Both exposure of stratum corneum to neutral pH buffers and blockade of acidification mechanisms disturb cutaneous permeability barrier homeostasis and stratum corneum integrity/cohesion, but these approaches all introduce potentially confounding variables. To study the consequences of stratum corneum neutralization, independent of hydration, we applied two chemically unrelated superbases, 1,1,3,3-tetramethylguanidine or 1,8-diazabicyclo [5,4,0] undec-7-ene, in propylene glycol:ethanol (7:3) to hairless mouse skin and assessed whether discrete pH changes alone regulate cutaneous permeability barrier function and stratum corneum integrity/cohesion, as well as the responsible mechanisms. Both 1,1,3,3-tetramethylguanidine and 1,8-diazabicyclo [5,4,0] undec-7-ene applications increased skin surface pH in parallel with abnormalities in both barrier homeostasis and stratum corneum integrity/cohesion. The latter was attributable to rapid activation (<20 min) of serine proteases, assessed by in situ zymography, followed by serine-protease-mediated degradation of cornedesmosomes. Western blotting revealed degradation of desmoglein 1, a key cornedesmosome structural protein, in parallel with loss of cornedesmosomes. Coapplication of serine protease inhibitors with the superbases, thus normalizing stratum corneum integrity/cohesion, also delayed permeability barrier recovery, attributable to decreased β-glucocerebrosidase activity, assessed zymographically, resulting in a lipid-processing defect on electron microscopy. These studies demonstrate unequivocally that stratum corneum neutralization alone provokes stratum corneum functional abnormalities, including aberrant permeability barrier homeostasis and decreased stratum corneum integrity/cohesion, as well as the mechanisms responsible for these abnormalities. Key words: cornedesmosome/permeability barrier function/serine protease/serine protease inhibitor/stratum corneum/superbase/transepidermal water loss.


Althought the skin has long been known to display an acid surface (“acid mantle”) (Schade and Marchionini, 1928), even today little is known about either the origin or the function(s) of this acidic surface. Prior studies on the acid mantle ascribed its origin principally to exogenous sources of microbial, sebaceous gland, and/or eccrine gland origin (Marchionini and Hausknecht, 1938). Furthermore, based upon a variety of indirect evidence, its function has been assumed to be principally antimicrobial (Ally et al, 1975; Puhvel et al, 1975).

Recent studies support alternative views of the origin of the acid mantle, as well as additional functions of stratum corneum (SC) acidity (Ohman and Vahlquist, 1994; 1998; Chikakane and Takahashi, 1995; Denda et al, 2000). In addition to exogenous mechanisms, three endogenous pathways have been identified as potential contributors to SC acidity: (1) generation of urocanic acid by histidase-catalyzed deamination of histidine (Krien and Kermici, 2000); (2) secretory phospholipase A2 (sPLA2) generation of free fatty acids from phospholipids (Fluhr et al, 2001); and (3) a nonenergy-dependent sodium–proton exchanger (NHE1) (Behne et al, 2002). Of these three mechanisms, the histidase pathway, though quantitatively capable of acidifying the SC (Krien and Kermici, 2000), appears least likely to mediate functions in the lower SC, because (1) lack of substrate would make this mechanism nonoperative at the high relative humidities of the stratum compactum (Scott and Harding, 1986), and (2) urocanic acid is a water-soluble metabolite, which may not reach lipophilic membrane domains from the cornocyte cytosol, where it is generated. Yet, it is in membrane sites in the lower SC that the permeability barrier is formed (Elias and Friend, 1975), and it is in these sites that SC integrity/cohesion localizes (Chapman and Walsh, 1990; Fartasch et al, 1993; Elias et al, 2001). Hence, our laboratory has focused recently on the role and functional importance of the sPLA2 and NHE1 pathways, which both appear to influence membrane acidity in the lower SC (Fluhr et al, 2001; Behne et al, 2002). Indeed, inhibition and/or blockade of either sPLA2 or NHE1 result in an elevated SC pH, and more importantly, interference with these mechanisms perturbs permeability.
barrier homeostasis and/or SC integrity/cohesion (Fluhr et al., 2001; Behne et al., 2002).

Direct evidence for the importance of pH for permeability barrier homeostasis was first shown by the delay in barrier recovery that occurs when acutely disrupted skin sites are immersed in neutral pH buffers (Mauro et al., 1998). Moreover, the barrier abnormality resulting from either sPLA2 or NHE1 blockade could be overridden by coexposure of inhibitor-treated sites to an acidic (normal pH) buffer (Fluhr et al., 2001; Behne et al., 2002). An acidic pH is critical for barrier homeostasis, in part because two key lipid-processing enzymes, β-glucocerebrosidase (βGlcCerαse) and acidic sphingomyelinase, which generate a family of ceramides from glucosylceramide and sphingomyelin precursors, respectively (Uchida et al., 2002), exhibit low pH optima (Vaccaro et al., 1985; Holleran et al., 1993; Jensen et al., 1993; Schmuth et al., 2000). It has also been proposed that an acidic pH directly impacts lipid interactions in the SC extracellular lamellar bilayers (Bouwstra et al., 1999). Together, these mechanisms appear to regulate the competence of the extracellular lamellar bilayer system.

An acidic SC pH also clearly promotes SC integrity and cohesion. Exposure to either neutral pH buffers or sPLA2 blockade results in an enhanced tendency for the SC to be removed by tape stripping (integrity), as well as increased amounts of protein removed per stripping (= cohesiveness) (Fluhr et al., 2001). In the case of sPLA2 blockade, the alterations in SC integrity/cohesion could be further attributed to a decreased density of corneodesmosomes (CD), and to a decline in at least one of its constituent proteins, desmoglein 1 (DSG1) (Fluhr et al., 2001). Furthermore, as both the chymotryptic and trypsin serine proteases (SCCE and SCTE), which mediate degradation leading to desquamation, exhibit neutral pH optima (Ekholm et al., 2000), they could become activated as SC pH increases.

All prior attempts to manipulate SC pH, and to assess the effects of pH on epidermal function, have utilized either buffers, which could exert effects on function independent of pH (e.g., from hydration or occlusion), or inhibitor/knockout models (e.g., a variety of unrelated changes can occur). To establish definitively the apparent link between SC pH and barrier homeostasis and SC integrity/cohesion, we developed a new model in which SC pH could be modulated directly, utilizing topical applications of low concentrations of two “superbases”, 1,3,3,7-tetramethyl-guanidine (TMG) and 1,8-diazabicyclo [5,4,0] undec-7-ene (DBU) (Kaljurand et al., 2000; Oyama and Kondo, 2003), to raise and sustain pH to specific levels within the SC, without evidence of toxicity at the low concentrations employed. Because superbases and superacids are by definition at least an order of magnitude more basic or more acidic than 1 N NaOH and 1 N HCl, these agents are by definition at least an order of magnitude more basic or more acidic than 1 N NaOH and 1 N HCl. Moreover, the barrier ab-

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Integrity (g per m2 per h ± SD)</th>
<th>Significance (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMG control</td>
<td>44.00 ± 5.56</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TMG alone</td>
<td>66.00 ± 4.33</td>
<td></td>
</tr>
<tr>
<td>TMG + soybean trypsin inhibitor</td>
<td>33.00 ± 4.58</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TMG + aprotinin</td>
<td>19.33 ± 0.07</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TMG + chymostatin</td>
<td>31.66 ± 5.85</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TMG + PMSF</td>
<td>20.50 ± 30.50</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Table I. SPI reverse superbase-induced abnormalities in SC integrity

**Materials and Methods**

**Animals and Materials** Male hairless mice (Skh1/Hr), 6–8 wk old, were purchased from Charles River Laboratories (Wilmington, MA) and fed Purina mouse diet and water ad libitum. Propylene glycol, ethanol, and HCl were from Fisher Scientific (Fairlane, NJ), whereas TMG, DBU, phenylmethylsulfonyl fluoride (PMSF), chymostatin, soybean trypsin inhibitor, and aprotinin were from Sigma Chemical (St Louis, MO). Rabbit polyclonal antibody against mouse DSG1 was a gift from Dr John Stanley (University of Pennsylvania). Horseradish peroxidase conjugated with anti-rabbit IgG was purchased from Vector Laboratories (Burlingame, CA). Exzet-Mch Proteinase Inhibitor Cocktail (Calbiochem) and N,N-dimethylglucopyranoside were purchased from Molecular Probes (Eugene, OR). 22 mm D-Squame-100 tapes were purchased from CuDerm (Dallas, TX). Bradford protein assay kit and bovine plasma γ globulin were purchased from Bio-Rad (Heracles, CA). Mice were anesthetized with chloral hydrate (Morton Grove Pharmaceuticals, Morton Grove, IL).

**Experimental procedures**

*Acute neutralization and pH recovery model* Normal hairless mice were treated topically with a single application of either TMG or DBU (dose range 1:000–1:0000 vol/vol) in propylene glycol/ethanol (7:3 vol/vol) on 5–6 cm2 areas on both flanks. Controls were treated with HCl-neutralized TMG (nTMG) in the same propylene glycol/ethanol vehicle. The general idea of using superbases is related to their ability to accept protons, thereby acting as “proton sponges”. The chelating function of superbasic TMG is based on its 1,8-diaminonaphthalene skeleton. TMG not only shows a high thermodynamic basicity (pKBH+ = value of 25.1), but it also reveals unusually high kinetics for basic reactions, which makes this superbase highly attractive for base-catalyzed applications. Superbases such as TMG and DBU break water to generate a hydroxyl group and chelate a proton to become positively charged. As noted above, the positively charged superbase, with its liposoluble core structure, confers on TMG and DBU physicochemical properties that allow penetration through the SC.

**Surface pH** Surface pH was measured with a flat, glass surface electrode from Mettler-Toledo (Giesen, Germany), attached to a pH meter (PH 900, Courage & Khazaka, Cologne, Germany), immediately before and at 1, 2, 3, 4, 6, 9, 12, 18, and 24 h after TMG, DBU, and nTMG applications. For the experiments with serine protease inhibitors (SPI), animals received coapplications of TMG or DBU with either 10 mM PMSF, chymostatin, aprotinin, or soybean trypsin inhibitor (concentrations specified in the legend to Table I).

**Post-barrier disruption, neutralization model** Normal hairless mice were treated topically with a single application of TMG (1:000 vol/vol) in propylene glycol/ethanol (7:3 vol/vol) on a 5–6 cm2 area on one flank versus nTMG, TMG plus PMSF, or vehicle alone to the contralateral flank, or to the flanks of littermates immediately after barrier disruption by sequential tape stripping (transepidermal water loss (TEWL) rates ≥ 4 mg per cm2 per h). Surface pH was measured immediately after disruption and at the same time points as above, followed by assessment of function and mechanistic studies (see below).

**Functional assessments**

**Permeability recovery** To assess the kinetics of epidermal permeability barrier recovery, TEWL levels were measured on the flanks of hairless mice using an electrolyte water analyzer (MEECO, Warrington, PA), immediately before and after, as well as 3 h after, acute barrier disruption by repeated D-Squame tape stripping, and after a single application of TMG, nTMG, DBU, or nDBU (see above).

**SC hydration** SC water content, as the sum of hydration of all SC layers, was determined by capacitance measurements with a corneometer (CM820, Courage & Khazaka, Cologne, Germany) 3 h after application of TMG, nTMG, DBU, or nDBU to intact skin.

**SC integrity** To study SC integrity (rate of change in TEWL with repeated stripings), sequential tape stripping was performed on the flanks of hairless mice 3 h after prior application of either TMG or nTMG. TEWL levels were measured after each tape stripping, until TEWL rates exceeded 4 mg per cm2 per h (five to six stripings in normal murine SC).
SC cohesion Three hours after superbase applications, and immediately before stripping the SC, the skin surface was cleaned with a single ethanol wipe. D-Squame tapes were then placed sequentially onto the test areas for about 3 s each, removed with forceps, and stored in glass scintillation vials at 5°C. SC cohesion is reflected by the amount of protein removed from pooled, sequential D-Squame strippings (whole SC down to the stratum compactum from one site per mouse), extracted in 2 ml of 1 N NaOH, and measured as previously described (Dreher et al., 1998). This microwax system was shown again to be linear for human SC in the range 1–10 µg per ml, using SC from human callus to generate standard curves (calculated slope R² = 0.9297 ± 0.00062; Spearman coefficient 0.999; p < 0.0000). The protein content per stripping was determined with the Bio-Rad Protein Assay Kit, as described recently, using bovine γ-globulin as the standard in all assays (Fluhler et al., 2000). Briefly, tapes were incubated with 1 ml of 1 N NaOH for 1 h at 37°C in an incubator shaker and neutralized thereafter with 1 ml of 1 N 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 polystyrene cuvettes, and absorption was measured with a Genesys 5 spectrophotometer (Spectronic, Rochester, NY) at 595 nm. Blank D-Squame tape was extracted and assayed as a negative control. The amount of protein removed was then normalized to skin surface area (μg per cm²). The amount of protein per D-Squame strip was compared to previous reports in untreated sites of hairless mice (i.e., range 2.5–4 µg per strip) (Dreher et al., 1998).

**In situ zymographic assays**

SP activity Biopsies were obtained from hairless mouse flanks after treatment with superbase or neutralized superbase, and the subcutaneous fat was removed by scraping with a #10 Bard-Parker blade. Frozen sections (10 µm thick) were rinsed with a washing solution (2% Tween 20 in deionized water) and incubated at 37°C for 2 h with 250 µl of BODIPY-FI-caspe (BODIPY, Molecular Probes, Eugene, OR), operated at 60 kV. In order to quantify CD density in electron micrographs, 10 or more pictures were taken by an independent observer from three or more blocks from three animals in each experiment at 31,500 magnification; i.e., a total of at least 30 micrographs. The ratio of the total length of intact CD to the total length of the cornified envelopes in the first and second cell layers of the lower SC was determined using a planimeter (Morris, 2000).

β-Glucerase activity Biopsies were obtained from treated sites as above, sectioned (6 µm), rinsed with the washing solution, and incubated with 250 µl of resorufin β-D-glucopyranoside in deionized water (1 mM) at 37°C for 2 h. Acidification of some superbase-treated sections was again performed with 10 mM MES buffer, pH to 5.20, as above. Sections were then visualized in the confocal microscope at an excitation wavelength of 588 nm and an emission wavelength of 644 nm.

**Western immunoblotting** SC was isolated from hairless mouse flanks, previously treated as above, using D-Squame sequential tape strippings until no further SC could be removed (= whole SC; typically five to six strippings). Hematoxylin and cosin staining was performed on paraffin sections (6 µm) from biopsies of the tape-stripped areas to ensure equivalent SC removal for each experiment group. D-Squame tapes were then incubated overnight at 4°C in 1% Triton X-100 and a protease inhibitor cocktail (Complete Mini, EDTA-Free, Roche, 1 tablet per 10 ml) in deionized water and sonicated for 5 min at room temperature to extract protein from the tapes. The protein content per stripping was then determined, as above. An equal amount of extracted protein from each experimental group was loaded onto 4%–12% Tris-glycine polyacrylamide gels (PAGE Gold Precast Gels, BioWhittaker Molecular Applications, Rockland, ME). After electrophoresis, proteins were transferred from slab gels onto nitrocellulose membranes and immunoblotted with the rabbit antimouse DSG1 antibody, and antibody binding to DSG1 was detected with the Western Lighting chemiluminescence kit (PerkinElmer Life Sciences, Boston, MA).

**Electron microscopy** Skin biopsy samples were taken at 1 and 3 h after the various treatments (n = 3 from each group) and processed for light and electron microscopy. Samples were minced to less than 0.5 mm³, fixed in modified Karnovsky’s fixative overnight, and postfixed in either ruthenium tetroxide (RuO₄) or 2% aqueous osmium tetroxide (OsO₄), both containing 1.5% potassium ferrocyanide (Hou et al., 1991). After postfixation, all samples were dehydrated in graded ethanol solutions and embedded in an Epon epoxy mixture. Ultrathin sections were examined, with or without further lead citrate contrast, in a Zeiss 10A electron microscope (Carl Zeiss, Thornwood, NY), operated at 60 kV. In order to quantify CD density in electron micrographs, 10 or more pictures were taken by an independent observer from three or more blocks from three animals in each experiment at 31,500 magnification; i.e., a total of at least 30 micrographs. The ratio of the total length of intact CD to the total length of the cornified envelopes in the first and second cell layers of the lower SC was determined using a planimeter (Morris, 2000).

![Figure 1](image_url) Figure 1. Single topical applications of two superbases acutely increase SC pH. (A), (B) Both TMG and DBU 1:100 in propylene glycol:ethanol, 7:3 vol/vol, increase SC pH. (C) The coapplication of an SPI (PMSF) did not prevent the TMG-induced increase in SC pH. (D) pH dose–response curve 3 h after TMG applications at concentrations from 1:100 to 1:1000 vol/vol. (E) Surface pH recovers to normal values by 24 h following applications of TMG (1:1000). (F) The TMG-related increase in SC pH is sustainable and extends throughout the depth of the SC, with a significant increase in comparison to untreated controls at all levels of the SC. Results shown represent mean ± SD (n = 4–6 animals in each group).
Statistical analyses  Nonparametric Mann–Whitney statistical analyses were performed to compare percentage ratios between different groups of treatments (Morris, 2000). Statistical analyses were performed using Prism 2 (GraphPad Software, San Diego, CA).

RESULTS

Superbase applications increase SC pH without altering epidermal morphology or basal barrier function  Before assessing the consequences of increased pH on SC function, we first measured SC pH after applications of two chemically unrelated superbases, TMG and DBU. Surface pH of hairless mouse skin, assessed 3 h after a single TMG or DBU application, increased significantly in comparison to nTMG and nDBU, respectively (Fig 1A–C). The changes in skin surface pH were concentration dependent at TMG concentrations between 1:100 and 1:1000 (vol/vol) (Fig 1D), and regardless of applied dose, recovery of an acidic surface pH occurred over the subsequent 24 h (Fig 1E). The superbase-induced increase in pH extended throughout the SC, with pH in TMG-treated sites deep within the SC remaining significantly higher than the pH at comparable depths of nTMG-treated sites (Fig 1F).

Despite the sustained elevations in surface pH, histologic sections, taken 3 and 24 h following TMG or nTMG treatment, revealed neither histologic abnormalities nor evidence of cytotoxicity or inflammation (Fig 2). Moreover, despite the sustained elevation in SC pH, both basal TEWL and SC hydration levels remained unchanged in superbase-treated sites. TEWL levels for TMG and DBU (1:100) remained around 2 mg per cm² per h (controls: 2.2 mg per cm² per h), whereas hydration levels in all groups remained between 31.5 and 36.8 (arbitrary units). These studies show that a single application of a topical superbases raises SC pH, in a sustainable and concentration-dependent manner, extending throughout the SC. Moreover, such superbase treatment does not produce evidence of toxicity or inflammation. Finally, such short-term increases in SC pH alter neither basal TEWL nor SC hydration.

A neutral pH provokes abnormalities in SC integrity and cohesion, attributable to SP activation  To assess superbase-associated changes in SC function, we first assessed the effects of a short-term elevation in pH on SC integrity and cohesion. After 3 h exposure of normal skin to either TMG or DBU, both SC integrity and cohesion deteriorated significantly in comparison to untreated skin (Fig 3). Moreover, the 1:100 and 1:1000 concentrations appeared to be equally effective in producing these alterations. Integrity and cohesion in acidiﬁed nTMG- or nDBU-treated sites remained comparable to untreated skin (Fig 3; untreated skin not shown).

Prior studies have shown that SC desquamation is mediated by two SP, the SCCE and SCTE, which both exhibit neutral pH

Figure 2. Superbase treatment produces neither toxicity nor inflammation. Hematoxylin and eosin stained paraffin sections (6 μM) of biopsies at 3 and 24 h after TMG (1%) and nTMG (1%) application show comparable, normal cellular and epithelial structure, without evidence of inflammation or cytotoxicity. Magnification bars: 10 μm.

Figure 3. Both superbases produce abnormalities in SC integrity and cohesion. SC integrity (A, B, C) and cohesion (D, E, F) 3 h after a single application of either TMG or DBU versus nTMG or nDBU (1:100 or 1:1000 vol/vol) to intact skin. Integrity reflects the rate of change of TEWL with each stripping. Cohesion reflects the total protein removed from pooled D-Squame stripings taken from each treatment site. Results are shown as the mean ± SD (n = 4–6 animals in each group).
To assess the basis for the pH-induced changes in integrity and cohesion, we next assessed whether SC neutralization alone (surface pH 7.4) activates SP, evaluated by in situ zymography of SP activity in sections from TMG- versus nTMG-treated skin sites. The increase in SP activity could be detected as early as 20 min after superbase applications, and remained elevated for at least 3 h (Fig 4). We next ascertained whether the superbase-induced increases in SP activation were reversible, with re-acidification of sections from TMG-treated skin sites. As shown in Fig 5, SP activity disappeared when the SC was re-acidified in an acidic (MES) buffer, evidence that the superbase-induced increase in SP activity reflects pH-related modulations in enzyme catalytic activity.

Finally, to determine whether the superbase-induced increases in SP activity account for the observed abnormalities in SC integrity/cohesion, we next assessed these functions when superbase-treated sites were cotreated with an SPI (Fig 4). All of the SPIs tested inhibited superbase-induced SP activation (Fig 4D, F). The abnormalities in SC integrity and cohesion were reversed by coapplications of PMSF (Fig 6) or several other tryptic and/or chymotryptic SPI, including soybean trypsin inhibitor, aprotinin, and chymostatin (Table I). Similarly, abnormalities in SC cohesion were reversed when PMSF was coapplied with TMG (data not shown). Yet, as shown in Fig 1(C), coapplication of the SPIs did not themselves alter surface pH; i.e. the superbase induced an increase in SC pH even in the presence of an SPI. Together, these results show first, that a superbase-induced increase in SC pH alters SC integrity and cohesion; and second, that these functional changes are due to reversible increases in SP activity.

**Superbase-induced abnormalities in SC integrity/cohesion correlate with accelerated CD degradation** We next assessed the structural basis for the abnormalities in SC integrity and cohesion that result from SC neutralization. A single application of TMG again induced an increase in SC pH (to 7.4), which, in turn, provoked a significant decrease in the density of CD in the lower SC, assessed by quantitative electron microscopy (Fig 6). This decrease in CD density could be observed as early as 1 h after

---

**Figure 4.** The superbase-induced increase in SP activity is inhibited by coapplied SPI. (A)–(C) *In situ* zymography of changes in SP activity 20 min, 1 h, and 3 h after superbase treatment demonstrates a progressive increase in enzyme activity in TMG- but not in nTMG-treated sites (D). (E), (F) Coapplication of two chemically unrelated SPI (soybean trypsin inhibitor and PMSF) with TMG inhibits superbase-induced SP activation. *En face* views of SC show SP activity to be localized to SC membrane domains (A versus D, insets). Magnification bar: 10 μm.
superbase applications (Fig 6). In contrast, nTMG applications did not change CD density in comparison to untreated SC (untreated data not shown). Furthermore, the decrease in CD could be further ascribed to the superbase-induced increase in SP activity (see above), because coapplications of PMSF with TMG conserved CD density (Fig 6). Finally, we assessed whether the superbase-induced decrease in CD density was attributable to loss of its constituent proteins, by assessing DSG1 in western blots of SC from TMG-, nTMG-, and (TMG + PMSF)-treated SC. Immunoblotting showed extensive proteolysis of DSG1 3 h after TMG treatment in comparison to either nTMG or (TMG + PMSF)-treated samples (Fig 7). Together, these results show that the decline in SC integrity and cohesion at a neutral pH can be ascribed to a rapid dissolution of CD and its constituent proteins in the lower SC.

Elevation of SC pH delays barrier recovery after acute insults Although exposure of intact skin to a neutral pH does not alter basal permeability barrier function, prior studies have shown that barrier recovery is delayed when acutely disrupted skin sites are immersed in a variety of neutral pH buffers (Mauro et al, 1998). To determine whether SC neutralization alone is responsible for the delay in the kinetics of recovery after acute barrier disruption, we next applied TMG immediately after acute barrier disruption by sequential tape stripping, and assessed TEWL between 0 and 6 h after disruption. At the 3 h time point, superbase (TMG) treated sites exhibited a significant delay in barrier recovery (11.48% ± 1.49%; p < 0.001) in comparison to nTMG-treated sites (40.00% ± 2.45%). PMSF coapplications with TMG did not reverse the superbase-induced delay in barrier recovery. However, (13.68% ± 1.48%; p < 0.001).

To explore the mechanism(s) responsible for the delay in barrier recovery, we next assessed the in situ activity of a key lipid-processing enzyme, β-GlcCerâse, with an acidic pH optimum, in TMG- versus nTMG-treated skin. β-GlcCerâse activity, assessed by in situ zymography, decreased markedly in superbase-treated skin sites. To ascertain whether the elevation in pH was affecting enzyme activity, rather than inducing enzyme degradation, we next assessed whether β-GlcCerâse activity reappeared with re-acidification of sections from TMG-treated skin, as above. As seen in Fig 8, β-GlcCerâse activity returned when sections from superbase-treated skin were re-acidified (cf. Fig 5, where re-acidification produced the opposite effect; i.e., SP activity disappeared with re-acidification). Finally, TMG-treated sites exhibited a delay in lipid processing, as indicated by the persistence of “immature”, partially processed, membrane structures within the interspaces of the lower SC (Fig 9). These results show that the neutral-pH-induced delay in barrier recovery can be attributed to a downregulation of at least one lipid-processing enzyme (β-GlcCerâse), which in turn results in defective lipid processing leading to a corresponding delay in maturation of SC membrane structures.

DISCUSSION

The skin has long been known to have an acidic surface that is thought to play a key role in preventing infection (Wilhelm and Maibach, 1990). In this paper, we demonstrate directly that acidification of the SC is important for at least two other essential functions of mammalian skin. Using two unrelated superfades that raise pH selectively within the SC without evidence of toxicity or inflammation, we demonstrated that both cutaneous permeability barrier homeostasis and SC integrity and cohesion are perturbed when SC pH is elevated. These results confirm and extend previous studies from our laboratory that employed less specific methods to increase SC pH (Mauro et al, 1998; Fluhr et al, 2001; Behne et al, 2002) (Fig 10). Specifically, in these prior studies from our laboratory that employed less specific methods to increase SC pH (Mauro et al, 1998; Fluhr et al, 2001; Behne et al, 2002) (Fig 10). Specifically, in these prior
In contrast, the superbase-induced increase in SC pH (1) was dose dependent; (2) affected all layers of the SC; (3) was reversible with acidic buffers; and (4) returned towards normal with time. Yet, pH remained elevated for 6–12 h following a single superbase application. Moreover, superbase treatment did not result in any skin abnormalities, either on inspection or on morphologic (light and electron microscopy) examination. The lack of toxicity is also shown by the rapid reversibility of the superbase-induced alterations by re-acidification and by the fact that the same molecules, when neutralized (acidified), produce no functional or structural abnormalities. Nor did the superbases alter SC hydration or basal permeability barrier function. Thus, the use of superbases to elevate SC pH represents a direct, apparently nontoxic approach to determine the effects of alterations of SC pH on cutaneous function. The results of this study, using superbases to raise SC pH, together with previous studies that raised pH with buffers, inhibitors, or transgenic knockout animals, demonstrate clearly that an acidic SC pH is essential for both normal permeability barrier function and SC integrity/cohesion.

This study also shed light on the mechanisms by which an acute increase in SC pH adversely impacts SC function. Several types of protease activity have been identified in the SC (Horikoshi et al., 1999; Watkinson, 1999), with a convincing link to desquamation for two SP – SCCE and SCTE – based upon in vitro/in vivo inhibitor studies (Brattsand and Egelrud, 1999; Horikoshi et al., 1999). SCCE is localized to the extracellular membrane domains, and delivered to the SC interstitices via lamellar body secretion (Hansson et al., 1994; Sondell et al., 1994). SCTE is distributed both intracellularly and extracellularly within the SC (Watkinson et al., 1994; Ekhollm et al., 2000). As both of these SP are most active at a neutral pH (Brattsand and Egelrud, 1999; Ekhollm and Egelrud, 2000), we postulate that the normal acidic environment of the SC would reduce the catalytic activity of these enzymes, and conversely, an increase in SC pH would increase SP activity. In support of this hypothesis, we demonstrated here that raising SC pH increased SP activity in membrane domains of the SC, and that this increase in activity was reversible with lowering of SC pH. Zymography in tissue sections provides information about changes in total catalytic activity and about the localization of such pH-induced changes. Furthermore, the superbase-induced increase in SP activity led to an increased degradation of the
proteins that form the CD, i.e., a reduction in DSG1, resulting in a reduction in CD density and hence, in abnormal SC integrity and cohesion (Fig. 10). Most importantly, inhibition of SP activity with several different SPI prevented this cascade of events; i.e., DSG1 levels did not decline; CD density was maintained; and SC integrity and cohesion remained normal, even in the face of an increase in SC pH.

Although the above sequence of events, initiated by a pH-induced increase in SP activity in the SC, explains why an elevation in SC pH produces an abnormally high pH in SC integrity and cohesion, it does not explain the superfase-induced abnormality in cutaneous permeability barrier function. In contrast to SC integrity and cohesion, SPI did not correct the defect in barrier homeostasis induced by neutralization of the SC. Prior studies by our laboratory have demonstrated that processing of lipids secreted by lamellar bodies in the extracellular spaces of the SC is essential for the formation of mature lamellar membranes and normal permeability barrier function (Kassner et al., 1999). Moreover, two of the enzymes that are responsible for this lipid processing, β-GlcCer’-ase, which hydrolyzes glucosylceramides to ceramides, and acid sphingomyelinase, which hydrolyzes sphingomyelin to ceramides, are most active at an acidic pH (Vaccaro et al., 1985; Holleran et al., 1993; Jensen et al., 1999). Hence, the increased pH of the SC would be expected to reduce the activity of these two key lipid-processing enzymes, leading to a failure to form functionally competent, lamellar bilayers. In this study, we demonstrate that processing enzymes, leading to a failure to form functionally barrier function (Rassner et al., 1998, 1999). As the normal environment of the SC is acidic, it is very likely that the activity of other proteins/enzymes in the SC will be altered by increases in SC pH. These studies were supported by NIH grants AR 19098, 39448 (PP), HD 29706, and the Medical Research Service, Department of Veterans Affairs.

Figure 10. Methods for experimental increase in SC pH and mechanistic consequences.

The harmful effects of an elevation in SC pH on cutaneous function could adversely impact a number of clinical situations. First, it is well recognized that many cutaneous disorders, including acute eczema, atopic dermatitis, and seborrheic dermatitis, are associated with an increased SC pH (Wilhelm and Maibach, 1990; Chikakane and Takahashi, 1995; Rippke et al., 2002). The increase in SC pH in these disorders, by adversely affecting permeability barrier function and disrupting SC integrity and cohesion, could further exacerbate these conditions, resulting in more severe and/or more prolonged clinical manifestations. For example, a pH-induced decline in SC pH and coagulation would further enhance the skin’s susceptibility to minor injuries, e.g., with exposure to solvents, detergents, or mechanical forces. Moreover, the pH-induced increase in barrier disruption, with further impairment in permeability barrier repair, is likely to have adverse clinical consequences (virtually all dermatoses exhibit abnormal barrier function). Previous studies by our laboratory and other laboratories have shown that disturbances in barrier function stimulate the epidermal production of inflammatory cytokines, such as interleukin-1 β and tumor necrosis factor α, which could lead to or exacerbate inflammation (Tsai et al., 1994; Wood et al., 1996). A second important clinical situation where an elevation in SC pH occurs is in neonates. At birth, the pH of the SC is elevated (Green et al., 1968), and normal acidification of the SC is not achieved for several weeks to months postbirth (Yosipovitch et al., 2000). During this period of development, an increased SC pH could adversely affect permeability barrier homeostasis and SC integrity and cohesion. As described above, such functional alterations could have adverse clinical consequences, and could contribute to the increased sensitivity of neonates to the development of dermatitis. Diaper dermatitis is an extremely common occurrence, and our studies support the hypotheses that the elevated SC pH plays a role in increasing the severity and duration of this disorder (Andersen et al., 1994; Berg et al., 1994). Finally, our studies are relevant for the well-known adverse effects of alkaline soaps on the skin (reviewed in Fuhler and Elias, 2002). Thus, our observations, which demonstrate the importance of an acidic pH for two key SC functions, could point to alternative preventive or therapeutic strategies for a wide variety of cutaneous disorders that are associated with an increase in SC pH.

These studies were supported by NIH grants AR 19098, 39448 (PP), HD 29706, and the Medical Research Service, Department of Veterans Affairs.

REFERENCES


