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Prostacyclin releases endothelium-derived relaxing factor and potentiates its action in coronary arteries of the pig

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1 The possible interactions between prostacyclin and endothelium-derived relaxing factor were examined, in isolated coronary arteries of the pig treated with indomethacin (10^{-5} M).

2 In organ chamber experiments, prostacyclin caused relaxations, which were potentiated in the presence of the endothelium; the potentiation was abolished by oxyhaemoglobin.

3 In bioassay experiments, prostacyclin caused minimal relaxations of bioassay rings without endothelium; these relaxations were potentiated when the bioassay ring was exposed to basally-released endothelium-derived relaxing factor (interaction between prostacyclin and basal endothelium-derived relaxing factor) and further augmented when the endothelial cells were exposed to the prostanoid (stimulated release of endothelium-derived relaxing factor). The endothelium-dependent, but not the direct effects of prostacyclin were augmented by superoxide dismutase plus catalase and abolished by oxyhaemoglobin.

4 Forskolin, a direct activator of adenylate cyclase, caused relaxations of rings without endothelium, which were augmented by the presence of the endothelium.

5 The relaxations induced by prostacyclin or forskolin also had an endothelium-dependent component in basilar and femoral arteries and in jugular veins of the pig.

6 The endothelium-dependent actions of prostacyclin probably reflect activation of adenylate cyclase.

Introduction

Prostacyclin (Moncada *et al.*, 1976) and endothelium-derived relaxing factor (Furchgott, 1983; Vanhoutte *et al.*, 1986) are potent vasodilator and anti-aggregatory substances (Azuma *et al.*, 1986; Furlong *et al.*, 1987; Radomski *et al.*, 1987a) released from the endothelium. The effects induced by prostacyclin are linked with an activation of adenylate cyclase, leading to a rise in intracellular levels of adenosine 3′: 5′-cyclic monophosphate (cyclic AMP) (Gorman *et al.*, 1977; Tateson *et al.*, 1977). In contrast, endothelium-derived relaxing factor causes vasodilatation by activating guanylate cyclase, resulting in a rise in intracellular levels of guanosine 3′: 5′-cyclic monophosphate (cyclic GMP) (Rapaport & Murad, 1983). Both substances have a relatively short half-life (Moncada *et al.*, 1976; Furchgott, 1983; Vanhoutte *et al.*, 1986), and an interaction between the two could considerably augment their effects. Thus, synergistic interactions between prostacyclin and endothelium-derived relaxing factor occur as regards inhibition of platelet aggregation (Radomski *et al.*, 1987b). The present study was designed to examine the possible interactions between prostacyclin and endothelium-derived relaxing factor in causing relaxation of isolated coronary arteries of the pig.

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Methods

Normal male Yorkshire pigs (43.5 ± 1.2 kg) were used. In a first protocol, the hearts were removed after anaesthesia with ketamine hydrochloride (300 mg, i.m., Bristol) followed by sodium pentobarbitone (12.5 mg kg^{-1}, i.v., Fort Dodge Laboratories, Inc.). The right and left anterior descending and circumflex coronary arteries were removed and

immersed in cold modified Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, Ca-EDTA 0.016 and glucose 11.1 (control solution). The left anterior descending coronary artery was used for organ chamber experiments and the right coronary and left circumflex coronary arteries were used for bioassay experiments.

Organ chamber experiments

Rings (3 to 4 mm long) were cleaned of loose connective tissue, with special care taken not to touch the luminal surface. In some rings, the endothelium was removed by gently rubbing the luminal surface with a cotton swab wetted with control solution (Shimokawa *et al.*, 1987a). The rings were suspended horizontally between two stirrups in organ chambers filled with 25 ml of control solution (37°C, pH 7.4) gassed with 95% O_2 and 5% CO_2. The preparations were attached to a strain gauge (Gould UC2) and isometric tension was recorded. The rings were then progressively stretched to 8–9 g, an optimal tension for pig isolated coronary arteries (Shimokawa *et al.*, 1987a). They were allowed to equilibrate for 30 min.

After equilibration, all rings were exposed to bradykinin (10^{-7} M) during a contraction caused by prostaglandin $F_{2\alpha}$ (2×10^{-6} M) to confirm the presence or absence of functional endothelial cells (Shimokawa *et al.*, 1987a). In all rings with endothelium, bradykinin caused more than 100% decrease in tension caused by prostaglandin $F_{2\alpha}$, while it caused no relaxation in rings without endothelium (Shimokawa *et al.*, 1987a). The rings then were incubated with indomethacin (10^{-5} M) for 40 min in order to prevent formation of endogenous prostaglandins (Shimokawa *et al.*, 1987a). Relaxations to prostacyclin or forskolin were examined during a contraction caused by prostaglandin $F_{2\alpha}$ (2×10^{-6} M).

In a second protocol, relaxations to prostacyclin or forskolin were examined in basilar, carotid and femoral arteries, and in jugular veins. In these experiments, the rings were also stretched to the optimal tension; [3 g (basilar artery), 14–16 g (carotid and femoral arteries) and 2 g (jugular vein); Shimokawa *et al.*, 1988; Vanhoutte & Shimokawa, 1987]. The same protocol was used as for the coronary arteries.

Bioassay experiments

In this system, the biological activity of endothelium-derived relaxing factor released from a coronary segment (4 cm long, right coronary artery) was bioassayed by a coronary ring (proximal left circumflex coronary artery), from which the endothelium had been removed mechanically (Rubanyi *et al.*, 1985). The donor coronary segment was perfused at constant flow (2 ml min^{-1}) with control solution maintained at 37°C. There was a transient delay of 1 s before the fluid reached the bioassay rings which were suspended below the donor segment. The tension developed in the rings was recorded. The bioassay rings were first superfused for 60 min with control solution pumped through a stainless steel cannula (direct superfusion). During this time, they were stretched in a stepwise manner to their optimal tension (8–9 g). Control perfusion solution was provided from an aerated tower. an adjacent aerated tower contained control solution plus prostaglandin $F_{2\alpha}$ at a concentration of 2×10^{-6} M. Relaxation responses were examined during a contraction caused by prostaglandin $F_{2\alpha}$. The absence of the endothelium in the bioassay ring was confirmed by the lack of relaxation to bradykinin (10^{-7} M) infused under direct superfusion. The bioassay rings could also be superfused with solution pumped through the coronary segment (endothelial superfusion). Prostacyclin or forskolin was infused either above or below the coronary segment. Indomethacin (10^{-5} M) was present throughout the experiment in order to prevent the formation of endogenous prostaglandins.

Drugs

The following drugs were used: bovine haemoglobin (Type 1), bradykinin, indomethacin, prostaglandin $F_{2\alpha}$, prostaglandin I_2 (prostacyclin), sodium nitroprusside (all from Sigma) and 1,9, dideoxy forskolin (Calbiochem). All drugs were prepared daily with distilled water except for indomethacin and prostacyclin, which were dissolved in Na_2CO_3 (10^{-5} M) and $NaHCO_3$ (1.5×10^{-1} M, pH = 8.5, adjusted to pH 9 with 1 N NaOH), respectively.

Bovine haemoglobin (Type 1) contains a mixture of oxyhaemoglobin and the oxidized derivative, methaemoglobin. Oxyhaemoglobin was prepared by adding 600 mg bovine haemoglobin to 10 ml distilled water containing 70 mg sodium dithionite ($Na_2S_2O_4$). Sodium dithionite was then removed by dialysis in 15 litres of distilled water (containing 0.001% EDTA) for 2 h at room temperature. During dialysis the water was bubbled with nitrogen. The percentage of oxyhaemoglobin was determined spectrophotometrically (Martin *et al.*, 1984).

Data analysis

Results are expressed as means ± s.e.mean. In organ chamber experiments, relaxations are expressed as percentage relaxation of the contraction obtained with prostaglandin $F_{2\alpha}$ (2×10^{-6} M). In bioassay experiments, relaxations are expressed as percentage change in tension relative to the maximal relaxation

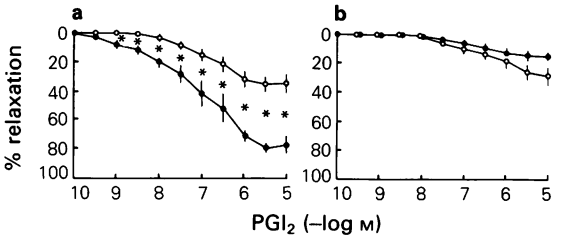

Figure 1 Cumulative concentration-response curves to prostacyclin during a contraction evoked by prostaglandin $F_{2\alpha}$ (2×10^{-6} M) in the presence of indomethacin (10^{-5} M) in organ chamber experiments; under control conditions (a, $n = 6$) and in the presence of oxyhaemoglobin (10^{-5} M) (b, $n = 6$). Data shown as means with s.e.mean indicated by vertical lines. The asterisk denotes a significant difference ($P < 0.05$) between rings with (●) and without (○) endothelium.

caused by sodium nitroprusside (10^{-5} M); for endothelial superfusion, they are expressed relative to the level of tension achieved in the presence of basally released endothelium-derived relaxing factor. Statistical evaluation of the data was performed by Student's t test for paired observations. When more than two means were compared, a one-way analysis of variance was used. If a significant value was found, Scheffe's test for multiple comparisons was used to identify differences among groups. Values were considered to be statistically significantly different when P was smaller than 0.05.

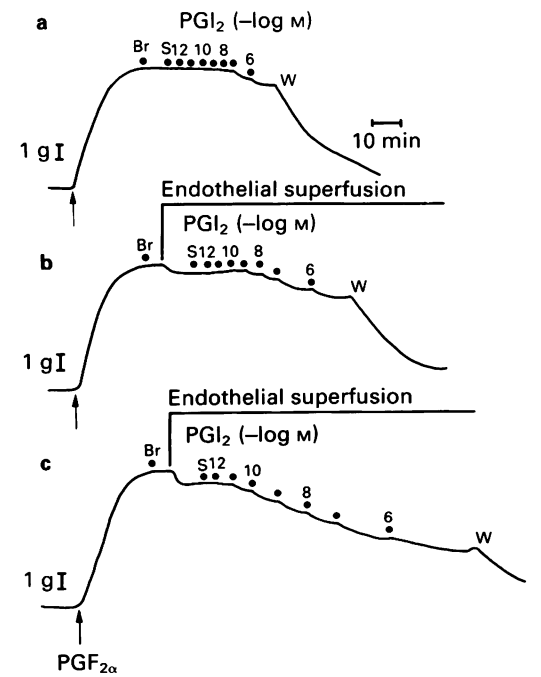

Figure 2 Original tracings of a bioassay experiment with prostacyclin in the presence of indomethacin (10^{-5} M); during direct superfusion (a), and during endothelial superfusion (b and c). Prostacyclin was infused below the coronary segment (site 2) (b), or above the coronary segment (site 1) (c). S = solvent, W = washout with control solution, Br = bradykinin (10^{-7} M) superfusion.

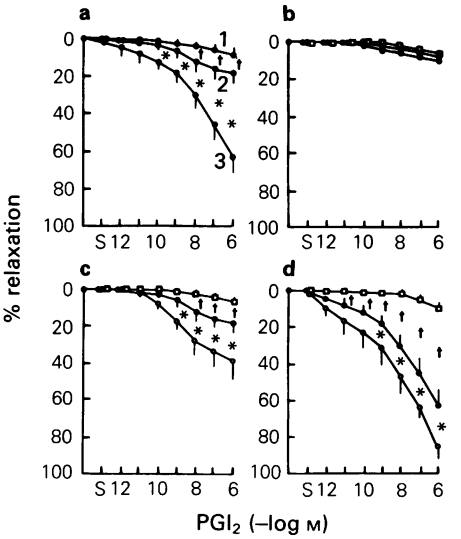

Figure 3 Cumulative concentration-response curves to prostacyclin (PGI_2) in the presence of indomethacin (10^{-5} M); (a) under control conditions (1 = direct superfusion; 2 = endothelial superfusion, prostacyclin given at site 2; 3 = endothelial superfusion, prostacyclin given at site 1); (b) during direct superfusion (● = solvent; ○ = superoxide dismutase (150 u ml^{-1}) plus catalase (1200 u ml^{-1}); □ = oxyhaemoglobin (10^{-5} M)); (c) during endothelial superfusion with prostacyclin infused below the coronary segments (site 2; symbols as in b); (d) during endothelial superfusion with prostacyclin infused above the coronary segments (site 1; symbols as in b). Data shown as means with s.e. mean indicated by vertical lines. *,† represent a significant difference ($P < 0.05$) between two points.

Results

Coronary artery

Prostacyclin In organ chamber experiments prostacyclin (10^{-10} to 10^{-5} M) caused relaxations which were greater in rings with, than in those without, endothelium (Figure 1). The solvent for prostacyclin had no relaxant effects ($n = 4$; data not shown). The endothelium-dependent component of the relaxations to prostacyclin was abolished by oxyhaemoglobin (10^{-5} M) (Figure 1).

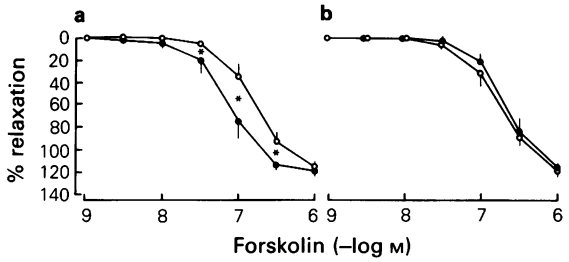

Figure 4 Cumulative concentration-response curves to forskolin during a contraction evoked by prostaglandin $F_{2\alpha}$ (2×10^{-6} M) in the presence of indomethacin (10^{-5} M) in organ chamber experiments, under control conditions (a, $n = 6$), and in the presence of oxyhaemoglobin (10^{-5} M) (b, $n = 6$). Data shown as means with s.e.mean indicated by vertical lines. * Significant difference ($P < 0.05$) between rings with (●) and without (○) endothelium.

In bioassay experiments, the relaxant effect of prostacyclin (10^{-12} to 10^{-6} M) under direct superfusion was minimal (Figures 2 and 3). Under endothelial superfusion, the infusion of prostacyclin below the donor segment (to determine possible interactions between prostacyclin and basally released endothelium-derived relaxing factor) caused larger relaxations (Figures 2 and 3) and infusion of

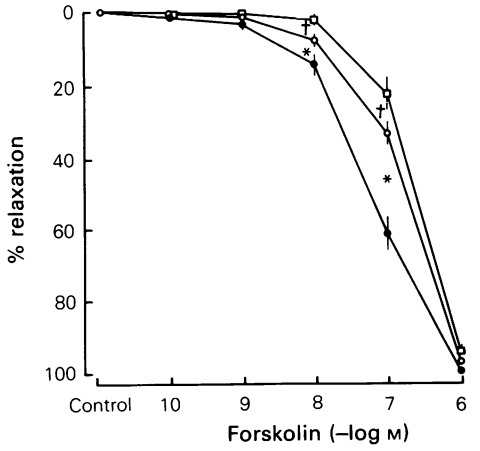

Figure 5 Cumulative concentration-response curves to forskolin during a contraction evoked by prostaglandin $F_{2\alpha}$ (2×10^{-6} M) in the presence of indomethacin (10^{-5} M) in bioassay experiments ($n = 6$, each). (□) = Direct superfusion, forskolin given at site 2; (○) = endothelial superfusion, forskolin given at site 2; (●) = endothelial superfusion, forskolin given at site 1. Data shown as means with s.e.mean indicated by vertical lines. *, † Represent a significant difference ($P < 0.05$) between the two points.

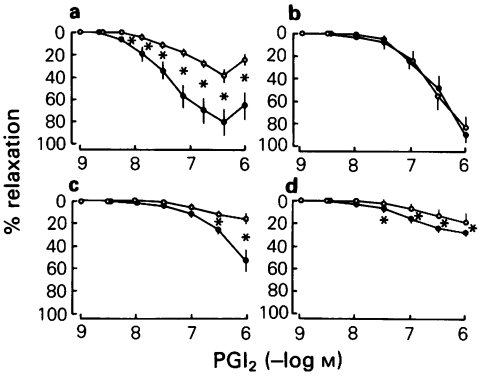

Figure 6 Cumulative concentration-response curves to prostacyclin during a contraction evoked by prostaglandin $F_{2\alpha}$ (2×10^{-6} M) in the presence of indomethacin (10^{-5} M) in organ chamber experiments ($n = 6$, each). (a) basilar artery; (b) carotid artery; (c) femoral artery; (d) jugular vein. Data shown as means with s.e.mean indicated by vertical lines. * Significant difference ($P < 0.05$) between rings with (●) and without (○) endothelium.

prostacyclin above the segment (to determine any stimulated release of endothelium-derived relaxing factor) caused even more pronounced relaxations (Figures 2 and 3). The endothelium-dependent potentiation of the prostacyclin-induced relaxation and the release of endothelium-derived relaxing factor by prostacyclin were augmented by superoxide dismutase (150 u ml^{-1}) plus catalase 1200 u ml^{-1} and abolished by oxyhaemoglobin (10^{-5} M) (Figure 3).

Forskolin In organ chamber experiments, the relaxations caused by forskolin (10^{-9} to 10^{-6} M) had both a direct and an endothelium-dependent component (Figure 4); the latter was abolished by oxyhaemoglobin (Figure 4). 1,9 Dideoxy forskolin, (10^{-9} to 10^{-6} M) the biologically inactive analogue of forskolin, did not cause significant changes in tension in rings with or without endothelium (data not shown). In bioassay experiments, forskolin (10^{-10} to 10^{-6} M) demonstrated three different relaxant effects: (a) it relaxed the coronary artery ring directly, (b) the relaxations were potentiated in the presence of basally-released endothelium-derived relaxing factor, and (c) they were further augmented during endothelial superfusion when the endothelial cells were exposed to forskolin (Figure 5).

Heterogeneity

The effects of prostacyclin and forskolin were examined in basilar, carotid and femoral arteries and in

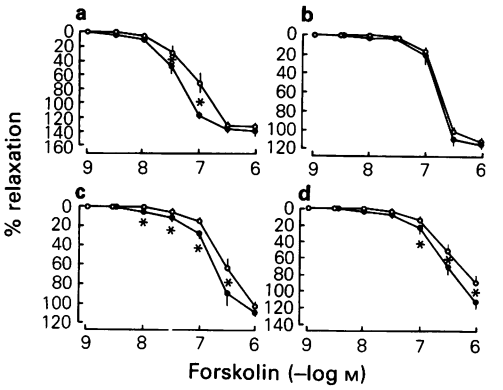

Figure 7 Cumulative concentration-response curves to forskolin during a contraction evoked by prostaglandin $F_{2\alpha}$ (2×10^{-6} M) in the presence of indomethacin (10^{-5} M) in organ chamber experiments ($n = 6$, each): (a) basilar artery; (b) carotid artery; (c) femoral artery; (d) jugular vein. Data shown as means with s.e.mean indicated by vertical lines. * Significant difference ($P < 0.05$) between rings with (●) and without (○) endothelium.

jugular veins of the pig. Prostacyclin caused relaxations in all arteries and in the veins; except for the carotid arteries, the relaxations were more pronounced in the presence of the endothelium (Figure 6). The solvent used had no relaxant effects in either blood vessel ($n = 3$; data not shown). The endothelium-dependent potentiation was significantly more pronounced in the basilar arteries (Figure 6). Forskolin caused relaxations in the four blood vessels; the relaxations were potentiated in the presence of the endothelium in all but the carotid arteries (Figure 7).

Discussion

The present study demonstrates that exogenously administered prostacyclin causes relaxations of coronary arteries of the pig which are potentiated by the endothelium. The potentiation was observed in the presence of indomethacin and abolished by oxyhaemoglobin, a selective inactivator of endothelium-derived relaxing factor (Martin *et al.*, 1984). These results are consistent with the interpretation that the potentiated relaxation can be attributed to endothelium-derived relaxing factor. The bioassay experiments provide evidence that the endothelium-dependency of the potentiation of the relaxation to prostacyclin consists of two components: (a) an interaction between prostacyclin and basally released endothelium-derived relaxing factor and (b) the stimulated release of endothelium-derived relaxing factor. Indeed, the endothelium-dependent effects of prostacyclin were abolished by oxyhaemoglobin (Martin *et al.*, 1984) and augmented by superoxide dismutase which by scavenging superoxide anions prolongs the half-life of endothelium-derived relaxing factor (Rubanyi & Vanhoutte, 1986; Gryglewski *et al.*, 1986), demonstrating that they are mediated by endothelium-derived relaxing factor. Thus, the present data indicate that prostacyclin exerts three different relaxant effects in the porcine coronary artery; direct relaxation of the smooth muscle, synergistic interactions with endothelium-derived relaxing factor, and stimulated release of the factor. The latter two mechanisms appear to be more important than the former.

Although most pig blood vessels studied exhibited an endothelium-dependent potentiation of the relaxation to prostacyclin, this was not the case for the carotid artery where the direct relaxant effect of prostacyclin was the most pronounced. We have no explanation for this heterogeneity. The data obtained in pig arteries and veins contrasts with previous reports that exogenously administered prostacyclin causes endothelium-independent relaxations in intrapulmonary arteries of the dog (Chand & Altura, 1981), no relaxations in the aorta of the pig (Gordon & Martin, 1983) or even endothelium-dependent contractions in basilar arteries of the dog (Katusic *et al.*, 1988).

The effects of prostacyclin are linked with an inactivation of adenylate cyclase, leading to a rise in intracellular levels of cyclic AMP (Gorman *et al.*, 1977; Tateson *et al.*, 1977). In order to examine further the mechanisms of the prostacyclin-induced relaxations, the relaxant effects of forskolin, a direct activator of adenylate cyclase (Muller & Baer, 1983; Jones *et al.*, 1984; Gerthoffer *et al.*, 1984), were examined. Both prostacyclin and forskolin activate adenylate cyclase, leading to a rise in cyclic AMP levels in endothelial cells (Karnushina *et al.*, 1983; Whorton *et al.*, 1985; Leitman *et al.*, 1986). The relaxant effect of forskolin had a similar endothelium-dependency to that of prostacyclin, suggesting that stimulation of the production of cyclic AMP could account for the endothelium-dependent relaxations caused by prostacyclin and thus that an increase in cyclic AMP in the endothelium facilitates the synthesis and/or release of the relaxing factor. The data with forskolin also indicate that the synergistic interactions between prostacyclin and endothelium-derived relaxing factor at the level of the smooth muscle involve a positive interaction between cyclic AMP and GMP at the level of the contractile machinery. Both prostacyclin and forskolin are highly lipid soluble and may increase the fluidity of lipid membranes, causing a release of endothelium-derived relaxing factor as do arachidonic acid and other unsaturated fatty acids (oleic acid, elaidic acid

and eicosapentaenoic acid) and platelet-activating factor (Furchgott, 1984; Rubanyi & Vanhoutte, 1985; Vanhoutte & Houston, 1985; Shimokawa *et al.*, 1987b). This interpretation is unlikely since the endothelium-dependent relaxations to the unsaturated fatty acids occur at higher concentrations than the ones described in the present study for prostacyclin and forskolin, and since 1,9 dideoxy forskolin did not cause endothelium-dependent relaxations.

The physiological significance of the present findings remains to be examined. It could be that prostacyclin and endothelium-derived relaxing factor act synergisticly to inhibit platelet aggregation (Radomski *et al.*, 1987b) and platelet-induced contractions of the vascular smooth muscle (Shimokawa *et al.*, 1987a,b; 1988). Prostacyclin is released mainly intraluminally, while endothelium-derived relaxing factor is released mainly abluminally (Busse *et al.*, 1985; Rubanyi *et al.*, 1986; Pohl *et al.*, 1986). Prostacyclin appears to be released more *in vivo* than *in vitro* (Busse *et al.*, 1985; Rubanyi *et al.*, 1986; Pohl *et al.*, 1986), and may interact with endothelial cells downstream of its site of production. It is interesting to note that the endothelium-dependent potentiations of the prostacyclin-induced relaxations are the most pronounced in the coronary and basilar arteries, where the endothelium-dependent inhibition of platelet-induced contractions is the largest (Shimokawa *et al.*, 1987b; Vanhoutte & Shimokawa, 1987). Impaired production of or interactions between prostacyclin and endothelium-derived relaxing factor may play an important role in pathological conditions such as vasospasm, thrombosis and atherosclerosis.

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