ELSEVIER

European Journal of Pharmacology 282 (1995) 255–258

ejp

Short communication

S-Adenosyl-L-homocysteine and 5′-methylthioadenosine inhibit binding of [^{3}H]flunitrazepam to rat brain membranes

Vadim Tsvetnitsky [a,1], Iain C. Campbell [b], William A. Gibbons [a,*]

[a] *University-Industry Centre for Pharmaceutical Research, School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK*

[b] *Department of Biochemistry, Institute of Psychiatry, De Crespigny Park, Denmark Hill, London SE5 8AF, UK*

Received 16 June 1995; accepted 20 June 1995

Abstract

The effect of inhibitors of transmethylation reactions, *S*-adenosylhomocysteine and 5′-methylthioadenosine, on [^{3}H]flunitrazepam-specific binding to the rat brain membranes has been investigated. Both *S*-adenosylhomocysteine and 5′-methylthioadenosine are able to inhibit binding with K_i values of 7.9 μM and 15.8 μM respectively. These compounds therefore may be candidate endogenous benzodiazepine-receptor ligands. In light of these and other data possible correlations between phospholipid methylation and γ-aminobutyric acid (GABA) receptor function are discussed.

Keywords: Methylation inhibitor; Benzodiazepine binding; *S*-Adenosylhomocysteine; 5′-Methylthioadenosine; (Rat)

1. Introduction

The γ-aminobutyric acid ($GABA_A$)-benzodiazepine receptor complex is embedded in the plasma membrane lipid bilayer and it has been shown that the treatment of membranes with phospholipases A_2 and C affects the relationship between the benzodiazepine and the GABA binding sites (Havoundjian et al., 1986; Ueno and Kuriyama, 1981). The three-step methylation of phosphatidylethanolamine to phosphatidylcholine has been implicated in a number of receptor-mediated events (Hirata and Axelrod, 1980) and in the process of phospholipid methylation *S*-adenosylmethionine is converted to *S*-adenosylhomocysteine, a competitive inhibitor for all methylation reactions for which *S*-adenosylmethionine is the methyl donor. 5′-Methylthioadenosine, which is metabolic derivative of decarboxylated *S*-adenosylmethionine, can also inhibit methylation.

A number of reports have suggested a close relationship between phospholipid methylation and [^{3}H]GABA and [^{3}H]diazepam binding in rat brain membranes. Inhibition of phospholipid methylation by injection of L-homocysteine (an *S*-adenosylhomocysteine precursor) into rats induced an increase in specific [^{3}H]muscimol (GABA receptor agonist) binding to rat brain without affecting [^{3}H]diazepam binding (Benistant et al., 1987). Phospholipid methylation in synaptic membranes increased the specific binding of GABA (Vartanyan and Aprikyan, 1991) and decreased its reuptake. The finding that incubation of brain membranes with *S*-adenosylmethionine increased both benzodiazepine and GABA binding (Di Perri et al., 1983) was recently studied in more details by Benistant et al. (1990). They claimed that the increase in basal benzodiazepine binding after *S*-adenosylmethionine treatment was, in fact, induced by the low residual levels of endogenous GABA. Nevertheless these different studies appear to agree on the close relationship between *S*-adenosylmethionine and its metabolites and GABA-benzodiazepine receptor binding. On the basis of these various studies, we have investigated the effect of *S*-adenosylmethionine, *S*-adenosylhomocysteine and 5′-methylthioadenosine on [^{3}H]flunitrazepam binding to

* Corresponding author. Tel. +44(0)171-753-5884, fax +44(0)171-278-1939.

[1] Current address: Institute of Biochemistry, Odense University, 55 Campusvej, DK-5230 Odense M, Denmark.

Elsevier Science B.V.
SSDI 0014-2999(95)00379-7

the benzodiazepine-recognition site of the $GABA_A$ receptor.

2. Materials and methods

5'-Methylthioadenosine, *S*-adenosylhomocysteine, *S*-adenosylmethionine, diazepam and GABA were purchased from Sigma and [*N*-methyl-^{3}H]flunitrazepam (85 Ci/mmol) was obtained from Amersham. All other chemicals were of analytical grade or better. Water was of MilliQ quality.

Wistar rats (180–220 g) were killed by CO_2 intoxication and the brain was removed. Two rats were used for each membrane preparation. Crude synaptic membranes were prepared according to the method described elsewhere (Enna and Snyder, 1976). The prepared membranes were washed 3 times with 50 mM Tris-HCl buffer (pH 7.4) and stored at −20°C for at least 12 h. After being thawed, membranes were resuspended in 50 mM Tris-HCl buffer (pH 7.4) and centrifuged at 48 000 × *g* for 20 min. The resultant pellet was resuspended in 40 ml of 50 mM Tris-HCl buffer (pH 7.4) to about 10–12 mg protein/ml and used in the binding assays. *S*-Adenosylmethionine, *S*-adenosylhomocysteine and 5'-methylthioadenosine stock solutions (10 mM) were made in 0.1 mM HCl to aid solubilisation.

Membrane preparations (400 μl) were incubated with 8 nM [^{3}H]flunitrazepam (which is about 4 times the K_d value for flunitrazepam binding (Squires and Braestrup, 1977)) together with varying concentrations of *S*-adenosylmethionine, *S*-adenosylhomocysteine or 5'-methylthioadenosine (or an equal volume of buffer for control) in a final volume of 1 ml for 1 h at 4°C. Incubations were terminated by rapid filtration under vacuum through Whatmann GF/B filter and the filters were washed with 3 × 2 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4). Radioactivity retained on the filters was measured by liquid scintillation counting in a Beckman LS 5000 CE spectrophotometer using OptiPhase scintillation cocktail (LKB). Specific binding was calculated by subtracting the amount of non-specific binding, determined by carrying out parallel incubations in the presence of 10^{-5} M diazepam, from total binding (Ueno and Kuriyama, 1981). Protein content was measured by the method of Bradford (1976) using BSA as a standard.

3. Results

Incubation of cerebral synaptic membranes with [^{3}H]flunitrazepam resulted in a high level of specific binding of the radiolabelled ligand. Under our experimental conditions the specific binding was about 6000 dpm (3170 fmol/g wet tissue) while non-specific values were around 120 dpm (63.4 fmol/g wet tissue). In addition, GABA at 10^{-5} M significantly enhanced [^{3}H]flunitrazepam binding (by over 30%, data not shown).

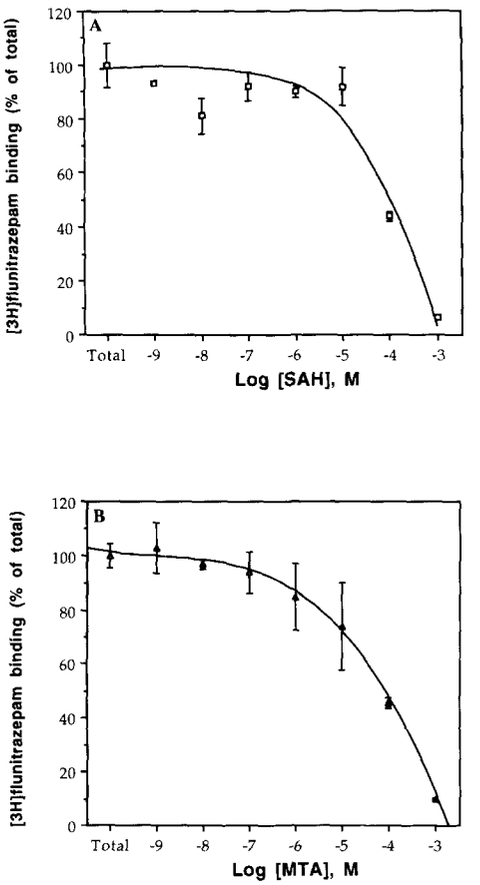

Fig. 1. Inhibition of [^{3}H]flunitrazepam binding to rat brain membranes by *S*-adenosylhomocysteine and 5'-methylthioadenosine. Membranes were incubated with different amount of *S*-adenosylhomocysteine (panel A) or 5'-methylthioadenosine (panel B) and 8 nM [^{3}H]flunitrazepam for 1 h at 4°C and the amount of radioactivity bound to membrane was determined as in text. Total and non-specific [^{3}H]flunitrazepam binding was 6000 ± 480 dpm (3170 fmol/g wet tissue) and 120 ± 20 dpm (63.4 fmol/g wet tissue), respectively. The results are the mean ± S.D. of three individual experiments performed in triplicate.

To examine the effect of *S*-adenosylmethionine and its metabolites on [^{3}H]flunitrazepam binding, increasing concentrations of either *S*-adenosylmethionine, *S*-adenosylhomocysteine or 5'-methylthioadenosine (from 10^{-9} to 10^{-3} M) were added to the membranes incubated with the radiolabelled ligand. Both 5'-methylthioadenosine and *S*-adenosylhomocysteine decreased [^{3}H]flunitrazepam binding with K_i values of 15.8 μM and 7.9 μM respectively, and virtually abolished it at 1 mM (Fig. 1). *S*-Adenosylmethionine in the same concentration range had no effect on specific binding (not shown).

4. Discussion

As mentioned in the Introduction, *S*-adenosylmethionine was reported (Di Perri et al., 1983) to increase the benzodiazepine binding. This effect was shown later to be mediated by low levels of residual GABA released from the membranes upon thawing (Benistant et al., 1990). A step in membrane preparation involving freeze-thawing and providing an extra wash was therefore employed in this study. Subsequent lack of an effect of *S*-adenosylmethionine on [^{3}H]flunitrazepam binding can be taken to support the Benistant et al. (1990) conclusions.

Several compounds have been proposed to function as endogenous benzodiazepines (Stephenson, 1989). The purines inosine and hypoxanthine inhibit diazepam binding with IC_{50} values 400–1300 μM and 700–3700 μM, respectively (Asano and Spector, 1979). *S*-Adenosylhomocysteine is an established precursor of and can be rapidly metabolised to adenosine and then to inosine and hypoxanthine. Adenosine can also inhibit benzodiazepine binding and the existence of a specific interaction between benzodiazepines and adenosine and its derivatives has been proposed (Phillis and Wu, 1980). However, a role for these compounds cannot be fully accepted or rationalised when the potency for the inhibition of binding is compared with the known brain concentrations (Braestrup and Nielsen, 1983).

Our data show that *S*-adenosylhomocysteine and 5′-methylthioadenosine have the capacity to inhibit [^{3}H]flunitrazepam binding more specifically than the other reported adenosine derivatives. Both *S*-adenosylhomocysteine and 5′-methylthioadenosine are present in the brain and their synthetic and degradation pathways have been also shown to occur in brain tissue. On the basis of these and the other observations (Fonteh et al., 1989), 5′-methylthioadenosine and *S*-adenosylhomocysteine can be proposed as candidate endogenous benzodiazepine-receptor ligands.

There is an apparent lack of correlation between our results and those of Benistant et al. (1987) who showed that injection of L-homocysteine had no effect on benzodiazepine binding. This, however, can be explained, at least in part, by the fact that although L-homocysteine is a non-direct precursor of *S*-adenosylhomocysteine, it can also be converted to taurine and cysteine amino acids (the route being influenced by *S*-adenosylmethionine content of the tissue). It is therefore possible that under some circumstances the resulting increase in *S*-adenosylhomocysteine concentration could be small enough so that it does not affect benzodiazepine binding. The concentration of *S*-adenosylhomocysteine in whole rat brain is reported to be 1 nmol/g wet tissue (Gharib et al., 1982) and since it is difficult to assess the local concentration of *S*-adenosylhomocysteine and 5′-methylthioadenosine achievable in certain circumstances (such as increased *S*-adenosylmethionine metabolism), the biological significance of our findings must await further research. The physiological roles of *S*-adenosylhomocysteine and 5′-methylthioadenosine in cells are not fully elucidated although both compounds have been shown to inhibit methylation (Cantoni, 1975; Kido et al., 1991) including phospholipid methylation. Interestingly, *S*-adenosylhomocysteine is a sleep-inducing agent and anticonvulsant (Gharib et al., 1982). On the other hand, *S*-adenosylmethionine has antidepressant activity (mechanism unknown) and potentiates barbiturate-induced sleep (Baldessarini, 1987). But since *S*-adenosylhomocysteine is a product of phospholipid methylation and may bind competitively to the benzodiazepine-recognition site, the possibility exists that other correlations between phospholipid methylation and GABA receptor exist. Phosphatidylethanolamine and phosphatidylcholine are the substrate and product of phospholipid *N*-methyltransferase, and phosphatidylethanolamine, but not phosphatidylcholine, has been shown to compete with the GABA for the GABA site (Johnston and Kennedy, 1978). These workers also remarked on the isosteric relationship between the phosphoethanolamine head group of phosphatidylethanolamine and GABA. So one may speculate that the locally increased rate of phospholipid methylation can convert phosphatidylethanolamine into phosphatidylcholine thereby increasing GABA binding. Concomitantly, *S*-adenosylhomocysteine concentration may achieve the level where it can bind to the benzodiazepine-recognition site and uncover high-affinity $GABA_A$ receptor (in the manner that benzodiazepines are proposed to act). In this way, the opening of the Cl^- ionophore becomes a more efficient process than in the presence of GABA alone (Skolnick and Paul, 1981). Iontophoretic application of *S*-adenosylhomocysteine not only potentiated the effect of GABA on chloride fluxes but also the homologous glycine receptor currents. In the same experiment it had no effect on acetylcholine receptor currents (Davis and Gibbons, unpublished data). It seems reasonable therefore that phospholipid methylation can be coupled to GABA receptor function by methylating phosphatidylethanolamine associated with the channel and via *S*-adenosylhomocysteine being an endogenous ligand. Besides, since benzodiazepines were reported to stimulate phospholipid methylation (Strittmatter et al., 1979), it is possible that *S*-adenosylhomocysteine can provide a feed-back mechanism.

Acknowledgements

V.T. was supported by the School of Pharmacy post-graduate studentship. We would like to thank N.

Bowery and M. Simmonds for critical reading of the manuscript.

References

Asano, T. and S. Spector, 1979, Identification of inosine and hypoxanthine as endogenous ligands for the brain benzodiazepine-binding sites, Proc. Natl. Acad. Sci. USA 76, 977.

Baldessarini, R.J., 1987, Neuropharmacology of *S*-adenosyl-L-methionine, Am. J. Med. 83 (Suppl. 5A), 95.

Benistant, C., C. Rey, P. Fonlupt and H. Pacheco, 1987, Increase of in vitro brain [^{3}H]muscimol binding after L-homocysteine administration to rat, Neurosci. Lett. 77, 237.

Benistant, C., C. Rey and P. Fonlupt, 1990, Increase of GABA-stimulated diazepam binding after lipid methylation in membrane preparations from rat brain, Neurosci. Lett. 110, 137.

Bradford, M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72, 248.

Braestrup, C. and M. Nielsen, 1983, Benzodiazepine receptors, in: Handbook of Psychopharmacology, Vol. 17, eds. I. Iversen, G. Iversen and F. Snyder (Plenum Press, New York) p. 285.

Cantoni, G.L., 1975, Biological methylation: selected topics, Annu. Rev. Biochem. 44, 435.

Di Perri, B., G. Calderini, A. Battistella, R. Raciti and G. Toffano, 1983, Phospholipid methylation increases [^{3}H]diazepam and [^{3}H]GABA binding in membrane preparations of rat cerebellum, J. Neurochem. 41, 302.

Enna, S.J. and S.H. Snyder, 1976, Influences of ions, enzymes and detergents on gamma-aminobutyric acid receptor binding in synaptic membranes of rat brain, Mol. Pharmacol. 13, 442.

Fonteh, A.N., K. McBride and W.A. Gibbons, 1989, Fundamental roles for ethanolamine and choline lipids in cell excitation and transmembrane signalling and anaesthesia, in: Proceedings of the 20th European Peptide Symposium, eds. G. Jung and E. Bayer (Walter de Gruyter, Berlin) p. 670.

Gharib, A., N. Sarda, B. Chabannes, L. Cronenberger and H. Pacheco, 1982, The regional concentration of *S*-adenosyl-L-methionine, *S*-adenosyl-L-homocysteine and adenosine in rat brain, J. Neurochem. 38, 810.

Havoundjian, H., R.M. Cohen, S.M. Paul and P. Skolnick, 1986, Differential sensitivity of central and peripheral type benzodiazepine receptors to phospholipase A_2, J. Neurochem. 46, 805.

Hirata, F. and J. Axelrod, 1980, Phospholipid methylation and biological signal transmission, Science 209, 1082.

Johnston, G.A.R. and S.M.E. Kennedy, 1978, GABA receptors and phospholipids, in: NATO Adv. Study Inst., Series A, Life Sci., Vol. 16, ed. F. Fonnum (Plenum Press, New York) p. 507.

Kido, J., Y. Ashida, K. Shinkai, H. Akedo, A. Isoai, H. Kumagai and H. Inoue, 1991, Effects of methylthiodeoxyadenosine and its analogs on in vitro invasion of rat ascites hepatoma cells and methylation of their phospholipids, Jpn. J. Canc. Res. 82, 1104.

Phillis, J.W. and P.H. Wu, 1980, Interaction between the benzodiazepines, methylxanthines, and adenosine, Can. J. Neurol. Sci. 7, 247.

Skolnick, P. and S.M. Paul, Benzodiazepine receptors, 1981, Annu. Rep. Med. Chem. 16, 21.

Squires, R.F. and C. Braestrup, 1977, Benzodiazepine receptors in rat brain, Nature 266, 732.

Stephenson, F.A., 1989, Endogenous ligands for modulatory sites, in: GABA: Basic Research and Clinical Applications, eds. N.G. Bowery and G. Nistico (Pythagora Press, Rome) p. 171.

Strittmatter, W.J., F. Hirata, J. Axelrod, P. Mallorga, J.F. Tallman and R.C. Henneberry, 1979, Benzodiazepine and β-adrenergic receptor ligands independently stimulate phospholipid methylation, Nature 282, 857.

Ueno, E. and K. Kuriyama, 1981, Phospholipids and benzodiazepine recognition sites of brain synaptic membranes, Neuropharmacology 20, 1169.

Vartanyan, A.A. and G.V. Aprikyan, 1991, Methylation of phospholipids in brain synaptosomes and GABA reception, Dokl. Akad. Nauk SSSR. 319, 745.