The Polarized Effect of Intracellular Calcium on the Renal Epithelial Sodium Channel Occurs as a Result of Subcellular Calcium Signaling Domains Maintained by Mitochondria*

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Background: Mitochondria can sequester calcium and regulate signaling processes in epithelia.
Results: Intracellular calcium regulates the renal epithelial sodium channel in a polarized fashion, and this polarity is dependent on mitochondrial calcium uptake.
Conclusion: Mitochondria maintain calcium polarity in renal epithelia.
Significance: Identifying subcellular calcium signaling domains in renal epithelium is essential to understanding ion channel regulation.

The renal epithelial sodium channel (ENaC) provides regulated sodium transport in the distal nephron. The effects of intracellular calcium ([Ca\textsuperscript{2+}]) on this channel are only beginning to be elucidated. It appears from previous studies that the [Ca\textsuperscript{2+}]i increases downstream of ATP administration may have a polarized effect on ENaC, where apical application of ATP and the subsequent [Ca\textsuperscript{2+}]i increase have an inhibitory effect on the channel, whereas basolateral ATP and [Ca\textsuperscript{2+}]i, have a stimulatory effect. We asked whether this polarized effect of ATP is, in fact, reflective of a polarized effect of increased [Ca\textsuperscript{2+}]i on ENaC and what underlying mechanism is responsible. We began by performing patch clamp experiments in which ENaC activity was measured during apical or basolateral application of ionomycin to increase [Ca\textsuperscript{2+}]i near the apical or basolateral membrane, respectively. We found that ENaC does indeed respond to increased [Ca\textsuperscript{2+}]i in a polarized fashion, with apical increases being inhibitory and basolateral increases stimulating channel activity. In other epithelial cell types, mitochondria sequester [Ca\textsuperscript{2+}]i, creating [Ca\textsuperscript{2+}]i signaling microdomains within the cell that are dependent on mitochondrial localization. We found that mitochondria localize in bands just beneath the apical and basolateral membranes in two different cortical collecting duct principal cell lines and in cortical collecting duct principal cells in mouse kidney tissue. We found that inhibiting mitochondrial [Ca\textsuperscript{2+}]i uptake destroyed the polarized response of ENaC to [Ca\textsuperscript{2+}]i Overall, our data suggest that ENaC is regulated by [Ca\textsuperscript{2+}]i in a polarized fashion and that this polarization is maintained by mitochondrial [Ca\textsuperscript{2+}]i sequestration.

The renal epithelial sodium channel (ENaC)2 is a known contributor to the development of salt-sensitive hypertension, particularly in African Americans (1–3). Its regulation, therefore, has been the subject of much research over recent years. The distal portion of the renal tubule functions to fine-tune sodium reabsorption to regulate plasma sodium concentration. This is done transcellularly by movement of sodium through ENaC channels on the apical surface of the cell, followed by basolateral movement through the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase. The epithelial cells in this segment must express different proteins on the apical versus basolateral surface of the cell to allow for regulated transcellular ion movement. Polarization of the single layer of epithelial cells lining the tubule is essential for the nephron to sense changes in plasma and tubular fluid composition and regulate ENaC and other membrane proteins appropriately (4–6). ENaC must, therefore, be regulated differently by hormones present in the blood versus the tubular fluid.

The role of intracellular calcium ([Ca\textsuperscript{2+}]) in ENaC regulation is beginning to emerge. P2Y2 receptors are G protein-coupled receptors located on the apical membrane in principal cells, where they function to inhibit ENaC by a Ca\textsuperscript{2+}-dependent mechanism (7, 8). [Ca\textsuperscript{2+}]i inhibition of ENaC is, in fact, a well-known phenomenon, and several publications by different investigators have proposed the following model. G\textsubscript{i}-coupled receptors activate phospholipase C, which causes release of Ca\textsuperscript{2+} through IP\textsubscript{3} receptors on the endoplasmic reticulum near the apical plasma membrane, where ENaC is expressed (7). Following an increase in [Ca\textsuperscript{2+}]i, PKC is activated (9), and, via a separate pathway, Ca\textsuperscript{2+} binds to calmodulin. ENaC is usually held in the membrane by phosphatidylinositol phosphates, particularly PIP\textsubscript{2} and phosphatidylinositol 1,4,5-trisphosphate (10, 11). A protein, myristoylated alanine-rich C kinase substrate (MARCKS), normally stabilizes PIP\textsubscript{2} to create an anchoring domain for ENaC (12). When MARCKS is bound by calmodulin or phosphorylated by PKC, this causes loss of MARCKS from the membrane, destabilization of PIP\textsubscript{2}, and endocytosis of ENaC. [Ca\textsuperscript{2+}]i can also activate the ubiquitin ligase Nedd4-2, causing ubiquitination and proteasomal degradation of ENaC (13).

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1 The abbreviations used are: ENaC, epithelial sodium channel; MARCKS, myristoylated alanine-rich C kinase substrate; MCU, mitochondrial calcium uniporter; PIP\textsubscript{2}, phosphatidylinositol 4,5-bisphosphate; IP\textsubscript{3}, inositol 1,4,5-trisphosphate; CCD, cortical collecting duct.
Although increases in $[\text{Ca}^{2+}]_i$ in the cytosol just beneath the apical plasma membrane most likely inhibit ENaC, research from our group and others suggests that increasing $[\text{Ca}^{2+}]_i$ in the cytosol very near the basolateral membrane of the cell may stimulate ENaC (14, 15). Basal P2X4 receptors stimulate ENaC in a Xenopus distal tubule cell line (14). P2X4 channels are known $\text{Ca}^{2+}$ channels, and chelating $[\text{Ca}^{2+}]_i$ with BAPTA (1,2 bis(o-aminophenoxy)ethane,N,N',N''-tetraacetic acid) decreased P2X4-induced ENaC stimulation, suggesting that increases in $[\text{Ca}^{2+}]_i$ are stimulating ENaC when they originate from the basal pole.

$[\text{Ca}^{2+}]_i$ spreading must somehow be prevented in epithelia of the distal nephron to observe a polarized effect of $[\text{Ca}^{2+}]_i$ on ENaC. In pancreatic acinar and airway epithelia, mitochondria can restrict $[\text{Ca}^{2+}]_i$ diffusion by sequestering $[\text{Ca}^{2+}]_i$ (16, 17). We tested the hypothesis that mitochondria in cortical collecting duct (CCD) function in a similar manner to create regions of high and low $[\text{Ca}^{2+}]_i$ within the cell ($\text{Ca}^{2+}$ pools) and that these pools allow the same second messenger to affect the same protein (i.e. ENaC) in opposing ways, depending on the origin (apical versus basal) of the signal. This allows the cell to respond differently to hormone signals that signal via $[\text{Ca}^{2+}]_i$, depending on whether the hormone is sensed in the serosal or luminal compartment.

**Materials and Methods**

**Cells**—Experiments used either the 2F3 clone of A6 Xenopus distal tubule cells or mouse mpkCCD cells from the cortical collecting duct. The type of cell used in each experiment was decided by ease of use. 2F3 cells are suited for electrophysiology because of their ability to remain at room temperature for extended periods of time, high expression of ENaC, and low expression of other channels. mpkCCD cells were used for microscopy experiments because they are easy to transfected and easily take up mitochondrial dyes. Cells were grown on permeable supports to confluency, and then cells were polarized, and tight junctions were fully developed. For 2F3 cells, medium was supplemented with aldosterone (1.5 μM) to increase ENaC activity. Ionomycin, when used, was dissolved in 0.05% ethanol. Although ethanol can stimulate ENaC, it has no effect at this concentration (18).

**Imaging**—To visualize mitochondria, cells were loaded with MitoTracker Red (Life Technologies) for 30 min before visualization. To visualize the ER, cells were loaded with ERTracker Blue (Life Technologies) for 10 min prior to imaging. To label the membrane, cells were transfected with PLCδ-1 PH-GFP (1.5 μg/well, Addgene) using the Xfect system (Clontech). pN1-Lck-GCaMP tagged with enhanced GFP was used to visualize changes in $[\text{Ca}^{2+}]_i$ near the plasma membrane of the cell (19). To detect changes in mitochondrial $\text{Ca}^{2+}$, cells were cold-loaded with Rhodamine-2/AM (Life Technologies) because cold loading has been shown previously to increase the specificity of the dye for mitochondrial versus cytosolic $\text{Ca}^{2+}$ (20). MpkCCD cells were incubated with Rhod2/AM for 1.5 h at 4 °C, followed by incubation in media without serum for 4 h at 37 °C. Cells were rinsed briefly prior to use. Cells were imaged using an Olympus FV-1000 confocal microscope. To resolve subcellular distribution of fluorescent markers, z-stacks were obtained using sequential optical slices starting at the basal membrane. All images were taken using the same parameter settings.

**Single-channel Patch Clamp**—Single-channel patch clamping was performed as described previously (14). Briefly, a microelectrode was filled with physiological buffer (96 mM NaCl, 0.8 mM CaCl$_2$, 0.8 mM MgCl$_2$, and 20 mM HEPES (pH 7.4)) and lowered to touch the apical or basolateral membrane of a single cell. Suction was applied gently so that the membrane remained intact and a >1 GΩ seal was formed. All current was recorded at a holding potential of 0 mV. ENaC was identified by its characteristic channel kinetics and current-voltage relationship. ENaC probability of opening was analyzed using ClampFit software. Empty patches (those with no apparent activity) comprised 30–50% of all patches and were excluded from the study.

**Data Analysis and Statistics**—To quantify the confocal microscopy experiments, ImageJ was used. The number of pixels in a given area was calculated before and after drug addition. Averages were compared using Student’s t-test with $p < 0.05$ considered significant.

**Results**

$[\text{Ca}^{2+}]_i$, Influences Renal ENaC in a Polarized Fashion—We sought to test the hypothesis that ENaC is affected differently by changes in $[\text{Ca}^{2+}]_i$, depending from which pole of the cell the signal originates. To do this, we performed single-channel patch-clamping as described previously (15), and cells were treated with either 5 μM apical ionomycin (Fig. 1A) or 15 μM basolateral ionomycin (Fig. 1B) to increase $[\text{Ca}^{2+}]_i$. After 1–3 min of apical application of ionomycin, we observed a significant decrease in channel activity measured as probability of opening. We saw that the decrease in ENaC activity was maintained through 4–6 min of ionomycin addition. In contrast, basolateral application of ionomycin increased ENaC activity, but only after 13–15 min. In other words, the effect of $[\text{Ca}^{2+}]_i$ on ENaC is dependent on the side of the cell in which the increase in $[\text{Ca}^{2+}]_i$ occurs. Note that the difference in time course and concentration here is likely due to limited diffusion of agents administered from the basal side of the cell (see “Discussion”). We hypothesize that this polarized effect is the result
of two different Ca\(^{2+}\)-mediated signaling pathways present in the same cell type. Beneath the apical surface of the cell, Ca\(^{2+}\) activates pathways that inhibit ENaC, but, beneath the basal surface, Ca\(^{2+}\) activates pathways that stimulate ENaC. Despite the relatively diffusibility of Ca\(^{2+}\), these two pools of Ca\(^{2+}\) must not interact for polarization to occur. We hypothesize that mitochondrial Ca\(^{2+}\) uptake prevents diffusion of [Ca\(^{2+}\)]\(_{i}\), within principal cells to allow for these two pathways to remain separated.

**Preventing Mitochondrial Ca\(^{2+}\) Transport Destroys the Polarized Effect of [Ca\(^{2+}\)]\(_{i}\) on ENaC**—We next tested whether the polarized effects of [Ca\(^{2+}\)]\(_{i}\) on ENaC function were dependent on mitochondrial Ca\(^{2+}\) uptake. Mitochondrial Ca\(^{2+}\) uptake is dependent on movement through the inner mitochondrial membrane, a process facilitated by the mitochondrial calcium uniporter (MCU) (21). Ru360 is a drug that inhibits the mitochondrial calcium uptake, apical ATP has a stimulatory effect on ENaC. ATP has been shown repeatedly to inhibit ENaC when applied apically (a process that is dependent on an increase in [Ca\(^{2+}\)]\(_{i}\)) (24). Fig. 2D shows that, in the absence of mitochondrial calcium uptake, apical ATP has a stimulatory effect on ENaC. Notice that, in each of the panels of Fig. 2, B–D, the shape of the response is the same. Although Ru360 alone has an inhibitory effect on ENaC that persists over time, adding any agent that increases [Ca\(^{2+}\)]\(_{i}\) on the apical or basolateral side of the cell has a stimulatory (or null effect that is likely Ru360 inhibition opposed by stimulation) effect on ENaC in the presence of Ru360. In these experiments, we apply Ru360 to the apical surface of the cell. The inability of mitochondria to take up Ca\(^{2+}\) would lead to an increase in regional [Ca\(^{2+}\)]\(_{i}\). We know from the data in Fig. 1 that this would inhibit ENaC.

**Mitochondria Form Bands in the Distal Nephron**—Mitochondrial bands in pancreatic acinar and airway epithelia have been observed by other groups to form barriers to prevent [Ca\(^{2+}\)]\(_{i}\) diffusion by sequestering [Ca\(^{2+}\)], and slowly releasing it (17, 18). We hypothesized that mitochondria may be localized in bands in the cortical collecting duct to allow for [Ca\(^{2+}\)]\(_{i}\) polarization and opposing effects on ENaC. We began testing this hypothesis by using MitoTracker Red to observe mitochondrial localization in cortical collecting duct cells. We used two live cortical collecting duct cell lines: A6 cells from Xenopus CCD and mpkCCD cells from mice. The z axis view of both mpkCCD cells (Fig. 3A) and A6 cells (Fig. 3B) demonstrates that, in fact, mitochondria localize in the cytosol very close to each membrane in the cortical collecting duct. To pinpoint the
Mitochondrial Regulation of ENaC

location of mitochondrial bands with respect to the plasma membrane (Fig. 3C), we used the fluorescent PIP₂ indicator PLC8-1 PH-GFP to label the apical and lateral membranes. We found that the apical mitochondrial bands are located just beneath the plasma membrane. Release of ER Ca²⁺ through IP₃ receptor channels is known to inhibit ENaC. We hypothesized that, for the Ca²⁺ exiting the ER through the IP₃ receptors to access ENaC and not be taken up by the mitochondria, sections of the ER would have to lie between the apical mitochondrial barrier and the apical plasma membrane. To observe ER localization in relation to mitochondria, we loaded mpkCCD cells with MitoTracker Red and ERTracker Blue to label the mitochondria and ER, respectively (Fig. 3D). We found that the ER does pass the mitochondrial barrier in spots in live cells. It is possible that this localization is necessary for G₉ receptor inhibition of ENaC because IP₃ receptor-mediated Ca²⁺ release would have to occur in the cytosol between the apical membrane, where ENaC is localized, and the mitochondrial band, where Ca²⁺ would be sequestered for the majority of the Ca²⁺ to access the channel. Fig. 3E shows transmission electron microscopy of a mouse cortical collecting duct principal cell. Note the localization of mitochondria, as indicated by black lines highlighting the bands. Location of ~900 mitochondria in more than 10 different cells is quantified in Fig. 3F.

[Ca²⁺], Does Not Spread in the Cortical Collecting Duct—In Fig. 3, we show that mitochondria in the CCD form bands underneath the apical and basal membranes. This morphology is similar to other epithelia, such as airway epithelia, in which mitochondrial bands slow Ca²⁺ movement across the cell. Because morphology does not necessarily imply function, we tested whether Ca²⁺ can move across live CCD cells from one membrane to the other (Fig. 4). To be able to clearly observe Ca²⁺ beneath the cell membrane at the apical and basal poles, we transfected cells with a membrane-tethered Ca²⁺ sensor, pN1-Lck-CaMP2 (19). This protein contains a membrane-tethering domain from Src and a GFP molecule that is fused to calmodulin so that an increase in [Ca²⁺], beneath the membrane enhances the fluorescence of the GFP molecule. In Fig. 4A, we applied ionomycin to the apical surface of the cell and found that [Ca²⁺], increased in the apical but not basal region of the cytosol. Conversely, Fig. 4B shows that basal ionomycin increases [Ca²⁺], in the basal but not apical portion of the cytosol, suggesting that [Ca²⁺], is compartmentalized in mpkCCD cells. Fig. 4C shows that, in the absence of mitochondrial Ca²⁺ uptake, [Ca²⁺], compartmentalization is lost and that apical application of ionomycin results in an increase in [Ca²⁺], in both the apical and basal portions of the cytosol.

Mitochondrial Bands Take Up Ca²⁺ following [Ca²⁺], Increases in CCD—To test whether mitochondria take up Ca²⁺ following an increase in [Ca²⁺], we cold-loaded mpkCCD cells with the dye Rhod2/AM, which has been shown to be specific for mitochondrial Ca²⁺ when cells are cold-loaded (20) (Fig. 5). After apical application of ionomycin, there was an increase in mitochondrial Ca²⁺ in the apical but not basal mitochondrial band. These data show that mitochondria in CCD cells do take up Ca²⁺ rapidly following an increase in [Ca²⁺], at the apical pole of the cell and also confirm that there is limited Ca²⁺ movement across the cell because there is no Ca²⁺ uptake by the basal band following an apical increase in [Ca²⁺],. Fig. 5B shows localization of Rhod2/AM in mitochondria, and Fig. 5C shows that Ru360 prevents Ca²⁺ uptake into mitochondria.

Discussion

The data shown here are the first to directly show that the effect of increasing [Ca²⁺], at the apical versus basolateral membrane produces a different effect on ENaC function in the kidney. Polarization of [Ca²⁺], implies that [Ca²⁺], is compartmentalized in renal epithelia and does not diffuse between compartments. We show that, in a principal cell line, apical application of the Ca²⁺ ionophore ionomycin inhibits ENaC activity (Fig. 1). The effects of increasing apical [Ca²⁺], on renal ENaC function have been well studied. The most accepted physiological mechanism by which Ca²⁺ inhibits ENaC occurs downstream of purinergic signaling via the P2Y2 receptor on the apical membrane (7). P2Y2 receptors signal via a G₉-coupled receptor pathway to induce Ca²⁺ release from ER stores. This release of Ca²⁺ can act via a variety of signaling pathways, including PKC activation, to inhibit ENaC (25). This is demonstrated physiologically by the fact that mice lacking PKC have overactive ENaC and salt-sensitive hypertension (9, 26). ENaC must be tethered to the apical membrane by PIP₂ or phosphati-dylinositol 1,4,5-trisphosphate. Although both PIPs and ENaC are rare, they are recruited together by MARCKS. Binding of calmodulin to MARCKS or phosphorylation by PKC leads to removal of MARCKS and, therefore, ENaC from the membrane. Ca²⁺ may also inhibit ENaC directly or activate the ubiquitin ligase Nedd4-2 to tag ENaC for proteasomal degradation (13, 27).
Interestingly, our data also show, for the first time, that ENaC is stimulated by basolaterally applied ionomycin (Fig. 1B). It is of note that stimulating ENaC requires a very high dose of ionomycin and takes much longer than apical ionomycin to cause an effect. This could imply that effects of basal $[\text{Ca}^{2+}]_i$ on ENaC are not physiologically relevant. It is likely that the delay in response and high dose of ionomycin are due to the larger volume of media on the basolateral side of the cells, small size of the pores in the polyester membranes used as a surface in our experiments, and invagination of the basal membrane which limit the diffusion of ionomycin so that a larger dose and more time are needed. Unfortunately determining whether this is the case would be difficult to test experimentally.

What is not clear from this work (or work published previously) are the signaling mechanisms dictating how basolateral increases in $[\text{Ca}^{2+}]_i$ on ENaC are not physiologically relevant. It is likely that the delay in response and high dose of ionomycin are due to the larger volume of media on the basolateral side of the cells, small size of the pores in the polyester membranes used as a surface in our experiments, and invagination of the basal membrane which limit the diffusion of ionomycin so that a larger dose and more time are needed. Unfortunately determining whether this is the case would be difficult to test experimentally.

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Using two cell culture lines derived from this kidney segment, we observe that, in a polarized, live cell culture system, mitochondria form distinct bands at the apical and basal poles of the cells (Fig. 3). Bands such as these have been identified previously in other epithelial cell types: pancreatic acinar cells and airway epithelia (16, 17). Interestingly, airway and renal epithelia are the primary sites of ENaC expression (29). Although the influence of airway mitochondrial bands on ENaC activity has not been addressed, it is interesting that all tissues in which banding has been observed rely heavily on ENaC for function. We also show that this banding occurs in vivo because transmission electron microscopy of mouse kidney shows banding in cortical collecting duct principal cells.

In this study, we observed the apical mitochondrial band to be situated just beneath the plasma membrane with portions of the ER jutting through the band. This particular localization is interesting in that it would allow the mitochondria to participate in $\text{Ca}^{2+}$ regulation near lipid raft domains. Indeed, ENaC is known to localize in such domains and proteins such as Nedd4–2 and MARCKS, known to regulate ENaC in these...
Mitochondrial Regulation of ENaC

FIGURE 5. Mitochondrial calcium before and after apical application of ionomycin. mpkCCD cells were stained with Rhod2/AM to visualize mitochondrial calcium. A, z stacks were obtained before (left panel) and after (right panel) apical treatment with 5 μM ionomycin. Apical changes in calcium were quantified before and after treatment. B, cells were co-stained with MitoTracker Green and Rhod2/AM. C, cells were stained with Rhod2/AM and treated with Ru360 for 20 min prior to apical addition of ionomycin. Apical changes in calcium were quantified before and after ionomycin treatment. n ≥ 3/group; *, p < 0.05 versus control.

domains are influenced by [Ca^{2+}], (12, 30). Future experiments should be aimed at determining whether disrupting mitochondrial Ca^{2+} uptake influences lipid raft Ca^{2+} signaling.

Using innovative Ca^{2+} sensors for [Ca^{2+}], beneath the membrane and mitochondrial Ca^{2+}, we show that [Ca^{2+}], is taken up by mitochondria following apical application of ionomycin and that [Ca^{2+}], stimulated by ionomycin does not spread throughout the cell (Fig. 4). To visualize mitochondrial Ca^{2+}, we used the dye Rhod2/AM (Fig. 5). This dye can sense [Ca^{2+}], as well but has a preference for mitochondrial Ca^{2+} when cold-loaded and seemed to localize in a band in our study (20). We were really only able to visualize the apical mitochondrial band using Rhod2/AM. This could be due to the low uptake of the dye and the faintness of the basal band compared with the apical one. For this reason, a limitation of our study is that we were unable to measure Ca^{2+} uptake by the basal mitochondrial band. We did see the Ca^{2+} in the apical mitochondrial band increase following apical ionomycin application, indicative of Ca^{2+} sequestration. To visualize [Ca^{2+}], we used the membrane-tethered [Ca^{2+}] sensor Lck-CaMP (19). This vector consists of an enhanced GFP molecule fused to calmodulin and the membrane domain of Src. When Ca^{2+} increases at the membrane, the conformation of calmodulin changes so that GFP is excited. This vector helped us to see [Ca^{2+}], just under the membrane and determine whether [Ca^{2+}], spread following ionomycin application. We found that mitochondria sequester [Ca^{2+}], following apical increases in [Ca^{2+}], and that, when [Ca^{2+}], is increased on one side of the cell, it does not migrate to the opposing pole.

Mitochondria transport Ca^{2+} across the inner mitochondrial membrane via the MCU (31). Ru360, a ruthenium compound used in this study to obliterate the polarized effects of [Ca^{2+}], on ENaC, inhibits MCU as well as a component of outer mitochondrial membrane Ca^{2+} uptake (22, 32). In the absence of mitochondrial Ca^{2+} uptake, a similar biphasic response of ENaC to [Ca^{2+}], was observed regardless of which pole ionomycin was added to (Fig. 2). These data suggest that mitochondrial Ca^{2+} uptake is required for the polarized response of ENaC to [Ca^{2+}].

It is interesting that mitochondrial Ca^{2+} uptake has been implicated in blood pressure regulation in a Chinese family that displays maternally inherited hypertension (33). The data presented here provide the groundwork for future investigations into the role of mitochondrial Ca^{2+} uptake in ENaC regulation in salt-sensitive hypertension.

Overall, we conclude that the renal epithelial sodium channel ENaC likely exists in microdomains of Ca^{2+} signaling and that these domains are maintained by belts of mitochondria within the renal epithelial cell. Because this is the first work suggesting the existence of mitochondrial barriers to [Ca^{2+}], movement in renal epithelia, much work remains to be done to determine what role these barriers play in the regulation of other proteins or systemic blood pressure.


References
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