Adenosine 3',5'-Monophosphorothioate (Rp-isomer) Induces Down-regulation of Surface Cyclic AMP Receptors without Receptor Activation in Dictyostelium discoideum*

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Cyclic AMP (cAMP) induces the activation and subsequent desensitization of adenylate cyclase in Dictyostelium discoideum. cAMP also induces down-regulation of surface cAMP receptors. Desensitization of adenylate cyclase is composed of a rapidly reversible component (adaptation) and a slowly reversible component related to down-regulation of surface cAMP receptors (Van Haastert, P. J. M. (1987) J. Biol. Chem. 262, 7700–7704). The agonistic and antagonistic activities of the cAMP derivative adenosine 3',5'-monophosphorothioate ((Rp)-CAMPS) for these responses were investigated. 1) (Rp)-CAMPS competes with cAMP for binding to different receptor forms with an apparent $K_i = 5 \mu M$. 2) (Rp)-CAMPS does not activate adenylate cyclase and antagonizes the cAMP-induced activation with an apparent $K_i = 5 \mu M$. 3) (Rp)-CAMPS induces down-regulation of surface cAMP receptors with $EC_{50} = 5 \mu M$. 4) (Rp)-CAMPS induces desensitization of adenylate cyclase, which is not rapidly reversible. These results indicate that desensitization of adenylate cyclase by (Rp)-CAMPS is due to down-regulation of surface cAMP receptors and not to adaptation. We conclude that down-regulation of surface cAMP receptors does not require their activation or modification involved in adaptation.

Extracellular cAMP is a first messenger in Dictyostelium discoideum and induces chemotaxis (1), morphogenesis (2), and cell differentiation (3). cAMP is detected by cell surface cAMP receptors (4), and the activation of the receptor leads to increases of guanylate and adenylate cyclase activity (5, 6). Cellular cGMP levels peak at 10 s after stimulation, and cAMP levels reach a maximal concentration after about 1 min (7, 8).

The activation of the cAMP receptor has been investigated by using cAMP derivatives (9–12). Binding of cAMP to the receptor requires an intact N-6-amino and 3'-oxygen, whereas activation of the receptor also requires a certain conformation of the exocyclic oxygen atoms (10). Thus, (Rp)-CAMPS, in which the axial oxygen is replaced by sulfur, still binds to the receptor but does not induce a response. In addition, (Rp)-CAMPS competitively inhibits the activation of the receptor by cAMP (11, 13). Chemotaxis and the cAMP-induced cGMP responses were investigated in these studies. This report describes the regulation of adenylate cyclase by (Rp)-CAMPS.

The cAMP-induced activation of adenylate cyclase is transient also when the cAMP concentration remains constant (14–16). This desensitization process is composed of multiple components (17), i.e. adaptation and down-regulation of surface receptors. Adaptation of adenylate cyclase is completed after several minutes (16). Cells deadapt after removal of cAMP with a half-time of about 2–4 min (15). Down-regulation of surface cAMP receptors takes place at a similar time scale as adaptation of adenylate cyclase (17, 18). However, in contrast to adaptation, down-regulation is not rapidly reversible after stimulus removal and shows a half-time of recovery of about 1 h (17–19).

Adaptation of adenylate cyclase has been correlated with the cAMP-induced alteration of the electrophoretic mobility of the cAMP receptor as observed in polyacrylamide gel electrophoresis and is presumably caused by receptor phosphorylation (20–22). In vertebrates it has been shown that receptor phosphorylation may lead to receptor-effector uncoupling (23–26). Desensitization of adenylate cyclase stimulation in vertebrates also includes down-regulation of surface receptors. Since receptor-effector uncoupling occurs generally more rapidly and at lower ligand concentrations than down-regulation of the surface receptor, it has often been suggested that down-regulation occurs after its modification that induces receptor-effector uncoupling (27, 28).

The relationship between down-regulation of surface cAMP receptors and cAMP-induced desensitization of adenylate cyclase was investigated in D. discoideum. The results show that the cAMP antagonist (Rp)-CAMPS induces down-regulation of surface receptors and desensitization of adenylate cyclase. However, (Rp)-CAMPS does not induce the activation of adenylate cyclase nor its rapidly reversible adaptation. This suggests that down-regulation of surface cAMP receptors can occur without receptor activation and its modification which is associated with adaptation.

EXPERIMENTAL PROCEDURES

Materials—[2,8-3H]cAMP (1.5 TBq/mmol) and the cGMP radioimmunoassay kit were obtained from Amersham Corp. LiChrosorb 2

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1 The abbreviations used are: (Rp)-cAMP, adenosine 3',5'-monophosphorothioate; (Rp)-isomer; (Sp)-cAMPs, adenosine 3',5'-monophosphorothioate; (Sp)-isomer; dcAMP, 2'-deoxygenosine 3',5'-monophosphate; DTT, dithiothreitol.

2 Desensitization, down-regulation, and adaptation are defined operationally. Desensitization is any cAMP-induced reduction of cellular responsiveness. Down-regulation is any cAMP-induced reduction of the number of detectable cAMP-binding sites. Adaptation is a form of desensitization by which cells lose responsiveness to trains of stimuli with equal concentrations, but remain responsive to higher cAMP stimuli; adaptation is reversible with a half-time of 2–4 min, while down-regulation and desensitization due to down-regulation are reversible with a half-time of 1 h (17).
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RP-18 was from Chrompack (Middelburg, the Netherlands). (Sp)-cAMPS and (Rp)-cAMPS were a generous gift of Drs. Barnanis, Stec, and J. B. Torff (29). DTT, cAMP, deAMP, and 8-bromo-cAMP were purchased from Sigma. Extracellular cyclic nucleotide phosphodiesterase was isolated from D. discoideum cells as described (30).

Purification of (Rp)-cAMPS—(Rp)-cAMPS was purified by high performance liquid chromatography on a Lichrosorb BP-18 column with 1 mM phosphate buffer, 10% methanol, pH 6.5, as the mobile phase. This purification removes (Sp)-cAMPS and cAMP (10). During storage of (Rp)-cAMPS small amounts of cAMP (but not (Sp)-cAMPS) are formed which were removed immediately before the experiment by degradation with cyclic nucleotide phosphodiesterase. The incubation contained 0.5 nM (Rp)-cAMPS and 2 µM cyclic nucleotide phosphodiesterase; this amount of cyclic nucleotide phosphodiesterase hydrolyzes 1 µM cAMP with a half-time of 1-2 min. The reaction was performed for at least 1 h and not terminated.

Thus, cells stimulated with (Rp)-cAMPS also received cyclic nucleotide phosphodiesterase; the amount of cyclic nucleotide phosphodiesterase added to the cells was maximally 10% of the activity the cells already contained.

Culture Conditions—D. discoideum, NC-4, was grown as described (30) and harvested in 10 mM sodium/potassium phosphate buffer, pH 6.5 (Pb buffer). Cells were starved for 5 h in suspension, harvested and washed in Pb buffer, and resuspended in this buffer at a density of 10^6 cells/ml.

cAMP Binding—The binding of cAMP to cells was measured in a mixture containing 4 × 10^6 cells, Pb buffer, 10 mM dithiothreitol, 2 or 100 nM [3H]cAMP, and the indicated concentrations of cAMP, (Sp)-cAMPS, or (Rp)-cAMPs. The incubation was a total volume of 350 µl. The incubation period was 45 s at room temperature and terminated by either direct centrifugation of the incubation mixture through silicone oil (equilibrium binding) or by centrifugation through silicone oil at 10 s or 2 min after the addition of 1 ml of 0.1 mM cAMP in Pb buffer (slowly dissociating sites (32, 33)). Nonspecific binding was determined by including 0.1 mM cAMP in the entire incubation.

cAMP Response—The incubation mixture (15 µl) contained 1.35 × 10^6 cells, 10 mM DTT, and the concentrations of cyclic nucleotides as indicated in the figures. Cells were lysed at the indicated times by the addition of 15 µl of perchloric acid (3.3% v/v), neutralized with 7.5 µl of potassium bicarbonate (50% saturated at 20 °C), and centrifuged for 2 min at 10,000 × g. cAMP levels were determined in 30 µl of the supernatant by an isotope dilution assay (34) as described (35). The competition of the cyclic nucleotides with the binding of [3H]cAMP to the binding protein was determined by adding the cyclic nucleotides after cells were lysed.

Down-regulation of cAMP Receptors and Desensitization of Adenylate Cyclase Stimulation—Down-regulation of cAMP receptors was induced in a mixture containing 3 × 10^6 cells, 2 mM caffeine, 10 mM dithiothreitol, and the indicated concentrations of cAMP, (Sp)-cAMPS, or (Rp)-cAMPS in a total volume of 350 µl. After 15 min at room temperature the incubation volume was transferred to a tube containing 15 ml of ice-cold Pb buffer, and cells were washed twice with 15 ml of Pb buffer and resuspended in 300 µl. cAMP binding was measured in triplicate and nonspecific binding in duplicate; the cAMP-induced accumulation was measured in triplicate determination at 0 s and 5 min after stimulation with 10 mM DTT and 10 µM deAMP.

RESULTS

Competition with cAMP Binding—Previously we have shown that (Rp)-cAMPS competed with cAMP binding to D. discoideum cells as was measured in ammonium sulfate (10). Recently we have shown that D. discoideum cells may contain multiple forms of the receptor which have different affinities and rate constants of dissociation (32, 33). These receptor forms were designated A_t with K_t = 60 nM and k_− = 0.4 s^−1, A_w with K_w = 450 nM and k_− = 1.0 s^−1, B with K_B = 15 nM and k_− = 0.05 s^−1, and B_8 with K_B = 15 nM and k_− = 0.005 s^−1. Fig. 1 shows the potency of cAMP, (Sp)-cAMPS, and (Rp)-cAMPS to compete with the binding of [3H]cAMP to D. discoideum cells. Equilibrium binding at 2 nM [3H]cAMP detects predominantly binding to A_t (Fig. 1A), while equilibrium binding of 100 nM [3H]cAMP detects mainly the A_w form of the receptor (Fig. 1B). At 10 s or 2 min after the onset of dissociation of the [3H]cAMP receptor complex the binding is predominantly to respectively B_w (Fig. 1C) and B_8 (Fig. 1D). The results show that (Rp)-cAMPs competes with the binding of 2 nM [3H]cAMP to all binding forms with an apparent IC_50 of about 5 nM (A, C, and D) and an IC_50 of about 100 µM for the competition with 100 nM [3H]cAMP (B). (Rp)-cAMPS is about 3-fold less active than (Sp)-cAMPS and about 100–300-fold less active than cAMP.
dependent protein kinase as the CAMP-binding protein content was determined with an isotope dilution assay using CAMP-protein kinase, which makes purification of cAMP unnecessary (35). 

~1 of 3.5% perchloric acid. Lysates were neutralized, and the cAMP of adenylate cyclase.

analog dcAMP was used because this analog has high affinity for the surface cAMP receptor in D. discoideum but low affinity for the protein kinase, which makes purification of cAMP unnecessary (35).

lated more rapidly. The effect of a receptor agonist on adenylate cyclase stimulation is most effectively measured at 90 s after stimulation in the presence of DTT. This protocol has the advantage that it consumes about 1000-fold less cyclic nucleotides than the perfusion experiments.

Dose-response curves of some cyclic nucleotides for the induction of adenylate cyclase activation are shown in Fig. 3A. cAMP induced a half-maximal activation at about 3.8 nM. The agonists dcAMP and (Sp)-cAMPS induced a half-maximal effect at about 7- and 90-fold higher concentrations, respectively, than cAMP, which is in close agreement with their lower affinity for the cAMP receptor (10). It has been shown that (Rp)-cAMPS, which was stored lyophilized for 2 years at -20 °C and was free of cAMP and (Sp)-cAMPS at the onset of the storage period, induced a partial activation of adenylate cyclase. An extensive analysis of the purity of a preparation of (Rp)-CAMPS, which was stored lyophilized (31). (Rp)-CAMPS contaminated with 0.15% cAMP, which was formed during the storage period (31). (Rp)-cAMPS no longer induced the activation of adenylate cyclase after removal of this cAMP with cyclic nucleotide phosphodiesterase in the presence of different amounts of cyclic nucleotide phosphodiesterase.

Control experiments indicate that the cyclic nucleotide phosphodiesterase itself had no effect, because it did not affect the response to the agonist (Sp)-cAMPS (Figs. 3A and B). It should be noted that both (Sp)-cAMPS and (Rp)-cAMPS are not effectively degraded by cyclic nucleotide phosphodiesterase (36).

The antagonistic potency of (Rp)-cAMPS in respect to the cAMP-induced activation of adenylate cyclase is shown in Fig. 3C. Cells were stimulated with 50 nM dcAMP in the presence of different concentrations of (Rp)-cAMPS, and the increase of cAMP levels was measured after 90 s. The results indicate that (Rp)-cAMPS inhibited the stimulation of adenylate cyclase. A half-maximal effect was observed at 10 μM (Rp)-cAMPS, suggesting that the apparent K_i is about 5 μM. These results indicate that (Rp)-cAMPS does not activate adenylate cyclase in D. discoideum and antagonized the agonist-induced stimulation of this enzyme.

Desensitization of Adenylate Cyclase—The stimulation of adenylate cyclase by cAMP rapidly decreases at longer time intervals after stimulation (14–16). This desensitization process can take place in the absence of adenylate cyclase activation (37). Therefore, we have analyzed whether (Rp)-cAMPS may induce desensitization of adenylate cyclase. Cells were incubated with 10 μM (Sp)-cAMPS or (Rp)-cAMPS for 15 min, extensively washed at 0 °C, and stimulated with 10 μM dcAMP and 10 mM DTT. (Sp)-cAMPS induced a 90% desensitization of adenylate cyclase stimulation (Fig. 4). Surprisingly, (Rp)-cAMPS also induced desensitization.

In the accompanying article it was shown that desensitization of adenylate cyclase stimulation is composed of at least two components (17). One component, called adaptation, is induced by low cAMP concentrations and reverses rapidly after removal of cAMP (half-time is 3–4 min). This component is not related to a reduction of the number of binding sites but is possibly mediated by the phosphorylation of the receptor (20–22). The second component of adenylate cyclase desensitization is due to receptor down-regulation. This com-

**FIG. 2. Microassay for the cAMP-agonist-induced activation of adenylate cyclase.** D. discoideum cells were stimulated with 5 mM DTT (a cyclic nucleotide phosphodiesterase inhibitor) or 5 mM DTT and 10 μM dcAMP in a total volume of 130 μl. At the times indicated 15 μl of the suspensions were added to a tube containing 19 μl of 5% perchloric acid. Lysates were neutralized, and the cAMP content was determined with an isotope dilution assay using cAMP-dependent protein kinase as the CAMP-binding protein (34). The analog dcAMP was used because this analog has high affinity for the surface cAMP receptor in D. discoideum but low affinity for the protein kinase, which makes purification of cAMP unnecessary (35).

**FIG. 3. Activation of adenylate cyclase by cyclic nucleotides.** Cells were stimulated with 10 mM DTT and the indicated concentrations of cyclic nucleotides in a total volume of 15 μl. Cells were lysed after 0 and 90 s, and the cAMP levels were measured. The data show the increase of cAMP levels and thus correct for the competition of the stimuli with [5H]cAMP in the isotope dilution assay; this competition was maximally the equivalent of 5 pmol/10^6 cells. A, the stimuli are cAMP (+), dcAMP (○), (Sp)-cAMPS (△), (Sp)-cAMPS preincubated with cyclic nucleotide phosphodiesterase (PDE) (▲), (Rp)-cAMPS contaminated with 0.15% cAMP (□), and (Rp)-cAMPS preincubated with cyclic nucleotide phosphodiesterase to remove the cAMP contamination (●). B, the stimuli are 0.3 μM (Sp)-cAMPS in the presence of different amounts of cyclic nucleotide phosphodiesterase. C, the stimuli are 50 nM dcAMP with different amounts of cyclic nucleotide phosphodiesterase-purified (Rp)-cAMPS. The stimuli mixtures containing cyclic nucleotide phosphodiesterase-purified (Rp)-cAMPS were prepared a few seconds before addition to the cells to prevent the formation of cAMP and the degradation of dcAMP by cyclic nucleotide phosphodiesterase in the preparation of (Rp)-cAMPS. The results are the means of triplicate determinations from a typical experiment reproduced once (B) or three times (A and C).
Fig. 4. Deensensitization of adenyly cyclase. Cells were preincubated with buffer (○), 10 μM (Sp)-cAMPS (○), or 10 μM (Rp)-cAMPS (△) for 15 min at 20 °C. Then cells were diluted 40-fold in ice-cold buffer, centrifuged for 1 min at 200 × g, and the cell pellet was resuspended in 10 mM DTT and 10 μM dcAMP at 20 °C. Duplicate samples were withdrawn at the times indicated, and the cAMP content was measured. The results shown are the means of three independent experiments.

Fig. 5. Relationship between down-regulation of surface receptors and desensitization of adenyly cyclase. A, down-regulation. Cells were preincubated with 2 mM caffeine, 10 mM DTT, and the indicated concentrations of cAMP (●), (Sp-)cAMPS (○), or (Rp)-cAMPS (△) for 15 min at 20 °C. Then cells were washed twice at 0 °C, resuspended in buffer, and cAMP binding was detected. B, specificity of down-regulation. Down-regulation of surface receptors was induced as described above. The cAMP derivatives are 1, cAMP; 2, dcAMP; 3, (Sp)-cAMPS; 4, (Rp)-cAMPS; and 5, 8-bromo-cAMP. The concentrations which induced half-maximal down-regulation were determined, and the reduction of the affinity relative to cAMP was calculated as ΔG values (10). The specificity of down-regulation is expressed against the specificity of the surface receptor (●) or cAMP-dependent protein kinase from D. discoideum (○). C, desensitization of adenyly cyclase. The cells used in panel A were also restimulated with 10 mM DTT and 10 μM dcAMP, and the cAMP accumulation was measured at 5 min after restimulation at 20 °C. The symbols are as in panel A. D, reversibility of desensitization of adenyly cyclase. Cells were preincubated with buffer, 3 μM (Sp)-cAMPS, or 30 μM (Rp)-cAMPS for 15 min at 20 °C. Then cells were washed twice at 0 °C, resuspended in buffer at 20 °C, and stimulated with 10 mM DTT and 10 μM dcAMP. cAMP levels were determined at 5 min after stimulation. Cells were stimulated immediately after resuspension (open bars) or at 15 min after resuspension (shaded bars). The results shown are the means of two experiments (A), three experiments (4 and C), and the means and standard deviations of five experiments (D).

**Discussion**

In this report experiments are presented which further investigate the actions of the putative antagonist of cAMP, (Rp)-cAMPS, in *D. discoideum*. Previously we have shown that (Rp)-cAMPS competes with cAMP for the binding to the cell surface cAMP receptor (10). This observation was further elaborated in this report, showing that (Rp)-cAMPS competes with cAMP for the binding to the multiple receptor forms which can be detected. (Rp)-cAMPS is 3-fold less active than the agonist (Sp)-cAMPS and about 100-300-fold less active than cAMP. Although (Rp)-cAMPS binds to the receptor, it does not induce a response. Previously we have shown that it did not induce chemotaxis or the rapid accumulation of cGMP levels (10, 11, 13). The present results indicate that (Rp)-cAMPS also does not stimulate adenyly cyclase.

The recent observation that this compound did induce a small activation of adenyly cyclase (12) was probably due to the small contamination of (Rp)-cAMPS with cAMP, which was formed during handling of the preparation. A detailed analysis of the chemical stability of (Rp)-cAMPS revealed (31) that (Rp)-cAMPS is stereochemically stable, i.e. the formation of (Sp)-cAMPS was not observed. However, (Rp)-cAMPS is slowly degraded to cAMP with a rate of 0.08%/year when stored lyophilized at −20 °C and a rate of 0.1%/year, 0.1%/day, and 0.1%/h at −20, 20, and 100 °C, respectively, when stored at pH 6.5 (31). This instability is sufficiently low to use the compound in cell stimulation ex-
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Experiments provided that the cAMP impurity is removed just before the experiment. (Rp)-cAMPS no longer activates adenylate cyclase after the removal of the cAMP contamination by degradation with cyclic nucleotide phosphodiesterase.

It has been shown that the surface cAMP receptor mediates the activation of guanylate cyclase, adenylate cyclase, and the induction of chemotaxis (10–12). Since (Rp)-cAMPS binds to this receptor but does not induce a response, it was suggested that it should be a competitive antagonist of cAMP. Indeed, this was shown for the induction of chemotaxis and the cGMP response (10, 11, 13). The present results indicate that this is also true for the activation of adenylate cyclase. These results suggest that (Rp)-cAMPS is an antagonist of cAMP for the induction of receptor-mediated responses.

cAMP does not only activate adenylate cyclase but also induces the desensitization of this enzyme activity (14–16). Unexpectedly, (Rp)-cAMPS induced the desensitization of adenylate cyclase. In the accompanying report (17) it was shown that desensitization of adenylate cyclase is composed of two components, adaptation and down-regulation. Adaptation is induced by low cAMP concentrations and is rapidly reversible; adaptation is not associated with a loss of cAMP binding to the cell surface. The second component of adenylate cyclase desensitization is the down-regulation of cell surface cAMP receptor (18), which requires higher cAMP concentrations and is slowly reversible. It should be noted that the kinetics of adaptation and down-regulation are similar, but not identical, showing a half-time of about 1–3 min. The present results show that (Rp)-cAMPS induces down-regulation of cAMP receptors with a half-maximal effect at 5 μM, which is similar to its affinity for the cAMP receptor.

The criteria, dose dependence and reversibility, were used to reveal the mechanism of desensitization of adenylate cyclase. The desensitization of adenylate cyclase by (Rp)-cAMPS requires relatively high concentrations and is not readily reversible. These observations strongly suggest that the desensitization of adenylate cyclase by (Rp)-cAMPS is due to down-regulation and not to adaptation.

It has been shown that (Rp)-cAMPS partially activates cAMP-dependent protein kinase from *D. discoideum*, which is in contrast to mammalian protein kinase where (Rp)-cAMPS is an antagonist (13). Does (Rp)-cAMPS induce down-regulation because it penetrates the cell and activates cAMP-dependent protein kinase? The cyclic nucleotide specificity of cAMP-induced down-regulation of surface receptors indicates that it is mediated by the surface receptor (Fig. 5B). Although agonists apparently act via the surface receptor, this does not exclude the possibility that the antagonist (Rp)-cAMPS acts via another route. A definite proof for the mechanism of action of (Rp)-cAMPS can be given when the selective analogs 8-bromo-(Rp)-cAMPS and 2′-deoxy-(Rp)-cAMPS are investigated. These analogs are presently being synthesized.

The observation that (Rp)-cAMPS specifically induces down-regulation of surface receptors and no activation or adaptation of adenylate cyclase may make this compound very useful in the study of the mechanism of down-regulation. It has been shown that adaptation of adenylate cyclase is associated with a shift of the apparent molecular weight in sodium dodecyl sulfate/polyacrylamide gel electrophoresis of a protein that was photoaffinity labeled with [3ZP]-azido-cAMP. This shift of the molecular weight is most likely due to the phosphorylation of the cAMP receptor (20–22). Does down-regulation require the phosphorylation of the receptor? Preliminary experiments indicate that (Rp)-cAMPS does not induce the shift of the molecular weight of the receptor. This observation does not exclude the possibility that (Rp)-cAMPS induces some phosphorylation of the receptor, because the shift of the molecular weight of the receptor is associated with the incorporation of multiple phosphates/receptor, and it is not clear how many phosphate molecules must be covalently labeled to induce the shift of the molecular weight of the receptor. Another interesting question is whether down-regulated receptors can be induced by cAMP to shift their apparent molecular weight. Preliminary experiments indicate that receptors which are down-regulated by (Rp)-cAMPS (and which have not shifted) shift after cAMP addition.

Finally, we face the possibility that down-regulation of surface receptors is part of a transmembrane signal transduction pathway. This would imply that (Rp)-cAMPS may induce another effect in *D. discoideum*. However, each effect induced by (Rp)-cAMPS must be analyzed carefully, because the compound activates cAMP-dependent protein kinase and because the compound is not chemically stable. Nevertheless, (Rp)-cAMPS appears to be the most interesting cAMP derivative, because it is not a cAMP antagonist sensu stricto; (Rp)-cAMPS is an antagonist for the activation and adaptation of adenylate and guanylate cyclase but an agonist for the induction of down-regulation.

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