Effects of ethylenediamine – a putative GABA-releasing agent – on rat hippocampal slices and neocortical activity in vivo

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ABSTRACT

The simple diamine diaminoethane (ethylenediamine, EDA) has been shown to activate GABA receptors in the central and peripheral nervous systems, partly by a direct action and partly by releasing endogenous GABA. These effects have been shown to be produced by the complexation of EDA with bicarbonate to form a carbamate. The present work has compared EDA, GABA and β-alanine responses in rat CA1 neurons using extracellular and intracellular recordings, as well as neocortical evoked potentials in vivo. Superfusion of GABA onto hippocampal slices produced depolarisation and a decrease of field epsps, both effects fading rapidly, but showing sensitivity to blockade by bicuculline. EDA produced an initial hyperpolarisation and increase of extracellular field epsp size with no fade and only partial sensitivity to bicuculline, with subsequent depolarisation, while β-alanine produces a much larger underlying hyperpolarisation and increase in fepsps, followed by depolarisation and inhibition of fepsps. The responses to β-alanine, but not GABA or EDA, were blocked by strychnine. In vivo experiments, recording somatosensory evoked potentials, confirmed that EDA produced an initial increase followed by depression, and that this effect was not fully blocked by bicuculline. Overall the results indicate that EDA has actions in addition to the activation of GABA receptors. These actions are not attributable to activation of β-alanine-sensitive glycine receptors, but may involve the activation of sites sensitive to adipic acid, which is structurally equivalent to the dicarbamate of EDA. The results emphasise the complex pharmacology of simple amines in bicarbonate-containing solutions.

Key-words: Ethylenediamine; 1,2-diaminoethane; GABA; bicuculline; kynurenic acid; hippocampus; cortical evoked potentials

1. INTRODUCTION

Ethylenediamine (1,2-diaminoethane, EDA) is used as a complexing and solubilising agent in preparations such aminophylline, which is a complex of EDA and theophylline. When aminophylline was applied by microiontophoresis to spontaneously firing neurones in vivo (Stone and Perkins, 1979) it produced a depression of neuronal firing, leading to the realisation that the inhibition was entirely attributable to the EDA, not the theophylline component of the complex (Perkins and Stone, 1980,1982a; Perkins et al., 1981). This conclusion was confirmed by others studying neuronal activity in the hippocampus (Blaxter and Cottrell 1985), cerebellum and invertebrate CNS (Bokisch et al., 1984).

Both EDA and GABA showed the same reversal potential, the depressant activity was blocked by bicuculline (Perkins et al., 1981; Perkins and Stone, 1982a) and potentiated by benzodiazepines (Bokisch et al., 1984). Neurochemical analyses showed that EDA could be transported into GABA-containing synaptic terminals, provoking the selective release of GABA but not dopamine (Lloyd et al., 1982a,b; Kerr and Ong, 1982, 1987; Sarthy, 1983). Overall, EDA was a GABA-mimetic, acting partly indirectly by GABA release (either by uptake and displacement of synaptic GABA or by promoting the exchange of GABA for EDA) and partly by direct activation of $GABA_A$ receptors (Morgan and Stone, 1982; Davies et al., 1982). Later work indicated that EDA can activate GABA receptors in smooth muscle (Ong and Kerr, 1987; Barbier et al, 1989; Maggi et al., 1989; Chaudhuri and Ganguly, 1994, Begg et al., 2002), partly via endogenous GABA release (Kerr and Ong, 1984; Erdo et al., 1986; Mckay and Krantis, 1991).

In order to explain this neuronal inhibition by a simple amine lacking any oxygen-containing functional group, it was proposed that the positively charged amine groups

of EDA might interact with bicarbonate ions in solution (Stone and Perkins, 1984) to generate an aliphatic structure resembling GABA. The existence of the postulated complex was subsequently confirmed and identified as EDA monocarbamate (Curtis and Malik, 1984; Kerr and Ong, 1987), which reproduced the activity of EDA. Analogues with longer or branched structures are much less effective as GABA-mimetics (Perkins and Stone 1982a).

The similarity between EDA and GABA pharmacology extended to the ability of nipecotic acid to inhibit EDA uptake in brain slices (Lloyd et al., 1982b) and the ability of depolarising stimuli to release EDA in a calcium-dependent fashion.

Overall, these results indicated that EDA might make a valuable tool to explore aspects of GABA synaptic pharmacology, promoting the effects of endogenous GABA released from synaptic terminals. However, there remain several unanswered questions. For example, GABA acts on CA1 apical dendrites with a hyperpolarisation that is not blocked by bicuculline (Alger and Nicoll, 1982) although it is still mediated via $GABA_A$ and not $GABA_B$ receptors. Therefore, the present study was designed to re-examine the pharmacology of EDA under the controllable conditions of hippocampal slices. In addition, it was of interest to examine the effects of EDA in vivo, where the intercellular milieu is more complex and adaptive than under in vitro conditions.

2. MATERIALS AND METHODS

2.1 General

All procedures performed in this work were in accordance with the regulations and recommendations of the Animals (Scientific Procedures) Act, 1986 of the United

Kingdom Home Office. Hippocampal slices were prepared as described previously (see Stone, 2007; Ferguson and Stone, 2008). Briefly, male Wistar rats weighing 100-150g were killed by administering an overdose of urethane (10ml/kg body weight i.p., as a 25% solution in water) followed by cervical dislocation. The brain was rapidly removed into ice-cold artificial cerebrospinal fluid (aCSF) of composition: (in mM) NaCl 115; KH_2PO_4 2.2; KCl 2; $MgSO_4$ 1.2; $NaHCO_3$ 25; $CaCl_2$ 2.5; D-glucose 10, gassed with 5%CO_2 in oxygen. The hippocampi were chopped into 450μm transverse slices using a McIlwain tissue chopper. The slices were preincubated at room temperature for at least 1 hour in a water-saturated atmosphere of 5%CO_2 in O_2 before individual slices were transferred to a 1 ml capacity superfusion chamber for recording.

For extracellular recording, submerged slices were superfused with aCSF at 28-30°C and a flow rate of 3-4 ml/min. The Schaffer collaterals and commissural afferents to the CA1 region were stimulated using a concentric bipolar electrode (Harvard Apparatus, Edenbridge, UK) positioned in the stratum radiatum, (0.1 Hz stimulation using a pulse duration of 50-300μs. The stimulating electrode tip was located immediately internal to the stratum pyramidale at the border between the CA1 and CA2 regions. For antidromically-evoked potentials, the stimulating electrode was placed in contact with the alvear white matter containing the axons of the pyramidal neurons. Recording electrodes were constructed from fibre-containing borosilicate glass capillary tubing (Harvard Apparatus, Edenbridge, Kent, UK), with the tips broken back under microscopic control to 2-4μM, DC resistance approximately 5MΩ. These electrodes were filled with a solution of 1M NaCl. The recording electrode tip was placed either in the stratum pyramidale (for population spike recordings) or internal to the CA1 pyramidal cell layer at the point of maximum

epsp amplitude. The potentials were amplified and captured on a micro1401 interface (CED, Cambridge Electronic Design, Cambridge, UK) for storage on computer and subsequent analysis using Signal software (CED, Cambridge, UK). The amplitude of population spikes was taken as the potential difference between the negative and positive peaks of the potential. The negative slope of the epsp was taken as the gradient of the line of best fit of all sampled points between approx. 25% and 75% after the start of the negative slope. The epsp amplitude was taken as the potential difference between the negative peak of the epsp and the pre-stimulus baseline.

Intracellular recordings were made as described previously (Stone, 2007), using sharp electrodes pulled from fibre-containing glass capillary tubing (Clark Electromedical, Reading, UK, and Harvard Apparatus, Edenbridge, UK) using a vertical Narashige puller. The electrodes were filled with 1M potassium acetate, and had tip resistances of 90-120 MΩ. Potentials were amplified via an Axoclamp-2 system or Neurolog DC amplifier in bridge balance mode, with a filter bandwidth between DC and 500Hz to reduce higher frequency components of epsp recordings. Following penetration, cells were allowed to reach a maximum resting potential and then left for 30 min before the addition of compounds into the superfusion line. Cells were only used if they exhibited stable resting potentials of at least –60mV. Neuronal excitability was tested by applying intracellular depolarising current pulses between 0.05 and 1.0nA amplitude. Input resistance was monitored using 0.05 to 1.0nA hyperpolarizing pulses. Responses were digitised at 20kHz via a CED (Cambridge Electronic Design, Cambridge, UK) micro1401 interface and stored on computer for later analysis using the Signal programme (CED). Only one cell was studied in each slice, to preclude the possibility that drug superfusion might alter the responsiveness of subsequent neurons in the same slice.

2.2 In vivo electrophysiology

The in vivo work was approved by the local Ethics Committee for the use of animals. Male Sprague Dawley rats weighing 200-320g were housed and maintained at the animal care facility of the university's veterinary hospital according to the standards of the local ethics committee. The rats had free access to food and water with a 12 hour light/dark cycle. The experimental procedures we used for recording somatosensory evoked potentials (SEPs) have been described in previous reports (Addae et al., 2000, 2007). Briefly, following anesthesia with urethane (1.5-1.7 g/kg) the rats were mounted in a stereotaxic frame and the body temperature maintained at 37^{o}C by means of a heating blanket and a rectal thermistor. A burr hole, 3-4 mm in diameter, was made to expose the cortical area that represents the forepaw and the dura mater and arachnoid mater were removed in this area to allow recording of the SEPs and application of drugs via a cortical cup. The contralateral forepaw was stimulated at 0.5 Hz using needle electrodes inserted into the skin of the forepaw, and the SEPs were recorded at 0.8-1.2mm below the cortical surface with a glass microelectrode filled with 3M NaCl (resistance of approximately 5 MΩ). Individual SEPs were recorded using a CEDmicro1401 computer interface and Signal software (Cambridge Electronic Design, UK). Changes in SEP amplitude were calculated as a percentage of the mean baseline value. Following application of drugs the cortex was washed by continuous superfusion of pre-gassed (95%O_2/ 5%CO_2) artificial cerebrospinal fluid (aCSF) with the following composition (in mM): NaCl 115.0; KCl 2.0; KH_2PO_4 2.2; $NaHCO_3$ 25.0; D-Glucose 10; $MgSO_4$ 1.2 and $CaCl_2$ 2.5. 2.5. EDA and bicuculline methobromide were dissolved in the aCSF, although when EDA was

being applied at high concentrations it was dissolved in aCSF with reduced concentrations of NaCl to maintain isotonicity of the solution.

Data are expressed as mean ± S.E.M.. Statistical comparisons of two data sets were made with an unpaired Student's t-test. Comparisons of multiple data sets were made using ANOVA.

2.3 Statistics

Data are presented throughout as mean ± standard error. Baseline values were obtained from a stable 10min period of evoked potential size prior to the addition of any drugs, with the first of those potentials being defined as 100%. This allowed the 10min pre-drug period to provide an indication of baseline variance. For statistical comparisons between two groups, unpaired, two-tailed t-tests were used, but where two or more data sets were compared with a common control, an analysis of variance (ANOVA) was performed, followed by Dunnett's test for comparisons with a common control, or the Bonferroni test for selected pairs of columns as appropriate. All analyses were conducted using Instat software.

2.4 Sources

Glutamate, 4-aminobutyric acid (GABA), glycine, β-alanine, adipic acid, α-amino-adipic acid, ethylenediamine dihydrochloride (EDA), strychnine and nipecotic acid, were obtained from Sigma-Aldrich Chemical Company, Poole, UK. (-)bicuculline methobromide (BIC), 3-((R)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), (3-aminopropyl)(diethoxymethyl)phosphinic acid (CGP35348) and gabazine were obtained from Tocris Chemicals, Bristol, UK.

3. RESULTS

3.1 Responses to EDA and GABA

The main response to EDA was a reduction in the size of fepsps (Fig. 1A,B). The time course of responses is illustrated by the family of curves obtained in response to maintained (10min) applications of EDA such that responses reached a plateau (Fig.1A). The corresponding log concentration-response curves are constructed in Fig.1C. When perfusion with EDA was maintained until this plateau of depression, a significant effect could be seen at a concentration as low as 50μM (Fig. 1B,C). None of these responses to EDA were associated with any changes in the size of the axonal volley (Fig.1B). In an initial series of 16 slices tested with EDA at 1mM or above, 4 slices showed a depression of fepsps which was preceded by a small increase in size which reached 120.9 ± 2.2% of the initial baseline (n = 4, significantly different from baseline, P = 0.006), and was not clearly related to concentration.

At concentrations below approximately 1mM, the effects of EDA were normally fully reversible, with potential size returning to levels not significantly different from the initial baseline values (Fig. 1A). At concentrations of 1 mM or above, a proportion of slices showed a long-lasting depression of fepsp size. Overall, of the initial group of 16 slices perfused with EDA alone at 1mM or above, 12 showed a fully reversible depressant effect of EDA on fepsps, while 4 showed persistent depression after recovering only partially towards baseline levels. This long-lasting depression was even more evident when slices were perfused for a shorter period of 5min (Fig. 1D).

For comparison, GABA was superfused over slices at a range of concentrations similar to those used for EDA (Fig. 1C). The responses obtained were of a broadly similar magnitude and time course to those seen with EDA, although there were no

instances of initial increases in fepsp size, and all responses returned to the baseline levels, with no maintained depression. Indeed, when applied at concentrations of 500μM or above for 10min, the fepsp size reached a peak of depression after about 1min, before this effect began to fade, starting to recover towards baseline values during the continued presence of GABA. In these cases, for the purpose of constructing the concentration-response curve in Fig. 1C, the amplitude of the response was taken as the maximum extent of the depression.

3.2 GABA receptor blockade

In order to examine the pharmacology of these responses, EDA was applied at a concentration of 1mM for periods of 2 min, once every 10-15 min. This protocol allowed the establishment of two consistent control responses, followed by the application of a potential modulator and then recovery of the original EDA response. The results were discarded from any slices in which the EDA response did not recover to within 10% of its initial value.

The application of bicuculline methobromide to slices tends to result in the development of multiple population spikes in the recordings of fepsp, together with the occurrence of spontaneous epileptiform bursting. These experiments were therefore conducted in slices in which a cut was made between the CA3 and CA1 regions, approximately at the border between CA2 and CA3. While this did not completely prevent signs of hyperexcitability, the changes seen did not interfere with the overall behaviour of the slices, or affect their eventual recovery.

Slices were superfused with bicuculline for 10 min before the addition of 1mM EDA for 2 min, with continued perfusion of bicuculline during subsequent applications of EDA. The application of bicuculline at a concentration of 20μM produced a slowly

developing reduction in the size of fepsp responses to EDA, with a maximal reduction after approximately 30 min of bicuculline (Fig. 2A), and with no changes of axonal volley amplitude (Fig. 2B). A higher concentration of 100μM bicuculline antagonised EDA more rapidly but did not produce any greater maximal degree of antagonism (Fig. 2C).

Since bicuculline is known to affect some receptors and channels in addition to those for GABA, experiments were also performed using gabazine, a highly selective antagonist at $GABA_A$ receptors. In these cases, a similar, partial antagonism of EDA was seen when gabazine (20μM) was used, a concentration known to block $GABA_A$ receptors (Lindquist et al., 2005) (Fig. 2C). A higher concentration of 100μM did not produce a significantly greater reduction of the EDA response (Fig. 2C).

In contrast to these results, bicuculline at 20μM produced a complete blockade of the responses to GABA (1mM; data not shown). On the other hand, neither strychnine (10 or 100μM) (Fig. 2C) nor the $GABA_B$ receptor antagonist CGP35348 (100μM; data not shown) were able to produce any significant reduction in the size of responses to EDA.

Similarly, perfusion with neither bicuculline nor strychnine produced any change in the size of the initial increase of fepsp size. On those slices showing this aspect of the response, the fepsp size was increased by 16.43 ± 1.20% with EDA alone (n = 3); 16.87 ± 2.89% with EDA and bicuculline (n = 3); and 16.77 ± 2.5% with EDA and strychnine (n = 3). These increases were not significantly different (ANOVA ($F(2,8)$ = 0.0096; P = 0.99).

3.3 Nipecotic acid

The GABA transport (GAT-1) inhibitor nipecotic acid (100μM) was applied for 10min before and during a series of applications of EDA. The concentration of 100μM was selected as one which had no effect alone on the fepsp size. With this protocol, nipecotic acid produced a gradual enhancement of the size (Fig. 3A,B,C) and particularly the duration of responses to EDA, with no change in the size of the axonal volley (Fig. 3B). The potentiation of EDA responses recovered to a level not significantly different from the original values (Fig. 3A,C,D)

3.4 Chelation

EDA is well-established as a chelator of metal ions, for which property it is widely used in the chemical synthesis of organo-metallic complexes, it was considered that the depression of evoked potentials, particularly that component of the depression that was not prevented by bicuculline or gabazine, might reflect the chelation of Ca++ ions. Doubling the calcium content of the aCSF increased the size of the baseline fepsp by approximately 20%, but did not reduce the percentage fepsp depression produced by 500μM EDA (41.6 ± 3.7%, n = 4 in normal aCSF, 38.8 ± 1.7%, n = 4 in elevated calcium, P = 0.53) . Similarly the possibility was also considered that the initial increase in fepsp slope seen in some slices (see above) might result from the chelation of Mg++, possibly resulting in an initial overdepolarisation of slices caused by increased sensitivity of NMDA receptors. When a slice was encountered in which EDA produced a clear and reproducible initial increase of fepsp size, the selective NMDA receptor antagonist CPP (10μM) was superfused. However, CPP did not prevent either the early hyperexcitability (control increase 18.23 ± 3.6%, with CPP 22.8 ± 3.8%, n = 3, P = 0.44) or the predominant depression of fepsp slope (control depression 50.9 ± 5.6%, with CPP 44.9 ± 6.3, n = 4, P = 0.51).

3.5 Antidromic potentials

The foregoing results suggest thatsome of the depression of fepsps produced by EDA could be attributed to activation of $GABA_A$ receptors, but the origin of the occasional increase of excitability, and of that fraction of neuronal inhibition that was not blocked by bicuculline, remained unclear. It was therefore decided to test EDA on non-synaptic potentials evoked by the antidromic activation of CA1 pyramidal cells. Even with this paradigm, EDA at 300μM continued to depress the amplitude of the antidromic population spikes (Fig. 4A,B). When these slices were superfused with kynurenic acid, a non-selective antagonist at glutamate receptors (Perkins and Stone, 1982b; Stone 2001), using a concentration of 2mM, there was no significant change in the size of the EDA-induced depression (Fig. 4A,B).

3.6 β-alanine

In addition to forming a GABA-like agonist complex, the structure of EDA might lend itself to the formation of a complex or carbamate compound similar in electronic structure to β-alanine, which has inhibitory activity on central neurons. Applications of β-alanine depressed orthodromic potentials with a potency comparable to that of EDA (Fig. 5A), producing a reduction in the fepsp of 83.05 ± 3.48% (n = 5) at a concentration of 2mM and 44.72 ± 3.94% (n = 4) at 500μM. Superfusion with bicuculline at 100μM did not change the depression of fepsps whereas strychnine (20μM) produced a selective block of the depressant response which developed during a strychnine application lasting 30min (Fig. 5A,B).

The depression of fepsps by β-alanine was preceded in most cases (7 of 8 slices tested using 500μM) by a significant increase in the slope of the potential (Fig. 5A).

This was larger, longer-lasting and appeared in a larger proportion of slices than was the initial increase sometimes seen with EDA. The initial increase of fepsp size was not reduced by superfusion with bicuculline or strychnine (Fig. 5A,C).

3.7 Intracellular recordings

Since the foregoing results presented a complicated picture of mixed excitatory and inhibitory components in the response to EDA, experiments were performed to address further the mechanism of action of EDA by intracellular recordings using classical sharp electrodes. A total of 22 neurones in the CA1 pyramidal layer were recorded (in separate slices), with resting membrane potentials ranging from -66 to -81mV. The application of EDA to the slices for 2min produced, in most cases (16 of 22), and at both the 300μM or 1mM concentrations examined, a rapid depolarisation of the neurones (Fig. 6A).

The depolarisations produced by EDA at 1mM reached 27.93 ± 2.0 mV (*n* = 16). This depolarisation was associated with a marked decrease of membrane input resistance from baseline values of 57.9 ± 3.8 MΩ to 32.3 ± 2.5 MΩ (*P* = 0.0002; *n* = 6)(Fig. 6A,B), the decreased resistance still being apparent when the membrane potential was clamped at the original resting potential (Fig. 6C). The application of concentrations greater than 1mM usually resulted in overdepolarisation of the neurones.

In several cases (6 of 22 cells), the depolarisation was preceded and followed by a marked hyperpolarisation (Fig. 6B) which reached 9.75mV ± 2.15 in those 6 neurons, all of which were treated with EDA at 1mM.

The mechanism of the EDA-induced depolarisation was probed by perfusing with kynurenic acid (2mM) for 10 min before, during and for 10min following EDA

(500μM). Kynurenic acid produced only small and non-significant changes in the size of the overall EDA depolarisations, which were reduced from 22.2 ± 3.6 mV to 21.1 ± 3.9 mV (P = 0.83, n = 4).

Superfusion with bicuculline (20μM) reduced the amplitude of EDA depolarisations elicited at 1mM (Fig. 7A,B) or 300μM concentrations, an effect which reached a maximum plateau after about 30 min of superfusion, consistent with the effects seen using synaptically-evoked potentials (above). Strychnine 20μM did not change the magnitude of the EDA depolarisations (controls 26.6mV ± 3.6mV, with strychnine 24.3 ± 2.9mV, n = 4, P = 0.64).

Applications of GABA for 2 min (1mM) generated complex responses which in most cases (5 of 7 cells) involved an early depolarisation which attained a peak within about 30secs and then declined substantially during the presence of GABA. These responses closely resembled in profile the changes of extracellularly recorded fepsps described above. The responses were fully blocked by superfusion with bicuculline (20μM), but not strychnine (20μM), and were unaffected by the $GABA_B$ receptor antagonist CGP35348 (100μM)(data not shown).

When applied during intracellular recording, β-alanine (1mM) produced a depolarisation of 22.3mV ± 3.1 (n = 4), preceded in 3 cases by an initial and subsequent hyperpolarisation (6.4mV ± 0.69, n = 3) very similar to those seen with EDA (above, Fig. 6B). Strychnine produced a gradual blockade of the depolarising phase of these responses (to 8.9mV ± 2.7, n = 4, P = 0.002 using paired t test), with no effect on the hyperpolarisations (6.0mV ± 0.67, n = 3, P = 0.72). Bicuculline (100μM) had no significant effect on either component of the β-alanine responses.

3.8 Adipic acid

Given the chemical structure of EDA, and previous evidence for its ability to form acidic complexes with bicarbonate ions, the possibility was considered that a similar di-carboxy adduct might be responsible for the bicuculline-resistant neuronal depolarisation. Such an adduct should most closely resemble adipic acid in structure, and this compound was therefore tested on slices.

Adipic acid itself produced little effect at 1mM, with small and inconsistent effects at 2mM. At a concentration of 10mM it produced changes of the fepsps which were either increased (22.6 $\pm$ 3.0%, n = 4, significantly different from baseline, P = 0.005), or decreased (21.8 $\pm$ 3.3%, n = 5, significantly different from baseline, P = 0.003). These effects were not only smaller than those produced by EDA (at 1mM), but were also slower in both onset and recovery (Fig. 8A). Intracellular recordings showed that, like EDA, the dominant effect of adipic acid on CA1 neurones was one of depolarisation with increased membrane conductance but these responses were unaffected by bicuculline (20μM) or strychnine (20μM) (data not shown).

In view of the structural similarity between EDA monocarbamate and adipic acid and α-amino-adipic acid (αAA), and the fact that the latter compound was one of the earliest antagonists to be identified at glutamate receptors (Biscoe et al., 1977), the possibility was considered that EDA carbamates and adipic acid might both act as weak NMDA receptor agonists, and might be antagonised by αAA. However, this was not the case, and when superfused at a concentration of 1mM, αAA had no significant activity against the responses to either EDA or adipic acid (Fig. 8A,B).

3.9 Role of extracellular bicarbonate

In order to pursue the question of whether the activity of EDA was attributable to complex formation with bicarbonate, 25% of the normal bicarbonate content of the

aCSF was replaced by HEPES. This substitution reduced significantly the depression of evoked potentials by 1mM EDA from 75 ± 5% (n = 6) to 40 ± 5% (n = 6) ($P < 0.001$), while there was no change in the depression produced by 1mM GABA (controls 40 ± 5%, with HEPES 40 ± 5%, n = 6, n.s.).

3.10 Channel modulation

4,4'-di-isothiocyano-stilbene-2,2'-disulphonic acid (DIDS), a blocker of chloride / bicarbonate exchange, is known to block the conductance changes induced by GABA and hence to reduce its depolarising and hyperpolarising actions (Bonnet and Bingmann, 1995). When tested against responses to EDA, DIDS (20μM) reduced substantially the depolarisation and depression of evoked potentials: the effect of 1mM EDA was reduced from 70 ± 5% (n = 8) to 20 ± 5% (n = 8) ($P < 0.001$), consistent with a similar site and mechanism of action between EDA and GABA.

3.11 In vivo experiments

In order to determine whether EDA had any effect on evoked potentials under more physiological conditions, the somatosensory evoked potential (SEP) was examined in anaesthetised rats. When applied for 5 min EDA (25mM) produced an initial short-lasting increase in the amplitude of the negative (N) phase of the SEP (by 12 ± 7%, *n* = 5), invariably followed by a decrease (by 30 ± 12%, *n* = 5) (Fig. 9A,B). A higher concentration produced a much lower initial increase in the SEP but subsequently decreased it by 74 ± 5% (*n* = 5) (Fig 9B). EDA at 5 mM did not affect the size or shape of the SEPs (*n* = 5).

To examine the role of GABA-A receptors in these effects, bicuculline was also applied topically at a concentration of 10μM for 15 min before and during the

application of EDA. This compound increased the overall amplitude of the SEP by 77 ± 34% (*n* = 7)(Fig. 9C), with the greatest change occurring in the positive (P) phase of the potential. However, the application of EDA (in the continued presence of bicuculline) still caused an immediate and complete abolition of the evoked positive wave and a gradual decrease in the overall amplitude of the SEP by 22 ± 15% (*n* = 7). There was no significant difference in the degree of SEP depression caused by EDA on its own or in the presence of bicuculline (two-tailed t test; P = 0.40).

4. DISCUSSION

Although several agents are available with which to explore the pharmacology and physiological roles of GABA receptors, uptake and metabolism, there are few with which to probe neuronal or behavioural responses to the selective release of endogenous GABA. As noted in the Introduction EDA appeared to be an efficient GABA-mimetic, partly by inducing GABA release and partly by direct activation of $GABA_A$ receptors in the CNS (Perkins and Stone, 1980, 1982a; Blaxter and Cottrell,1985; Bokisch et al., 1984; Lloyd et al., 1982a,b). The effects of EDA extended to the modulation of benzodiazepine binding (Morgan and Stone, 1982; Davies et al., 1982).

The ability of EDA to release GABA is a result of the uptake of EDA itself by a nipecotic acid-sensitive transporter, possibly producing an exchange with GABA since the GABA release is calcium-independent (Lloyd et al., 1982a,b). Here, nipecotic acid increased the size and duration of the EDA effects on fepsps, implying that the balance of nipecotic acid's activity is to prevent re-uptake of GABA rather

than to reduce the initial uptake of EDA (since the EDA response would then be reduced).

The observation that EDA could produce a long-lasting depression in some slices could imply a modification of receptors – such as those for glutamate – involved in the generation of long-term depression (LTD), or could simply reflect the accumulation of EDA within the tissue. We consider the former explanation more likely given that the early time course of recovery was normal, the plateau of depression could be maintained for up to one hour, and that shorter (5min) applications of EDA appeared more likely to produce this LTD. Present experiments are being directed at further understanding of the mechanisms of the LTD.

Following the proposal that this GABA-mimetic activity might involve a complex formed between EDA and bicarbonate (Stone and Perkins, 1984), strong supporting evidence for this hypothesis was obtained by demonstrating, physiologically and spectroscopically, the existence of EDA monocarbamate in solutions of EDA gassed with 5%CO_2 in oxygen (Curtis and Malik, 1984; Myers and Nelson, 1990). Ejecting the active monocarbamate ion onto individual neurones resulted in a larger and more rapid depression of firing than was seen with EDA alone.

However, there has been little detailed systematic or quantitative work on the pharmacology of EDA and the present work was designed to pursue this. The profile of extracellular and intracellular recordings is consistent with previous work showing that somatic applications of GABA and EDA generate bicuculline-sensitive hyperpolarisations, while dendritic applications produce bicuculline-sensitive depolarisations (Blaxter and Cottrell, 1985). The intracellular depolarisation presumably corresponds to the extracellularly recorded depression of fepsps.

Equally, the increase of fepsp size is most probably a result of the intracellularly recorded hyperpolarisation, both effects being resistant to bicuculline.

These results, together with the reduced responses to EDA in lowered extracellular bicarbonate therefore support the postulated activation of GABA receptors by EDA, probably via an EDA-bicarbonate complex. Perhaps more importantly, the present study has revealed an additional mechanism of action of EDA. Structurally, EDA monocarbamate is similar not only to GABA, but also to β-alanine (Fig. 10), and EDA can release preloaded β-alanine as well as GABA from neurones (Davies et al., 1983). Indeed, EDA is a more effective inhibitor of β-alanine uptake than of GABA uptake (Davies et al., 1982; Forster et al., 1981), possible suggesting that EDA may be a better inhibitor of glial transport (β-alanine) than of neuronal uptake (GABA) (Davies et al., 1982).

It is, therefore, intriguing to discover in this study that the effects of EDA are closely mimicked by applications of β-alanine, an amino acid recognised primarily for its ability to activate glycine receptors. Both EDA and β-alanine produced an initial increase of fepsp size preceding the reduction in their size, and both also produced intracellularly recorded depolarisations, often preceded by an underlying hyperpolarisation, with no fade being seen in the presence of the agonist. In contrast GABA itself was able to produce only a decrease of fepsp size and intracellular depolarisation, with both the extracellular and intracellular responses exhibiting marked fade after about several seconds of the GABA applications. However, despite these similarities, the effects of EDA and β-alanine remain largely distinct since the EDA-induced depression of fepsps and the intracellular depolarisation were blocked to a large extent, though not completely, by bicuculline, whereas these effects of β-alanine were blocked by strychnine.

The simplest interpretation of these data is that while EDA, almost certainly in the form of its monocarbamate, is able to activate $GABA_A$ receptors, and β-alanine is able to activate strychnine-sensitive, conventional, $glycine_A$ receptors, there is a third site which is resistant to bicuculline and strychnine at which both EDA and β-alanine, again in the form of their monocarbamates, produce hyperpolarisation when recorded intracellularly, and the increased fepsp size when recorded extracellularly. This response would correspond to the dendritic, bicuculline-resistant, hyperpolarisation observed by Blaxter and Cottrell (1985) when GABA was applied locally to the dendritic region. It has been proposed by others, based on a substantial amount of evidence on uptake, release and actions, that β-alanine could function as an independent neurotransmitter (DeFeudis and del Rio, 1977; Sandberg and Jacobson, 1981).

The activity of EDA at sites which are resistant to blockade by bicuculline was noted both in hippocampal slices and at the somatosensory cortical surface in vivo. In order to explain this bicuculline-resistant fraction of responses to EDA, two hypotheses were considered and tested in the slice preparation. Firstly, it is conceivable that EDA might form, in addition to the monocarbamate structure, a dicarbamate analogue which would resemble adipic acid in structure (Fig. 10). Such a complex might then activate classical excitatory amino acid receptors, or a distinct dicarboxylic acid receptor. The former possibility was tested using a high concentration of the general glutamate receptor antagonist kynurenic acid (Perkins and Stone 1982b; Stone and Darlington 2002), but this compound failed to block the depression of antidromically-induced potentials or the intracellularly recorded depolarising responses to EDA, suggesting that activation of glutamate receptors cannot account for the results.

A role for an adipic acid receptor was also tested by applying adipic acid itself to the slices, where it did produce a depression of fepsps and an intracellular depolarisation. Those effects were not blocked by either bicuculline or strychnine, leaving open the possibility that EDA dicarbamate and adipic acid could act partly by a novel receptor site or transporter. Neither the EDA receptor nor the adipic acid site were blocked by α-aminoadipic acid, an antagonist at glutamate receptors (Biscoe et al., 1977; Stone, 1979).

The blockade of EDA responses by DIDS strongly supports the involvement of common ionic mechanisms between GABA and EDA. In addition, it may be that the poor blockade of EDA effects by bicuculline reflects a direct action on GABA receptors distal to the site of action of GABA itself, explaining the common dependence on chloride but with recognisably distinct pharmacology. A direct action of some kind is certainly needed to account for the GABA-like effects in the absence of synaptic terminals (Morgan and Stone, 1982; Davies et al., 1982) and such an action, rather than activation of sites sensitive to agents such as β-alanine or adipic acid, could account for many of the present results.

The work in vivo provides an interesting comparison with the hippocampus in vitro. The SEPs result from the depolarization of pyramidal cells by thalamo-cortical afferents. A subsequent positive wave due to the spread of depolarization to superficial cortical layers (I – III) is increased by the removal of tonic inhibition from GABAergic interneurons in these superficial layers (Addae et al. 2007; Jellema et al, 2004; Reyes et al. 1998), confirming a role for endogenous GABA release. The results provide strong support to the findings in vitro. The profile of the trendplot of the SEPs shows an initial slight increase in the SEP size and a subsequent more prolonged decrease followed by recovery. The profile is similar to that seen in some

of the in vitro recordings (e.g. Fig 3).The in vivo profile is consistent with the report that depolarizing GABA responses are found in the superficial cortical layers where topical EDA is likely to exert its initial effect (DeFazio et al., 2005). It is clear that EDA is able to exert a profound inhibitory action on SEPs similar to the inhibition noted in vitro. Also, and perhaps more importantly, the effects of EDA are at least partially resistant to bicuculline, since they are not prevented by concentrations of bicuculline that induce an increase of neuronal excitability sufficient to double the amplitude of an SEP and to induce multiple PS and spontaneous epileptiform activity in the neocortex. Indeed, this failure of bicuculline to block the EDA effects in vivo suggests that the direct effects of EDA on GABA receptors or channels noted above is relatively more important in vivo than it is in vitro.

Following the initial reports concerning EDA, it became clear that an interaction with bicarbonate or carbon dioxide was required for the excitatory and toxic action of β-N-methylamino-L-alanine (BMAA) at excitatory amino acid receptors (Myers and Nelson, 1990). Also, high concentrations of neutral amino acids such as L-alanine and L-proline, can activate glutamate receptors (Pace et al., 1992). The formation of carbamates or similar complexes of these amino acids in bicarbonate-containing media could generate molecules able to mimic glutamate, making them relevant to excitotoxicity in physiological or pathological conditions in which their concentrations are elevated.

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Figure legends:

Figure 1. Responses of hippocampal slices to EDA.

A*.** illustrates the effects of EDA when applied for 10 min to allow the attainment of a plateau depression. Responses to three concentrations are illustrated: 100μM (*n* = 5), 300μM (*n* = 4) and 1000μM (*n* = 16). ***B. is of field epsps from one slice showing the baseline potential (the largest epsp) and sample potentials recorded at the peak depression induced by 50, 100, 300, 500 and 1000μM EDA (progressively smaller epsps). The traces indicate that the depression of the epsps was not accompanied by any change of axonal volley. ***C.*** shows the log concentration-response curves for EDA (filled circles, *n* = 6-8) and GABA (open circles, *n* = 5-6). The measurements were based on the mean of the last 10 epsps recorded during superfusion with EDA or GABA for 10 min. ***D***. shows the mean data for 4 slices in which EDA (1mM) produced a long-lasting depression of the fepsp.

Calibrations for B: 0.5mV and 5ms.

Data are shown as mean ± S.E.M..

Figure 2. Effects of bicuculline and gabazine on responses to EDA.

A. The time course of the depression of fepsps by EDA (1000μM, *n* = 5) applied for 2 min, and its partial blockade by bicuculline methobromide (BIC, 20μM, *n* = 4).

B. illustrates records of epsps from a slice treated with bicuculline 100μM, showing the baseline potential, the depression produced by EDA 1000μM, and the partial blockade by bicuculline.

C. shows the overall data in which bicuculline at 20μM (*n* = 5) and 100μM (*n* = 3) or gabazine at 20μM (*n* = 3) and 100μM (*n* = 3) produced a partial blockade of the

responses to EDA (1mM). There is no significant difference between the blockade produced by the two concentrations of antagonist, suggesting that the blockade is maximal. Strychnine at 10 or 100μM failed to modify the responses to EDA.

Data are shown as mean ± S.E.M..

** P < 0.01 compared with the control response to EDA (Dunnett's test for multiple comparisons).

Figure 3. Effects of nipecotic acid on responses to EDA.

A. Amplitudes of the first of a series of fepsp depressions induced by EDA 300μM (filled circles), the increase in amplitude and duration of the response sampled as the maximum response to EDA in the presence of nipecotic acid 100μM (open circles), and recovery towards the original response profile (filled triangles). Nipecotic acid was applied for 15 min before, during and for 30 min following EDA.

B. Sample traces showing the fepsps recorded (a) at baseline, (b) at the peak of depression produced by the first application of EDA and (c) at the peak of depression produced by EDA in the presence of nipecotic acid.

C. summarises the time course of change in the mean size of the depression produced by EDA when slices were superfused with nipecotic acid (100μM).

D. summarises the overall data in which nipecotic acid produced a significant increase in the amplitude of the EDA response.

Data are shown as mean ± S.E.M. (*n* = 6).

** P < 0.01 compared with the control response to EDA.

Figure 4. Effects of kynurenic acid on antidromically-evoked spikes.

A. The depression of antidromic spike amplitude produced by EDA 1mM in the absence (filled circles) and presence (open circles) of kynurenic acid 2mM. Kynurenic acid was present for 15 min before, during and for 20 min following the application of EDA, but failed to modify the EDA response.

B. Sample antidromic spikes recorded (a) at baseline, (b) at the peak depression produced by EDA and (c) at the peak depression produced by EDA in the presence of kynurenic acid.

Data are shown as mean ± S.E.M. (*n* = 5).

Figure 5. Effects of β-alanine on fepsps.

A. When applied for 5min at concentrations of 500μM, β-alanine (closed circles, *n* = 4) induced a depression of fepsp slope which was smaller and slower than that produced by EDA. Strychnine 100μM (open triangles, *n* = 4), applied for 15 min before, during and for 30 min after β-alanine reduced the size of depressant response but did not change the initial increase in fepsp size.

B. illustrates sample fepsps at baseline (a), at the time of maximum inhibition by β-alanine (b) and when blocked by strychnine (c).

C. chart summarising the decreased fepsp size produced by β-alanine (β-ala), the failure of bicuculline (bic) to modify that depression, and the blockade by strychnine (stry), and failure of bicuculline or strychnine to modify the initial increase in fepsp size produced by β-alanine.

** P < 0.01 (*n* = 4) compared with the control response to β-alanine alone.

Calibrations: 1mV, 10ms.

Figure 6. Intracellular recordings of the effects of EDA on a CA1 pyramidal neuron. ***A***. illustrates a typical record of the depolarisation produced by EDA 300μM. The depolarisation is associated with an increase in membrane conductance, tested using 500ms pulses of hyperpolarising current (downward deflections). The upward deflections on the trace are spontaneous epsps which are suppressed by the membrane depolarisation. ***B.*** illustrates both the larger depolarisation and conductance change produced at 1mM EDA, and an underlying hyperpolarisation which occurs before and following the depolarisation (6 neurons). ***C***. illustrates a neuron depolarised by EDA (500μM) in which, during the period indicated by the hatched bar, the membrane potential was manually clamped at the resting potential to confirm that the decrease of membrane resistance was still observed under these conditions.

Calibrations in ***A:*** 5mV and 1min; calibrations in ***B, C***: 10mV and 1 min.

Figure 7. Blockade of EDA depolarisation by bicuculline.

A. (a) illustrates the initial depolarisation induced by EDA, 1mM, while (b) shows the response after the application of bicuculline methobromide (20μM, 20min). In (c) the response to EDA recovers substantially following removal of the antagonist. ***B***. The effect of bicuculline was statistically significant, with recovery reaching an amplitude not significantly different from the initial value (n = 4). Calibrations in ***A:*** 20mV and 2min. Action potentials triggered in this cell have been cut off.

**P < 0.01 relative to EDA alone.

Figure 8. Responses to EDA and adipic acid.

A. Control responses are shown (open circles) for typical responses to an application of EDA at 500μM followed by the smaller and slower effect of adipic acid (2mM). Superimposed on that plot are responses after superfusion with α-aminoadipic acid (1mM, closed circles). There is no effect of α-aminoadipic acid on the response to adipic acid, although there is a small apparent reduction in the response to EDA. ***B.*** The overall analysis indicated that α-aminoadipic acid had no significant effect on the response to EDA or adipic acid. Data are shown as mean ± S.E.M. for *n* = 4.

Figure 9. Effects of EDA on somatosensory evoked potentials (SEPs) in vivo.

A. A typical individual record showing a response to topical EDA (25 mM for 5 min) causing an initial increase and then a decrease in the SEPs. ***B.*** Graphs of the pooled data showing a concentration dependent effect of EDA at 25 mM ***(black circles)*** and 50 mM ***(open circles)*** (n = 5 at each concentration). The initial increase (more marked at the lower concentration) was followed by the decrease which recovered slowly to the control level. ***C.*** Bicuculline (10 μM for 15 min) caused an increase in the SEPs but did not significantly affect the decrease in the size of the SEP caused by EDA. Note especially the immediate and complete abolition by EDA of the increased positive wave caused by bicuculline. The graphs show the changes in amplitude of the first negative (downward) wave of the SEP with each point being an average of 30 SEPs recorded over 1 min. The representative waveforms represent individual sample SEPs taken at the times (a,b,c,d) indicated on the time graph.

Figure 10 Chemical structures of relevant molecules.

Figure 1

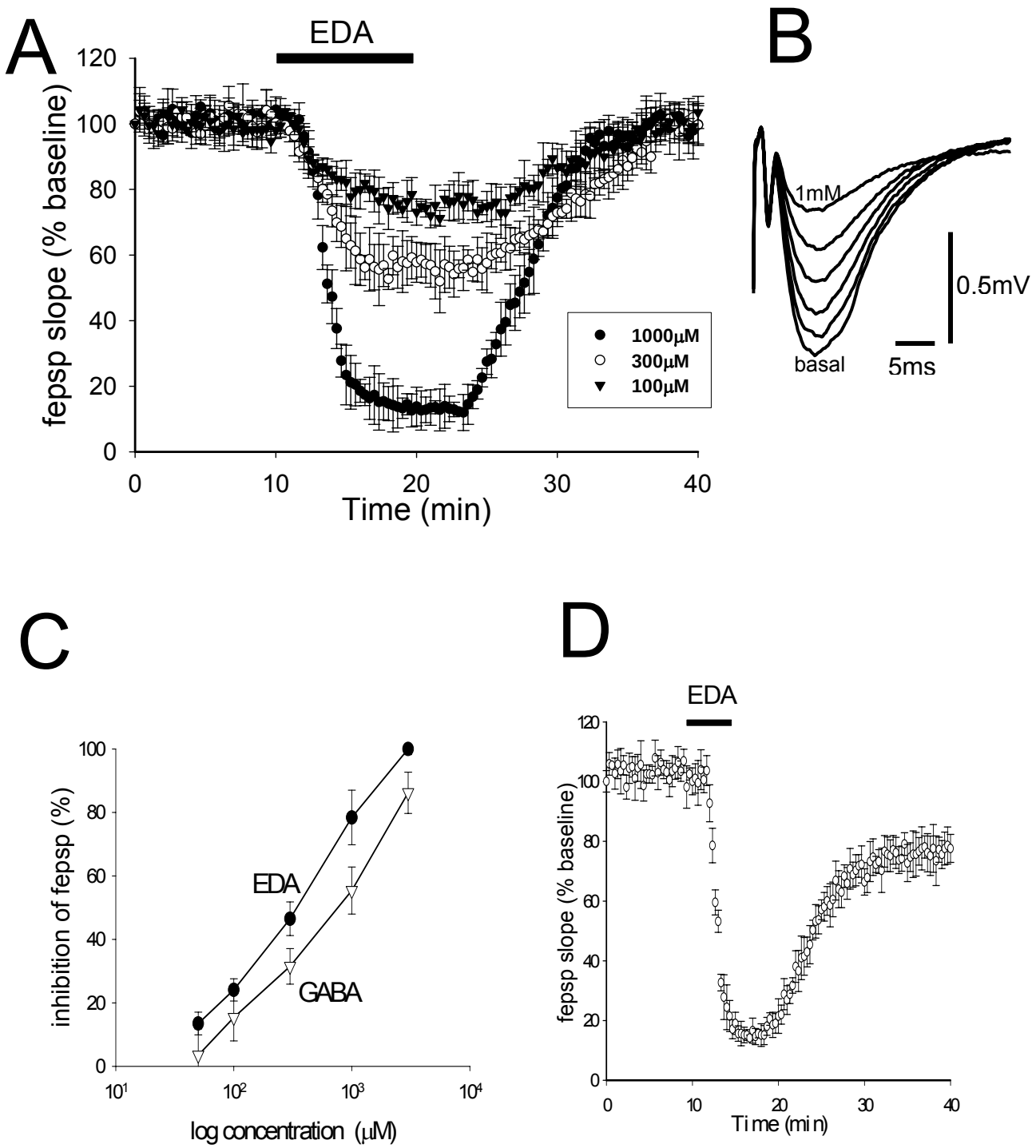

A
EDA
fepsp slope (% baseline)
Time (min)
1000μM
300μM
100μM
B
1mM
0.5mV
basal
5ms
C
inhibition of fepsp (%)
EDA
GABA
log concentration (μM)
D
EDA
fepsp slope (% baseline)
Time (min)

Figure 2

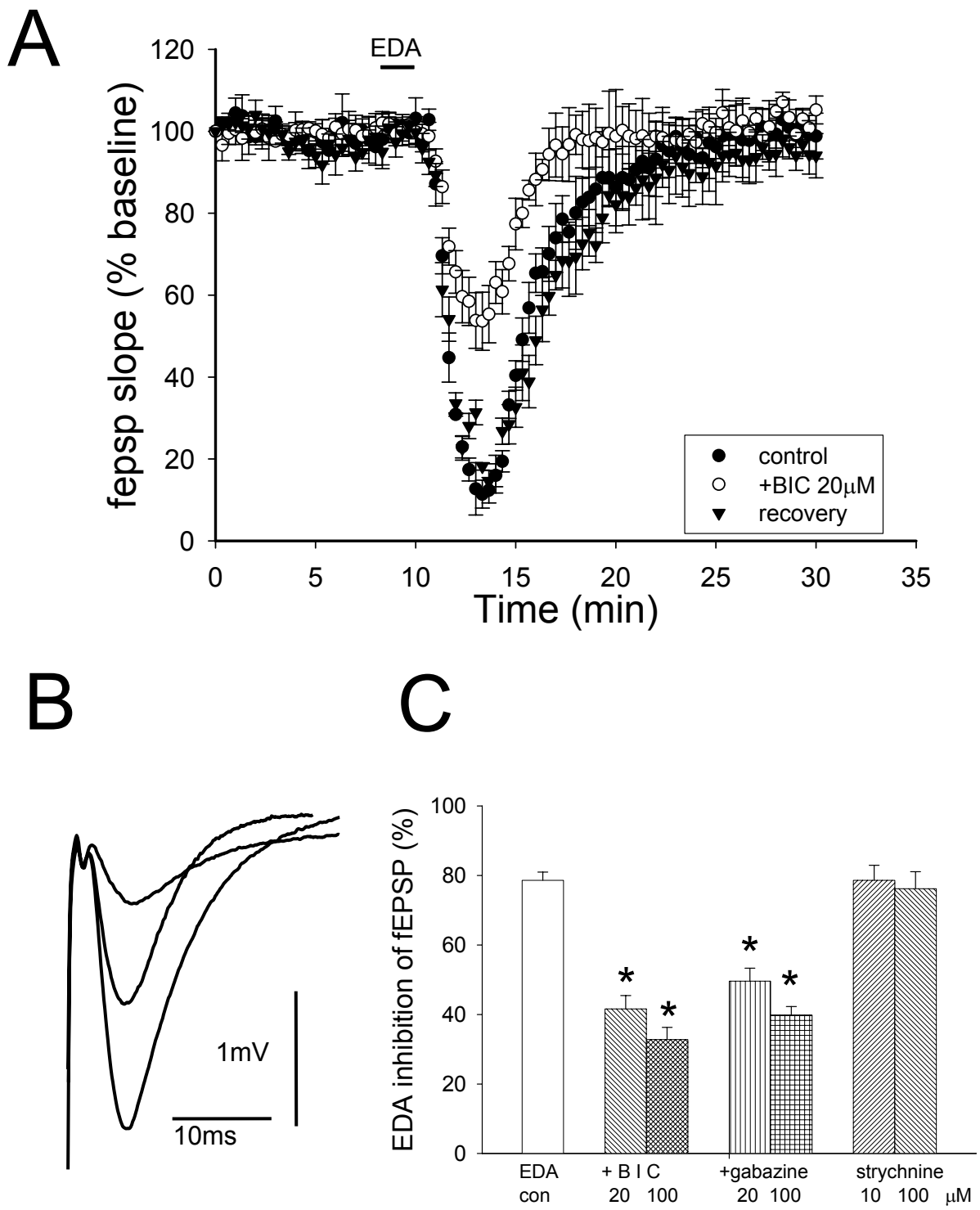

A
EDA
fepsp slope (% baseline)
Time (min)
control
+BIC 20μM
recovery
B
1mV
10ms
C
EDA inhibition of fEPSP (%)
EDA
con
+ B I C
20 100
+gabazine
20 100
strychnine
10 100 μM

Figure 3

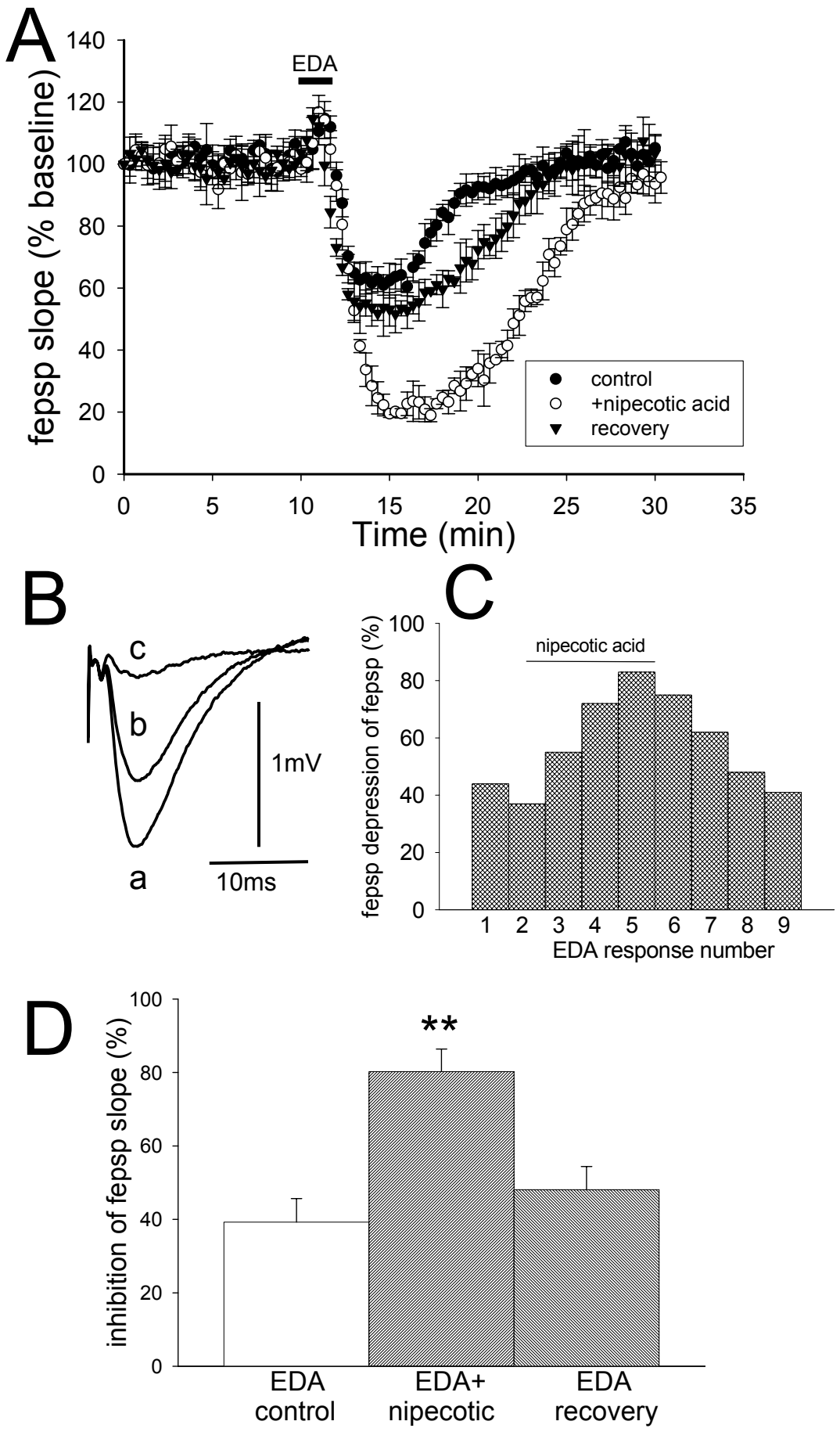
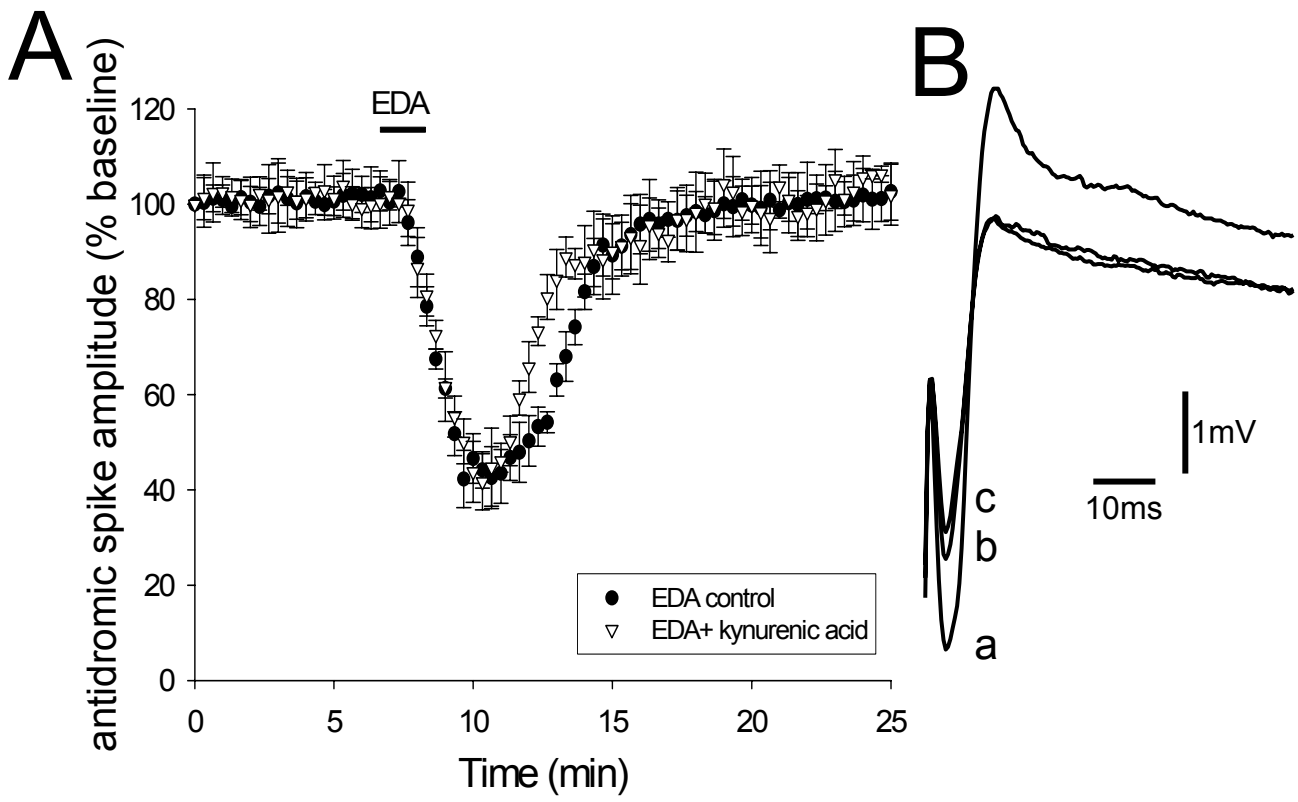

A
EDA
fepsp slope (% baseline)
Time (min)
control
+nipecotic acid
recovery
B
c
b
a
1mV
10ms
C
nipecotic acid
fepsp depression of fepsp (%)
EDA response number
D
**
inhibition of fepsp slope (%)
EDA control
EDA+ nipecotic
EDA recovery

Figure 4


A
antidromic spike amplitude (% baseline)
EDA
120
100
80
60
40
20
0
0
5
10
15
20
25
Time (min)
EDA control
EDA+ kynurenic acid
B
1mV
10ms
c
b
a

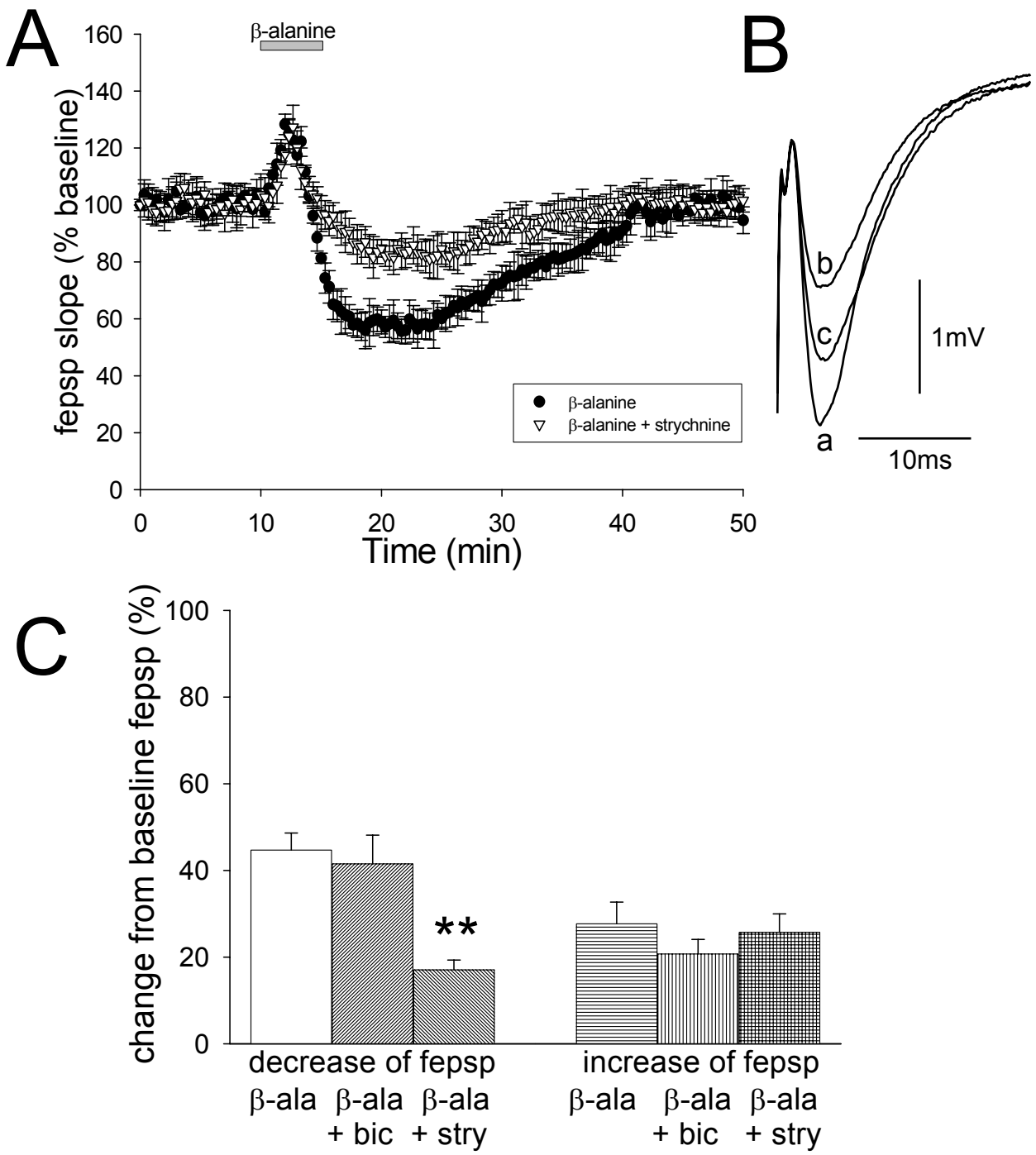

A
β-alanine
fepsp slope (% baseline)
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
Time (min)
β-alanine
β-alanine + strychnine
B
b
c
a
1mV
10ms
C
change from baseline fepsp (%)
100
80
60
40
20
0
**
decrease of fepsp
β-ala
β-ala + bic
β-ala + stry
increase of fepsp
β-ala
β-ala + bic
β-ala + stry

FIGURE 5

Figure 6

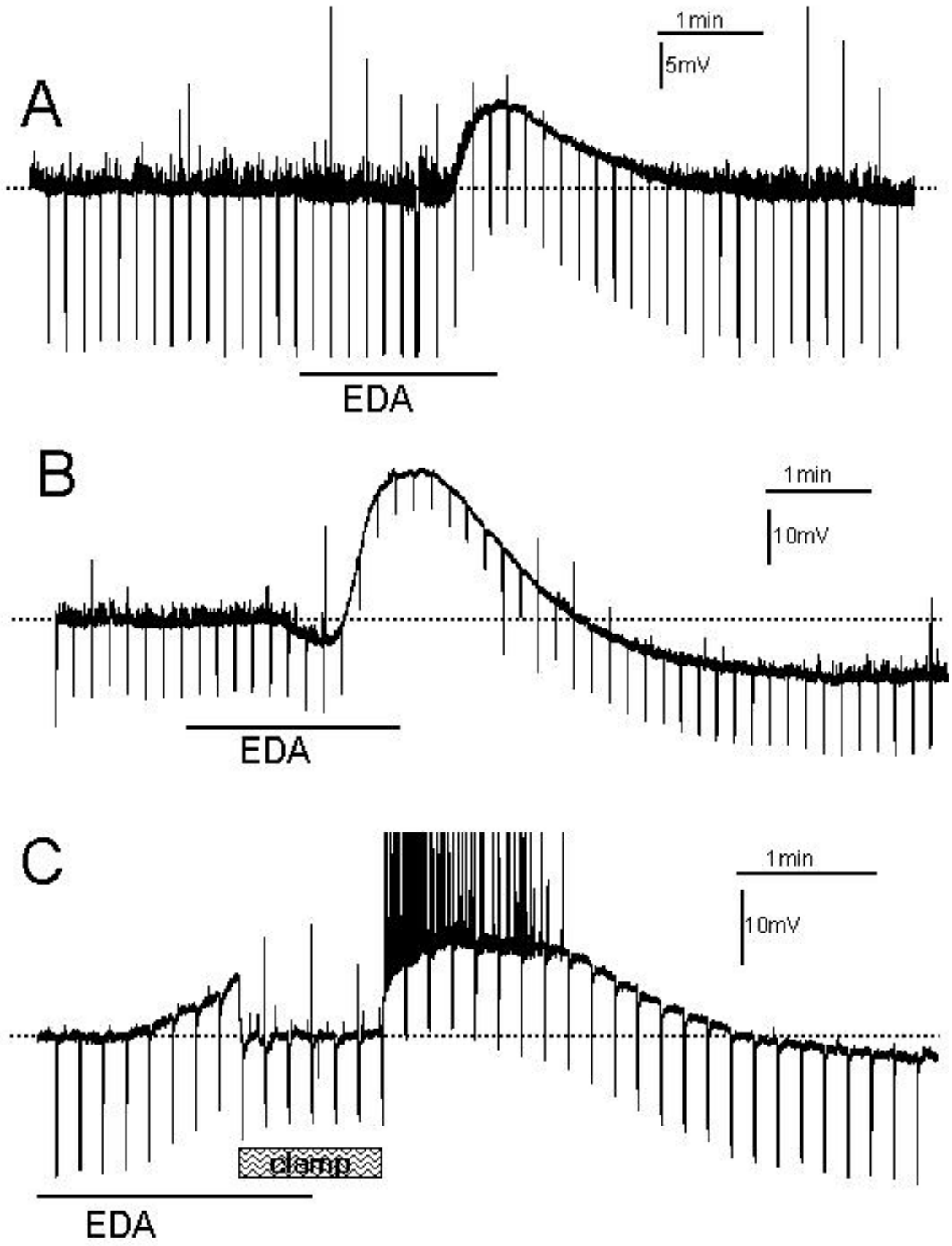


Figure 7

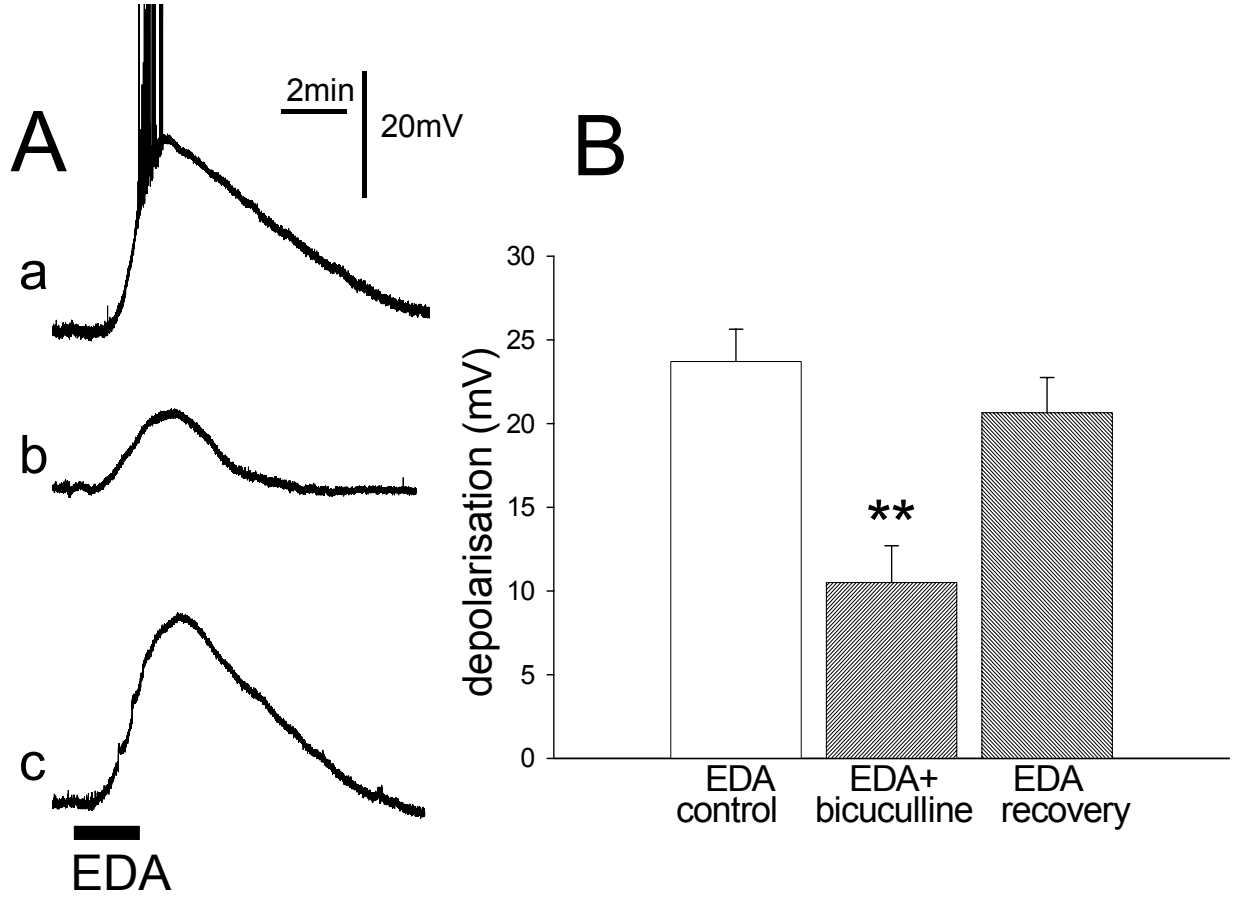

A
2min
20mV
a
b
c
EDA
B
depolarisation (mV)
30
25
20
15
10
5
0
**
EDA control
EDA+ bicuculline
EDA recovery

Figure 8

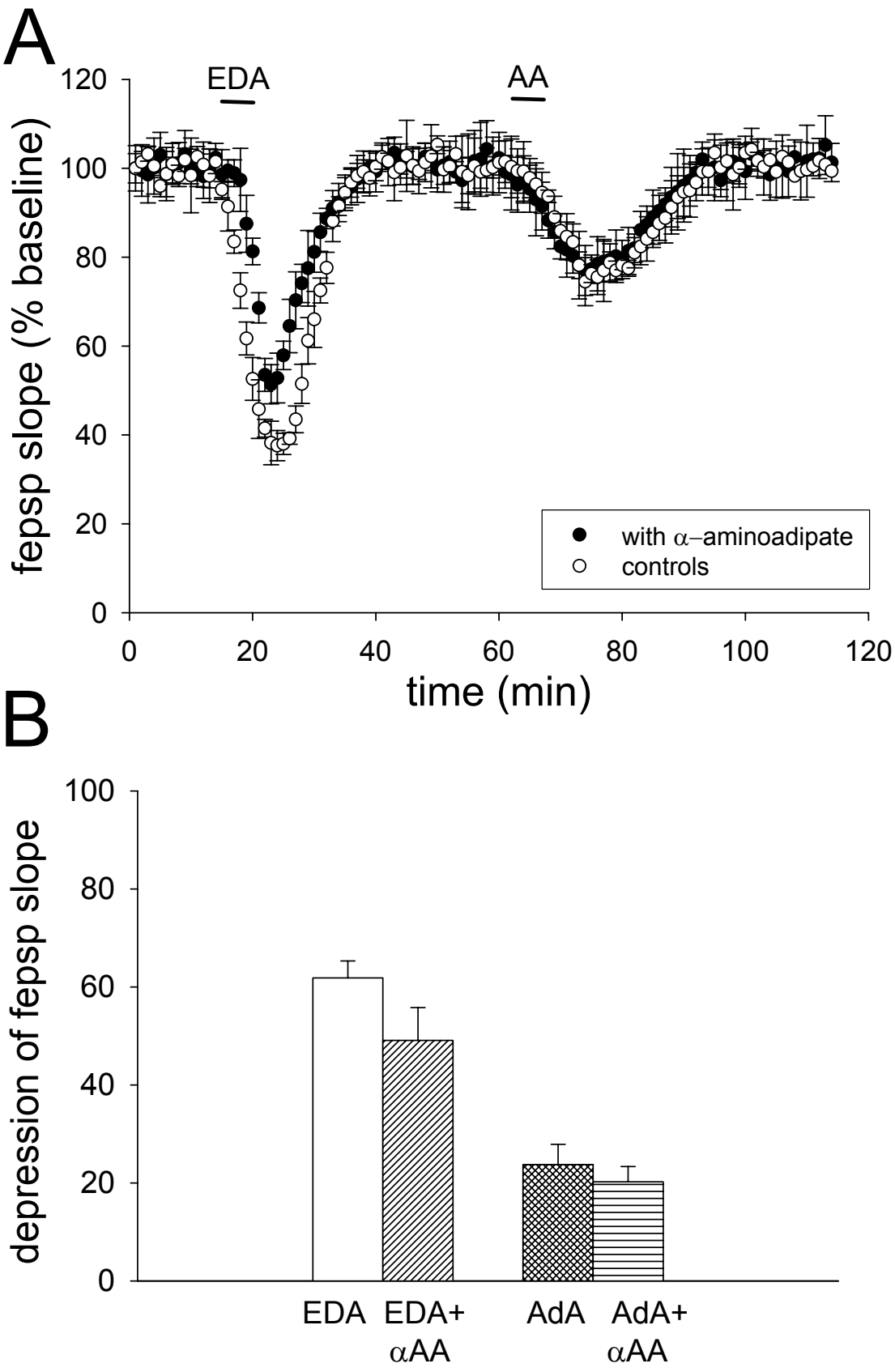

A
EDA
AA
fepsp slope (% baseline)
120
100
80
60
40
20
0
0
20
40
60
80
100
120
time (min)
with α–aminoadipate
controls
B
depression of fepsp slope
100
80
60
40
20
0
EDA
EDA+ αAA
AdA
AdA+ αAA

Figure 9

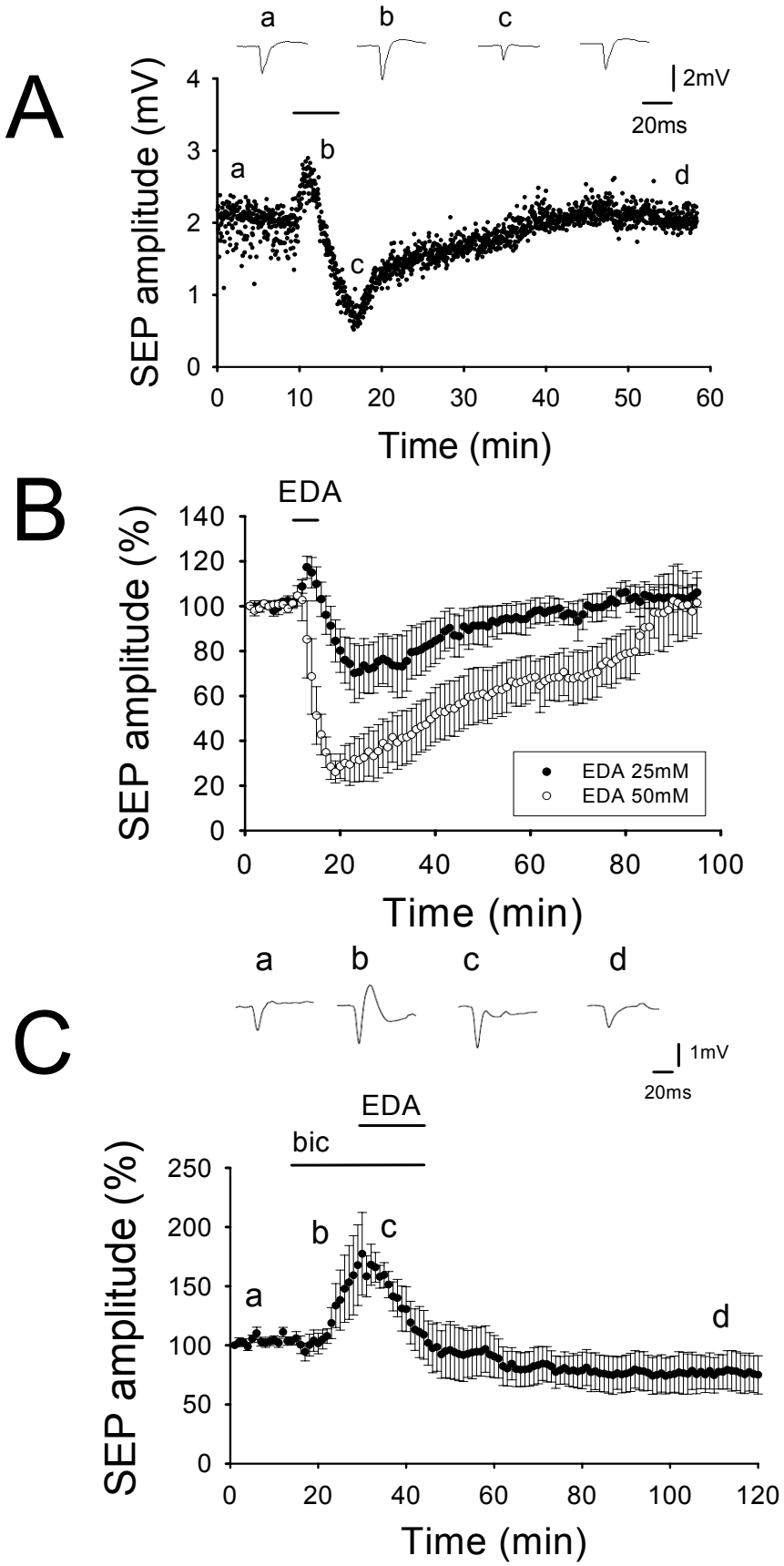
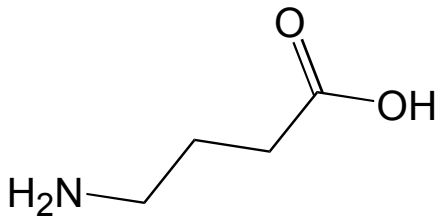
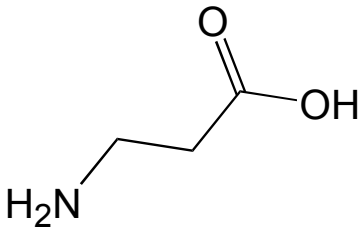

A
a
b
c
2mV
20ms
SEP amplitude (mV)
Time (min)
B
EDA
SEP amplitude (%)
EDA 25mM
EDA 50mM
Time (min)
C
a
b
c
d
1mV
20ms
EDA
bic
SEP amplitude (%)
Time (min)

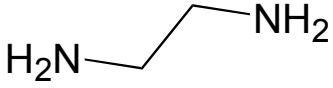
Figure 10



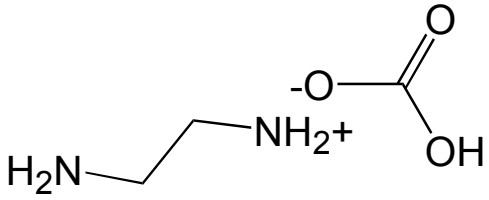
4-aminobutyric acid (GABA)



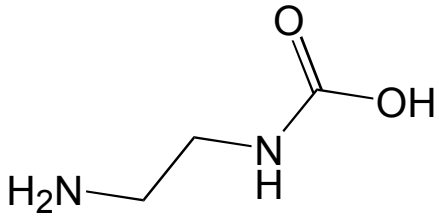
β–alanine



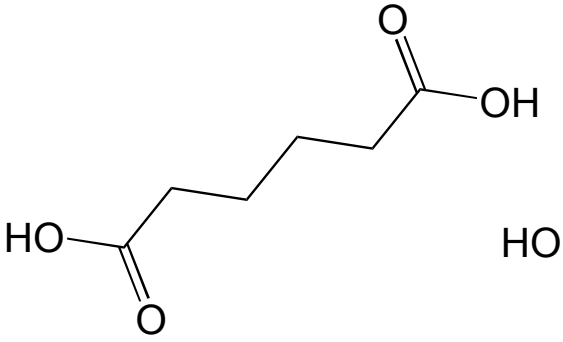
ethylenediamine



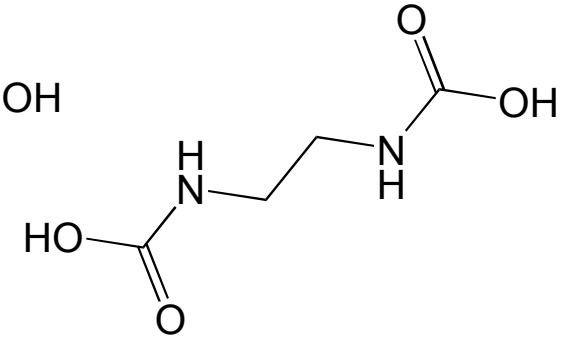
ethylenediamine bicarbonate complex



ethylenediamine carbamate



adipic acid



ethylenediamine di-carbamate