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Xanthine Derivatives that Selectively Inhibit Cyclic GMP Hydrolysis Potentiate Cardiac Contractile Effects of Isoproterenol but Not Those of Bethanecol

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SUMMARY

MUSHLIN, P., R. C. BOERTH, AND J. N. WELLS. Xanthine derivatives that selectively inhibit cyclic GMP hydrolysis potentiate cardiac contractile effects of isoproterenol but not those of bethanecol. *Mol. Pharmacol.* **20**:190–194 (1981).

This study examines the abilities of xanthines to alter contractile and cyclic nucleotide responses to isoproterenol or bethanecol in left atria isolated from rabbits. Two of the xanthines studied (1-methyl-3-isobutyl-8-methoxymethylxanthine, and 1-methyl-3-isobutyl-8-*t*-butylxanthine) inhibited cyclic GMP hydrolysis more potently than cyclic AMP hydrolysis; another xanthine, theophylline, was equipotent at inhibiting the hydrolysis of the two cyclic nucleotides. All of the xanthines studied comparably potentiated contractile and cyclic AMP responses to isoproterenol (10^{-8} M), regardless of their potencies to inhibit cyclic GMP hydrolysis. These data indicate that cyclic GMP is not antiadrenergic. The selective inhibitor, 1-methyl-3-isobutyl-8-methoxymethylxanthine, at a concentration that elevated cyclic GMP content to a level that was 3-fold above that observed with bethanecol alone, failed to depress contractile force, alter cardiodepressant effects of bethanecol, or alter atrial cyclic GMP content in the presence of this choline ester. These results demonstrate that the inhibition of cyclic GMP phosphodiesterase neither produces negative inotropic effects nor enhances the cardiodepressant effects of cholinergic agents.

INTRODUCTION

George *et al.* (1), using isolated, spontaneously beating rat hearts, were the first to demonstrate that acetylcholine can elevate the myocardial cyclic GMP concentration. Subsequently, a number of investigators have suggested that a causal relationship exists between the abilities of choline esters to elevate cyclic GMP concentrations in heart and to depress contractile force (2–4). This concept, however, is highly controversial. For example, Brooker (5) has reported that, in guinea pig atria, carbachol produces 90% of its negative inotropic effect without significantly increasing cardiac cyclic GMP content. Also, large increases in cyclic GMP have been observed with agents such as nitroprusside without concomittant negative inotropic effects (6).

If cholinergic agonists do indeed produce cardiac depressant effects via guanylate cyclase stimulation (3), agents that impede the hydrolysis of cyclic GMP should be expected to enhance both the elevation of cyclic GMP and the negative inotropic effects of cholinergic compounds. In accordance with this idea, papaverine, an inhibitor of phosphodiesterase, has been reported to enhance the negative inotropic effects of acetylcholine in rat atria (7). In many tissue preparations, however, papaverine is more potent as an inhibitor of cyclic AMP than of cyclic GMP hydrolysis (8).[2] It would seem more appropriate to conduct interaction studies between cholinergic agents and phosphodiesterase inhibitors that are relatively selective for cyclic GMP hydrolysis. We have recently reported that 8-methoxymethyl MIX[3] is 16 times more potent as an inhibitor of the hydrolysis of cyclic GMP than of cyclic AMP [(9), and see accompanying paper (10)]. Using this compound, the present study addresses the question of whether or not selective inhibitors of cyclic GMP hydrolysis can potentiate cardiodepressant effects of the cholinergic agonist, bethanecol. This study also addresses the hypothesis that cyclic GMP can antagonize the positive inotropic effects of cyclic AMP (11).

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[2] P. Mushlin, R. C. Boerth, and J. N. Wells, unpublished observations.

[3] The abbreviations used are: 8-methoxymethyl MIX, 1-methyl-3-isobutyl-8-methoxymethylxanthine; DF, developed force; 8-*t*-butyl MIX, 1-methyl-3-isobutyl-8-*t*-butylxanthine.

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MATERIALS AND METHODS

The materials and methods used in this study were as described in the preceding paper (10).

RESULTS

Bethanecol and 8-methoxymethyl MIX. After a 2- to 3-hr equilibration period, atrial strips were incubated with 8-methoxymethyl MIX (405 μM) or buffer for 12 min. This concentration of 8-methoxymethyl MIX is 3-fold higher than that required to inhibit by 50% the hydrolysis of 1 μM cyclic AMP and 48-fold higher than that required to inhibit by 50% the hydrolysis of 1 μM cyclic GMP by the soluble fraction from rabbit atria (10). Bethanecol (0.3 μM or 1 μM) or buffer was then added to the baths. Bethanecol produced time-related reductions of peak DF (Fig. 1). These reductions, apparent immediately after the addition of either concentration of bethanecol, approached a plateau at 3 min. By 3 min, the mean DF of atria treated with bethanecol alone were 10% (0.3 μM; $p < 0.10$) or 28% (1 μM; $p < 0.01$) lower than the respective control (Fig. 2, *top panel*). More important, 8-methoxymethyl MIX (which lacked inotropic effects) failed to enhance negative inotropic responses to bethanecol, despite the fact that the cyclic GMP contents of groups treated with this xanthine were 3-fold higher than those of groups studied in its absence (Fig. 2, *middle panel*) ($p < 0.05$). The effects of 405 μM 8-methoxymethyl MIX on cyclic GMP levels were submaximal; 40% greater effects were observed at 1 mM (data not shown). Nevertheless, bethanecol failed to alter the effects of 8-methoxymethyl MIX on cyclic GMP content (Fig. 2, *middle panel*). The group treated with 8-methoxymethyl MIX alone contained significantly more cyclic AMP than groups treated with bethanecol alone ($p < 0.05$; Fig. 2, *bottom panel*).

TREATMENTS A	TREATMENTS B	N	DF (mg, $\bar{x} \pm$ SEM) AT TIME 0
△—△ BUFFER	BETH. (1 μM)	6	1075 ± 211
▲—▲ 8-MeoMe MIX (405 μM)	BETH. (1 μM)	6	777 ± 200
□—□ BUFFER	BETH. (0.3 μM)	6	945 ± 248
■—■ 8-MeoMe MIX (405 μM)	BETH. (0.3 μM)	6	1102 ± 199
○—○ 8-MeoMe MIX (405 μM)	BUFFER	6	888 ± 254

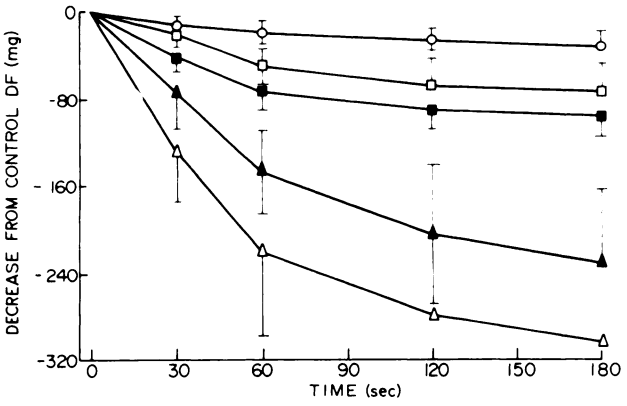

FIG. 1. *Time-related decreases in the peak developed force of left atrial strips treated with bethanecol (BETH.) or bethanecol plus 8-methoxymethyl MIX (8-MeoMe MIX)*

Left atrial strips were loaded with 1000 mg of force and paced electrically at 60 beats/min. Atria were exposed to either 8-methoxymethyl MIX or buffer (*treatment Column A*). Control values, taken 12 min later, show mean DFs for the five treatment groups immediately preceding the additions shown in *treatment Column B*. Buffer or bethanecol was added at 0 sec. The symbols show mean decreases in DF with time. *Vertical bars* show standard error of the mean for six experiments.

Isoproterenol and xanthines. These experiments uti-

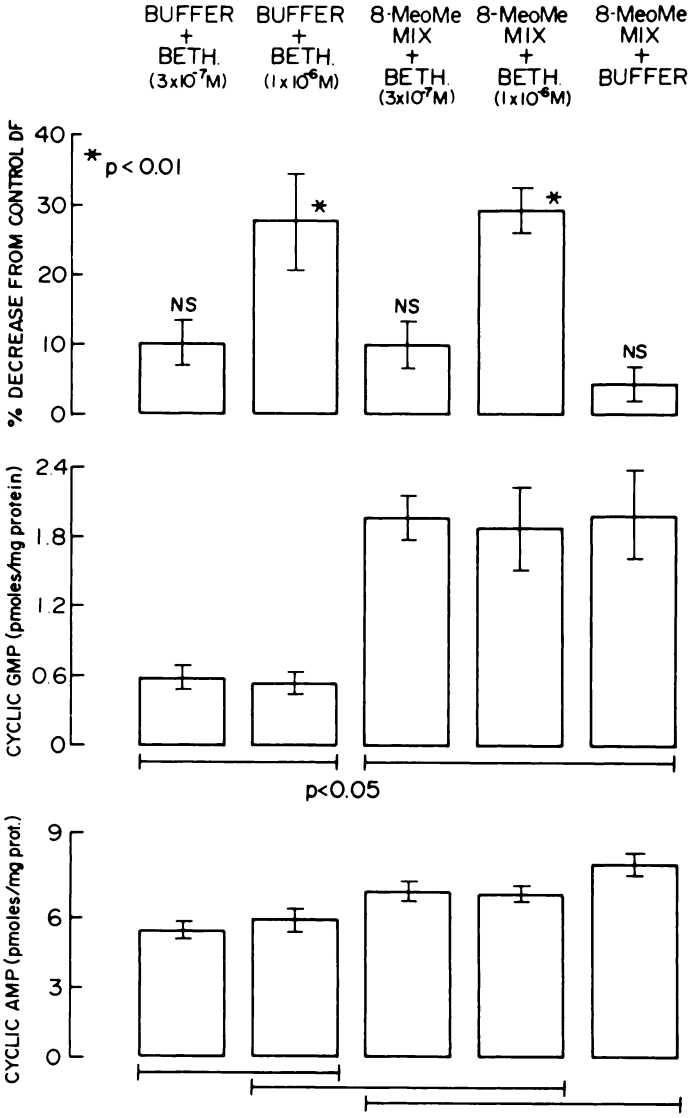

FIG. 2. *Effects of bethanechol (BETH.) on peak developed force, cyclic AMP content, and cyclic GMP content of left atrial strips in the absence and presence of 8-methoxymethyl MIX (8-MeoMe MIX)*

Left atrial strips were loaded with 1000 mg of force and paced electrically at 60 beats/min. After exposing atrial strips to buffer or 8-methoxymethyl MIX (405 μM) for 12 min, BETH. (0.3 μM or 1 μM) or buffer was added to baths. Strips were freeze-clamped 3 min later. *Bar heights* show mean percentage decreases from control (preBETH.) developed forces (*top panel*), mean cyclic GMP contents (*middle panel*), and mean cyclic AMP contents (*bottom panel*) at the end of the 3-min period. *Vertical brackets* show standard error of the mean of six experiments. Analysis of variance with Duncan's New Multiple Range Test was used to compare treatment group means. Mean developed forces of the five treatment groups were not statistically different before adding BETH. BETH. (1 μM) significantly decreased developed force (*, $p < 0.01$). In the *bottom two panels*, *bars* not underscored by a common line were statistically different from one another at the 5% level. NS, nonsignificant difference.

lized xanthines that are relatively selective inhibitors of cyclic GMP hydrolysis (8-*t*-butyl MIX and 8-methoxymethyl MIX) and a nonselective xanthine, theophylline (see preceeding paper, ref. 10). These xanthines were each used at concentrations 3-fold above the concentrations that inhibit by 50% the hydrolysis of 1 μM cyclic AMP by the 48,000 × *g* supernatant of rabbit atria. Left atrial strips, loaded with 1,000 mg of force and paced electrically at 30 beats/min, were allowed to equilibrate for 2–3 hr before xanthine or buffer was added to the bath. Twelve minutes after xanthine additions, the mean DF was increased by 47 ± 15% in atria treated with theophylline ($p < 0.01$); it was unchanged in the group treated with 8-methoxymethyl MIX, and it was decreased by 24 ± 9% in atria treated with 8-*t*-butyl MIX ($p < 0.05$; Fig. 3, *top portion*).

Subsequent addition of isoproterenol (10^{-8} M) produced time-related increases of DF in all treatment groups (Fig. 3, *lower portion*). By 3 min, isoproterenol had increased the mean DF by more than 100% in the control group (buffer) and by more than 390% in each xanthine-treated group. The mean contractile response to isoproterenol (417 ± 84 mg, $N = 6$) was enhanced 231% by 8-*t*-butyl MIX (864 ± 199 mg, $N = 6$), 300% by 8-methoxymethyl MIX (1251 ± 206 mg, $N = 6$) and 305% by theophylline (1272 ± 192 mg, $N = 6$) (Fig. 4, *top panel*).

TREATMENTS A	B	N	DF (mg, x̄ ± SEM) PRE-MeX	POST-MeX	% OF CONTROL (x̄ ± SEM) MeX EFFECT
8 MeoMe MIX	+ ISO	6	442 ± 167	432 ± 130	105 ± 5 NS
8-t-But-MIX	+ ISO	6	226 ± 74	142 ± 28	76 ± 9 *
Theophylline	+ ISO	6	321 ± 74	421 ± 164	147 ± 15 **
BUFFER	+ ISO	6	343 ± 129	334 ± 125	98 ± 4

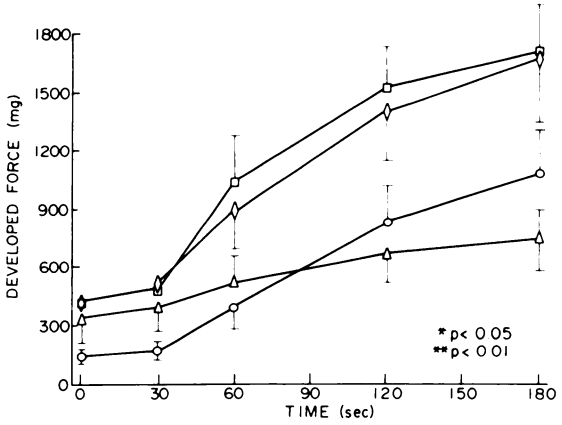

FIG. 3. *Time-related increases in peak developed force of left atrial strips treated with isoproterenol or isoproterenol plus xanthine*

Xanthines were used at three times their I_{50} cyclic AMP concentrations; I_{50} was defined as the concentration of xanthine producing a 50% inhibition of the hydrolysis of 1 μM substrate (cyclic AMP) by the 48,000 × *g* supernatant fraction of rabbit atria. Atrial strips were loaded with 1000 mg of force and were paced electrically at 30 beats/min. Premethylxanthine (*Pre-MeX*) and postmethylxanthine (*Post-MeX*) columns give mean peak DFs ± standard error of the mean before, and 12 min after, adding treatments specified in *Column A*. *N* = number of experiments. MIX = 1-methyl-3-isobutylxanthine. The inotropic effects of 8-methoxymethyl MIX (*8MeoMe MIX*; 405 μM), 8-*t*-butyl MIX (*8-t-But-MIX*; 77 μM), or theophylline (710 μM) in each atrial strip prior to addition of isoproterenol were determined using the relationship: (Post-MeX/Pre-MeX) × 100 = percentage of control. The mean percentage of control of each xanthine-treated group was compared statistically to that of the buffer-treated group. *, $p < 0.05$; **, $p < 0.01$; *NS* = nonsignificant difference. After atrial strips had been exposed to xanthines or buffer for 12 min, isoproterenol (final concentration = 10 nM; Treatment B) was added at 0 sec; *symbols* show mean developed forces as a function of time after addition of isoproterenol. *Vertical bars* show standard error of the mean.

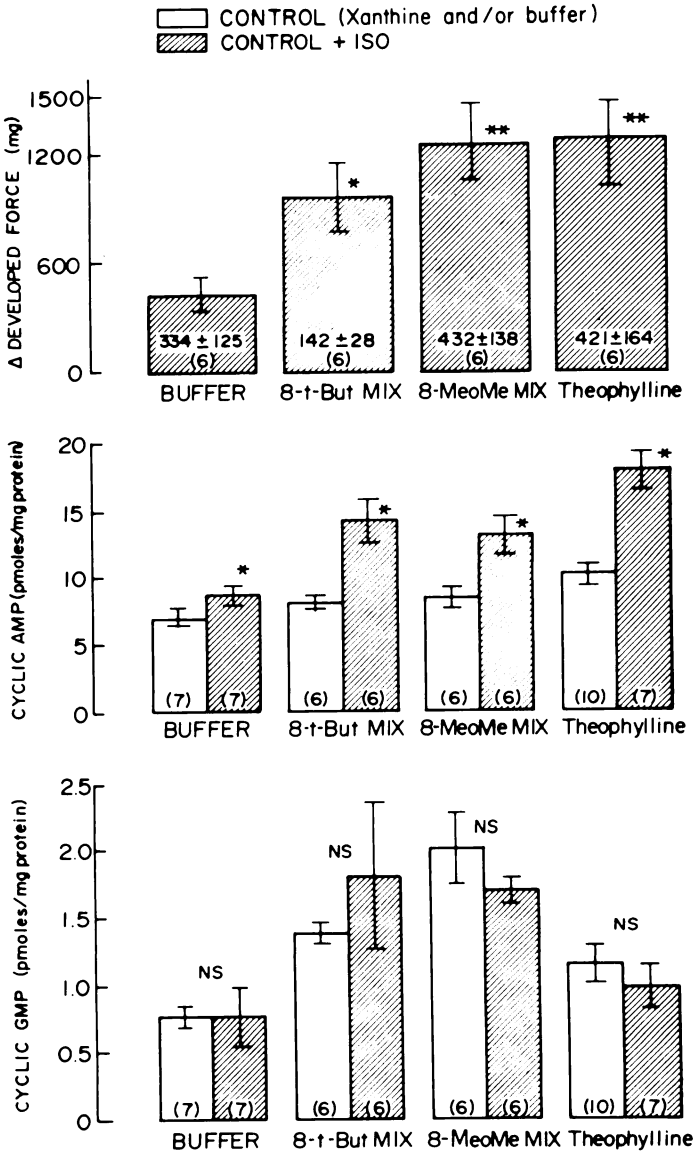

FIG. 4. *Effects of isoproterenol on peak developed force, cyclic AMP content, and cyclic GMP content of left atrial strips in the absence and presence of xanthines*

Left atrial strips of rabbits were loaded with 1000 mg of force and paced electrically at 30 beats/min. Numbers inside bars (*top panel*) give mean DFs ± standard error of the mean of atrial strips after a 12-min exposure to buffer, 8-*t*-butyl MIX (*8-t-But MIX*; 77 μM), 8-methoxymethyl MIX (*8-MeoMe MIX*; 405 μM), or theophylline (710 μM). The number of experiments are shown inside *parentheses*. MIX = 1-methyl-3-isobutyl-xanthine. Immediately after this 12-min exposure, buffer or isoproterenol (*ISO*, final concentration of 10 nM) was added to baths. Strips were freeze-clamped 3 min later. Bar heights show mean changes in DF (*top panel*), mean cyclic AMP contents (*middle panel*), and mean cyclic GMP contents (*bottom panel*) at the end of this 3-min period. *Hatched bars* show values from preparations in which a 3-min exposure to buffer was substituted for treatment with ISO. Vertical extensions from *bars* show standard error of the mean. Analysis of variance with Duncan's New Multiple Range Test was used to compare responses to ISO in groups treated with xanthine or buffer. *, $p < 0.05$; **, $p < 0.01$; *NS*, nonsignificant difference.

The isoproterenol-induced increase in the cyclic AMP content of buffer-treated atria was small but significant ($p < 0.05$) after a 3-min exposure to this agent (Fig. 4, *middle panel*). This response to isoproterenol was potentiated 3-fold by 8-methoxymethyl MIX, 4-fold by 8-*t*-butyl MIX, and 5-fold by theophylline. The extent to which the selective inhibitors of cyclic GMP hydrolysis and theophylline potentiated contractile responses and cyclic AMP responses to isoproterenol did not differ significantly. All xanthines increased mean atrial cyclic GMP content, but higher elevations were observed with the selective inhibitors of cyclic GMP hydrolysis than with theophylline. Isoproterenol did not alter the cyclic GMP contents of buffer- or xanthine-treated atria.

DISCUSSION

The present data demonstrate that cyclic GMP hydrolysis can be selectively inhibited without (a) concomitant negative inotropic effects, (b) potentiation of the cardiodepressant effects of cholinergic agents or (c) antagonism of the positive inotropic effects of *beta*-adrenergic agents. The xanthine, 8-methoxymethyl MIX, produced more than a 3-fold elevation of atrial cyclic GMP concentration (Fig. 4 and ref. 10) but failed to significantly alter either cyclic AMP concentration or peak developed force (Fig. 3). Despite its greater effect on cyclic GMP than on cyclic AMP levels, this agent potentiated contractile responses to isoproterenol but not bethanecol. Using xanthines at concentrations that produced an equivalent amount of cyclic AMP phosphodiesterase inhibition (3 times I_{50} values for inhibition of cyclic AMP hydrolysis), both the selective inhibitors of cyclic GMP hydrolysis (8-*t*-butyl MIX and 8-methoxymethyl MIX) and the nonselective inhibitor, theophylline, comparably potentiated contractile and cyclic AMP responses to isoproterenol (Fig. 4). These results corroborate the hypothesis that when xanthines potentiate contractile responses to isoproterenol, they do so by inhibiting the hydrolysis of cyclic AMP (12). However, these results are incompatible with the view that cyclic GMP antagonizes positive inotropic effects of agents that act via cyclic AMP (11).

The question of whether or not cholinergic agents produce their negative inotropic effects by stimulating guanylate cyclase has yet to be resolved. Consistent with this idea, Nawrath (7) reported that papaverine, a potent inhibitor of phosphodiesterase, enhanced the negative inotropic effects of acetylcholine in rat atria. In addition, Endoh and Honma (13) reported that carbachol (3 μM) antagonized positive inotropic effects of papaverine in dog ventricles, an antagonistic effect that was accompanied by a 4-fold potentiation of the carbachol-induced elevation in myocardial cyclic GMP concentration. However, the interpretation of the papaverine studies is not straightforward, since this agent produced negative inotropic effects that may be mediated by mechanisms independent of phosphodiesterase inhibition (14). On the other hand, 8-methoxymethyl MIX did not depress contractile function at concentrations that were nearly 300 times its I_{50} value for inhibition of cyclic GMP hydrolysis (see preceeding paper, ref. 10). In addition, this agent did not enhance the cyclic GMP levels response or the negative inotropic response to bethanecol. The simplest explanation of these data is that cyclic GMP is not involved in the negative inotropic effects of cholinergic agents. However, one possibility for the apparent dissociation between cyclic GMP responses and negative inotropic responses is that guanylate cyclase may be compartmentalized with the physiologically important enzyme being relatively unimportant in terms of its contribution to whole tissue cyclic GMP concentrations. This compartmentalization could be within the myocytes or could represent differences between the responsiveness of the cyclase of myocytes and of other cell types in the atrial preparations. This compartmentalization, whether intracellular or due to heterogeneity of cell types, could explain the failure to observe increases in whole tissue cyclic GMP levels at bethanecol concentrations that decreased DF by 28% in rabbit atria (Fig. 2) or at carbachol concentrations that decreased DF by 90% in cat atria (5). Moreover, it could explain the failure of nitroprusside to produce negative inotropic effects in the presence of 10-fold (15) to 17-fold (6) elevations in cardiac cyclic GMP. Lincoln and Keely (15) proposed that functionally distinct pools of cyclic GMP exist in heart, since nitroprusside elevated whole tissue cyclic GMP concentrations 8- to 10-fold without activating the cyclic GMP-dependent protein kinase activity, whereas acetylcholine activated the kinase while increasing cardiac cyclic GMP levels by only 2- to 3-fold. Assuming that the xanthines gain access to all intracellular phosphodiesterases, and there is no evidence that they do not, 8-methoxymethyl MIX should at least enhance the cyclic GMP signal in the vicinity of the guanylate cyclase that is stimulated by choline esters. Since 8-methoxymethyl MIX fails to enhance negative inotropic responses to bethanecol, it seems unlikely that cyclic GMP is intimately involved in this cholinergic response. Thus, the present results suggest that inhibition of cyclic GMP hydrolysis neither enhances the cardiodepressant effects of cholinergic agonists nor antagonizes the positive inotropic effects of *beta*-adrenergic agents.

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