0013-7227/97/$03.00/0
Endocrinology
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Vol. 138, No. 8
Printed in U.S.A.

Aldosterone Biosynthesis in the Rat Brain*

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ABSTRACT

Messenger RNA (mRNA) for enzymes involved in adrenal steroid biosynthesis are expressed in the brain, and the coded enzymes have been shown to be active. The expression of mRNA for the cytochrome P-450 enzyme aldosterone synthase, crucial for the final step in the synthesis of aldosterone and the synthesis of aldosterone was studied in several anatomic areas of the rat brain. Expression of the mRNA for the aldosterone synthase was demonstrated by RT-PCR/Southern blot in adrenal, aorta, hypothalamus, hippocampus, amygdala, cerebrum, and cerebellum. Incubation of brain minces from intact and adrenalectomized rats demonstrated the synthesis of corticosterone and aldosterone from endogenous precursors. Incubations of brain minces with [1,2^{3}H]-deoxycorticosterone, followed by extraction and three different successive TLCs, demonstrated the presence of labeled aldosterone, corticosterone, and 18-hydroxy-deoxycorticosterone. Incubation, in the presence of 10 μM cortisol or metyrapone, inhibited the synthesis of aldosterone or both aldosterone and corticosterone, respectively. These studies indicate that the rat brain has the enzymatic machinery for the synthesis of adrenal corticosteroids and is capable of synthesizing aldosterone. Aldosterone synthesized in the brain might play a paracrine role in the regulation of blood pressure. (*Endocrinology* **138:** 3369–3373, 1997)

MINERALOCORTICOIDS are crucial for fluid, electrolyte, and blood pressure homeostasis in vertebrates (1). Aldosterone, the primary mineralocorticoid, is synthesized in the zona glomerulosa of the adrenal cortex and released to the circulation to be carried to target organs, where it binds the mineralocorticoid receptor to exert its actions. Target organs include epithelia involved in vectorial transfer of sodium, vascular smooth muscle, and select areas of the brain, where aldosterone action modulates blood pressure and behavior related to sodium acquisition (2). Enzymes of the adrenal steroidogenic cascade, down through corticosterone, have been demonstrated in the brain (3–10). Cytochrome P-450-aldosterone synthase, the final enzyme in the aldosterone biosynthetic pathway, hydroxylates deoxycorticosterone (DOC) successively to corticosterone, 18-hydroxycorticosterone, and aldosterone and is expressed in the zona glomerulosa of the adrenal gland (11). Extraadrenal synthesis of aldosterone and the expression of the aldosterone synthase messenger RNA (mRNA) has been demonstrated in aortic endothelial cells and in vascular smooth muscle (12). The aldosterone synthase mRNA could not be demonstrated in the rat brain using an ribonuclease protection assay (7). We present our studies on the expression of the aldosterone synthase in the rat brain, using RT-PCR/Southern blot, and demonstrate aldosterone biosynthesis in brain minces.

Materials and Methods

Materials

Oligonucleotides were synthesized at the University of Missouri-Columbia DNA core. Ham F12 culture medium and steroids were obtained from Sigma Chemical Company (St. Louis, MO). Solvents were reagent grade and obtained from Fisher Scientific Company (St. Louis, MO). RT, Superscript II, was purchased from Life Technologies (Gaithersburg, MD). Male and female Sprague-Dawley rats, weighing 180–240 g, were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and were kept on a normal rat chow. Rats were adrenalectomized under Isoflurane anesthesia and were maintained on 0.9% saline, as drinking fluid, until used.

RT-PCR of the aldosterone synthase

Total RNA from adrenal and extraadrenal tissues from six male and female rats (180–200 g), placed on a low-sodium diet, was extracted using RNAZol (13). Reverse transcription was performed using Superscript II and a poly-T primer. PCR was performed using the primers: sense GGA TGT CCA GCA AAG TCT CTT C, antisense CCT GAG TTA TTA GTG CTG CCA C (amplified a 332-bp specific fragment of the aldosterone synthase) to amplify a 332-bp fragment from exons 3–5 (the genomic DNA will have 717 bp). A total of 27 cycles were run and the product was electrophoresed in agarose and then transferred to a nylon membrane for Southern blotting. The biotin-labeled fragment for hybridization was generated using the PCR Nonradioactive Labeling System from Life Technologies. Negative controls comprised a water blank and tubes in which the RNA and all of the reagents for RT-PCR, except RT, were present. The amount of RNA from the adrenal sample was 1/50 that of the other tissues. RT-PCR of a hypothalamic sample was run for 40 cycles and the band sequenced using the *Taq* DyeDeoxy Terminator Cycle sequencing Kit and ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA).

Incubation of rat brain minces

Minces from various brain sections (~100 mg) from eight intact male rats and eight male rats, 5 days after bilateral adrenalectomy, were incubated in 1 ml of Ham F12 medium (n = 3) at 37 C in an atmosphere of 5% CO_2 in air for 3 h. The supernatant (50 μl) was assayed for

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Received December 30, 1996.

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* This work was supported by the Medical Research Service of the Department of Veterans Affairs and NIH Grants HL-27255 and -27737.

† Recipient of a J. William Fulbright International Scholarship.

aldosterone by enzyme-linked immunosorbent assay (ELISA) using specific antibodies (14). The experiment was repeated twice.

Incubation of rat brain minces with [1,2^{3}H]-DOC and [1,2^{3}H]-corticosterone.

Minces (~100 mg) from various brain areas of eight intact male rats (180–200 g) in triplicate were incubated in Ham F12 medium containing 10 μM DOC plus 10 μCi [1,2^{3}H]-DOC at 37 C in an atmosphere of 5% CO$_2$ in air for 3 h. The supernatant was separated and extracted initially with 7% dichloromethane in hexane to remove nonpolar steroids (DOC), followed by extraction of the aqueous phase with dichloromethane. The organic extract was evaporated under air and purified using TLC with Silica Gel GF254 plates and chloroform:methanol:water (300:20:1). The areas corresponding to aldosterone and corticosterone were scraped and eluted twice with 1 ml chloroform:methanol (3:1). Eluates were then chromatographed in chloroform:acetone (82:18). The areas corresponding to aldosterone, corticosterone, 18-OH-DOC, and 11-dehydrocorticosterone were eluted and processed as above and rechromatographed in benzene:acetone (2:1) for aldosterone and benzene:acetone (3:1) for corticosterone, 18-OH-DOC, and 11-dehydrocorticosterone. Recoveries were estimated by incubating tissues in a similar way with unlabeled DOC, and at the end of the incubation, known amounts of tritiated corticosterone, 11-dehydrocorticosterone, 18-OH-DOC, and aldosterone were added and handled as above. The recoveries varied between 35–45%. After correction for recoveries, the production was expressed as mol/mg of wet tissue. Similar incubations and purifications were done with [1,2^{3}H]-corticosterone.

To further demonstrate that ^{3}H-aldosterone was formed from ^{3}H-DOC, 200 mg of cerebellar minces were incubated with 1 μCi ^{3}H-DOC in 5 ml Ham F12 medium for 3 h at 37 C. The medium was extracted with 40 ml of dichloromethane, washed with 2 ml N NaOH and water, and evaporated. The extract was then subjected to TLC using chloroform: methanol:water (300:20:1). The area corresponding to aldosterone was eluted as above, evaporated, and dissolved in 5 ml dichloromethane and treated with 0.5 ml of 0.1 M periodic acid for 1 h in the dark. The dichloromethane was then washed with 1 ml N NaOH twice and water. The evaporated extract was then subjected to reverse-phase HPLC using a C-18 column (Whatman EQC S100A ODS 5 μ, 5 × 250 mm) and eluted with methanol water 50%, and 1-ml fractions were collected and counted in a liquid scintillation counter.

Inhibition of steroid synthesis by cortisol and metyrapone

Cerebellar minces were incubated in triplicate with [1,2^{3}H]-DOC in the presence and absence of 10 μM cortisol and 10 μM metyrapone and the supernatants assayed as described above.

Measurement of aldosterone by ELISA

ELISA for aldosterone was done using a monoclonal antibody as previously described (14). The sensitivity of the assay is 1 pg/well. The blank of the assay, when using control incubations with Ham F12 medium, varied between undetectable amounts to 2 pg/50 μl of medium and were less than 10% of that measured in the various samples. The data are presented as the mean ± SEM.

Results

Expression of the aldosterone synthase mRNA was demonstrated in this study by RT-PCR, with amplification for 27 cycles, followed by Southern blot hybridization from RNA extracted from the adrenal, aorta, hypothalamus, hippocampus, amygdala, and cerebellum (Fig. 1). The RT-PCR Southern blot gave a strong band corresponding to the 332-bp fragment of the aldosterone synthase and several other smaller bands of unknown origin. A sample from hypothalamus was amplified, using 40 cycles, and the 332-bp band sequenced, confirming that the amplified band was truly the mRNA for aldosterone synthase. RT-PCR controls done similarly, but without using RT, gave no bands. mRNA was not detected in mesenteric artery, and only faint bands were seen in the atrium and ventricles of the heart, even under the condition of low sodium intake, which maximally stimulates adrenal aldosterone synthase mRNA and activity and production of aldosterone. RT-PCR of tissues from female rats gave similar results.

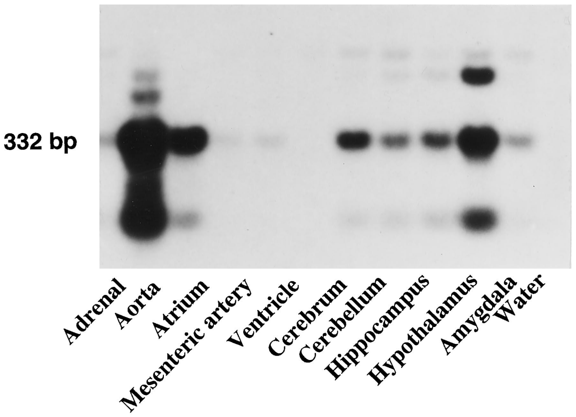

FIG. 1. RT-PCR Southern blot hybridization of aldosterone synthase mRNA in male rats with a low sodium intake. The 332-bp band corresponds to the aldosterone synthase confirmed by sequencing.

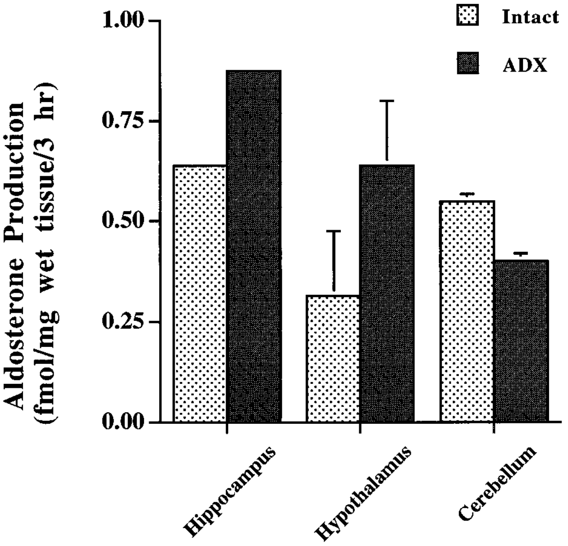

FIG. 2. Aldosterone and corticosterone production from incubations of minces from various brain sections from male intact and adrenalectomized rats. The results are triplicates from an experiment that was done twice.

To investigate aldosterone synthase activity in the brain, minces of hippocampus, hypothalamus, and cerebellum from intact rats and rats adrenalectomized for 5 days were incubated (Fig. 2). Hippocampus, hypothalamus, and cerebellum from both intact and adrenalectomized rats secreted similar amounts of aldosterone into the medium, suggesting that aldosterone is formed *de novo* in the brain from endogenous precursors. Brain minces also were incubated with 10 μM of DOC containing 10 μCi [1,2 ^{3}H]-DOC at 37 C for 3 h. The supernatant was extracted and subjected to three successive TLCs. Aldosterone and corticosterone were formed in hippocampus, hypothalamus, brain stem, and cerebellum with corticosterone (and its metabolite 11-dehydrocorticosterone) formation predominating (Fig. 3a). Incubations of cerebellar minces, with similar amounts of cold and tritiated corticosterone, yielded similar results (Fig. 3b). The formation of 11-dehydrocorticosterone from corticosterone was very prominent, as would be expected, because no attempt was made to inhibit the 11β-hydroxysteroid dehydrogenases. Aldosterone formation from ^{3}H-DOC was confirmed by treating the extract corresponding to the aldosterone band with periodic acid to form the etiolactone, which was then subjected to HPLC. A labeled peak corresponding to aldosterone-etiolactone was demonstrated clearly (Fig. 4). Periodic acid oxidation of steroids with the hydroxy ketone side chain are transformed to etienic acids, which are eliminated by the NaOH wash. The etiolactones of aldosterone and 18-hydroxylated steroids remain in the organic solvents but have different elution characteristics.

Minces from cerebellum also were incubated with 10 μM of DOC containing 10 μCi [1,2 ^{3}H]-DOC at 37 C for 3 h with and without 10 μM cortisol or 10 μM metyrapone. Cortisol is a competitive inhibitor of the aldosterone synthase and decreased the formation of aldosterone (Fig. 5) but not of corticosterone or 18-OH-DOC (15). Metyrapone is a cytochrome P-450 inhibitor (16) and, as expected, inhibited the formation of aldosterone, corticosterone, and 18-OH-DOC (Fig. 5).

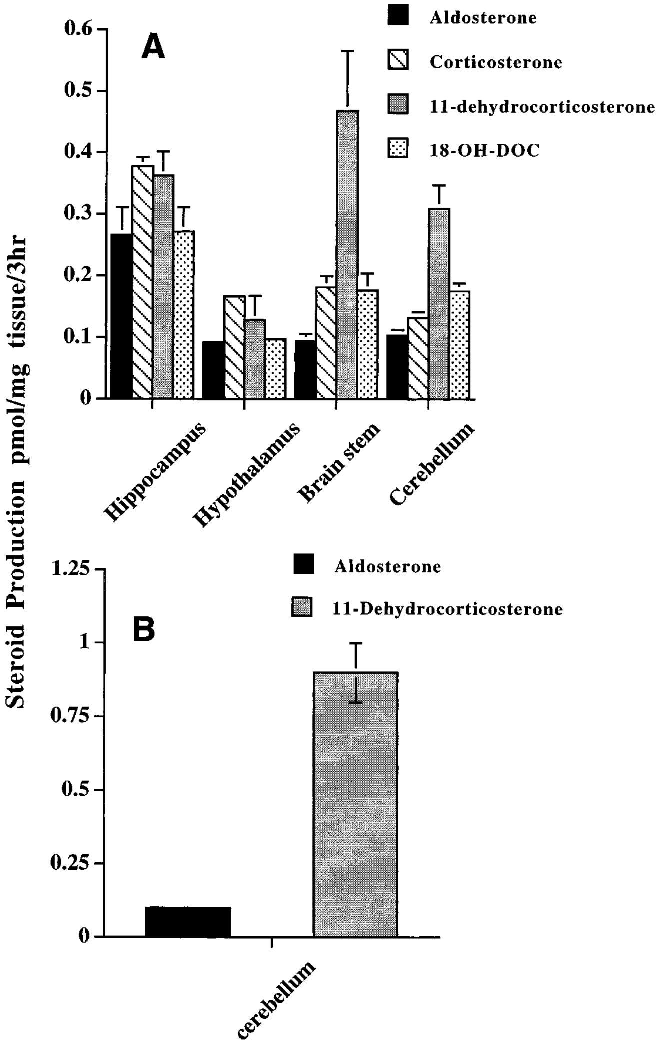

FIG. 3. A, Conversion of [1,2^{3}H]-DOC to aldosterone, corticosterone, 18-hydroxy-DOC, and 11-dehydrocorticosterone from incubations with minces from various brain areas of intact rats; B, conversion of [1,2^{3}H]-corticosterone to aldosterone and 11-dehydrocorticosterone from incubation of minces from cerebellum.

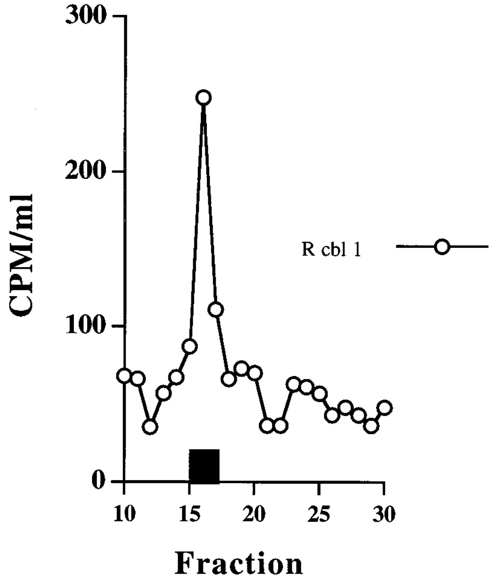

FIG. 4. HPLC elution of ^{3}H-aldosterone-etiolactone from incubation of cerebellum with ^{3}H-DOC. The extract was purified by TLC, and the area corresponding to aldosterone was oxidized with periodic acid, washed with NaOH, and subjected to reverse-phase HPLC eluted with 50% methanol. The *bar* corresponds to the elution of authentic aldosterone-etiolactone.

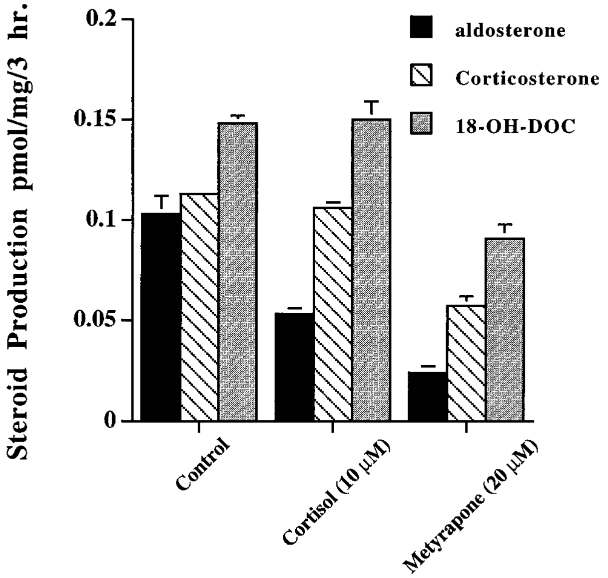

FIG. 5. Inhibition of the conversion of [1,2^{3}H]-DOC to aldosterone, 18-hydroxy-DOC, and corticosterone from incubations of minces from cerebellum of intact male rats in the presence and absence of 10 μM cortisol or 10 μM metyrapone.

Discussion

The brain, an important target organ for most circulating steroid hormones, has been known to possess steroidogenic capabilities for over 50 yr (17). In the last two decades, many of the enzymes involved in steroid synthesis have been demonstrated in the central nervous system (CNS). The term, neurosteroids, was coined by Baulieu and Robel to refer to the synthesis of pregnenolone, progesterone, and 20α-hydroxy-pregnenolone from cholesterol within the brain (4), but the term has been extended to encompass the biosynthesis of any steroid within the CNS (3). Though most research on brain biosynthesis of steroids has focused on pregnenolone, progesterone, DHEA, and their derivatives, there is increasing evidence that adrenal steroids also are synthesized within the CNS (3, 6, 7, 18). The first regulated step in steroid biosynthesis is the conversion of cholesterol to pregnenolone by the cytochrome P-450 side-chain cleavage (scc) enzyme. In addition to adrenal and gonadal cells, this reaction has been reported to occur in oligodendrocytes, glial cells, and rat C6 glioma cells (4, 7). The mRNA expression of the scc enzyme is very low, requiring RT-PCR, combined with Southern blotting, for its demonstration (7); however, the enzyme can be demonstrated rather easily using immunocytochemistry or Western blots (19, 20), suggesting that the protein is very stable in the CNS. The next step in steroid synthesis, conversion of pregnenolone to progesterone by the 3β-hydroxysteroid dehydrogenase Δ4–5 isomerase, also has been demonstrated in glial and Schwann cells (9). Activity and immunoreactivity of the microsomal cytochrome P-450–21-hydroxylase, the enzyme responsible for the hydroxylation of progesterone and 17-hydroxy-progesterone to 11-DOC and 11-deoxycortisol, respectively, have been demonstrated in the brain, especially in the myelinated tracts of the ascending reticulothalamic fibers (8).

Expression of the 11β-hydroxylase genes, CYP11B1 and CYP11B3 (7, 21, 22), but not the CYP11B2 gene (7), have been demonstrated previously in the brain by ribonuclease protection assays, *in situ* hybridization, and RT-PCR (7, 22, 23). The cytochrome P-450 11β-hydroxylase, product of the CYP11B1 gene, converts 11-DOC to corticosterone and 18-hydroxy-DOC in the rat, and 11-deoxycortisol to cortisol in the human. 11β-Hydroxylase immunoreactivity has been found in the myelinated tracts in the same general areas of the brain where the P450$_{scc}$ has been located (6); however, unlike the P450$_{scc}$, the 11β-hydroxylase was not found in cultured glia, suggesting that it may be found in neurons (7). The production of corticosterone plus its metabolic product, 11-dehydrocorticosterone, was significantly greater than that of 18-OH-DOC. The CYP11B3 mRNA is expressed in similar amounts in the adrenal gland and brain (22). It is not known if the gene product of the CYP11B3, the 18/11β-hydroxylase mRNA is translated into protein in the brain or adrenal; however, if this enzyme were present in significant quantities, one would have expected a greater proportion of 18-OH-DOC, compared with corticosterone and 11-dehydrocorticosterone, to have been formed (22).

The aldosterone synthase message and activity has been reported to be expressed in human endothelial cells and rat mesenteric arteries (12), but we could not demonstrate it in mesenteric artery. We cannot explain this discrepancy (12), and further studies need to be done. Aldosterone has been measured previously in various areas of the brain, but its source was assumed to be the adrenal (24). Our studies show the presence of the mRNA for the CYP11B2 gene in various areas of the brain and the synthesis of aldosterone from endogenous substrate and exogenous DOC and corticosterone. The demonstration of the CYP11B2 gene product and aldosterone synthase activity in the brain may have important implications for the control of blood pressure under certain conditions. In addition to increasing sodium retention by the kidney and vascular smooth muscle reactivity, aldosterone produces hypertension via mineralocorticoid receptors in the brain (25). The SS/jr rat is an inbred strain of the Dahl Salt Sensitive rat, which is spontaneously hypertensive, given enough time, but which develops malignant hypertension if fed a high-salt diet. We have shown that the salt-induced hypertension in this strain can be prevented by the intracerebroventricular infusion of a mineralocorticoid receptor antagonist at doses that are too low to be effective when infused systemically (26), yet circulating aldosterone is not elevated in these animals. An analogy to the blood pressure-lowering response to mineralocorticoid antagonist in the SS/jr rat may be present in a significant subset of people with essential hypertension, who respond to mineralocorticoid antagonist therapy, even though their plasma renin and aldosterone levels are normal or low. 19-Ethynyldeoxycorticosterone is a mechanism-based inhibitor of various 11β-hydroxylases (27), which was shown to decrease salt-induced blood pressure in the SS/jr rat when administered as an sc implant (28). The intracerebroventricular infusion of doses of 19-ethynyldeoxycorticosterone, which were too low to have a systemic effect, resulted in the mitigation of the increase in blood pressure produced by increasing salt intake in the SS/jr rat (21).

The amounts of mRNA for steroidogenic enzymes of the late adrenocorticosteroid synthetic pathways are quite low, as are the amounts of the steroids measured in the supernatant from tissue incubations. If aldosterone synthesized in the CNS is relevant to blood pressure control, it almost certainly acts in an paracrine fashion, directly or indirectly in the areas that have been identified by ablation and infusion studies to be important in blood pressure regulation (2). These studies did not show impressive differences in the regional synthesis of aldosterone in the brain; however, the minces of the various brain regions that were used comprise several nuclei, whereas aldosterone may be synthesized in only a few cells and act in a paracrine fashion. The relatively large and indiscriminate anatomic areas harvested would mask differential secretion in discrete nuclei or their member cells. Aldosterone paracrine actions might explain why mineralocorticoid antagonists effectively lower blood pressure in some low-renin, low/normal aldosterone forms of essential hypertension in man and genetic and experimental hypertension models in animals in which circulating mineralocorticoids are not elevated (29).

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