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Design, synthesis and pharmacology of model compounds for indirect elucidation of the topography of AMPA receptor sites

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Summary — Based on structure–activity studies on excitatory amino acids with specific agonist effect at (*RS*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) receptors we have earlier proposed a simple model of the AMPA receptor pharmacophore. In order to judge the capacity of this empirical model we have now synthesized and tested 3 model compounds derived from the AMPA receptor agonists, AMPA and (*RS*)-3-hydroxy-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-7-carboxylic acid (7-HPCA). These model compounds, (*RS*)-2-amino-3-(5-ethyl-3-hydroxy-4-isoxazolyl)propionic acid (Et-AMPA), (*RS*)-2-amino-4-(3-hydroxy-5-methyl-4-isoxazolyl)butyric acid (Homo-AMPA) and (*RS*)-3-hydroxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[5,4-*c*]azepine-8-carboxylic acid (Homo-7-HPCA) were tested electrophysiologically and in receptor binding assays. Et-AMPA was slightly more potent than AMPA as an AMPA agonist (EC_{50} = 2.3 μM compared to 3.5 μM for AMPA) and as a specific inhibitor of [^{3}H]AMPA binding (IC_{50} = 0.030 μM compared with 0.040 μM for AMPA), whereas Homo-AMPA was essentially inactive. Homo-7-HPCA was much weaker than 7-HPCA. These data support the view that the AMPA recognition site(s) comprise a confined region, which tightly binds the charged structure-elements of agonists molecules, and a cavity capable of accommodating bulky lipophilic groups in such compounds.

excitatory amino acids / AMPA receptors / AMPA agonist pharmacophore / structure–activity studies / 3-hydroxyisoxazoles / cortical wedge

Introduction

There is an interest in the central excitatory amino acid (EAA) receptors as targets for therapeutic intervention in neurodegenerative disorders [1–4]. Furthermore, EAA receptors seem to play a key role in long-term potentiation (LTP), which may be an important mechanism in learning and memory processes [5, 6]. These aspects have brought EAA receptors into the focus of drug design projects in Alzheimer's disease and related disorders characterized by progressive impairments of short- and long-term memory [7–9].

Extensive pharmacological and molecular biological studies have disclosed the existence of at least 5 main classes of EAA receptors, most, if not all, of which comprise a number of isoreceptors [3, 4, 10–12]. Although selective agonists and, to some extent, antagonists for each of these EAA receptor families are available [10, 13, 14], there is an urgent need for specific ligands for precise pharmacological characterization not only of these receptor families but also of the individual isoreceptors.

It is well established that the *N*-methyl-D-aspartic acid (NMDA) receptor is a receptor complex containing a co-transmitter glycine site and several other binding sites assumed to have modulatory functions [15]. Similarly, the (*RS*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) type of

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Abbreviations used: ABPA, (*RS*)-2-amino-3-[5-(bromomethyl)-3-hydroxy-4-isoxazolyl]propionic acid; 4-AHCP, (*RS*)-2-amino-3-(3-hydroxy-7,8-dihydro-6*H*-cyclohept[1,2-*d*]isoxazol-4-yl)propionic acid; AMPA, (*RS*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid; APPA, (*RS*)-2-amino-3-(3-hydroxy-5-phenyl-4-isoxazolyl)propionic acid; ATPA, (*RS*)-2-amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid; CPP, (*RS*)-3-(2-carboxy-4-piperazinyl)propyl-1-phosphonic acid; D-AP5, D-2-amino-5-phosphonovaleric acid; EAA, excitatory amino acid; Et-AMPA, (*RS*)-2-amino-3-(5-ethyl-3-hydroxy-4-isoxazolyl)propionic acid; Homo-AMPA, (*RS*)-2-amino-4-(3-hydroxy-5-methyl-4-isoxazolyl)butyric acid; Homo-7-HPCA, (*RS*)-3-hydroxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[5,4-*c*]azepine-8-carboxylic acid; 7-HPCA, (*RS*)-3-hydroxy-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-7-carboxylic acid; KAIN, kainic acid; MK-801, (*RS*)-5-methyl-10,11-dihydro-5*H*-dibenzo-[*a*,*d*]cyclohepta-5,10-imine; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline; NMDA, *N*-methyl-D-aspartic acid.

ionotropic EAA receptor may be a receptor complex comprising a regulatory unit and binding sites through which receptor function and/or desensitization may be modulated [16, 17]. Compounds acting at the AMPA receptors may have therapeutic interest in Alzheimer's disease, including partial agonists, ligands with AMPA-potentiating properties or agonists acting directly at the AMPA recognition site [18–20]. In order to design such agents on a rational basis, information about the topography of the AMPA recognition site would be of major interest.

Although the primary structures of AMPA isoreceptors are known [11, 12, 21], the localizations and 3-dimensional structures of their recognition sites are unknown. Future X-ray structure determination of AMPA receptor proteins may shed light on these aspects of key importance for rational drug design. Until this kind of structural information becomes available, it is important to develop simple models of AMPA recognition sites based on studies of the relationship between 3-dimensional structures and pharmacological effects of specific AMPA receptor ligands. On the basis of such structure–activity studies on a series of specific AMPA agonists, including AMPA, (*RS*)-2-amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid (ATPA), (*RS*)-2-amino-3-(3-hydroxy-7,8-dihydro-6*H*-cyclohept[1,2-*d*]isoxazol-4-yl)propionic acid (4-AHCP) and (*RS*)-3-hydroxy-4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-7-carboxylic acid (7-HPCA) [22–26] a simple hypothetical model of the AMPA recognition site has been developed [20, 27]. As shown in figure 1, the fully charged 'acidic amino acid moieties' of the agonist molecules are proposed to bind tightly to elements of the receptor macromolecules assumed to contain complementary charges. In addition, the AMPA recognition site appears to contain a pocket or cavity capable of accommodating lipophilic structure elements of AMPA agonists of considerable size such as the *tert*-butyl and cycloheptenyl groups of ATPA and 4-AHCP, respectively (fig 1). The nature of this proposed lipophilic cavity is unknown, but replacement of, for example, the spherical *tert*-butyl group of ATPA by a planar phenyl group leads to the relatively weak partial AMPA agonist, (*RS*)-2-amino-3-(3-hydroxy-5-phenyl-4-isoxazolyl)propionic acid (APPA) [18].

In order to judge the capacity of this simple receptor model as a template for semirational design of agonists and partial agonists showing different levels of potency and efficacy, at AMPA receptors we have now synthesized and tested 3 model compounds derived from the specific AMPA agonists, AMPA and 7-HPCA. Each of these model compounds (*RS*)-2-amino-3-(5-ethyl-3-hydroxy-4-isoxazolyl)propionic acid (Et-AMPA), (*RS*)-2-amino-4-(3-hydroxy-5-methyl-4-isoxazolyl)butyric acid (Homo-AMPA) and

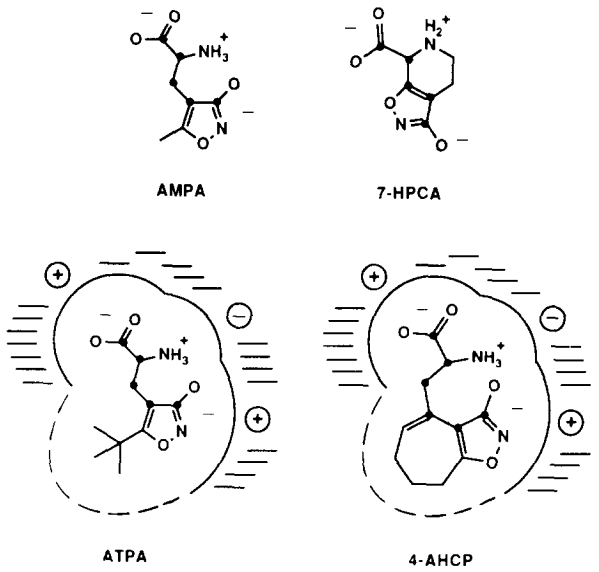
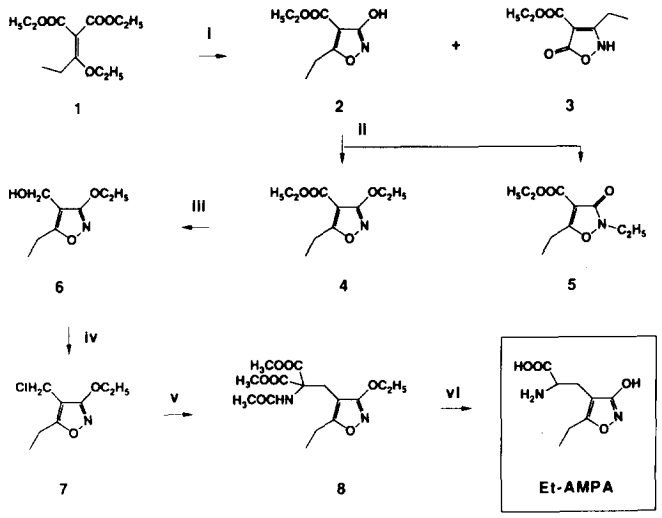


Fig 1. Structures of the AMPA receptor agonists AMPA, 7-HPCA, ATPA, and 4-AHCP, and illustration of the binding of the latter two to a hypothetical model of the AMPA receptor, assumed to contain a lipophilic cavity.

(*RS*)-3-hydroxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[5,4-*c*]azepine-8-carboxylic acid (Homo-7-HPCA) (see fig 5) contain an additional CH_2-group and are estimated to have electronic properties that are very similar to those of the parent agonist molecules. Thus, any difference between the effects of the parent agonists AMPA and 7-HPCA and the respective model compounds on AMPA receptors can be attributed primarily to steric and conformational effects.

Chemistry

The AMPA analogue Et-AMPA was synthesized from ethyl 3-ethoxy-2-(ethoxycarbonyl)-2-pentenoate **1** (scheme 1). Compound **1** was synthesized following a procedure analogous with that described for ethyl 3-ethoxy-2-(ethoxycarbonyl)-2-butenoate [28], using triethyl orthopropionate and diethyl malonate. Cyclization of the enol ether **1** with hydroxylamine afforded the 3-hydroxyisoxazole **2** and a more polar compound, assumed to be the isomeric isoxazolin-5-one **3**, which was not isolated and characterized. Alkylation of compound **2** with ethyl bromide afforded the products **4** and **5** in approximately 60 and 30% yields, respectively. Reduction of the ester group in compound **4** with $LiAlH_4$ to give **6** followed by treatment with thionyl chloride gave compound **7**. A Sorensen synthesis converted **7** into **8**, which was

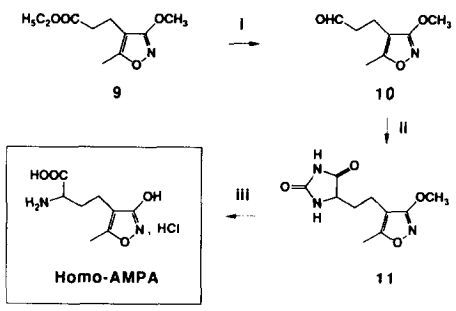


Scheme 1. i): NH_2OH; ii): C_2H_5Br, K_2CO_3; iii): $LiAlH_4$; iv): $SOCl_2$; v): $H_3CCONHCNa(COOCH_3)_2$; vi): 48% HBr/H_2O.

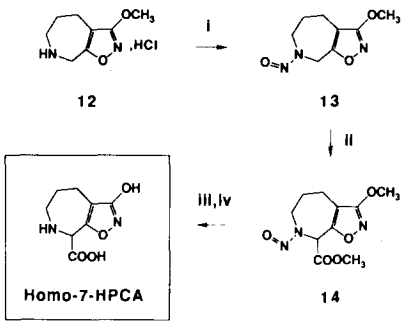
deprotected using concentrated hydrobromic acid. The final product, Et-AMPA, was isolated as a zwitterion.

The homologue of AMPA, Homo-AMPA, was synthesized *via* the hydantoin **11**, obtained by reduction of the ester **9** [29] with diisobutylaluminum hydride followed by treatment of the intermediate aldehyde **10** with potassium cyanide and ammonium carbonate (scheme 2). Deprotection of **11** by reflux with 6 M hydrochloric acid afforded Homo-AMPA as a hydrochloride.

Homo-7-HPCA was synthesized from 3-methoxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[5,4-*c*]azepine hydrochloride **12** (scheme 3) prepared from methyl 3-hydroxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[5,4-*c*]-azepine-7-carboxylate [30] by methylation with



Scheme 2. i) Diisobutylaluminum hydride; ii): KCN, $(NH_4)_2CO_3$; iii) 6 M HCl.



Scheme 3. i): $NaNO_2$; ii): BuLi, Cl-$COOCH_3$; iii): HBr_{gas}, HBr/CH_3COOH; iv): IRA-400.

diazomethane and *N*-deprotection with potassium hydroxide. The methoxycarbonyl group of compound **14** was introduced regiospecifically *via* the *N*-nitroso intermediate **13** by treatment with butyllithium and methyl chloroformate. Deprotection of compound **14** was carried out stepwise, using HBr_{gas} to remove the nitroso group followed by treatment with HBr in glacial acetic acid, and finally ester hydrolysis on a strongly basic ion exchange column to give Homo-7-HPCA.

In vitro pharmacology

The receptor affinities of the new compounds were evaluated in a number of receptor binding assays, using the ligands [^{3}H]AMPA [31], [^{3}H]kainic acid ([^{3}H]KAIN) [32], [^{3}H](*RS*)-3-(2-carboxy-4-piperazinyl)propyl-1-phosphonic acid ([^{3}H]CPP) [33], [^{3}H](*RS*)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepta-5,10-imine ([^{3}H]MK-801) (baseline and fully stimulated binding) [34] and [^{3}H]glycine [35]. Neither Et-AMPA, Homo-AMPA nor Homo-7-HPCA showed significant affinity (IC_{50} > 100 μM) in the [^{3}H]KAIN binding assay or in the assays representing binding sites at the NMDA receptor complex: [^{3}H]CPP, [^{3}H]MK-801, and [^{3}H]glycine binding. The IC_{50} values in [^{3}H]AMPA, [^{3}H]KAIN, and [^{3}H]CPP binding assays are shown in table I, where Et-AMPA is seen to be a very effective inhibitor of [^{3}H]AMPA binding (IC_{50} = 0.030 μM), slightly more potent than AMPA itself (IC_{50} = 0.040 μM). Homo-7-HPCA showed very weak [^{3}H]AMPA binding affinity (IC_{50} = 100 μM), whereas Homo-AMPA was inactive (IC_{50} > 100 μM).

These receptor binding data are in agreement with the results of electrophysiological experiments using the rat cortical slice model (table I; fig 2). In this rat brain tissue preparation [36], Et-AMPA (EC_{50} = 2.3 μM) also proved to be slightly more potent than

Table I. Receptor binding and *in vitro* electrophysiological data.

Compound	*IC_{50} (μM)*			*EC_{50} (μM)*
	[^{3}H]AMPA	*[^{3}H]KAIN$_{binding}$*	*[^{3}H]CPP*	*Electrophysiol*
AMPA	0.040 ± 0.014	> 100	> 100	3.5 ± 0.2
7-HPCA	0.15 ± 0.05	> 100	> 100	35 ± 3
4-AHCP	0.74 ± 0.16	> 100	98 ± 4	10 ± 3
KAIN	4.0 ± 1.2	0.007 ± 0.002	> 100	nt
Et-AMPA	0.030 ± 0.015	> 100	> 100	2.3 ± 0.2
Homo-AMPA	> 100	> 100	> 100	> 1000
Homo-7-HPCA	100[a]	> 100	> 100	1000[a]

IC_{50} and EC_{50} values ± SEM, n = 3–4; nt: not tested; [a]n = 2 (only small amounts of compound available).

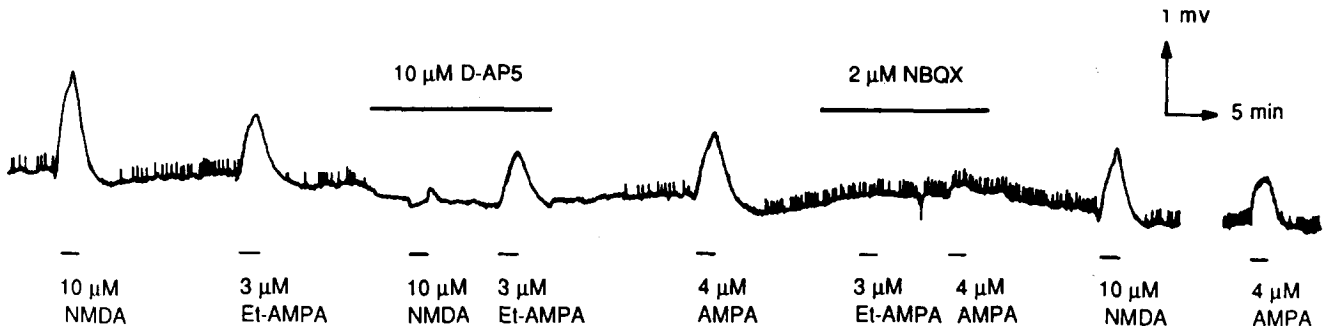


Fig 2. Recordings from cortical neurones depolarized by administration of NMDA, Et-AMPA, and AMPA. Effects of the selective EAA antagonists, D-AP5 or NBQX, and recovery are illustrated.

AMPA (EC_{50} = 3.5 μM). The depolarising effects of Et-AMPA and AMPA could be fully antagonized by the selective non-NMDA antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline (NBQX) [37], whereas no antagonist effect was observed when using the selective NMDA antagonist D-2-amino-5-phosphonovaleric acid (D-AP5) [38]. Homo-7-HPCA showed very weak NBQX sensitive depolarising activity in this model (EC_{50} = 1 mM), whereas Homo-AMPA was without detectable activity even at 1 mM. Homo-AMPA was also tested for possible antagonist effects, but showed no significant antagonism at 100 μM towards the excitatory activity elicited by NMDA (10 μM), AMPA (5 μM) or KAIN (10 μM) (not illustrated). The EC_{50} values determined in the rat cortical slice preparation are shown in table I, and the dose–response curves for Et-AMPA, AMPA, 7-HPCA and Homo-7-HPCA are illustrated in figure 3.

Discussion

The ligand of choice for pharmacological studies of the AMPA receptors is the potent and specific agonist, AMPA [13, 22]. Replacement of the 5-methyl group of AMPA by various other groups has provided a number of potent and selective AMPA receptor agonists. The bromomethyl analogue, (*RS*)-2-amino-3-[5-(bromomethyl)-3-hydroxy-4-isoxazolyl]propionic acid (ABPA) was shown to be an agonist at the AMPA receptors, without receptor alkylating properties *in vivo* or *in vitro* [25, 39]. The *tert*-butyl analogue ATPA proved to be a fairly potent agonist at AMPA receptors [24], whereas the phenyl analogue APPA was shown to be a weak partial agonist [18]. The relationship between structure and biological activity of these and other analogues of AMPA containing alkyl groups of very different size and

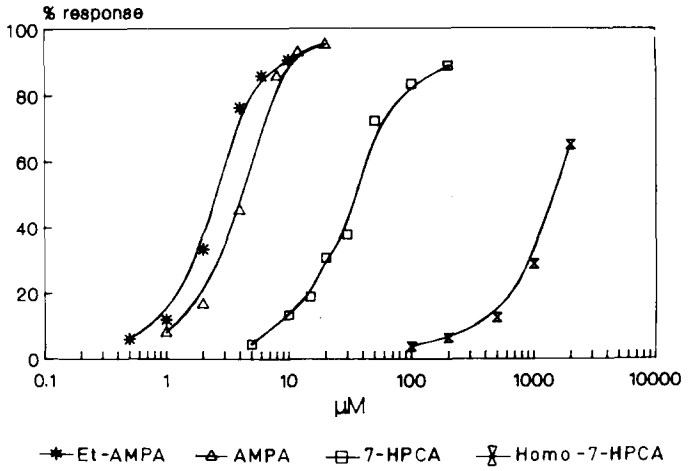


Fig 3. Dose–response curves obtained from *in vitro* electrophysiological experiments on Et-AMPA, AMPA, 7-HPCA, and Homo-7-HPCA. Each curve represents data obtained from single experiments, which have been repeated 1–3 times according to table I.

shape indicate that the AMPA receptor(s) contain a lipophilic cavity, which can accommodate rather bulky substituents [27]. These studies have been extended in this paper in order to examine the validity of a previously suggested simple model of the AMPA receptor agonist pharmacophore as illustrated in figure 1. AMPA and 7-HPCA were chosen as lead structures, and the model compounds Et-AMPA, Homo-AMPA and Homo-7-HPCA were designed by incorporation of a methylene group into selected positions of the lead molecules (see fig 5).

Et-AMPA was synthesized and shown to be slightly more active than AMPA, both in [^{3}H]AMPA binding studies and in electrophysiological experiments (table I). This enhanced activity is interesting, as other AMPA analogues, *eg* ATPA, with larger substituents in the 5-position of the isoxazole ring, have shown decreased activity compared to AMPA. Obviously, this extra steric bulk of the 5-ethyl substituent compared with that of the methyl group of AMPA, is very well tolerated by the AMPA receptor. The structural similarity between AMPA and Et-AMPA is illustrated in figure 4 showing low-energy conformations of the 2 compounds. On the assumption that the 5-ethyl group has a similar effect on the electronic properties of the 3-hydroxyisoxazole ring as the 5-methyl group in AMPA, this enhanced activity of Et-AMPA can be ascribed to a beneficial effect of the additional steric bulk of the ethyl group. This effect may reflect that the ethyl group fits very well into a lipophilic cavity at the binding site of the AMPA receptor (see fig 5), or alternatively, that the ethyl group of Et-AMPA, more effectively than the methyl group of AMPA, forces the amino acid side chain into a conformation recognizable by the receptor.

Studies on the relationship between structure, conformational mobility and effects on NMDA receptors of a series of piperidinedicarboxylic acids indicate that a certain degree of conformational flexibility is required for potent NMDA receptor agonist activity [40]. This does, however, not seem to be a general requirement for all classes of NMDA agonists [41], and it certainly is not the case for AMPA receptor agonists. Thus, the conformationally immobilized analogue 7-HPCA (fig 5), is an effective and specific AMPA receptor agonist (table I) [23, 25]. Homo-7-HPCA was shown to have a markedly lower activity (EC_{50} = 1 mM) at AMPA receptors compared to 7-HPCA (EC_{50} = 35 μM). This may be explained by differences in the conformations of Homo-7-HPCA and 7-HPCA, although the orientation of the functional groups in the 2 compounds are quite similar. This similarity is illustrated for low-energy conformations of 7-HPCA and Homo-7-HPCA in figure 4, both with pseudo-equatorial orientation of the carboxylate group. It should be noted though that the 6-membered ring of 7-HPCA is considerably more planar compared to the 7-membered ring of Homo-7-HPCA. This is reflected by the partially hidden amino group of Homo-7-HPCA when viewed from the angle illustrated in figure 4. The extra CH_2-group in Homo-7-HPCA has not only afforded a less planar ring system, but it has also been incorporated into a part of the molecule, assumed to be intimately involved in the binding to the AMPA receptor site (fig 5). In contrast, the bulky 7-membered ring of the very potent AMPA agonist, 4-AHCP, is assumed to be accommodated by the proposed lipophilic cavity at the AMPA receptor in a manner similar to that of the *tert*-butyl group of ATPA [26] (fig 1). In this model the orientation of the 3-OH group of 7-HPCA is somewhat different from that of the 3-OH group of AMPA (fig 5). This may to some extent explain the 10-fold lower activity of 7-HPCA in the electrophysiological model as compared to AMPA (table I). However, it should be noted that deprotonation of the OH group and delocalization of the anion at physiological pH may reduce the influence of the somewhat different orientations of the isoxazole bioisosteric groups.

Homo-AMPA is a side-chain homologue of AMPA. The low-energy conformation of Homo-AMPA illustrated in figure 4 indicates that Homo-AMPA cannot easily obtain a bend conformation with

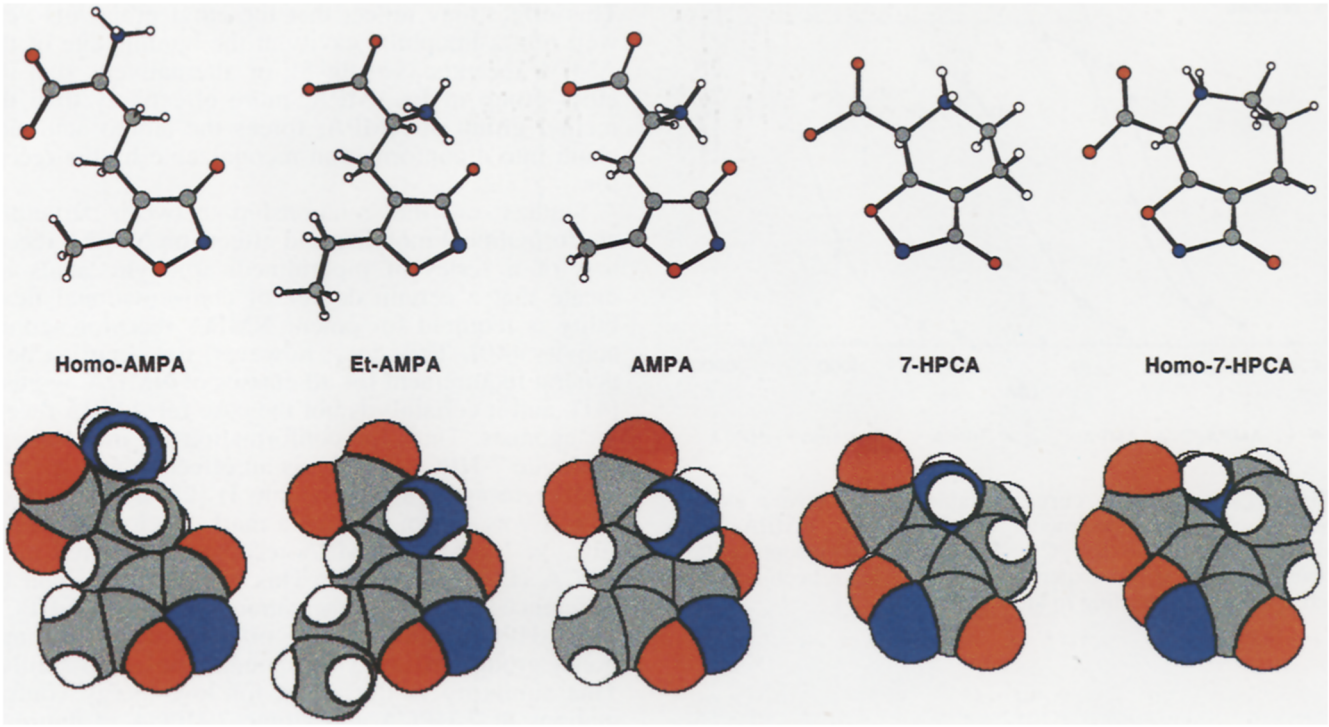


Fig 4. Low-energy conformations in ball-and-stick and space-fill models obtained using the program MacMimic and subsequent MM2 energy minimization of the molecules using the program Chem3D. The conformation of AMPA corresponds to a low-energy conformation obtained in previous modelling studies [26] and Et-AMPA is illustrated in a similar conformation. 7-HPCA is illustrated in a conformation corresponding to previous X-ray and NMR studies [25] and Homo-7-HPCA is shown in a similar conformation. A complete conformational analysis has not been performed on Homo-AMPA, and the conformation shown is therefore only one example of possible low-energy conformations, shown for illustrative purposes.

the functional groups spatially arranged in a manner similar to that of AMPA. Whether the inactivity of Homo-AMPA reflects a direct steric effect, destructive for receptor binding, or whether the preferred conformation(s) of the side chain of Homo-AMPA are not recognizable by the AMPA receptor, as indicated in figure 5, is at present not known.

Et-AMPA was shown to be a specific AMPA agonist, slightly more potent than AMPA itself. The 3 new compounds synthesized and tested have contributed to development of the AMPA receptor agonist pharmacophore models. There are strict requirements for the spatial arrangement of the α-amino acid moiety and the distal acidic group, and the present studies seem to support the hypothesis that the AMPA receptors contain a lipophilic cavity. It must be emphasized that the suggested pharmacophore outlined in figures 1 and 5 is a primitive working model.

The recent developments regarding molecular biology and sequence analysis of receptor isoforms, including AMPA receptor isoforms [11, 42, 43] represent major steps towards structure determination of receptors. Until more direct studies, including X-ray crystallographic approaches can provide more precise information about the 3-dimensional structures of receptor sites, simple receptor models may prove to be useful in semirational agonist and antagonist drug design.

Experimental protocols

Chemistry

Melting points were determined in capillary tubes and are uncorrected. All compounds were detected as single spots on TLC plates and visualized using UV light and $KMnO_4$ spraying reagent. Compounds containing amino groups were also visual-

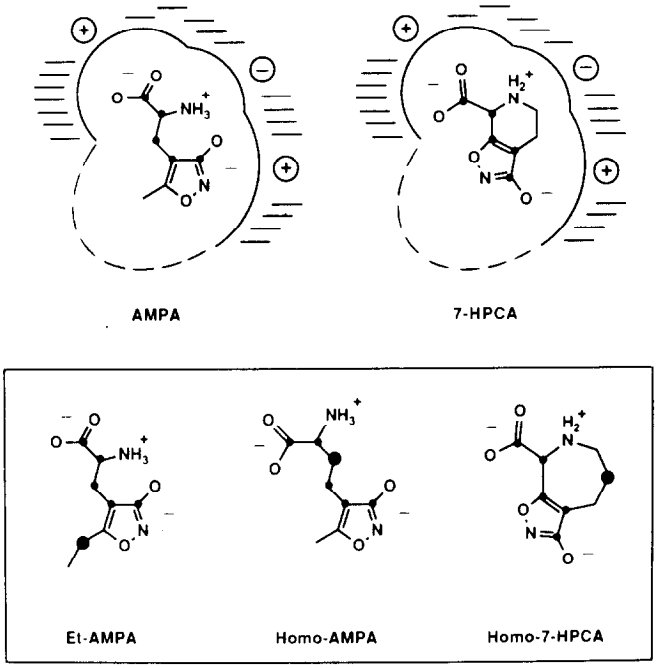


Fig 5. Illustration of the binding of the agonists AMPA and 7-HPCA to a hypothetical model of the AMPA receptor (top). At the bottom are shown the structures of the new compounds Et-AMPA, Homo-AMPA and Homo-7-HPCA with indications (large dots) of the extra CH_2-groups incorporated into the molecules of the parent structures, AMPA and 7-HPCA.

ized using a ninhydrin spraying reagent. IR were recorded on a Perkin–Elmer 781 spectrophotometer in KBr pellets for solids and between NaCl disks for liquids. ^{1}H-NMR spectra were obtained on a Bruker AC-200 F (200 MHz) or a Varian EM360L (60 MHz) spectrometer in $CDCl_3$ solutions using TMS as an internal standard, unless otherwise indicated. Microanalyses were within ± 0.4% of calculated values, unless otherwise indicated, and were performed by G Cornali, Microanalytical Laboratory, Leo Pharmaceutical Products, or P Hansen, Department of General and Organic Chemistry, University of Copenhagen.

Ethyl 5-ethyl-3-hydroxyisoxazole-4-carboxylate **2**
To a solution of sodium methoxide, prepared from sodium (3.95 g; 0.17 mol) and MeOH (60 ml), was added a solution of hydroxylamine hydrochloride (6.26 g; 0.09 mol) in MeOH (70 ml). This mixture was added at 0°C to a solution of ethyl 3-ethoxy-2-(ethoxycarbonyl)-2-pentenoate **1** [28] (20 g; 0.08 mol) in MeOH (10 ml). The reaction mixture was stirred overnight at room temperature. After cooling to 0°C, concentrated hydrochloric acid (8 ml) was added. The reaction mixture was filtered, evaporated and subjected to a Soxhlet extraction for 4 h with light petroleum (300 ml). Evaporation of the extract gave crude **2** (8.7 g; 57%). A small sample was recrystallized (ether/light petroleum) to give an analytical sample of **2**, mp: 82–83°C; IR 3200–2500 (multiple, m-s), 1700 (s), 1620 (s), 1530 (s), 1510 (s) cm^{-1}; ^{1}H-NMR (60 MHz) δ 8.2 (1 H, br s), 4.45 (2H, q, J = 7.5 Hz), 3.0 (2 H, q, J = 7.5 Hz), 1.3 (3 H, t, J = 7.5 Hz), 1.2 (3 H, t, J = 7.5 Hz). Anal $C_8H_{11}NO_4$ (C, H, N).

Ethyl 3-ethoxy-5-ethylisoxazole-4-carboxylate **4** *and ethyl 2,5-diethyl-3-oxo-isoxazoline-4-carboxylate* **5**
To a solution of **2** (7.3 g; 39 mmol) in acetone (150 ml) was added potassium carbonate (16.4 g; 118 mmol), and the mixture stirred at 60°C for 1 h. Ethyl bromide (8.5 g; 78 mmol) was added to the mixture, which was stirred overnight at 60°C. The reaction mixture was cooled, filtered, evaporated and subjected to column chromatography (toluene/ethyl acetate, 10:1, containing 1% glacial acetic acid) to give crude **4** (5.0 g; 59%) as a yellow oil: ^{1}H-NMR (60 MHz) δ 4.35 (2 H, q, J = 7.5 Hz), 4.3 (2 H, q, J = 7 Hz), 3.0 (2 H, q, J = 7.5 Hz), 1.4 (9 H, m). Further elution (toluene/ethyl acetate, 1:1, containing 1% glacial acetic acid) gave crude **5** (2.6 g; 30%) as a yellow oil: ^{1}H-NMR (60 MHz) δ 4.35 (2 H, q, J = 7.5 Hz), 4.0 (2 H, q, J = 7.5 Hz), 3.0 (2 H, q, J = 7.5 Hz), 1.3 (9 H, m).

3-Ethoxy-5-ethyl-4-(hydroxymethyl)isoxazole **6**
To a suspension of lithiumaluminum hydride (1.4 g; 36 mmol) in dry ether (150 ml) was slowly added a solution of **4** (5.1 g; 24 mmol) in dry ether (70 ml). The reaction mixture was stirred at room temperature for 90 min. After cooling to 0°C, water (50 ml) was added. The aqueous phase was acidified with 4 M hydrochloric acid, and after separation of the phases, the aqueous phase was extracted twice with ether. The organic phases were dried ($MgSO_4$), filtered and evaporated to give crude **6** (3.6 g; 87%). A small sample was distilled in a Kugelrohr apparatus (140°C, 0.3 mmHg) to give an analytical sample of **6** as a colourless oil: IR 3500–3200 (br, m-s), 2980 (s), 2940 (s), 2880 (m), 1645 (s), 1515 (s) cm^{-1}; ^{1}H-NMR (200 MHz) δ 4.40 (2 H, s), 4.35 (2 H, q, J = 7.0 Hz), 2.70 (2 H, q, J = 7.5 Hz), 1.75 (1 H, s), 1.45 (3 H, t, J = 7.0 Hz), 1.30 (3 H, t, J = 7.5 Hz). Anal $C_8H_{13}NO_3$ (C, H, N).

4-(Chloromethyl)-3-ethoxy-5-ethylisoxazole **7**
A mixture of **6** (3.6 g; 21 mmol) and thionyl chloride (15 ml) was heated to 90°C for 90 min. After evaporation the residue was subjected to column chromatography (toluene/ethyl acetate, 2:1) to give crude **7** (2.9 g; 73%). A small sample was distilled in a Kugelrohr apparatus (120°C, 0.1 mmHg) to give an analytical sample of **7** as a yellow oil: IR 2980 (s), 2940 (m), 2900 (w), 2880 (w), 1645 (s), 1515 (s) cm^{-1}; ^{1}H-NMR (200 MHz) δ 4.35 (2 H, s), 4.33 (2 H, q, J = 7.0 Hz), 2.75 (2 H, q, J = 7.5 Hz), 1.45 (3 H, t, J = 7.0 Hz), 1.30 (3 H, t, J = 7.5 Hz). Anal $C_8H_{12}NO_2Cl$ (C, H, N, Cl).

Methyl 2-acetamido-2-(methoxycarbonyl)-3-(3-ethoxy-5-ethyl-4-isoxazolyl)propionate **8**
To a solution of sodium methoxide in MeOH prepared from sodium (160 mg; 6.8 mmol) and MeOH (20 ml), was added dimethyl acetamidomalonate (1.3 g; 6.8 mmol) and afterwards a solution of **7** (1.3 g; 6.8 mmol) in MeOH (10 ml). After reflux for 4 h the reaction mixture was cooled, evaporated and, upon addition of water (15 ml), extracted with dichloromethane (3 x 15 ml). The combined organic phases were washed with 1 M NaOH (30 ml) cooled to 0°C, dried ($MgSO_4$) and evaporated. Column chromatography (toluene/ethyl acetate, 2:1) afforded **8** (1.2 g; 51%); mp: 102–103°C (toluene); IR 3350 (m), 2980 (m), 2950 (m), 1750 (s), 1640 (s), 1510 (s) cm^{-1};

^{1}H-NMR (200 MHz) δ 6.65 (1 H, s), 4.25 (2 H, q, J = 7.0 Hz), 3.80 (6 H, s), 3.35 (2 H, s), 2.53 (2 H, q, J = 7.5 Hz), 2.0 (3 H, s), 1.40 (3 H, t, J = 7.0 Hz), 1.22 (3 H, t, J = 7.5 Hz). Anal $C_{15}H_{22}N_2O_7$ (C, H, N).

(RS)-2-Amino-3-(5-ethyl-3-hydroxy-4-isoxazolyl)propionic acid zwitterion (Et-AMPA)
Compound **8** (300 mg; 0.88 mmol) was suspended in hydrobromic acid (10 ml, 48%) and kept at reflux for 30 min. After evaporation, the residue was dissolved in water (0.2 ml), EtOH (5 ml) was added, and pH of the solution adjusted to *ca* 5 with triethylamine. The precipitate was filtered off and recrystallized (water) to give Et-AMPA (81 mg; 46%); mp: 230–233°C (decomp); IR 3300–2300 (multiple, w-m), 2100 (w), 1655 (m), 1605 (s), 1535 (s), 1510 (s) cm^{-1}; ^{1}H-NMR (200 MHz, D_2O) δ 3.9 (1 H, t, J = 7 Hz), 2.85 (2 H, d, J = 7 Hz), 2.6 (2 H, q, J = 7.5 Hz), 1.15 (3 H, t, J = 7.5 Hz). Anal $C_8H_{12}N_2O_4 \cdot 1/8H_2O$ (C, H, N).

*3-(3-Methoxy-5-methyl-4-isoxazolyl)propionaldehyde **10***
To a solution of ethyl 3-(3-methoxy-5-methyl-4-isoxazolyl)propionate **9** [29] (1.5 g; 6.9 mmol) in dry toluene (30 ml) at −78°C was added diisobutylaluminum hydride (17.4 ml, 1 M in hexane; 17.4 mmol) under a nitrogen atmosphere. After stirring for 6 min, MeOH (6 ml) and Rochelles salt solution (18 ml) was added. The reaction mixture was slowly heated to room temperature and extracted with ether. The organic phases were dried ($MgSO_4$) and evaporated, followed by flash chromatography (toluene/ethyl acetate, 4:1) to give crude **10** (1.03 g; 88%) as a yellow oil: ^{1}H-NMR (60 MHz) δ 9.9 (1 H, s), 4.0 (3 H, s), 2.8–2.6 (4 H, m), 2.3 (3 H, S).

*(RS)-5-[2-(3-Methoxy-5-methyl-4-isoxazolyl)ethyl]hydantoin **11***
A mixture of **10** (1.0 g; 6.1 mmol), potassium cyanide (1.0 g; 15 mmol), ammonium carbonate (3.0 g; 30 mmol), water (7.5 ml), and MeOH (7.5 ml) was refluxed for 6 h. Water was added (3 ml) and the reaction mixture left at 5°C for 5 d for crystallization. After filtration and recrystallization (95% EtOH) **11** (406 mg; 28%) was obtained; mp: 204–205°C; IR 3600–2800 (multiple, m-s), 1775 (m), 1740–1700 (multiple, s), 1660 (m), 1520 (s) cm^{-1}; ^{1}H-NMR (200 MHz, DMSO) δ 8.05 (2 H, s), 4.05 (1 H, dd, J = 7.5 and 5.5 Hz), 3.95 (3 H, s), 2.4 (2 H, t, J = 7.5 Hz), 2.35 (3 H, s), 1.95–1.65 (2 H, m). Anal $C_{10}H_{13}N_3O_4$ (C, H, N).

(RS)-2-Amino-4-(3-hydroxy-5-methyl-4-isoxazolyl)butyric acid (Homo-AMPA) hydrochloride
Compound **11** (86 mg; 0.36 mmol) was refluxed in 6 M hydrochloric acid (5 ml) for 48 h. The reaction mixture was evaporated, re-evaporated from water and recrystallized (glacial acetic acid) to give crude Homo-AMPA (51 mg). This product was subjected to ion-exchange chromatography (IRA-400), and the evaporated fractions dissolved in 4 M hydrochloric acid, gave after repeated recrystallizations (glacial acetic acid) Homo-AMPA hydrochloride (9 mg; 11%); mp: 180–181°C (decomp); IR 3420 (br, m), 3300–2500 (multiple, m-s), 1735 (s), 1660 (s), 1525 (s) cm^{-1}; ^{1}H-NMR (200 MHz, D_2O) δ 3.9 (1 H, t, J = 7.0 Hz), 2.4 (2 H, t, J = 7.5 Hz), 2.2 (3 H, s), 2.0 (2 H, m). Anal $C_8H_{13}N_2O_4Cl \cdot 1/3H_2O$ (C, H, N).

*3-Methoxy-5,6,7,8-tetrahydro-4H-isoxazolo[5,4-c]azepine hydrochloride **12***
A solution of methyl 3-methoxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[5,4-*c*]azepine-7-carboxylate [30] (700 mg; 3.09 mmol) in potassium hydroxide in MeOH/water (5:1) (3.2 M, 7 ml) was heated at 80°C for 20 h. After evaporation, water (25 ml) was added to the residue, and the mixture was extracted with chloroform. The combined and dried ($MgSO_4$) organic phases were evaporated and the residue was dissolved in ethyl acetate (4 ml). Addition of hydrogen chloride in ethyl acetate (2.2 M, 10 ml) gave **12** (440 mg; 72%); mp: 195–198°C (decomp); IR 3460 (broad, m), 2960 (m), 2820 (s), 2740 (s), 1540 (s), 1415 (s) cm^{-1}; ^{1}H-NMR (200 MHz, D_2O) δ 4.40 (2 H, s), 3.91 (3 H, s), 3.56–3.49 (2 H, m), 2.55–2.48 (2 H, m), 2.09–1.94 (2 H, m). Anal $C_8H_{13}N_2O_2Cl \cdot 1/4H_2O$ (C, H, N). Cl: calcd: 16.95; found: 16.33.

*3-Methoxy-7-nitroso-5,6,7,8-tetrahydro-4H-isoxazolo[5,4-c]azepine **13***
To a solution of **12** (439 mg; 2.16 mmol) in acetate buffer (pH 4, 10 M, 16 ml) was added dropwise and with stirring a solution of sodium nitrite (2.98 g; 43 mmol) in water (6 ml). The solution was heated at 90°C for 30 min and after cooling to 25°C, the solution was extracted with dichloromethane. The organic phase was washed with 2 M sodium carbonate and evaporated. Column chromatography (toluene/ethyl acetate, 2:3 containing 1% glacial acetic acid) gave **13** (332 mg; 80%) as a yellow oil: IR 2950 (m), 1710 (m), 1520 (s), 1460 (br, s) cm^{-1}; ^{1}H-NMR (200 MHz) δ 5.50 (1.1 H, s), 4.92 (0.9 H, s), 4.62–4.55 (0.9 H, m), 3.96 (s) + 3.92 (s) + 3.91–3.88 (m) (a total of 4.1 H), 2.51–2.40 (2 H, m), 2.55–2.12 (0.9 H, m), 1.99–1.85 (1.1 H, m). Anal $C_8H_{11}N_3O_3$ (C, H, N).

*Methyl (RS)-3-methoxy-7-nitroso-5,6,7,8-tetrahydro-4H-isoxazolo[5,4-c]azepine-8-carboxylate **14***
To a solution of **13** (250 mg; 1.27 mmol) in dry THF (6 ml), kept under a nitrogen blanket at −78°C, was added butyllithium (1.6 M in hexane, 900 µl; 1.4 mmol), and after 1 min was added methyl chloroformate (977 µl; 12.7 mmol) during a 1-min period. Stirring was continued at −78°C for 5 min, and upon addition of glacial acetic acid (50 µl) the solution was evaporated. Water (5 ml) was added to the residue and the mixture extracted with dichloromethane. The combined and dried organic phases were evaporated. Column chromatography (dichloromethane/ethyl acetate, 19:1) gave **14** (62 mg; 19%); mp: 85–86°C (ethyl acetate/light petroleum): IR 3400 (br, w), 2960 (w), 2905 (w), 1745 (s), 1660 (m), 1530 (s) cm^{-1}; ^{1}H-NMR (200 MHz) δ 6.85 (0.6 H, s), 6.76 (0.4 H, s), 4.97 (0.6 H, ddd, J = 14.7, J = 7.1 and J = 2.5 Hz), 4.70 (0.4 H, ddd, J = 14.8, J = 8.0 and J = 2.5 Hz), 4.40 (0.6 H, ddd, J = 14.7, J = 9.4 and J = 2.0 Hz), 4.00 (1.8 H, s), 3.98 (1.2 H, s), 3.83 (1.2 H, s), 3.74 (1.8 H, s), 3.31 (0.4 H, ddd, J = 14.8, J = 8.8 and J = 2.0 Hz), 2.59–2.39 (2 H, m), 2.39–2.25 (0.7 H, m), 2.21–1.90 (1 H, m), 1.89–1.71 (0.3 H, m). Anal $C_{10}H_{13}N_3O_5$ (C, H, N).

(RS)-3-Hydroxy-5,6,7,8-tetrahydro-4H-isoxazolo[5,4-c]azepine-8-carboxylic acid (Homo-7-HPCA) hydrate
Through a solution of **14** (60 mg; 0.23 mmol) in glacial acetic acid (5 ml), kept at 0°C was passed a stream of hydrogen bromide gas for 5 min, and stirring was continued at room temperature for 15 h. The solution was evaporated and the residue recrystallized (methanol/ethyl acetate) to give methyl (*RS*)-3-hydroxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[5,4*c*]azepine-8-carboxylate hydrobromide (45 mg). This was dissolved in water (1 ml) placed on an ion exchange column (IRA-400). After 4 h, the column was eluted with acetic acid (1 M) and recrystallized (water) to give Homo-7-HPCA hydrate (18 mg; 40%); mp: 148–150°C (decomp); IR 3500–2300 (multiple, m), 1630 (s), 1575 (s), 1480 (s), 1360 (s) cm^{-1}; ^{1}H-NMR

(200 MHz, D_2O) δ 5.17 (1 H, s), 3.60–3.51 (2 H, m), 2.60–2.30 (2 H, m), 2.15–1.85 (2 H, m). Anal $C_8H_{10}N_2O_4 \cdot H_2O$ (C, H, N).

Receptor binding assays

[^{3}H]AMPA, [^{3}H]CPP, [^{3}H]glycine, [^{3}H]KAIN and [^{3}H]MK-801 were purchased from NEN, Denmark. [^{3}H]AMPA binding was studied using a modified version of an earlier described method [31]. The assay was carried out using 5 nM [^{3}H]AMPA (60 Ci/mmol) in 30 mM Tris–HCl buffer (pH 7.1) with 2.5 mM $CaCl_2$ and 100 mM KSCN added. Following incubation at 0–4°C for 30 min, bound ligand was isolated using filtration through Whatman GF/C filters. The method used for studies of the binding of [^{3}H]KAIN was a modified version of the procedure described by Braitman and Coyle [32]. The assay was carried out using 5 nM [^{3}H]KAIN (60 Ci/mmol) in 50 mM Tris–HCl buffer (pH 7.1). Following incubation at 0–4°C for 60 min, bound ligand was isolated using filtration through Whatman GF/C filters. [^{3}H]CPP binding was studied using a modified version of the method described by Murphy *et al* [33]. The assay was carried out using 5 nM [^{3}H]CPP (30 Ci/mmol) in 30 mM Tris–HCl buffer (pH 7.1) with 2.5 mM $CaCl_2$ added. Following incubation at 0–4°C for 30 min, bound ligand was isolated using filtration through Whatman GF/B filters. [^{3}H]MK-801 baseline binding was studied using a modified version of the method described by Wong *et al* [34]. The assay was carried out using 5 nM [^{3}H]MK-801 (29 Ci/mmol) in 5 mM Tris–HCl buffer (pH 7.4) in the presence of 100 nM glycine. Following incubation at 25°C for 120 min, bound ligand was isolated using filtration through Whatman GF/B filters. [^{3}H]MK-801 binding in fully stimulated membranes was studied using a modified version of the method described by Wong *et al* [34]. The assay was carried out using 5 nM [^{3}H]MK-801 (29 Ci/mmol) in 5 mM Tris–HCl buffer (pH 7.4) in the presence of 1 μM glycine and 30 μM glutamate. Following incubation at 25°C for 120 min, bound ligand was isolated using filtration through Whatman GF/B filters. [^{3}H]glycine binding was carried out by a modified version of the method described by Kemp *et al* [35], using filtration through Whatman GF/C filters instead of centrifugation to isolate bound ligand.

In vitro electrophysiology

A rat cortical slice preparation for testing the depolarizing activity of EAAs described by Harrison and Simmonds [36] was used in a modified version. Wedges (500 μm thick) of rat brain containing cerebral cortex and corpus callosum were placed with the cortex part between 2 layers of absorbent fiber and the corpus callosum part between 2 other layers of absorbent fiber. The 2 halves were electrically insulated from each other with a grease gap. The cortical part was constantly perfused with a Mg^{2+}-free, oxygenated Krebs buffer to which the compounds tested were added, whereas the corpus callosum part was perfused with a Mg^{2+}- and Ca^{2+}-free Krebs buffer. The 2 parts were each in contact with an Ag/AgCl electrode through which DC potentials were measured and *via* a DC amplifier plotted on a chart recorder. NMDA and KAIN were purchased from Sigma Chemical Co, USA and D-AP5 from Tocris Neuramin, UK. AMPA, 4-AHCP and 7-HPCA were synthesized as described earlier [22, 23]. NBQX was a gift from T Honoré, Novo-Nordisk, Denmark.

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