Rat Strain Effects of AT$_1$ Receptor Activation on D$_1$ Dopamine Receptors in Immortalized Renal Proximal Tubule Cells

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Abstract—The dopaminergic and renin-angiotensin systems regulate blood pressure, in part, by affecting sodium transport in renal proximal tubules (RPTs). We have reported that activation of a D$_1$-like receptor decreases AT$_1$ receptor expression in the mouse kidney and in immortalized RPT cells from Wistar-Kyoto (WKY) rats. The current studies were designed to test the hypothesis that activation of the AT$_1$ receptor can also regulate the D$_1$ receptor in RPT cells, and this regulation is aberrant in spontaneously hypertensive rats (SHRs). Long-term (24 hours) stimulation of RPT cells with angiotensin II, via AT$_1$ receptors increased total cellular D$_1$ receptor protein in a time- and concentration-dependent manner in WKY but not in SHR cells. Short-term stimulation (15 minutes) with angiotensin II did not affect total cellular D$_1$ receptor protein in either rat strain. However, in the short-term experiments, angiotensin II decreased cell surface membrane D$_1$ receptor protein in WKY but not in SHR cells. D$_1$ and AT$_1$ receptors colocalized (confocal microscopy) and their coimmunoprecipitation was greater in WKY than in SHRs. However, AT$_1$/D$_1$ receptor coimmunoprecipitation was decreased by angiotensin II (10$^{-8}$M/24 hours) to a similar extent in WKY (22% decrease) and SHRs (12% decrease). In summary, these studies show that AT$_1$ and D$_1$ receptors interact differently in RPT cells from WKY and SHRs. It is possible that an angiotensin II-mediated increase in D$_1$ receptors and dissociation of AT$_1$ from D$_1$ receptors serve to counter regulate the long-term action of angiotensin II in WKY rats; different effects are seen in SHRs. (Hypertension. 2005;46:799-805.)

Key Words: dopamine • kidney • receptors, angiotensin II • rats, spontaneously hypertensive

Angiotensin II and dopamine are important regulators of sodium and water transport across the renal proximal tubule (RPT). Angiotensin II receptor subtypes (AT$_1$, AT$_2$, and AT$_4$) are expressed in brush border and basolateral membranes of RPTs. The activation of AT$_1$ receptors by low concentrations of angiotensin II (picomolar) causes an increase in sodium reabsorption in RPTs. All the dopamine receptor subtypes, D$_1$-like (D$_1$ and D$_5$) and D$_2$-like (D$_2$, D$_3$, and D$_4$) are also expressed in brush border and basolateral membranes of RPTs. In contrast to the stimulatory effect of AT$_1$ receptors on renal sodium transport, activation of D$_1$ and D$_3$ receptors decreases renal sodium reabsorption.

Several reports have shown an interaction between the dopamine and renin-angiotensin system. Intrarenally produced angiotensin II opposes the natriuretic action of the D$_1$-like dopamine receptor agonist, fenoldopam, in rats. D$_1$-like receptor agonists also antagonize the stimulatory effect of angiotensin II, acting via AT$_1$ receptors, on renal proximal tubular luminal sodium transport. When angiotensin II generation is inhibited or AT$_1$ receptors are blocked, the natriuretic effect of dopaminergic drugs is enhanced.

The AT$_1$ receptor mediates the anti-natriuretic effect of angiotensin II, whereas the D$_1$ dopamine receptor is responsible for ~80% of D$_1$-like receptor activity in RPTs. We have reported that in RPT cells of Wistar-Kyoto (WKY) rats, the D$_1$-like agonist, fenoldopam, increased D$_1$ receptor but decreased AT$_1$ receptor protein expression. In contrast, in RPT cells of spontaneously hypertensive rats (SHRs), fenoldopam also decreased AT$_1$ receptor expression but no longer stimulated D$_1$ receptor expression. We also reported that AT$_1$ and D$_1$ receptors coimmunoprecipitate in RPT cells; fenoldopam increased this coimmunoprecipitation in WKY RPT cells but decreased it in SHR RPT cells. We hypothesize that the AT$_1$ receptor may also regulate the D$_1$ receptor, including its expression. Therefore, we studied the effect of angiotensin II on D$_1$ receptor expression and AT$_1$ and D$_1$
receptor interaction in immortalized rat RPT cells. We have previously shown that the D<sub>1</sub> receptor effects on signal transduction and sodium transporters in immortalized RPT cells are similar to those obtained in freshly obtained renal proximal tubules. Parenti et al have also reported that the AT<sub>1</sub> receptor behaves similarly in freshly obtained renal tubules and immortalized RPT cells.

We now report that AT<sub>1</sub> and D<sub>1</sub> receptors colocalize in RPT cells. D<sub>1</sub> and AT<sub>1</sub> receptor coimmunoprecipitation was greater in WKY than in SHRs, in agreement with our previous report. In this earlier study, long-term fenoldopam stimulation (24 hours) increased the coimmunoprecipitation of D<sub>1</sub> and AT<sub>1</sub> receptors in WKY but decreased it in SHRs. We now show that angiotensin II decreases D<sub>1</sub> and AT<sub>1</sub>, coimmunoprecipitation similarly in WKY and SHRs. However, D<sub>1</sub> receptor expression is increased by angiotensin II in WKY but not in SHRs. In contrast, short-term angiotensin II, via AT<sub>1</sub> receptors (15 minutes), decreases cell surface membrane expression of D<sub>1</sub> receptors in WKY but not in SHRs. These studies show that angiotensin II differentially affects total cellular and surface membrane D<sub>1</sub> receptor expression in RPT cells from WKY and SHRs.

**Methods**

**Cell Culture**

Immortalized RPT cells from 4- to 8-week-old WKY and SHRs were cultured at 37°C in 95% air/5% CO<sub>2</sub> atmosphere in DMEM/F-12 culture media, as previously described. The cells (80% confluence) were extracted in ice-cold lysis buffer (phosphate-buffered saline with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin), sonicated, kept on ice for 1 hour, and centrifuged at 16 000 g for 30 minutes. The supernatants were stored at −70°C until use for immunoblotting and/or immunoprecipitation.

**Immunoblotting**

The antibodies are polyclonal purified antipeptides. The amino acid sequence for the immunogenic peptide (rabbit anti-human AT<sub>1</sub> receptor antibody) is QDDCPKAGRHC, amino acids 15 to 24 of the sequence for the immunogenic peptide (rabbit anti-human AT<sub>1</sub> receptor antibody) is QDDCPKAGRHC, amino acids 15 to 24 of the amino acid sequence of rat D<sub>1</sub> receptor (amino acids 299 to 307) (Research Genetics). Monoclonal mouse anti-human AT<sub>1</sub> receptor was purchased from Abcam Limited (Cambridge, UK).

**Immunofluorescence Confocal Microscopy of Double-Stained RPT Cells**

RPT cells, grown on coverslips, were fixed with 3% paraffinmaldehyde (30 minutes) and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (15 minutes). Reactions with antibodies were performed as described previously. The D<sub>1</sub> and AT<sub>1</sub> receptors were visualized by using a rabbit antipeptide polyclonal IgG affinity-purified rat D<sub>1</sub> receptor antibody followed by fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody (Molecular Probes) or by a mouse anti-human AT<sub>1</sub> receptor monoclonal antibody followed by the secondary goat anti-mouse antibody labeled with Alexa 568 (Molecular Probes). Cells on coverslips were mounted with the ProLong Antifade Kit (Molecular Probes). The immunofluorescence densities and images were acquired (Olympus AX70) at an excitation wavelength of 488 nm and 568 nm; emission was detected at 535 and 645 nm.

**Immunoprecipitation**

RPT cells were incubated with vehicle or angiotensin II (10<sup>−8</sup> M) for 24 hours, as described. The cells were lysed with ice-cold lysis buffer for 1 hour and centrifuged at 16 000 g for 30 minutes. The supernatants from the cell lysate was immunoprecipitated with the AT<sub>1</sub> receptor antibody (positive control) were used as immunoprecipitates, instead of the D<sub>1</sub> receptor antibody. To determine the specificity of the bands, preimmune serum of D<sub>1</sub> receptor antibody (negative control) and AT<sub>1</sub> receptor antibody (positive control) were used as immunoprecipitants, instead of the D<sub>1</sub> receptor antibody. The densities of the bands were quantified by densitometry using Quantiscan (Ferguson, Mo) as previously reported.

**Cell Surface D<sub>1</sub> Receptor Expression**

Cultured RPT cells were starved in serum-free medium for 2 hours, and then treated with angiotensin II (10<sup>−5</sup> M) for 15 minutes. Surface membrane proteins were biotinylated by adding sulfo-NHS-LC-biotin (final concentration 250 μg/mL) into the medium 5 minutes before adding angiotensin II. The cells were washed 3 times with wash buffer. Then, the cells were lysed with lysis buffer, sonicated and placed on ice for 1 hour. The supernatant from the cell lysate was immunoprecipitated with the anti-rat D<sub>1</sub> receptor antibody, followed by immunoblotting. The membrane sheets were blocked with 5% milk overnight and after washing (3 times), the sheets were incubated with peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratory, Inc, in wash buffer at 1/5000 dilution for 30 minutes. The biotinylated protein bands were visualized by enhanced chemiluminescence (Western Blotting Detection Kit; Amersham, Arlington Heights, Ill).

**Materials**

Rabbit anti-human AT<sub>1</sub> receptor antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, Calif). Rabbit anti-rat D<sub>1</sub> receptor antibody was produced against a synthetic oligopeptide from the amino acid sequence of rat D<sub>1</sub> receptor (amino acids 299 to 307) (Research Genetics). Monoclonal mouse anti-human AT<sub>1</sub> receptor was purchased from Abcam Limited (Cambridge, UK). Angiotensin II was purchased from Peninsula Laboratory, Inc (St Louis, Mo). Losartan was a gift from Merck & Co (Philadelphia, Pa). Peroxidase-conjugated streptavidin was purchased from Jackson ImmunoResearch Laboratory, Inc (West Grove, Pa). Sulfo-NHS-LC-biotin was purchased from PIERCE (Rockford, Ill). Other chemicals for various buffers were of the highest purity available and purchased either from Sigma or Gibco.

**Statistical Analysis**

The data are expressed as mean±SEM. Comparison within groups was made by ANOVA for repeated measures, and comparison among groups was made by factorial ANOVA with Duncan’s test. P<0.05 was considered significant.

**Results**

Activation of AT<sub>1</sub> Receptors Increases D<sub>1</sub> Receptor Expression in RPT Cells From WKY Rats, But Not From SHRs

In RPT cells from WKY rats, angiotensin II increased D<sub>1</sub> receptor expression in a concentration- and time-dependent...
manner. The stimulatory effect was evident at $10^{-7}$ M (Figure 1A) as early as 16 hours and maintained for at least 30 hours (Figure 1B). Consistent with the stimulatory effect of angiotensin II on D1 receptor expression (Figure 1A and 1B), a 24 hour-incubation with angiotensin II ($10^{-8}$ M) increased D1 receptors in RPT cells from WKY rats. In contrast, this effect was not observed in RPT cells from SHRs (WKY: control $= 0.77 \pm 0.15$, angiotensin II $= 1.33 \pm 0.19$; SHR: control $= 0.87 \pm 0.13$, angiotensin II $= 1.04 \pm 0.17$; n = 7) (Figure 1C).

To determine the specificity of angiotensin II action on AT1 receptors, we studied the effect of the AT1 receptor antagonist, losartan. Consistent with the results in WKY rats in Figure 1A, 1B, and 1C, angiotensin II ($10^{-8}$ M/24 hours) increased D1 receptor expression (control $= 0.83 \pm 0.15$, angiotensin II $= 1.48 \pm 0.15$; n = 8). The AT1 receptor antagonist, losartan ($10^{-8}$ M), by itself, had no effect on D1 receptor expression ($0.8 \pm 0.13$) but reversed the stimulatory effect of angiotensin II on D1 receptor expression ($0.7 \pm 0.08$) (Figure 1D).

To investigate whether there is a right-shift of the concentration-response curve in SHRs, their RPT cells were incubated with varying concentrations of angiotensin II ($10^{-9}$ M to $10^{-5}$ M) for 24 hours. Consistent with the results in Figure 1C, angiotensin II had no effect on D1 receptor expression in SHRs (Figure 1E).

**Figure 1.** Effect of angiotensin II on D1 receptor protein in RPT cells from WKY and SHRs. A, Concentration-response of D1 receptor protein in RPT cells from WKY rats treated with angiotensin II. Immunoreactive D1 receptor protein was determined after 24 hour incubation with the indicated concentrations of angiotensin II. Results are expressed as relative density units (DU) (n = 8, *P < 0.05 vs control (C), ANOVA, Duncan’s test). B, Time-course of D1 receptor protein in RPT cells from WKY rats treated with angiotensin II. The cells were incubated for the indicated times with $10^{-8}$ M angiotensin II. Results are expressed as relative density units (DU) (n = 8, *P < 0.05 vs control [0 time], ANOVA, Duncan’s test). C, Effect of angiotensin ($10^{-6}$ M/24 hours) on D1 receptor protein in RPT cells from WKY and SHRs. Results are expressed as the ratio of D1 receptor to a–actin densities (n = 7, *P < 0.05 vs control, ANOVA, Duncan’s test). D, Effect of angiotensin II (Ang) and an AT1 receptor antagonist (losartan) on D1 receptor protein in WKY rat RPT cells. The cells were incubated with the indicated reagents (angiotensin II, $10^{-8}$ M; losartan, $10^{-8}$ M) for 24 hours. Results are expressed as the ratio of D1 receptor to a–actin densities (n = 8, *P < 0.05 vs others, ANOVA, Duncan’s test). E, Concentration-response of D1 receptor protein in SHR RPT cells treated with angiotensin II. Immunoreactive D1 receptor protein was determined after a 24 hour-incubation with the indicated concentrations of angiotensin II. Results are expressed as relative density units (DU) (n = 6, P = NS vs control (O), ANOVA, Duncan’s test).

**AT1 Receptor Colocalizes With the D1 Receptor in Rat RPT Cells**

Immunofluorescence laser confocal microscopy revealed a colocalization of D1 and AT1 receptors in WKY rat RPT cells (Figure 2). To determine whether there is a physical interaction between the D1 and the AT1 receptor, additional experiments were performed. As shown in Figure 3, in the basal state, the band of 45 kDa, representing the coimmunoprecipitated D1 and AT1 receptors in RPT cells from WKY rats and SHRs (WKY: control $= 37 \pm 1$ DU) than in those from SHRs ($23 \pm 3$ DU, n = 7, P < 0.05), similar to the findings in our previous report.19 A 24-hour incubation with angiotensin II ($10^{-8}$ M) decreased D1/AT1 receptor coimmunoprecipitation to a similar degree in RPT cells from WKY rats and SHRs (WKY: control $= 37 \pm 1$ density units (DU), angiotensin II $= 28 \pm 2$ DU; SHR: control $= 23 \pm 3$ DU, angiotensin II $= 17 \pm 3$ DU; n = 7, P < 0.05).
Activation of AT1 Receptors Decreases the D1 Receptor Expression in Surface Membranes of RPT Cells From WKY Rats But Not From SHRs

The effect of short-term stimulation with angiotensin II on cell surface D1 receptors is shown in Figure 4. Angiotensin II (10^{-8} M) decreased the quantity of D1 receptors on the cell surface membranes in WKY RPT cells, but not in SHR RPT cells (WKY: control = 45 ± 5 DU, angiotensin II = 17 ± 5 DU; SHR: control = 19 ± 5 DU, angiotensin II = 19 ± 5 DU, n=6).

Discussion

Polygenic essential hypertension is associated with increased sodium transport in renal proximal tubules. As mentioned, dopamine and angiotensin II are important regulators of sodium and water reabsorption in the kidney, serving contrasting functions in the proximal tubule. Both dopamine and renin-angiotensin systems exist in the RPT, and the components of the renin-angiotensin system, including angiotensinogen mRNA, renin, and angiotensin-converting enzyme, have been localized to the proximal tubule. The proximal tubule is also the site of local dopamine production. Urinary dopamine and angiotensin II concentrations exceed circulating levels, suggesting both systems may have autocrine and/or paracrine effects in RPTs. Angiotensin II stimulates sodium reabsorption via activation of sodium transporting proteins that are inhibited by dopamine (NHE3, Cl/HCO3 exchanger, Na/HCO3 cotransporter, and Na+/K+ ATPase). Dopamine and D1-like and D2-like receptor agonists have been reported to antagonize the actions of angiotensin II on NHE3 and Na+/K+-ATPase activities in brush border and basolateral membranes of renal proximal tubules. Dopamine, via D1-like receptors, has also been reported to decrease the AT1 receptor mRNA and protein, as well as angiotensin II binding sites in renal proximal tubules. The effect of angiotensin II on D1-like receptors has not been reported.

In WKY RPT cells, stimulation of the AT1 receptor with angiotensin II for 2 different periods (short-term or long-term) yields opposite results: short-term stimulation decreases cell surface D1 receptor expression, whereas long-term stimulation increases whole cell D1 receptor expression. It is unlikely that the change in the short-term could account for the long-term result. A decrease of cell surface D1 receptor expression would remove a restraint allowing AT1 receptor activation to increase sodium reabsorption to a greater extent. However, the increase in D1 receptor expression would tend to counterbalance the prolonged angiotensin II effect.

Cell surface membrane localization of G protein-coupled receptors is decreased by phosphorylation of the receptor. We have reported that G protein-coupled receptor kinases (GRKs), GRK2 and GRK4, are involved in D1 receptor phosphorylation. Among the GRKs, GRK2 is most impor-

Figure 3. Effect of angiotensin II on the coimmunoprecipitation of D1 and AT1 receptors in rat RPT cells. The cells were incubated with angiotensin II (10^{-8} M) for 24 hour. Results are expressed as relative density units (DU) (∗∗P<0.05 vs control, ∗P<0.05 vs WKY, n=7, ANOVA, Duncan’s test). One immunoblot (45 kDa) is depicted in the inset: (lane 1=positive control, lane 2=negative control, lane 3=vehicle-treated RPT cells from WKY rats, lane 4=angiotensin II-treated RPT cells from WKY rats, lane 5=vehicle-treated RPT cells from SHRs, lane 6=angiotensin II-treated RPT cells from SHRs).

Figure 4. Effect of angiotensin II on cell surface D1 receptors in rat RPT cells. The cells were incubated with angiotensin II (10^{-8} M) for 15 minutes. Results are expressed as relative density (DU) (∗P<0.05 vs control, ∗∗P<0.05 vs WKY, n=6, ANOVA, Duncan’s test). One immunoblot is depicted on top of the bar graphs.
tant in promoting AT₁ receptor phosphorylation. It is possible that the GRK2 activation subsequent to AT₁ receptor stimulation also results in the alteration of the phosphorylation and membrane localization of the D₁ receptor. Such a scenario can explain the ability of AT₁ receptors to decrease the membrane localization of the D₁ receptor in WKY. This does not occur in SHRs because the D₁ receptor is already hyperphosphorylated and fewer D₁ receptors are found on renal cell surface membranes in SHR. Although GRK4 is the major GRK regulating D₁ receptor function, GRK4 is not involved in the desensitization of the AT₁ receptor. The possibility exists that the AT₁ receptor can cause heterologous desensitization of the D₁ receptor via stimulation of effector enzymes, but it is not clear which one.

The dephosphorylation of D₁ receptors in renal proximal tubules is also different in WKY and SHRs. We have reported that short-term D₁-like receptor stimulation increases protein phosphatase 2A activity in renal proximal tubules in WKY but not in SHRs. It is possible that in the WKY, the AT₁ receptor inhibits protein phosphatase 2A activity and thus prevents dephosphorylation and recycling of the D₁ receptor to the cell surface membrane. Although there is no report to support this mechanism, AT₁ receptor has been reported to stimulate protein phosphatase 2A activity.

Long-term stimulation of AT₁ receptors increases total cellular D₁ receptor expression in WKY. This finding in vitro was also found in vivo; chronic angiotensin II infusion in mice also caused an increase in D₁ receptor expression (unpublished data). Presumably, cell surface D₁ receptor expression is also increased. Although the mechanism by which AT₁ receptor stimulation increases D₁ receptor expression remains to be determined, such an effect could serve as a physiological negative feedback under normal circumstances (in WKY). However, in SHR cells, this upregulation of D₁ receptors by stimulation of AT₁ receptors is lost because of decreased responsiveness of the hyperphosphorylated D₁ receptor.

We have reported that the renal D₁ receptor is hyperphosphorylated in SHRs as a consequence of increased activity of GRK4 and decreased protein phosphatase 2A activity.

Long-term incubation with angiotensin II decreases D₁/AT₁ receptor coimmunoprecipitation to the same degree in cells from SHR and WKY rats. Our previous study showed that long-term exposure to angiotensin II decreases AT₁ receptor protein expression in RPT cells from WKY rats but increases it in SHRs. The decreased interaction between D₁ and AT₁ receptors in renal proximal tubules in WKY rats is, therefore, not the result of decreased AT₁ receptor expression, per se, because a similar response is seen in SHRs where AT₁ expression is increased by angiotensin II. Thus, angiotensin II, via the AT₁ receptor, probably causes a decrease in the physical interaction of AT₁ and D₁ receptors in both WKY and SHR cells. Further studies are needed to determine whether the decreased interaction between these 2 receptors is direct or indirect, possibly by the alteration of an adaptor gene or adaptor proteins.

In both SHR and Dahl salt-sensitive rats, dopamine and D₁-like receptor agonist-mediated natriuretic and diuretic responses are impaired. The impaired D₁-like receptor function in hypertension is not caused by abnormalities in G proteins, effectors, or ion transporting proteins, such as adenyl cyclase, NHE3 or Na⁺/K⁺ ATPase. Rather, the renal D₁-like receptor is uncoupled from G protein subunits, leading to decreased D₁-like receptor interaction with G protein subunits, and resulting in decreased production of second messengers and decreased interaction between G protein subunits, effector enzymes, and ion transporters.

To determine whether the impaired D₁ receptor function occurs at the receptor level, we investigated the expression of D₁ receptor expression in cell surface membranes and whole cells. We found that cell surface membrane expression of D₁ receptors is lower and D₁ receptor phosphorylation is higher in SHRs than in WKY cells, although total and D₁ receptor expression are not different in the 2 cell lines. Renal D₁ receptor phosphorylation and GRK4 activity impact on the regulation of blood pressure. Inhibition of renal GRK4 activity by antisense oligonucleotides attenuates the increase in blood pressure with age in SHRs without affecting blood pressure in WKY rats.

In summary, we have demonstrated that long-term stimulation of AT₁ receptors positively regulates the expression of D₁ receptors in RPT cells from WKY but not from SHRs. AT₁ and D₁ receptors colocalize in RPT cells from both rat strains, but the basal level of cell surface membrane D₁ receptor expression is higher in WKY than in SHR RPT cells. It appears that a greater proportion of D₁ receptors are located on the cell surface membrane in WKY cells than in SHR cells. Short-term AT₁ receptor stimulation decreases cell surface membrane D₁ receptor expression in WKY rats but not in SHRs. It is possible that differences in AT₁ receptor regulation of D₁ receptor expression and cell surface expression may participate in the abnormal regulation of renal proximal sodium transport in genetic hypertension.

**Perspectives**

The dopaminergic and renin-angiotensin system are 2 important systems that regulate blood pressure. Dopamine promotes natriuresis, whereas angiotensin II decreases sodium excretion. The major D₁-like receptor subtype mediating the increase in sodium excretion is probably the D₁ receptor, whereas the major angiotensin II receptor mediating the decrease in renal sodium excretion is the AT₁ receptor. In SHRs, renal proximal tubular D₁ receptor function is impaired. Regardless of the mechanism involved in the interaction between D₁ and AT₁ receptors, the dissociation of D₁ from AT₁ receptors after angiotensin II stimulation may allow the AT₁ and D₁ receptor to exert their functions separately. Renal proximal tubular D₁ receptors are functional in WKY but not in SHRs. In contrast, AT₁ receptor function is enhanced in SHRs. The inability of AT₁ receptors to increase D₁ receptor expression in renal proximal tubules in SHRs and a decreased D₁ and AT₁ receptor interaction could lead to enhanced AT₁ receptor function.

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