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Release of platelet-derived growth factor from human platelets by arachidonic acid

(prostaglandin pathways/atherosclerosis/platelet aggregation/platelet factor 4/β-thromboglobulin)

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ABSTRACT **Platelet α-granules contain a factor that stimulates the proliferation of arterial smooth muscle cells and may play a role in atherogenesis. We have studied the role of arachidonic acid in mediating the release of the platelet-derived growth factor (PDGF) from human platelets. PDGF was assayed by stimulation of [^{3}H]thymidine incorporation into DNA of mouse 3T3 cells. Platelet aggregation and the release of platelet factor 4, β-thromboglobulin, and serotonin were also studied. A biphasic response pattern was observed when gel-filtered platelets were incubated with arachidonate over the concentration range 0.01–0.4 mM. At low arachidonate levels (approximately 0.025–0.1 mM), specific concentration-dependent aggregation and release of PDGF and of the other components were observed. This effect was not seen with any of five other fatty acids tested and was suppressed by indomethacin (25 μM). At higher arachidonate concentrations (approximately 0.15–0.35 mM), a concentration-dependent turn-off of both aggregation and release occurred. At these concentrations the platelets remained functional, and no release of lactate dehydrogenase was observed. A similar biphasic pattern of arachidonate-induced aggregation and release was observed with platelet-rich plasma, over a similar range of arachidonate to albumin mole ratios. These studies demonstrate that PDGF and other α-granule constituents can be released from platelets specifically by arachidonate via an indomethacin-sensitive pathway, most probably involving the platelet cyclooxygenase and conversion of arachidonate to prostaglandin metabolites. The mechanisms responsible for the turn-off of the specific arachidonate-mediated responses at higher arachidonate concentrations remain to be defined.**

Human platelets contain a growth factor that stimulates the proliferation of a variety of cells in tissue culture, including arterial smooth muscle cells (1–3), fibroblasts (2–5), and human glial cells (6). It has been suggested that this platelet-derived growth factor (PDGF) may play a significant role in the pathogenesis of atherosclerosis (7, 8) by promoting intimal smooth muscle cell proliferation and the development of early atherosclerotic lesions. Evidence in support of this hypothesis has been obtained in experimental studies with rabbits (9, 10) and baboons (11).

Platelets contain several different populations of granules. Recent studies have shown that platelet α-granules most likely contain the PDGF, as well as platelet factor 4 (PF4) and β-thromboglobulin (βTG) (3, 12). The dense granules are known to contain ADP, ATP, and serotonin.

Dense granule release and platelet aggregation are mediated, in part, by the metabolism of arachidonic acid to the prostaglandin endoperoxides (13–16) and thromboxanes (17). Although numerous investigators have examined the arachidonic acid-mediated release of dense granule constituents, no information is available on the effects of arachidonic acid on release of α-granule components. We now report studies on the arachidonic acid-mediated release of PDGF from human platelets. These studies aimed to explore the role of prostaglandin-related pathways in the regulation of the release of PDGF and other α-granule constituents from platelets.

MATERIALS AND METHODS

Platelet Preparation. Platelet-rich plasma (PRP) was prepared, as described by Holmsen *et al.* (18), from venous blood drawn from normal healthy donors who had not taken any medication within the preceding 2 weeks. The platelet dense granule pool was labeled with [^{14}C]serotonin, and labeled gel-filtered platelets (GFP) were prepared as described (3). The procedure yielded 30–40 ml of labeled GFP containing 1–3 × 10^8 platelets per ml.

Fatty Acid Salt Solutions. Aqueous solutions of the potassium salts of arachidonic acid (Nu Chek Prep, Elysian, MN) and of palmitic, oleic, linoleic, icosatrienoic, and docosahexaenoic acids (Sigma) were prepared as described (19).

Platelet Aggregation and Preparation of Samples for Assay. Platelet aggregation was monitored photometrically at 37°C using a Payton dual channel aggregation module with a Riken-Denshi vertical two-channel recorder. The platelet response was studied at final fatty acid concentrations between 0.01 and 1.0 mM. The necessary dilutions of the fatty acid salt solutions were prepared in phosphate-buffered saline (137 mM NaCl/2.7 mM KCl/10.6 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.4) just prior to their addition to GFP. Aggregation was allowed to proceed for 3 min after the addition of the fatty acid salt and was terminated by immersing the samples in an ice-water bath. After aggregation, samples were centrifuged at 12,350 × *g* for 30 min at 4°C and the supernatant fluids were decanted and retained for assay of the released products. For inhibition studies, a weighed amount of indomethacin (Sigma) was dissolved in 60% (vol/vol) ethanol. GFP were incubated with indomethacin solution or 60% ethanol alone for 1 min prior to the addition of arachidonic acid. The final concentration of indomethacin was 25 μM and that of ethanol was 0.12%. In preliminary experiments it was observed that a final concentration of 0.5% ethanol significantly altered platelet function and reactivity, whereas 0.12% had little, if any, such effect.

PDGF Assay. PDGF activity was determined by measuring growth stimulation of Swiss albino mouse 3T3 cells (ATCC CCL 92, American Type Culture Collection). Preparation of samples and the assay procedure, which measures stimulation of [^{3}H]-thymidine incorporation into DNA, have been described in detail (3).

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Abbreviations: PDGF, platelet-derived growth factor; PF4, platelet factor 4; βTG, β-thromboglobulin; PRP, platelet-rich plasma; GFP, gel filtered platelets; LDH, lactate dehydrogenase; PG, prostaglandin.

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Assay of Other Released Products. PF4 and βTG were measured by radioimmunoassay as reported (3, 20). The release of serotonin was determined by measuring ^{14}C in platelet supernatants, as described (3). Lactate dehydrogenase (LDH) was assayed by the method of Wroblewski and LaDue (21). The reagents for this assay were purchased from Sigma.

Controls. During each experiment, supernatant fluids were prepared from equivalent GFP samples that were frozen and thawed three times. The amount of each component released during an experiment was expressed as a percent of the total amount that could be released by repeated freeze-thawing. The amount released by freeze-thawing was not intended to quantitate the absolute content of each component in the platelets, but rather to provide a standard reference point within each experiment. In each experiment, control incubations of GFP with phosphate-buffered saline alone were also carried out so as to be able to correct the PDGF data for the small amount of unstimulated background release that was sometimes observed; background release was quantitatively negligible for the other released components measured.

RESULTS

Effects of Arachidonate Compared to Those of Other Fatty Acid Anions. Eight experiments were carried out to investigate the concentration-dependent effects of arachidonate on platelet aggregation and release, and to compare these effects with those obtained with other fatty acid anions. In each experiment, the release of PDGF was determined and correlated with platelet aggregation and the release of serotonin. Release of βTG and PF4 was also determined in two experiments, to complement the PDGF data. In four experiments the effects of arachidonate were compared with those of oleate; the effects of arachidonate were compared with those of palmitate, linoleate, icosatrienoate, and docosahexaenoate in single experiments with each of these fatty acids.

Similar results were observed in each experiment. Fig. 1 illustrates the results obtained in two representative experiments. The upper left panel shows the PDGF release observed in an experiment comparing the effects of arachidonate with those of linoleate. Arachidonate induced the release of PDGF in a dose-dependent manner. Low concentrations of arachidonate specifically induced PDGF release, with release first seen at 0.04 mM arachidonate and peaking in a plateau fashion from 0.06 to 0.1 mM. At these concentrations, linoleate did not induce PDGF release. As the concentration of arachidonate was further increased, the release of PDGF declined; by 0.25 mM arachidonate, no PDGF release was observed.

The upper right panel of Fig. 1 shows the PDGF release observed in a dose–response study comparing the effects of arachidonate with those of oleate. Again, arachidonate resulted in a specific, concentration-dependent, biphasic ("on–off") pattern of PDGF release. The arachidonate concentrations at which release was stimulated or inhibited, however, were not identical with those observed in experiment 1.

Platelet aggregation and release of serotonin, βTG, and PF4 also exhibited concentration-dependent biphasic patterns of response to arachidonate (see Fig. 1). Although qualitatively similar, within a given experiment the arachidonate dose-dependent release patterns of the various granule constituents were not exactly parallel. In general, these intra-experimental variations did not reveal a consistent difference among particular granule constituents.

In all experiments conducted, the same biphasic pattern of platelet responses to arachidonate was observed. Although there was some variation from experiment to experiment in the precise arachidonate concentrations at which particular events

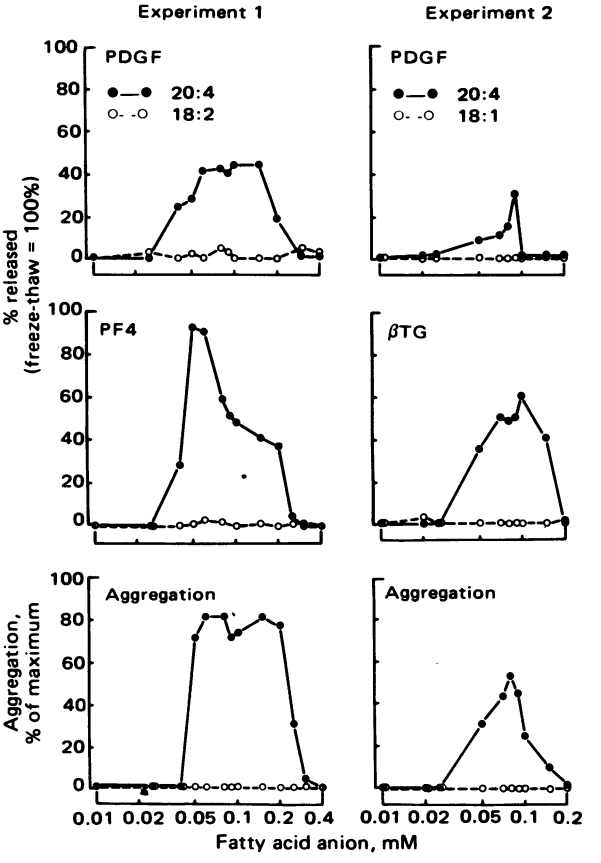

FIG. 1. The effects of arachidonate (20:4), compared with those of other fatty acid anions (linoleate, 18:2, and oleate, 18:1) on platelet release and aggregation. The data show the fatty acid anion concentration dependence (dose–response) of platelet aggregation and of the release of PDGF, βTG, and PF4 from GFP. The data for the released components are expressed as the percent of the amount of each component released from a sample of GFP that had been frozen and thawed three times. A separate freeze-thawed sample of GFP was used for each experiment. Each data point is the mean of duplicate determinations. The data for aggregation were determined from aggregation tracings and are expressed as a percent of the maximum aggregation observed within each experiment. In both experiments, the patterns of release of serotonin (data not shown) were similar to those of aggregation.

occurred, qualitatively similar platelet responses were consistently observed within each of two concentration ranges of arachidonate. These two arachidonate concentration ranges, which define the biphasic dose–response pattern, were (*i*) low (0.03–0.1 mM) and (*ii*) high (0.15–0.35 mM). In the low arachidonate concentration range, specific arachidonate-mediated platelet responses were observed. Release declined and was abolished in the high arachidonate concentration range.

At concentrations greater than 0.4 mM, nonspecific fatty acid-mediated effects on GFP were observed both with arachidonate and with all the other fatty acids tested. In this concentration range (0.4–1.0 mM), all of the platelet components assayed (serotonin, PDGF, βTG, and PF4) were found in the GFP supernatants. However, the appearance of these platelet components in the GFP supernatants was accompanied by the release of LDH, a cytoplasmic marker, which was found in the GFP supernatants at a concentration of about 250 units/ml. In contrast, LDH release was never observed throughout the fatty acid concentration range characterizing the arachidonate-specific biphasic platelet response. The release of LDH at the very high fatty acid levels indicated that platelet lysis occurred (22) under these conditions, with concomitant leakage of

platelet granule contents. It is unlikely that these toxic nonspecific effects seen at very high fatty acid levels have any physiological meaning.

Effects of Indomethacin on Arachidonate-Mediated Effects. The effects of indomethacin, a known inhibitor of the enzyme (fatty acid cyclooxygenase) that converts arachidonic acid to prostaglandin endoperoxides, were examined in three experiments. Aggregation and the release of PDGF and serotonin were determined in all experiments; release of βTG was also measured in one experiment, the results of which are presented in Fig. 2.

In the absence of indomethacin, all parameters of platelet function studied exhibited a biphasic response to increasing concentrations of arachidonate (see also Fig. 1). Indomethacin completely inhibited these specific effects of arachidonate. Thus, indomethacin blocked the arachidonate-induced release of PDGF (Fig. 2, upper left panel), βTG (Fig. 2, lower left), and serotonin (Fig. 2, upper right), as well as platelet aggregation (Fig. 2, lower right). Similar results were obtained in all three experiments with indomethacin.

Direct Effects of Arachidonate on the PDGF Bioassay. Although platelet aggregation and the release of PF4, βTG, and serotonin paralleled PDGF release, we nevertheless investigated the effects of arachidonic acid on the bioassay in order to rule out any possible direct contribution of arachidonate in the test samples to the PDGF assay results. Three experiments were carried out in which arachidonate, in concentrations corresponding to those employed in the platelet experiments, was added directly to 3T3 cells with and without a mitogen, to determine the effects of arachidonate itself on both basal and stimulated thymidine incorporation. The source of the mitogen was a crude, but partially purified, preparation of PDGF obtained from outdated human PRP. At concentrations of 0.01–0.5 mM, arachidonate had no effects on [^{3}H]thymidine incorporation. Thus, arachidonic acid did not interfere with

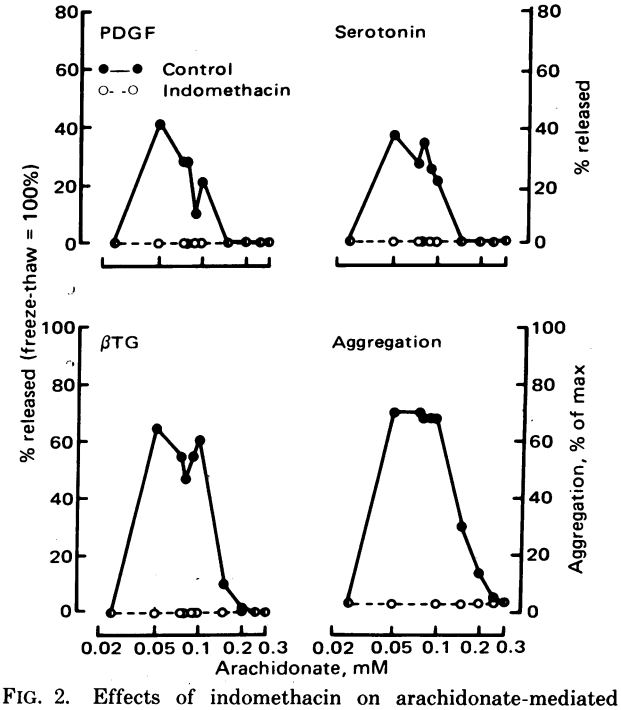

FIG. 2. Effects of indomethacin on arachidonate-mediated platelet release and aggregation. The arachidonate concentration-dependent release of PDGF, βTG, and serotonin and the aggregation of GFP were studied in the presence and absence of 25 μM indomethacin. The data for the released components and aggregation are expressed as described in the legend to Fig. 1.

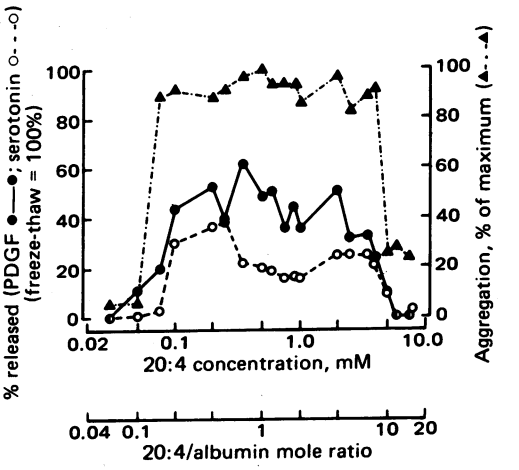

FIG. 3. The arachidonate concentration-dependence of platelet aggregation and of the release of PDGF and serotonin from PRP. See legend to Fig. 1 for further details.

the PDGF bioassay at concentrations of arachidonate corresponding to those that induced the turn-off of platelet aggregation and release (approximately 0.15–0.35 mM in the experiments with GFP).

Effects of Arachidonate on PRP. Although it is known that arachidonate can specifically induce platelet aggregation and dense granule release, the observation that increasing concentrations of arachidonate can turn off platelet function has not been reported previously. Most other investigators have used PRP in their studies. Accordingly, two experiments were conducted to examine the concentration-dependent effects of arachidonate in PRP instead of GFP. A higher range of arachidonate concentrations was studied in these experiments, in order to examine a range of arachidonate to albumin molar ratios similar to those studied with GFP. The albumin concentration of PRP is about 20 times that which was present in the experiments with GFP (see *Discussion*).

Virtually identical results were obtained in the two experiments with PRP; the results of one of these experiments are shown in Fig. 3. As illustrated, with PRP, platelet aggregation and the release of both PDGF and serotonin again demonstrated the characteristic biphasic response to increasing concentrations of arachidonate. With PRP, the turn-off of these platelet functions occurred at arachidonate concentrations between 5 and 7.5 mM, approximately 20 times higher than the concentrations at which the turn-off was seen with GFP. The arachidonate to albumin mole ratio at which this effect was observed was, however, similar with PRP and GFP. As with GFP, no platelet LDH release was observed throughout the range of arachidonate concentrations associated with this biphasic response. Thus, normal plasma levels of LDH activity were observed (*ca.* 200 units/ml) in the PRP supernatants in the concentration range of 0.075–7.5 mM arachidonate.

DISCUSSION

This study was undertaken to examine the role of arachidonic acid in mediating the release of PDGF from human platelets. The data presented indicate that PDGF and other α-granule constituents (PF4 and βTG) can be released from platelets by low concentrations (0.02–0.1 mM) of arachidonate. The failure of five other fatty acids to induce these effects suggested that α-granule release was due to the specific effects of arachidonic acid or of its metabolites.

To explore whether arachidonate itself might be the direct mediator of PDGF release, experiments were conducted in the presence of indomethacin, which blocks the conversion of

arachidonic acid to the prostaglandin (PG) endoperoxides PGG_2 and PGH_2 and, thereby, inhibits arachidonate-induced platelet aggregation and dense granule release (15, 16). The prostaglandin endoperoxides have been shown to stimulate platelet aggregation and dense granule release (13, 14); these compounds are also precursors of thromboxane A_2, a potent mediator of these platelet functions (17, 23). As reported here, indomethacin completely inhibited the arachidonate-mediated release of PDGF and βTG, as well as the release of serotonin and platelet aggregation. These data suggest that the specific arachidonate-mediated α-granule release, as well as dense granule release, is dependent on the conversion of arachidonic acid to its active prostaglandin metabolites.

In these experiments, the onset of PDGF release occurred approximately in parallel with the onset of platelet aggregation and the release of βTG, PF4, and serotonin. Thus, α-granule and dense granule release appear to be similarly sensitive to the concentration of arachidonate. In contrast, previous studies showed that α-granule release was more sensitive to thrombin concentration than was dense granule release (3).

In the absence of a specific chemical assay for PDGF, caution must be exercised in the quantitative interpretation of the PDGF release data. We believe, however, that the results obtained from appropriate controls and from other release studies clearly support the reliability of the bioassay used in the study of PDGF release. In other studies (12), we have found that almost all of the mitogenic activity (as assayed here) resides in the α-granule, and that there appear to be no other platelet subcellular components that possess significant activity. Furthermore, as described in *Results*, we have demonstrated that any potential carry-over of arachidonic acid from the test sample to the culture system does not affect the bioassay. At the present time, we cannot rule out the possibility that other inhibitory or stimulatory substances may be generated by the platelets during the course of our experiments. However, the observed release of PDGF in parallel with other known α-granule components (βTG and PF4, measured directly by specific radioimmunoassays) suggests strongly that such other substances did not contribute significantly to our results. Finally, the serotonin release and platelet aggregation data, taken together with the βTG and PF4 data, clearly eliminate bioassay artifact as an explanation for the biphasic release pattern of PDGF.

In the high concentration range of arachidonate (0.15–0.35 mM), aggregation and release of both α- and dense granule constituents progressively declined, until these responses were completely abolished. This phenomenon has not been reported previously in studies on arachidonate-mediated aggregation and dense granule release utilizing PRP as the source of platelets. One of the major differences between the GFP preparations used here and PRP is the albumin concentration. The albumin concentration of human PRP (*ca.* 4 g/dl) is approximately 20 times that of GFP (0.2 g/dl, provided as a component of the elution buffer used in gel filtering the platelets). It is well known that albumin binds fatty acid anions with high affinity and that the concentration of unbound fatty acid anion in aqueous solution is a function of the fatty acid to albumin molar ratio (24, 25). As shown in Fig. 3, a turn-off of platelet aggregation and release in PRP occurred at a mole ratio of arachidonate to albumin (approximately 7:1 to 15:1) that was identical to that found for the turn-off with GFP. These experiments with PRP clearly showed that the turn-off of platelet function with increasing concentrations of arachidonate was not peculiar to GFP; rather, this turn-off was dependent on concentrations of arachidonate such that the arachidonate to albumin mole ratio was high (>5:1). Thus, the concentration of albumin appears to critically affect the dose–response of platelets to arachidonic acid.

The mechanism by which arachidonate effects the turn-off of platelet function at high concentrations is not known. The question might be raised as to whether the platelets are physiologically intact during this apparent inhibition, or whether the turn-off reflects platelet damage by high concentrations of unbound fatty acid. Although the lack of LDH release in this arachidonate concentration range argues against severe platelet damage, the possibility exists that the progressive decline in platelet response represents an increasing functional injury to the platelets. Preliminary experiments in our laboratory, however, indicate that the platelets are, in fact, functional during the turn-off phase. These experiments have shown that platelets preincubated with an "inhibitory" dose of arachidonate (0.25 mM) are capable of undergoing aggregation and release in response to collagen. In these studies, arachidonate-treated platelets were inhibited, compared with control platelets, at low collagen concentrations (<2.5 μg/ml). At higher collagen concentrations, however, the arachidonate-treated platelets showed a concentration-dependent progressively increasing aggregation and granule release. No LDH release was observed. These data suggest that the turn-off phenomenon reported here does not reflect platelet damage.

Two classes of possible mechanisms by which arachidonate effects the turn-off of platelet function can be considered: (*i*) formation of inhibitory products or (*ii*) inhibition of critical enzymes. Arachidonate metabolism proceeds through the cyclic endoperoxides to a number of products. One endoperoxide derivative is thromboxane A_2, a stimulator of platelet function (17, 23); another product is PGD_2, a potent inhibitor of platelet aggregation (26, 27). It has been proposed that formation of PGD_2 during platelet aggregation might represent a mechanism of feedback inhibition of aggregation (26). The fact that PGD_2 is formed rapidly (26) and is more stable than either the cyclic endoperoxides or thromboxane A_2 suggests the possibility that PGD_2 may play a role in the presently observed inhibition of platelet function by high arachidonate.

The possibility of inhibition of key enzymes in the prostaglandin pathway is suggested by the work of Smith and Lands (28) and of Egan *et al.* (29), who demonstrated that the cyclooxygenase can be inactivated during the course of metabolism of arachidonate to other products. Whereas these investigators utilized ram seminal vesicle preparations, Lapetina and Cuatrecasas (30) have recently demonstrated a similar phenomenon for the platelet cyclooxygenase. Furthermore, as suggested by the work of Hammarström and Falardeau (31), platelet thromboxane synthetase may similarly be sensitive to inactivation during the course of arachidonate metabolism.

Could the high arachidonate levels associated with the turn-off of platelet function be relevant to normal platelet physiology? Endogenous arachidonic acid is released from platelet phospholipids when platelets are stimulated with an agent such as thrombin (32, 33). As much as 20 nmol per 10^9 platelets is released by maximal stimulation (32). Depending on the volume of distribution of the released arachidonic acid, concentrations considerably in excess of 1 mM could conceivably be locally achieved. This estimate assumes near maximal arachidonate release and distribution only within an average platelet volume (10^{-9} μl). Although concentrations of free arachidonate actually achieved in the platelet are undoubtedly much less than this, these local concentrations might well be within the arachidonate concentration range for the turn-off of platelet function.

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