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PURIFICATION AND CHARACTERIZATION OF AN ENDOGENOUS PROTEIN MODULATOR OF RADIOLIGAND BINDING TO "PERIPHERAL-TYPE" BENZODIAZEPINE RECEPTORS AND DIHYDROPYRIDINE Ca^{2+}-CHANNEL ANTAGONIST BINDING SITES

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Abstract—Acidified extracts of rat antral stomach chromatographed on octadecylsilane cartridges contained material that inhibited the binding of [^{3}H]Ro 5-4864 (4′-chlorodiazepam) and [^{3}H]nitrenidipine to "peripheral-type" benzodiazepine receptors and dihydropyridine Ca^{2+}-channel antagonist binding sites respectively. This material reduced the apparent affinites of both radioligands without significantly affecting the maximum number of binding sites. In contrast, the binding of [^{3}H]diazepam, [^{3}H]Ro 15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo [1,5*a*][1,4]benzodiazepine-3-carboxylate), and [^{3}H]3-carbomethoxy-β-carboline to "brain-type" benzodiazepine receptors and [^{3}H]dihydroalprenolol binding to β-adrenergic receptors were unaffected by this material. Subsequent column chromatography on hydroxylapatite purified this material by >2000-fold. This semi-purified substance was resolved by reverse phase HPLC as one u.v. adsorbing peak that inhibited both [^{3}H]Ro 5-4864 and [^{3}H]nitrendipine binding. The activity of this 16,000 dalton substance was destroyed completely by both heat treatment and pronase and partially reduced by trypsin. Furthermore, the inhibitory activity of this substance was enhanced by Ca^{2+} in a concentration-dependent fashion (0.1 to 10 mM). Comparison of TLC scans of 2–9,10[^{3}H]dipalmitoyl-phosphatidylcholine incubated with either the HPLC purified material or authentic phospholipase A_2(PLA_2) (*Naja naja*) revealed that this substance has enzymatic properties indistinguishable from PLA_2. These findings suggest that this endogenous protein may be a PLA_2 isoenzyme which may modify both "peripheral-type" benzodiazepine receptors and dihydropyridine Ca^{2+}-channel antagonist binding sites.

Shortly after the discovery of benzodiazepine receptors in the central nervous system [1, 2], binding sites for [^{3}H]diazepam were also described in a number of peripheral tissues and transformed cells of neural origin [3, 4]. These "peripheral-type" benzodiazepine binding sites are physically and pharmacologically [5–8] distinct from the benzodiazepine receptors that are present solely in tissues derived from the neural crest. The "peripheral-type" binding sites have been termed "acceptor" sites denoting a lack of physiologic or pharmacologic function [9, 10]. However, more recent studies have demonstrated neural or hormonal control of the density of these sites in a number of tissues [11–18] and a stereoselective inhibition of radioligand binding [19]. Moreover, ligands with high affinity for these sites such as Ro 5-4864‖ and PK 11195 can affect both the electrical and mechanical properties of guinea pig myocardium at concentrations consistent with a receptor-mediated action [20, 21]. Thus, these sites fulfill many of the requirements of a pharmacologic receptor.

In a preliminary report [22], we described the presence of both high ($M_r > 10{,}000$) and low ($M_r < 2000$) molecular weight materials isolated in crude form from rat antral stomach that inhibit the binding of [^{3}H]Ro 5-4864 (4′-chlorodiazepam) to "peripheral-type" benzodiazepine receptors (PBR) but do not affect the binding of [^{3}H]diazepam to "brain-type" benzodiazepine receptors. We now report an efficient scheme for purification and subsequent characterization of this 16,000 dalton protein, termed antralin. Antralin has also been found to inhibit dihydropyridine Ca^{2+}-channel (DHP) antagonist binding, and its activity is depen-

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‖ Abbreviations: Ro 5-4864, (4′-chlorodiazepam) 7-chloro-1,3-dihydro-1-methyl-5-(*p*-chlorophenyl)-2*H*-1,4-benzodiazepine-2-one; PK 11195, 1-(2-chlorophenyl)-*N*-methyl-(1-methylpropyl)-3-isoquinoline carboxamide; Ro 15-1788, ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5*a*][1,4]benzodiazepine-3-carboxylate; Tris, Tris (hydroxymethyl) aminomethane; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TBPS, *t*-butylbicyclophosphorothionate; β-CCM, 3-carbomethoxy-β-carboline; PBR, "peripheral-type" benzodiazepine receptors;and PLA_2, phospholipase A_2.

dent on the Ca^{2+} concentration. Incubation of antralin with [^{3}H]phospholipid demonstrated that it could hydrolyze this substrate in a manner identical to that of phospholipase A_2 (PLA_2) from *Naja naja*. Thus, antralin may be an isoenzyme of PLA_2 which affects radioligand binding to both PBR and DHP binding sites.

MATERIALS AND METHODS

Tissue extraction. Male Sprague–Dawley rats (175–225 g, Taconic Farms, Germantown, NY) were killed by decapitation, and the peritoneal cavity was rapidly exposed. The stomach was cut in a line perpendicular to the longitudinal axis at the level of the greater curvature along the line of transition from cutaneous to glandular mucous membrane. The caudoventral portion of the stomach, the pyloris, and about 1 cm of the descending duodenum were removed (1.6 to 1.8 g/rat) and washed with cold isotonic saline. The tissues were immediately frozen on solid CO_2. Antral stomach from 25 to 30 rats was routinely used for each batch preparation. Frozen tissues were weighed and thawed in 5 vol. of an ice-cold acidic extraction solvent consisting of: formic acid (5% v/v), TCA (5% w/v), 1 N HCl, and sodium chloride (1% w/v) as modified from Bennett *et al.* [23]. The tissue was homogenized for 1 min in a Waring blender at room temperature. The homogenate was centrifuged at 20,000 *g* for 20 min, and the resulting supernatant fraction was filtered through gauze. The filtered supernatant was kept on ice for subsequent purification.

Batch purification procedure. Batch purification of tissue extracts was performed by absorption onto octadecylsilyl-silica cartridges (Sep-pak C_{18}, Waters Associates, Milford, MA). Cartridges were first activated with 10 ml methanol, then washed with 10 ml water followed by 10 ml of 0.1% trifluoroacetic acid (TFA) before applying the tissue extract. Twenty-five milliliter portions of extract were passed through a single Sep-pak C_{18} cartridge followed by a 5-ml wash with 0.1% TFA. Each cartridge was eluted with 3 ml of aqueous 80% acetonitrile containing 0.1% TFA into silinized glass tubes. Column eluates were dried in a Speed-Vac concentrator (Savant Instruments, Hicksville, NY). The residue was reconstituted in approximately 0.01% of the original extraction volume with 0.05% TFA and stored frozen (−20°) for binding studies and further purification.

Hydroxylapatite chromatography and HPLC. Sep-pak C_{18} isolated material was further purified by adsorption onto hydroxylapatite. For this procedure, a 1 × 7.5 cm Biogel HT (Bio-Rad, Rockville Centre, NY) column was prepared and washed with 50 ml of 0.4 M sodium phosphate buffer, pH 7.4. The column was then equilibrated with 50 ml of 0.4 mM sodium phosphate buffer, pH 7.4, to achieve a pH of 3.5 to 5.5 at low ionic strength. Antralin was eluted from the column with a linear gradient of 0 to 0.5 M NaCl in 0.4 mM sodium phosphate buffer, pH 7.4. Fractions inhibiting [^{3}H]Ro 5-4864 binding were pooled and lyophilized. The residue was reconstituted in 0.01% of the original extract volume with 0.05% TFA and stored at (−20°) before it was desalted by reverse phase HPLC.

Reverse phase high pressure liquid chromatography (HPLC) was carried out using a Gilson (Middleton, WI) model 302 dual pump system with an Hitachi variable wavelength u.v. detector and a Waters μBondapak C_{18} column (10 mm; 3.9 mm × 30 cm). The mobile phase was pumped at a flow rate of 1 ml/min of 0.05% TFA in acetonitrile (Burdick & Jackson, Muskegon, MI). The acetonitrile concentration started at 5% and was increased 5 min after sample injection to 95% in a linear gradient over the next 45 min. Fractions (1 ml) were collected into silinized tubes and dried in a vacuum centrifuge. To each tube, 500 μl of 0.05% TFA was added, the tube was vortexed, and an aliquot was taken for binding studies. Fractions that inhibited [^{3}H]Ro 5-4864 binding to kidney membranes were pooled and stored frozen at −20°.

Molecular weight determination. HPLC gel exclusion chromatography of antralin obtained by reverse phase HPLC was performed using a Bio-Rad Bio-Sil TSK 125 column (300 × 7.5 mm) with a guard column (75 × 7.5 mm) of the same material. The column was eluted with 40% acetonitrile/0.1% TFA [24] on a Beckman HPLC System (340 organizer, 114M solvent delivery module, 421 controller). The flow rate was 0.5 ml/min. The eluate was detected at 280 nm with a Beckman 160 absorbance detector in a 18 μl flow cell. Fractions were dried in a vacuum centrifuge and analyzed for inhibition of radioligand binding.

The amino acid composition of antralin was determined with a Beckman 6300 amino acid analyzer by the method of Spackman *et al.* [25] following hydrolysis for 24 hr in 6 N HCl at 110°. The values obtained are the mean of two determinations.

Radioligand binding assays. Male Sprague–Dawley rats (175–225 g) were killed by decapitation, and tissues were removed for use in binding assays. The binding of [^{3}H]Ro 5-4864 and [^{3}H]PK 11195 to PBR was determined using kidney membranes. Kidneys, which were frozen previously in 0.32 M sucrose, were thawed and homogenized in 25 vol. of cold 50 mM Tris–HCl buffer (pH 7.4) with a Brinkmann Polytron at setting 6–7 for 30 sec. The homogenate was centrifuged at 20,000 *g* at 4° for 10 min, the supernatant fraction was decanted, and the pellet was resuspended in the same volume of buffer. This washing procedure was repeated twice. The tissue pellet was resuspended in 200 vol. of buffer for radioligand binding. The activity of antralin was determined in a standard assay consisting of [^{3}H]Ro 5-4864 (1 nM) in a 1-ml assay volume containing 50 mM Tris–HCl buffer, pH 7.4, and 300 μl of washed kidney membranes. The samples were incubated for 60 min at 0° [26]. Nonspecific binding was determined in the presence of 1 μM Ro 5-4864. Some assays included 10 mM calcium where indicated. A standard semi-logarithmic curve of the per cent inhibition of specific binding by unlabeled Ro 5-4864 was constructed and inhibition of binding produced by tissue extracts converted to units of activity where 1 unit was equal to 50% inhibition.

Binding of [^{3}H]diazepam, [^{3}H]Ro 15-1788, and [^{3}H]β-CCM binding was determined using five times

washed rat cerebral cortical membranes with a final radioligand concentration of 1 nM in an assay previously described [26]. Frozen rat cerebral cortical membranes were prepared and assayed for [^{35}S]TBPS binding at a 1 nM concentration as described elsewhere [27]. The binding of [^{3}H]nitrendipine (80 pM) was determined as outlined by Bolger *et al.* [28] in a 2-ml assay volume using 500 μl of rat heart membranes prepared as follows: ventricular tissue was dissected free of atria, minced, and homogenized with a Polytron (setting 6–7) for 20 sec. The homogenate was centrifuged at 1,100 *g* for 10 min, and the supernatant fraction obtained was recentrifuged at 25,000 *g* for 20 min. The final pellet was resuspended in 30 vol. of 50 mM Tris–HCl buffer, pH 7.4. [^{3}H]Ouabain binding [29] at 9 nM was performed using guinea pig heart ventricular membranes. The total assay volume was 500 μl. Ventricular tissue was homogenized with a Polytron (setting 6–7) and centrifuged at 20,000 *g* for 15 min. The pellet obtained was washed twice and finally suspended in 10 vol. of 50 mM Tris–HCl buffer, pH 7.4. A 200-μl aliquot was used in the assay. [^{3}H]Dihydroalprenolol binding was assayed in rat brain cortical tissue as described [30].

Determination of phospholipase A_2 activity. One unit of antralin in 9 μl of 0.05% TFA was dried in siliconized glass tubes under a stream of N_2. After dissolving the residue in 100 μl of 50 mM Tris–HCl + 5 mM $CaCl_2$, pH 7.4, 1.8 nmol 2–9,10[^{3}H]dipalmitoyl-phosphatidylcholine in 1 μl toluene–ethanol (1:1) was added. For controls, 9 μl of 0.005% TFA or PLA_2 in 10 μl of 1 mM HCl was dried under N_2. Incubations were carried out at 0 or 37° for 60 min for antralin and control and for 30 min at 37° for PLA_2 (*Naja naja*, Sigma, St. Louis, MO). Rat kidney membrane preparations (1:100, w/v) replaced the buffer in some experiments. The reactions were stopped by adding 1.7 ml of chloroform–methanol (2:1) and the mixture was centrifuged for 5 min at 1000 *g*. The aqueous phase was aspirated, and 1 ml of the chloroform phase was dried over a stream of N_2. The residue was resuspended twice in 100 μl benzene–ethanol (2:1) and redried before it was finally dissolved in 10 μl chloroform–methanol. The thin-layer chromatography (TLC) was performed on 1000 μl silica plates GF using chloroform–methanol–29% NH_4OH (65:35:5) as a solvent. The TLC plates were scanned using a Berthold LB 2760 scanner.

Materials. Radioligands were purchased from New England Nuclear (Boston, MA) at the following specific activities: [^{3}H]Ro 5-4864 (74.9 Ci/mmol), [^{3}H]PK 11195 (85 Ci/mmol), [^{3}H]β-CCM (86.4 Ci/mmol), [^{3}H]diazepam (92.3 Ci/mmol), [^{35}S]TBPS (91.7 Ci/mmol), [^{3}H]nitrendipine (81.3 Ci/mmol), [^{3}H]ouabain (20.9 Ci/mmol), and 2–9,10[^{3}H]-dipalmitoyl-phosphatidylcholine (57 Ci/mmol). [^{3}H]Dihydroalprenolol (78 Ci/mmol) was purchased from Amersham (Des Plaines, IL). Benzodiazepines were supplied by Dr. Peter Sorter, Hoffmann-LaRoche, Nutley, NJ. PK 11195 was the gift of Dr. Gerard LeFur, Pharmuka Laboratories, Gennevilliers, France. *T*-Butylbicyclophosphorothionate was purchased from New England Nuclear. Enzymes were purchased from the Sigma Chemical

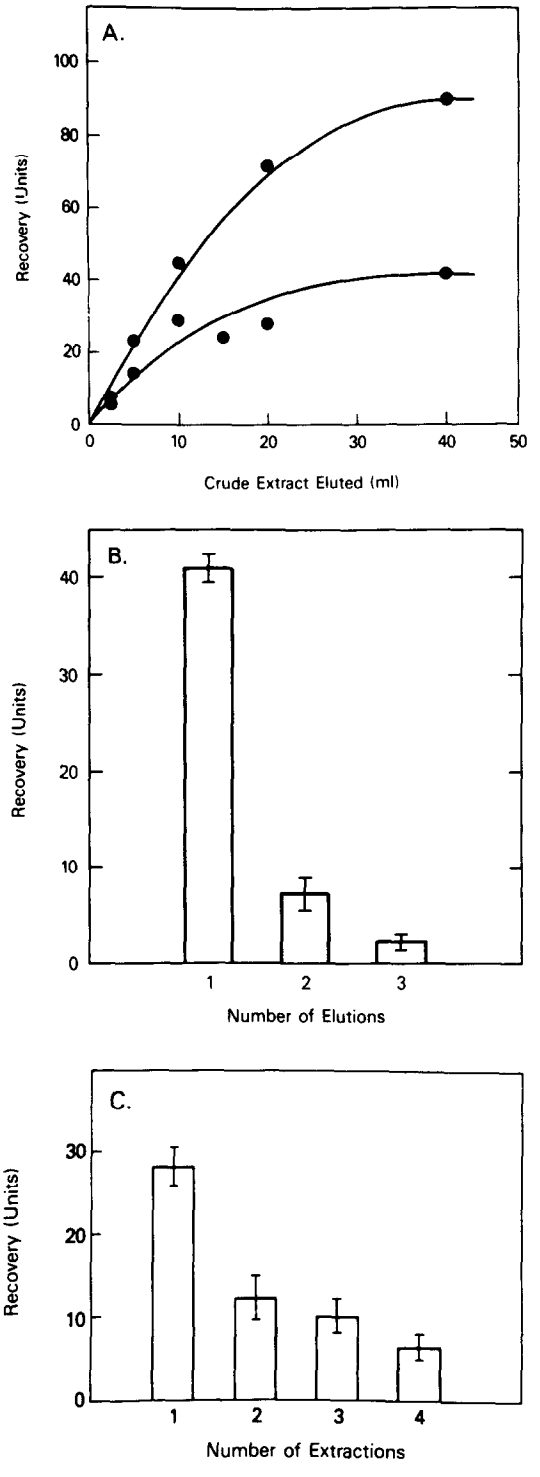

Fig. 1. Isolation of PBR inhibitory material from crude tissue extracts on Sep-pak C_{18} cartridges. Antral stomach was obtained from rats and a crude tissue supernatant was prepared as described in Materials and Methods. (A) Recovery of antralin from two separate batches of antral stomach with increasing volumes of supernatant passed through one cartridge. (B) Recovery of antralin from a single cartridge after 25 ml of 80% acetonitrile/0.1% TFA. (C) Recovery of antralin in single cartridges after repeated application of the same 25 ml of supernatant. Values in panels B and C represent the mean ± SEM of three experiments.

Co. (St. Louis, MO). Polypeptide standards used for HPLC were purchased from Pharmacia (Uppsala, Sweden).

RESULTS

Isolation of antralin on Sep-pak C_{18} cartridges. Tissues were extracted with an aqueous, highly acidic salt solution [23] to obtain material that inhibited [^{3}H]Ro 5-4864 binding to kidney membranes. The adsorption of this inhibitory material onto Sep-pak C_{18} cartridges was saturable using 25–30 ml of a crude supernatant prepared from a 20% (w/v) homogenate of antral stomach tissue (Fig. 1A). Saturation of the cartridge appeared to be independent of the intrinsic activity of the individual batch or tissue source and may represent saturation of the packing material by proteins and other tissue constituents. In subsequent experiments, 25 ml of the crude supernatant fraction was routinely used per cartridge. More than 80% of the activity added could be eluted with one 3-ml volume of 80% acetonitrile/0.1% TFA (Fig. 1B). Elution of the crude supernatant fraction through one Sep-pak C_{18} cartridge did not, however, adsorb all of the inhibitory activity (Fig. 1C). Thus, 75 ml of the pooled, once-extracted crude supernatant fraction was re-extracted through a second Sep-pak C_{18} cartridge to enhance recovery of material. Elution of Sep-pak C_{18} cartridges with solvent alone did not yield material that inhibited [^{3}H]Ro R-4864 binding (results not shown).

Characterization of inhibitory material eluted from Sep-pak C_{18} cartridges. Substances present in antral stomach and isolated on Sep-pak C_{18} cartridges inhibited the binding of [^{3}H]Ro 5-4864 to PBR in a concentration-dependent manner with no effect on nonspecific binding (Fig. 2A). Scatchard analysis demonstrated that inhibition of specific binding was the result of a decrease in the apparent affinity (increased K_d) with no concomitant alteration in the maximum number of binding sites (Fig. 2B). Extraction and isolation of PBR inhibitory material starting with equal weights of brain, antral stomach, kidney, and skeletal muscle yielded an uneven distribution of this material (Table 1). Whole brain contained the highest, stomach and kidney intermediate, and muscle the lowest level of inhibitory activity.

The ability of this material to inhibit radioligand binding to other types of receptors was also studied (Table 2). At a concentration of inhibitor that decreased [^{3}H]Ro 5-4864 binding by nearly 90%, no effect was found on [^{3}H]PK 11195 binding. Nor was any significant inhibition of [^{3}H]diazepam, [^{3}H]Ro 15-1788, or [^{3}H]β-CCM binding observed. Little inhibition of [^{3}H]ouabain binding and no inhibition of [^{3}H]dihydroalprenolol binding were detected. However, a 25% inhibition of [^{35}S]TBPS binding occurred at about a 20-fold higher concentration of material than necessary to inhibit [^{3}H]Ro 5-4864 binding by 100%. The Sep-pak C_{18} eluates were found to produce a concentration-dependent inhibition of [^{3}H]nitrendipine binding equal in potency to that seen with [^{3}H]Ro 5-4864 binding.

The biochemical nature of antralin was studied by examining its susceptibility to inactivation by proteolytic enzymes and heat (Table 3). A 50% reduction of activity was observed after a 15-min exposure to boiling water (data not shown), and a complete loss of activity was found after a 30-min exposure (Table 3). Incubation with pronase destroyed 80% of inhibitory activity, whereas trypsin reduced the activity by 34%. Other enzymes such as carboxypeptidase and leucine aminopeptidase, endonuclease and exonuclease, and β-glucuronidase did not affect the activity of antralin under the incubation conditions employed. The ability of mono- and divalent cations to affect the potency of the Sep-pak C_{18} isolated antralin to [^{3}H]Ro 5-4864 binding to rat kidney membranes was studied. While Na^+ and Li^+ were without effect, Ca^{2+} (0.1 to 10 mM) enhanced the potency of this material in a concentration-dependent manner (Table 4), while Mg^{2+} (1 mM) was without effect. The binding of [^{3}H]Ro 5-4864 in the absence of antralin was not affected by

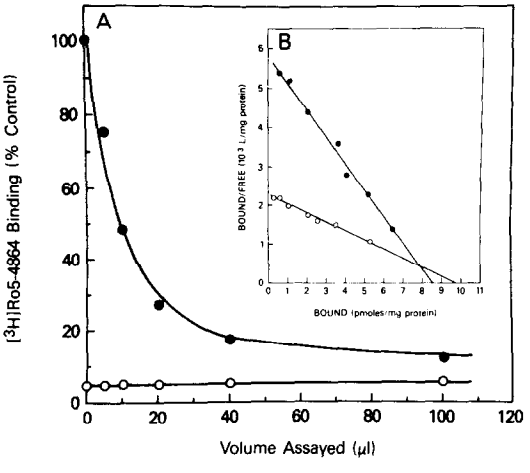

Fig. 2. Inhibition of [^{3}H]Ro 5-4864 binding by Sep-pak C_{18} eluates. Kidney membranes were prepared as described in Materials and Methods and incubated with [^{3}H]Ro 5-4864 (1 nM) at 0° for 60 min in 50 mM Tris–HCl buffer, pH 7.4 (A) Aliquots of material isolated on Sep-pak C_{18} from rat antral stomach and dissolved in 0.05% TFA were tested for inhibition of total (●) and nonspecific (○) binding. A volume of up to 300 μl 0.05% TFA did not decrease total binding. (B) Representative Scatchard plot of [^{3}H]Ro 5-4864 binding in the absence (●) (K_d 1.5 nM, B_{max} 8.5 pmol/mg protein) or presence of 1 unit of antralin (○) (K_d 4.5 nM, B_{max} 9.8 pmol/mg protein).

Table 1. Tissue distribution of antralin in the rat

Tissue	Total activity recovered (units/g tissue)
Whole brain	15.6 ± 0.61
Stomach	6.6 ± 0.29
Kidney	1.5 ± 0.06
Skeletal muscle	0.7 ± 0.6

Activity was measured in tissue extracts that were purified on Sep-pak C_{18} cartridges as described in Materials and Methods. Total activity was based on the inhibition of [^{3}H]Ro 5-4864 binding and expressed in terms of units as described in the text. Values represent the mean ±SEM of three separate tissue preparations, using about 4 g of tissue per preparation.

Table 2. Effect of antralin on radioligand binding

Ligand (nM)	Tissue source	Percent inhibition of specific binding Units/ml 0.25	1	4
[^{3}H]Ro 5-4864	Kidney	23 ± 10	57 ± 7	83 ± 7
[^{3}H]PK 11195 (1)	Kidney	—	—	<5
[^{3}H]Diazepam (1)	Brain	—	—	<5
[^{3}H]Ro 15-1788 (1)	Brain	—	—	<5
[^{35}S]TBPS (1)	Brain	7 ±4	16 ± 2	25 ± 1
[^{3}H]β-CCM (1)	Brain	—	—	<5
[^{3}H]Nitrendipine (0.8)	Heart	22 ± 2	40 ± 3	77 ± 3
[^{3}H]Ouabain (9)	Heart	—	—	18
[^{3}H]Dihydroalprenolol (1)	Heart	—	—	<5

Antralin was prepared from extracts of antral stomach and purified on Sep-pak C_{18} cartridges as described in Materials and Methods. Units were based on inhibition of [^{3}H]Ro 5-4864 specific binding by non-radioactive Ro 5-4864 as described in the text. Concentrations of antralin inhibiting [^{3}H]Ro 5-4864 binding by 20–80% were tested on specific radioligand binding to various receptors at the final ligand concentration indicated. Tissue membranes from different sources were prepared as outlined in Materials and Methods. Values represent the mean ± SEM of three separate assays performed in triplicate.

Ca^{2+}. Calcium also enhanced the inhibitory activity of Sep-pak C_{18} material toward [^{3}H]nitrendipine binding (data not shown).

Purification and molecular weight determination. Material isolated on Sep-pak C_{18} cartridges was further purified by adsorption onto hydroxylapatite and eluted with an NaCl gradient. A representative profile of activity obtained by chromatographing 1000 units of antralin is shown in Fig. 3. The PBR inhibitory material was strongly bound to hydroxylapatite and required about 0.25 to 0.28 M NaCl for desorption from the column. This step resulted in a >2000-fold purification of Sep-pak C_{18} material (Table 5). The active fractions were pooled, lyophilized, and resuspended in 0.05% TFA. Reverse phase HPLC of hydroxylapatite purified antralin yielded essentially one major u.v. absorbing peak (Fig. 4A). As seen in panels B and C of Fig. 4, the inhibition of both [^{3}H]Ro 5-4864 and [^{3}H]nitrendipine binding coincided with the retention time of the major u.v. peak. While reverse phase HPLC did not result in further purification of antralin (Table 5), it desalted the active fractions. However, this step did produce about a 50% loss in total activity despite nearly complete recovery of total protein. The active fractions were pooled, and a portion was chromatographed on gel exclusion HPLC for molecular weight estimation. Figure 5 illustrates that

Table 3. Effects of degradative enzymes and temperature on the activity of antralin

Treatment	Enzyme Control	Enzyme + Antralin	% Inhibition
	[^{3}H]Ro 5-4864 Binding (pmol/mg protein)		
None	3.47	0.54	84
Heat (95°)	3.60	3.68	0
Pronase	2.72	2.29	16
Trypsin	3.44	1.17	66
Carboxypeptidase C	3.51	0.34	90
Leucine aminopeptidase	2.81	0.22	92
Endonuclease	3.31	0.47	86
Exonuclease	3.17	0.23	93
β-glucuronidase	2.99	0.24	92

Antralin was prepared from extracts of rat antral stomach and purified on Sep-pak C_{18} cartridges as described in Materials and Methods. For each treatment, antralin was diluted in 50 mM Tris–HCl buffer, pH 7.4, to a concentration of 4 units/ml. One-milliliter samples (in triplicate) were incubated at 37° for 18 hr in the presence or absence of various degradative enzymes each at a concentration of 1–4 units per tube, also containing 1 mM Mg^{2+} and 2 mM Ca^{2+}. All tubes were then lyophilized, and each tube was analyzed for inhibition of [^{3}H]Ro 5-4864 binding (1 mM) using kidney membranes in a total assay volume of 1 ml as described in the text. Heat inactivation was done in a boiling water bath for 30 min and processed as outlined above.

Table 4. Effect of cations on the potency of antralin to inhibit [^{3}H]Ro 5-4864 binding to PBR

Addition	Ion concn (mM)	Specific binding (cpm)	Activity (units)
None	0	21,200	
Antralin	0	9370	1.3
Antralin plus			
NaCl	150	9030	1.4
LiCl	150	8280	1.7
Choline Cl	1	8920	1.4
$MgCl_2$	1	8720	1.5
$CaCl_2$	0.001	9390	1.3
	0.01	8400	1.5
	0.1	5450	3.0
	1.0	2880	5.3
	10.0	1410	7.5

Antralin was prepared from extracts of antral stomach and purified on Sep-pak C_{18} cartridges as described in Materials and Methods. Approximately one unit of antralin was incubated with [^{3}H]Ro 5-4864 (1 nM) in 50 mM Tris–HCl buffer, pH 7.4, using kidney membranes as a receptor source. Assays were performed in triplicate. In other samples, the same amount of antralin was incubated with different cations at the concentrations indicated. Control samples included the ions alone and in no case did these salts, including $CaCl_2$, affect [^{3}H]Ro 5-4864 binding at the concentrations shown.

the active fractions obtained by reverse phase HPLC comprised one-well resolved u.v. peak with an estimated M_r = 16,000 ± 1000 (x ± SEM, N = 8) which inhibited both [^{3}H]nitrendipine and [^{3}H]Ro 5-4864 binding.

Amino acid composition of antralin. The amino acid composition of antralin (from two separate batches) normalized to five alanine residues is represented in Table 6. This analysis corresponded to an apparent molecular weight of 13,682 daltons. Based upon these results, antralin is rich in aspartate (22 residues/molecule, 18% of amino acids).

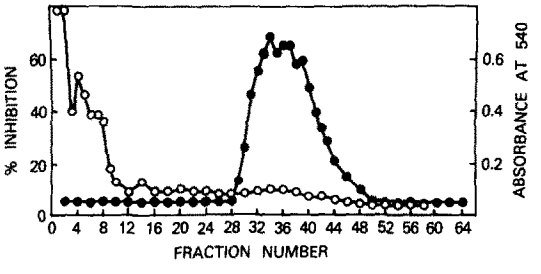

Fig. 3. Representative elution profile of Sep-pak C_{18} eluates applied to Biogel HT column. A 1 × 7.5 cm hydroxylapatite column was prepared as described in Materials and Methods, and 1000 units (100–150 mg protein) of antralin was applied in 0.4 mM sodium phosphate buffer, pH 7.4. The column was eluted with a linear gradient of 0–0.5 M NaCl in 0.4 mM sodium phosphate buffer, pH 7.4, at a flow rate of 8 ml/hr, and 2-ml fractions were collected. Aliquots from each tube (25 μl) were assayed for inhibition of [^{3}H]Ro 5-4864 binding (1 nM) in the presence of 10 mM Ca^{2+} using kidney membranes as a receptor source. Percent inhibition of binding in each sample (●) was calculated based on binding to pre-sample column control tubes. The amount of protein in each fraction was determined by the method of Lowry *et al.* [31] and the absorbance plotted (○). Active fractions taken for lyophilization in this example included tubes 31–41.

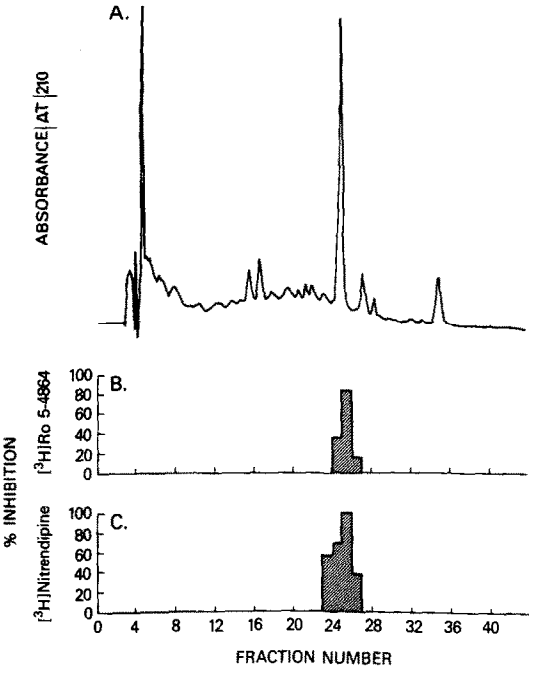

Fig. 4. Representative elution profile of BioGel HT purified antralin applied to reverse phase HPLC column. Approximately 500 units of antralin (30 μg protein) in 0.5 ml of 0.05% TFA was applied to a μBondapak column (3.9 mm × 30 cm) and eluted with an acetonitrile gradient as described in Materials and Methods. Fractions were dried overnight in a vacuum centrifuge and reconstituted in 0.5 ml of 0.05% TFA; 10-μl aliquots were taken for testing in binding assays. (A) Ultraviolet absorption profile of chromatographic run. (B) Inhibition of [^{3}H]Ro 5-4864 specific binding (1 nM) in the presence of 10 mM Ca^{2+} using kidney membranes. (C) Inhibition of [^{3}H]nitrendipine specific binding (80 pM) in the presence of 10 mM Ca^{2+} using heart ventricular membranes for both radioligand binding assays. Controls consisted of the amount of binding found in the last three column samples.

Table 5. Purification of antralin from rat antral stomach

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Crude supernatant	236	21,712	508	0.02	
Sep-pak C_{18}	3	402	2,300	7.46	100
Hydroxylapatite	2	0.12	2,101	17,517	91
Reverse phase HPLC	2	0.10	1,147	11,470	50

Antral stomach tissue was obtained from 30 male rats (175–225 g), and a crude supernatant fraction was prepared as described in Materials and Methods. Protein was determined by the method of Waddell [32]. Activity was established by assaying several different volumes of each fraction for inhibition of [^{3}H]Ro 5-4864 specific binding (1 nM) in the presence of 10 mM Ca^{2+} using rat kidney membranes. Inhibition of binding between 30 and 70% was converted to units as described in the text. Percent recovery was based on the activity obtained in Sep-pak C_{18} purified material since in separate experiments it was determined that the extraction solvent used to prepare the crude supernatant interfered with [^{3}H]Ro 5-4864 binding, even when the solvent was neutralized (unpublished data).

Phospholipase activity. 2-9,10[^{3}H]Dipalmitoyl-phosphatidylcholine incubated with rat kidney membranes was not degraded as judged by TLC (Fig. 6, panel A), whereas when the substrate was coincubated with antralin, at both 0 and 37°, it was no longer detected. A spot corresponding to the product of PLA_2 (i.e. the free fatty acid) was monitored at a higher R_f value (Fig. 6, panels C and D). Ten units of PLA_2 reduced, but did not abolish, the original 2-9,10[^{3}H]dipalmitoyl-phosphatidylcholine peak (Fig. 6, panel B). Addition of tissue to antralin reduced the rate of hydrolysis of 2-9,10[^{3}H]dipalmitoyl-phosphatidylcholine at both temperatures, so that the original compound was still detectable. In contrast to this finding, PLA_2 activity was increased by the addition of tissue (data not shown).

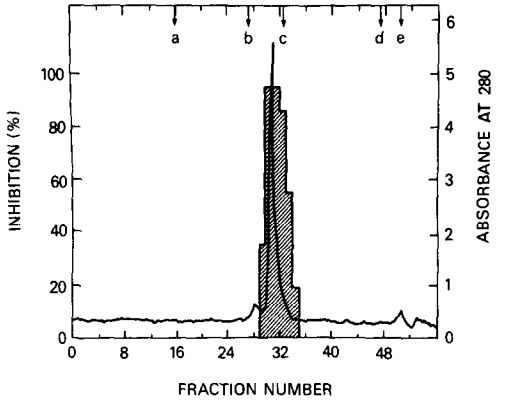

Fig. 5. Representative elution profile of reverse phase HPLC purified antralin applied to a gel exclusion HPLC column. Solid line represents u.v. absorption of the chromatographic run. The shaded histogram shows the percent inhibition of [^{3}H]nitrendipine specific binding (80 pM) with 10 mM Ca^{2+} using heart ventricular membranes. The same fractions that inhibited dihydropyridine radioligand binding also inhibited [^{3}H]Ro 5-4864 binding (1 nM) determined in the presence of 10 mM Ca^{2+} using kidney membranes (data not shown). Arrows marked a, b, c, d, and e indicate the eluted volume of myoglobin polypeptide molecular weight markers: (a) 17,201, (b) 14,631, (c) 8,235, (d) 6,383, and (e) 2,556.

DISCUSSION

Substances that inhibit [^{3}H]Ro 5-4864 binding to PBR have been described in extracts of stomach, brain, kidney, blood, and urine [22, 33, 34]. These studies used acidified methanol or TCA for the initial extraction step. However, methanol proved an unsatisfactory solvent for extensive purification since it is not easily lyophilized, and traces of this solvent inhibit [^{3}H]Ro 5-4864 binding. Lyophilization of TCA extracts resulted in a viscous residue which proved unsuitable for large scale chromatography. Recently we showed that endogenous inhibitors of [^{3}H]Ro 5-4864 binding are retained on a μBondapak C_{18} column [35]. Thus, an extraction procedure was devised using a highly acidic salt medium to prevent possible proteolysis during homogenization followed by isolation on octadecylsilane-silica (Sep-pak C_{18}) cartridges. This scheme resulted in reproducible yields of an inhibitor of [^{3}H]Ro 5-4864 binding which appeared identical to, but purer than, the form of

Table 6. Amino acid composition of purified antralin

Amino acid	Residues*
Asp	21.50 (22)†
Thr	6.57 (7)
Ser	7.10 (7)
Glu	9.98 (10)
Pro	7.90 (8)
Gly	8.05 (8)
Ala	5.00 (5)
Cys	5.02 (5)
Val	4.52 (5)
Ile	5.73 (6)
Leu	6.95 (7)
Tyr	7.53 (8)
Phe	4.07 (4)
His	3.18 (3)
Lys	11.53 (12)
Arg	4.25 (4)

* Mean number of residues following 24-hr hydrolyses from two separate batches of antralin.

† Values in parentheses correspond to the integer amino acid composition of antralin.

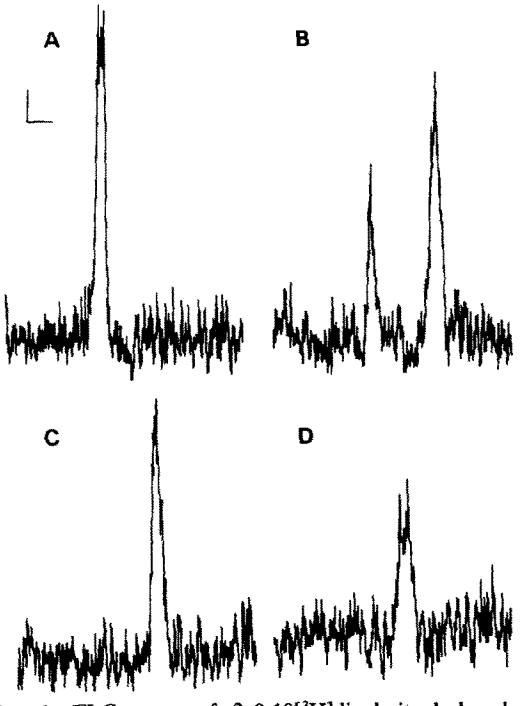

Fig. 6. TLC scans of 2-9,10[^{3}H]dipalmitoyl-phosphatidylcholine after incubation with antralin and PLA_2. 2-9,10[^{3}H]Dipalmitoyl-phosphatidylcholine was incubated and extracted as described in Materials and Methods. Key: (A) control without any addition; (B) 10 units of PLA_2 incubated for 30 min at 37°(C) 1 unit antralin incubated for 60 min at 37° or (D) at 0°. The horizontal bar corresponds to 1 cm of TLC plate and the vertical bar to 100 cpm. The counting efficiency was less than 10%. The R_f values for the peak in panel A and the first peak in panel B were 0.45 and 0.47 respectively. The second peak in panel B and the peaks in panels C and D had R_f values of 0.76, 0.68, and 0.66 respectively.

this material first obtained by methanol extraction of rat stomach tissue.

The material eluted from Sep-pak C_{18} reduced specific, but not nonspecific [^{3}H]Ro 5-4864 binding in a concentration-dependent manner (Fig. 2). Parallel experiments where solvent alone was passed through the Sep-pak C_{18} cartridge did not result in the isolation of PBR binding inhibitory material. Marked differences in the total amount of inhibitory material extracted from equal weights of tissue (Table 1) confirm earlier reports from our laboratory [22] demonstrating differences in the distribution of this PBR inhibitory material and support the view that this inhibition of radioligand binding cannot be attributed to sequestration of ligand by ubiquitous nonspecific binding proteins extracted from these tissues.

The pharmacological specificity of this material was demonstrated initially by its lack of effect on [^{3}H]diazepam [22], [^{3}H]Ro 15-1788, and [^{3}H]β-CCM binding to "central-type" benzodiazepine receptors (Table 2). However, Sep-pak C_{18} purified material was also found to potently inhibit [^{3}H]nitrendipine binding to dihydropyridine Ca^{2+}-channel antagonist binding sites in the heart. In highly purified preparations, the inhibitory activity toward both radioligands was shown to coelute in the same fractions that corresponded to the major peak of u.v. absorbance (Figs. 4 and 5). This demonstrated that one substance was responsible for interaction with these two distinct binding sites.

Inclusion of leupeptin (50 μM) in binding assays did not affect the ability of antralin to inhibit PBR binding, suggesting that this substance was not a protease. However, the relatively large size of antralin coupled with the apparent activation by Ca^{2+} did not rule out that this protein could affect radioligand binding by enzymatic modification of the receptors involved. This notion was supported by preliminary experiments demonstrating that the effect of antralin on [^{3}H]nitrendipine binding was not reversed by extensive washing of the tissue (results not shown).

Recently, we reported [36, 37] that phospholipase A_2 from *Naja naja* and other mammalian and non-mammalian sources inhibits radioligand binding to PBR and DHP Ca^{2+}-channel antagonist sites, and [^{35}S]TBPS binding to GABA-gated chloride channels. When purified antralin was incubated with [^{3}H]dipalmitoyl-phosphatidylcholine, complete degradation occurred. This suggests that antralin has PLA_2 activity. However, there are differences in the relative potencies between PLA_2 from *Naja Naja* and antralin at these binding sites. Particularly interesting is the lack of inhibition of [^{3}H]PK 11195 binding to PBR by PLA_2 from *Naja naja* venom and the same correspondence with antralin. The molecular weight of antralin (16 kD) is consistent with reported molecular weights of PLA_2 from a number of sources [38]. The inhibitory activity of antralin is destroyed by heating, whereas other phospholipases are reported to be relatively resistant to heat treatment [38]. Thus, antralin may be a PLA_2 isoenzyme.

Since antralin is found in blood and is widely distributed in tissues, the possibility exists that this form of mammalian PLA_2 may regulate Ca^{2+} channels through its action on PBR and DHP binding sites. While the precise relationship between PBR and DHP Ca^{2+}-channel binding sites remains unknown, it has been shown both pharmacologically [20, 39] and electrophysiologically [20, 40] that PBR are coupled to Ca^{2+}-dependent phenomena. Thus, antralin may have a role in regulating biological activity in a number of tissues. Whether such a potential function of antralin is ultimately through its phospholipase activity will come from comparison of its amino acid homology to known phospholipase enzymes.

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