Elsevier Biomedical Press b.v. P.O. Box 1527 Amsterdam

Date: 10-8 Slips 1-5 1st proof

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BRES 20473

Brain Research, 000 (1984) 000–000
Elsevier

BRE 20473

Quantitative determination of angiotensin II binding sites in rat brain and pituitary gland by autoradiography

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(Accepted July 5th, 1984)

Key words: angiotensin II binding sites — radioautography — quantitation of binding sites — brain — pituitary gland — organon subfornicalis

Rat brain and pituitary angiotensin II (AII) binding sites were quantitated by incubation of tissue sections with ^{125}I-[Sar^1]-AII, ultrofilm radioautography, computerized densitometry, and comparison with ^{125}I-standards at appropriate film exposure times. The highest number of AII binding sites was found in anterior pituitary and the circumventricular organs, organon subfornicalis and organon vasculosum laminae terminalis.

Blood-borne angiotensin II (AII) acts in the brain to stimulate drinking, increase blood pressure, and release pituitary hormones[2,5,13]. These effects are mediated, at least partially, through stimulation of AII receptors in circumventricular organs[2,13,16].

All components of the angiotensin system are present in the central nervous system[5], suggesting that local synthesis of AII could also occur in brain[5]. AII receptors have been characterized in membrane fractions of mammalian brain, including areas not accessible to peripheral AII[1,7,8,10,14,16].

Binding sites for drugs and neurotransmitters can be localized and quantitated in brain by radioautography using [^{3}H]ultrofilm, computerized densitometry and appropiate ligands[3,9,11,15]. Precise quantitation is essential for studies on the kinetic properties and physiological regulation of binding sites. In vitro studies demonstrated that the AII agonist ^{125}I-[Sar^1]AII binds to a single class of high affinity, saturable sites in rat brain, with ligand specificity similar to that or ^{125}I-AII[8]. In addition, AII and [Sar^1]AII showed complete cross-displacement in binding experiments to rat hypothalamic membranes[7,8]. These characteristics made the [Sar^1]AII analogue a suitable ligand for AII binding site studies. AII binding sites have been localized in specific rat brain nuclei by radioautographic non-quantitative techniques with the use of ^{125}I-[Sar^1]AII[8].

We report the quantitative determination of AII binding sites in rat brain and pituitary gland after incubation with ^{125}I-[Sar^1]AII. This method involves the use of ^{3}H-sensitive film for radioautography[11,15], selection of appropriate film exposure times, quantitation of optical densities by computerized microdensitometry[3], and comparison to ^{125}I-standards prepared from brain tissue.

Rats were sacrificed by decapitation and brains and pituitary glands immediately removed and frozen by immersion in isopentane at −30 °C. Frozen, 8 μm-thick sections were cut in a cryostat at −14 °C and thaw-mounted onto subbed glass slides. Binding sites for AII were labeled in vitro by incubation with ^{125}I-[Sar^1]AII (a gift from Dr. M. Khosla, Cleveland Clinic, Cleveland, OH, iodinated by a modified Cloramine-T method at Melloy Laboratories, Springfield, VA, act. 1666 Ci/mmol). Tissue sections were

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preincubated for 15 min at 20 °C in 5 ml of 10 mM sodium phosphate buffer, pH 7.4, containing NaCl (120 mM), NA_2 EDTA (5 mM), bacitracin (0.1 mM) and bovine serum albumin (0.2%), and then incubated for 60 min in fresh buffer containing ^{125}I-[Sar^1]AII (0.469 μCi) at a 56 pM final concentration. In preliminary experiments, this was sufficient time for ^{125}I-[Sar^1]AII to equilibrate with AII binding sites. Non-specific binding was determined in the presence of 1 μM unlabeled AII (Sigma Chemicals, MO). After incubation, the slides were washed 4 times (60 s each) with ice-cold 50 mM Tris-HCl buffer, pH 7.56, and dried under a cold stream of air. Preliminary experiments also showed that the use of a low ligand concentration maximized the differences between specific and non-specific binding and allowed us to selectively study the high-affinity binding sites present in the brain[1,8,14].

Sets of ^{125}I-standards were prepared as described for ^{3}H-standards[15]. Known amounts of increasing concentrations of ^{125}I-AII (spec. act. 1280 ci/mmol, New England Nuclear, Boston, MA) were thoroughly mixed with brain tissue aliquots previously ground to a paste, placed as blocks of tissue on microtome specimen holders, and frozen on dry ice. Tissue sections, 8 μm thick, were cut in a cryostat at −14 °C and thaw-mounted onto subbed glass slides[11,15]. Parallel sets of standards were used for determination of protein concentrations[6] and radioactivity.

Tissue sections after incubation and ^{125}I-standards were placed in cassettes (CGR Med., Baltimore, MD) and opposed against [^{3}H]ultrofilm (LK8 Industries, Rockville, MD) at room temperature for varied periods. The films were developed at 20 °C for 5 min with undiluted D19 Kodak developer[15] and optical densities were quantitated by computerized densitometry[3]. Cresyl violet-stained brain sections were used for reference[15]. The optical densities observed in the brain regions and pituitary gland were related to the concentration of radioactivity present by comparison with standard curves generated by processing sets of standards with each of the radioautograms[15]. Each set of standards contained concentrations of radioactivity/μg protein varying from 9.76 to 1008 dpm. A typical radioautographic image of standards, with computerized densitometry, after 4 days exposure to [^{3}H]ultrofilm is shown in Fig. 2 (left panel). There was a linear relationship between the

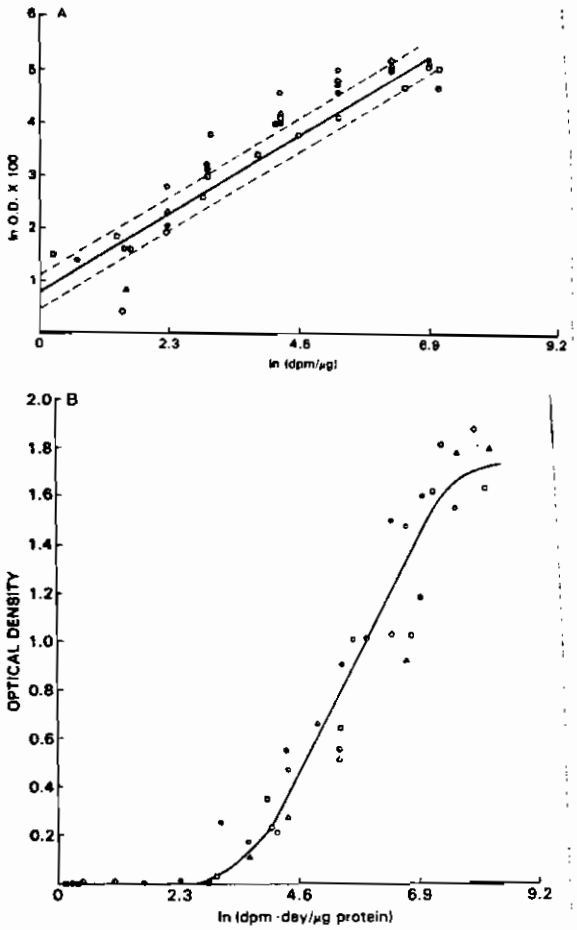
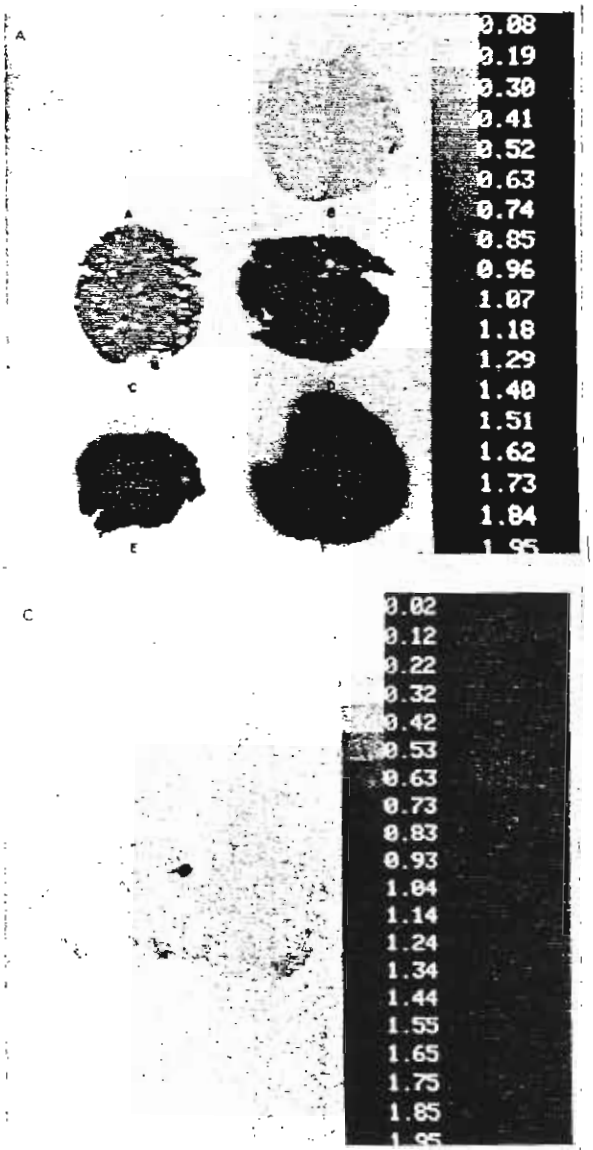


Fig. 1. A: ln–ln plot of o.d. vs radioactivity (dpm/μg protein) in standards. Exposure times were 0.3, 1, 3 and 4 days. Each point represents the average of 6 optical density readings from radioautograms generated from triplicate standard curves and prepared for each film uitilized for the quantitation of AII receptors. Dashed lines represent the standard error of estimate of x from y. The general equation of the straight line was, for 39 pairs of data: y: 0.6654X + 0.73; r = 0.8931; F = 68.4 ($P < 0.0001$). B: characteristic curve of [^{3}H]ultrofilm with ^{125}I-standards; o.d. are plotted as a function of ln of dpm/μg protein × exposure time. Symbols are the same as in left panel.

ln of o.d. and the ln of the concentration of radioactivity at any exposure of [^{3}H]ultrofilm to the standards (Fig. 1, left panel). The molar quantities of ligand bound/mg of tissue were determined by interpolating the o.d. in the straight line obtained from the ln–ln standard curve (Fig. 1, left panel) and correcting according to the equation:

$$\frac{\text{mmol}}{\text{Ci (spec. act.}} \times \frac{1\ \text{Ci}}{2.22 \times 10^{12}\ \text{dpm}} \times 10^3 = \text{fmol/mg}$$



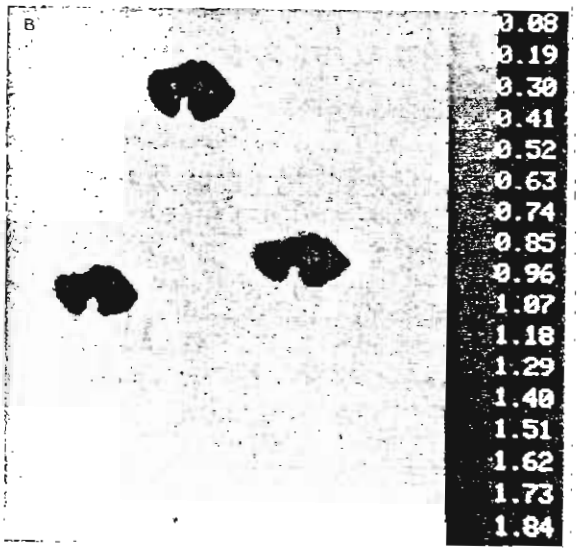


Fig. 2. A: autoradiographic image with computerized densitometry of a set of ^{125}I-standards after 4 days exposure to [^{3}H]ultrofilm. Each section contains a different amount of radioactivity/μg of protein. A, 9.76 dpm; B; 20.8 dpm; C, 72.8 dpm; D, 200.5 dpm; E, 536.4 dpm; F, 1008.0 dpm. (See also Fig. 1A). B: autoradiographic image with computerized densitometry of AII binding sites in rat forebrain. The exposure time of ultrofilm was 3 days. Arrow points to subfornical organ. (See also Table I). C: autoradiographic image with computerized densitometry of AII binding sites in rat pituitary gland. Exposure time of ultrofilm was 1 day. Arrow points to posterior pituitary.

The semi-log plot of the optical densities read from the film vs the exposure time and amount of radioactivity present in the standards generated the so-called characteristic curve of the film. This relationship was similar to that established for ^{3}H-standards[15] (Fig. 1, right panel). The slope of the curve gives a measure of the photographic contrast. At o.d. of 0.2 units or less, the changes in the slope as a function of exposure time are very small, and discrimination between different values is difficult. At o.d. greater than 1.6 units, the film rapidly saturates and no useful data can be obtained. A linear relationship ($r = 0.934$) exists between o.d. and dpm/μg of protein × time of exposure for values between 0.3 and 1.6 o.d. units. In this case, the contrast is high between different o.d. values, making it easy to discriminate between small differences in concentration of radioactivity, i.e. between different concentrations of binding sites for a particular structure (Fig. 1, right panel, and Fig. 2, left panel). With this information, the exposure time which produces the optimal contrast can be estimated for a particular tissue section. It is therefore desirable, in preliminary experiments, to expose the labelled tissue sections for varying lengths of time to determine the adequate conditions for optimum contrast (o.d. between 0.6 and 1.4 units for each particular structure).

The accuracy of this method was tested by studying the regional distribution of AII binding sites in forebrain areas and in the pituitary gland. Our results confirm and expand previous observations[1,2,5,8,10,14], indicating a very specific neuroanatomical distribution for AII binding sites. High number of AII binding sites occurred in specific nuclei of the hypothala-

TABLE I

Quantitative determination of AII binding sites in rat brain nuclei and pituitary gland

Results are means ± S.E.M. of 4 determinations from individual rats. Non-specific binding (5–10% for brain areas, 2% for anterior pituitary) was substracted from all readings. ^{125}I-[Sar^1]AII concentration was 56 pM. o.d. measurements were converted into fmol/mg protein as described in text.

Region	*Apparent concentration of AII binding sites (fmol/mg protein)*
Brain	
Organon subfornicalis (A 5780 μm)*	33.2 ± 3.8
Organon vasculosum laminae terminalis (A 7470 μm)	14.3 ± 0.3
Nucleus suprachiasmatis (A 6060 μm)	6.7 ± 0.5
Nucleus paraventricularis (A 5660 μm)	6.2 ± 0.2
Nucleus septalis lateralis (A 7470 μm)	0.81 ± 0.25
White matter (corpus callosum, A 6570 μm)	0.35 ± 0.08
Pituitary gland	
Anterior lobe	40.5 ± 0.8
Anterior lobe (non-specific)	0.70 ± 0.07

* Antero-posterior axis, according to König and Klippel[4]

mus and in circumventricular organs (Table I). Specific binding of AII was low or absent in most other forebrain areas studied, including the cerebral cortex and striatum (not shown) and the white matter (Table I). Highest AII binding site concentrations were found in the organon subfornicalis (95-fold higher than in white matter, Fig. 2, middle panel), and in the organon vasculosum laminae terminalis (40-fold higher than in white matter), followed by the nuclei suprachiasmatis and paraventricularis (20-fold higher than in the white matter) (Table I). The anterior pituitary gland had a very high concentration of AII binding sites, of the same order of magnitude than that of the organon subfornicalis (Table I and Fig. 2, right panel). In contrast, no substantial amounts of AII binding sites were detected in the posterior pituitary (Fig. 2, right panel).

The AII binding sites described here correspond to brain sites of action for AII[2,5,13]. Both the organon subfornicalis and the organon vasculosum laminae terminalis are involved in the drinking and blood pressure responses to peripheral AII[2,13]. These receptor-rich areas, lacking blood–brain barrier, are accessible to blood-borne AII[13]. Other areas, such as the nuclei paraventricularis and suprachiasmatis, are not accessible to circulating AII and may represent binding sites for centrally formed AII.

It is of interest to note that the organon subfornicalis contains the highest angiotensin-converting enzyme activity in the rat brain[12], as well as the highest concentration of AII binding sites. This area may represent a point of linkage between the peripheral and brain angiotensin systems. The use of the present technique will allow the detailed kinetic study and precise quantitation of changes in AII binding sites in selective brain nuclei and areas of the rat brain under different physiological conditions.

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