and GluR5 mutants, affecting the selectivity for ATPA. A, current traces recorded from a two-electrode, voltage-clamped *X. laevis* oocyte expressing GluR5(S721T, S741M). The first response was induced after application of 10 μM kainate and the following from increasing concentrations of (*R*,*S*)-ATPA. The oocyte was pretreated with conA. B, dose response relationship of (*R*,*S*)-ATPA on GluR1 (○), GluR5 (●), GluR1(M722S) (□), and GluR5(S741M) (■). Responses were normalized to an estimated I_{max} value set to 1. C, relative I_{max} for (*RS*)-ATPA, when tested on the different receptors. The estimated I_{max} was normalized to the response elicited by 300 μM kainate.

TABLE 3

EC_{50} and n_H coefficients for AMPA analogs characterized on GluR1 and GluR5 wild type and mutant receptors

EC_{50} and n_H coefficients for GluR1 and GluR5 wild-type and mutant receptors expressed in *X. laevis* oocytes and analyzed by two-electrode voltage clamp. Oocytes expressing GluR5 were pretreated with conA to remove desensitization. Min/max are ± 1 × S.E.M., assuming a logarithmic distribution. All the characterized compounds are racemic mixtures except for (*S*)-2-Me-Tet-AMPA, which is the resolved *S*-enantiomer. Values represent data from at least three individual oocytes.

	GluR1wt			GluR1(M722S)			GluR5wt			GluR5(S741M)		
	EC_{50}	Min/Max	n_H	EC_{50}	Min/Max	n_H	EC_{50}	Min/Max	n_H	EC_{50}	Min/Max	n_H	Min/Max
	μM			μM			μM			μM			
ATPA[a]	62	57/68	0.61	4.6	4.0/5,0.3	0.74	0.66	0.61/0.72	1.1	9.5	8.7/10	1.1	1.1/1.2
Isopropyl-AMPA	21	14/31	0.75	1.2	0.88/1.5	0.44	3.3	2.7/3.9	0.80	18	16/21	1.2	1.1/1.3
Propyl-AMPA	7.9	5.2/12	0.78	0.53	0.43/0.65	0.61	27	19/38	0.79	85	66/110	0.89	0.85/0.93
Isobutyl-AMPA	26	19/38	0.67	38	26/54	0.44	28	23/33	0.88	250	220/290	0.77	0.76/0.78
(*S*)-2-Me-Tet-AMPA[b]	0.16	0.13/0.18	0.77	0.15	0.11/0.20	1.7	8.7	5.6/13	0.79	15	13/18	1.6	1.4/1.7

wt, wild-type.
[a] Wild-type values for ATPA are from Stensbøl et al. (1999).
[b] GluR1wt and GluR5wt values are taken from Vogensen et al. (2000).

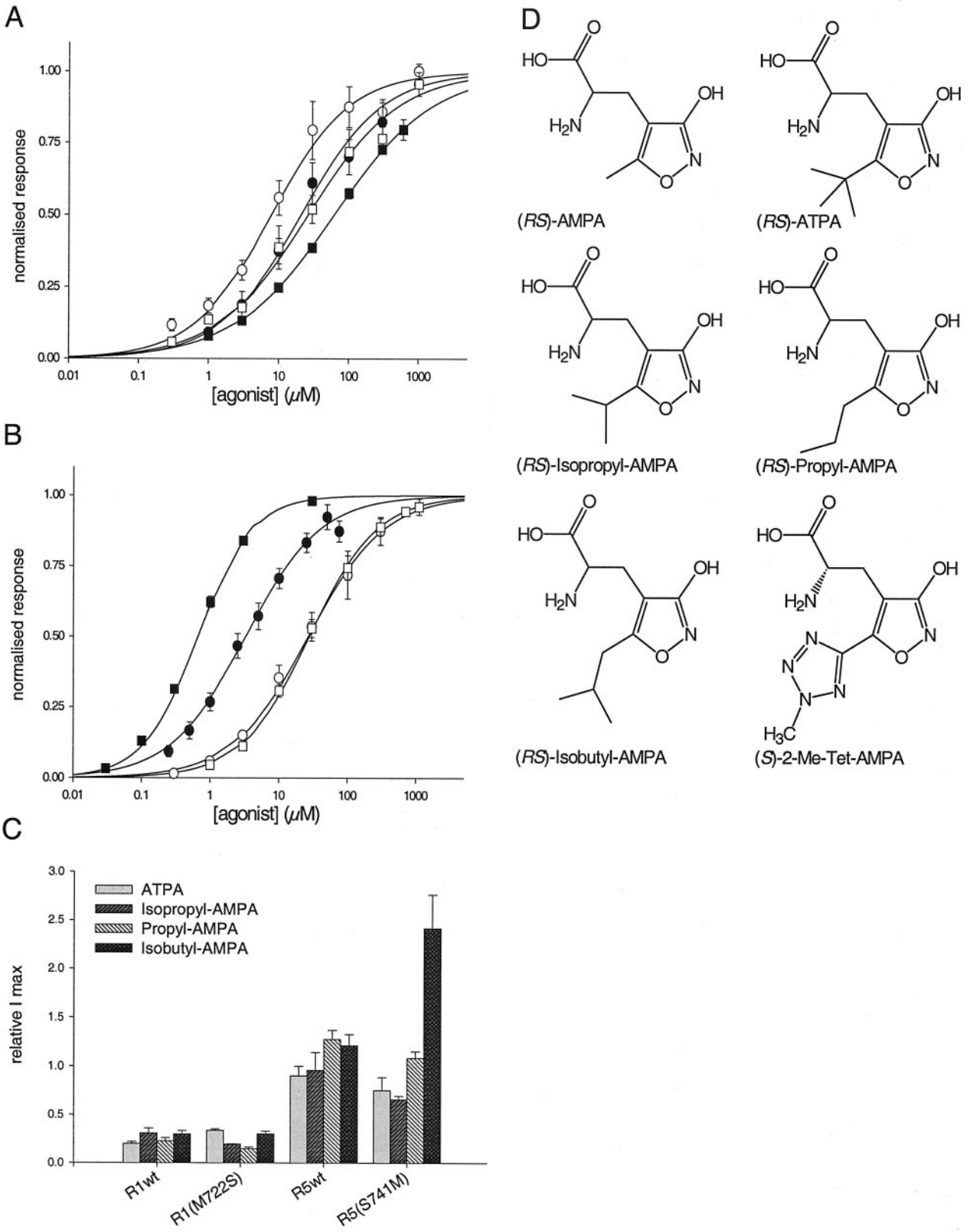

Fig. 2. Activity of AMPA analogs with aliphatic substituents in the 5-position of the isoxazole ring. A and B, dose-response curves for (*R*,*S*)-ATPA (■), (*R*,*S*)-propyl-AMPA (○), (*R*,*S*)-isopropyl-AMPA (●), and (*R*,*S*)-isobutyl-AMPA (□) on the wild-type receptors expressed in *X. laevis* oocytes and characterized by the two-electrode voltage clamp technique. Dose-response relationship on GluR1 (A) or GluR5 (B). Responses were normalized to an estimated I_{max} value set to 1. C, relative estimated maximal current of GluR1wt, GluR1(M722S), GluR5wt and R5(S741M) for the AMPA analogs, normalized to the current elicited by 300 μM kainate. D, structures of AMPA and the AMPA analogs used in the present study.

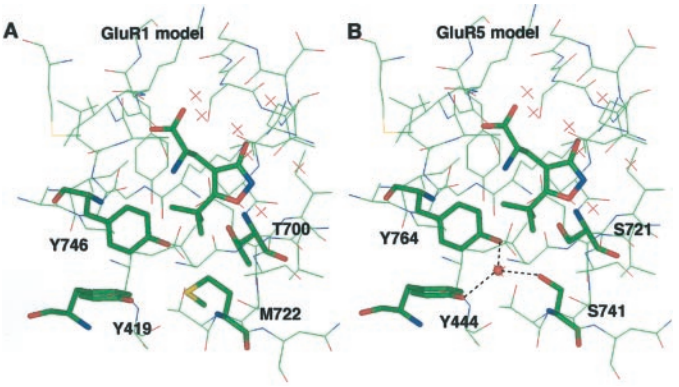

Fig. 3. A, a model of (*S*)-ATPA in the binding site of GluR1. B, a model of (*S*)-ATPA in the binding site of GluR5. (*S*)-ATPA and the residues involved in the selective interactions between ATPA and the receptor are highlighted. The two tyrosines $Y419_{R1}/Y444_{R5}$ and $Y746_{R1}/Y764_{R5}$ correspond to Y405 and Y732 in GluR2, respectively. The red crosses correspond to water molecules present in the X-ray structure of the AMPA-GluR2 complex. The red sphere indicates the proposed water molecule interacting with S741, Y414, and Y734, and the suggested hydrogen bond interactions are indicated by dashed lines.

ATPA. Consequently, only isopropyl-AMPA and ATPA exhibit higher selectivity for GluR5 than GluR1, although the selectivity was less pronounced for isopropyl-AMPA. Similar to ATPA, both isopropyl-AMPA and propyl-AMPA showed decreased potencies at GluR5(S741M) compared with GluR5 and increased potencies at the GluR1(M722S) compared with GluR1. For isobutyl-AMPA, the GluR1(M722S) mutation did not affect the potency significantly, indicating that the more distant β-branching might exceed the binding pocket and interact in a different manner than the other compounds.

The maximal steady-state currents elicited by the derivatives were normalized to the response elicited by 300 μM kainate. No significant ($p = 0.05$) differences in the relative steady-state currents were observed for the wild-type GluR1, whereas isopropyl-AMPA and propyl-AMPA (Fig. 2) showed a 42 and 50% reduction, respectively, in relative maximal current for GluR1(M722S). On the GluR5 receptors, only isobutyl-AMPA on GluR5(S741M) shows significantly different relative maximal current compared with ATPA (Fig. 2C).

To obtain more detailed information, molecular modeling and energy minimizations were performed using the coordinates from the (*S*)-AMPA–GluR2 crystal structure, including amino acids located closer than 12 Å from the AMPA molecule (see *Materials and Methods*). To avoid high energy distortions of (*S*)-ATPA, removal of the water molecule located between the 3-hydroxy group on AMPA and lobe 2 of the binding domain ($Ser654_{R2}$ and $Thr655_{R2}$) was required, suggesting a glutamate-like binding mode for (*S*)-ATPA (Armstrong and Gouaux, 2000). When the models for GluR1 and GluR5 were generated, a water molecule could be modeled into the binding site of GluR5 by substitution of the amino acids differing between the subunits (see Fig. 3B). This water molecule displays hydrogen bonding distances (measured as O-O distances) to Ser741, Tyr764 and Tyr444 in the range 2.7 to 2.9 Å. Thus, a water molecule in this position is predicted to display a strong hydrogen bond to each of the three residues (Fig. 3B), whereas similar interactions are not possible for GluR1 (Fig. 3A) or GluR5(S741M).

To examine whether the water-mediated stabilization is ligand-dependent, we tested (*S*)-2-Me-Tet-AMPA, which, as we have shown previously, exhibits selectivity for AMPA receptors with an unprecedented high potency (Vogensen et al., 2000). The 2-methyltetrazole substituent at the 5-position did not imply any significant changes in the potencies between the wild type and GluR1(M722S) (Table 3), suggesting that the almost planar structure of the tetrazole ring can be accommodated in the binding pocket without selective interaction with the amino acid at the 708_{R2} position. We also tested glutamate, which does not reach into the pocket (Armstrong and Gouaux, 2000) and found no significant ($p > 0.05$) difference between GluR1 and GluR1(M722S), supporting the model in that the $M708_{R2}$-mediated selectivity might be ATPA-dependent.

Discussion

Examination of the agonist-binding cleft based on the crystal structure of kainate and the soluble binding domain of GluR2 revealed no obvious hydrophobic pocket, but three amino acids near kainate differed between GluR1 and GluR5. Modeling pointed at the positions 686_{R2} and 708_{R2} as the critical residues. An asparagine at the 686_{R2} position in GluR6 had previously been shown to prevent AMPA interaction (Swanson et al., 1997). However, the potency and selectivity of ATPA were not significantly changed by mutation at position 686_{R2} in either GluR1 or GluR5.

Our studies suggest that the major determinant for the difference in ATPA potencies between the AMPA receptor GluR1 and the kainate receptor GluR5 is the presence of a serine in GluR5 at position 708_{R2}. We hypothesized that the lower potency of ATPA at the AMPA receptors might result from a steric clash between the *tert*-butyl group and the methionine (at 708_{R2}) in GluR1 compared with the smaller serine in GluR5. However, all the examined GluR5 substitutions, including smaller and larger hydrophobic residues, at the 708_{R2} position resulted in reduced potencies of ATPA, suggesting that the lower potency of ATPA at GluR1 cannot be attributed to a steric clash alone but also to an additional stabilization of ATPA by the hydrophilic serine. By introducing the serine in GluR1, we observed a similar stabilization of isopropyl- and propyl-AMPA but not of the potent AMPA receptor agonist (*S*)-2-Met-Tet-AMPA, suggesting that increased potency at the GluR1(M722S) mutant depends on both the agonist and the specific nature of the substituent at the 5-position.

The GluR1(M722S) mutation does not fully convert the potency of ATPA to that for GluR5, and the EC_{50} at GluR5(S741M) is 6-fold lower compared with the value found at GluR1. The remaining discrepancy in ATPA potency might result from small differences in the interactions with the conserved residues in the binding pocket. The potencies were determined from the steady-state currents recorded in *X. laevis* oocytes, suggesting that the remaining differences in potencies are more probably caused by unequal energetic requirements for activation of the individual receptor subtypes and/or varying degrees of desensitization between GluR1 and GluR5 receptors.

We did observe some differences in the relative maximal current between the mutants, indicating that they might desensitize to various degrees, but these changes cannot account for all of the differences we found in the EC_{50} values. In particular, GluR5(S741M) exhibited the same maximal

current for ATPA as GluR5wt, whereas the EC_{50} is 15-fold higher. Interestingly, some of the mutants displayed Hill coefficients less than 1, suggesting either negative cooperativity between the agonist binding sites or, more likely, a concentration-dependent desensitization, where high agonist concentrations and, consequently, full occupancy at all the binding sites might increase the degree of desensitization. Studies using faster agonist applications might resolve the mechanism underlying the low Hill coefficient.

The position equivalent to 708_{R2} has been mutated in the NR2B *N*-methyl-D-aspartic acid receptor subunit (Laube et al., 1997). Mutation of the wild-type valine to an alanine reduced the potency of glutamate and D-(−)-2-amino-5-phosphonopentanoic acid by 30- and 6-fold, respectively. Interestingly, ATPA also exhibited lower potency for GluR5(S741A) than for GluR5(S741V), supporting the view that the selectivity cannot only be determined by steric interference.

The structural studies of the agonist interactions with the GluR2 binding domain show almost identical interactions of the protein with the α-carboxyl and α-amino groups of kainate, glutamate, and AMPA; surprisingly, however, the isoxazole moiety of AMPA was not directly bioisosteric with the kainate and glutamate γ-carboxyl groups. The direct interaction between the glutamate γ-carboxyl group and the backbone of $Ser654_{R2}$ and $Thr655_{R2}$ is replaced by a water-mediated interaction in the AMPA complex, resulting in a displacement of the isoxazole moiety toward $Met708_{R2}$. The 5-methyl group is accommodated by a reorientation of $Met708_{R2}$ relative to the extended form observed in the kainate and glutamate complexes (Armstrong and Gouaux, 2000). A direct replacement of AMPA with (*S*)-ATPA would generate strong steric clashes between the *tert*-butyl group and $Tyr450_{R2}$, $Pro477_{R2}$, and $Met708_{R2}$, suggesting that (*S*)-ATPA and AMPA are bound differently. This is supported by docking of (*S*)-ATPA into the binding cavity of the AMPA-GluR2 complex and prediction of its binding position by energy minimization (for further details, see *Materials and Methods*). The predicted binding position of ATPA in GluR5 (and GluR1) is, in contrast to that displayed by AMPA, similar to the binding mode of glutamate in the glutamate-GluR2 complex (Armstrong and Gouaux, 2000). Thus, the binding of the ring nitrogen atom and the exocyclic oxygen atom in (*S*)-ATPA corresponds to that displayed by the γ-carboxyl group of glutamate. This binding mode relieves the steric clashes described above.

Modeling proposes a water-mediated stabilization of the ATPA-induced closed conformation of the binding domain of GluR5. The water-mediated interaction might stabilize the closed form of the binding domain because it involves residues located on each lobe (Ser741 and the tyrosines Tyr764 and Tyr444), similar to the stabilizing interlobe interactions, $Glu402_{R2}$-$Thr686_{R2}$ and $Lys449_{R2}$-$Asp651_{R2}$, $Ser652_{R2}$, observed in the kainate-GluR2 crystal structure (Armstrong et al., 1998). Interestingly, the peptide bond rearrangement in the AMPA-bound structure allows two additional interdomain hydrogen bonds, namely $Gly451_{R2}$-$Ser652_{R2}$ and $Tyr450_{R2}$-$Asp651_{R2}$, the latter of which is through a water molecule, correlating with AMPA displaying higher potency than kainate on this receptor family (Armstrong and Gouaux, 2000). The potency of neither glutamate nor (*S*)-2-Me-Tet-AMPA is affected by the mutation at GluR1(M722S), which supports the model that the presence and/or strength of these water-mediated interactions depend on the properties of the ligand and, in particular, the moiety, at the 5-position of the AMPA structure. This difference might be used for future development of more selective and potent glutamate receptor ligands.

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