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The Role of the Calcium Sensing Receptor in Regulating Intracellular Calcium Handling in Human Epidermal Keratinocytes

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Calcium is critical for controlling the balance of proliferation and differentiation in epidermal keratinocytes. We previously reported that the calcium sensing receptor (CaR) is required for mediating Ca^{2+} signaling and extracellular Ca^{2+} (Ca_o^{2+})-induced differentiation. In this study, we investigated the mechanism by which CaR regulates intracellular Ca^{2+} (Ca_i^{2+}) and its role in differentiation. Membrane fractionation, fluorescence immunolocalization, and co-immunoprecipitation studies were performed to assess potential interactions between CaR and other regulators of Ca^{2+} stores and channels. We found that the glycosylated form of CaR forms a complex with phospholipase C γ1, IP_3 receptor (IP_3R), and the Golgi Ca^{2+}-ATPase, secretory pathway Ca^{2+}-ATPase 1, in the *trans*-Golgi. Inactivation of the endogenous CaR gene by adenoviral expression of a CaR antisense cDNA inhibited Ca_i^{2+} response to Ca_o^{2+}, decreased Ca_i^{2+} stores, decreased Ca_o^{2+}-induced differentiation, but augmented store-operated channel activity and Ca^{2+} uptake by intracellular organelles. Our results indicate that CaR regulates keratinocyte differentiation in part by modulating Ca_i^{2+} stores via interactions with Ca^{2+} pumps and channels that regulate those stores.

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INTRODUCTION

It is well established that extracellular Ca^{2+} (Ca_o^{2+}) suppresses proliferation and promotes differentiation in epidermal keratinocytes (Yuspa *et al.*, 1989; Menon *et al.*, 1992; Bikle and Pillai, 1993). Elevation of Ca_o^{2+} triggers an acute and then a sustained increase in intracellular Ca^{2+} (Ca_i^{2+}), and subsequently initiates expression of early differentiation markers genes (Sharpe *et al.*, 1989; Bikle *et al.*, 1996). Previous studies demonstrated the requirement for calcium sensing receptor (CaR), a G-protein-coupled receptor, in mediating the Ca^{2+} sensing and signaling in keratinocytes (Oda *et al.*, 2000; Tu *et al.*, 2001). Activation of CaR with Ca^{2+} activates phospholipase C (PLC), which converts phosphatidylinositol 4,5-bisphosphate into diacylglycerol and IP_3. IP_3 binds to its receptors, IP_3R, in the endoplasmic reticulum (ER) and Golgi apparatus membrane and triggers release of Ca^{2+} from internal stores, resulting in an acute increase in Ca_i^{2+} (Berridge, 1993). Emptying of Ca_i^{2+} stores activates the store-operated channel (SOC)-mediated capacitative Ca^{2+} entry (Putney *et al.*, 2001). Raised Ca_i^{2+} directly activates plasma membrane channels, amplifying the Ca^{2+} influx and thus prolonging the rise in Ca_i^{2+}. The Ca_i^{2+} level is tightly regulated to prevent overstimulation of cellular responses and cytotoxicity owing to prolonged elevation of cytosolic Ca^{2+}. Cytosolic Ca^{2+}-binding proteins, Ca^{2+} pumps (Ca^{2+}-ATPases), and Ca^{2+}–Na^+ exchangers in the plasma membrane, ER, and Golgi all contribute to Ca^{2+} homeostasis. Repetitive cycles of release and uptake generate oscillations of Ca^{2+}, leading to specific cellular responses and gene transcription (Berridge, 2001). Inhibition of CaR expression in keratinocytes diminishes the increase of Ca_i^{2+} in response to Ca_o^{2+} and the subsequent expression of differentiation related genes (Tu *et al.*, 2001).

Conceivably, the abrogated Ca_i^{2+} response following CaR inhibition is caused by disrupted Ca^{2+} release from intracellular stores or Ca^{2+} influx through channels in the plasma membrane. ER is generally considered as the major agonist-sensitive Ca_i^{2+} store (Berridge, 2002). However, recent studies revealed that in many cell types the Golgi apparatus also functions as a Ca^{2+} store (Pinto *et al.*, 1998; Surroca and Wolff, 2000), using both sarco/endoplasmic

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Abbreviations: Ca_i^{2+}, intracellular Ca^{2+}; Ca_o^{2+}, extracellular Ca^{2+}; CaR, calcium sensing receptor; ER, endoplasmic reticulum; HDV, high-density vesicle; HEPES, N-2-hydroxyl piperazine-N'-2-ehane sulfonic acid HHD, Hailey–Hailey disease; IP_3R, IP_3 receptor; LDV, low-density vesicle; NHK, normal human keratinocyte; PLC, phospholipase C; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SOC, store-operated channel SOCE, store-operated Ca^{2+} entry; SPCA, secretory pathway Ca^{2+}-ATPase; TRPC, canonical transient receptor potential channel

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reticulum Ca^{2+}-ATPase (SERCA) and secretory pathway Ca^{2+}-ATPase (SPCA) Ca^{2+} pumps to accumulate Ca^{2+} (Taylor *et al.*, 1997; Rojas *et al.*, 2000). The importance of ER and Golgi in mediating Ca^{2+} signaling and maintaining normal epidermis physiology is signified in two skin disorders, Darier's disease (Sakuntabhai *et al.*, 1999) and Hailey–Hailey disease (HHD) (Hu *et al.*, 2000), which are caused by mutations inactivating *ATP2A2* and *ATP2C1* genes, which encode SERCA2 and SPCA1, respectively. Keratinocytes from the patients' skin display defective cell–cell adhesion and impaired differentiation (Dhitavat *et al.*, 2004; Foggia and Hovnanian, 2004). Previous studies have shown that in keratinocytes CaR localizes predominantly in an intracellular perinuclear compartment with lower levels in the plasma membrane (Tu *et al.*, 2001, 2004). This cytoplasmic localization of CaR is often observed in other cell types, for example, osteoblasts, articular and growth plate chondrocytes (Chang *et al.*, 1999), and pancreatic acinar cells (Bruce *et al.*, 1999). Although it has been hypothesized that CaR mediates Ca^{2+} sensing not only in the plasma membrane but also within the intracellular stores (Brown and MacLeod, 2001), the mechanism by which this occurs is unclear.

A persistent increase in Ca_i^{2+} is necessary for induction and maintenance of differentiation in keratinocytes. Blocking the rise in Ca_i^{2+} with an Ca_i^{2+} chelator blocked the ability of Ca_o^{2+} to induce differentiation (Li *et al.*, 1995). Moreover, an agent such as ATP, which triggers only an acute increase of Ca_i^{2+} fails to induce differentiation (Pillai and Bikle, 1992). PLCγ1 activity is required for sustaining the rise in Ca_i^{2+} and initiating differentiation (Xie and Bikle, 1999). Raising Ca_o^{2+} stimulates the expression level and lipase activity of PLCγ1 (Xie *et al.*, 2005), resulting in increased IP_3 and Ca^{2+} release from stores. After emptying internal Ca^{2+} stores, PLCγ1 and IP_3R further increase Ca_i^{2+} by activating Ca^{2+} entry via direct interactions with SOCs (Boulay *et al.*, 1999; Patterson *et al.*, 2002; Tu *et al.*, 2005). Whether CaR regulates Ca_i^{2+} by directly modulating Ca^{2+} influx through channels, for example, SOC, on the plasma membrane is uncertain.

In this study, we investigated the hypothesis that CaR mediates the Ca_i^{2+} response to Ca_o^{2+} via interactions with other critical Ca^{2+} modulators of internal stores and channels. We found that glycosylated CaR forms a protein complex with PLCγ1, IP_3R, and SPCA1 in *trans*-Golgi. Inhibition of CaR expression led to a steep decline of Ca_i^{2+} pools with enhanced Ca^{2+} uptake by stores *in vitro* and Ca^{2+} influx through SOC. These changes in Ca^{2+} handling disabled the Ca_i^{2+} response to Ca_o^{2+}, and consequently impeded differentiation in keratinocytes.

RESULTS

CaR localizes in *trans*-Golgi in keratinocytes

In previous studies, we had demonstrated that most of the CaR in keratinocytes was intracellular. The functional significance of this observation was unclear, although this intracellular location suggested that CaR might interact with the Ca^{2+} pumps and channels in the subcellular organelles to regulate their activities. As an initial test of this hypothesis, membrane fractionation was performed to assess whether CaR and these Ca^{2+} regulators localize to the same subcellular compartments. The membrane preparations of keratinocytes cultured in 0.03 mM $CaCl_2$ were fractionated on a 15–50% discontinuous sucrose gradient. The fractions were collected and analyzed by immunoblotting using antibodies against specific organelle markers and various Ca^{2+} regulators, including CaR, PLC, IP_3R, and the Ca^{2+} pumps SERCA2 and SPCA1. As shown in Figure 1a, the ER (Bip and calreticulin) and the *cis*-Golgi marker (GM130) were primarily in the low-density (15 and 25% sucrose) fractions, the *trans*-Golgi markers (TGN38 and p230) were in the high-density (40 and 50% sucrose) fractions, and the plasma membrane markers (α2-integrin and canonical transient receptor potential channel 4 (TRPC4)) were in the intermediate-density (25 and 35% sucrose) fractions. We next compared the sucrose fractions collected from cells grown in either 0.03 or 1.2 mM $CaCl_2$ to examine whether Ca_o^{2+} changes the distribution pattern of the various Ca^{2+} regulators including CaR. As shown in Figure 1b, the distribution of the 120 kDa non-glycosylated CaR was limited to the ER/*cis*-Golgi fractions, whereas the 140 and 160 kDa glycosylated forms of the CaR were mainly in the *trans*-Golgi fractions. Only a small fraction of CaR was present in the plasma membrane fractions. Raising Ca_o^{2+} to 1.2 mM increased the levels of the non-glycosylated CaR in the ER fractions and glycosylated CaR in the plasma membrane fractions. PLCβ1 was strictly localized in the plasma membrane fractions regardless of the concentration of Ca_o^{2+}. In cells cultured in 0.03 mM $CaCl_2$, PLCγ1 was found mainly in the *trans*-Golgi fractions, whereas the level of PLCγ1 in the plasma membrane fractions increased in cells grown in 1.2 mM $CaCl_2$. Three forms of IP_3R were found in keratinocytes. The 260 kDa form of IP_3R was present mostly in the ER/*cis*-Golgi fractions, the 310 kDa form was widely distributed in various subcellular compartments, and the 340 kDa form was found primarily in the plasma membrane and *trans*-Golgi fractions. The ER Ca^{2+}-ATPase, SERCA2, localized to the ER/*cis*-Golgi fractions, and the Golgi Ca^{2+}-ATPase, SPCA1, localized to the *trans*-Golgi fractions as expected. These results indicate that the glycosylated CaR, PLCγ1, SPCA1, and high molecular weight forms of IP_3R localize to *trans*-Golgi fractions. Fluorescence immunolocalization of CaR, PLCγ1, IP_3R, and SPCA1 demonstrated that these calcium regulators extensively colocalize with the *trans*-Golgi protein TGN38 (Figure 1c), confirming the membrane fractionation results. In contrast, as shown in Figure 1d, minimal colocalization of CaR and the ER protein Bip was detected.

CaR, IP_3R, PLCγ1, and SPCA1 form a protein complex in *trans*-Golgi

To determine whether CaR interacts directly with IP_3R, PLCγ1, and SPCA1, we performed co-immunoprecipitation experiments. The membrane proteins in the *trans*-Golgi fractions from keratinocytes cultured in 0.03 mM Ca^{2+} were incubated with mAbs against IP_3R, PLCγ1, or SPCA1, and precipitated with protein G-conjugated Sepharose beads. The immunoprecipitates were then analyzed for the presence of CaR, IP_3R, PLCγ1, and SPCA1 by immunoblotting. As shown

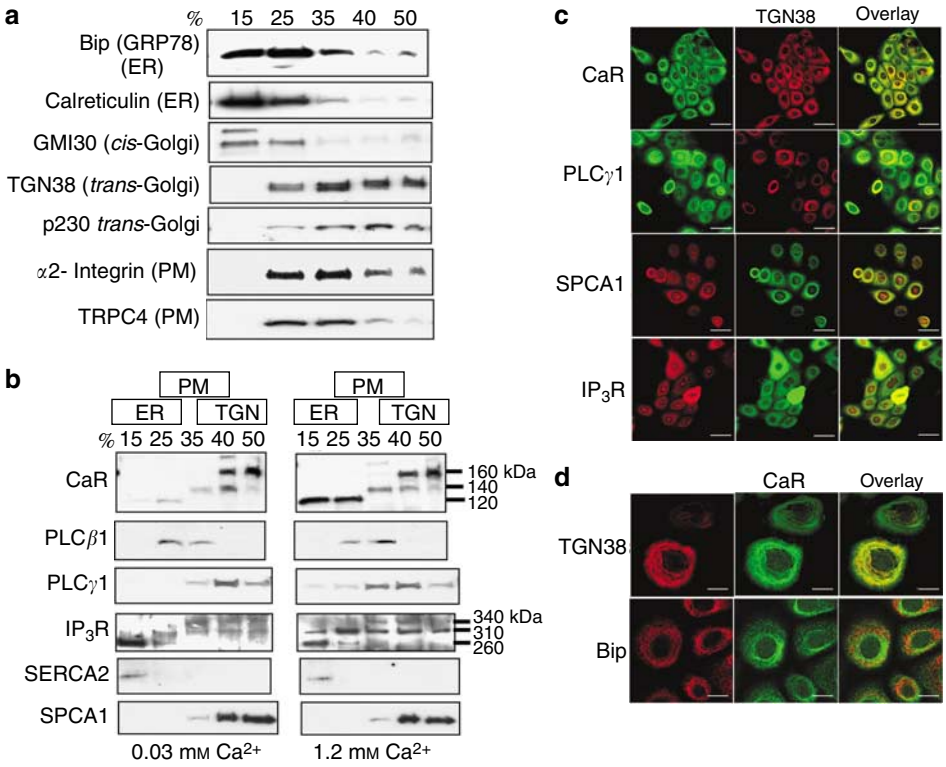

Figure 1. Localization of CaR and other Ca_i^{2+} regulators in the *trans*-Golgi. Crude keratinocyte membranes were separated by a discontinuous sucrose gradient (15–50%), and the fractions were collected and analyzed for markers of (**a**) subcellular organelles and (**b**) proteins of interest. The unglycosylated CaR (120-kDa form) and SERCA localize to the ER fractions, whereas the glycosylated CaR (the 140- and 160-kDa forms) colocalizes with the 310-kDa form of IP_3R, PLCγ1, and the Golgi Ca^{2+}-ATPase, SPCA1, in the *trans*-Golgi (TGN) fractions. (**c**) Fluorescence immunostaining of CaR, IP_3R, PLCγ1, SPCA1, and TGN38. Keratinocytes grown in 0.03 mM Ca^{2+} were stained with polyclonal antibodies against CaR, PLCγ1, IP_3R, and SPCA1 and a mAb against TGN38, a *trans*-Golgi structural protein, followed by the appropriate FITC- or Texas Red-conjugated secondary antibodies. Fluorescent signals were detected with a confocal microscope (original magnification ×40). Substantial colocalization of CaR, IP_3R, PLCγ1, SPCA1, and TGN38 in Golgi was observed. (**d**) CaR extensively colocalized with TGN38. On the contrary, minimal colocalization was detected between CaR and Bip, an ER protein. The original magnification of these images is ×100. Bars = (**c**) 50 μm, (**d**) 20 μm. Similar observations were made in at least two separate cell preparations.

in Figure 2a, each antibody not only precipitated its own target protein but also brought down CaR and the other calcium regulators, indicating that CaR, PLCγ1, IP_3R, and SPCA1 form a protein complex within the *trans*-Golgi. However, none of the antibodies precipitated TGN38, demonstrating that formation of this protein complex was not due to the coincidental localization of these proteins in the trans-Golgi. Fluorescence immunolocalization by confocal microscopy verified the colocalization of CaR with PLCγ1, IP_3R, and SPCA1 in a perinuclear compartment, likely the *trans*-Golgi, in keratinocytes (Figure 2b).

Both ER and Golgi function as internal Ca^{2+} stores in keratinocytes

In the membrane fractionation scheme used for these experiments, the low-density fractions are enriched with ER/*cis*-Golgi vesicles, whereas the high-density fractions are enriched with *trans*-Golgi vesicles. To investigate whether these vesicles operate as internal Ca^{2+} stores, we measured the Ca^{2+} uptake and Ca^{2+} release in these two groups of vesicles. Low-density vesicles (LDVs) and high-density vesicles (HDVs) were collected from keratinocytes grown in 0.03 or 1.2 mM Ca^{2+}, then ATP-dependent Ca^{2+} uptake was measured. As shown in Figure 3a, HDV had a higher ATP driven Ca^{2+} uptake than LDV ($P<0.01$, $n=3$). Whereas HDVs from cells cultured in 1.2 mM Ca^{2+} (2801±218 c.p.m.) were less efficient in accumulating Ca^{2+} than those from cells grown in 0.03 mM Ca^{2+} (4698±65 c.p.m.) ($P<0.01$, $n=3$), the higher Ca_o^{2+} had the opposite effect on the Ca^{2+}-uptake ability of LDV (1.2 *vs* 0.03 mM Ca^{2+}, 659±46 *vs* 474±18 c.p.m.; $P<0.01$, $n=3$). To test whether the release of Ca^{2+} from these vesicles is regulated, we compared the ability of IP_3 to induce Ca^{2+} release from HDV and LDV. In this experiment (Figure 3b), the IP_3-induced Ca^{2+} release was expressed as % of the maximal release induced by the Ca^{2+} ionophore, ionomycin. IP_3 stimulated more Ca^{2+} release from the LDV (80–85% of total) than the HDV (50% of total) ($P<0.05$, $n=3$). These results demonstrate that these membrane vesicles have the ability to accumulate and release Ca^{2+} in a controlled manner, indicating that both ER and Golgi serve as regulated Ca_i^{2+} stores in keratinocytes.

To determine whether CaR regulates function of Ca^{2+} pumps, we next examined the impact of inhibiting CaR expression on the ability of intracellular stores to accumulate Ca^{2+}. Preconfluent keratinocytes were infected with an

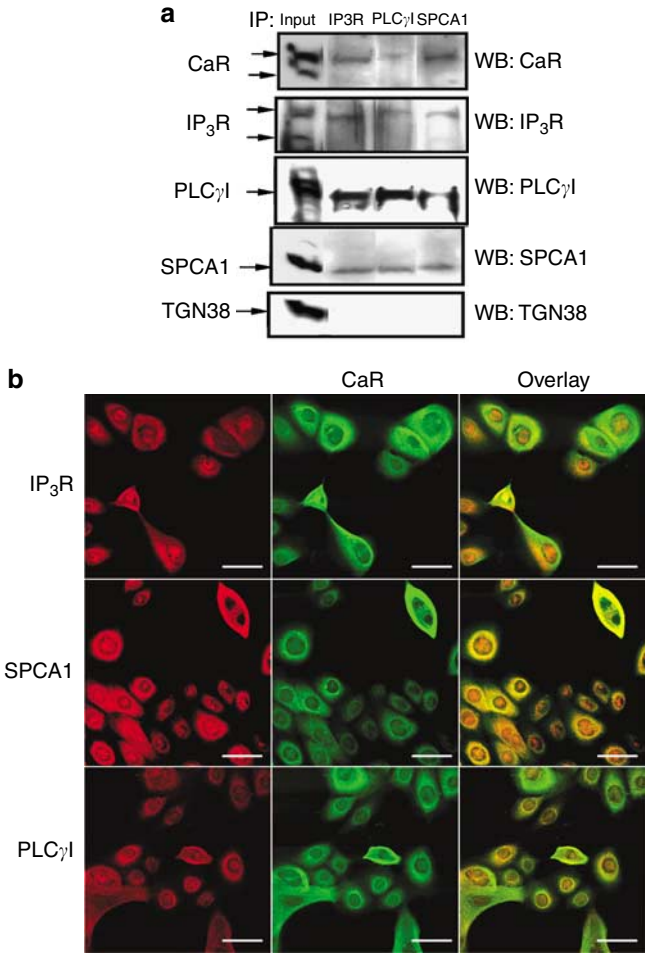

Figure 2. CaR forms a protein complex with IP_3R, PLCγ1, and SPCA1 in *trans*-Golgi. (**a**) Co-immunoprecipitation of CaR with IP_3R, PLCγ1, and SPCA1 but not TGN38. The *trans*-Golgi membrane fraction was immunoprecipitated with antibodies for IP_3R, PLCγ1, and SPCA1. The immunoprecipitates were then analyzed by immunoblotting for these proteins, CaR, and the *trans*-Golgi marker TGN38 as a negative control. Each antibody immunoprecipitated its own target protein and the other three calcium regulators, but not TGN38, indicating that CaR, IP_3R, PLCγ1, and SPCA1 form a protein complex in the *trans*-Golgi. (**b**) Fluorescence immunostaining of CaR, IP_3R, PLCγ1, and SPCA1 in human keratinocytes. Keratinocytes grown in 0.03 mM Ca^{2+} were stained with mAbs against PLCγ1, IP_3R, and SPCA1 and a polyclonal antibody against CaR, followed by FITC-conjugated anti-rabbit and Texas Red-conjugated anti-mouse antibodies. Fluorescent signals were detected with a confocal microscope (original magnification ×40). Bar = 50 μm. Substantial colocalization of CaR with IP_3R, PLCγ1, and SPCA1 was detected in a perinuclear intracellular compartment, likely Golgi. Similar observations were made in three separate cell preparations.

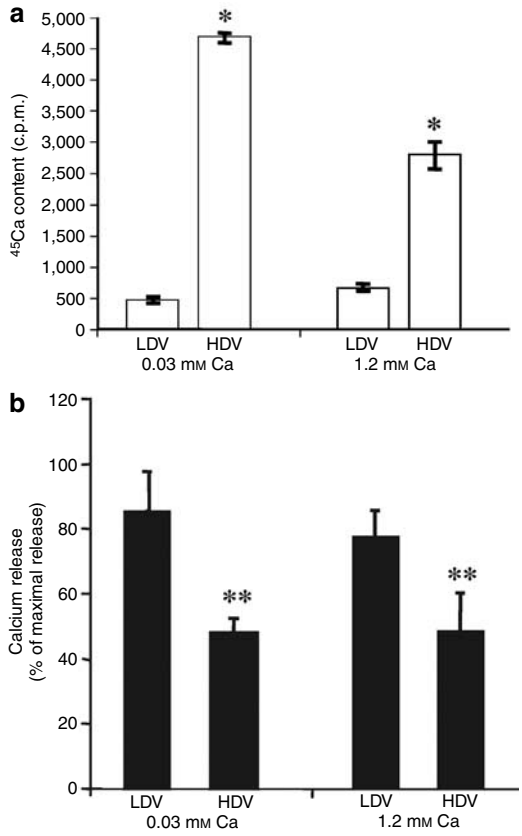

Figure 3. Ca^{2+} flux in low- and high-density vesicles. (**a**) ATP-dependent Ca^{2+} uptake by and (**b**) IP_3-dependent Ca^{2+} release from ER and *trans*-Golgi of keratinocytes. Membrane preparations from keratinocytes cultured in 0.03 or 1.2 mM Ca^{2+} were separated on a 15–50% sucrose gradient. For Ca^{2+} uptake measurements, 30 μg of membrane vesicles proteins collected from the ER-rich low-density (LDV) and the *trans*-Golgi-rich high-density (HDV) fractions were incubated in an EGTA-buffered Ca^{2+} solution containing 1 μM free Ca^{2+}. Ca^{2+} uptake was initiated by the addition of 5 mM ATP in the presence of Ca^{45}. For Ca^{2+} release assays, LDV and HDV were collected and loaded with Ca^{45} as described in the Materials and Methods. Ca^{2+} release was initiated by the addition of either 1 μM IP_3 or 20 μM ionomycin. The IP_3-induced Ca^{2+} release was expressed as % of the maximal release induced by ionomycin. Each data point shows the mean ± SD of triplicate determinations. Asterisks denote statistical significance as compared to LDV. $*P<0.01$, $**P<0.05$. Whereas HDV were more efficient in accumulating Ca^{2+} than LDV, IP_3 stimulated a greater percentage of Ca^{2+} release from LDV than HDV. The data presented are representative of at least three independent experiments.

adenovirus carrying a CaR antisense cDNA (Ad-ASCaR), and the expression of endogenous CaR was measured by immunoblotting (Figure 4a) and immunostaining (Figure 4b). Ad-ASCaR nearly completely blocked the endogenous CaR protein level as compared with the cells infected with a control adenovirus (Ad-DNR). LDV and HDV were then collected from keratinocytes infected with Ad-ASCaR or Ad-DNR, and assessed for ATP-dependent Ca^{2+} uptake. As shown in Figure 5a, a reduction in CaR with Ad-ASCaR promoted the ATP-dependent Ca^{2+} uptake in both LDV and HDV. This was accompanied by an increase of SERCA2 and SPCA1 protein levels (Figure 5b). However, we did not find a consistent effect of inhibiting CaR expression on Ca^{2+} release from these vesicles.

Inhibition of CaR expression reduced Ca_i^{2+} stores

To determine whether inhibition of CaR expression altered Ca_i^{2+} stores, we knocked down endogenous CaR expression with Ad-ASCaR and examined its impact on Ca_i^{2+} response to Ca_o^{2+} and Ca_i^{2+} store. Consistent with our previous study (Tu *et al.*, 2001), knockdown of CaR inhibited the Ca_i^{2+} response to raised Ca_o^{2+}. As shown in Figure 6a, raising Ca_o^{2+}

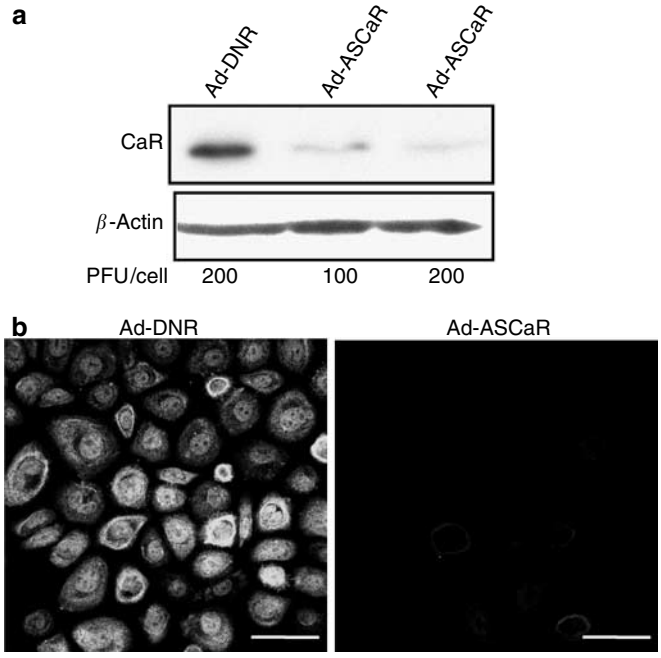

Figure 4. Inhibition of the expression of endogenous CaR by a CaR antisense cDNA. Human keratinocytes were infected by an adenovirus carrying a full-length CaR antisense cDNA (Ad-ASCaR) or an Ad-DNRcontrol virus (Ad-DNR) at a titer of 100 or 200 plaque-forming units (p.f.u.)/cell. Seven days after infection, endogenous CaR protein levels were examined (**a**) by immunoblotting of total cell lysates and by (**b**) fluorescence immunostaining using a polyclonal antibody against CaR. Bar = 50 μm. Infection of the Ad-ASCaR efficiently decreased the expression of endogenous CaR proteins as compared with cells infected with control adenovirus (Ad-DNR).

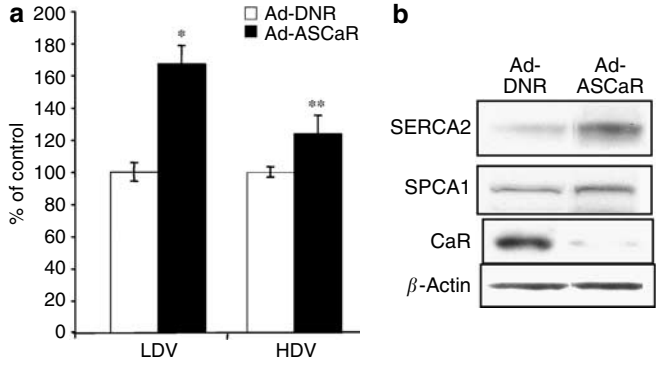

Figure 5. CaR knockdown enhanced Ca^{2+} uptake by ER and *trans*-Golgi of Keratinocytes. (**a**) Increased ATP-dependent Ca^{2+} uptake into keratinocyte vesicles and (**b**) the increased expression level of SERCA2 and SPCA1 proteins after CaR depletion. Keratinocytes were infected with an Ad-ASCaR or a control virus (Ad-DNR) at a titer of 200 plaque-forming units (p.f.u.)/cell. Cells were maintained in medium containing 0.03 mM Ca^{2+}. (**a**) Seven days after infection, membrane vesicles in the LDV and the HDV fractions from sucrose gradients were collected, and ATP-dependent Ca^{2+} uptake was measured as described in the legend to Figure 3. The ATP driven Ca^{2+} uptake in vesicles from keratinocytes infected with Ad-ASCaR was expressed as % of the Ca^{2+} uptake in vesicles from Ad-DNR infected cells. Each data point shows the mean ± SD of triplicate determinations. Asterisks denote statistical significance as compared to Ad-DNR infected cells. *$P<0.01$, **$P<0.05$. The data presented are representative of three independent experiments. (**b**) Total lysates were collected from keratinocytes infected with Ad-ASCaR or Ad-DNR for 7 days. The protein levels of SERCA and SPCA1 were examined by immunoblotting, using β-actin as a loading control.

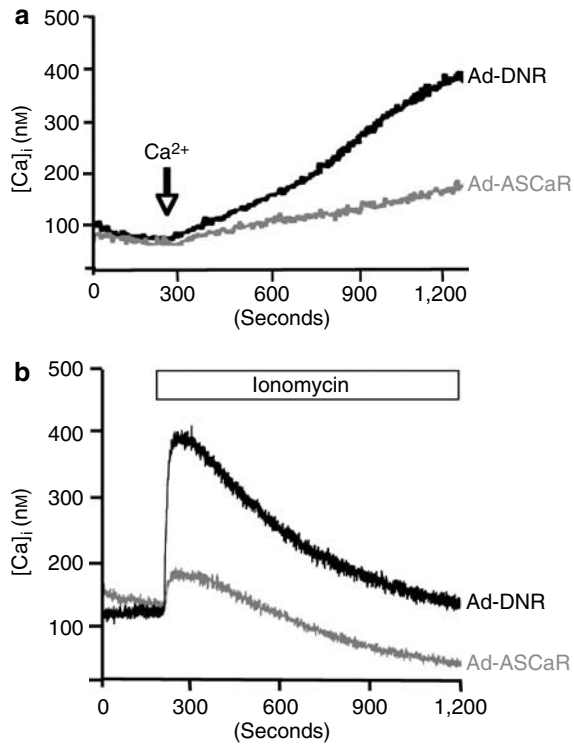

Figure 6. CaR knockdown (a) blunted the acute Ca_i^{2+} response to elevated Ca_o^{2+} and (b) reduced the Ca_i^{2+} pool in keratinocytes. Keratinocytes were grown on glass coverslips and infected with an Ad-ASCaR or a control virus (Ad-DNR) in medium containing 0.03 mM Ca^{2+} then loaded with Fura-2. (**a**) Ca_i^{2+} was initially measured in buffer containing 0.03 mM Ca^{2+} and then following the addition of 2 mM Ca^{2+}. (**b**) Keratinocytes were cultured, infected with adenoviruses, and loaded with Fura-2 in medium containing 0.03 mM Ca^{2+}. Ca_i^{2+} was measured before and after the addition of ionomycin (20 μM) in the absence of Ca_o^{2+}. The trace shown in the figure represents the average Ca_i^{2+} of 21–50 individual keratinocytes during recording. The CaR antisense cDNA significantly reduced the Ca_i^{2+} response to Ca_o^{2+} and the ionomycin-sensitive releasable Ca_i^{2+}. The results are representative of four different experiments.

from 0.03 to 2 mM induced an increase in Ca_i^{2+} in keratinocytes infected with the control virus Ad-DNR from 91 ± 2 to 392 ± 43 nM (mean ± SD; $n = 29$). Keratinocytes infected with the Ad-ASCaR virus had comparable resting Ca_i^{2+} (81 ± 11 nM; $n = 21$), but had a marked reduction in the rise of Ca_i^{2+} (to 182 ± 33 nM) in response to 2 mM Ca_o^{2+}. The blunted Ca_i^{2+} response was correlated with a decrease in the Ca_i^{2+} pool, as revealed by ionomycin administration in the absence of Ca_o^{2+} (Figure 6b). Ionomycin (20 μM) induced Ca^{2+} release from internal stores and, as a result, a rise in Ca_i^{2+} (increased from 118 ± 12 to 394 ± 44 nM, $n = 50$) in cells infected with Ad-DNR. Knockdown of CaR expression by Ad-ASCaR led to a substantial reduction of the rise in Ca_i^{2+} (from 116 ± 20 to 180 ± 21 nM, $n = 48$). Thus, lack of CaR caused an approximate 75% decrease in the Ca_i^{2+} pools, suggesting that CaR regulates Ca_i^{2+} levels in part by modulating internal Ca^{2+} stores. This decrease in Ca^{2+} stores may have led to the compensatory increase in SERCA2 and SPCA1 levels and activity shown in Figure 5.

Inhibition of CaR expression increased store-operated Ca^{2+} entry

In most cell types, including keratinocytes, reduction of Ca^{2+} content in internal stores by agonist stimulation or store-depletion agents, such as the SERCA blocker thapsigargin (Tg), triggers calcium influx through channels in the plasma membrane, a process known as store-operated Ca^{2+} entry (SOCE) (Putney *et al.*, 2001; Tu *et al.*, 2005). To assess the role of CaR in regulation of Ca^{2+} entry, we compared SOCE in keratinocytes infected with Ad-ASCaR to that in keratinocytes infected with Ad-DNR. Adenovirus-infected keratinocytes were treated with Tg to deplete internal stores, and SOCE was monitored by measuring the Ca_i^{2+}. In the presence of 0.1 mM EGTA, 1 μM Tg elicited an initial increase in Ca_i^{2+} (from 105 ± 10 to 220 ± 18 nM, $n=50$) in the Ad-DNR-infected cells owing to passive release from Ca^{2+} stores. After Ca_i^{2+} recovered to resting levels, addition of 2 mM Ca_o^{2+} induced a second phase of increased Ca_i^{2+} (to 212 ± 15 nM) resulting from Ca^{2+} influx through SOC in the plasma membrane (Figure 7a). Consistent with the decline in internal Ca^{2+} stores demonstrated in Figure 6, the Tg-induced Ca^{2+} release in keratinocytes infected with Ad-ASCaR was reduced (Ca_i^{2+} from 107 ± 16 to 170 ± 13 nM, $n=42$) compared to Ad-DNR-infected cells (from 105 ± 10 to 220 ± 18 nM). However, these cells responded to Ca^{2+} replenishment with an amplified Ca^{2+} entry, resulting in a greater rise in Ca_i^{2+} (to 313 ± 19 nM) (Figure 7b) than in cells infected with Ad-DNR (to 212 ± 15 nM). This enhancement was not because of changes in SOC expression, as the protein levels of TRPC1 and TRPC4, which we previously had shown were the major SOCs in keratinocytes (Tu *et al.*, 2005), were not affected by CaR knockdown (Figure 7c).

CaR depletion suppressed Ca_o^{2+}-induced keratinocyte differentiation

To investigate whether these changes in Ca^{2+} handling following reduction in CaR expression affected differentiation, we examined the impact of CaR depletion on Ca_o^{2+}-induced expression of the intermediate and late differentiation markers, transglutaminase type I (TG-1) and filaggrin, respectively. As shown in Figure 8, 72 hours of incubation in 1.2 mM Ca^{2+} significantly increased the protein levels of transglutaminase and filaggrin in keratinocytes infected with Ad-DNR. However, the stimulation of transglutaminase and filaggrin expression by Ca_o^{2+} was blocked in cells infected with Ad-ASCaR.

DISCUSSION

Intracellular perinuclear distribution of CaR is commonly observed in many cell types, including keratinocytes. It has been long speculated that instead of simply nascent receptor protein being processed through the biosynthetic pathway, the cytoplasmic CaR plays a distinctive role in regulating cellular Ca^{2+} homeostasis, for instance, by serving as a store Ca^{2+} sensor (Brown and MacLeod, 2001). Whether it regulates Ca^{2+} stores had not previously been established. In this study, we demonstrate that in addition to its function as a membrane receptor sensing the changes in Ca_o^{2+} and relaying the Ca^{2+} signal into cells, CaR directly interacts with several Ca_i^{2+} modulators, that is, PLCγ1, IP_3R, and SPCA1, in the *trans*-Golgi and regulates the functions of Ca^{2+} channels and Ca^{2+} pumps which control Ca^{2+} stores. Inhibition of CaR expression in keratinocytes caused a conspicuous decline in the Ca^{2+} content of intracellular stores. The decrease in Tg-induced Ca^{2+} release and consequential augmentation in SOC activity featured by these cells are

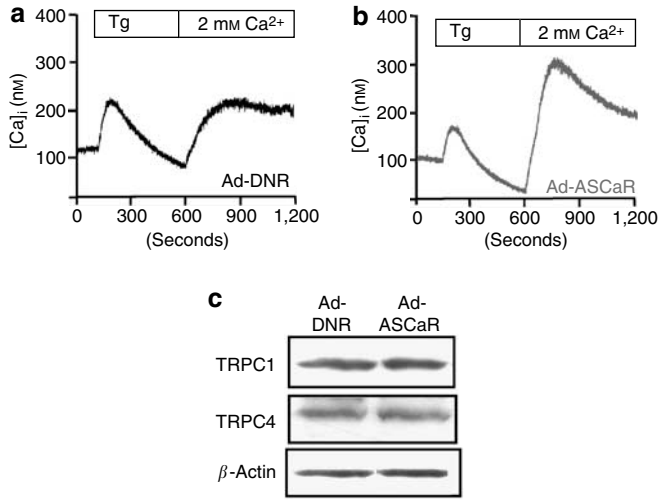

Figure 7. CaR knockdown enhances the SOCE in keratinocytes. Keratinocytes are cultured on cover slips, infected with an (**b**) Ad-ASCaR or (**a**) a control virus (Ad-DNR) in medium containing 0.03 mM Ca^{2+}, and loaded with Fura-2. Internal Ca^{2+} stores were depleted with 1 μM Tg in the absence of Ca_o^{2+}. Ca^{2+} influx was initiated by addition of 2 mM of Ca^{2+} to the medium. Ca_i^{2+} measurement was performed as described previously. The results are representative of three separate experiments. (**c**) Total lysates were collected from keratinocytes infected with Ad-ASCaR or Ad-DNR. The protein levels of endogenous SOC were determined by immunoblotting using polyclonal antibodies against TRPC1 and TRPC4, the major SOCs in keratinocytes. β-Actin was measured as a loading control.

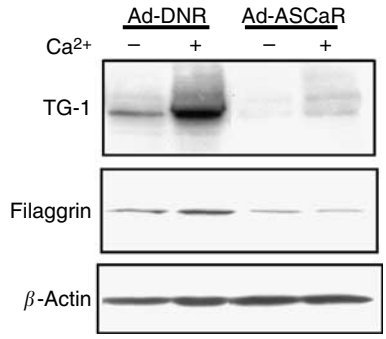

Figure 8. Inhibition of CaR expression blocked Ca_o^{2+}-stimulated expression of differentiation markers, transglutaminase and filaggrin. Keratinocytes were infected with an Ad-ASCaR or a control virus (Ad-DNR) for 7 days. Keratinocytes were either maintained in 0.03 mM or switched to 1.2 mM Ca^{2+} for 72 hours, and total lysates were collected and analyzed for transglutaminase type I (TG-1) and filaggrin by immunoblotting. The Ca_o^{2+}-stimulated expression of transglutaminase and filaggrin was blocked in cells infected with Ad-ASCaR. The data are representative of two independent experiments.

consistent with reduced Ca_i^{2+} pools, including the ER and Golgi. Although ER is generally believed to be the major Ca^{2+} store in most cell types, emerging evidence indicates that the Golgi functions as the main Ca^{2+} reservoir in keratinocytes (Behne *et al.*, 2003; Callewaert *et al.*, 2003) and mediates Ca^{2+} signaling in these cells (Foggia *et al.*, 2006). This may explain why the defective SPCA1 caused by mutations in the *ATP2C1* gene in HHD preferentially affects the skin (Hu *et al.*, 2000). CaR abrogation enhanced the ability of ER and Golgi to accumulate Ca^{2+}, in part, owing to upregulation of SERCA2 and SPCA1. This is probably a compensatory mechanism for the decreased Ca^{2+} stores in these organelles. Nevertheless, one must consider the concept that CaR plays a part in the feedback mechanism that regulates Ca^{2+}-ATPase activity corresponding to the Ca^{2+} level in the store lumen. When the lumen Ca^{2+} level is high, CaR might inhibit the Ca^{2+} uptake by forming a protein complex with a Ca^{2+}-ATPase, such as SPCA1. As the lumen Ca^{2+} level decreases after agonist-induced Ca^{2+} release, Ca^{2+}-ATPase would be liberated from the interaction with CaR and resume Ca^{2+} uptake into stores. Therefore, in the keratinocytes lacking CaR, Ca^{2+} uptake activities by the ER and Golgi would be enhanced.

Consistent with its effect to deplete internal stores, inhibition of CaR expression also promoted the SOC-mediated Ca^{2+} influx. It is unclear how CaR regulates the SOC, but it may involve its interactions with PLCγ1 and IP_3R. PLCγ1 is crucial for instigation of agonist-induced Ca^{2+} entry and SOCE, as inhibition of PLCγ1 completely abolished activation of SOC in keratinocytes (Tu *et al.*, 2005). PLCγ1 physically interacts with several TRPC channels (Patterson *et al.*, 2002; Tu *et al.*, 2005), the molecular basis of SOC (Clapham *et al.*, 2001). We previously demonstrated that activation of SOC requires the direct interaction of PLCγ1 and IP_3R with the TRPC1 channel, one of the major SOC in keratinocytes (Tu *et al.*, 2005). It is conceivable that lack of CaR would free PLCγ1 and IP_3R from the protein complex in the internal Ca^{2+} stores, that is, *trans*-Golgi, enabling PLCγ1 and IP_3R to interact with TRPC channels on the plasma membrane and trigger Ca^{2+} influx. Interestingly, CaR depletion caused diminished total Ca^{2+} in keratinocytes, but these cells maintain a normal resting Ca_i^{2+} probably as a result of increased Ca^{2+} influx via SOC. It is unclear what causes the reduction in Ca_i^{2+} pools in CaR-deficient keratinocytes. As the activities of store Ca^{2+} pumps and SOC were not inhibited by CaR depletion, downregulation of other Ca^{2+} channels critical for store refilling is suspected. This hypothesis will be tested in our future studies.

Inhibition of CaR altered several aspects of keratinocyte physiology: loss of Ca_i^{2+} response to Ca_o^{2+}, defective cell–cell adhesion (unpublished observation), and decreased cell differentiation. Similar phenotypic changes have been observed in keratinocytes from the HHD patients (Missiaen *et al.*, 2004). Because of SPCA1 deficiency, HHD keratinocytes feature low intra-Golgi Ca^{2+} and high cytosolic Ca^{2+} concentrations, and do not respond to raised Ca_o^{2+} (Hu *et al.*, 2000). Despite high cytosolic Ca^{2+}, these cells are characterized by loss of cell–cell adhesion and abnormal actin reorganization (Aronchik *et al.*, 2003). Thus, reduced internal Ca^{2+} stores contribute to the loss of Ca^{2+}-mediated Ca^{2+} response and consequent impairment of terminal differentiation in CaR-deficient and HHD (i.e. SPCA1 deficient) keratinocytes. It is not clear how the Ca_i^{2+} stores affect differentiation. The defective Ca^{2+} signaling in keratinocytes lacking CaR could influence gene expression or alter post-translational modification of target proteins critical for differentiation and survival. Alternatively, the low levels of luminal Ca^{2+} in the ER and the Golgi apparatus could impair translation, processing, maturation, folding, and trafficking of membrane-associated proteins important in cell adhesion and Ca^{2+} transport.

In summary, we demonstrated the *trans*-Golgi localization of CaR in keratinocytes, and its involvement in regulating Ca^{2+} signaling and differentiation. The effect of CaR depletion on differentiation might be secondary to defective Ca_i^{2+} stores and changes in Ca^{2+} handling, which are regulated by CaR via interactions with other Ca_i^{2+} modulators. Thus, the role of the CaR in maintaining cellular Ca^{2+} homeostasis remains open for further investigation, but this study indicates that it has such a role.

MATERIALS AND METHODS

Materials

Ionomycin and Tg were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Stock solutions of these compounds were prepared in DMSO. All other chemicals were purchased form Sigma-Aldrich (St Louis, MO). All DNA constructs used in generation of adenoviruses were prepared using Qiagen Maxi-prep columns (Chatsworth, CA), according to the manufacturer's protocol. mAbs for organelle markers Bip (GRP78), GM130, TGN38, p230, and α2-integrin were obtained from BD Biosciences (Palo Alto, CA). The polyclonal antibody for CaR, ADDR, was raised against the peptide corresponding to amino acids 215–236 of the human keratinocyte CaR (Tu *et al.*, 2001). Polyclonal antibodies and mAbs against IP_3R were from Calbiochem-Novabiochem Corp. Antibodies against PLCβ1, PLCγ1, SERCA2, SPCA1, and calreticulin were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against TRPC1 and TRPC4 were purchased from Chemicon International (Temecula, CA). The antibodies for human transglutaminase type I and filaggrin were purchased from Biomedical Technologies (Stoughton, MA) and Covance Research Product Inc. (Denver, PA), respectively.

Cell culture

Normal human keratinocytes (NHKs) were isolated form neonatal human foreskins and grown in serum-free keratinocyte growth medium (154CF, Cascade Biologics, Portland, OR) as described (Gibson *et al.*, 1996). Briefly, keratinocytes were isolated from newborn human foreskins by trypsinization (0.25% trypsin, 4°C, 16 hours), and primary cultures were established in growth medium containing 0.07 mM $CaCl_2$. Second passage keratinocytes were plated in medium containing 0.03 mM $CaCl_2$ and used in the experiments described. The study was conducted according to the Declaration of Helsinki Principles, and the use of human keratinocytes was approved by the Committee for Human Research at the University of California San Francisco and the San Francisco Veteran Affair medical Center.

Generation of adenoviral stocks and infection of keratinocytes
Adenoviruses carrying the antisense human CaR cDNA were made using an Adeno-X Expression System II kit (BD Biosciences), according to the manufacturer's instructions. A 3.3 kb human CaR cDNA encompassing the full-length open reading frame was subcloned in an antisense orientation into a pDNR-CMV donor vector containing two loxP sites flanking the cloning site. The antisense CaR cDNA was then transferred by Cre/lox recombination to an adenoviral acceptor cosmid to generate the Ad-ASCaR construct. A control Ad-DNR construct was constructed by transferring empty pDNR-CMV vector to the adenoviral acceptor cosmid. After amplifying in human embryonic kidney 293 cells, viral particles were collected and titered using an Adeno-X rapid titer kit (BD Biosciences) and used to infect NHKs. For inactivation of the CaR, subconfluent NHKs were infected with an adenovirus carrying the Ad-ASCaR construct (100–200 plaque-forming units (p.f.u.)/cell) in growth medium containing 0.03 mM $CaCl_2$ and cultured for 5–7 days before subcellular fractionation or exposure to 1.2 mM $CaCl_2$ for 72 hours to induce differentiation. Three days after infection, the viral supernatant was replaced with fresh culture medium containing 0.03 mM $CaCl_2$. No additional adenovirus was provided after the initial infection. Control cells were infected with an adenovirus carrying the Ad-DNR vector.

Measurement of cytosolic Ca^{2+}
The Ca_i^{2+} responses to elevated Ca_o^{2+}, ionomycin, and the SOCE were measured using a Dual-wavelength Fluorescence Imaging System (Intracellular Imaging Inc., Cincinnati, OH) as described (Oda *et al.*, 2000). Preconfluent keratinocytes were infected with an Ad-ASCaR or a control virus (Ad-DNR) on a coverslip in keratinocyte growth medium containing 0.03 mM Ca^{2+}. Five to 7 days later, cells were loaded with 5 μM Fura-2/AM (Molecular Probes, Eugene, OR) in 0.1% Pluronic F127 in buffer A (20 mM *N*-2-hydroxyl piperazine-*N*′-2-ehane sulfonic acid (HEPES), 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mg/ml sodium pyruvate, 1 mg/ml glucose) containing 0.07 mM Ca^{2+}. Cells were then washed and measured in buffer A containing 0.03 mM Ca^{2+} before exposure to 2 mM Ca^{2+}. The cells were alternately illuminated with 340 and 380 nm light, and the fluorescence at emission wavelength 510 nm was recorded. For SOCE measurement, Ca^{2+} stores were depleted by 1 μM Tg in 0.1 mM EGTA, and Ca^{2+} entry in Fura2-loaded keratinocytes was determined in the presence of 2 mM Ca^{2+}. All experiments were performed at room temperature. The signals from 20 to 50 single cells for each measurement were recorded. Each sample was calibrated by the addition of 20 μM ionomycin (R_{max}) followed by 20 mM EGTA/Tris, pH 8.3 (R_{min}). Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated from the ratio of emission at the two excitation wavelengths based on the formula $[Ca^{2+}]_i = K_d Q(R-R_{min})/(R_{max}-R)$, $R = F_{340}/F_{380}$, $Q = F_{min}/F_{max}$ at 380 nm, and K_d for Fura-2 for Ca^{2+} is 224 nM. The data presented are representative of three independent experiments.

Membrane fractionation
Membrane fractionation of keratinocytes was carried out as described (Bourguignon *et al.*, 1993) with modifications. Briefly, NHKs were suspended in cold homogenizing buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 1 mM dithiothreitol) supplemented with Complete protease inhibitors (Roche Diagnostics, Indianapolis, IN), and were homogenized with Polytron homogenizer at 27,000 r.p.m., 4°C for 30 seconds. After homogenization, nuclei were removed by centrifugation at 500 × *g* for 5 minutes. The resulting supernatant was centrifuged at 100,000 × *g* for 30 minutes at 4°C. The crude membrane pellets were resuspended in homogenizing buffer and were layered on a discontinuous sucrose gradient consisting of 0, 15, 25, 35, 40, and 50% sucrose (w/w) in a buffer containing 10 mM HEPES, pH 7.0, 50 mM KCl, 1 mM dithiothreitol, 2 mM $MgCl_2$, and protease inhibitors. The gradient was centrifuged at 100,000 × *g* for 16 hours at 4°C. The membranous materials located in various sucrose layers were collected for immunoblotting analyses or used in Ca^{2+} uptake measurements.

Immunoblotting
Membrane proteins of various organelle origins were prepared by sucrose fractionation as described above. Total cell lysates were prepared from NHKs 5–7 days after adenoviral infections. Cells were extracted with radioimmunoprecipitation assay buffer (20 mM HEPES, pH7.4, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 20 μg/ml phenylmethylsulfonyl fluoride and protease inhibitors). The protein concentrations in the various membrane fractions and total lysates were determined by the BCA Protein Assay Kit (Pierce Corp., Rockford, IL). Equal amounts of protein samples were electrophoresed through 5 or 4–15% gradient polyacrylamide gels and electroblotted onto polyvinylidene fluoride membranes (Immobilon-P, 0.45 μm; Millipore Corp., Bedford, MA). After blocking with 5% milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA), the blots were incubated with various primary antibodies at 4°C overnight. Subsequently, the blots were incubated with appropriate HRP-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 hour at room temperature. The bound antibody was visualized using the SuperSignal West Dura Chemiluminescent Kit ((Pierce Corp.) and subsequent exposure to X-ray film. The data presented are representative of three independent experiments.

Co-immunoprecipitation
Membrane proteins in the high-density fractions following subcellular membrane fractionation were collected and used in immunoprecipitation experiments. Five hundred micrograms protein aliquots were immunoprecipitated by 5 μg of mAbs against PLCγ1, SPCA1 (Santa Cruz Biotechnology Inc.) or IP_3R (Calbiochem-Novabiochem Corp.), followed by Sepharose-conjugated protein G (ImmunoLink Immobilized Protein G, Pierce Corp.) in 0.5 ml of cold lysis buffer (1% NP-40 in Tris-buffered saline with protease inhibitors) at 4°C with gentle tumbling overnight. Immunoprecipitates were collected, washed, eluted, and analyzed by immunoblotting.

Fluorescence immunostaining
Keratinocytes were cultured on coverslips, fixed with 4% paraformaldehyde for 20 minutes at room temperature, and permeablized with 1% NP-40 in phosphate-buffered saline for 5 minutes. After blocking with 5% goat serum in phosphate-buffered saline/0.01% Tween-20, cells were incubated with 10 μg/ml of primary antibodies at 4°C for overnight. Subsequently, cells were incubated with the appropriate fluorescein or Texas Red-conjugated secondary antibody (Molecular Probes) at room temperature for 1 hour. For actin staining, cells were incubated with Texas Red-conjugated phalloidin

at room temperature for 1 hour. Finally, coverslips were washed in phosphate-buffered saline, mounted on glass slides using Gel-Mount (Biomeda, Foster City, CA) and examined with a Leica TCS NT/SP confocal microscope (Leica Microsystems, Heidelberg, Germany).

Ca^{2+} flux measurement in LDV and HDV

In Ca^{2+} uptake experiments, membrane vesicles in the ER-rich low-density fractions and the *trans*-Golgi-rich high-density fractions from the subcellular fractionation were collected and resuspended (0.6 mg protein/ml) in a reaction mixture containing 100 mM KCl, 30 mM imidazole (pH 6.8), 5 mM $MgCl_2$, 10 mM phosphocreatine, 35 U/ml creatine kinase (Roche Diagnostics), 0.44 mM EGTA, 5 mM NaN_3, 10 μM each of oligomycin and actimycin A. $CaCl_2$ was added to this solution to generate 1 μM free Ca^{2+}. Subsequently, $^{45}Ca^{2+}$ (10 μCi/ml, Amersham-Pharmacia Biotech) and 5 mM ATP were added to the reaction mixture and incubated at 30°C for 40 minutes. In Ca^{2+} release experiments, 1 μM IP_3 or 20 μM ionomycin was added to the $^{45}Ca^{2+}$-containing vesicles. The maximal amount of Ca^{2+} release occurred 40 seconds after the addition of IP_3. The amount of Ca^{2+} uptake or release by the vesicles was determined by filtering an aliquot of the reaction mixture (containing 30 μg vesicles) through HAWP nitrocellulose filters (0.45 μm, Millipore Corp.) and washing with a buffer containing 120 mM KCl, 20 mM HEPES (pH 7.2), and 1 mM EGTA. The filter-associated radioactivity was analyzed by liquid scintillation counting.

The medical ethical committees of Veteran Affairs Medical Center and University of California approved all described studies.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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