ORIGINAL PAPER

Complex Actions of Ionomycin in Cultured Cerebellar Astrocytes Affecting Both Calcium-Induced Calcium Release and Store-Operated Calcium Entry

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Abstract The polyether antibiotic ionomycin is a common research tool employed to raise cytosolic Ca^{2+} in almost any cell type. Although initially thought to directly cause physicochemical translocation of extracellular Ca^{2+} into the cytosol, a number of studies have demonstrated that the mechanism of action is likely to be more complex, involving modulation of intrinsic Ca^{2+} signaling pathways. In the present study we assessed the effect of ionomycin on primary cultures of murine cerebellar astrocytes. Ionomycin concentrations ranging from 0.1 to 10 μM triggered a biphasic increase in cytosolic Ca^{2+}, consisting of an initial peak and a subsequent sustained plateau. The response was dependent on concentration and exposure time. While the plateau phase was abolished in the absence of extracellular Ca^{2+}, the peak phase persisted. The peak amplitude could be lowered significantly by application of dantrolene, demonstrating involvement of Ca^{2+}-induced Ca^{2+}-release (CICR). The plateau phase was markedly reduced when store-operated Ca^{2+}-entry (SOCE) was blocked with 2-aminoethoxydiphenyl borate. Our results show that ionomycin directly affects internal Ca^{2+} stores in astrocytes, causing release of Ca^{2+} into the cytosol, which in turn triggers further depletion of the stores through CICR and subsequently activates SOCE. This mechanistic action of ionomycin is important to keep in mind when employing it as a pharmacological tool.

Introduction

Ionomycin belongs to a group of polyether antibiotics that were believed to exert their antimicrobial effect through an ability to form lipid-soluble complexes with inorganic cations, thereby transporting them across biological membranes [1]. The dibasic ionomycin molecule selectively binds Ca^{2+} in a 1:1 stochiometry, but it can also form complexes with Mg^{2+} although with a lower affinity [2]. Due to its ionophoric properties and selectivity towards Ca^{2+}, ionomycin is a common pharmacological tool employed to study Ca^{2+} dynamics in virtually all cell types by raising cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$). However, while early studies indicated that the primary mechanism by which ionomycin increases $[Ca^{2+}]_c$ is a physiochemical translocation of extracellular Ca^{2+} through the lipid bilayer, it has since been shown to activate intrinsic Ca^{2+} modulating pathways and, hence, the response to ionomycin can vary significantly amongst cell types. Ionomycin was first shown to mobilize intracellular Ca^{2+} stores in fibroblasts and adrenal chromaffin cells [3, 4]. In a human epithelial cell line ionomycin was reported to evoke a biphasic response; initially Ca^{2+} was released from intracellular stores yielding a spike in $[Ca^{2+}]_c$, subsequently followed by a plateau phase, probably caused by store-operated Ca^{2+} entry (SOCE) [5]. This biphasic response was later confirmed by Dedkova et al. [6] in a range of other cell types although it was also established that the nature and composition of the $[Ca^{2+}]_c$ response was dependent on cell type and ionomycin concentration. The same study also for the first time reported ionomycin-resistant cells, underlining the improbability of unspecific physiochemical translocation across the plasma membrane as the primary mode of action.

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Astrocytes, a subtype of glial cells, were originally thought to merely provide structural support for neurons. By now we know that their functions not only extend to providing neurons with an optimal metabolic and homeostatic environment, but that they are crucial players in neurotransmission and higher cognitive functions like learning and memory [reviewed in 7]. Interestingly, though astrocytes are not classically electrically excitable cells, they experience evoked and spontaneous heterogeneous oscillations in $[Ca^{2+}]_c$ that can spread into several neighboring cells in so called astrocytic Ca^{2+} waves [reviewed in 8].

The present study was aimed at assessing the cytosolic Ca^{2+} response to ionomycin in primary cultures of murine cerebellar astrocytes. By employing a number of pharmacological tools we demonstrate that ionomycin triggers a concentration- and time-dependent biphasic calcium response that involves both Ca^{2+}-induced Ca^{2+}-release (CICR) and SOCE.

Materials and Methods

Materials

Seven-day-old mice were purchased from Harlan. Plastic culture dishes were from Thermo Scientific (formerly Nunc, Roskilde, Denmark), fetal calf serum and Fura-2 AM from Invitrogen A/S (Taastrup, Denmark) and penicillin from Leo Pharma (Ballerup, Denmark). Corning cellBIND surface 96-well plates, culture medium, and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Cultures

Primary cell cultures of cerebellar astrocytes were cultured consistent with the protocol described by Hertz et al. [9]. In brief, 7-day-old mice were decapitated and cerebella were isolated under aseptic conditions, passed through a nylon mesh (80 μm pore size) and immersed in modified Dulbecco's modified eagle's medium (DMEM) as defined by Hertz et al. [10], supplemented with 2.5 mM L-glutamine, 6 mM glucose, penicillin and 20 % fetal calf serum. The cell suspension was triturated three times using a 50 ml syringe with a steel cannula and plated either into 96-well plates or onto glass coverslips contained in 6-well multi-dish plates. Subsequently, cells were incubated at 37 °C in a humidified atmosphere of 5 % CO_2/95 % air. Medium was exchanged twice a week and serum concentration reduced by 5 % each week. In the third week of culturing, medium was supplemented with 0.25 mM dibutyryl-cAMP [11]. Astrocytes were used for experiments after 3 weeks in culture. The use of such cultures of astrocytes as a model of astrocytes in situ has recently been discussed by Lange et al. [12].

Measurement of Cytosolic Ca^{2+}

Astrocytes were loaded with 5.2 μM fura-2/acetoxymethyl ester by adding it to the culture medium and incubating for 45 min at 37 °C. Thereafter, cells were washed in tris-buffered saline (TBS) (15 mM Tris–HCl, 1 mM $MgSO_4$, 140 mM NaCl, 3.5 mM KCl, 1.8 mM $CaCl_2$, 2.5 mM glucose, 1.2 mM Na_2PO_4, pH 7.4) and left at room temperature for 10 min to equilibrate. Experiments were carried out in TBS with ionomycin in concentrations ranging from 0.01 to 10 μM and supplemented with either 30 μM dantrolene, 25 μM verapamil or 100 μM 2-aminoethoxydiphenyl borate (2-APB). Emission was recorded at 510 nm (10 nm bandwidth filter) with excitation at 340 and 380 nm using either a NOVOstar plate reader (BMG LABTECH GmbH, Offenburg, Germany) for monitoring cell populations or for single cell recordings a CCD-camera C4742-98 (Hamamatsu, Hersching, Germany) attached to a DMIRB microscope (Leica, Wetzlar, Germany) controlled via simple PCI (Hamamatsu, Hersching, Germany). Data were plotted as 340/380 nm ratio changes normalized to the fluorescence ratio baseline.

Statistics

Each data point is the average of 2–4 batches of astrocytes with n denoting the number of wells of a 96-well-plate in case of population assays ($n = 8$–23) and individual cells in cases of single cell measurements ($n > 200$). All data are presented as mean ± standard error of the mean (SEM). Comparison across concentrations within a single condition was conducted using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to compare individual means. Two-way ANOVA succeeded by Bonferroni's post hoc test was used to compare conditions across concentrations. $p < 0.05$ was considered statistically significant. GraphPad Prism v. 6 was used for all statistical analysis.

Results

Ionomycin Evokes a Biphasic Ca^{2+} Response

Murine cerebellar astrocytes in primary culture were treated with 1 μM ionomycin and the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) was monitored by recording changes of the fura-2 340/380 ratio in cell populations plated in 96-well plates. Ionomycin evoked an instant increase in $[Ca^{2+}]_c$ which was sustained in a plateau for at least 35 min (Fig. 1). Interestingly, an initial response could still be evoked in the absence of extracellular Ca^{2+}, though markedly lower in amplitude and decaying within a few minutes. Accordingly, the increase in $[Ca^{2+}]_c$ caused by

ionomycin consisted of at least two components of distinct origins: an initial peak and a sustained plateau phase. The persistence of a peak response in the absence of extracellular Ca^{2+} indicates that ionomycin initially mobilized intracellular stores, while the plateau component of the Ca^{2+} signal was clearly due to influx from extracellular space.

Ionomycin Increases Cytosolic Ca^{2+} in a Concentration- and Time-Dependent Manner

In order to further characterize the Ca^{2+} response to ionomycin, populations of astrocytes were exposed to a range of ionomycin concentrations and the exposure time was varied. The experiment was carried out in the presence of extracellular Ca^{2+}, in order to analyze both the amplitude of the initial peak and the amplitude of the plateau as measured after 35 min. The peak Ca^{2+} response increased with ionomycin concentrations from 0.01 to 10 µM (Fig. 2). At concentrations ≤ 1 µM $[Ca^{2+}]_c$ slowly declined after the initial peak, reaching significantly lower levels after 35 min. However, at 2 µM of ionomycin the peak level of Ca^{2+} was sustained in the plateau phase, and at concentrations >2 µM ionomycin $[Ca^{2+}]_c$ was significantly higher after 35 min than at the initial peak.

Taking into account these concentration-dependent differences in response, subsequently all experiments were carried out with 1 and 5 µM ionomycin to assess possible diversity in mode of action.
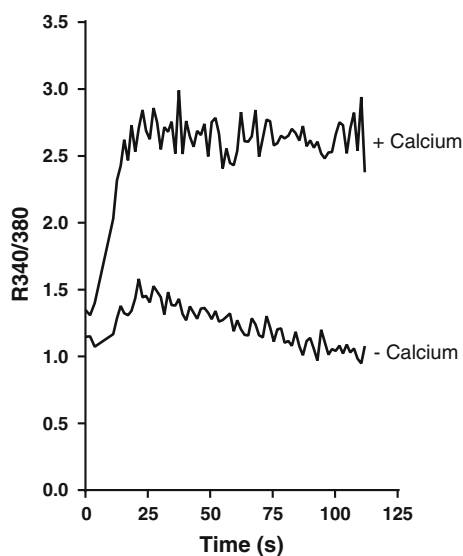

Fig. 1 Ionomycin caused a bi-phasic increase in cytosolic Ca^{2+} ($[Ca^{2+}]_c$). $[Ca^{2+}]_c$ was monitored in a population of cerebellar astrocytes upon treatment with 1 µM ionomycin in the presence or absence of 1.8 mM extracellular Ca^{2+}. Individual traces of typical populations loaded with fura-2 are shown, the fura-2 340/380 ratio is normalized to baseline

Ca^{2+}-Induced Ca^{2+}-Release is a Component of the Initial Ca^{2+} Peak

CICR is facilitated by channels in the endoplasmatic reticulum (ER)-membrane, which are activated upon binding of Ca^{2+} on their cytosolic site. CICR channels can be inhibited by ryanodine and dantrolene [13]. In order to assess the role of CICR in the astrocytic Ca^{2+} response to ionomycin, cell populations treated with 30 µM dantrolene were monitored. In the presence of 1 and 5 µM ionomycin, dantrolene significantly inhibited the initial rise in $[Ca^{2+}]_c$ (Fig. 3a), demonstrating that the peak response is partly due to CICR. In contrast, the plateau phase was not affected by dantrolene (Fig. 3b).

L-Type Voltage-Gated Ca^{2+} Channels are not Affected by Ionomycin

Since some types of astrocytes have been shown to express L-type voltage-gated Ca^{2+} channels (VGCCs), we used the channel blocker verapamil to assess a possible involvement of these channels in the ionomycin response. Cultured
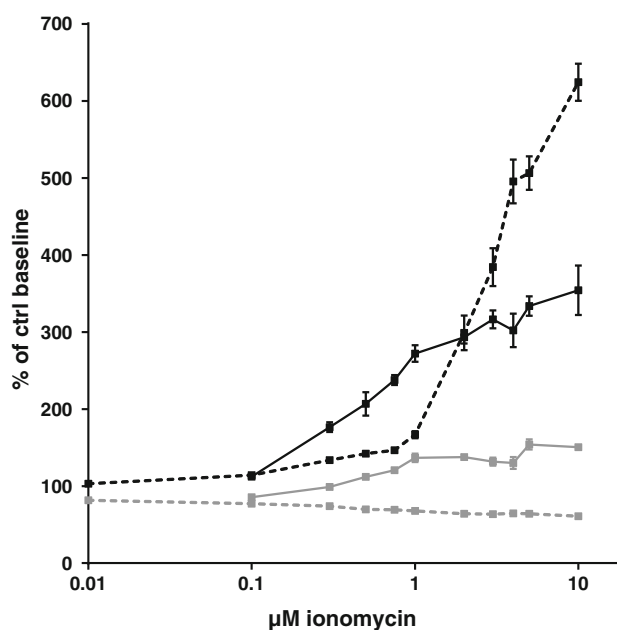

Fig. 2 Effects of ionomycin on cytosolic Ca^{2+} ($[Ca^{2+}]_c$) are dependent on concentration and exposure time. Fura-2 loaded cerebellar astrocytes were exposed to concentrations of ionomycin ranging from 0.01 to 10 µM and changes in 340/380 ratio were recorded over the course of 35 min and plotted as percent of baseline fluorescence ratio. The peak amplitude increased with concentration both in the presence (*black trace*) and absence (*grey trace*) of 1.8 mM extracellular Ca^{2+}, while a plateau phase (measured as amplitude after 35 min) was only evident with extracellular Ca^{2+} present (*black dotted trace*) and $[Ca^{2+}]_c$ declined directly after the peak in conditions with Ca^{2+}-free buffer (*grey dotted trace*). Data points represent mean ± SEM of astrocyte populations ($n = 8$–23)

astrocytes were incubated with 25 μM verapamil in the presence of extracellular Ca^{2+} and Ca^{2+} changes in cell populations were recorded. Blocking L-type VGCC did not have an effect on either peak or plateau phase of the Ca^{2+} signal (Fig. 4). This data suggests that VGCCs do not contribute to the astrocytic Ca^{2+} response to ionomycin.

Inhibition of Store-Operated Ca^{2+}-Entry Markedly Reduces the Plateau Phase

2-APB is commonly employed as an inhibitor of store-operated Ca^{2+}-entry (SOCE), suppressing Ca^{2+} influx upon store depletion in a concentration and cell-type dependent manner. Consistent with protocols in earlier studies on pancreatic cell lines [14], we pre-incubated astrocytes with 100 μM 2-APB prior to application of 1 or 5 μM ionomycin. The fura-2 340/380 ratio was monitored on a single cell level. After 5 min of pre-incubation with 2-APB, the peak amplitude of the biphasic Ca^{2+} response to 1 μM ionomycin was unchanged compared to control. However, 2-APB reduced the Ca^{2+} peak evoked by 5 μM ionomycin and drastically reduced the plateau phase of the response to both 1 and 5 μM ionomycin (Fig. 5). This result strongly indicates that SOCE is a major component of the plateau phase while at high concentrations of ionomycin SOCE also seems to be involved in the initial peak response.

Discussion

While ionomycin was initially thought to increase cytosolic calcium via physicochemical translocation of ions across the plasma membrane, there have been a number of publications indicating that it acts by directly modulating cellular calcium signaling pathways.

The present study demonstrates that astrocytes respond to ionomycin with a bi-phasic increase in $[Ca^{2+}]_c$, consisting of an initial peak and a subsequent sustained plateau. The temporal outline of the $[Ca^{2+}]_c$ response to ionomycin in cultured astrocytes strongly suggests that the underlying mechanism of action involves intrinsic Ca^{2+}
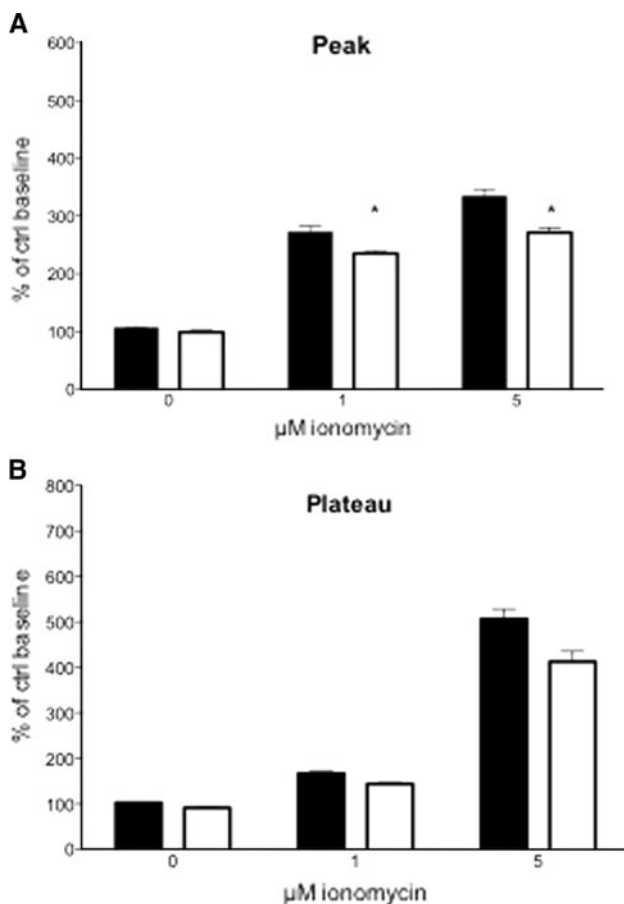
Fig. 3 The peak Ca^{2+} response to ionomycin is due in part to Ca^{2+}-induced Ca^{2+}-release (CICR). Changes in fura-2 340/380 ratio in populations of astrocytes treated with 1 and 5 μM ionomycin in the presence (*white bars*) or absence (*black bars*) of 30 μM dantrolene are plotted as percent of baseline fluorescence ratio. The plateau represents the amplitude after 35 min incubation. Each bar represents mean $\pm$ SEM of astrocyte populations ($n = 8$–23). *$p < 0.05$
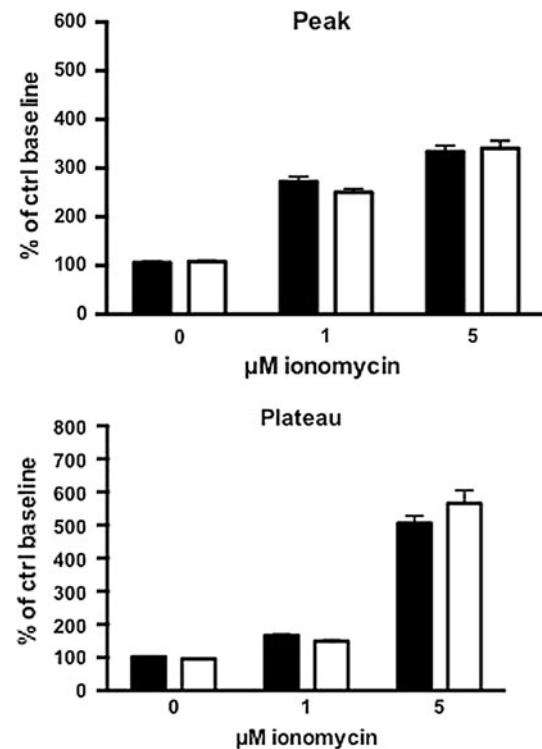
Fig. 4 The astrocytic ionomycin response is independent of VGCC. Fura-2 340/380 ratios in populations of astrocytes incubated with 1 and 5 μM ionomycin and 25 μM verapamil (*white bars*). Bars show mean $\pm$ SEM in fluorescence ratio plotted as percent of baseline fluorescence ratio. The plateau represents the amplitude after 35 min incubation. $n = 8$–23 populations
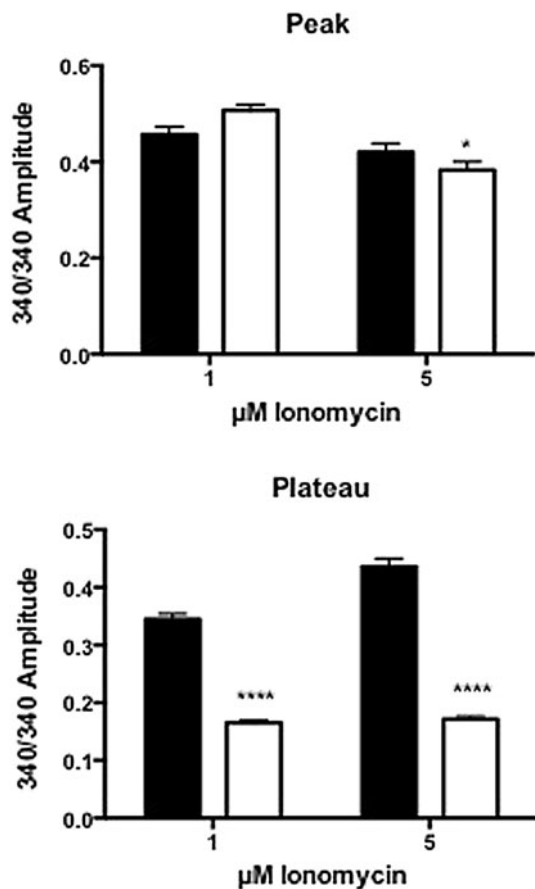
Peak

Plateau

Fig. 5 Store-operated Ca^{2+}-entry (SOCE) is responsible for the plateau phase. SOCE was blocked in astrocytes treated with 1 or 5 μM ionomycin by 5 min of pre-incubation with 100 μM 2-APB (*white bars*). Changes in fura-2 340/380 fluorescence ratio were monitored on the single cell level. Plateau represents the amplitude 3 min after peak. Bars represent mean amplitude normalized to baseline fluorescence ratio ± SEM ($n > 200$). *$p < 0.05$; ****$p < 0.0001$

modulating pathways. Our data indicates that the initial peak is partly due to depletion of intracellular stores while the plateau is caused by subsequent Ca^{2+} influx via SOCE. Thus, it seems likely that the primary site of action for ionomycin in astrocytes is not the plasma membrane, but the ER, where it seems to trigger Ca^{2+} release into the cytosol. The initial cytosolic Ca^{2+} increase causes CICR, which can be inhibited with dantrolene, and further depletes internal stores. This emptying of Ca^{2+} stores subsequently triggers SOCE, which is the cause of the plateau phase of the ionomycin Ca^{2+} signal.

Our results complement earlier work showing that ionomycin affects intracellular Ca^{2+} stores [5, 6]. Consistent with our data these previous studies show that internal stores are the source of the Ca^{2+} peak, while influx of Ca^{2+} across the plasma membrane is responsible for the plateau phase.

The concept of SOCE was first developed in the 1980s [15] with evidence for the idea accumulating in the

following years [16–18]. However, while SOCE is far from a novel concept, the molecular machinery facilitating this mode of Ca^{2+} entry has only been discovered in recent years. In 2005, stromal interacting molecule 1 (STIM 1) was identified as a Ca^{2+} sensor inside the ER [19–21]. In the following year, three different groups almost simultaneously discovered Orai1 as the pore forming subunit of the channel conducting the SOCE Ca^{2+} current [22–24]. Once the proteins involved had been identified, further studies elucidated the two-fold mechanism underlying SOCE: upon efflux of Ca^{2+} from the ER, Ca^{2+} dissociates from the EF-hand domain of STIM1, triggering an aggregation of STIM1 molecules at sites close to the plasma membrane, forming discrete puncta [25]. Subsequently, Orai1 accumulates in clusters at corresponding sites of the plasma membrane [26]. An ever increasing amount of studies has demonstrated direct interaction of STIM1 and Orai1 following the formation of puncta and clusters, suggesting that a direct activation of Orai1 by STIM1 leads to opening of the channel pore and subsequent Ca^{2+} entry [27–31]. Additionally, there have been reports indicating a possible involvement of transient receptor potential (TRP) channels in SOCE and the interaction between STIM1 and Orai1 [32].

The identification of the proteins involved in SOCE led to the characterization of new pharmacological tools, amongst them 2-APB, which has been demonstrated to block SOCE by inhibiting STIM1 translocation as well as puncta formation [14, 33]. Consistent with these studies, pre-treatment of astrocytes with 2-APB inhibited SOCE in our experiments and almost completely abolished the plateau phase of the ionomycin response.

Thus, we can conclude that ionomycin acts directly on the internal Ca^{2+} stores in astrocytes, causing an increase in cytosolic Ca^{2+}, which in turn triggers further release of Ca^{2+} via CICR. The depletion of internal Ca^{2+} stores subsequently causes SOCE. This mechanism of action should be kept in mind when routinely using ionomycin as a pharmacological tool.

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