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Original Article

Actions of Benzaldehyde Hydrazones and Semicarbazones on Biogenic Amine Receptors in the Silkworm *Bombyx mori*

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Four hydrazones (HZs) and six semicarbazones (SCZs) of substituted benzaldehydes were synthesized and examined for their ability to control insect adenylate cyclase through their interaction with biogenic amine receptors. Among the compounds synthesized, two with a hydroxyl group at the 4-position of the phenyl moiety, HZ-01 and SCZ-03, were found to reduce the basal levels of cAMP in head membrane homogenates of fifth instar larvae of the silkworm *Bombyx mori*. The semicarbazone SCZ-03 dose-dependently attenuated not only basal but also forskolin-stimulated cAMP levels. Tyramine (TYR) and dopamine (DPM) also produced a dose-dependent reduction in basal cAMP levels. DPM and TYR receptor antagonists abolished the attenuating effects of SCZ-03. These findings suggest that SCZ-03 acts as a non-selective agonist for DPM and TYR receptors to inactivate adenylate cyclase in *B. mori* larvae.

Key words: tyramine receptor, octopamine receptor, dopamine receptor, adenylate cyclase, cyclic AMP, *Bombyx mori*.

INTRODUCTION

Biogenic amines are a group of compounds derived from aromatic amino acids, and have neurotransmitter, neuromodulator, and neurohormonal roles. Much attention has been paid to biogenic amines because of their involvement in numerous important physiological processes and neurological disorders. In invertebrates, five out of seven known members of this group have apparent physiological significance.[1] Biogenic amines exert their effects primarily through interaction with G-protein-coupled receptors, although biogenic amine-gated ion channels are also known to exist.

Adrenaline and noradrenaline, physiologically important vertebrate biogenic catecholamines, have no physiological role in invertebrates. In invertebrates, two phenolic amines, octopamine (OCT; Fig. 1) and tyramine (TYR; Fig. 1), are utilized instead of the catecholamines. Though the functional roles of OCT in invertebrates have been studied extensively,[2] little is known about TYR. Together with several reported physiological effects of TYR[1,3] and its fulfillment of the criteria to be a neurotransmitter in the locust brain,[4] however, the unequivocal identification of TYR receptors in several insect species[5–8] supports the notion that TYR is not only the precursor for the biosynthesis of OCT but also functions as a neurotransmitter or neuromodulator. OCT and TYR generally elicit opposite effects on intracellular cAMP levels through their interaction with distinct receptors; OCT activates adenylate cyclase via G_s-protein to increase cAMP levels, while TYR inactivates adenylate cyclase via G_i-protein to reduce cAMP levels. Dopamine (DPM; Fig. 1), a catecholamine, is present in both vertebrates and invertebrates. Our knowledge of the physiological role of DPM in insects is fragmental. The detection of DPM-activated adenylate cyclase activity in insect tissue indicates that some of the effects of DPM are mediated through an increase in intracellular cAMP levels.[9–11] Two types of DPM receptors, both of which activated adenylate cyclase to elevate intracellular cAMP levels, were isolated from the fruit fly *Drosophila melanogaster*[12–15] and the honeybee *Apis mellifera*.[16] On the other hand, DPM receptors, the activation of which leads to decreased production of cAMP, have also been identified[17,18] and cloned.[19]

We have recently reported that OCT and TYR regulate adenylate cyclase positively and negatively, respectively, in the membrane homogenates of the heads of silkworm (*Bombyx mori*) larvae. Using this *B. mori* assay system, we discovered 5-(4-hydroxyphenyl)oxazole as a possible tyramine agonist, which affects adenylate cyclase

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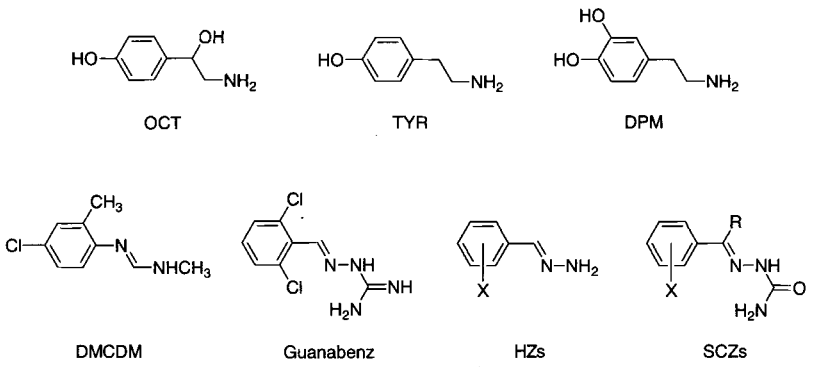

Fig. 1. Structures of biogenic amines and synthetic agonists.

negatively. The new discovery of ligands would facilitate research into the biogenic amine receptor, particularly because no specific tyramine receptor agonists have been found to date. In our continued research, we found that 4-hydroxybenzaldehyde hydrazone and semicarbazone have similar negative effects on *B. mori* adenylate cyclase. We report here the ability of 4-hydroxybenzaldehyde semicarbazone to reduce cAMP levels in the membrane homogenates of the heads of *B. mori* larvae.

MATERIALS AND METHODS

1. *Chemicals*

Racemic OCT·HCl was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). TYR·HCl and DPM·HCl were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Chlorpromazine·HCl (CPZ), *R*(+)-SCH-23390·HCl (SCH), yohimbine·HCl (YHM), and spiperone (SP) were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). [^{3}H]cAMP (20 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA).

2. *General Procedures for the Synthesis of Hydrazones and Semicarbazones*

Hydrazones (HZs; Fig. 1) and semicarbazones (SCZs; Fig. 1) were readily prepared by the reactions of substituted benzaldehydes with hydrazine and semicarbazine, respectively. For the synthesis of HZs, substituted benzaldehyde (4.2 mmol) in ethanol (3 ml) was added to hydrazine hydrate (16.6 mmol) and heated at 80°C for 24 hr. After the heating, the reaction mixture was cooled in an ice bath for 2 hr. The resulting solid was collected on filter paper and washed with ethanol (100 ml) to afford a substituted HZ as a crystalline product.

For the synthesis of SCZs, a solution of semicarbazine·HCl (0.5 g) plus sodium acetate (0.75 g) in water (5 ml) and a solution of substituted benzaldehyde (0.5 g) in ethanol (3 ml) were prepared. After each solution was heated in a steam bath for several minutes, the aqueous solution was poured into the alcohol solution. After the mixture had been swirled in the steam bath for 5 min, it was poured into water (5 ml). The resulting mixture was cooled in an ice bath until precipitates appeared. Oily products were solidified by rubbing them with a glass rod. The solids were collected on filter paper and washed with water (100 ml). The residue was recrystallized from methanol to give an SCZ.

The chemical structures and purity of HZs and SCZs thus synthesized were confirmed by TLC, mass spectrometry (MS), and ^{1}H NMR spectrometry. ^{1}H NMR spectra were measured, using tetramethylsilane as an internal standard on a JEOL JNM-A400 (400 MHz) instrument. Mass spectra were obtained at 70 eV on a Hitachi M-80B spectrometer. Melting points were determined with a Yanako MP-500D apparatus and are uncorrected. HZ-01: mp 285.5-287.6°C; ^{1}H NMR δ_H (DMSO-d_6): 6.39 (2H, bs, NH_2), 6.71 (2H, d, J = 8.5 Hz, Ph), 7.29 (2H, d, J=8.5 Hz, Ph), 7.62 (1H, s, CH), 9.51 (1H, s, OH); EIMS *m/z*: 136 (M^+). SCZ-03: mp 221.2-223.6°C; ^{1}H NMR δ_H (DMSO-d_6): 6.37 (2H, bs, NH_2), 6.76 (2H, d, J=8.5 Hz, Ph), 7.52 (2H, d, J=8.5 Hz, Ph), 7.73 (1H, s, CH), 9.73 (1H, s, OH), 10.02 (1H, s, NH); EIMS *m/z*: 179 (M^+).

3. *Insects*

Eggs of *B. mori* (Kinshu-Showa) and an artificial diet were purchased from Ueda Silkworm-Eggs Coop. (Ueda, Japan). The larvae were reared on the artificial diet at 25°C.

4. *Preparation of B. mori Head Homogenates*

Heads (stored frozen at −80°C) of eight fifth instar larvae of *B. mori* were homogenized in 3 ml of 10 mM Tris-maleate buffer (pH 7.4) with a glass-Teflon homogenizer. The homogenate was centrifuged at 500 *g* for 10 min, and the pellets were again homogenized and centrifuged in the same manner. The combined supernatant was centrifuged at 25,000 *g* for 10 min. The pellets were superficially washed with the buffer, resuspended in the buffer, and allowed to stand in an ice-bath for 15 min. The suspension was recentrifuged at 25,000 *g* for 10 min. The pellets were washed superficially and resuspended in the buffer. Lastly, the protein concentration was determined as described by Bradford.[20]

5. Adenylate Cyclase Assay

The initial reaction medium consisted of (a) 50 μl of 190 mM Tris-maleate buffer (pH 7.4) containing 25 mM theophylline, 20 mM $MgCl_2$, 0.25 mM GTP, and 1.25 mM EGTA; (b) 12.5 μl of an aqueous solution containing a compound(s); and (c) 50 μl of the *B. mori* head membrane suspension (20 μg protein). After the medium had been preincubated at 30°C for 1 min, the reaction was started by the addition of 12.5 μl of 20 mM ATP. The reaction mixture was incubated at 30°C for 10 min, and then the reaction was stopped by heating the mixture in boiling water for 2 min. The mixture was centrifuged at 14,000 *g* for 10 min, and 50 μl of the supernatant was used to determine the cAMP level. The level of cAMP production was determined by the method of Munirathinam and Yoburn.[21] The radioactivity was measured using an LS 6000SE liquid scintillation counter (Beckman Coulter, Inc., CA). Test compounds were first dissolved in dimethyl sulfoxide (DMSO) and diluted with distilled water. The final concentration of DMSO was 0.4%, which did not affect adenylate cyclase activity. The assay was repeated three times or more.

RESULTS

1. Effects of HZs and SCZs on B. mori Adenylate Cyclase

Four HZs and six SCZs were synthesized, with the structure of the α_2-adrenergic receptor agonist guanabenz (Fig. 1) in mind. The synthesized compounds were examined for their ability to modulate the levels of cAMP generated by adenylate cyclase in the head membrane homogenates of the fifth instar larvae of *B. mori*. Figure 2 shows the effects of 100 μM HZs and SCZs on basal cAMP production, where the production stimulated by 100 μM OCT is presented as 100%. In HZs, the 4-hydroxyl analogue HZ-01 produced an approximately 36% reduction in cAMP levels. Three other HZs were only marginally effective or almost inactive, although the 2,6-dichloro analogue (HZ-02), bearing the same substitution on the benzene ring as that of guanabenz, caused a slight increase in cAMP levels as compared to basal levels. Among SCZs, SCZ-03 with a 4-hydroxyl group on the benzene ring showed a more remarkable attenuating effect on cAMP production than did HZ-01. The inhibitory effect of SCZ-03 on basal cAMP production (9.5 $\pm$ 2.5 pmol/mg protein/min) was dose-dependent in the range from 10 nM to 1 mM, as shown in Fig. 3a. We further tested whether SCZ-03 attenuates the production of cAMP stimulated by forskolin, a direct activator of adenylate cyclase. As shown in Fig. 3b, SCZ-03 (100 nM–1 mM) dose-dependently depressed the 5 μM forskolin-stimulated

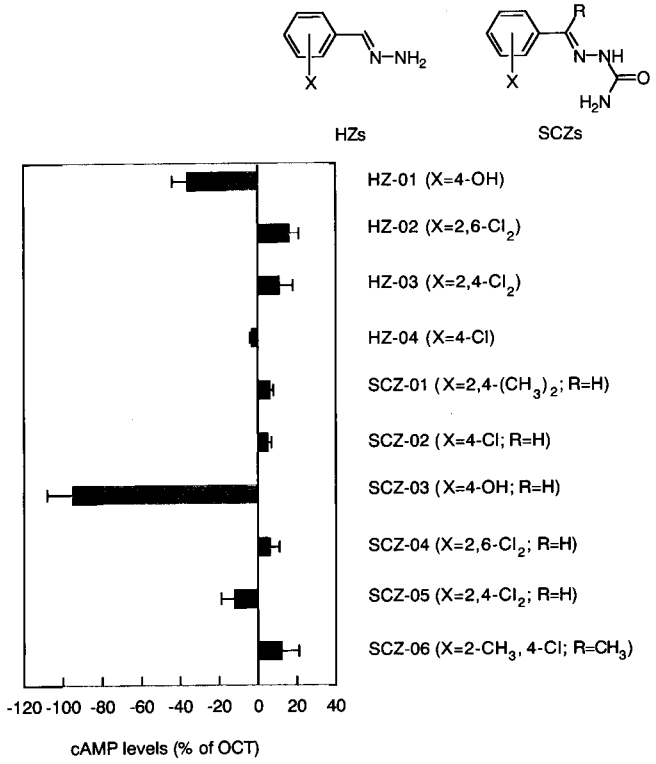

Fig. 2. Effects of 100 μM HZs and SCZs on basal cAMP production in *B. mori* head homogenates.

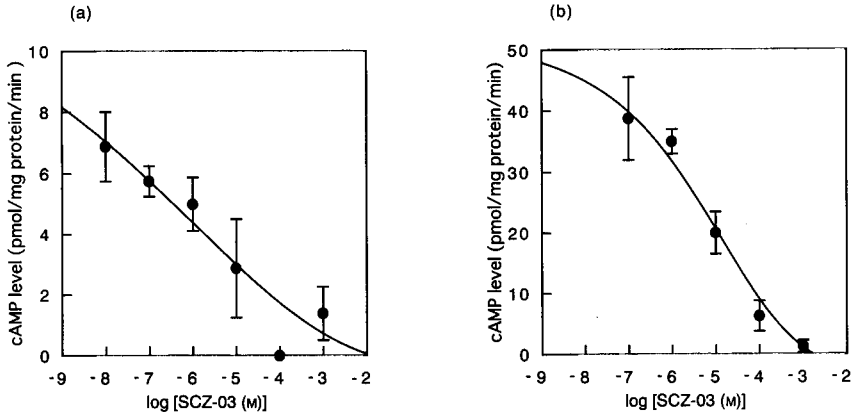

Fig. 3. Dose-response curves of SCZ-03 in attenuating (a) basal and (b) forskolin (5 μM)-stimulated cAMP levels in *B. mori* head homogenates.

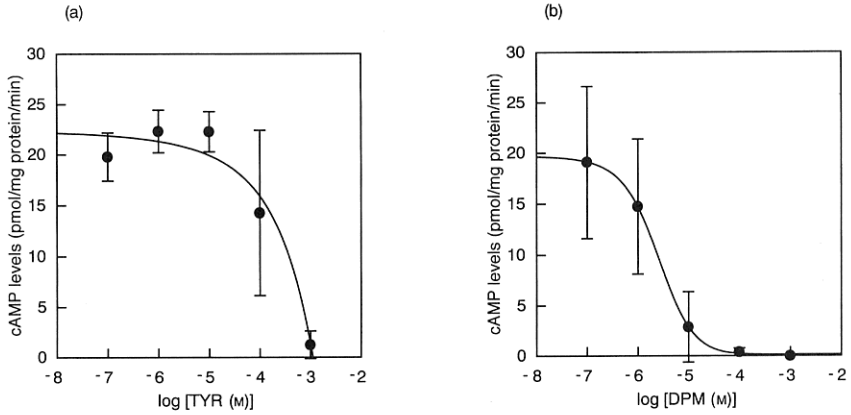

Fig. 4. Dose-response curves of (a) TYR and (b) DPM in attenuating basal cAMP production in *B. mori* head homogenates.

cAMP levels (57.5 ± 3.3 pmol/mg protein/min). Other SCZs showed little activity. A 2-methyl-4-chloro substitution was introduced into the phenyl moiety to mimic the substitution pattern of the OCT receptor agonist demethylchlordimeform (DMCDM; Fig. 1), but this substitution turned out to be ineffective.

2. Effects of TYR and DPM on B. mori Adenylate Cyclase

As SCZ-03 showed a remarkable negative effect on cAMP production, we conducted experiments to determine whether biogenic amines regulate *B. mori* adenylate cyclase positively or negatively. In a previous study,[22] we were able to show that OCT elevates cAMP levels in the head membrane homogenates of *B. mori* larvae. In contrast to OCT, TYR had negative effects. TYR at 1 mM reduced the basal level of cAMP production (24.8±4.8 pmol/mg protein/min) to 1.3±1.4 pmol/mg protein/min (Fig. 4a). On the other hand, DPM at 100 μM attenuated the basal level of cAMP production (18.9±6.0 pmol/mg protein/min) to 0.4±0.4 pmol/mg protein/min (Fig. 4b). These findings indicate that both TYR and DPM receptors that are negatively coupled to adenylate cyclase are expressed in the heads of the fifth instar larvae of *B. mori*, although considering that the potency of TYR is two orders of magnitude lower than that of DPM, the possibility that TYR cross-reacts with DPM receptors cannot be ruled out.

3. Effects of Antagonists on SCZ-03-Attenuated cAMP Levels in B. mori Head Membranes

To examine which receptor mediates the negative effect of SCZ-03 on cAMP production, we examined the effects of the TYR receptor antagonist YHM as well as the DPM receptor antagonists CPZ, SCH and SP on SCZ-03 (10 μM)-attenuated cAMP production in *B. mori* head homogenates. As shown in Fig. 5, all antagonists tested at 100 μM almost restored the SCZ-03-induced 38% reduction of cAMP production to levels close to basal levels (SP could not be tested at 100 μM because of solubility problems). When tested at 10 μM, however, all antagonists appeared to be ineffective.

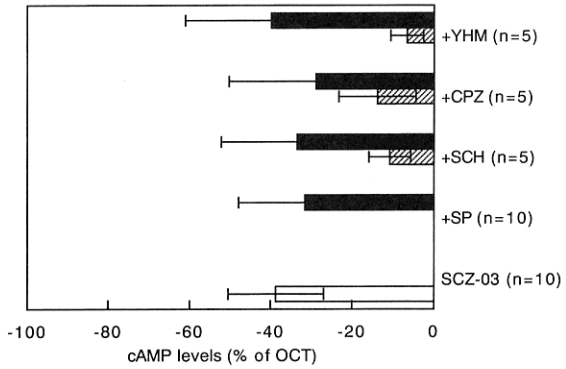

Fig. 5. Effects of TYR and DPM receptor antagonists on SCZ-03 (10 μM)-attenuated cAMP levels (open bar) in *B. mori* head homogenates. Solid and hatched bars indicate cAMP levels in the presence of 10 μM and 100 μM antagonists, respectively. SP could not be tested at 100 μM because of solubility problems.

DISCUSSION

In the present study we performed experiments to discover agonists for biogenic amine receptors. Benzaldehyde hydrazones and semicarbazones were designed as analogues of the α_2-adrenergic receptor agonist guanabenz. A hydrazone (HZ-02), bearing the same substitution on the benzene ring (i.e., 2,6-Cl_2) as that of guanabenz, displayed an adenylate cyclase activation only approximately 16% of that induced by 100 μM OCT in *B. mori* head homogenates (Fig. 2). With respect to the activation of adenylate cyclase, other compounds including SCZs were even less active than this compound. However, two compounds, HZ-01 and SCZ-03, were found to have the ability to reduce basal cAMP production, and the latter compound reduced forskolin-stimulated cAMP levels as well (Fig. 3). The basal cAMP production was brought about by the activation of adenylate cyclase probably with an unidentified endogenous factor(s) or substance(s), as we observed a linear increase in cAMP levels with time during the incubation of *B. mori* head

membranes alone (data not shown). These findings clearly show that SCZ-03 exerts an attenuating effect on adenylate cyclase.

We have reported in a previous paper that 5-(4-hydroxylphenyl)oxazole and the 4-cyanophenyl congener reduced basal and OCT-stimulated cAMP production, and that the effect of the former compound was abolished by the TYR antagonist YHM.[23] The DMP antagonists CPZ and SCH had no effect (unpublished data). This finding indicates that these compounds might be TYR receptor agonists. Both HZ-01 and SCZ-03 possess a hydroxyl group at the 4-position of the phenyl moiety. All of these compounds that showed attenuating effects on cAMP production had a hydroxyl group or a cyano group, both of which can work as a hydrogen bond acceptor. This fact leads to speculation that the hydrogen bond formation with an amino acid residue(s) in the binding site of biogenic amine receptors is involved in the negative effects of these compounds.

There are numerous reports that biogenic amines modulate adenylate cyclase in various ways through interaction with their G-protein-coupled receptors. OCT generally activates adenylate cyclase to raise intracellular cAMP levels through interaction with cloned OCT receptors[24-26] as well as native OCT receptors in the nervous tissue.[9] A negative effect of TYR on adenylate cyclase resulting in decreased cAMP levels has been observed in *Drosophila* head membrane homogenates[27] and cloned TYR receptor preparations.[5-8] DPM elevated cAMP production in various tissues of insects.[9,10,28] In experiments using the corpora allata and brain of the fifth instar *Manduca sexta* larvae, DPM had stage-specific effects on cAMP production; DPM stimulated cAMP production in day 0 larvae but inhibited the production in day 6 larvae.[18] cDNAs encoding two different DPM receptors, both of which elicited the production of cAMP after the application of DPM and so might correspond to vertebrate D_1-like DPM receptors, were isolated from *Drosophila*,[12-15] and a homologue for one of the two *Drosophila* DPM receptors was isolated from *A. mellifera*.[16] cDNAs encoding *A. mellifera* DPM receptors that are negatively coupled to adenylate cyclase and so might correspond to vertebrate D_2-like DPM receptors have also been identified.[17] More recently, cDNAs encoding D_2-like DPM receptors have been isolated from *D. melanogaster*.[19]

As mentioned above, not only TYR receptors but also DPM receptors have been implicated in the negative modulation of adenylate cyclase in some insect species. The data presented here also indicate that both TYR and DPM receptors that negatively regulate adenylate cyclase can be readily detected in the heads of the fifth instar larvae of *B. mori* (Fig. 4). To determine which of the receptors is involved in the negative effects of SCZ-03, we examined the ability of several antagonists to restore SCZ-03-attenuated cAMP levels. SCH is a selective antagonist for vertebrate D_1-like DPM receptors, whereas CPZ and SP are selective antagonists for vertebrate D_2-like DPM receptors.[29] In *A. mellifera* D_1-like DPM receptors,[16] the rank order of the potency of antagonists was: CPZ > SP > SCH. SCH was also less active than SP in one of the cloned *Drosophila* D_1-like DPM receptors,[12,13] but in another of the cloned *Drosophila* D_1-like DPM receptors the order was reversed.[14] On the other hand, SP had no activity in *Drosophila* D_2-like DPM receptors.[19] DPM receptor antagonists such as SP and CPZ were also reported to cross-react with TYR receptors.[5,30] When tested at 100 μM, the DPM receptor antagonists SCH and CPZ as well as the TYR receptor antagonist YHM showed antagonistic activity for the SCZ-03-induced decrease in cAMP levels, as shown in Fig. 5. In an attempt to identify which receptor is most related to the negative effect of SCZ-03, we performed similar assays using a lower concentration (10 μM) of antagonists, but failed to determine the rank order of the potency of antagonists because of variation in data. Our data suggest that SCZ-03 appears not to be a selective agonist for DPM receptors or TYR receptors, although the negative effect of SCZ-03 is mediated through its interaction with the biogenic amine receptors.

In conclusion, we showed that a hydrazone and semicarbazone of 4-hydroxybenzaldehyde dose-dependently elicit a decrease in cAMP levels in the heads of *B. mori* larvae. The hydroxyl group of these compounds appears to be responsible for the action, because no other analogues with other substituents showed such effects. The attenuating effect of SCZ-03 was abolished by DPM and TYR receptor antagonists, indicating that at least two biogenic amine receptors, DPM and TYR receptors, are involved in the attenuation of cAMP levels by SCZ-03. The cAMP assay system using *B. mori* larvae might provide an opportunity to discover selective agonists for biogenic amine receptors. The *in vivo* activity of SCZ-03 and related compounds will be reported elsewhere.

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