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Prejunctional muscarinic (M_1)-receptor interactions on guinea-pig ileum: lack of effect of cisapride

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1 Cisapride stimulates gastrointestinal motility, probably by enhancing the release of acetylcholine from myenteric nerve endings. Such an effect could be mediated via presynaptic muscarinic (M_1)-receptors. Our aim was to determine whether cisapride could antagonize the inhibitory effects of a M_1-agonist, McN-A-343 or mimic the effects of a M_1-antagonist, pirenzepine.

2 Longitudinal segments were suspended in Krebs solution (95% O_2, 5% CO_2, 37.5°C) for isometric tension recording (preload 1 g) during electrical transmural stimulation (0.1 Hz, 1 ms, sub- or supramaximal current).

3 McN-A-343 (2.0×10^{-6} M) reduced the contractile response to supramaximal stimulation ($EC_{50} = 1.6 \times 10^{-6}$ M), but had no effect on the contractions induced by exogenous acetylcholine.

4 The inhibitory effect of McN-A-343 on the contractile response to electrical stimulation could be reversed by pirenzepine ($EC_{50} = 1.6 \times 10^{-8}$ M) but not by atropine. At these concentrations pirenzepine itself did not modify the contractile response to electrical stimulation. However, at 50 times higher concentrations pirenzepine inhibited the response to electrical stimulation as well as the response to exogenous acetylcholine ($EC_{50} = 8.5 \times 10^{-7}$ M).

5 Cisapride enhanced the contractile response to submaximal electrical stimulation by 49 ± 10%. This stimulating effect of cisapride was not affected by the presence of pirenzepine but was reduced in the presence of McN-A-343 (22 ± 7%).

6 In conclusion: the effects of McN-A-343 and pirenzepine on the electrically stimulated guinea-pig ileum are compatible with an interaction on presynaptic muscarinic-(M_1)-receptors. Cisapride enhances the twitch amplitude via mechanisms independent of such M_1-receptor interactions.

Introduction

Cisapride is a new gastrointestinal prokinetic compound that accelerates gastric and intestinal transit in animals (Schuurkes *et al.*, 1984; Schuurkes & Van Nueten, 1987) and human volunteers and patients (Reyntjens *et al.*, 1986). Its mechanism of action is ascribed to an enhanced release of acetylcholine from postganglionic myenteric nerve endings (Van Nueten *et al.*, 1984; Schuurkes *et al.*, 1985). Direct evidence for an enhanced release of acetylcholine was obtained on guinea-pig preparations from ileum (Pfeuffer-Friederich & Kilbinger, 1984) and stomach (Chen *et al.*, 1986). A pharmacological analysis of the actions of cisapride on the ileum excluded the following mechanisms of action: an effect on muscarinic (M_2)-receptors, a ganglionic mechanism, a postjunctional direct effect on the smooth muscle cells and an inhibition of acetylcholinesterase activity (Schuurkes *et al.*, 1985).

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Theoretically the possibility remained that cisapride enhanced the release of cholinergic transmitter by an effect on prejunctional muscarinic (M_1)-receptors located in myenteric ganglia (Buckley & Burnstock, 1986). The aim of the present study was to determine whether cisapride enhanced the twitch responses of the guinea-pig ileum to electrical stimulation via an effect on muscarinic (M_1) receptors, making use of the M_1-agonist, McN-A-343 and the M_1-antagonist, pirenzepine (Hammer & Giachetti, 1982; Gilbert *et al.*, 1984; Birdsall & Hulme, 1985).

Methods

Pirbright guinea-pigs (350–450 g, fasted overnight) of either sex were killed by cervical dislocation. The ileum (distal 10 cm discarded) was removed for experimentation. The intraluminal contents were

removed by repeated washing. Segments, 4.5 cm long, were suspended vertically with a preload of 1 g in 100 ml of Krebs solution (37.5°C), gassed with a mixture of 95% O_2 and 5% CO_2. Contractions were measured isometrically (Statham UC2). Transmural excitation was applied over the whole length of the ileum strip by means of two platinum electrodes, the anode threaded through the lumen of the ileum, the cathode in the bath solution. The preparation was excited with single rectangular stimuli (1 ms, 0.1 Hz, supramaximal current), known to release acetylcholine from intramural nerve endings (Paton, 1957). After a stabilization period, McN-A-343 was administered cumulatively (1.3×10^{-7} M–3.2×10^{-5} M). McN-A-343 (2×10^{-6} M–3.2×10^{-5} M) was also studied for its effect on contractions induced by exogenous acetylcholine (2.2×10^{-7} M). This concentration of acetylcholine evoked contractions of similar strength to those induced by electrical stimulation.

In a second series of experiments, the effects of single concentrations of pirenzepine (1.4×10^{-9}–1.5×10^{-6} M), cisapride (5.4×10^{-9}–5.4×10^{-6} M) and atropine (1.8×10^{-9}–2.9×10^{-8} M) were determined on the contractile responses to electrical stimulation in the presence of 2.0×10^{-6} M McN-A-343 (administered 10 min previously). In addition the effects of pirenzepine (2.3×10^{-8}–2.3×10^{-5} M) on exogenously added acetylcholine (2.2×10^{-7} M) were determined.

To validate the use of acetylcholine at a concentration of 2.2×10^{-7} M, a full dose-response curve was constructed either in the absence or the presence of McN-A-343 (2.2×10^{-6} M) or pirenzepine (9.0×10^{-8} M).

In a third series of experiments the effects of cisapride (3.4×10^{-7} M) and pirenzepine (9.0×10^{-8} M) or their combination were compared when administered after a stabilization period of 10 min either in the presence of McN-A-343 (2.0×10^{-6} M) or in conditions during which the current was reduced to a level producing twitch responses similar to those after 2.0×10^{-6} M McN-A-343 (50–60% of maximal stimulation).

Drugs

Cisapride (Janssen, Belgium), pirenzepine (Thomae, Germany), McN-A-343 ([4-(*m*-chlorophenylcarbamoyloxy)-bur-2-ynyl]trimethylammonium, McNeil, U.S.A.), atropine (Pugh, Belgium), were added to the bath solution in volumes of 1 ml. They were dissolved in distilled water except for cisapride which was dissolved in distilled water acidified with tartaric acid. This acidified water (pH $\geqslant$ 3) was used for the solvent experiments. The Krebs solution contained (mM): KCl 4.69, $CaCl_2.2H_2O$ 2.51; $NaHCO_3$ 25.0, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.18, NaCl 118.06 and glucose 5.55.

Statistical analysis

Data were expressed as mean percentage of initial value $\pm$ s.e. mean before drug administration for graphical representation. To allow calculation of EC_{50} values, the maximal effect obtained after drug administration was set at 100%. Data were expressed as a percentage of this maximal effect and least square regression line analysis was used to determine the concentration needed to obtain 50% of the maximal effect (i.e. EC_{50} value). Differences between mean values were tested by analysis of variance [Statistical Analysis System (SAS)]. In the results section only statistically significant effects are described ($P \leqslant 0.05$).

Results

Administration of McN-A-343 reduced the amplitude of the contractions elicited by supramaximal electrical stimulation (Figure 1). Subsequent administration of pirenzepine reversed the McN-A-343-induced inhibition (Figure 1). The dose-response relation for this inhibitory effect of McN-A-343, was determined by administering the compound cumulatively. The EC_{50} value obtained was 1.6×10^{-6} M (lower limit 1.4×10^{-6} M, upper limit 1.8×10^{-6} M) (Figure 2). At concentrations that completely blocked the responses to electrical stimulation ($\geqslant 7.9 \times 10^{-6}$ M), McN-A-343 did not reduce the response to exogenously added acetylcholine (Figure 2).

After reduction of the contractile response to supramaximal stimulation with McN-A-343 at a concentration of 2.0×10^{-6} M, which did not affect the full dose-response curve to acetylcholine (Figure 3), single doses of pirenzepine were added (Figure 4). Pirenzepine reversed the McN-A-343-induced inhibition up to a concentration of 9.0×10^{-8} M. The EC_{50} value of pirenzepine as an antagonist of the McN-A-343-induced inhibition was 1.6×10^{-8} M (lower limit 1.3×10^{-8} M, upper limit 2.0×10^{-8} M). At a concentration of 9.0×10^{-8} M, pirenzepine did not affect the responses to exogenous acetylcholine (Figures 3, 4). Concentrations above 9.0×10^{-8} M that reduced the contractile response to exogenous acetylcholine also reduced the response to electrical stimulation (Figure 4). In comparison to the concentrations needed to reverse the McN-A-343-induced inhibition, 50 times higher concentrations of pirenzepine were required to antagonize acetylcholine-induced contractions of similar strength ($EC_{50} = 8.5 \times 10^{-7}$ M, lower limit 6.6×10^{-7}, upper limit 1.1×10^{-8} M).

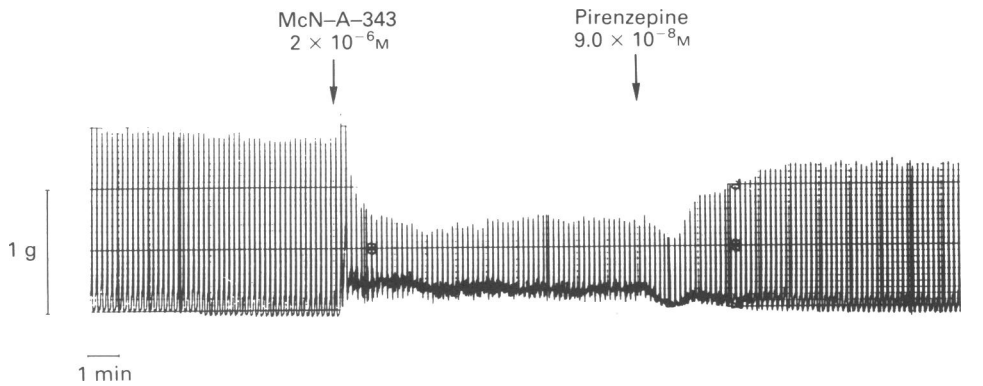

Figure 1 Original experiment showing the effect of pirenzepine (9.0×10^{-8} M) on McN-A-343-(2.0×10^{-6} M)-induced inhibition of the contractile response of the guinea-pig ileum to electrical stimulation (1 ms, 0.1 Hz, supramaximal current).

In contrast to pirenzepine, atropine did not reverse the McN-A-343-induced inhibition (Figure 5). Cisapride only slightly reversed the inhibition at concentrations above those needed for pirenzepine. The EC_{50} value for cisapride was 6.3×10^{-8} M (lower limit 1.6×10^{-8}, upper limit 2.5×10^{-7} M). The optimal concentrations to reverse the McN-A-343-induced inhibition were 9.0×10^{-8} M for pirenzepine and 3.4×10^{-7} M for cisapride (Figure 5).

These concentrations were tested after inhibition of the twitch contractions by either McN-A-343 (2.0×10^{-6} M) or by reduction of the stimulus current (Figure 6). Pirenzepine did enhance the amplitude after McN-A-343 but had no effect after submaximal stimulation. In contrast, cisapride enhanced the contractions after McN-A-343 as well as after submaximal stimulation; the latter response being larger (49 ± 10%) than the response in the presence of McN-A-343 (22 ± 7%). Moreover, the presence of pirenzepine did not affect the response to cisapride after submaximal stimulation (Figure 6).

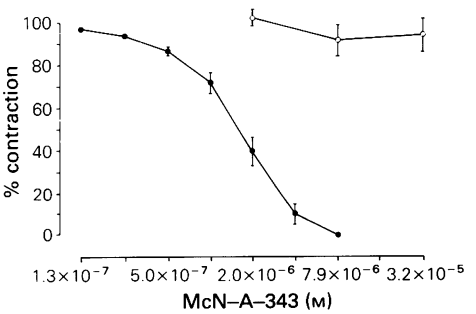

Figure 2 Effect of McN-A-343 on the amplitude of contractions of the guinea-pig ileum induced by electrical stimulation (1 ms, 0.1 Hz, supramaximal current; 100% = 3.1 ± 0.2 g) (●) or by acetylcholine (2.2×10^{-7} M; 100% = 3.6 ± 0.2 g) (○). McN-A-343 abolishes the twitch reponses at concentrations not affecting the response to exogenous acetylcholine; mean values are shown with vertical lines indicating s.e. mean, ($n = 6$).

Discussion

The existence of inhibitory prejunctional muscarinic receptors is generally accepted (Fosbraey & Johnson, 1980a; Kilbinger *et al.*, 1984; Fox *et al.*, 1985; North *et al.*, 1985). However, the concept of M_1/M_2-receptor classification is still a matter of

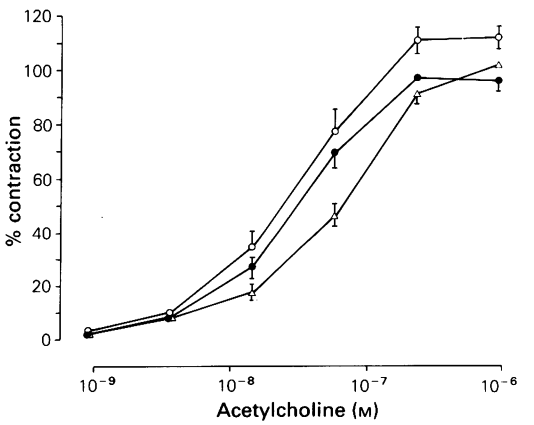

Figure 3 Lack of effect of McN-A-343 (2.0×10^{-6} M) (○) or pirenzepine (9.0×10^{-8} M) (△) on the amplitude of the contractions of the guinea-pig ileum induced by a full dose-range of acetylcholine (●). Values are mean with s.e. mean shown by vertical lines ($n = 6$, 100% = 3.8 ± 0.3 g).

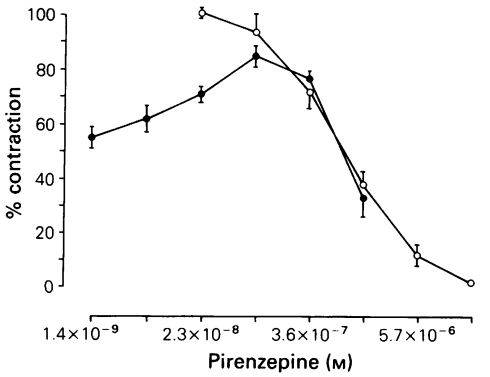

Figure 4 Effect of pirenzepine on the amplitude of contractions of the guinea-pig ileum induced by electrical stimulation (1 ms, 0.1 Hz, supramaximal current; 100% initial value = 3.1 ± 0.1 g) in the presence of the inhibitor McN-A-343 (2.0×10^{-6} M) (●) or by acetylcholine (2.2×10^{-7} M; 100% initial value = 3.9 ± 0.1 g) (○). Pirenzepine reverses the McN-A-343-induced inhibition (overall rest response 54.1 ± 1.3%) at lower concentrations, but inhibits the contractions induced by electrical stimulation or acetylcholine at higher concentrations. Values are mean with s.e. mean shown by vertical lines ($n = 6$).

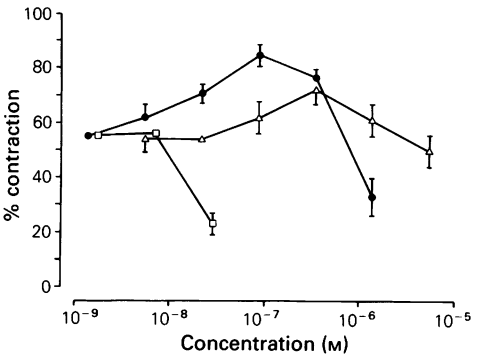

Figure 5 Effect of pirenzepine (●), cisapride (△) and atropine (□) on the amplitude of contractions of the guinea-pig ileum induced by electrical stimulation (1 ms, 0.1 Hz, supramaximal current; 100% initial value = 3.1 ± 0.1 g) in the presence of the inhibitor McN-A-343 (2.0×10^{-6} M). McN-A-343 reduced the amplitude to a value of 54.1 ± 1.3% of the initial value. In contrast to atropine, pirenzepine and to a minor extent cisapride, partly reverse the McN-A-343-induced inhibition. Values are mean with s.e. mean shown by vertical lines ($n = 6$).

dispute (Eglen & Whiting, 1985), due to the limited number of pharmacological selective compounds and to the small number of functional correlates to challenge this concept. In this study McN-A-343 and pirenzepine were used as M_1-agonist and antagonist respectively (Hammer & Giachetti, 1982) to try and find a functional correlate for the concept of receptor subclassification on the electrically stimulated guinea-pig ileum. Electrical transmural stimulation elicits twitch contractions that can be abolished by either tetrodotoxin or atropine (Schuurkes & Van Nueten, 1987), indicating that the twitch contraction is caused by the release of acetylcholine from intramural nerves (Paton, 1957). Unfortunately, the instability of the responses of the guinea-pig ileum to lower stimulus currents did not allow us to construct a stimulus-response curve on this preparation. Only submaximal stimuli above 50% of supramaximal values could be used. Therefore, our conclusions cannot be extrapolated to lower stimulus levels.

McN-A-343 effectively reduced the twitch responses. The inhibitory effect of McN-A-343 is due to a prejunctional mechanism since high concentrations of McN-A-343 did not affect the contractile response to acetylcholine on non-stimulated preparations.

Pirenzepine reversed the McN-A-343-induced inhibition in a dose-dependent manner. The EC_{50} for this effect against a single concentration of McN-A-343 was 50 times less than the EC_{50} value against acetylcholine-induced contractions, indicating that pirenzepine can be used to distinguish between pre- and postjunctional muscarinic receptors. The concentrations of pirenzepine needed to reverse the McN-A-343-induced inhibition (EC_{50}: 1.6×10^{-8} M) were somewhat higher than the concentrations reported for the specific interaction of pirenzepine with M_1-muscarinic receptors e.g. the A_2 (i.e. the concentration of the antagonist needed to shift the dose-response curve for the agonist twofold) for neuronal M_1-receptor interaction was $\sim 4 \times 10^{-9}$ M (Brown *et al.*, 1980; Kilbinger & Nafziger, 1985; North *et al.*, 1985). If this difference is not due to the comparison of EC_{50} with A_2-values, it may be explained by the observation that whereas the inhibitory effect of pirenzepine is only evident at higher concentrations, its intrinsic postjunctional M_2-blocking properties affect the dose-response relation even at lower concentrations. The IC_{50} value of pirenzepine against acetylcholine-induced contractions was 8.5×10^{-7} M. These concentrations are in good agreement with reported A_2-values for the effect of pirenzepine on M_2-muscarinic receptor subtypes (Brown *et al.*, 1980; Kilbinger & Nafziger, 1985). Pirenzepine did not enhance the twitch amplitude in the absence of McN-A-343, illustrating that the prejunctional M_1-receptors, under normal conditions do not contribute to the twitch response.

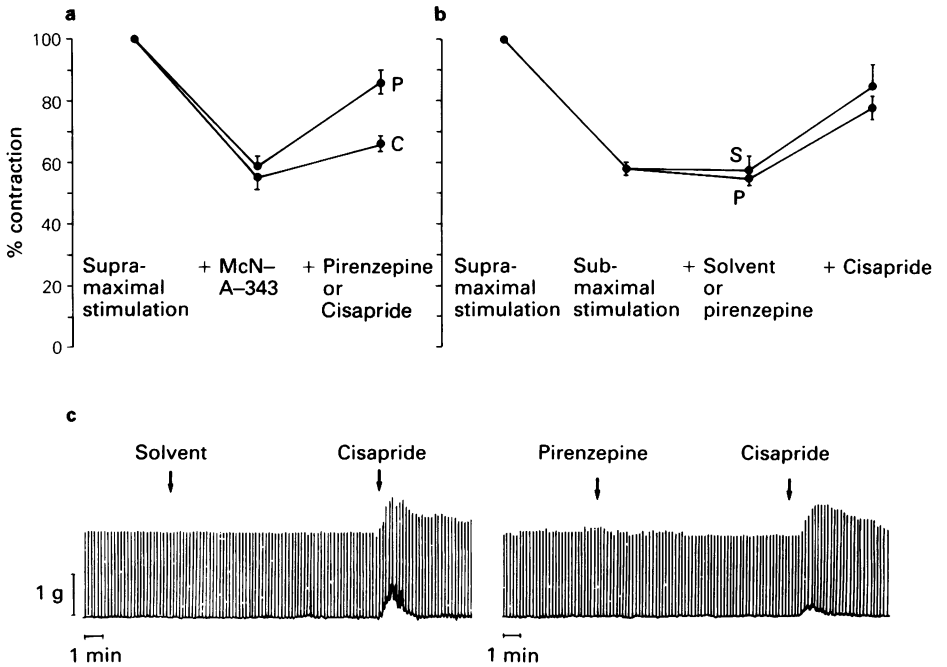

Figure 6 Effect of pirenzepine (P, 9.0×10^{-8} M) and cisapride (C, 3.4×10^{-7} M) on the amplitude of contractions of the guinea-pig ileum, evoked by supramaximal electrical stimulation in the presence of McN-A-343 (2.0×10^{-6} M) [1 ms, 0.1 Hz, supramaximal current, initial value 100% = 3.3 ± 0.2 g reduced by McN-A-343 to a rest response of 59 ± 4% (pirenzepine-series) and 55 ± 4% (cisapride series)] (a, trace see Figure 1) or after reduction of the current to a submaximal level resulting in amplitudes similar to those after administration of McN-A-343 [initial value 100% = 3.4 ± 0.4 at supramaximal stimulation reduced to 58 ± 4% (pirenzepine-series) and 58 ± 2% (cisapride series) at submaximal stimulation] (b, trace c) (S = solvent). Values are mean with s.e. mean shown by vertical lines ($n = 6$).

Our results can be explained by a prejunctional effect of McN-A-343 on M_1-receptors leading to a reduced release of cholinergic transmitter; an effect that can be specifically reversed by pirenzepine. Previous work suggested that presynaptic release-inhibiting muscarinic receptors were similar to those located on the smooth muscle cells (Halim *et al.*, 1981; Kilbinger *et al.*, 1984; North *et al.*, 1985; Fox *et al.*, 1985). However, the fact that McN-A-343 and pirenzepine can distinguish between pre- and post-junctional receptors, whereas atropine cannot, illustrates the existence of 2 different receptor populations. Kilbinger & Nafzinger (1985) reported the presence of a ganglionic M_1-receptor in the myenteric plexus of the guinea-pig ileum (also shown by Buckley & Burnstock, 1986), functionally involved in enhanced release of acetylcholine. In contrast Gilbert *et al.* (1984) showed the presence of a neuronal M_1-receptor via which McN-A-343 induced a pirenzepine-sensitive inhibition of tone (relaxation) in the lower oesophageal sphincter. Our results provide the first indication that functional inhibitory neuronal M_1-receptors are also present on the guinea-pig ileum.

The effects of cisapride in this study are in agreement with the hypothesis that the cisapride-induced enhancement of the twitch response of the guinea-pig ileum to electrical stimulation is due to enhanced release of cholinergic transmitter (Van Nueten *et al.*, 1984; Schuurkes *et al.*, 1985). Cisapride enhanced the contractile response to electrical stimulation in the presence but also, and to a larger extent, in the absence of McN-A-343. Thus, the reversal of the McN-A-343-induced inhibition may be explained by a functional antagonism by cisapride. Indeed, if cisapride had M_1-receptor antagonistic properties, its stimulatory effect should be more pronounced in the presence of McN-A-343 than after submaximal stimulation. However, the opposite is true. The presence of McN-A-343 reduces the stimulatory response to cisapride to a similar extent to that by which it reduces the twitch amplitude. The EC_{50} value for this effect of cisapride in the presence of McN-A-343 was 6.3×10^{-8} M, a concentration

higher than reported for its effect in the absence of McN-A-343 (9.2×10^{-9} M; Schuurkes *et al.*, 1985). In contrast, pirenzepine only reverses the McN-A-343-induced inhibition, without affecting the twitch amplitude after submaximal stimulation. Thus, we may conclude that the effects of cisapride are not mediated by blockade of inhibitory prejunctional muscarinic receptors. The observation that the stimulating effect of cisapride is not reduced by the presence of an M_1-receptor blocking concentration of pirenzepine strengthens this hypothesis. Our findings on cisapride are in agreement with previous work, showing that the effects of metoclopramide, another substituted benzamide, cannot be explained by interaction with prejunctional muscarinic receptors (Fosbraey & Johnson, 1980b; Kilbinger *et al.*, 1982; Lobbezoo *et al.*, 1985).

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