Endoplasmic Reticulum Calcium Regulates Epidermal Barrier Response and Desmosomal Structure

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Ca^{2+} fluxes direct keratinocyte differentiation, cell-to-cell adhesion, migration, and epidermal barrier homeostasis. We previously showed that intracellular Ca^{2+} stores constitute a major portion of the calcium gradient especially in the stratum granulosum. Loss of the calcium gradient triggers epidermal barrier homeostatic responses. In this report, using unfixed ex vivo epidermis and human epidermal equivalents we show that endoplasmic reticulum (ER) Ca^{2+} is released in response to barrier perturbation, and that this release constitutes the major shift in epidermal Ca^{2+} seen after barrier perturbation. We find that ER Ca^{2+} release correlates with a transient increase in extracellular Ca^{2+}. Lastly, we show that ER calcium release resulting from barrier perturbation triggers transient desmosomal remodeling, seen as an increase in extracellular space and a loss of the desmosomal intercellular midline. Topical application of thapsigargin, which inhibits the ER Ca^{2+} ATPase activity without compromising barrier integrity, also leads to desmosomal remodeling and loss of the midline structure. These experiments establish the ER Ca^{2+} store as a master regulator of the Ca^{2+} gradient response to epidermal barrier perturbation, and suggest that ER Ca^{2+} homeostasis also modulates normal desmosomal reorganization, both at rest and after acute barrier perturbation.

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INTRODUCTION

A competent epidermal barrier is required for terrestrial survival. A variety of components such as secreted and processed lipid, adherens or tight junctions, and desmosomes contribute to epidermal barriers to water loss, shear forces, and external toxins or microbes. These components must reorganize in response to both normal epidermal differentiation and epidermal perturbation such as barrier disruption or wounding. Ca^{2+} controls several of the processes that keep the epidermis in equilibrium. The epidermal Ca^{2+} gradient controls barrier recovery by stimulating lipid release after barrier perturbation. Ca^{2+} fluxes also control keratinocyte migration during wound healing (Trollinger et al., 2002). On the cellular level, Ca^{2+} controls desmosome formation and remodeling, via endoplasmic reticulum (ER) Ca^{2+} release and protein kinase C-alpha activation (Hobbs et al., 2011; Thomason et al., 2012; Wallis et al., 2000). Desmosome formation requires Ca^{2+}. However, contrary to other cell-to-cell junctions such as adherens junctions, fully formed desmosomes adopt a stronger, calcium-independent hyperadhesive state, which is characterized functionally by resistance to disruption by EGTA calcium chelation, and morphologically by the presence of a dense intercellular midline at the electron microscopy level (Garrod et al., 2005). Desmosomes in the hyperadhesive state retain plasticity (Tariq et al., 2015) and have been shown to respond to wounding by reverting to a non-hyperadhesive, calcium-dependent state through a protein kinase C-alpha-dependent process (Garrod et al., 2005) to allow cell migration and re-epithelialization of the wound. Hobbs et al. (2011) demonstrated that ER calcium release causes changes in desmosomal assembly and intercellular adhesive strength of fully formed desmosomes in cultured human keratinocytes.

Although Ca^{2+} fluxes regulate crucial processes in epidermal differentiation and barrier homeostasis (Baek et al., 2013; Elias et al., 2002a, 2002b; Yuspa et al., 1989), the signaling pathways by which the Ca^{2+} gradient responds to barrier perturbation is not well understood. In normal human and murine epidermis, Ca^{2+} levels are low in the basal layer, increase to a maximum in the stratum granulosum (SG), and drop to a minimum in the stratum corneum (SC) (Menon and Elias, 1991; Menon et al., 1992). This Ca^{2+} gradient depends on a competent epidermal barrier (Mauro et al., 1998; Menon and Elias, 1991; Menon et al., 1994), which in turn is formed by keratinocyte Ca^{2+} uptake, release, and influx (Feingold and Denda, 2012; Hu et al., 2000; Man et al., 1997). Studies from our group (Celli et al., 2010) and others (Adams et al., 2012; Cornelissen et al., 2007; Denda et al., 2012) have identified intracellular, rather than extracellular, calcium stores as the main components of the epidermal calcium gradient, and shown that ER Ca^{2+} release alone can control barrier repair processes (Celli et al., 2011).

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Abbreviations: CFP, cyan fluorescent protein; ER, endoplasmic reticulum; FLIM, fluorescence lifetime imaging; FRET, Förster resonance energy transfer; HEE, human epidermal equivalent; SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; TG, thapsigargin; YFP, yellow fluorescent protein

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Although we previously have shown that the Ca^{2+} gradient in unperturbed epidermis largely is formed by organelle (i.e., ER) stores (Celli et al., 2010), how ER Ca^{2+} responds to barrier perturbation to shape changes in the Ca^{2+} gradient has not yet been defined. In this report, we show that ER Ca^{2+} is released in response to barrier perturbation, resulting in a transient increase in tissue [Ca^{2+}], followed by later depletion. The time course of this transient increase has not been detected or defined by previous studies. Further, we find that barrier perturbation causes widening of the intercellular spaces and desmosome remodeling in the SG and stratum spinosum (SS), similar to that seen in tissue wounding (Garrod et al., 2005), likely allowing Ca^{2+} to freely diffuse out of the epidermis, and allowing transitional cells to move upward in the epidermis during barrier repair. Pharmacologic ER Ca^{2+} release with topical thapsigargin (TG) reproduces desmosome remodeling, even though the epidermal barrier is not perturbed. These data demonstrate that regulated ER Ca^{2+} release controls the epidermal Ca^{2+} gradient and desmosomal reorganization after barrier perturbation.

RESULTS

Calcium levels are transiently elevated after barrier perturbation in human skin

We first used human skin explants stained with calcium green 5N to monitor total free calcium fluxes after barrier perturbation using the fluorescence lifetime imaging (FLIM) technique previously described in Celli et al. (2010). This technique allowed us to image the full depth of the unsectioned tissue within the first 10 minutes after barrier abrogation. To control for changes due to simple mechanical stress with tape stripping, we also perturbed the barrier by acetone lipid extraction. Morphology of control and perturbed epidermal strata and false color images representing their respective calcium concentrations as measured by phasor-FLIM analysis are shown in Supplementary Figure S1 online. Because the epidermal calcium concentration exceeds the sensitivity of our method, a mean calcium concentration value cannot be calculated from our images. Therefore, to quantify and compare calcium levels from separate experiments, we calculated calcium concentration distribution histograms per each image and the calcium concentration corresponding to the peak of the distribution. Figure 1a and b represent the averaged position of the calcium concentration peaks in each epidermal layer. We observed a dramatic shift of the calcium concentration distribution toward higher calcium levels immediately after barrier perturbation in all epidermal layers (Figure 1a and b, respectively, and Supplementary Figure S1) in agreement with data previously reported in mice (Behne et al., 2011). To monitor calcium fluxes during barrier recovery, we imaged tape-stripped and acetone-treated samples 4 and 24 hours after perturbation. Four hours after perturbation, calcium levels were lower than baseline in all viable layers of tape-stripped samples in agreement with previous reports (Man et al., 1997; Menon et al., 1994), and in the SG of acetone-treated samples. Elevated calcium levels in the SS and stratum basale of acetone-treated samples (Figure 1b and Supplementary Figure S1) persist 4 hours after perturbation, likely as a consequence of persistent acetone exposure. Within 10–24 hours after barrier perturbation, calcium levels in the viable epidermal layers returned to baseline values, whereas calcium levels in the SC of the acetone-treated samples remained slightly elevated. Localization of epidermal calcium pools before and after perturbation is shown in Supplementary Figure S1.

Morphological analysis of FLIM images showed that immediately after barrier perturbation, calcium levels are elevated both intracellularly and extracellularly in the viable epidermis (Figure 2a and Supplementary Figure S1). Moreover, we noticed that, although extracellular domains are well below the optical resolution of the microscope in the granular layer of intact epidermis, they become apparent immediately after barrier perturbation (Figure 2a) and display total calcium levels that are higher than in unperturbed skin, suggesting large calcium fluxes through the extracellular space. Analysis of low-magnification (×4,800) micrographs revealed extended wider gaps between cells in the SG and SS after barrier perturbation by either tape stripping or acetone (Figure 2b). Interestingly, whereas extracellular spaces present wider gaps after barrier perturbation, desmosomes maintain a tightly closed conformation as shown in Figure 3c and discussed below. We used ×4,800 and ×10,000 electron microscopy micrographs to semiquantify changes in cell-to-cell distances, and found that barrier perturbation results in statistically significant widening of intercellular spaces (Table 1).

ER calcium is released after barrier perturbation

To test the hypothesis that increased intracellular calcium was due to release from intracellular stores, we used fully differentiated human epidermal equivalents (HEEs), which can be transfected with organelle-targeted calcium indicators. Our HEEs display a calcium concentration profile as measured by FLIM (Figure 3a and Sun et al., 2015) and ion-capture cytochemistry (Petrova et al., 2014), and barrier recovery after tape stripping (Sun et al., 2015) similar to that of human and murine epidermis.

We then transfected HEEs with the cameleon-based ER targeted calcium sensor D1ER (Palmer et al., 2006) to monitor the ER calcium profile at baseline and after perturbation. In the presence of calcium, Förster resonance energy transfer (FRET) between the cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) moieties of D1ER occurs, and the ratio between the YFP and CFP intensities ($R = I_{(YFP)}/I_{(CFP)}$) increases. In fully differentiated and unperturbed HEEs, the ratio (R) was significantly higher in the SG cells than in the SS and stratum basale cells, indicating higher calcium sequestration in the ER in the upper epidermal layers (Figure 3b). Because ER calcium release leads to lipid secretion and physiological apoptosis in SG keratinocytes (Celli et al., 2012), we specifically monitored the ER calcium levels in SG cells in perturbed and unperturbed HEEs over a course of 40 minutes (Figure 3c). Although unperturbed HEEs displayed constant ER calcium levels in the SG over this time interval, we observed a 20% decrease in the FRET ratio in the SG immediately after tape stripping, demonstrating that calcium is, in fact, released from the ER into the cytosol of granular cells after barrier perturbation. The average FRET ratio then increased until plateauing above the baseline, indicating active store refill. In a separate experiment, we