Human Aortic Smooth Muscle Cells Containing Angiotensin II Type 1 Receptors

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ABSTRACT—We characterized the angiotensin II (AII) receptor in human aortic smooth muscle cells (HASMCs). This receptor binds [^{125}I]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 K_i values, the ranking order of [^{125}I]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

Angiotensin II (AII) plays an important role in the regulation of blood pressure, salt and water homeostasis (1-3). These events appear to be exerted by diverse AII-induced actions, including vascular smooth muscle contraction and aldosterone secretion from the adrenal cortex, through specific receptors in target cells. Two AII receptor subtypes have been demonstrated by using selective nonpeptide ligands. A subtype sensitive to Dup 753 was classified as type 1 (AT_1), and another sensitive to PD 123319 was designated as type 2 (4-6). Recently, several investigators have suggested the involvement of at least three different signal transduction pathways in the cellular responses to AII (phosphoinositide breakdown, activation of dihydropyridine-sensitive voltage-dependent Ca^{2+} channels and inhibition of adenylate cyclase activity) (7). A rat angiotensin II type 1 receptor cDNA was cloned and shown to be a member of the 7-transmembrane, G-protein-coupled family of receptors (8, 9). Although the effects of AII have been partially elucidated in experimental animals, there is yet little information about its effects in human derived cells (10). Recently, however, human aortic smooth muscle cells (HASMCs) have been used for experiments. In the present study, we characterized AII receptors in these cells by binding studies and measurement of intracellular Ca^{2+}.

MATERIALS AND METHODS

Materials

Angiotensin II, Sar^1,[^{125}I]-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'-(1*H*-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-1*H*-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% CO_2 - 95% air at 37℃. 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-cm^2 plastic dish were washed twice with phosphate-buffered saline, scrapped into a solution containing 250 mM sucrose, 10 mM $MgCl_2$, and 50 mM tris/HCl (pH 7.4), and homogenized with a polytron at 4℃. After centrifugation at $2,000 \times g$ for 10 min at 4℃, the supernatant was centrifuged at $40,000 \times g$ for 20 min at 4℃, and the resulting pellet was resuspended in a 50 mM tris/HCl (pH 7.4) and 10 mM $MgCl_2$ and stored at −80℃ until use.

Binding assay

Incubations were carried out at 37℃ for 60 min in 0.5 ml of incubation buffer (10 mM Na_2HPO_4, 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 [^{125}I]Sar^1,Ile^8-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 γ-counter (ARC-950, Aloka, Tokyo). Nonspecific binding was determined in the presence of 1 μM Sar^1, Ile^8-AII. Data were analyzed by the previously described method (11).

Measurements of Ca^{2+}

Confluent HASMCs grown on a coverslip were incubated for 60 min with 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℃) cell holder. Excitation wavelength were 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 $[Ca^{2+}]_i$ signal stabilized and basal $[Ca^{2+}]_i$ measurement was obtained. Thereafter, cells were subjected to a specific agonist (e.g., AII), and $[Ca^{2+}]_i$ signals were recorded for an additional 5 min. The maximum ratio (R_{max}) for the fura-2 in each coverslip was measured by adding 10 μM ionomycin, and the ratio in the absence of Ca^{2+} (R_{min}) was measured by then adding 3 mM EGTA. From the ratio of fluorescence at 340 and 380 nm, the $[Ca^{2+}]_i$ was determined as described Grynkiewicz et al. (12) using the following equation:

$$[Ca^{2+}]_i\ (nM) = K_d \times [(R - R_{min})/(R_{max} - R)] \times \beta$$

The term β is the ratio of fluorescence of fura-2 at 380 nm in zero and saturating Ca^{2+}. K_d is the dissociation constant of fura-2 for Ca^{2+}, 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 [^{125}I]Sar^1,Ile^8-AII. Figure 1 shows the specific binding of [^{125}I]Sar^1,Ile^8-AII at the concentration of 0 to 1.5 nM in HASMC membrane. Scatchard plots revealed that the [^{125}I]-AII,Sar^1 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 [^{125}I]-AII,Sar^1, as the AII receptor antagonist. Radioligand binding was potently inhibited by AII, Sar^1,Ile^8-AII and the AT_1 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 [^{125}I]-Sar^1,Ile^8-AII at doses as high as 1×10^{-5} M (Table 1).

AII-induced Ca^{2+} mobilization in HASMCs was examined by measuring changes in intracellular free Ca^{2+} concentration with fura-2 as a Ca^{2+} indicator. The addition of 1×10^{-8} M AII evoked a rapid, transient increase in $[Ca^{2+}]_i$ followed by a lower, sustained phase which persisted for at least 5 min. This $[Ca^{2+}]_i$ response was blocked

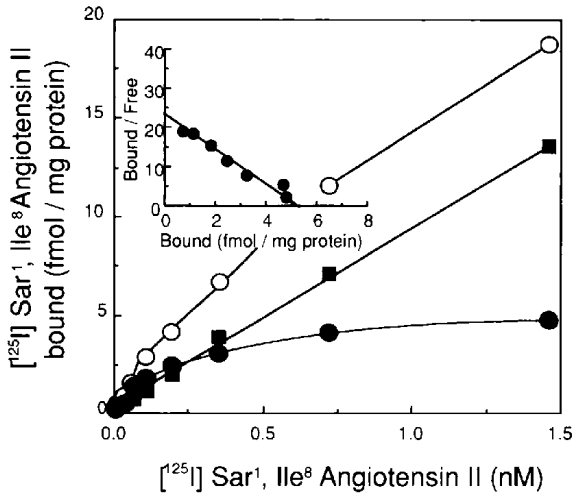

Fig. 1. Binding of [^{125}I]-AII,Sar^1,Ile^8 to membranes prepared from HASMCs. The saturation curve for the total (○), nonspecific binding (■) and specific binding (●) of [^{125}I]-AII,Sar^1,Ile^8 from one typical experiment is shown. Each point showed the mean of triplicate assays. Similar results were obtained from 2 different experiments. Inset shows a Scatchard plot from the same data.

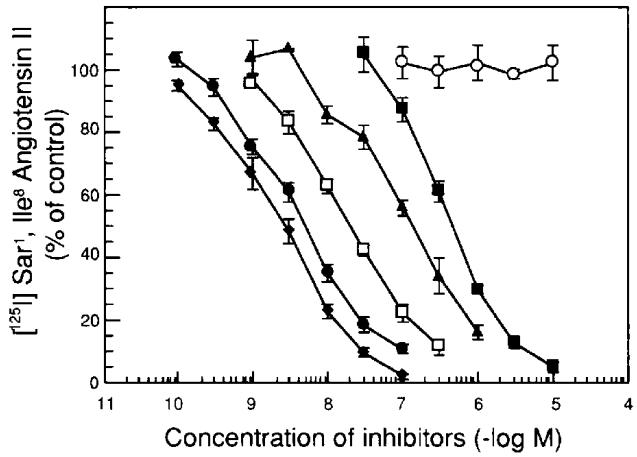

Fig. 2. Competitive inhibition curves for [^{125}I]-AII,Sar^1,Ile^8 binding to HASMC membranes. HASMC membranes were incubated with [^{125}I]Sar^1,Ile^8-angiotensin II in the presence of the indicated concentrations of unlabeled Sar^1,Ile^8-angiotensin II (◆), angiotensin II (●), angiotensin I (■), angiotensin III (▲), Dup 753 (□), and PD 123319 (○). Each point represents the mean ±S.E. of three or four experiments.

Table 1. K_i values of angiotensin analogues in HASMC membranes

Ligand	K_i (nM)	Hill	n
AI	372 (346−401)	1.00 (0.87−1.14)	3
AII	3.6 (3.4−3.8)	0.73 (0.67−0.80)	3
Sar^1,Ile^8-AII	1.6 (1.5−1.6)	0.81 (0.75−0.87)	4
AIII	87 (82−91)	0.72 (0.62−0.87)	3
Dup 753	17 (16−17)	0.84 (0.74−0.91)	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 AT_1 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 $[Ca^{2+}]_i$ response was not changed. When the cell was pretreated with 1×10^{-5} 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 [^{125}I]Sar^1,Ile^8-AII to AII receptors present in HASMC membranes. Scatchard analysis of specific [^{125}I]Sar^1,Ile^8-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_i values, the ranking order of [^{125}I]Sar^1,Ile^8-AII binding inhibition was as follows: Sar^1,Ile^8-AII > AII > Dup 753 > AIII > AI ≫ PD 123319 (Table 1). This inhibition profile agreed well with those obtained using expressed AT_1 receptors from rats (9) and humans (13). These results suggest that the AII receptors in HASMCs are of the AT_1 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, AT_1 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 AT_1 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 AT_1 ligand in hepatocytes (19) and rat mesanginal cells (20) also suggests that AT_1 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-

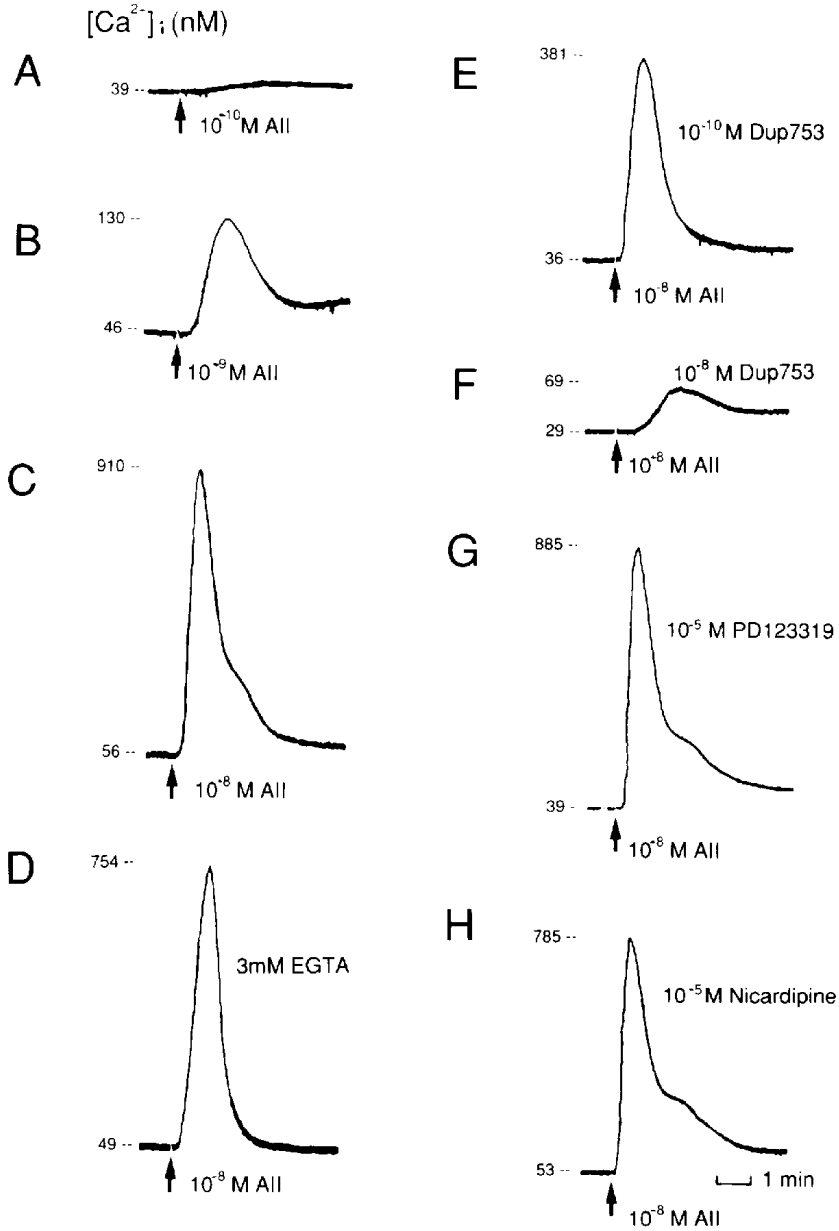

Fig. 3. The effect of AII on intracellular free Ca^{2+}. Fura-2-loaded HASMCs were exposed to 1×10^{-10} M AII (A), 1×10^{-9} M AII (B), or 1×10^{-8} M AII (C–H) in the presence of 3 mM EGTA (D), Dup 753 (1×10^{-10} M, 1×10^{-8} M) (E, F), 1×10^{-5} M PD 123319 (G), or 1×10^{-5} M nicardipine (H). EGTA, 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.

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.

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