Evidence for the AT₁ Subtype of the Angiotensin II Receptor in the Rat Submandibular Gland

Shigeru MATSUBARA,^a Kiyoshige SAITO,^b Yasuo KIZAWA,^b Masakazu SANO,^c Masami OSAWA,^d Keishi IWAMOTO,^a and Hajime MURAKAMI^{*,b}

Department of Physical Education^a and Physiology & Anatomy,^b Nihon University College of Pharmacy, Funabashi, Chiba 274–8555, Japan, Department of Oral Physiology, Showa University School of Dentistry,^c Shinagawa-ku, Tokyo 142–8555, Japan, and Division of Health & Sport Sciences, Nihon University College of Industrial Technology,^d Mimomi Campus, Shinsakae, Narashino, Chiba 275–8576, Japan. Received March 6, 2000; accepted June 29, 2000

We have characterized angiotensin II (Ang II) receptor subtypes on rat submandibular gland membranes using a radioligand binding assay. [³H]Ang II binding to the membrane fractions exhibited both high ($K_d=0.08$ nM, $B_{max}=2.19$ fmol/mg protein) and low ($K_d=4.19$ nM, $B_{max}=13.7$ fmol/mg protein) affinity. Ang II, Ang III and saralasin completely displaced the [³H]Ang II binding, whereas CV-11974, an AT₁ receptor antagonist and PD123319, an AT₂ receptor antagonist maximally displaced up to approximately 87 and 13% of the total binding, respectively. [³H]DuP753 binding to the membrane fractions exhibited a single population of binding site with a K_d of 4.22 nM and B_{max} of 3.77 pmol/mg protein. Ang II, Ang III and CV-11974 completely displaced the [³H]DuP753 binding with slope factors near unity, but PD123319 did not. These findings suggest that rat submandibular gland membranes contain predominantly the AT₁ receptor subtype.

Key words angiotensin II; AT₁ receptor subtype; DuP753; CV-11974; submandibular gland

Angiotensin II (Ang II) is an important regulatory peptide with multiple physiological functions. A stimulatory effect of Ang II on electrolyte transport has been observed in a number of exocrine glands.^{1–3} Ang II stimulates the release of endothelin-1 (ET-1) from endothelial cells.⁴ Ang II and ET-1 act as growth factors in producing the submandibular gland hypertrophy that develops in rats treated repeatedly with β_1 -adrenoceptor agonists. Ang II stimulates ET-1 production in the glandular tissue.⁵ The multiple effects of Ang II are mediated through different Ang II receptor subtypes that are expressed in a tissue- and species-specific manner.⁶ Two main subtypes of Ang II receptors, AT₁ and AT₂, have been identified based on differences in their affinity for selective antagonists. AT₁ receptors have a high affinity for losartan (DuP753), while AT₂ receptors have a high affinity for PD123319 and CGP42112A.^{6–8} Candesartan (CV-11974) recently reported by Noda *et al.*⁹ is a powerful AT₁ receptor-selective antagonist in rabbit aortic membranes and has no appreciable affinity for AT₂ receptors found in bovine cerebellum. In the isolated submandibular gland of the rat, Ang II-stimulated ET-1 production is inhibited by an AT₁/AT₂ receptor antagonist, saralasin, but not by a specific AT₂ receptor antagonist, PD123319, suggesting that the production is mediated through AT₁ receptors.¹⁰ Ang II-induced ETs production in human mesangial cells in the presence of insulin is prevented by DuP753, suggesting that this tissue response is mediated through AT₁ receptors.¹¹ Ang II and arginine vasopressin stimulate ET-1 production in cultured rat mesangial cells. The Ang II-induced ET-1 production is mediated through AT₁ receptors.¹²

These findings prompted us to investigate Ang II receptors present in rat submandibular gland membranes. The present study was designed to investigate directly the subtypes of Ang II receptors using radioligand binding assay.

MATERIALS AND METHODS

Materials Seven-week-old male Wistar rats (Nihon Clea

Inc. Japan) were used throughout the present experiment. [³H]Angiotensin II (1.94 TBq/mmol) and [³H]DuP753 (1.62 TBq/mmol) were obtained from NEN Life Science Products Inc. (Boston, MA., U.S.A.); PD123319 ditrifluoroacetate was from Research Biochemicals Int. (Natick, MA., U.S.A.); leupeptin and pepstatin A were from Peptide Inst. Inc. (Osaka, Japan); angiotensin II acetate salt, angiotensin III and [Sar¹, Val⁵, Ala⁸]-angiotensin II were from Sigma Chemical Co. (St. Louis, MO. U.S.A.); 30% polyethylenimine p-70 solution was from WAKO Pure Chemicals Inc. (Tokyo, Japan); ethylenediamine-*N,N,N',N'*-tetraacetic acid disodium salt dihydrate (EDTA) was from Dojindo Laboratories Co. Ltd. (Kumamoto, Japan). Candesartan (CV-11974) was donated by Takeda Chemical Industries Ltd. (Osaka, Japan). All other chemicals used were of analytical grade and obtained from commercial sources.

Preparation of Gland Membranes Membranes from rat submandibular glands were prepared by homogenization of the minced glands in 15–20 volume of buffer A [50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 μ M leupeptin, 10 μ M pepstatin A] using a glass-Teflon homogenizer, and then the homogenized tissues were centrifuged at 1000 *g* for 30 min at 4 °C. The resulting supernatant was further centrifuged at 48000 *g* for 30 min at 4 °C. The resulting pellets were washed twice in buffer B [buffer A containing 5 mM MgCl₂], and finally resuspended in buffer C [buffer B containing 250 mM sucrose]. The membrane preparations were stored at –80 °C until use. The protein concentrations of the membrane preparations were determined according to the method of Lowry *et al.*¹³ using bovine serum albumin (BSA) as a standard.

Binding Assay The membrane preparations (40–100 μ g/tube) were incubated for 60 min at 25 °C with various concentrations of [³H]Ang II (0.25–12 nM) or [³H]DuP753 (1–10 nM) for the saturation binding experiments, and with 2 nM [³H]Ang II or 2 nM [³H]DuP753 for displacement binding experiments, in the presence of unlabelled Ang II, Ang III, saralasin, PD123319 or CV-11974 in 250 μ l of buffer [50

* To whom correspondence should be addressed. e-mail: hmurajyo@pha.nihon-u.ac.jp

Fig. 1. Saturation Binding (A) and Scatchard Plot (B) of [^3H]Ang II Binding to Rat Submandibular Gland Membrane Fractions

Increasing concentrations of [^3H]Ang II (0.25–12 nM) were bound to membrane fractions. The results of Scatchard analysis were subjected to nonlinear regression analysis using the LIGAND program to obtain the two asymptotes. The data shown are the means of triplicate determinations in a representative experiment.

mm Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM MgCl_2 , 0.2% (w/v) BSA, 10 μM leupeptin and 10 μM pepstatin A]. The incubation mixtures were immediately filtered through Whatman GF/B glass fiber filters presoaked in buffer [50 mM Tris-HCl (pH 7.4), 0.1% (w/v) BSA, 0.4% polyethylenimine] and dried. The radioactivity on the filter was counted in 5 ml of scintillator (ASC II, Amersham) using a liquid scintillator spectrometer (TRI-CARB 2000 CA, Packard). Nonspecific binding was determined in the presence of 2 μM unlabelled Ang II. Each experiment was performed in triplicate. Under these experimental conditions, the nonspecific binding was 20–30% of total binding. The 50% inhibitory concentration (IC_{50}) of a drug that displaced 50% of the specific binding of [^3H]Ang II or [^3H]DuP753 was determined by linear regression analysis of the displacement curve converted through logit transformation.

Statistical Analysis Data are expressed as means $\pm$ S.E.M. for 3 to 5 experiments. The data were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test with PRISM3 software program (GraphPad Software Inc., San Diego, CA, U.S.A.). Differences were considered to be statistically significant when the p value was less than 0.05. Saturation binding data were analyzed by the iterative curve fitting program LIGAND¹⁴) to determine the maximum number of binding sites (B_{max}) and the affinity (K_d) of the labeled ligand.

RESULTS

[^3H]Ang II Binding to Rat Submandibular Gland Membrane Fractions [^3H]Ang II binding to rat submandibular gland membrane fractions was specific and saturable (Fig. 1A). The Scatchard plots of the saturation binding data was curvilinear, suggesting the presence of two or more distinct binding sites with different affinities (Fig. 1B). Nonlinear regression analysis of the data indicated a high-affinity binding site with a dissociation constant (K_d) of 0.08 ± 0.03 nM and a maximum binding (B_{max}) of 2.19 ± 0.58 fmol/mg protein ($n=3$), and a low-affinity binding site with a K_d of 4.19 ± 1.28 nM and a B_{max} of 13.7 ± 3.72 fmol/mg protein ($n=3$), as shown in Fig. 1B.

Displacement of [^3H]Ang II Binding by Ang II, Ang III,

Fig. 2. Displacement of [^3H]Ang II Binding to Membrane Fractions by Ang II (●), Ang III (○), CV-11974 (Δ), Saralasin (◇) or PD123319 (■)

Each curve represents the mean of 3–5 independent experiments.

CV-11974, Saralasin or PD123319 Ang II, Ang III and saralasin displaced completely the [^3H]Ang II binding in a monophasic and concentration-dependent manner. The slope factors (pseudo Hill-coefficient), however, were less than unity, as shown in Fig. 2 and Table 1. On the other hand, CV-11974 and PD123319 displaced the [^3H]Ang II binding maximally up to 87.2 ± 2.87 and $13.1 \pm 1.95\%$ respectively. The slope factor for CV-11974 was not significantly different from unity, as shown in Fig. 2 and Table 1.

[^3H]DuP753 Binding to Rat Submandibular Gland Membrane Fractions [^3H]DuP753 binding to the membrane fractions was specific and saturable. Scatchard analysis of the saturation binding data indicated a straight line with a K_d of 4.22 ± 0.70 nM and B_{max} of 5.12 ± 0.57 pmol/mg protein. The Hill-coefficient of the data was not significantly different from unity ($n_H = 0.99 \pm 0.07$). These data indicate a single population of [^3H]DuP753 binding site in the gland membranes, as shown in Figs. 3A and B.

Displacement of [^3H]DuP753 Binding by Ang II, Ang III, CV-11974 or PD123319 Ang II, Ang III and CV-11974 displaced completely the [^3H]DuP753 binding in a monophasic and concentration-dependent manner respectively. The slope factors were not significantly different from

Table 1. 50% Inhibitory Constants (IC₅₀) and Slope Factors of Various Ligands for Displacement of [³H]Ang II or [³H]DuP753 Binding to Rat Submandibular Gland Membrane Fractions^{a)}

Ligand	Angiotensin II			DuP753		
	<i>n</i>	IC ₅₀ (nM)	Slope factor	<i>n</i>	IC ₅₀ (nM)	Slope factor
Angiotensin II	5	2.05±0.23	0.65±0.04 [#]	3	3.36±1.51	1.06±0.07
Saralasin	3	8.15±0.79	0.56±0.16 [#]		n.d.	
Angiotensin III	3	82.4±3.92*	0.67±0.14 [#]	4	170±26.7*	1.04±0.07
CV-11974	3	10.3±3.67	0.94±0.04	3	5.50±0.79	1.00±0.03
PD123319	4	>10000		3	>10000	

a) IC₅₀ values were obtained by linear regression of log-logit plots of displacement binding curves. Each value represents the mean±S.E.M. of 3–5 independent experiments (*n*). * Significant difference from IC₅₀ of Ang II for receptors labeled by [³H]Ang II or [³H]Dup753 at *p*<0.05. [#] Significant difference from unity at *p*<0.05. n.d.; not determined.

Fig. 3. Saturation Binding (A) and Scatchard Plot (B) of [³H]DuP753 Binding to Rat Submandibular Gland Membrane Fractions
Increasing concentrations of [³H]DuP753 (1.0–16 nM) were bound to membrane fractions. The data shown are means of triplicate determinations in a representative experiment.

Fig. 4. Displacement of [³H]DuP753 Binding to Membrane Fractions by Ang II (●), CV-11974 (Δ), Ang III (○) or PD123319 (■)
Each curve represents the mean of 3 independent experiments.

unity, indicating a single population of binding site. However, PD123319 showed no displacement even at a concentration of up to 10 μM, as shown in Fig. 4 and Table 1.

DISCUSSION

In our previous studies, we found that Ang II causes an increase in the content of ET-1,⁵⁾ and that the increase in the intensity of immunoreactive ET-1 induced by Ang II is inhibited by saralasin, but not by PD123319, in rat submandibular gland tissues.¹⁰⁾ These findings suggest that the Ang II-in-

duced ET-1 production in the gland tissues is mediated by the AT₁ subtype of Ang II receptors.

In the present study, therefore, we investigated Ang II receptors present in the submandibular gland membranes. [³H]Ang II binding to the membrane fractions exhibited two binding sites, having two dissociation constants (*K_d*) of high and low affinity for Ang II.

In order to explain the above results, displacement experiments for [³H]Ang II binding were performed using Ang II, Ang III, saralasin, an AT₁/AT₂ subtype receptor antagonist, CV-11974, a selective AT₁ receptor subtype antagonist,^{9,15)} or PD123319, a selective AT₂ receptor subtype antagonist. Ang II, Ang III and saralasin completely displaced the [³H]Ang II binding to the membrane fractions with the following order of potency for binding displacement: Ang II>saralasin>Ang III. The AT₁ receptor subtype is characterized by the angiotensins with the following order of potency for displacement of the binding: Ang II≥saralasin>Ang III>Ang I, whereas the AT₂ receptor subtype is characterized as: Ang III>Ang II≥saralasin>Ang I.^{16,17)} The order of displacement potencies obtained by Ang II, Ang III and saralasin using the gland membrane fractions were commensurate with those for [³H]Ang II to the AT₁ receptor subtype, but their slope factors were significantly different from unity (Table 1), suggesting that the membranes contain two different binding sites for Ang II. In addition, CV-11974 and PD123319 produced a maximal displacement up to approximately 87% and 13% of the total [³H]Ang II binding, respectively, and moreover the slope factor of the displacement curve for CV-11974 was not significantly different from unity. These re-

sults suggest that approximately 87% of all Ang II binding sites may be the AT₁ subtype and 13% may be the AT₂ subtype. [³H]DuP753 is a potent and highly specific radioligand for the AT₁ receptor subtype^{16,18)} and its affinity is affected only slightly by changes in the protein content of the assay.¹⁸⁾ Therefore, [³H]DuP753 is utilized to localize the AT₁ receptor subtype present in the gland membrane.

The binding of [³H]DuP753 to the membrane fractions was specific and saturable. Scatchard analysis of the binding data gave a straight line, indicating the presence of a single population of binding site. Ang II, Ang III and CV-11974 completely eliminated the binding in a monophasic and concentration-dependent manner and their slope factors were not significantly different from unity, indicating that these ligands bind to the AT₁ receptor subtype present in the gland membranes; however, PD123319 did not have any effect.

These findings show that the gland membranes contain predominantly the AT₁ receptor subtype, thus supporting our previous data indicating that the Ang II-stimulated ET-1 production in the gland may be mediated through the AT₁ receptor subtype.¹⁰⁾ The AT₁ receptor subtype may be related to the development of isoproterenol-induced submandibular gland hypertrophy in the rat, since Ang II and ET-1 presumably act as growth factors in response to isoproterenol in rats.⁵⁾

In conclusion, the present study has demonstrated that Ang II receptors present in the rat submandibular gland membranes are predominantly of the AT₁ receptor subtype.

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