The Journal of Experimental Biology 200, 2669–2674 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JEB1025

CYCLIC AMP INDUCES A RELAXATION RESPONSE IN THE BULLFROG *RANA CATESBEIANA*, BUT NITRIC OXIDE DOES NOT

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Accepted 23 July 1997

Summary

Cholecystokinin octapeptide (CCK), acetylcholine (ACh) and ceruletide have been shown to produce contraction in bullfrog (*Rana catesbeiana*) gallbladder strips. Agents capable of relaxing the bullfrog gallbladder are less numerous. Calcitonin gene-related peptide reduced the amount of both CCK- and ACh-induced tension in bullfrog gallbladder strips. The purpose of this study was to determine whether vasoactive intestinal peptide (VIP), nitric oxide (NO) and the second messengers cyclic GMP or cyclic AMP had any effect on gallbladder motility in the bullfrog. *In vitro* tension studies using L-N^G-nitro-arginine methyl ester, Methylene Blue, sodium nitroprusside and N^2,2′-*O*-dibutyryl guanosine 3′,5′-cyclic monophosphate suggested that nitric oxide did not modulate gallbladder motility in the bullfrog gallbladder. Histochemical staining for NADPH diaphorase (nitric oxide synthase) failed to demonstrate nerve fibers containing nitric oxide synthase in the bullfrog gallbladder. *In vitro* studies demonstrated that VIP had no effect on CCK-induced tension. However, *in vitro* studies using either 8-bromoadenosine 3′,5′-cyclic monophosphate or forskolin demonstrated that both agents relaxed strips precontracted with CCK. The results of this study suggested that, while neither NO nor VIP had a role in modulating bullfrog gallbladder motility, cyclic AMP was capable of modulating bullfrog gallbladder motility.

Key words: vasoactive intestinal peptide, nitric oxide, cyclic AMP, cyclic GMP, gallbladder, smooth muscle contraction, bullfrog, *Rana catesbeiana*.

Introduction

Calcitonin gene-related peptide (CGRP) is a neuropeptide that is an alternative product of the calcitonin gene preferentially expressed in nervous tissues (Rosenfeld *et al.* 1983). CGRP induces a concentration-dependent relaxation of cholecystokinin octapeptide (CCK)-induced contraction in guinea pig gallbladder strips. This relaxation is due to both a direct action of CGRP on the smooth muscle and an indirect action caused by induced activity in nonadrenergic noncholinergic pathways (Kline and Pang, 1992). CGRP has vasorelaxant effects in the bullfrog (*Rana catesbeiana*) femoral artery (Kline *et al.* 1988).

CGRP-like material has been demonstrated in bullfrog sympathetic ganglia (Kuramoto and Fujita, 1986) and in the dorsal horn of the spinal cord of *Rana esculenta* (Venesio *et al.* 1987). CGRP affects synaptic transmission at the neuromuscular junction of the frog *Rana temporaria* (Caratsch and Eusebi, 1990). CGRP is present in nerves supplying the internal gills of larval bullfrogs (Kusakabe and Kawakami, 1992; Kusakabe *et al.* 1993) and in the pharynx, the lungs and the carotid labyrinth (Kusakabe *et al.* 1994) of the bullfrog. CGRP may thus have a role in regulating the respiratory systems of some amphibians throughout their life (Kusakabe *et al.* 1995).

It has been suggested that nitric oxide (NO) is an important regulatory transmitter in the gastrointestinal system of several species. In the rat, inhibitors of NO synthesis induced dose-dependent increases in the intraluminal pressure of the gut and initiated phasic intestinal contractions (Calignano *et al.* 1992; Kanada *et al.* 1992). In the guinea pig gallbladder, NO derived from the NO donor sodium nitroprusside (SNP) abolished the contraction in response to low doses of CCK and reduced the response to a higher dose (1.2 nmol kg^{-1}) of CCK. The use of inhibitors of NO synthesis led to a significant enhancement of the contractile response to CCK or bethanecol (Mourelle *et al.* 1993). In the guinea pig gallbladder, part of the CGRP-mediated relaxation response was mediated by NO (Kline and Pang, 1994*a*). NO stimulates guanylyl cyclase to increase intracellular cyclic GMP concentrations, which leads to relaxation of smooth muscle (Archer *et al.* 1994).

The presence of CGRP-like material in the small intestine and gallbladder of the green iguana *Iguana iguana* has been demonstrated using immunocytochemical techniques (Ohtani *et al.* 1989; Kline and Pang, 1994*b*). In the iguana, CGRP was shown to relax CCK-induced tension. The relaxation was mediated by CGRP acting directly on the smooth muscle. The

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relaxation may also have been mediated, in part, by NO (Kline and Pang, 1994*b*). When strips were treated with CGRP prior to treatment with either acetylcholine (ACh) or CCK, the amount of tension in bullfrog gallbladder strips was less than in strips not treated with CGRP (Kline *et al.* 1992).

In addition to NO and CGRP, vasoactive intestinal peptide (VIP) has also been shown to have relaxant properties in the gallbladder of several species. Aldman and Holmgren (1992) demonstrated *in vivo* that CCK-induced contractions of the gallbladder were reduced in a dose-dependent manner by VIP in the rainbow trout *Oncorhychus mykiss*. VIP induced a dose-dependent relaxation of both the feline gallbladder and canine gallbladder strips (Dahlstrand *et al.* 1989; Kalfin and Milenov, 1991).

The relaxant action of cyclic AMP on both rabbit and guinea pig gallbladder strips precontracted with CCK was first described by Amer (1972) and Andersson *et al.* (1972). Both cyclic AMP and N^6-2′-*O*-dibutyryl adenosine 3′,5′-cyclic monophosphate (db-cAMP) were reported to produce a relaxation.

Little has been reported on the mechanisms regulating bullfrog gallbladder motility, nor has much been reported on the role of NO in regulating smooth muscle activity in the gastrointestinal tract of the bullfrog. The purpose of this study was to determine whether NO, VIP or cyclic AMP had a role in mediating bullfrog gallbladder contraction.

Materials and methods

Mature bullfrogs (*Rana catesbeiana* Shaw, 1802) were used (body mass 213±11 g) throughout these investigations.

In vitro *studies*

Bullfrogs were anesthetised in tricaine methanesulfonate and pithed, and the gallbladder was removed and placed in frog Ringer's solution (FRS) containing (in mmol l^{-1}), NaCl, 80; KCl, 2.5; $CaCl_2$, 1.8; $NaHCO_3$, 24; NaH_2PO_4, 0.12; and glucose, 11.0 g l^{-1}. Each gallbladder was cut into strips (1.5 mm×5 mm) which were secured in Sawyer-Bartlestone chambers containing FRS, gassed with 95 % O_2/5 % CO_2 and maintained at room temperature. A resting tension of 0.2 g was found to provide an optimal baseline tension (Kline *et al.* 1992).

The tension developed by the strips was measured using Grass FT-03D force displacement transducers and recorded on a Grass 7D polygraph. Isolated strips were equilibrated for 45 min prior to determining whether they were suitable for use. The test for suitability was performed using 10^{-4} mol l^{-1} carbachol. A minimum tension of 0.1 g had to be generated before the strips were used further. The test was repeated twice with an equilibration time of 30 min between tests.

Five series of experiments were performed on the bullfrog gallbladder strips to determine whether NO had a role in mediating gallbladder motility. The first series of experiments examined the effects of CGRP (300 ng ml^{-1}) on CCK-induced tension. CCK induced tension in the gallbladder strips which reached a steady level after 3 min (Kline *et al.* 1992). This tension remained steady for approximately another 8 min. Four minutes after the addition of CCK, CGRP was added to the chambers to determine whether CGRP relaxed the strips, as had been observed in the guinea pig (Kline and Pang, 1992) and iguana (Kline and Pang, 1994*b*). In these and all the other experiments, the agents were added directly to the chambers. The next series of experiments examined the effects of L-N^G-nitro-arginine methyl ester (L-NAME, 10 and 100 µmol l^{-1}) on the CCK-induced tension. L-NAME was added to the chambers 10 min prior to the CCK, which induced tension in the strips. The third series of experiments examined the effects of Methylene Blue (30 or 60 µmol l^{-1}) on the CCK-induced tension. Methylene Blue was added to the chambers 10 min prior to the CCK in the same manner as was the L-NAME. The next series of experiments used sodium nitroprusside (SNP) to determine whether NO had a role in mediating relaxation of the bullfrog gallbladder. SNP was added in a stepwise manner (25, 50, 100 or 200 µmol l^{-1}) to determine whether a concentration-dependent effect occurred. The last series of experiments used N^2,2′-*O*-dibutyryl guanosine 3′,5′-cyclic monophosphate (db-cGMP). After the CCK-induced tension had reached a steady level, db-cGMP (0.1, 1.0 and 10 µmol l^{-1}) was added to the chambers to determine whether the gallbladder smooth muscle relaxed.

Three other series of experiments were performed to determine whether an agent might affect bullfrog gallbladder motility. The first series of experiments used forskolin. Initially, forskolin was added to the chambers following the same protocol as CGRP. After observing a significant relaxation using 1 µmol l^{-1} forskolin, the experiments were repeated using 1 µmol l^{-1}, 0.1 µmol l^{-1}, 0.01 µmol l^{-1} and 1 nmol l^{-1} forskolin. The second series of experiments used 8-Br-cAMP (1 mmol l^{-1}). The strips were precontracted with CCK. After 4 min, the 8-Br-cAMP was added to the chambers and the relaxation measured. The last series used VIP to determine whether it would induce relaxation of gallbladder strips precontracted with CCK. Two concentrations were used, 0.1 and 1 µmol l^{-1}. VIP was added to the chambers exactly as in the protocol described for CGRP.

Histochemical studies

Gallbladders from three bullfrogs and three green iguanas (*Iguana iguana*) were stained for NADPH diaphorase (nitric oxide synthase, NOS) using a method modified from Scherer-Singler *et al.* (1983), Vincent and Kimura (1992) and Morin and Stanboli (1993). The iguana gallbladders were used as positive controls since NO has been shown to be involved in CGRP-induced relaxation (Kline and Pang, 1994*b*). The gallbladders of both species were carefully pealed into three layers and fixed with 4 % paraformaldehyde in 0.1 mol l^{-1} phosphate-buffered saline (PBS) for 2 h. The tissues were then preincubated with 3 % Triton X-100 in PBS and incubated in a dark chamber with NADPH (1 mmol l^{-1}), Nitroblue Tetrazolium (0.5 mol l^{-1}) and 0.3 % Triton X-100 in PBS for

1 h at 37 °C. After rinsing the tissues with PBS, the tissues were mounted for viewing under light microscopy.

Green iguana gallbladder strips have been reported to respond to L-NAME. This suggested that NO may mediate part of the observed CGRP-induced relaxation response (Kline and Pang, 1994*b*). Six young green iguanas (body mass 150±10 g) were used to compare the response of gallbladder strips, which probably respond to NO, with the responses of the bullfrog strips. The iguana gallbladder strips were prepared and tested for use in a manner similar to the bullfrogs, except that reptilian Ringer's solution was used (Kline and Pang, 1994*b*). The iguana strips were used in a series of experiments using the same concentrations of SNP (25, 50, 100 and 200 μmol l^{-1}) as with the bullfrog gallbladder strips in order to determine whether a concentration-dependent relaxation of CCK-induced tension could be measured. A second series of experiments using 30 μmol l^{-1} Methylene Blue was performed to determine whether Methylene Blue had an effect on the CGRP-induced relaxation response in the iguana gallbladder strips.

Statistical analysis was performed using either the paired *t*-test or two-way analysis of variance (ANOVA) and the Newman–Kuels test.

Results

Nitric oxide and cyclic GMP studies

No relaxation was observed when CGRP was added to the chambers after the CCK-induced tension in the bullfrog gallbladder strips had reached a steady level. The use of two concentrations of L-NAME (10 or 100 μmol l^{-1}), which blocks NOS, did not significantly increase CCK-induced tension. If NO mediates relaxation, the use of a NOS blocker frequently results in an increase in smooth muscle tension when stimulated with an agonist. Similarly, the use of two concentrations of Methylene Blue (30 or 60 μmol l^{-1}), an inhibitor of soluble guanylyl cyclase, had no significant effect on the CCK-induced tension. Methylene Blue produced a significant (P<0.01) decrease in the CGRP-induced relaxation in the iguana gallbladder strips (CGRP only, 59±4.8 %; with Methylene Blue, 39±4.1 %; values are means ± S.E.M., N=8). The NO donor SNP, when used in a cumulative concentration manner, had no significant relaxation effect on the bullfrog gallbladder strips. In comparison with the bullfrog gallbladder strips, the use of SNP had a significant relaxation effect in the iguana gallbladder strips (Fig. 1). When db-cGMP (0.1, 1.0 or 10 μmol l^{-1}) was administered in a stepwise manner, to obtain a cumulative concentration effect, no significant effect on CCK-induced tension was observed (data not shown).

Histochemistry

There was little, if any, evidence of NOS in the smooth muscle layers of the bullfrog gallbladder (Fig. 2). However, NOS-like material was observed in the iguana gallbladder. An abundant network of nerve fibers staining for NOS was observed in the smooth muscle layers (Fig. 3). This further confirmed the *in vitro* results which suggested that NO had no role in mediating bullfrog gallbladder motility.

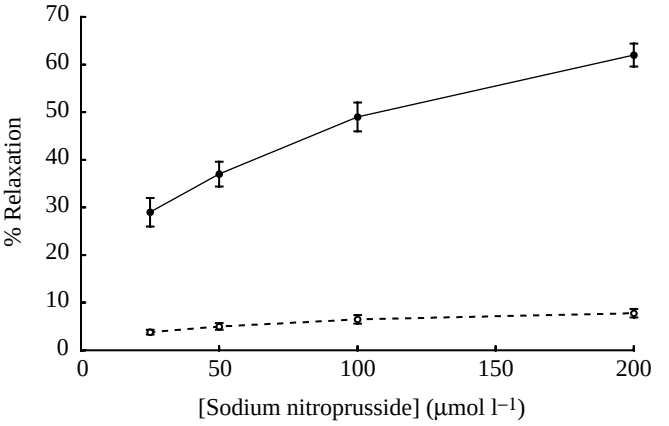

Fig. 1. The cumulative addition of sodium nitroprusside, a nitric oxide donor, had no significant effect on bullfrog *Rana catesbeiana* gallbladder strips (broken line). Iguana *Iguana iguana* gallbladder strips (solid line) responded to the addition of sodium nitroprusside with a concentration-dependent relaxation in CCK-induced tension. Values are means ± S.E.M., N=8.

Forskolin, 8-Br-cAMP and VIP

Forskolin, which activates adenylyl cyclase, induced relaxation of bullfrog gallbladder strips precontracted with CCK. Fig. 4 demonstrates that forskolin produced a concentration-dependent relaxation of CCK-induced tension. Since forskolin exerted its actions by activating adenylyl cyclase, 8-Br-cAMP, a membrane-permeable analog of cyclic AMP, was used to confirm that the relaxation observed was mediated by increases in intracellular cyclic AMP concentration. The use of 1 mmol l^{-1} 8-Br-cAMP produced a 30.3±8.2 % (N=8) relaxation in the tension induced by 1 nmol l^{-1} CCK. Since the effects of VIP have been shown to be mediated by cyclic AMP, cyclic AMP was used to determine whether VIP was able to relax the bullfrog gallbladder. Neither concentration of VIP used had an effect on strips precontracted with CCK (data not shown, N=8).

Discussion

There have been few reports on the regulation of bullfrog gallbladder motility. A report on the toad *Bufo marinus* described the presence of CGRP-containing neurons in the toad gallbladder. The nerves also stained for substance P. VIP-containing fibers were also described in the toad gallbladder (Davies and Campbell, 1994). In addition, NOS-containing nerves have been described in the gastrointestinal tract of *Bufo marinus* (Li *et al.* 1992; Murphy *et al.* 1993). In the guinea pig, VIP-containing nerves were also found to contain NOS (Talmage and Mawe, 1993). These authors suggested that VIP/NADPH-diaphorase-containing neurons could represent an intrinsic inhibitory system of motor neurons which relaxed the gallbladder muscles while it was filling. A similar situation could exist in the toad.

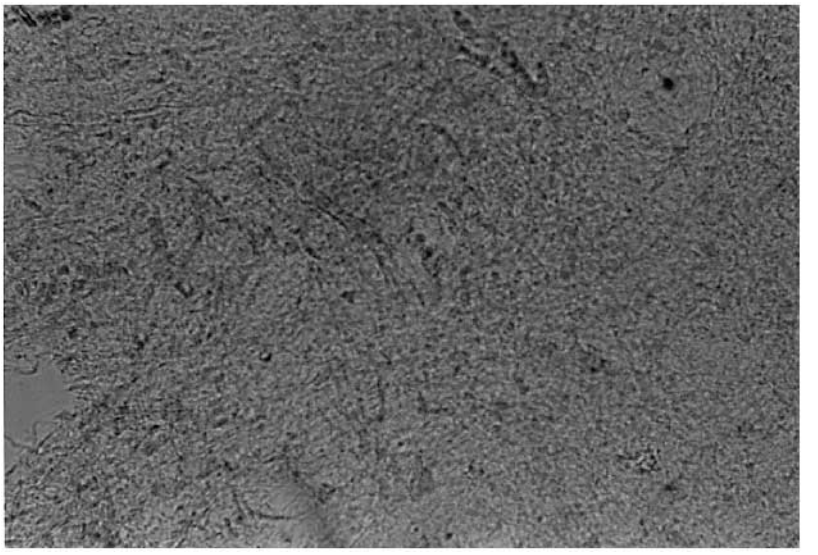

Fig. 2. A bullfrog *Rana catesbeiana* gallbladder stained for NADPH diaphorase (nitric oxide synthase). No nerve fibers containing nitric oxide synthase were observed. Magnification ×30.

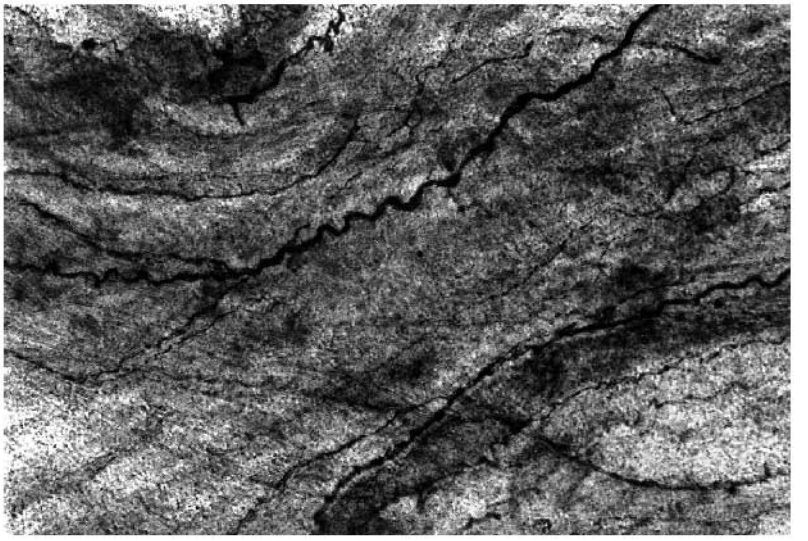

Fig. 3. Iguana *Iguana iguana* gallbladder strips stained for NADPH diaphorase (nitric oxide synthase). A rich network of fibers can be seen in the smooth muscle layers. Magnification ×60.

In the present study using the bullfrog, the use of CGRP did not cause a relaxation response during CCK-induced contraction. In the iguana and guinea pig gallbladder, the use of a NOS inhibitor, L-NAME, caused an increase in the amount of CCK-induced contraction. In addition, Methylene Blue, an inhibitor of soluble guanylyl cyclase, caused an increase in the amount of CCK-induced contraction in the guinea pig gallbladder (Kline and Pang, 1994*a*,*b*; Mourelle *et al.* 1993). However, in the bullfrog gallbladder, these agents had no significant effect on CCK-induced tension. This lack of a response suggested that NO may not mediate bullfrog gallbladder motility. The NO donor SNP did not relax CCK-induced contraction in the bullfrog. This result is different from the effects of NO in the guinea pig strips (Mourelle *et al.* 1993; Kline and Pang, 1994*a*). In iguana gallbladder strips, the NO donor SNP produced a concentration-dependent relaxation. This suggested that in the bullfrog gallbladder, unlike the iguana gallbladder, NO does not have a role in regulating motility. The lack of histochemical localization of NOS in the bullfrog gallbladder further confirmed the *in vitro* results. The positive staining for NOS in the iguana gallbladder, which is known to use NO to regulate its motility (Kline and Pang, 1994*b*), confirmed the utility of the methodology used. Lastly, the addition of db-cGMP had no effect on CCK-induced tension. The lack of response suggested that bullfrog gallbladder smooth muscle does not contain an NO-sensitive guanylyl cyclase. In addition, the bullfrog gallbladder does not utilize a cyclic-GMP-dependent pathway to relax.

In contrast to results in the rainbow trout, cat and dog (Aldman and Holmgren, 1992; Dahlstrand *et al.* 1989; Kalfin and Milenov, 1991), VIP produced no reduction in the CCK-induced tension in bullfrog gallbladder strips. Since, in the guinea pig, VIP-containing nerves were found to contain NOS (Talmage and Mawe, 1993), the lack of a response to VIP in the bullfrog may be due to its inability to respond to NO or because the bullfrog gallbladder does not have receptors for

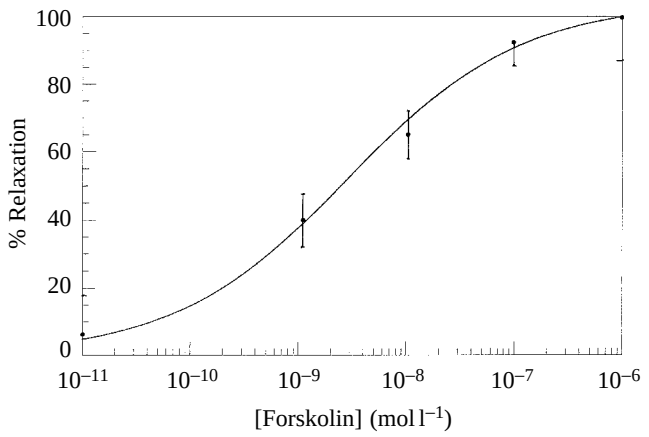

Fig. 4. The concentration-dependent response of bullfrog *Rana catesbeiana* gallblader strips to forskolin. Normalized data are fitted to the Hill function. Values are means ± S.E.M., *N*=8.

VIP. The concentration of VIP may not have been high enough to initiate a response.

It has been shown in the rabbit and guinea pig that cyclic AMP or its derivative db-cAMP can relax strips precontracted with CCK (Amer, 1972; Andersson *et al.* 1972). In our hands, bullfrog gallbladder strips also relaxed when treated with a cyclic AMP derivative 8-Br-cAMP, or with an agent that stimulates intracellular cyclic AMP production, forskolin. While a physiological agent capable of stimulating cyclic AMP production in the bullfrog gallbladder has not yet been described, the actions of cyclic AMP in the bullfrog gallbladder are consistent with observations in rabbit and guinea pig gallbladder strips.

It has been shown in the bullfrog that CGRP, when added prior to either CCK or ACh, reduced the subsequent amount of agonist-induced tension (Kline and Pang, 1992). CGRP, when added after the agonist-induced tension was well established, did not induce a relaxation of this tension. Since it has been shown that NOS is not present in the bullfrog gallbladder nor does the gallbladder respond to a well-known NO donor (SNP), it is unlikely that the actions of CGRP are mediated through NO. Using a rat tail artery helical strip, it has been shown that CGRP blocked intracellular Ca^{2+} release, leading to a relaxation in the vascular smooth muscle (Kline and Pang, 1988). Edvinsson *et al.* (1985) observed that CGRP elevated cyclic AMP levels in the middle cerebral arteries of the cat. This increase in cyclic AMP levels led to a decrease in intracellular Ca^{2+} release. Further examination of the effects of CGRP on Ca^{2+} fluxes in bullfrog gallbladder smooth muscle may assist in determining how the actions of CGRP are mediated in the bullfrog gallbladder.

In conclusion, both *in vitro* and histochemical studies suggested that the bullfrog gallbladder does not use NO, VIP or cyclic GMP to mediate gallbladder motility. However, it has been shown that cyclic AMP can mediate bullfrog gallbladder motility.

The authors thank Mr R. Ter Heide and Mr J. Jefferson, of Canada Safeway, Ltd, for the generous donation of vegetables to feed the iguanas.

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