The Ca\(^{2+}\)-ATPase Isoforms of Platelets Are Located in Distinct Functional Ca\(^{2+}\) Pools and Are Uncoupled by a Mechanism Different from That of Skeletal Muscle Ca\(^{2+}\)-ATPase*

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Simone Engelender, Herman Wolosker, and Leopoldo de Meis‡

From the Instituto de Ciencias Biomedicas, Departamento de Bioquimica, Universidade Federal do Rio de Janeiro, Cidade Universitaria, Ilha do Fundao, RJ 21941-590, Brasil

Vesicles derived from the dense tubular system of platelets possess a Ca\(^{2+}\)-ATPase that can use either ATP or acetyl phosphate as a substrate. In the presence of phosphate as a precipitating anion, the maximum amount of Ca\(^{2+}\) accumulated by the vesicles with the use of acetyl phosphate was only one-third of that accumulated with the use of ATP. Vesicles derived from the sarcoplasmic reticulum of skeletal muscle accumulated equal amounts of Ca\(^{2+}\) regardless of the substrate used.

When acetyl phosphate was used in platelet vesicles, the transport of Ca\(^{2+}\) was inhibited by Na\(^{+}\), Mg\(^{2+}\), and K\(^{+}\); in sarcoplasmic reticulum vesicles, only Na\(^{+}\) caused inhibition. When ATP was used as substrate, the different monovalent cation had no effect on either sarcoplasmic reticulum or platelet vesicles.

The catalytic cycle of the Ca\(^{2+}\)-ATPase is reversed when a Ca\(^{2+}\) gradient is formed across the vesicle membrane. The stoichiometry between active Ca\(^{2+}\) efflux and ATP synthesis was one in platelet vesicles and two in sarcoplasmic reticulum vesicles.

The coupling between ATP synthesis and Ca\(^{2+}\) efflux in sarcoplasmic reticulum vesicles was abolished by arsenate regardless of whether the vesicles were loaded with Ca\(^{2+}\) using acetyl phosphate or ATP. In platelets, uncoupling was observed only when the vesicles were loaded using acetyl phosphate. In both sarcoplasmic reticulum and platelet vesicles, the effect of arsenate was antagonized by thapsigargin (2 μM), micromolar Ca\(^{2+}\) concentrations, Pi (5-20 mM), and MgATP (10-100 μM). Trifluoperazine also uncoupled the platelet Ca\(^{2+}\) pump but, different from arsenate, this drug was effective in vesicles that were loaded using either ATP or acetyl phosphate. Trifluoperazine enhanced Ca\(^{2+}\) efflux from both sarcoplasmic reticulum and platelet vesicles; thapsigargin, Ca\(^{2+}\), Mg\(^{2+}\), or K\(^{+}\) antagonized this effect in sarcoplasmic reticulum but not in platelet vesicles.

The data indicate that the Ca\(^{2+}\)-transport isoforms found in sarcoplasmic reticulum and in platelets have different kinetic properties.

The Ca\(^{2+}\)-ATPase found in the endoplasmic reticulum plays an important role in the maintenance of a low cytosolic Ca\(^{2+}\) concentration in different cells. At least three genes encode the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA). The SERCA\(_1\) ATPase isoform is expressed in fast skeletal muscle (1). SERCA\(_2\) gives rise to SERCA\(_{2a}\) and SERCA\(_{2b}\) isoforms by alternative splicing. SERCA\(_{2b}\) is expressed in cardiac and slow skeletal muscle (2), while SERCA\(_{2b}\) is expressed in smooth muscle and represents a generic "endoplasmic reticulum" form that, together with SERCA\(_{3}\), is found in several non-muscle cells (3, 4).

The Ca\(^{2+}\)-ATPase (SERCA\(_1\)) found in vesicles derived from the sarcoplasmic reticulum has been studied extensively. This enzyme catalyzes the formation of a Ca\(^{2+}\) gradient by translocating Ca\(^{2+}\) from the medium into the vesicle lumen using energy derived from the hydrolysis of ATP (5). The entire catalytic cycle of the ATPase can be reversed, so that the enzyme synthesizes ATP from ADP and Pi, using the energy derived from the Ca\(^{2+}\) gradient. The synthesis of ATP is coupled with the release of Ca\(^{2+}\) from the vesicles into the medium (5-9). The reversal of the Ca\(^{2+}\) pump is uncoupled by different drugs. This was first observed with arsenate, a phosphate analog that interacts with the catalytic site of the Ca\(^{2+}\) ATPase, increasing the rate of Ca\(^{2+}\) efflux and inhibiting the synthesis of ATP (10-12). Similar to arsenate but in concentrations two orders of magnitude lower, a wide variety of hydrophobic drugs such as phenothiazines (13-16), local anesthetics (17), and fatty acids (18) can also uncouple the Ca\(^{2+}\) pump, greatly increasing the efflux of Ca\(^{2+}\) from the vesicles. Ligands and substrates of the ATPase block the Ca\(^{2+}\) efflux through the Ca\(^{2+}\) pump promoted both by arsenate and hydrophobic drugs (11, 13, 14, 16).

The dense tubular system found in blood platelets is a membranous network that retains a Ca\(^{2+}\) transport ATPase. Like the enzyme found in muscle, it can catalyze both the hydrolysis and the synthesis of ATP (19-29). Recently it has been shown that platelets express both SERCA\(_{2b}\) and SERCA\(_{3}\) Ca\(^{2+}\)-ATPase isoforms (30-32). It is not clear why different Ca\(^{2+}\)-ATPase isoforms coexist in platelets and the possibility is then raised that the two isoforms may be located in functionally distinct Ca\(^{2+}\) storage pools of the cell.

In this report we compare the kinetic properties of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA\(_1\)) with those of platelets (SERCA\(_{2b}\) and SERCA\(_{3}\)). It is shown that arsenate and trifluoperazine uncouple the Ca\(^{2+}\) pump of these two membrane systems in different manners. In platelets, but not in muscle, the substrate specificity and the sensitivity to arsenate indicate the possible existence of two different Ca\(^{2+}\) storage pools.

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† To whom correspondence should be addressed. Fax: 5521-270-8647.

1 The abbreviations used are: SERCA, sacro\(endo\)plasmic reticulum Ca\(^{2+}\)-ATPase; IP\(_3\), inositol 1,4,5-trisphosphate; MOPS, 4-morpholino propanesulfonic acid.
Two Functional Ca\(^{2+}\) Pools in Platelets

MATERIALS AND METHODS

Outdated platelets were obtained from a blood bank in a concentrated form. Platelet vesicles derived from the dense tubular system were prepared as described by Le Peuch et al. (28). Light sarcoplasmic reticulum vesicles derived from the longitudinal tubules of rabbit skeletal muscle were prepared according to Eletr and Inesi (33). This vesicle preparation does not contain significant amounts of ryanodine-sensitive Ca\(^{2+}\) channels, nor does it exhibit the phenomenon of "Ca\(^{2+}\) -induced Ca\(^{2+}\) release" found in the heavy fraction of sarcoplasmic reticulum (14, 34). The vesicles were stored in liquid nitrogen until use.

Protein concentration was estimated by the procedure of Lowry et al. (35) using bovine serum albumin as a standard.

The Ca\(^{2+}\) uptake was measured in medium containing 50 mM MOPS-Tris, pH 7.0, 1 mM MgCl\(_2\), 10 mM ATP, 50 mM KCl, 5 mM MgCl\(_2\), P\(_i\), and 0.1 mg of protein/ml. After 30–45 min of incubation at 35°C, the reaction was arrested by filtration through Millipore filters (0.45 μm) (36). The protein retained on the filter was washed four times with 5 ml of 3 mM La(NO\(_3\))\(_3\), and the remaining radioactivity was counted in a scintillation counter.

For the Ca\(^{2+}\) efflux experiments, the vesicles were preloaded with 45Ca in a medium containing 50 mM MOPS-Tris, pH 7.0, 5 mM MgCl\(_2\), 20 mM P\(_i\), 0.04–0.3 mM CaCl\(_2\), 3 mM ATP or 10 mM acetyl phosphate, and 0.1 mg of protein/ml. After 30–45 min of incubation at 35°C, the vesicles were centrifuged at 40,000 X g for 40 min, the supernatant was discarded, and the walls of the tubes were blotted to minimize contamination by the residual Ca\(^{2+}\)-loading medium. The pellets were immediately resuspended in ice-cold water with four strokes of a glass homogenizer and diluted to a final concentration of 0.05–0.1 mg of protein/ml into a medium containing 50 mM MOPS-Tris, pH 7.0, and additions specified in the figure legends.

In order to determine ATP and Pi, 45CaCl\(_2\), MgCl\(_2\), P\(_i\), and ATP or acetyl phosphate as substrate (Fig. 1) was measured at 35°C in a medium containing 50 mM MOPS-Tris, pH 7.0, 5 mM MgCl\(_2\), 10 mM hexokinase, 10 mM glucose, 5 mM 32Pi, and 0.2 mM ADP. The reaction was arrested with trichloroacetic acid to a final concentration of 10% (w/v). After centrifugation, an aliquot of the supernatant was used to measure ATP and Pi, 45CaCl\(_2\), MgCl\(_2\), P\(_i\), and ATP or acetyl phosphate as substrate (5). The rate of Ca\(^{2+}\) accumulation by the vesicles was the same regardless of the vesicle preparation used. In platelet preparations, with nonradioactive Ca\(^{2+}\) as described above and diluted in a medium containing 50 mM MOPS-Tris, pH 7.0, 5 mM EGTA, 5 mM MgCl\(_2\), 10 units/mI hexokinase, 10 mM glucose, 5 mM 32Pi, and 0.2 mg of protein/ml, it was found that vesicles prepared from platelets can also use acetyl phosphate and ITP as substrate, but different from the muscle Ca\(^{2+}\)-ATPase, the maximum level of Ca\(^{2+}\) accumulated with these substrates was 2–3-fold smaller than that measured with ATP (Fig. 1A).

With the use of acetyl phosphate, but not with ATP, a difference in the sensitivity to monovalent cations was observed. With ATP, monovalent cations had practically no effect in the Ca\(^{2+}\) accumulation measured with either platelet or sarcoplasmic reticulum vesicles (Table I). However, the Ca\(^{2+}\) uptake supported by acetyl phosphate was inhibited to different extents depending on the vesicle preparation used. In platelet vesicles, inhibition was observed with all three cations, so that Na\(^{+} -> Li^{+} -> K^{+}\) in order of inhibitory activity (Table I).

In sarcoplasmic reticulum vesicles, only Na\(^{+}\) significantly inhibited Ca\(^{2+}\) uptake.

\underline{Thapsigargin} is a highly specific inhibitor of SERCA isoforms and has no effect on plasma membrane Ca\(^{2+}\) ATPase (40–42). Nanomolar concentrations of thapsigargin abolished the Ca\(^{2+}\) uptake of platelet (Fig. 2) and sarcoplasmic reticulum vesicles (not shown) loaded with the use of either ATP or acetyl phosphate. This indicates that the Ca\(^{2+}\) accumulation measured in Fig. 1 and Table I was mediated by SERCA isoforms in the dense tubules of platelets and not by plasma membrane contaminants.

\underline{RESULTS}

\underline{Ca\(^{2+}\) Uptake—}\(\text{The Ca}^{2+}\)-ATPase found in sarcoplasmic reticulum vesicles can use different triphosphates, nucleosides, and acetyl phosphate as substrate (5). The rate of Ca\(^{2+}\) accumulation by sarcoplasmic reticulum vesicles varied depending on whether ATP, ITP, or acetyl phosphate was used, but when the steady-state was reached, the maximum amount of Ca\(^{2+}\) accumulated by the vesicles was the same regardless of the substrate (Fig. 1B). We found that vesicles prepared from platelets can also use acetyl phosphate and ITP as substrate, but different from the muscle Ca\(^{2+}\)-ATPase, the maximum level of Ca\(^{2+}\) accumulated with these substrates was 2–3-fold smaller than that measured with ATP (Fig. 1A).

With the use of acetyl phosphate, but not with ATP, a difference in the sensitivity to monovalent cations was observed. With ATP, monovalent cations had practically no effect in the Ca\(^{2+}\) accumulation measured with either platelet or sarcoplasmic reticulum vesicles (Table I). However, the Ca\(^{2+}\) uptake supported by acetyl phosphate was inhibited to different extents depending on the vesicle preparation used. In platelet vesicles, inhibition was observed with all three cations, so that Na\(^{+} -> Li^{+} -> K^{+}\) in order of inhibitory activity (Table I). In sarcoplasmic reticulum vesicles, only Na\(^{+}\) significantly inhibited Ca\(^{2+}\) uptake.

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Passive Ca\(^{2+}\) Efflux—A slow release of Ca\(^{2+}\) is observed when vesicles previously loaded with Ca\(^{2+}\) are diluted in medium containing EGTA and none of the ligands of the ATPase (5). In earlier reports (9, 13, 14) it has been shown that this passive Ca\(^{2+}\) efflux from sarcoplasmic reticulum vesicles is decreased when either Ca\(^{2+}\), Mg\(^{2+}\), K\(^+\), or thapsigargin is added to the efflux medium (Table II). As previously discussed (9, 13, 14), this indicates that, in sarcoplasmic reticulum vesicles, passive Ca\(^{2+}\) efflux occurs through the Ca\(^{2+}\)-ATPase. The passive Ca\(^{2+}\) efflux from platelet vesicles was much slower and was not inhibited by Ca\(^{2+}\), Mg\(^{2+}\), or K\(^+\) (Table II). Thapsigargin inhibited the efflux from platelet vesicles but its effect was less pronounced than that measured in sarcoplasmic reticulum (Table II). The effects observed in Table II were the same regardless of whether the vesicle preparations were loaded using ATP or acetyl phosphate as substrate (data not shown).

Reversal of the Ca\(^{2+}\) Pump—The rate of Ca\(^{2+}\) efflux is enhanced when the loaded vesicles are incubated in a medium that contains ADP, P\(_i\), and Mg\(^{2+}\) in addition to EGTA. The difference between the efflux in this case and the passive efflux with only EGTA present is termed active Ca\(^{2+}\) efflux and is coupled to the synthesis of ATP (5). In sarcoplasmic reticulum vesicles, the stoichiometry between Ca\(^{2+}\) release and ATP synthesis is two (5, 8) (Fig. 3B). The catalytic cycle of platelet Ca\(^{2+}\) ATPase could also be reversed (29), but different from sarcoplasmic reticulum, the stoichiometry found between active Ca\(^{2+}\) efflux and ATP synthesis was one (Fig. 3A), regardless of the substrate used to load the vesicles. For platelet vesicles loaded with acetyl phosphate, the stoichiometry found was 1.20 ± 0.10 (X ± S.E., n = 4) (Fig. 3A), and for vesicles loaded with ATP it was 1.19 ± 0.08 (n = 5).

Uncoupling of the Ca\(^{2+}\) Pump by Arsenate—Arsenate uncouples the reversal of the sarcoplasmic reticulum pump. It competes with P\(_i\) for the catalytic site of the SERCA ATPase and enhances the rate of passive Ca\(^{2+}\) efflux at the same time that it inhibits the synthesis of ATP (10–12). This is observed regardless of whether the vesicles are loaded with ATP or acetyl phosphate (12). Unlike vesicles derived from muscle, platelet vesicles respond to arsenate with a pronounced increase in Ca\(^{2+}\) efflux only after loading with acetyl phosphate (Figs. 4 and 5). Note that, in these experiments, the substrate used in the loading mixture was removed by centrifugation before the addition of preloaded vesicles to the efflux medium. These data and those of Fig. 1 suggest that platelet vesicles are composed of two distinct populations in which only the fraction of platelet vesicles that are loaded with acetyl phosphate (Fig. 1A) can be uncoupled by arsenate (Fig. 4A). The difference in the effect of arsenate on platelet vesicles loaded with acetyl phosphate and ATP is probably not related to the amount of Ca\(^{2+}\) retained by the vesicles. The rate of Ca\(^{2+}\) efflux depends on the concentration of free Ca\(^{2+}\) inside the vesicles. Inorganic phosphate was used as a calcium-precipitating agent when the vesicles were loaded. Thus, the total calcium accumulated refers to the calcium phosphate retained by the vesicles, but the free Ca\(^{2+}\) concentration will always be determined by the solubility product of calcium phosphate and should be the same regardless of the amount of calcium phosphate inside the vesicles (34). Ac-

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### Table II

Effect of cations and thapsigargin on the Ca\(^{2+}\) efflux rate from platelet and sarcoplasmic reticulum vesicles

| Additions          | Calcium efflux rate |
|--------------------|---------------------|
|                    | Platelet            | Sarcoplasmic reticulum |
|                    | nmol/mg min         | nmol/mg min           |
| None               | 3.7 ± 0.3 (100)     | 348 ± 33 (100)        |
| KCl, 100 mM        | 4.2 ± 0.4 (114)     | 125 ± 10 (36)         |
| MgCl\(_2\), 10 mM  | 4.1 ± 0.4 (111)     | 140 ± 13 (40)         |
| CaCl\(_2\), 100 μM | 3.8 ± 0.3 (103)     | 110 ± 9 (32)          |
| Thapsigargin, 2 μM | 2.8 ± 0.2 (76)      | 175 ± 12 (50)         |

* Numbers in parentheses are percentages.
Accordingly, the slower Ca$^{2+}$ efflux of platelet vesicles when compared to sarcoplasmic reticulum vesicles (Table II) is probably not related to different amount of Ca$^{2+}$ in the vesicles, but to a difference of the number of ATPase units available in the membrane of the two vesicle preparations. In fact, the Ca$^{2+}$-ATPase of platelet vesicles accounts for 2-4% of the total membrane protein (43), while in sarcoplasmic reticulum vesicles it represents 70-80% of the total protein (5).

In sarcoplasmic reticulum, the concentration of arsenate required to promote half-maximum stimulation of Ca$^{2+}$ efflux was 4.2 mM, regardless of the substrate used to load the vesicles (Fig. 5B). Similarly, in platelet vesicles loaded with acetyl phosphate, half-maximum stimulation was obtained with 3.6 mM arsenate (Fig. 5A).

In sarcoplasmic reticulum vesicles, the effect of arsenate requires magnesium and is antagonized by micromolar Ca$^{2+}$ concentrations, Pi, and MgATP (10, 11). The same was observed for the fraction of platelet vesicles loaded by acetyl phosphate (Figs. 5–8), with the only difference being that the concentration of MgATP required to antagonize the effect of arsenate in platelet vesicles was one order of magnitude smaller than that required for sarcoplasmic reticulum vesicles (compare Fig. 8, A and B).

For platelet vesicles loaded with ATP, arsenate did not activate the efflux of Ca$^{2+}$ even after the addition of hexokinase (50 units/ml) and glucose (20 mM) to the efflux medium to drain residual ATP.

Effect of Trifluoperazine—Hydrophobic drugs such as trifluoperazine are more effective than arsenate in uncoupling the sarcoplasmic reticulum Ca$^{2+}$-ATPase. In addition to increasing passive Ca$^{2+}$ efflux, trifluoperazine inhibits Ca$^{2+}$ uptake and ATPase activity in sarcoplasmic reticulum vesicles (13–16, 44). These effects were also observed with platelet vesicles, where trifluoperazine inhibited both Ca$^{2+}$ uptake and ATPase activity in lower concentrations than those necessary for sarcoplasmic reticulum vesicles (Fig. 9). Half-maximal inhibition of Ca$^{2+}$ uptake (Fig. 9A) was attained with one-fifth the concentration of trifluoperazine required for inhibition of ATPase activity (Fig. 9B). White and Raynor (45) reported that trifluoperazine inhibits Ca$^{2+}$ uptake in platelet vesicles and proposed that this effect was related solely to inhibition of Ca$^{2+}$ influx, since it did not affect Ca$^{2+}$ efflux. In our experimental conditions, inhibition of Ca$^{2+}$ uptake is probably related to enhancement of Ca$^{2+}$ efflux (Figs. 10 and 11). Like arsenate, trifluoperazine inhibited the synthesis of ATP and active Ca$^{2+}$ efflux from platelet vesicles (data not shown). A puzzling finding was that, different from arsenate, trifluoperazine uncoupled the Ca$^{2+}$ pump of platelet vesicles regardless of whether the vesicles were loaded using ATP or acetyl phosphate (data not shown).

In contrast to its effects in sarcoplasmic reticulum, the effect...
Inhibition of Ca$^{2+}$ uptake and Ca$^{2+}$-ATPase activity by trifluoperazine in platelet and sarcoplasmic reticulum vesicles. 

A. Ca$^{2+}$ uptake was measured at 35°C in medium containing 50 mM MOPS-Tris, pH 7.0, 2 mM MgCl$_2$, 2 mM ATP, 50 μM $^{45}$CaCl$_2$, 10 mM P$_i$, increasing concentrations of trifluoperazine and either 0.1 mM platelet vesicles (○) or 0.01 mg/ml sarcoplasmic reticulum vesicles (●). The reaction time was 40 min for platelet vesicles and 5 min for sarcoplasmic reticulum vesicles. The values represent the percent of Ca$^{2+}$ uptake without trifluoperazine: 180 nmol of Ca$^{2+}$/mg for platelet vesicles and 2.8 μmol of Ca$^{2+}$/mg for sarcoplasmic reticulum vesicles. B. ATPase activity was measured in medium containing [γ-32P]ATP instead of radioactive Ca$^{2+}$. The values represent the percent of ATPase activity without trifluoperazine: 540 nmol of P$_i$/mg for platelet vesicles and 1.4 μmol of P$_i$/mg for sarcoplasmic reticulum vesicles. The values are representative of three different experiments performed with three different vesicle preparations.

The present study shows kinetic differences between the Ca$^{2+}$-ATPase isoforms of skeletal muscle and platelets. Modulation of Ca$^{2+}$ uptake and Ca$^{2+}$ efflux by cations (Tables I and II), inhibition of passive Ca$^{2+}$ efflux by thapsigargin (Table II) and the Ca$^{2+}$/ATP stoichiometry measured during reversal of the pump (Fig. 3) distinguished the sarcoplasmic reticulum Ca$^{2+}$-ATPase isoform from that found in platelet vesicles.

The uncoupling effect of arsenate differentiated platelet vesicles loaded using ATP from those loaded using acetyl phosphate. Arsenate increased passive Ca$^{2+}$ efflux only from platelet vesicles loaded with acetyl phosphate (Fig. 4), and this efflux was antagonized by thapsigargin (Fig. 4), micromolar Ca$^{2+}$ concentrations (Fig. 6B), P$_i$ (Fig. 7), and MgATP (Fig. 8). These data indicate that vesicles loaded with acetyl phosphate contain a Ca$^{2+}$-ATPase isoform with properties different from those found in vesicles loaded with ATP. Other cells, such as bovine chromaffin cells (48) possess two Ca$^{2+}$ stores with distinct Ca$^{2+}$-ATPases in each of them.

Trifluoperazine also uncoupled the Ca$^{2+}$ pump of platelet vesicles, but unlike arsenate, it did not distinguish vesicles loaded with ATP from those loaded with acetyl phosphate. However, this drug did differentiate between muscle and platelet Ca$^{2+}$-ATPase isoforms, since thapsigargin and cations did not antagonize the effect of trifluoperazine in platelet vesicles (Figs. 10 and 11). The trifluoperazine concentrations used are at least 150-200-fold in excess in molar basis to the Ca$^{2+}$-ATPase content of either muscle or platelet vesicles preparations. Thus, the differences in sensitivity to trifluoperazine of the two vesicle preparations are not related to differences in Ca$^{2+}$-ATPase content.

Uncoupling of the platelet Ca$^{2+}$-ATPase by arsenate and trifluoperazine produced a greater increase in Ca$^{2+}$ efflux than that promoted by IP$_3$. This suggests that the number of Ca$^{2+}$-ATPase units found in the vesicle membranes is greater than the number of IP$_3$ receptors.

The effects of arsenate and trifluoperazine indicate that Ca$^{2+}$ can be released from platelet vesicles through the Ca$^{2+}$-ATPase. This pathway might serve to mobilize Ca$^{2+}$ in the cell. It is noteworthy that arsenate and trifluoperazine exhibit distinct effects depending on the tissue studied, even allowing identification of different functional compartments in platelets. The physiological implications of different Ca$^{2+}$ pools in platelets and other tissues, such as bovine chromaffin cells, are not clear at present.
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