Modulations in Epidermal Calcium Regulate the Expression of Differentiation-Specific Markers

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Mammalian epidermis normally displays a distinctive calcium gradient, with low levels in the basal/spinous layers and high levels in the stratum granulosum. Although changes in stratum granulosum calcium regulate the lamellar body secretory response to permeability barrier alterations, whether modulations in calcium also regulate the expression of differentiation-specific proteins *in vivo* remains unknown. As acute barrier perturbations reduce calcium levels in stratum granulosum, we studied the regulation of murine epidermal differentiation after loss of calcium accompanying acute barrier disruption and by exposure of such acutely perturbed skin sites to either low (0.03 M) or high (1.8 M) calcium. Three hours after acute barrier disruption, coincident with reduced calcium and ultrastructural evidence of accelerated lamellar body secretion, both northern analyses and *in situ* hybridization revealed decreased mRNA levels for loricrin, profilaggrin, and involucrin in the outer epidermis, but protein levels did not change significantly. Moreover, exposure of acutely disrupted skin sites to low calcium solutions sustained the reduction in mRNA levels, whereas exposure to high calcium solutions restored normal mRNA levels (blocked by the L-type calcium channel inhibitor, nifedipine). Finally, with prolonged exposure to a low (<10% relative humidity) or high (>80% relative humidity) humidity, calcium levels increased and declined, respectively. Accordingly, mRNA and protein levels of the differentiation-specific markers increased and decreased at low and high relative humidity, respectively. These results provide direct evidence that acute and sustained fluctuations in epidermal calcium regulate expression of differentiation-specific proteins *in vivo*, and demonstrate that modulations in epidermal calcium coordinately regulate events late in epidermal differentiation that together form the barrier. ***Keywords: Ca channels/calcium/filaggrin/involucrin/loricrin/permeability barrier. J Invest Dermatol 119:1128–1136, 2002***

Epidermal differentiation is a vertically directed, vectorial process, with sequential expression of epidermis-specific, structural proteins by suprabasal keratinocytes, terminating in cornification (Eckert *et al*, 1997). Involucrin, loricrin, and several other peptide precursors are enzymatically crosslinked by transglutaminase 1 and other transglutaminases into a mechanically and chemically resistant scaffold, the cornified envelope (Polakowska and Goldsmith, 1991; Reichert *et al*, 1993; Simon, 1994). Additional cytosolic proteins (e.g., filaggrin, keratins 1 and 10) also associate with the cornified envelope during terminal differentiation (Steinert and Marekov, 1995). In cultured keratinocytes, most of these differentiation-specific proteins are regulated both at a transcriptional and, in some cases, at a post-transcriptional level (e.g., transglutaminase 1 and profilaggrin) by external calcium (Ca) levels (Hennings *et al*, 1980; Yuspa *et al*, 1989; Pillai *et al*, 1990; Resing *et al*, 1993; Yamazaki *et al*, 1997). Under basal conditions, mammalian epidermis displays a Ca gradient, with low levels of Ca in the basal and lower spinous layers, followed by an increase in extracellular and intracellular Ca that peaks in the stratum granulosum (SG) (Menon *et al*, 1985; Forslind *et al*, 1995; Mauro *et al*, 1998). Perturbations in permeability barrier function transiently alter this epidermal Ca gradient, producing a steep decline in Ca levels in the outer epidermis, followed by a restoration of Ca levels to normal over 6–24 h in parallel with barrier recovery (Mao-Qiang *et al*, 1997). Loss of Ca from the outer epidermis after acute barrier disruption stimulates the secretion of a preformed pool of lamellar bodies (LBs) from the outermost SG cell, a response that facilitates normal barrier recovery. Furthermore, using alternative methods, we showed that changes in Ca in the outer epidermis directly regulate LB secretion, independent of barrier perturbation (Menon *et al*, 1994a; Lee *et al*, 1998), by a mechanism that involves Ca transport through L-type Ca^{2+} channels (Lee *et al*, 1992).

Whereas these studies show that modulations in epidermal Ca regulate LB secretion, the increased levels of Ca that are present normally in the outer epidermis correlate spatially with the sites where epidermis generates late, differentiation-specific proteins, such as involucrin, filaggrin, and loricrin. Yet, whether modulations in epidermal Ca would dominate over a variety of other

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Abbreviations: LB, lamellar body; RH, relative humidity; SG, stratum granulosum; TEWL, transepidermal water loss.

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endogenous factors that coregulate keratinocyte differentiation (Eckert *et al*, 1997) is not known. Thus, despite repeated notations to the likely relationship of these two phenomena, there is no direct evidence that links fluctuations in epidermal Ca levels with changes in epidermal differentiation. We provide here direct evidence that experimental alterations in epidermal Ca directly regulate the expression of certain differentiation-linked, keratinocyte structural proteins. Together with prior evidence for the stimulation of LB secretion by reductions in epidermal Ca, these studies demonstrate that barrier-induced fluctuations in epidermal Ca coordinately, but divergently, regulate the two major differentiation programs that generate a normal stratum corneum: (i) the lipid-enriched extracellular matrix; and (ii) the corneocyte.

METHODS

Experimental perturbations The epidermal Ca gradient was disrupted in 6–8-wk-old, male hairless mouse skin (Charles River Laboratories, Philadelphia, PA) by either repeated cellophane tape stripping or acetone wipes until transepidermal water loss (TEWL) levels were 4 mg per cm^2 per h, while animals were under general anesthesia (chloral hydrate, 6–9 mg in normal saline, administered intraperitoneally, hourly). Saline-wiped or untreated sites on the contralateral flank of the same animals served as controls. Our methods for acute barrier disruption and TEWL measurements have been described in detail elsewhere (Grubauer *et al*, 1989; Menon *et al*, 1992b). In one model, samples were obtained immediately and 3 h after acute perturbations from treated and control sites, and assessed for (i) status of the Ca gradient by ion capture cytochemistry, (ii) extent of LB secretion, and (iii) mRNA levels by *in situ* hybridization, northern blotting, and immunohistochemical assessment of profilaggrin, involucrin, and loricrin levels (see below). In a second model, the barrier was first disrupted as above, and then flanks of some animals were exposed to an external bath containing isotonic sucrose with either 0.03 mM or 1.8 mM Ca ($+40$ mM K^+) for 2.5 h (Lee *et al*, 1992). In parallel bath experiments, the L-type Ca channel inhibitor, nifedipine (0.1 μM), was added to the 1.8 mM Ca solution (Lee *et al*, 1992). Samples were taken at the end of 2.5 h incubations for *in situ* hybridization and northern blotting, as well as for assessment of LB secretion and the Ca gradient (see below). In a third model, we subjected hairless mice to extremes in relative humidity (RH) for 2 wk. Animals were kept separately in 7.2 l cages in which the RH was maintained at either 10% with a stream of dry air or 80% with humid air, as described previously (Denda *et al*, 1998). The temperature remained between 22°C and 25°C, and fresh air was circulated 100 times per hour, with diversion of the air stream away from the animals. Finally, the levels of ammonia were always below 1 ppm. Prior studies have shown that barrier function changes in response to extremes in environmental humidity, with deterioration at high and improvement at low humidities (Denda *et al*, 1998). After 2 wk, biopsies were obtained for ion capture cytochemistry, *in situ* hybridization, and immunohistochemistry, as below.

Assessment of epidermal calcium gradient Samples from treated and control skin were removed and processed for ion capture cytochemistry, as described previously (Menon *et al*, 1985). For Ca visualization by ion capture precipitation, biopsies were taken from the flanks of each treated and control group at 30 min (≈15–25 min from the end of acetone treatment), 60 min, 180 min, 360 min, and 24 h after barrier disruption, as well as 2.5 h after bathing in iso-osmolar sucrose, with added 0.03 or 1.8 mM Ca (Lee *et al*, 1992). Samples were minced finely (<0.1 mm^3), and immediately immersed in an ice-cold fixative, containing 2% glutaraldehyde, 2% formaldehyde, 90 mM potassium oxalate, and 1.4% sucrose, pH 7.4. After overnight fixation at 4°C in the dark, samples were postfixed in 1% osmium tetroxide (OsO_4), containing 2% potassium pyroantimonate, at 4°C in the dark for 2 h, rinsed in cold distilled water (adjusted to pH 10 with potassium hydroxide), and routinely processed and embedded in an Epon-epoxy resin mixture (see below). Ultrathin (60–80 nm) sections were double-stained with uranyl acetate lead citrate, and examined with a Zeiss electron microscope operating at 60 kV. Prior studies have shown that this ultrastructural method demonstrates modulations in Ca levels similarly to quantitative methods, such as ion analysis by X-ray diffraction, ion probe microanalysis, and electron probe X-ray emission (Forslind *et al*, 1985; Mauro *et al*, 1998). The number of particles per area was quantified from randomly obtained micrographs (>15 each) from dry and humid-exposed animals ($n=4$ each) using computer software (NIH image).

Assessment of lamellar body secretion Biopsy samples were minced to 1 mm^3 pieces and fixed overnight (≈16 h) at 4°C in 2% glutaraldehyde and 2% paraformaldehyde with 0.06% calcium chloride in 0.1 M sodium cacodylate buffer, pH 7.3. Specimens were then placed in 0.1 M sodium cacodylate buffer prior to further processing. Tissue samples were postfixed in 1% OsO_4 with potassium ferrocyanide (1.5%) in 0.1 M sodium cacodylate, pH 7.4, at room temperature in the dark for 1 h. After rinsing in buffer, tissue samples were dehydrated in a graded ethanol series, and embedded in a low-viscosity, epoxy resin mix of DER 736 and Epon 812 (1:1). Thin sections were examined using a Zeiss 10 A electron microscope operating at 60 kV, after double staining with lead citrate and uranyl acetate.

***In situ* hybridization** Digoxygenin (DIG)-labeled RNA probes to detect loricrin (3′ noncoding region, 200 bases) (Mehrel *et al*, 1990) and profilaggrin (coding region 300 bases) (Yuspa *et al*, 1989) mRNAs were made from linearized cDNA sequences (gifts from Dr. Stuart Yuspa, National Institute of Health), using reagents supplied by Boehringer-Mannheim (Indianapolis, IN). *In situ* hybridization was performed as described previously (Komuves *et al*, 1998). Briefly, the sections were hybridized at 40°C and the hybridization of DIG-labeled probes to the endogenous mRNA was detected by anti-DIG-alkaline phosphatase (Boehringer-Mannheim). Alkaline phosphatase activity was revealed with 5-bromo-4-chloro-3-indolyl phosphate/nitrotetrazolium blue substrate (Chemicon, Temecula, CA), containing 2 mM levamisole (Sigma, St. Louis, MO). Hybridization with DIG-labeled sense control probes resulted in no signal, indicating the specificity of hybridization with the antisense probe. Omitting the DIG-labeled antisense probes from the hybridization cocktail resulted in no signal, which demonstrated that only DIG-containing RNA hybrids were detected. Moreover, incubation with the 5-bromo-4-chloro-3-indolyl phosphate/nitrotetrazolium blue substrate reagents alone resulted in no staining, showing that endogenous alkaline phosphatase activity did not contribute to the signal obtained.

Immunohistochemistry Affinity-purified rabbit antimouse antibodies (BabCo, Berkeley, CA), were used for immunohistochemical detection of involucrin and loricrin, as described previously (Kömüves *et al*, 1998). Affinity-purified biotinylated goat antirabbit IgG, affinity-purified biotinylated goat antimouse IgG, and avidin–biotin complex peroxidase were purchased from Vector Laboratories (Burlingame, CA). Peroxidase activity was revealed with diaminobenzidine (QualTek Laboratories, Santa Barbara, CA), followed by counterstaining with methyl green. Omission of the first antibodies, preabsorption of the antibodies with specific proteins, or incubation with the substrate solution alone resulted in no signal, showing that neither nonspecific antibody binding, the avidin–biotin complex alone, exogenous peroxidase alone, nor endogenous peroxidase contributed to the observed signals.

Northern blotting Total RNA was isolated with TRIZOL® Reagent (Life Technologies, Grand Island, NY) following the manufacturer's protocol. Poly(A)$^+$ mRNA was isolated, and size-fractionated through a 1% agarose gel containing 2.2 M formaldehyde, as described previously (Harris *et al*, 1998). The integrity of the mRNA was visualized following acridine orange staining of the electrophoresed gel. The subfractionated mRNA was transferred onto a nylon membrane fixed in place using a UV Stratalinker, model 2400 (Stratagene, La Jolla, CA), and hybridized with appropriate ^{32}P-labeled probes overnight at 65°C. Blots subsequently were washed twice with 0.1% sodium citrate/chloride buffer and 0.1% sodium dodecyl sulfate for 20 min at room temperature followed by 30 min at 65°C. Autoradiographs were incubated at −70°C for appropriate times using Fuji RX X-ray film. Blots were probed with cyclophillin to assure equal loading, and to provide a denominator for quantitation by scanning densitometry. The cDNA probes for mouse filaggrin, rat involucrin, and mouse loricrin were gifts from Drs Robert Rice, U.C. Davis, and S.H. Yuspa, National Institute of Health.

Statistics Statistical comparisons were made using the Student's *t* test. Where more than two groups were compared, a further ANOVA analysis was performed.

RESULTS

Calcium depletion after acute barrier disruption results in a transient decrease in the expression of differentiation-specific proteins We initially assessed mRNA levels after acute barrier disruption, which results in a loss of epidermal Ca for up to 6 h after barrier abrogation (Menon *et al*, 1992a). As

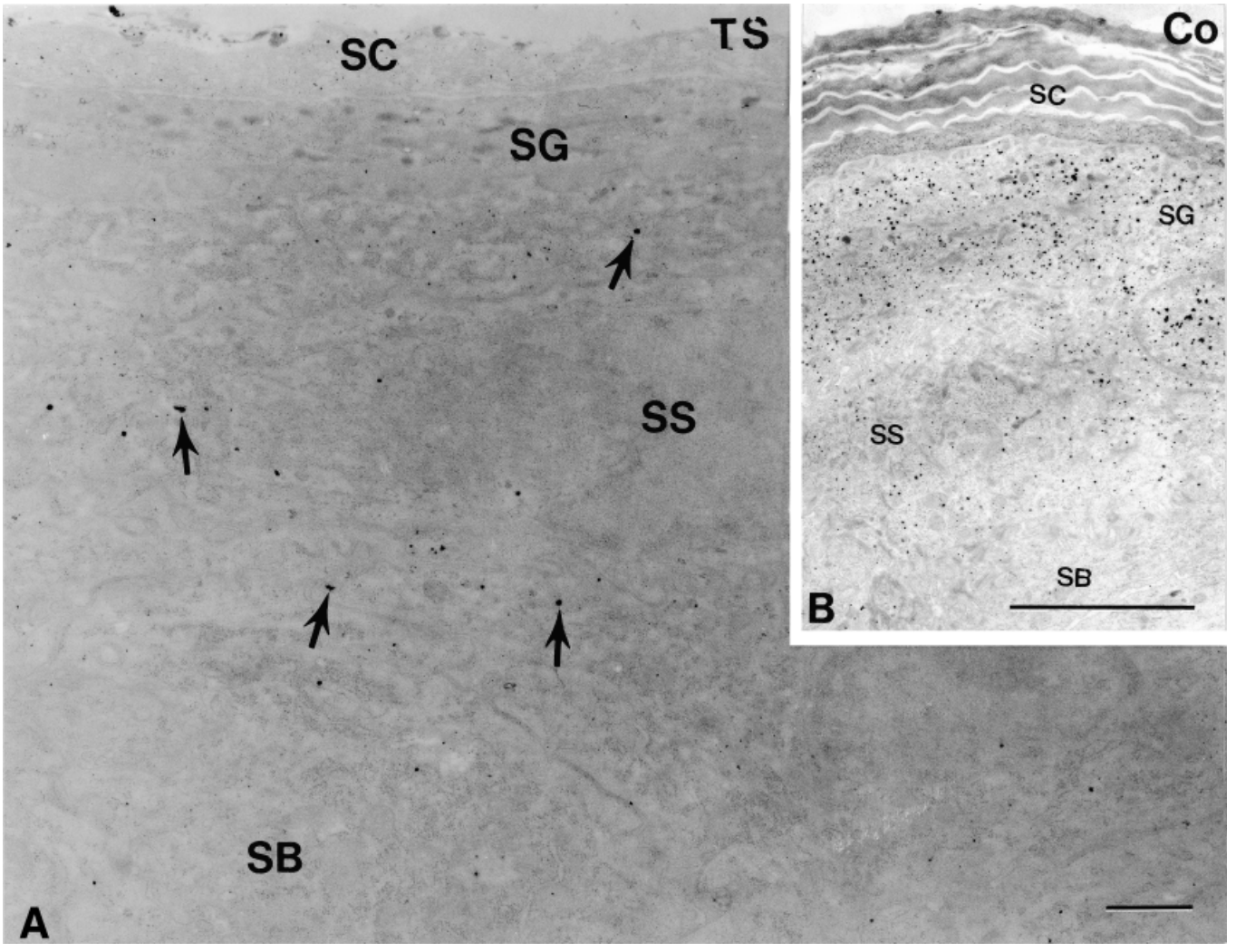

Figure 1. Acute barrier disruption results in loss of calcium from outer epidermis. Ion precipitation depicts Ca as electron-dense deposits. (*A*) Immediately after tape stripping (TS), the density of Ca precipitates in the outer epidermis is very low in comparison to normal, untreated epidermis (*B*). Greatest density of precipitates in control epidermis is in the outer SG, whereas levels appear very low in stratum corneum (SC), and decline in the stratum spinosum (SS) and basal layer (SB). *Bars*: 2 μm. Sodium pyroantimonate precipitation, followed by osmium tetroxide postfixation.

observed previously (Menon *et al*, 1992a; 1992b; Mauro *et al*, 1998), acute barrier disruption by either sequential tape stripping or acetone treatment was accompanied by a precipitous loss of Ca from the outer epidermis (**Fig 1*A*** *vs* **1*B***), as well as rapid secretion of the preformed pool of LBs from the outermost SG cell (**Fig 2*A*** *vs* **2*B***). Ca levels remained subnormal in the outer epidermis 3 h after acute disruption, but returned towards normal between 6 and 24 h (not shown; see Menon *et al*, 1992a; Mao-Qiang *et al*, 1997; Mauro *et al*, 1998).

The loss of Ca from the outer epidermis after acute barrier disruption by sequential tape stripping was paralleled by a decline in mRNA levels for loricrin, filaggrin, and involucrin, as assessed by northern blotting (**Fig 3*A***). Acetone treatment, an unrelated method of acute barrier disruption, produced a comparable loss of Ca from the outer epidermis (not shown; see Menon *et al*, 1992a; Mauro *et al*, 1998), with comparable reductions in loricrin, filaggrin, and involucrin mRNA levels by northern blotting (**Fig 3*B***). In contrast, the mRNA levels of cyclophillin, a housekeeping protein, did not change after either method of barrier disruption. These experiments show that acute, barrier-induced reductions in epidermal Ca are associated with a decrease in mRNA levels for selected differentiation-linked proteins.

Acute barrier disruption results in loss of Ca primarily from the outer, nucleated layers of the epidermis. Therefore, we next assessed whether the decline in mRNA expression for the differentiation-linked proteins occurs in those epidermal cell layers that become depleted in Ca. At the same time point after acute barrier disruption that mRNA levels for profilaggrin, loricrin, and involucrin declined on northern blotting (i.e., 2–3 h), mRNA became nondetectable in the SG layer, as assessed by *in situ* hybridization, returning to normal by 24 h (**Fig 4*A***, ***B***). Yet, because of their long half-lives, immunostaining for these proteins remained unchanged at these early time points; i.e., 2–3 h after acute barrier disruption, and continued to remain unchanged at later time points ($\geqslant$12 h) (**Table I**). These results show that the loss of Ca produced by acute barrier disruption is accompanied by transient downregulation of the mRNAs of differentiation-specific epidermal proteins, whereas protein levels do not change under these acute conditions.

Exposure of acutely disrupted skin sites to high *versus* low Ca directly regulates protein expression The above results demonstrate a correlation between a decline in mRNA levels for the differentiation-specific proteins with loss of Ca from the outer epidermis following acute barrier disruption. We next assessed directly whether changes in Ca are responsible for these alterations by immersing acutely perturbed (tape-stripped) flanks of hairless mice either in low (0.03 mM) or high (1.8 mM) Ca (Lee *et al*, 1992). The controls for these studies included

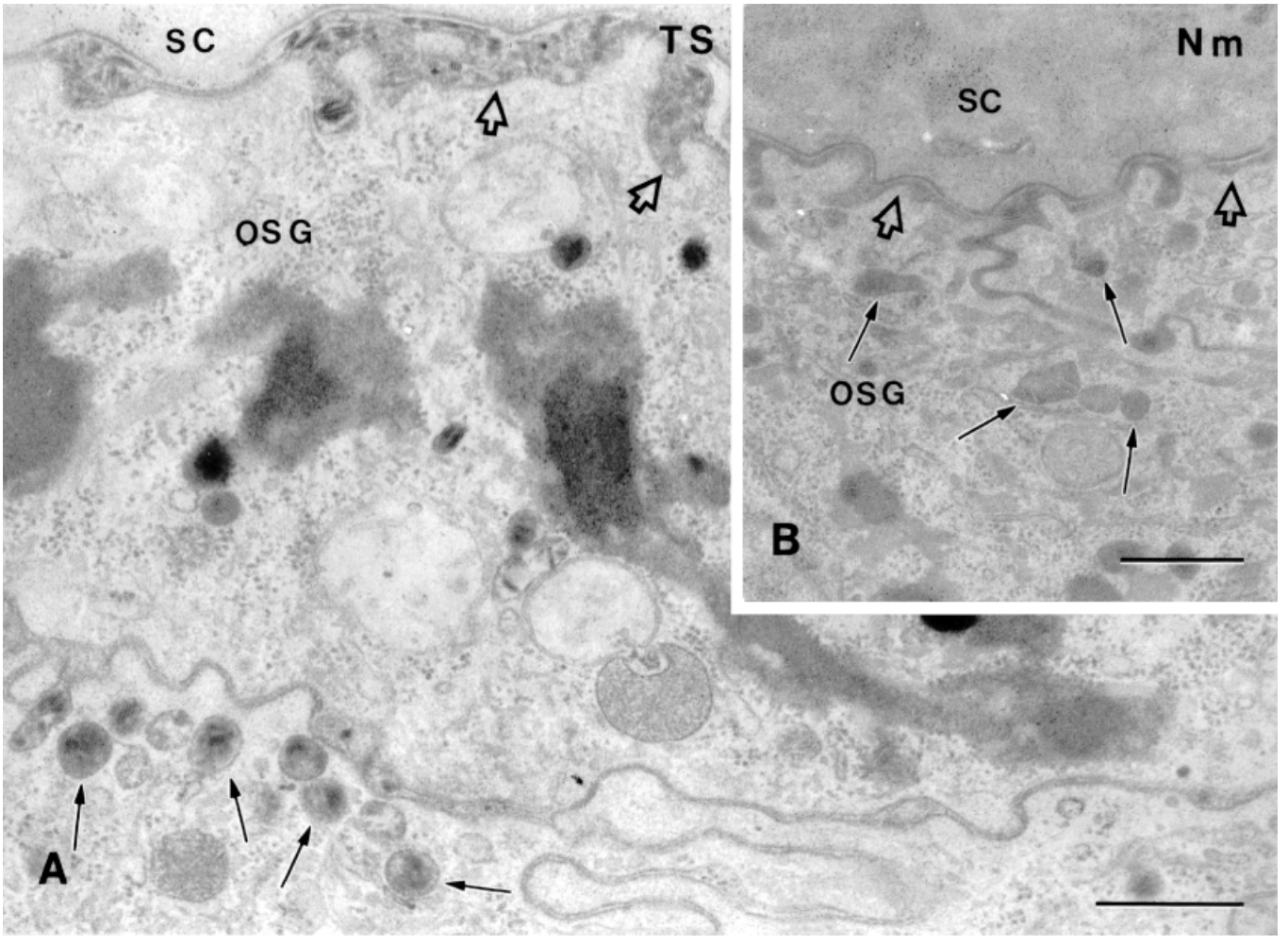

Figure 2. Acute barrier disruption stimulates an immediate secretion of epidermal LBs. Hairless mice were sequentially tape stripped (TS) until TEWL $\geqslant$ 4 mg per cm^2 per h. (*A*) Immediately after tape stripping, cytosol of outer granular cells (OSG) is largely devoid of LBs, though abundant organelles (*single arrows*) are present in subjacent SG layers. Note abundant, secreted LB contents at OSG–stratum corneum (SC) interface (*open arrows*). (*B*) Normal, nonstripped control epidermis shows numerous LBs in OSG (*single arrows*), and less secreted organelle contents at the OSG–SC interface (*open arrows*). *Bars*: 0.5 μm. Osmium tetroxide postfixation.

tape-stripped and air-exposed flanks, normal untreated flanks, and normal flanks (with intact barrier) immersed in low or high Ca solutions. As reported previously (Lee *et al*, 1992; 1994), the permeability barrier recovered at rates comparable to air-exposed sites, following 2.5 h in low Ca (**Table II**). Moreover, LBs continued to be secreted in an accelerated fashion from the outermost SG layer (**Fig 5*A***), whereas Ca levels both surrounding and within SG cells remained low (**Fig 6*A***; compare with **Fig 1*A***). Conversely, with exposure to the high Ca buffer, barrier recovery was blocked (**Table II**); very few LBs were secreted (**Fig 5*B***), and substantial Ca was present in the outer epidermis (**Fig 6*B***). These results show that addition of low or high Ca concentrations to acutely pertubated skin sites directly regulates LB secretion.

Exposure of acutely disrupted skin sites to the low Ca bath also resulted in a reduction in mRNA levels of loricrin, i.e., to levels that were comparable to those in air-exposed epidermis at 3 h after acute disruption (**Fig 7**). In contrast, exposure to high Ca prevented the decrease in loricrin mRNA, resulting in levels that were comparable to those observed in untreated skin (**Fig 7**). Pertinently, immersion of nondisrupted, normal skin in low or high Ca for 3 h altered neither epidermal Ca (**Fig 6*C***) nor loricrin mRNA levels (**Fig 7**). These results demonstrate directly that experimental modulations in epidermal Ca levels produce parallel changes in mRNA levels for the differentiation-specific protein, loricrin.

Prior studies have shown that Ca-regulated, downregulation of both LB secretion and barrier recovery requires the participation of L-type Ca channels (Lee *et al*, 1992; 1994). Therefore, we next assessed whether the changes in epidermal Ca that regulate epidermal differentiation also require translation of extracellular Ca into the cytosol via L-type Ca channels. We examined whether nifedipine reverses the previously noted, high-Ca-induced, normalization of epidermal differentiation following barrier disruption. As reported previously (Lee *et al*, 1992), treatment with nifedipine normalized barrier recovery (**Table II**) and, seen in **Fig 5(*C*)**, LB secretion was normalized as well. Finally, under the same conditions, nifedipine blocked the high Ca-induced normalization of loricrin mRNA expression (**Fig 7**). Together, these results (i) show that restoration of high Ca to acutely disrupted skin sites normalizes mRNA expression of the differentiation-specific epidermal protein loricrin, independent of changes in barrier function, whereas, in contrast, maintenance of low Ca after acute barrier disruption results in low levels of loricrin mRNA; (ii) also indicate that the Ca-induced regulation of epidermal differentiation occurs via L-type Ca channels.

Humidity-induced changes in epidermal Ca regulate epidermal differentiation Whereas the studies above demonstrate that acute changes in Ca regulate mRNA levels for the differentiation-specific proteins, we next assessed whether more

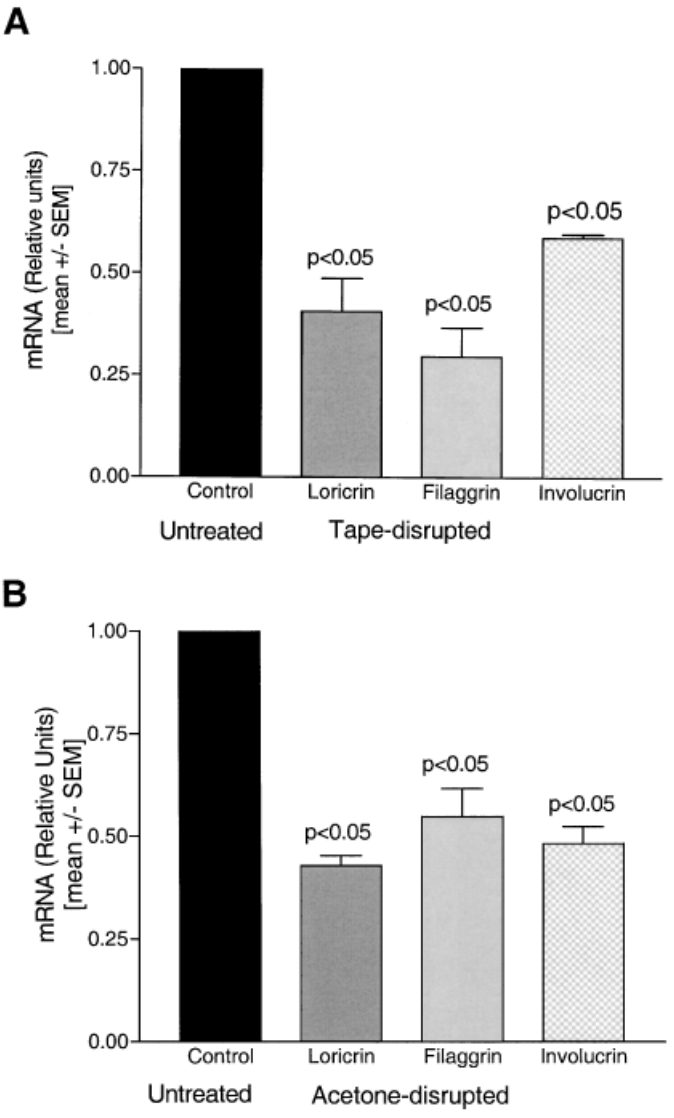

Figure 3. Acute barrier disruption downregulates differentiation-specific mRNA. Hairless male mice were tape stripped (*A*) or acetone swabbed (*B*) until TEWL $\geqslant 4$ mg per cm^2 per h and allowed to recover for 3 h in air. Poly(A)$^+$ mRNA was isolated and analyzed as described in *Methods*. Quantitation by scanning densitometry of loricrin, filaggrin, and involucrin mRNA levels is shown in acutely disrupted *versus* control animals.

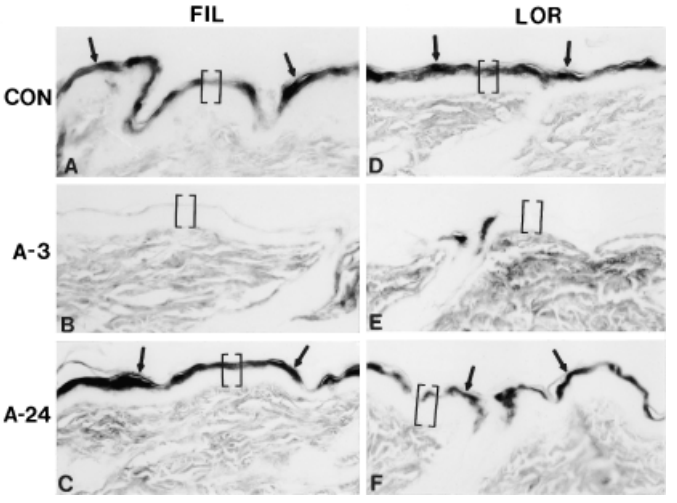

Figure 4. Acute barrier disruption results in transient loss of granular layer signal for differentiation-specific mRNA. DIG-labeled antisense riboprobes were used to detect filaggrin (FIL) (*A, B, C*) and loricrin (LOR) (*D, E, F*) mRNAs in control and acetone (A) treated murine epidermis, 3 and 24 h after acute disruption. The hybridized probes were localized using anti-DIG antibody, followed by labeling with alkaline phosphatase. Enzyme activity was revealed with BCIP/NBT substrate reaction. DIG-labeled sense riboprobes were applied to parallel sections to ensure the specificity of hybridization (not shown). Brackets indicate the full thickness of murine epidermis. *Original magnification*: 200 ×.

Table I. Changes in protein expression and localization after acute barrier disruption[a]

Protein	Time	SB	SS	SG	SC
Loricrin	Pre	−	−	+ +	−
	2–3 h	−	−	+ +	−
	12 h	−	−	+ +	−
Involucrin	Pre	−	−	+ +	+ −
	2–3 h	−	−	+ +	+ −
	12 h	−	+ −	+ +	+

[a]Paraffin-embedded sections were labeled with antimouse polyclonal antibodies, visualized by avidin-peroxidase histochemistry, and compared in an observer-blinded fashion. Intensity of labeling was evaluated as follows: −, no visible signal; +, minimal signal; + +, moderate signal; + + +, intense (maximal) signal. SB, stratum basale; SS, stratum spinosum; SC, stratum corneum.

Table II. Barrier recovery after tape stripping followed by air exposure or immersion in high/low calcium ± nifedipine (% at 3 h)[a]

Conditions	Mean ± SEM	Significance
Air-exposed ($n = 9$)	48.8 ± 4.4	–
Immersed, low Ca ($n = 10$)	43.6 ± 4.8	NS
Immersed, high Ca ($n = 10$)	5.2 ± 1.7	$p < 0.001$
Immersed, high Ca + nifedipine ($n = 9$)	31.8 ± 6.3	NS

[a]Hairless mice were stripped on one flank, and the treated flanks were either left air-exposed or immersed immediately in isotonic sucrose, containing either low (0.03 mM) or high Ca (1.8 mM + 40 mM K^+), with and without nifedipine (0.1 μM). p-values represent comparisons between air-exposed and other conditions by Student's *t* test, followed by ANOVA.

sustained changes in Ca also regulate protein expression. Prior studies have shown that permeability barrier function progressively deteriorates with prolonged exposure to high RH, but improves with sustained exposure to reduced RH (i.e., 40%–50% acceleration of barrier recovery rates) (Denda *et al*, 1998). Therefore, we next employed this model as an independent measure of the effects of sustained, barrier-induced changes in Ca on epidermal differentiation. **Figure 8** shows representative electron micrographs of Ca distribution in the epidermis of animals exposed to low (dry) *versus* high (humid) RH for a prolonged period (2 wk). Whereas the density of Ca precipitates appeared to increase with dry exposure (**Fig 8*A***), Ca levels, instead, appeared to decline in the outer epidermis with prolonged exposure to a high RH (**Fig 8*B***). We next quantitated the density of Ca precipitates in the two humidity groups, in an observer-blinded fashion. These measurements confirmed that the Ca gradient becomes more prominent with changes in depth within the epidermis with dry exposure, whereas the gradient is lost with sustained humid exposure (**Fig 9**). Parallel *in situ* hybridization and immunohistochemical studies, following prolonged exposure to either a high or a low RH, demonstrated reduced levels of loricrin and filaggrin mRNA and protein in the outer nucleated layers at a high RH, whereas the low humidity milieu stimulated expression of these proteins (**Figs 10, 11**). These studies show, in another unrelated model, (i) that barrier-linked changes in epidermal Ca correlate with modulations in epidermal

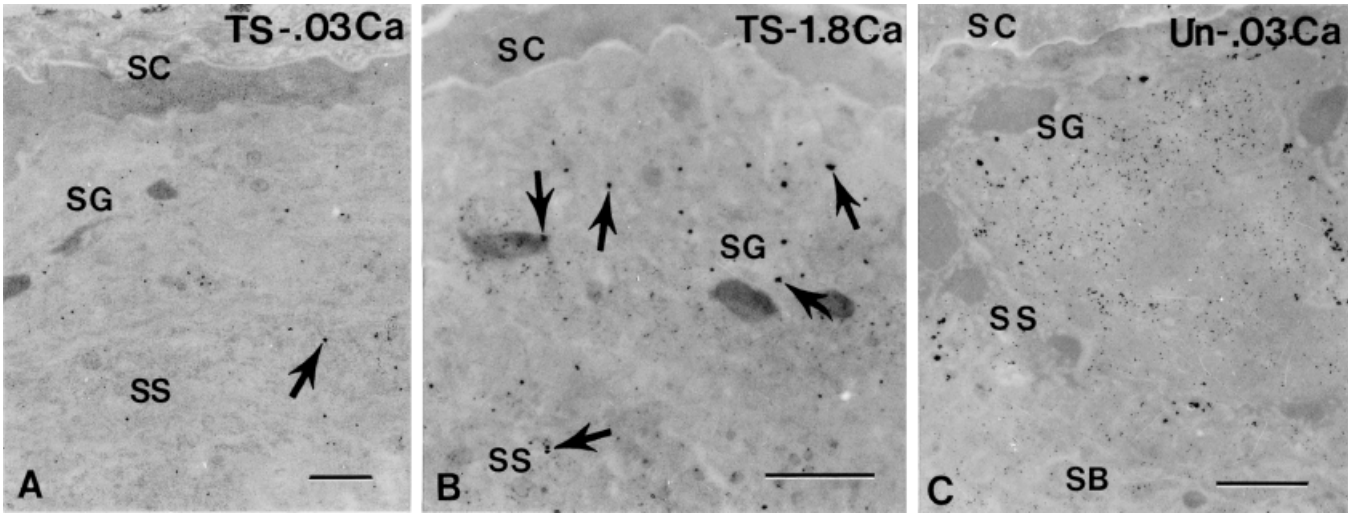

Figure 5. Low *versus* high Ca immersion regulates Ca levels in the outer epidermis. (*A*) After acute disruption by tape stripping and immersion in low Ca for 2.5 h, the density of Ca deposits remains low, i.e., comparable to Ca levels immediately after acute disruption (cf. **Fig 1*A***). (*B*) In contrast, immersion in high Ca increases the levels of Ca in the outer epidermis (*arrows*), although Ca levels are lower than in either normal, untreated skin (cf. **Fig 1*B***) or normal skin immersed in low external Ca (*C*). *Bar*: 1 μm. Sodium pyroantiminate precipitation, followed by osmium tetroxide postfixation.

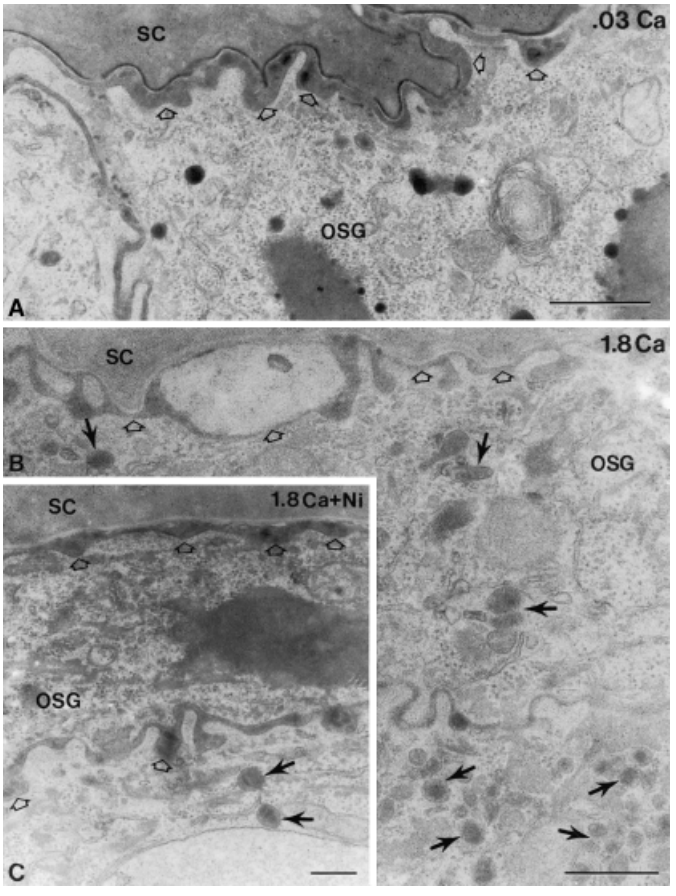

Figure 6. Immersion in high *versus* low Ca oppositely regulates LB secretion. Acute barrier disruption was achieved by tape stripping (TS), followed by immersion in low (0.03 mm) *versus* high (1.8 mM) Ca for 2.5 h. In low Ca (*A*), LB secretion is stimulated, with low numbers of organelles (*arrows*) in the outer SG (OSG) and abundant secreted LB contents (*open arrows*) at the stratum corneum (SC)–OSG interface. In contrast, exposure to high Ca (*B*) leaves a higher density of LBs (*arrows*) in the cytosol of the OSG, and less secreted contents at the SC–OSG interface. Extent of LB secretion is comparable to that in untreated, control samples when nifedipine is added to high Ca (*C*). *Bar*: 1 μm. Osmium tetroxide postfixation.

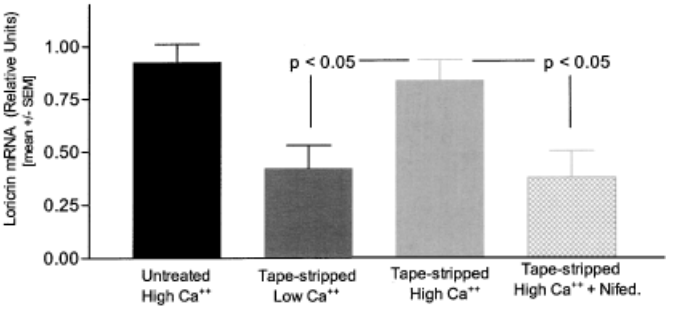

Figure 7. Immersion of acutely disrupted skin sites in low *versus* high Ca oppositely regulates loricrin expression. Hairless mice were tape stripped (TEWL $\geqslant$4 mg per cm^2 per h) and immersed in a low or high Ca bath at 37°C for 2.5 h ($n = 4$). Nifedipine (10 μM) was added to the high Ca bath in one group of animals ($n = 4$). Animals that had not been tape stripped were immersed in a high Ca bath and are included as controls. In nontape-stripped animals with an intact barrier exposure to a low or high Ca bath results in loricrin mRNA levels that are similar. Poly(A)$^+$ mRNA was isolated from the epidermis and northern blots were prepared and probed for loricrin as described in *Methods*. Data are presented as mean $\pm$ SEM normalized for cyclophillin expression.

differentiation; and (ii) that sustained changes in epidermal Ca levels regulate not only mRNA but also protein levels of the differentiation-specific proteins.

DISCUSSION

Prior studies have shown both indirectly (Menon *et al*, 1992a; Mao-Qiang *et al*, 1997) and directly (Menon *et al*, 1994a; Lee *et al*, 1998) that fluctuations in Ca in the outer epidermis regulate LB secretion, a key event in the homeostatic sequence that maintains and restores permeability barrier homeostasis (Elias and Menon, 1991). Whereas abrupt reductions in Ca levels in the outer epidermis trigger LB secretion (Menon *et al*, 1992b; Lee *et al*, 1998), the high levels of Ca present in unperturbed epidermis restrict organelle secretion to low rates that are, nevertheless, sufficient to maintain barrier homeostasis under basal (maintenance) conditions (Menon *et al*, 1994a). We hypothesized here that fluctuations in Ca would similarly, but oppositely, regulate epidermal

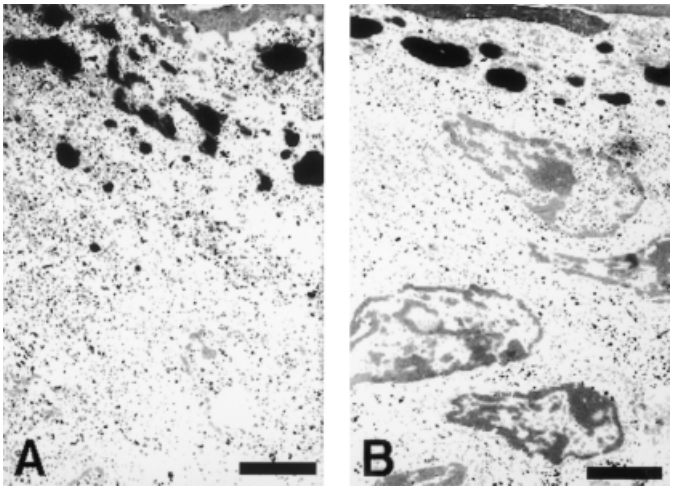

Figure 8. Prolonged exposure to high *versus* low humidity changes epidermal Ca levels. (*A*) Following 2 wk exposure to <10% external RH (low), the density of Ca precipitates appears greater than in normal controls (cf. **Fig 1*B***). (*B*) In contrast, 2 wk exposure to >80% external humidity (high) appears to decrease Ca levels in the outer epidermis. *Bars*: 1.0 μm.

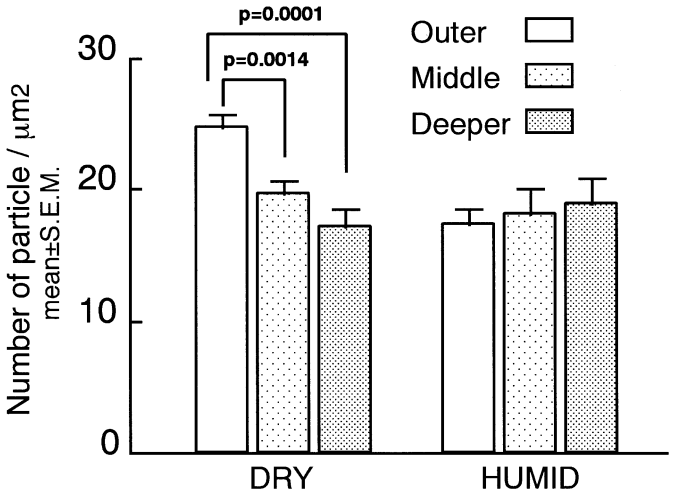

Figure 9. Quantitation of Ca precipitation reveals a selective loss of Ca from the outer epidermis with humid exposure. The number of particles per area was quantified using computer software (NIH image). The outer layer includes the area 3 μm from the surface, the middle layer is 3–6 μm from the surface, and the deep layer is 6 μm and 9 μm from the surface. The data are presented as the mean ± SEM. Twenty sections from three mice were randomly selected for analysis.

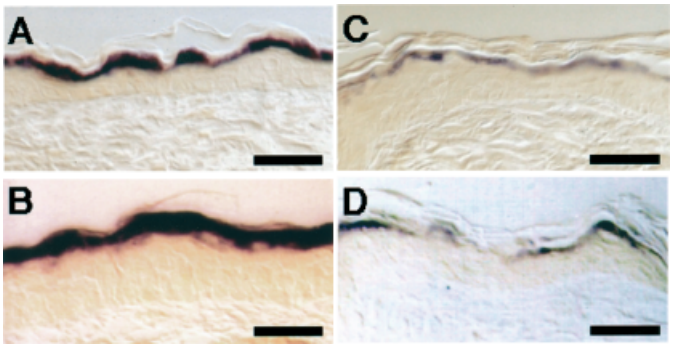

Figure 10. Epidermal loricrin and profilaggrin mRNA levels change in response to humidity extremes oppositely to the response to acute barrier disruption. Hairless mice were exposed to low (dry) *versus* high (humid) humidity for 2 wk. *In situ* hybridization for loricrin and profilaggrin was performed, as described in *Methods* and in the legend to **Fig 4**. Prolonged exposure to low humidity increases loricrin (*A*) and profilaggrin (*B*) mRNA levels in the outer epidermis in comparison to normal (cf. **Fig 4**). In contrast, prolonged exposure to high humidity decreases loricrin (*C*) and profilaggrin (*D*) mRNA expression in comparison to both low (dry) humidity (*A*, *B*) and normal (cf. **Fig 4**).

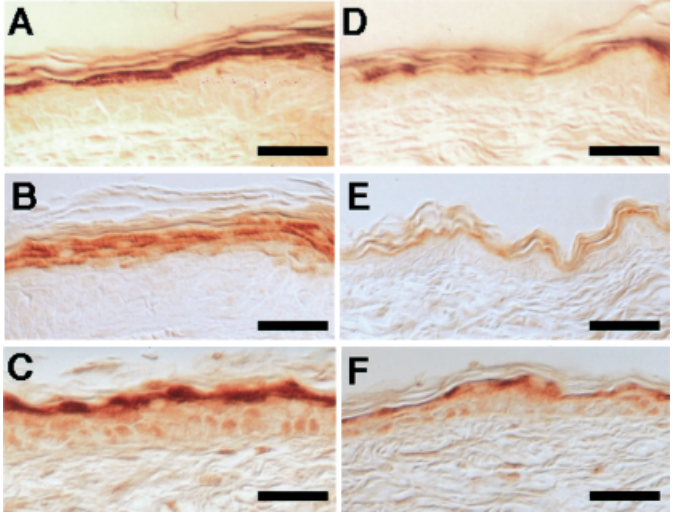

Figure 11. Epidermal differentiation-specific protein levels change oppositely in response to humidity extremes. Hairless mice were exposed to low *versus* high humidity for 2 wk. Immunohistochemistry for involucrin, profilaggrin, and loricrin was performed, as described in *Methods*. With prolonged exposure to low humidity (dry environment) loricrin (*A*), profilaggrin (*B*), and involucrin (*C*) protein levels increase in the outer epidermis. In contrast exposure to a high humidity environment results in a decrease in loricrin (*D*), profilaggrin (*E*), and involucrin (*F*) levels.

differentiation (**Fig 12**). When cultured keratinocytes are exposed to high (>0.1 mM) Ca, most of the structural proteins of the corneocyte are upregulated transcriptionally, and in some cases post-transcriptionally as well (Hennings *et al*, 1980; Yuspa *et al*, 1989; Pillai *et al*, 1990; Resing *et al*, 1993; Yamazaki *et al*, 1997). Yet, although the high levels of Ca that are present in the outer nucleated cell layers under basal conditions could provide a comparable stimulus for expression of these proteins *in vivo*, the observation that changes in Ca alone regulate epidermal differentiation *in vivo* is not self-evident, as a wide variety of other differentiation-inducing stimuli [e.g. ligands of class II nuclear hormone receptors, several transcription factors, activators of protein kinase C isoforms, and endogenous lipids (e.g. cholesterol sulfate)] are known to regulate epidermal differentiation *in vivo* (Dlugosz and Yuspa 1993; Eckert *et al*, 1997; Kömüves *et al*, 1998; Hanley *et al*, 2000; 2001). Whether changes in Ca alone would dominate over one or more of these influences *in vivo* is not known.

To address this question, we modulated Ca levels in the outer nucleated layers of the epidermis in a series of unrelated models, and assessed the resultant changes in differentiation using three different assays (northern hybridization, *in situ* hybridization, and/or immunohistochemistry). In both an acute model (i.e., postdisruption with either tape stripping or acetone) and an unrelated chronic model (i.e., prolonged exposure to either high or low RH), we showed that the mRNA levels of epidermal differentiation-specific proteins change in parallel with alterations in the levels of Ca in the outer epidermis. In organotypic cultures of human epidermis, Vicanova *et al* (1998) showed similarly that barrier function, Ca levels, and keratinocyte differentiation change in parallel. As all of these approaches provide largely correlative data, however, we utilized a third model as a direct test of

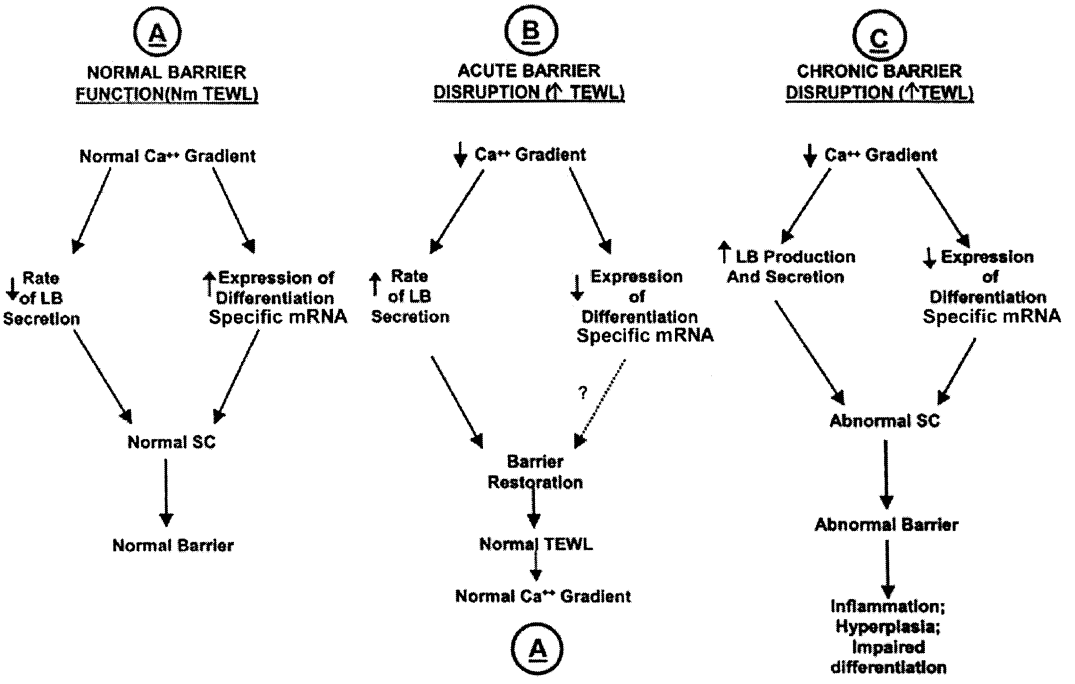

Figure 12. Modulations in Ca levels coordinately regulate both lamellar body secretion and epidermal differentiation.

the hypothesis that changes in Ca regulate epidermal differentiation (i.e., a bath model, with exposure of acutely disrupted skin sites to either low or high Ca) (Lee *et al*, 1992). These results showed directly that changes in Ca regulate mRNA expression of the differentiation-specific protein loricrin, i.e., exposure to low *versus* high Ca suppressed and normalized mRNA levels, respectively. Thus, changes in mRNA levels occur when Ca levels change both indirectly (i.e., with barrier disruption) or directly (i.e., with exposure to a low/high Ca bath, independent of barrier status). Finally, we showed that coexposure to high Ca plus the selective, dihydropyridine, L-type Ca channel inhibitor nifedipine blocks the normalization of mRNA levels for loricrin. This experiment indicates further that Ca signaling of the differentiation-specific proteins involves Ca translocation across L-type Ca channels (Abernethy and Schwartz, 1999). Pertinently, a nifedipine-sensitive, L-type Ca channel likewise is required for inverse Ca regulation of barrier recovery (Lee *et al*, 1992). Together, these results show unequivocally that both acute and sustained modulations in Ca levels *in vivo* regulate the expression of differentiation-specific epidermal proteins, and that such Ca regulation involves the participation of L-type Ca channels.

The observation that mRNA but not protein levels are regulated in parallel with acute changes in epidermal Ca levels can be explained by the long half-lives of these proteins (Polakowski and Goldsmith, 1991; Simon, 1994). In contrast, with more sustained, humidity-associated changes in Ca, we showed that not only mRNA but also protein levels change. Several observations suggest that the changes in protein levels that occur with sustained changes in epidermal Ca levels could have pathophysiologic significance. Prior studies have shown that the sustained abnormality in barrier function that occurs in essential fatty acid deficiency is paralleled by loss of the epidermal Ca gradient (Menon *et al*, 1994a). Accordingly, Ca levels are reduced in the outer epidermis in other, cutaneous pathologic entities that are accompanied by a barrier abnormality, e.g., atopic dermatitis and psoriasis (Menon *et al*, 1991; Pallon *et al*, 1996). The link between decreased barrier function and loss of epidermal Ca, in turn, could contribute to the decreased expression of differentiation-linked proteins in these disorders (Detmar *et al*, 1990; Schroeder *et al*, 1992; Ishida-Yamamoto and Iizuka, 1995; Ishida-Yamamoto *et al*, 1996; Fujimoto *et al*, 1997). Furthermore, because the differentiation-specific proteins provide a critical scaffold for LB-derived, extracellular lamellar bilayers (Elias *et al*, in press), decreased differentiation could, in turn, further aggravate the barrier abnormality. Accordingly, a barrier abnormality (Elias *et al*, 1981) accompanies the well-documented downregulation of differentiation-specific proteins that occurs with administration of supraphysiologic doses of retinoids (Cline and Rice, 1983; Rubin and Rice, 1986; Floyd and Jetten, 1989; Hohl *et al*, 1991; Liew and Yamanishi, 1992; Marvin *et al*, 1992; Monzon *et al*, 1996).

Finally, our results suggest that the lipid and protein arms of epidermal differentiation are linked and coregulated in parallel by barrier-imposed changes in epidermal Ca levels (**Fig 12**). Under basal (maintenance) conditions, high levels of Ca in the outer epidermis promote epidermal protein expression, and simultaneously restrict LB secretion. In contrast, after acute barrier perturbations, epidermal Ca levels decrease in parallel with a steep, though transient, decline in mRNA levels for the differentiation-specific protein, whereas LB secretion simultaneously is stimulated. Thus, in all three models where the expression of differentiation-specific proteins is regulated by changes in Ca, parallel, but opposite, changes occur in LB secretion. LB secretion and enhanced lipid synthesis represent two key metabolic responses that lead to barrier recovery after acute insults (Elias, 1996). Whereas the transient downregulation in mRNA levels that occurs after barrier disruption could represent a specific adaptation that directly facilitates barrier recovery, it could, instead, support barrier recovery indirectly by acting as a form of metabolic conservation.

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