Pharmacology, Biochemistry and Behavior 94 (2010) 341–345

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Neurochemical changes on oxidative stress in rat hippocampus during acute phase of pilocarpine-induced seizures

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ARTICLE INFO

Article history:
Received 16 August 2009
Received in revised form 13 September 2009
Accepted 21 September 2009
Available online 1 October 2009

Keywords:
Seizures
Gangliosides
Phospholipids
Na^+, K^+-ATPase
Glutathione reduced

ABSTRACT

Using the epilepsy model obtained by systemic administration of pilocarpine in rats in the present study we investigated the changes caused by seizures on content and species of gangliosides and phospholipids, as well as on cholesterol concentration, glutathione reduced contents, Na^+, K^+-ATPase activity and lipid peroxidation levels in rat hippocampus. Wistar rats received pilocarpine hydrochloride (400 mg/kg, i.p., pilocarpine group), and other group received 0.9% saline (i.p., control group). Results showed that seizures significantly decreased the total content of lipids and glutathione reduced concentration in rat hippocampus. We also observed that seizures significantly reduced the absolute quantity of the major brain gangliosides (GM1, GD1a, GD1b and GT1b) and phospholipids (sphingomyelin, phosphatidylcholine and phosphatidylethanolamine). Our data also showed a decreased Na^+, K^+-ATPase activity and an increased TBARS levels in hippocampus of seized rats. If confirmed in human beings, these data could suggest that the alteration in lipid composition, Na^+, K^+-ATPase activity, glutathione reduced content and TBARS levels caused by seizures might contribute to the neurophysiopathology of seizures observed in epileptic patients.

1. Introduction

Previous studies have showed that plasma membrane lipids of the central nervous system (CNS) include gangliosides, phospholipids and cholesterol (Dal-Pizzol et al., 2000). Gangliosides are glycosphingolipids containing sialic acid in the molecule. They are related to cell to cell signaling and interaction, cellular growth and differentiation and may be involved in neural development (synaptogenesis myelinogenesis), since neural membranes are enriched in these lipids (Nagai, 1995).

It has been also shown that lipids, such as gangliosides, phospholipids and cholesterol present a variety of important physiological functions in neurons (Agranoff and Hajra, 1994). Gangliosides, a complex family of sialylated glycosphingolipids, are particularly abundant in the neuronal membranes acting on proliferation and neuronal differentiation, myelination and synaptic transmission. It has also been proposed that gangliosides may play significant role in learning/memory mechanisms (She et al., 2005).

Phospholipids are a heterogeneous group of compounds that constitute the backbone of neural membranes. They provide the membrane with suitable environment, fluidity and ion permeability, and are required for the function of integral membrane proteins, receptors and ion channels (Farooqui et al., 2004). Cholesterol is a structural component of membranes and is required for viability and cellular proliferation (Ohvo-Rekila et al., 2002). It and glycosphingolipids are concentrated in detergent-resistant microdomains or lipid rafts, which are seen as platforms for the signal transduction events (Paratcha and Ibanez, 2002). Cholesterol is also involved in membrane trafficking, myelin formation and synaptogenesis (Valenza and Cattaneo, 2006).

There are some evidence showing that alterations in the content and composition of gangliosides occur in experimental models of neurodegenerative diseases (Trindade et al., 2002; Ramirez et al., 2003). Additionally, it has been demonstrated that brain phospholipid and cholesterol contents decrease with advancing age (Svennerholm and Gottfries, 1994) and in patients with neurodegenerative diseases (Farooqui et al., 2004). However, the changes in the content and composition of gangliosides, phospholipids and cholesterol in hippocampus of adult rats yet no were demonstrated during acute phase of seizures induced by pilocarpine.

Based in this, the aim of this study was to investigate whether seizures affect the content and the species of gangliosides and phospholipids, as well as the concentration of cholesterol in hippocampus of rats. Additionally, considering that Na^+, K^+-ATPase and thiobarbituric acid-reactive substances (TBARS) are marker of neuronal membrane (Jones and Matus, 1974) and lipid peroxidation (Ohkawa et al., 1979; Fighera et al., 2006), respectively, we also evaluated the changes caused by seizures on these parameters in rat hippocampus. Our hypothesis is that

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doi:10.1016/j.pbb.2009.09.015

seizures could alter lipid content, Na^+, K^+-ATPase activity, glutathione reduced concentration and lipid peroxidation in rat hippocampus. We used hippocampus because this structure is involved in memory/learning mechanisms (Izquierdo et al., 1998) and several patients with epilepsy present cognitive impairment (Stella, 1999).

2. Experimental procedures

2.1. Animals and reagents

Male Wistar rats (250–280 g; 2-month-old) were obtained from Central Animal House of the Federal University of Piaui, Piaui, Brazil. They were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in air conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20% (w/w) protein commercial chow. Animal care followed the official governmental guidelines in compliance with the Society Policy and was submitted by the Ethics Committee of the Federal University of Piaui, Brazil. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Experimental procedure

The following substance was used: pilocarpine hydrochloride (Sigma, Chemical USA). All doses are expressed in milligrams per kilogram and were administered in a volume of 10 mL/kg injected intraperitoneally (i.p.). In a set of experiments, the animals were divided in two groups and treated with 0.9% saline (i.p., $n = 24$) and 30 min later, they received pilocarpine hydrochloride (400 mg/kg, i.p.), and in this 30-min interval rats were observed for the occurrence of any change in behavior. The treatment previously described represents the pilocarpine group. Other group received 0.9% saline (i.p., $n = 36$, control group). After the treatments, the animals were placed in 30 cm × 30 cm chambers to record: latency to first seizure (any one of the behavioral indices typically observed after pilocarpine administration: wild running, clonus, tonus, clonic–tonic seizures (Turski et al., 1983), number of animals that died during 1 h after pilocarpine administration.

Previous work have shown that the numbers of convulsions and deaths occurring within 1 and 24 h post injection always follow the same pattern, so we decided to observe the animals for 1 h as pilocarpine-induced convulsions occur in 1 h and deaths within 1 h after pilocarpine injection. The survivors were killed by decapitation and their brains dissected on ice to remove hippocampus for determinations content and species of gangliosides and phospholipids, as well as on cholesterol concentration, glutathione reduced contents, Na^+, K^+-ATPase activity and lipid peroxidation levels. The pilocarpine group was constituted by those rats that presented seizures; SE for over 30 min and that did not died within 1 h.

The drug dosages were determined from both dose–response studies, including pilocarpine (data not shown), and observations of the doses currently used in animals studies in the literature (Oliveira et al., 2007; Freitas, 2009). The doses used are not equivalent to those used by humans because rats have different metabolic rates.

2.3. Lipid extraction

The hippocampus was weighed and homogenized in a 2:1 mixture of chloroform:methanol (*C*:*M*, 2:1, v/v) to a 20-fold dilution of tissue mass and centrifuged at 800 ×*g* for 10 min. The pellet was re-homogenized in *C*:*M* (1:2) to a 10-fold dilution of original sample mass (Folch et al., 1957). The *C*:*M* extracts were combined and this pool was used for the following determinations.

2.4. Total gangliosides, phospholipids and cholesterol determinations

Aliquots from the total lipid extracts were used for ganglioside determination by the N-acetyl-neuraminic acid (NeuAc) quantification with the thiobarbituric acid assay described by Skoza and Mohos (1976). Phospholipid and cholesterol were quantified in aliquots from total lipid extracts according to the method of Bartlett (1959) and to the Trinder-enzymatic technique (Bergmeyer, 1974), respectively.

2.5. Thin layer chromatography (TLC) analysis

Ganglioside species were analyzed by TLC and this technique was performed on 10 cm × 10 cm Merck plates of silica gel 60 using a developing tank described by Nores et al. (1994). Aliquots of the total lipid extracts containing 4 nmol of NeuAc suspended in 10 mL *C*:*M* (1:1) were spotted on 8 mm lanes. TLC was developed, sequentially, with two mixtures of solvents, firstly *C*:*M* (4:1, v/v) and secondly *C*:*M*: 0.25% $CaCl_2$ (60:36:8, v/v/v). Ganglioside profile was visualized with resorcinol reagent (Svennerholm, 1957). The chromatographic bands were quantified by scanning densitometry at 580 nm with a CS 9301 PC SHIMADZU densitometer. Individual ganglioside values, expressed as nmol NeuAc/mg protein, were calculated by relating their respective percentage to the absolute total quantity of ganglioside-NeuAc. The terminology used herein for gangliosides is that recommended by Svennerholm (1963).

Phospholipid species were analyzed by TLC using chloroform: methanol: acetic acid:water (*C*:*M*:Aac:W, 86:14:4:1, v/v/v/v) as the solvent system which is a modification of the theoretical under phase (Folch et al., 1957). Aliquots of total lipid extracts containing a quantity equivalent to 7 mmol of inorganic phosphorus (Pi) suspended in 10 mL of *C*:*M* (2:1) were spotted on same plate size described above. Phospholipid bands were visualized with Comassie-Blue R250 (Nakamura and Handa, 1984). The chromatographic bands were quantified by scanning densitometry at 500 nm with a CS 9301 PC SHIMADZU densitometer. Individual phospholipid values, expressed as nmol Pi/mg protein, were calculated by relating their respective percentage to the absolute total quantity of phospholipid-Pi.

2.6. Preparation of synaptic plasma membrane

The hippocampus was homogenized in 10 volumes (1:10, w/v) of 0.32 M sucrose solution containing 5.0 mM HEPES and 0.1 mM EDTA, pH 7.4. After homogenization, synaptic plasma membranes were prepared according to the method of Jones and Matus (1974) with some modifications (Wyse et al., 1995). These membranes were isolated using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 M. After centrifugation at 69,000 ×*g* for 2 h, the fraction between 0.8 and 1.0 M sucrose interface was taken as the membrane enzyme preparation.

2.7. Na^+, K^+-ATPase activity assay

The reaction mixture for Na^+, K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in final volume of 200 mL. The reaction was initiated by addition of ATP to a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays, as described by Wyse et al. (2000). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein.

2.8. Measurements of glutathione reduced (GSH) levels

GSH levels were evaluated to estimate endogenous defenses against oxidative stress. The method was based on Ellman's reagent (DTNB) reaction with free thiol groups. Hippocampus homogenates 10% (w/v) in EDTA 0.02 M were added to a 50% trichloroacetic acid solution. After centrifugation (3000 rpm/15 min), the supernatant of homogenate was collected and the production levels of GSH were

Table 1
Determinations of concentrations of ganglioside, phospholipid and cholesterol in rat hippocampus after pilocarpine-induced seizures.

Groups	Lipid contents		
	Gangliosides (nmol NeuAc/mg protein)	Phospholipids (nmol Pi/mg protein)	Cholesterol (mg cholesterol/mg protein)
Control	215.55 ± 21.43 (8)	696.09 ± 33.17 (8)	143.86 ± 2.95 (8)
Pilocarpine	112.12 ± 1.95 (7)[a]	525.91 ± 15.14 (7)[a]	90.89 ± 1.18 (7)[a]

Male rats (250–280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg/kg, i.p., $n=7$) and the control group with 0.9% saline ($n=8$). Animals were observed for 1 h and then killed. Results are mean ± S.E.M. for the number of animals shown inside the parentheses. The differences in experimental groups were determined by analysis of variance.
[a] $p<0.05$ as compared with control group (ANOVA and Student–Newman–Keuls test).

determined as described by Sedlak and Lindsay (1998). Briefly, the samples were mixed with 0.4 M Tris–HCl buffer, pH 8.9 and 0.01 M DTNB. GSH level was determined by the absorbance at 412 nm and was expressed as ng of GSH/g wet tissue.

2.9. Thiobarbituric acid-reactive substances (TBARS) levels

For all of the experimental procedures, 10% (w/v) homogenates of the area of the brain investigated were prepared for all groups. Lipid peroxidation levels in the pilocarpine group and control animal were analyzed by measuring the thiobarbituric acid-reactive substances (TBARS) in homogenates, as previously described by Draper & Hadley (1990). Briefly, the samples were mixed with 1 mL 10% trichloroacetic acid and 1 mL 0.67% thiobarbituric acid. They were then heated in a boiling water bath for 15 min and butanol (2:1, v/v) was added to the solution. After centrifugation (800 ×*g*, 5 min), thiobarbituric acid-reactive substances were determined from the absorbance at 535 nm. The results above were expressed as nmol of malondialdehyde (MDA)/g wet tissue (nmol of MDA/g wet tissue).

2.10. Protein determination

Protein concentration was measured by the method of Lowry et al. (1951). Bovine serum albumin was used as standard.

2.11. Statistical analysis

Results of neurochemical alterations were compared using ANOVA and the Student–Newman–Keuls test as post hoc test, because these results show a parametric distribution. In all situations statistical significance was reached at *p* less-than-or-equals, slant 0.05. All analyses were performed using the software GraphPad Prism, Version 3.00 for Windows, GraphPad Software (San Diego, CA, USA).

3. Results

According our previous studies (Freitas et al., 2005), immediately after pilocarpine administration, animals persistently show behavioral changes, including initial akinesia, ataxic lurching, peripheral cholinergic signs (miosis, piloerection, chromodacriorrhea, diarrhea and masticatory automatisms), stereotyped movements (continuous sniffing, paw licking, rearing and wet dog shakes that persist for 10–15 min), clonic movements of forelimbs, head bobbing and tremors. These behavioral changes progress to motor limbic seizures as previously described by Turski et al. (1983). Limbic seizures persist for 30–50 min, progressing to SE. In the latter experiments, pilocarpine induced the first seizure at 35.00 ± 0.70 min. All the animals studied showed generalized tonic–clonic convulsions with status epilepticus (SE), and 50% survived the seizures during the 1 h observation period. No one animal that received injections of isotonic saline (control) alone showed seizure activity.

We initially evaluated the changes caused by seizures on total lipid content in hippocampus of rats. As can be seen in Table 1, seizures

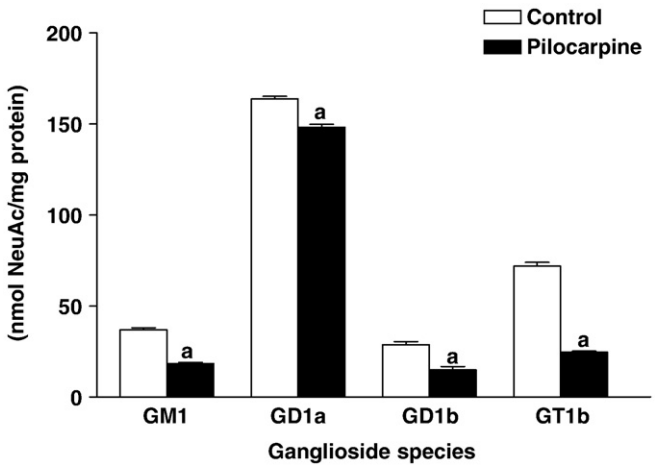

Fig. 1. Determinations of ganglioside species contents in rat hippocampus after pilocarpine-induced seizures. Male rats (250–280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg/kg, i.p., $n=7$) and the control group with 0.9% saline ($n=8$). Animals were observed for 1 h and then killed. Individual ganglioside values were calculated by relating their respective percentage to the absolute total quantity of ganglioside-NeuAc (see Table 1). The percentages were obtained by scanning densitometry of the TLC. Results are mean ± S.E.M. for the number of animals shown inside the parenthesis. The differences in experimental groups were determined by analysis of variance. [a] $p<0.0001$ as compared with control group (ANOVA and Student–Newman–Keuls test).

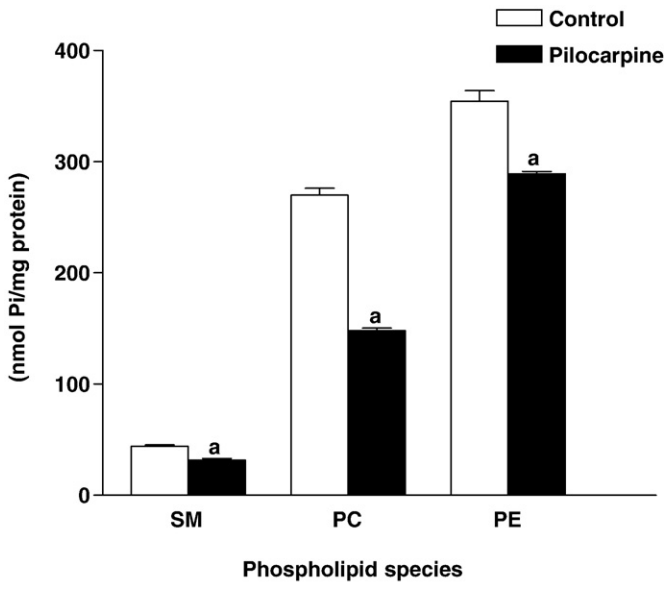

Fig. 2. Determinations of phospholipid species contents in rat hippocampus after pilocarpine-induced seizures. Male rats (250–280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg/kg, i.p., $n=7$) and the control group with 0.9% saline ($n=8$). Animals were observed for 1 h and then killed. Individual phospholipid values were calculated by relating their respective percentage to the absolute total quantity of phospholipid-Pi (see Table 1). The percentages were obtained by scanning densitometry of the TLC. Results are mean ± S.E.M. for the number of animals shown inside the parenthesis. The differences in experimental groups were determined by analysis of variance. [a] $p<0.0001$ as compared with control group (ANOVA and Student–Newman–Keuls test).

Table 2
Determinations of Na^+, K^+-ATPase activity, glutathione reduced (GSH) and thiobarbituric acid-reactive substances (TBARS) levels in rat hippocampus after pilocarpine-induced seizures.

Groups	Determinations		
	Na^+, K^+-ATPase activity (nmol Pi/min mg protein)	GSH level (μg/g tissue)	TBARS levels (nmol of MDA/g wet tissue)
Control	991.5 ± 6.94 (8)	240.33 ± 4.72 (8)	1.29 ± 0.02 (8)
Pilocarpine	758.3 ± 55.76 (7)[a]	127.16 ± 3.27 (7)[a]	1.88 ± 0.02 (7)[a]

Male rats (250–280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg/kg, i.p., $n=7$) and the control group with 0.9% saline ($n=8$). Animals were observed for 1 h and then killed. Results are mean ± S.E.M. for the number of animals shown inside the parentheses. The differences in experimental groups were determined by analysis of variance.

[a] $p<0.0001$ as compared with control group (ANOVA and Student–Newman–Keuls test).

significantly decreased gangliosides [$p<0.0006$], phospholipids [$p<0.0007$] and cholesterol [$p<0.0001$] content around 48, 24 and 37%, respectively.

We also evaluated the content of the different gangliosides and phospholipids (sphingomyelin (SM), phosphatidylcholine (PC) and phosphatidylethanolamine (PE)) in hippocampus of rats after pilocarpine-induced seizures. Fig. 1 shows that seizures significantly decreased the gangliosides contents studied in 51, 10, 48 and 66% [GM1 ($p<0.0001$); GD1a ($p<0.0001$); GD1b ($p<0.0001$); GT1b ($p<0.0001$)], respectively, when compared to control group (Fig. 1). We also observed a reduction of 28, 45 and 18% in all classes of phospholipids studied [SM ($p<0.0001$); PC ($p<0.0001$); PE ($p<0.0001$)], when compared to control group, respectively (Fig. 2).

Na^+, K^+-ATPase activity, glutathione reduced content and TBARS levels were also studied in hippocampus of rats after pilocarpine-induced seizures. Table 2 shows that seizures caused a significant reduction of (25 and 47%) in Na^+, K^+-ATPase activity [$p<0.0007$] and glutathione reduced content [$p<0.0001$], respectively. Moreover, seizures caused a significant increase (46%) of TBARS values [$p<0.0001$], when compared to control group.

4. Discussion

There is a growing body of evidence suggesting that elevated lipid peroxidation levels and/or its metabolites are potentially neurotoxic (Sgaravatti et al., 2009; Dal-Pizzol et al., 2000). In this context, we have previously demonstrated that pilocarpine-induced seizures produce oxidative stress and increases nitrite content in rat hippocampus (Freitas et al., 2004). Besides, we also have showed that seizures decreases glutathione reduced concentration and induces lipid peroxidation in rat hippocampus (Freitas et al., 2005).

In the present study we initially investigated the changes caused by seizures on total lipid content in rat hippocampus, by using an experimental model of seizures induced by pilocarpine (Turski et al., 1983; Cavalheiro et al., 1996). Results demonstrated that seizures significantly reduced the total content of gangliosides, phospholipids and cholesterol in rat hippocampus. Therefore, considering that seizures can alters the development of central nervous system and intense cellular proliferation and growth (Gary and Clark, 2002; Trindade et al., 2002), and that this phase of seizures studied is also characterized by a progressive accumulation of free radical particularly nitrite and nitrate (Oliveira et al., 2007; Freitas, 2009), it is conceivable that seizures could alter normal brain development in rats.

The profile of gangliosides in rat hippocampus after seizures was also evaluated in our study. Results showed that seizures significantly reduced the quantity of the major gangliosides (GM1, GD1a, GD1b and GT1b). These results could indicate that seizures cause alterations in brain plasma membranes, since gangliosides are closely associated with neuronal membranes and participate in many neuronal functions. We also determined the phospholipid classes in hippocampus after seizures. We observed a reduction in the content of the different phospholipids studied (SM, PC, and PE). In agreement with our study, alterations in brain phospholipid composition have been also reported in other models of brain injury, such as epilepsy (Gerasimova and Nikitina, 2007), hypoxia/ischemia (Kikuchi et al., 2006), schizophrenia (du Bois et al., 2005) and others.

Since seizures alter membrane lipid composition that can alter membrane fluidity and permeability and Na^+, K^+-ATPase is considered a marker of neuronal membrane, in the present study we also investigated the changes caused by seizures on this enzyme activity in synaptic plasma membrane from rat hippocampus. Results showed that Na^+, K^+-ATPase activity was significantly reduced during acute phase of seizures. This result is in agreement with previous studies in epilepsy data showing that Met inhibits hippocampal Na^+, K^+-ATPase activity (Streck et al., 2006; Folbergrova, 1986). It has been demonstrated that the oxidative membrane damage can affect the cellular dynamic properties, and consequently the function of membrane-bound enzymes, which may have serious consequences on neuronal functioning (Kamboj et al., 2006). We observed here that seized rats present a decrease in glutathione reduced level and an increase of TBARS (index of lipid peroxidation) in hippocampus. These results are in agreement with previous studies showing that seizures increases lipid peroxidation in hippocampus of rats (Freitas et al., 2005; Oliveira et al., 2007). We propose that the alterations caused by seizures could be, at least in part, due to oxidative damage of the membrane lipids. This fact could provoke changes in lateral assembly of glycosphingolipids, unsaturated glycerophospholipids and cholesterol and alter Na^+, K^+-ATPase activity as suggested by other investigators (Molander-Melin et al., 2005; Welker et al., 2007). In conclusion, the present study shows that seizures decreases the content of the major categories of lipids (gangliosides, cholesterol and phospholipids), reduces glutathione reduced level and the activity of the Na^+, K^+-ATPase and increases lipid peroxidation in rat hippocampus. If confirmed in human beings, our results in association with other studies might contribute, at least in part, to the neurophysiopathology observed in epileptic patients. However, more studies are necessary to investigate additional mechanisms involved in acute phase of seizures.

Acknowledgements

We would like to thank Stenio Gardel Maia for her technical assistance. This work was supported in part by grants from CNPq, Brazil.

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