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COMPARISON OF THE EFFECTS OF ETHYLENEDIAMINE ANALOGUES AND γ-AMINOBUTYRIC ACID ON CORTICAL AND PALLIDAL NEURONES

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1 The actions of ethylenediamine (EDA) and structurally related compounds were investigated by microiontophoresis in Wistar rats.

2 EDA inhibited, via a bicuculline-sensitive mechanism, the spontaneous firing rate of all cortical and pallidal cells tested.

3 The results with the analogues suggest that two amine groups are required for this neuronal depressant action whereas a carboxyl grouping is not. N-substitution reduces the depressant effect. The length of the molecule is also critical, more than 3 methylene components seriously reducing its effectiveness. A rigid analogue of EDA, piperazine, was also active. In addition the apparent transport numbers of EDA and γ-aminobutyric acid (GABA) were calculated, showing a close similarity between the two.

4 The results are discussed with respect to the possibility that EDA may represent a new class of GABA-mimetics, or may indicate the existence of a novel diamine receptor mediating bicuculline-sensitive inhibition in the rat CNS.

Introduction

We have previously shown that ethylenediamine (EDA) ($NH_2.CH_2.CH_2.NH_2$) has powerful depressant actions on central neuronal excitability, and that this action is antagonized by bicuculline (Forster, Lloyd, Morgan, Perkins & Stone, 1981; Perkins, Bowery, Hill & Stone, 1981). The inhibitory actions of γ-aminobutyric acid (GABA) on single unit firing rate have been well documented (see Kelly, 1975; Kelly & Beart, 1975) and structure-activity studies have shown that the electronegative carboxyl group is of great importance in this respect (Curtis & Watkins, 1960; see Kelly & Beart, 1975). It was of interest therefore, that EDA had such marked inhibitory action on cell firing although it lacked any such electronegative moiety. In addition the antagonism of such actions by bicuculline, widely regarded as a specific GABA antagonist (Curtis, Duggan, Felix & Johnston, 1970; Curtis, Duggan, Felix & Johnston, 1971) raised the possibility that EDA was acting on the GABA receptor.

Furthermore, EDA has proved able to inhibit GABA uptake into rat cortical slices, induce release and depolarize sympathetic ganglia (Perkins *et al.*, 1981) again by a bicuculline-sensitive mechanism, and displace GABA from its binding sites in the central nervous system (CNS) (Forster *et al.*, 1981, Bowery & Hill personal communication).

In view of the possibility that EDA may be a GABA-mimetic compound, we have undertaken a more detailed microiontophoretic study of the structure-activity relationships existing between various analogues and derivatives of EDA. The compounds were chosen on three grounds. The necessity or otherwise of two amine groups was investigated by the case of either substituted compounds, for example 2-aminoethanol, or structures where one amine group was partially masked as in N-methylethylenediamine (NMEDA). Secondly, the distance between the amine groups was varied by increasing the carbon chain length. Lastly an attempt was made to look at some more rigid analogues of EDA, using piperazine and piperidine. Pyrazine was also used as it provided a planar version of piperazine. The recordings were made from the cortex and the pallidum. The latter was chosen because of the high regular firing rate of many of the cells (Perkins & Stone, 1980) and the observation that glycine (a control depressant) was much more effective in pallidum than cortex.

Methods

Male Wistar rats were anaesthetized with urethane (1.3 g/kg) and placed in a stereotaxic frame designed for use with DeGroot's stereotaxic atlas (DeGroot, 1959). The scalp was incised and the skull removed around the bregma suture to expose the somatosensory areas of cerebral cortex, and expose an area through which electrodes were inserted into the pal-

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lidum at cordinates AP 6.6–7.6, L 2–5, H 5.0 according to DeGroot.

Seven-barrelled micropipettes were used to deliver the following drugs by conventional microiontophoretic techniques: γ-aminobutyric acid (GABA), 100 mM pH 4.0; glycine hydrochloride 100 mM, pH 2.5–3.0; EDA, 100 mM, pH 4.0–4.5; N-methyl-EDA (NMEDA), 100 mM, pH 4.0; 1,3-diaminopropane (DAP), 200 mM, pH 4.0–4.5; 1,4-diaminobutane (DAB) 100 mM, pH 4.5–5.0; 1,5-diaminopentane, 100 mM, pH 4.0; diethylenetriamine (DELTA), 100 mM, pH 8.0; 2,3-diaminopropionic acid (DAPA) 100 mM, pH 4.0; 2-aminoethanol, 200 mM, pH 4; piperazine citrate, (Pz), 100 mM, pH 4.0; piperidine, 100 mM, pH 4.0; pyrazine 100 mM, pH 4.0; picrotoxin, saturated solution in 165 mM NaCl; bicuculline methobromide, 10 mM, pH 5.0; strychnine sulphate, 10 mM, pH 4.0. All drugs were obtained from Sigma except bicuculline which was a gift from J.F. Collins. The pH was adjusted, when necessary, with 0.5 M HCl or NaOH.

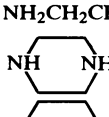
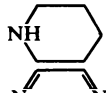
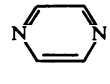
Usually only 3 agonists were used in any one electrode to ensure that the antagonist could be separated from any one agonist by either an empty barrel or one containing 165 mM NaCl, used for current balancing. This was to avoid any possibility of electrical coupling between agonist- and antagonist-containing barrels. All drugs were expelled with a cationic (i.e. positive) current. Drugs were always applied in a constant time cycle via a Neurophore (Medical System Incorp.) unit to prevent variations of response size due to the retaining currents of 4 to 10 nA (Bradshaw, Roberts & Szabadi, 1973).

Recording of extracellular unit activity in cortex and pallidum was accomplished with a single glass microelectrode containing 1 M potassium acetate attached to the side of the multibarrelled pipette by epoxy resin and protruding 2–10 μm beyond the tip (Stone, 1973). Extracellular unit activity was amplified and displayed on an oscilloscope using conventional techniques and passed through an amplitude discriminator. Output pulses of the latter, corresponding to action potentials, were counted and recorded on a polygraph by a resetting integrator as spikes per second or as an instantaneous ratemeter record. Drugs were considered to be inactive on a cell only after they had been tested with currents of at least 80 nA for 30 s.

Estimation of apparent transport number of ethylenediamine and γ-aminobutyric acid

[^{14}C]-EDA (sp.act. 25 μCi/μmol) and [^{14}C]-GABA (sp.act. 226 μCi/μmol), both supplied by the Radiochemical Centre, Amersham, were made up in distilled water to a final concentration of 0.29 mM (pH 4.0). In the case of GABA this required the addition of 'cold' GABA; this dilution of the radioactive label being taken into account in the calculation of the number of moles released from the pipettes.

Table 1 Table showing the structures and relative potencies of the agonists studied, together with the number of cells responding

Compound	*Structure*	*pH*	*No. of cells inhibited no. tested*	*n coulombs charge for just maximal response (mean ± s.e.)*	*Potency ratio*
γ-Aminobutyric acid	$NH_2CH_2CH_2CH_2COOH$	3.5–4.0	76/76	273 ± 61	1.0
Ethylenediamine	$NH_2CH_2CH_2NH_2$	4.0–4.5	73/73	318 ± 46	0.86
Diaminopropane	$NH_2CH_2CH_2CH_2NH_2$	4.0–4.5	17/17	357 ± 43	0.76
Diaminobutane	$NH_2CH_2CH_2CH_2CH_2NH_2$	4.5–5.0	3/13	1232 ± 15	0.22
Diaminopentane	$NH_2CH_2CH_2CH_2CH_2CH_2NH_2$	4.0–4.5	1/7	2075	0.13
N-methyl-ethylene-diamine	$CH_2NHCH_2CH_2NH_2$	4.0	8/24	1142 ± 102	0.24
Diaminopropionic acid	$NH_2CH_2CH(NH_2)COOH$	4.0	5/14	1284 ± 163	0.21
Diethylenetriamine	$NH_2CH_2CH_2NHCH_2CH_2NH$	8.0	6/16	2581 ± 725	0.11
2-Amino-ethanol	$NH_2CH_2CH_2OH$	4.0	0/5*		
Piperazine		4.0	31/33	453 ± 127	0.60
Piperidine		4.0	0/8		
Pyrazine		4.0	1/7*	1380	0.20

*See text; 1 or more of these cells was excited.

The potency ratio is the ratio of the mean charge GABA for just maximal response to mean charge test compound for just maximal response.

As it was necessary to eject sufficient label to give reasonable counts (a particular problem with EDA because of its low specific activity) the two drugs were ejected from 5 barrels of the micropipettes simultaneously. Two such electrodes were used for the measurement of the transport number for GABA and 5 in the case of EDA.

The drugs were expelled from each electrode into 500 μl samples of 165 mM NaCl, for two time periods of 1 and 10 min and at two current intensities of 40 and 80 nA. In addition a sample was taken with the compounds held by a retaining current of −10 nA for 1 min in order to estimate leakage. Blanks were run with each electrode and the counts of these subtracted from the total before calculating the apparent transport number/barrel. The samples were placed in 5 ml of Fisofluor No. 1 (Fisons Ltd) scintillation fluid and counted in an SL 3000 beta counter (Intertechnique) for 10 min each.

The apparent transport number (n) was calculated using the following formula (Curtis, 1964):

$$n = \frac{\text{mol ejected}.F}{\text{coulombs}}$$

where F is Faraday's constant.

Results

Comparison of ethylendiamine, γ-aminobutyric acid and glycine

The results are summarized in Table 1.

In order to gain quantitative measure of relative potencies the mean total charge, in n-coulombs, required to achieve a maximal response to each agonist was calculated. A potency ratio was then derived with GABA as unity (see Table 1). With the exception of glycine (see below) the responses to the agonists were similar in cortex and pallidum and thus no distinction is made between the two recording areas.

EDA was tested on 73 spontaneously active cells with currents from 31–64 nA, and for times of up to 10 s, typically about 5–7 s. On all cells tested it produced a clear inhibition of firing rate (Figure 1); the time course of this response was very similar to that of the response to GABA (76/76 cells inhibited) with onset and recovery usually occurring within 1–3 s after the beginning and terminating of the current pulse.

When expressing the dosage of drug used, the coulombs passed (ampere × time) was chosen in order to include the duration of application of drug in the measure of dose. This implies an equivalency of time and current intensity in determining the amount of drug ejected which may or may not be valid. However, as some of the weaker agonists required applications of up to 30 s at the same current intensity as that used with GABA for 4–6 s it was felt to be important to include the time factor in the measure of dose.

EDA appeared to be approximately equipotent to GABA having a potency ratio of 0.86 with respect to GABA, based on the coulombs required for a maximal response. In addition, any variability in the responses to EDA and GABA between cells occurred in parallel for the two agonists. Thus, if a greater than

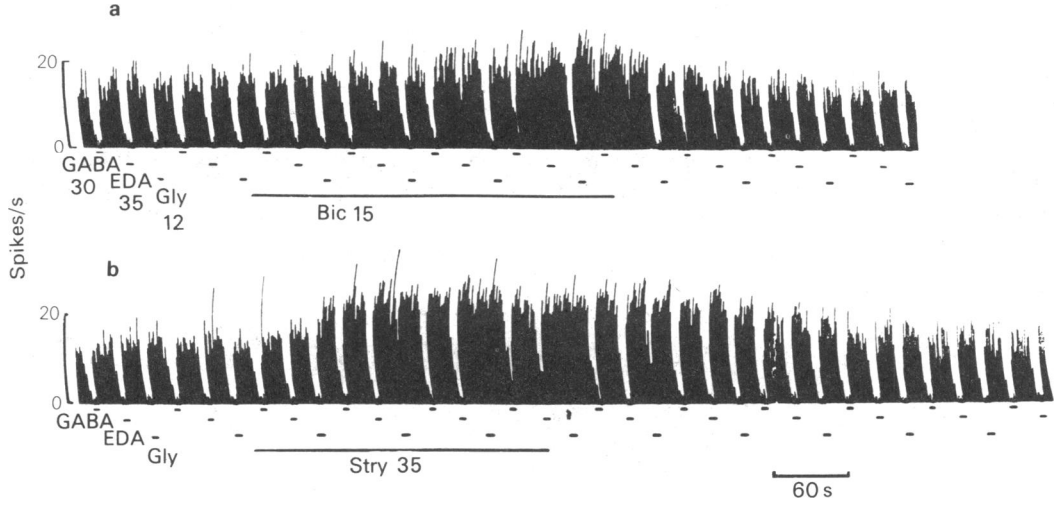


Figure 1 Ratemeter records of the firing rate of a pallidal cell. Horizontal bars refer to microiontophoretic drug applications. (a) Antagonism of responses to γ-aminobutyric acid (GABA + 30 nA) and ethylenediamine (EDA + 35 nA), but not to glycine (Gly + 12 nA), by bicuculline (Bic + 15 nA). (b) (Trace continuous with (a)) Antagonism of the responses to glycine by strychnine (Stry + 35 nA): responses to GABA and EDA are unaffected. Note that the baseline firing rate was raised by the antagonists. Ordinate scale: spikes/s. Time bar: 60 s.

average amount of current was required for inhibition of spontaneous activity for GABA, then EDA would similarly require higher currents and *vice versa*. This parallelism in response characteristics between GABA and EDA was emphasized by the lack of such a correlation in the case of glycine. Glycine-evoked inhibitions were typically very weak or non-existent in the cortex (3 of 22 cells were depressed) and strong in the pallidum (27 of 27 cells), and this variation was independent of the strength or weakness of GABA or EDA responses.

Effect of bicuculline on responses to ethylenediamine, γ-aminobutyric acid and glycine

Bicuculline was tested on responses to EDA and GABA or glycine on 24 cortical and pallidal cells (see Figure 1).

Glycine was included as a control for non-specific antagonism of inhibitions in 16 cells. On 2 cells only glycine and EDA were pulsed; bicuculline antagonizing only EDA. On 11 of the 19 cells, glycine-evoked inhibitions were unaffected (see Figure 1) and on one cell slightly reduced at the time of blockade of EDA and GABA. On 2 cells glycine was blocked as well as EDA and GABA.

On 5 cells only EDA and GABA were pulsed and bicuculline blocked both responses on all 5. In these cases and all subsequent ones, unless otherwise stated, recovery from bicuculline antagonism was always obtained. Typically, low doses of bicuculline were required, usually under 15 nA for up to 3 min.

Bicuculline had no effect on any agonist on 1 cell, blocked EDA, but not GABA on two units and in one case blocked GABA whilst sparing EDA.

Effect of picrotoxin on responses to ethylenediamine and γ-aminobutyric acid

Picrotoxin was tested on depressant responses evoked by EDA on 9 cells, on 5 of which responses to GABA were also obtained. Picrotoxin needed to be applied with high currents (up to 120 nA) and for up to 6–8 min.

On 4 cells when EDA and GABA were pulsed and on 3 cells when only EDA was present, picrotoxin reduced all responses by 50% or more. However recovery was typically slow and poor taking up to 10–15 min, and was absent in 2 cases.

Responses to GABA and EDA were unaffected on one cell and on another unit picrotoxin failed to antagonize responses to EDA applied on its own.

Effect of strychnine on responses to ethylenediamine, γ-aminobutyric acid and glycine

Strychnine was applied with up to 30 nA for 1–3 min and reversed glycine-evoked inhibitions on 7 cells whilst not affecting EDA and/or GABA evoked inhibitions (see Figure 1).

Straight chain analogues and substituted derivatives of ethylenediamine

Diaminopropane (DAP) depressed the spontaneous firing rate of all 17 cells tested, with currents of up to 80 nA for 8–12 s. It was slightly less potent than EDA, having a potency ratio compared to GABA of 0.76 (see Table 1) and a potency ratio compared to EDA of 0.89. However, the time course of the response was similar to EDA with a latency of response of 2–5 s and recovery within 2–6 s after termination of the current pulse.

Bicuculline was tested on DAP-evoked inhibitions on 12 cells; an example is shown in Figure 2. On 3 cells only GABA and DAP were applied; responses to DAP and GABA were equally antagonized. On 5 cells both GABA and glycine were applied in addition to DAP; in all cases glycine-evoked inhibitions were unaffected when responses to GABA and DAP were blocked. Glycine and EDA were included as agonists with DAP on 4 cells, on 3 of which antagonism of responses to EDA and DAP was observed, while responses to glycine were not affected (see Figure 2). On the remaining cell, responses to all three agonists were blocked by bicuculline.

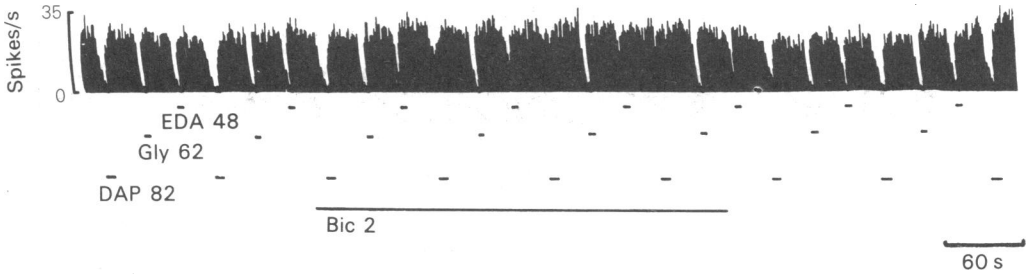


Figure 2 Ratemeter records of the firing rate of a pallidal neurone (as in Figure 1) showing antagonism of the response to diaminopropane (DAP) and ethylenediamine (EDA) by bicuculline (Bic); the response to glycine (Gly) was not affected.

Diaminobutane (*putrescine*) weakly inhibited 3 out of 13 cells when tested up to the criterion dose of 80 nA for 30 s. Responses on the three responding cells were antagonized by bicuculline together with responses to GABA and DAP.

Diaminopentane (cadaverine) evoked only a weak inhibitory response in 1 out of 7 cells.

2-Amino-ethanol and substituted analogues

2-Amino-ethanol was tested on 5 cells. The only response was a slight excitation of one cell when applied with 80 nA for 10 s or more. N-methyl-EDA produced inhibition of 8/24 cells tested (see Figure 3). Diaminopropionic acid (DAPA) produced only a

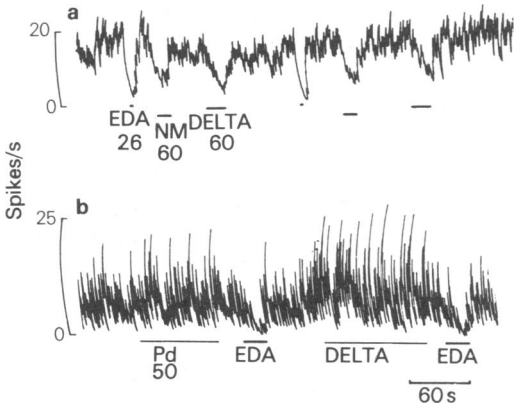


Figure 3 Ratemeter records of the firing rate of two cortical cells (as in Figure 1). (a) Inhibitory responses to N-methyl ethylenediamine (NM + 60 nA) and diethylenetriamine (DELTA + 60 nA) and ethylenediamine (EDA + 26 nA). (b) Lack of effect of piperidine (Pd + 50 nA) and the more typical absence of effect of DELTA (+ 50 nA) although the cell still responds to EDA (+ 30 nA).

weak inhibition of 5 out of 14 cells. Diethylenetriamine ($NH_2.(CH_2)_2.NH_2(CH_2)_2.NH_2$) the likeliest contaminant of EDA (personal communication from Sigma Chemicals) was only occasionally effective, inhibiting 6 out of 16 cells tested (see Figure 3). Furthermore the currents required to evoke responses were 60 nA or more, lower currents being ineffective.

Rigid and cyclic analogues of ethylenediamine

Piperazine inhibited the spontaneous activity of 31/33 cells tested with currents of 40–80 nA for up to 12 s. These inhibitions were slower in onset (2–5 s) and recovery (2–5 s) than those produced by EDA. Piperazine responses were blocked by bicuculline when glycine-induced inhibitions were unaffected or slightly reduced on 10 cells tested and in 2 of these cells bicuculline was able to antagonize reversibly responses to piperazine at doses that did not affect responses to GABA (Figure 4). In 3 cases the final responses to glycine during the application of bicuculline was also blocked. Strychnine blocked responses to glycine but not to piperazine on 2 of 3 cells, the remaining cell showing a reduction in the response to both agonists in the presence of strychnine.

Pyrazine, the unsaturated analogue of piperazine, produced excitation of 4 to 7 cells, and caused a weak depression on 1.

Piperidine, on the other hand, was inactive with currents of up to 120 nA on all 8 cells tested. For ease of comparison the results are summarized in Table 1.

Apparent transport numbers of ethylenediamine and γ-aminobutyric acid

Owing to the low [^{14}C]-EDA counts obtained with the 40 nA ejecting current, the apparent transport number has been calculated from the results obtained with 80 nA. Using the mean counts for each electrode the amount of drug released at 80 nA for 1 and 10 min was calculated. With 80 nA for 1 and 10 min this gave a release of 9.2 and 76 pmol [^{14}C]-EDA per barrel respectively. The [^{14}C]-GABA counts gave a release of 6.4 pmol/barrel at 80 nA for 1 min and 76 pmol/barrel when ejected for 10 min. This gave an apparent transport number for EDA of 0.18 and

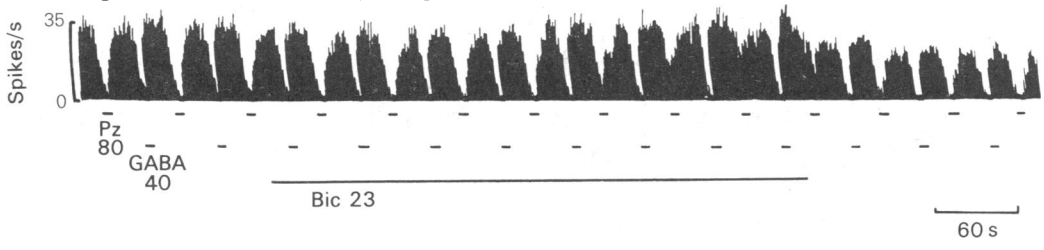


Figure 4 Ratemeter record of the firing rate of a cortical cell (as in Figure 1). The cell was depressed by piperazine (Pz + 80 nA) and γ-aminobutyric acid (GABA + 48 nA): the response to piperazine was antagonized by bicuculline (Bic + 23 nA), while the response to GABA was not affected.

0.15/barrel for the 1 and 10 min sample respectively. GABA had an apparent transport number of 0.13 based on the 1 min sample and 0.15 when ejected for 10 min.

Discussion

Bicuculline is widely considered to be a relatively specific antagonist of the neuronal depressant effects of GABA (Curtis *et al.*, 1970; 1971; Johnston, Beart, Curtis, Game, McCulloch & Maclachlan, 1972). The reversible blockade of EDA-evoked inhibitions in parallel with GABA by bicuculline, while responses to glycine were not affected, could suggest a direct agonist action of EDA on the GABA receptor. This view is supported by other work indicating that EDA can inhibit GABA uptake into cortex slices, induce a bicuculline-sensitive depolarization of sympathetic ganglia, and even displace [^{3}H]-GABA from binding sites on rat brain synaptosomes (Perkins *et al.*, 1981, Bowery & Hill personal communication).

The fact that in two instances, EDA was blocked before GABA is consistent with the finding that in an *in vitro* system EDA is approximately three times more sensitive to bicuculline than GABA (Perkins *et al.*, 1981). However, at present we have no explanation for the single case where GABA was preferentially antagonized to EDA.

The antagonism, albeit partial, of EDA and GABA by picrotoxin would also suggest that inhibitory actions of EDA are mediated via GABA receptor mechanisms, although as picrotoxin is more likely to act at the level of the chloride ionophore linked to the GABA receptor (Ticku & Olsen, 1978; Simmonds, 1980) it is not as specific a pharmacological tool as bicuculline. The difficulty of obtaining clear reversible antagonism with picrotoxin could reflect the lower solubility of picrotoxin and hence difficulty in iontophoretic ejection.

The bicuculline reversible response to EDA could be due to release or inhibition of uptake of GABA, or direct activation of the GABA receptor mechanisms. Although these experiments cannot definitely preclude any one or more of these actions it is possible that the rapid onset and recovery of the responses to iontophoretically applied EDA reflects a direct action on the GABA receptor and not an indirect effect by blocking uptake or evoking release.

As EDA possesses two basic amino groups and therefore will carry a strong positive charge at physiological pH, whereas GABA exists largely as a zwitterion at such pH, it was necessary to consider the possibility that EDA was more easily ejected iontophoretically. In such a case EDA could be in effect a weaker agonist than GABA but more would be ejected for a given amount of current. However, the transport numbers are very similar for the two compounds, implying a real similarity of pharmacological efficacy. Our value for the transport number for GABA is similar to that found by previous authors (see Krnjević, 1971; Kelly, 1975) although no allowance has been made here for any electro-osmotic ejection, hence the use of the term 'apparent' transport number (Bevan, Bradshaw, Pun, Slater & Szabadi, 1979).

If the bicuculline sensitivity of the depressant responses to EDA and derivatives reflects an action at GABA receptors, as we suggest, then the simplest assumption is that one amino group of EDA interacts with the molecular acceptor for the GABA amino group. This could be consistent with the loss of activity with increasing chain length, as the second amino group of, for example, diaminobutane or diaminopentane approaches the presumably electropositive grouping normally interacting with the GABA carboxyl moiety. The activity shown by DAP, however, probably reflects the fact that it is only one carbon longer than EDA and therefore there is no significant steric or electrochemical hindrance between it and the molecular acceptor groupings. The same arguments that apply to bicuculline reversal of EDA can therefore be applied to DAP and this antagonist. The simple addition of a carboxyl group, as in 2,3-diaminopropionic acid however, reduces rather than improves activity. Diaminobutyric acid has similarly been shown to be only a weak depressant of cell firing (Curtis & Watkins, 1960). On the other hand, the monoamines methylamine, ethylamine and propylamine were found to be inactive on cell firing rates (Curtis & Watkins, 1960) and in the present study the replacement of one amino by hydroxyl (2-aminoethanol) or the masking of one nitrogen as in N-methyl EDA reduces or abolishes activity.

In attempting to explain these findings it may be noted that numerous authors have suggested that two or more molecules of GABA may be required to activate the receptors (Takeuchi & Takeuchi, 1969; Feltz, 1971; Brookes & Werman, 1979). If this is so, then EDA may have the correct amount of spacing between the aminonitrogens to fit onto two adjacent GABA-amine acceptors.

An alternative explanation for the effects of EDA and its analogues is that they are interacting with a novel, bicuculline-sensitive receptor to produce inhibition of cell firing, and this interaction requires two amine groups at a critical distance apart.

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