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Identification of a subunit-specific antagonist of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate/kainate receptor channels

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ABSTRACT **Excitatory synaptic transmission in the mammalian central nervous system is mediated predominantly by glutamate receptor (GluR) channels of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate/kainate (AMPA/KA) receptor type. A major improvement in our understanding of glutamatergic synaptic transmission has been achieved after the identification of quinoxalinediones (e.g., 6-cyano-7-nitroquinoxaline-2,3-dione) as specific antagonists of AMPA/KA receptors. In addition to their effects on neurons, quinoxalinediones were also shown to block glutamate-induced responses mediated by recombinant AMPA/KA receptor channels expressed in heterologous systems, irrespective of their particular subunit composition. Here we report the identification of an AMPA/KA receptor antagonist that selectively blocks a subset of AMPA/KA receptors. We found that Evans blue, a biphenyl derivative of naphthalene disulfonic acid, blocks at low concentrations (IC_{50} = 355 nM for the subunit combination GluR1,2) KA-mediated responses of the subunits GluR1, GluR1,2, GluR1,3, and GluR2,3 expressed in *Xenopus* oocytes but not responses of GluR3 or GluR6. The blocking action of Evans blue was partially reversible and did not compete with KA for the agonist binding site. These findings suggest not only that Evans blue is a potent tool for elucidating the functional role of specific AMPA/KA receptor subtypes for excitatory synaptic transmission but also that it may also represent a powerful starting point for clinically useful drugs that are able to reduce the excitatory drive in specific neuronal populations of the central nervous system.**

Excitatory synaptic transmission in the mammalian central nervous system is mediated by different types of glutamate receptors (GluRs) that are commonly distinguished by their sensitivity to different GluR agonists (1–3). Synaptic transmission is primarily mediated by GluRs of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate/kainate (AMPA/KA) receptor type that are characterized by a linear current–voltage relationship and a competitive block by quinoxalinediones in the submicromolar concentration range (4–6). On the molecular level, the isolation of AMPA/KA receptor clones and their functional expression in heterologous expression systems have demonstrated a large variety of AMPA/KA receptors. Their functional properties depend critically on the particular subunit composition (7–12). Thus, it has been shown that AMPA/KA receptors containing the subunit GluR2 display a linear current–voltage relationship and a low calcium permeability, whereas recombinant receptors composed of subunits GluR1 and GluR3 or the subunit combination GluR1,3 show an inwardly rectifying current–voltage relationship and are highly permeable for calcium ions (8, 13–15). Despite these impressive functional differences, all subunit combinations of AMPA/KA receptors investigated so far are sensitive to quinoxalinediones, the best known high-affinity class of antagonists of excitatory glutamatergic synaptic transmission in the brain (16). In this report, we demonstrate that different AMPA/KA receptor subtypes are also pharmacologically distinct. They can be pharmacologically separated by an antagonist, a biphenyl derivative of 1,3-naphthalene disulfonic acid ($C_{34}H_{24}N_6Na_4O_{14}S_4$) commonly known as Evans blue (EB).

MATERIALS AND METHODS

Preparation of Oocytes and mRNA. The procedures of preparing and maintaining *Xenopus* oocytes followed the procedures described by Methfessel *et al.* (17). Briefly, oocytes were removed from frogs and injected the same day with 2 ng of complementary RNA encoding for GluR1, GluR2, and GluR3. One day later, oocytes were incubated in collagenase (clostridiopeptidase A) from *Clostridium histolyticum* type I (Sigma) for 1–4 hr and the residual follicle cell layers were skinned by hand. After 2–3 days, skinned oocytes were used for experiments.

The preparation of mRNA was performed as described by Hollmann *et al.* (7, 8). To avoid the formation of GluR1 homomers after coinjection of GluR1 and GluR2, the RNA ratio 1:10 was chosen for expression of receptors GluR1,2. The same rationale was applied for the formation of GluR2,3. Indeed, if the RNA ratio of GluR2 and GluR3 was chosen as 1:1, a more rectifying current voltage relationship and a smaller blocking effect of EB indicated a substantial formation of GluR3 homomers.

Electrophysiology and Data Analysis. Current recordings were performed by using the two-electrode voltage clamp system Turbo Tec 01C ("npi electronic"; Tamm, F.R.G.) as described (17, 18). During measurements, the recording chamber was continuously perfused with saline at room temperature (21–24°C). Normal saline (frog Ringer) contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM Hepes·NaOH (pH 7.2). Perfusing solutions also contained 150 μM niflumic acid (Sigma) to block the transient component of intrinsic Ca^{2+}-activated chloride currents in *Xenopus* oocytes. EB (C.I. 23860, pure; M_r = 960.8) was obtained from Serva at a purity of 81% observed with thin layer chromatography. Chicago acid was bought from Aldrich (21,686-0) and Congo red (C.I. 22120), Chicago sky blue (C.I. 24410), and naphthol were from Sigma. Drugs were applied by continuous bath perfusion, which yielded a complete change of solution within 5 s. Recording electrodes were made of fine glass pipettes (1–2 MΩ) filled with 2 M KCl. Signals were filtered at 0.1–5 KHz with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) and stored on video tapes by a PCM/VCR recording device (Instrutech, Mineola, NY) or directly on computer by using the Luigs and Neu-

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; KA, kainate; EB, Evans blue; GluR, glutamate receptor.

mann software (Ratingen, F.R.G.). The software used for analysis was from Instrutech or Luigs and Neumann.

RESULTS

EB Blocks AMPA/KA Receptors Composed of GluR1,2. Recombinant AMPA/KA receptors were expressed in *Xenopus* oocytes and after 2–3 days their functional properties were studied by conventional electrophysiological techniques (17). Fig. 1 displays current responses obtained from oocytes expressing recombinant AMPA/KA receptors composed of GluR1 and GluR2 (termed GluR1,2). By using the two-electrode voltage-clamp configuration (17, 18), KA-induced currents through GluR1,2 were investigated either by clamping the holding potential to 0 mV and monitoring the agonist-induced currents during brief negative voltage pulses as indicated in Fig. 1*a* or simply by evaluating the blocking effect of EB on the inward current evoked directly at negative values (between −60 and −100 mV). In general, we preferred the protocol indicated in Fig. 1*a*, as it minimized possible secondary effects (e.g., desensitization) resulting from the accumulation of internal calcium passing through open AMPA/KA receptor channels (18).

EB Block Is Specific for Particular Subunit Combinations. Fig. 1*b* shows that 1 μM EB blocked the KA-induced currents through GluR1,2 by ≈90%. This block developed quite fast (within 30 s after switching to the EB-containing solution) and was partially reversible upon washout of EB. The concentration of EB that inhibited 50% of the current response (IC_{50}) was 356 ± 27 nM (mean ± SD, $n = 6$; see also Fig. 1*c*). The corresponding Hill coefficient was 1.96 ± 0.26 (mean ± SD, $n = 6$), suggesting a significant cooperativity in the binding of EB to the AMPA/KA receptor complex. Current responses to glutamate and AMPA were also reduced by EB in a similar concentration range (data not shown). As indicated in Table 1, EB was most effective in blocking currents through GluR1,2, but it also blocked currents through GluR1 and the subunit combinations GluR1,3 and GluR2,3. For all these subunits, the properties of the blocking action were quite similar, except for a significant variation in the IC_{50} values found for different subunit combinations. Surprisingly, EB displayed a remarkable subunit specificity of its blocking effect at low concentrations. Thus, currents through the subunits GluR3 or GluR6 were not blocked. Instead, responses through GluR3 were slightly increased by submicromolar EB concentrations. This is shown in Fig. 2, which compares the results of GluR3 with GluR1,2, the subunit combination with the highest affinity for EB.

Table 1. EB-mediated block for various AMPA/KA receptor subunit combinations

Subunit combination	IC_{50}, nM	Oocytes, no.
GluR1	535 (430–600)	5
GluR3	Not blocked	8
GluR1,2	355 (316–388)	11
GluR2,3	380 (329–500)	4
GluR1,3	680 (590–940)	6
GluR6	Not blocked	4

Numbers in parentheses are the range.

EB Is a Noncompetitive Antagonist of AMPA/KA Receptor Channels. What is the mechanism of the EB-blocking effect observed for GluR1,2? To address this question, we established dose–response relations for KA-induced currents in the absence and in the presence of 400 nM EB. The KA concentration producing a half-maximal current response (EC_{50}) in the presence of EB was 95 ± 34 μM (mean ± SD, $n = 3$, GluR1), which was similar to the value obtained under control conditions 110 ± 44 μM (mean ± SD, $n = 3$; also Fig. 3). This finding strongly suggests that the block by EB is noncompetitive, implying that EB acts on sites that are distinct from the binding site of the agonist. In an additional series of experiments, we tested several compounds that are structurally related to EB for their inhibitory effects. As demonstrated in Table 2, EB is by far the most potent substance despite the small structural differences between EB and the other compounds tested. For example, Congo red, a substance that shares the overall structure of EB, had virtually no effect at a concentration of 1 μM. Similar results were found for the substance Chicago sky blue, which is identical to EB except for the replacement of two central methyl side chains with two methoxy groups. The small current reduction of Chicago sky blue (<5%) was not reversible and could be explained by the "rundown" of the current amplitudes commonly observed in these experiments. In addition to the blocking action of EB at low micromolar

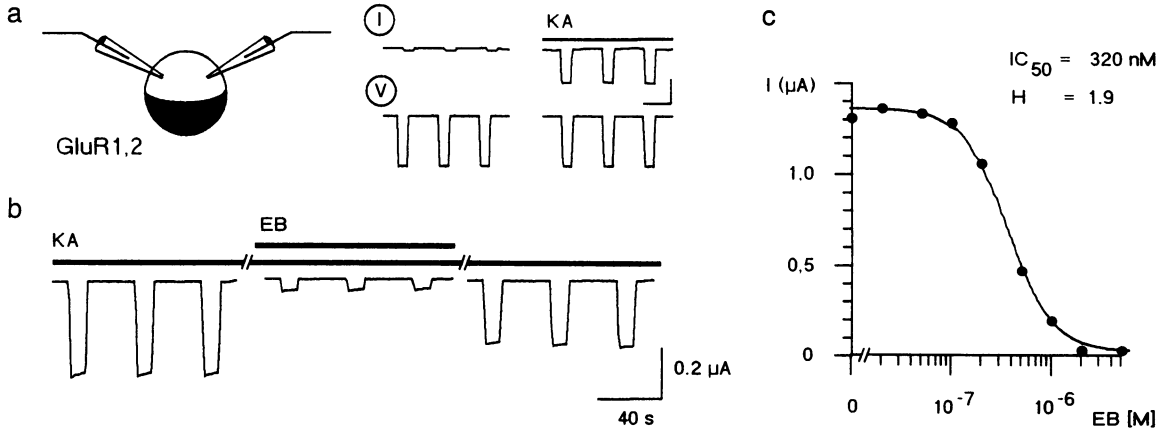


FIG. 1. Block of AMPA/KA receptor channel GluR1,2 by EB. (*a*) KA-induced currents through GluR1,2 were measured in the two-electrode voltage-clamp configuration (17, 18) according to the voltage protocol described to the right in *a*. The membrane voltage was held at 0 mV and repetitively stepped to −100 mV. Minute current steps registered before the application of KA reflect the background current of the oocyte (<100 nA at −100 mV). Bath application of 300 μM KA induced inward currents through GluR1,2 at −100 mV with a current reversal potential around 0 mV. For these experiments, GluR complementary RNA for GluR1 and GluR2 were injected in *Xenopus* oocytes at a ratio 1:10 to avoid the formation of GluR1 homomers. Calibration bars correspond to 20 s (horizontal) and 0.2 μA (vertical). (*b*) Simultaneous bath application of 1 μM EB blocked KA-induced currents by 90% in this oocyte. The block could be partially reversed after washout of EB from the bath solution, with a 70% recovery after a washout time >5 min. (*c*) Dose–response curve obtained for GluR1,2 (ratio 1:10) after bath application of increasing concentrations of EB (between 200 nM and 5 μM). Current values were fitted according to least squares, yielding a half-maximum inhibition (IC_{50}) of 320 nM and a Hill coefficient (H) of 1.9 for this experiment.

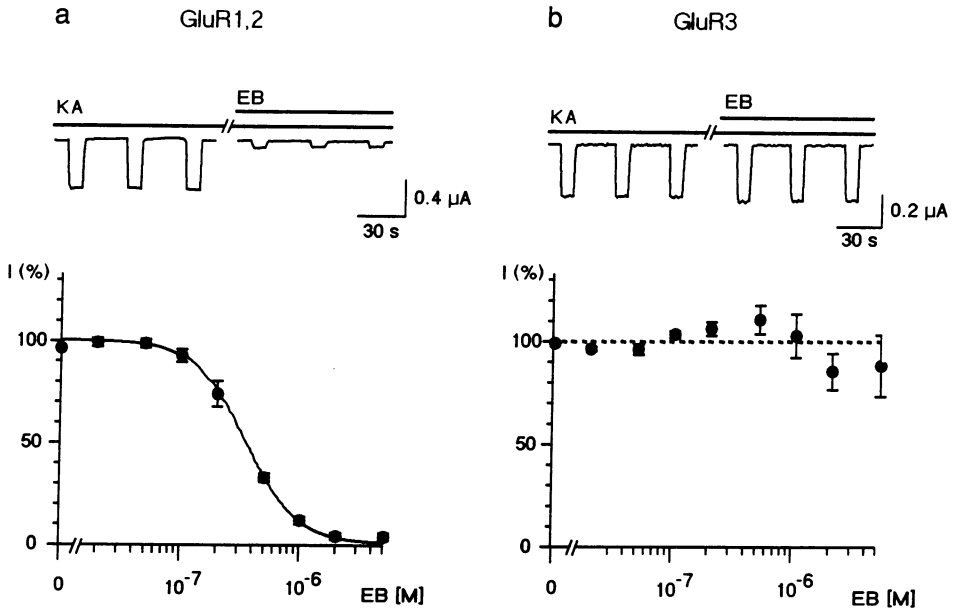


FIG. 2. Subunit-specific block of AMPA/KA receptor channels. (*a*) (*Upper*) Experimental protocol used to measure the current responses to bath application of 300 μM KA before (to the left) and after (to the right) perfusion with 1 μM EB to an oocyte expressing GluR1,2. (*Lower*) Averaged dose–response relationship for oocytes expressing GluR1,2 (RNA ratio, 1:10). Each data point represents the average of six current measurements obtained after normalization to the maximum current response at 0 μM EB (= 100%). (*b*) (*Upper*) Current responses observed after bath application of 300 μM KA before (to the left) and after (to the right) perfusion with 1 μM EB to an oocyte expressing GluR3. Current recordings show a small but significant potentiation of current responses after EB application. (*Lower*) Averaged current responses obtained for six oocytes normalized to the current response at 0 μM EB (= 100%). Note the increase in the variability of the results for higher concentrations of EB that may result from a potentiation effect (see also *Upper*) combined with a small variable rundown of the currents for long agonist applications.

concentrations, we observed a massive potentiation of KA-induced currents at EB concentrations >50 μM. These effects were observed for all subunit combinations tested, independent of their blocking properties in the submicromolar concentration range. In the present analysis, this potentiation was not studied in more detail, but it should be an interesting area for future investigations.

DISCUSSION

By performing electrophysiological experiments on recombinant AMPA/KA receptors expressed in *Xenopus* oocytes, we have identified a subunit-specific antagonist of AMPA/KA receptor channels. A biphenyl derivative of naphthalene disulfonic acid, commonly termed EB, has been shown to block a subpopulation of AMPA/KA receptors in the submicromolar concentration range. Among the AMPA/KA receptor subunit combinations tested so far, all receptors were blocked with the exception of homomeric receptors composed of either GluR3 or GluR6. These results demonstrate that besides their profound differences in electrophysiological properties, recombinant AMPA/KA receptors are also pharmacologically distinct.

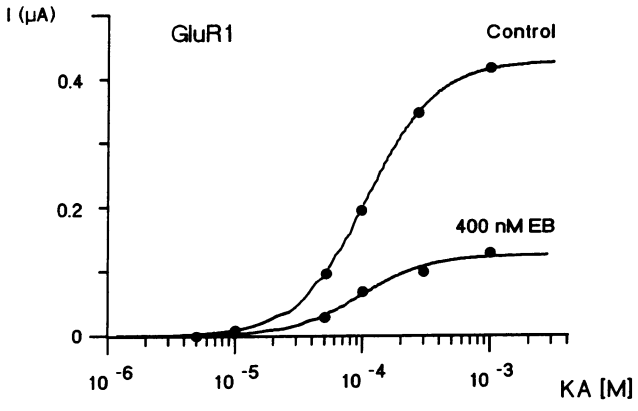
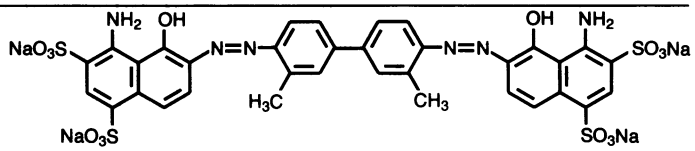
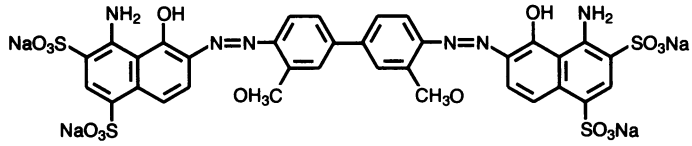
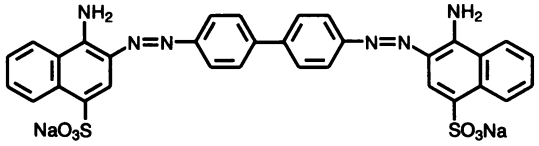
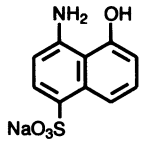
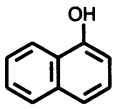


FIG. 3. EB as a noncompetitive antagonist of AMPA/KA receptor channels. Dose–response curves obtained from current measurements for an oocyte expressing GluR1,2 (GluR1/GluR2 RNA ratio, 1:10) in the absence (control) and presence of 0.4 μM EB. Application of EB reduced the KA response for all kainate concentrations investigated but had little effect on the apparent EC_{50} for kainate, which was 95 ± 34 μM (mean ± SD, $n = 3$) under control conditions and 110 ± 44 μM (mean ± SD, $n = 3$) after application of 0.4 μM EB. Both results are in agreement with a noncompetitive action of EB on the AMPA/KA receptor complex.

So far, the binding site for EB on the AMPA/KA receptor complex is essentially unknown. The elimination of the blocking effect by replacement of two methyl with methoxy side chains (Table 2) implies that either hydrophobic or steric interactions between the binding site and the central biphenyl region of EB are fundamental for the blocking effect. An alternative possibility is that the decrease in hydrophobicity imposed by the two methoxy side chains prevents the molecule from reaching a binding site within the transmembrane region of the receptor. The existence of a secondary potentiating action of EB at higher concentrations in fact suggests a rather complex heterogeneous interaction between EB and AMPA/KA receptors. In future studies, however, the formation of chimeric receptors combining EB-sensitive and -insensitive subtypes should allow the EB binding site(s) on the AMPA/KA receptor complex to be specified in more detail.

Subunit-specific antagonists may serve as powerful tools to identify the functional role of different AMPA/KA receptor subunits during excitatory synaptic transmission and, perhaps, to discriminate between different types of glutamatergic synapses in the central nervous system. Indeed, micromolar concentrations of EB have been shown to affect excitatory synapses in cultured neurons from thalamus, mainly by slowing both activation and decay of miniature synaptic currents recorded with the patch-clamp technique

Table 2. Specificity of the EB-mediated blocking effect on GluR1,2

Compound	Inhibition by 1 μM antagonist, %	Structure
EB	85–90	
Chicago sky blue	<5	
Congo red	<5	
Chicago acid	<5	
Naphthol	<5	

(19). Applied at higher concentrations (100 μM), EB abolished the fast desensitization of AMPA/KA receptors in response to extracellularly applied glutamate and, more importantly, completely blocked miniature excitatory synaptic currents in thalamic neurons. So far, the mechanism of EB action in this system is not well understood. One explanation for the relatively high EB concentration necessary to block synaptic currents is provided by significant diffusion barriers preventing the drug from entering the synaptic cleft. Although, due to differences in the experimental protocol, these results are not directly comparable to our data on recombinant channels, they provide clear evidence for a functional role of EB-sensitive AMPA/KA receptor channels at some types of excitatory synapses in the central nervous system.

In conclusion, our data demonstrate that EB is a potent subunit-specific antagonist that binds to a site on the AMPA/KA receptor complex that is different from the site of "classical" agonists. The molecular determinants of the binding site and the mechanisms of interaction between EB and the AMPA/KA receptor complex remain to be elucidated. A potentially important implication, however, is related to the involvement of AMPA/KA receptors in various neurological diseases, such as Parkinson and Alzheimer diseases (20–23) or some forms of epilepsy (24–27). Thus, a recent report (28) presented evidence for a therapeutic strategy of Parkinson disease based on AMPA/KA receptor antagonists. The finding of subunit-specific compounds combined with the heterogeneous distribution of AMPA/KA receptors (8, 29) suggests that EB might preferentially act on selected brain regions or neuron types. This opens the possibility that this or a related compound may serve as a basis for clinically useful substances designed to specifically reduce excitatory activity in brain regions associated with pathological processes.

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