Br. J. Pharmac. (1983), **79**, 973–978

Structure-activity studies on the potentiation of benzodiazepine receptor binding by ethylenediamine analogues and derivatives

P.F. Morgan & T.W. Stone

Department of Physiology, St. George's Hospital Medical School, University of London, London, SW17 0RE

1 The effect of ethylenediamine analogues on *in vitro* binding of [^{3}H]-diazepam to crude cerebral cortical synaptosomal membranes in the rat was studied.

2 Ethylenediamine significantly increased [^{3}H]-diazepam binding to a maximum potentiation of 154% control ($EC_{50} = 1.8 \times 10^{-4}$ M) and was the most active compound studied in terms of both potency and the maximum potentiation observed.

3 Potentiation of [^{3}H]-diazepam binding by ethylenediamine analogues is dependent on carbon-chain length, appears to require two terminal amino groups, and is not observed in the rigid analogues studied.

4 Potentiation of [^{3}H]-diazepam binding by ethylenediamine analogues is mediated largely by a change in receptor number and not receptor affinity.

5 Results are discussed in terms of the possible nature of the ethylenediamine binding site.

Introduction

Ethylenediamine (EDA) causes a marked depression of neuronal activity when applied iontophoretically to central neurones (Anderson, Haas & Hösli, 1973; Phillis, 1977; Perkins & Stone, 1980; 1982; Perkins, Bowery, Hill & Stone, 1981) and this action can be antagonized by bicuculline (Forster, Lloyd, Morgan, Parker, Perkins & Stone, 1981; Perkins *et al.*, 1981). EDA can also release [^{3}H]-γ-aminobutyric acid (GABA) and inhibit [^{3}H]-GABA uptake in rat brain slices (Forster *et al.*, 1981; Lloyd, Perkins, Gaitonde & Stone, 1982a; Lloyd, Perkins & Stone, 1982b), depolarize the superior cervical ganglion with a pharmacological profile of action similar to GABA (Perkins *et al.* 1981) and displace [^{3}H]-GABA, [^{3}H]-muscimol, and [^{3}H]-baclofen binding to rat synaptic membrane (Bowery, Hill, Hudson, Perkins & Stone, 1982). Taken collectively the evidence strongly suggests that EDA acts upon GABA receptors (Perkins & Stone, 1982).

Structure-activity studies of the GABA receptor using GABA analogues have emphasized the importance of the carboxyl group in GABA receptor ligands (Curtis & Watkins, 1960; Kelly & Beart, 1975) and thus since EDA lacks this electronegative group it presents an interesting compound. Electrophysiological structure-activity studies of EDA analogues (Perkins & Stone, 1982) indicate that the GABA-mimetic activity of EDA analogues is rapidly lost with increasing carbon chain length (thus the rank order EDA > 1,3-diaminopropane > 1,4-diaminobutane > 1,5-diaminopentane was observed). Activity is apparently also dependent on two terminal amine groups since replacing or substituting one amino group drastically reduces activity and activity is demonstrable in rigid analogues (eg. piperazine) of EDA.

Benzodiazepines are intimately linked to GABA systems (Polc, Möhler & Haefely, 1974; Curtis, Lodge, Johnston & Brand, 1976; Geller, Taylor & Hoffer, 1978; Tallman, Paul, Skolnick & Gallagher, 1980) and GABA and some GABA-mimetics potentiate benzodiazepine binding to neuronal benzodiazepine receptors (Tallman, Thomas & Gallagher, 1978; Briley & Langer, 1978; Martin & Candy, 1978; Wastek, Speth, Reisine & Yamamura, 1978). We have recently shown that EDA can potentiate [^{3}H]-diazepam binding to crude synaptosomal membrane preparations by an apparently different mode of action from that of GABA. Thus EDA was found to potentiate [^{3}H]-diazepam binding by increasing the apparent number of binding sites whereas GABA increases benzodiazepine binding by increasing the affinity of receptors for their ligands (Morgan & Stone, 1982). Clearly either potentiation

of [^{3}H]-diazepam binding by EDA is distinct from its GABA-mimetic action *in vivo* (Forster *et al.*, 1981; Perkins *et al.*, 1981; Perkins & Stone, 1982) and *in vitro* (Forster *et al.*, 1981; Perkins *et al.*, 1981; Bowery *et al.*, 1982; Hughes, Morgan & Stone, 1982) or such differences in the type of potentiation observed must reflect different ways in which EDA and GABA bind to the GABA-receptor-ionophore complex. One possibility is that stimulation of the acceptor site for the electronegative GABA carboxyl moiety is necessary for potentiation of benzodiazepine binding by a change in affinity whereas stimulation of the acceptor site for the GABA amine moiety alone (e.g. by EDA) will lead to a potentiation of benzodiazepine binding by a change in apparent number of binding sites.

We decided to explore these possibilities by examining the structure activity relationships in the potentiation of benzodiazepine binding by EDA analogues and derivatives.

Methods

Tissue preparation

Naive male Wistar rats (200–250 g) were killed by a blow to the head and decapitated. The whole cerebral cortex was isolated, homogenized (Braun motor-driven Potter teflon-glass homogenizer, 12 strokes at 800 rev min^{-1}) in 20 volumes ice cold 0.32 M sucrose and centrifuged at 5,000 *g* for 10 min. The supernantant was further centrifuged at 25,000 g for 20 min and resuspended in 5 mM Tris citrate buffer, pH 7.6. This suspension was then centrifuged at 45,000 *g* for 20 min and resuspended in 12 volumes Tris citrate buffer twice before being frozen overnight at −20°C. After being allowed 30 min to thaw, the tissue was resuspended in 12 volumes Tris citrate buffer and centrifuged at 45,000 *g* for 20 min a further 3 times before again being frozen. Tissue left at −20°C for at least 48 h was then allowed to thaw (30 min), resuspended in 12 vols Tris citrate buffer, centrifuged at 45,000 *g* for 20 min and finally resuspended in Tris citrate buffer to form a crude synaptosomal membrane preparation.

Binding assay

The [^{3}H]-diazepam binding assays were performed by incubating [^{3}H]-diazepam (free concentration 1.5 nM except in Scatchard analysis) at room temperature (23°C) with crude synaptosomal membranes and test compounds in a 5 mM Tris citrate (pH 7.6) medium in a total assay volume of 0.6 ml and at a protein concentration of approximately 0.6 mg ml^{-1}. Test drugs were pre-incubated with the crude synaptosomal membrane preparations for 1 h before incubation (15 min) with [^{3}H]-diazepam. After 15 min incubation samples were vortex mixed and 0.3 ml samples were pipetted directly onto Whatman GF/B glassfibre filters under low vacuum. The filters were washed with 20 ml of 5 mM Tris citrate buffer at room temperature and radioactivity measured by liquid scintillation spectrophotometry (efficiency 38–44%) in Fisofluor scintillant. Aliquots of 100 μl of [^{3}H]-diazepam solution were also taken and radioactivity measured by scintillation spectrophotometry from which the free concentration of ligand was calculated. [^{3}H]-diazepam bound in the presence of 20 μM flurazepam was termed non-specific and was subtracted from total binding to obtain an estimate of specific binding. Non-specific binding thus defined represented approximately 10% of total binding.

Protein assay

Protein concentration was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as a standard.

Materials

N-methyl-[^{3}H]-diazepam (71 Ci mmol^{-1}) was purchased from Amersham International Ltd. Ethylenediamine analogues and derivatives and Tris buffer were obtained from Sigma Corp.

Results

EDA potentiated specific [^{3}H]-diazepam binding to a maximum of 154% control (Figure 1) and from linear regression analysis of a log probit plot of the data in Figure 1 the EC_{50} for EDA was estimated at 1.8×10^{-4} M. This compares to a maximum potentiation of 175% control binding and an EC_{50} of 4.6×10^{-7} M for GABA (data not shown).

Increasing the carbon chain length decreased the ability of the analogues to potentiate [^{3}H]-diazepam binding in terms of both potency and the maximum observed potentiation. Thus while all of the straight-chain analogues significantly ($P < 0.05$, Student's *t* test) potentiated [^{3}H]-diazepam binding, the rank order EDA > 1,3-diaminopropane > 1,4-diaminobutane > 1,5-diaminopentane was observed (Figure 1). It should be noted that the maximum effects of 1,4 diaminobutane and 1,5 diaminopentane have not been attained in the concentration range used, due to solubility limitations.

The analogues 2-amino-ethanol and 2,3-diaminopropionic acid were both inactive as was the substituted diamine molecule N-methyl-

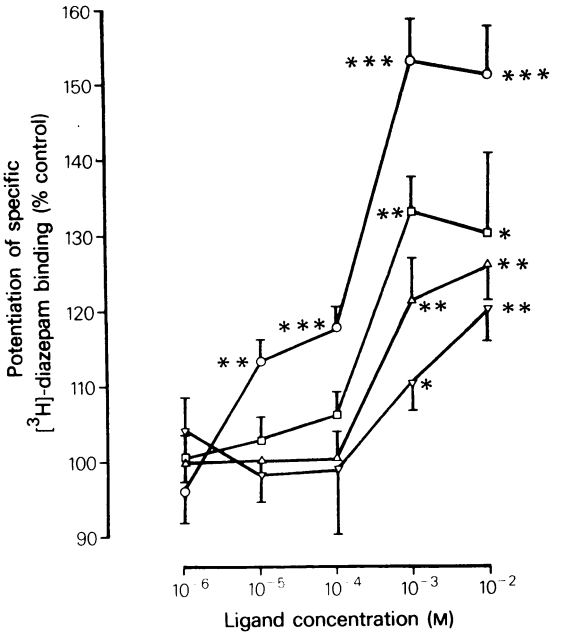

Figure 1 The effect of increasing carbon chain length on the activity of ethylenediamine (EDA) analogues in potentiating specific [^{3}H]-diazepam binding: (○) EDA; (□) diaminopropane; (△) diaminobutane; (▽) diaminopentane. Crude synaptosomal membranes were prepared and incubated as described in Methods. Values are the mean of 6 experiments performed in duplicate; vertical lines show s.e.mean. $^*P<0.05$; $^{**}P<0.01$; $^{***}P>0.005$; paired Student's t test.

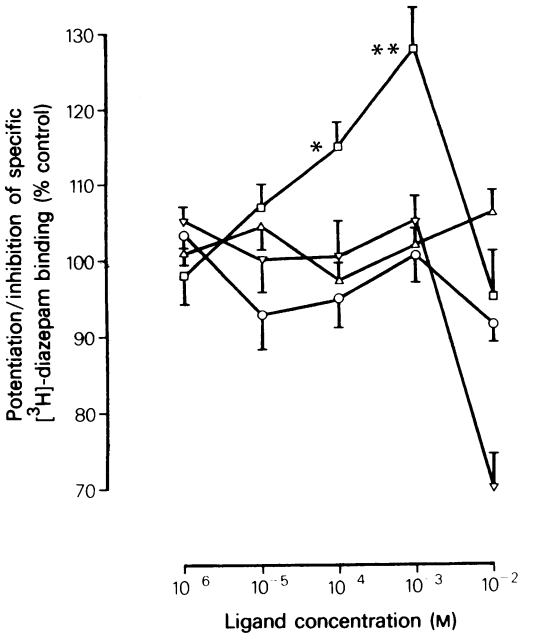

Figure 2 The effect of substituted and masked analogues of ethylenediamine on specific [^{3}H]-diazepam binding: (○) diaminoproprionic acid; (□) diethylenetriamine; (△) 2-amino ethanol; (▽) N-methyl-ethylenediamine. Crude synaptosomal membranes were prepared and incubated as described in Methods. Values are the mean of 6 experiments performed in duplicate; vertical lines show s.e.mean. $^*P<0.05$; $^{**}P<0.01$; paired Student's t test.

ethylenediamine. However, diethylenetriamine significantly ($P<0.05$, Student's t test) potentiated [^{3}H]-diazepam binding (Figure 2).

The rigid analogues of EDA, piperazine and piperidine were completely inactive as was the unsaturated, planar version of piperazine, pyrazine (data not shown).

Scatchard analysis of [^{3}H]-diazepam binding indicates that the potentiation of [^{3}H]-diazepam by ethylenediamine analogues is largely mediated by a change in receptor number (B_{max}). Thus ethylenediamine, diaminopropane, diaminobutane and diethylenetriamine all significantly ($P<0.05$) increased the estimated number of binding sites for [^{3}H]-diazepam whilst having no significant effect on [^{3}H]-diazepam binding affinity (K_d) (Table 1).

Discussion

If EDA acts directly at the GABA binding site of the GABA receptor one of the simplest assumptions about the EDA-GABA receptor interaction is that EDA binds only to the electronegative acceptor site for the GABA amino moiety. This might explain the loss of potency with increasing carbon chain length since steric hindrance with the electropositive GABA carboxyl acceptor moiety might be increased with increasing carbon chain length (see Perkins & Stone, 1982). However, the lack of activity of the analogues of EDA in which one amino group is replaced or substituted (where steric hindrance with the GABA carboxyl moiety acceptor site should be reduced) argues against this possibility. If on the other hand, GABA acts in dimers as has been suggested (Takeuchi & Takeuchi, 1969; Feltz, 1971; Brookes & Werman, 1979) then EDA may act between adjacent (paired?) GABA amino moiety acceptor sites which would explain the ineffectiveness of the analogues of EDA.

The rank order of potency of the EDA analogues is very similar to their rank order of potency in depressing cortical and pallidal neurones reported by Perkins & Stone (1982). These authors obtained the

Table 1 Effect of ethylenediamine analogues on [^{3}H]-diazepam/benzodiazepine receptor binding parameters

Compound	K_d (nM)	Bmax *(fmol mg^{-1} protein)*	r
Control	28.3 ± 3.1 (15)	836 ± 27 (15)	0.90 ± 0.03 (15)
Ethylenediamine (1 mM)	22.6 ± 3.8 (3)	1104 ± 66 (3)**	0.97 ± 0.02 (3)
Diaminopropane (1 mM)	28.8 ± 2.6 (3)	1074 ± 92 (3)**	0.96 ± 0.01 (3)
Diaminobutane (1 mM)	28.1 ± 4.5 (3)	1028 ± 71 (3)*	0.92 ± 0.05 (3)
Diaminopentane (1 mM)	28.7 ± 0.2 (3)	925 ± 67 (3)	0.88 ± 0.02 (3)
Diethylenetriamine (1 mM)	36.2 ± 3.3 (3)	1060 ± 28 (3)*	0.92 ± 0.01 (3)

Estimates of the equilibrium dissociation constant (K_d) and the maximum number of binding sites (B_{max}) were derived from Scatchard plots of [^{3}H]-diazepam binding to crude synaptic membranes. The membranes were prepared and incubated as described in Methods except that increasing concentrations of [^{3}H]-diazepam diluted 10 fold with unlabelled diazepam to give a final specific activity of 7.1 mCi mmol^{-1} (final concentrations 5–150 nM) were used. Each Scatchard curve was obtained by linear regression analysis of 8 data points performed in duplicate. Values represent the mean ± s.e.mean with number of experiments in parentheses. r = mean correlation coefficient of linear regression slopes. *$P < 0.05$; **$P < 0.005$, compared to control; unpaired Student's t test.

same rank order of potency for the diamines of varying carbon chain length and similar relative potencies for the analogues in which one amino group is replaced or substituted. However, Perkins & Stone (1982) report that electrophysiologically the rigid analogue of EDA, piperazine was almost equipotent with EDA whereas we found it to be inactive in potentiating benzodiazepine binding. In this respect it is interesting to note that piperazine may only act upon a sub-population of GABA-receptors (Constanti & Nistri, 1976; Martin, 1982) and thus GABA and EDA potentiation of benzodiazepine binding may be mediated only via the piperazine-insensitive sub-population of GABA receptors.

Modulation of benzodiazepine receptors by a change in receptor number, but not affinity, is not unique to EDA and some of its analogues. Picrotoxin, picrotoxinin and the convulsant 4-(isopropyl)-1-phospho-2,6,7-trioxabicyclo-(2, 2, 2)-octane-1-oxide (IPTBO) have also been reported to increase *in vitro* benzodiazepine binding by a change in receptor number, but not affinity (Karobath, Drexler & Supavilai, 1981). Although EDA and its analogues, picrotoxin, picrotoxinin and IPTBO all appear to be intimately related to a GABA receptor-ionophore complex (Morgan & Stone, 1982; Skolnick & Paul, 1982; Olsen, 1982) no evidence suggests that these compounds share a common mechanism of action in increasing benzodiazepine receptor number. Indeed, it is not easy to envisage models for the mechanism underlying a ligand-induced increase in B_{max} especially as, in the case of EDA at least, this is a reversible phenomenon (Morgan & Stone, 1982). Nevertheless such *in vitro* observations may be of physiological or pathological significance since very rapid but transient increases of benzodiazepine receptor number have been reported *in vivo* following electroconvulsive shock treatment (Paul & Skolnick, 1978) and cold water stress (Soubrie, Thiebot, Jobert, Montrastru, Henry & Hamon, 1980).

P.F.M. is an M.R.C. Student. We are grateful to the S K & F Foundation and London University Central Research Fund for equipment.

References

ANDERSON, E.G., HAAS, H. & HÖSLI, L. (1973). Comparison of effects of noradrenaline and histamine with cyclic AMP on brain stem neurons. *Brain Res.*, **49**, 471–475.

BOWERY, N.G., HILL, D.R., HUDSON, A.L., PERKINS, M.N. & STONE, T.W. (1982). GABA receptor binding in physiological salt solution. *Br. J. Pharmac.*, **76**, 47P.

BRILEY, M.S. & LANGER, S.Z. (1978). Influence of GABA receptor agonists and antagonists on the binding of ^{3}H-diazepam to the benzodiazepine receptor. *Eur. J. Pharmac.*, **52**, 129–132.

BROOKES, N. & WERMAN, R. (1979). The cooperativity of GABA action on the membrane of locust muscle fibres. *Molec. Pharmac.*, **9**, 571–579.

CONSTANTI, A. & NISTRI, A. (1976). A comparative study of the action of γ-aminobutyric acid and piperazine on the lobster muscle fibre and the frog spinal cord. *Br. J. Pharmac.*, **57**, 347–358.

CURTIS, D.R. & WATKINS, J.C. (1960). The excitation and depression of spinal neurons by structurally related amino acids. *J. Neurochem.*, **6**, 117–141.

CURTIS, D.R., LODGE, D., JOHNSTON, G.A.R. & BRAND, S.J. (1976). Central action of benzodiazepines. *Brain Res.*, **118,** 344–347.

FELTZ, A. (1971). Competitive interaction of β-guanidinopropionic acid and GABA on the muscle fibre of the crayfish. *J. Physiol.*, **216,** 381–401.

FORSTER, P., LLOYD, H.G.E., MORGAN, P., PARKER, M., PERKINS, M.N. & STONE, T.W. (1981). Ethylenediamine acts upon GABA receptors and uptake sites. *Br. J. Pharmac.*, **74,** 274P.

GELLER, H.M., TAYLOR, D.A. & HOFFER, B.J. (1978). Benzodiazepines and central inhibitory mechanisms. *Naunyn-Schmiedebergs Arch. Pharmac.*, **304,** 81–88.

HUGHES, P.R., MORGAN, P.F. & STONE, T.W. (1982). Inhibitory action of γ-aminobutyric acid on the excitatory but not inhibitory innervation of the rat anococcygeus muscle. *Br. J. Pharmac.*, **77,** 691–695.

KAROBATH, M., DREXLER, G. & SUPAVILAI, P. (1981). Modulation by picrotoxin and IPTBO of ^{3}H-flunitrazepam binding to the GABA/benzodiazepine receptor complex of rat cerebellum. *Life Sci.*, **28,** 307–313.

KELLY, J.S. & BEART, P.M. (1975). Amino acid receptors in C.N.S. 11. GABA in supraspinal regions. In *Handbook of Psychopharmacology*, Vol. 4. ed. Iversen, L.C., Iversen, S.D. & Snyder, S.H. pp. 129–198. New York: Plenum Press.

LLOYD, H.G.E., PERKINS, M.N., GAITONDE, M.K. & STONE, T.W. (1982a). Uptake and calcium-dependent release of Ethylenediamine (1, 2-diamino ethane) by rat brain slices. *J. Neurochem.*, **38,** 1118–1122.

LLOYD, H.G.E., PERKINS, M.N. & STONE, T.W. (1982b). Ethylenediamine as a specific releasing agent of γ-aminobutyric acid in rat striatal slices. *J. Neurochem.*, **38,** 1168–1169.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent *J. biol. Chem.*, **193,** 265–275.

MARTIN, I.L. & CANDY, J.M. (1978). Facilitation of benzodiazepine binding by sodium chloride and GABA *Neuropharmac.*, **17,** 993–998.

MARTIN, R.J. (1982). Electrophysiological effects of piperazine and diethylcarbamazine on *Ascaris suum* somatic muscle. *Br. J. Pharmac.*, **77,** 255–265.

MORGAN, P.F. & STONE, T.W. (1982). Ethylenediamine and GABA potentiation of ^{3}H-diazepam binding to benzodiazepine receptors in rat cerebral cortex. *J. Neurochem.*, **39,** 1446–1451.

OLSEN, R.W. (1982). Drug interactions at the GABA receptorionophore complex. *A Rev. Pharmac. Tox.*, **22,** 245–77.

PAUL, S. & SKOLNICK, P. (1978). Rapid changes in brain benzodiazepine receptors after experimental seizures *Science*, **202,** 892–894.

PERKINS, M.N., BOWERY, N.G., HILL, D.R. & STONE, T.W. (1981). Neuronal responses to ethylenediamine: preferential blockade by bicuculline. *Neurosci. Lett.*, **23,** 325–327.

PERKINS, M.N. & STONE, T.W. (1980). Blockade of striatal neurone responses to morphine by aminophylline: evidence for adenosine mediation of opiate action. *Br. J. Pharmac.*, **69,** 131–138.

PERKINS, M.N. & STONE, T.W. (1982). Comparison of the effects of ethylenediamine analogues and γ-aminobutyric acid on cortical and pallidal neurones. *Br. J. Pharmac.*, **75,** 93–99.

PHILLIS, J.W. (1977). The role of cyclic nucleotides in the CNS. *Canad. J. Neurolog. Sci.*, **4,** 151–195.

POLC, P., MÖHLER, H. & HAEFELY, W. (1974). The effects of diazepam on spinal cord activities: Possible sites and mechanism of action. *Naunyn-Schmiedbergs Arch. Pharmac.*, **284,** 319–327.

SKOLNICK, P. & PAUL, S.M. (1982). Benzodiazepine receptors in the central nervous system. *Int. Rev. Neurobiol.*, **23,** 103–140.

SOUBRIE, P., THIEBOT, M., JOBERT, A., MONTRASTRUC, J., HENRY, F. & HAMON, H. (1980). Decreased convulsant potency of picortoxin and pentetrazol and enhanced ^{3}H-flunitrazepam cortical binding following stressful manipulations in rats. *Brain Res.*, **189,** 505–517.

TAKEUCHI, A. & TAKEUCHI, N. (1969). A study of the action of picrotoxin on the inhibitory neuromuscular junction on the crayfish. *J. Physiol.*, **205,** 377–391.

TALLMAN, J.F., THOMAS, J.W. & GALLAGER, D.W. (1978). GABAergic modulation of benzodiazepine binding site sensitivity. *Nature*, **274,** 383–385.

TALLMAN, J.F., PAUL, S.M., SKOLNICK, P. & GALLAGER, D.W. (1980). Receptors for the age of anxiety: pharmacology of the benzodiazepines. *Science*, **207,** 274–281.

WASTKEK, G.W., SPETH, R.C., REISINE, T.D. & YAMAMURA, H.I. (1978). The effects of γ-aminobutyric acid on ^{3}H-flunitruzepam binding in the rat brain. *Eur. J. Pharmac.*, **50,** 445–447.

(Received February 24, 1983.
Revised March 29, 1983.)