

Polyamines stimulate the phosphorylation of phosphatidylinositol in rat mast cell granules

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Rat mast cell granules were obtained by homogenization of highly purified rat mast cells and isolated in a Percoll gradient. Diphosphoinositide (DPI) synthesis in rat mast cell granules was assayed by measuring the incorporation of ^{32}P from $[\gamma^{32}\text{P}]\text{ATP}$ into DPI in the absence of exogenous phosphatidylinositol (PI). Lipids were isolated with methanol/chloroform/HCl and were separated by thin-layer chromatography on oxalic acid impregnated silica gel plates. DPI areas were identified by staining with iodine, scraped and measured for ^{32}P radioactivity. The addition of polyamines, spermine and spermidine, to the granules caused an increase of DPI synthesis, which can be catalyzed by PI kinase. This effect of polyamines in the DPI synthesis was in a dose-dependent manner and maximal effects were observed at 1 mM spermine and 10 mM spermidine, respectively.

Key words: mast cell; granule; phosphatidylinositol; polyamine.

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Release of mediators from mast cells has been considered to occur by exocytosis. During exocytosis cytoplasmic granule membranes fuse with each other and with the mast cell plasma membranes, permitting the release of granules and their contents. However, little is known about the mechanisms of exocytosis from mast cells. Our recent studies have indicated that $^{32}\text{PO}_4$ incorporation into phosphatidylinositol 4-phosphate or diphosphoinositide (DPI) and phosphatidylinositol 4,5-bisphosphate or triphosphoinositide (TPI) was increased during mediator release from rat mast cells, and these changes in the metabolism of polyphosphoinositides may be an intrinsic part of the biochemical mechanisms that control mediator release from mast cells (7). Control studies in the investigation in unstimulated rat mast cells labeled with $^{32}\text{PO}_4$ suggested that at least 50% of total cellular radiolabelled polyphosphoinositide was in the granules (7). Rat mast cell granules were shown to contain a highly active phosphatidylinositol (PI) kinase which catalyzes the

formation of DPI from endogenous PI in the granule membranes (8). This enzyme is stimulated by Mg^{2+} or Mn^{2+} , which are not known to change dramatically in the cytosol during granule exocytosis from mast cells, and there is no obvious mechanism for producing rapid alterations in PI kinase activity *per se* in activated mast cells.

Polyamines, such as spermine and spermidine, are naturally occurring cations. Their specific functions are still obscure, but their ubiquitous distribution and the complexity of the mechanisms that regulate their intracellular concentrations suggest for these compounds important roles in normal cellular growth and differentiation (5, 15, 21). Vogel & Hoppe (23) showed that polyamines caused an increase in the concentrations of DPI and TPI in plasma membranes from A431 cells and half-maximum stimulation by spermine occurred under near physiological conditions at 1.9 mM. In the present report, effect of polyamines on ^{32}P incorporation into DPI in rat mast cell granules

was studied. Evidence will be presented that polyamines cause an increase of DPI synthesis in a dose-dependent manner.

MATERIAL AND METHODS

[$\gamma^{32}\text{P}$]ATP (4.5×37 MBq/mmol) (ICN Pharmaceuticals, Irvine, CA, U.S.A.); adenosine 5'-triphosphate (ATP), trisma base, L- α -phosphatidylinositol-4-phosphate (diphosphoinositide [DPI]), L- α -phosphatidylinositol-4,5-bisphosphate (triphosphoinositide [TPI]), bovine serum albumin (BSA), iodine, toluidine blue, spermine tetrahydrochloride, spermidine trihydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.); Percoll (Pharmacia Co., Uppsala, Sweden); Li-quifluor (New England Nuclear, Boston, MA, U.S.A.); silica gel plates (20×20 cm, 250 μm layer) (Macherey-Nagel, Duren, West Germany); sodium dodecyl sulfate (Gallard-Schlesinger, Carle Place, NY, U.S.A.); acrylamide, NN'-methylene-bisacrylamide, NNN'-N'-tetramethyl-ethylenediamine, bromophenol blue, Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA, U.S.A.); oxalic acid, potassium oxalate and others (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Rat mast cell preparations

Rat mast cells mixed with leukocytes and serosal cells were obtained by peritoneal and pleural lavage of male Sprague-Dawley rats (250 to 300 g; Shizuoka Laboratory Animals, Shizuoka, Japan) in amounts of about 1×10^6 mast cells/rat as described previously (7, 8). The lavage medium was a heparinized (10 U/ml) balanced salt solution (BSS) (4.0 mM Na_2HPO_4 , 2.7 mM KH_2PO_4 , 150 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl_2 , pH 7.2) containing 0.046% (w/v) BSA. The mast cells in this mixture were purified on a Percoll gradient (4) and were more than 90% pure by toluidine blue staining reactions.

Isolation of rat mast cell granules

Granules were isolated from sonicated rat mast cells on a Percoll gradient as described previously

(8) by the method of Kruger et al. (6). Granules with intact membranes revealed by electron microscopy and ruthenium red staining (6) were washed twice with BSS after recovery from the gradient and were used in the experiments below. Each experiment was performed separately on the day when rat mast cell granules were prepared.

Measurements of phosphorylating activity in isolated granules

Incubations were carried out in conical polypropylene tubes in a total volume of 100 μl . Standard incubation mixtures contained 30 μl medium with 20 mM Tris/HCl, pH 7.4, 20 mM MgCl_2 , 10 μM [$\gamma^{32}\text{P}$]ATP (diluted with unlabelled ATP to 5×10^9 cpm/ μmol) mixed with 60 μl of resuspended mast cell granules in BSS and 10 μl of polyamine solution. Each tube contained intact membrane granules from 1.5 to 2.3×10^6 mast cells, uncorrected for losses during purification. Based on direct protein measurements by the method described below, this corresponds to approximately 15 to 16 μg granule protein per tube. In routine assays incubations were conducted at 30°C for 15 min and the reaction was stopped by the addition of organic solvents to the entire granule suspension as described below.

Isolation of rat mast cell granule phosphoinositide and chromatography

Lipids in the mast cell granules were isolated from granule suspensions by using a modified Bligh & Dyer (1) extraction procedure: 0.5 ml methanol, 0.25 ml chloroform and 0.1 ml 0.2 N HCl were added directly to the reaction mixtures. Tubes were capped, shaken and centrifuged at $1000 \times g$ for 10 min. The upper aqueous phase was carefully removed by aspiration and aliquots of the organic phase were dried under N_2 gas at room temperature. Dried samples were dissolved in 60 μl chloroform and were stored at -80°C overnight. Phospholipids were separated by one-dimensional thin-layer

chromatography (TLC) on silica gel plates as described previously (8). Before use, the plates were developed with either 1% (w/v) aqueous solution of oxalic acid or methanol/water (2:3, v/v) containing 1% (w/v) potassium oxalate. The oxalic acid- or potassium oxalate-impregnated plates were dried at room temperature and activated at 110°C for 15 min. After the application of samples, oxalic acid-impregnated plates were developed with chloroform/methanol/4 N NH_4OH (9:7:2, v/v/v); the potassium oxalate-impregnated plates with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, v/v/v/v/v). Lipid-containing areas were identified by staining with iodine, scraped into vials and measured for radioactivity in a liquid scintillation counter after addition of 10 ml of scintillation fluid (Liquifluor). The added unlabelled phosphoinositides were used both as internal standards and as isolated standards in an adjacent lane on the TLC plate. The radioactivities recovered in the DPI areas on oxalic acid- and potassium oxalate-impregnated silica gel plates in replicate samples corrected closely (less than 10% differences) with one another. Unless otherwise indicated, regular experiments were

performed with the oxalic acid-impregnated silica gel plates.

Verification of DPI in rat mast cell granule

To verify that labelled DPI was indeed present when radioactivity was demonstrated in the DPI area, the presumed DPI spot on 1% (w/v) oxalic acid-impregnated plates was scraped into tubes and extracted a second time. A portion was measured for radioactivity and the remainder was chromatographed in the other TLC system on 1% (w/v) potassium oxalate-impregnated plates. Extraction was performed as described above except that 0.5 ml methanol, 0.25 ml chloroform and 0.25 ml 0.1 N HCl were used in the monophasic system and the extraction with chloroform in the two-phase system was repeated three times. After drying and autoradiography, the radioactivity on the second plate was recovered in the DPI area. When samples were reextracted by a more conventional extraction procedure for the major phospholipid species involving the use of chloroform-methanol in the presence of 0.04% (w/v) CaCl_2 without dilute HCl, less than 1% of the radioactivity was recovered. Conventional phospholipids but not DPI are recovered almost quantitatively in this extraction system.

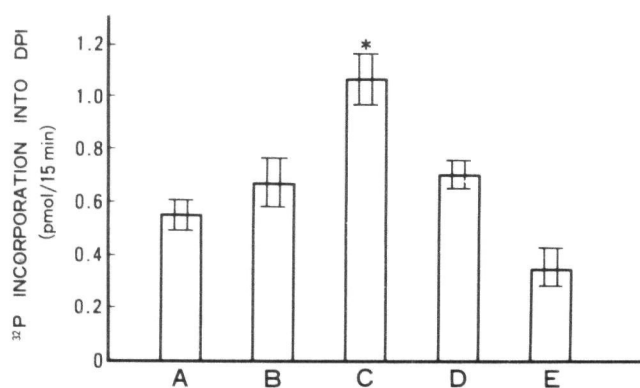


Fig. 1. Effect of spermine on ^{32}P incorporation into DPI in rat mast cell granules. Rat mast cell granules were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and Mg^{2+} for 15 min in the absence or presence of different concentrations of spermine. Each column represents the mean and standard error of the mean (SEM) of 3 experiments. A: control; B, C, D, E: 0.1, 1, 10, 100 mM spermine, respectively. * Statistical significance from the control at $P < 0.01$.

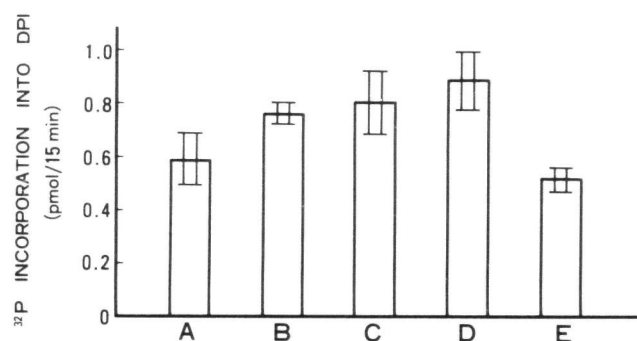


Fig. 2. Effect of spermidine on ^{32}P incorporation into DPI in rat mast cell granules. Rat mast cell granules were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and Mg^{2+} for 15 min in the absence or presence of different concentrations of spermidine. Each column represents the mean and standard error of the mean (SEM) of 3 experiments. A: control; B, C, D, E: 0.1, 1, 10, 100 mM spermidine, respectively.

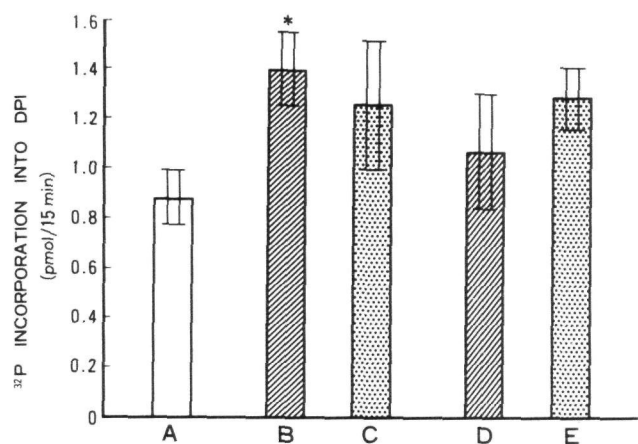


Fig. 3. Effect of spermine or spermidine on ^{32}P incorporation into DPI in rat mast cell granules. Rat mast cell granules were incubated with $[\gamma^{32}\text{P}]\text{ATP}$ and Mg^{2+} for 15 min in the absence or presence of 1 mM or 10 mM polyamines. Each column represents the mean and standard error of the mean (SEM) of 3 experiments. A: control; B: 1 mM spermine; C: 1 mM spermidine; D: 10 mM spermine; E: 10 mM spermidine. * Statistical significance from the control at $P < 0.05$.

Protein assay of granules

Protein concentrations of granules were determined by the spectrometric method of Bradford (2) with crystalline BSA as a standard.

Standardization of granule concentrations

Concentrations of granules were standardized with respect to one another on the basis of major granule membrane proteins. Granule proteins were separated by electrophoresis in the presence of sodium dodecyl sulfate in polyacrylamide slab gels, basically following the method of Laemmli (12). A linear gradient of polyacrylamide, from 6% (w/v) to 18% (w/v), was used as the separating gel with a 3% (w/v) acrylamide stacking gel. In all gels the ratio of acrylamide to bisacrylamide was 37:1 by weight. Stained proteins were quantitated by scanning in a Zeineth Soft Laser scanning densitometer (Biomed Instruments, Inc., Chicago, IL, U.S.A.).

Analysis of data

Unless otherwise stated, each point illustrated in each figure represents the mean and standard

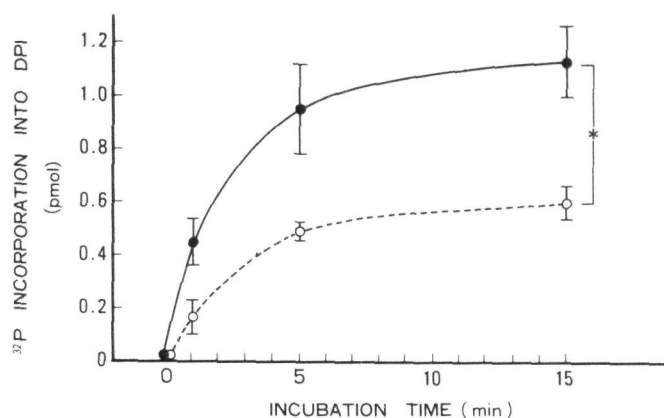


Fig. 4. Effect of incubation time with 1 mM spermine on ^{32}P incorporation into DPI in rat mast cell granules. Rat mast cell granules were incubated with $[\gamma^{32}\text{P}]\text{ATP}$ and Mg^{2+} for the indicated time in the absence or presence of 1 mM spermine. ----: control; —: with 1 mM spermine. * Statistical significance from the control at $P < 0.05$.

error of the mean (SEM) of three experiments (each done in duplicate; differences between duplicates were less than 6%). Significance was determined by employing the independent Student t-test (two-tailed).

RESULTS

As shown in Figs. 1 and 2, ^{32}P incorporation into DPI in rat mast cell granules incubated with Mg^{2+} and $[\gamma^{32}\text{P}]\text{ATP}$ was enhanced by spermine and spermidine, respectively, in a dose-dependent manner. 1 mM spermine stimulated the ^{32}P incorporation significantly compared with the control ($P < 0.01$) and 10 mM spermidine showed a tendency to increase the ^{32}P incorporation ($P < 0.1$). In another experiment, as shown in Fig. 3, ^{32}P incorporation into DPI in rat mast cell granules incubated with Mg^{2+} and $[\gamma^{32}\text{P}]\text{ATP}$ was increased significantly in the presence of 1 mM spermine ($P < 0.05$) and showed a tendency to increase in the presence of 10 mM spermidine ($P < 0.1$), but there were no differences in the increasing effects among the polyamines at either concentrations of 1 mM and 10 mM. When the effect of 1 mM spermine on ^{32}P incorporation into DPI in rat mast cell granules was investigated serially, the amount of ^{32}P incorporation was increased in a time-dependent manner and ^{32}P incorporation into DPI in rat mast cell granules

incubated with 1 mM spermine for 1 min and 5 min showed a tendency to increase ($P < 0.1$) and that for 15 min was enhanced significantly ($P < 0.05$) compared with the ^{32}P incorporation in the granules in the absence of spermine (Fig. 4).

DISCUSSION

Polyamines are ubiquitously distributed in animal cells, tissues and extracellular fluids and strongly associated with membranes and implicated in membrane function (19). Polyamines are polycations associated with nucleic acids and recognized as important regulators of cell functions, such as cell differentiation and proliferation (5, 15, 21). They are also shown to interact with polyphosphoinositides (22). Polyamines, spermine and spermidine, are found in human blood in concentrations that vary from 2.3 to 3.8 μM (17) and increased concentrations of the polyamines in human body fluids has been documented in several physiological and pathological conditions such as cystic fibrosis (18) and tumors (5).

The addition of polyamines, spermine and spermidine, to rat mast cell granules incubated with Mg^{2+} and $[\gamma^{32}\text{P}]\text{ATP}$ caused an increase in ^{32}P incorporation into DPI in rat mast cell granules, which can be catalyzed by PI kinase. This effect of polyamines in the DPI synthesis was dose-dependent and maximal effects were observed at 1 mM spermine and 10 mM spermidine, respectively, showing that the increase was seen only at relatively high concentrations of polyamines. About the relevance of the concentrations of the polyamines used in this study, stimulation of the phosphorylation of PI in the membranes by spermine and spermidine under the near concentrations have been reported already (23). PI kinase in rat mast cell granules is stimulated by Mg^{2+} or Mn^{2+} (8), which are not known to change dramatically in the cytosol during granule exocytosis from mast cells. Even though there is no obvious mechanism for producing rapid alterations in PI kinase activity *per se* in activated mast cells, these data suggest that polyamines may alter the PI kinase activity as natural polycations.

DPI kinase in rat mast cell granules, which catalyzes the formation of TPI from DPI, has been demonstrated in the presence of exogenous DPI (9). The increase in ^{32}P incorporation into DPI in rat mast cell granules was not accompanied by an increase in ^{32}P incorporation into TPI (data not shown), indicating that polyamines stimulate an increased phosphorylation of PI to form more DPI than TPI without an increase in the breakdown of the phosphoinositides since no increased formation of ^{32}P -labelled phosphatidic acid was detected. Thus, the increase in ^{32}P incorporation into DPI in rat mast cell granules by polyamines could be due to enhanced activity of PI kinase rather than an inhibition of phospholipase C activity, although inhibitory activity of polyamines on phospholipase C in human platelets and neutrophils has been suggested (3, 14).

Evidence is accumulating that phosphorylation of inositol lipids may generally be under the control of protein phosphorylating enzymes. Certain tyrosine kinases produced by transforming genes of Rous sarcoma virus (pp60^{V-src}) and avian sarcoma virus (UR 2 p68^{V-ros}) were shown to phosphorylate inositol lipids, and phosphorylation of PI has been suggested to play a role in transformation since PI turnover was increased in the transformed cells by these viruses (13, 20). Polyamines were shown to have an inhibitory effect on adenylate cyclase activity in platelet with a decrease of cyclic AMP levels (16), and inhibitory effect of cyclic AMP as well as adenine nucleotides on PI kinase in rat mast cell granules has been reported (8, 10). Thus results from the present experiments suggest that polyamines may directly affect PI kinase or indirectly regulate the kinase through the regulation of unknown cyclic AMP-dependent protein phosphorylating enzymes involved in, for example, a cyclic AMP-dependent phosphorylation of a 44 K protein in the granules (11). The stimulation of inositol-lipid phosphorylation by polyamines may have significant implications for the mechanisms of mediator release from rat mast cells because the metabolisms of phosphoinositides in the activated rat mast cells can be increased (7). However, further studies are required for conclu-

sion, as the mechanism behind the increase in radioactivity in DPI could not be investigated directly in this study.

REFERENCES

1. Bligh, E. & Dyer, W.: A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **3**, 911-917, 1959.
2. Bradford, M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248-254, 1976.
3. Das, I., de Belleruche, J. & Hirsh, S.: Inhibitory action of spermidine on formyl-methionyl-leucyl-phenylalanine stimulated inositol phosphate production in human neutrophils. *Life Sci.* **41**, 1037-1041, 1987.
4. Enerbach, L. & Svenson, I.: Isolation of rat peritoneal mast cells by centrifugation on density of Percoll. *J. Immunol. Methods* **39**, 135-145, 1980.
5. Janne, J., Poso, H. & Raina, A.: Polyamines in rapid growth and cancer. *Biochim. Biophys. Acta* **473**, 241-293, 1978.
6. Kruger, P. G., Lagunoff, D. & Wan, H.: Isolation of rat mast cell granules with intact membranes. *Exp. Cell. Res.* **129**, 83-93, 1980.
7. Kurosawa, M. & Parker, C. W.: Changes in polyphosphoinositide metabolism during mediator release from stimulated rat mast cells. *Biochem. Pharmacol.* **38**, 431-437, 1989.
8. Kurosawa, M. & Parker, C. W.: A phosphatidylinositol kinase in rat mast cell granules. *J. Immunol.* **136**, 616-622, 1986.
9. Kurosawa, M., Okayama, Y. & Kobayashi, S.: A di-phosphoinositide kinase in rat mast cell granules. *J. Allergy Clin. Immunol.* **84**, 118-128, 1989.
10. Kurosawa, M., Okayama, Y. & Kobayashi, S.: Inhibitory effect of adenine nucleotides and anti-allergic drugs on phosphorylation of phosphatidylinositol in rat mast cell granules. *Allergy* **44**, 576-587, 1989.
11. Kurosawa, M. & Parker, C. W.: Protein phosphorylation in rat mast cell granules. Cyclic AMP dependent phosphorylation of a 44 K protein associated with broken granules. *Biochem. Pharmacol.* **36**, 131-140, 1987.
12. Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. *Nature* **227**, 680-685, 1970.
13. Macara, I. G., Marinetti, G. V. & Balduzzi, P. C.: Transforming protein of avian sarcoma virus UR2 is associated with phosphatidylinositol kinase activity: possible role in tumorigenesis. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2728-2732, 1984.
14. Nahas, N. & Graff, G.: Inhibitory activity of polyamines on phospholipase C from human platelets. *Biochem. Biophys. Res. Commun.* **109**, 1035-1040, 1982.
15. Pegg, A. E. & McCann, P. P.: Polyamine metabolism and function. *Am. J. Physiol.* **243**, C212-C221, 1982.
16. Rennert, O., Buehler, B. & Miale, T.: Polyamines and platelet aggregation. *Life Sci.* **19**, 257-264, 1976.
17. Rennert, O. & Shukla, J.: Polyamines in health and disease. In Campbell, R., Morris, D., Bartos, D., Daves, G. & Bartos, F. (eds.): *Advances in polyamines research*, pp. 196-209. Raven Press, New York, 1978.
18. Rennert, O., Frias, J. & Shukla, J.: Polyamines metabolism in cystic fibrosis. *Tex. Rep. Biol. Med.* **34**, 187-196, 1976.
19. Schindler, M., Koppel, D. E. & Sheetz, M. P.: Modulation of membrane protein lateral mobility by polyphosphates and polyamines. *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1457-1461, 1980.
20. Sugimoto, Y., Whitman, M., Cantley, L. C. & Erikson, R. L.: Evidence that Rous sarcoma virus transforming gene product phosphorylates phosphatidylinositol and diacylglycerol. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2117-2121, 1984.
21. Tabor, C. W. & Tabor, H.: Polyamines in microorganism. *Microbiol. Rev.* **49**, 81-99, 1985.
22. Tadolini, B. & Varani, E.: Interaction of spermine with polyphosphoinositides containing liposomes and myo-inositol 1, 4, 5 triphosphate. *Biochem. Biophys. Res. Commun.* **135**, 58-64, 1986.
23. Vogel, S. & Hoppe, J.: Polyamines stimulate the phosphorylation of phosphatidylinositol in membranes from A431 cells. *Eur. J. Biochem.* **154**, 253-257, 1986.

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