Possible physiological role of guanosine triphosphate and inositol 1,4,5-trisphosphate in Ca^{2+} release in macrophages stimulated with chemotactic peptide

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The release of Ca^{2+} induced by inositol 1,4,5-trisphosphate (InsP_3) in the presence of GTP was examined by using saponin-permeabilized macrophages. The origin and the amount of mobilized Ca^{2+} in intact macrophages stimulated with chemotactic peptide were also examined to assess the physiological significance of GTP and InsP_3 on Ca^{2+} -releasing activities. The total amount of Ca^{2+} released by 20 μM -A23187 from the unstimulated intact macrophages was 1.4 nmol/ 4×10^6 cells, and the mitochondrial uncoupler did not cause an efflux of Ca^{2+} from the cells. The Ca^{2+} accumulation by the non-mitochondrial pool(s) was inhibited by the presence of GTP, and the total amount of releasable Ca^{2+} (1.4 nmol/ 4×10^6 cells) was comparable with that accumulated by the non-mitochondrial pool(s) in the presence of GTP at a free Ca^{2+} concentration of 0.14 μM . The mobilized and subsequently effluxed Ca^{2+} in cells stimulated with chemotactic peptide was estimated to be 0.3 nmol/ 4×10^6 cells. Much the same amounts were released by about the half-maximal dose of InsP_3 from the non-mitochondrial pool(s) of saponin-treated macrophages that had accumulated Ca^{2+} at a free concentration of 0.14 μM in the presence of GTP. These results suggest that the Ca^{2+} -releasing activity induced by GTP may play a role in the long-term regulation of Ca^{2+} content in the non-mitochondrial pool(s) of macrophages, and that released by InsP_3 can explain, quantitatively, the chemotactic-peptide-induced mobilization of Ca^{2+} .

INTRODUCTION

A general feature of hormones and neurotransmitters which exert their physiological effects through changes in the cytosolic free Ca^{2+} concentrations, is the stimulated phosphodiesteric hydrolysis of phosphatidylinositol 4,5-bisphosphate, thereby elevating the concentrations of two intracellular messengers, diacylglycerol and inositol 1,4,5-trisphosphate (InsP_3) (for review, see Berridge & Irvine, 1984; Nishizuka, 1984). The latter compound mobilizes Ca^{2+} from the intracellular stores, particularly the endoplasmic reticulum (Streb *et al.*, 1983; Suematsu *et al.*, 1984; Hirata *et al.*, 1984a; Berridge & Irvine, 1984).

Dawson and colleagues were apparently the first to demonstrate the enhancement by GTP in releasing Ca^{2+} from the endoplasmic-reticulum-rich microsomal fraction of rat liver by InsP_3 (Dawson, 1985; Dawson *et al.*, 1986). On the other hand, GTP in itself can induce a remarkable release of Ca^{2+} in the microsomal fraction or in permeabilized N1E-115 cells (Ueda *et al.*, 1986; Gill *et al.*, 1986). Essentially the same conclusion was reached with the microsomal fraction or permeabilized cells of parotid glands (Henne & Söling, 1986), liver (Nicchitta *et al.*, 1986), the neuronal cell line NG 108 $\times$ 15 (Jean & Klee, 1986), pancreatic-islet cells (Wolf *et al.*, 1987) and peritoneal macrophages (Hamachi *et al.*, 1987).

The remarkable release of Ca^{2+} induced by GTP required the simultaneous presence of poly(ethylene glycol) (PEG). We previously reported that GTP not only possesses the inherent activity of releasing Ca^{2+}

from the endoplasmic reticulum of saponin-permeabilized macrophages, but it also stimulates the Ca^{2+} -accumulating activity. The latter activity is eliminated by the presence of PEG (Hamachi *et al.*, 1987). Therefore we assumed that the simultaneous presence of PEG with GTP produced an apparent domination of Ca^{2+} -releasing activity. On the other hand, Nicchitta *et al.* (1986) reported that the enhancing effect by PEG on the Ca^{2+} release mediated by GTP is closely associated with its enhancing effect on the GTPase activity of the endoplasmic reticulum of rat liver, since the effect of GTP seems to require its hydrolysis (Dawson, 1985; Gill *et al.*, 1986; Hamachi *et al.*, 1987). There are reports on the relation between GTP- and InsP_3 -mediated release of Ca^{2+} in the microsomal fraction or permeabilized cells in a variety of cell types (Ueda *et al.*, 1986; Chueh & Gill, 1986; Jean & Klee, 1986; Wolf *et al.*, 1987). They concluded that these two compounds, GTP and InsP_3 , function via distinct mechanisms to activate the release of Ca^{2+} , and that the Ca^{2+} pools sensitive to these compounds may differ, because the releasing activity of Ca^{2+} was approximately additive. However, the physiological significance of these activities in stimulus-response coupling in a variety of cells is unknown. In macrophages, the chemotactic peptide *N*-formyl-methionyl-leucylphenylalanine (fMet-Leu-Phe) stimulated the formation of InsP_3 by breakdown of phosphatidylinositol bisphosphate, through receptor activation. Thus InsP_3 seems to play the role of a physiological messenger to mobilize intracellular Ca^{2+} (Kukita *et al.*, 1986). However, the distribution of Ca^{2+}

Abbreviations used: InsP_3 , inositol 1,4,5-trisphosphate; PEG, poly(ethylene glycol); CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; fMet-Leu-Phe, *N*-formylmethionyl-leucylphenylalanine (chemotactic peptide).

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in unstimulated macrophages, and the origin and amount of Ca^{2+} mobilized by the fMet-Leu-Phe stimulation, have remained unclear. The InsP_3 -mediated Ca^{2+} release is not more than 25–35 % of the Ca^{2+} accumulated by the intracellular non-mitochondrial pool(s) at $0.14 \mu\text{M}$ free Ca^{2+} (Hirata *et al.*, 1984a), whereas the GTP-mediated Ca^{2+} release amounts to about 60–70 % in the presence of PEG (Hamachi *et al.*, 1987). Whether the amount of Ca^{2+} mobilized by fMet-Leu-Phe stimulation could be explained by that released by InsP_3 has to be clarified. In the present study, we examined the physiological significance of these Ca^{2+} -releasing mechanisms in macrophages stimulated with fMet-Leu-Phe.

EXPERIMENTAL

Materials

$^{45}\text{Ca}^{2+}$ (38.16 mCi/mg) was obtained from New England Nuclear. GTP was obtained from Yamasa Shoyu Co. and was used without further purification. PEG (average M_r 6500) was from Wako Pure Chemical Co. A23187 and fMet-Leu-Phe were from Calbiochem-Behring Corp. The bovine brain phosphoinositide fraction and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were from Sigma. Antipyrilazo III was from Nakarai Chemicals. RPMI 1640 was from Nissui Seiyaki Co. Other reagents were of the highest grade available.

Methods

Preparation of macrophages and saponin treatment. Macrophages were collected from peritoneal cavities of guinea pigs intraperitoneally injected with sterilized liquid paraffin and treated with saponin as described previously (Hirata & Koga, 1982; Hirata *et al.*, 1983a,b).

Preparation of InsP_3 . InsP_3 was prepared by alkaline hydrolysis of the bovine brain phosphoinositide fraction, by the method of Grado & Ballou (1961), and was further purified by cellulose t.l.c. as described by Hirata *et al.* (1985).

Assays of Ca^{2+} uptake and Ca^{2+} release by InsP_3 in the presence or absence of GTP, using saponin-permeabilized macrophages. Assays were carried out as described by Hirata *et al.* (1984a) and Hamachi *et al.* (1987). The Ca^{2+} uptake was initiated by adding 2 mM-ATP. After 10 min, a sample of the reaction mixture was passed through a glass-fibre filter (Whatman GF/C, pore size $1.2 \mu\text{m}$). The filter was washed twice with the reaction mixture without $^{45}\text{Ca}^{2+}$, dried, and the radioactivity was counted. The amount of Ca^{2+} accumulated was thus determined. To measure Ca^{2+} release, InsP_3 in 0.01 vol. of the reaction mixture was added after the accumulation of Ca^{2+} had reached a plateau (routinely after 11 min). At an appropriate time, a sample of the reaction mixture was passed through a glass-fibre filter. The filter was treated as described above, and the Ca^{2+} remaining in saponin-treated macrophages was determined.

Assay of Ca^{2+} efflux from intact macrophages. Ca^{2+} efflux from macrophages was assayed by using a Ca^{2+} -indicator dye, antipyrilazo III, as described by Hamachi *et al.* (1986). Briefly, macrophages were preincubated in RPMI 1640 for at least 30 min at 37°C , washed once with a Ca^{2+} -free physiological salt solution containing

Fig. 1. Ca^{2+} uptake without PEG in the absence (○) or presence (●) of $100 \mu\text{M}$ -GTP at various free Ca^{2+} concentrations

The Ca^{2+} uptake was carried out after 10 min, when accumulation of Ca^{2+} had reached a plateau. The ATP-independent Ca^{2+} uptake, amounting to 7–15 % of the ATP-dependent Ca^{2+} uptake, was subtracted from the amount of ATP-dependent Ca^{2+} uptake. Each point represents the mean of five experiments.

130 mM-NaCl, 5 mM-KCl, 20 mM-Hepes/Tris buffer (pH 7.2), 5.5 mM-glucose and 1 mM- MgCl_2 . Hepes/Tris buffer was prepared as follows: approx. 1 M-Tris was added to 0.2 M-Hepes to give pH 7.2 at 37°C , and 0.2 M-Hepes was diluted to 20 mM. The washed cells were resuspended in Ca^{2+} -free solution and transferred to a cuvette (thermostatically maintained at 37°C) containing $100 \mu\text{M}$ -antipyrilazo III. Changes in the absorbance of antipyrilazo III at wavelengths of 720 and 790 nm were continuously monitored with a Hitachi 557 dual-wavelength spectrophotometer. The absorbance changes were linear up to a Ca^{2+} concentration of 35 nmol/ml. However, a full scale of 0.01 absorbance unit was routinely used, so that Ca^{2+} concentrations up to 5 nmol/ml could be measured. The total content of releasable Ca^{2+} was expressed as the amount of Ca^{2+} released by $20 \mu\text{M}$ -A23187. The application of a much higher dose of A23187, $50 \mu\text{M}$, gave the same result as did $20 \mu\text{M}$.

RESULTS

Effects of GTP without PEG on Ca^{2+} uptake and Ca^{2+} release mediated by InsP_3 in non-mitochondrial pool(s) of saponin-permeabilized macrophages

We previously reported that the Ca^{2+} uptake enhanced by MgATP in the presence of NaN_3 is due to non-mitochondrial pool(s) of saponin-permeabilized macrophages (Hirata & Koga, 1982; Hirata *et al.*, 1983a,b, 1984a). We also reported that application of GTP gradually induced a slight release of Ca^{2+} from the non-mitochondrial pool(s) in the absence of PEG, whereas a remarkable release of Ca^{2+} was observed in the presence of PEG (Hamachi *et al.*, 1987). In the present study, we examined the effect of GTP on the Ca^{2+} uptake of saponin-permeabilized macrophages. Fig. 1 shows the Ca^{2+} uptake by non-mitochondrial pool(s) at various free Ca^{2+} concentrations, in the presence or absence of GTP, without PEG. The presence of GTP ($100 \mu\text{M}$)

Fig. 2. InsP_3 -induced release of Ca^{2+} without PEG in the absence (●) or presence (■) of 100 μM -GTP

(a) Time course. Ca^{2+} was accumulated by saponin-treated macrophages at a free Ca^{2+} concentration of 0.14 μM , in the absence (○) or presence (□) of GTP. Arrow indicates the addition of 5 μM - InsP_3 . (b) Dose-dependence. Various doses of InsP_3 were added 11 min after the addition of ATP for Ca^{2+} uptake, in the absence (●) or presence (■) of GTP. Ca^{2+} released 5 min after addition of InsP_3 was plotted against the dose of InsP_3 . (c) Ca^{2+} released 5 min after the addition of InsP_3 was plotted against the amount of accumulated Ca^{2+} . Ca^{2+} accumulation was carried out without (●) or with (■) GTP. The accumulation of Ca^{2+} was carried out at free Ca^{2+} concentrations of 0.014, 0.037, 0.14, 0.37, 1.5, 3.5, 24 and 47 μM . The ATP-independent Ca^{2+} uptake, amounting to 7–15% of the value of ATP-dependent Ca^{2+} uptake, was subtracted from the amount of ATP-dependent Ca^{2+} uptake. Data are representative of at least nine experiments.

evenly inhibited the Ca^{2+} uptake at free Ca^{2+} concentrations ranging from 15 nM to 47 μM . The inhibitory effect of GTP may be due to increases in the releasing activity of Ca^{2+} (Hamachi *et al.*, 1987). A lower concentration of GTP (10 μM) gave a similar inhibition to that seen with 100 μM . At a free Ca^{2+} concentration of 0.14 μM [the intracellular free Ca^{2+} concentration of unstimulated macrophages, as assessed by the loading of quin 2 (Hirata *et al.*, 1984b)], the Ca^{2+} uptake in the presence of GTP (100 μM) amounted to 1.4 $\text{nmol}/4 \times 10^6$ cells, yet the uptake in its absence was 2.2 $\text{nmol}/4 \times 10^6$ cells.

When the Ca^{2+} accumulation by the non-mitochondrial pool(s) was carried out in the presence of GTP with PEG, the amount was further decreased, to about 0.5 or 1.6 $\text{nmol}/4 \times 10^6$ cells at a free Ca^{2+} concentration of 0.14 μM or 24 μM respectively (results not shown).

We then examined the release of Ca^{2+} mediated by InsP_3 without PEG, in the presence or absence of GTP, with respect to the time course, dose-dependence and the dependence on the free Ca^{2+} concentrations. As shown in Fig. 2(a), when the Ca^{2+} accumulation was measured at a free Ca^{2+} concentration of 0.14 μM in the presence or absence of GTP, the amount of Ca^{2+} released by the

Fig. 3. Measurement of total releasable Ca^{2+} and Ca^{2+} effluxed by fMet-Leu-Phe from intact macrophages

Macrophages (1×10^7 cells) were suspended in the Ca^{2+} -free solution containing $100 \mu\text{M}$ -antipyrylazo III after preincubation in RPMI 1640 for more than 30 min. Changes in the external Ca^{2+} were measured with a wavelength pair of 720 and 790 nm. (a) A23187 ($20 \mu\text{M}$) was added at 2 min, followed by the addition of CCCP ($10 \mu\text{M}$). The trace is representative of at least 15 traces. (b) fMet-Leu-Phe ($0.2 \mu\text{M}$) was added, followed by the addition of A23187 ($20 \mu\text{M}$). The trace is a typical example of at least 10 traces.

maximally effective dose of InsP_3 was much the same (about $0.5 \text{ nmol}/4 \times 10^6$ cells). The dose-response relationship for InsP_3 -induced Ca^{2+} release in the presence of GTP was the same as that in the absence of GTP (Fig. 2b). In the presence or absence of GTP, the maximal release by InsP_3 was observed when the saponin-permeabilized cells accumulated Ca^{2+} to about 1.4 or $2.2 \text{ nmol}/4 \times 10^6$ cells respectively (Fig. 2c).

Amount of total releasable Ca^{2+} and Ca^{2+} mobilized by fMet-Leu-Phe in intact macrophages

The release of Ca^{2+} by GTP and by InsP_3 may involve different pools and different mechanisms. To determine the physiological significance of the Ca^{2+} release mediated by these compounds in macrophages stimulated with chemotactic peptide, we examined the distribution of Ca^{2+} in unstimulated macrophages and the amount of Ca^{2+} mobilized in the cells stimulated with chemotactic peptide by measuring the absorbance change of antipyrylazo III.

The addition of $20 \mu\text{M}$ -A23187 to cells led to an increase in external Ca^{2+} within 6 min (Fig. 3a). This increase may be due to the release of Ca^{2+} from internal store(s) and the subsequent efflux through the plasma membrane, but not to the simple dissociation of extracellularly bound Ca^{2+} , because the external Ca^{2+} increased equally when a washing procedure was performed in the presence of La^{3+} , which effectively displaced extra-macrophage Ca^{2+} (Hirata *et al.*, 1983c). Since macrophages have two types of Ca^{2+} store(s), mitochondrial and non-mitochondrial, and A23187 can act on both stores (Hirata *et al.*, 1983a,b), the effect of a mitochondrial uncoupler on the absorbance change was examined. Application of the mitochondrial uncoupler CCCP ($10 \mu\text{M}$) had no effects on absorbance change of antipyrylazo III (Fig. 3a). These results indicate that the increase in external Ca^{2+} by application of A23187 is derived from the release of Ca^{2+} from non-mitochondrial store(s) and that the mitochondrial pool contains little releasable Ca^{2+} . The amount of A23187-releasable Ca^{2+}

was calculated to be $1.4 \pm 0.2 \text{ nmol}/4 \times 10^6$ cells (mean $\pm$ S.E.M. for ten determinations). We reported that Ca^{2+} exchangeable with $^{45}\text{Ca}^{2+}$ in macrophages was not affected by the mitochondrial inhibitors oligomycin and NaN_3 , and amounted to $1.6 \text{ nmol}/4 \times 10^6$ cells (Hirata *et al.*, 1983c). The value of releasable Ca^{2+} in intact macrophages was much the same as that of the Ca^{2+} uptake at a free Ca^{2+} concentration of $0.14 \mu\text{M}$ in saponin-permeabilized macrophages in the presence of GTP (Fig. 1). Therefore we assume that, in unstimulated macrophages, most of the releasable Ca^{2+} may be located in the non-mitochondrial pool(s), if the Ca^{2+} -uptake activity of saponin-permeabilized macrophages is quantitatively the same as in the intact cells and the Ca^{2+} -releasing activity by GTP is continuously activated by the concentrations of GTP present in macrophages. We next examined, quantitatively, the Ca^{2+} mobilized by fMet-Leu-Phe. The addition of fMet-Leu-Phe ($0.2 \mu\text{M}$) evoked the Ca^{2+} efflux within 1 min, by about $0.3 \pm 0.05 \text{ nmol}/4 \times 10^6$ cells (mean $\pm$ S.E.M. for eight determinations), and the subsequent application of A23187 evoked a further efflux of the remaining Ca^{2+} (Fig. 3b). After the first application of fMet-Leu-Phe, macrophages were washed and freshly suspended in solution for assay of the absorbance change of antipyrylazo III. A second application of fMet-Leu-Phe to such cells no longer evoked the Ca^{2+} efflux (results not shown). Therefore, most of the Ca^{2+} released by fMet-Leu-Phe may not be re-accumulated into the store site(s), but rather an efflux through the surface membrane may occur.

DISCUSSION

InsP_3 seems to play the role of a physiological messenger to mobilize intracellular Ca^{2+} . Many investigators have reported that GTP could induce the release of Ca^{2+} in various cells, and that the releasing activities of Ca^{2+} mediated by GTP or by InsP_3 might function on different Ca^{2+} pool(s) through distinct mechanisms

(Ueda *et al.*, 1986; Gill *et al.*, 1986; Chueh & Gill, 1986; Jean & Klee, 1986; Wolf *et al.*, 1987).

In fact, we examined the relation between GTP- and InsP_3 -mediated Ca^{2+} release using saponin-permeabilized macrophages (Y. Kimura, M. Hirata, T. Hamachi & T. Koga, unpublished work). The results obtained with guinea-pig macrophages were essentially the same as noted in the above-mentioned reports.

The intracellular contents of GTP in macrophages are unknown, but in the rat liver or brain the cellular amount of GTP was estimated to be about $320 \mu\text{M}$ or $23.8 \mu\text{mol}/100 \text{ g}$ wet wt. of tissue respectively (Kleineke *et al.*, 1979; Mandel & Edel-Harth, 1966). In the latter case, the cellular ATP amounted to $199.8 \mu\text{mol}/100 \text{ g}$ wet wt. of tissue. If such is the case in macrophages, and there is no compartmentation of intracellular GTP pools and the cellular GTP is evenly distributed in the cytoplasm (but see Henne & Söling, 1986), the Ca^{2+} -releasing activity by GTP may be continuously activated. The permanent activation of Ca^{2+} release by GTP without PEG resulted in an apparent inhibition of Ca^{2+} -accumulating activity by non-mitochondrial pool(s). The amount of releasable Ca^{2+} in intact macrophages (which was about equal to the Ca^{2+} exchangeable with $^{45}\text{Ca}^{2+}$ at equilibrium) was much the same as that of the Ca^{2+} accumulated by non-mitochondrial pool(s) of saponin-permeabilized cells in the presence of GTP at a free Ca^{2+} concentration of $0.14 \mu\text{M}$, thereby pertaining to the cytoplasmic free Ca^{2+} concentration in the unstimulated state. These results indicate that most of the releasable Ca^{2+} is located in the non-mitochondrial pool(s) and that the GTP-mediated release of Ca^{2+} is continuously activated. This is based on the assumption that the intracellular Ca^{2+} pools of saponin-permeabilized cells are qualitatively and quantitatively much the same as in the intact cells. Therefore the GTP effect in releasing Ca^{2+} may play a physiological role in the long-term regulation of Ca^{2+} content in non-mitochondrial pool(s) of macrophages, as previously reported (Hamachi *et al.*, 1987).

The addition of fMet-Leu-Phe evoked the efflux of Ca^{2+} . The amount of effluxed Ca^{2+} is probably about equal to that mobilized by the fMet-Leu-Phe stimulation. Most of the intracellularly mobilized Ca^{2+} may be effluxed through the plasma membrane, without a re-accumulation into the internal pool(s), for the following reasons. A second addition of fMet-Leu-Phe to macrophages that had once been challenged with this agent no longer evoked an efflux of Ca^{2+} . The response to the second challenge of fMet-Leu-Phe, in changes in intracellular free Ca^{2+} concentrations, as assessed by the loading of quin 2, only gradually recovered in Ca^{2+} -containing medium, but not in Ca^{2+} -free medium (Hirata *et al.*, 1984b). This finding is in contrast with the recovery from down-regulation, as the fMet-Leu-Phe binding to neutrophils was rapid (binding experiments of fMet-Leu-Phe to macrophages have yet to be done). More than 90% of the down-regulated sites re-appeared within 1 min, irrespective of the presence of the external Ca^{2+} (Hamachi *et al.*, 1986).

The amount of Ca^{2+} intracellularly mobilized and then effluxed through the plasma membrane by fMet-Leu-Phe stimulation was about $0.3 \text{ nmol}/4 \times 10^6$ cells, thereby indicating that the partial release of Ca^{2+} by InsP_3 is sufficient to explain the amount of Ca^{2+} intracellularly mobilized by fMet-Leu-Phe stimulation. The maximal

dose of InsP_3 induced a release of Ca^{2+} of about $0.5 \text{ nmol}/4 \times 10^6$ cells in the presence of GTP, when Ca^{2+} had been loaded in the non-mitochondrial pool(s) to about $1.4 \text{ nmol}/4 \times 10^6$ cells, a value equivalent to the cellular content of releasable Ca^{2+} in unstimulated macrophages. The InsP_3 was rapidly formed in macrophages, with fMet-Leu-Phe stimulation. The absolute amount of InsP_3 formed has not been determined. Bradford & Rubin (1986) reported that, within 10 s of stimulation with fMet-Leu-Phe, the InsP_3 content of the peritoneal neutrophils increased from 0.05 to $0.55 \text{ pmol}/10^6$ cells, equivalent to a change in intracellular concentration from 100 nM to $1.1 \mu\text{M}$. If this value can be extrapolated to macrophages, then $1.1 \mu\text{M-InsP}_3$ could induce a release of Ca^{2+} of about $0.32 \text{ nmol}/4 \times 10^6$ cells, a value corresponding to the amount of Ca^{2+} effluxed by fMet-Leu-Phe stimulation.

In conclusion, the present study shows the physiological significance of the Ca^{2+} release mediated by GTP and InsP_3 . A GTP-mediated release of Ca^{2+} may be continuously activated by the concentrations of GTP always present inside the cells, consequently there would be a decrease in Ca^{2+} content in the non-mitochondrial pool(s). This mechanism may play a physiological role in the long-term regulation of the intravesicular content of Ca^{2+} in macrophages. An InsP_3 -mediated release of Ca^{2+} could explain, with respect to the time course and the determination of Ca^{2+} , the chemotactic-peptide-induced mobilization of Ca^{2+} from non-mitochondrial pool(s). InsP_3 may be the sole physiological messenger for the mobilization of Ca^{2+} in macrophages stimulated with chemotactic peptide.

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