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Evans Blue Antagonizes Both α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate and Kainate Receptors and Modulates Receptor Desensitization

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SUMMARY

The biphenyl derivative of 1,3-naphthalene disulfonic acid, known as Evans blue (EB), has been shown previously to specifically antagonize currents mediated by the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) subtype of glutamate receptors (1). In contrast, we demonstrate herein that EB potently inhibits glutamate-evoked currents mediated by the kainate-type receptor GluR6 (IC_{50} 150 nM) as well as the AMPA-type receptor GluR1 (IC_{50} = 220 nM) in whole-cell patch clamp recordings from transfected human embryonic kidney 293 cells. In addition to diminishing GluR6-mediated peak current amplitude, EB significantly altered receptor desensitization by slowing the rate of onset by ~2-fold (1 μM EB), slowing the rate of recovery by ~2-fold (0.1 μM EB), and increasing the steady state to peak current amplitude ratio by ~50-fold (1 μM EB). Interestingly, relatively little EB inhibition of GluR6 currents was observed in recordings from cells pretreated with the lectin concanavalin A, which eliminates kainate receptor desensitization. Similarly, currents recorded from GluR1-transfected cells were also relatively insensitive to EB inhibition if desensitization was first blocked by cyclothiazide. Moreover, for both GluR6 and GluR1, EB inhibition of agonist-evoked current was largely reversed if transfected cells were subsequently exposed to concanavalin A or cyclothiazide, respectively. Although EB may not be as selective an antagonist as previously believed, the relationship between EB-induced peak current inhibition and effects on receptor desensitization may be useful in further elucidating structures or mechanisms involved in the rapid desensitization of AMPA- and kainate-type glutamate receptors.

Ionotropic GluRs mediate most fast synaptic transmission in the mammalian brain, playing important roles in development, learning, memory, and sensorimotor processing, as well as in certain neuropathological conditions (2, 3). These receptors are divided into three subtypes (AMPA, kainate, and NMDA) according to physiological and pharmacological criteria (4, 5). Cloning studies have revealed that ionotropic GluRs are composed of oligomeric complexes of homologous subunits. Combinations of GluR1(A), GluR2(B), GluR3(C), and GluR4(D) compose AMPA receptors; GluR5,6,7 and KA-1,2 are constituents of kainate receptors; and NR1 in combination with NR2A, NR2B, NR2C, or NR2D forms NMDA receptors (for review, see Refs. 6–8). This information has facilitated the detailed analysis of the structure, function, and modulation of these receptors. From experiments using heterologous expression of recombinant receptors in *Xenopus laevis* oocytes or mammalian cell lines, correlations can be made between ion channel properties and subunit composition (8, 9). This ultimately will provide insight into the properties of native GluR channels in the nervous system.

In situ hybridization and immunocytochemical studies indicate that the subunits that compose AMPA, kainate, and NMDA receptors are distributed differentially in the central nervous system (for review, see Ref. 8). However, it is difficult to determine the significance of receptor subunit composition in synaptic transmission or in neurotoxicity without the aid of subunit-specific and subtype-specific antagonists. Whereas the NMDA receptors have a well defined and specific array of antagonists, there are relatively few inhibitors that discriminate between AMPA and kainate receptors; of these inhibitors, only the 2,3-benzodiazepines (e.g., GYKI 53655 and 52466) show more than a 10-fold difference in IC_{50} values for these two receptor subtypes (10, 11).

It has been suggested that EB, a biphenyl derivative of 1,3-naphthalene disulfonic acid, is an antagonist specific for

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ABBREVIATIONS: AMPA, α-amino-3-hyroxy-5-methyl-4-isoxazolepropionate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EB, Evans blue; GYKI 52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine; HEK, human embryonic kidney; con A, concanavalin A; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; NMDA, *N*-methyl-D-aspartate; GluR, glutamate receptor; BAPTA 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid

AMPA-preferring receptors (1). In the study by Keller *et al.* (1), experiments were performed using recombinant receptors expressed in *X. laevis* oocytes, and EB was shown to antagonize kainate-evoked currents in oocytes injected with combinations of the AMPA receptor subunits GluR1, GluR2, and GluR3. The effect of EB was potent (IC_{50} values of 355 nM), partially reversible, and did not seem to involve competitive binding with kainate. However, in that study, inhibition by EB of kainate-evoked current was not observed in oocytes expressing the kainate receptor subunit GluR6.

We have repeated these experiments using recombinant GluR subunits expressed in HEK 293 cells. In contrast to previous results, we found that EB antagonized both GluR1- and GluR6-mediated currents. Moreover, our results suggest that in addition to its inhibitory effects on peak current amplitude, EB modulates regions of these receptors involved in agonist-induced desensitization. Agents that eliminate receptor desensitization, such as cyclothiazide and con A (12–15), blocked the inhibitory activity of EB on GluR1 and GluR6, respectively. As well, after application of EB, the residual GluR1- and GluR6-mediated currents showed slowing of desensitization. Therefore, whereas the role of EB as a specific AMPA receptor antagonist has been diminished, it may become an important tool for the further study of mechanisms involved in GluR desensitization.

Materials and Methods

Maintenance and transfection of HEK 293 cells. HEK 293 cells were maintained in minimal essential medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO), L-glutamine, penicillin, streptomycin, and sodium pyruvate in an incubator at 37°, 5% CO_2. HEK 293 cells were transfected by the method of calcium-phosphate precipitation (16) using 10 or 20 μg of DNA per 10-cm plate. Cells were washed and transferred onto glass coverslips in fresh medium 12–16 hr later. cDNA encoding the flip splice variant of GluR1 (17) in prK5 was a gift from P. Seeburg (University of Heidelberg, Heidelberg, Germany). cDNA encoding the fully RNA-edited version of GluR6 (V;C;R) was a gift from S. Heinemann and J. Egebjerg (Salk Institute, La Jolla, CA); this DNA was subcloned into a mammalian expression vector containing a cytomegalovirus promoter (18).

Electrophysiology. At 40–72 hr after transfection, cells were transferred to a recording chamber on the stage of an inverted microscope (Carl Zeiss, Thornburg, NY) and perfused continuously with external saline (145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 11 mM glucose, and 10 mM HEPES, pH 7.3). Agonist-induced currents from recombinant GluRs were recorded under voltage-clamp (V_H = −60 mV) in the whole-cell configuration of the patch clamp recording technique (19). Electrodes were pulled from thin-walled borosilicate glass (Warner Instruments) and heat polished. When filled with pipette solution (145 mM KCl, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 4 mM MgATP, 2 mM tetraethylammonium chloride, 5.5 mM BAPTA, and 10 mM HEPES, pH 7.2), electrode resistance was between 3 and 6 MΩ. Currents were amplified with either an EPC 7 (List Electronics, Darmstadt-Eberstadt, Germany) or Axopatch 200A (Axon Instruments, Foster City, CA). Data were acquired and analyzed using pCLAMP software (Axon Instruments). Rapid application of agonist was accomplished via computer-triggered solenoid valves (Neptune Research, Northboro, MA), which controlled gravity-fed flow of agonist and control solutions from the two sides of a θ tube (Hilgenberg, Malsfeld, Germany) positioned within 50–100 μm of the cell (18). Cyclothiazide was prepared as a 20-mM or 50-mM stock solution in dimethylsulfoxide and stored at −20°; the final concentration of dimethylsulfoxide in external solution for all experiments was less than 0.5%. Cyclothiazide was a gift from Eli Lily (Indianapolis, IN). Unless indicated, all other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Results

EB inhibits both GluR1- and GluR6-mediated currents

Fig. 1 demonstrates the effect of 1 μM EB on glutamate-activated currents recorded from cells expressing either GluR6 or GluR1 (flip variant). For both receptors, EB application resulted in a marked reduction in the amplitude of glutamate-evoked peak current, by 94 ± 0.01% (GluR6; eight experiments) and 93 ± 1.84% (GluR1; seven experiments) (mean ± standard error), a result in apparent contradiction to that obtained in *X. laevis* oocytes, in which inhibition of GluR6-mediated currents was not observed (1). Although kainate was used as the agonist in the study by Keller *et al.* (1), we have observed similar inhibition of agonist-evoked currents by EB recorded from GluR6-transfected HEK 293 cells using either glutamate or kainate (two experiments; data not shown) as the agonist. Inhibition by EB seemed to be irreversible, because little to no recovery of the agonist-evoked current response was observed after 30–60 min of washing.

To compare EB inhibition of the kainate-type receptor GluR6 with that of the AMPA-type receptor GluR1, dose-response measurements were made for the reduction in peak current amplitude. In our system (in which receptor desensitization was intact), EB seemed to be an even more potent inhibitor than reported previously (1). The IC_{50} values for EB inhibition were 150 nM for GluR6 and 220 nM for GluR1 (Fig. 2). The time constant for achieving a stable decrease in peak current amplitude during continuous superfusion with EB was also dose dependent. For both receptor types, time to

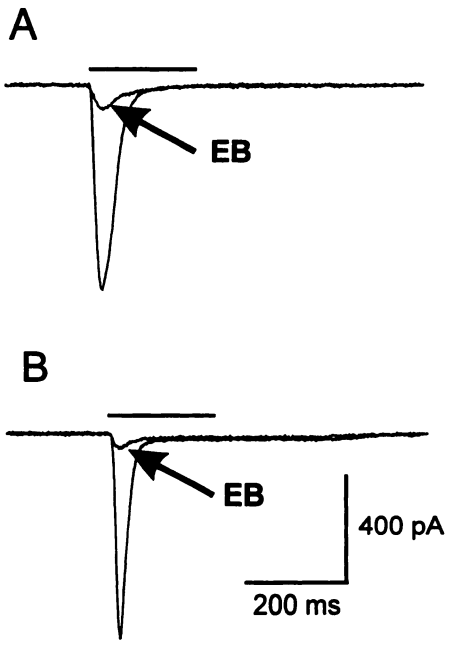

Fig. 1. EB inhibits glutamate-evoked current recorded from both GluR6 and GluR1. A, Currents evoked in an HEK 293 cell expressing GluR6 before and during exposure to 1 μM EB. B, Currents evoked in a HEK 293 cell expressing GluR1 before and during exposure to 1 μM EB. *Bar*, duration of 1 mM glutamate application.

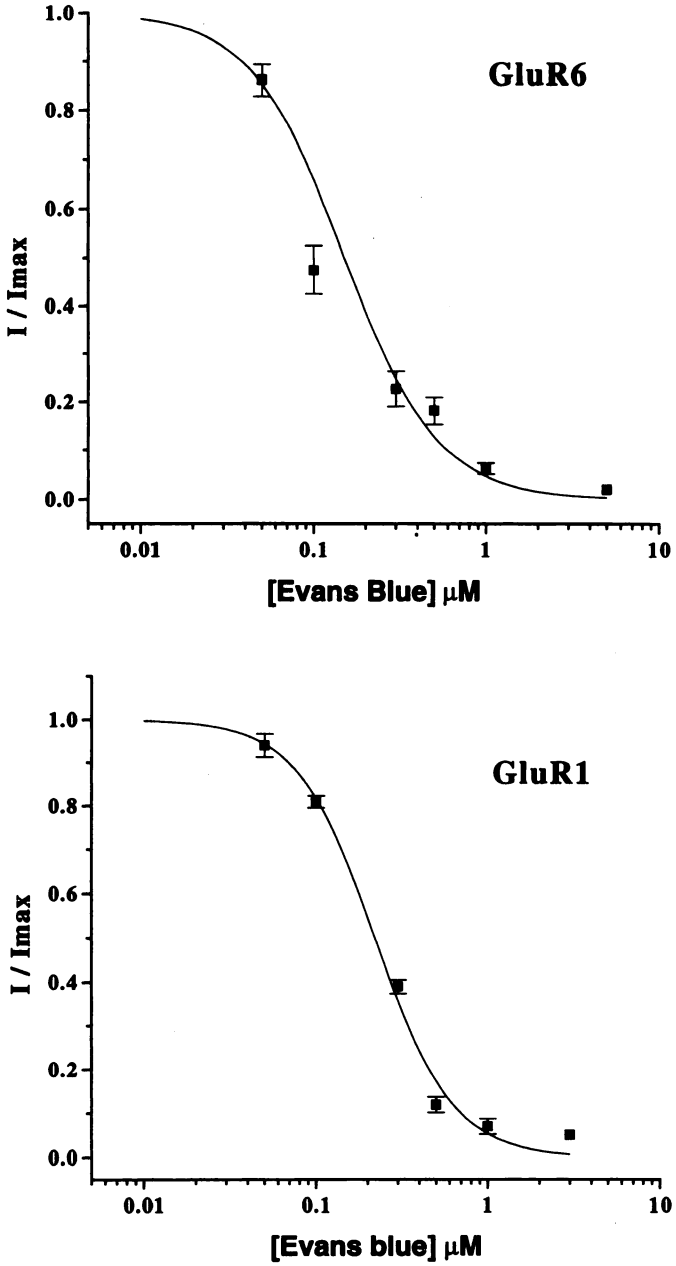

Fig. 2. Dose-response relationship for EB inhibition of glutamate (1 mM)-evoked current in GluR6- (*top*) and GluR1- (*bottom*) expressing cells. ■, peak current during EB exposure normalized to that before exposure (mean ± standard error). *I*, Peak current amplitude measured after a stable response was attained in the presence of EB (see text). *Curve*, fitted to the equation $I = 1/(1 + ([IC_{50}/[EB])^{n_H})$, *where* n_H is the Hill coefficient (28). *Points (top)*, data from 3–16 different cells; the IC_{50} value was 150 nM and n_H = 1.6. *Points (bottom)*, data from three to seven different cells; IC_{50} value was 220 n_H and nM = 1.9.

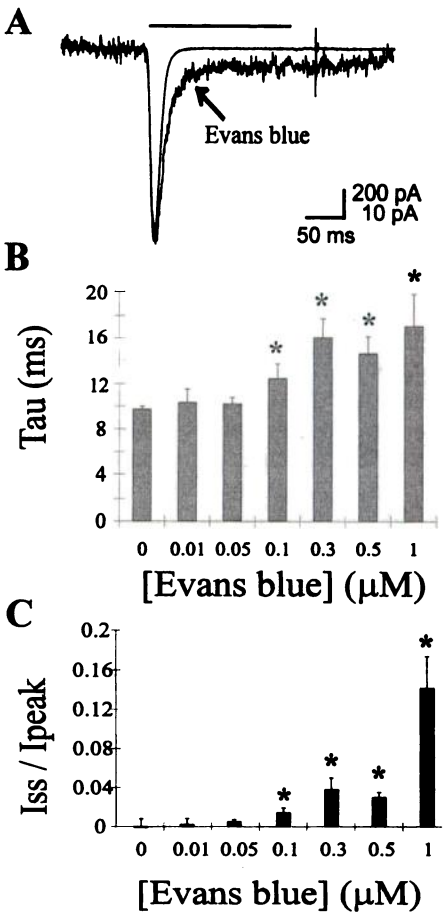

Fig. 3. Residual glutamate-evoked current, during exposure to EB, differs from the control in τ_D and the ratio of steady-state-to-peak-current for GluR6-transfected cells. A, Current evoked by rapid application of glutamate (1 mM) before and during exposure of a GluR6-transfected cell to 1 μM EB. The EB trace has been scaled to the amplitude of the control trace to allow for direct comparison between the two, revealing the increase in τ_D and in the ratio of steady-state-to-peak-current. B, Dose-dependent increase in τ_D during exposure to EB. *Bars*, mean τ_D (mean ± standard error) measured from 3–16 different cells. C, Dose-dependent increase in the ratio between the steady state current (*Iss*) and peak current (*Ipeak*) amplitudes before and during exposure to EB (mean ± standard error). *, Significance at 95% confidence interval after *t* test analysis.

reach a half-maximal effect ranged from ~300 sec (0.05 μM EB) to <30 sec (1 μM EB).

EB affects desensitization of glutamate receptors. By scaling the amplitude of the current remaining during exposure to 1 μM EB to match the original current amplitude recorded from GluR6-transfected cells, additional effects of EB became evident (Fig. 3A). First, an increase in the decay time constant (τ_D) for the onset of desensitization was apparent. This action of EB was dose dependent and, at the highest concentration tested (1 μM), EB treatment resulted in a 1.82 ± 0.27-fold increase in τ_D (Fig. 3B; eight experiments). Second, the ratio of steady-state-to-peak-current amplitude increased with application of EB in a dose-dependent manner (Fig. 3C). In fact, during treatment with 1 μM EB, there was an increase in the absolute amplitude of steady state current for six of eight cells (2.4 ± 0.82 pA before and 9.2 ± 2.63 pA during EB), and overall, the ratio of steady-state-to-peak-current increased significantly (by ~50-fold) from 0.003 ± 0.001 to 0.142 ± 0.032 (paired *t* test, $p < 0.005$; eight experiments). Whereas the dose-response relationship for the increase in the τ_D was similar to that for the reduction in peak current amplitude, the effect on the ratio of steady-state-to-peak-current seemed to require higher concentrations of EB. EB (1 μM) also slowed the onset of agonist-induced desensitization in recordings from GluR1-transfected cells. However, this effect was observed less consistently (only five of eight cells showed a significant change in τ_D) and smaller (τ_D increased by 1.38 ± 0.07-fold; $p < 0.05$ by paired *t* test) than that observed for GluR6-transfected cells.

It was possible that the observed effects of EB on the desensitization kinetics of GluR1 and GluR6 receptors were nonspecific, perhaps due to errors involved in measuring τ_D and the steady state and peak from the small amplitude currents remaining after ~95% inhibition by 1 μM EB. The possibility that slower desensitization kinetics were correlated with small amplitude currents was investigated by generating a plot of τ_D versus peak current amplitude measured from GluR6-transfected cells in the absence of EB. Such a plot showed no correlation between these two variables (r = 0.068; 50 cells). In addition, we investigated whether inhibition of GluR1-mediated current by CNQX, a competitive antagonist with an IC_{50} value of around 0.3 μM for AMPA receptors (20), resulted in slowing of the desensitization kinetics of the glutamate-evoked response. In contrast to our results with 1 μM EB, there was no significant change in τ_D or steady state to peak current ratio after >95% inhibition by 10 μM CNQX of the GluR1-mediated glutamate-evoked current response (four experiments; data not shown). Together, these results suggest that the slowing of desensitization of GluR1- and GluR6-mediated responses seen with EB is a specific effect of the drug.

In addition to its effects on the time constant for the onset of desensitization, EB also altered the time course of recovery from desensitization for glutamate-activated GluR6 currents. This effect was demonstrated by varying the time interval between two successive 1 mM glutamate pulses. As illustrated in Fig. 4, the amount of time required to fully recover from desensitization was prolonged in the presence of 0.1 μM EB. (In these experiments, we chose to use a relatively low concentration of EB to ensure sufficient residual current amplitude to allow a reasonable measurement of the partially recovered currents.) Under control conditions, the recovery time constant was 1.84 sec and increased to 3.08 sec after application of 0.1 μM EB.

Modifiers of desensitization inhibit EB activity. The lectin con A is known to inhibit the rapid glutamate-induced desensitization of GluR6-mediated currents (13, 21). Interestingly, when GluR6-transfected cells that had been pretreated with 5–10 μM con A were exposed to EB, even high concentrations of this drug resulted in little apparent reduction of glutamate-activated current (four experiments; Fig. 5A). Moreover, when the experiment was performed in reverse order, EB inhibition of glutamate-evoked currents was reversed largely by subsequent application of 5 μM con A (three experiments; Fig. 6A). These results offer an explanation why the inhibitory effects of EB on GluR6-mediated currents were not seen originally (1), because in those experiments, oocytes expressing GluR6 were always pretreated with 10 μM con A before testing for EB inhibition.

In light of the interaction between con A and EB on GluR6-mediated currents, experiments were performed to determine whether cyclothiazide, a drug that eliminates desensitization of AMPA-type subunits (14), would also interfere with EB inhibition of GluR1-mediated currents. As we found for GluR6, when GluR1-transfected cells were exposed to 50 μM cyclothiazide and then treated with high concentrations of EB, the latter drug had only a small inhibitory effect on glutamate-evoked currents (three experiments; Fig. 5B). Similarly, glutamate-activated currents that had been inhibited by EB subsequently were restored to amplitudes exceeding those of the initial currents (before application EB) by exposure of the cell to cyclothiazide (seven experiments; Fig. 6B).

From Figs. 5B and 6B, it is clear that EB inhibition of glutamate-activated current was impaired in the presence of

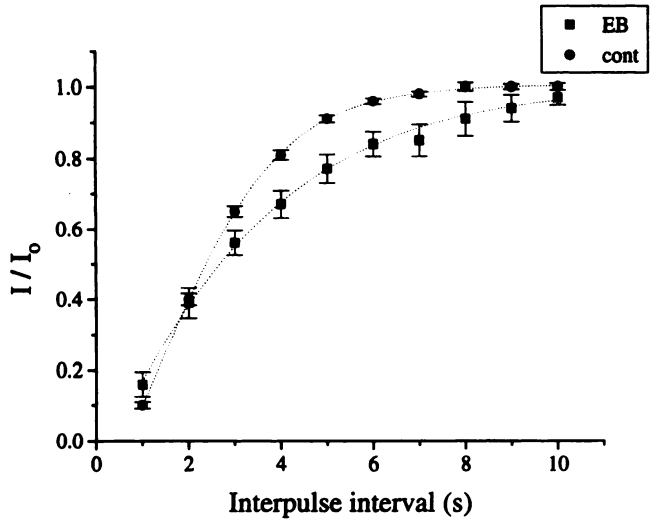

Fig. 4. EB (0.1 μM) decreases the rate of recovery from desensitization for glutamate-evoked currents in HEK 293 cells expressing GluR6. Glutamate-activated currents (1 mM) were evoked in pairs, with an interval ranging from 1–10 sec between glutamate pulses. *Points*, amplitude of the second current of each pair normalized to that of the first (mean ± standard error). The recovery time constants were 1.84 sec for control and 3.08 sec in the presence of 0.1 μM EB.

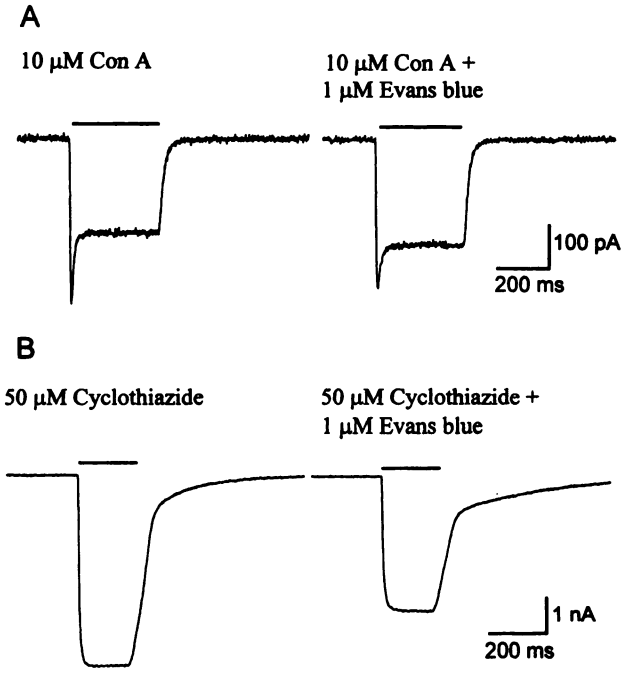

Fig. 5. EB inhibition of glutamate-evoked current is blocked by modifiers of receptor desensitization. A, Glutamate-activated currents recorded from a GluR6-transfected cell in the presence of con A (10 μM) before (*left*) and during (*right*) exposure to 1 μM EB. B, Glutamate-activated currents recorded from a GluR1-transfected cell in the presence of cyclothiazide (50 μM) before (*left*) and during (*right*) exposure to 1 μM EB. EB application resulted in a small decrease in current amplitude that stabilized after 5–10 min; *right trace*, recorded 10 min after onset of coperfusion with EB and cyclothiazide; *bars*, duration of 1 mM glutamate application.

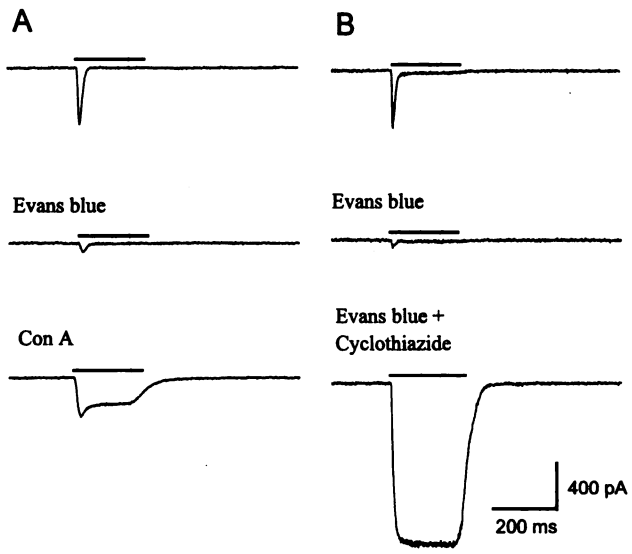

Fig. 6. EB inhibition of glutamate-activated currents is reversed by modifiers of receptor desensitization. A, *Top*, Glutamate-activated current recorded from a GluR6-transfected cell. *Middle*, 0.5 μM EB inhibition of glutamate-activated current in the same cell. *Bottom*, EB inhibition of glutamate-activated current was reversed largely upon exposure of the cell to 5 μM con A. B, *Top*, Glutamate-activated current recorded from a GluR1-transfected cell. *Middle*, 1 μM EB inhibition of glutamate-activated current in the same cell. *Bottom*, EB inhibition of glutamate-activated current was reversed upon exposure of the cell to 50 μM cyclothiazide (in the continued presence of EB). *Traces*, 1 mM application of glutamate; *bars*, duration of glutamate application.

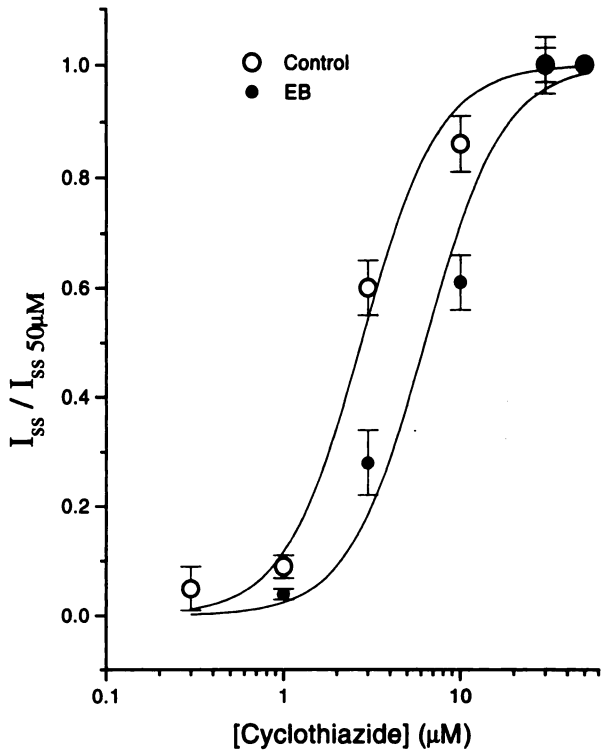

Fig. 7. Interaction between EB and cyclothiazide. Dose-response relationships were determined for the cyclothiazide-induced increase in 1 mM glutamate-evoked steady state current in the presence and absence of 0.5 μM EB. GluR1-transfected cells were exposed to increasing concentrations of cyclothiazide, with 50 μM always the final concentration. The steady state current amplitude measured at 50 μM cyclothiazide was used to normalize data from the lower concentrations. *Points*, mean ± standard error of normalized steady state current for each concentration (3–6 cells); *curves*, fitted to the equation $I_{ss} = I_{ss\ 50\ \mu M}/(1 + (EC_{50}/[CTZ])^{n_H}$, where ss is steady state and n_H is the Hill coefficient. The EC_{50} value was 2.8 μm (two experiments) for the control curve, whereas the EC_{50} value was 6.3 μM for the EB curve (two experiments). The EB curve was also well described by a competitive inhibition model (27), using 0.28 μM as the K_i value for EB (not shown).

a saturating concentration of cyclothiazide. However, because of the difficulties in fully washing out high concentrations of EB (>1 μM) from our perfusion system, we were unable to characterize the effect of cyclothiazide on the EB dose-response relationship. Therefore, to investigate the possibility that EB and cyclothiazide share a common binding site, we measured the effect of 0.5 μM EB on the dose-response relationship for cyclothiazide in recordings from GluR1-transfected cells. When compared with the dose-response relationship for cyclothiazide alone, a small, rightward shift in the EC_{50} values was observed (EC_{50} = 2.8 μM versus 6.3 μM; Fig. 7) in the presence of EB. This suggests that competition for a common binding site occurs. However, because the cyclothiazide-insensitive GluR6 subunit is EB-sensitive, an allosteric mechanism probably accounts for this result.

One interpretation of the above results is that EB exerts its inhibitory effect on only the peak (desensitizing portion) of the GluR1- or GluR6-mediated currents. In contrast, however, the nondesensitizing portion of kainate (1 mM)-evoked currents recorded from GluR1-transfected cells also was inhibited potently by EB (three experiments; data not shown), as previously described (1). Together, these results suggest (not surprisingly) that the mechanisms by which con A and cyclothiazide eliminate desensitization of GluR6- and GluR1-mediated currents, respectively, differ from that underlying the largely nondesensitizing current evoked by kainate at AMPA-preferring receptors.

Discussion

In this study, we have shown that EB is a potent inhibitor of currents mediated by kainate-type GluRs (GluR6) as well as AMPA-type receptors. In fact, our results indicate that GluR6 is actually more sensitive to EB inhibition than the AMPA receptor GluR1. Therefore, we conclude that EB is not a useful agent for discriminating between these two subtypes of non-NMDA GluRs, as originally proposed by Keller *et al.* (1). Consistent with our results, Ruano *et al.* (22) also reported an inhibitory effect of EB (at μM concentrations) on fast-desensitizing kainate-evoked currents recorded from embryonic/early postnatal dissociated hippocampal neurons.

Apart from peak current inhibition, we have demonstrated additional effects of EB on the agonist-induced desensitization of AMPA and kainate receptors, as well as interactions between EB and agents that block this desensitization, such as con A and cyclothiazide. It is not surprising that Keller *et al.* (1) saw no inhibition by EB of kainate-evoked currents recorded from GluR6-expressing *X. laevis* oocytes; they used con A to inhibit desensitization of these currents, and we have found that pretreatment with con A blocks the inhibitory effect of EB on GluR6-mediated currents.

One possible explanation for blockade of EB inhibition by con A and cyclothiazide is that these agents may sterically hinder access of EB to its binding site on GluR6 and GluR1, respectively. Because con A is a high molecular-weight, bulky molecule that has no defined region of interaction on GluR6

(but probably binds at glycosylated residues) (23, 24), the fact that con A blocks EB inhibition provides little information regarding EB's site of action on GluR6. However, cyclothiazide is a much smaller molecule, and its region of interaction with GluR1 is better defined. Recently, it has been shown that the flip variant of GluR1 [nomenclature of Sommer *et al.* (17)] is more sensitive to cyclothiazide inhibition of desensitization than the flop variant (25) and that a single amino acid within the flip/flop module largely determines sensitivity to cyclothiazide (26). Therefore, if steric hindrance by cyclothiazide (or con A) is involved in blocking EB activity, then the region in or around the flip/flop module may be the site of EB's actions as well. Our experiments demonstrate that EB affects a small shift in the EC_{50} values of cyclothiazide, which is consistent with the sharing of a common binding site by EB and cyclothiazide. However, initial studies also indicated a competitive interaction between the non-NMDA antagonist GYKI 52466 and cyclothiazide (27); more recent evidence, however, has revealed that these two drugs interact through an allosteric mechanism (28, 29). Similarly, we suspect that the interaction between cyclothiazide and EB is allosteric rather than competitive, because GluR6 is potently inhibited by EB but is unaffected by cyclothiazide, which suggests that these drugs bind at distinct sites on GluR1.

As well, the alteration in desensitization kinetics seen with EB may be a result of allosteric modulation of the flip/flop module; in addition to reducing the peak amplitude of agonist-evoked currents mediated by GluR6 and GluR1, EB also altered their desensitization kinetics, an effect not observed with CNQX (see Results), or GYKI 52466 (28, 30). Specifically, EB slowed the rate of onset as well as the rate of recovery from desensitization. A similar effect has been demonstrated for AMPA subunits that are RNA edited (from arginine to glycine) at a position near the start of the flip/flop module (31). However, it must also be noted that other regions (as yet unidentified) of the GluRs clearly contribute to desensitization (32). Further experiments are required to investigate whether EB interacts directly with regions known to be involved in desensitization, or if its actions are mediated by an allosteric mechanism.

The noncompetitive nature (1) as well as the relative irreversibility of EB inhibition, coupled with its effects on desensitization, suggest that EB's actions may be more complex than simple inhibition of receptor-channel activation. Instead, EB may act to modify the open state conformation of the channel and shift the equilibrium between open and desensitized states. In support of this hypothesis, at concentrations of EB that were saturating with respect to its inhibitory effects on GluR6, ~5% of the original agonist-evoked peak current amplitude remained, and this current showed a single exponential for onset of desensitization that was significantly slower than the initial (untreated) current. As well, the single-exponential time course for recovery from desensitization (at relatively low EB concentrations) was significantly slowed compared with control. Moreover, in addition to an increase in the ratio of steady-state-to-peak-current amplitude, in most cases for GluR6 the absolute amplitude of steady state current was increased in the presence of high concentrations of EB. Previous studies have also reported effects of EB on non-NMDA GluR desensitization and/or steady state current amplitude. Similar to our results, Ruano *et al.* (22) noted both slowing of desensitization onset and an increase in steady state current amplitude in a subset of dissociated hippocampal neurons that originally showed fast-desensitizing current responses to kainate. Furthermore, in the presence of EB, Leßmann *et al.* (33) observed a reduction in desensitization of non-NMDA glutamate currents in cultured thalamic neurons, and Keller *et al.* (1) noted a slight potentiation of kainate-evoked currents for GluR3-expressing oocytes.

In conclusion, although our results have diminished the role of EB as a specific inhibitor of AMPA-preferring GluRs, this agent may still prove useful as a tool for probing receptor structure-function relationships. Specifically, the identification of the receptor site(s) involved in the action of EB may provide additional clues to regions responsible for desensitization. Moreover, further investigation into the multiple effects of EB on non-NMDA GluR desensitization may reveal new insights into mechanisms underlying the process of agonist-induced desensitization of these receptors.

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