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Stimulation of phospholipase C activity by norepinephrine, *t*-ACPD and bombesin in LA-N-2 cells

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Abstract

The release of [^{3}H]inositol phosphates from myo-[^{3}H]inositol-prelabeled LA-N-2 cells was measured in the presence of β-adrenoceptor, metabotropic glutamate and bombesin agonists. Norepinephrine and isoproterenol increased the formation of [^{3}H]inositol phosphates in a dose-dependent manner, with an EC_{50} of 100 μM for norepinephrine and an EC_{50} of 5 μM for isoproterenol. These stimulations were abolished by propranolol, a β-adrenoceptor antagonist, with an IC_{50} in the range of 50–55 μM for both norepinephrine and isoproterenol. The stimulation of [^{3}H]inositol phosphate appearance occurred with varying concentrations of *trans*-1-amino-1,3-cyclopentanedicarboxylic acid (*t*-ACPD), a metabotropic glutamate receptor agonist. This release of [^{3}H]inositol phosphates was blunted by its antagonist, 2-amino-3-phosphonopropionic acid (AP-3). Bombesin and neuromedin-B, a bombesin-like peptide, also increased the appearance of [^{3}H]inositol phosphates. This was blunted by the antagonist [Tyr^4, D-Phe^{12}]bombesin. The appearance of [^{3}H]inositol phosphates stimulated by *t*-ACPD was coupled through a cholera toxin-sensitive G-protein and the bombesin-stimulated appearance of [^{3}H]inositol phosphates was coupled through a pertussis toxin-sensitive G-protein. The norepinephrine-stimulated appearance of [^{3}H]inositol phosphates was toxin insensitive. The stimulation of the [^{3}H]inositol phosphate appearance by these three agonists was protein kinase and Ca^{2+} independent.

Keywords: Phospholipase C; Norepinephrine; *t*-ACPD (*trans*-1-amino-1,3-cyclopentanedicarboxylic acid); Bombesin; LA-N-2 cell; β-Adrenoceptor; Metabotropic

1. Introduction

The phospholipase C-catalyzed hydrolysis of phosphoinositides (PtdIns) yields two products that are intracellular messengers (Fisher and Agranoff, 1987). The stimulation of [^{3}H]inositol phosphate formation, a measure of phospholipase C activity, by norepinephrine binding to α_1-adrenoceptor has been reported in a variety of cell types (Burch et al., 1986; Gu et al., 1992; Steinberg et al., 1989; Slivka and Insel, 1987, Glowinski et al., 1984; Drouva et al., 1991; Wu et al., 1992; Cotecchia et al., 1990) and in a variety of tissues (Cheung et al., 1990; Weglicki and Low, 1987; Strosznajder et al., 1994; Duman et al., 1986; Holler et al., 1994; Chandler and Crews, 1990). β-Adrenoceptor-mediated stimulation of phospholipase C activity is uncommon (Cooper et al., 1991); however, this does occur (Rooney et al., 1991; James et al., 1994, Vaziri and Downes, 1992; Zhu et al., 1994).

Agonist binding to two of the five cloned metabotropic glutamate receptors activated phospholipase C (Masu et al., 1991; Houamed et al., 1991; Tanabe et al., 1992, 1993; Abe et al., 1992). Similarly, bombesin-stimulated appearance of [^{3}H]inositol phosphates has been reported (Brown et al., 1984; Trepel et al., 1988; Swope and Schonbrunn, 1988; Patel and Schrey, 1990, Imoto et al., 1993; Pelvin et al., 1990; Hoshijima et al., 1988).

In the present study we document the adrenoceptor, metabotropic and bombesin agonist-mediated stimulation of phospholipase C activity in LA-N-2 cells.

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2. Materials and methods

2.1. Materials

Myo-[2-^{3}H]inositol (17.6 Ci/mmol) was purchased from Amersham Canada (Oakville, Ontario, Canada). L-Norepinephrine bitartrate, *trans*-1-amino-1,3,-cyclopentanedicarboxylic acid (*t*-ACPD), propranolol-HCl, isoproterenol HCl, phentolamine mesylate, 2-amino-3-phosphonopropionic acid (AP-3) and prazosin HCl were obtained from Research Biochemicals International (Natick, MA, USA). Bombesin, neuromedin-B and [Tyr^4-D-Phe^{12}]bombesin were obtained from Sigma Chemicals (St. Louis, MO, USA). BAPTA/AM (1,2-bis-(0-amino-5,5'-difluorophenoxyl)-ethane-*N*,*N*,*N'*,*N'*-tetra acetic acid tetra-(acetoxymethyl)-ester], TMB-8 [8-(diethylamino)-octyl-3,4,5,-trimethoxy-benzoate, HCl], pertussis toxin, cholera toxin, tyrophostin, genistein and U73122 (phospholipase C inhibitor) were obtained from Calbiochem Corporation (La Jolla, CA, USA). GTPγS and GDPβS were obtained from Boehringer Mannheim Canada (Laval, Quebec, Canada). Leibovitz's L-15 medium and heat-inactivated fetal calf serum were obtained from Flow Laboratories (Mississauga, Ontario, Canada). Dowex AG1x-8 formate (200–400) mesh was obtained from Bio-Rad Labs (Hercules, CA, USA). The human neuroblastoma cell line, LA-N-2 (passage 48) was obtained from Dr. R. Seeger (UCLA, Los Angeles, CA, USA) and maintained as previously described (Singh et al., 1990).

2.2. Cell labelings and measurements of [^{3}H]inositol phosphate appearance

Confluent monolayer cultures of LA-N-2 cells were incubated with myo-[^{3}H]inositol (2 μCi/ml) for 48 h in L-15 medium containing 15% fetal-bovine serum in order to label the cellular PtdIns. The cells were harvested, washed twice with L-15 medium containing 10 mM LiCl, and these [^{3}H]inositol-prelabeled cells usually containing about 0.6–0.8 mg protein were incubated in 1.0 ml L-15 medium containing 10 mM LiCl at 37°C for 10 min with the various agonists and antagonists. The reactions were terminated by adding 200 μl of 50% ice-cold trichloroacetic acid. Samples were then transferred to Eppendorf centrifuge tubes and allowed to stand in ice for 15 min. They were centrifuged and the trichloroacetic acid extracts were transferred to 13 × 100 mm glass tubes. The trichloroacetic acid extracts were washed five times with diethylether (1 : 1 ratio) and the pH adjusted to neutral with NaOH. The pellet obtained from the trichloroacetic acid precipitation was dissolved in 0.8 N NaOH and protein was quantitated by a standard procedure (Lowry et al., 1951). The neutralized samples were applied to Dowex-1-8-formate columns, and the labeled [^{3}H]inositol phosphates were eluted according to a published procedure (Berridge et al., 1983).

3. Results

3.1. Agonist-induced stimulation of the appearance of [^{3}H]inositol phosphates by norepinephrine, ACPD, bombesin and neuromedin-B

The [^{3}H]inositol-prelabeled LA-N-2 cells were exposed to varying concentrations of norepinephrine, an α,β-adrenoceptor agonist. There was a progressive increase in the appearance of [^{3}H]inositol phosphates, the expected products of phospholipase C activation, with a plateau at 100 μM concentration (Fig. 1). Isoproterenol, a β-adrenoceptor agonist, increased the appearance of [^{3}H]inositol phosphates in a dose-dependent fashion ranging from 0.5 to 25 μM and reached a plateau at 5 μM concentration (data not shown). There was a linear increase in the appearance of [^{3}H]inositol phosphates with increasing concentrations of *t*-ACPD, with a plateau at 500 μM (Fig. 2). Exposure of [^{3}H]inositol-prelabeled LA-N-2 cells to increasing concentrations of bombesin resulted in increased appearance of [^{3}H]inositol phosphates with a plateau at 100 nM (Fig. 3). There was a progressive increased appearance of [^{3}H]inositol phosphates with increasing concentrations of neuromedin-B with a plateau at 100 nM (Fig. 3). Norepinephrine and *t*-ACPD, at 100 μM concentrations, were capable of activating phospholipase C over 2-fold, bombesin and neuromedin-B at 100 nM concentration activated phospholipase C 2- to 3-fold.

3.2. Antagonists blunting of agonist activations

Propranolol, a β-adrenoceptor antagonist, blunted the release of [^{3}H]inositol phosphates by norepinephrine and

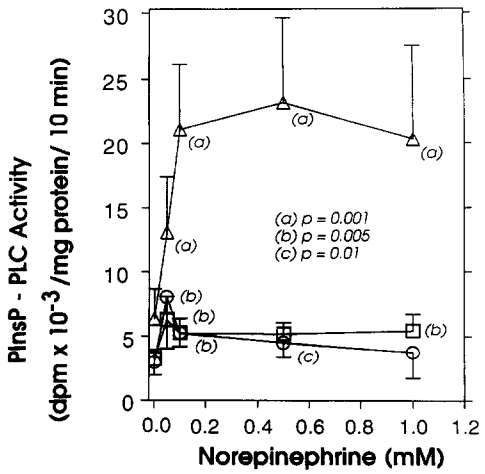

Fig. 1. Effect of varying concentrations of norepinephrine on phospholipase C activity. [^{3}H]Inositol-prelabeled LA-N-2 cells were exposed to different concentrations of norepinephrine for 10 min and the release of [^{3}H]inositol mono- ($InsP_1$) (△—△), di- ($InsP_2$) (□—□) and triphosphates ($InsP_3$) (○—○) were estimated as described in Materials and methods. The experiment was conducted in duplicate on three separate occasions.

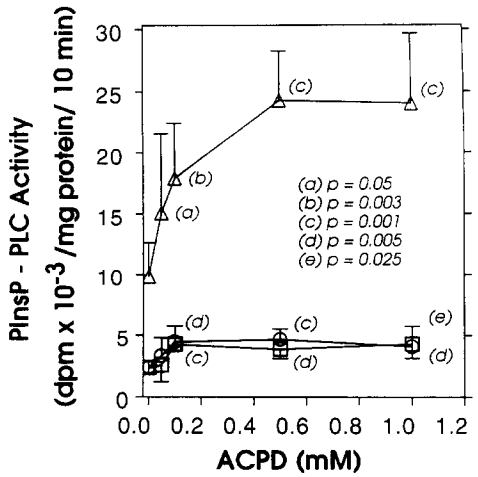

Fig. 2. Effect of varying concentrations of *t*-ACPD on phospholipase C activity. The experimental details are provided in Materials and methods. The experiment was conducted in duplicate on three separate occasions. △—△, $InsP_1$; □—□, $InsP_2$; and ○—○, $InsP_3$.

isoproterenol, suggesting that the phospholipase C activation was coupled to a β-adrenoceptor (Table 1). Prazosin and phentolamine mesylate, which are α-adrenoceptor antagonists, were ineffective in blunting the activation of phospholipase C by norepinephrine and isoproterenol (Table 1). The IC_{50} of propranolol for norepinephrine-stimulated [^{3}H]inositol phosphate appearance was 55 μM and for isoproterenol was 4.5 μM (data not shown). The IC_{50} of AP-3 for *t*-ACPD-stimulated appearance of [^{3}H]inositol phosphates was 25 μM. The IC_{50} of [Tyr^4, D-Phe^{12}]bombesin for bombesin-stimulated [^{3}H]inositol

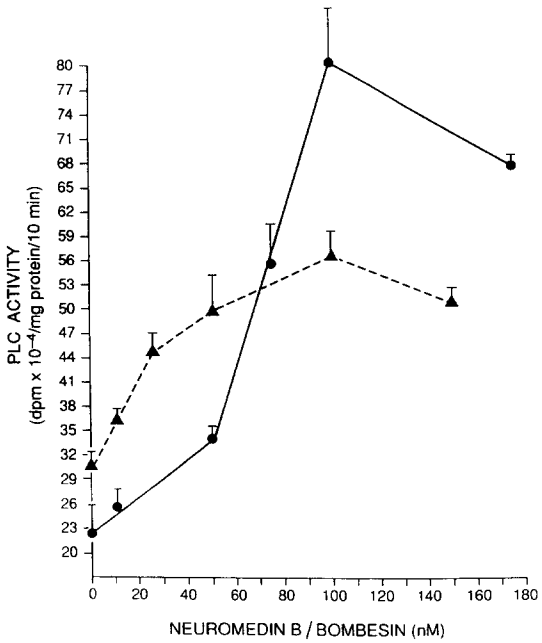

Fig. 3. Effect of varying concentrations of neuromedin-B (▲---▲) and bombesin (●—●) on phospholipase C activity. The experiment was conducted in triplicate on two separate occasions and the experimental details are provided in Materials and methods.

Table 1
The effect of adrenergic antagonists on the norepinephrine and isoproterenol stimulation of [^{3}H]inositol phosphate appearance [a]

	[^{3}H]Inositol phosphates
Control	226094 ± 32490
Norepinephrine (100 μM)	419745 ± 44542 [b]
Norepinephrine (100 μM) + propranolol (250 μM)	263902 ± 30526
Norepinephrine (100 μM) + phentolamine mesylate (250 μM)	500303 ± 27429 [b]
Norepinephrine (100 μM) + prazosin (250 μM)	567435 ± 77573 [b]
Isoproterenol (5 μM)	387419 ± 83813 [b]
Isoproterenol (5 μM) + propranolol (10 μM)	217074 ± 22114
Isoproterenol (5 μM) + phentolamine mesylate (250 μM)	288246 ± 40624
Isoproterenol (5 μM) + prazosin (250 μM)	280282 ± 65105

[a] Values are expressed as mean dpm/mg protein/10 min ± S.D. The data are from two separate experiments conducted in triplicate.
[b] $P < 0.001$ compared to controls, others were not significant as analyzed by Student's *t*-test.

phosphate appearance was 25 nM and for neuromedin-B was 75 nM (data not shown). The IC_{50} estimates were obtained from two independent experiments conducted in triplicate.

3.3. G-protein involvement in the agonist stimulation of [^{3}H]inositol phosphate appearance

The involvement of a GTP binding protein in the agonist stimulations of [^{3}H]inositol phosphate appearance was investigated. [^{3}H]Inositol-prelabeled LA-N-2 cells were incubated with 50 μg/ml saponin for 4 min in the presence of either 100 μM GTPγS or 1 mM GDPβS. The cells were rinsed twice with L-15 medium and their response to the various agonists measured (Table 2). GTPγS stimulated the appearance of [^{3}H]inositol phosphates. Significantly more [^{3}H]inositol phosphates were found with a combination of GTPγS and norepinephrine or GTPγS and *t*-ACPD or GTPγS and bombesin as compared to the agonist or GTPγS alone. The presence of 1 mM GDPβS blunted the responses to norepinephrine, *t*-ACPD and bombesin (Table 2). The [^{3}H]inositol-prelabeled LA-N-2 cells remained in the presence of either 200 ng/ml of cholera toxin or 200 ng/ml of pertussis toxin for 18 h, the toxins were removed, and the cells were washed twice with L-15 medium. The ability of norepinephrine, *t*-ACPD and bombesin to stimulate [^{3}H]inositol phosphate appearance was measured. The norepinephrine-stimulated [^{3}H]inositol phosphate appearance was unaffected in cholera toxin- and pertussis toxin-treated cells. The *t*-ACPD-stimulated [^{3}H]inositol phosphate appearance was blunted in the cholera toxin-treated cells. The bombesin-stimulated [^{3}H]inositol phosphate appearance was blunted

Table 2
The effect of GTPγS and GDPβS on the norepinephrine, ACPD and bombesin stimulation of [^{3}H]inositol phosphate appearance [a]

	[^{3}H]Inositol phosphates
Controls	202716 ± 25731
GTPγS (100 μM)	271458 ± 16749 [b]
Norepinephrine (100 μM)	307086 ± 19346 [b]
Norepinephrine (100 μM) + GTPγS (100 μM)	386970 ± 84449 [c]
Norepinephrine (100 μM) + GDPβS (1 mM)	248122 ± 26175
t-ACPD (100 μM)	357307 ± 21095 [b]
t-ACPD (100 μM) + GTPγS (100 μM)	416254 ± 20659 [c]
t-ACPD (100 μM) + GDPβS (1 mM)	280401 ± 25278
Bombesin (100 nM)	437683 ± 87148 [b]
Bombesin (100 nM) + GTPγS (100 μM)	493171 ± 11162 [c]
Bombesin (100 nM) + GDPβS (1 mM)	352901 ± 87055

[a] Values are expressed as mean dpm inositol phosphates/mg protein/10 min ± S.D. The data are from two separate experiments carried out in triplicate.
[b] $P < 0.001$ compared to controls; [c] $P < 0.005$ compared to norepinephrine or *t*-ACPD or bombesin, respectively.

in the pertussis toxin-treated cells (Table 3). These results show that the stimulation of [^{3}H]inositol phosphate appearance by these three agonists are coupled through different G-proteins.

3.4. Agonist-stimulated [^{3}H]inositol phosphate appearance, Ca^{2+} and protein kinases

The omission of Ca^{2+} from the incubations with the inclusion of 10 mM EDTA and 10 mM EGTA did not influence the stimulation of [^{3}H]inositol phosphate appearance by norepinephrine, *t*-ACPD and bombesin. Inclusion of BAPTA/AM, a chelator of internal Ca^{2+}, and TMB-8 [8-(diethylamino)-octyl-3,4,5-trimethoxy-benzoate, HCl], a Ca^{2+} channel antagonist, did not influence the [^{3}H]inositol phosphate appearance with the three agonists (data not shown). These results indicate that the norepinephrine, *t*-ACPD and bombesin stimulation of [^{3}H]inositol phosphate appearance was a Ca^{2+}-independent process.

In order to determine if the agonist-stimulated [^{3}H]inositol phosphate appearance was dependent upon protein kinase activity, a series of inhibitors were utilized. Pretreatment of the prelabeled cells with the protein kinase C inhibitor staurosporine (50 μM), the tyrosine kinase inhibitor genistein (200 μM) and the Ca^{2+} calmodulin-dependent protein kinase inhibitor calmidazolium (20 μM) did not influence the phospholipase C stimulations by norepinephrine, *t*-ACPD and bombesin (data not shown). These results suggest that the activation of phospholipase C by these agonists is protein kinase independent.

Table 3
The effect of cholera toxin and pertussis toxin upon the appearance of [^{3}H]inositol phosphates by norepinephrine, *t*-ACPD and bombesin [a]

	[^{3}H]Inositol phosphates
Controls, untreated cells	296075 ± 24391
Norepinephrine (100 μM)	573753 ± 54245
t-ACPD (100 μM)	593774 ± 71693
Bombesin (100 nM)	807921 ± 75345
Cholera toxin-treated cells	404842 ± 41023
Cholera toxin-treated + norepinephrine (100 μM)	676104 ± 20726
Cholera toxin-treated + *t*-ACPD (100 μM)	354934 ± 46671
Cholera toxin-treated + bombesin (100 nM)	607195 ± 76327
Pertussis toxin-treated cells	380122 ± 37555
Pertussis toxin-treated + norepinephrine (100 μM)	668709 ± 57830
Pertussis toxin-treated + *t*-ACPD (100 μM)	622451 ± 51328
Pertussis toxin-treated + bombesin (100 nM)	362031 ± 54829

[a] Values are expressed as mean dpm inositol phosphates/mg protein/10 min ± S.D. The data is from a representative experiment carried out in triplicate. Similar results were obtained on two other occasions but the absolute values varied.

Table 4
The effects of combinations of norepinephrine, *t*-ACPD and bombesin on the stimulation of [^{3}H]inositol phosphate appearance [a]

	[^{3}H]Inositol phosphates
Control	268610 ± 43686
Norepinephrine (100 μM)	454952 ± 63876
t-ACPD (100 μM)	452302 ± 47541
Bombesin (100 nM)	515975 ± 49200
Norepinephrine (100 μM) + *t*-ACPD (100 μM)	442510 ± 56446
t-ACPD (100 μM) + bombesin (100 nM)	515034 ± 50394
Norepinephrine (100 μM) + bombesin (100 nM)	541766 ± 42787
Norepinephrine (100 μM) + *t*-ACPD (100 μM) + bombesin (100 nM)	553569 ± 55391

[a] Values are expressed as mean dpm inositol phosphates/mg protein/10 min ± S.D. The data was obtained from two separate experiments carried out in triplicate.

3.5. Agonist combination and [^{3}H]inositol phosphate appearance

It was of interest to determine if combinations of these agonists resulted in greater [^{3}H]inositol phosphate appearance than each individually. The prelabeled LA-N-2 cells were exposed to either norepinephrine, or *t*-ACPD, or bombesin, or norepinephrine + *t*-ACPD, or norepinephrine + bombesin, or *t*-ACPD + bombesin or norepinephrine + *t*-ACPD + bombesin and the appearance of [^{3}H]inositol phosphates measured. The results showed that there were no increases in [^{3}H]inositol phosphate appearance with any combination of agonists compared to the agonists individually (Table 4). This suggests that norepinephrine, *t*-ACPD and bombesin activate a common phospholipase C.

4. Discussion

The stimulation of phospholipase C activity by β-adrenoceptor, metabotropic glutamate and bombesin-coupled receptors in the human neuronal cell line, LA-N-2 was

investigated. β-Adrenoceptor-mediated phospholipase C activation was reported in turkey erythrocyte membranes (Rooney et al., 1991; James et al., 1994; Vaziri and Downes, 1992; Zhu et al., 1994) by a G-protein-dependent (Cooper et al., 1991; Rooney et al., 1991; James et al., 1994), cholera toxin-insensitive (James et al., 1994) and pertussis toxin-insensitive (Rooney et al., 1991) mechanism. The isoprenaline activation of phospholipase C in turkey erythrocyte membranes was not blunted by the α-adrenoceptor antagonists, yohimbine and prazosin, but was inhibited in a dose-dependent manner by β-adrenoceptor antagonists (James et al., 1994; Vaziri and Downes, 1992). In LA-N-2 cells, norepinephrine- and isoproterenol-stimulated phospholipase C activity was blunted by propranolol, a β-adrenoceptor antagonist, but was unaffected by the α-adrenoceptor antagonists, phentolamine mesylate and prazosin, indicating that β-adrenoceptor occupancy activates phospholipase C. This activation is G-protein dependent, cholera toxin and pertussis-toxin insensitive in LA-N-2 cells.

The effects on phospholipase C activity of a novel glutamate analogue, *t*-ACPD, was examined in primary cultures of striatal neurons and *Xenopus* oocytes (Manzoni et al., 1990, 1992; Lonart et al., 1992) and shown to be dependent on extracellular Ca^{2+} (Lonart et al., 1992). The results also showed that ACPD stimulates quisqualate phosphoinositide-coupled receptors but not ionotropic glutamate receptors. The *t*-ACPD-stimulated [^{3}H]inositol phosphate appearance was shown to be sensitive to pertussis toxin (Sugiyama et al., 1987; Furuya et al., 1989), intracellular Ca^{2+} (Furuya et al., 1989), and was GTPγS dependent with post-mortem human brain membranes (Jope et al., 1994). The *t*-ACPD-stimulated release of [^{3}H]inositol phosphates from [^{3}H]inositol-prelabeled cells is sensitive to cholera-toxin treatment (Table 3).

Bombesin-induced phospholipase C activation was found to be GTPγS dependent (Piiper et al., 1993) and partially inhibited by tyrosine kinase inhibitors in pancreatic acini (Piiper et al., 1994). Differential sensitivity to phorbol esters and pertussis toxin (Hoshijima et al., 1988) and cholera toxin (Milligan et al., 1989) of bombesin-induced phospholipase C activity was also reported in NIH 3T3 cells. In rat glioblastoma C-6 cells phospholipase C activation was coupled to neuromedin-B receptors and this activation was mediated by intracellular Ca^{2+} (Wang et al., 1992). The bombesin-stimulated [^{3}H]inositol phosphate appearance was coupled to guanine nucleotides and intracellular Ca^{2+} in rat pancreatic acinar cells (Piiper et al., 1993). The pretreatment of [^{3}H]inositol-prelabeled cells with 200 ng/ml pertussis-toxin blunted phospholipase C activation by bombesin (Table 3). Synergistic activation of phosphoinositide hydrolysis induced by norepinephrine and *t*-ACPD was reported in rat brain slices (Kolasa and Jope, 1993). There is no additive effect by combinations of norepinephrine, *t*-ACPD and bombesin in LA-N-2 cells (Table 4).

The activation of phospholipase C by this triad of agonists in a clonal cholinergic cell line has not been previously reported. The β-adrenergic agonist activation of phospholipase C is another unusual feature of the LA-N-2 cells. These three agonists appear to be coupled to a common phospholipase C by separate types of G-proteins.

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