# Isolation of an endogenous clonidine-displacing substance from rat brain

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An endogenous substance which specifically displaces clonidine, yohimbine and rauwolscine from rat brain $\alpha_2$-adrenergic receptors, has been isolated. The new compound, designed clonidine-displacing-substance (CDS), has been partially purified by ion exchange chromatography, zone electrophoresis and high performance liquid chromatography (HPLC). CDS binds specifically to $\alpha_2$-adrenergic receptors by competing with either $\alpha_2$-adrenergic agonists or $\alpha_2$-antagonists, but has no effect on the specific binding of [$^3$H]prazosin to $\alpha_1$-adrenergic receptors in rat brain membranes. In the course of isolation, CDS was shown to be neither the endogenous neurotransmitter (−)norepinephrine (NE) nor the guanyl nucleotide GTP which lowers the specific binding of $\alpha_2$-agonists to the $\alpha_2$-adrenergic receptors.

*Clonidine* *$\alpha_2$-Adrenergic receptor* *Yohimbine* *Hypertension*

## 1. INTRODUCTION

The wide-spread interest in $\alpha_2$-adrenergic agonists is mostly due to their clinical application as antihypertensive drugs. Clonidine, guanabenz, guanadrel, labaz, guanoxan and tramazoline are but a few representatives of numerous imidazoline and guanido-containing $\alpha_2$-agonists (review [1–4]). Their potent antihypertensive activity results from stimulation of $\alpha_2$-adrenergic receptors in the central nervous system (CNS). Clonidine is an imidazoline which is defined as being exclusively an $\alpha_2$-agonist, and it is shown to compete with NE, the endogenous $\alpha_2$-agonist [5,6]. $\alpha$-Methyldopa, considered to be among the most specific $\alpha_2$-agonists [7–9], was recently shown to be ineffective as a hypotensive agent at the nucleus reticularis lateralis (NRL) which is the main site of hypotensive action of clonidine in the medulla oblongata [10,11]. The abundance of guanido-containing substances in the spinal fluid and in the CNS (guanido acetic acid, guanido glutaric acid, guanido succinic acid, etc.) [12], as well as the existence of a structure-activity relationship for imidazoline structure in the NRL at the medulla oblongata [10], prompted us to look for an endogenous clonidine-like substance in the brain.

## 2. EXPERIMENTAL

### 2.1. *Chemicals*

[$^3$H]Clonidine (25.5 Ci/mmol; Ci = $3.7 \times 10^{10}$ becquerels), [$^3$H]prazosin (40 Ci/mmol), [$^3$H]yohimbine (80 Ci/mmol), [$^3$H]NE (24.6 Ci/mmol) and [$^3$H]rauwolscine (79 Ci/mmol) were purchased from New England Nuclear. The following were generous gifts: phentolamine, from Ciba Geigy, and clonidine, from Boehringer Ingelheim. (−)NE was purchased from Sigma. Other chemicals and biochemicals were of the highest purity available. In high performance liquid chromatography (HPLC) studies, the water was distilled 3 times and the solvents were of HPLC grade.

### 2.2. *Binding of [$^3$H]clonidine, [$^3$H]yohimbine, [$^3$H]rauwolscine and [$^3$H]prazosin to rat brain $P_2$ membranes*

The membrane preparation and the binding assays were carried out essentially as in [13].

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2.2.1. $\alpha_2$-Adrenergic receptors

Binding to $\alpha_2$-adrenergic receptors was evaluated in an assay volume of 0.3 ml, containing 200–300 $\mu$g membrane protein from rat brain, in 50 mM Tris–HCl (pH 7.5), using either 3–5 nm [$^3$H]clonidine, 1 nM [$^3$H]yohimbine or [$^3$H]rauwolscine, respectively. Specific binding which was 70–80% in [$^3$H]clonidine binding, 70% in [$^3$H]yohimbine binding and 60% in [$^3$H]rauwolscine binding, was determined by subtracting non-specific binding at 10 $\mu$M NE [13] from total binding.

2.2.2. $\alpha_1$-Adrenergic receptors

The same binding procedure as described above for the $\alpha_2$-adrenergic receptors was followed, with 1 nM [$^3$H]prazosin, an $\alpha_1$-specific radioligand. Phentolamine (33 $\mu$M) was used to determine non-specific binding.

2.3. *Protein assay*

Protein concentration determinations were carried out as in [14], using bovine serum albumin as the standard.

2.4. *HPLC studies*

These studies were performed by reverse-phase liquid chromatography using RP-8 or RP-18 columns (10 $\mu$m, 4.6 × 250 mm, Alltech, IL).

## 3. RESULTS

### 3.1. *Isolation and partial purification of an endogenous compound which binds to $\alpha_2$-adrenergic receptors*

During the purification procedure, at each step samples were withdrawn and their biological activity as well as the yield of isolation were determined. All activity measurements were carried out by displacement studies of [$^3$H]clonidine from rat brain $P_2$-membrane preparations, using 10 $\mu$M (−)NE as a reference for non-specific binding. One unit of activity is defined as the amount needed to displace 50% of specifically bound [$^3$H]clonidine under standard conditions (3.5 nM [$^3$H]clonidine and 250 $\mu$g protein per assay, 40 min at 25°C).

3.1.1. Step 1: crude methanolic extract

Calf brains (~300 g wet wt, after removal of the cerebellum) were excised into small pieces (1–3 cm) and placed into 3 vols (w/v) of 10 mM Tris–HCl buffer (pH 7.7) at 4°C. The slices were subjected to 25 s of homogenization in Polytrone, followed by centrifugation at 100000 × $g$ for 30 min at 4°C. The pellet was discarded and the supernatant (~60% of the total volume) was boiled for 15 min over a water bath. After cooling, the boiled soup was centrifuged at 100000 × $g$ for 15 min at 4°C. Extraction of the dry lyophilizate (2.9 g, ~1% yield; yields varied from 1.2–0.9% of the brains' wet wt) was performed with 20 vols methanol. The methanolic extract contains an average of 3000 units/300 g (an average of 1500 ± 100 units/150 g wet wt).

3.1.2. Step 2: DEAE–Sephacel column

The crude methanolic extract was chromatographed on a DEAE–Sephacel column (1.25 cm$^2$ × 20 cm). The activity was eluted at 0.11–0.14 M in a gradient of 0.0–0.25 M ammonium bicarbonate. [$^3$H]NE was included in one of the column runs and eluted with the water washings prior to the initiation of the gradient.

3.1.3. Step 3: zone electrophoresis

Column electrophoresis in agarose suspension (0.18%, w/v) was carried out as in [15]. The activity eluted from DEAE–Sephacel (not exceeding 20 mg dry wt) was dissolved in 0.6 ml of the agarose suspension in 0.01 M ammonium bicarbonate (pH 8.1) and applied to the column (0.9 × 65 cm). The electrophoresis was carried out for 20 h at 490 V at a current of 4 mA. Samples of 0.6 ml were taken out by suction from the top. The samples were assayed for activity and their absorbance at 275 nm was recorded (fig.1). Ninety to 100% of the biological activity was recovered.

3.1.4. Step 4: HPLC

Lyophilized samples, after zone electrophoresis, were injected into a reverse-phase column and eluted from it with *n*-propanol (0–50%) for 1 h in 0.1% TFA. The biological activity was eluted with 36–42% *n*-propanol (fractions 21–23) (fig.2). The 80–90% of the recovered biological activity was ninhydrin and fluorescamine negative. GTP, incorporated in one of the runs, was not retained on the column. Displacement of specifically bound

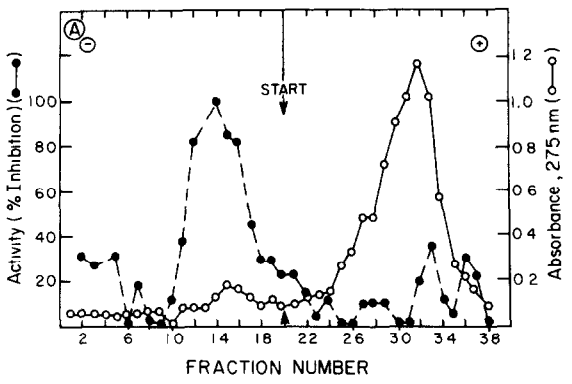


Fig.1. Column electrophoresis in agarose suspension. Extracted CDS activity from DEAE–Sephacel column (10–20 mg dry powder), dissolved in 0.6 ml of 0.18% agarose suspension of 0.01 M ammonium bicarbonate (pH 8.4), was applied to the column (start). An electrical current of 4 mA was applied at 490 V for 20 h. The activity was determined on aliquots (1–3%) of the supernatant after centrifugation of 0.6 ml withdrawn samples (15000 × *g*, 2 min) (●—●). Absorbance at 275 nm was determined (○—○).

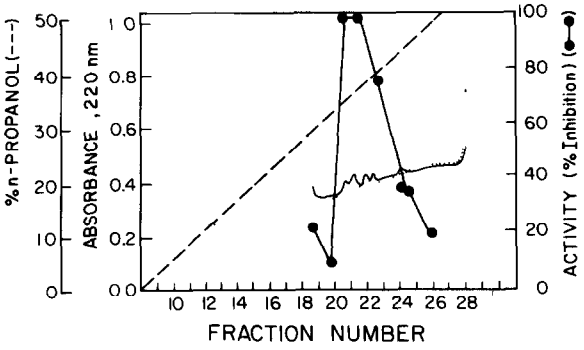


Fig.2. HPLC of the activity eluted from zone electrophoresis run. The lyophilizate preparation (100 units after zone electrophoresis) was applied to the HPLC column (size 4.1 mm interior diameter × 25 cm) and eluted at a rate of 0.8 ml/min in a linear gradient of 0.1% TFA and 0–50% *n*-propanol. Absorbance was detected by Instrumentation Specialists Co., model 1840, recorder. Activity of 5% of each fraction after lyophilization was determined. CDS eluted at 36–42% *n*-propanol. Control runs show an identical pattern (marked by dotted line) of the absorbance at 220 nm but without any activity.

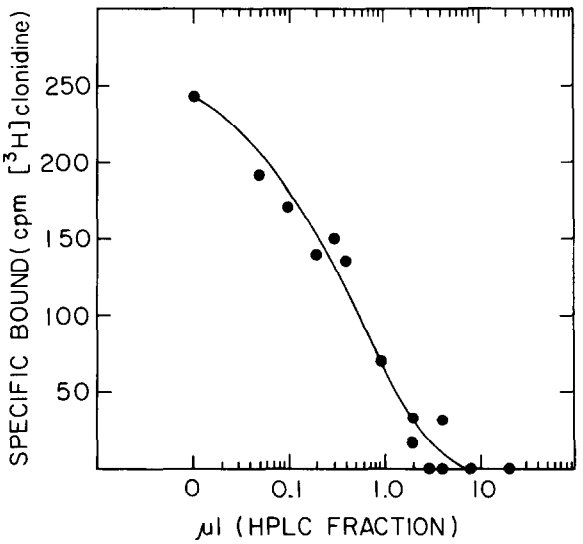


Fig.3. Displacement of specifically bound [$^3$H]clonidine in rat brain membranes by CDS (HPLC fraction). Displacement of [$^3$H]clonidine from rat brain membrane was performed as described in section 2. Aliquots of CDS (0.5–20 µl) were added to a binding mixture containing 8.8 nM [$^3$H]clonidine, 150 µg protein membrane, in a final volume of 0.25 ml. Non-specific binding was determined in the presence of 10 µM (−)NE. Total binding is 144 fmol/mg protein and non-specific binding is 62 fmol/mg protein.

Table 1

Steps in the purification of CDS: calculations of percent yield based on weight

| Purification step | Weight (g) | Yield (%) |
|---|---|---|
| Calf brain | 300 (wet wt) | 100 |
| Methanolic extract | 3.0 ± 0.2 | 1.0 ± 0.2 |
| DEAE–Sephacel | 0.6 ± 0.2[a] | 0.2 ± 0.06 |
| Zone electrophoresis | not detectable[b] | |

[a] Most probably contains $NH_4HCO_3$
[b] Amount too low to be weighed

[$^3$H]clonidine in rat brain membranes by CDS (HPLC fraction) is presented in fig.3.

### 3.2. *Absence of $\alpha_1$-adrenergic activity*

One and 2 units of CDS activity did not affect the binding of 1 mM [$^3$H]prazosin to rat brain. Phentolamine (33 µM) and NE (33 µM) were used for the determination of non-specific binding (fig.4).

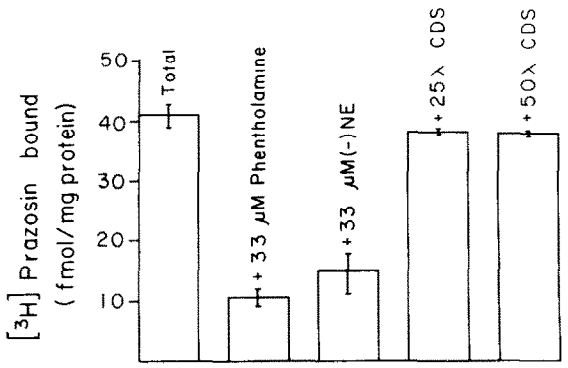


Fig.4. The effect of CDS on $\alpha_1$-adrenergic receptors. Displacement of [$^3$H]prazosin from rat brain membranes was performed as described in section 2. One unit (25 $\mu$l) and 2 units (50 $\mu$l) of CDS were added to the binding mixture, and the effect of specific binding was determined in the presence of either 33 $\mu$M phentolamine or 33 $\mu$M (−)NE.

## 4. DISCUSSION

A new activity (CDS) is purified in a four-step procedure: methanolic extraction, ion exchange chromatography (DEAE), zone electrophoresis and HPLC. Although similar activity was obtained from rat brain, due to the small quantities available, the studies were shifted to calf brain. The endogenous compound (CDS) extensively purified from calf brain has $\alpha_2$-adrenergic properties. After HPLC, CDS is obtained in minute amounts which are not detectable by weight and are insufficient for determination of molecular mass or for carrying out NMR analysis or chemical analysis. Scaling up the production of the compound will be necessary before a final structure may be determined.

The new substance displaces specifically bound [$^3$H]clonidine, [$^3$H]yohimbine and [$^3$H]rauwolscine in rat brain membranes. These are three specific $\alpha_2$-adrenergic ligands which are commonly used to probe $\alpha_2$-adrenergic receptors. It is therefore suggested that the new activity which can specifically displace these ligands might have some structural resemblance that permits the interaction at the $\alpha_2$-adrenergic sites. No effect on the specific binding to $\alpha_1$-adrenergic receptors was observed using [$^3$H]prazosin, a specific $\alpha_1$-antagonist. CDS has negative ninhydrin and negative fluorescamine reactions.

The possibility of another endogenous ligand with adrenergic properties, present in the brain, can meet some of the difficulties to explain the effects of adrenergic drugs on arterial pressure [16] or the insensitivity of junctional receptors to phentolamine or prazosin in smooth muscle organ [17]. Our present effort is directed toward scaling up the production of the compound for determination of its chemical structure, and expanding its biological characteristics by application of CDS in electrophysiological studies into specific nuclei in the brain.

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