ABSTRACT—We characterized the angiotensin II (AII) receptor in human aortic smooth muscle cells (HASMCs). This receptor binds [125I]Sar^1,Ile^8-angiotensin II with a high affinity of 0.20±0.04 nM and a low capacity of 5.3±0.4 fmol/mg protein (230±17 sites/cell). Based on the Kᵢ values, the ranking order of [125I]Sar^1,Ile^8-AII binding inhibition was as follows: Sar^1,Ile^8-AII > AII > Dup 753 > AII > AI > PD 123319. The addition of AII to HASMCs induced a rapid, transient increase in intracellular free Ca^{2+} concentration followed by a lower, sustained phase. When extracellular Ca^{2+} was removed by adding 3 mM EGTA, this initial transient increase was not changed, but the sustained phase was abolished. These results revealed AII receptors in HASMCs to be of the type 1 receptor subtype, which induce Ca^{2+} mobilization mainly from intracellular Ca^{2+} stores.

Keywords: Angiotensin II, Receptor, Aorta (human), Smooth muscle cell

Materials and Methods

Materials

Angiotensin II, Sar^1,[125I]-Tyr^4,Ile^8-, (S.A. 81.4 TBq/mmol) was purchased from NEN-Du Pont (Boston, MA, USA). Human aortic smooth muscle cells (HASMCs) were obtained from Kurabo (Osaka). The nonpeptide antagonists Dup 753, (2-n-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)-methyl]imidazole) and PD 123319, ((S)-1-[[4-dimethylamino)-3-methylphenyl]methyl-5-(diphenylacetyl)-4, 5, 6, 7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid) were prepared by Yamanouchi Pharmaceutical Co., Ltd.

Cell culture

The HASMCs were cultured to confluence in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum plus 4 μg/ml of gentamicin under 5% CO₂ - 95% air at 37°C. The medium was changed every 2–3 days and the cells subcultured every 5–7 days after trypsinization. All cells used in these experiments were under 15–25 population doubling level (PDL). For experimental purposes, confluent cells were serum-deprived by culture in DMEM containing 0.3% bovine serum albumin (BSA) for 24 hr. HASMCs were identified by their typical "hill-and-valley" morphology and by immunofluores-
cence using a monoclonal antibody against human α-smooth muscle actin, which is a specific marker of differentiated smooth muscle cells.

**Membrane preparation**

HASMCs in a 500-cm² plastic dish were washed twice with phosphate-buffered saline, scrapped into a solution containing 250 mM sucrose, 10 mM MgCl₂, and 50 mM tris/HCl (pH 7.4), and homogenized with a polytron at 4°C. After centrifugation at 2,000 × g for 10 min at 4°C, the supernatant was centrifuged at 40,000 × g for 20 min at 4°C, and the resulting pellet was resuspended in a 50 mM tris/HC1 (pH 7.4) and 10 mM MgCl₂ and stored at -80°C until use.

**Binding assay**

Incubations were carried out at 37°C for 60 min in 0.5 ml of incubation buffer (10 mM Na₂HPO₄, 5 mM EDTA, 100 mM NaCl, 0.1 mM PMSF, 0.2 mg/ml soybean trypsin inhibitor, 0.018 mg/ml o-phenanthroline, 2 mg/ml BSA, 0.14 mg/ml bacitracin, pH = 7.4). The assay was started by the addition of [125I]Sar¹,11e⁸-AII, (60 pM) to the membranes from HASMCs (100 µg protein). Incubation was terminated by rapid filtration through Whatman GF/C filters (Maidstone, Kent, UK) using a Brandel cell harvester (Gaithersburg, MD, USA). The filter was then rinsed 3 times with 3 ml of 10 mM tris-HCl (pH 7.4), 0.15 M NaCl and 0.01% BSA. Radioactivity retained on the filters was counted by a r-counter (ARC-950, Aloka, Tokyo). Nonspecific binding was determined in the presence of 1 pM Sar¹, 11e⁸-AII. Data were analyzed by the previously described method (11).

**Measurements of Ca²⁺**

Confluent HASMCs grown on a coverslip were incubated for 60 min in 5 µM fura-2/AM in Hanks’ balanced salt solution (HBSS) containing 0.05% BSA and 10 mM glucose. They were washed twice with HBSS, and the coverslips were secured in a quartz cuvette in a CAF-100 fluorescence spectrometer (Japan Spectrometer Co., Tokyo) equipped with a thermostatically controlled (30°C) cell holder. Excitation wavelength was set at 340 and 380 nm and emission wavelengths, at 500 nm. The ratio of fluorescence (R) of the sample at 340 and 380 nm was monitored for 1–3 min until the [Ca²⁺], signal stabilized and basal [Ca²⁺], measurement was obtained. Thereafter, cells were subjected to a specific agonist (e.g., AII), and [Ca²⁺], signals were recorded for an additional 5 min. The maximum ratio (Rmax) for the fura-2 in each coverslip was measured by adding 10 µM ionomycin, and the ratio in the absence of Ca²⁺ (Rmin) was measured by then adding 3 mM EGTA. From the ratio of fluorescence at 340 and 380 nm, the [Ca²⁺], was determined as described by Grynkiewicz et al. (12) using the following equation:

\[
[Ca^{2+}] = K_d \times [\text{(R - R}_{\text{min}})/\text{(R}_{\text{max}} - \text{R})] \times \beta
\]

The term \( \beta \) is the ratio of fluorescence of fura-2 at 380 nm in zero and saturating Ca²⁺. Kd is the dissociation constant of fura-2 for Ca²⁺, assumed to be 224 nM (12).

**RESULTS**

To assess the pharmacological properties of the AII receptor in human-derived cells, HASMCs were tested for their ability to bind to [125I]Sar¹,11e⁸-AII. Figure 1 shows the specific binding of [125I]Sar¹,11e⁸-AII at the concentration of 0 to 1.5 nM in HASMC membrane. Scatchard plots revealed that the [125I]-Sar¹,1le⁸-AII binding site consisted of only a mono-component with high affinity (0.20 ± 0.04 nM) and low capacity (5.3 ± 0.4 fmol/mg protein, 230 ± 17 sites/cell). Figure 2 shows the competition curves of various ligands with [125I]-Sar¹,1le⁸-AII as the AII receptor antagonist. Radioligand binding was potent inhibited by AII, Sar¹,1le⁸-AII and the AT₁ receptor-selective antagonist Dup 753 (6). In contrast, PD 123319 (9), a type 2 AII receptor-selective antagonist, had no effect on the binding of [125I]-Sar¹,1le⁸-AII at doses as high as 1 × 10⁻⁵ M (Table 1).

AII-induced Ca²⁺ mobilization in HASMCs was examined by measuring changes in intracellular free Ca²⁺ concentration with fura-2 as a Ca²⁺ indicator. The addition of 1 × 10⁻⁴ M AII evoked a rapid, transient increase in [Ca²⁺], followed by a lower, sustained phase which persisted for at least 5 min. This [Ca²⁺], response was blocked
Fig. 2. Competitive inhibition curves for [125I]-AII,Sar',Ile8 binding to HASMC membranes. HASMC membranes were incubated with [125I]Sar',Ile8-angiotensin II in the presence of the indicated concentrations of unlabeled Sar',Ile8-angiotensin II (●), angiotensin II (●), angiotensin I (●), angiotensin III (●), Dup 753 (●), and PD 123319 (●). Each point represents the mean ±S.E. of three or four experiments.

Table 1. \( K_s \) values of angiotensin analogues in HASMC membranes

| Ligand     | \( K_s \) (nM) | Hill | n   |
|------------|----------------|------|-----|
| AII        | 372 (346–401)  | 1.00 | 3   |
| AII        | 3.6 (3.4–3.8)  | 0.73 | 3   |
| Sar',Ile8-AII | 1.6 (1.5–1.6)  | 0.81 | 4   |
| AIII       | 87 (82–91)     | 0.72 | 3   |
| Dup 753    | 17 (16–17)     | 0.84 | 3   |
| PD 123319  | >10,000        |      | 3   |

Data are the mean and 95% confidence limits of three or four separate experiments.

by Dup 753 but not PD 123319, indicating an AT1 receptor-mediated event (Fig. 3). In contrast, when extracellular Ca\(^{2+}\) was removed by adding 3 mM EGTA, the sustained phase was completely abolished, but the initial transient [Ca\(^{2+}\)] response was not changed. When the cell was pretreated with 1 × 10\(^{-3}\) M nicardipine, a blocker of voltage-dependent L-type (dihydropyridine-sensitive) Ca\(^{2+}\) channels, neither phase was affected (Fig. 3).

**DISCUSSION**

The major aim of the present study was to characterize the binding of \([^{25}\text{I}]\text{Sar}'\text{Ile}^8\text{-AII}\) to AII receptors present in HASMC membranes. Scatchard analysis of specific \([^{25}\text{I}]\text{Sar}'\text{Ile}^8\text{-AII}\) binding revealed that its binding sites on the HASMC membrane was mono-component with high affinity and low capacity (Figs. 1 and 2). Based on the \( K_s \) values, the ranking order of \([^{25}\text{I}]\text{Sar}'\text{Ile}^8\text{-AII}\) binding inhibition was as follows: Sar',Ile8-AII > AII > Dup 753 > AIII > A > PD 123319 (Table 1). This inhibition profile agreed well with those obtained using expressed AT1 receptors from rats (9) and humans (13). These results suggest that the AII receptors in HASMCs are of the AT1 type.

Agents that fully inhibit AII receptor binding should also completely antagonize second messenger production. The effects of Dup 753 and PD 123319 on the increase in [Ca\(^{2+}\)]i, induced by AII, were examined, and the results were consistent with the data from the binding experiments.

AII-induced Ca\(^{2+}\) mobilization was separated into two phases. The transient increase in [Ca\(^{2+}\)]i results from mobilization of intracellular Ca\(^{2+}\) stores mediated by inositol-1,4,5 triphosphate, whereas the sustained phase is dependent on Ca\(^{2+}\) influx across the plasma membrane via dihydropyridine-insensitive Ca\(^{2+}\) channels. In HASMCs, the AII-induced [Ca\(^{2+}\)]i increment resulted mostly from the mobilization of intracellular Ca\(^{2+}\) stores. The Ca\(^{2+}\) channel agonist BayK 8644 was used to confirm the presence of dihydropyridine-sensitive Ca\(^{2+}\) channels, but no marked sustained increase in [Ca\(^{2+}\)]i was observed (data not shown). Furthermore, a slight increase in [Ca\(^{2+}\)]i in response to 100 mM KCl was seen (data not shown). These results appeared to indicate the presence of scattered voltage-dependent L-type (dihydropyridine-sensitive) Ca\(^{2+}\) channels in the HASMC membrane. In rat aortic vascular smooth muscle cells, AT1 receptors are reported to bind with voltage-dependent L-type Ca\(^{2+}\) channels (7, 14). Gardner et al. (15) reported that density or activity of voltage-sensitive Ca\(^{2+}\) channels in cultured human umbilical artery vascular smooth muscle cells is small. They suggest that a small increase in Ca\(^{2+}\) uptake induced by depolarization was taken up by the endoplasmic reticulum or recycled back to the medium. The lack of Ca\(^{2+}\) channels in our study might be explained by loss during culture or a species difference.

Previous reports have demonstrated that only the AT1 receptor mediates the known biologic effects of AII, such as blood pressure increases in vivo (16, 17), aldosterone secretion (17), and contraction of the aorta (18) in vitro. The inhibition of AII-dependent formation of inositol triphosphate by a nonpeptide AT1 ligand in hepatocytes (19) and rat mesangial cells (20) also suggests that AT1 receptors are linked to phospholipase C. These observations are supported by our AII receptor finding in HASMCs.

In summary, our study provides the first information on AII binding sites in human aortic smooth muscle derived normal cells. This receptor is of the type 1 AII recep-
tor subtype and mediates the well-known effects of AII in these preparations, including Ca\(^{2+}\) mobilization. The availability of these cells will facilitate studies on physiological and pathological conditions in humans.

Acknowledgments
The authors are thankful to Takako Ichizawa for technical assistance.

Fig. 3. The effect of AII on intracellular free Ca\(^{2+}\). Fura-2-loaded HASMCs were exposed to 1 x 10\(^{-10}\) M AII (A), 1 x 10\(^{-9}\) M AII (B), or 1 x 10\(^{-8}\) M AII (C–H) in the presence of 3 mM EGTA (D), Dup 753 (1 x 10\(^{-10}\) M, 1 x 10\(^{-9}\) M) (E, F), 1 x 10\(^{-5}\) M PD 123319 (G), or 1 x 10\(^{-5}\) M nicardipine (H). EGTA, Dup 753 and PD 123319 were added to the bathing solution 5 min before stimulation with AII. Nicardipine was added 10 min before the addition of AII. Representative tracings are shown; similar results were obtained from four separate experiments.

REFERENCES
1 Regoli, D., Park, W.K. and Rioux, F.: Pharmacology of angiotensin. Pharmacol. Rev. 26, 69–123 (1974)
2 Peach, M.J.: Renin-angiotensin system: biochemistry and mechanisms of action. Physiol. Rev. 57, 313–370 (1977)
3 Barrett, P.Q., Bollag, W.B., Isales, C.M., McCarthy, R.T., and Rasmussen, H.: Role of calcium in angiotensin II mediated aldosterone secretion. Endocr. Rev. 10, 496–518 (1989)
4 Chang, R. and Lotti, V.: Two distinct angiotensin II receptor binding sites in rat adrenal revealed by new selective nonpeptide ligands. Mol. Pharmacol. 29, 347–351 (1990)
5 Dudley, D.T., Panek, R.L., Major, T.C., Lu, G.H., Bruns, R.F., Klinkefus, B.A., Hodges, J.C. and Weishaar, R.E.: Subclasses of angiotensin II binding sites and their functional significance. Mol. Pharmacol. 38, 370–377 (1990)
6 Chiu, A.T., Herblin, W.F., McCall, D.F., Ardecky, R.J., Carini, D.J., Duncia, J.V., Pease, L.J., Wong, P.C., Wexler, R.R., Johnson, A.L. and Timmermans, P.B.M.W.M.: Identification of angiotensin II receptor subtypes. Biochem. Biophys. Res. Commun. 165, 196–203 (1989)
7 Ohnishi, J., Ishido, M., Shibata, T., Inagami, T., Murakami, K. and Miyazaki, H.: The rat angiotensin II AT1A receptor couples with three different signal transduction pathways. Biochem. Biophys. Res. Commun. 186, 1094–1101 (1992)
8 Murphy, T.J., Alexander, R.W., Griendling, K.K., Runge, M.S. and Bernstein, K.E.: Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. Nature 351, 233–236 (1991)
9 Iwai, N., Yamano, Y., Chaki, S., Konishi, F., Bardhan, S., Tibbetts, C., Sasaki, K., Hasegawa, M., Matsuda, Y. and Inagami, T.: Rat angiotensin II receptor: cDNA sequence and regulation of the gene expression. Biochem. Biophys. Res. Commun. 177, 299–304 (1991)
10 Chansel, D., Czekalski, S., Pham, P. and Ardaillou, R.: Characterization of angiotensin II receptor subtypes in human glomeruli and mesangial cells. Am. J. Physiol. 262, F432–F441 (1992)
11 Yazawa, H., Takanashi, M., Sudoh, K., Inagaki, O. and Honda, K.: Characterization of [3H]YM617, 5-[2-[2[ethoxyring(n)-3H] (o-ethoxyphenoxy)ethyl]amino]-propyl]-2-methoxybenzenesulfonamide HCl, a potent and selective alpha1-adrenoceptor radioligand. J. Pharmacol. Exp. Ther. 263, 201–206 (1992)
12 Grynkiewicz, G., Poenie, M. and Tsien, R.Y.: A new generation of Ca2+ indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450 (1985)
13 Bergsma, D.J., Ellis, C., Kumar, C., Nuthulaganti, P., Kersten, H., Elshourbagy, N., Griffin, E., Stadel, J.M. and Aiyar, N.: Cloning and characterization of human angiotensin II type 1 receptor. Biochem. Biophys. Res. Commun. 183, 989–995 (1992)
14 Andrawis, N.S. and Abernethy, D.R.: Verapamil blocks basal and angiotensin II-induced RNA synthesis of rat aortic vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 186, 767–773 (1992)
15 Gardner, J.P., Tokudome, G., Tomonari, H., Maher, E., Hollander, D. and Aviv, A.: Endothelin-induced calcium responses in human vascular smooth muscle cells. Am. J. Physiol. 262, C148–C155 (1992)
16 Wilkes, B.M., Dion, I., Sollott, S., Michaels, S. and Kiesel, G.: Intrarenal renin-angiotensin system modulates glomerular angiotensin receptors in the rat. Am. J. Physiol. 254, F345–F350 (1988)
17 Wong, P.C., Hart, S.D., Zaspel, A.M., Chiu, A.T., Ardecky, R.J., Smith, R.D. and Timmermans, P.B.M.W.M.: Functional studies of nonpeptide angiotensin II receptor subtype-specific ligand: Dup 753 (AII-1) and PD 123177 (AII-2). J. Pharmacol. Exp. Ther. 255, 584–592 (1990)
18 Chiu, A.T., McCall, D.E., Price, W.A., Wong, P.C., Carini, D.J., Duncia, J.V., Wexler, R.R., Yoo, S.E., Johnson, A.L. and Timmermans, P.B.M.W.M.: Nonpeptide angiotensin II receptor antagonists. VII. Cellular and biochemical pharmacology of Dup 753, an orally antihypertensive agent. J. Pharmacol. Exp. Ther. 252, 711–718 (1990)
19 Garcia-Sainz, J.A. and Macias-Silva, M.: Angiotensin II stimulates phosphoinositide turnover and phosphorylase through A II-1 receptors in isolated rat hepatocytes. Biochem. Biophys. Res. Commun. 172, 780–785 (1990)
20 Pfeilschifter, J.: Angiotensin II B type receptor mediates phosphoinositide hydrolysis in mesangial cells. Eur. J. Pharmacol. 184, 201–202 (1990)