Bimodal distribution of vasodilator responsiveness to adenosine due to difference in nitric oxide contribution: implications for exercise hyperemia

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Submitted 9 June 2005; accepted in final form 29 March 2006

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cAMP and cGMP vasodilator pathways, respectively, in a subgroup of the subjects. Finally, given that Ado-mediated vasodilation may also have a NO-mediated component (37), we used the NOS inhibitor N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA) in a second protocol to measure the effect of inhibition of NO production on the Ado- and exercise-mediated vasodilation.

**METHODS**

**Subjects**

All protocols and procedures were approved by the Institutional Review Board at Mayo Clinic. A total of 27 young healthy subjects (15 men and 12 women) participated in the study after giving written, informed consent. All subjects were nonsmokers, nonobese, normotensive, and not taking any medications other than oral contraceptives. Female subjects were not pregnant, as determined by a pregnancy test <24 h before the study. Female subjects were studied during the placebo phase of oral contraceptive use, or in the early follicular phase of their menstrual cycle to minimize possible confounding influences of reproductive hormones on control of blood flow (2, 23). The subjects fasted overnight and refrained from caffeine 48 h before the study and alcohol and exercise 24 h before the study.

**General Methods**

**Arterial catheterization.** The brachial artery of the nondominant forearm was catheterized under aseptic conditions after local anesthesia (1% lidocaine). A standard 5-cm, 20-gauge Teflon catheter was inserted and connected to a three-port connector system for simultaneous measurements of arterial pressure and local administration of study drugs (5).

**FBF and vascular conductance.** A 4-MHz pulsed Doppler probe (model 500V, Multigon Industries, Mt. Vernon, NY) was used to measure brachial artery mean blood velocity (MBV) with the probe securely fixed to the skin over the brachial artery proximal to the catheter insertion site as previously described (40). The probe transducer angle was 60°. A linear 7.0-MHz echo Doppler ultrasound probe (model 128XP Acuson, Mountain View, CA) was placed in a holder securely fixed to the skin immediately proximal to the velocity probe to measure brachial artery diameter. FBF was calculated as:

\[
\text{FBF} = \frac{\text{MBV} \times \text{brachial artery diameter}^2}{2} \times 60
\]

where FBF is in milliliters per minute, MBV is in centimeters per second, the brachial diameter is in centimeters, and 60 is used to convert from milliliters per second to milliliters per minute. Forearm vascular conductance (FVC) was calculated as [FBF/mean arterial pressure (MAP)] × 100, and it is expressed as milliliters per minute per 100 millimeters of Hg.

**Rhythmic handgrip exercise.** Rhythmic forearm handgrip exercise was performed at three different workloads using a 2.3-, 4.6-, or 6.9-kg weight for female subjects or a 3.2-, 6.4-, or 9.6-kg weight for male subjects. The weight was lifted 5 cm over a pulley at a duty cycle of 1 s of contraction-2 s relaxation (20 contractions/min) using audio and visual signals to ensure the correct timing. These workloads averaged 6.8 ± 0.2, 13.7 ± 0.5, 20.6 ± 0.7% of maximum voluntary contraction for the low, medium, and high workloads, respectively, for all subjects.

**Reactive hyperemia.** Maximum FBF was estimated using reactive hyperemia. FBF was measured for 1 min after 5 min of brachial artery occlusion via inflation of a suprasystolic (220 mmHg) blood pressure cuff on the upper arm.

**Brachial Artery Infusions**

Drugs were infused on the basis of forearm volume (FAV; water displacement) via the brachial artery catheter. The absolute infusion rate was <3 ml/min in every trial.

Ado. Ado was infused into the brachial artery at three doses: 3,125, 6,25, and 12.5 \(\mu\text{g}\cdot\text{min}^{-1}\cdot\text{dl FAV}^{-1}\). Our goal was to match FBF levels during low, medium, and high doses of Ado with the corresponding exercise workload. Ado doses and exercise workloads were chosen according to previous studies from our laboratory (6, 40).

**Isoproterenol and ACh.** Because Ado-mediated vasodilation can occur via both the cAMP and cGMP pathways (25, 26), we assessed the integrity of the cAMP vasodilator pathway with dose-response determinations using isoproterenol, a nonspecific \(\beta\)-adrenergic agonist, and the integrity of the endothelial (NO) cGMP pathway with dose-response determinations using ACh, a nonspecific muscarinic agonist (protocol 2, below). Isoproterenol was infused at 1.0, 6.0, and 12.0 ng \(\text{min}^{-1}\cdot\text{dl FAV}^{-1}\), and ACh was infused at 1.0, 4.0, and 8.0 \(\mu\text{g}\cdot\text{min}^{-1}\cdot\text{dl FAV}^{-1}\).

**L-NMMA.** Because Ado-mediated vasodilation may contain a significant NO-mediated component (37), L-NMMA was infused to measure the effect of inhibition of NO production on Ado-mediated vasodilation and exercise hyperemia (protocol 3, below). L-NMMA was infused at a loading dose of 5 mg/min for 5 min and then at a maintenance dose of 1 mg/min for the remainder of the experiment.

**Specific Protocols**

**General experimental approach.** Each trial consisted of 1 min of baseline measurement with saline infusion, followed either by sequential intra-arterial infusions of low, medium, and high doses of vasodilator drugs or by rhythmic handgrip exercise at low, medium, and high workloads (4 min at each dose or workload). All subjects participated in more than one protocol.

**PROTOCOL 1.** Twenty-seven subjects participated in protocol 1 as shown in Fig. 1A. We conducted two Ado dose-response trials and two exercise workload-response trials. Successive trials were separated by 20 min of rest to allow FBF to return to baseline values. The order of the Ado dose-response trials and forearm handgrip exercise trials was randomized.

**PROTOCOL 2.** Twenty-two of the subjects from protocol 1 participated in protocol 2 as shown in Fig. 1B. We conducted one isoproterenol dose-response trial and one ACh dose-response trial. Successive trials were separated by 20 min of rest to allow FBF to return to baseline values. The order of the trials was randomized.

**PROTOCOL 3.** Seventeen of the subjects in protocol 1 also participated in protocol 3 as shown in Fig. 1C. In this protocol, we conducted one Ado dose-response, one exercise workload-response, and one reactive hyperemia trial before L-NMMA infusion and one of each of these trials after blockade of NOS with infusion of L-NMMA. Successive trials were separated by 20 min of rest to allow FBF to return to baseline values. L-NMMA was loaded during the rest period after the first reactive hyperemia trial and continued at a maintenance dose for the remainder of the experiment.

**Data Acquisition and Analysis**

Data were collected and stored on computer at 250 Hz and analyzed offline with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH). MAP was determined from the arterial pressure waveform. FVC is reported as the change above baseline FVC values. Baseline FVC and MAP values reported represent 60-s averages during baseline. Other FVC values represent the change above baseline FVC values presented as 30-s averages taken during the last minute of each dose or workload. For protocol 1, FVC values from the first Ado and first exercise trials are reported.

**Statistics**

All values are reported as means ± SE. Subject demographics were compared using a rank sum test, and gender was compared using Fisher’s exact test. Repeated-measures ANOVA was used to assess differences between treatment groups and levels. Two-sample t-test
was used to compare pairs of group means over levels of a factor. When significance was detected, Tukey’s post hoc test was used to identify individual differences and to adjust \( P \) values to account for multiple comparisons, to preserve an overall type I error rate of 0.05. Significance was set at \( P < 0.05 \).

**RESULTS**

**Subjects**

Group mean age, body mass index (BMI), and FAV were 27 ± 1 yr, 23.1 ± 0.6 kg/m\(^2\), and 1,005 ± 38 ml, respectively, for all subjects. Based on criteria outlined below, we identified subjects who had robust vasodilator responses to both Ado infusions and handgrip exercise as “responders” and those had blunted vasodilator responsiveness to Ado infusion, compared with their vasodilator responsiveness to handgrip exercise, as “nonresponders.” Ado nonresponders were defined as subjects in whom the FVC during each exercise workload was twice the FVC during each Ado infusion dose. Group mean age, BMI, and FAV were 27 ± 1 yr, 22.8 ± 0.7 kg/m\(^2\), and 1,008 ± 52 ml, respectively, for Ado responders and were 25 ± 2 yr, 23.6 ± 0.9 kg/m\(^2\), and 1,000 ± 52 ml, respectively, for Ado nonresponders. There were no statistical differences in subject

![Diagram A](image1)

![Diagram B](image2)

![Diagram C](image3)
demographics, including caffeine use or exercise training between Ado responders and nonresponders or between subjects in protocol 1 vs. protocol 2 (P > 0.45).

Baseline FVC and MAP before each set of infusions or exercise workloads did not differ between trials or between Ado responders and nonresponders (P > 0.05). Average baseline FVC was 179 ± 20 ml·min⁻¹·100 mmHg⁻¹, and average baseline MAP was 91 ± 2 mmHg.

Protocol 1

Vasodilator responses to Ado and exercise. Fifteen subjects from both protocols showed robust vasodilator responses to both Ado infusion and exercise, and they were identified as Ado responders. The other 12 subjects showed blunted vasodilator responses to Ado infusion compared with vasodilator responses to handgrip exercise (FVC during each exercise workload was more than twice the FVC during the corresponding dose of Ado), and they were identified as Ado nonresponders. For the low, medium, and high doses of Ado, respectively, the change in FVC above baseline for the 15 Ado responders was 209 ± 33, 419 ± 57, and 603 ± 75 ml·min⁻¹·100 mmHg⁻¹, and for the 12 Ado nonresponders it was 102 ± 36, 113 ± 42, 151 ± 54 ml·min⁻¹·100 mmHg⁻¹ (P < 0.04 at low dose and P < 0.001 at medium and high doses for Ado responders vs. nonresponders), as shown in Fig. 2. As shown in the figure, the change in FVC above baseline during each exercise workload was similar to the corresponding Ado infusion dose in Ado responders (P > 0.58), but it was different in Ado nonresponders (P < 0.01).

Repeatability of adenosine and exercise trials. In protocol 1, the two Ado trials and two exercise trials were repeatable (P > 0.05 between trials; data not shown). Additionally, the order of the trials had no impact on the magnitude of vasodilator responsiveness.

Protocol 2

Vasodilator responses to isoproterenol. The mean vasodilator responses to isoproterenol infusion were significantly higher in Ado responders compared with nonresponders, as shown in Fig. 4A. The change in FVC above baseline for the low, medium, and high doses, respectively, was 135 ± 31, 314 ± 67, and 446 ± 94 ml·min⁻¹·100 mmHg⁻¹ for the 11 Ado responders, and it was 20 ± 5, 86 ± 32, and 110 ± 37 ml·min⁻¹·100 mmHg⁻¹ for the 11 Ado nonresponders (P < 0.01).

Vasodilator responses to ACh. The mean vasodilator responses to ACh infusion were higher in Ado responders compared with nonresponders, although this was only significant at the medium dose, as shown in Fig. 4B. The change in FVC above baseline for the low, medium, and high doses, respectively, was 421 ± 106, 590 ± 116, and 686 ± 137 ml·min⁻¹·100 mmHg⁻¹ for the 11 Ado responders and was 240 ± 108, 240 ± 106, and 307 ± 143 ml·min⁻¹·100 mmHg⁻¹ for the 11 Ado nonresponders (P = 0.25, 0.04, and 0.07 for the low, medium, and high doses, respectively).

Protocol 3

Blunting of Ado responses in Ado responders by L-NMMA. L-NMMA significantly decreased baseline FVC in all subjects (P < 0.05 pre- vs. post-L-NMMA), as reported previously (10).

Fig. 2. Change (Δ) in FVC above baseline for low, medium, and high Ado doses or exercise workloads. Values are means ± SE. Ado responses were significantly greater in Ado responders (n = 15) vs. Ado nonresponders (n = 12; P < 0.05). Exercise responses were similar between Ado responders and nonresponders (P > 0.05).

Fig. 3. ΔFVC above baseline for low, medium, and high adenosine (Ado) doses. Individual data points and averages are shown for the Ado responders (n = 15) and Ado nonresponders (n = 12). *P < 0.05 for every Ado dose in responders vs. nonresponders.
Furthermore, as shown in Fig. 5A, L-NMMA significantly blunted vasodilator responsiveness to Ado at all Ado infusion doses, but this occurred only in Ado responders. In the 10 Ado responders, for the low, medium, and high workloads, respectively, change in FVC above baseline was 217 ± 51, 404 ± 77, and 527 ± 92 ml·min⁻¹·100 mmHg⁻¹ before L-NMMA, and it was 218 ± 59, 426 ± 94, and 558 ± 114 ml·min⁻¹·100 mmHg⁻¹ after L-NMMA infusion (P > 0.05 at each dose pre- vs. post L-NMMA infusion).

No blunting of exercise hyperemia by L-NMMA. As shown in Fig. 5B, L-NMMA failed to blunt exercise hyperemia at any workload in either Ado responders or nonresponders. In the 10 Ado responders, for the low, medium, and high workloads, respectively, change in FVC above baseline was 217 ± 51, 404 ± 77, and 527 ± 92 ml·min⁻¹·100 mmHg⁻¹ before L-NMMA, and it was 218 ± 59, 426 ± 94, and 558 ± 114 ml·min⁻¹·100 mmHg⁻¹ after L-NMMA.

Fig. 4. A: ΔFVC above baseline for low, medium, and high isoproterenol (Iso) doses. Iso vasodilator responses were significantly higher in Ado responders. *P < 0.05 for Ado responders vs. nonresponders. B: ΔFVC above baseline for low, medium, and high acetylcholine (ACh) doses. ACh vasodilator responses were significantly higher in Ado responders only at the medium dose. †P < 0.05 for Ado responders vs. nonresponders.

Fig. 5. A: ΔFVC above baseline for low, medium, and high Ado doses before and after L-NMMA infusion. L-NMMA significantly blunted Ado-mediated vasodilation in Ado responders (n = 10) but not in nonresponders (n = 7). *P < 0.05 for Ado responders vs. nonresponders. †P < 0.01 for Ado responders vs. Ado responders + l-NMMA. B: ΔFVC above baseline for low, medium, and high exercise workloads before and after L-NMMA infusion. There were no differences between subgroups in exercise vasodilation, and there was no effect of L-NMMA on this response (P > 0.05 for all).
ml·min⁻¹·100 mmHg⁻¹ after L-NMMA infusion. For the seven Ado nonresponders, these values were 261 ± 79, 418 ± 101, and 561 ± 142 ml·min⁻¹·100 mmHg⁻¹ before L-NMMA, and they were 167 ± 44, 382 ± 86, and 519 ± 133 ml·min⁻¹·100 mmHg⁻¹ after L-NMMA infusion (P > 0.05 for Ado responders vs. nonresponders and pre- vs. post-L-NMMA in both subgroups).

No difference in maximum FBF. We found no difference in maximum FBF between Ado responders and nonresponders as obtained during the reactive hyperemia trial. Change in FVC above baseline after 5 min of brachial artery occlusion was 960 ± 151 ml·min⁻¹·100 mmHg⁻¹ for Ado responders and 899 ± 145 for nonresponders before L-NMMA. (P > 0.76 for Ado responders vs. nonresponders.) These numbers did not change after L-NMMA infusion (P > 0.60).

DISCUSSION

The major new finding of the present study was our identification of an apparent bimodal distribution of vasodilator responses to Ado among human subjects, such that some subjects demonstrated robust vasodilation (Ado responders) and others much less vasodilation (Ado nonresponders). No significant difference in subject demographics, caffeine use, exercise training, maximum FBF, or exercise hyperemia was found between these two subgroups. We found significantly different vasodilator responses to isoproterenol infusion between Ado responders and nonresponders at all doses, and we found nearly significant differences in vasodilator responses to ACh. We also found that NOS inhibition blunted FVC responses to Ado infusion in Ado responders, but it had no effect in Ado nonresponders. L-NMMA infusion failed to blunt exercise hyperemia in either group.

Although intersubject variability is a common observation in human studies and previous studies have reported variability to Ado infusion (6) and ACh infusion (3), this is the first report, to our knowledge, of a bimodal distribution of vasodilator responsiveness to intra-arterial infusion of Ado among human subjects, despite similar responses to exercise and reactive hyperemia. These findings may explain previous contradictory reports concerning the contribution of NO to Ado-mediated vasodilation, and they may have important implications for our understanding of the mechanisms of exercise hyperemia, as discussed below. The existence of a bimodal distribution provides evidence that the contribution of Ado to exercise hyperemia might have a variability with a consistent biological basis.

In both animal and human studies, there is evidence to both support and reject a key role for Ado in exercise hyperemia. Recent enthusiasm for Ado comes primarily from human studies showing that when administered exogenously, Ado evokes vasodilator responses similar to those seen during exercise (30, 31, 34). However, the present data suggest that this relationship is more complex. Our results partially agree with the study by Rådegran and Calbet (31), in which arterial infusion of exogenous Ado evoked a vasodilator response that mimicked the increase in blood flow observed in response to exercise in previous studies in their laboratory (30, 34). However, these authors did not report variability in the vasodilator responses to Ado infusions among subjects. Our present results may suggest that Ado is not an obligatory role for exercise hyperemia, because 12 of our 27 subjects demonstrated blunted vasodilator responses to Ado infusion, despite robust vasodilator responses to exercise.

There are several possible explanations for our findings. First, there might be a nonspecific difference in downstream vasodilator mechanisms between Ado responders and nonresponders, because Ado-mediated vasodilation can occur via both cAMP and cGMP pathways (25, 26). We did observe a significant difference in vasodilator responses to isoproterenol infusion between Ado responders and nonresponders. Although isoproterenol does not work exclusively via cAMP-mediated vasodilation (41), it has a strong cAMP component and may also evoke NO release. In this context, perhaps Ado responders have a greater cAMP component of Ado-mediated vasodilation perhaps resulting from stimulation of a greater number of Gs stimulatory protein-mediated Ado receptor subtypes (Ado receptors A2A and A2B), which are present on both endothelial and skeletal muscle cells (21).

Along these lines, ACh does not work exclusively via cGMP-mediated vasodilation (8), but it does clearly have cGMP-mediated NO release and vasodilation. However, we only observed a significant difference in vasodilator responses to ACh infusion at the medium dose between Ado responders and nonresponders, although the means were different at each dose. Although ACh has multiple physiological effects (3, 8), these data may suggest a difference in cGMP-component of Ado-mediated vasodilation between Ado responders and nonresponders, especially when combined with the Ado + L-NMMA data, as discussed below.

Our data may suggest that there is a difference in the NO contribution to Ado-mediated vasodilation between subgroups. Some previous studies have shown blunting of Ado-induced skeletal muscle vasodilation by NOS inhibition (32, 37), whereas other studies demonstrated no effect of NOS inhibition Ado-induced skeletal muscle vasodilation (3, 18, 19). In the present study, we demonstrate a statistically significant contribution of NO to Ado-mediated vasodilation in Ado responders only. These data are consistent with the greater vasodilator response to ACh observed in Ado responders, because ACh acts in part via NO (3, 8). Therefore, these data may clear up confusion concerning the role of NO in Ado-mediated vasodilation, because the previous studies did not comment on intersubject variability in Ado vasodilator responses.

Another area of conflicting evidence concerns the role of NO in exercise hyperemia. Inhibition of NOS blunted exercise hyperemia (9, 11) in some studies, whereas others demonstrated no effect of NOS inhibition Ado-induced skeletal muscle vasodilation (3, 18, 19). In the present study, we demonstrate a statistically significant contribution of NO to Ado-mediated vasodilation in Ado responders only. These data are consistent with the greater vasodilator response to ACh observed in Ado responders, because ACh acts in part via NO (3, 8). Therefore, these data may clear up confusion concerning the role of NO in Ado-mediated vasodilation, because the previous studies did not comment on intersubject variability in Ado vasodilator responses.

The use of specific l-arginine analogs [l-NMMA vs. ΔG²-nitro-l-arginine methyl ester (l-NAME)] in these studies may have contributed to discrepant findings, although previous studies from our laboratory have failed to find a difference in efficacy of l-NMMA or l-NAME to block NOS (6). Additionally, when NOS inhibitors are given via the brachial artery before contraction (as in the present study), the impact on the exercise-induced rise in blood flow above baseline is generally minimal. Along these lines, in the present study, we found no
significant NO component to exercise hyperemia at any workload in either subgroup.

The present study does not exclude the possibility of other specific differences in mechanisms of vasodilation between Ado responders and nonresponders. First, there might be a structural difference that limits the ability to vasodilate in Ado nonresponders. However, there were no differences in exercise hyperemia or peak reactive hyperemic blood flow between Ado responders and nonresponders, suggesting that both subgroups have the capacity to vasodilate to the same magnitude. Second, there may be a genetic variation in some element of Ado metabolism or the Ado-mediated vasodilator pathway might contribute to the responses we observed (4, 12, 15), such as genetic polymorphisms in the Ado receptor or Ado transporter in either of the two subgroups. Third, differences in Ado receptor or Ado transporter density may exist between subgroups. These situations could either prevent adequate amounts of Ado from binding Ado receptor in nonresponders compared with responders, or they could result in more efficient clearance and metabolism of Ado from the interstitial space in nonresponders compared with responders.

**Experimental Considerations**

First, we chose in this experiment to compare Ado-induced increases in FVC with exercise-induced increases in FVC. This is a nontraditional method of investigating the contribution of Ado to exercise hyperemia. However, we believe that, by identifying Ado responders and nonresponders, our approach sheds important new light on the possible role of Ado in exercise hyperemia, as well as a potential confound in interpretation of previous negative or conflicting reports.

Second, the drug doses and exercise workloads were chosen according to previous studies from our laboratory (6, 40). In this context, the majority of subjects demonstrated significantly smaller increases in FVC between the medium and high doses of Ado than between baseline and low or baseline and medium Ado doses. Therefore, increasing the doses would likely have produced a “ceiling” or plateau effect on FVC and would confounded the results.

Third, we used the L-arginine analog l-NMMA rather than L-NAME to block NOS. We believe that this had no effect on our results, because previous studies from our laboratory have failed to find a difference in efficacy of l-NMMA or L-NAME to block NOS during exercise (6). Furthermore, the use of L-NMMA to block NOS during Ado infusion has both blunted (37) and fail to blunt (3) Ado-mediated vasodilation in different human blood flow studies, suggesting that the choice of NOS inhibitor did not contribute to the discrepant findings.

It could be argued that delivery of exogenously infused Ado into the interstitial space may be limited if there is a significant endothelial barrier to exogenous Ado. However, MO and Ballard (24) have shown that interstitial concentrations of Ado increased when sufficient Ado was infused arterially into dog skeletal muscle. The Ado infusion doses used in this study are likely large enough to account for much of the vasodilator response we observed during the Ado infusion trials, because the resting interstitial concentration of Ado in human skeletal muscle is very low (20).

Finally, our subjects refrained from caffeine use 48 h before the study. This was done to prevent confounding effects of methylxanthine antagonism of Ado receptors during the study. We also considered possible differences in caffeine use among subjects, which could contribute to variable responses to Ado, but after querying all subjects about caffeine use, we found no difference between Ado responders and nonresponders, eliminating the possibility that sensitivity or tolerance to Ado infusion could be related to excessive caffeine use in either subgroup of subjects.

In summary, we investigated the potential role of Ado in exercise hyperemia by comparing Ado-mediated vasodilation with vasodilation during voluntary forearm contractions in human subjects. We report an apparent bimodal distribution of Ado-mediated vasodilation in the human forearm, such that Ado-mediated vasodilation was equally as robust as exercise-mediated vasodilation in about half the subjects in each of two protocols, and the remaining subjects were only minimally responsive to Ado. This difference in Ado vasodilator responsiveness may be due to a larger NO, or endothelial, component of Ado-mediated vasodilation in the Ado responders. The observation that exercise-mediated vasodilation was similar between subgroups suggests that the contributions of both Ado and NO to skeletal muscle vasodilation with exercise may be nonobligatory. Differences between these subgroups in structure or density of Ado receptors, or in Ado transporters, could account for this bimodal distribution and require further investigation.

**ACKNOWLEDGMENTS**

We thank Shelly Roberts, Karen Krucker, Pam Engrav, Branton Walker, Chris Johnson, Dr. Niki Dietz, and Dr. Francis Haddy for their contributions and insights, and the subjects who volunteered for this study.

**GRANTS**

This research was supported by National Institutes of Health (NIH) Grant HL-46493 (to M. J. Joyner), NIH Grant GM-08685 (to W. T. Nicholson), and NIH General Research Center Grant RR-00585 (to the Mayo Clinic, Rochester, MN).

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