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Biochemistry

Activation of cardiac adenylate cyclase: Hormonal modification of the magnesium ion requirement

(3′:5′-cyclic AMP/epinephrine/histamine/fluoride ion/guanine nucleotides)

ROBERT ALVAREZ AND JOHN J. BRUNO

Institute of Biological Sciences, Syntex Research, Palo Alto, California 94304

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ABSTRACT **Histamine and epinephrine stimulate the activity of guinea pig heart adenylate cyclase [ATP pyrophosphate-lyase (cyclizing) EC 4.6.1.1], in part, by decreasing the requirement for Mg^{2+} as an activator. This effect may represent an increase in affinity for Mg^{2+} and/or a decrease in sensitivity of the enzyme towards inhibition by free ATP. Both of these inotropic hormones also increase maximum velocity. Pretreatment of the membrane-bound enzyme with EDTA, to remove available divalent cations, almost eliminates persistent stimulation by guanyl-5′-yl imidodiphosphate [Gpp(NH)p]. Addition of Mg^{2+} to the preincubation medium restores the capacity of Gpp(NH)p to acutely activate the enzyme. These results indicate that Mg^{2+} interacts with the nucleotide (GTP) regulatory site. Persistent stimulation of the enzyme by either Gpp(NH)p or fluoride ion also involves a decrease in the requirement for Mg^{2+} and an increase in maximum velocity.**

The increase in cardiac contractility following the administration of catecholamines, histamine, and other inotropic hormones is mediated, in part, by the activation of a membrane-bound adenylate cyclase [ATP pyrophosphate-lyase (cyclizing) EC 4.6.1.1] (1, 2). This enzyme system is thought to include hormone-specific receptors at the cell surface, a nucleotide (GTP) regulatory site, and a catalytic site (2, 3). Several studies have demonstrated that magnesium ion (Mg^{2+}), in excess of the concentration required to form the substrate (MgATP), increases adenylate cyclase activity (4–7). This effect was initially attributed to the binding of Mg^{2+} to an activation site associated with the enzyme (4–7). More recently, de Haën (8) has proposed that Mg^{2+} increases activity by forming a complex with low concentrations of ATP. In this model, free ATP is a potent inhibitor of the binding of substrate to the catalytic site.

Birnbaumer *et al.* (4) observed that the apparent affinity of rat fat cell adenylate cyclase for Mg^{2+} was increased in the presence of corticotropin (ACTH) and fluoride ion. At high Mg^{2+} concentrations the maximum velocity under basal conditions was almost as high as that of the stimulated enzyme. In contrast, Drummond *et al.* (5, 6) reported that the primary effect of epinephrine and fluoride ion on guinea pig heart adenylate cyclase was to increase the maximum velocity without significantly influencing the affinity for Mg^{2+}. From an analysis of the data obtained with fat cell (4) and heart (6) adenylate cyclases, de Haën (8) proposed that hormonal or fluoride ion stimulation did not require a Mg^{2+} site but involved a lower sensitivity of the enzyme to inhibition by free ATP. Experimental evidence in support of this model has been presented by Rodbell and his colleagues (9–12). Recently, Hammes and Rodbell (13) have proposed that hormonal activation involves an alteration in the pK values of ionizable groups at the active site and in the affinity for Mg^{2+}. The available data, however, do not permit a distinction between Mg^{2+} activation and the removal of free ATP as an inhibitor of substrate binding (3, 14). Garbers and Johnson (14) obtained kinetic evidence for a Mg^{2+} binding site with rabbit heart adenylate cyclase and did not detect potent inhibition of detergent-dispersed brain adenylate cyclase by free ATP.

The primary objective of the present study was to examine the role of Mg^{2+} in the activation of guinea pig heart adenylate cyclase as it relates to stimulation of the enzyme by histamine, epinephrine, and guanine nucleotides.

Abbreviations: cyclic AMP, adenosine 3′:5′-cyclic monophosphate; Gpp(NH)p, guanyl-5′-yl imidodiphosphate.

MATERIALS AND METHODS

[α-^{32}P]ATP (10–20 Ci/mmol) and cyclic [8-^{3}H]AMP (21 Ci/mmol) were purchased from New England Nuclear. Nucleotides, monopotassium 2-phospho*enol*pyruvate, pyruvate kinase, histamine, and *l*-epinephrine were from Sigma Chemical Co. Guanyl-5′-yl imidodiphosphate [Gpp(NH)p] was from P-L Biochemicals, Inc. EDTA was from Aldrich Chemical Co. $MgSO_4$ (ultra pure) was from Schwarz/Mann.

Preparation of Enzyme. Guinea pig heart adenylate cyclase was prepared and lyophilized according to the procedure described by Drummond and Duncan (5) (Method I), or by the following modification (Method II). Guinea pigs (0.2–0.5 kg) were sacrificed by CO_2 asphyxiation; the hearts were excised and placed in ice-cold Tris·HCl buffer, pH 7.7, 10 mM EDTA (Tris–EDTA buffer). All subsequent isolation procedures were performed at 4°. The hearts were perfused with Tris–EDTA buffer to remove blood and homogenized in 10 volumes of Tris–EDTA buffer in a commercial Waring blendor for 1 min. Connective tissue was removed by straining through two layers of cheesecloth. The homogenate was centrifuged at 3000 × *g* for 15 min. The pellet was washed once in Tris–EDTA buffer by resuspending and centrifuging at 3000 × *g* for 15 min. The particulate preparation was then washed three times as described above with 10 mM Tris·HCl buffer, pH 7.7. The final pellet was lyophilized and stored at −20°. Prior to use, 100 mg of lyophilized powder was suspended in 4–9 ml of cold Tris buffer and brought to a smooth suspension with a glass homogenizer. In some experiments the lyophilized enzyme was resuspended in Tris–EDTA buffer to remove additional divalent cations. The enzyme was washed free of EDTA as described above before use in the adenylate cyclase assay.

Persistent Activation. In all experiments with Gpp(NH)p or fluoride ion, the enzyme was *preincubated* at 37° with the persistent activators. After preincubation, the solutions (1 ml) were cooled in an ice-water bath and 5 ml of cold Tris·HCl buffer were added to each tube. The particulate enzyme was washed twice by centrifugation at 8000 × *g* for 10 min. The final pellets were resuspended in 0.5 ml of Tris·HCl buffer prior to use in the assay.

Adenylate Cyclase Assay. The components of the incubation medium in a total volume of 150 μl were: 40 mM Tris·HCl

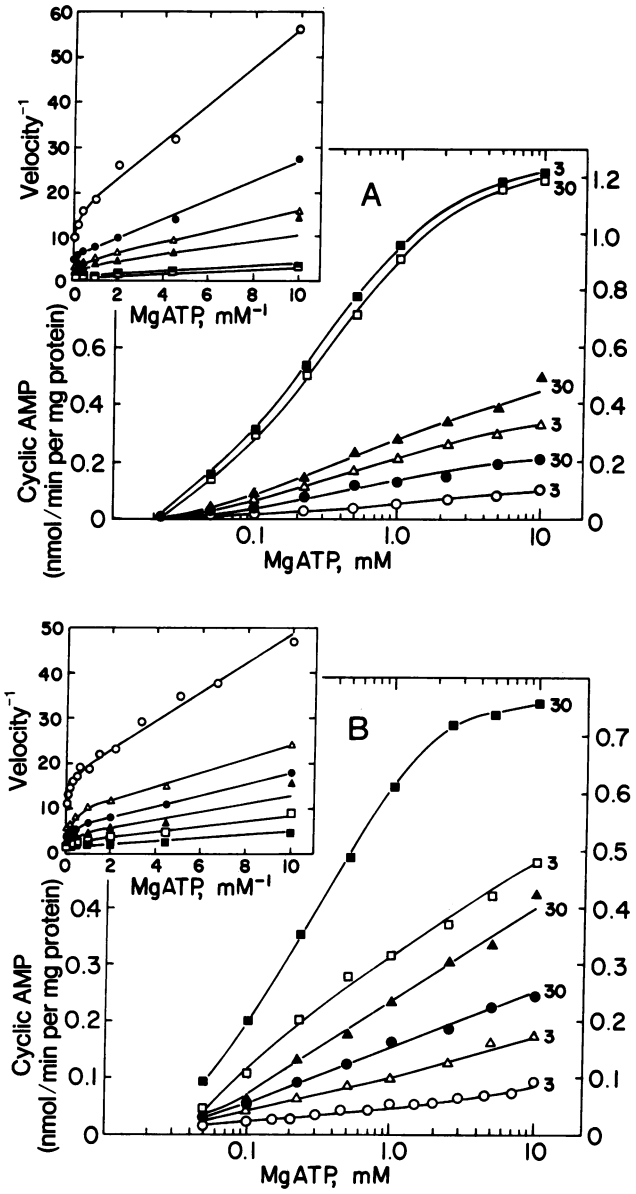

FIG. 1. Guinea pig heart adenylate cyclase activity as a function of substrate concentration. Enzyme was prepared in the absence (A) or presence (B) of EDTA (Methods I and II, respectively) as described in *Materials and Methods*. Basal enzyme activity (○, ●) is compared to enzyme preactivated for 5 min at 37° with 10 mM NaF (△, ▲) or 0.1 mM Gpp(NH)p (□, ■). The calculated free Mg^{2+} concentrations (3 and 30 mM) are indicated by the numbers on each curve.

buffer, pH 7.7 at 25° (pH 7.4 at 37°), 8 mM theophylline, 2 mM cyclic AMP, 5.5 mM KCl, 20 mM phospho*enol*pyruvate, 130 μg/ml of pyruvate kinase, 1 mM [α-^{32}P]ATP (0.5 μCi), and 3 mM $MgSO_4$. The reaction was initiated by the addition of enzyme and incubations were for 10 minutes at 37°. The reaction was terminated with a 5 min boiling-water bath. When ATP or Mg^{2+} concentrations were varied, they were adjusted to the same concentration after the boiling-water bath. Fifty microliters of cyclic [^{3}H]AMP (0.05 μCi) was then added to each tube to monitor recovery of the product. Labeled cyclic AMP was isolated from neutral alumina columns (15). Assays were performed in triplicate. Enzyme activity was linear for at least 10 min and proportional to protein concentration in the range of 100–600 μg. Protein was estimated with bovine serum albumin as a standard (16). ATP concentrations were verified by ultraviolet absorption at 259 nm (molar absorbancy index = 15.4 × 10^3). Concentrations of $MgATP^{2-}$, ATP^{4-}, and Mg^{2+} were calculated from the stability constant (15,000 M^{-1}) reported for reaction conditions at pH 7.4 at 37° (17).

RESULTS AND DISCUSSION

Enzyme activity as a function of substrate concentration

To examine the effect of Mg^{2+} and ATP^{4-} on adenylate cyclase activity, velocity was measured as a function of MgATP at two fixed concentrations of free Mg^{2+}. Under these conditions, ATP^{4-} was present in constant proportion to the variable substrate. A 10-fold increase in Mg^{2+} elevated basal and fluoride-stimulated enzymatic activity at all substrate concentrations tested (Fig. 1A). The reciprocal plots of these data were nonparallel and concave downward. This kinetic pattern corresponds to that expected if ATP^{4-} interacts as a partial competitive inhibitor and Mg^{2+} binds to an activation site (14). The evidence for inhibition by ATP^{4-}, however, is equivocal because the hyperbolic curves could also be due to substrate activation, negative cooperativity, or to the presence of multiple adenylate cyclases (14).

Activation of adenylate cyclase by Gpp(NH)p has been demonstrated with homogenates from several tissues (18). The guanine nucleotide binds to the GTP regulatory site and persistently activates the enzyme. When guinea pig heart adenylate cyclase was pre-activated with Gpp(NH)p, typical Michaelis–Menten kinetics were obtained. The reciprocal plots of these data were linear and intersected to the left of the ordinate (K_m = 0.3 mM MgATP). In contrast to basal activity, apparent inhibition by ATP^{4-} was not detected and 3 mM Mg^{2+} was as effective as 30 mM. The latter observation suggested that Gpp(NH)p influences the requirement for Mg^{2+} as an activator.

Pretreatment of the enzyme with EDTA, to remove available enzyme-bound magnesium, slightly increased basal activity and reduced the persistent activation by Gpp(NH)p and fluoride ion (Fig 1B). Under these conditions, a 10-fold increase in Mg^{2+} enhanced activity of the Gpp(NH)p-stimulated enzyme.

Mg^{2+} requirement for persistent activation by Gpp(NH)p

The influence of Mg^{2+} on persistent activation was examined by preincubating the enzyme with Gpp(NH)p and varying the concentration of Mg^{2+}. The particulate preparation was then washed free of the metal ion and unbound Gpp(NH)p prior to the enzyme assay. Thus, effects of Mg^{2+} on ATP complex formation (as part of the activation process) were eliminated.

Activation of guinea pig heart adenylate cyclase by 0.1 mM Gpp(NH)p was increased by the addition of Mg^{2+} to the preincubation medium in the range of 0.05–0.5 mM in a dose-dependent manner. Higher Mg^{2+} concentrations (0.5–100 mM) did not result in further increases in activity (data not shown). When the enzyme was pretreated with EDTA, persistent activation by Gpp(NH)p was sharply reduced. Gpp(NH)p activation was restored in a biphasic pattern by the addition of Mg^{2+} to the preincubation medium (Fig. 2).* Activation coincided with the formation of the MgGpp(NH)p complex at 0.05–0.5 mM Mg^{2+} and was not diminished by higher concentrations (100 mM). It is likely, therefore, that MgGpp(NH)p is an activating species. These results also indi-

* Persistent stimulation by 10 mM sodium fluoride was abolished by EDTA pretreatment and reversed by the addition of Mg^{2+} (0.3–3 mM) to the preincubation medium (unpublished results).

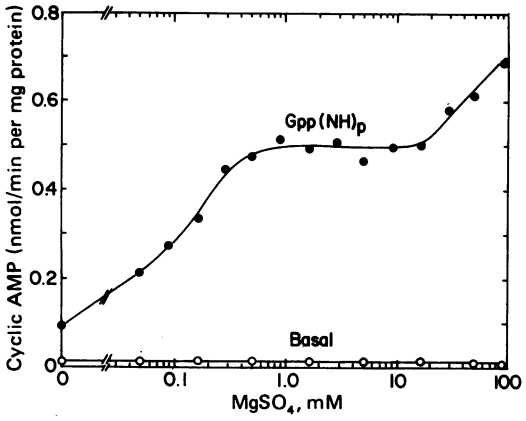

FIG. 2. Effect of Mg^{2+} on persistent activation of adenylate cyclase. Lyophilized enzyme, prepared by Method II, was suspended in Tris–EDTA buffer to remove available divalent cations. The particulate enzyme was then centrifuged and washed with Tris·HCl buffer as described in *Materials and Methods*. The washed enzyme was preincubated for 15 min at 37° in the absence (○) or presence (●) of 0.1 mM Gpp(NH)p. $MgSO_4$ was varied as indicated on the abscissa. After preincubation the enzyme was washed as described in *Materials and Methods*. The final pellets were assayed for adenylate cyclase activity with 1 mM ATP and 3 mM $MgSO_4$.

cate the presence of at least one Mg^{2+} binding site associated with the enzyme which influences the efficiency of Gpp(NH)p as an activator. Preincubation with Mg^{2+} alone had no significant effect on enzymatic activity. The enhancement of Gpp(NH)p persistent activation was only observed if the guanine nucleotide and divalent cation were present simultaneously during the preincubation.

The augmentation of Gpp(NH)p activation by Mg^{2+} has been attributed to chelation of protonated ATP ($HATP^{3-}$) as an inhibitor and Gpp(NH)p^{4-} has been described as the sole activating species (11, 19). The results presented here demonstrate that Mg^{2+} interacts with and, perhaps, binds to the nucleotide (GTP) regulatory site. In addition, optimal persistent activation by Gpp(NH)p requires Mg^{2+} or other divalent cations that are tightly bound to the enzyme.

Enzyme activity as a function of Mg^{2+} concentration

Mg^{2+} increased basal activity in the range of 0.2–30 mM (0.5 V_{max} = 5.7 mM) and was essential to the expression of enzyme activity (Fig. 3A and B). Under basal conditions, maximum velocity did not plateau above 30 mM Mg^{2+}. Instead, a reproducible decrease in velocity occurred. Additional studies are required to explain this inhibitory effect, which may obscure the true maximum velocity and interfere with the determination of apparent enzyme–Mg^{2+} association constants.

Double reciprocal plots of velocity versus Mg^{2+} were concave downward under basal conditions and linear or almost linear when the enzyme was stimulated by Gpp(NH)p, fluoride ion, histamine, or epinephrine (not shown). These observations were similar to those obtained when velocity was measured as a function of substrate concentration (Fig. 1) and again suggest the possibility that ATP^{4-} inhibits substrate binding.

Activation of adenylate cyclase by Gpp(NH)p or histamine was associated with a reduced requirement for Mg^{2+} as an activator (Figs. 3B and 4B). The dose–response curves were displaced towards lower Mg^{2+} concentrations with respect to activation, maximum velocity, and inhibition. Similar results were obtained when the enzyme was activated in the presence of 0.1 mM epinephrine (0.5 V_{max} = 0.78 mM) or preactivated with 3.3 mM sodium fluoride (0.5 V_{max} = 0.64 mM). Activation by histamine and epinephrine was accompanied by a relatively small increase in maximum velocity. In contrast, Gpp(NH)p decreased the requirement for Mg^{2+} and increased maximum velocity by approximately the same magnitude (12-fold). The fold increase in activity in response to either hormonal or persistent stimulation was greatest when the Mg^{2+} concentration was sufficient to form the substrate but less than that required for maximal basal activity.

Indirect evidence that Mg^{2+} binds to a single activation site was obtained by measuring the slope of Hill plots. Although Hill coefficients were approximately 1 at 0.5 V_{max}, the slopes were not linear throughout the range of 0.1–0.9 V_{max} under basal conditions. Upon stimulation with Gpp(NH)p the Hill plot was biphasic and approached a slope of 2 at high Mg^{2+} concentrations. These deviations from conventional Hill plots may represent a significant feature of cardiac adenylate cyclase.

In a previous study of guinea pig heart adenylate cyclase, Drummond and Duncan (5) did not detect a change in the apparent affinity for Mg^{2+} when fluoride ion was added directly to the final incubation medium. Perkins (3), on the other hand, observed an increase in apparent affinity for Mg^{2+} when rabbit heart adenylate cyclase was preactivated with fluoride ion. The discrepancy may be explained by the formation of

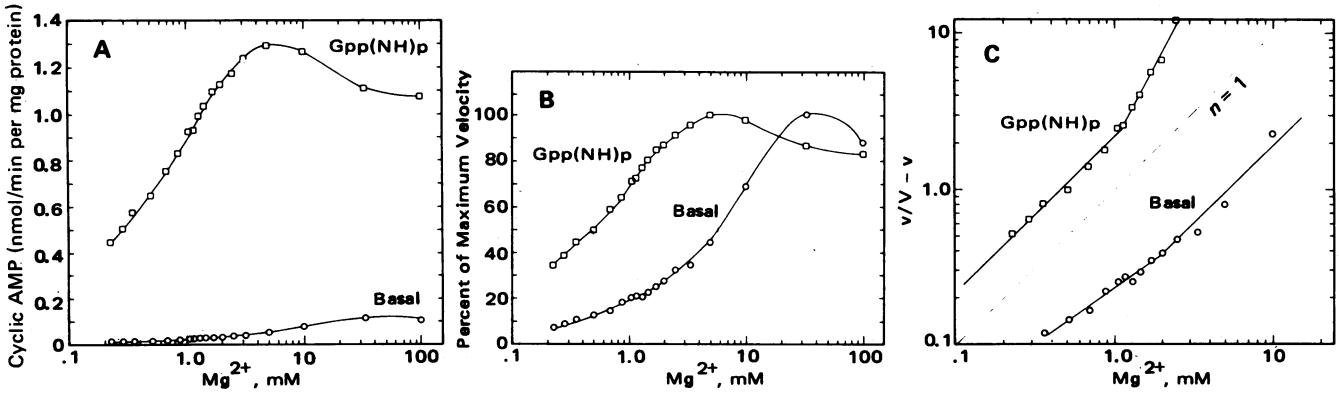

FIG. 3. Effect of Mg^{2+} ion concentration on basal and Gpp(NH)p-activated adenylate cyclase. Lyophilized enzyme (Method II) was suspended in 33 μM $MgSO_4$ and preincubated in the absence (○) or presence (□) of 0.33 mM Gpp(NH)p for 15 min at 37°. The membrane preparation was washed as described in *Materials and Methods*. The assay was performed under standard conditions with 1 mM MgATP and variable Mg^{2+} concentrations as indicated. (A) Adenylate cyclase activity as a function of Mg^{2+} concentration. (B) Percent of maximum velocity obtained with basal and Gpp(NH)p-activated enzyme versus Mg^{2+} concentration. (C) Hill plots (v = initial velocity and V for V_{max} = maximum velocity). Mg^{2+} concentrations required for half-maximal velocity were: basal, 5.7 mM and Gpp(NH)p, 0.48 mM.

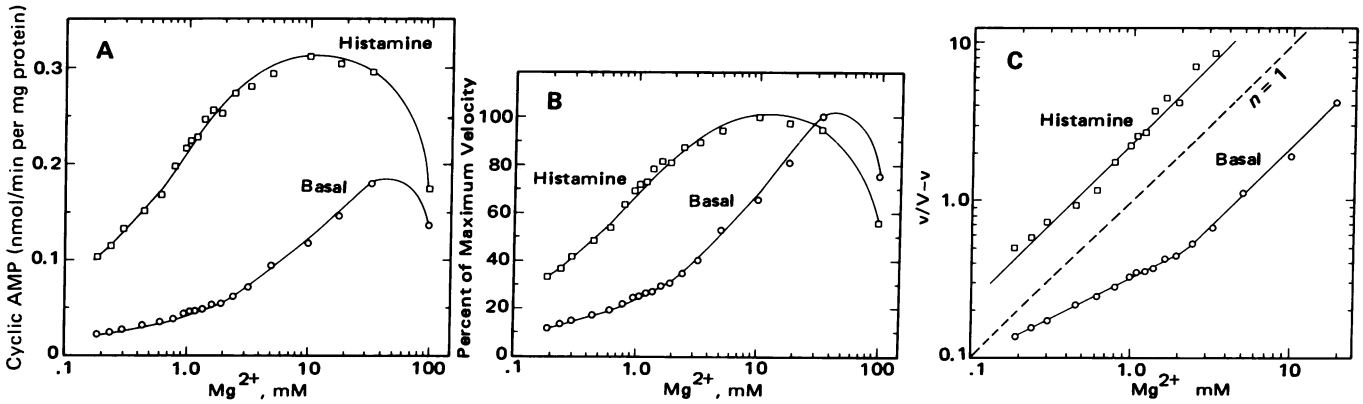

FIG. 4. Effect of Mg^{2+} ion concentration on basal and histamine-activated adenylate cyclase. Enzyme (Method II) was preincubated in the absence (○) or presence (□) of 1 mM histamine and various $MgSO_4$ concentrations for 15 min at 37° in a total volume of 75 μl. The tubes were cooled at 4° and adenylate cyclase incubation medium (75 μl) containing 0.1 mM GTP was added to each tube. The adenylate cyclase assay was performed under standard conditions with 1 mM ATP and various Mg^{2+} concentrations as indicated. Legends for (A), (B), and (C) are described in Fig. 3. Mg^{2+} concentrations required for half-maximal velocity were: basal, 4.6 mM and histamine, 0.47 mM.

complexes between Mg^{2+} and fluoride ion as previously suggested (3). In addition, when velocity data are plotted versus *total* Mg^{2+} on a linear scale, the full influence of an agonist on the requirement for free Mg^{2+} is not revealed.

The results reported here indicate that Mg^{2+} binds to an activation site associated with guinea pig heart adenylate cyclase and that the concentration of Mg^{2+} required for maximum activity is reduced in the presence of either histamine or epinephrine. The evidence also suggests that Mg^{2+} interacts with the nucleotide (GTP) regulatory site.

It is clear that the role of Mg^{2+} is manifold. In addition to forming the substrate and binding to an activation site, the divalent cation may also increase activity by chelating free ATP and thus remove a putative potent inhibitor. The kinetic data, while equivocal, support the proposal that free ATP acts as a partial competitive inhibitor of substrate binding under basal conditions. For these reasons, the evidence that hormones increase the affinity of adenylate cyclase for Mg^{2+} can also be interpreted as representing a decrease in sensitivity of the enzyme towards inhibition by free ATP. It is also conceivable that both events occur simultaneously. Additional studies are required to distinguish between these alternatives.

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