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Differential modulation of AMPA receptor mediated currents by Evans Blue in postnatal rat hippocampal neurones

Britta Schürmann, Xueqing Wu, Irmgard D. Dietzel & [1]Volkmar Leßmann

Lehrstuhl für Molekulare Neurobiochemie, Ruhr-Universität Bochum, NC7/170 44780 Bochum, Germany

1 The modulation of non-N-methyl-D-aspartate (NMDA) receptor-mediated whole cell currents and of glutamatergic synaptic transmission by purified Evans Blue (EB) was investigated in rat cultured postnatal hippocampal neurones by use of patch clamp recordings and a fast drug application system.

2 Three different groups of neurones could be distinguished with respect to the type of modulation obtained with 10 μM EB: EB was either a predominant inhibitor of desensitization (13% of the neurones), a predominant inhibitor of current amplitudes (42%) or a mixed inhibitor of both properties (45%). Both effects were not use-dependent and reached maximal levels after 30 s of pre-equilibration with the diazo dye.

3 Dose-response curves obtained from glutamate activated whole cell currents yielded an IC_{50} value for EB of 13.3 μM (Hill coefficient: 1.3) for the inhibition of desensitization, and an IC_{50} value of 10.7 μM (Hill coefficient: 1.2) for the inhibition of current amplitudes.

4 Chicago acid SS (100 μM) which is one of the synthesis precursors of EB had no effect on current amplitudes of glutamate activated whole cell currents but was a weak inhibitor of desensitization in all hippocampal neurones investigated, irrespective of the type of modulation obtained with EB in the same neurone.

5 Oxidatively modified EB (the so-called VIMP (10 μM)) had no effect on the kinetics but was a partial inhibitor of glutamate-activated whole cell currents in all hippocampal neurones investigated.

6 EB (10 μM) inhibited the amplitudes of non-NMDA receptor mediated autaptic currents to the same extent (to 39 ± 19% of control) as observed for glutamate activated whole cell currents (to 41 ± 17% and 56 ± 20%). However, the decay of the autaptic responses remained uninfluenced upon EB application, indicating that either receptor desensitization does not dominate the time course of the synaptic response or that the non-NMDA receptors sensitive to modulation of desensitization by EB are not present in the postsynaptic membrane.

7 In conclusion, EB differentially modulates α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor gating in different subsets of neurones. Upon identification of the cellular determinants for the differential modulation (e.g. AMPA receptor subunit composition) EB could become a useful tool to investigate receptor subtypes during electrophysiological recordings.

Keywords: Desensitization; glutamate receptors; AMPA receptors; non-NMDA receptors; Evans Blue; autapses; Chicago acid SS; hippocampal cell culture; whole cell currents

Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and detailed knowledge of the properties of glutamate receptors is essential to understand excitatory synaptic transmission in the brain. Glutamatergic transmission at excitatory synapses is mediated by three different classes of receptor gated ion channels: (i) AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) prefering receptors consisting of (hetero)oligomeric complexes of the glutamate receptor subunits GluR1-4 (also named GluR A-D), (ii) kainate prefering receptors consisting of the subunits GluR 5-7 and KA 1-2 and (iii) N-methyl-D-aspartate (NMDA) receptors assembled from heterooligomeric complexes of the subunits NR1 and NR2A-D, respectively (for reviews see Seeburg, 1993; Hollmann & Heinemann, 1994). An additional degree of complexity of possible subunit compositions of AMPA receptors results from alternative processing of each of the 4 AMPA receptor subunit mRNAs (GluR1-4), yielding either flip or flop splice variants (Sommer *et al*., 1990).

A number of electrophysiological studies indicate that neurones from different brain areas possess distinct kinetic properties of non-NMDA receptor mediated currents (see e.g. Colquhoun *et al*., 1992; Leßmann & Gottmann, 1994), suggesting the presence of distinct types of non-NMDA receptors in these brain regions. These receptor subtypes could differ either in their receptor subunit composition, in their post-translational modifications (e.g. phosphorylation, glycosylation), in intracellular protein-protein interactions or in their lipid microenvironment. Among these possibilities, thus far, only receptor subunit composition has been proven to modulate the kinetics of AMPA receptor-mediated currents: combined electrophysiological and molecular biological studies have revealed that specific AMPA receptor subunits and subunit splice variants differentially influence the kinetic properties of AMPA receptor mediated currents (Sommer *et al*., 1990; Mosbacher *et al*., 1994; Lomeli *et al*., 1994). However, receptor subtype specific antagonists and modulators of AMPA receptors (whether they sense differential subunit compositions or other differences in AMPA receptor properties) would be beneficial to identify directly AMPA receptor subtypes during electrophysiological recordings. Several drugs (Evans Blue, cyclothiazide, concanavalin A) have been shown previously to have the potential to discriminate between different non-NMDA glutamate receptor complexes: Evans Blue (EB) was found to reduce desensitization and partially block the peak amplitudes of non-NMDA-receptor mediated currents in rat thalamic neurones (Leßmann *et al*., 1992), and to block kainate evoked currents of heterologously expressed recombinant AMPA receptors containing either subunit

[1] Author for correspondence.

GluR1 or GluR2 (Keller *et al*., 1993). On the other hand, cyclothiazide and concanavalin A selectively inhibit desensitization of either AMPA or kainate receptors, respectively (Mayer & Vyklicky, 1989; Yamada & Tang, 1993; Partin *et al*., 1993; Wong & Mayer, 1993). Cyclothiazide was shown to have a higher potency at the flip splice variants as compared to the respective flop subunits of AMPA receptors (Partin *et al*., 1994). In addition, Joro spider toxin has been shown to block specifically current amplitudes of heterologously expressed AMPA receptors lacking the GluR2 subunit (Blaschke *et al*., 1993). However, a drug that shows distinct types of effects on different classes of AMPA receptors has so far not been described.

Previous experiments on thalamic neurones had shown that the antagonism by EB at non-NDMA receptors is purely non-competitive whereas the reduction of desensitization shares competitive and non-competitive aspects (V.L., unpublished). These data suggested that either (i) two different compounds of the commercially available impure dye (that has been used in former studies; purity $\geqslant 80\%$) independently modulated desensitization and current amplitudes, (ii) different residues of the EB molecule mediate the two distinct effects at non-NMDA receptors or (iii) EB can mediate both effects via binding to different classes of non-NMDA receptors.

Our study of the modulation of non-NMDA receptor-mediated currents by purified EB now reveals that the dye mediates both, the reduction of peak current amplitudes and the inhibition of desensitization at AMPA receptors. Secondly, different subsets of cells can be distinguished with respect to the type of modulation observed with EB, leading either to predominant modulation of desensitization, predominant inhibition of current amplitudes or to a mixture of both effects. Finally, since EB does not change the kinetics of glutamatergic synaptic currents, we provide evidence that in cultured hippocampal neurones either desensitization does not dominate the decay of excitatory postsynaptic currents or that the non-NMDA receptors sensitive for modulation of desentization by EB are not located in the postsynaptic membrane.

Methods

Cell culture

Hippocampal cultures were prepared from postnatal Wistar rats (P0–P5) essentially as described previously (Leßmann *et al*., 1994). In brief, hippocampi were dissected in ice-cold phosphate buffered saline with Ca^{2+} and Mg^{2+} (PBS+/+) and incubated for 10–15 min at 37°C in 0.25% trypsin. After being rinsed with PBS+/+ the tissue was dissociated with plastic pipettes of decreasing tip diameters and the cell suspension was injected into a three fold excess of DMEM/10% FCS. Cells were centrifuged at $300 \times g$ (10 min, 4°C) and resuspended in 3–5 ml DMEM/10% FCS. Viable cells were counted and 50,000 cells per dish were plated in 3.5 cm dishes (Nunc) coated with polyornithine (0.5 mg ml^{-1}, overnight). After 3 h the medium was changed to serum-free DMEM/B18 medium (Brewer & Cotman, 1989). Cultures were maintained in a humidified incubator with 10% CO_2 at 37°C.

Microisland cultures: we modified (V.L., unpublished) the microisland technique initially introduced by Segal & Furshpan (1990). In brief, cortical hemispheres (Wistar rats P1–P5) were trypsinized and dissociated as described above. Depending on the age of the rat pups 80,000–200,000 cortical cells were plated in uncoated plastic dishes. Glial cells preferentially attached to the plastic dishes and the supernatant was removed after 1–2 h. These glial cells were cultured for 7 days in DMEM/10% FCS, and supplemented with 10 μM cytosine-β-D-arabinofuranoside (ARAC) starting at 4 days *in vitro* (DIV). Under these conditions neatly spaced astrocytic islands of 100–300 μm diameter were formed. At 7 DIV 30,000–70,000 dissociated hippocampal neurones (see above) were plated into these dishes. On the next day the medium was exchanged to DMEM/B18, or to Neurobasal medium supplemented with B27 (Gibco). Cultures were maintained in an incubator with 5% CO_2 at 37°C. All experiments were performed at room temperature (i.e. 22°C).

Electrophysiological recording

Whole cell patch clamp recordings of agonist-induced currents were obtained from hippocampal cultures at 7–12 DIV by employing an EPC-7 patch clamp amplifier (List Medical Ins.). Autaptic currents were recorded in the whole cell configuration of the patch clamp technique at 10–14 DIV. Somatic voltage activated Na^+ currents were elicited with short (1 ms) depolarizing current pulses injected through the patch pipette. This paradigm evoked action potentials in more distal parts of the cell leading to transmitter release (Bekkers & Stevens, 1991). Patch electrodes were fabricated from borosilicate glass (GB150-8P, Science Products) and had typical resistances of 4–7 MΩ when filled with the standard pipette solution (in mM): K-gluconate 140, NaCl 5, $MgCl_2$ 1, $CaCl_2$ 0.2, EGTA 2, HEPES 15, pH adjusted to 7.3 with KOH. Standard extracellular solution was (in mM): NaCl 145, KCl 4, $CaCl_2$ 3, $MgCl_2$ 2, HEPES 10 and glucose 10, pH adjusted to 7.3 with NaOH. The holding potential was corrected for a junction potential of -10 mV resulting from this combination of intra- and extracellular solutions. The access resistance in the whole cell configuration was in the range of 9–13 MΩ and was compensated to 40–60% by a feedback circuit of the patch clamp amplifier. For recordings of current voltage relationships K-gluconate in the intracellular solution was replaced by CsCl (120 mM). During recordings of agonist-induced currents 1 μM TTX was added to all extracellular solutions to block synaptic activity. Agonists were dissolved in extracellular solution and applied as described below.

Currents were filtered at 3 kHz, digitized (Digidata 1200, Axon Ins.) and stored on the hard disk of a personal computer. Data analysis was performed by AUTESP software (Version 0.93, Garching Ins.). If not stated otherwise, all current traces shown are the average of 3–6 consecutive agonist applications or evoked autaptic currents, respectively. If not stated otherwise, all data are given as mean$\pm$s.d. Significance of differences of mean values were analysed by unpaired Student's *t*-test at *P* levels as indicated in the text and figure legends.

Drug application

Fast transmitter application was achieved by a superfusion system consisting of 3 glass capillaries as described in detail previously (Leßmann *et al*., 1992; Leßmann & Dietzel, 1995). In brief, opposing inflow and outflow pipettes created a laminar stream of agonist solution lateral to the cell. The stream was rapidly extended to superfuse the cell soma by transiently increasing the inflow pressure. Solution exchange measured as diffusion current at the tip of a patch pipette was $\leqslant 1$ ms. Solution exchange at the cell during whole cell recordings was <20 ms as judged from the relaxation current obtained when a cell was depolarized to elicit voltage activated K^+ currents and the extracellular K^+ concentration was switched to 20 mM by the superfusion system. Cells were equilibrated with the modulators (e.g. EB, VIMP) by means of a second inflow pipette. This second stream of solution was replaced by the fast extending jet of agonist solution during transmitter application. Removal of solution was achieved by sudden application of negative pressure in the inflow pipette.

The concentration of L-glutamate was 1 mM in all experiments shown in this study. This facilitates the direct comparison of the results obtained from agonist-induced whole cell currents with the autaptic recordings, since the peak glutamate concentration in the synaptic cleft of hippocampal neurones has been estimated to be >300 μM (Jonas & Sakmann, 1992) and $\geqslant 1$ mM (Clements *et al*., 1992). In addition 1 mM glutamate is close to the EC_{50} values (0.4–1.1 mM) for gluamate-induced whole cell and outside-patch currents in rat central

neurones when using fast transmitter applications systems (see e.g. Kiskin *et al*., 1986; Hestrin, 1992; Jonas & Sakmann, 1992; Leßmann & Gottmann, 1994).

Dye purification and analysis

Since commercially available Evans Blue (EB) is indicated by the manufacturers to contain impurities of ~10% we developed a purification method, to assure that the modulatory effects of EB were mediated by the main compound of this raw material. Briefly, 1 g of EB was dissolved in 4 ml H_2O, mixed with an equal amount of silicagel and dried. This solid was placed on top of a silicagel column (4 × 20 cm, silicagel, 70–230 mesh) that had been equilibrated with ultrapure acetone (reagent grade). The separation was performed by elution with successive acetone:water dilutions of 50 : 1, 20 : 1 and 6 : 1, respectively (a detailed description of the purification protocol is accessible upon request). This procedure yielded one major compound (>90%) spectroscopically identified as Evans Blue. The purity of this fraction was determined by nuclear magnetic resonance (n.m.r.) spectroscopy to be ⩾99%. This purified EB was used in all experiments for the preparation of recording solutions. The only impurity present in the commercially available EB in sizeable amounts was violet and amounted to about 5% of the raw EB. This violet impurity (VIMP) was shown to be generated from EB by oxidation with dissolved molecular oxygen and occurred during extensive stirring of solutions or in the presence of surface catalysts (see Figure 5). VIMP was not generated during preparation or use of our EB recording solutions as judged by silicagel chromatography that was regularly performed at the end of a recording session. As judged from n.m.r. spectroscopic data, VIMP is an asymmetric molecule created most likely by oxidation of the C-5 and/or C-8 atoms (see asterisks in Figure 5) of the Chicago acid residues in EB (B.S., unpublished). The molecular weight of this oxidized EB was estimated to 1000 g mol^{-1} (compared to 960 g mol^{-1} for EB) and the concentrations for VIMP given in this study are based on this estimated value.

A more detailed chemical analysis of VIMP was hindered by the inaccessibility of the molecule to the different protocols of mass spectroscopy that we tried so far. In addition, VIMP still contained some minor impurities that, nevertheless, hindered the chemical analysis by 2-D-n.m.r. spectroscopy or X-ray-crystallography.

L-Glutamate, kainate and Evans Blue were purchased from Sigma, AMPA from Tocris Cookson (U.K.), suramin from RBI and Chicago acid SS was a gift kindly provided by Dr Oberkirch from Bayer AG (Leverkusen, Germany).

Results

Non-NMDA receptor mediated whole cell currents

The application of 1 mM L-glutamate at a holding potential of −70 mV evoked whole cell currents with a mean rise time (0–100% of peak current) of 11.1 ± 2.4 ms and a mean peak amplitude of 477 ± 333 pA (n = 58). At this negative holding potential and in the absence of glycine and the presence of 2 mM Mg^{2+} in the extracellular solution, glutamate evoked whole cell currents are almost exclusively mediated by non-NMDA receptors. This was confirmed in a subset of experiments where 10 μM of the non-NMDA receptor specific antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) reversibly blocked the glutamate evoked currents to 6.3 ± 2.3% (n = 7) of control amplitudes. The glutamate evoked responses showed pronounced desensitization to a steady state value (measured 180 ms after the peak current) of 81 ± 62 pA (i.e. 17% of peak amplitudes). Peak currents showed a linear dependence on the holding potential in the range of −50 to +30 mV, with a mean reversal potential of 0.4 ± 3.0 mV (n = 12, not shown). The desensitizing phase of the whole cell currents could be fitted monoexponentially with a mean time constant of 26.1 ± 6.9 ms (n = 58). The time constant of desensitization varied considerably from cell to cell (Figure 1a; range 12–45 ms). This variability could in principle result from different time courses in the rise of the glutamate concentration at the cell membrane, leading to more or less asynchronous activation of glutamate receptors depending on the specific cell under investigation. In this case cells showing a larger rise time should also show a larger time constant of desensitization. This is not the case under our experimental conditions as shown by a plot of the rise time versus the corresponding time constant of desensitization for each of

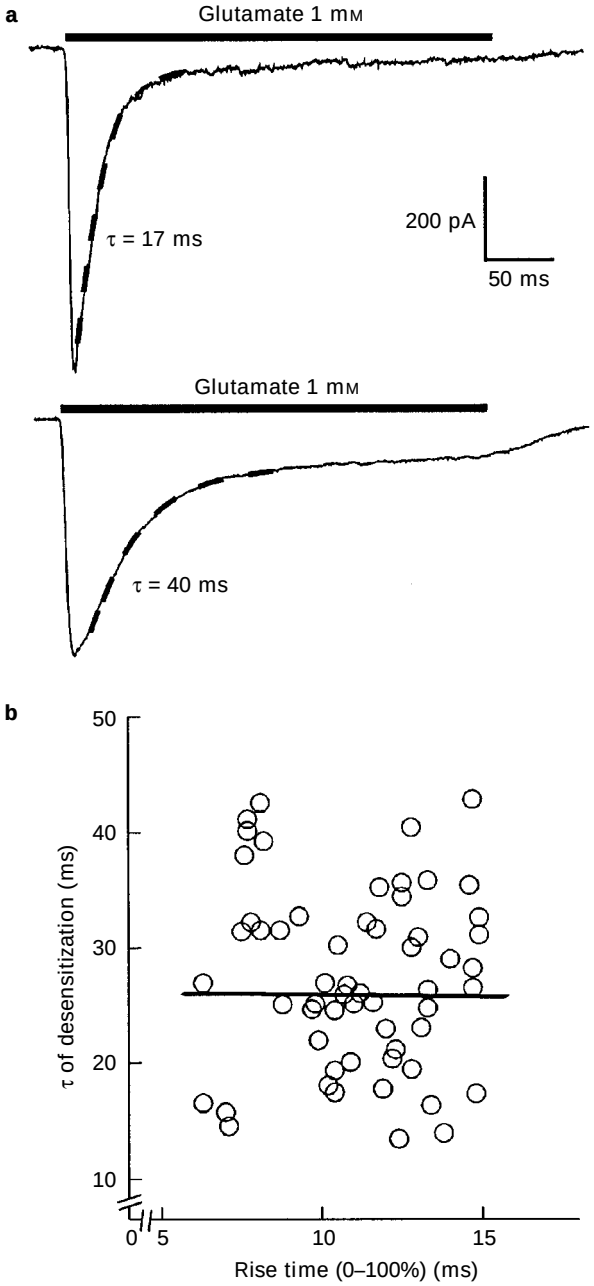

Figure 1 Scatter in time constants of desensitization in cultured hippocampal neurones. L-Glutamate-induced (1 mM) whole cell currents obtained from 2 different cultured postnatal hippocampal neurones (a). Although the rise time (0–100% of peak current) was <10 ms for both cells the time constants of macroscopic desensitization (τ) show marked differences. The stippled lines show the results of the monoexponential fit to the desensitizing phase of the currents. A plot of the time constant of desensitization vs the respective rise time (b) for 58 different hippocampal cells shows the weak correlation (solid line, correlation coefficient = 0.042) between both parameters, suggesting cell specific differences in the time course of desensitization.

58 cells (Figure 1b). Since no correlation between these two parameters is evident ($r=0.042$), we assume that the variability of the time constant of desensitization reflects at least in part cell specific differences in the time course of desensitization.

Effects of purified EB on native non-NMDA receptors in hippocampal cultures

L-Glutamate (1 mM for 360 ms) was applied to the cells in 15–30 s intervals starting 1 min after obtaining the whole cell configuration. Several glutamate-activated currents were elicited to assure stable amplitudes and kinetics under control conditions. Preincubation of the neurones with 10 μM purified EB (purity $\geqslant$99%) differentially modulated non-NMDA receptor mediated currents in different subsets of cells: (A) 22 out of 52 cells investigated showed a partial block of peak current amplitudes to 41 ± 17% of control values with a recovery to 73 ± 17% of the initial amplitude upon washout of EB (Figure 2a). Whereas the time constant of desensitization (τ) remained unaffected, the steady state current value (residual current after 180 ms) slightly decreased (Table 1) and the rise times significantly increased to 14.4 ± 4.8 ms (control 11.1 ± 2.4 ms, $P<0.05$). (B) Seven cells showed a nearly complete inhibition of desensitization ($\tau=427\pm679$ ms compared to 26 ± 5 ms under control) accompanied by increased maximal current amplitudes (126 ± 18% of control; Figure 2b, Table 1). In addition the rise time of the whole cell currents was reversibly prolonged to 38.4 ± 13.2 ms ($P<0.001$). (C) The remaining 23 cells displayed a combination of the effects described under (A) and (B): peak current amplitudes were reduced to 56 ± 20% (range: 28–91%) of control whereas steady state current amplitudes were increased three fold. In addition, the time constant of desensitization was prolonged to 69.2 ± 54.9 ms (see Table 1) and the rise time was increased significantly to 16.1 ± 6.7 ms ($P<0.01$).

Our criterion to assign a neurone to group (C) instead of (A) was a change of $\geqslant$10% in the time constant of desensitization. As an alternative to the fit of desensitization time constants, desensitization of glutamate receptor currents can be quantified by calculating the per cent desensitization (i.e. $100\times$(peak current$-$steady state current)/(peak current $-$baseline current). A plot of the normalized peak current inhibition vs the normalized inhibition of the percent desensitization by EB for the cells classified in the three groups (A), (B) and (C) is shown in Figure 2e. The dominant modulation of either desensitization (group B) or peak current amplitudes (group A) by EB is clearly visible. As expected, the values for the group C neurones are scattered along a line that would connect the two extreme groups, reflecting the cell specific variation in the proportions of the effects of EB on desensitization and current amplitudes.

Both effects of EB could also be observed when using lower agonist concentrations (e.g. 100 μM L-glutamate) and the relative inhibition of desensitization and current amplitudes by

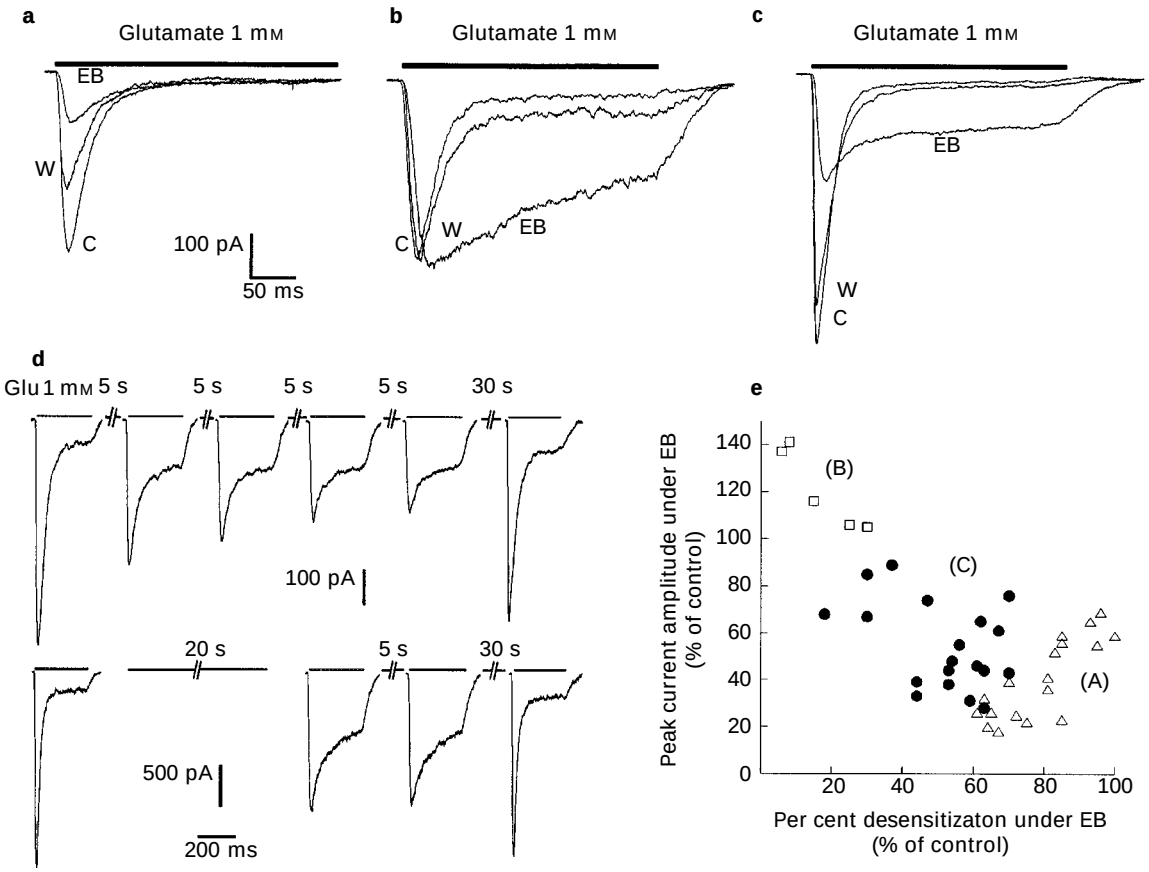

Figure 2 Distinct types of modulation of glutamate activated whole cell currents by Evans Blue (EB). L-Glutamate (1 mM)-induced currents from three different hippocampal neurones (a–c, correspond to groups A, B and C in text). Upon pre-equilibration of the neurones with 10 μM Evans Blue (EB, 20 s) either a predominant block of current amplitudes (a; 42% of the cells), a predominant inhibition of desensitization (b; 13%) or a mixed block of amplitude and desensitization (c; 45%) were observed. C, control; EB, 10 μM EB; W, wash. Pre-equilibration (20 s) of a neurone with 10 μM EB in the absence of channel openings (d, lower trace) was sufficient to obtain the full effect. Shorter EB incubation interrupted by glutamate applications resulted in the successive development of the full EB effect (d, upper trace). In either case the recovery was complete within 30 s of washout. In (d) non-averaged original currents are shown. (e) Plot of the peak current amplitude seen in the presence of EB vs the block by EB of the per cent desensitization for individual hippocampal neurones given as per cent of control (i.e. in the absence of EB). The neurones with either the predominant block of desensitization (□, group B), the predominant block of current amplitudes (△, group A) or the mixed effect (●, group C) are indicated by the different symbols.

Table 1 Modulation of AMPA receptor mediated whole cell currents by Evans Blue and related substances

Test substance (μM)	n	*Amplitude* (% of control)		τ (desensitization) (ms)			*Steady state* (% of control)	
		Test	*Wash*	*Control*	*Test*	*Wash*	*Test*	*Wash*
EB(A) (10)	22	41±17*	73±17	26.9±7.1	27.7±9.3	28.0±8.3	82±18*	77±17
EB(B) (10)	7	126±18*	105±33	26.0±5.4	427±679*	26.1±5.8	477±228*	132±62
EB(C) (10)	23	56±20*	76±14	23.7±6.8	69.2±54.9*	27.3±7.2	258±236*	101±33
VIMP (10)	9	57±16*	77±17	29.3±7.4	27.3±6.5	27.9±6.2	60±34*	76±30
CASS (100)	16	96±10	90±14	22.2±5.6	29.6±7.6*	21.6±4.1	170±59*	115±43
Suramin (10)	13	97±16	102±14	28.9±9.4	29.5±10.7	30.2±9.5	88±37	83±43
AMPA + EB (10)	4	56±15*	86±35	31.0±11.6	72.7±15.8*	34.1±18.5	169±29*	93±37
KA + EB (10)	10	50±20*	76±13					

Neurones were equilibrated ($\geqslant$30 s) with either Evans Blue (EB), oxidized EB (VIMP), Chicago acid SS (CASS) or suramin. L-Glutamate (1 mM), S-AMPA (200 μM) or kainate (KA, 500 μM) evoked whole cell current traces were averaged ($n=3-6$). Amplitudes of peak currents, steady state currents (180 ms after the peak) and the time constant of desensitization (τ) were calculated from this average currents ('test'). Current amplitudes are given as percentage of 'control' before the application of a modulator. The extent of recovery ('wash') is indicated. Data are given as means±s.d. *Significantly different from control with $P<0.01$ (unpaired Student's t test).

EB was comparable to the effects observed when using 1 mM glutamate (not shown).

Except for the only partial recovery of current amplitudes, all modulatory effects of EB were reversible upon washout. Either of the 3 effects had reached maximal levels after 20 s of preincubation of the neurones with the dye and was independent of channel opening (see Figure 2d). All effects were also independent of the presence of EB during the glutamate application. No correlation between the cell specific desensitization time constants (Figure 1) and the type of modulation obtained with EB was observed. The recovery after washout of EB was also non use-dependent and was complete within 20–40 s.

We also investigated the effect of EB on non-NMDA receptor mediated currents recorded from outside-out patches. Glutamate (1 mM) activated currents showed a mean amplitude of 170±147 pA and a mean time constant of desensitization of 11.0±1.5 ms ($n=6$, not shown). Upon application of EB (10 μM) we observed either a pure inhibition of current amplitudes ($n=3$) or a mixed inhibition of current amplitudes and desensitization ($n=3$), indicating that the two distinct effects of EB can be observed independent of the presence of an intact intracellular environment of the receptors. We next investigated the potency of EB for the inhibition of desensitization and current amplitudes by determining dose-response relationships. The data obtained were fitted to the Hill equation by a least squares fitting procedure. The IC_{50} value for the inhibitory action on peak current amplitudes was 10.7±1.0 μM (mean±s.e.). The corresponding Hill coefficient was 1.2±0.1 (Figure 3a). The dose-response curve for EB for the per cent desensitization yielded an IC_{50} value of 13.3±1.7 μM and a Hill coefficient of 1.3±0.2 (Figure 3b). Thus, the two separate effects of EB on glutamate activated currents are elicited at very similar concentrations.

We wanted to find out whether the two distinct effects of EB can also be observed when only AMPA receptors are activated. Therefore, we performed an additional series of experiments with AMPA, which specifically activates channels assembled of GluR1-4 subunits. (S)-AMPA (200 μM) was applied to neurones preincubated with 10 μM EB. Out of the 6 cells investigated 2 neurones displayed a predominant block of current amplitudes (Figure 4a) whereas the remaining 4 cells showed the combined desensitization/amplitude effect (Figure 4b; Table 1). This strongly suggests that the specific interaction of EB with different classes of AMPA receptors accounts for the 2 distinct modulatory effects of the dye observed with glutamate as the agonist. Kainate-evoked (500 μM) currents in the absence of EB never displayed any detectable sign of desensitization which would have been expected if large amounts of kainate receptors (composed of the subunits GluR5-7 and KA1-2) were present in our cultures. The kainate (500 μM)-induced whole cell currents were reversibly inhibited to 50±20% of control in the presence of 10 μM EB (see Table 1). This value almost exactly mirrors the extent of peak current reduction obtained in more than 80% of the neurones when glutamate was the agonist. Thus, the inhibition of current amplitudes by EB was independent of the agonist used to activate the non-NMDA receptors.

Evans Blue and suramin have been shown to be potent antagonists at P_{2X}-purinoceptors (Bültmann & Starke, 1993, Bültmann *et al*., 1995). Hence, if the effects of EB on non-NMDA receptors were mediated via binding to these purinoceptors, suramin should have comparable effects. In contrast, preincubation of neurones with 10 μM suramin left L-glutamate induced whole cell currents unaltered (Figure 4d, Table 1). Likewise, ATP (500 μM) which is the natural agonist of the P_{2X}-purinoceptors had no significant effect on non-NMDA receptor mediated whole cell currents (data not shown).

Effects of derivatives of EB on whole cell currents

VIMP The purification of EB (see Methods) yielded 2 main compounds: EB which made up ~95% of the unpurified dye and a violet fracation (VIMP) which amounted to ~5% of the unpurified dye. By use of n.m.r.-spectroscopy and silicagel chromatography we found that VIMP is an oxidatively modified derivative of EB most probably resulting from oxidation of the lateral Chicago acid SS moieties of EB by molecular oxygen (see Figure 5). Previous studies of EB-mediated effects on glutamate receptors (Leßmann *et al*., 1992; Keller *et al*., 1993) were performed with the unpurified dye and, thus, in the presence of VIMP. Therefore, we sought to determine, whether VIMP has an effect on non-NMDA-receptor mediated currents on its own. Preincubation (30 s) of neurones with 10 μM VIMP (the concentration of VIMP was calculated based on the estimated molecular weight of 1000 g mol^{-1}, see Methods) resulted in a reversible reduction of peak (57±16% of control) and steady state (60±34% of control) current amplitudes (Table 1) in all neurones investigated ($n=9$). Activation and desensitization time courses of the glutamate-activated currents were unaffected. As expected, the inhibitory effect of VIMP on AMPA (200 μM)-evoked currents was indistinguishable from the effect on glutamate-evoked currents (not shown). The IC_{50} value for the peak reduction by VIMP was estimated to be below 3 μM since 10 and 3 μM VIMP showed comparable antagonistic potency (not shown). In contrast to the differential modulation by EB, identical effects of VIMP were observed in cells showing either predominant amplitude block or predominant inhibition of desensitization upon EB application (compare Figure 6a and b). This suggests that the oxidation of the Chicago acid residues in VIMP prevents the inhibitory effect on desensitization.

Chicago acid SS (CASS) The results obtained with VIMP indicated that the CASS moieties of EB are critical for the modulation of desensitization by EB. Consequently, we determined the effects of free CASS on non-NMDA receptor-mediated currents.

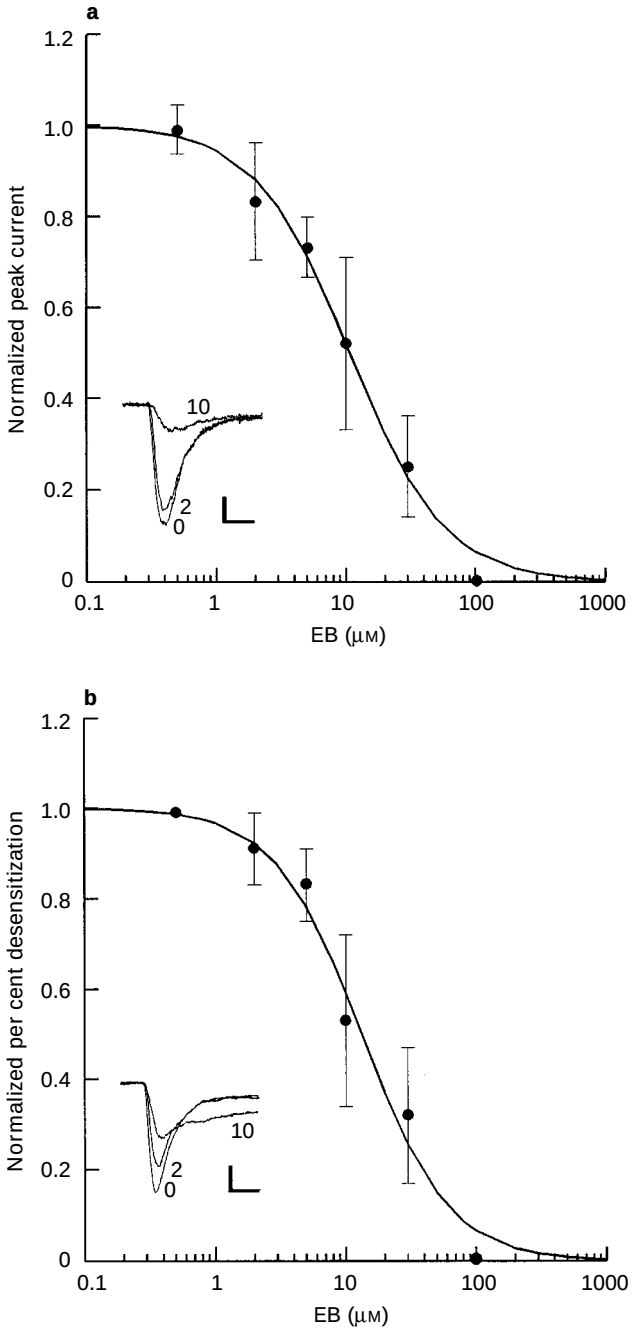

Figure 3 Dose-response relationship for the two modulatory effects of Evans Blue (EB). (a) Plot of the reduction of current amplitudes of L-glutamate (1 mM) -induced whole cell currents vs the EB concentration. Each point represents the mean ($n \geqslant 4$ cells; all cells from either group EB(A) or EB(C), compare Table 1) peak current amplitude obtained in the presence of the indicated EB concentration divided by the respective control current amplitude of the same cell. (b) Plot of the mean ($n \geqslant 4$ cells, all cells from group EB(C)) per cent desensitization (i.e. $100 \times$(peak current − steady state current)/(peak current − baseline current) in the presence of various concentrations of EB divided by the respective control value (in the absence of EB) of the same cell. Data points were fitted by the Hill equation (n_H = Hill coefficient, IC_{50} = concentration producing half maximal inhibition). In (a): $IC_{50} = 10.7\ \mu M$, $n_H = 1.2$; in (b): $IC_{50} = 13.3\ \mu M$, $n_H = 1.3$. Insets in both (a) and (b): family of currents obtained from two typical cells at indicated EB concentrations (in μM). Bars represent 40 ms and 20 pA, respectively.

In all cells investigated ($n = 16$) preincubation (60 s) of the neurones with 100 μM CASS significantly prolonged the time constants of desensitization of glutamate-evoked whole cell currents (from $22.2 + 5.6$ ms to 29.6 ± 7.6 ms, $P < 0.01$) and increased steady-state current amplitudes to $170 \pm 59\%$ of control values (Table 1). The rise time and peak current amplitudes were not affected by CASS (Figure 6). Preincubation of neurones for 1 min was obligatory to obtain the steady state effect of CASS. For incubation times < 5 min the modulatory effect of CASS was fully reversible.

The effect of CASS was the same irrespective of the type of modulation obtained with EB in the same cell. Thus, CASS modulated desensitization even in cells that showed the predominant amplitude block with EB (see Figure 6b). Preincubation of the neurones with only 10 μM CASS had no detectable effect on any of the inspected properties of glutamate-induced currents. Hence, although the modulation of desensitization by EB seems to depend on the presence of unaltered Chicago acid moieties in the dye molecule, free CASS only weakly modulated desensitization. Thus, the chemical configuration of CASS in the EB molecule most likely improves the potency and confers the differential modulation of non-NMDA receptors on EB.

Modulation of glutamatergic synapses by EB and related compounds

We wanted to determine whether the modulation of synaptic non-NMDA receptors by EB reflected the pharmacological profile obtained for glutamate-evoked whole cell currents. Evoked glutamatergic autaptic currents were investigated in microisland cultures (see Methods) of postnatal rat hippocampal neurones after 10–14 DIV. At a holding potential of

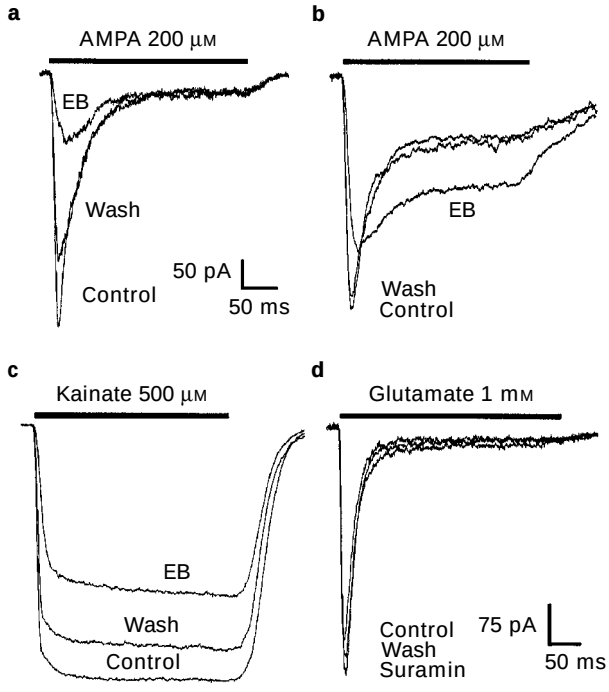

Figure 4 Binding to AMPA receptors mediates the two distinct effects of Evans Blue (EB). AMPA (200 μM) evoked whole cell currents in postnatal rat hippocampal neurones in the presence of 10 μM EB (a,b). Similar to glutamate, AMPA evoked currents showed the predominant block of peak currents (a) and the mixed block of amplitudes and desensitization (b) in different subsets of cells. Kainate (500 μM) induced whole cell currents (c) showed no indication of macroscopic desensitization. Following pre-equilibration of the cells with 10 μM EB, kainate-induced currents were reversibly reduced in amplitude. The antagonism of EB at P_{2X}-purinoceptors does not account for the modulation of AMPA receptors since another such antagonist (suramin 10 μM) left glutamate induced whole cell currents unaltered (d).

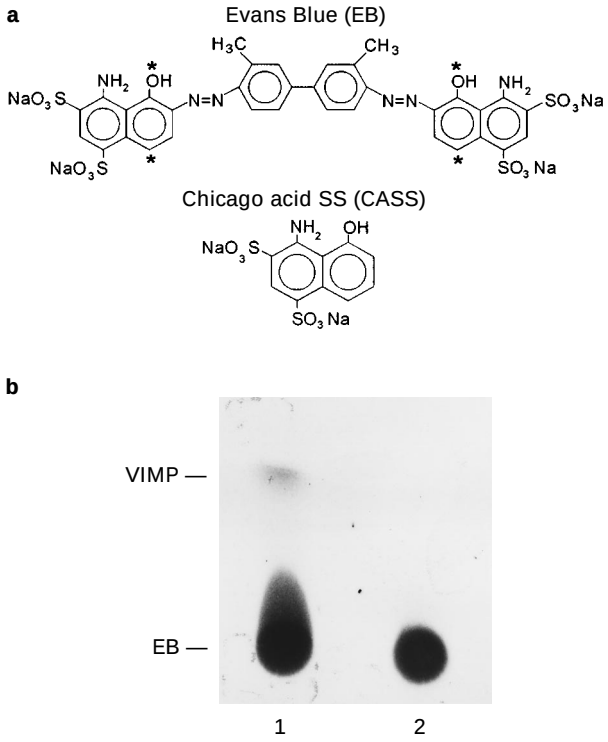

Figure 5 Chemical structure of the AMPA receptor modulators and silica gel chromatography. (a) Whereas EB mediates both effects on glutamate activated currents (i.e. block of desensitization and amplitude block), Chicago acid SS (CASS, same compound as lateral residues in EB) is a pure inhibitor of desensitization (see text). The asterisks in the EB formula indicate the candidate C-atoms of EB that are oxidized in VIMP. (b) Silica gel chromatogram of purified EB manipulated in the presence (1) or absence (2) of molecular oxygen. In the presence of surface catalysts, such as silica gel, molecular oxygen oxidizes the CASS residues in EB, yielding VIMP (see Methods).

−70 mV somatic voltage-activated Na^+ currents were elicited to trigger transmitter release. Under control conditions evoked glutamatergic autaptic currents had a mean current amplitude of 537 ± 340 pA ($n = 14$) and monoexponentially decayed with a mean time constant of 5.2 ± 1.1 ms (range: 3.4–6.5 ms). Autaptic currents were elicited in regular intervals (0.1 Hz) until stable baseline values were obtained. Superfusion of the microisland cultures with EB (10 μM) resulted in a reversible reduction of the autaptic currents to $39 \pm 19\%$ of control values in all cells investigated (Figure 7a; $n = 12$), which is close to the amplitude block obtained for glutamate-evoked whole cell currents (inhibition to $41 \pm 17\%$ and $56 \pm 20\%$ of control; see Table 1). Interestingly, the time constant of decay of the autaptic responses remained unchanged in all cells investigated (see Table 2), although in the same preparation more than half of the cells showed pronounced modulation of desensitization of glutamate-evoked whole cell currents by EB. Superfusion of the autapses with VIMP (10 μM, Figure 7b) caused a reversible reduction of the autaptic responses to the same extent as observed for glutamate-evoked currents (see Table 2). Likewise, the kinetic properties of autaptic responses were also not affected.

Finally, unlike the small reduction of desensitization in whole cell recordings, superfusion of neurones with 100 μM CASS left the decay of autaptic currents unchanged. The lack of effect of CASS on amplitudes of autaptic currents is in agreement with the respective results obtained for its effect on glutamate-evoked currents (Figure 7c; Table 2). Neither of the EB derivatives affected the amplitude or time course of voltage-activated somatic Na^+ or Ca^{2+} currents ($n = 15$, not shown).

Thus, whereas the potencies of EB and VIMP to block the amplitude of autaptic currents were nearly identical to the respective results observed for the glutamate-evoked currents, neither EB nor CASS changed the decay of the autaptic responses. If one assumes that the non-NMDA receptors sensitive to modulation of desensitization by EB are located postsynaptically, these results suggest that desensitization does not contribute significantly to the decay of glutamatergic synaptic currents in cultured hippocampal neurones.

Discussion

AMPA receptor subtype specific antagonists or modulators would be a valuable tool to characterize the receptors involved in cellular responses to glutamate application and in glutamatergic synaptic transmission in different neuronal populations. The diazo dye Evans Blue (EB) has been shown previously to be a promising candidate to modulate specifically non-NMDA receptors (Leßmann *et al*., 1992; Keller *et al*., 1993). Conflicting results existed with respect to the exact mode of action of EB. Our results now show that EB has two distinguishable effects on AMPA receptor mediated currents, namely block of current amplitudes and inhibition of desensitization. Secondly, we provide evidence that the major impurity (VIMP) of the commercially available unpurified EB potently inhibits glutamate evoked whole cell currents in all cells investigated. Finally we show that the decay of glutamatergic synaptic currents remains unaffected upon EB or CASS application, suggesting that either receptor desensitization does not contribute significantly to the termination of the synaptic response or the non-NMDA receptors sensitive to modulation of desensitization by EB are not located in the post-synaptic membrane.

Non-NMDA receptor-mediated whole cell currents

The properties of non-NMDA receptor-mediated whole cell currents in this study are comparable to previously published results from postnatal rat hippocampal neurones. More specifically, our mean time constant of desensitization (26.1 ± 6.9 ms) relates to the 40 ms (Kiskin *et al*., 1986), 70 ms (Thio *et al*., 1991) and 10–30 ms (Patneau & Mayer, 1991) found by others. However, direct comparison of such data is complicated by the fact that different (concentrations of) agonists were employed in all these studies. In addition, the time constant of desensitization in the whole cell recording mode is always limited by the speed of solution exchange at the cell membrane because delayed activation of neighbouring receptors artifically prolongs macroscopic desensitization. This interpretation is corroborated by the finding that the same experimental setup allowed us to observe two fold faster desensitization time constants in outside-out patch recordings (i.e. 11.0 ± 1.5 ms) from the same neurones, which is almost identical to the respective desensitization time constants described by Colquhoun *et al*. (1992). However, the relatively large scatter in the whole cell desensitization time constants between different hippocampal neurones in our cultures (12–45 ms) still seems to reflect, at least in part, cell specific differences in non-NMDA receptor properties, since we could not detect a correlation of the time constants of desensitization with the speed of the glutamate application (see Figure 1). In favour of this interpretation Fleck *et al*. (1996) have recently obtained a similar scatter in the time constants of desensitization for hippocampal neurones. Cell specific differences in glutamate uptake kinetics are not likely to account for the heterogeneity of desensitization, since the fastest time constants of desensitization in our whole cell recordings (12 ms) are close to the values observed in outside-out patch recordings from non-NMDA receptor channels (see e.g. Colquhoun *et al*., 1992), and glutamate uptake should decrease rather than increase the time constants of desensitization in the whole cell configuration as is observed here. Whether the heterogeneity in

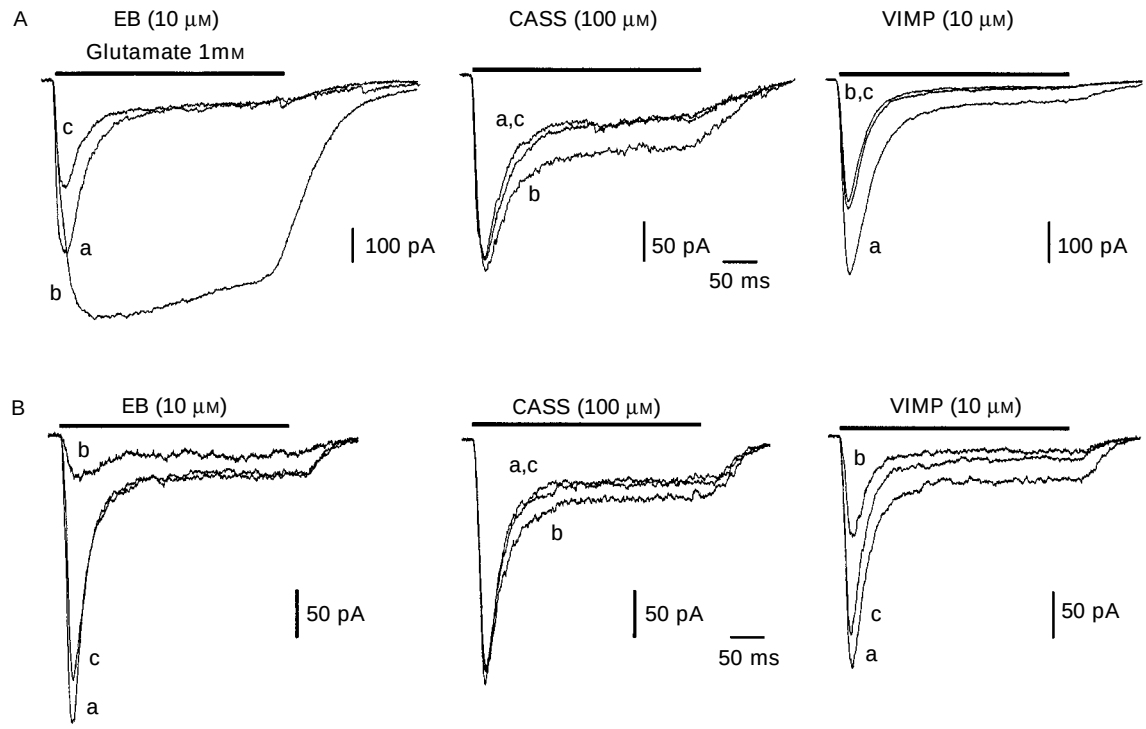

Figure 6 Modulation of glutamate induced currents by VIMP and CASS. Hippocampal neurones showing block of desensitization with 10 μM EB (A, same cell for all currents) showed reduced desensitization with CASS (Chicago acid SS, 100 μM) and a block of peak current amplitudes in the presence of 10 μM VIMP (violet impurity, oxidatively modified EB in the CASS residues); a: control, b: in the presence of the modulator; c: wash. Hippocampal neurones showing block of current amplitudes with 10 μM EB (B, same cell for all currents) also showed reduced desensitization with 100 μM CASS and a block of peak currents with 10 μM VIMP. Thus, CASS was a pure modulator of desensitization and VIMP a pure modulator of current amplitudes irrespective of whether EB was an inhibitor of desensitization (A) or an inhibitor of current amplitudes (B) of glutamate activated currents in the same neurone.

the time constants of desensitization reflects differential expression of AMPA receptor subunits in different cells, as has been shown directly with single-cell PCR analysis in hippocampal and cortical neurones *in vitro* (Bochet *et al*., 1994; Jonas *et al*., 1994; Geiger *et al*., 1995; Lambolez *et al*., 1996), cannot be deduced from our experiments.

Effects of EB and derivatives on whole cell currents

The modulation of glutamate activated whole cell currents by EB as shown here is in agreement with the effects found for thalamic neurones (Leßmann *et al*., 1992). However, in this previous study the mixed desensitization/amplitude effect of EB (5 μM) was found in all cells investigated. This difference corroborates the finding that thalamic and hippocampal non-NMDA receptor mediated currents differ with respect to their kinetic properties (Colquhoun *et al*., 1992; Leßmann & Gottmann, 1994), and could result from distinct AMPA receptor subunit compositions in neurones from these brain regions. Since even purified EB (purity >99%) still exerts both effects on non-NMDA receptors, the differential effects of EB on desensitization and current amplitudes cannot be explained by the presence of different components in the unpurified dye.

In the present study, the neurones were assigned to different groups (A through C) depending on the type of effect observed with EB (see Figure 2 and Table 1). This necessarily tends to be arbitrary at the edges of the groups. The criterion to assign a neurone to group C (mixed effect) instead of B (predominant desensitization effect) was a reduction of the peak current amplitude. The criterion to assign a neurone to group C instead of A (predominant peak inhibition) was a change in the time constant of desentitization. As is evident from Figure 2e the quantification of desensitization by a different procedure (per cent desensitization) would justify the assignment of some neurones to the neighbouring group as well. However, the presence of 2 clearly distinguishable types of effects produced by EB (groups A and B) and an intermediate group C between these extremes is evident from our experiments whatever method of analysis of the currents is selected. It could be argued that even in the group B neurones there still was a peak current reduction by EB that was masked by the large inhibition of desensitization by EB. Single channel recordings will have to be performed to solve this issue definitely. However, the fact that the proportions of the effects of EB on desensitization versus current amplitudes varied between different neurones is not questioned by this hypothesis. Irrespective of the assignment of the neurones to the groups A through C, EB prolonged the rise time of the glutamate induced currents in all neurones investigated. A prolongation of the rise time is an expected finding for a drug that affects the gating of the non-NMDA receptors. Whether the modulation of rise times is a third independent effect of EB or whether it is correlated with its effects on desensitization and current amplitudes was not investigated systematically here. This kind of correlation has to be investigated in outside-out patch recordings, where the solution exchange is fast enough to resolve the activation kinetics of the non-NMDA receptor channels reliably and will have to be addressed in future studies.

The differential modulation of non-NMDA receptors by EB showed no correlation with distinct properties of glutamate-activated control currents (e.g. slow or fast desensitization time constants; see above). In contrast, Fleck *et al*. (1996) have shown recently that the sensitivity of AMPA receptors for cyclothiazide directly correlates with the time constant of desensitization. Taken together, both results suggest that the receptor properties sensed by EB are different from those distinguished by the differential sensitivity to cyclothiazide, suggesting that more than two distinguishable groups of non-NMDA receptor subtypes are present in hippocampal neurones.

Under our experimental conditions glutamate activated predominantly AMPA receptors and AMPA-induced whole

Table 2 Modulation of AMPA receptor mediated autaptic currents by Evans Blue and related substances

Test substance (μM)	n	*Amplitude* (% of control) *Test*	*Wash*	*Control*	τ (decay) (ms) *Test*	*Wash*
EB (10)	12	39±19*	72±11	5.3±1.2	5.0±1.2	4.9±1.2
VIMP (10)	6	64±24*	92±11	4.9±0.7	3.9±0.8*	3.7±0.4
CASS (100)	6	91±15	82±16	5.6±1.5	5.6±1.7	5.6±1.7

Neurones were equilibrated ($\geqslant$30 s) with either Evans Blue (EB), oxidized EB (VIMP) or Chicago acid SS (CASS). Evoked glutamatergic autaptic currents ($n=3-6$) were averaged. Amplitudes of peak currents and the time constant of the decay of autaptic currents (τ) were calculated from this average currents ('test'). Current amplitudes are given as percentage of 'control' before the application of a modulator. The extent of recovery ('wash') is indicated. Data are given as means±s.d. *Significantly different from control with $P<0.01$ (unpaired Student's t test).

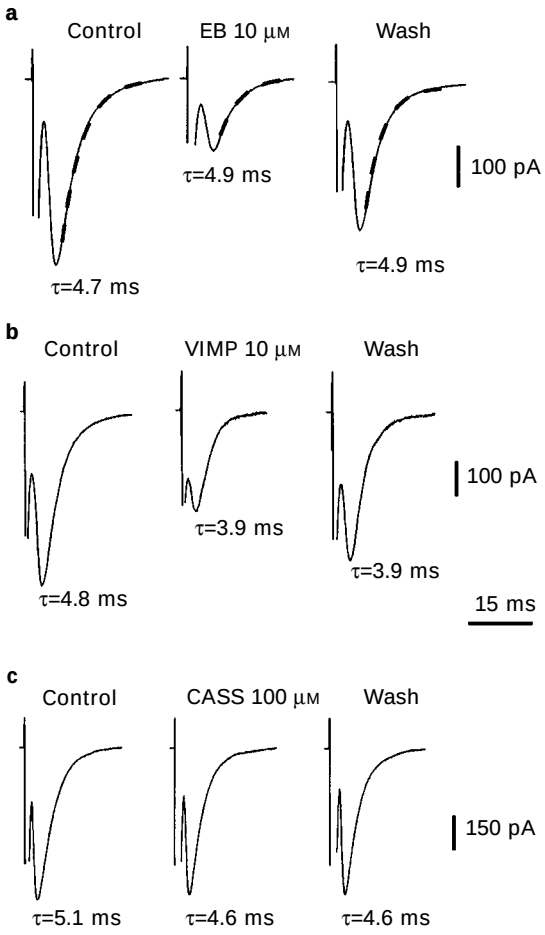

Figure 7 Modulation of glutamatergic autaptic currents by Evans Blue (EB) and related substances. Autaptic currents were elicited by short depolarizing current pulses leading to somatic voltage activated Na^+ currents (truncated downward deflections). The hippocampal microcultures were preincubated for at least 1 min with either of the indicated modulators (a–c). Each current is the average of 3 individual traces obtained before the application of a modulator (Control), after equilibration with a modulator (middle trace) and after washout (Wash) of the respective substance. The decay time constants (τ) represent the result of a monoexponential fit to each average current. The resulting fit curves are shown as stippled lines in (a), and were omitted in (b) and (c). Whereas the block of peak current amplitudes by EB and VIMP was comparable to the corresponding effects on whole cell currents, neither EB nor CASS changed the decay of the glutamatergic autaptic currents.

cell currents were also differentially modulated by EB. Thus the simplest explanation for the cell specific effects of EB is the differential expression or assembly of the AMPA receptor subunits (GluR1-4) in different subsets of cells. This interpretation could in principle be supported by single cell RT-PCR analysis, relating different patterns of AMPA receptor subunit compositions to the distinct modulatory effects of EB. However, the differential modulation of desensitization and current amplitudes by EB observed in the present study appears to be a graded phenomenon (compare Figure 2e). Thus, it seems likely that the differences in glutamate receptors sensed by EB rather result from quantitative than from qualitative variations in AMPA receptor subunit expression. To obtain convincing results from quantitative single cell PCR analysis is a very difficult task and was beyond the scope of the present study. Our future experiments will focus on heterologously expressed homomeric and heteromeric recombinant AMPA receptors to reveal a possible involvement of specific AMPA receptor subunits in the differential modulation by EB.

Amplitude reduction by EB of kainate-induced currents through heterologously expressed AMPA receptors containing either GluR1 or GluR2 subunits has been shown to occur with an IC_{50} of $\sim$0.5 μM (Keller *et al*., 1993). The higher potency of EB in the oocyte expression system as compared to the native hippocampal receptors analysed here ($IC_{50}\sim10$ μM) might result from inherent differences in these distantly related expression systems. In spite of these differences in IC_{50} values the amplitude blocking effect of EB in our study could in principle be attributed to the presence of GluR1 and/or GluR2 subunits in the native hippocampal AMPA receptors. As expected by the presence of either GluR1 or GluR2 subunits in virtually all hippocampal neurones *in vitro* (Craig *et al*., 1993; Eshhar *et al*., 1993) and *in vivo* (Sato *et al*., 1993), we found the amplitude block of EB in the majority of cells (45 of 52 cells=87%). Interestingly, the reduced desensitization in response to EB was found in only 30 out of 52 cells (58%), suggesting that less prominent AMPA receptor subunits or subunit splice variants could confer the sensitivity of desensitization to EB on AMPA receptors. A receptor subunit specific modulation of desensitization of non-NMDA receptors has been shown for cyclothiazide and concanavalin A (Partin *et al*., 1993; Wong & Mayer, 1993). These two agents discriminate between AMPA receptor- (GluR1-4, sensitive to cyclothiazide) and kainate receptor-subunits (GluR5-7 and KA1-2, sensitive to concanavalin A). In addition, the desensitization properties of the flip splice variants of the heterologously expressed AMPA receptor subunits GluR1-4 in HEK293 cells are more sensitive to cyclothiazide than the respective flop splice variants (Partin *et al*., 1994). Provided that specific AMPA receptor subunit(s) sensitive to the desensitization and/or amplitude effect of EB will be identified, the dye could facilitate the identification of AMPA receptor subunits during electrophysiological recordings.

The differential modulation of AMPA receptors by EB must not necessarily result from differences in receptor subunit expression. Differences in the phosphorylation or glycosylation status of AMPA receptors, in the lipid microenvironment or in the interaction with attached proteins could equally well explain the differential effects of EB. However, to our knowledge such differences have not yet been described to modulate the kinetics and amplitudes of AMPA receptor mediated currents.

In an attempt to deduce the residues of EB which are critical for the 2 modulatory effects, we performed experiments with

VIMP and CASS. VIMP, which is a closely related derivative of EB, oxidized in the lateral CASS residues, retained only the blocking effect of EB on current amplitudes. This suggests that unaltered CASS residues in EB are critical for the modulation of desensitization. However, isolated CASS modulates desensitization less effectively and with lower potency than EB. In addition, CASS does not show differential modulation of desensitization. Thus, the chemical environment in EB confers a higher potency and the cell specific effects on the CASS residues.

Effects of EB and derivatives on glutamatergic synaptic transmission

The time constant of the decay of postsynaptic currents in our preparation (5.2 ± 1.1 ms; range: 3.4–6.5 ms) closely matches the respective values that can be obtained from hippocampal neurones *in situ* (3–9 ms (Keller *et al*., 1991); 3–7 ms (Jonas *et al*., 1993); 4–8 ms (Hestrin *et al*., 1990)). Although it is likely that the decay time constants in our study (as in others) are slowed by dendritic filtering, we believe that our results obtained with EB justify the conclusion that non-NMDA receptor desensitization does not dominate the synaptic decay in our preparaiton: we show that EB significantly increases the time constant of desensitization by a factor of 3–20 and increases the steady state current component of glutamate-induced whole cell currents in 58% of the hippocampal neurones (see Table 1). If desensitization does contribute significantly to the termination of the glutamatergic synaptic current in our preparation, the synaptic current decay should be slowed to a comparable extent. However, EB does not show any sign of prolongation of the decay of glutamatergic autaptic currents in the same preparation although the accessibility of the subsynaptic non-NMDA receptors to EB is indicated by the peak inhibition of the synaptic responses, that occurs to the same extent as the inhibition of the glutamate-evoked whole cell responses (compare Tables 1 and 2). Taken together, these results indicate that non-NMDA receptor desensitization does not contribute significantly to the decay of glutamatergic synaptic currents in hippocampal neurones. This conclusion is in line with previous results obtained from the comparison of the time constants of desensitization of glutamate-induced currents in outside-out patch recordings with the decay of synaptic responses in the hippocampus (Colquhoun *et al*., 1992). Alternatively, we cannot exclude the possibility that the non-NMDA receptor subtypes, sensitive for modulation of desensitization by EB are not located in the postsynaptic membrane. In addition, we cannot definitely rule out that EB also has some presynaptic effects, although voltage activated Na^+ and Ca^{2+} currents were not affected and the synaptic amplitude block can be entirely explained by the effect of EB on postsynaptic non-NMDA receptors.

EB has been shown to inhibit vesicular glutamate uptake in rat central neurones (Roseth *et al*., 1995). Since synaptosomal uptake was not affected in this study and EB does not penetrate the plasma membrane, presynaptic effects on glutamate uptake are also not likely to have influenced our results. At first glance, the results presented here seem to be contradictory to the slowing of the decay of glutamatergic synaptic currents by 5 μM EB in thalamic neurones (Leßmann *et al*., 1992). However, it has been shown previously that the contribution of desensitization to the decay of glutamatergic synaptic responses varies greatly, depending on the particular neuronal circuit investigated. For example, the desensitization kinetics in outside-out patches from neurones of different brain regions in response to a step application of glutamate were compared with the respective decay time constants of synaptic currents from the same brain area: whereas in hippocampal and neocortical neurones desensitization proceeds too slowly to contribute to the decay of the synaptic response (Colquhoun *et al*., 1992; Hestrin, 1992), desensitization in thalamic neurones, spinal cord neurones and neurones from the nucleus magnocellularis is fast enough to contribute significantly to the decay of synaptic transmission (Trussell & Fischbach, 1989; Trussell *et al*., 1993; Leßmann & Gottmann, 1994). By use of different channel modulators (aniracetam, cyclothiazide, EB) desensitization of non-NMDA glutamate receptors has been shown to contribute to the termination of synaptic transmission in various preparations (cultured hippocampal neurones: Tang *et al*., 1991; Yamada & Tang, 1993; hippocampal slice: Isaacson & Nicoll, 1991; nMAG: Trussell *et al*., 1993; cultured thalamic neurones: Leßmann *et al*., 1992). The modulatory action of aniracetam and cyclothiazide at hippocampal synapses might at least in part be explained by the additional slowing of channel closure by both drugs (Tang *et al*., 1991; Hestrin, 1992; Yamada & Tang, 1993). In favour of this interpretation a recent pharmacological study (Rammes *et al*., 1996) has shown that the slowing of glutamatergic synaptic responses by cyclothiazide at least in part reflects delayed channel closure upon removal of glutamate from the synaptic cleft. If desensitization does not affect the decay of glutamatergic synaptic currents in our hippocampal preparation (as suggested by the results obtained with EB), deactivation of non-NMDA receptors must be responsible for the synaptic decay. Provided that the non-NMDA receptor complexes sensitive for the modulation of desensitization by EB are expressed subsynaptically, this could be interpreted such that, at the concentrations used here, EB does not modulate the deactivation of non-NMDA receptors. However, to find out whether EB has an effect on non-NMDA receptor deactivation, future studies with very short (1 ms) glutamate pulses (that cannot be achieved with our superfusion system) applied to outside-out patches must be performed.

In conclusion, the diazo dye Evans Blue (EB) modulates desensitization and current amplitudes of AMPA receptor-mediated currents to various extents in different hippocampal neurones. The chemical configuration of the lateral Chicago acid residues of EB is crucial for this differential modulation to be observed. Glutamatergic autaptic currents show only sensitivity to the amplitude block exerted by EB, thus suggesting that desensitization does not significantly affect the decay of the synaptic currents.

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