In Vivo Blockade of Platelet ADP Receptor P2Y₁₂ Reduces Embolus and Thrombus Formation but Not Thrombus Stability

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Objective—ADP is a key platelet agonist in thromboembolism. One of the receptors involved in ADP-induced platelet activation is the P2Y₁₂ receptor, which is a target for antithrombotic drugs.

Methods and Results—Here, we present first evidence for a differential role of this receptor in thrombus and embolus formation in vivo. Anesthetized rabbits were treated with the selective P2Y₁₂ antagonists AR-C69931 MX (3 μg·kg⁻¹·min⁻¹ IV) or clopidogrel (25 mg/kg orally). Efficacy of these treatments was monitored by aggregation and thrombin generation measurements in blood samples ex vivo. Mesenteric arterioles were mechanically injured; thrombus growth and subsequent embolus formation were visualized by real-time intravital microscopy. AR-C69931 MX and clopidogrel significantly (P<0.05) reduced the total duration of embolization (by 52% and 36%, respectively), and fewer and smaller emboli were produced. The size of the initial thrombus was significantly reduced (P<0.005), but its stability was unaffected: plug formation was still effective.

Conclusions—These findings demonstrate that ADP and its P2Y₁₂ receptor are involved in thrombus growth and especially in the formation of emboli on the downstream side of the initial thrombus. This may explain the beneficial effects of P2Y₁₂ receptor antagonists in secondary prevention of ischemic events in patients with arterial thrombosis. (Arterioscler Thromb Vasc Biol. 2003;23:●●●●●●●.)

Key Words: vessel wall injury ■ in vivo thromboembolism ■ platelet activation ■ adenosine diphosphate ■ antithrombotic drugs

Interactions between activated platelets and the vessel wall play a key role in normal hemostasis but also in vascular diseases such as arterial thrombosis. When platelets adhere to a damaged or diseased vessel wall, they become activated and recruit other platelets into an aggregate. Adherent platelets also expose a procoagulant surface on which thrombin can be formed; the resulting fibrin stabilizes the aggregate, and bleeding is stopped. However, this primary thrombus often remains highly thrombogenic, resulting in the subsequent production of ≥1 embolus. These secondary emboli may cause downstream vascular occlusions and lead to ischemia. Therefore, especially the embolization phase of the thromboembolic process is potentially hazardous. For instance, in patients with atherothrombotic disease, thrombi may form at the surface of an atherosclerotic plaque, and embolization may lead to myocardial or cerebral infarction or peripheral arterial ischemia.

ADP is likely to play an important role in thromboembolism. Once released from dense granules on platelet activation, ADP amplifies the responses to other platelet agonists such as collagen and thrombin.² ADP-induced platelet activation involves 2 receptors, P2Y₁ and P2Y₁₂, both of which contribute to full aggregation in vitro.³ The P2Y₁ receptor is coupled to the Gq protein, causing mobilization of Ca²⁺ ions to the cytosol, leading to shape change and initiation of aggregation.⁴,⁵ The recently cloned platelet-specific P2Y₁₂ receptor⁶ signals the inhibition of adenylate cyclase via Gi and serves to complete and amplify the aggregation response to ADP.⁶,⁷ The P2Y₁₂-mediated signaling pathway involves the phosphoinositide 3-kinase–dependent activation of the platelet fibrinogen receptor α₃β₃,⁸ and it has also been implicated in stabilization of platelet aggregates induced by thrombin receptor activation.⁹

Pharmacological agents inhibiting P2Y₁₂ receptors have found wide applications as antithrombotic drugs. For instance, the thienopyridine derivatives ticlopidine and clopidogrel, forming a metabolite with potent anti-P2Y₁₂ receptor activity,¹¹ significantly reduce the risk of ischemic events in symptomatic atherothrombosis.¹²,¹³ Other novel P2Y₁₂ antagonists of the AR-C series¹⁴,¹⁵ also have demonstrated efficacy as antithrombotic drugs. However, not much is known about how these drugs interfere in the dynamic process of thrombus

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formation and subsequent embolization, because most of the
in vivo studies on the role of the P2Y12 receptor in thrombotic
processes provide information only on end-point parameters
such as bleeding time6 or time to occlusion of a vessel.16
Therefore, it was the aim of the present study to investigate
the mechanism underlying the antithrombotic effects of P2Y12
receptor inhibition in an established rabbit model of real-time
thromboembolism in vivo17–20 by using the specific antago-
nist AR-C69931 MX (AR-CMX) and clopidogrel. For the
first time, we provide evidence that blocking the P2Y12
receptor reduces thrombus formation and substantially dimin-
ishes embolization, without influencing the stability of the
thrombus that sheds the emboli.

Methods
Animal Preparation and Intravital Microscopy
Experiments using laboratory animals were approved by the local
ethics committee. These experiments were performed on New
Zealand White rabbits (1.8 to 2.8 kg, n=33) of either sex as
described previously.17–20 Rabbits were anesthetized by intramuscu-
lar injections of 40 mg ketamine hydrochloride (Nimatek, Eurovet)
and 4 mg xylazine hydrochloride (Sedamun, Eurovet) per kilogram
body weight. Anesthesia was maintained by continuous intravenous
infusion of ketamine (40 mg/kg per hour) and xylazine (5 mg/kg per
dissolved in lactetrol (15 mL/h, Eurovet). Body temperature
was kept at 37°C to 38°C. Arterial pressure and heart rate were
continuously monitored by using an external pressure transducer
(Uniflow, Baxter) connected to a catheter in the femoral artery.
Rabbits were ventilated through a tracheal cannula with a mixture of
nitrogen (74%), oxygen (25.5%), and carbon dioxide (0.5%) to
control systemic arterial pH (7.46±0.01 [mean±SEM]; PO2
(83±3 mm Hg), and PEO2 (46±1 mm Hg). Arterial platelet counts and
hematocrit values were assessed before the start of experimen-
tation (Coulter Counter, Coulter Electronics). Ear bleeding time
was determined by making incisions (1 mm deep and 10 mm long)
parallel to the long axis of the ear, avoiding macroscopically visible
vessels. The incision site was carefully blotted with filter paper at
30-second intervals. Bleeding time was assessed from incision until
the paper was no longer stained with blood.

Through a midline abdominal incision, a segment of the distal
ileum was exteriorized. The mesentery was continuously superfused
with buffered Tyrode’s solution (37°C, pH 7.35 to 7.40) and
visualized with an intravital microscope (Leitz) with a long working
distance objective (Leitz L1.25, numerical aperture 0.35). Images
were projected on a charge-coupled-device camera (Hamamatsu)
and stored on videotape for offline analysis.

Vessel Wall Puncture and Thromboembolic Reaction
A thromboembolic reaction was induced in selected mesenteric
arterioles (diameter 20 to 40 μm).17–20 After a stabilization period of
30 minutes, arterioles were mechanically injured by wall puncture
using a glass micropipette with tip diameter of 6 to 8 μm. Puncture
was considered successful if red blood cells were seen leaving the
vessel, indicating that all vessel wall layers were damaged. In all
vessels, bleeding stopped after a few seconds by the formation of a
white platelet-rich thrombus. Circulating platelets continuously ad-
ered to the downstream side of this stationary thrombus, and these
newly formed aggregates embolized repeatedly. Embolization
stopped after some time, while the thrombus remained unchanged
at the site of injury during the observation period of 600 seconds per
vessel. Arterioles were punctured up to 3 hours after the stabilization
period.

The following parameters were quantified offline: duration of
bleeding (microvessel bleeding time), occurrence of rebleedings,
maximal thrombus height relative to local vessel diameter, total
duration of embolization, number of emboli produced, size of
individual emboli, and median embolus production time per vessel.
Emboli were counted when their short axis, perpendicular to the
vessel wall, was >5 μm. Aggregates of smaller dimensions could
not be distinguished from the background with sufficient accuracy.
Sizes of individual emboli were measured along their short axes and
categorized on a semiquantitative scale: 5 to 10 μm, 10 to 15 μm,
etc. Vascular diameter was measured offline with an image-shearing
device. Mean red blood cell velocity was measured online21 for 1
minute directly preceding puncture; wall shear rate was calculated by
using the velocity and diameter data according to Tangelder et al.22

Administration of AR-CMX and Clopidogrel In Vivo
The optimally effective dose of AR-CMX was determined ex vivo by
whole-blood aggregometry in 4 rabbits. AR-CMX (Astra Zeneca)
was infused via a catheter in an ear vein. Its dose was increased
stepwise from 0 (saline=baseline) to 0.01, 0.03, 0.1, 0.3, 1, and 10
μg/kg per minute. After each infusion step (20 minutes), 0.9 mL
arterial blood was collected in 0.1 mL trisodium citrate (0.129
mol/L) via the arterial catheter. Whole-blood aggregation was
induced by 20 μmol/L ADP and measured with an impedance
aggregometer (Chronolog) at 37°C in the presence of H-Phe-Pro-Arg
chloromethyl ketone (PPACK, 40 μmol/L) and CaCl2 (16.6 mmol/L)
to achieve physiological concentrations of free Ca2+. Inhibition of
the maximum level of aggregation by AR-CMX was determined
relative to baseline level and calculated as follows: [(baseline−AR-
CMX)/baseline]×100%. On the basis of these ex vivo measure-
ments, an AR-CMX dose of 3 μg/kg per minute was selected for
wall puncture experiments.

In a group of 8 rabbits, the compound was continuously infused
via an ear vein (AR-CMX group). Continuous infusion was used
because AR-CMX effects have been shown to rapidly recover on
termination of infusion.14 Control rabbits (n=8) received continuous
infusion of saline. In all rabbits, infusion (5 mL/h) was started at least
20 minutes before the first vessel was punctured. Rabbits were
randomly assigned to the groups. The in vivo experiments and the
offline analyses were performed blindly.

To compare the effects of AR-CMX with those of another
well-known P2Y12 antagonist, an additional group of 3 rabbits was
pretreated with an effective dose of clopidogrel (25 mg/kg).23 Clopi-
dogrel was administered orally 2 hours before vessel puncture
started. Arterial blood was collected in trisodium citrate (0.129
mol/L, 1/10 vol) before and 2 and 5 hours after clopidogrel
administration. The maximal level of ADP-induced platelet aggre-
gation (measured in platelet-rich plasma (PRP) as described below)
was reduced by 62% after 2 hours and by 47% after 5 hours.

Platelet Aggregation and Ca2+ Measurements
Blood was collected in 1/10 vol trisodium citrate (0.129 mol/L) from
a central ear artery of 4 rabbits to prepare PRP (2×108 platelets/mL).
PRP was recalified with 16.6 mmol/L CaCl2, and PPACK (40
μmol/L) was added. ADP (20 μmol/L)–induced platelet aggrega-
tion was measured at 37°C by using a Chronolog optical aggregometer.
To measure Ca2+ responses, isolated rabbit platelets were loaded
with fluo 3 as described.28 After immobilization on fibrinogen-
covered coverslips, platelets were stimulated with 3 μmol/L ADP and
2 mmol/L CaCl2, and fluorescence intensity changes in single
platelets were measured in a fluorescence microscope. In addition, fluo 3–labeled platelets were injected into anesthetized rabbits (n=3), and fluorescence intensity changes in platelets participating in the thromboembolic process
were measured as described previously.20

Thrombin Generation Assay
Thrombin generation was measured in PRP by using the Thrombo-
gram method.23 Blood was collected from 3 rabbits before (baseline)
and after 30 minutes of infusion of AR-CMX at 3 μg/kg per minute.
PRP (1.5×108 platelets/mL) was prepared and added to wells of a
96-well plate containing rabbit thromboplatin (1/30 000 vol
Thromboplatin-S, Biopool International). Thrombin formation was
started by adding CaCl2 (16.6 mmol/L) and fluorescent thrombin
substrate (417 μmol/L Z-Gly-Gly-Arg-AMC, Bachem) and contin-
uously measured in time with a microtiter plate fluorometer (Fluo-
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Effects of P2Y12 Receptor Blockade on Rabbit Platelets

To demonstrate whether P2Y12 mediates the activation of rabbit platelets, ADP-induced platelet aggregation in PRP was measured in vitro. AR-CMX reduced maximal aggregation by 44±5% at 1 μmol/L and by 66±6% at 100 μmol/L. Complete inhibition of aggregation was achieved only when a P2Y1 receptor antagonist was added as well, indicating that P2Y1 receptor activation accounted for the residual ADP-induced aggregation. To confirm that AR-CMX is specific for P2Y12, we investigated the effect of AR-CMX on Ca2+ responses in rabbit platelets. As expected, ADP-induced Ca2+ responses of single platelets in vitro were not influenced by preincubation with AR-CMX. In vivo, fluo 3–labeled platelets were seen adhering to growing emboli. During AR-CMX infusion, the Ca2+ responses of these platelets were not different from control (data not shown). Hence, AR-CMX seems specific for the P2Y12 receptor in rabbit platelets.

The optimal AR-CMX dose for in vivo experiments was established ex vivo by use of whole blood aggregometry (Figure 1). Doses >1 μg/kg per minute significantly (P<0.05) decreased maximal aggregation: infusion of 1 μg/kg per minute reduced aggregation by 49±14%; infusion of 3 μg/kg per minute, by 71±6%; and infusion of 10 μg/kg per minute, by 80±5%. Because infusion of AR-CMX at 3 μg/kg per minute caused an almost maximal inhibition of aggregation (Figure 1), this dose was selected for subsequent in vivo experiments.

Effect of P2Y12 Blockade on Thromboembolic Reaction In Vivo

After the puncture of mesenteric arterioles (control, n=29; AR-CMX, n=36; and clopidogrel, n=19), bleeding and thrombus formation started immediately. Formation of the primary thrombus was completed within 1 to 2 seconds. AR-CMX reduced thrombus height by ~20% (control median, 66%; AR-CMX, 54%; P<0.005; Figure 2). Microvessel bleeding time was not influenced (control, 4.9 seconds; interquartile range 2.3 to 7.7 seconds; AR-CMX, 2.9 seconds, interquartile range 1.3 to 10.7 seconds; P=0.57). After initial thrombus formation, rebleeding occurred in some vessels, but this frequency was similar in both groups (control, 24% of vessels; AR-CMX, 28% of vessels). Clopidogrel had similar effects: thrombus height was reduced to 37% (P<0.001, Figure 2) without significantly influencing microvessel bleeding time (3.1 seconds, interquartile range 1.9 to 5.8 seconds; P=0.29) or rebleeding frequency (37%, P=0.52). Thus, although the thrombus was smaller during P2Y12 blockade, its effectiveness to stop bleeding and prevent rebleeding was not influenced.

The formation of emboli on the downstream side of the thrombus was markedly reduced by AR-CMX (Figure 3). The total duration of embolization was decreased from 469 seconds in control to 228 seconds in AR-CMX arterioles (P<0.001, Figure 3a). In 11 of 29 control vessels, embolization continued for >600 seconds, whereas it stopped within 600 seconds in all but 1 AR-CMX arteriole. During the embolization period, on average, 14 visible emboli (short axis >5 μm) were produced in control arterioles, but only 8 were produced in AR-CMX vessels (P=0.001, Figure 3b). Moreover, AR-CMX decreased the size of these emboli: overall median embolus size per vessel was 10 to 15 μm in control arterioles and only 5 to 10 μm in AR-CMX vessels (P<0.01, Figure 3c). Because these observed emboli tended to get smaller during the course of embolization, individual emboli were compared as well. Of the first 10 emboli produced per
vessel, 9 were significantly smaller in the AR-CMX arterioles compared with control arterioles. In clopidogrel-treated animals, embolization duration was reduced to 303 seconds (rmP<0.05 compared with control, Figure 3a); 8 emboli were produced per vessel (rmP<0.05, Figure 3b), with a median size of 5 to 10 μm (rmP<0.01, Figure 3c).

To investigate whether the smaller size of the emboli is due to decreased platelet adhesion during P2Y12 blockade, the rate of adherence of individual platelets was determined in the experiments with fluo 3–labeled platelets. Individual labeled platelets adhered to the growing emboli at a similar rate in control animals (4.8 [range 2.9 to 9.7] platelets/min, ) and AR-CMX–treated animals (3.9 [range 2.4 to 5.5] platelets/min, ). The seemingly incompatible combination of an unchanged platelet adherence rate and the formation of smaller emboli at a similar embolus production time (for control, median 21.2 seconds, interquartile range 13.8 to 29.7 seconds; for AR-CMX, 21.6 seconds, interquartile range 13.8 to 31.7 seconds) indicates that AR-CMX caused detachment of small platelet aggregates that did not reach the visible embolus size of 5 μm, in addition to the visible emboli.

Figure 2. Effects of AR-CMX or clopidogrel (clop) on thrombus formation induced by wall puncture of rabbit arterioles. Thrombus height is expressed as percentage of local vessel diameter; medians and interquartile ranges are shown. **rmP≤0.01 and ***rmP≤0.001 vs control by Mann-Whitney U test.

Figure 3. Effects of AR-CMX and clop on embolization after wall puncture in rabbit arterioles. a and b, Medians and interquartile ranges of duration of embolization (a) and number of emboli produced per vessel (b). c, Median embolus size of first, second, and third embolus per vessel and overall median per vessel. Closed bars indicate control; open bars, AR-CMX; and dashed bars, clop. *rmP≤0.05, **rmP≤0.01, and ***rmP≤0.001 vs control by Mann-Whitney U test.
Preincubation of control blood with 10 μmol/L thrombin substrate at time 0.

These values are within normal ranges for anesthetized rabbits.17,26

Figure 4. Inhibition of platelet-dependent thrombin generation by AR-CMX. Typical examples of thrombin generation in PRP from blood collected before (control) and after (AR-CMX) infusion of 3 μg · kg · min⁻¹ AR-CMX. The reaction was started by adding fluorescent thrombin substrate at time 0.

Platelet-Dependent Thrombin Generation
The effect of P2Y₁₂ blockade on platelet-dependent thrombin formation was tested ex vivo. Infusion of AR-CMX at 3 μg/kg per minute reduced the peak value of thrombin generation by 25±7% (P=0.06, Figure 4). This parameter was not further reduced when PRP from blood collected after infusion was preincubated with AR-CMX (10 μmol/L) in vitro, indicating that infusion of AR-CMX at 3 μg/kg maximally reduced P2Y₁₂-dependent thrombin formation. Preincubation of control blood with 10 μmol/L AR-CMX in vitro caused a reduction of thrombin generation of 42±11%, which was not significantly different from the decrease caused by AR-CMX infusion (P=0.24). This suggests that the effective plasma concentration of AR-CMX during the in vivo experiments was in the micromolar range, near 10 μmol/L.

Systemic Parameters and Fluid Dynamic Conditions
Ear bleeding time increased from 7.0 minutes (control) to 20.5 minutes (AR-CMX) during P2Y₁₂ inhibition. Mean arterial blood pressure, heart rate, hematocrit, and platelet count were not different from control during AR-CMX or clopidogrel treatment. Overall medians and ranges were as follows: mean arterial pressure 69 (46 to 93) mm Hg, heart rate 148 (116 to 192) bpm, hematocrit 40% (32% to 53%), and platelet count 520×10⁹/L (268×10⁹/L to 836×10⁹/L). These values are within normal ranges for anesthetized rabbits.17,26

In addition, P2Y₁₂ blockade had no significant effect on local fluid dynamic parameters: arteriolar diameter (29 [19 to 46] μm), mean red blood cell velocity (1.8 [0.3 to 6.1] mm · s⁻¹), and wall shear rate (943 [181 to 3605] s⁻¹) were not different between groups. These parameters were not significantly correlated with any of the thromboembolic parameters.

Discussion
This is the first study to show that (in damaged rabbit arterioles) blocking the platelet ADP receptor P2Y₁₂ reduces initial thrombus formation and especially reduces the number and size of emboli shed by the initial thrombus, without influencing thrombus stability. The rate of embolization and the rate of adherence of individual platelets to growing emboli are not decreased. The seemingly incompatible combination of an unchanged platelet adherence rate and the formation of smaller emboli indicates that even smaller and hence less harmful aggregates (<5 μm) are produced during P2Y₁₂ blockade as well. This occurrence is probably due to relatively loose adhesion of platelets to the primary thrombus.

P2Y₁₂ is the target of many antithrombotic drugs.12–15 The present in vivo findings provide insight into the way these drugs interfere in the dynamic process of thromboembolism. The present study shows that blockade of P2Y₁₂ is especially effective during the embolization phase of a thromboembolic process. The duration of embolization was reduced by 40% to 50% with a correspondingly lower number of emboli; moreover, emboli were significantly smaller. If it is assumed that the process of platelet adhesion and aggregation that underlies embolization is similar in small and large vessels, these data may explain the effectiveness of P2Y₁₂-inhibiting drugs in reducing the risk of ischemic events, such as myocardial or cerebral infarction or peripheral arterial ischemia in patients with atherothrombotic disease.12,13 because such ischemic events are considered to be the result of relatively large emboli shed from a thrombus.

If it is taken into account that P2Y₁₇-mediated ADP effects are not influenced, the effects of P2Y₁₂ blockade (by AR-CMX or clopidogrel) on embolization clearly indicate a prominent role for ADP during this phase of the thromboembolic process. Because emboli are of a loose nature and consist of weakly activated platelets compared with platelets in the thrombus,20 it is indeed likely that embolization involves platelet activation caused by relatively weak platelet agonists such as ADP. Activated platelets and vascular cells can also release thromboxane A₂ (TXA₂), which stimulates platelet aggregation. Previously, we have shown that in the same animal model, specific blockade of TXA₂ receptors by sulotroban (BM 13.177) significantly reduces embolization in arterioles by >55%, without influencing thrombus size and stability.18 These effects of TXA₂ receptor blockade on embolization (which can also be achieved by low doses of aspirin) are similar to those of P2Y₁₂ blockade. The involve-
ment of both TXA₂ and ADP in embolization may explain why combined blockade of ADP and TXA₂ pathways in patients is more effective in reducing ischemic events than is blocking the TXA₂ pathway alone.¹³

In addition to the pronounced inhibition of embolization, P₂Y₁₂ blockade resulted in a reduction of thrombus size. The biological relevance of this reduction can be questioned because rebleeding frequency was not influenced, indicating that the stability of the thrombus was unaffected. The reduction in thrombus size during P₂Y₁₂ blockade may be the consequence of a decrease in thrombus formation at the surface of procoagulant platelets. Because (in contrast to emboli) the primary thrombus is a stable aggregate with tightly packed, heavy, shape-changed, and degranulated platelets that may be procoagulant,²⁰ it is likely that thrombin is mainly formed in the thrombus. This is confirmed by pilot experiments in which a low molecular weight heparin was used: thrombus stability decreased, but embolization was unaffected (authors’ unpublished data, 2002). Thrombin can play a dual role in thrombus formation: (1) by activating platelets, it is (partly) responsible for thrombus size; and (2) by converting fibrinogen into fibrin, it stabilizes the thrombus. In the present study, we show that AR-CMX infusion indeed reduces the thrombin-generating capacity by ≈25%, which may explain the decrease in thrombus size. Because thrombus stability is not influenced by P₂Y₁₂ blockade, the remaining thrombin is apparently able to produce sufficient fibrin. The reduction in thrombus size may also be due to inhibition of collagen-induced platelet activation, because in vitro studies have shown that collagen-induced platelet aggregation is impaired in platelets from P₂Y₁₂-deficient mice.⁶

In addition, in perfusion studies over collagen surfaces, thrombus size was found to be reduced when human platelets were deficient in P₂Y₁₂ or when this receptor was blocked by AR-CMX (in vitro)²⁷ or clopidogrel (ex vivo).²⁸

The microvessel bleeding times presented the present study are relatively short and unaffected by P₂Y₁₂ receptor blockade. Bleeding times as measured in mice⁶ and humans¹⁵ are substantially longer and prolonged when blocking the P₂Y₁₂ receptor. This discrepancy can likely be explained by the different types of vessel damage between the models used, being small in our model of vessel wall puncture and more severe in mice and humans, in whom larger vessels are transected, and surrounding tissue is opened as well. This is supported by our observation that P₂Y₁₂ blockade substantially increased bleeding time when there was more severe damage to the ear.

In summary, this is the first study in which the role of the ADP receptor P₂Y₁₂ is investigated in the thromboembolic process in vivo. Inhibition of this receptor especially reduces the production of potentially harmful emboli, without affecting the stability of the initial thrombus. Although our data were obtained in small rabbit arterioles, they may represent a general mechanism of action for P₂Y₁₂-inhibiting drugs in diseased human arteries. By shortening embolization duration and decreasing the size of emboli, the risk of downstream ischemia will be clearly reduced.

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