Endogenous inhibitors of 4′-[^{3}H]chlorodiazepam (Ro 5-4864) binding to 'peripheral' sites for benzodiazepines

Charles R. Mantione, Ben Avi Weissman, Mark E. Goldman*, Steven M. Paul+ and Phil Skolnick†

*Laboratory of Bioorganic Chemistry, NIADDK, *Laboratory of Chemistry, NHLBI,* and *+Clinical Neuroscience Branch, NIMH, National Institutes of Health, Bethesda, MD 20205, USA*

Received 14 August 1984

'Peripheral' binding sites for benzodiazepines are under neural or homonal control in the pineal gland, olfactory bulb, and kidney. These observations prompted a search for an endogenous substance which could modulate these sites under physiological conditions. Acidified methanol extracts from several tissues (e.g. stomach, kidney, lung) were found to inhibit the binding of [^{3}H]Ro 5-4864 to 'peripheral' binding sites, but did not significantly affect the binding of [^{3}H]diazepam to 'brain' benzodiazepine receptors. Fractionation of a crude extract prepared from antral stomach by either ultrafiltration or gel filtration chromatography yielded high ($M_r > 10000$) and low ($M_r < 1000$) M_r fractions which competitively inhibited [^{3}H]Ro 5-4864 binding to 'peripheral' sites. These observations suggest the presence of endogenous substances in several rat tissues which may represent physiologically important ligands for 'peripheral' binding sites for benzodiazepines.

Benzodiazepine receptor *5-4864* *Antral stomach* *Kidney* *Diazepam* *4′-Chlorodiazepam (Ro 5-4864)*

1. INTRODUCTION

The demonstration that psychopharmacologically active benzodiazepines bind to unique recognition sites (receptors) in the mammalian central nervous system [1,2] prompted a search for endogenous ligand(s) which could physiologically subserve these sites. Proteins, peptides, purine, indole derivatives and lipids which inhibit [^{3}H]diazepam or [^{3}H]flunitrazepam binding to brain membranes have been identified [3–5]. Such substances appear to interact with 'brain' benzodiazepine receptors [6,7], but not with binding sites for benzodiazepines which were first described in peripheral tissues [8,9], transformed cells of neural origin [10] and more recently in the central nervous system [11].

While the physiological and pharmacological importance of 'brain' benzodiazepine receptors is generally acknowledged [12], the biological relevance of 'peripheral' binding sites for benzodiazepines (PBS) is unknown. Recently, we have found that lateral olfactory tract lesions decrease the density of PBS in olfactory bulb, and that PBS in pineal gland are under neural control [13,14]. Others have shown that destruction of the olfactory nerve with $ZnSO_4$ decreases the density of PBS binding in the olfactory bulb [15]. Moreover, PBS in kidney have also been shown to be altered in several animal models of hypertension [16–18], further supporting the presence of a neural and hormonal regulation of these sites. These observations stimulated a search for an endogenous substance which could physiologically regulate PBS.

† To whom correspondence should be addressed

Portions of this work were presented at the first International Symposium on Endocoids, Fort Worth, TX (May, 1984)

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00 © 1984 Federation of European Biochemical Societies

We now report the presence of high (M_r> 10 000) and low (M_r< 1000) m_r substances isolated from rat tissues which selectively inhibit the binding of [^{3}H]Ro 5-4864 to PBS in a competitive fashion. There is more than a 50-fold variation in the concentration of these substances in the tissues examined. These results suggest that endogenous substances present in the rat may physiologically regulate PBS.

2. MATERIALS AND METHODS

2.1. *Extraction and isolation procedures*

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) (200–225 g) were killed by decapitation and the tissues removed and quickly frozen on solid CO_2. When appropriate, fat and connective tissue were removed prior to freezing. The tissue was weighed and allowed to thaw in 3 vol. of cold acidified methanol (20 μl glacial acetic acid/ 100 ml methanol). Tissues were minced and homogenized for 30 s with a Polytron (setting 7–8) (Brinkmann Instruments, Westbury, NY). The resulting homogenates were centrifuged at 20 000 × g for 20 min (4°C). The supernatants were transferred to 50 ml vacuum flasks, and the sample volume reduced under vacuum at room temperature until the volume was reduced to the approximate original tissue weight. Samples were then heated for 15 min (55°C) and centrifuged at 40 000 × g for 30 min (4°C). The resulting supernatant was transferred to test tubes, frozen on solid CO_2 and lyophilized. The lyophilized residue was then reconstituted in 10% of the original volume with cold distilled water (this material is referred to as the 'crude extract'). The crude extract was filtered through an Amicon PM-10 filter (Amicon Corp., Danvers, MA), and the resulting filtrate passed through an Amicon UM-05 filter. Alternatively, the crude extract was applied to a Biogel P2 column, and eluted with sodium phosphate buffer (20 mM, pH 7.4) (fig.1).

The amount of inhibitor present in the various tissue preparations was expressed as 'units' of inhibitory activity. One 'inhibitory unit' was equal to that concentration of Ro 5-4864 which inhibited the binding of [^{3}H]Ro 5-4864 (1 nM) to rat cerebral cortical membranes by 50% (IC_{50}). Under standard incubation conditions, 0.25 nM (0.25 pmol/ assay) of Ro 5-4864 usually produced a 50% inhibition. Inhibition of [^{3}H]Ro 5-4864 due to the presence of endogenous substances was transformed into the appropriate concentration of Ro 5-4864 from the standard curve, and divided by the IC_{50} concentration to yield units of activity.

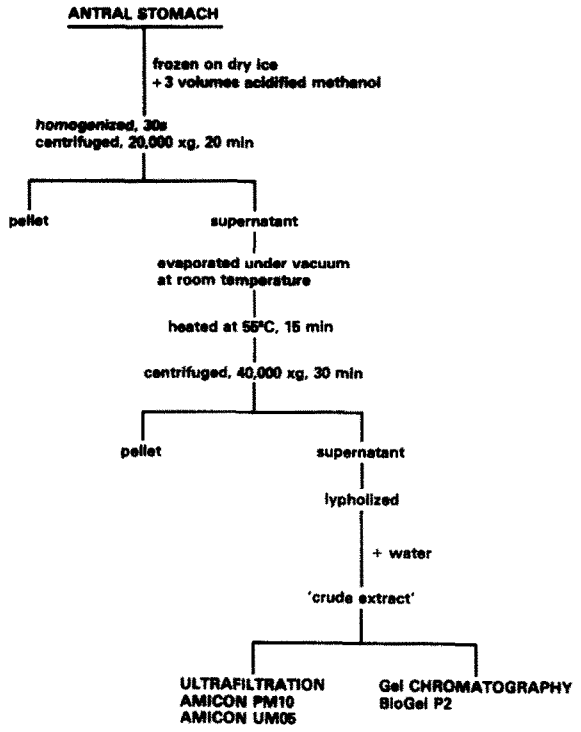

Fig. 1. Flow scheme for partial purification of endogenous substances inhibiting [^{3}H]Ro 5-4864 binding to PBS.

2.2. *Assay of [^{3}H]benzodiazepine binding*

The binding of [^{3}H]diazepam to 'brain' benzodiazepine receptors and [^{3}H]Ro 5-4864 binding to PBS was determined as in [19]. In brief, rat cerebral cortex was homogenized in 100 vol. of 50 mM Tris–HCl buffer (pH 7.4) and centrifuged at 20 000 × g for 20 min (4°C). Tissue was resuspended in 100 vol. of the same buffer. Replicate tubes contained 0.6 ml of this tissue suspension, 0.1 ml radioligand, and 0.1–0.3 ml of drug, buffer, or column fraction. The reaction was initiated by addition of tissue and terminated after 60 min (0–4°C) by rapid filtration through Whatman GF/B filter strips using a Brandel M-24R Cell Harvester (Brandel Instruments, Gaithersburg, MD). Filters were washed twice with 5 ml of ice

cold buffer. The filters were suspended in 8 ml Ready-Solv MP (Beckman Instrument Co., Fullerton, CA), and the radioactivity measured in a Packard B-2450 liquid scintillation spectrometer. PK 11195 and Ro 15-1788 were used to determine the nonspecific binding of [^{3}H]Ro 5-4864 and [^{3}H]diazepam, respectively.

2.3. *Materials*

Radioactive ligands were purchased from New England Nuclear, Boston, MA. Benzodiazepines were donated by Dr Peter Sorter, Hoffmann-La Roche, Nutley, NJ. PK 11195 was the gift of Dr Gerard Le Fur, Pharmuka Co., Gennevilliers, France. Other chemicals were obtained from standard commercial sources.

3. RESULTS AND DISCUSSION

Acidified methanol extracts of several tissues were far more potent inhibitors of [^{3}H]Ro 5-4864 (4′-chlorodiazepam) binding than [^{3}H]diazepam binding to rat cortical membranes (fig.2). The specificity of the tissue extracts for these two closely related radioligands strongly suggests a selectivity for PBS. These tissue extracts also inhibited [^{3}H]Ro 5-4864 binding to kidney membranes (not shown), which suggests that the inhibitory action of these extracts is independent of the source of PBS.

If endogenous inhibitors of [^{3}H]Ro 5-4864 binding are physiologically important, then an uneven distribution of these substances in various tissues might be predicted. We examined the tissue distribution of this factor(s) by measuring the amount of inhibitor present in crude extracts from several tissues (table 1). These experiments revealed more than a 50-fold difference in the concentration of inhibitor present. Further, there appears to be an inverse relationship between the concentration of inhibitor present and the density of PBS in each tissue (table 1), suggesting that the density of PBS may be regulated by the amount of endogenous inhibitor present in the tissue. Alternatively, endogenous inhibitors of PBS may be produced in one or more tissues and reach other organs via the circulation. The presence of inhibitory activity in serum would seem to support the latter hypothesis, although both possibilities are currently under investigation.

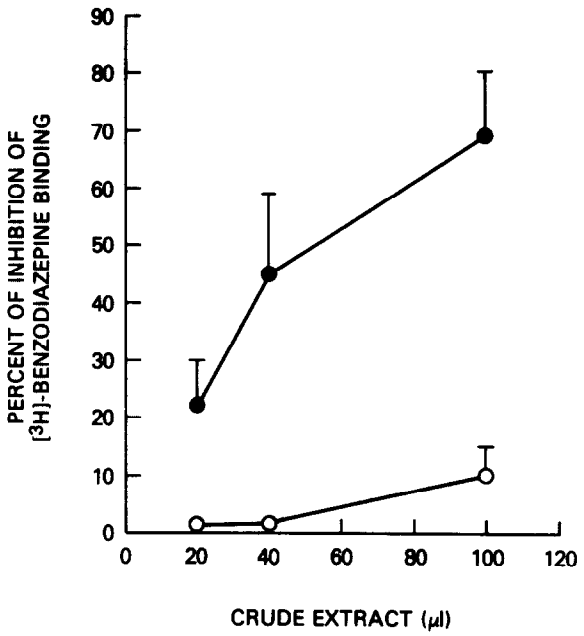

Fig. 2. Inhibition of [^{3}H]benzodiazepine binding by acidified methanol extracts from antral stomach. Extracts were prepared as section 2 (see fig. 1). Aliquots of the nonlyophilized extract were incubated for 60 min (4°C) with either 1 nM [^{3}H]Ro 5-4864 (●) or 5 nM [^{3}H]diazepam (○) using rat cortical membranes as in section 2. Values are expressed as percent of specific binding relative to control incubations and represent the $\bar{x} \pm$ SE for 3–6 independent experiments.

When the crude extract (fig.1) was filtered through an Amicon PM-10 membrane (nominal molecular mass exclusion limit of 10 000 Da) about 70% of the original activity was retained by the filter (table 2). The remaining activity was not retained by a UM-05 membrane (table 2) which has a nominal exclusion limit of 500 Da. The loss in activity following PM-10 filtration supports the notion that both high and low molecular mass inhibitors are present in the crude extract. The filtration membranes used in these experiments have been reported to adsorb both hydrophobic macromolecules and steroids [20]. Since tissue extraction in methanol would favor the isolation of hydrophobic substances, it is possible that a large quantity of a low molecular mass species could have been bound to the PM-10 filter. However, a crude extract prepared from rat antral stomach and chromatigraphically separated on a Biogel P2 column

Table 1

Tissue distribution of endogenous inhibitors of PBS

Tissue	Inhibitor (units/g tissue)	[^{3}H]Ro 5-4864 binding[a] (fmol/mg protein)
Brain	31.0	86
Stomach	18.0	
Liver	17.4	186
Serum	15.3[b]	
Lung	13.5	379
Pancreas	8.9	
Heart	6.9	169
Kidney	2.9	552
Olfactory bulb	0.5	725

[a] Comparative binding data were taken from [7] and [13] using [^{3}H]Ro 5-4864 (1 nM) as radioligand

[b] Serum activity is expressed as units/ml

Tissues were homogenized in acidified methanol, centrifuged, and the supernatants concentrated under vacuum. Extracts were heated (55°C), recentrifuged, and the second supernatant lyophilized as in section 2. Residues were reconstituted in 3 vol. of distilled water, and aliquots analyzed for inhibition of [^{3}H]Ro 5-4864 binding using rat cortical membranes as a source of PBS

Table 2

Effect of ultrafiltration of acidified methanol extracts on inhibitory activity of a crude extract

Preparation	Activity (units/ml)	Crude extract (%)
Crude extract	74 ± 3.4	100
PM-10 (M_r cut-off 10^4) Filtrate	16 ± 1.2	22
UM-05 (M_r cut-off 500) Filtrate	24 ± 3.0	32

Frozen antral stomach tissue was homogenized in acidified methanol, centrifuged, and the supernatant volume reduced under vacuum as described in section 2. The extract was heated (55°C), recentrifuged, and the second supernatant lyophilized. The residue was reconstituted in 3 volumes of water and sequentially passed through Amicon membrane filters. Aliquots were measured for inhibition of [^{3}H]Ro 5-4864 binding at each stage. Values are the mean ± SE of two preparations

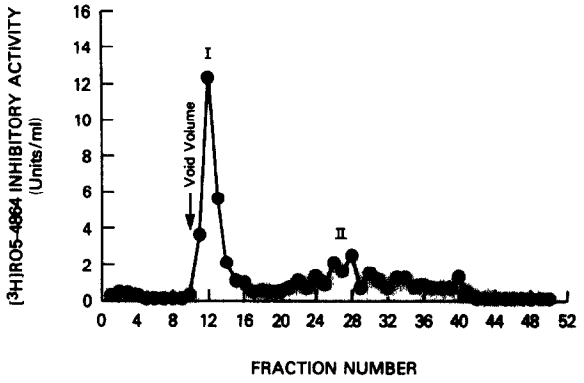

Fig. 3. BioGel P2 column chromatography of rat antral stomach extracts. One milliliter of crude extract was applied to a 2.5 × 50 cm Biogel P2 column which was eluted at a flow rate of 20 ml/h in 20 mM sodium phosphate buffer, pH 7.4. Five ml fractions were collected and 300 μl aliquots assayed for inhibition of [^{3}H]Ro 5-4864 binding as in section 2.

resulted in two discrete peaks of inhibitory activity (fig.3). Peak I eluted essentially with the void volume of this column, while a second fraction (Peak II) eluted with a retention time slightly greater than that of cyanocobalamin (M_r 1350). Thus, the results obtained by gel filtration are in agreement with data obtained using ultrafiltration, and suggest the presence of at least two factors which inhibit the binding of [^{3}H]Ro 5-4864 to PBS.

The sensitivity of these endogenous substances to proteolytic enzymes was then examined. The inhibitory activity of a crude extract prepared from antral stomach (which contains both high and low molecular mass factors) was unaffected by extended incubation with pronase, a relatively nonselective proteolytic enzyme (results not shown). The inability of pronase to affect the inhibitory activity present in the crude extract is striking, since that preparation contains the majority of inhibitory activity as a relatively high molecular mass substance as measured by both ultrafiltration and chromatographic techniques (table 2). If the inhibitory material is a peptide, the inability of pronase to alter the activity of the preparation suggests that either the cleaved fragments retain activity or that the amino acids are highly protected by other groups. It is presently not known if the high molecular mass inhibitor(s) is related to the lower molecular mass species. However, preliminary

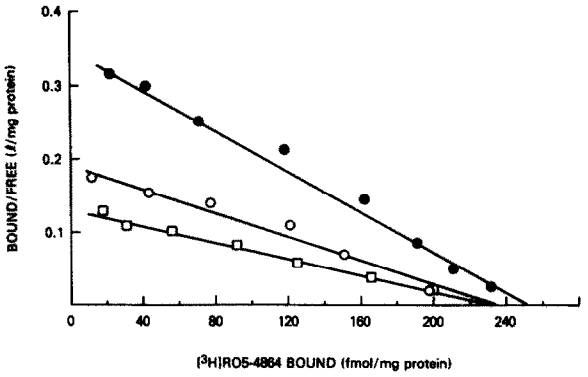

Fig. 4. Inhibition of [^{3}H]Ro 5-4864 binding to PBS by BioGel P2 Peak II. Chromatographic fractions corresponding to Peak II (see fig. 3) were lyophilized and reconstituted in 10% of their original volume with distilled water. The apparent affinity of [^{3}H]Ro 5-4864 (K_D = 0.73 nM, B_{max} = 254 fmol/mg protein, ●—●) was reduced in the presence of 50 μl (K_D = 1.22 nM, B_{max} = 238 fmol/mg protein, ○—○) and 100 μl (K_D = 1.74 nM, B_{max} = 234 fmol/mg protein □—□) of inhibitor. Peak I was also found to competitively inhibit [^{3}H]Ro 5-4864 binding (not shown). Rat cerebral cortex was used to assay PBS.

evidence with a Sephadex G-50 column suggests the molecular mass of the 'high' molecular mass species to be between 10000–15000 Da (unpublished).

Several lines of research also support the notion that an endogenous ligand(s) for PBS may be present in mammalian tissue. For example, [^{3}H]Ro 5-4864 binding in kidney membranes was noted to increase if the kidneys were perfused with saline prior to assay [11] which could remove or dilute an endogenous inhibitor. In other studies on the autoradiographic localization of [^{3}H]PK III95 binding to adrenal glands, kinetic analysis demonstrated that the affinity of this ligand for PBS was approximately 10-fold lower in adrenal slices than in homogenates [21]. Homogenization of this tissue may have also diluted an endogenous inhibitor retained by slices [21]. Certainly, the competitive inhibition of [^{3}H]Ro 5-4864 binding to PBS by both Peaks I and II (fig.4) is consistent with a physiologically relevant substance(s).

The present studies have provided evidence that several tissues from the rat contain endogenous inhibitors of PBS. Whether the substances characterized here are identical with those reported to be present in human urine and plasma [22] is currently under investigation. Further purification and characterization of endogenous inhibitors of PBS should aid in clarifying the physiological role of these sites.

REFERENCES

[1] Moehler, H. and Okada, T. (1977) Science 198, 849–851.
[2] Squires, R.F. and Braestrup, C. (1977) Nature 266, 732–734.
[3] Skolnick, P., Marangos, P.J. and Paul, S.M. (1983) in: Anxiolytics: Neurochemical, Behavioral and Clinical Perspectives (Malick, J.B., Enna, S.J., and Yamamura, H.I., eds) pp. 41–53, Raven Press, New York.
[4] Guidotti, A., Corda, M.G. and Costa, E. (1983) in: Benzodiazepine Recognition Site Ligands: Biochemistry and Pharmacology (Biggio, G. and Costa, E., eds) Adv. Biochem. Psychopharmacol., vol. 38, pp. 95–103, Raven Press, New York.
[5] Hamon, M. and Soubrie, P. (1983) Neurochem. Internat. 5, 663–672.
[6] Asano, T. and Spector, S. (1979) Proc. Natl. Acad. Sci. USA 76, 977–981.
[7] Marangos, P.J., Patel, J., Boulenger, J.-P. and Clark-Rosenberg, R. (1982) Mol. Pharmacol. 22, 26–32.
[8] Braestrup, C. and Squires, R.F. (1977) Proc. Natl. Acad. Sci. USA 74, 3805–3809.
[9] Williamson, M., Paul, S. and Skolnick, P. (1978) Life Sci. 23, 1935–1940.
[10] Syapin, P.J. and Skolnick, P. (1979) J. Neurochem. 32, 1047–1051.
[11] Schoemaker, H., Boles, R.G., Horst, D. and Yamamura, H.I. (1983) J. Pharmacol. Exp. Ther. 225, 61–69.
[12] Usdin, E., Skolnick, P., Tallman, J.F., Greenblatt, D. and Paul, S.M. (1982) in: Pharmacology of Benzodiazepines, Macmillan Press Ltd., London, England.
[13] Bolger, G., Mezey, E., Cott, J., Weissman, B.A., Paul, S. and Skolnick, P. (1984) Eur. J. Pharmacol., in press.
[14] Weissman, B.A., Skolnick, P. and Klein, D. (1984) Pharmacol. Biochem. Behav., submitted.
[15] Anholt, R.R.H., Murphy, M.M., Mack, G. and Snyder, S.H. (1984) J. Neurosci. 4, 593–603.
[16] Regan, J.W., Yamamura, H.O., Yamada, S. and Roeske, W.R. (1981) Life Sci. 28, 991–998.
[17] Taniguchi, T., Wang, J.K.T. and Spector, S. (1981) Eur. J. Pharmacol. 70, 587–590.

[18] Del Zompo, M., Bocchetta, A., Corsini, G.U., Tallman, J.F. and Gessa, G.L. (1983) in: Benzodiazepine Recognition Site Ligands: Biochemistry and Pharmacology (Biggio, G. and Costa, E., eds) Adv. Biochem. Physiopharmacol., vol. 38, pp. 239–248, Raven Press, New York.

[19] Weissman, B.A., Bolger, G.T., Isaac, L., Paul, S.M. and Skolnick, P. (1984) J. Neurochem. 42, 969–975.

[20] Amicon Corporation (1980) Publication No. 550, Lexington, MA.

[21] Benavides, J., Malgouris, F., Imbault, F., Begassat, F., Uzan, A., Renault, C., Dubroeucq, M.C., Gueremy, C. and Le Fur, G. (1983) Arch. Int. Pharmacodyn. 266, 38–49.

[22] Beaumont, K., Cheung, A.K., Geller, M.L. and Fanestil, D.D. (1983) Life Sci. 33, 1375–1384.