Purification of a high-affinity inositol 1,3,4,5-tetrakisphosphate receptor from brain

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Ins(1,3,4,5) P_4 has been suggested to be involved in cellular Ca^{2+} signalling. A receptor from pig cerebellar membranes, which binds Ins P_4 with high affinity and selectivity [Donié & Reiser (1989) FEBS Lett. **254**, 155–158], has been solubilized and purified about 20000-fold by chromatography using CM-cellulose, heparin-agarose and hydroxyapatite. The Ins P_4 receptor, identified by SDS/PAGE, had an apparent molecular mass of 42 kDa and bound 4.6 nmol of Ins P_4 per mg of protein, with a dissociation constant of 5.6 nM.

INTRODUCTION

Inositol oligophosphate synthesis is a characteristic of intracellular signal transduction processes activated by various hormones and sensory stimuli in a multitude of cells [1]. The key event is the activation of a phosphoinositide-specific phospholipase C. This enzyme yields two products with second messenger function, diacylglycerol and Ins(1,4,5) P_3 (Ins P_3). Ins P_3 releases Ca^{2+} from internal stores which are part of the endoplasmic reticulum [2]. For inactivation, Ins P_3 is metabolized by dephosphorylation to Ins(1,4) P_2 [3]. Alternatively Ins P_3 is phosphorylated by a 3-kinase [4]. The product Ins(1,3,4,5) P_4 (Ins P_4) also appears to be involved in Ca^{2+} regulation. Physiological and biochemical experiments indicate that Ins P_4 might act in concert with Ins P_3 [5,6]. However, the actual function of Ins P_4 is still a matter of debate.

The Ins P_3 receptor [7], a protein of apparent molecular mass 250 kDa purified from rat cerebellum [8], has been reconstituted into lipid vesicles. This reconstituted preparation has been shown to mediate Ca^{2+} release after activation by Ins(1,4,5) P_3 [9].

The uncertainties about the function of Ins P_4 are related to the lack of knowledge about the cellular site of action of this inositol oligophosphate. It is still unknown whether Ins P_4 acts on the plasma membrane, on internal Ca^{2+} stores or on a structural element which connects the plasma membrane and the Ca^{2+} store. In order to attempt to answer these questions, we solubilized the high-affinity Ins P_4 receptor found in the cerebellum [10]. Similarly, Ins P_4 receptors have been described in HL-60 cells [11] and in parathyroid glands [12]. However, the Ins P_4 receptor has not yet been purified.

Recently we described that the Ins P_4 receptor in a membrane preparation from pig cerebellum binds Ins(1,3,4,5) P_4 , with a high selectivity for Ins(1,3,4,5) P_4 among several inositol phosphates tested [10]. We used this binding protein preparation for quantifying cellular Ins P_4 by a radioreceptor assay [13]. Subsequently, we enriched the solubilized membrane-bound Ins P_4 receptor from pig cerebellum by heparin-agarose chromatography and separated the Ins P_4 receptor from the Ins P_3 -binding activity [14]. The enriched preparation of the high-affinity Ins P_4 receptor (300 pmol/mg of protein) showed very low specific Ins P_3 binding. By a different procedure, Theibert *et al.* [15] separated an Ins P_4 -binding protein solubilized from rat cerebellum from the Ins P_3 receptor and the enzymes Ins P_3 5-phosphatase and Ins P_3 3-kinase.

Heparin suppresses high-affinity Ins P_3 binding [16,17] and blocks Ins P_3 -induced Ca^{2+} release [18,19]. Therefore, disappearance of a cellular effect after injection of heparin has frequently been taken as evidence for the involvement of Ins(1,4,5) P_3 . However, our results [10,14] showed that Ins P_4 binding was also inhibited by heparin (IC_{50} (half-maximal inhibitory concentration) 15 ng/ml [14]) and related polyglycans with high potency. Therefore suppression by heparin is ambiguous, since it could imply involvement of Ins(1,4,5) P_3 or of Ins(1,3,4,5) P_4 in the activation process. In this context, the reason for the failure of heparin (50 μ g/ml) to inhibit the Ca^{2+} resequestration induced by Ins(1,3,4,5) P_4 [20] still has to be investigated.

Here we describe the purification and molecular identification of the Ins P_4 receptor.

MATERIALS AND METHODS

Materials

[3H]Ins(1,3,4,5) P_4 (specific radioactivity 1020–1330 GBq/mmol) was from Amersham, Braunschweig, Germany. Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 (potassium salts) and Ins(1,2,3,4,5,6) P_6 (sodium salt) were from Calbiochem, Frankfurt, Germany. Ins(1,4,5) P_3 , Ins(1,3,4,5) P_4 and Ins(1,3,4,5,6) P_5 (ammonium salts) were from Boehringer, Mannheim, Germany. Heparin-agarose, Amido Black, Bio-Gel HT and Bio-Gel P-4 were from Bio-Rad, München, Germany. CM-cellulose was from Whatman (Maidstone, Kent, U.K.). Brij 58 (polyoxyethylene monooctyl ether) and chemicals for electrophoresis were from Serva, Heidelberg, Germany. Molecular mass standards were from Pharmacia, Freiburg, Germany. All other chemicals were of analytical grade and were obtained from Merck, Darmstadt, Germany.

Methods

Preparation of membranes and solubilization were carried out as described [14], with some minor modifications to improve the yield of active receptor. Pig cerebella freshly collected from the local abattoir were kept on ice for about 1 h before being homogenized in buffer containing (mM) Tris/HCl (50), EDTA (1) and mercaptoethanol (1), pH 7.7, at 4 °C at a ratio of 100 g of tissue to 400 ml of buffer, using a Waring Blendor (medium setting, 3 $\times$ 20 s) and centrifuged (30 min, 35000 *g*). The pellet was resuspended in homogenization buffer containing 0.4 M-

Abbreviation used: IC_{50} , half-maximal inhibitory concentration.

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Fig. 1. Purification of the InsP_4 receptor protein by CM-cellulose (a), heparin-agarose (b), and hydroxyapatite (c)

Protein concentration (A_{280}), NaCl concentration used for elution and InsP_4 -binding activity are shown. (a) The membrane extract obtained with Brij 58 was applied to a CM-cellulose column and eluted with NaCl at the concentrations indicated on the right ordinate (broken lines). InsP_4 -binding activity is given as total binding of 0.1 nM [^3H] InsP_4 . (b) The fractions marked by a horizontal bar in (a) were applied to heparin-agarose and eluted by buffer A containing NaCl at the concentrations shown. After the first elution step the sensitivity of the photometer was increased by a factor of 10, resulting in a broader baseline. (c) The fractions indicated by the horizontal bar in (b) were dialysed in buffer B, applied to hydroxyapatite and eluted with NaCl in buffer B as indicated. The scale for NaCl concentrations in (c) also applies to (b). Fraction sizes were 5 ml (a), 1.5 ml (b) and 0.6 ml (c). Protein concentrations and the values for the specific InsP_4 -binding activity of the pooled fractions obtained in (a), (b) and (c) are included in Table I.

NaCl and centrifuged. Subsequently the pellet was washed twice with homogenization buffer by resuspension and centrifugation (30 min , $35000 g$). The final pellet, resuspended in buffer at a concentration of $20\text{--}30 \text{ mg}$ of protein/ml, was homogenized using a Potter-Elvehjem homogenizer and kept at -20°C .

For solubilization, the membrane suspension was centrifuged (10 min , $27000 g$). The membrane pellet was mixed with solubilization buffer at a ratio of 5 ml of buffer to 1 g of membranes. The solubilization buffer contained (mM) Tris/HCl (50), EDTA (1), NaCl (400) and mercaptoethanol (5), pH 7.5 , plus Brij 58 (1.5% , w/v). The mixture was stirred at 4°C for 1 h , and centrifuged (1 h , $100000 g$). The supernatant was dialysed in buffer A, and was applied to a CM-cellulose column ($3 \text{ cm} \times 5 \text{ cm}$) equilibrated with buffer A. Buffer A contained (mM) Tris/HCl (50), EDTA (1) and mercaptoethanol (1), pH 7.5 , plus Brij 58 (0.1% , w/v). After washing with buffer A, proteins were eluted by increasing the NaCl concentration (see Fig. 1). The fractions with InsP_4 -binding activity were pooled and applied to a heparin-agarose column ($2 \text{ cm} \times 1.7 \text{ cm}$) equilibrated with buffer A. This column was eluted by increasing the NaCl concentration discontinuously (see Fig. 1). The fractions of the 0.8 M -NaCl eluate

with InsP_4 -binding activity were pooled, dialysed in buffer B and subjected to hydroxyapatite chromatography ($1 \text{ cm} \times 0.5 \text{ cm}$ column). Buffer B contained (mM) $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (25), EDTA (1) and mercaptoethanol (1), pH 7.5 , plus Brij 58 (0.1% , w/v). After washing with buffer B containing 0.1 M -NaCl, the fractions eluted with buffer B containing 1 M -NaCl showing InsP_4 -binding activity were pooled, dialysed in buffer A and applied to a second heparin-agarose column ($1 \text{ cm} \times 0.3 \text{ cm}$). The column was eluted with stepwise increases of the NaCl concentration in buffer A to 0.4 or 0.8 M -NaCl. Fractions with InsP_4 -binding activity obtained with 0.8 M -NaCl were used for analysis.

InsP_4 -binding activity was assayed in solubilized and purified preparations by spun-column chromatography. Samples were incubated for 20 min at 4°C in incubation buffer containing (mM): sodium acetate (25), pH 5.0 , potassium phosphate (25), pH 5.0 , EDTA (1) and mercaptoethanol (1), plus Brij 58 (0.1% , w/v) and [^3H] $\text{Ins}(1,3,4,5)\text{P}_4$ ($0.08\text{--}0.10 \text{ nM}$). A sample of the incubation mixture was applied to columns containing Bio-Gel P-4 (1 ml of packed resin) and centrifuged (2 min , $3500 g$) to separate bound from unbound ligand. Bound [^3H] $\text{Ins}(1,3,4,5)\text{P}_4$ was determined by measuring the radioactivity in the void volume [14].

Protein contents of samples of detergent-solubilized membranes or of detergent-containing eluates from column chromatography were measured after precipitation with trichloroacetic acid by Amido Black, as described by Schaffner & Weissman [21] modified for Brij-58-containing samples. Discontinuous SDS/PAGE was carried out according to Laemmli [22]. The gel was stained with Coomassie Brilliant Blue R250 or silver [23].

RESULTS

By treatment with the non-ionic detergent Brij 58 we could solubilize the high-affinity InsP_4 receptor from pig cerebellar membranes [10]. Only high concentrations of the detergent (above 1%) allowed solubilization. Furthermore, the yield of the solubilized receptor was increased by addition of 0.4 M -NaCl (results not shown). Thus the protein responsible for high-affinity InsP_4 binding seems to be an integral membrane protein.

InsP_4 -binding activity was purified by a series of chromatographic steps. Fig. 1 shows the results from column runs during a typical purification experiment. In the solubilized preparation, Scatchard analysis revealed InsP_4 receptors with a K_d of 2 nM and a maximal binding capacity (B_{max}) of 1.5 pmol/mg of protein. When the solubilized membranes were applied to CM-cellulose, about 60% of the InsP_4 -binding activity passed through the column. The solubilized fraction contained various InsP_4 -binding proteins with different affinities, some of which adhered to the cation exchanger. Using the partially purified InsP_4 receptor described previously [14], we observed that the high-affinity InsP_4 receptor is retained by CM-cellulose under the conditions used here (results not shown). Therefore the CM-cellulose column was eluted with increasing NaCl concentrations. The fractions eluted with 50 mM -NaCl in buffer A contained proteins with no detectable InsP_4 -binding activity. The subsequent elution with a salt gradient from 100 to 300 mM -NaCl gave a complex profile of the protein concentration, with several peaks (Fig. 1a). InsP_4 binding displayed a single peak in the fractions eluted at around 150 mM -NaCl. The proteins eluted by the subsequent step of 0.8 M -NaCl were devoid of InsP_4 -binding activity.

The fractions with maximal InsP_4 -binding activity from the CM-cellulose column were combined and subjected to heparin-agarose chromatography (Fig. 1b). The fractions eluted with 0.4 M -NaCl in buffer A contained the bulk of the proteins (28 mg out of 40 mg applied to the column). The maximal InsP_4 -binding

Table 1. Chromatographic purification of the high-affinity Ins(1,3,4,5) P_4 receptor from cerebellum

Membranes from pig cerebellum (67 g), prepared as described [10], were solubilized and centrifuged, as described in the Materials and methods section. The 100 000 g supernatant was applied to CM-cellulose, heparin-agarose and hydroxyapatite chromatography, as shown in Figs 1(a)–1(c). The fractions of the hydroxyapatite column containing Ins P_4 -binding activity indicated by a horizontal bar (Fig. 1c) were dialysed in buffer A and applied to a second heparin-agarose column. The column was eluted by stepwise increases in the NaCl concentration to 0.4 and 0.8 M. Ins P_4 -binding activities were determined with 0.1 nM-[3 H]Ins(1,3,4,5) P_4 in the incubation mixture and analysed by spun-column chromatography [14]. For measuring unspecific binding, 0.5 μ M-Ins(1,3,4,5) P_4 was added. Specific binding activities (total binding minus unspecific binding) of the dialysed samples assayed in duplicate are given. Results are representative of three experiments.

Fraction	Protein (mg)	Ins P_4 -binding activity (pmol/ml)	Specific binding activity (pmol/mg of protein)	Total binding activity (pmol)	Yield (%)	Purification (fold)
Solubilized membranes	1540	49.6	10.3	15870	100	1
CM-cellulose						
Flow-through	1440	27.9	6.2			
50 mM-NaCl eluate	6	0	0			
100–300 mM-NaCl eluate	40	156	213	8580	54	21
800 mM-NaCl eluate	3	0	0			
Heparin-agarose 1						
Flow-through	11	0	0			
0.4 M-NaCl eluate	25	39.5	14			
0.5 M-NaCl eluate	0.7	14.9	199			
0.8 M-NaCl eluate	0.3	405	11910	4050	25	1150
Hydroxyapatite						
Flow-through	0.02	0	0			
1 M-NaCl eluate	0.08	860	47780	4020	25	4625
Heparin-agarose 2						
Flow-through	0.04	0	0			
0.4 M-NaCl eluate	0.017	96.6	5750			
0.8 M-NaCl eluate	0.03	684	42750	1370	8.6	4140

activity was found in the fraction eluted with 0.8 M-NaCl, where only 0.3 mg of protein was left. These fractions were dialysed in phosphate buffer and passed through a hydroxyapatite column, which retained all of the Ins P_4 -binding activity. Fractions eluted with buffer B containing 1 M-NaCl were enriched in the high-affinity Ins P_4 -binding activity, with 80 μ g of protein in the active fraction. This eluate from the hydroxyapatite column was dialysed in buffer A and subjected to a second heparin-agarose column for further purification and concentration. Some of the activity could be found in the fraction eluted with 0.4 M-NaCl in buffer A, but most of it was present in the eluate obtained at 0.8 M-NaCl.

Table 1 summarizes the data of the purification scheme for the Ins P_4 receptor from pig cerebellum. The values for binding activity given in Table 1 are used to assess the success of the purification. However, binding activity is an arbitrarily chosen value, since it represents the difference between the amount of Ins P_4 bound at a single concentration of [3 H]Ins P_4 (0.1 nM) and unspecific binding, measured in the presence of 0.5 μ M-Ins P_4 . It should be emphasized that binding activity does not give the number of binding sites ($B_{\max}$). The latter was calculated from Scatchard analysis (see below). In the experiment presented in Table 1, we started with 67 g of crude membranes (wet weight), which corresponds to 3.6 g of protein. The active fraction derived by elution from the CM-cellulose column with a salt gradient still contained 40 mg of protein from 1.54 g of protein in the solubilized membranes, with 54 % of binding activity remaining. After the hydroxyapatite chromatography, the total binding activity of 4020 pmol was virtually the same as after the previous heparin-agarose column, but the amount of protein was decreased from 300 to 80 μ g. After the second heparin-agarose column we obtained an overall yield of 30 μ g of receptor protein, corresponding to 8.6 % of the binding activity contained in the solubilized membranes. The purification factors in Table 1 are

given with reference to the solubilized preparation and do not include the initial purification achieved by solubilizing the membranes.

Scatchard analysis of Ins P_4 binding as seen in the eluate from the hydroxyapatite column or in the final fraction from the second heparin-agarose column revealed a dissociation constant (K_d) for Ins P_4 varying between 2 and 9 nM (5.6 ± 3.5 nM, $n = 4$). The maximal binding capacity ($B_{\max}$) was 1500 pmol/mg of protein for the fraction eluted from the hydroxyapatite column and 4600 pmol/mg of protein for the active fraction (0.8 M-NaCl) from the second heparin-agarose column. Thus, in comparison with the original membrane preparation (0.22 pmol of binding sites per mg of protein [10]), we finally obtained an overall purification factor of 20000.

The specificity of binding to the purified Ins P_4 receptor was tested. Binding of [3 H]Ins P_4 could be displaced by Ins(1,3,4,5) P_4 and Ins(1,3,4,5,6) P_5 with a comparable affinity and to the same extent, whereas Ins P_6 displaced [3 H]Ins P_4 binding with an IC_{50} about 80 times higher. Ins(1,4,5) P_3 , however, could not displace Ins P_4 binding at concentrations up to 10 μ M (results not shown). Changing the pH from 5 to 7 altered the K_d for Ins P_4 from 2 to 14 nM and enhanced the amount of unspecific binding from 13 to 45 % (results not shown).

The fractions obtained during the purification of Ins P_4 -binding activity were analysed by SDS/PAGE. Fig. 2 shows the protein bands after staining with Coomassie Brilliant Blue or silver. The active fraction from the CM-cellulose column (lane C) displayed a most prominent band at 46 kDa and another less intensely stained band at 37 kDa, apart from a series of bands with lower molecular masses. In the fraction eluted from the first heparin-agarose column at 0.8 M-NaCl, where maximal Ins P_4 -binding activity was present, only three protein bands were revealed (lane F), with approximate molecular masses of 46, 42 and 37 kDa. In the eluate from the hydroxyapatite column (lane G), the 46 kDa

Fig. 2. SDS/PAGE analysis of fractions obtained during purification of the high-affinity InsP_4 receptor

Analysis was by electrophoresis in 10 % polyacrylamide gels (3 % polyacrylamide stacking gel), stained first with Coomassie Brilliant Blue R250 (a) and afterwards with silver (b). Lane B, 5 μg of protein from cerebellar membranes solubilized with Brij 58; lane C, 36 μg of protein from the pooled fractions eluted between 100 and 300 mM-NaCl from the CM-cellulose; lanes D (F), 18 (6.8) μg eluted by 0.4 (0.8) M-NaCl from the first heparin-agarose column; lane G, 3.6 μg eluted from the hydroxyapatite column by 1 M-NaCl; lanes H (I), eluates from the second heparin-agarose column, 0.85 (0.8) μg eluted by 0.4 (0.8) M-NaCl. Lanes A, E and K contain molecular mass markers α -lactalbumin (14.4 kDa, 605 ng), trypsin inhibitor (20.1 kDa, 400 ng), carbonic anhydrase (30 kDa, 415 ng), ovalbumin (43 kDa, 735 ng), bovine serum albumin (67 kDa, 415 ng) and phosphorylase b (94 kDa, 320 ng). Lanes G, H, I and K in (b) represent the corresponding lanes after silver staining. The intensity of the marker proteins in lane K in (b) indicates the length of development with silver stain, revealing the impurities of the marker proteins. For electrophoresis, proteins were precipitated by incubating with a 4-fold volume of methanol/acetone (1:1, v/v) for 10 min at 37 °C, centrifuged at 10000 g and incubated overnight in sample buffer [final concentrations Tris (125 mM), pH 6.8, urea (4 M), mercaptoethanol (5%), SDS (2%) and Bromphenol Blue (0.002 %)] under reducing conditions at 4 °C.

band was less prominent, whereas the two other bands were still present at a comparable density.

Comparison of the protein bands in the two fractions obtained from the second heparin-agarose column make the identification of the InsP_4 receptor possible. In the 0.8 M-NaCl fraction, even with intense silver staining (Fig. 2b), only two bands (37 and 42 kDa) were visible. The relative amount of the 37 kDa band was greatly decreased in the 0.8 M-NaCl fraction (Fig. 2a, lane I) in comparison with the 0.4 M-NaCl fraction. Since specific binding activity was 8-fold higher in the sample obtained at 0.8 M-NaCl, the protein with approximate molecular mass 42 kDa represents the high-affinity InsP_4 receptor from cerebellum.

DISCUSSION

In the present work, we achieved purification of the InsP_4 receptor. The first purification step using the cation exchanger CM-cellulose substantially decreased the amount of protein left in the active fraction. With CM-cellulose and heparin-agarose chromatography we achieved a 1200-fold purification compared with the solubilized membrane preparation. After chromatography on a subsequent hydroxyapatite column, the yield of active receptor remained the same but the specific activity (referred to protein) was increased by a factor of four. The

fraction with InsP_4 -binding activity obtained after hydroxyapatite chromatography, analysed by SDS/PAGE, revealed still three dominant protein bands. The second heparin-agarose column lowered the contributions of contaminating proteins, despite the decrease in specific activity observed in some experiments (see Table 1). This chromatographic step discriminated between the three protein bands mentioned above. The coincidence between InsP_4 -binding activity and the relative abundance of the protein bands allowed us to unequivocally identify the high-affinity InsP_4 receptor as the 42 kDa protein. At present we cannot exclude the possibility that the 42 kDa protein might be a proteolytic fragment of a larger InsP_4 receptor.

For a 42 kDa protein, the maximum specific binding is 24 nmol/mg of protein, assuming a stoichiometry of one ligand (InsP_4)-binding site per receptor molecule. The value obtained here (4.6 nmol/mg of protein) is one-fifth of the theoretical maximum. This stoichiometry of 1:5 could be partly due to the artificial detergent environment or to non-accessible binding sites.

For the partially purified InsP_4 receptor [14], we had already reported a selectivity for the various inositol phosphates similar to that detected with the membrane preparation [10]. The purified receptor described here displayed the same selectivity. For $\text{Ins}(1,3,4,5,6)\text{P}_5$ in the membrane preparation the affinity was 100 times lower than for $\text{Ins}(1,3,4,5)\text{P}_4$. After purification, however, $\text{Ins}(1,3,4,5,6)\text{P}_5$ inhibited [^3H] $\text{Ins}(1,3,4,5)\text{P}_4$ binding with an IC_{50} close to that for non-radioactive $\text{Ins}(1,3,4,5)\text{P}_4$. Thus the selectivity of the receptor appears to be regulated by an accessory protein. This is an intriguing possibility because (i) in many cells InsP_5 is present at high concentration [24], and (ii) InsP_5 can mimic some of the cellular effects of $\text{Ins}(1,3,4,5)\text{P}_4$ [25].

A further, possibly important, regulatory property of the $\text{Ins}(1,3,4,5)\text{P}_4$ receptor is indicated by the influence of pH. At pH 7 the InsP_4 receptor has lowered affinity for $\text{Ins}(1,3,4,5)\text{P}_4$ and increased unspecific binding (apparently reflecting a binding site with very low affinity), whereas at acidic pH the affinity increases. Such cellular pH changes occurring during Ca^{2+} regulation might modify the binding properties of the InsP_4 receptor.

The InsP_4 receptor is retained by CM-cellulose at pH 7.5. Therefore the receptor is expected to be a basic protein, whereas the InsP_3 receptor is an acidic protein [8]. These opposite overall electrical charges could favour a mutual binding of the two receptors. This is compatible with the notion of a direct interaction of the InsP_4 receptor with the InsP_3 receptor, predicted by the model of InsP_4 action proposed by Irvine [25].

The high-affinity InsP_4 receptor described here is most likely not the $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase, which is mostly soluble, or a kinase, as there is no evidence that under physiological conditions $\text{Ins}(1,3,4,5)\text{P}_4$ is phosphorylated [1,3]. The purified InsP_4 receptor should provide us with the tools to localize this receptor in cells and to determine the actual functions of InsP_4 in cellular Ca^{2+} homeostasis. Subcellular localization may give clues to solve the problem of whether InsP_4 induces resequestration of Ca^{2+} into internal stores, as suggested by Hill and co-workers [20], or whether InsP_4 regulates Ca^{2+} entry into the cytoplasm through the plasma membrane [25].

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