Generation of Free Fatty Acids from Phospholipids Regulates Stratum Corneum Acidification and Integrity

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There is evidence that the "acid mantle" of the stratum corneum is important for both permeability barrier formation and cutaneous antimicrobial defense. The origin of the acidic pH of the stratum corneum remains conjectural, however. Both passive (e.g., eccrine/sebaceous secretions, proteolytic) and active (e.g., proton pumps) mechanisms have been proposed. We assessed here whether the free fatty acid pool, which is derived from phospholipase-mediated hydrolysis of phospholipids during cornification, contributes to stratum corneum acidification and function. Topical applications of two chemically unrelated secretory phospholipase sPLA$_2$ inhibitors, bromphenacylbromide and 1-hexadecyl-3-trifluor-oethylglycero-sn-2-phosphomethanol, for 3 d produced an increase in the pH of murine skin surface that was paralleled not only by a permeability barrier abnormality but also altered stratum corneum integrity (number of strippings required to break the barrier) and decreased stratum corneum cohesion (protein weight removed per stripping). Not only stratum corneum pH but also all of the functional abnormalities normalized when either palmitic, stearic, or linoleic acids were coapplied with the inhibitors. Moreover, exposure of intact murine stratum corneum to a neutral pH for as little as 3 h produced comparable abnormalities in stratum corneum integrity and cohesion, and further amplified the inhibitor-induced functional alterations. Furthermore, short-term applications of an acidic pH buffer to inhibitor-treated skin also reversed the abnormalities in stratum corneum integrity and cohesion, despite the ongoing decrease in free fatty acid levels. Finally, the secretory-phospholipase-inhibitor-induced alterations in integrity/cohesion were in accordance with premature dissolution of desmosomes, demonstrated both by electron microscopy and by reduced desmoglein 1 levels in the stratum corneum (shown by immunofluorescence staining and vizualized by confocal microscopy). Together, these results demonstrate: (i) the importance of phospholipid-to-free-fatty-acid processing for normal stratum corneum acidification; and (ii) the potentially important role of this pathway not only for barrier homeostasis but also for the dual functions of stratum corneum integrity and cohesion. *Key words: acidification/epidermis/fatty acids/free fatty acids/pH/phospholipases/phospholipids/secretory phospholipases/stratum corneum. J Invest Dermatol 117:44–51, 2001*

A lthough first recognized several decades ago (Schade and Marchionini, 1928; Blank, 1939; Draize, 1942; Beare *et al*, 1958; Baden and Pathak, 1967; Dikstein and Zlotogorski, 1994; Ohman and Vahlquist, 1994, 1998), the origin and function of the "acid mantle" of the stratum corneum (SC) remains conjectural. One function of SC acidification appears to be antimicrobial, because an acidic pH inhibits colonization by pathogenic bacteria such as *Staphylococcus aureus* (Aly *et al*, 1975; Puhvel *et al*, 1975). Conversely, alkalinization of the SC, as occurs in the urea-soaked skin of diaper

dermatitis, is an important antecedent of bacterial and yeast infections (Leyden and Kligman, 1978; Brook, 1992). A second, key function of an acidic SC relates to the activation of lipid hydrolases, which process secreted polar lipids, a sequence required for normal barrier function (Elias and Menon, 1991). Barrier recovery after acute injuries is impeded at a neutral *versus* acidic pH (Mauro *et al*, 1998), apparently because one or more secreted hydrolases, e.g., β-glucocerebrosidase (Holleran *et al*, 1992; Takagi *et al*, 1999), display an acidic pH optimum. β-Glucocerebrosidase, with acid sphingomyelinase, represent two such critical processing enzymes, with acidic pH optimum, that generate a family of ceramides required for epidermal permeability barrier homeostasis (Holleran *et al*, 1993; Jensen *et al*, 1999; Schmuth *et al*, 2000).

Both active (energy-requiring) and passive (nonenergy-requiring) mechanisms have been proposed for the origin of the acid mantle of the SC. Passive exogenous mechanisms include: (i) free fatty acids (FFA), generated by lipases of bacterial and/or pilosebaceous origin (Puhvel *et al*, 1975); or (ii) eccrine-gland-derived products such as lactic acid (Ament *et al*, 1997). Passive processes, mediated by hydrolytic enzymes, include bulk proteo-

lysis that accompanies catabolic processes late in terminal differentiation (Freinkel and Traczyk, 1983). Another passive mechanism, i.e., generation of *cis*-urocanic acid from histidine, was proposed recently to be the major, if not the sole, source of the acidification of the SC aqueous compartment (Krien and Kermici, 2000). This mechanism, however, may not be relevant either for hydrolytic processes that occur deep in the SC, or for those within the hydrophobic, extracellular compartments of the SC. It is in these domains that the processing events leading to the dual SC functions of barrier formation and desquamation largely occur (Elias and Menon, 1991). Moreover, to what extent this mechanism is operative under conditions of high humidity, when less histidine would be available as a precursor for *cis*-urocanic acid generation (Scott *et al*, 1982; Scott and Harding, 1986), is unknown.

A third, passive, enzymatic mechanism could come from the generation of proton-donating FFA, resulting from the bulk hydrolysis of epidermal phospholipids (PL) or other complex lipids late in terminal differentiation. PL disappear during cornification (Elias *et al*, 1988), and a family of nonessential FFA, required for normal barrier homeostasis, results from the bulk hydrolysis (processing) of PL within the SC interstices (Mao-Qiang *et al*, 1995). Although the responsible phospholipase(s) has (have) not been identified (Long and Yardley, 1972; Freinkel and Traczyk, 1980; Forster *et al*, 1983; 1985; Bergers *et al*, 1988; Andersen *et al*, 1994; Li-Stiles *et al*, 1998; Mazereeuw-Hautier *et al*, 2000), applications of inhibitors of the 14 kDa family of secretory phospholipases (sPLA$_2$) block the processing of PL, while inducing abnormalities in permeability barrier homeostasis (Mao-Qiang *et al*, 1995; 1996). We assessed here whether applications of these sPLA$_2$ inhibitors also induce abnormalities in SC acidity, as well as the potential functional consequences of such alterations. Our studies demonstrate that PL-to-FFA processing is an important source of SC acidification. Furthermore, we show that inhibition of FFA generation from PL results not only in altered barrier function, but also in abnormalities in SC integrity and cohesion.

MATERIALS AND METHODS

Materials Male hairless mice (Skh1/Hr), 8–12 wk old, were purchased form Charles River Laboratories (Wilmington, MA) and fed Purina mouse diet and water *ad libitum*. Propylene glycol, ethanol, NaOH, and HCl were from Fisher Scientific (Fairlane, NJ), whereas palmitic acid (PA), stearic acid, linoleic acid, HEPES buffer, and bromophenacyl bromide (BPB) were from Sigma Chemical (St. Louis, MO). 1-hexadecyl-3-trifluoroethylglycero-sn-2-phosphomethanol (MJ33) was synthesized as described previously (Jain *et al*, 1991). The stripping for the protein assay was performed with 22 mm D-Square 100 tapes purchased from CuDerm (Dallas, TX). Bradford protein assay kits (Bio-Rad Protein Assay Dye), as well as lyophilized, bovine plasma gamma globulin were purchased from Bio-Rad (Hercules, CA).

Experimental procedures, pH determinations, and transepidermal water loss (TEWL) measurement Normal hairless mice were treated topically twice daily for 3 d with BPB (4 mg per ml) or MJ33 (4 mg per ml), both in propylene glycol:ethanol (7:3 vol/vol) vehicle, or the vehicle alone on an area of 5–6 cm^2 on the backs and flanks, as described previously (Mao-Qiang *et al*, 1996). The doses that we employed were shown previously to be nontoxic to murine skin (Mao-Qiang *et al*, 1995; 1996), and to inhibit secretory PLA$_2$ activity selectively in different tissues and cell types (Jain *et al*, 1991; Gelb *et al*, 1994). For the override experiments, animals received coapplications of either palmitic acid, stearic acid, or linoleic acid (10 mg per ml), at the same time that these sites were treated with the inhibitor in one solution. The acidification experiments were performed with 10 mM HEPES buffer, adjusted to either pH 5.5 or pH 7.4, as follows. One flank of the anesthetized mice was immersed on a mesh netting, as described previously (Lee *et al*, 1992). The mice were anesthetized with chloral hydrate (Morton Grove Pharmaceuticals, Morton Grove, IL).

After 3 h of immersion at 37°C, the mice were removed, and the remaining buffer was gently blotted off. After 15 min, barrier function was determined by measurement of TEWL with an electrolytic water analyzer (MEECO®; Warrington, PA). Surface pH was measured with a flat, glass surface electrode from Mettler-Toledo (Giessen, Germany),

attached to a pH meter (Skin pH Meter PH 900; Courage & Khazaka, Cologne, Germany).

Immunofluorescence staining Hairless mouse skin was excised with a 6 mm punch biopsy, and the subcutaneous fat was removed. Tissue sections were incubated for 1 h in blocking buffer (1% bovine serum albumin, 0.1% cold water fish gelatin in phosphate-buffered saline, PBS) and were then incubated for 1 h further at room temperature with 1:500 dilution of polyclonal rabbit antimouse desmoglein 1 (DSG 1) antibody (gift of Dr. John Stanley, University of Pennsylvania) diluted in blocking buffer. The tissue was then washed with blocking buffer and incubated for 1 h at room temperature with fluorescein-labeled, isothiocyanate-conjugated, goat antirabbit IgG antibody (DAKO, Carpinteria, CA), diluted in blocking buffer. Either preincubation of DSG 1 antibody with DSG-1-recombinant protein (gift of Dr. Masayuki Amagai, Keio University, Tokyo, Japan), or omission of the DSG 1 primary antibody eliminated specific staining. Tissue sections were then washed with PBS and coverslipped before visualization under a confocal microscope (Leica TCS SP, Heidelberg, Germany) using fluorescein isothiocyanate at an excitation wavelength of 494 nm and an emission wavelength of 518 nm.

Protein assay on sequential tape strips The protein assay utilized the Bradford dye-binding procedure for quantification of total protein (Bradford, 1976). HEPES buffer and propylene glycol:ethanol, the two vehicles used in this study, are known to be compatible with this assay (Protein assay, Product Information, Bio-Rad, Hercules, CA). Before stripping the SC, the skin surface was cleaned with a single ethanol wipe. D-Square tapes were then placed sequentially to the test areas for about 3 s each, removed with forceps, and stored in glass scintillation vials at 5°C.

The amount of protein removed per D-Square was measured by a modification of the method of Dreher *et al* (1998). The microassay system was shown to be linear in the range 1–10 μg per ml, using human SC removed from a heel callosity. The calculated slope $R_f \pm$ SD is 0.0297 ± 0.00062; Spearman coefficient 0.999; p < 0.0001.

The protein content per stripping was determined with the Bio-Rad Protein Assay Kit. Lyophilized, bovine gamma globulin was used as the standard in all assays, because it correlated best with human SC. Each tape was incubated with 1 ml of 1 M NaOH for 1 h at 37°C in an incubator shaker at 80 rpm, and neutralized thereafter with 1 ml of 1 M HCl in the scintillation vials. Subsequently 0.2 ml of this solution was incubated in 0.6 ml distilled water plus 0.2 ml of the Bio-Rad protein dye for 5 min in borosilicate tubes. After incubations, the reagents were transferred to polysterene cuvettes, and absorption was measured with a Genesys 5 spectrophotometer (Spectronic, Rochester, NY) at 595 nm. An empty D-Square tape, as well as distilled water incubated with the Bio-Rad dye, served as negative controls. The amount of calculated protein was then normalized to skin surface area (μg per cm^2). The amount of removed protein per D-Square strip agreed with previous reports in untreated skin of hairless mice (i.e., range 2.5–4 μg per strip) (Weber *et al*, 1999).

Statistical analyses Statistical analyses were performed using Prism 2 (GraphPad Software, San Diego, CA). Normal distribution was tested before calculating the comparison. In the three group comparisons we calculated an ANOVA followed by a *posthoc* test (Bonferroni). Two groups were compaired with an unpaired *t* test.

RESULTS

Inhibition of PL-to-FFA processing increases the pH of the SC, while altering barrier homeostasis and SC integrity The surface pH of hairless mouse skin increased significantly after 1 d of BPB treatment, and continued to increase up to 3 d in comparison to vehicle-treated sites (**Fig 1***A*; p < 0.01). As previously reported (Mao-Qiang *et al*, 1995), daily topical applications of the group-1-specific sPLA$_2$ inhibitor BPB produce a concurrent, progressive abnormality in barrier function of normal skin, which becomes evident by day 2 (**Fig 1***B*). The integrity of the SC, quantitated as the number of tape strippings required to produce elevated TEWL levels, was also markedly abnormal after 3 d of BPB treatment. As seen in **Fig 1**(*C*), a significant abnormality was already present at the first stripping, and SC integrity continued to deteriorate thereafter. These changes in integrity were paralleled by an increase in the amount of protein removed per D-Square stripping (a measure of SC cohesion),
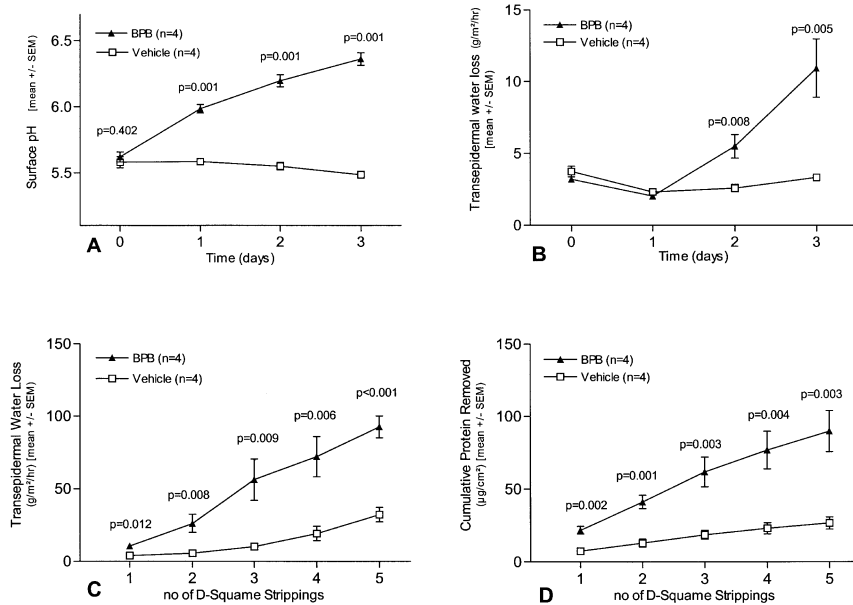
Figure 1. Alteration of stratum corneum pH with an sPLA$_2$ inhibitor correlates with altered barrier function, integrity, and cohesion. (*A*) Three days of topical BPB to intact skin results in a significant increase in SC surface pH. (*B*) Barrier function, assessed as TEWL, increases with 2 d of BPB applications. (*C*) SC integrity, assessed as number of tape strippings in relation to barrier function (TEWL), declines significantly (data shown are for 3 d of BPB). (*D*) SC cohesion, assessed as protein removed per D-Square strip, also declines significantly (data shown are for 3 d of BPB).

which again was significant by the first strip, and continued to diverge from vehicle-treated sites with further stripping (**Fig 1*D***). These results show that repeated applications of the sPLA$_2$ inhibitor BPB result in an increased skin surface pH, a change that is accompanied not only by altered barrier function, but also by altered SC integrity and cohesion in comparison to the vehicle-treated control group.

Because BPB is an alkylating agent, which could nonspecifically affect other cellular processes, we performed additional studies with a chemically unrelated, competitive inhibitor of sPLA$_2$, MJ33, which is a highly specific, competitive inhibitor of group 1 sPLA$_2$. MJ33 treatment also produced a modest increase in SC surface pH *versus* vehicle (5.87 ± 0.06 *vs* 5.60 ± 0.05; p = 0.0023). Moreover, as seen previously (Mao-Qiang *et al*, 1995; 1996), repeated applications of MJ33 produced a progressive barrier abnormality, with a 2–3-fold increase in TEWL levels by day 3 (5.19 ± 0.81 *vs* 2.97 ± 0.16; p < 0.001, for MJ33 *versus* vehicle-treated animals). As with BPB treatments, 3 d of MJ33 applications also progressively altered SC integrity (**Fig 2*A***), reflected by a parallel change in SC cohesion (**Fig 2*B***). These results show that a second, chemically unrelated, sPLA$_2$ inhibitor produces comparable changes in skin surface pH, in parallel with altered SC barrier function, integrity, and cohesion.

The acidification and cohesion abnormalities are due to reduced generation of FFA Prior studies have shown that the barrier abnormality induced by both of the topical sPLA$_2$ inhibitors can be overridden by coapplications of certain end-products of PL hydrolysis, i.e., nonessential FFA (Mao-Qiang *et al*, 1995; 1996). We confirmed here, again, that coapplications of either PA (C16:0) or stearic acid (18:0), with either BPB or MJ33, prevented emergence of the barrier abnormality (data not shown). Coapplications of PA, stearic acid, and to a lesser extent linoleic acid prevented the BPB-induced increase in the pH of the SC (PA, 5.40 ± 0.14 *vs* 5.92 ± 0.05; stearic acid, 5.73 ± 0.09 *vs* 6.17 ± 0.08; linoleic acid, 5.77 ± 0.10 *vs* 6.28 ± 0.10). Furthermore, coapplications of either stearic acid, PA, and to a lesser extent linoleic acid also prevented emergence of the sPLA$_2$-inhibitor-induced abnormalities in SC integrity and cohesion (**Fig 3*A–D***; linoleic acid data not shown). Likewise, coapplications of PA with MJ33 prevented emergence of the MJ33-induced abnormality in SC integrity (**Fig 2*A*, *B***). These results show that the sPLA$_2$-inhibitor-induced abnormalities in integrity and cohesion are linked to increased pH, resulting from decreased generation of FFA, the end-products of PL hydrolysis by sPLA$_2$.
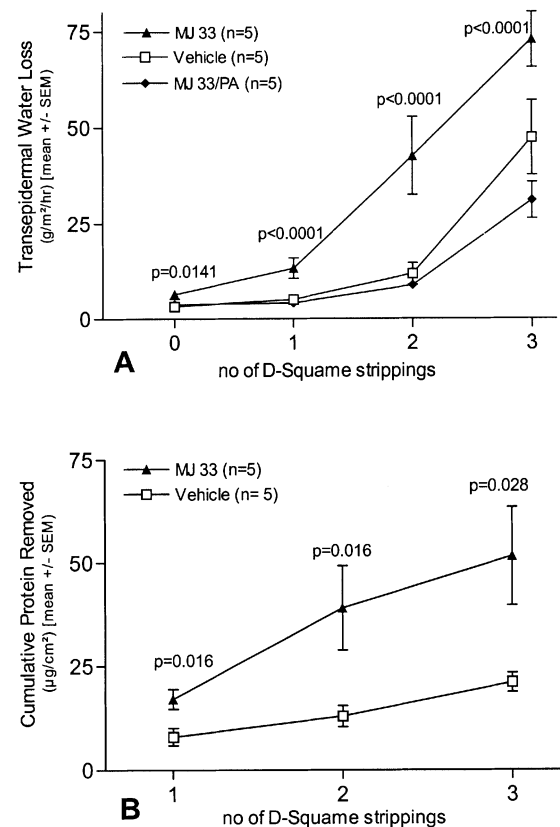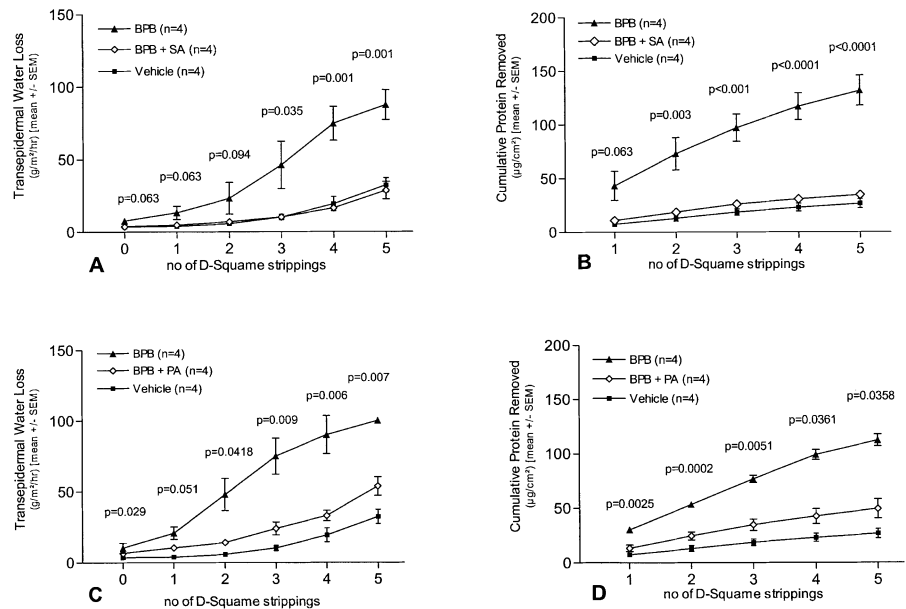

Figure 2. A chemically unrelated sPLA$_2$ inhibitor produces similar changes in SC integrity and cohesion. (*A*) Three days of topical MJ33 to intact skin results in a significant increase in TEWL, but coapplications of FFA (PA) could override this effect; indicated p-values were determined by ANOVA; the *posthoc* (Bonferroni) test revealed p < 0.05 for MJ33 *versus* vehicle at all data points, and p < 0.01 MJ33 *versus* MJ33/PA at all data points except at zero D-Square strippings (which was not significant). (*B*) Integrity and cohesion of SC after 3 d of MJ33 applications to intact skin were significantly reduced; p-values determined by *t* test.

Figure 3. Coapplication of FFA (stearic acid, palmitic acid) with sPLA$_2$ inhibitor normalizes SC integrity and cohesion. (*A*, *B*) Integrity and cohesion data, respectively, after 3 d of BPB ± stearic acid application to intact skin. (*C*, *D*) Similar data on integrity and cohesion after 3 d of BPB ± PA application to intact skin. Indicated p-values for (*A*)–(*D*) were derived by ANOVA. (*A*) *Posthoc* (Bonferroni) test revealed p < 0.01 for BPB *versus* both groups for four and five D-Square strippings, (*B*) p < 0.01 for BPB *versus* both groups for two, three, four, and five D-Squame strippings, and p < 0.05 for BPB *versus* vehicle for one D-Squame stripping. (*C*) Bonferroni test: p < 0.01 for BPB *versus* vehicle at all data points and p < 0.05 for BPB *versus* BPB/ PA for three, four, five D-Squame strippings. (*D*) Bonferroni test: p < 0.01 for BPB *versus* both groups for all data points.

Exposure of normal skin to a neutral pH also produces abnormalities in pH, barrier function, and integrity/cohesion To ascertain more directly whether the sPLA$_2$-inhibitor-induced functional alterations could be due to the associated pH changes, we next assessed SC integrity after short-term exposure of normal skin to a neutral *versus* acidic pH. After 3 h of exposure to a neutral pH buffer (HEPES), the surface pH of the SC rose from 5.86 ± 0.21 to 6.41 ± 0.20 (**Fig 4***A*, p = 0.0257). Whereas exposure to both buffers increased basal TEWL levels, a slightly greater increase in TEWL occurred following exposure to the neutral pH buffer for 3 h (all changes within normal range; data not shown). Even with such short-term exposures, SC integrity and cohesion declined significantly after 3 h of treatment with the neutral pH buffer (**Fig 4***B*, *C*). These studies show that short-term exposure to a neutral pH buffer alone begin to produce functional abnormalities that mimic those induced by the sPLA$_2$ inhibitors.

Exposure to a neutral pH buffer amplifies the sPLA$_2$-inhibitor-induced alterations in SC integrity As a further test of the importance of PL-derived FFA for SC functions, we next assessed whether exposure of BPB-treated skin to a neutral or acidic buffer could amplify or override the functional abnormalities produced by the inhibitor. Exposure of skin sites to BPB for 3 d provoked an increase in SC pH, which was amplified further by exposure of the BPB-treated sites to a neutral pH buffer for 3 h. Exposure to an acidic buffer lowered the surface pH from 5.95 ± 0.05 to 5.72 ± 0.06 (**Fig 5***A*). In parallel with the increase in pH, exposure to the neutral pH buffer also accentuated the BPB-induced abnormality in SC integrity. In contrast, integrity remained unchanged in BPB-treated sites exposed to an acidic pH buffer (**Fig 5***B*). These studies demonstrate further that the abnormalities in SC integrity/cohesion induced by sPLA$_2$ blockage can be attributed largely to the acidification abnormality produced by inhibitor treatment.

Premature desmosomal dissolution accounts for the abnormality in SC integrity/cohesion We next assessed the basis for the abnormalities in SC integrity and cohesion that result from application of sPLA$_2$ inhibitors to murine skin. Both inhibitors provoked a dramatic decrease in the density of desmosomes in the lower SC and at the stratum granulosum (SG)–SC interface (**Figs 6**, **7**). Moreover, coapplication of free fatty acids with the inhibitors reversed the decrease in desmosome density in the lower SC (**Figs 6**, **7**). **Figure 8** shows the reduction

of corneodesmosomes after 3 d of MJ33 treatment, an effect that could be prevented by coapplication of FFA (PA). BPB treatment resulted in a comparable reduction of corneodesmosomes (data not shown). A similar reduction in desmosomes was also shown by immunohistochemical assessment of DSG-1-positive structures in the lower SC of BPB *versus* vehicle-treated SC. On laser confocal microscopy, the density of DSG-1-positive clusters, which are presumed to correspond to intact desmosomes, declined dramatically in BPB-treated SC, whereas DSG-1-positive staining in vehicle-treated SC was comparable to control (**Fig 6**). These results show that the inhibitor-induced decline in SC integrity and cohesion can be attributed to a premature dissolution of desmosomes in the lower SC.

DISCUSSION

The acidic pH of the SC could be derived from one or more of several potential sources (Ohman and Vahlquist, 1998). Until recently, extrinsic sources were most commonly invoked, e.g., secreted eccrine/sebaceous gland products or metabolic byproducts of resident, cutaneous microflora (Aly *et al*, 1975; Puhvel *et al*, 1975). Among potential intrinsic (endogenous) sources, both passive (catabolic enzymes) and active (proton pumps or exchangers) sources have been postulated. Recently, Krien and Kermici (2000) proposed that *cis*-urocanic acid generation, the end-product of the filaggrin-to-histidine-to-urocanic acid proteolytic pathway, could alone explain the acidic pH of the SC. Their assay method, however, employed an externally applied aqueous receptor, which probably samples neither the lower SC nor hydrophobic micro-domains within the SC interstices. It should also be noted that filaggrin hydrolysis is regulated by changes in external humidity (Scott *et al*, 1982; 1986), which, in turn, could influence the amount of histidine that would be available for *cis*-urocanic acid generation. Accordingly, we showed here that an alternative enzymatic mechanism, i.e., generation of FFA from sPLA$_2$-mediated catabolism of PL, regulates SC acidification. Topical applications of two chemically unrelated sPLA$_2$ inhibitors increased SC pH. As the inhibitor-induced increases, however, did not achieve a neutral pH, it is likely either that sPLA$_2$ blockade is incomplete or that other acidification mechanisms compensate, in part, for sPLA$_2$ blockade. Yet, it is also important to note that such potential, compensatory acidifying mechanisms, if operative, did not normalize pH during these experiments. We then demonstrated the specificity of this reaction through the ability of
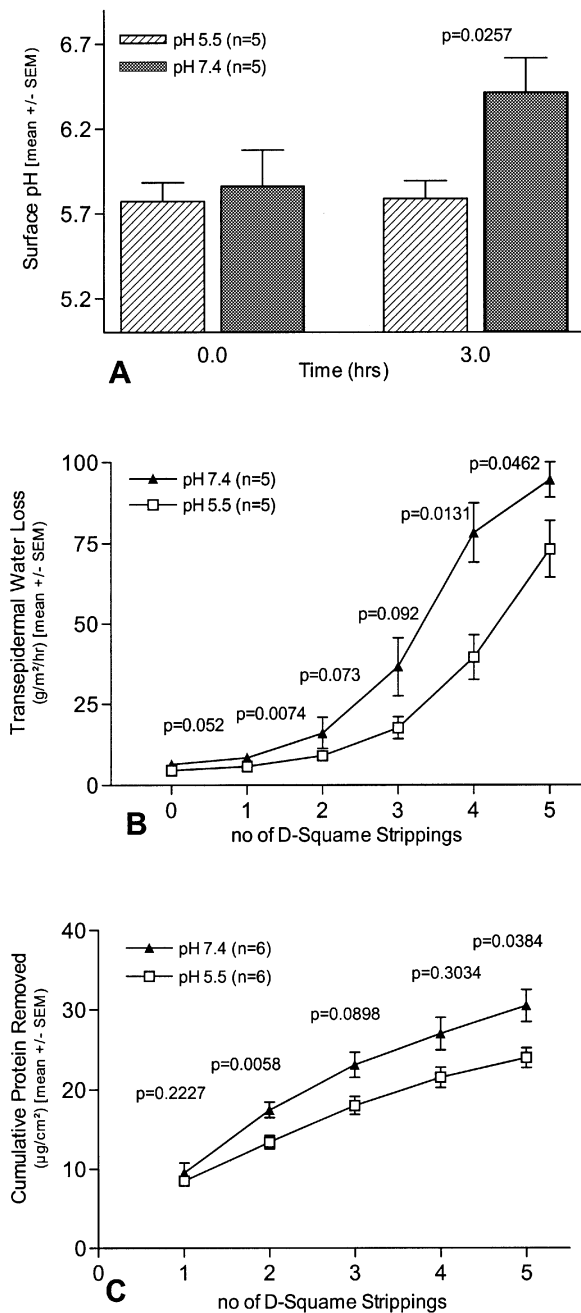
Figure 4. Exposure of intact skin to neutral pH buffer produces similar changes in SC pH, barrier function, and integrity. Anesthetized mice were immersed for 3 h in HEPES buffer, adjusted to pH 7.4 or pH 5.5. (*A*) After exposure to HEPES buffer pH 7.4 the pH of SC is significantly elevated in comparison to skin surfaces exposed to HEPES buffer pH 5.5. (*B*) An abnormality in SC integrity is also apparent. (*C*) The changes in SC cohesion after 3 h exposure to HEPES buffer, pH 7.4 *vs* pH 5.5, are also significant.

Figure 5. A neutral pH buffer amplifies the sPLA$_2$-inhibitor-induced alteration in pH, whereas an acidic buffer overrides inhibitor-induced alterations in barrier function and integrity. The hairless mice were first treated with the sPLA$_2$ inhibitor BPB for 3 d and then exposed to HEPES buffer, either pH 7.4 or pH 5.5, for 3 h. (*A*) Exposure to HEPES buffer pH 7.4 for 3 h amplifies the BPB-induced barrier abnormality, whereas exposure to HEPES buffer pH 5.5 prevents this change. The calculated ANOVA was not significant and thus a *posthoc* comparison was not performed. (*B*) After 3 d of BPB treatment, a significant barrier abnormality is present (cf. **Fig 2**). This abnormality is amplified by 3 h exposure to HEPES buffer at pH 7.4, but not at pH 5.5. (*C*) Three days of BPB treatment plus 3 h of exposure to HEPES buffer at either pH 7.4 or pH 5.5. Both treatments cause a further decline in SC integrity, but the defect is exacerbated significantly at pH 7.4 *vs* pH 5.5.

coapplications of either FFA or an acidic pH buffer to restore a normal acidic pH to inhibitor-treated SC. Thus, it can be assumed that sPLA$_2$-mediated catabolism of PL to FFA represents an important acidifying mechanism in normal SC.

FA are one of the three major lipid species of the SC, which together account for about 10% of SC by weight (Yardley and Summerly, 1981; Schurer and Elias, 1991). Cytochemical studies have shown that sPLA$_2$ activity (Freinkel and Traczyk, 1985) is codelivered to the SC interstices, along with PL (Grayson *et al*, 1985), through the secretion of epidermal lamellar body contents at
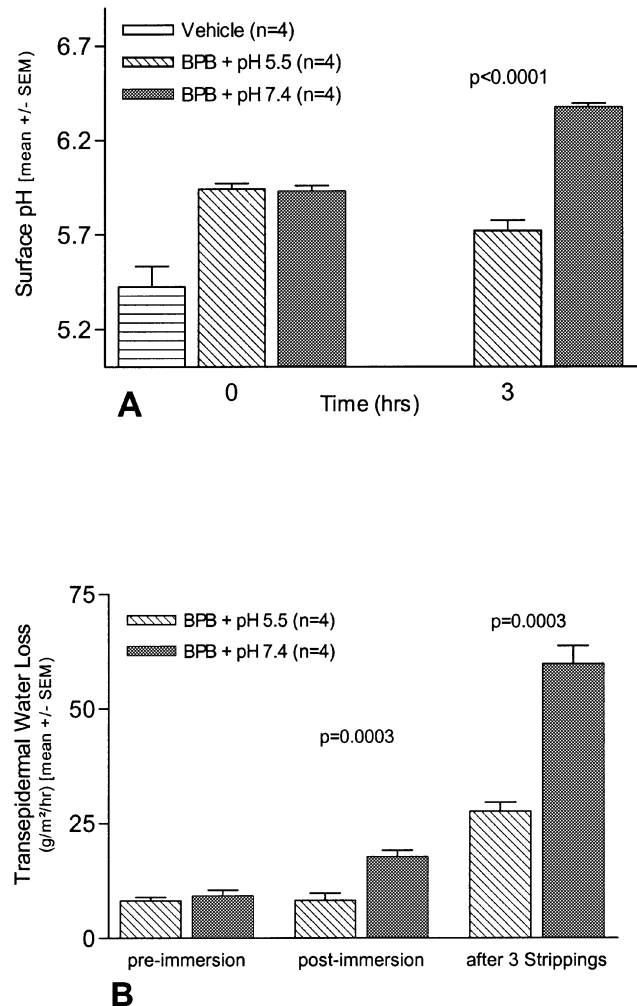
the SG–SC interface (Elias *et al*, 1988). Moreover, there is also evidence that PL-to-FFA generation is an early catabolic event in a sequence of biochemical reactions that transforms secreted polar lipids into their nonpolar products (Long *et al*, 1985; Elias *et al*, 1988; Mao-Qiang *et al*, 1995; 1996). Although there are no other sources of FFA among the secreted, lamellar-body-derived polar lipids, in theory FFA could also be generated in the outer SC from the catabolism of nonlamellar-body-derived triglycerides by the abundant triacylglycerol lipase that is delivered to the SC interstices by lamellar body secretion (Grayson *et al*, 1985; Menon *et al*, 1986). Moreover, whereas only small quantities of triglycerides are present in SC (Hedberg *et al*, 1988; Schurer and Elias, 1991), large amounts of triglycerides are synthesized in both the nucleated layers of the epidermis (Monger *et al*, 1988) and sebaceous glands (Thody and Shuster, 1989; Stewart and Downing, 1991). Yet, although
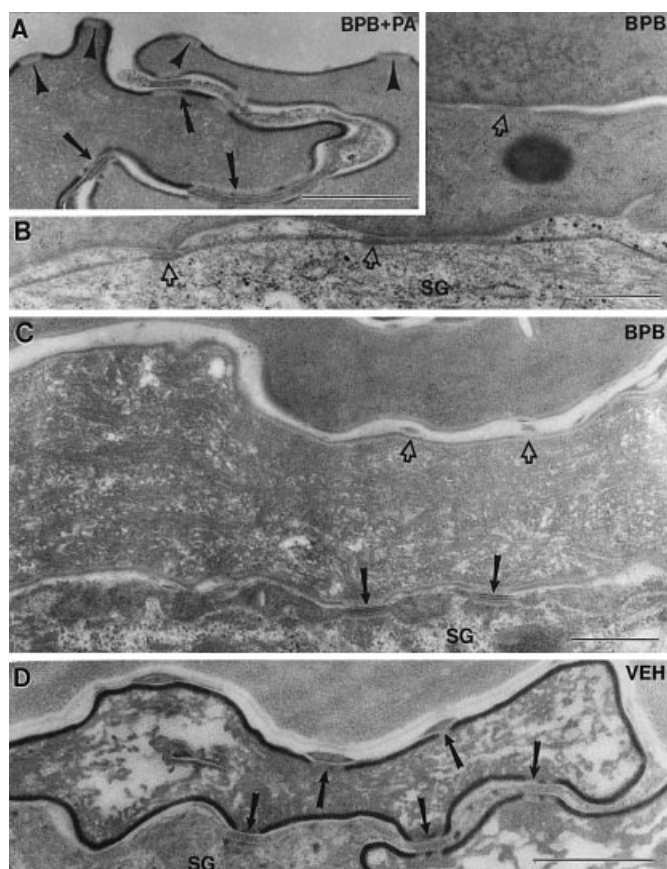
Figure 6. Treatment with two sPLA$_2$ inhibitors reduced desmosome density in the lower SC. (*B, C*) Hairless mouse skin treated with the sPLA$_2$ inhibitor BPB. Note the diminution of intact desmosomes (*solid arrow*) at the SG–SC interface with the presence of desmosomal remnants (*open arrows*). In contrast, coapplication of PA with BPB maintains normal desmosome numbers (*A, solid arrows* and *arrowheads*). (*D*) Vehicle (VEH)-treated SC − desmosome numbers in the SC appear to be normal (*D, solid arrows*). (*A*)–(*D*) OsO4 postfixation. *Magnification bars*, 0.5 μm.

Figure 7. Treatment with the sPLA$_2$ inhibitor BPB reduces desmosoglein labeling in the lower SC. Reduction in desmosomes in inhibitor-treated skin is also shown by the decrease in DSG 1 immunolabeling in the lower SC of BPB *versus* vehicle-treated SC, on laser confocal microscopy (*A*). In contrast DSG 1 staining in vehicle-treated SC remained prominent (*B*).

Figure 8. Treatment with the sPLA$_2$ inhibitor MJ33 reduces corneodesmosomes in the lower SC. Reduction of corneodesmosomes after MJ33 treatment, an effect that could be prevented by coapplication of the FFA PA. Indicated p-value was determined by ANOVA; the *posthoc* (Bonferroni) test revealed $p < 0.05$ for MJ33 *versus* MJ33 + PA and MJ33 *versus* vehicle.

some FFA could, in theory, derive from epidermal (nonlamellar body) and/or sebaceous gland sources, we found no changes in SC pH either in asebia (Asb-J) mice that lack sebaceous glands nor in animals treated with the topical lipase inhibitor orlistat (Elias PM, Mao-Qiang M, Kao J, Feingold KR, unpublished observations). Thus, a specific PL-derived pool of FFA accounts for the observed, FFA-induced changes in skin surface pH.

Regardless of the origin of FFA, these lipids are segregated within the interstices of normal SC. Within this compartment, they could regulate the pH selectively, resulting in modulation of pH-sensitive functions of the SC (**Fig 9**). Indeed, we showed here that certain key functions of the SC are regulated in parallel with the pH changes induced by a lack of PL-derived FFA. As shown previously, we showed again that inhibition of sPLA$_2$ results in a permeability barrier abnormality, and that coapplications of FFA normalize barrier function (Mao-Qiang *et al*, 1995, 1996). These prior studies showed that depletion of PL-derived FFA results in membrane structural abnormalities attributable to an altered ratio of the three key SC lipids function (Mao-Qiang *et al*, 1995, 1996). The question remains, however, whether an acidic pH alone could compensate for depletion of FFA in correcting the barrier. In fact, an alteration in pH alone could theoretically alter barrier homeostasis by at least two different mechanisms (**Fig 9**). First, barrier recovery after acute perturbations is delayed at a neutral pH, attributable to altered extracellular processing of secreted lamellar body polar lipids (Mauro *et al*, 1998). Moreover, both β-
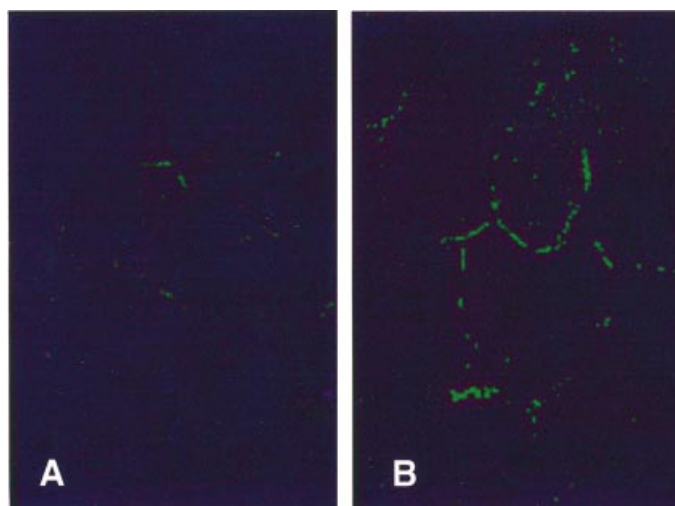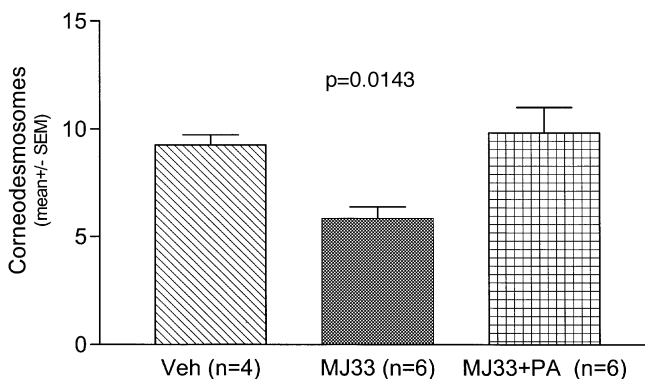
glucocerebrosidase and acid sphingomyelinase, enzymes that are required for the generation of ceramides from glucosylceramides and sphingomyelin, respectively, exhibit acidic pH optima (Holleran *et al*, 1992; Jensen *et al*, 1999; Takagi *et al*, 1999). Accordingly, an elevated SC pH could alter barrier function by blockade of ceramide generation from their two polar lipid precursors. Second, a neutral pH could directly impair the structural organization of lamellar membrane structures. Bouwstra *et al* (1998, 1999) have shown that the pKa resulting from an acidic pH allows FFA to assemble more efficiently into ceramide and cholesterol-containing membrane bilayers. Yet, whereas the BPB-induced barrier abnormality could be prevented by coapplied FFA, it could not be reversed by an acidic pH buffer alone: barrier recovery after acute perturbation is delayed comparably when BPB is applied in either a neutral or an acidic buffer (Fluhr JW, Feingold KR, Elias PM, unpublished observations). Thus, whereas the pH-related mechanism clearly is important for barrier homeostasis, it cannot substitute for PL-derived generation of FFA.

A second functional consequence of FFA-mediated acidification relates to modulations of SC integrity/cohesion (**Fig 9**). Elevation
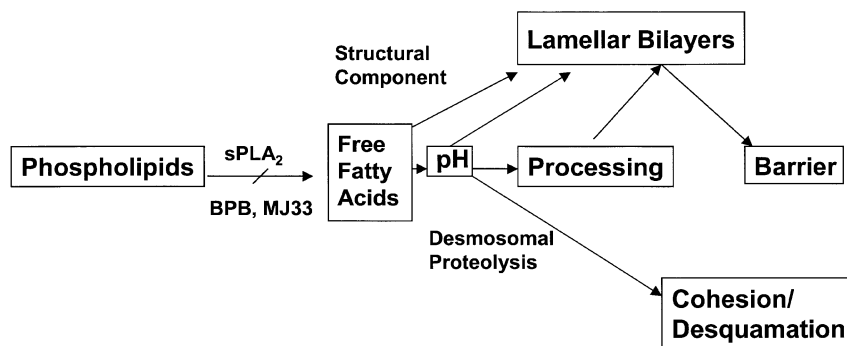
Figure 9. Structural and functional consequences of PL-to-FFA pathway. Lamellar body secretion delivers both PL and sPLA$_2$ to the SC interstices. Degradation of PL to FFA has distinct, separate downstream consequences for both permeability barrier function and SC integrity/cohesion.

of pH through inhibition of sPLA$_2$ compromised both SC integrity and SC cohesion, as evidenced by an sPLA$_2$-inhibitor-induced decrease in resistance to sequential strippings, and an increase in the quantity of protein removed per stripping, respectively. Again, the link between sPLA$_2$ inhibition and diminished SC integrity was shown by the normalization of SC integrity/cohesion that followed reacidification with either coapplied FFA or exposure to an acidic buffer. Conversely, integrity/cohesion deteriorated further when inhibitor-treated skin was exposed to a neutral pH buffer. We also showed that the basis for diminished SC cohesion with sPLA$_2$ blockade correlates with a premature dissolution of corneodesmosomes. Corneodesmosomes normally persist into the mid SC, but in sPLA$_2$-inhibitor-treated skin extensive degradation/dissolution was evident in the mid to lower SC, extending even to the SG–SC interface. Conversely, coapplication of FFA with the inhibitors restored normal corneodesmosome densities. Accordingly, desmoglein immunolabeling of corneocytes decreased abruptly above the SG–SC interface in inhibitor-treated skin. The accelerated dissolution of desmosomes in sPLA$_2$-inhibitor-treated skin could reflect increased activities of the SC chymotryptic and tryptic enzymes, which display neutral-to-alkaline pH optima (Brattsand and Egelrud, 1999; Egelrud, 2000). In contrast, the low desquamation rates (increased cohesion) that occur in intact skin could reflect a decrease in activity of one or both of these two enzymes. Further studies will be needed to ascertain the enzymatic basis for the sPLA$_2$-inhibitor-induced alterations in SC integrity and cohesion.

In summary, topical applications of two chemically unrelated sPLA$_2$ inhibitors produced an increase in the skin surface pH of murine SC that was paralleled by a permeability barrier abnormality, altered SC integrity, and decreased SC cohesion. Not only SC pH but also all of the functional abnormalities normalized when FFA were coapplied with the inhibitors. Short-term applications of a neutral pH buffer to inhibitor-treated skin further amplified the abnormalities in SC integrity and cohesion. Finally, the alterations in integrity/cohesion were linked to premature dissolution of corneodesmosomes.

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