Intracellular Sodium Modulates the Expression of Angiotensin II Subtype 2 Receptor in PC12W Cells

Masaaki Tamura, Yoshio Wanaka, Erwin J. Landon, Tadashi Inagami

Abstract—Although the angiotensin II subtype 2 receptor (AT2-R) is expressed abundantly in the adrenal medulla, its physiological significance has not yet been determined. To obtain fundamental knowledge of the regulation of AT2-R expression in the adrenal medulla, we investigated the effects of modulating several ion channels on AT2-R expression in PC12W cells. Experiments were performed after 24 hours of serum depletion under subconfluent conditions. After 48 hours of treatment with various agonists or antagonists, the receptor density and mRNA level of AT2-Rs were quantified by [125I]-[Sar1,Ile8]angiotensin II binding and Northern blot analysis. Ouabain (10 to 100 nmol/L) and insulin (10 to 100 nmol/L) dose-dependently increased receptor density and mRNA level. Analysis of the binding characteristics revealed that the ouabain-dependent increase in AT2-R levels was due to an increase in binding capacity without a change in the KD value. These increases were blocked by lowering the Na+ concentration in the medium. A low concentration of the sodium ionophore monensin (10 nmol/L), the K+ channel blocker quinidine (10 µmol/L), and the ATP-sensitive K+ channel blocker tolbutamide (100 µmol/L) also significantly increased receptor density, but the ATP-sensitive K+ channel agonist cromakalim (100 µmol/L) decreased receptor density significantly (P<0.01). Nifedipine (10 µmol/L) decreased basal receptor density and completely blocked the increase in receptor density caused by these agents. The increase in receptor density caused by an increase in intracellular Na+ was accompanied by an increase in mRNA level, whereas the ATP-sensitive K+ channel blockers did not change mRNA level. Nifedipine slightly decreased mRNA level. These results suggest that AT2-R expression is sensitively regulated by intracellular cation levels. The change in intracellular Na+ level transcriptionally regulates AT2-R expression, whereas the K+ channel blocker–dependent upregulation appears to be at least in part posttranslational. (Hypertension. 1999;33:626-632.)

Key Words: sodium channels ■ ATP-sensitive K+ channel ■ AT2 receptor ■ angiotensin II ■ PC12 cells ■ calcium channels

The renin-angiotensin system plays an important role in the regulation of blood pressure, body fluid and electrolyte homeostasis, the facilitation of adrenergic nerve activity, and drinking behavior.1–4 These biological actions are initiated through the binding of angiotensin II (Ang II) to specific receptors.1–4 There are at least two subtypes of Ang II receptors.5–7 Most of the known biological effects ascribed to Ang II, such as vasoconstriction, aldosterone release, and cell proliferation, are mediated by the subtype 1 Ang II receptor (AT1-R).1–4 However, the subtype 2 Ang II receptor (AT2-R) is present in a variety of tissues and is implicated in several biological functions, such as inhibition of cell growth,8,9 pressure natriuresis,10 production of prostaglandins,11–13 apoptosis,14 and AT2 regulation of vascular tone.15–18 In neuronal cells, Ang II regulates K+ currents19 and T-type Ca2+ channels20 and promotes differentiation21,22 through AT2-R. The mechanisms by which these actions occur remain incompletely understood, mainly because of the unclear signaling mechanism(s) and the lower AT2-R expression levels compared with AT1-R levels.

Among several peripheral tissues in adult rats that have been reported to express AT2-Rs, adrenal medulla expresses them most abundantly.6,7 However, no physiological significance has yet been attributed to this AT2-R. In adrenal medulla, Ang II stimulates catecholamine production through AT1-R,23,24 a mechanism for which extracellular Ca2+ entry into the cytoplasm is necessary.25,26 AT2-Rs, however, do not transmit the Ang II signal for intracellular Ca2+ mobilization in adrenal medullary cells.27 If AT2-R expression is regulated in association with intracellular Na+ or K+ levels, AT2-R is anticipated to play a role in signaling that is eventually associated with Na+ or K+ metabolism. Clarification of AT2-R expression, in conjunction with intracellular cation mobilization, may therefore provide us with a clue to the physiological significance of AT2-R in adrenal medulla. We conducted the present study to obtain fundamental knowledge...
of the potential physiological function of AT₂-R using rat pheochromocytoma-derived PC12W cells, which have been reported to express AT₂-R exclusively and abundantly.²⁷,²⁸ In the present article, we report on intracellular cation (particularly Na⁺)-dependent regulation of AT₂-R expression in PC12W cells.

**Methods**

**Chemicals and Reagents**

Ang II and [Sar¹,Ile⁸]Ang II were purchased from Peninsula Laboratories. Ouabain, insulin, monensin, nifedipine, tolbutamide, glybenclamide, and cromakalim were from Sigma Chemical Laboratories. Ouabain, insulin, monensin, quinidine, nifedipine, tolbutamide, and fetal bovine serum (FBS) were purchased from GIBCO-BRL. All other chemicals were of analytical grade.

**Cell Culture**

The rat pheochromocytoma cell line PC12 was derived from clonal isolation of an adrenal chromaffin cell tumor.²⁹ A substrain, PC12W, previously.³¹ The cells were cultured in DMEM supplemented with 10% FBS at 37°C and under a humidified atmosphere of 95% air/5% CO₂. For determination of receptor density, cells were plated in 24-well plates at a density of 2.5×10⁵ cells and cultured for 2 to 3 days. The medium was changed to serum-free DMEM 24 hours before the binding assay, which was performed when the culture was approximately 80% to 90% confluent. When the effect of extracellular Na⁺ concentration on AT₂-R expression levels was studied, PC12W cells were cultured in DMEM supplemented with 10% FBS for 2 to 3 days. They were then cultured further in a modified DMEM consisting of 145 mmol/L NaCl, 1.8 mmol/L KCl, 1.2 mmol/L KH₂PO₄, 5 mmol/L NaHCO₃, 0.5 mmol/L MgSO₄, 1.8 mmol/L CaCl₂, 25 mmol/L glucose, 4% MEM vitamin solution (50×), 4% MEM vitamin solution (100×), 1% MEM nonessential amino acid solution (100×), 0.5% FBS, and 0.5 mg/mL bovine serum albumin (BSA) (pH 7.4) at 37°C in a CO₂ incubator. The modified DMEM with low Na⁺ (35 mmol/L NaCl) was prepared by substituting 110 mmol/L NaCl with choline chloride.

Vascular smooth muscle cells (VSMCs) were obtained from thoracic aorta tissue of male spontaneously hypertensive rats as described previously.³² VSMCs were cultured for 5 to 6 days in the same medium and under the same conditions as described for PC12W cells. COS-7 cells stably expressing AT₂-R were prepared by the electroporation transfection method with pRC/CMV (Invitrogen) containing a 2.9-kb insert derived from AT₁, cDNA as described previously.³³ The cells were cultured in DMEM supplemented with 10% FBS under the same conditions as described for PC12W cells for 2 to 3 days before the experiment.

**125I-[Sar¹,Ile⁸]Ang II Binding Assay**

The radioligand receptor binding assay was performed using intact cultured cells and ¹²⁵I-[Sar¹,Ile⁸]Ang II. ¹²⁵I-[Sar¹,Ile⁸]Ang II was prepared from [Sar¹,Ile⁸]Ang II and ¹²⁵I-Na by the lactoperoxidase method. Subconfluent PC12W cells in 24-well plates were washed twice with Hanks' balanced salt solution (HBSS) and incubated with 0.5 mmol/L ¹²⁵I-[Sar¹,Ile⁸]Ang II with or without 1 μmol/L unlabeled [Sar¹,Ile⁸]Ang II for 3 hours at 4°C in the presence of 1 μmol/L losartan and 0.5 mg/mL BSA. Unbound ligand was thoroughly washed out with HBSS at 4°C. Cells were solubilized with 0.5 mol/L NaOH, and the remaining radioactivity was counted. Specific binding was estimated by subtracting the nonspecific binding obtained in the presence of 1 μmol/L unlabeled ligand from the total binding. An aliquot of the solubilized cells was subjected to protein assay (BCA protein assay method, Pierce Chemical Co). Specific binding was normalized by protein quantity per well. Saturation isotherm data were analyzed according to the Scatchard method.³⁴

**Northern Blot Analysis**

The total RNA in PC12W cells was isolated by the acid guanidinium-phenol-chloroform extraction method.³⁵ Twenty micrograms of the total RNA was then electrophoresed on a 1.0% agarose/1.0% formaldehyde gel and transferred to a Hybond N⁺ membrane (Amersham). A full-length cDNA of the mouse AT₂ gene was labeled with ³²P by a Prime It kit (Stratagene) and used as a probe after heat denaturation. The filter was then exposed to Kodak X-OMAT film at −70°C. The hybridized filter was stripped and hybridized to a ³²P-labeled GAPDH probe to obtain a reference for the amount of applied RNA. Autoradiographic analysis was performed by an image scanner (ES-800C scanner, Epson America, Inc.) and a computer program (Image 1.59, National Institutes of Health).

**Statistical Analysis**

Data obtained from the binding assay were averaged and are presented as mean±SE. Significant differences between groups were evaluated by one-way analysis of variance with the Student-Newman-Keuls test. A value of P<0.05 was considered significant.

**Results**

The cardiac glycoside ouabain is a well-known Na⁺ pump-specific inhibitor and has been widely used for the modulation of intracellular Na⁺ levels. As illustrated in Figure 1, low concentrations of ouabain (10 to 100 nmol/L) dose- and time-dependently increased AT₂-R expression in PC12W cells. A maximal increase (50%) was observed at a concentration of 50 nmol/L ouabain in the medium. PC12W cells easily detach from culture plates when the conditions are serum-free for longer than 24 hours. Therefore, ouabain was added to the 10% serum-containing medium, and the cells were initially cultured for 24 hours under these conditions before changing to the serum-free medium for an additional 24 hours. This ouabain effect was also studied with serum in the medium (0.5% or 10% serum in the DMEM) for 48 hours, but the AT₂-R stimulation pattern by ouabain was consistent (data not shown).

Since PC12W cells express AT₂-R exclusively,²⁷,²⁸ the receptor subtype specificity of the ouabain effect was investigated using VSMCs, which predominantly express AT₇-Rs (no detectable AT₂-Rs were expressed), and COS-7 cells, which contain permanently transfected AT₂-R coding regions. In VSMCs, AT₂-R expression was slightly decreased by low concentrations of ouabain (10 and 25 nmol/L, Figure 2A). In COS-7 cells, AT₂-R expression was significantly increased at low concentrations of ouabain (25 and 50 nmol/L, Figure 2B). These results, together with the results shown in Figure 1, clearly demonstrate that the ouabain effect on Ang II receptors is specific to AT₂-R. The involvement of intracellular Na⁺ level in AT₂-R expression was further examined using insulin and a mixture of insulin and ouabain. Insulin alone dose-dependently (10 to 100 nmol/L) increased AT₂-R expression (Figure 3). When insulin and ouabain were added together, they showed additive effects on the increase in AT₂-R expression in PC12W cells (Figure 3). To clarify whether the ouabain-dependent increase in receptor density is due to an increment in the receptor density or induction of another type of receptor protein, we analyzed ¹²⁵I-[Sar¹,Ile⁸]Ang II binding characteristics using intact whole cells. Data in Figure 4 indicate that AT₂-R in PC12W cells possesses two types of binding sites (Kᵣ=1.7 nmol/L, high-affinity site; Kᵢ=17.4 to 19.1 nmol/L, low-affinity site).
Ouabain treatment increased the binding maximum of both high- and low-affinity sites by approximately 55%. These data suggest that the intracellular Na\(^{+}\)– dependent increase in \(\text{AT}_2\)-R levels is due to an increment in receptor density.

The above experiments suggest that intracellular Na\(^{+}\) level is an important factor in the regulation of \(\text{AT}_2\)-R expression. To clarify this hypothesis, we manipulated intracellular Na\(^{+}\) level with the sodium ionophore monensin. A very low concentration of monensin (10 nmol/L) significantly increased \(\text{AT}_2\)-R expression by approximately 75%. Although a higher concentration of monensin (\(\geq 100 \text{ nmol/L}\)) is known to be cytotoxic, the cytotoxicity of monensin at 10 nmol/L for 48 hours of treatment was not significant. This result confirms that intracellular Na\(^{+}\) level is tightly associated with the regulation of \(\text{AT}_2\)-R expression. Since an increase in intracellular Na\(^{+}\) level was shown to upregulate \(\text{AT}_2\)-R expression, we studied the reverse effect by lowering extracellular Na\(^{+}\) concentration. In this experiment, osmolarity in the low-sodium-modified medium was maintained at the same level as in the high-sodium-modified medium by substituting the removed NaCl with choline chloride. When PC12W cells were cultured in modified DMEM containing 0.5% FBS for 48 hours, the increase in \(\text{AT}_2\)-R expression caused by ouabain or insulin in the 145 mmol/L Na\(^{+}\) medium was abolished by lowering the Na\(^{+}\) concentration in the medium to 35 mmol/L (Figure 5). These results again suggest that increased intracellular Na\(^{+}\) upregulates \(\text{AT}_2\)-R expression.

In addition to investigating \(\text{AT}_2\)-R expression in PC12W cells by manipulating intracellular Na\(^{+}\) level, we investigated such expression by using a K\(^{+}\)-channel agonist and a variety of antagonists. The ATP-sensitive K\(^{+}\)-channel blockers tolbutamide and glybenclamide dose-dependently increased \(\text{AT}_2\)-R expression, whereas the ATP-sensitive K\(^{+}\)-channel agonist cromakalim significantly downregulated receptor density (Figure 6A). Although the nonspecific K\(^{+}\)-channel blockers tetraethylammonium and 4-aminopyridine increased receptor level significantly, both blockers required much higher concentrations (higher than millimoles per liter) than the specific channel blockers to increase the receptor level in
However, another type of nonspecific K⁺-channel blocker, quinidine (10 μmol/L), significantly increased AT₂-R expression over a narrow concentration range (Figure 6B). The Ca²⁺-sensitive K⁺-channel blocker charybdotoxin (∼1 μmol/L) did not show any effect on AT₂-R expression levels (data not shown). These results demonstrate that the changing K⁺ currents are also an important factor affecting AT₂-R expression levels in PC12W cells.

K⁺-channel blockade and Na⁺ pump inhibition promote decreased membrane polarization in neuronal-type cells such as PC12W cells. This in turn activates the L-type voltage-dependent Ca²⁺ channels, which could in turn account for AT₂-R upregulation. Nifedipine (10 μmol/L), a specific blocker of the L-type Ca²⁺ channel, slightly but significantly lowered basal AT₂-R expression level and almost abolished the effect of 50 nmol/L ouabain, 10 μmol/L quinidine, or 0.5 mmol/L tolbutamide (Figure 7).

Receptor protein expression is a summation of transcriptional and translational regulation and protein degradation. To evaluate the involvement of transcriptional regulation in AT₂-R expression, we estimated AT₂-R mRNA levels using Northern blot analysis. Treatment with ouabain (50 nmol/L), monensin (10 nmol/L), quinidine (10 μmol/L), or insulin (100 nmol/L) for 48 hours significantly increased AT₂-R mRNA levels in PC12W cells (Figure 8), whereas the ATP-sensitive K⁺-channel blocker tolbutamide and the K⁺-channel agonist cromakalim (100 μmol/L) at levels effective for receptor upregulation did not change mRNA levels in PC12W cells. Nifedipine (10 μmol/L) slightly decreased mRNA level. These results suggest that an increase in intracellular Na⁺ may stimulate transcriptional regulation of AT₂-R expression, whereas a K⁺-channel blocker–dependent increase in AT₂-R expression may be due to posttranslational regulation.
Discussion

Although AT2-R is expressed abundantly in rat adrenal medulla, its physiological significance has not yet been clarified. The present study focused on determining the basic regulation mechanism of AT2-R expression in adrenal medullary cells. PC12W cells are a substrain of the rat pheochromocytoma cell line and are capable of differentiating to neuronal cells under appropriate culture conditions.34 PC12W cells do not produce all of the catecholamines. 35 However, this cell line is a good model for the study of the regulation of AT2-R expression because these cells express high levels of AT2-R exclusively. 27,28

In the present study intracellular Na\(^+\) level was manipulated by inhibition of the Na\(^+\) pump with ouabain, by activation of sodium entry with insulin, or by treatment with the sodium ionophore monensin; then AT2-R expression was evaluated. In these three types of experiments, AT2-R expression was upregulated (see Figures 1 and 3 and Results). Thus, AT2-R expression appears to be upregulated when intracellular Na\(^+\) level is increased. This result was supported by another experiment, in which the intracellular Na\(^+\)-dependent AT2-R upregulation caused by ouabain was abolished by lowering extracellular Na\(^+\) level (Figure 5). In agreement with the present study, insulin has been reported to increase AT2-R expression in R3T3 cells. 36

Changes in intracellular Na\(^+\) influence membrane potential. Since increases in intracellular Na\(^+\) sensitively upregulated AT2-R expression, changes in K\(^+\) currents were also anticipated to regulate AT2-R expression. Indeed, our study demonstrated that manipulation of K\(^+\) currents by a variety of K\(^+\)-channel blockers increased AT2-R expression. Among many K\(^+\)-channel blockers tested, the ATP-sensitive K\(^+\)-channel blockers toltubutamide and glybenclamide and the

![Figure 6](http://hyper.ahajournals.org/)

**Figure 6.** Effects of ATP-sensitive K\(^+\)-channel blockers toltubutamide (Tol) and glybenclamide (Gly) and agonist cromakalim (Cro) (A) and nonspecific K\(^+\)-channel blocker quinidine (Qui) (B) on AT2-R expression levels. Each value represents mean±SE of 6 incubations. *P<0.05, **P<0.01 compared with basal receptor density level.

![Figure 7](http://hyper.ahajournals.org/)

**Figure 7.** Effect of nifedipine (Nif) on basal AT2-R expression and expression stimulated by ouabain (Oua), quinidine (Qui), or toltubutamide (Tol) in PC12W cells. Experimental conditions were identical to conditions described in Figure 1. Nifedipine was added to medium 15 minutes before stimulus. Each value represents mean±SE of 6 incubations. *P<0.05, **P<0.01 compared with basal or corresponding chemical-stimulated receptor density level.

![Figure 8](http://hyper.ahajournals.org/)

**Figure 8.** Effects of various ion channel inhibitors and chemicals on AT2-R mRNA expression in PC12W cells. Cells were grown to subconfluence for 2 days and then treated with various chemicals for 48 hours as described in Figure 1. Total RNA was isolated, and 20 μg was subjected to Northern blot analysis (A). AT2-R mRNA was detected as described in Methods. AT2-R mRNA expression level with respect to GAPDH was estimated (B). Data are representative of 3 experiments.
classically nonspecific K⁺-channel blocker quinidine most effectively upregulated AT₂-R expression. The ATP-sensitive K⁺-channel agonist cromakalim dose-dependently downregulated AT₂-R expression. These results indicate that as with Na⁺, changes in K⁺ currents in the cell membrane regulate AT₂-R expression (Figure 6).

K⁺ channels and the Na⁺ pump alter membrane polarization and Ca²⁺ entry through voltage-dependent Ca²⁺ channels. Neuronal cells possess voltage-dependent Ca²⁺ channels, so it is of interest to evaluate the effect of intracellular Ca²⁺ on AT₂-R expression. In the experiment with the voltage-dependent Ca²⁺-channel blocker nifedipine, lowering Ca²⁺ entry from extracellular sources decreased basal AT₂-R expression as well as the intracellular Na⁺- or K⁺-current-dependent upregulation of AT₂-R (Figure 7). Nifedipine (10 μmol/L) treatment for 48 hours also decreased AT₂-R mRNA level by approximately 25%. These results suggest that there could be intracellular Ca²⁺-dependent regulation of AT₂-R downstream of the intracellular Na⁺ and K⁺ current-dependent regulation mechanism. However, since all of the chemicals used for the present study are known to modulate membrane potential, modification of the membrane potential may contribute to the regulation of AT₂-R expression.

The binding characteristics of radiolabeled Ang II or its analogues to AT₂-R have been studied in many types of cells.²⁷,³¹,³⁸–⁴⁰ Many of these studies have reported that AT₂-R contains a single, saturable binding site.²⁷,³¹,³⁸–³⁹ The present study, however, revealed that AT₂-R in PC12W cells contains distinct high- and low-affinity binding sites (Figure 4). In agreement, Siemens et al.⁴⁰ have reported that neuroblastoma cells (NIE-115) possess two distinct AT₂-Rs. Despite the use of different cells and different procedures (Siemens et al used solubilized membrane fractions, whereas the present study used intact cells), the Kᵥ values from their experiment and from the present study are almost identical (=1.7 nmol/L). The discrepancy between the present study and other studies, which have reported a single binding site on AT₂-R, can be explained by the following. In the present study, saturation curves were drawn using a wide range of [¹²⁵I]-[Sar¹,Ile⁸]Ang II concentrations (0 to 15 nmol/L), whereas most other reports have used only a very narrow concentration range of the radiolabeled ligands (<5 nmol/L). The present study showed a low-affinity binding site that emerged at approximately 4 nmol/L [¹²⁵I]-[Sar¹,Ile⁸]Ang II (Figure 4A), so most of the other reports would not have been able to detect this low-affinity binding site. The results of the present study clearly indicate that the binding capacities at both binding sites were increased to almost an identical extent by ouabain treatment. This may suggest that the two binding sites belong to a single molecule. AT₁-R and AT₂-R have been shown to be derived from different genes located on different chromosomes.⁴¹ Their mRNA and protein expressions are apparently regulated by different mechanisms. In the present study, AT₁-R expression in VSMCs was not upregulated by ouabain (Figure 2), suggesting that the intracellular cation level or an alteration of the membrane potential is not a signal for the regulation of AT₁-R expression. Although nonspecific K⁺-channel blockers such as tetraethylammonium have been reported to inhibit agonist-induced receptor desensitization in seven transmembrane-type receptors,⁴² it has been shown that AT₂-R is not internalized by Ang II.²⁷,³⁸,³⁹ In the present study, K⁺-channel blocker–dependent increases in AT₂-R expression were not accompanied by an increment in mRNA level. These results may suggest that AT₂-R upregulation by K⁺-channel blockers is in part due to a decrease in the receptor turnover rate.

Protein expression is the sum result of transcription and translation. In the present study we measured mRNA levels by Northern blot analysis. We also determined the relative and functional quantities of the expressed protein by measuring ligand-receptor binding. The results from both determinations suggest that an increase in intracellular Na⁺-sensitive upregulates AT₂-R expression. The mechanism underlying this upregulation is possibly transcriptional regulation, whereas a K⁺-channel blocker–dependent increase in AT₂-R level is postulated to be at least in part translational/posttranslational regulation. The results provide a potential approach to determination of the physiological significance of AT₂-R-mediated signals. It is of interest to determine whether similar mechanisms occur in vivo. To the best of our knowledge, the present study is the first to demonstrate that AT₂-R is regulated by intracellular cations, particularly the sodium ion.

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Intracellular Sodium Modulates AT₂ Receptor Expression


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