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The mechanism of Ba^{2+}-induced exocytosis from single chromaffin cells

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Dialysis of Ba^{2+} into voltage-clamped single bovine chromaffin cells produced a concentration-dependent increase in cell capacitance, reflecting an enhanced rate of exocytotic events. Between 0.1 and 1 mM, Ba^{2+} linearly increased both the rate and the total amount of exocytosis. In unclamped cells also, extracellular Ba^{2+} induced the release of catecholamines, as assayed with a carbon-fibre electrode in the amperometric mode. Additionally, extracellular application of Ba^{2+} increased the apparent internal Ca^{2+} concentration ($[Ca^{2+}]_{app}$) in fura-2-loaded chromaffin cells. These observations were made both in the presence and absence of external Ca^{2+} (Ca_o^{2+}), as well as after depletion of the intracellular Ca^{2+} stores with ionomycin. Under current-clamp conditions, Ba^{2+} induced pronounced depolarization of the cells. These results are compatible with the following conclusions: by blocking K^+ channels, Ba^{2+} causes depolarization of chromaffin cells. This results in opening of voltage-gated Ca^{2+} channels and Ba^{2+} entry into the cytosol. Ba^{2+} then directly triggers exocytotic events, although it induces exocytosis only at concentrations more than a 100-fold higher than Ca^{2+}. Various effects contribute to the generally observed greater secretory responses with Ba^{2+} as compared with Ca^{2+}; these are the depolarizing effects of extracellular Ba^{2+}, its greater entry through non-inactivating Ca^{2+} channels and its poor intracellular buffering largely arising from its weak affinity for plasmalemmal Ca^{2+} extrusion mechanisms. In some cases, Ba^{2+} additionally induces release of Ca^{2+} from internal stores, as evidenced by its effect on fura-2 fluorescence at different wavelengths.

Barium; Exocytosis; Secretion; Calcium release; Amperometry; Chromaffin cell

1. INTRODUCTION

Ba^{2+} mimics the effect of Ca^{2+} in the regulation of various Ca^{2+}-dependent processes. In fact, a well-established criterion when trying to characterize a Ca^{2+}-regulated step is to determine whether Ba^{2+} can maintain such an event in the absence of Ca^{2+}. However, Ba^{2+} differs from Ca^{2+} in many respects both at plasmalemmal and intracellular sites. For instance, Ba^{2+} generally permeates high voltage-activated (HVA) Ca^{2+}-channels better than Ca^{2+} [1,2]; these channels are inactivated by Ca^{2+} but not by Ba^{2+} [3]. On the other hand, Ca^{2+} activates a class of K^+ channels of large [4] or small [5] conductance, while Ba^{2+} is a high affinity blocker of numerous K^+ channels [6–8]. Finally, Ba^{2+} exhibits a poor affinity for the Ca^{2+} pump [9,10] and also for intracellular Ba^{2+} buffering systems [11].

In exocytosis, Ba^{2+} substitutes for Ca^{2+} in maintaining the release of catecholamines in response to K^+ depolarization [12]; however, the time–course of activation and inactivation is much slower with Ba^{2+} than with Ca^{2+} [13,14]. In addition, Ba^{2+} itself (in contrast to Ca^{2+}) can trigger a drastic secretory response in the absence of imposed cell depolarization [12,15,16]. In trying to elucidate the mechanism of activation of secretion by Ba^{2+}, conflicting reports have recently appeared. TerBush and Holz [17] conclude that Ca^{2+} and Ba^{2+} stimulate secretion from digitonin-permeabilized chromaffin cells through similar pathways. In contrast, Heldman et al. [18] conclude that Ba^{2+} acts by a mechanism independent of Ca^{2+}. Furthermore, it has been proposed for other cell systems that Ba^{2+} simply triggers exocytosis by releasing Ca^{2+} from internal stores [19]. In trying to understand the mechanism of action of Ba^{2+} on secretion we have measured the release of catecholamines from single bovine chromaffin cells. The secretory signal in response to intracellular or extracellular application of Ba^{2+} was correlated with changes in fura-2 fluorescence and the membrane potential.

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2. MATERIALS AND METHODS

Bovine adrenal chromaffin cells were isolated as described by Moro et al. [20]. They were plated on polylysine-treated coverslips suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 50 μg/ml streptomycin and 50 IU/ml penicillin, and kept in an incubator under a water-saturated atmosphere of 5% CO_2/95% air at 37°C. 1–5-day-old cells were used.

Electrophysiological experiments were carried out using either the voltage- or current-clamp mode of the patch-clamp technique [21]. Fluorescence measurements were carried out using fura-2 as an indicator both for Ca^{2+} and Ba^{2+} [22]. Catecholamine release was measured in two ways: (i) by monitoring membrane capacitance as described by Lindau and Neher [23]; and (ii) through electrochemical amperometric detection using carbon fiber electrodes, as described previously [24,25].

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The extracellular solution contained in mM: 140 NaCl; 2.8 KCl; 2 $CaCl_2$; 2 $MgCl_2$; 50 glucose; pH 7.2 and the pipette solution: 145 caesium glutamate; 8 NaCl; 1 $MgCl_2$; 2 Mg-ATP; 0.3 GTP; 10 Na-HEPES; 0.1 fura-2. Experiments were performed at room temperature (around 25°C).

3. RESULTS

3.1. *Changes in capacitance induced by intracellular Ba^{2+} dialysis*

Bovine chromaffin cells were clamped at a holding potential of −60 mV and capacitance was monitored as different concentrations of Ba^{2+} were dialysed into the cell through the patch pipette. At 0.1 mM Ba^{2+}, a total capacitance increase of 0.73 ± 0.13 pF was seen in 5 cells (mean ± S.E.M.). This response increased with $[Ba^{2+}]$ to 5.5 ± 1.5 pF at a concentration of 1 mM Ba^{2+} (n = 5; Fig. 1a). When analyzing the slopes of capacitance increase (Fig. 1b) we observed that the initial rates of increase at 0.1 and 0.3 mM Ba^{2+} were 0.9 ± 0.3 and 3.7 ± 1.3 pF/s, respectively; 3 min later these rates increased to values of 1.8 ± 0.8 and 7.4 ± 4.8 pF/s, respectively. This pattern was inverted, however, when the cells were dialyzed with 1 mM Ba^{2+}; the initial slope was

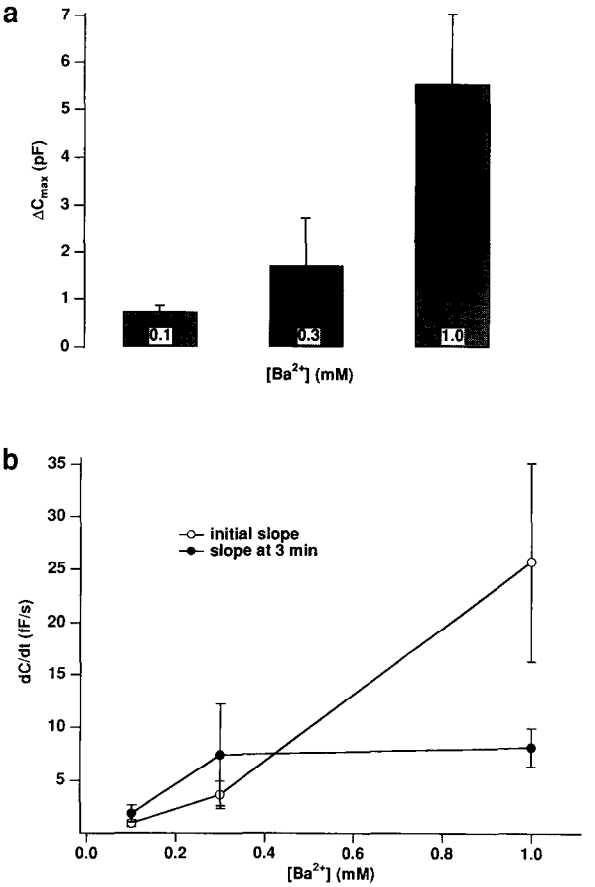

Fig. 1. (a) Maximum capacitance response after dialysis of 0.1, 0.3 and 1 mM Ba^{2+} through the patch pipette. Cells were held at a constant holding potential of −60 mV. (b) Slopes of capacitance increase initially and after 3 min of three different Ba^{2+} concentrations. Each point corresponds to the mean ± S.E.M. (n = 5).

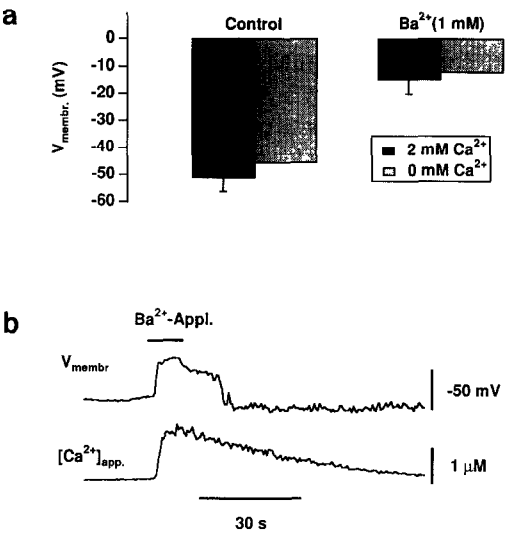

Fig. 2. (a) Changes in membrane potential recorded from cells under current-clamp conditions after external application of 1 mM Ba^{2+}. Results correspond to the mean ± S.E.M. of 7 different cells (2 mM Ca^{2+}) or the mean of two cells (0 Ca^{2+}). (b) An example of the depolarization (V_{membr}) and the change in $[Ca^{2+}]_{app}$ induced by the application of 1 mM Ba^{2+}. Exocytosis also occurred during the Ba^{2+} transient; the capacitance after the transient was increased by 620 fF as compared to the value before.

25.7 ± 9.4 pF/s but 3 min later it had decreased to 8.1 ± 1.9 pF/s.

3.2. *Effects of external Ba^{2+} on membrane potential*

Experiments under current-clamp conditions showed that external application of 1 mM Ba^{2+} (in the presence of external Ca^{2+}) depolarized the cells from −51.5 ± 4.8 mV (resting potential) to −15.3 ± 5.0 mV (n = 7 cells; Fig. 2a). This change in membrane potential was accompanied by an increase in the apparent calcium concentration ($[Ca^{2+}]_{app}$), although with a delay of a few seconds. In most cases, the depolarization induced by Ba^{2+} recovered almost completely when the application of this cation ended. In the absence of external Ca^{2+}, the changes in both membrane potential and $[Ca^{2+}]_{app}$ induced by Ba^{2+} occurred more rapidly. This faster depolarization might simply be due to the frequently observed voltage shift in channel activation exerted by different external Ca^{2+} concentrations. In a 0 Ca^{2+} solution, relatively more Na^+ and Ca^{2+} channels would open to progressively depolarize the cell. Nevertheless, the absolute values of total depolarization induced by external Ba^{2+} were not statistically different from those obtained in the presence of Ca^{2+}; thus the membrane potential went from −46.0 to −12.4 mV. Fig. 2b gives an example of such a record.

3.3. *Effects of extracellular Ba^{2+} on secretion*

Electrochemical detection of catecholamine release from single cells was achieved using carbon fiber electrodes located less than 1 μm from the cell surface and

held at a constant voltage of 800 mV. External application of 1 mM Ba^{2+} to chromaffin cells bathed in Ringer solution containing 2 mM Ca^{2+} induced the prompt appearance of secretory events with a high frequency (Fig. 3a); secretion was accompanied by a parallel increase in the $[Ca^{2+}]_{app}$. At a higher time resolution, individual release events similar to those previously described [24,25] could be observed (data not shown). The same findings were made when the cells were bathed in the absence of external Ca^{2+} (Fig. 3b); again, the secretory events were accompanied by parallel increase in $[Ca^{2+}]_{app}$. When secretion ceased in these cells, a second application of Ba^{2+} re-initiated the appearance of secretory spikes. To test whether Ba^{2+} itself was inducing secretion by acting directly on the secretory machinery, or through the release of Ca^{2+} from internal stores, cells were first treated with ionomycin (2 μg/ml) in the absence of external Ca^{2+} (Fig. 3c). The ionophore was applied twice; upon the first application there was a transient increase in $[Ca^{2+}]_i$; however, the second ionophore application gave no further Ca^{2+} increase, suggesting that a complete depletion of internal stores had been achieved. Under these conditions, cells exposed to 1 mM Ba^{2+} gave secretory responses persisting for several minutes after the removal of external Ba^{2+}. At the same time a pronounced increase in $[Ca^{2+}]_{app}$ was observed upon Ba^{2+} addition. Since Ca^{2+} stores had been depleted using ionomycin, this suggests that Ba^{2+} was entering the cell and activating the exocytotic machinery independent of internal Ca^{2+} release. Interestingly, in the cells which had been loaded with the membrane-permeable fura-2 AM, $[Ca^{2+}]_{app}$ remained elevated a long time after the removal of external Ba^{2+}, decaying with a time constant of 446 ± 3.6 s (n = 3). This indicates that extrusion of Ba^{2+} from the cell interior was much slower than that of Ca^{2+}, probably because the cell is not endowed with a specific mechanism to extrude or to buffer Ba^{2+}. On the other hand, Ba^{2+} does not prevent extrusion of Ca^{2+} from the cells. When external Ca^{2+} was allowed to enter the cytosol through Ca^{2+} channels in cells which were internally dialyzed with Ba^{2+}, the $[Ca^{2+}]_i$ decayed with a τ of 3.4 ± 0.4 s (n = 9), which is much faster than the time–constant of exchange with the patch pipette in the same cells (τ = 95 ± 29 s; n = 4). Since any Ca^{2+} released from internal stores should be captured with the same time–course, this is further evidence that Ba^{2+} is indeed capable of inducing secretion on its own. The fact that in intact cells Ba^{2+} is extruded so slowly may thus explain why secretion proceeds long after the removal of external Ba^{2+} (Fig. 3b,c).

3.4. *Ba^{2+}-induced release of Ca^{2+} from internal stores*

Even though Ba^{2+} can trigger exocytosis on its own, it might additionally do so by releasing Ca^{2+} from internal stores. Ba^{2+} bound to fura-2 shifts the isosbestic point of this complex relative to Ca^{2+}-bound fura-2 [22], which we were able to follow. When Ca^{2+} and Ba^{2+} influx is activated in sequence, Ca^{2+} influx through voltage-dependent Ca^{2+} channels leads only to a decrease in the fluorescence at 390 nm (Fig. 4a; 1), while Ba^{2+} application concomitantly provokes an increase at 360 nm (Fig. 4a; 2). A plot of the fluorescence at 390 nm vs. that at 360 nm (Fig. 4b) reveals that the main part of the signal is due to Ba^{2+} rather than Ca^{2+} binding to fura-2. The theoretical fluorescence response of a given ion species is shown as a dotted line. For Ca^{2+} this is expected to be a straight line parallel to the y-axis. The theoretical relative fluorescences at different Ba^{2+} concentrations have been derived from spectra in Murray and Kotlikoff [22] and are shown as open circles. Since there is some deviation of the response to Ba^{2+} application from the theoretical line, a small part of the signal might in fact be due to release of Ca^{2+} from internal stores.

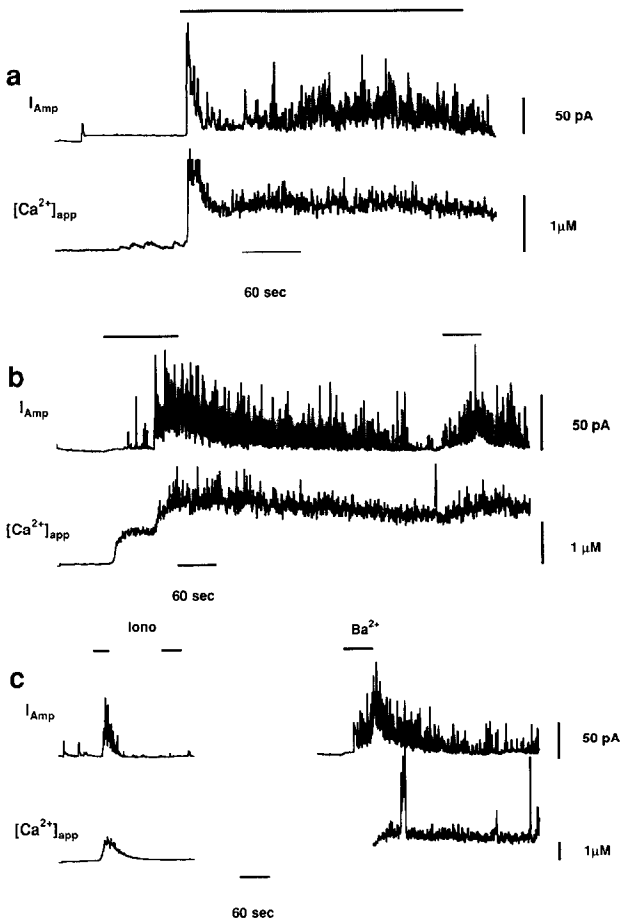

Fig. 3. Secretory events recorded from single adrenal chromaffin cells with a carbon fiber electrode in the amperometric mode (I_{Amp}) and simultaneously measured changes in the apparent Ca^{2+} concentration ($[Ca^{2+}]_{app}$) in cells bathed in 2 mM Ca^{2+}–Ringer (a), Ca^{2+}-free Ringer (b) or in 0 Ca_o^{2+} after pretreatment with 2 μg/ml ionomycin (c). Application bars indicate the time of Ba^{2+} (1 mM) application except for c where they indicate either ionomycin (Iono.) or Ba^{2+} (Ba^{2+}) application. In c the time during which the measurement was not valid has been blanked out.

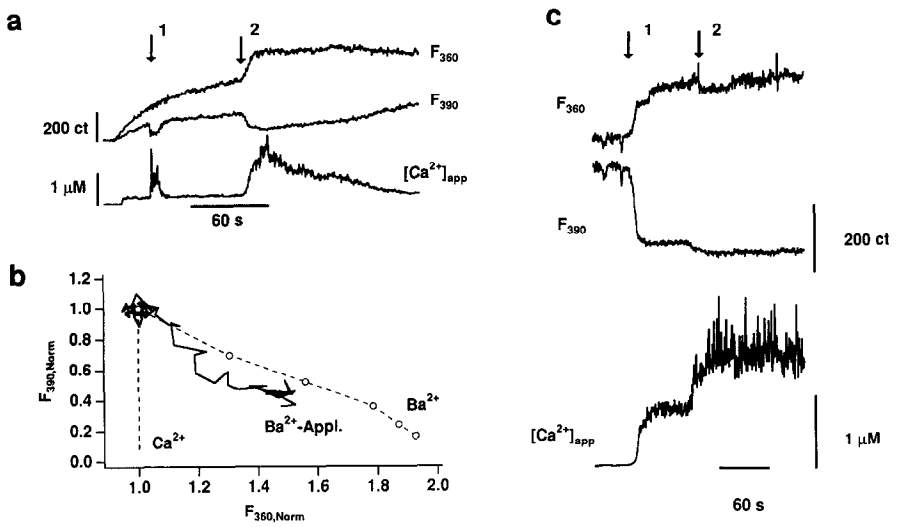

Fig. 4. Ba^{2+}-evoked release of Ca^{2+} from internal stores. In (a) the relative changes in the fluorescence at 360 nm (F360) and 390 nm (F390), as well as the calculated Ca^{2+} concentration ($[Ca^{2+}]$), are shown both for depolarization-induced Ca^{2+} influx (1; voltage clamp) and Ba^{2+} influx (2; current clamp). (b) The fluorescence at 390 nm plotted against the fluorescence at 360 nm for the second transient from Fig. 4a (solid line), as well as theoretical expectations derived from spectra given in [22] (dotted lines). (c) The occasionally observed biphasic increase in $[Ca^{2+}]_{app}$ following the application of Ba^{2+}. The first phase (1) is due to influx of Ba^{2+} and the subsequent one (2) to release of Ca^{2+} from internal stores.

In some cells two phases in the change of $[Ca^{2+}]_{app}$ can be observed (e.g. in Fig. 3b). When inspecting the fluorescence traces of this particular response in detail, it becomes apparent that one phase is accompanied by an increase at 360 nm (Fig. 4c, 1), indicating Ba^{2+} influx, while the other one is not (Fig. 4c, 1), suggesting the occurrence of Ca^{2+} release from internal stores. These two phases were found even if there was no external Ca^{2+} present (like in the presented example). The order of these two phases varied from cell to cell, usually causing secretory events of higher frequency with phase 2, regardless of whether that was due to Ca^{2+} release or to Ba^{2+} influx. This provides evidence that although Ba^{2+} is capable of evoking secretion on its own, it additionally can release Ca^{2+} from internal stores.

4. DISCUSSION

In single bovine chromaffin cells we have observed the following actions of Ba^{2+} ions: (i) intracellular dialysis of Ba^{2+} produced catecholamine release as reflected by an increase in the cell capacitance; (ii) extracellular application of Ba^{2+} also triggered secretion, independent of both external Ca^{2+} and depletion of intracellular Ca^{2+} stores; (iii) extracellular application of Ba^{2+} caused a drastic depolarization of the cells; the rate of depolarization was faster in the absence of Ca^{2+}; (iv) in addition to its action on secretion, Ba^{2+} occasionally induced release of Ca^{2+} from internal stores.

The differences in the kinetic behavior of secretion as induced by distinct internal Ba^{2+} concentrations might reflect its differential effects on several steps in the pathway of regulated exocytosis. Recently it has been shown that Ca^{2+} not only triggers in the final step of fusion between vesicular and plasma membrane but also an earlier step in exocytosis [26]. The fact that the total amount of exocytosis increases concomitantly with the concentration of intracellularly applied Ba^{2+} indicates that, in principle, this cation is capable of replacing Ca^{2+} at all steps of this pathway, especially since the total amount of capacitance increase is about the same as that evoked by high Ca^{2+} concentrations [27]. Although the maximal rate of exocytosis decreases with time at high Ba^{2+} concentrations, as is the case for Ca^{2+}, the secretory rates, however, show a significant delay in reaching their maximal rates at low concentrations. This indicates that compared with Ca^{2+}, Ba^{2+} has a lower relative affinity for the actual fusion machinery than for the vesicle transport step.

A further question is whether Ba^{2+} acts on the same [18] or on a different target [17] when substituting for Ca^{2+} as the trigger of secretion. Although an action of Ba^{2+} at a target different to Ca^{2+} cannot be excluded, there is some evidence that they act on the same sites. When dialyzing single chromaffin cells with high (1 mM) concentrations of Ba^{2+}, additional depolarizations in the presence of Ca^{2+} evoked almost no further increases in capacitance; in contrast at low internal Ba^{2+} (100 μM), such depolarizations led to pronounced increases in both $[Ca^{2+}]_{app}$ and capacitance (data not shown). This might be taken as evidence that the effects of both cations are additive at low concentrations, but not at high concentrations when the secretory apparatus is already saturated.

It has been suggested previously that the mechanism of Ba^{2+}-induced secretion is mediated by release of Ca^{2+}

from internal stores [19]. We obtained evidence, however, that the most important component of the Ba^{2+} secretory response was a direct action on the secretory machinery. First, secretion due to Ba^{2+} is observed even in the absence of external Ca^{2+} and after depletion of the internal Ca^{2+} stores with ionomycin. Second, application of Ba^{2+} caused changes of the fura-2 fluorescence signals very close to the ones expected for a pure Ba^{2+} signal. Third, due to the low affinity of Ba^{2+} for Ca^{2+} pumps [9,10] it prevents its own extrusion but not that of Ca^{2+}. Therefore, any Ca^{2+} released from internal stores should be efficiently extruded even in the presence of Ba^{2+}. These findings do not rule out the possibility that the Ba^{2+} effect might be partially mediated by release of Ca^{2+} from internal stores, since such a release was actually observed. In summary, our results make previous findings seem less contradictory since Ba^{2+} can evoke secretion both directly through the activation of the secretory machinery, and indirectly, by releasing Ca^{2+} from internal stores. Furthermore they explain why Ba^{2+} is such a good secretagogue, even though it has 100-times less affinity than Ca^{2+} for the secretory machinery. In addition, the results suggest that any putative Ca^{2+}-binding protein supposedly involved in triggering exocytosis should display a sufficient Ba^{2+} sensitivity in order to be a good candidate for a Ca^{2+} sensor of the exocytotic machinery.

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