Effects of calcium on phosphatidylserine- and saposin C-stimulated glucosylceramide β -glucosidase activity

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Glucosylceramide β -glucosidase is a membrane-bound lysosomal hydrolase that is activated by acidic lipids, the most effective of which is phosphatidylserine (PtdSer), and an activator protein, saposin C. This report documents effects of Ca^{2+} ions on PtdSer- and saposin C-enhanced β -glucosidase activity. Ca^{2+} either increased or decreased enzyme activity, depending on (1) the concentration of phospholipid, and (2) the presence or absence of saposin C. At PtdSer concentrations between 7.6 and 76 μM , in the absence of saposin C, Ca^{2+} caused an increase in β -glucosidase activity up to 3 times that measured with PtdSer

alone; this was due to both an increase in V_{max} and a decrease in K_m . In contrast, at PtdSer concentrations greater than 100 μM , Ca^{2+} inhibited β -glucosidase activity by 50 %, due to a 2-fold increase in K_m . Ca^{2+} was inhibitory at all PtdSer concentrations tested when both PtdSer and saposin C were present in the assay. Ca^{2+} ions were also shown to cause changes in the aggregation states of PtdSer. These results suggest that changes in Ca^{2+} concentration may play a role in regulating β -glucosidase activity *in vivo*, thereby modulating sphingolipid metabolism. The implications of these findings are discussed.

INTRODUCTION

Glucosylceramide β -glucosidase (EC 3.2.1.45) is a membrane-bound lysosomal hydrolase that participates in the degradation of glycosphingolipids by cleaving glucocerebroside to yield glucose and ceramide. A deficiency of this enzymic activity results in Gaucher disease, a lysosomal storage disorder characterized by the accumulation of glucocerebroside [1].

Acidic lipids have been shown to be potent activators of purified β -glucosidase from liver, spleen and brain. One of the most effective physiological lipid activators is the acidic phospholipid, phosphatidylserine (PtdSer) [2]. In addition to the acidic lipids, mammalian tissues contain a low-molecular-mass protein which acts synergistically with acidic lipids to activate β -glucosidase [3,4]. This protein, saposin C, has been shown to have a positive effect on β -glucosidase activity in the absence of lipid [5], but the highest activities are obtained when saposin C and an acidic lipid are both present in the assay [5,6].

In general, β -glucosidase activity increases with increasing concentration of the activator lipid. In the presence of saposin C, less acidic lipid is needed to stimulate the enzyme maximally [5,6]. The physiological relevance of saposin C activation of glucosylceramide β -glucosidase is evident by the identification of patients with a form of Gaucher disease caused by a deficiency of saposin C [7].

Activation by acidic lipids and saposin C is a result of an increase in the enzyme's V_{max} , as well as a decrease in K_m [6,8]. Kinetic analyses indicate that glucosylceramide β -glucosidase has two activator-binding sites: one that affects the maximum rate of the reaction, and another that affects the enzyme's affinity for substrate [9]. In another study, the binding sites for saposin C and PtdSer were shown to be distinct [5].

The effects of acidic lipids and saposin C have been shown to be due to direct interaction with the enzyme, and not to direct interactions with each other or with the substrate [5,6]. However, saposin C clearly affects the interaction of acidic lipids with the enzyme. Saposin C has been shown to increase binding of PtdSer to β -glucosidase, probably by affecting the enzyme's conformation [5].

The fact that specific lipids and saposin C alter β -glucosidase activity *in vitro* suggests that this enzyme is regulated similarly *in vivo*, as well. Glucosylceramide β -glucosidase resides in the lysosomal membrane, surrounded by other membrane proteins and multiple species of lipids. Saposin C is also a lysosomal protein [10], present in the lumen of the organelle. Although much is known about the kinetic effects of β -glucosidase activation, little is known about the regulation of activation. For example, it is not known how saposin C, which is soluble, comes in contact with β -glucosidase, which is membrane-bound.

In theory, the regulation of β -glucosidase activity in the lysosome could be accomplished by changes in the local concentrations of lysosomal membrane lipids or saposin C, or alterations in the interaction between the enzyme and its activators. The elucidation of such regulatory mechanisms is important for understanding the pathogenesis of the different clinical forms of Gaucher disease and for developing maximally effective therapies. Additionally, it has been shown that ceramide, a product of the β -glucosidase reaction, affects multiple cellular functions such as growth, differentiation and viability [11]. Thus studies on the regulation of β -glucosidase activity potentially have a still broader significance.

In the present report, the effects of Ca^{2+} ions on PtdSer- and saposin C-stimulated β -glucosidase activity were investigated. Ca^{2+} is a known regulator of various intracellular events, and has been shown to interact directly with PtdSer [12] to regulate the activity of other enzymes that bind PtdSer [13–15]. We found that Ca^{2+} affects PtdSer- and saposin C-stimulated β -glucosidase activity in both positive and negative ways, depending on the concentration of the phospholipid and the presence or absence of saposin C.

EXPERIMENTAL

Materials

Phosphatidylserine was obtained from Supelco (Bellefonte, PA, U.S.A.). 4-Methylumbelliferyl β -D-glucopyranoside (MUGlc)

was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Octyl- and phenyl-Sepharose were purchased from Pharmacia (Piscataway, NJ, U.S.A.). Frozen rat livers were obtained from Pel-Freez (Rogers, AR, U.S.A.). All other reagents were of the highest quality available.

Purification of rat liver glucosylceramide β -glucosidase

Frozen rat livers were thawed, minced, and processed through the sodium cholate, ammonium sulphate, and butan-1-ol extraction steps as described by Furbish et al. [16]. The resulting preparation (purified 9-fold) was dialysed against 0.1 M sodium acetate, pH 6.0, containing 5 mM EDTA and 1 mM 2-mercaptoethanol (buffer A).

The dialysed preparation was subjected to octyl-Sepharose chromatography. β -Glucosidase was eluted with 0.5 M NaSCN and 60 % ethylene glycol in buffer A. The resulting preparation (purified 480-fold) was dialysed against 10 % ethylene glycol in 0.1 M sodium acetate, pH 6.0, 5 mM EDTA and 1 mM 2-mercaptoethanol (buffer B).

The dialysed octyl-Sepharose pool was then subjected to phenyl-Sepharose chromatography. β -Glucosidase was eluted with 0.5 M NaSCN and 60 % ethylene glycol in buffer A. Fractions containing β -glucosidase activity were pooled (1800-fold purification) and dialysed against buffer B.

Finally, an affinity column of immobilized PtdSer and saposin C in polyacrylamide, made by a modification of the method of Uchida and Filburn [17], was employed. The enzyme was eluted with 0.5 M NaSCN and 60 % ethylene glycol in buffer A. The β -glucosidase-containing fractions were pooled and dialysed against 90 % ethylene glycol in 10 mM sodium acetate, pH 6.0, and 1 mM 2-mercaptoethanol. The dialysed enzyme preparation was stored at -30°C . This protocol resulted in a final purification of 22000-fold. Samples were diluted 2-fold with distilled deionized water immediately before use in this study.

β -Glucosidase assay

Glucosylceramide β -glucosidase activity was determined with MUGlc as substrate. The standard assay contained 5 mM MUGlc, 0.2 M sodium acetate, pH 5.5, and the indicated amounts of PtdSer and CaCl_2 , in a final volume of 0.1 ml. The incubation was carried out for 30 min at 37°C , and fluorescence was determined as described elsewhere [6]. All assays were performed in duplicate. One unit of β -glucosidase activity is the amount of enzyme that releases 1 nmol of glucose from MUGlc/h at 37°C .

In this paper, β -glucosidase activity refers to glucosylceramide β -glucosidase acting on the water-soluble substrate, MUGlc. Use of this non-physiological substrate is necessary when studying lipid effects on glucosylceramide β -glucosidase because the natural substrate is insoluble in the absence of lipids or detergents. In studies in which the effects of a lipid on enzyme activity are examined, it is essential to avoid interference from other lipids or detergents that are usually added to the β -glucosidase assay mixture [6]. Therefore, detergents and lipids other than PtdSer were not included in the assay mixture in this study.

Addition of 0.25 mM conduritol B-epoxide (CBE), a specific active-site inhibitor of glucosylceramide β -glucosidase [18], to the assay medium resulted in greater than 95 % inhibition of the β -glucosidase activity of the purified enzyme preparation. Therefore, the activity measured in this study is due to glucosylceramide β -glucosidase, and not some other β -glucosidase.

Preparation of saposin C

Saposin C was purified from human Type 1 Gaucher-disease spleen as previously described [4]. In the present paper, one unit of saposin C activity is defined as the amount of saposin C required to stimulate β -glucosidase activity 2-fold in the presence of $13\text{ }\mu\text{M}$ PtdSer. The preparation of saposin C used in this study had a specific activity of 2778 units/mg.

Determination of kinetic parameters, K_m and V_{max}

β -Glucosidase determinations were performed at ten different MUGlc concentrations covering a 20-fold range as described by Segel [19]. K_m and V_{max} values were determined by fitting the data to a rectangular hyperbola with a computer-analysis method described by Cleland [20].

Preparation of aqueous solutions of PtdSer

A chloroform/methanol (2:1, v/v) solution of PtdSer was dried under nitrogen gas, and the residue was suspended in water and sonicated for 1 min at 4°C , with the standard microtip on a Heat-Systems Ultrasonics cell disruptor. The phosphorus content of the phospholipid suspension was determined by the method of Bartlett [21], with potassium phosphate as standard.

Examination of aggregation states of PtdSer

The aggregation of PtdSer at different concentrations was examined with the fluorescent dye *N*-phenyl-1-naphthylamine as described by Kovatchev et al. [22] Aggregation studies were carried out at 37°C in 0.2 M sodium acetate buffer, pH 5.5.

Protein determination

The protein concentration of β -glucosidase preparations was estimated by the method of Bradford [23], with BSA as standard.

RESULTS

Effect of Ca^{2+} concentration on β -glucosidase activity

Ca^{2+} ions can interact directly with acidic phospholipids, thereby either enhancing or disrupting protein-lipid interactions. Thus we were interested in determining whether Ca^{2+} affects PtdSer-enhanced β -glucosidase activity. As shown in Figure 1, Ca^{2+} did alter this activity; the effects depended on the concentration of PtdSer and, to a lesser extent, the concentration of Ca^{2+} in the assay medium. At a low PtdSer concentration ($3\text{ }\mu\text{M}$), Ca^{2+} had no effect on β -glucosidase activity. However, at a phospholipid concentration of $13\text{ }\mu\text{M}$, Ca^{2+} stimulated β -glucosidase activity greater than 2-fold, and the stimulation was maximal at 5 mM Ca^{2+} . In contrast, Ca^{2+} inhibited β -glucosidase activity by 50 % in the presence of $380\text{ }\mu\text{M}$ PtdSer. No effect of Ca^{2+} on β -glucosidase activity was detected in the absence of PtdSer (results not shown). Also, the activation and inhibition were due to the cation and not to Cl^- , since 1–10 mM NaCl had no effect on β -glucosidase activity (results not shown).

PtdSer concentration-dependence of Ca^{2+} effects

The effects of Ca^{2+} on β -glucosidase activity measured over an extensive range of PtdSer concentrations were examined next.

Figure 1 Effects of increasing Ca^{2+} concentrations on glucosylceramide β -glucosidase activity

β -Glucosidase activity was determined with the standard assay and the addition of 3 ($\square$), 13 ($\circ$), or 380 ($\bullet$) μM PtdSer and the indicated concentrations of CaCl_2 in a final volume of 0.1 ml. The control activity is the activity measured in the absence of CaCl_2 at each PtdSer concentration.

Figure 2 Effects of 5 mM Ca^{2+} on the activation of glucosylceramide β -glucosidase by various concentrations of PtdSer

β -Glucosidase was assayed with 5 mM MUGlc in 0.2 M sodium acetate, pH 5.5, with the addition of the indicated amounts of PtdSer in the absence ($\circ$) or presence ($\bullet$) of 5 mM CaCl_2 .

Figure 2 shows a comparison of β -glucosidase activity measured over an 800-fold range of PtdSer concentrations in the absence and presence of 5 mM Ca^{2+} . At PtdSer concentrations between 7.6 and 76 μM , Ca^{2+} caused a significant increase in β -glucosidase activity over that seen with PtdSer alone. In contrast, at PtdSer concentrations greater than 100 μM , Ca^{2+} inhibited β -glucosidase activity.

Effect of Ca^{2+} on saposin C activation of β -glucosidase

Figure 3 shows the effect of saposin C on β -glucosidase activity in the presence of PtdSer. Although high concentrations of PtdSer alone were capable of stimulating β -glucosidase activity, the presence of saposin C resulted in much greater stimulation at

Figure 3 Stimulation of β -glucosidase by saposin C

β -Glucosidase was assayed as described for Figure 2 in the presence of the indicated concentrations of PtdSer and in the absence ($\circ$) and presence ($\bullet$) of 0.01 mg/ml saposin C.

Table 1 Effect of Ca^{2+} on saposin C stimulation of β -glucosidase

β -Glucosidase was assayed as described in the text in the presence of 0.01 mg/ml saposin C and the indicated concentrations of PtdSer with and without the addition of 5 mM CaCl_2 .

[PtdSer] (μM)	β -Glucosidase activity (units/ μg)	
	Minus Ca^{2+}	Plus Ca^{2+}
0	38	33
13	213	55
380	322	79

all PtdSer concentrations tested. When Ca^{2+} was included in the β -glucosidase assay mixture with saposin C and PtdSer, the stimulatory effect of saposin C was abolished (Table 1) at both low (13 μM) and high (380 μM) PtdSer concentrations.

Evaluation of the kinetic basis for Ca^{2+} effects

In order to study the basis of the observed activation and inhibition of β -glucosidase activity by Ca^{2+} , the effects of Ca^{2+} on the kinetic parameters of β -glucosidase were examined (Table 2). In the absence of PtdSer, Ca^{2+} had no apparent effect on K_m and V_{max} . However, in the presence of 13 μM PtdSer, the addition of Ca^{2+} resulted in a higher V_{max} (143 versus 89 units/ μg of protein) and lower K_m (3.8 versus 5.8 mM) than were observed when only the lipid was present. Therefore the activation of β -glucosidase by Ca^{2+} can be attributed to both an increase in V_{max} and a decrease in K_m . In contrast, when 130 μM PtdSer was used (the concentration at which the curves in Figure 2 intersect), no effect on either of the kinetic parameters was observed.

Additionally, in the presence of 380 μM PtdSer, Ca^{2+} caused a 2-fold increase in the K_m (from 2.0 to 4.2 mM), but had no effect on the V_{max} , indicating that the inhibition by Ca^{2+} observed in Figure 2 was due to a decreased affinity of the enzyme for the substrate.

Table 2 Effects of Ca^{2+} on kinetic parameters of PtdSer-enhanced β -glucosidase activity

Kinetic parameters were determined as described in the Experimental section, in the absence or presence of 5 mM CaCl_2 , at the indicated concentrations of PtdSer. Three separate determinations were performed for each condition. K_m and $V_{\max}$ values are listed as means $\pm$ 1 S.E.M.

[PtdSer] (μM)	No Ca^{2+}		Plus Ca^{2+}	
	$V_{\max}$ (units/ μg)	K_m (mM)	$V_{\max}$ (units/ μg)	K_m (mM)
0	57	> 15*	53	> 15*
13	89 $\pm$ 15	5.8 $\pm$ 1.7	143 $\pm$ 18**	3.8 $\pm$ 0.9†
130	427 $\pm$ 18	2.4 $\pm$ 0.2	421 $\pm$ 51	2.9 $\pm$ 0.8
380	507 $\pm$ 23	2.0 $\pm$ 0.2	510 $\pm$ 54	4.2 $\pm$ 0.8†

* The actual K_m and $V_{\max}$ values could not be determined for these assay conditions, due to the insolubility of the MUGic substrate at high concentrations. The $V_{\max}$ value listed is the actual velocity observed at a MUGic concentration of 15 mM.

** Significantly different from the value obtained in the absence of CaCl_2 at $P \leq 0.001$ by Student's t test.

† $P \leq 0.01$, relative to the value obtained in the absence of CaCl_2 .

Table 3 Effects of Ca^{2+} on kinetic parameters of saposin C- and PtdSer-enhanced β -glucosidase activity

Kinetic parameters were determined as described in the Experimental section in the presence of 0.01 mg/ml saposin C and the indicated amounts of PtdSer, and in the absence and presence of 5 mM CaCl_2 . Three separate determinations were performed for each condition. $V_{\max}$ and K_m values are listed as means $\pm$ 1 S.E.M.

[PtdSer] (μM)	No Ca^{2+}		Plus Ca^{2+}	
	$V_{\max}$ (units/ μg)	K_m (mM)	$V_{\max}$ (units/ μg)	K_m (mM)
0	65.8 $\pm$ 4.4	3.5 $\pm$ 0.6	52.3 $\pm$ 3.2	2.9 $\pm$ 0.5
13	464 $\pm$ 52	5.0 $\pm$ 1.0	80.1 $\pm$ 8.6*	3.2 $\pm$ 0.8
380	660 $\pm$ 64	4.0 $\pm$ 0.8	144 $\pm$ 7.4*	4.3 $\pm$ 0.4

* Significantly different from the value obtained in the absence of Ca^{2+} at $P \leq 0.001$ by Student's t test.

When saposin C was included in the assay medium, the effects of Ca^{2+} on the kinetic parameters were different (Table 3). In the absence of PtdSer, there was no effect of Ca^{2+} on the kinetic parameters measured in the presence of saposin C. When 13 μM PtdSer and saposin C were present, the addition of Ca^{2+} caused a decrease in the $V_{\max}$ from 464 to 80 units/ μg of protein. Ca^{2+} also caused a decrease in the $V_{\max}$ when 380 μM PtdSer and saposin C were used (660 versus 144 units/ μg). This is in contrast with the results obtained with 380 μM PtdSer alone, where the Ca^{2+} inhibition was due to a change in the K_m .

Effect of Ca^{2+} ions on the aggregation state of PtdSer

The kinetic experiments demonstrated that the effects of Ca^{2+} on PtdSer-stimulated β -glucosidase activity were dependent on the concentration of PtdSer used in the assay. We were therefore interested in determining whether Ca^{2+} affected the aggregation of PtdSer at different PtdSer concentrations.

The fluorescent dye *N*-phenyl-1-naphthylamine can be used to monitor changes in the aggregation state of a lipid, and is used in determining the critical micellar concentrations of lipids [22].

Figure 4 Effects of Ca^{2+} on the aggregation of PtdSer

PtdSer was incubated with *N*-phenyl-1-naphthylamine at 37 °C in 0.2 M sodium acetate, pH 5.5, in the absence (○) or presence (●) of 5 mM CaCl_2 . Fluorescence was measured as described by Kovatchev et al. [22].

The fluorescence of a constant amount of the dye changes concordantly with alterations in the aggregation state of the lipid present in the solution. A change in slope in the plot of fluorescence versus lipid concentration indicates a change in the aggregation state of the lipid.

When *N*-phenyl-1-naphthylamine was incubated with PtdSer, in the presence and absence of Ca^{2+} , several features of PtdSer aggregation were noted (Figure 4). First, in the absence of Ca^{2+} , PtdSer appears to exist in different aggregation states, depending on its concentration, as shown by the lack of linearity of the graph. Second, the shape of the graph changed when Ca^{2+} was included in the incubation. Below 8 μM PtdSer both curves were nearly identical. However, between 8 and 60 μM PtdSer there was a slight, although significant, difference in the curves. That is the range of PtdSer concentrations at which Ca^{2+} enhanced β -glucosidase activity (Figure 2). Additionally, at PtdSer concentrations greater than 100 μM there was a pronounced difference in the curve obtained in the presence of Ca^{2+} , corresponding to the PtdSer concentration range at which Ca^{2+} inhibited β -glucosidase activity.

DISCUSSION

This study demonstrates that Ca^{2+} can influence PtdSer- and saposin C-stimulated glucosylceramide β -glucosidase activity. Both stimulatory and inhibitory effects of Ca^{2+} were noted, depending on the concentration of PtdSer and the presence or absence of saposin C. Stimulation of β -glucosidase activity by Ca^{2+} ions was observed at low PtdSer concentrations, and was due to both an increase in $V_{\max}$ and a decrease in K_m . Inhibition of β -glucosidase activity by Ca^{2+} was noted in the presence of high concentrations of PtdSer, and when PtdSer and saposin C were present together. The inhibition in the presence of PtdSer alone was due to an increase in K_m , whereas the inhibition observed in the presence of PtdSer and saposin C was due to a decrease in $V_{\max}$.

Ca^{2+} ions were shown to affect the aggregation state of PtdSer under the conditions of the β -glucosidase assay. Effects on PtdSer aggregation were observed in the two PtdSer concentration ranges at which Ca^{2+} affected β -glucosidase activity. These results indicate that the stimulation of β -glucosidase

activity by Ca^{2+} at low PtdSer concentrations is a result of a change in the structure of the PtdSer aggregates that creates a more effective form of PtdSer. Likewise, the inhibition of β -glucosidase activity by Ca^{2+} at high PtdSer concentrations is probably due to the formation of PtdSer structures that are less effective activators.

The inhibitory effects of Ca^{2+} on β -glucosidase activity in the presence of saposin C are more complicated; they can be explained by effects on PtdSer aggregation and effects on the saposin. Saposin C has been shown to bind to β -glucosidase, but not to PtdSer or the substrate [5,6]. However, in the presence of saposin C lower concentrations of PtdSer are required for activation, indicating that saposin C affects the binding of PtdSer aggregates to the enzyme. It is possible that a very low concentration of Ca^{2+} is present endogenously in the saposin C preparation, and may contribute to the stimulatory effect of the saposin on β -glucosidase activity.

The change in the aggregation state of PtdSer that occurs in the presence of 5 mM Ca^{2+} may create forms of the lipid that are not optimal for binding to the enzyme-saposin C complex. If that is the case, negative effects on β -glucosidase activity, such as those presented in Tables 1 and 3, would be expected in the presence of Ca^{2+} .

The inhibitory effects of Ca^{2+} on PtdSer- and saposin C-stimulated β -glucosidase activity might also be due to an effect on the binding of the saposin to the enzyme. However, this is unlikely since Ca^{2+} did not affect the ability of saposin to lower K_m in the absence of PtdSer (compare Tables 2 and 3).

One possible mechanism for the intracellular modulation of β -glucosidase activity by Ca^{2+} is alterations in Ca^{2+} concentrations at the lysosomal membrane or inside the lysosome. In the context of β -glucosidase located in a lysosomal membrane, Ca^{2+} could facilitate the interaction between β -glucosidase and nearby PtdSer molecules that are also a part of the membrane. Alternatively, Ca^{2+} could affect saposin C-mediated enhancement of lysosomal β -glucosidase activity by modulating saposin and/or PtdSer binding.

Regulation of β -glucosidase activity is especially important to understand, in view of the current use of enzyme replacement therapy for the treatment of Gaucher disease [24]. In order to achieve maximal therapeutic advantage, aspects of the enzyme's regulation *in vivo* should be taken into consideration. It has been noted that saposin C levels increase in tissues of persons with Gaucher disease [4], indicating that synthesis of the activator protein is regulated by the concentration of glucocerebroside and/or the level of β -glucosidase, and is a point of regulation for β -glucosidase activity. It has also been shown that minimal changes in intralysosomal pH can alter β -glucosidase activity, and that different Gaucher-disease genotypes are affected differentially by variations in pH [25].

Regulated breakdown of glucosylceramide could also be physiologically relevant as a means of controlling the intracellular levels of various sphingolipid metabolites that, in turn, affect critical processes required for normal cell function and growth. Sphingosine and lysosphingolipids have been shown to be potent inhibitors of protein kinase C [26], and are thought to be involved in the regulation of that enzyme *in vivo* [27]. Ceramide, a product of the β -glucosidase reaction, has been shown to affect many different cell functions, including growth and differen-

tiation, and has been shown to be cytotoxic in some circumstances [11].

Also, accumulation of lysosphingolipids, and the resulting inhibition of protein kinase C, in neural tissue of persons afflicted with sphingolipid storage diseases is believed to be responsible for the extensive neurological dysfunction noted in some of those diseases [26]. The skeletal pathology associated with Gaucher disease might also be due to the effects of accumulated glucocerebroside and/or glucosylsphingosine in skeletal tissues.

Continued investigation into the regulation of β -glucosidase activity within the lysosome and the role of sphingolipids in cellular physiology is essential to provide information necessary for the further development of therapies for Gaucher disease and other sphingolipidoses. This study suggests that Ca^{2+} plays a role in the regulation of β -glucosidase activity and, consequently, plays a role in sphingolipid metabolism that can have wide-ranging effects on cells.

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