

Basic Study

P2X7 receptor activation causes phosphatidylserine exposure in canine erythrocytes

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Author contributions: Kuit TA and Sluyter R designed the research; Faulks M, Sophocleous RA and Jurak LM performed the research; Faulks M, Sophocleous RA and Sluyter R analysed the data; Curtis BL and Curtis SJ contributed samples; Sluyter R wrote the paper; Faulks M, Sophocleous RA, Curtis BL, Curtis SJ and Jurak LM reviewed the paper.

Supported by The Centre for Medical and Molecular Bioscience (University of Wollongong); and the American Kennel Club Canine Health Foundation.

Institutional review board statement: The study was reviewed and approved by the Human and Animal Ethics Committees of the University of Wollongong.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Animal Ethics Committee of the University of Wollongong (Protocol AE14/09). Consent from pet owners was reviewed and approved by the Human Ethics Committee of the University of Wollongong (Protocol HE10/063).

Conflict-of-interest statement: The authors declare no conflicts of interest regarding this manuscript.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external

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Manuscript source: Invited manuscript

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Received: July 20, 2016

Peer-review started: July 21, 2016

First decision: August 5, 2016

Revised: August 31, 2016

Accepted: September 21, 2016

Article in press: September 23, 2016

Published online: November 6, 2016

Abstract

AIM

To determine if activation of the ATP-gated P2X7 receptor channel induces phosphatidylserine (PS) exposure in erythrocytes from multiple dog breeds.

METHODS

Peripheral blood was collected from 25 dogs representing 13 pedigrees and seven crossbreeds. ATP-induced PS exposure on canine erythrocytes *in vitro* was assessed using a flow cytometric Annexin V binding assay.

RESULTS

ATP induced PS exposure in erythrocytes from all dogs

studied. ATP caused PS exposure in a concentration-dependent manner with an EC₅₀ value of 395 μmol/L. The non-P2X7 agonists, ADP or AMP, did not cause PS exposure. The P2X7 antagonist, AZ10606120, but not the P2X1 antagonist, NF449, blocked ATP-induced PS exposure.

CONCLUSION

The results indicate that ATP induces PS exposure in erythrocytes from various dog breeds and that this process is mediated by P2X7 activation.

Key words: Adenosine triphosphate; Dog; P2X1 receptor; P2X7 receptor; Phospholipid; Purinergic receptor; Red blood cells

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Core tip: Phosphatidylserine (PS) exposure in erythrocytes has potential roles in erythrocyte clearance and thrombus formation. Activation of the ATP-gated P2X7 receptor channel induces PS exposure in human erythrocytes, but whether this process occurs in erythrocytes from other mammals remained hitherto unknown. The current study shows that extracellular ATP causes PS exposure in dog erythrocytes from 13 pedigrees and seven crossbreeds. Notably, the current study shows that this process is mediated by P2X7 activation. These results suggest that P2X7-mediated PS exposure on erythrocytes may have important roles in red blood cell biology in dogs.

Faulks M, Kuit TA, Sophocleous RA, Curtis BL, Curtis SJ, Jurak LM, Sluyter R. P2X7 receptor activation causes phosphatidylserine exposure in canine erythrocytes. *World J Hematol* 2016; 5(4): 88-93 Available from: URL: <http://www.wjgnet.com/2218-6204/full/v5/i4/88.htm> DOI: <http://dx.doi.org/10.5315/wjh.v5.i4.88>

INTRODUCTION

Exposure of the plasma membrane lipid, phosphatidylserine (PS), to the outer leaflet is an important physiological and pathophysiological signal^[1]. In erythrocytes, PS exposure serves emerging roles in the clearance of senescent, damaged and diseased erythrocytes from the circulation^[2]. Moreover, PS exposure can serve as a substrate for thrombin formation and incorporation of erythrocytes into thrombi^[3]. PS exposure also serves as a parameter for stored erythrocyte integrity^[4] and may be important in the removal of such cells following transfusion^[5]. Thus, it remains important to understand the mechanisms by which PS becomes exposed on the surface of erythrocytes.

The P2X7 receptor is a trimeric ligand-gated channel activated by extracellular ATP^[6] at concentrations at least 10-fold greater than that required for other purinergic receptors^[7]. Functional P2X7 has been reported in

humans, dogs, rodents and other species^[8]. P2X7 and other purinergic receptors, namely P2X1, P2Y1 and P2Y13, are present on the plasma membrane of erythrocytes^[9]. P2X7 activation induces PS exposure in human erythrocytes^[10,11], but it remains unknown if P2X7 activation mediates PS exposure in erythrocytes from other species. ATP can induce PS exposure in erythrocytes obtained from English springer spaniels^[12], but whether this process occurs in other dog breeds and whether it is mediated by P2X7 activation remains to be determined. P2X7, however, is present in leukocytes from various dog breeds^[13,14] suggesting that P2X7 activation may mediate PS exposure in canine erythrocytes.

Using a flow cytometric Annexin V binding assay, the current study aimed to determine if ATP induces PS exposure in erythrocytes from multiple dog breeds and whether this process is mediated by P2X7 activation.

MATERIALS AND METHODS

Materials

Nucleotides were from Sigma Chemical Co. (St. Louis, MO). AZ10606120 was from Tocris Bioscience (Ellisville, MO). NF499 was from Cayman Chemical (Ann Arbor, MI).

Blood samples

Peripheral blood was collected from either pedigree or crossbreed dogs into VACUETTE lithium heparin tubes (Greiner Bio-One, Frickenheisen, Germany). All samples were collected from privately owned dogs presenting at the Albion Park Veterinary Hospital (Albion Park, Australia), with informed consent of owners, and in accordance with and approval from the Animal and Human Ethics Committees of the University of Wollongong (Wollongong, Australia). The animal protocol was designed to minimize pain or discomfort to the animals, and conducted according to standard veterinary practices.

PS exposure assays

Erythrocytes from peripheral blood were isolated and resuspended in NaCl medium (147.5 mmol/L NaCl, 2.5 mmol/L KCl, 5 mmol/L glucose, 20 mmol/L HEPES, pH 7.4) at a final haematocrit of 2% as described^[12]. Erythrocytes were then incubated in 96-well U-bottom plates (Greiner Bio-One) in the absence or presence of nucleotide (as indicated) for 24 h at 37 °C/5% CO₂. In some experiments, erythrocytes were pre-incubated in the absence or presence of AZ10606120 or NF449 for 15 min at 37 °C prior to ATP addition. Following nucleotide incubation, 20 μL of resuspended erythrocytes were washed once in 1 mL Annexin V Binding Buffer (BioLegend, San Jose, CA) (450 × g for 3 min) and labeled with fluorescein isothiocyanate (FITC)-conjugated Annexin V (BioLegend, San Diego, CA) according to the manufacturer's instructions. Data was collected using a BD (San Jose, CA) LSR II or LSRFortessa flow cytometer

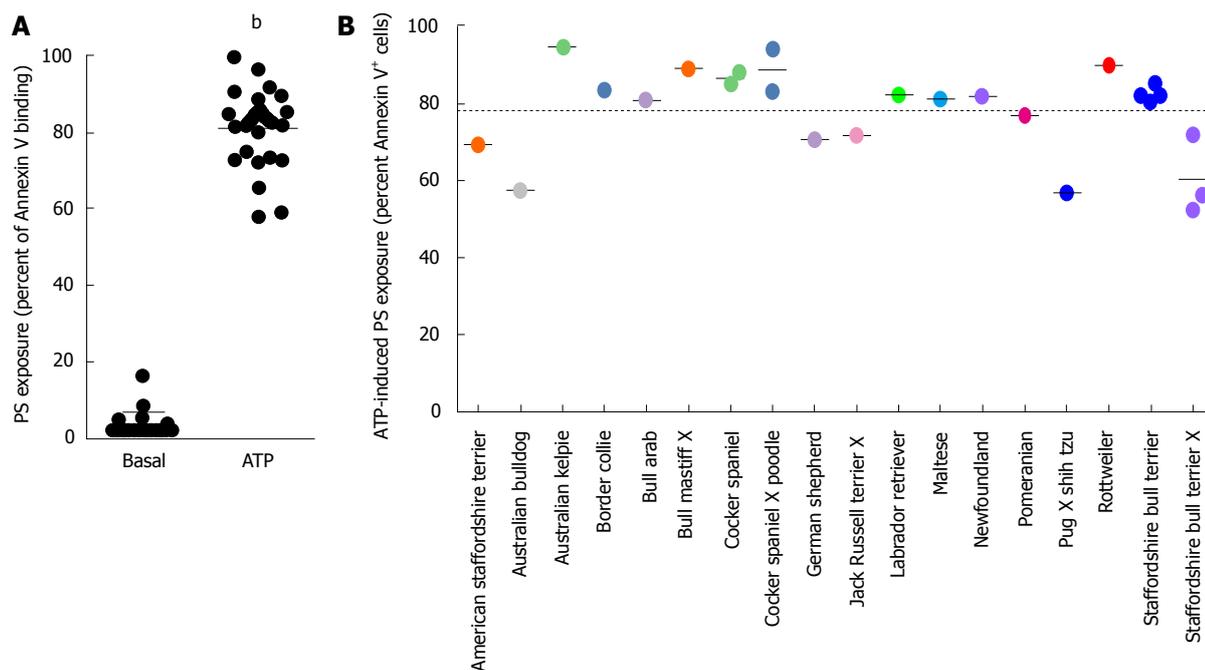


Figure 1 ATP induces phosphatidylserine exposure in erythrocytes from multiple dog breeds. Erythrocytes, from 17 pedigree and eight crossbreed (X) dogs, in NaCl medium were incubated for 24 h at 37 °C in the absence or presence of 1 mmol/L ATP. Cells were labeled with FITC-conjugated Annexin V and analyzed by flow cytometry. The bars represent group means (A and B). A: The symbols represent the percentage of Annexin V⁺ erythrocytes, from each dog, following incubation in the absence (basal) or presence of ATP; ^b*P* < 0.0001 ATP vs basal; B: The symbols represent the percentage of ATP-induced PS exposure in erythrocytes, from each dog, determined as the difference in the percentage of Annexin V⁺ erythrocytes following incubation in the presence and absence of ATP. The broken line represents the mean ATP-induced PS exposure from all dogs. The symbols for Staffordshire bull terrier X represent a Staffordshire bull terrier and Australian kelpie cross, Staffordshire bull terrier and bull terrier cross, or a Staffordshire bull terrier cross. PS: Phosphatidylserine.

and FACSDiva software. The percentage of Annexin V⁺ cells (PS exposure) was determined using FlowJo software (Tree Star, Inc., Ashland, OR).

Statistical analysis

Data is presented as mean ± SD. Statistical comparisons were performed using Prism 5 for Mac OS X (GraphPad Software, San Diego, CA). Differences between two or more groups were compared using a paired student's *t*-test or an ANOVA (using Tukey's multiple comparison test), respectively. Concentrations curves were fitted using the log(agonist) vs normalized response (variable slope) method.

RESULTS

To determine if ATP could induce PS exposure in erythrocytes in dog breeds other than English springer spaniels, erythrocytes, from 25 dogs representing 13 pedigrees and seven crossbreeds, were incubated in the absence or presence of ATP and the percent of Annexin V⁺ cells (PS exposure) determined by flow cytometry. Incubation in the absence of ATP led to a mean PS exposure of 3.3% ± 3.2% (Figure 1A). In contrast, incubation with ATP caused a 25-fold increase in the mean PS exposure to 81.0% ± 10.4% (Figure 1A). Collectively, this resulted in an average ATP-induced PS exposure of 77.7% ± 11.8% (Figure 1B). Notably, ATP caused PS exposure in erythrocytes from all dogs

studied (Figure 1B). ATP incubation also caused visible hemolysis compared to cells incubated in the absence of ATP (results not shown), but neither this nor other changes in erythrocyte morphology were investigated further.

To determine if P2X7 activation mediates exposure of PS in canine erythrocytes, erythrocytes were incubated with increasing concentrations of ATP and subsequent PS exposure assessed as described above. ATP induced PS exposure in a concentration-dependent manner with a maximum response at 2 mmol/L ATP and with an EC₅₀ value of 395 ± 45 μmol/L (Figure 2A).

To further establish if P2X7 activation mediates PS exposure in canine erythrocytes, erythrocytes were incubated with ATP, as well as ADP and AMP, which do not activate canine P2X7^[12,15]. Again ATP caused robust PS exposure in erythrocytes compared to erythrocytes incubated in the absence of nucleotide (Figure 2B). In contrast, ADP and AMP did not induce PS exposure in erythrocytes, with binding of Annexin V similar to that of erythrocytes incubated in the absence of nucleotide (Figure 2B).

Finally, canine erythrocytes were pre-incubated in the absence or presence of AZ10606120, which impairs canine P2X7^[15], or NF449, which impairs human and rodent P2X1^[16,17], prior to ATP incubation. Pre-incubation with AZ10606120 impaired ATP-induced PS exposure by 79%, while pre-incubation with NF449 had minimal effect on ATP-induced PS exposure (Figure 2C). Neither

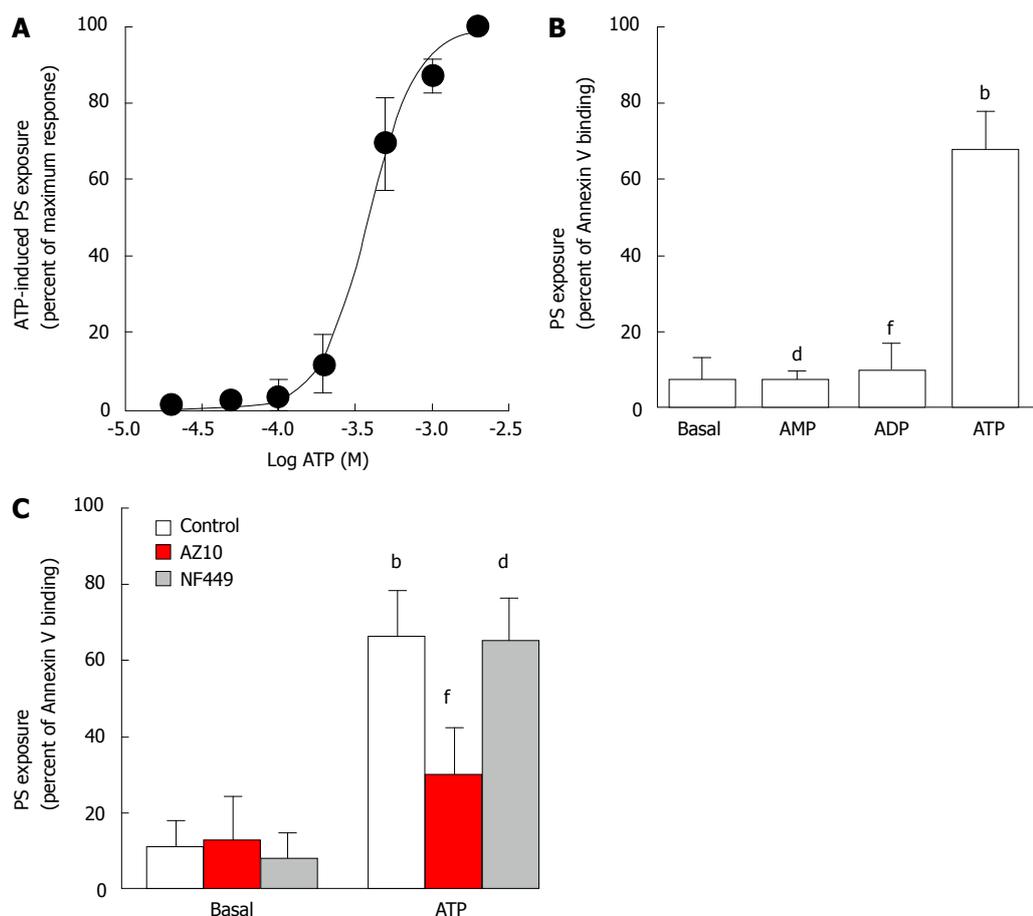


Figure 2 ATP induces phosphatidylserine exposure in canine erythrocytes in a concentration-dependent manner. Erythrocytes in NaCl medium were incubated for 24 h at 37 °C in the absence or presence of ATP (A) or 1 mmol/L nucleotide (B and C) (as indicated). Prior to ATP incubation, cells were pre-incubated for 15 min in the absence or presence of 10 μ mol/L AZ10606120 (AZ10) or 10 μ mol/L NF449 (C). Following nucleotide incubation, cells were labeled with FITC-conjugated Annexin V and analyzed by flow cytometry (A-C). A: The data represent percent maximum response to 2 mmol/L ATP (mean \pm SD, $n = 4$ dogs); B: The data represent mean \pm SD ($n = 5$ dogs); ^b $P < 0.001$ ATP vs basal; ^d $P < 0.001$ AMP vs ATP; ^f $P < 0.001$ ADP vs ATP; C: The data represent mean \pm SD ($n = 5$ dogs); ^b $P < 0.001$ ATP vs basal; ^d $P < 0.001$ ATP with NF449 vs NF449; ^f $P < 0.001$ ATP with AZ10606120 vs ATP.

AZ10606120 nor NF449 affected PS exposure in the absence of ATP (Figure 2C).

DISCUSSION

The current study demonstrated that ATP induces PS exposure in erythrocytes from 25 dogs representing 13 pedigrees and seven crossbreeds. On average, ATP caused PS exposure on 78% of erythrocytes from these dogs. This value is similar to that of ATP-induced PS exposure previously observed in erythrocytes from English springer spaniels (88%)^[12]. Combined, these data indicate that ATP can induce PS exposure in erythrocytes from multiple dog breeds and suggests that this is likely to be a common phenomenon in all breeds of dogs. Moreover, these data confirm that ATP-induced PS exposure in canine erythrocytes is about six-fold greater than that observed for ATP-induced PS exposure in human erythrocytes^[12], which corresponds to the increased expression and activity of P2X7 in canine erythrocytes compared to human erythrocytes^[12,18].

Similar to human erythrocytes^[10,11], the current study also demonstrates that ATP-induced PS exposure

in canine erythrocytes is predominately mediated by P2X7 activation. First, the EC₅₀ value for ATP-induced PS exposure (395 μ mol/L) is similar to that observed for native and recombinant canine P2X7-mediated cation fluxes in English springer spaniel erythrocytes^[12,18] and transfected HEK-293 cells^[15,19], respectively; second, the non-P2X7 agonists, ADP and AMP, did not cause PS exposure; last, the P2X7 antagonist, AZ10606120, but not the P2X1 antagonist, NF449, impaired ATP-induced PS exposure. It should be noted that blockade with AZ10606120 was not complete indicating that either other purinergic receptors have an additional role in this process, or that AZ10606120 has limited efficacy in the conditions tested and that P2X7 remains solely responsible for ATP-induced PS exposure in canine erythrocytes. The latter is supported by at least three points. First, the concentration response curve for ATP-induced PS exposure revealed a simple, not biphasic, sigmoidal curve suggesting involvement of only one purinergic receptor subtype. Second, ATP concentrations below 100 μ mol/L, which are sufficient to activate other ATP-responsive purinergic receptors^[7], failed to cause PS exposure. Last, ADP, which can activate P2X1, P2Y1 and

P2Y13, but not P2X7^[7], all of which are present in human or rodent erythrocytes^[20,22], did not induce PS exposure.

It remains unknown why the relative amounts of P2X7 differ between canine and human erythrocytes, but we have previously speculated^[12] that this difference may be due to alterations in the proteolytic systems mediating maturation-associated degradation in reticulocytes between these two species. Differences in erythrocyte P2X7 activity between these two species are unlikely to be due to altered expression of splice variants. Previous immunoblotting studies using an antibody to the extracellular loop of P2X7, which is predicted to bind all known splice variants of canine P2X7 (URL: <http://www.ncbi.nlm.nih.gov/gene/448778>) and human P2X7^[23,24], demonstrated only the full-length receptor in erythrocytes from both species^[12]. Notably, the lifespans of canine and human erythrocytes are similar (approximately 115 d)^[25] suggesting that P2X7-induced PS exposure in erythrocytes is unlikely to influence the removal of senescent cells.

In the current study, ATP caused visible hemolysis of canine erythrocytes, however this was not formally investigated. We have previously observed that 24 h ATP incubation induces a small but significant amount hemolysis of erythrocytes from English springer spaniels compared to those incubated in the absence of ATP (16% vs 1%, respectively)^[12]. Future studies are required to explore if this ATP-induced hemolysis is mediated by P2X7 or other purinergic receptors, such as P2X1 or P2Y1, which can also mediate hemolysis^[20,21]. Also, it remains unknown if 24 h ATP incubation causes other changes in erythrocyte morphology. Five minutes incubation with 1 mmol/L ATP of beagle erythrocytes increases cell viscosity as assessed by filterability of packed cells, but not changes in cell shape as observed by light microscopy^[26]. Therefore, further studies could explore if activation of P2X7 or other purinergic receptors alters canine erythrocyte morphology.

In conclusion, the current study indicates that P2X7 activation induces PS exposure in canine erythrocytes and that this phenomenon is common to many, if not all, dog breeds. The physiological importance of P2X7-induced PS exposure in canine erythrocytes, as for human erythrocytes, remains to be established. The tendency of human erythrocytes to undergo ATP-induced PS exposure does not change with erythrocyte age^[11], further supporting the concept that P2X7-induced PS exposure in erythrocytes is unlikely to be involved in the removal of aged cells. Instead, it remains plausible, that P2X7-induced PS exposure in erythrocytes is responsible for the clearance of these cells during cell stress, damage or disease. Alternatively, but not mutually exclusive to this point, P2X7-induced PS exposure in erythrocytes may facilitate thrombus formation to promote wound healing and immunity during tissue injury or infection, or to inadvertently cause vasocclusion in disorders such as malaria, sickle cell disease or diabetes. The robust PS exposure in canine erythrocytes following P2X7 activation

will provide a valuable experimental model to understand further the role of this receptor in red blood cell biology. Finally, whilst PS exposure is routinely reported in canine platelets^[27,28] and to some extent canine leukocytes^[29,30], to the best of our knowledge PS exposure in canine erythrocytes is limited to our preliminary^[12] and current observations. Thus, these studies support a rationale for exploring the physiological and pathophysiological roles and consequences of PS exposure in erythrocytes within dogs.

ACKNOWLEDGMENTS

The authors are grateful to Vanessa Sluyter (University of Wollongong) for technical assistance and pet owners for samples.

COMMENTS

Background

Exposure of phosphatidylserine (PS) in erythrocytes has roles in erythrocyte clearance and thrombus formation. Activation of P2X7 by extracellular adenosine triphosphate (ATP) induces PS exposure in human erythrocytes, but whether this process occurs in erythrocytes from dogs was unknown. Therefore this study aimed to determine if ATP can induce PS exposure in erythrocytes from dogs and if so, whether this process is mediated by activation of P2X7.

Research frontiers

The mechanisms by which PS exposure on dog erythrocytes and the function of P2X7 on these cells occurs remain poorly characterised. Moreover, there are limited reports of PS exposure on dog erythrocytes in any context.

Innovations and breakthroughs

This study demonstrated that extracellular ATP causes PS exposure in dog erythrocytes from multiple breeds and that this process is mediated by activation of P2X7.

Applications

This study suggests that P2X7-mediated PS exposure on erythrocytes may have important roles in red blood cell biology in dogs. This may have potential therapeutic or biomarker applications. Moreover, the relatively high amount of P2X7-mediated PS exposure on dog erythrocytes may provide a model to study this process, including its biological significance, in greater detail.

Terminology

PS is a phospholipid that is predominately localized to the inner layer of the lipid bilayer of the plasma membrane of healthy cells, but can become localized to the outer layer (exposed) following cellular activation. Annexin V is a PS-binding protein that can be conjugated to a fluorescent label and used to study cellular PS exposure by fluorescent techniques such as flow cytometry. The P2X7 receptor is a plasma membrane ligand-gated channel activated by extracellular ATP.

Peer-review

It is a well written interesting paper studying ATP-induced PS exposure, which has potential roles in erythrocyte clearance and thrombus formation, from various dog breeds and showing that this process is mediated by P2X7 activation.

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P- Reviewer: Classen CF, Moschovi MA S- Editor: Ji FF

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