Decanoyl lysophosphatidic acid induces platelet aggregation through an extracellular action

Evidence against a second messenger role for lysophosphatidic acid

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Platelets rapidly convert 1,2-didecanoyl-*sn*-glycerol into its corresponding phosphatidic acid and lysophosphatidic acid derivatives, thereby providing a means of introducing these two compounds into platelets. 1-Decanoyl-2-lyso-3-*sn*-phosphatidic acid, when added directly to platelets, induced platelet aggregation and raised intracellular Ca^{2+} levels at concentrations of 0.3 μM upwards, but was without effect when formed intracellularly from 1,2-didecanoylglycerol at an estimated concentration of approx. 47 μM . This indicates that the site of platelet activation by lysophosphatidic acid is extracellular. A concentration of thrombin (0.2 unit/ml), which produced maximal platelet aggregation, caused an estimated intracellular formation of 20 μM -lysophosphatidic acid in the presence of 2 mM- Ca^{2+} ; however, there was no detectable release of lysophosphatidic acid into the bathing medium. Lysophosphatidic acid, therefore, may not be an intracellular second messenger involved in platelet aggregation by thrombin.

INTRODUCTION

A great deal of attention has focused on the possible role of phosphatidic acid as an intracellular messenger generated, indirectly, through receptor-stimulated hydrolysis of inositol phospholipids (Putney, 1981; Lapetina *et al.*, 1981a). In support of this, phosphatidic acid has been suggested to be a Ca^{2+} ionophore because of its ability to translocate Ca^{2+} into organic solvents (Tyson *et al.*, 1976; Putney, *et al.*, 1980), across liposomes (Serhan *et al.*, 1981) and through phospholipid bilayers (Nayar *et al.*, 1984); in addition, it induces the contraction of a variety of smooth muscles (Vogt, 1963; Salmon & Honeyman, 1980; Tokumura *et al.*, 1980), stimulates $^{86}\text{Rb}^{+}$ efflux from the rat parotid gland (Putney *et al.*, 1980), causes Ca^{2+} release from a platelet membrane fraction (Gerrard *et al.*, 1978), and potentiates the activity of phospholipase A_2 (Apitz-Castro *et al.*, 1981).

More recent work, however, has questioned these results. Holmes & Yoss (1983) have suggested that the original observation of phosphatidic acid-induced translocation of Ca^{2+} across liposomes was most probably due to the presence of contaminating oxidized fatty acids. In their hands, freshly prepared phosphatidic acids were inactive in moving Ca^{2+} across liposomes. Further, Benton *et al.* (1982) have observed that lysophosphatidic acids, but not phosphatidic acids, are potent inducers of human platelet aggregation and have therefore suggested that lysophosphatidic acids, rather than phosphatidic acids, may be important intracellular messengers.

In support of this hypothesis, lysophosphatidic acid has been shown to accumulate in thrombin-stimulated human (Mauco *et al.*, 1978; Gerrard & Robinson, 1984) and horse (Lapetina *et al.*, 1981a, b) platelets, presumably through the action of either a phosphatidic acid-specific phospholipase A_2 (Billah *et al.*, 1981) or phosphorylation

of monoacylglycerol generated by diacylglycerol lipase (Bell *et al.*, 1979). Further, Benton *et al.* (1982), using the data of Lapetina *et al.* (1981a) in horse platelets, have calculated that the peak intracellular concentration of lysophosphatidic acid reached in platelets lies in the range 14–77 μM . This amount is maximally effective in producing platelet aggregation (Gerrard *et al.*, 1979; Tokumura *et al.*, 1981; Benton *et al.*, 1982; MacIntyre & Shaw, 1983; Hallam *et al.*, 1983; Simon *et al.*, 1984). In addition, lysophosphatidic acids have been previously shown to contract smooth muscles (Vogt, 1963; Tokumura *et al.*, 1980) and to potentiate production by thrombin of arachidonic acid metabolites in horse platelets (Lapetina *et al.*, 1981b).

The mechanism by which lysophosphatidic acids bring about these effects, however, is unclear. It was originally thought that they were also Ca^{2+} ionophores (Gerrard *et al.*, 1979), but Holmes & Yoss (1983) demonstrated that they are unable to translocate Ca^{2+} across liposomes. Furthermore, no direct evidence has been presented demonstrating that lysophosphatidic acids activate platelets through Ca^{2+} mobilization, and indeed this possibility has been questioned (Simon *et al.*, 1984). The present study has therefore been undertaken to investigate further the mechanism of platelet activation by lysophosphatidic acid by using platelets loaded with the intracellular Ca^{2+} indicator quin2.

METHODS

Platelet preparation

Blood (200 ml) was obtained from drug-free volunteers on the day of the experiment with 0.15 vol. of ACD (85 mM-trisodium citrate/111 mM-glucose/71 mM-citric acid) as an anti-coagulant. Platelet-rich plasma was

obtained by centrifugation at 200 *g* for 20 min. Separation of platelets from plasma was carried out by centrifugation at 800 *g* for 10 min; in some experiments prostacyclin (5 ng/ml) was included during this centrifugation (Siess *et al.*, 1983). All experiments, with the exception of the quin 2 fluorescence studies, were performed in a platelet aggregometer with stirring at a platelet concentration of 1.4×10^8 /ml (quin2-loaded platelets) or 8×10^8 /ml (all others). Indomethacin (20 μ M) was included in all studies.

Labelling with [32 P]P_i and [3 H]inositol

Platelets were resuspended in a modified Tyrode-Hepes buffer (134 mM-NaCl, 12 mM-NaHCO₃, 2.9 mM-KCl, 0.36 mM-NaH₂PO₄, 1 mM-MgCl₂, 5 mM-Hepes, 5 mM-glucose, pH 7.4) containing 1 mM-EGTA. Prostacyclin (5 ng/ml) was added followed by either [32 P]P_i (5 mCi/10 ml) or [3 H]inositol (65–250 μ Ci/ml). Platelets were then left in a shaking water bath at 37 °C for 1 h and 3 h, respectively. More prostacyclin (5 ng/ml) was added and the platelets were washed twice with buffer. They were finally resuspended in EGTA-free buffer unless stated otherwise. Ca²⁺ (1 mM) was added 10 min before experimentation.

Pre-warmed aliquots (0.49 ml) of platelets were added to aggregometer tubes and placed in an aggregometer at 37 °C. The platelets were stirred for 60 s before additions (10 μ l) of thrombin, 1,2-didecanoylglycerol, LPA₁₀, PA₁₀ or buffer were made. The reaction was stopped at various times by transferring the contents of the aggregometer tubes either to 1.11 ml of butan-1-ol saturated with water in order to measure [32 P]lysophosphatidic acid (Gerrard & Robinson, 1984), or to 1.88 ml of chloroform/methanol/HCl (50:100:1, by vol.) in order to monitor [3 H]inositol phosphates.

The possible release of [32 P]lysophosphatidic acid from platelets challenged with thrombin was also investigated. Platelets were incubated with thrombin (0.2 unit/ml) for 60 s at 37 °C in a Chronolog Aggregometer, and then transferred to a bench microfuge and centrifuged at 10000 *g* for 30 s. The supernatant was rapidly removed into 1.11 ml of butan-1-ol saturated with water; 0.5 ml of water was then added to the remaining pellet followed by 1.11 ml of butan-1-ol and the samples were sonicated for 60 s. [32 P]lysophosphatidic acid was then measured by t.l.c. as described below.

Separation of [32 P]lysophosphatidic acid and [32 P]ATP

EDTA (2.5 mM) was added to the butanol extract, the tubes were vortex-mixed and then centrifuged. The upper butanol-containing phase was removed to fresh tubes and dried under N₂. Subsequently, butan-1-ol (50 μ l) was added, the tubes were vortex-mixed and the samples were spotted on oxalate-impregnated silica gel G-25 t.l.c. plates which were then developed in chloroform/methanol/HCl (174:26:1, by vol.) (Cohen & Derksen, 1969). Labelled compounds were identified by autoradiography and characterized by co-chromatography with unlabelled standards visualized either by impregnation with I₂ or by phosphate spray (Vaskovsky & Latyshev, 1975). For some samples, PA₁₀ was also separated using t.l.c. as described by Watson *et al.* (1984a).

The bottom phase of the control samples was also removed and used to estimate [32 P]ATP levels by h.p.l.c. Separations were performed on a Whatman analytical Sax column using a gradient of ammonium phosphate, pH 3.35, from 0.02 to 0.8 M at a flow rate of 1 ml/min. The

effluent was monitored at 260 nm and calibrated using standards of ATP. The [32 P]ATP levels were measured by scintillation counting and the specific radioactivity of [32 P]ATP was determined as described by Holmsen *et al.* (1984).

Separation of [3 H]inositol phosphates

Chloroform (0.6 ml) and water (0.6 ml) were added to the chloroform/methanol/HCl extract and the tubes were vortex-mixed. The upper phase was then applied to Dowex anion-exchange columns and [3 H]inositol phosphates were separated as originally described by Berridge *et al.* (1982) and modified by Watson *et al.* (1984b).

Quin2 studies

Platelets were loaded with quin2 in platelet-rich plasma as described by Rink *et al.* (1982). Platelets were then separated from plasma as described above and resuspended in EGTA-free medium. Ca²⁺ was added 10 min prior to experimentation. Quin2 fluorescence was measured as based on the method of Tsien *et al.* (1982). In brief, platelets were incubated at 37 °C without stirring in a spectrophotometer for 3 min; quin2 fluorescence was monitored for the last 1 min. Additions were then made and the cuvette was then inverted four times before replacing into the spectrophotometer and monitoring fluorescence. Control experiments were performed on non-quin2-loaded platelets in order to enable corrections to be made for the fluorescence exhibited by high concentrations of A23187 and the lysophosphatidic acids. Parallel aggregation experiments were also carried as described above.

Materials

LPA₁₀ and PA₁₀ were purchased from Serdary and dissolved in ethanol before use. In some studies, LPA₁₀ and PA₁₀ were purified by h.p.l.c. on a μ Porasil Silica column. The following solvents were used: solvent A (hexane/propan-2-ol/H₂SO₄, 25000:750:1, by vol.) and solvent B (hexane/propan-2-ol/water/H₂SO₄, 5758:1250:180:1, by vol.). Samples were run using a 20 min gradient of 25% B to 100% B; LPA₁₀ and PA₁₀ were eluted at 17 min and 9 min respectively. Quin2, tetraacetoxymethyl ester, was from Amersham International. Other reagents were from previously described sources (Watson *et al.*, 1984b; Siess *et al.*, 1983).

RESULTS

Thrombin-induced formation of lysophosphatidic acid

There was no detectable formation of [32 P]lysophosphatidic acid in thrombin-stimulated human platelets maintained in the presence of EGTA (1 mM), but absence of Ca²⁺ (Fig. 1a). In contrast, in the presence of 2 mM-Ca²⁺, thrombin caused a significant formation of [32 P]lysophosphatidic acid which was detectable within 5 s and reached a peak at approx. 60 s (Fig. 1b). Previously, Gerrard & Robinson (1984) have also reported that thrombin induces formation of lysophosphatidic acid in Ca²⁺-containing but not in Ca²⁺-free medium. On the other hand, Lapetina *et al.* (1981a) have observed thrombin-induced formation of lysophosphatidic acid in horse platelets in Ca²⁺-free medium, suggesting a species difference. There was no detectable release of this [32 P]lysophosphatidic acid, or any other

Fig. 1. Time course of thrombin (0.2 unit/ml)-stimulated formation of [³²P]lysophosphatidic acid and [³²P]phosphatidic acid in human platelets

Platelets prelabelled with [³²P]Pi were stimulated with thrombin (0.2 unit/ml) for various times and lysophosphatidic acid (LPA) and phosphatidic acid (PA) were subsequently separated by t.l.c. as described in the Methods section. Results are shown from one experiment which is representative of three others. Each point represents the mean $\pm$ S.E.M. of a triplicate determination. (a) Platelets maintained in EGTA (1 mM) and no Ca²⁺; (b) platelets maintained in EGTA (1 mM) and Ca²⁺ (2 mM).

Fig. 2. Lysophosphatidic acid is not released from platelets challenged with thrombin

Platelets were challenged with thrombin (0.2 unit/ml) for 60 s, and then centrifuged in a microfuge at 10000 *g* for 30 s. The presence of [³²P]lysophosphatidic acid in the platelet pellet or supernatant was investigated by extraction with butanol and separation by t.l.c. as described in the

[³²P]phospholipids, from platelets challenged with thrombin (0.2 unit/ml) for 60 s (Fig. 2).

Based on the specific radioactivity of [³²P]ATP determined as described in the Methods section and assuming that 8×10^8 platelets occupy a volume of 8 μ l (Billah & Lapetina, 1982), it was calculated that after 60 s thrombin (0.2 units/ml) induces the intracellular formation of $20.2 \pm 6.3 \mu$ M of lysophosphatidic acid and $154 \pm 4 \mu$ M of phosphatidic acid ($n = 3$). The level of lysophosphatidic acid, therefore, is 13% of that of phosphatidic acid at this time. Previously, Holmsen *et al.* (1984) had calculated, also based on the specific radioactivity of [³²P]ATP, that thrombin (0.2 unit/ml) induces the intracellular formation of 128–242 μ M of phosphatidic acid after 60 s, while Broekman *et al.* (1980) had calculated, from mass determinations, that thrombin (5 units/ml) induces the intracellular formation of 220 μ M of phosphatidic acid after 60 s.

Conversion of 1,2-didecanoylglycerol to its corresponding phosphatidic acid and lysophosphatidic acid derivatives

Since thrombin only causes formation of lysophosphatidic acid in human platelets maintained in the presence of Ca²⁺ (2 mM), all subsequent experiments were performed under these conditions. Fig. 3 shows that 1,2-didecanoylglycerol (100 μ M) was rapidly converted to both LPA₁₀ and PA₁₀ in platelets. Identification of these two products was through co-chromatography with standards on h.p.l.c. and t.l.c. (Fig. 4) as described in the Methods section. After 60 s, the concentrations of LPA₁₀ and PA₁₀ in platelets, calculated as described above, were $46.6 \pm 17 \mu$ M and $343 \pm 77 \mu$ M, respectively. Again, the

Methods section. Shown above is a representative autoradiogram of control and thrombin-stimulated platelets, representative of two other experiments.

Fig. 3. Time course of metabolism of 1,2-didecanoylglycerol to ³²P-labelled PA₁₀ and LPA₁₀ in human platelets

Experimental procedures were as described in Fig. 1. Experiments were performed in EGTA (1 mM) and Ca²⁺ (2 mM) containing medium. Shown is one experiment which is representative of three others. Results are expressed as the mean ± S.E.M. for three determinations.

level of lysophosphatidic acid was approx. 13% of that of phosphatidic acid.

Platelet aggregation studies

Fig. 5 shows that LPA₁₀ caused a concentration-dependent (0.4–12 μM) shape change of human platelets followed by a reversible aggregation; maximal aggregation was reached within 60 s.

In contrast, 1,2-didecanoylglycerol (100 μM) caused a much slower, full aggregation response in platelets; there was no aggregation until at least 60 s after the levels of LPA₁₀ had reached a peak (compare Figs. 3 and 5). PA₁₀ (100 μM) had no effect on platelet aggregation or shape change responses (Fig. 5).

Quin2 studies

The estimated intracellular Ca²⁺ concentration in quin2-loaded platelets maintained in the presence of Ca²⁺ (2 mM) was 111 ± 3.4 nM (*n* = 3). The addition of 1,2-didecanoylglycerol (100 μM) or the buffer it was dissolved in (ethanol) raised the intracellular Ca²⁺ levels to a peak of 167 ± 14 nM (*n* = 3) and 157 ± 18 nM (*n* = 3) respectively; these values are not significantly different (*P* > 0.05). In contrast, concentrations of LPA₁₀ (0.3–1 μM) or A23187 which just elicited shape change raised the intracellular Ca²⁺ concentration to 508 ± 48 nM and 534 ± 60 nM, respectively.

Action on [³H]inositol phosphate levels in platelets

LPA₁₀ (100 μM) had no significant effect on the levels of [³H]inositol phosphates in indomethacin-treated human platelets (Table 1). The same results were observed with the Ca²⁺ ionophore A23187 (10 μM), while,

Fig. 4. Separation of LPA₁₀ and PA₁₀ by t.l.c.

Silica gel G-25 plates were developed in chloroform/methanol/HCl (174:26:1, by vol.) and radioactive spots were detected by autoradiography. Shown is an example of an autoradiogram from an experiment in which platelets were treated with thrombin (0.2 unit/ml) for 60 s or 1,2-didecanoylglycerol (DC₁₀) for 10 and 30 s. PA₁₀ and LPA₁₀ migrated behind their endogenous counterparts and were easily separated. Their identity was indicated by co-chromatography with synthetic standards and verification was through h.p.l.c. as described in the Methods section. In many experiments, it was observed that the *R_F* values of the phosphatidic acids and lysophosphatidic acids varied slightly between the individual lanes of the t.l.c. plate, and this is exemplified in the present Figure for the migration of the endogenous phosphatidic acid in the lanes corresponding to thrombin for 60 s or 1,2-didecanoylglycerol for 5 s and 30 s.

Fig. 5. Platelet aggregation studies

Platelets were prepared from platelet-rich plasma by centrifugation and suspended in EGTA-free and Ca²⁺ (1 mM)-containing medium. Both LPA₁₀ and PA₁₀ were purified by h.p.l.c. as described in the Methods section. Shown are traces from one experiment which is representative of three others.

Table 1. Action of LPA₁₀ on [³H]inositol phosphate levels in indomethacin-treated platelets

Platelets that had been labelled with [³H]inositol and treated with indomethacin (10 μ M) were placed in aggregometer tubes with stirring and exposed to various agonists for 60 s. The reaction was stopped by transferring to chloroform/methanol/HCl (50:100:1, by vol.) and [³H]inositol phosphates were analysed as described in the Methods section. Results are shown as mean $\pm$ S.E.M. relative to controls (i.e. 100% = no effect) of three experiments performed in duplicate.

Agonist	Inositol monophosphate	Inositol bisphosphate	Inositol trisphosphate
LPA ₁₀ (100 μ M)	90 $\pm$ 4	109 $\pm$ 5	110 $\pm$ 8
A23187 (10 μ M)	115 $\pm$ 5	111 $\pm$ 4	102 $\pm$ 2
Thrombin (1 unit/ml)	251 $\pm$ 36	1249 $\pm$ 239	491 $\pm$ 90

in contrast, thrombin (1 unit/ml) caused a marked formation of all three [³H]inositol phosphates (Table 1): The use of thrombin in these studies is to serve as a positive control since thrombin has been previously reported to cause inositol phosphate formation in platelets (Watson *et al.*, 1984b).

DISCUSSION

Mechanism of platelet activation by LPA₁₀

The present study has demonstrated that concentrations of LPA₁₀ and A23187 which just produce shape change of platelets increase the intracellular Ca²⁺ level from a resting value of 150 nM to approx. 500 nM as estimated with the Ca²⁺ indicator quin2. This value is very similar to that previously determined by Rink *et al.* (1982) for the Ca²⁺ ionophore ionomycin, but contrasts markedly with the much lower intracellular Ca²⁺ levels induced by doses of thrombin and collagen which are also threshold for platelet activation (Rink *et al.*, 1982, 1983; Watson *et al.*, 1985). These data therefore strongly suggest that LPA₁₀ induces platelet activation through a similar mechanism to A23187, namely through Ca²⁺ mobilization. The mechanism of this increase in intracellular Ca²⁺, however, is not known but is unrelated to the formation of inositol trisphosphate, the putative Ca²⁺ second messenger (Streb *et al.*, 1983; O'Rourke *et al.*, 1985; Berridge & Irvine, 1984), as LPA₁₀ does not induce its formation in indomethacin-treated platelets.

LPA₁₀ induces platelet activation through an extracellular action

The present study has shown that 1,2-didecanoylglycerol is rapidly converted to its corresponding phosphatidic acid and lysophosphatidic acid derivatives in human platelets, and can therefore be used as a means of introducing these two compounds to intracellular sites. Using this procedure, it was estimated that 100 μ M-1,2-didecanoylglycerol induced a peak formation of 47 μ M-LPA₁₀ in platelets without raising intracellular Ca²⁺ levels. Additionally, this concentration of 1,2-didecanoylglycerol induced a very slow, full aggregation response in platelets, the onset of which lagged behind the formation of LPA₁₀ by several minutes.

These observations are in marked contrast to the rapid, reversible aggregation response observed when LPA₁₀ is added directly to platelets at a similar concentration (12 μ M) to that formed in the above experiments. Moreover, extracellularly administered LPA₁₀ can produce

shape change of platelets and raise the intracellular Ca²⁺ concentration from 100 to 500 nM at considerably lower concentrations (0.3–1 μ M). These data therefore suggest that the site of action of LPA₁₀ is extracellular, and that formation of intracellular LPA₁₀ does not play a role in platelet aggregation by 1,2-didecanoylglycerol. The mechanism of aggregation by 1,2-didecanoylglycerol is most probably through protein kinase C stimulation since, like other diacylglycerols (Kaibuchi *et al.*, 1983), it has been shown to phosphorylate a 40000 Da protein in platelets which is a substrate for this enzyme (Watson *et al.*, 1984a).

These results have important implications for the mechanism of platelet activation by thrombin. The present study has demonstrated that a concentration of thrombin (0.2 unit/ml) which produces a rapid, maximal aggregation response in platelets in Ca²⁺ medium induces a peak formation of 20 μ M-lysophosphatidic acid. The time course of formation of this lysophosphatidic acid resembles that for the formation of LPA₁₀ from 100 μ M-1,2-didecanoylglycerol, and the peak concentration of lysophosphatidic acid formed by thrombin in these studies was 43% of the peak concentration of LPA₁₀ formed from 100 μ M-1,2-didecanoylglycerol. As shown above, this intracellular concentration of LPA₁₀ causes no Ca²⁺ mobilization in platelets. It is a possibility, therefore, that the lysophosphatidic acid produced in thrombin-stimulated platelets also plays no role in Ca²⁺ mobilization and platelet activation, thereby suggesting that it is not a second messenger. It should be remembered, however, that thrombin induces the formation of a different species of lysophosphatidic acid and, conceivably, this lysophosphatidic acid may be considerably more potent in raising intracellular Ca²⁺ levels. This possibility appears unlikely though, since Tokumura *et al.* (1981) have shown that 1-palmitoyl-2-lysophosphatidic acid was the most potent of a number of lysophosphatidic acids at inducing platelet aggregation, and 1-palmitoyl-2-lysophosphatidic acid is only approx. 10 times more potent than LPA₁₀ (results not shown). (Thrombin increased the intracellular concentration of lysophosphatidic acid to 20 μ M, while directly administered LPA₁₀ induced platelet activation at approx. 100 times lower concentrations.)

A further argument that lysophosphatidic acid is not an intracellular second messenger involved in platelet activation by thrombin arrives from the observation that it is not formed in the presence of EGTA and absence of Ca²⁺. Yet, under these conditions, thrombin can produce full platelet aggregation (Lapetina, 1984). The increase in

intracellular Ca^{2+} in human platelets levels induced by thrombin (Rink *et al.*, 1982), therefore, may be related to the generation of inositol trisphosphate (Agranoff *et al.*, 1983; Watson *et al.*, 1984b) which has been shown to mobilize Ca^{2+} in a number of tissues (for review, see Berridge & Irvine, 1984), including the platelet (O'Rourke *et al.*, 1985).

Finally, it should be stressed that the arguments presented here against a second messenger role for lysophosphatidic acid in human platelets are also applicable to phosphatidic acid. That is, the present study has shown that $100\ \mu\text{M}$ -1,2-didecanoylglycerol caused a peak formation of approx. $350\ \mu\text{M}$ - PA_{10} in human platelets without an apparent increase in the intracellular concentration of Ca^{2+} . The high concentration of PA_{10} formed presumably reflects the rapid diffusion of 1,2-didecanoylglycerol into platelets followed by its metabolism, thereby maintaining a concentration gradient. This concentration of phosphatidic acid was approx. 2.4 times greater than that induced by thrombin. Again, however, it should be remembered that thrombin induces the formation of a different species of phosphatidic acid.

In summary, the present study has shown that concentrations of LPA_{10} which produce platelet aggregation and raise intracellular Ca^{2+} levels when added directly to platelets are ineffective when added intracellularly through the use of 1,2-didecanoylglycerol. Further, thrombin, in concentrations which produce maximal platelet aggregation, caused the formation of a similar concentration of lysophosphatidic acid to that induced by 1,2-didecanoylglycerol. Taken together, these results suggest that the mechanism of platelet aggregation by lysophosphatidic acid is extracellular, and that lysophosphatidic acid is not an intracellular second messenger involved in thrombin-induced platelet activation.

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