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Inhibition of zymosan-induced air-pouch inflammation by rat seminal vesicle protein and by its spermidine derivative

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Abstract

The anti-inflammatory effect of one of the major proteins secreted by rat seminal vesicles (SV_{IV}) and of its spermidine derivative (Spd_2-SV_{IV}) was evaluated by measuring polymorphonuclear leukocyte migration, protein release, platelet-activating factor (PAF) and prostaglandin E_2 levels in the mouse air-pouch exudate following zymosan treatment. Both proteins were found to markedly reduce dose dependently PAF and prostaglandin E_2 levels in the exudate as well as the other parameters. Concurrent injection of either arachidonic acid or PAF, directly into the pouch, significantly counteracted the anti-inflammatory effect of SV_{IV} and of its polyaminated derivative. These results support the notion that the molecular mechanism of the anti-inflammatory activity of SV_{IV} and Spd_2-SV_{IV} is linked to the inhibition of both phospholipase A_2 and acetyl:lyso-PAF acetyltransferase.

Keywords: Seminal vesicle protein; Transglutaminase; Polymorphonuclear leukocyte migration; Air-pouch inflammation

1. Introduction

SV_{IV} [seminal vesicle protein No. 4, according to its mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)] is a 90-residue long protein (Mr = 9758) that is secreted under androgen control in large amounts in the lumen of adult rat seminal vesicles (Ostrowski et al., 1979; Mansson et al., 1981; Pan and Li, 1982). Numerous studies have demonstrated that SV_{IV} is endowed with powerful immunosuppressive, anti-inflammatory, antiphagocytic and antichemotactic properties (Metafora et al., 1989a,b; Galdiero et al., 1989; Peluso et al., 1994; Romano-Carratelli et al., 1995). Moreover, the protein was also found to inhibit the process of platelet aggregation induced by different agents both in vivo and in vitro (Persico et al., 1990), to markedly accelerate human blood clotting in vitro (Di Micco et al., 1994), to inhibit platelet-activating factor (PAF) biosynthesis (Camussi et al., 1990) and to exert a protective effect against PAF-elicited hypotension, bronchoconstriction and gastric mucosal injury in vivo (Persico et al., 1993).

Since SV_{IV} and its cyanogen bromide fragments were shown to act as acyl donor and acceptor substrates for the enzyme transglutaminase (E.C.; 2.3.2.13) (Porta et al., 1990, 1994; Aeschlimann and Paulsson, 1994), a spermidine (Spd) derivative of the native protein was synthesized in vitro (Porta et al., 1991) and its pharmacological properties investigated. The modified molecular form of SV_{IV} (Spd_2-SV_{IV}), which contains two spermidine molecules covalently bound, showed a more marked immunosuppressive activity in comparison with the native protein, while retaining unchanged other activities of SV_{IV} (Porta et al., 1993).

The in vivo anti-inflammatory effect of both SV_{IV} and Spd_2-SV_{IV} was, however, tested only by inducing hind-limb oedema by plantar injection of carrageenin in the rat paw (Porta et al., 1993). In the present study, we have investigated the anti-inflammatory activity of both SV_{IV} and Spd_2-SV_{IV} using an experimental model of inflammation caused by zymosan in an air-pouch prepared on the dorsum of mice. To characterize the drug anti-inflamma-

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tory effects in such a model, we measured polymorphonuclear leukocyte migration, protein release and PAF and prostaglandin E_2 levels in the pouch exudate.

2. Materials and methods

2.1. Drugs

Zymosan A, arachidonic acid sodium salt, carboxymethylcellulose sodium salt, phosphate-buffered saline (PBS), heparin sodium salt, bovine serum albumin, crystal violet, acetic acid, acetone, methoxyamine hydrochloride and L-α-phosphatidylcholine-β-acetyl-γ-*O*-alkyl (PAF) were obtained from Sigma (St. Louis, MO, USA), the PAF receptor antagonist [9*H*-1,7a-(epoxymethano)-1*H*,6a*H*-cyclopenta-(*c*)furo-(2,3-*b*)-furo-(3',2':3,4)-cyclopenta-(1,2-*d*)furan-5,9,12-(4*H*)-trione,3-*t*-butylhexahydro-8-ethyl] (BN52021) was obtained from Ipse (30 Rue Cambronne Paris, France), dexamethasone (Decadron) was obtained from Merck Sharp and Dohme (Haarlem), and indomethacin was obtained from Chiesi Farmaceutici (Milan, Italy). The protein SV_{IV} was purified to homogeneity from male adult rat (Fisher-Wistar) seminal vesicle secretions as described by Ostrowski et al. (1979). The purity of the protein was evaluated by 15% PAGE under denaturing and nondenaturing conditions (Metafora et al., 1987), fingerprint technique, amino acid composition analysis, and fast atom bombardment mass spectrometry (Porta et al., 1991). Spd_2-SV_{IV} was enzymatically obtained by a transglutaminase-catalyzed reaction as previously described (Porta et al., 1993).

2.2. Animals

Male Swiss albino mice (20–25 g; Nossan) were used for all experiments. Food and water were available ad libitum.

2.3. Mouse air-pouch model

An air-pouch was formed by s.c. injection of 2.5 ml of air on dorsum under light halothane anaesthesia on day 0 and day 3. On day 6, either 0.5 or 1 mg of zymosan was suspended in 0.5 ml of PBS, containing 0.5% carboxymethylcellulose sodium salt, and injected into the air-pouch. Control mice received the vehicle only. At various times after zymosan administration mice were killed with CO_2 and the pouches were washed with 2 ml of PBS containing 50 U/ml heparin. The lavage fluids were centrifuged at 220 × g for 10 min and the pellets resuspended in 2 ml of PBS containing 50 U/ml heparin. Leukocytes were counted after staining (1 : 10) in Turk's solution (crystal violet 0.01% in 3% acetic acid) using a Neubauer hemocytometer. The number of polymorphonuclear leukocytes recovered from each pouch was then calculated. Protein concentration in cell-free lavage fluids was measured according to the methodology described by Bradford (1976).

The effects of SV_{IV}, Spd_2-SV_{IV}, dexamethasone and BN52021 dissolved in 0.1 ml PBS were assessed by i.v. injection into a tail vein 2 h after zymosan administration. Both PAF dissolved in 0.5 ml of PBS containing 0.25% bovine serum albumin and arachidonic acid, dissolved in 0.5 ml of PBS, were injected directly into the pouch 2 h after zymosan treatment. Pouches were washed after 4 h and polymorphonuclear leukocyte number and protein concentration were measured as described above.

2.4. PAF assay

The PAF content in the pouch exudates was determined by a modification of the methods described by Calignano et al. (1988). The exudates were suspended in 2.0 ml of 154 mM NaCl containing 0.25% bovine serum albumin. After being vortexed for 20 s, the mixture was added to 2 ml of cold acetone and centrifuged at 2000 × *g* for 5 min. 2 ml of chloroform was then added and after vortexing for 10 s and centrifugation of the mixture for 10 min at 2000 × *g* the upper aqueous phase was discarded. The organic phase containing the extracted PAF was evaporated to dryness, redissolved in 60 μl of chloroform/methanol (1 : 1), applied to thin layer chromatography plates and developed in chloroform/methanol/water (65 : 35 : 6) together with authentic standard PAF. Zones corresponding to PAF were located by UV light and were re-extracted. The dried organic phase was resuspended in 25 mM Tris buffer pH 8.0 containing 0.25% bovine serum albumin and the activity was bioassayed by aggregation of rabbit washed platelets (Whittle et al., 1987).

2.5. Prostaglandin E_2 assay

The prostaglandin E_2 amount was determined by adding a 50 μl aliquot of exudate to 25 μM indomethacin to stop any further eicosanoid synthesis. Preformed eicosanoids were then converted to their methyl oximate derivatives by further addition of 50 μl methoxyamine hydrochloride. The amount of the methyl oxime derivative of prostaglandin E_2 was determined by RIA (Amersham) and expressed as total ng per mouse (lowest detectable amount of prostaglandin E_2 = 8 pg/ml; intrassay sensitivity at midrange 29 ± 0.7 pg/tube CV = 2.3%).

2.6. Statistical analysis

The results were expressed as means ± S.E.M. The means were compared using an analysis of variance (ANOVA) plus Bonferonni's test and a P value of less than 0.05 was considered significant.

3. Results

Fig. 1 shows the inflammatory effect induced by two different doses of zymosan and the magnitude of inflammation expressed as polymorphonuclear leukocyte infiltration and protein release in the mouse air-pouch at different times from drug treatment. Polymorphonuclear leukocyte infiltration and protein release reached the maximum at 8 h after zymosan treatment. We observed the effect of SV_{IV} and its spermidine derivative 4 h after zymosan treatment because at this time the inflammatory process is still evolving and the inflammation is submaximal. Fig. 2 shows that submaximal doses of either dexamethasone (5 μg/pouch) or BN52021 (300 μg/pouch) inhibited both polymorphonuclear leukocyte infiltration (44.1 ± 1.4% and 25.6 ± 1.8%, respectively) and protein release (31.4 ± 0.7% and 35.6 ± 1.1%, respectively) into the mouse air-pouch. When given in combination the effect of the two agents was cumulative (73.6 ± 2.0%). These results suggested a possible involvement of arachidonic acid metabolites and of PAF in the zymosan-induced mouse air-pouch inflammation. Either SV_{IV} or Spd_2-SV_{IV} at the doses 5–100 μg exerted a dose-related antinflammatory effect on zymosan-induced inflammation, reducing polymorphonuclear leukocyte infiltration and protein release (Table 1), PAF and prostaglandin E_2 levels (Table 3). Administration of a different protein with a similar molecular weight (ribonuclease, 100 μg) did not affect the zymosan-induced inflammation, thus indicating the specific antinflammatory effect of SV_{IV} (data not shown). To better clarify the role played by the arachidonic acid metabolites we directly injected into the pouch 5 μg of arachidonic acid. As reported in Table 1 arachidonic acid, administered at the same time of SV_{IV} and Spd_2-SV_{IV}, and at a dose that by itself had no effect, was able to counteract the anti-inflammatory effect of 5–50 μg of both SV_{IV} and Spd_2-SV_{IV}.

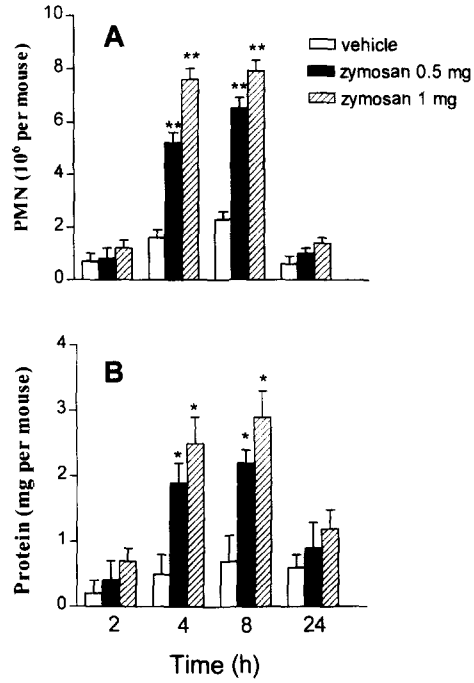

Fig. 1. Time course of zymosan-induced polymorphonuclear leukocyte (PMN) infiltration (panel A) and protein release (panel B) into the mouse air-pouch. Either 0.5 or 1 mg of zymosan in 0.5 ml PBS containing 0.5% carboxymethylcellulose sodium was injected at time 0 directly in the air-pouch. At different times following treatment mice were killed and polymorphonuclear leukocyte infiltration and protein release were measured. Values are means ± S.E.M. for 8 mice per group. * $P < 0.01$ and ** $P < 0.001$ vs. vehicle. Further experimental details are given in the text.

Higher doses of arachidonic acid (10 μg) were not used since significant increases in both polymorphonuclear leukocyte infiltration and protein release into the mouse air-pouch were produced (data not shown). However, the injection of 1 ng of PAF, directly into the pouch, drasti-

Table 1
Inhibitory effect of SV_{IV} and Spd_2-SV_{IV} administration on zymosan-induced polymorphonuclear leukocyte infiltration and protein release into the mouse air-pouch and its reversal by arachidonic acid

Treatment	Polymorphonuclear leukocyte migration (10^6 per mouse)		Protein release (mg per mouse)	
	Saline	+ arachidonic acid	Saline	+ arachidonic acid
None (control)	7.6 ± 0.4	8.1 ± 0.4	2.5 ± 0.4	2.8 ± 0.2
SV_{IV}, 5 μg	5.3 ± 0.4 [c]	7.8 ± 0.2 [f]	1.8 ± 0.4	2.8 ± 0.3
SV_{IV}, 10 μg	4.3 ± 0.3 [c]	6.0 ± 0.5 [c,e]	1.3 ± 0.2	2.5 ± 0.1 [d]
SV_{IV}, 50 μg	3.1 ± 0.4 [c]	5.5 ± 0.3 [c,f]	1.1 ± 0.4 [a]	2.2 ± 0.4
SV_{IV}, 100 μg	2.1 ± 0.2 [c]	2.6 ± 0.2 [c]	0.6 ± 0.2 [c]	1.8 ± 0.3
Spd_2-SV_{IV}, 5 μg	6.1 ± 0.3	7.8 ± 0.3 [d]	2.0 ± 0.2	2.7 ± 0.3
Spd_2-SV_{IV}, 10 μg	4.2 ± 0.2 [c]	6.3 ± 0.4 [a,d]	1.2 ± 0.2	2.6 ± 0.1 [e]
Spd_2-SV_{IV}, 50 μg	3.4 ± 0.4 [c]	5.6 ± 0.6 [c,e]	0.7 ± 0.3 [b]	1.8 ± 0.4
Spd_2-SV_{IV},100 μg	2.1 ± 0.4 [c]	2.8 ± 0.2 [c]	0.5 ± 0.2 [c]	1.0 ± 0.2 [c]
Dexamethasone, 5 μg	4.2 ± 0.3 [c]	6.0 ± 0.4 [b,e]	1.7 ± 0.2	2.5 ± 0.1 [d]

SV_{IV}, Spd_2-SV_{IV} or dexamethasone was i.v. injected 2 h after zymosan (1 mg) administration; 5 μg of arachidonic acid were directly injected into the pouch at the same time. At 4 h time following zymosan treatment the mice were killed and polymorphonuclear leukocyte infiltration and protein release were measured. The values are means ± S.E.M. for 8 mice per group. [a] $P < 0.05$, [b] $P < 0.01$ and [c] $P < 0.001$ vs. control; [d] $P < 0.05$, [e] $P < 0.01$ and [f] $P < 0.001$ vs. the respective values of the experiments performed without arachidonic acid treatment.

Table 2
Reversal effect of PAF on the SV_{IV} and Spd_2-SV_{IV} inhibition of polymorphonuclear leukocyte infiltration and protein release induced by zymosan into the mouse air-pouch

Treatment	Polymorphonuclear leukocyte migration (10^6 per mouse)		Protein release (mg per mouse)	
	Saline	+PAF	Saline	+PAF
None (control)	7.6 ± 0.4	8.4 ± 0.5	2.5 ± 0.4	2.7 ± 0.5
SV_{IV}, 5 μg	5.3 ± 0.4 [c]	7.9 ± 0.3 [f]	1.8 ± 0.4	2.9 ± 0.4
SV_{IV}, 10 μg	4.3 ± 0.3 [c]	6.5 ± 0.4 [a, e]	1.3 ± 0.2	2.8 ± 0.2 [d]
SV_{IV}, 50 μg	3.1 ± 0.4 [c]	6.1 ± 0.2 [c, f]	1.1 ± 0.4 [a]	2.6 ± 0.1 [d]
SV_{IV}, 100 μg	2.1 ± 0.2 [c]	4.5 ± 0.4 [c, f]	0.6 ± 0.2 [c]	2.0 ± 0.3
Spd_2-SV_{IV}, 5 μg	6.1 ± 0.3	8.1 ± 0.3 [c]	2.0 ± 0.2	2.8 ± 0.4
Spd_2-SV_{IV}, 10 μg	4.2 ± 0.2 [c]	7.4 ± 0.3 [f]	1.2 ± 0.2	2.6 ± 0.3 [d]
Spd_2-SV_{IV}, 50 μg	3.4 ± 0.4 [c]	6.3 ± 0.2 [b, f]	0.7 ± 0.3 [b]	2.2 ± 0.2 [c]
Spd_2-SV_{IV}, 100 μg	2.1 ± 0.4 [c]	4.5 ± 0.3 [c, f]	0.5 ± 0.2 [c]	1.9 ± 0.2 [d]
BN52021, 300 μg	5.6 ± 0.3 [b]	6.8 ± 0.5 [d]	1.6 ± 0.2	2.1 ± 0.4 [d]

SV_{IV}, Spd_2-SV_{IV} or BN52021 was i.v. injected 2 h after zymosan (1 mg) administration; 1 ng of PAF was directly injected into the pouch at the same time. At 4 h time following zymosan treatment the mice were killed and polymorphonuclear leukocyte infiltration and protein release were measured. The values are means ± S.E.M. for 6 mice per group. [a] $P < 0.05$, [b] $P < 0.01$ and [c] $P < 0.001$ vs. control; [d] $P < 0.05$, [e] $P < 0.01$ and [f] $P < 0.001$ vs. the respective values of the experiments performed without PAF treatment.

cally reduced the anti-inflammatory effect produced by 5–100 μg of SV_{IV} and Spd_2-SV_{IV} (Table 2). Higher doses of PAF (5–10 ng) alone produced polymorphonuclear leukocyte infiltration and protein release in the mouse air-pouch and increased the inflammatory effect induced by zymosan (data not shown). Moreover we observed that the injection of 1 ng of PAF directly into the pouch on dexamethasone-treated mice resulted in a partial reversal of the effect of dexamethasone on polymorphonuclear leukocyte infiltration (27.3 ± 2.1% of reversal vs. dexamethasone, $P < 0.05$). These results suggest that the anti-inflammatory effect of SV_{IV} and Spd_2-SV_{IV} is related to the modulation of arachidonic acid pathway and PAF biosynthesis.

To confirm that these two proteins are active in reducing inflammation by inhibiting phospholipase A_2, we measured the prostaglandin E_2 and PAF levels in the mouse air-pouch exudate after zymosan treatment. The results reported in Table 3 indicate that both PAF and prostaglandin E_2 levels are reduced in a dose-related manner by

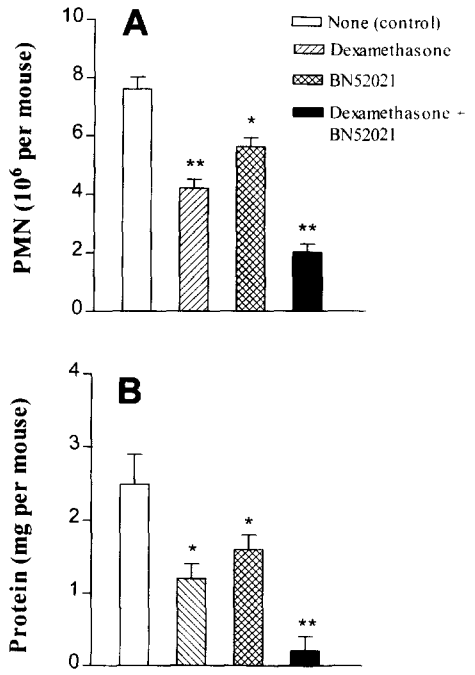

Fig. 2. Inhibitory effect of dexamethasone and BN52021 on zymosan-induced polymorphonuclear leukocyte (PMN) infiltration (panel A) and protein release (panel B) into the mouse air-pouch. Dexamethasone (5 μg) or BN52021 (300 μg) was injected i.v. 2 h after zymosan (1 mg) administration. At 4 h time point following zymosan treatment the mice were killed and polymorphonuclear leukocyte infiltration and protein release were measured. The values are means ± S.E.M. for 6 mice per group. * $P < 0.05$, ** $P < 0.01$ vs. control. Further experimental details are given in the text.

Table 3
Inhibitory effect of SV_{IV} and Spd_2-SV_{IV} on zymosan-induced PAF and prostaglandin E_2 (PGE_2) release into the mouse air pouch

Treatment	PAF (pg per mouse)	PGE_2 (pg per mouse)
None (control)	2200 ± 220	880 ± 50
SV_{IV}, 5 μg	1400 ± 180	608 ± 72
SV_{IV}, 10 μg	1050 ± 350 [a]	509 ± 42 [b]
SV_{IV}, 50 μg	800 ± 220 [b]	403 ± 65 [c]
SV_{IV}, 100 μg	650 ± 240 [c]	320 ± 71 [c]
Spd_2-SV_{IV}, 5 μg	1350 ± 220	612 ± 54
Spd_2-SV_{IV}, 10 μg	1020 ± 270 [a]	470 ± 55 [b]
Spd_2-SV_{IV}, 50 μg	814 ± 170 [b]	410 ± 70 [c]
Spd_2-SV_{IV}, 100 μg	635 ± 200 [c]	350 ± 67 [c]
BN52021, 300 μg	560 ± 210 [c]	720 ± 59
Dexamethasone, 5 μg	780 ± 120 [b]	328 ± 67 [c]

SV_{IV}, Spd_2-SV_{IV}, BN52021 or dexamethasone was i.v. injected 2 h after zymosan (1 mg) administration. At 4 h time following zymosan treatment the mice were killed and PAF and prostaglandin E_2 levels were measured. The values are means ± S.E.M. for 6 mice per group. [a] $P < 0.05$, [b] $P < 0.01$ and [c] $P < 0.001$ vs. control.

both SV_{IV} and Spd_2-SV_{IV}. It is worthy to note that dexamethasone inhibited both PAF and prostaglandin E_2 formation, confirming that phospholipase A_2 is involved in both biosynthetic pathways.

4. Discussion

Previous investigations have demonstrated that SV_{IV} possesses anti-inflammatory activity, probably due to a block of the arachidonic acid cascade at the level of the phospholipase A_2-catalyzed step (Metafora et al., 1989a; Camussi et al., 1990). Similar results were obtained when the pharmacological properties of a spermidine derivative of the protein, synthesized in vitro by using the enzyme transglutaminase, were examined (Porta et al., 1993). In fact, both proteins were found able (1) to inhibit porcine pancreatic phospholipase A_2 activity assayed in vitro, (2) to decrease the synthesis and the release of arachidonic acid and PAF from stimulated polymorphonuclear leukocytes and of prostaglandin E_2 from activated leukocytes, (3) to reduce the intensity of the oedema induced in the rat hind limb by plantar injection of carrageenin. The present study confirms these observations in a different inflammation model: we found that SV_{IV} and Spd_2-SV_{IV} markedly inhibit polymorphonuclear leukocyte infiltration and protein release into the mouse air-pouch following zymosan treatment. The antinflammatory effect of SV_{IV} and Spd_2-SV_{IV} could be considered specific because ribonuclease, a similar molecular weight protein, had no effect on zymosan-induced inflammation. Since the zymosan-induced air-pouch inflammation was partially inhibited by both dexamethasone and the PAF antagonist BN52021, we tested SV_{IV} and Spd_2-SV_{IV} for anti-inflammatory properties under concurrent injection of either arachidonic acid or PAF directly into the pouch of the mice. Moreover, both proteins were able to reduce PAF and prostaglandin E_2 levels in the pouch exudate. It is interesting to note that BN52021 treatment reduced not only the effect of PAF but also the PAF level. A conceivable explanation for the reduction of PAF level in the pouch exudate after BN52021 treatment could be related to the reduced polymorphonuclear leukocyte infiltration, which also resulted in a low amount of PAF. These results strengthened our preliminary hypothesis (Camussi et al., 1990) that the inhibition of both phospholipase A_2 and acetyl:lyso-PAF acetyltransferase activities is involved in the molecular mechanism underlying the anti-inflammatory properties of SV_{IV} and Spd_2-SV_{IV}.

Although the actions of dexamethasone and BN52021 were reversed by arachidonic acid and PAF, we cannot exclude that dexamethasone participates in the control of inflammation by modulating the effects of histamine.

Computer and immunological analyses have shown that SV_{IV} has a significant degree of homology with uteroglobin, a progesterone-induced binding protein that is widespread in many tissues and body fluids of rabbits (Metafora et al., 1987). In addition, both uteroglobin and SV_{IV} were shown to possess anti-inflammatory properties and have been found to effectively inhibit chemotaxis and phagocytosis of macrophages and neutrophils (Galdiero et al., 1989).

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