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Inhibition by abruquinone A of phosphoinositide-specific phospholipase C activation in rat neutrophils

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Abstract

In rat neutrophils, formyl-Met-Leu-Phe (fMLP)-induced inositol phosphate formation was inhibited by abruquinone A (IC_{50} value about 32.7 ± 6.4 μM) as well as by a putative phospholipase C inhibitor, [6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122) (IC_{50} value about 11.3 ± 1.2 μM). The reduction in inositol phosphate levels appeared to reflect inhibition of phospholipase C activity because the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) catalyzed by a soluble fraction from neutrophils was also inhibited by abruquinone A (IC_{50} value about 31.4 ± 5.6 μM) over the same range of concentrations. Although abruquinone A alone induced Ca^{2+} and Mn^{2+} influx into neutrophils in Ca^{2+}-containing medium, abruquinone A, like U73122, inhibited Ca^{2+} release (IC_{50} value about 23.5 ± 0.5 μM) from internal stores in Ca^{2+}-free medium. These results indicate that abruquinone A inhibits the activity of phosphoinositide-specific phospholipase C in neutrophils.

Keywords: Abruquinone A; Neutrophil; Inositol phosphate; Phospholipase C; Ca^{2+} concentration, intracellular

1. Introduction

Various extracellular signaling molecules, when binding to their cell surface receptors, elicit transmembrane signal transduction mechanisms. These include the activation of phospholipase C through a G-protein-dependent or -independent step, and catalysis of the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) (Rana and Hokin, 1990; Majerus, 1992). Diacylglycerol is the physiological activator of protein kinase C, and IP_3 induces the release of Ca^{2+} from internal stores (Nishizuka, 1986; Berridge and Irvine, 1989). This pathway is known to regulate several cellular processes, including metabolism, secretion, contraction, motility and proliferation (Rhee and Choi, 1992).

Abruquinone A (Fig. 1), a natural isoflavanquinone isolated from the roots of *Abrus precatorius* L. (Alessandro et al., 1979), antagonizes the plasma extravasation in the mouse ear elicited by several inflammatory mediators (histamine, serotonin, bradykinin, and substance P) and by hypersensitivity (passive cutaneous anaphylactic reaction) (Wang et al., 1995a). It has been reported that many inflammatory mediators utilize phosphoinositide turnover as part of their signal transduction pathway (Berridge and Irvine, 1984; Abdel-Latif, 1986), and that inhibition of phospholipase C partly accounts for the anti-inflammatory actions of manoalide (Bennett et al., 1987). In this study we examined the inhibitory effects of abruquinone A on both inositol phosphate formation in neutrophils and phospholipase C activity in crude cytosolic preparations. Furthermore, the effect of abruquinone A on the intracellular Ca^{2+} concentrations of neutrophils was also investigated.

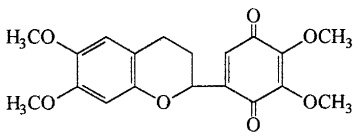

Fig. 1. Chemical structure of abruquinone A.

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2. Materials and methods

2.1. Materials

Abruquinone A was isolated and purified from *Abrus precatorius* as previously described (Alessandro et al., 1979). All chemicals were purchased from Sigma (St. Louis, MO, USA), except for the following: Dextran T-500 (Pharmacia Biotech, Uppsala, Sweden); Hanks' balanced salt solution (Life Technologies Gibco, Gaithersburg, MD, USA); [6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122) and cyclopiazonic acid (CPA) (Biomol Research, Plymouth Meeting, PA, USA); AG 1-X8 resin (Bio-Rad, Hercules, CA, USA); L-α-phosphatidyl-D-*myo*-inositol-4,5-bisphosphate (PIP_2) (Boehringer-Mannheim, Mannheim, Germany); L-3-phosphatidyl[2-^{3}H]inositol 4,5-bisphosphate ([^{3}H]PIP_2) and *myo*-[^{3}H]inositol (Amersham International, Amersham, UK); fura-2/AM (Molecular Probes, Eugene, OR, USA). Abruquinone A, U73122 and CPA were dissolved in dimethyl sulfoxide (DMSO).

2.2. Preparation of neutrophils

Rat blood was collected from the abdominal aorta and neutrophils were purified by dextran sedimentation, hypotonic lysis of erythrocytes, and centrifugation through Ficoll-Hypaque (Wang et al., 1995b). Purified neutrophils containing $>95\%$ viable cells were normally resuspended in Hanks' balanced salt solution containing 4 mM $NaHCO_3$ and 10 mM HEPES, pH 7.4.

2.3. Determination of inositol phosphates

Neutrophils (3×10^7 cells/ml) were incubated with *myo*-[^{3}H]inositol (83 Ci/mmol) at 37°C for 2 h and then washed (Wang et al., 1994). After incubation with formyl-Met-Leu-Phe (fMLP) for 10 s, the reaction was stopped by the addition of $CHCl_3/CH_3OH$ (1:1, v/v) mixture and acidification with 2.4 M HCl. The aqueous phase was removed, neutralized with 0.4 M NaOH, and then applied to AG 1-X8 resin (formate) column. Inositol monophosphate (IP_1), inositol bisphosphate (IP_2) and IP_3 were eluted sequentially using 0.2, 0.4, and 1.0 M ammonium formate in 0.1 M formic acid, respectively, as eluents, and then counted in dpm as described in detail elsewhere (Downes and Michell, 1981).

2.4. Measurement of cytosolic phospholipase C activity

Neutrophils (4×10^7 cells/ml) were disrupted by sonication in relaxing buffer (115 mM KCl, 5 mM KH_2PO_4, 2 mM EGTA, 0.91 mM $MgSO_4$, 10 mM HEPES, pH 7.4) supplemented with 0.1 mM dithiothreitol, 1 μM leupeptin, 1 μM aprotinin, 1 mM phenylmethylsulphonyl fluoride and 3 mM benzamidine. The neutrophil lysate was centrifuged at $100\,000\times g$ for 60 min at 4°C and the supernatant fluid was retained for phospholipase C assays. Substrate stock was prepared by mixing PIP_2 and 5 μCi [^{3}H]PIP_2 (1 Ci/mmol) in $CHCl_3/CH_3OH$ (1:1, v/v). The mixture was evaporated to dryness under N_2, redissolved in solvent mixture (20% sodium cholate, 250 mM 2-mercaptoethanol, 1 M PIPES, pH 6.8, 2.5 M NaCl) to produce a 0.361 mM PIP_2 solution, sonicated on ice, and then stored at −20°C. Phospholipase C activity was assayed by measuring the hydrolysis of PIP_2 into inositol phosphates (Cockcroft et al., 1994). Briefly, assay tubes contained test drugs, 10 mM HEPES buffer, pH 7.4, and cytosolic phospholipase C (about 0.04 mg protein). The assay mix was mixed well and incubated at 37°C for 3 min, and then 3 μl of 5 mM $CaCl_2$ and 20 μl of substrate stock were added to a total 150 μl of assay volume. Ten minutes later, the reaction was quenched with 0.75 ml ice-cold $CHCl_3/CH_3OH/HCl$ (50:50:1, v/v/v) and the mixture was acidified with 1 M HCl. After vigorous mixing and centrifugation at $2000\times g$ for 5 min at 4°C, a portion (400 μl) of the aqueous upper layer was collected for liquid scintillation counting.

2.5. Measurement of $[Ca^{2+}]_i$

Neutrophils (1×10^7 cells/ml) were suspended in HEPES buffer (124 mM NaCl, 4 mM KCl, 0.64 mM Na_2HPO_4, 0.66 mM KH_2PO_4, 15.2 mM $NaHCO_3$, 5.56 mM dextrose and 10 mM HEPES, pH 7.4), and loaded with 5 μM fura-2/AM at 37°C for 15 min as described previously (Wang et al., 1995b). The cells were then diluted 5-fold with the same buffer and incubated for an additional 15 min. Cell suspensions were then centrifuged at $900\times g$ for 10 min at 4°C, and the pellets were resuspended in HEPES buffer with 0.05% bovine serum albumin in the presence or absence of 1 mM $CaCl_2$. In some experiments, EDTA (1 mM) was present in the reaction mixture in which $CaCl_2$ was absent. Cell suspensions were placed in a double-wavelength fluorescence spectrophotometer (PTI, Deltascan 4000) equipped with a thermostated cuvette holder and stirrer. The fluorescence was monitored at 510 nm with excitation wavelengths of 340 and 360 nm in the ratio mode. Calibration of the excitation ratio in terms of Ca^{2+} concentration was performed by using fura-2-Ca^{2+} standards according to a previously described method (Grynkiewicz et al., 1985).

2.6. Assessment of Mn^{2+} influx

Entry of Mn^{2+} into cells was measured by the fura-2 fluorescence quenching technique. Fluorescence was monitored in fura-2-loaded cells in Ca^{2+}-containing medium at excitation 360 nm, the isosbestic point where fura-2 was insensitive to changes in $[Ca^{2+}]_i$, and emission 510 nm. $MnCl_2$ (0.5 mM) was added following the preincubation of the neutrophils with 10 μM CPA (Demaurex et al.,

1992) or various concentrations of abruquinone A. Diethylenetriamine pentaacetic acid (2 mM) was added at the end of an experiment, which indicated that less than 5% of the total fluorescence quenched by Mn^{2+} was due to leakage of fura-2.

2.7. Statistical analysis

Statistical analyses were performed using the Bonferroni *t*-test method after analysis of variance. A P value less than 0.05 was considered significant for all tests. Analysis of the regression line test was used to calculate IC_{50} values.

3. Results

3.1. Effect of abruquinone A on inositol phosphate formation

After addition of 0.3 μM fMLP to *myo*-[^{3}H]inositol-loaded neutrophils, a significant increase ($P < 0.01$) in cytosolic IP_2 and IP_3 levels was observed in comparison with the resting levels (the levels of IP_1, IP_2 and IP_3 were counted to be 34.1 ± 5.3, 67.7 ± 7.9 and 148.2 ± 13.7 dpm, respectively, in the resting cells, and 40.9 ± 5.5, 133.4 ± 8.7 and 345.1 ± 17.4 dpm, respectively, in the fMLP-stimulated cells). Abruquinone A as well as U73122, a putative phospholipase C inhibitor (Smith et al., 1990), reduced both the IP_2 and IP_3 levels in a concentration-dependent manner (Fig. 2). A significant inhibition was observed at concentrations of abruquinone A ≥ 8.3 μM and U73122 ≥ 3 μM in the suppression of IP_2 and IP_3 formation. The IC_{50} values of abruquinone A and U73122 for the inhibition of IP_3 formation were estimated to be about 32.7 ± 6.4 μM and 11.3 ± 1.2 μM, respectively.

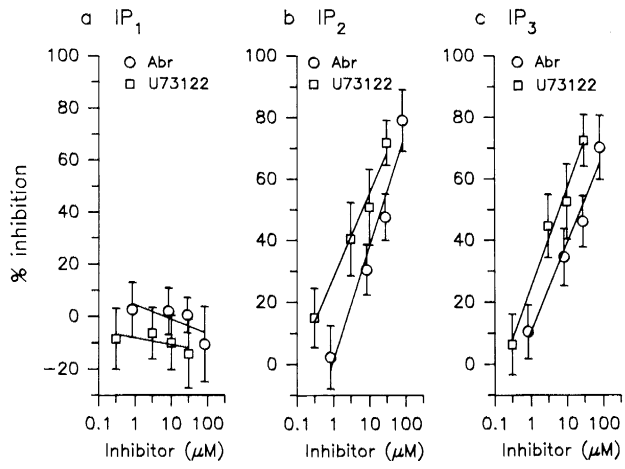

Fig. 2. Effect of abruquinone A (Abr) and U73122 on inositol phosphate formation in neutrophils. *myo*-[^{3}H]Inositol-loaded cells were treated with abruquinone A or U73122 at various concentrations for 3 min at 37°C before stimulation with 0.3 μM fMLP. Reaction was stopped by the addition of $CHCl_3/CH_3OH$ (1:1, v/v) mixture. Inositol phosphates were separated on an AG 1-X8 column, (a) IP_1, (b) IP_2 and (c) IP_3 were eluted, and the radioactivity was counted in dpm. Values are mean percent inhibition ± S.E.M. of 8 independent experiments.

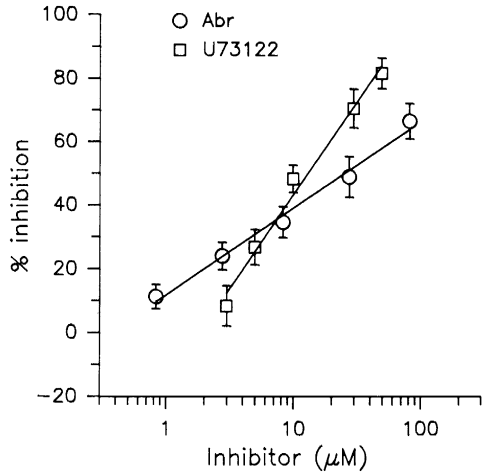

Fig. 3. Effect of abruquinone A (Abr) and U73122 on neutrophil cytosolic phospholipase C activity. Cytosolic phospholipase C was preincubated with abruquinone A or U73122 at various concentrations for 3 min at 37°C before addition of 100 μM $CaCl_2$ and 48 μM PIP_2/[^{3}H]PIP_2. Reaction was stopped by the addition of ice-cold $CHCl_3/CH_3OH/HCl$ (50:50:1, v/v/v) mixture. Inositol phosphate formation in the upper aqueous phase was determined. Values are mean percent inhibition ± S.E.M. of 8 independent experiments.

3.2. Effect of abruquinone A on cytosolic phospholipase C activity

On the addition of $CaCl_2$ and PIP_2 to the reaction mixture, neutrophil cytosolic phospholipase C was activated and used PIP_2 as substrate to produce inositol phosphates at the rate of 0.64 ± 0.04 nmol PIP_2 hydrolysis/min per mg protein. Both abruquinone A and U73122 suppressed the phospholipase C activity in a concentration-dependent fashion (Fig. 3). A significant inhibition was

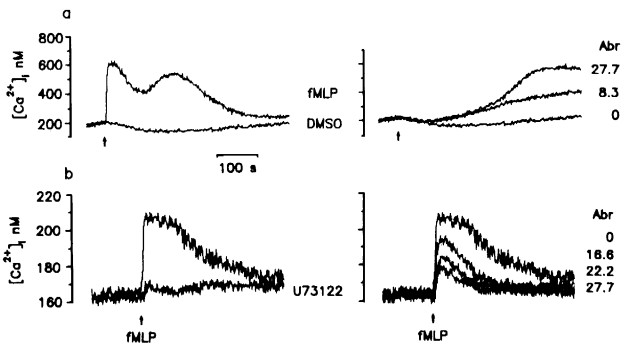

Fig. 4. Effect of abruquinone A (Abr) on $[Ca^{2+}]_i$ of neutrophils. Fura-2-loaded cells in (a) 1 mM Ca^{2+}-containing medium were challenged with dimethyl sulfoxide (DMSO), 0.1 μM fMLP, 8.3 or 27.7 μM abruquinone A; and in (b) Ca^{2+}-free medium with 1 mM EDTA were preincubated with DMSO, 1 μM U73122, 16.6, 22.2 or 27.7 μM abruquinone A for 3 min before stimulation with 0.1 μM fMLP. Similar results were obtained in 4–5 independent experiments.

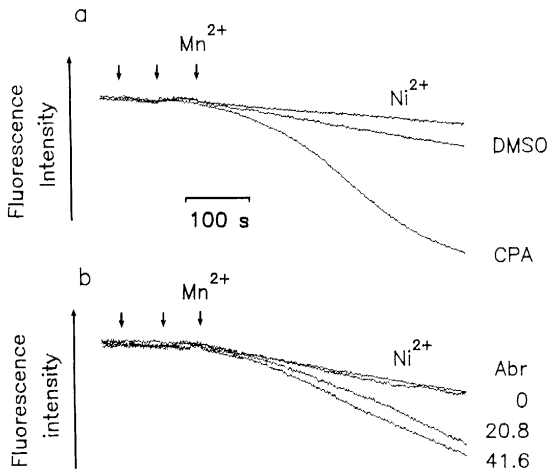

Fig. 5. Effect of abruquinone A (Abr) on Mn^{2+} influx in neutrophils. Fura-2-loaded cells were preincubated with or without 5 mM $NiCl_2$ (first arrow), then exposed subsequently (second arrow) to (a) dimethyl sulfoxide (DMSO) or 10 μM CPA; and to (b) DMSO, 20.8 or 41.6 μM abruquinone A, and then 0.5 mM $MnCl_2$ (third arrow). Similar results were obtained in 4 independent experiments.

observed at 8.3 μM abruquinone A ($P < 0.01$) with an estimated IC_{50} value of about 31.4 ± 5.6 μM and at 10 μM U73122 ($P < 0.01$) with an IC_{50} value estimated to be about 14.5 ± 2.3 μM.

3.3. Effect of abruquinone A on $[Ca^{2+}]_i$

In fura-2-loaded neutrophils, 0.1 μM fMLP induced a rapid and transient elevation of $[Ca^{2+}]_i$ in the Ca^{2+}-containing medium. A significant increase in $[Ca^{2+}]_i$ was also observed in the cell preparations treated with 8.3 and 27.7 μM abruquinone A alone (both $P < 0.01$), but with a slow and long-lasting profile of $[Ca^{2+}]_i$ elevation which was quite different from that of the fMLP-induced response (Fig. 4a). In the presence of 1 mM EDTA, abruquinone A alone even at concentrations up to 27.7 μM failed to increase $[Ca^{2+}]_i$ (data not shown). Moreover, abruquinone A produced a concentration-dependent inhibition of $[Ca^{2+}]_i$ elevation in neutrophils stimulated with 0.1 μM fMLP in Ca^{2+}-free medium (Fig. 4b), and the IC_{50} value was about 23.5 ± 0.5 μM.

Mn^{2+}-mediated quenching of cytosolic fura-2 has proved to be a useful model system for investigating Ca^{2+} influx. Like 10 μM CPA, abruquinone A at 20.8 and 41.6 μM significantly induced (both $P < 0.01$) Mn^{2+} influx and subsequently increased the rate of fura-2 quenching (Fig. 5). Both CPA- and abruquinone A-induced Mn^{2+} influx was abolished in the presence of 5 mM $NiCl_2$.

4. Discussion

A variety of responses of neutrophils to receptor-specific agonists, including exocytosis, $O_2^{\cdot -}$ and leukotriene B_4 formation, are inhibited by U73122, and the mechanism of inhibition by U73122 involves inhibition of phospholipase C activity (Smith et al., 1990). Abruquinone A was demonstrated to be an inhibitor of the fMLP-induced $O_2^{\cdot -}$ formation in rat neutrophils (data not shown). It has been reported that G proteins play a role in the transduction of signals from certain receptors to phospholipase C-β (Rhee and Choi, 1992). The inhibition by abruquinone A of fMLP-induced inositol phosphate (IP_2 and IP_3) formation in rat neutrophils was probably not a result of either a decrease in the agonist-receptor binding ability or interference with receptor-G protein coupling because the hydrolysis of PIP_2 catalyzed by the cytosol fraction of neutrophils was inhibited by abruquinone A over the same range of concentrations as for the inhibition of inositol phosphate formation of intact cells. The observation that abruquinone A exerted a similar inhibitory profile as U73122 did suggests that the inhibition of inositol phosphate formation by abruquinone A was due to the phospholipase C inhibition. Abruquinone A was less potent an inhibitor of phosphoinositide-phospholipase C than U73122.

The $[Ca^{2+}]_i$ response elicited by activation of receptors coupled to PIP_2 hydrolysis is composed of three phases: an initial spike, supported primarily by IP_3-induced release of Ca^{2+} from specific intracellular stores, followed by a plateau phase, which is sustained by Ca^{2+} influx from the extracellular medium (Meldolesi et al., 1991), and then a Ca^{2+} pump-mediated return to resting Ca^{2+} levels (Lagast et al., 1984). In the absence of extracellular Ca^{2+}, the agonist-induced increase in $[Ca^{2+}]_i$ is the result mainly of the release of Ca^{2+} from IP_3-sensitive intracellular stores. Therefore, suppression of phospholipase C activity resulted in a decrease in fMLP-induced IP_3 formation and $[Ca^{2+}]_i$ elevation. In support of the latter proposal was the finding that abruquinone A effectively suppressed the fMLP-induced $[Ca^{2+}]_i$ elevation in Ca^{2+}-free medium over a range of concentrations similar to those causing inhibition of fMLP-induced accumulation of IP_3. Manoalide, a marine natural product, is a nonsteroidal sesterterpenoid isolated from the sponge *Luffariella variabilis* (De Silva and Scheuer, 1980) and also inhibits both norepinephrine-induced inositol phosphate formation and Ca^{2+} transients in a smooth muscle cell line over the same range of concentrations (Bennett et al., 1987). However, U73122 is better able to inhibit the $[Ca^{2+}]_i$ elevation than IP_3 formation in neutrophils challenged with fMLP (Smith et al., 1990), and this result has been explained by the cooperative characteristic of IP_3-dependent mobilization of Ca^{2+} (Meyer et al., 1988). So far, we do not have evidence to explain the difference in susceptibilities of IP_3 formation and Ca^{2+} mobilization between abruquinone A and U73122.

Interestingly, abruquinone A alone slowly increased the $[Ca^{2+}]_i$ in Ca^{2+}-containing medium, as reflected by the increase in fura-2 fluorescence. However, in the absence of extracellular Ca^{2+}, abruquinone A alone had a negligible

effect on the increase in cytosolic free Ca^{2+}. Since abruquinone A alone increased the $[Ca^{2+}]_i$, experiments for the evaluation of the novel phospholipase C inhibitor on the suppression of fMLP-induced $[Ca^{2+}]_i$ elevation in the presence of extracellular Ca^{2+} could not be carried on. Like CPA, a Ca^{2+}-ATPase inhibitor (Seidler et al., 1989), abruquinone A induced Mn^{2+} influx, as measured by the fura-2 fluorescence quenching experiment. Since Ni^{2+}, a specific Ca^{2+} channel blocker (Shibuya and Douglas, 1992), abolished the abruquinone A-induced response, the influx of Mn^{2+} occurred through Ca^{2+}-permeable channels. Thus, in addition to the inhibition of agonist-induced Ca^{2+} release from internal Ca^{2+} stores, abruquinone A may also increase Ca^{2+} influx. Although the nature of the Ca^{2+} entry pathway is unclear yet, it has been reported that the release of Ca^{2+} from internal Ca^{2+} stores can activate Ca^{2+} entry (Berridge and Irvine, 1989; Putney et al., 1989). The profile of a slow increase in $[Ca^{2+}]_i$ caused by abruquinone A alone in Ca^{2+}-containing medium is similar to that caused by the Ca^{2+}-ATPase inhibitor CPA or thapsigargin (Seidler et al., 1989; Takemura et al., 1989). The Ca^{2+}-ATPase inhibitor provokes the release of Ca^{2+} from intracellular stores and the influx of Ca^{2+} from the extracellular space without increasing cellular IP_3 levels (Takemura et al., 1989; Demaurex et al., 1992). In the absence of extracellular Ca^{2+}, CPA still increases $[Ca^{2+}]_i$ (Wang, 1996), whilst abruquinone A failed to affect the $[Ca^{2+}]_i$ under the same conditions. These results indicate that the Ca^{2+} influx induced by depletion of internal Ca^{2+} stores, either through the increase in IP_3 or the blockade of Ca^{2+}-ATPase, did not occur in response to abruquinone A. Recently, a soluble cellular factor(s), extracted from neutrophils and lymphocyte cell line, has been reported to directly stimulate Ca^{2+} entry (Randriamampita and Tsien, 1993; Davies and Hallett, 1995). The precise mechanisms underlying the effect of abruquinone A on Ca^{2+} influx are unclear as yet and need further investigation. In conclusion, the present study indicates that the natural anti-inflammatory compound abruquinone A can inhibit phosphoinositide-specific phospholipase C activation in neutrophils.

Acknowledgements

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