Activity of the hSPCA1 Golgi Ca^{2+} pump is essential for Ca^{2+}-mediated Ca^{2+} response and cell viability in Darier disease

Lucie Foggia[1], Ida Aronchik[2], Karin Aberg[2], Barbara Brown[2], Alain Hovnanian[1,3,*] and Theodora M. Mauro[2,4*]

[1]INSERM U563, Purpan Hospital, Place du Dr Baylac, BP 2028, 31034 Toulouse CEDEX 3 and Université Paul Sabatier, 31062 Toulouse, France
[2]Department of Dermatology, University of California and [4]Dermatology Service, Department of Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, CA 94131, USA
[3]Department of Medical Genetics, Purpan Hospital, Place du Dr Baylac, 31059 Toulouse CEDEX 3, France
*Authors for correspondence (e-mail: tmauro@itsa.ucsf.edu; alain.hovnanian@toulouse.inserm.fr)

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Summary

Keratinocyte differentiation, adhesion and motility are directed by extracellular Ca^{2+} concentration increases, which in turn increase intracellular Ca^{2+} levels. Normal keratinocytes, in contrast to most non-excitable cells, require Ca^{2+} release from both Golgi and endoplasmic reticulum Ca^{2+} stores for efficient Ca^{2+} signaling. Dysfunction of the Golgi human secretory pathway Ca^{2+}-ATPase hSPCA1, encoded by *ATP2C1*, abrogates Ca^{2+} signaling and causes the acantholytic genodermatosis, Hailey-Hailey disease. We have examined the role of the endoplasmic reticulum Ca^{2+} store, established and maintained by the sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase SERCA2 encoded by *ATP2A2*, in Ca^{2+} signaling. Although previous studies have shown acute SERCA2 inactivation to abrogate Ca^{2+} signaling, we find that chronic inactivation of *ATP2A2* in keratinocytes from patients with the similar acantholytic genodermatosis, Darier disease, does not impair the response to raised extracellular Ca^{2+} levels. This normal response is due to a compensatory upregulation of hSPCA1, as inactivating *ATP2C1* expression with siRNA blocks the response to raised extracellular Ca^{2+} concentrations in both normal and Darier keratinocytes. *ATP2C1* inactivation also diminishes Darier disease keratinocyte viability, suggesting that compensatory *ATP2C1* upregulation maintains viability and partially compensates for defective endoplasmic reticulum Ca^{2+}-ATPase in Darier disease keratinocytes. Keratinocytes thus are unique among mammalian cells in their ability to use the Golgi Ca^{2+} store to mediate Ca^{2+} signaling.

Key words: Darier, Hailey-Hailey, SERCA2, hSPCA1, Calcium, Keratinocytes

Introduction

Changes in extracellular Ca^{2+} concentrations, as are seen in normal unperturbed epidermis or after epidermal permeability perturbation, control epidermal functions such as differentiation, barrier repair, keratinocyte cell-to-cell adhesion and keratinocyte motility (Fang et al., 1998; Mao-Qiang et al., 1997; Vasioukhin et al., 2000). Thus, transducing changes in extracellular Ca^{2+} levels into intracellular Ca^{2+} signals that control these processes is essential for keratinocyte and epidermal viability.

Increasing extracellular Ca^{2+} concentration in cultured keratinocytes mimics many of the changes in keratinocyte differentiation and cell-to-cell adhesion seen in vivo (Pillai et al., 1990; Stanley and Yuspa, 1983; Vasioukhin et al., 2000), through a well-defined pathway that uses a plasma-membrane-bound Ca^{2+} receptor (CaR) to signal intracellular Ca^{2+} release, and subsequent long-lasting Ca^{2+} influx (Fatherazi et al., 2003; Oda et al., 1998). The coordinated responses to raised extracellular Ca^{2+} levels establish a new resting cytosolic concentration that continues to increase slowly (days to weeks) as the cells are exposed to continuing high levels of extracellular Ca^{2+} (Pillai et al., 1990). Initial studies found that emptying the endoplasmic reticulum (ER) Ca^{2+} store by pharmacologically inhibiting the Ca^{2+}-ATPase, SERCA2, responsible for sequestering Ca^{2+}, impaired the cells' response to raised extracellular Ca^{2+} (Li et al., 1995). These studies suggested that keratinocytes, like most other non-excitable cells, used only the ER Ca^{2+} store.

However, more recent studies have demonstrated that the Golgi Ca^{2+} store, sequestered by a related Ca^{2+}-ATPase, hSPCA1, also is required for Ca^{2+} signaling in response to raised extracellular Ca^{2+} concentrations. Mutations in the Golgi hSPCA1 cause another inherited skin disorder, Hailey-Hailey disease (HHD). Keratinocytes from HHD patients do not respond to raised extracellular Ca^{2+} levels and suffer from depleted Golgi Ca^{2+} stores, even though ER Ca^{2+} stores are normal (Hu et al., 2000). Further, experimental manipulation of the hSPCA1 demonstrates that keratinocytes, but not COS cells, require the hSPCA1-mediated Golgi Ca^{2+} store for normal signaling (Callewaert et al., 2003). Keratinocytes may therefore be unique among mammalian cells in requiring intact Golgi Ca^{2+} stores for Ca^{2+} signaling.

Darier disease (DD) is an acantholytic skin disease in which the ER Ca^{2+}-ATPase, SERCA2 encoded by *ATP2A2*,

is mutated (Sakuntabhai et al., 1999). Like HHD, this disease is characterized by impaired differentiation and abnormal cell-to-cell adhesion. Keratinocytes with these naturally occurring mutations allow us to determine how keratinocytes adapt to impaired ER Ca^{2+} stores, and also to examine the relative contributions of ER vs Golgi Ca^{2+} stores in the Ca^{2+}-signaling cascade in response to raised extracellular Ca^{2+} levels.

Three isoforms of SERCA2 are generated by alternative splicing of exon 20 and exon 21. Isoform 'a' is the cardiac sarco-endoplasmic Ca^{2+} pump whereas isoform 'b' is the major SERCA isoform in non-muscle cells, including epidermal cells (Wuytack et al., 1989; Wuytack et al., 2002). SERCA2c, a recently described splice variant, is expressed in epithelial, mesenchymal and hematopoietic cell lines, and in monocytes (Gelebart et al., 2003). SERCA2b contains an additional transmembrane domain, which places the C-terminus of the protein in the lumen of the ER, whereas this terminus is cytosolic in SERCA2a. Both the SERCA2a and SERCA2b isoforms are highly expressed in cultured keratinocytes but only SERCA2b is found in the epidermis. Loss of the SERCA2b isoform is sufficient to cause DD (Dhitavat et al., 2003). SERCA2 sequesters Ca^{2+} in the ER whereas hSPCA1 localizes to the trans-Golgi membrane and sequesters Ca^{2+} in the Golgi (Behne et al., 2003). hSPCA1 transports Ca^{2+} with an affinity comparable to that of the ER SERCA2b pump (Lytton et al., 1992; Ton et al., 2002). hSPCA1 also transports Mn^{2+} ions with high affinity (Fairclough et al., 2003; Ton et al., 2002). hSPCA1 is responsible for 67% of the Ca^{2+} uptake into the Golgi (Callewaert et al., 2003), which is consistent with the observation that *ATP2C1* transcripts are highly expressed in keratinocytes (Hu et al., 2000).

In this report, we show that DD keratinocytes overexpress hSPCA1 in compensation for decreased SERCA2, and thereby retain a near-normal response to raised extracellular Ca^{2+} concentrations. These studies demonstrate that the Golgi Ca^{2+}-ATPase hSPCA1 mediates a compensatory mechanism for the functional deficiency in the ER Ca^{2+}-ATPase seen in DD keratinocytes and confirm that keratinocytes use the hSPCA1-controlled Golgi Ca^{2+} store to mediate Ca^{2+} signaling.

Results

SERCA2 mutations decrease SERCA2 protein levels and deplete SERCA2-controlled and total cellular Ca^{2+} stores

We first confirmed that SERCA2 protein was decreased in the DD keratinocytes (Fig. 1). Preliminary studies demonstrated that *ATP2A2* mRNA synthesis was tightly grouped among keratinocytes taken from four unrelated adult donors (data not shown); thus keratinocytes from one normal adult were used as controls in the following experiments. The S920Y (DD2) and N767S (DD5) mutations led to a minor decrease (about 25%) in the amount of total endogenous SERCA2 protein (WT + mutated protein), whereas mutations Y203del (DD1), A998fsX33 (DD3) and S346F (DD4) caused a more profound decrease (up to 50%). These results are consistent with previous functional analysis of the S920Y and A998fsX33 mutations using overexpression of a mutated cDNA (Ahn et al., 2003; Dhitavat et al., 2003; Dode et al., 2003). We next assessed the SERCA2-controlled ER Ca^{2+} store by measuring the Ca^{2+} released by thapsigargin, which specifically and irreversibly inhibits SERCA pumps. ER Ca^{2+} stores were much lower in DD versus normal keratinocytes (Table 2). Both Ca^{2+} stored in the ER by SERCA, measured after exposure to 500 nM thapsigargin, and also total stored Ca^{2+}, measured after exposure to 1 μM ionomycin, were decreased in DD keratinocytes (Table 2). Increases in cytosolic Ca^{2+} following ionomycin or thapsigargin treatment did not differ among keratinocytes taken from different DD patients (data not shown).

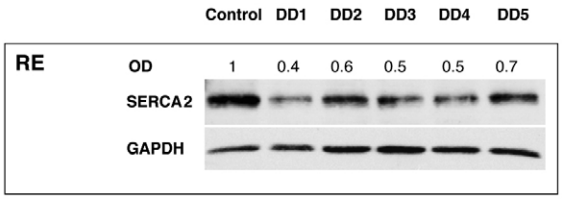

Fig. 1. SERCA2 protein is decreased in keratinocytes from all DD patients. Total protein extracts were analysed by immunoblotting using specific antibodies to SERCA2. Internal controls were GAPDH. The OD values represent densitometric analysis of the bands.

Table 1. Causative mutations in patients with Darier disease

Patient	*ATP2A2* mutation	Affected SERCA2 domain
DD1	Y203del	Actuator domain
DD2	S920Y	Eighth transmembrane domain
DD3	A998fsX33	C-terminal segment in SERCA2b
DD4	S346F	Phosphorylation domain
DD5	N767S	Fifth transmembrane domain

DD keratinocytes preserve a robust response to Ca^{2+}

Increased extracellular Ca^{2+} concentrations activate the plasma membrane Ca^{2+} receptor (CaR), resulting both in Ca^{2+} release from intracellular stores and subsequent Ca^{2+} influx through plasma membrane ion channels. Keratinocytes again seem to differ from other mammalian cells in that both the hSPCA1-controlled Golgi Ca^{2+} stores and the SERCA2-controlled ER Ca^{2+} stores are central to intracellular Ca^{2+} release and subsequent Ca^{2+} influx (Behne et al., 2003; Hu et al., 2000). We therefore tested whether the Ca^{2+} response was preserved in DD keratinocytes. Although overall Ca^{2+} homeostasis is not identical in DD and normal keratinocytes, as evidenced by abnormal resting Ca^{2+} concentrations, DD keratinocytes nevertheless responded robustly to raised extracellular Ca^{2+} concentrations (Fig. 2), with an identical absolute increase in cytosolic Ca^{2+} levels and a significantly higher increase in response to raised extracellular Ca^{2+}. The rate of increase in intracellular Ca^{2+} did not differ between normal and DD keratinocytes (Table 3).

Resting cytosolic Ca^{2+} concentrations varied among different patients, although Ca^{2+} values from each patient were tightly grouped, suggesting that different types of mutations might produce higher or lower cytosolic Ca^{2+} concentrations. However, simply classifying the mutations into nonsense or missense mutations did not predict resting Ca^{2+} values in our small sample (Table 3).

Table 2. ER and total Ca^{2+} stores are diminished in DD keratinocytes

	Control	DD
Baseline (nM)	84.05±30.97 (*n*=80)	37.26±14.54 (*n*=111)*
Peak response to thapsigargin (0.06 mM extracellular Ca^{2+}) (nM)	364.58±86.33 (*n*=19)	103.14±42.22 (*n*=29)*
Peak Ca^{2+} response to ionomycin (nM)	450.03±141.42 (*n*=33)	235.16±51.40 (*n*=51)*

ER Ca^{2+} stores were assessed by measuring peak cytosolic Ca^{2+} concentrations after exposure to 500 nM thapsigargin, whereas total Ca^{2+} stores were assessed by measuring peak cytosolic Ca^{2+} concentrations after exposure to 1 μM ionomycin. *n*=number of cells. Data are presented as the mean ± s.d. *P<0.05 compared with control values, assessed using a two-tailed Student's *t*-test.

Capacitive Ca^{2+} influx in DD keratinocytes is preserved

James Putney (Putney, 1986) first advanced the 'capacitive Ca^{2+} entry' model, proposing that Ca^{2+} entered the cell in response to the emptying of intracellular Ca^{2+} stores. Thus, intracellular Ca^{2+} release is amplified by Ca^{2+} influx from the extracellular compartment, passing through ion channels located in the plasma membrane. Earlier keratinocyte studies using Ca^{2+}-sensitive dyes have demonstrated that significant Ca^{2+} entry follows the emptying of the ER Ca^{2+} stores (Csernoch et al., 2000; Fatherazi et al., 2003). Since DD ER Ca^{2+} stores are depleted relative to normal keratinocytes, we compared capacitive Ca^{2+} entry in DD versus normal keratinocytes. Standard protocols for measuring capacitive Ca^{2+} entry first remove extracellular Ca^{2+} (to block non-capacitive Ca^{2+} entry), then release SERCA-dependent Ca^{2+} stores by adding thapsigargin, and finally restore extracellular Ca^{2+} and quantify capacitive Ca^{2+} influx by measuring increases in cytosolic Ca^{2+} concentrations. Because millimolar extracellular Ca^{2+} concentrations not only support capacitive Ca^{2+} influx but also might activate Ca^{2+} signaling via the CaR-mediated pathway, we restored extracellular Ca^{2+} to a final concentration of 0.06 mM (the same concentration in which the cells were grown), which does not significantly activate the keratinocyte plasma membrane CaR (Oda et al., 1998). When extracellular Ca^{2+} was restored after emptying Ca^{2+} stores, we found that capacitive Ca^{2+} entry was larger in DD relative to normal keratinocytes, consistent with diminished ER stores and normal signaling between intracellular Ca^{2+} stores and plasma membrane channels (Table 4, Fig. 3). Unlike pancreatic cells containing mutated

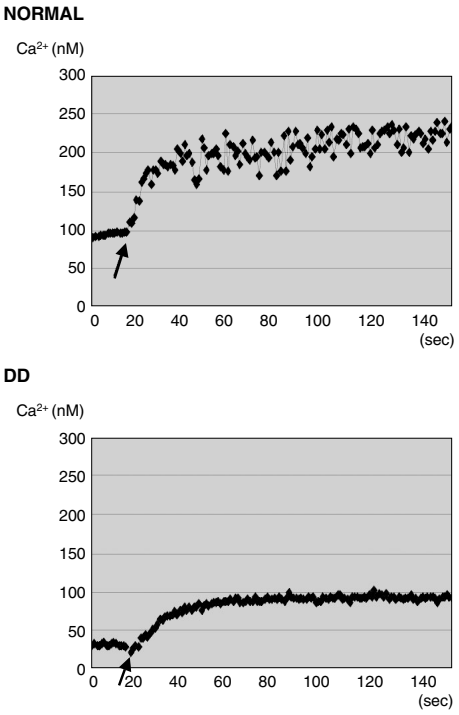

Fig. 2. The Ca^{2+} response is preserved in DD keratinocytes. Normal and DD keratinocytes were cultured on glass coverslips in medium containing 0.06 mM Ca^{2+} until they were ~50% confluent, then loaded with the Ca^{2+}-sensitive dye Fura-2 (see Materials and Methods). The cells initially were superfused with control solution containing: 138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1.0 mM Na_2HPO_4, 0.06 mM $CaCl_2$; 10 mM glucose, pH 7.4, 286 mOsm, then switched to this solution with 1.0 mM Ca^{2+} added (arrow). Although the baseline Ca^{2+} concentrations were much lower in DD keratinocytes, both normal and DD keratinocytes responded with a sustained increase in intracellular Ca^{2+} after exposure to raised extracellular Ca^{2+} levels. The absolute increase in intracellular Ca^{2+} was similar between DD and control keratinocytes, whereas the fold increase was significantly higher in DD keratinocytes (see Table 2).

Table 3. DD keratinocytes preserve a robust response to Ca^{2+}

	Control	DD
Baseline (nM)	84.05±30.97 (*n*=80)	37.26±14.54 (*n*=111)*
Peak response to 1.0 mM Ca^{2+} (nM)	142.60±44.10 (*n*=48)	90.63±23.78 (*n*=40)
Absolute increase (nM)	57.52±13.40 (*n*=48)	53.36±9.65 (*n*=40)
Fold increase	1.98±0.63 (*n*=48)	3.15±1.17 (*n*=40)*
Rate increase (nM/second)	1.08±1.18 (*n*=48)	0.84±0.69 (*n*=40)

Cytosolic Ca^{2+} was measured in normal and DD keratinocytes cultured at 0.06 mM Ca^{2+} at baseline (extracellular Ca^{2+} concentration 0.06 mM) and after increasing extracellular Ca^{2+} levels to 1.0 mM. *n*, number of cells. Data are presented as the mean ± s.d. *P<0.05, assessed using a two-tailed Student's *t*-test. Baseline cytosolic Ca^{2+} (nM) grouped by patient: DD1, 28.06±4.86 (*n*=16); DD2, 59.38±10.06 (*n*=13); DD3, 40.92±12.24 (*n*=12); DD4, 31.15±7.36 (*n*=26); DD5, 36.48±15.71 (*n*=44).

Table 4. Capacitive Ca^{2+} influx is increased in DD keratinocytes

	Control	DD
Cytosolic Ca^{2+} in EGTA (nM)	31.44±12.15 (*n*=50)	34.76±12.12 (*n*=17)
Thapsigargin peak (in EGTA) (nM)	92.83±29.20 (*n*=48)	41.82±16.51 (*n*=17)*
Peak capacitive Ca^{2+} (nM)	108.55±60.41 (*n*=33)	144.38±62.82 (*n*=17)*

Capacitive Ca^{2+} influx is larger in DD vs control keratinocytes, consistent with diminished ER Ca^{2+} stores. Note also that the response to thapsigargin is almost absent in DD keratinocytes, confirming that DD ER keratinocyte Ca^{2+} stores are severely depleted. *n*=number of cells. Data are presented as the mean ± s.d. *P<0.05 compared with control values, assessed using a two-tailed Student's *t*-test.

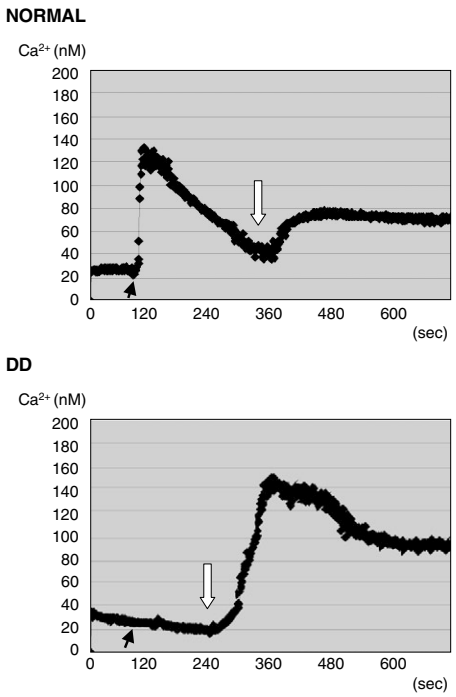

Fig. 3. Thapsigargin-releasable Ca^{2+} stores are decreased and capacitive Ca^{2+} influx is increased in DD keratinocytes. Normal and DD keratinocytes were cultured and loaded with Fura-2 as detailed in Fig. 1. Cells initially were superfused with solution containing: 138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1.0 mM Na_2HPO_4, 0.0 mM $CaCl_2$, 0.05 mM EGTA, 10 mM glucose, pH 7.4, 286 mOsm. After equilibration, 500 nM thapsigargin was applied (black arrows). Although the resulting ER Ca^{2+} release substantially increased intracellular Ca^{2+} in normal keratinocytes, minimal Ca^{2+} release was noted in DD keratinocytes, consistent with depletion of the SERCA-dependent ER Ca^{2+} stores (Table 2). After recovery, the extracellular Ca^{2+} was increased to 0.06 mM (white arrows), initiating capacitive Ca^{2+} entry. Both normal and DD keratinocytes displayed robust capacitive Ca^{2+} entry; however, the capacitive Ca^{2+} entry was proportionately larger in DD keratinocytes (Table 2).

ATP2A2 (Zhao et al., 2001), rates of Ca^{2+} increase or decrease were not different between normal and DD keratinocytes, suggesting that extrusion mechanisms such as that mediated by the plasma membrane Ca^{2+}-ATPase (PMCA) or by Na^+/Ca^{2+} exchangers were not different between DD and normal keratinocytes.

Taken together, these findings demonstrate that the Ca^{2+} signaling in response to raised extracellular Ca^{2+} is intact in DD keratinocytes, despite the loss of SERCA2-controlled Ca^{2+} stores. These findings suggest that other Ca^{2+}-signaling mechanisms are likely to be upregulated in response to decreased SERCA2 levels and function. We therefore examined other mechanisms that might compensate for SERCA2 loss.

The normal Ca^{2+} response of DD keratinocytes is not due to changes in Ca^{2+} influx or efflux

After extracellular Ca^{2+} stimulates an acute increase in intracellular Ca^{2+}, cytosolic Ca^{2+} is then stabilized at a new, increased Ca^{2+} concentration by the coordinated interaction between Ca^{2+} extrusion through the Na^+/Ca^{2+} exchangers and PMCA and re-uptake of Ca^{2+} into the ER (via the SERCA2), and into the Golgi (via the hSPCA1). Ca^{2+} signaling in DD keratinocytes therefore might be due to compensatory upregulation of Ca^{2+}-permeable ion channels, plasma membrane transporters such as PMCA4, the major PMCA expressed in keratinocytes (Cho and Bikle, 1997), or intracellular Ca^{2+} ATPases such as the hSPCA1, localized to the Golgi in keratinocytes. We first examined whether DD Ca^{2+} signaling was due to upregulation of plasma membrane ion channels or transporters by assessing Ca^{2+} influx or efflux across the plasma membrane, measured using $^{45}Ca^{2+}$ influx or efflux (Table 5). Neither Ca^{2+} influx nor Ca^{2+} efflux was significantly different in DD vs. normal keratinocytes. These experiments suggest that plasma membrane components, such as the PMCA, do not compensate for decreased SERCA2 in DD keratinocytes.

hSPCA1, but not PMCA, is upregulated in DD keratinocytes

hSPCA1 is central to keratinocyte Ca^{2+} signaling and regulation of resting Ca^{2+} concentrations (Behne et al., 2003; Hu et al., 2000). Because hSPCA1 constitutes the second major mechanism for sequestering intracellular Ca^{2+} in keratinocytes, we next investigated whether decreases in SERCA2 stimulated a compensatory upregulation of hSPCA1. We found that hSPCA1 was markedly upregulated in all DD keratinocytes studied (Fig. 4). These data, combined with the functional signaling characteristics of DD keratinocytes, suggested that hSPCA1 upregulation might compensate for SERCA2 dysfunction.

Plasma membrane Ca^{2+}-ATPases (PMCA) also are known to regulate Ca^{2+} extrusion. However, of the five DD patients studied, only two, DD2 (S920Y) and DD3 (A998fsX33), overexpressed total PMCA or PMCA-4. The others displayed a normal (DD4, S346F; DD5, N767S) or slightly decreased (DD1, Y203del) level of PMCA, indicating that PMCA expression was variable among DD patients. These data, coupled with the functional Ca^{2+} efflux data above, suggest that changes in hSPCA1, but not PMCA or PMCA-4, underlie the compensatory changes in DD keratinocytes that allow normal Ca^{2+} signaling in response to raised extracellular Ca^{2+} concentrations.

Table 5. Net Ca^{2+} flux in control and DD keratinocytes

	Control	DD1	DD2
Ca^{2+} influx rate (counts/minute/well)	250.17±7.34 (*n*=6)	265.50±17.74 (*n*=6)	224.00±34.05 (*n*=6)
Ca^{2+} efflux rate (counts/minute/well)	188.88±10.68 (*n*=6)	196.50±6.75 (*n*=6)	182.25±9.65 (*n*=6)

Ca^{2+} homeostasis and signaling are not due to differences in net Ca^{2+} flux across the plasma membrane, as Ca^{2+} influx and efflux rates are identical in normal and DD keratinocytes. *n*, number of experiments. Data are presented as the mean ± s.d.

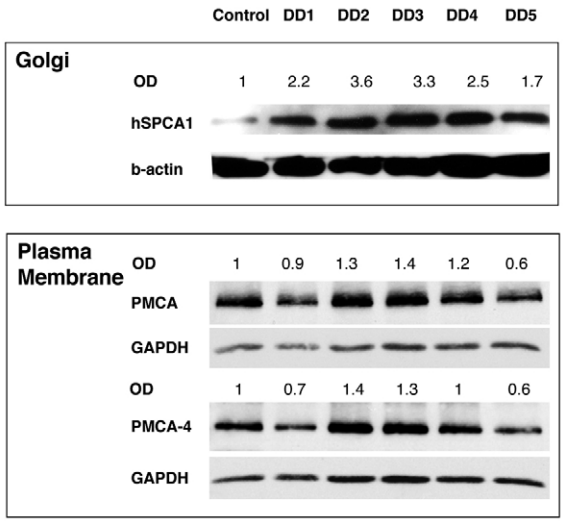

Fig. 4. hSPCA1, but not PMCA, is increased in DD keratinocytes. Total protein extracts were analyzed by immunoblotting using specific antibodies to PMCA1 and PMCA4 and hSPCA1. Internal controls were GAPDH or β-actin. The OD values result from the densitometric analysis of the bands. These data are representative of two separate experiments.

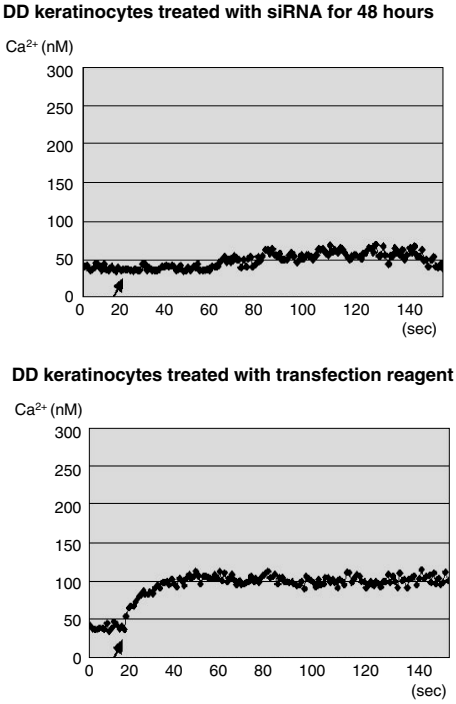

Fig. 5. Inactivating *ATP2C1* with siRNA blocks the Ca^{2+} response in DD keratinocytes. DD keratinocytes were cultured on glass coverslips until ~40% confluent, then treated with *ATP2C1* siRNA (see Materials and Methods). Forty-eight hours after treatment, at ~50% confluence, cells were loaded with Fura-2 and intracellular Ca^{2+} was measured by the protocol detailed in Fig. 2. Ca^{2+} in the superfusing fluid was raised from 0.06 to 1.0 mM (black arrow). The Ca^{2+} response was blunted in DD keratinocytes treated with siRNA to *ATP2C1* (top), whereas control DD keratinocytes responded normally to raised extracellular Ca^{2+} (bottom). The *P* value (Table 6) was calculated by a two-tailed *t*-test comparing vector control and siRNA. All values are expressed as mean ± s.d.

hSPCA1 controls Ca^{2+}-mediated Ca^{2+} signaling in normal and DD keratinocytes and is essential for cell viability in DD keratinocytes

We previously found that hSPCA1 dysfunction abrogates the Ca^{2+} response in keratinocytes containing *ATP2C1* mutations (Behne et al., 2003; Hu et al., 2000). To test whether compensatory hSPCA1 upregulation enables DD keratinocytes to respond to extracellular Ca^{2+} levels, even in the face of SERCA2 dysfunction, we inactivated *ATP2C1* in DD keratinocytes using small interfering RNA (siRNA). RNA interference (RNAi) is a strategy whereby genes are silenced post-translationally by treatment with sequence-specific double-stranded RNA. Cellular exposure to siRNA results in the selective degradation of the complementary native RNA (McManus and Sharp, 2002). Our siRNA oligonucleotides were designed to avoid any overlap between *ATP2C1* and *ATP2A2* (confirmed by BLAST analysis). To assess non-specific effects, we measured *ATP2A2* cDNA expression after treatment with *ATP2C1* siRNA. Only a 10% reduction in *ATP2A2* cDNA was observed after treatment with *ATP2C1* siRNA in normal keratinocytes, confirming that any changes in Ca^{2+} signaling were due specifically to decreases in hSPCA1. Normal and DD human keratinocytes were cultured in six-well plates to 75% confluence, then treated with siRNA (8 μg/well). Control samples were treated with transfection reagent and annealing buffer only. In preliminary experiments, we found that siRNA decreased *ATP2C1* mRNA by approximately 80% within 24 hours after treatment.

Since a drop in protein levels generally lags behind a decrease in mRNA, we next assessed protein levels and compared these with changes in Ca^{2+} signaling in normal and DD keratinocytes treated with siRNA to *ATP2C1*. Normal keratinocytes treated with siRNA displayed normal Ca^{2+} signaling, confirmed by measuring normal hSPCA1 protein levels, 48 hours after siRNA treatment (data not shown). By 72 hours, however, hSPCA1 protein levels dropped, and Ca^{2+} signaling in normal keratinocytes correspondingly changed to resemble the abnormal Ca^{2+} signaling seen in Hailey-Hailey keratinocytes, in which the hSPCA1 is mutated (Hu et al., 2000). Specifically, baseline cytosolic Ca^{2+} concentrations increased, and the ability to respond to raised extracellular Ca^{2+} levels diminished, after exposure to siRNA (Fig. 5 and Table 6). DD keratinocytes responded more quickly to siRNA, demonstrating decreased hSPCA1 levels and abnormal Ca^{2+}-signaling parameters within 48 hours of siRNA treatment. Resting Ca^{2+} concentrations, although increased, did not reach cytosolic Ca^{2+} levels seen in normal keratinocytes, demonstrating that Ca^{2+} signaling changes at 48 hours were due to selective inhibition of hSPCA1 synthesis and not non-specific toxicity. In contrast to normal keratinocytes, DD keratinocytes became pyknotic and did not cleave the Fura-2 dye to its active form at 72 hours, demonstrating that these cells were no longer viable. In both normal and DD keratinocytes, transfection-reagent-treated control cells did not differ from untreated keratinocytes (compare Table 6 to Table 3). These data confirm that hSPCA1 is essential for Ca^{2+}-mediated Ca^{2+} responses. Further, these data suggest that increased hSPCA1 compensation for dysfunctional SERCA2 is essential for DD

Table 6. Inactivation of *ATP2C1* changes baseline Ca^{2+} and Ca^{2+} signaling

	Control keratinocytes			DD keratinocytes		
	Transfection control	siRNA-treated for 72 hours	*P* value	Transfection control	siRNA-treated for 48 hours	*P* value
Baseline 0.06 mM Ca^{2+} (nM)	70.30±15.69 (*n*=20)	102.19±17.89 (*n*=16)	<0.001	34.58±11.85 (*n*=17)	48.94±16.72 (*n*=17)	<0.01
1.0 mM Ca^{2+} (nM)	144.53±27.43 (*n*=20)	125.87±22.12 (*n*=16)	0.030	91.67±22.75 (*n*=13)	59.82±19.23 (*n*=17)	<0.001
Fold increase	2.08±0.54 (*n*=20)	1.21±0.18 (*n*=16)	<0.0001	3.05±1.15 (*n*=13)	1.25±0.26 (*n*=17)	<0.0001

keratinocyte cell viability, because DD keratinocytes treated with siRNA to *ATP2C1* died 48-72 hours after *ATP2C1* was inactivated.

Discussion

In this study, we investigated Ca^{2+} homeostasis in DD keratinocytes with five different causative mutations of *ATP2A2*. These cells all displayed decreased total and ER Ca^{2+} stores owing to decreased SERCA2 levels. In spite of decreased ER Ca^{2+} stores, we found that the DD cells preserved their Ca^{2+}-signaling capability and were able to respond to raised extracellular Ca^{2+} levels. Because we know that ER Ca^{2+} is decreased in DD keratinocytes (Tables 2 and 4), it is likely that Ca^{2+} release from this compartment in response to raised extracellular Ca^{2+} is decreased in DD cells. As the hSPCA1 is upregulated in these DD keratinocytes, it appears that increased Ca^{2+} signaling via the Golgi Ca^{2+} store compensates for defective SERCA2-dependent signaling. The proposed changes in Ca^{2+} signaling pathways in DD vs normal keratinocytes are described in Fig. 6.

To date, 22 mutations of the *ATP2A2* gene have been studied by site-directed mutagenesis of the *ATP2A2* cDNA and overexpression in COS-1 or HEK293 cells (Ahn et al., 2003; Dode et al., 2003; Sato et al., 2004). Nonsense (Q790X, E917X) and frame-shift (1625delAG) mutations studied resulted in truncated pump proteins that were susceptible to degradation by the proteasome (Ahn et al., 2003). Most DD mutants (10 out of 12) displayed decreased expression and/or stability with the exception of two mutants (C344Y and V843F) (Ahn et al., 2003). Kinetic analyses of SERCA2 missense variants revealed that any one of the SERCA2 catalytic cycle steps (Ca^{2+} binding, autophosphorylation from ATP, conformational change, release of bound Ca^{2+} into the ER lumen and dephosphorylation) could be affected. Depending on the location of the amino acid substitution, the activity of the mutated pump may be decreased (most of the missense mutations), increased (S920Y), or abolished when the phosphorylation step is affected (T357K, G769R) (Dode et al., 2003; Sato et al., 2004). Furthermore, some missense mutants (N39D, N39T, C344Y, F487S, S920Y) inhibit the activity of native and recombinant wild-type SERCA2b by directly interacting with the wild-type pump (Ahn et al., 2003). Patient DD2 in this work bears the S920Y mutation studied by others (Ahn et al., 2003; Dode et al., 2003). Our results show that the resting cytosolic Ca^{2+} concentration in these cells was lower than that of the control but higher than in the four other DD cells studied. The kinetic analyses by Dode et al. revealed that S920Y is a unique DD mutant displaying an enhanced molecular Ca^{2+}-transport activity relative to wild-type SERCA2b. The apparent affinity for Ca^{2+}, however, was threefold lower relative to wild-type SERCA2b and this mutant was insensitive to the feedback inhibition of the transport cycle by accumulated lumenal Ca^{2+}. Ahn et al. have shown a decreased stability of S920Y, preferential co-immunoprecipitation of S920Y with the wild-type pump, and a reduction by S920Y of the activity of wild-type SERCA2b (Ahn et al., 2003). This could explain the intermediate level of resting Ca^{2+} concentrations in DD2 cells.

All these results demonstrate that DD is caused in most cases by haploinsufficiency of SERCA2b, which could be amplified when the mutated pump has a dominant-negative effect. The analysis of L321F (Sato et al., 2004) revealed that a decreased Ca^{2+} affinity and insensitivity to the feedback inhibition without decreased expression are sufficient to cause abnormal

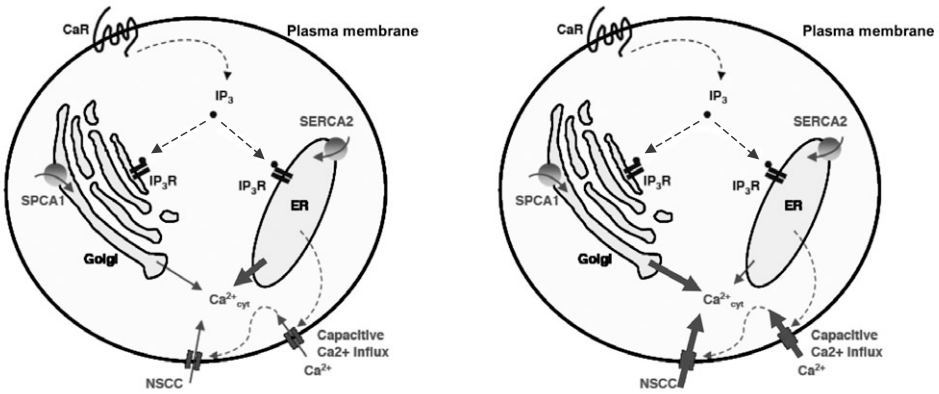

Fig. 6. Pathways for Ca^{2+} signaling in normal and DD keratinocytes. Raised extracellular Ca^{2+} binds to a Ca^{2+} receptor (CaR), located in the plasma membrane, producing the second messenger inositol-1,4,5-trisphosphate (IP3). IP3 causes Ca^{2+} release from the endoplasmic reticulum (ER) and the Golgi by binding to IP3 receptors (IP3R). Emptying of intracellular Ca^{2+} stores activates Ca^{2+} influx through several pathways, including capacitive Ca^{2+} influx and influx through a Ca^{2+}-permeable, Ca^{2+}-activated non-selective cation channel (NSCC). In this drawing, calcium fluxes are denoted by solid arrows. Thick and thin arrows represent relative increases and decreases, respectively, in calcium flux between normal and DD keratinocytes: Ca^{2+} release from the ER decreases, whereas Ca^{2+} release from the Golgi and capacitive Ca^{2+} influx increase in DD cells relative to normal cells.

Ca^{2+} homeostasis and DD. The range of functional alterations observed among *ATP2A2* variants could account for the variable clinical features of DD.

Although Ca^{2+}-mediated Ca^{2+} signaling was preserved in DD keratinocytes (Fig. 6), Ca^{2+} homeostasis was not completely normal, as evidenced by the abnormally low resting cytosolic Ca^{2+} concentrations (Table 2). Baseline cytosolic Ca^{2+} concentrations are maintained by the constant interaction of proteins that control Ca^{2+} release or influx with proteins that control Ca^{2+} extrusion or reuptake. Loss of one of the reuptake mechanisms, such as the Ca^{2+}-sequestering SERCA2, resulting from DD mutations or from acute pharmacologic SERCA2 inactivation (Hu et al., 2000; Jones and Sharpe, 1994), may be expected to increase cytosolic Ca^{2+} levels, as seen in HHD keratinocytes (Behne et al., 2003; Hu et al., 2000). In fact, DD keratinocytes respond to long-term SERCA2 dysfunction by decreasing resting cytosolic Ca^{2+} levels (Table 2). These cytosolic Ca^{2+} concentrations, although lower than those of most mammalian cells, are comparable to those seen in healthy mouse keratinocytes (Li et al., 1995); and we find that these DD keratinocytes appeared to retain comparable proliferative capability even though (or perhaps because) their resting cytosolic Ca^{2+} concentrations are abnormally decreased. Decreased baseline cytosolic Ca^{2+} levels were unlikely to result from changes in plasma membrane channels or transporters, as Ca^{2+} influx and efflux were unchanged in DD compared with normal keratinocytes. However, increased hSPCA1 may contribute to changes in resting cytosolic Ca^{2+}, as inactivating *ATP2C1* with siRNA, in addition to abrogating the Ca^{2+} response, also increased baseline cytosolic Ca^{2+} in both normal and DD keratinocytes (compare Table 6 with Table 3), consistent with changes seen in HHD keratinocytes (Behne et al., 2003; Hu et al., 2000), in which the *ATP2C1* is mutated. According to Liu and co-workers, in cells overexpressing the plasma membrane Ca^{2+}-ATPase, PMCA1a, the activity of the Ca^{2+} release-activated channels (CRAC) pathway is upregulated, whereas the inositol 1,4,5-trisphosphate receptor (IP3R) and the SERCA pumps are downregulated (Liu et al., 1996). In Chinese hamster ovary (CHO) cells overexpressing PMCA4, a reduction in SERCA2b expression levels has been described (Guerini et al., 1995). It is conceivable that a compensatory mechanism relates Ca^{2+} efflux by the cell membrane ATPases to the activity of the ER Ca^{2+}-ATPases, through the regulation of the respective genes. Our data, however, show that overexpression of PMCA proteins is at best small and non-uniformly observed across the range of DD mutations studied; apart from the *ATP2A2* mutations, the DD keratinocyte donors differed regarding age, gender and genetic background. In addition, Ca^{2+} efflux was not increased in DD keratinocytes, suggesting that PMCA does not play a significant compensatory role.

Conversely, we have found evidence of several-fold overexpression of hSPCA1 in DD keratinocytes. Moreover, we found that compensatory overexpression of this pump in response to chronic SERCA2 deficiency appears to be essential for a normal Ca^{2+} response to raised extracellular Ca^{2+} levels and partially compensates for SERCA2 functional deficiency by enabling DD keratinocyte survival. Thus, this report adds to the growing body of information demonstrating that this previously obscure (to mammalian cell biologists) Ca^{2+}-ATPase in fact plays an essential role in mammalian cell Ca^{2+} signaling. For example, Reinhardt and co-workers observed that overexpression of rat SPCA in COS-7 cells caused significant alterations in several of the cell Ca^{2+} transport molecules and dramatically increased the cell division rate (Reinhardt et al., 2004). Total PMCA protein expression was reduced along with the expression of the ER Ca^{2+}-binding protein calreticulin. These changes suggest a redundant flexibility in the cell Ca^{2+} homeostatic mechanism. The pumping of excess cytoplasmic Ca^{2+} out of the cell or into the ER was necessarily reduced to compensate for increased Ca^{2+} movement into the Golgi. The Golgi of these cells compensated for this increased Ca^{2+} influx by increased expression of the Golgi Ca^{2+}-binding protein CALNUC. This would seem to be a necessary adaptation to prevent Ca^{2+} cytotoxicity.

Taken together, the yeast and human data strongly suggest that SPCA is the mammalian Golgi Ca^{2+}-ATPase. The opposing argument put forth by Taylor and co-workers (Taylor et al., 1997) is that the Golgi complex does not contain a unique resident Ca^{2+}-ATPase. They state that all Ca^{2+} uptake into Golgi can be attributed to PMCAs in transit to the plasma membrane and to SERCAs that are not restricted to the ER. This is at odds with the data from the present study, where we show that specific inhibition of hSPCA1 abolishes Ca^{2+} response in DD keratinocytes. A major role for hSPCA1 in keratinocyte Ca^{2+} homeostasis is thus warranted.

The subcellular specialization of hSPCA1 may underlie the differences in clinical manifestations between DD and HHD. hSPCA1 deficiency causes acantholysis, whereas mutations in the gene encoding SERCA2 cause both acantholysis and apoptosis. In turn, the clinical phenotype of acantholysis plus apoptosis in DD probably reflects the varied functions of the keratinocyte hSPCA1- versus SERCA2-controlled Ca^{2+} stores.

Materials and Methods

DD patients and molecular diagnosis

All patients (Table 1) gave informed consent. Biopsies were taken from the unaffected abdominal skin. Control biopsies of normal skin were obtained from two age-matched controls. Patients were screened for mutations in the *ATP2A2* gene. PCR amplification of the 21 exons and flanking splice sites of *ATP2A2* was performed using previously published primers and protocols (Sakuntabhai et al., 1999). PCR products were sequenced on an ABI 377 automated sequencer using the BigDye chemistry (Applied Biosystems).

Mutations

The S920Y mutation found in patient DD2 was identified previously and the functional analysis of this mutation has been described (Ahn et al., 2003; Dode et al., 2003). The A998fsX33 mutation found in DD3 occurs in the region specific to the SERCA2b isoform and was identified previously (Dhitavat et al., 2003). The N767S missense mutation found in DD5 has been reported previously by others (Jacobsen et al., 1999; Ruiz-Perez et al., 1999). The previously unpublished mutations, Y203del (DD1, deletion) and S346F (DD4, missense), affect highly conserved amino acid positions, respectively in the actuator domain of SERCA2 and the phosphorylation domain next to the phosphorylation site (Asp351).

Cell culture

Biopsies were explanted from adult normal skin (surgical skin margins) or clinically normal DD skin (punch biopsies) and primary keratinocytes were isolated and grown as previously described (Rheinwald and Green, 1975). Second to fifth passage cultured human keratinocytes were grown in 0.06 mM Ca^{2+} EpiLife medium (Cascade Biologics, Eugene, Oregon) to approximately 60-80% confluence (Boyce and Ham, 1983).

$^{45}Ca^{2+}$ measurements

To study calcium influx (Ca_i), normal and DD keratinocytes were cultured in six-well plates to 70-80% confluence (Grando et al., 1996). Cells were washed for 10 minutes in Ca^{2+}-free buffer (20 mM HEPES, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mg/dl glucose, pH 7.4), then incubated for 1.5 hours at 37°C in EpiLife

plus 0.06 mM Ca^{2+} plus 0.05 μCi $^{45}CaCl_2$ (specific activity ~0.8 mCi/mmol). Subsequently they were washed four times, quickly, with Ca^{2+}-free buffer plus 2 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) then lysed with 0.5N NaOH. The lysate was analyzed with scintillation spectroscopy.

To study Ca^{2+} efflux, cultured cells were washed for 10 minutes with Ca^{2+}-free buffer, then incubated for 1.5 hours at 37°C in EpiLife plus 0.06 mM Ca^{2+} plus 0.05 μCi $^{45}CaCl_2$. Subsequently, they were washed four times, quickly, with Ca^{2+}-free buffer plus 2 mM EGTA, then incubated for 1.5 hours at 37°C in EpiLife plus 0.06 mM Ca^{2+}. Media then were collected and analyzed by scintillation spectroscopy.

Intracellular Ca^{2+} measurements

Keratinocytes grown on glass coverslips were incubated in 6.26 μM cell-permeant Fura-2 acetomethoxy ester (Molecular Probes, Eugene, OR) at 37°C for 15-30 minutes, rinsed in the control solution (138 mM NaCl, 2.7 mM KCl, 0.01 M Na phosphate, 0.06 mM $CaCl_2$, 10 mM glucose, pH 7.4), and intracellular Ca^{2+} was monitored by ratiometric method, using the InCytIM2 Imaging System (Intracellular Imaging, Cincinnati, OH). For experiments measuring capacitive Ca^{2+} influx, cells were initially perfused with a solution containing 138 mM NaCl, 2.7 mM KCl, 0.01 M phosphate, 10 mM glucose, 0 mM $CaCl_2$ plus 0.5 M EGTA, pH 7.4., then perfused with the same solution with the Ca^{2+} concentration increased to 0.06 mM, without EGTA. A calibration curve was constructed using a standard calibration kit (Molecular Probes, Eugene, OR).

Table 2 relates to cells from all five DD mutants. Tables 3 and 4 contain data from DD1, DD2 and DD3. Because of the technical complexity and numbers of cells needed to perform the $^{45}Ca^{2+}$ and siRNA experiments, the $^{45}Ca^{2+}$ experiments were done with cells from DD1 and DD2, whereas the siRNA experiments were done with cells from a single mutant, DD3. DD3 was chosen for siRNA studies because its mutation occurs in the region specific to the SERCA2b isoform, which we previously have shown is sufficient to cause Darier disease (Dhitavat et al., 2003).

The rate of change (increase or decrease) was calculated for each cell as: (Peak Ca^{2+} value – baseline or recovery Ca^{2+} value)/(Peak Ca^{2+} time – baseline or recovery Ca^{2+} time), leading to a rate of increase or decrease measured as ΔnM Ca^{2+}/second. Data are presented as the mean ± s.d. Statistical analysis was performed using an unpaired two-tailed Student's *t*-test.

SDS-PAGE and immunoblotting

For PMCA and SERCA2 detection, cell lysates in 2× Laemmli SDS sample buffer (2 μg total protein per well) were fractionated on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in PBS with 5% non-fat dry milk, 1% BSA and 0.5% Tween-20, and incubated at room temperature with the relevant primary antibody for 1 hour: mouse monoclonal anti-human SERCA2 antibody, clone IID8 (Biomol), 1:5000; mouse monoclonal anti-human PMCA, clone 5F10 (Abcam), 1:4000; and rabbit anti-human GAPDH polyclonal antibody (Abcam), 1:20,000. Chemiluminescent detection was performed using secondary reagents from the ECL Plus western blotting detection system (Amersham). The protein contents of the samples were quantified to ensure equal loading of protein into each well. For hSPCA1 detection, cells were lysed in a buffer containing 50 mM Tris-HCl pH 7.4, 150 mM KCl, 250 mM sucrose, with addition of 1 tablet of protease inhibitors per 10 ml of buffer (Complete Mini EDTA-free, Roche Diagnostics) and 30 μg total protein per well was fractionated by SDS-PAGE. Following transfer of proteins to PVDF membranes and blocking with 5% milk, 0.5% Tween-20 in PBS, samples were incubated overnight at 4°C with anti-human hSPCA1 primary antibody (Santa Cruz Biotechnology), 1:1000 in the blocking solution. Protein expression also was normalized using an antibody to β-actin (Sigma, clone AC-74), 1:5000 dilution. To quantify calcium pump expression in DD keratinocytes, densitometry was performed on the chemiluminescence photo images, using a Bio-Rad GS-710 scanner and Quantity One analysis software. Density values were normalized to loading control expression (GAPDH or β-actin) within same samples, and a percentage value for the difference between conditions was calculated.

Small interfering RNA

The effectiveness of potential siRNA candidates was first predicted by choosing a G/C content of approximately 50%, and homology to *ATP2C1* or *ATP2A2* was checked by running the sequence of the designed siRNA against the full genome sequence in BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). After testing, one optimum sequence to silence *ATP2C1* (codon position: 150, 47% G/C content, 5′-agg cuc gcc uau gac uaa cTT-3′) and one sequence to silence *ATP2A2* (codon position: 2515, 57% G/C content; 5′-gcg ccg acg uaa cag cca aTT-3′) were selected. The oligoribonucleotides were synthesized by TriLink Biotechnologies (San Diego, CA). At 75% confluence, primary normal and DD keratinocytes were treated with siRNA suspended in 0.03 mM Ca^{2+}, serum-free medium using the Trans-Messenger Transfection Kit (Qiagen). Control normal and DD keratinocytes were treated using only the transfection reagents. No signs of cytotoxicity were observed with this protocol in experimental or control keratinocytes. The medium was changed after 4 hours, and cells were harvested after 48 hours (DD keratinocytes) and 72 hours (normal keratinocytes). RNA was extracted using the RNAeasy Mini Kit (Qiagen) and quantified using a spectrophotometer at 260 nm. cDNA was synthesized from RNA of each sample using the TaqMan Reverse Transcription kit (Applied Biosystems) and measured by the ABI Prism 7900 HT instrument using SYBR Green as gene-amplification detection in quantitative PCR (Applied Biosystems). The 18S RNA was used as a control, housekeeping gene. Protein levels were determined on total lysates using western analysis (see above).

Statistics

Statistical analysis was performed using unpaired two-tailed Student's *t*-tests.

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