Secretory Phospholipase A_2 Activity Is Required for Permeability Barrier Homeostasis

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The extracellular bilayers which mediate the epidermal permeability barrier are enriched in ceramides, free fatty acids, and cholesterol. Yet, the epidermal lamellar body, the source of these lipids, is enriched in a more polar mixture; i.e., glucosylceramides and phospholipids, which it delivers to the stratum corneum (SC) interstices. Whereas the extracellular processing of glucosylceramides to ceramides has been shown to be required for barrier homeostasis, the requirement for phospholipid degradation to free fatty acids is not yet established. In this study, we ascertained that topical applications of two chemically unrelated inhibitors of secretory phospholipase A_2 (PLA_2), bromphenacyl bromide and MJ-33, produced a progressive perturbation in barrier function in intact murine skin, first appearing at 5 d, preceded by the development of epidermal hyperplasia. Moreover, the defect in barrier homeostasis could be reversed by topical co-applications of the nonessential fatty acid, and of palmitic acid, but not by linoleic acid, both products of phospholipid catabolism. Furthermore, the barrier abnormality was accompanied by a reduction in free fatty acid levels in the stratum corneum, while phospholipid levels remained unchanged. These biochemical alterations were accompanied by the appearance of immature, incompletely processed lamellar body-derived membranes in the SC interstices, and depletion of histochemically detectable neutral lipid. Both the abnormalities and the epidermal hyperplasia were reversed by co-applications of palmitic acid (but not linoleic acid) with either inhibitor. These results demonstrate that processing of phospholipids to nonessential free fatty acids, by a yet-to-be-identified extracellular phospholipase, is required for the maintenance of barrier homeostasis in intact skin. Furthermore, our studies show that the barrier abnormalities induced by the PLA_2 inhibitors are due to a failure to generate free fatty acids rather than to phospholipid accumulation. *Key words: epidermal lipids/barrier function/epidermal ultrastructure. J Invest Dermatol 106:57–63, 1996*

A major function of the epidermis is to generate a barrier that prevents excess water loss. This permeability barrier is localized within the extracellular, lipid-enriched domains of the stratum corneum (SC) (reviewed in [1–3]). The lipid composition of the SC differs greatly from the lipids present in the nucleated layers of the epidermis, consisting primarily of a nonpolar mixture of cholesterol, ceramides, and free fatty acids (reviewed in [4,5]). These lipids are derived primarily from the exocytosis of lamellar body-derived contents from stratum granulosum (SG) cells [1,3,6]. Since the major lipids in lamellar bodies comprise a relatively polar mixture of cholesterol, glucosylceramides, and phospholipids [3,7,8], secreted lamellar body lipid precursors must be metabolized within the extracellular spaces into more hydrophobic lipid products during barrier formation.

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Abbreviations: SC, stratum corneum; SG, stratum granulosum; PLA_2, phospholipase A_2; BPB, bromophenacyl bromide; TEWL, transepidermal water loss; HPTLC, high-performance thin layer chromatography.

Lamellar bodies also contain an array of hydrolytic enzymes, including sphingomyelinase, triacylglycerol hydrolase, β-glucosidases, phospholipase A, acid phosphatase, and certain proteases [7,9], which are co-secreted with the lipids into the extracellular spaces of the SC [10–12]. Recent studies have shown that certain of these enzymes process the relatively polar lipids derived from lamellar bodies, within the extracellular spaces, into the more nonpolar mixture that predominates in the SC. For example, conversion of glucosylceramides to ceramides by β-glucocerebrosidase is essential for normal barrier homeostasis: instead of the compact, membrane bilayer unit structures normally present in the intercellular domains of the SC, immature and loosely organized membrane structures appear when β-glucocerebrosidase activity is inhibited or deleted [13,14].

Very recent studies have shown that blockade of the conversion of phospholipids to free fatty acids by inhibition of phospholipase A_2 (PLA_2) activity with the topical application of either of two chemically unrelated suicide inhibitors of secretory PLA_2, bromophenacyl bromide (BPB) or MJ33, acutely alters barrier homeostasis. A single topical application of either of these inhibitors delays barrier repair following acute barrier disruption with acetone [15]. Moreover, this delay in barrier recovery is associated with an increase in phospholipids and a decrease in free fatty acids within inhibitor-treated SC, without significant effects on fatty acid syn-

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thesis. Furthermore, blockade of the conversion of phospholipids to free fatty acids also results in the appearance of abnormal, extracellular membrane bilayer structures within the SC interstices. Most importantly, the functional, biochemical, and structural abnormalities that result from the acute inhibition of PLA_2 activity could be normalized by a single co-application of palmitic acid, but not linoleic acid, a product of phospholipid catabolism, suggesting that a deficiency of nonessential free fatty acids accounts for these abnormalities [15]. Finally, the ability of palmitic acid to override inhibitor blockade correlated with the formation of mature extracellular membrane bilayer structures in isolated, inhibitor-treated stratum corneum [15]. Thus, in the setting of acute barrier repair, the conversion of phospholipids to free fatty acids plays a crucial role in barrier homeostasis. In the present study, we determined whether sustained inhibition of extracellular PLA_2 activity by repeated applications of the inhibitors to otherwise intact skin would produce alterations in SC structure, function, and lipid composition. In this model, as in the parallel acute model, extracellular processing of secreted phospholipids into nonessential fatty acids appears to be required for barrier homeostasis.

MATERIALS AND METHODS

Materials Male hairless mice (Hr/Hr), 8–12 wk old, were purchased from Simonsen Laboratories (Gilroy, CA), and fed Purina mouse diet and water *ad libitum*. Acetone and propylene glycol were from Fisher Scientific (Fairlane, NJ), while BPB and palmitic acid were from Sigma Chemical Co. (St. Louis, MO). One-hexadecyl-3-trifluroethylglycero-sn-2-phosphomethanol (MJ33) was synthesized as described previously [16]. High-performance thin layer chromatography (HPTLC) silica-gel pre-coated plates were purchased from Brinkmann Instruments (Westbury, NY). Ruthenium tetroxide and osmium tetroxide were from Polyscience, Inc. (Warrington, PA).

Experimental Protocols Animals were treated topically once daily with BPB (1 mg/ml; 20 μg total), MJ33 (1 mg/ml; 20 μg total) in a propylene glycol:ethanol vehicle (7:3 vol), or vehicle alone to a 5- to 6-cm^2 area on the backs of normal hairless mice. These doses have been shown both to be nontoxic in acetone-treated murine skin [15], and to inhibit secretory PLA_2 activity selectively in several other tissues and cell types [17]. In other experiments, a parallel group of inhibitor-treated animals also simultaneously received co-applications of palmitic acid or linoleic acid (10 mg/ml; 20 μg total). Barrier function was determined by measurement of transepidermal water loss (TEWL) with a Meeco electrolytic water analyzer (Meeco, Warrington, PA), every other day prior to the next application of inhibitor. The raw functional data were converted to $g/m^2/h$, and expressed as mean ± SEM.

Lipid Biochemistry Seven days after vehicle, BPB or MJ33 treatment, epidermal sheets were separated from the dermis by heat separation, as described previously [18,19]. Epidermal sheets then were incubated for 2 h at 37°C in a 0.5% trypsin solution in calcium- and magnesium-free phosphate-buffered saline (trypsin containing less than 2 ng of PLA_2 activity per 100 μg trypsin. After incubations, the samples were gently vortexed to remove residual nucleated cells, the remaining intact SC sheet was washed with distilled water four times, and sample weights were obtained after the SC sheets were blotted dry with Kimwipes. Total lipids were extracted from the SC, using Bligh-Dyer solvents, followed by fractionation and quantitation by HPTLC, as described briefly [20]: Two micrograms of lipid was applied onto plates for neutral lipid analysis, with both 20 or 100 μg utilized for polar lipid analysis. Both to generate standard curves, and to identify the major species, 0.2 to 1.0 μg of a polar lipid standard and 0.12 to 1.0 μg of a neutral lipid standard were applied in parallel. Neutral lipids were fractionated by developing the plates in petroleum ether:diethyl ether:acetic acid (80:20:1, vol), as described previously [20]. Polar lipids were developed to 35 and 55 mm in chloroform:ethyl acetate:ethy-methylketone:2-propanol:ethanol:methanol:glacial acetic acid:hexyl acetate (34:4:4:6:20:28:4:1, vol), and then to 70 mm in choroform:ethyl acetate:2-propanol:ethanol:methanol:H_2O (46:4:4:6:6:20:28:6, vol), and then to the top of the plate in chloroform:methanol:acetone (80:10:10, vol). The plates were painted with a charring solution, charred, and then scanned densitometrically, and the lipid fractions were quantitated by using CATS II software, as described in detail previously.

Light and Fluorescence Microscopy Biopsy samples, taken on day 0, 4, and 7 from both vehicle- and inhibitor-treated animals, were embedded in paraffin and stained with hemotoxylin-eosin followed by visualization with a Leitz Ortholux II microscope. The number of nucleated cell layers in 10 cross-sections, chosen at random from each group, was assessed as a measure of hyperplasia. Fresh biopsy samples were also obtained in parallel for lipid histochemistry and snap-frozen immediately in liquid nitrogen. Five-micrometer frozen sections were stained with nile red for depiction of polar and neutral lipid localization [21], and examined by fluorescence microscopy in the Leitz microscope equipped for epifluorescence.

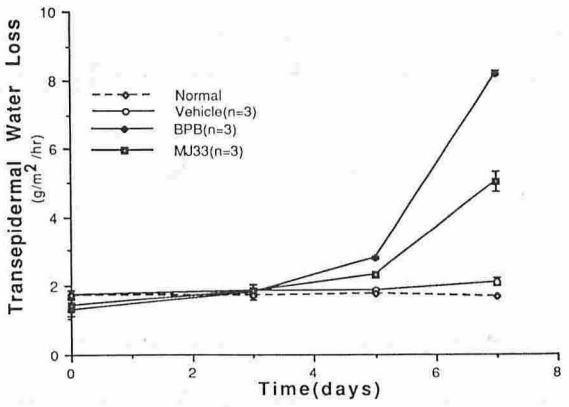

Figure 1. Repeated Applications of PLA_2 Inhibitors Disrupt Barrier Function. Following five daily applications of either of the two PLA_2 inhibitors (20 μg/day) to intact skin, barrier function, as assessed by TEWL, begins to deteriorate. In contrast, the vehicle alone does not disrupt the barrier. *Error bars*, SEM; n = 5 separate sites on three animals in each group.

Electron Microscopy Seven days after applications of either vehicle or PLA_2 inhibitors, biopsy samples were taken from treated areas, minced to 0.5 mm^3, fixed in modified Karnovsky's fixative overnight, washed in 0.1 M cacodylate buffer, and post-fixed in either 0.2% ruthenium tetroxide (RuO_4) or 1% osmium tetroxide, containing 0.5% potassium ferrocyanide, as described in detail previously [22]. Ultrathin sections were examined in a Zeiss 10A electron microscope operated at 60 kV.

Statistical Analysis Statistical significances were determined using a two-tailed Student's t test.

RESULTS

PLA_2 Inhibitors Disrupt Barrier Function in Intact Skin Our initial studies determined whether repeated topical applications of one or both PLA_2 inhibitors would disrupt barrier function in intact skin. No significant change in barrier function, measured as TEWL rates, occurs in animals treated with vehicle alone for 7 d **(Fig 1)**. In contrast, TEWL increases significantly after five consecutive daily applications of either BPB or MJ33 **(Fig 1)**. Moreover, the extent of barrier disruption progresses with time, so that by 7 d TEWL rates are markedly abnormal in both BPB- and MJ33-treated animals. Thus, repeated applications of either of the two, chemically unrelated PLA_2 inhibitors perturb barrier homeostasis in intact skin.

The Ability of PLA_2 Inhibitions to Disrupt the Barrier Is Due to Enzyme Inhibition To determine whether the effects of the phospholipase A_2 inhibitors on barrier function can be attributed specifically to inhibition of the conversion of phospholipids to free fatty acids, we next co-applied a product of phospholipid catabolism, palmitic acid, with each of the inhibitors. As shown in **Fig 2**, the abnormality in barrier function induced by either BPB or MJ33 is prevented by simultaneous co-applications of palmitate. In contrast, co-applications of linoleic acid had no effect; i.e., they did not prevent or delay emergence of the barrier abnormality (data not shown). These data indicate first, that the disruption of barrier function seen in BPB- or MJ33-treated animals can be attributed to the impaired conversion of phospholipids to nonessential free fatty acids, rather than to nonspecific toxic effects of these compounds. In addition, these studies demonstrate a requirement for nonessential free fatty acids, an end product of phospholipid catabolism, for barrier homeostasis in intact skin.

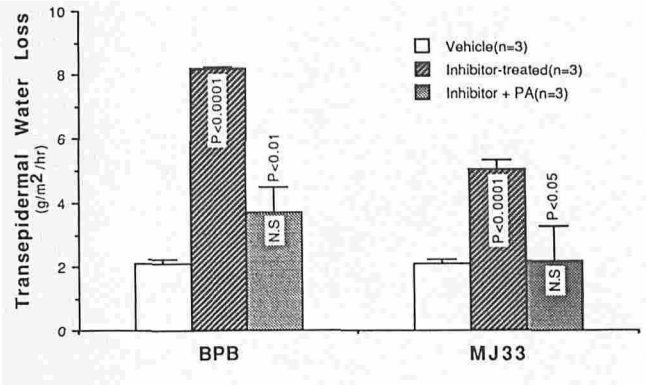

Figure 2. Coapplications of Palmitic Acid Block Emergence of the Barrier Abnormality. Data shown are for seven daily applications of inhibitor ± palmitic acid (20 μg/day) to intact skin. Each inhibitor alone again produces a highly significant barrier abnormality (c.f., **Fig 1**), which is reduced towards vehicle levels significantly by co-applications of palmitic acid (*PA*); i.e., differences of inhibitor + PA *versus* inhibitor are significant at <0.01 and <0.05 for BPB and MJ33, respectively. Both BPB + PA and MJ33 + PA water loss rates are not significantly different from vehicle alone. *Error bars*, SEM; n = 5 sites on three animals in each group.

PLA$_2$ Inhibitors Alter SC Lipid Content We next assessed whether topical applications of BPB and MJ33 alter the lipid content of the SC. As shown in **Fig 3**, 7 d of treatment with BPB resulted in an approximately 50% reduction in free fatty acid content in the SC ($p < 0.001$). In contrast, the quantities of free sterols, phospholipids, and sphingolipids are not changed significantly by BPB administration. A comparable decrease in free fatty acids is seen with MJ33 treatment **(Table I)**; phospholipid content remains unaffected (data not shown). These results indicate that repeated applications of either of the two, chemically unrelated PLA$_2$ inhibitors to intact skin result in a marked decrease in SC free fatty acid content.

PLA$_2$ Inhibitors Alter the Delivery of Stainable Neutral Lipids to the SC Interstices To determine whether inhibition of PLA$_2$ alters the generation of free fatty acids within the SC interstices, we next visualized the distribution of neutral lipids with nile red **(Fig 4*A*)**; a major portion of nile red–stainable lipids that exhibit green-gold fluorescence in the SC are free fatty acids) [1]. In vehicle-treated animals (7 d), bright gold fluorescence is observed in the SC in a membrane pattern, indicating the presence of

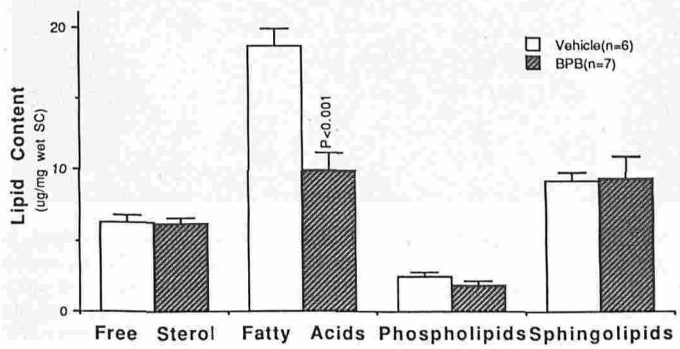

Figure 3. BPB Applications Deplete the Stratum Corneum of Free Fatty Acids. Data shown are from animals treated with seven daily applications of BPB (20 μg/day) to intact skin (n = 6 in vehicle and n = 7 in BPB groups). Stratum corneum is obtained by heat separation (60°C, 60 sec) followed by trypsinization. Total lipids were extracted with Bligh-Dyer solvents, and fractionated by quantitative HPTLC. Whereas free fatty acids content is reduced significantly, other major lipid classes, including phospholipids, are not significantly affected (c.f., **Table I**). *Error bars*, SEM.

Table I. Effect of MJ33 on Stratum Corneum Neutral Lipid Content[a]

Fraction (μg/mg SC)	Vehicle	Sign	MJ33
Neutral lipids			
Free sterols	6.32 ± 0.49	NS	5.48 ± 0.46
Free fatty acids	18.64 ± 1.26	<0.001	10.96 ± 1.05
Sterol esters	8.53 ± 2.68	NS	3.45 ± 0.60
Total	33.48 ± 3.63	<0.01	19.89 ± 1.82

[a] Hairless mice were treated with either MJ33 (20 μg/d) or vehicle for 7 d. Stratum corneum sheets were harvested by heat separation followed by trypsinization. Total lipids were extracted with Bligh-Dyer solvents, fractionated by HPTLC, and quantitated by photodensitometry. Results are mean ± SEM; n = 6 for vehicle and n = 5 for MJ33 groups. NS, not significant.

abundant neutral lipids **(Fig 4*B*)**. In contrast, in both BPB- and MJ33-treated samples, nile red–stainable material is markedly reduced in the SC, and no membrane pattern is observed **(Fig 4*C,D*)**. Moreover, co-applications of palmitic acid with either of the inhibitors normalized the pattern of lipid staining in the SC **(Fig 4*E,F*)**. These findings support the biochemical results, by providing further evidence that treatment with PLA$_2$ inhibitors decreases the content of stainable neutral lipid of the SC. In addition, they show that co-application of palmitic acid with the PLA$_2$ inhibitors normalizes the pattern of stainable neutral lipid in the SC.

PLA$_2$ Inhibition Results in Epidermal Hyperplasia The effects of BPB and MJ33 on cutaneous morphology is shown in **Fig 5*A–E***. In comparison with vehicle-treated skin **(Fig 5*E*)**, at 7 d, BPB- and MJ33-treated skin displays a marked increase in the number of nucleated cell layers **(Fig 5*A,B*)**, from an average of three to four in vehicle-treated samples to six to seven in inhibitor-treated samples (hyperplasia is also evident in **Fig 4*C,D***). The hyperplasia can be ascribed to PLA$_2$ inhibition, because: (i) it is induced by both of the chemically unrelated inhibitors; and (ii) its development is prevented by topical co-applications of palmitic acid (**Fig 5*C,D***; reversal of hyperplasia also is seen in **Fig 4*E,F***). Moreover, the hyperplasia cannot be ascribed to barrier disruption, because it appears at 4 d **(Fig 5*F,G*)**, prior to development of the barrier abnormality (c.f., **Fig 1**). Furthermore, development of hyperplasia is blocked by co-application of palmitate, even prior to development of the barrier abnormality **(Fig 4*H*)**. Finally, in contrast to the results with palmitic acid, the emergence of the barrier abnormality is not blocked by co-applications of linoleic acid (LA) (5 d TEWL: 2.14 ± 0.38 *vs* 2.46 ± 0.21 mg/cm^2/h, BPB alone *versus* BPB + LA, respectively). These results demonstrate first, that epidermal hyperplasia occurs as a consequence of inhibition of epidermal PLA$_2$ activity. Second, since epidermal hyperplasia appears before emergence of the barrier abnormality, hyperplasia cannot be attributed to prior barrier disruption. Third, the nonessential fatty acid, palmitic acid, prevents the emergence of both the barrier abnormality and hyperplasia.

PLA$_2$ Inhibitors Alter SC Extracellular Membrane Structure In order to determine whether the biochemical abnormalities described above are responsible for the barrier abnormality, we next examined SC lamellar membrane structure in inhibitor- *versus* vehicle-treated skin. As in previous studies (e.g., [13,15,20,23]), vehicle applications alone to intact skin alter neither the number nor the appearance of lamellar bodies in SG cells (not shown). Moreover, neither the quantities of secreted lamellar material at the SG-SC interface, nor the appearance of extracellular membrane bilayers in the SC interstices are altered (not shown). Furthermore, inhibitor-treated animals do not exhibit abnormalities in either the nucleated layers of the epidermis or in lamellar body structure and number (not shown; see [15]). In BPB-treated animals, unprocessed, secreted lamellar body–derived membranes persist for several cell layers above the SG-SC interface **(Fig 6*A*)**. In contrast,

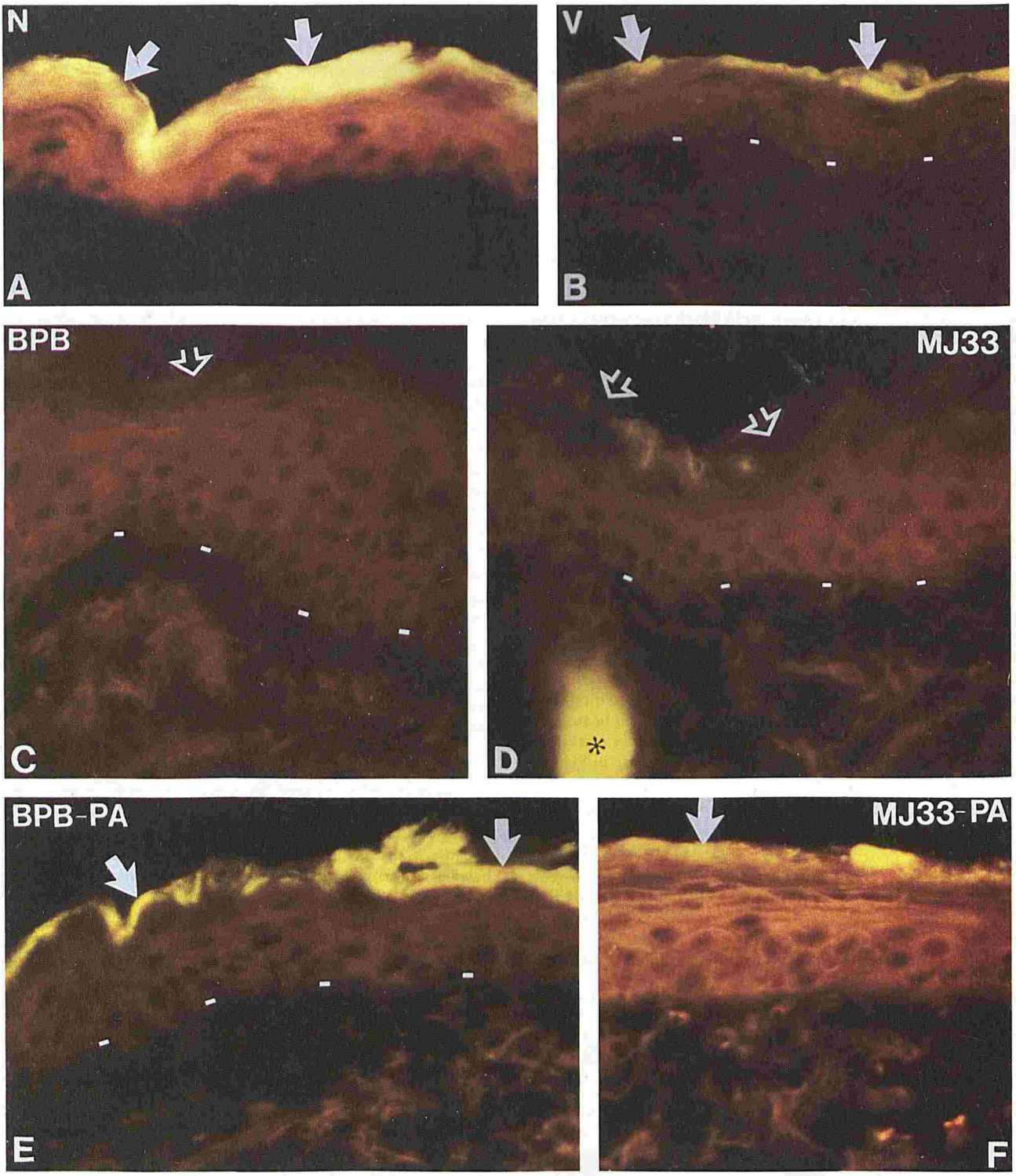

Figure 4. Both PLA_2 Inhibitors Deplete the Stratum Corneum of Stainable Neutral Lipids. Nile red fluorescence of stratum corneum lipids in 5-μm frozen sections. *A*) Untreated control hairless mouse epidermis (*N*) shows bright straining for neutral lipids in stratum corneum (arrows). *B*) After seven daily applications of vehicle (*V*) alone, neutral lipid staining in stratum corneum is moderately decreased (*arrows*; *dashes*, basement membrane). After seven applications (20 μg daily) of either *BPB* (*C*) or *MJ33* (*D*), neutral lipid staining is markedly diminished-to-absent (*open arrows*; *positive sebaceous lipid staining for comparison). Epidermal hyperplasia is evident after treatment with both inhibitors. Daily applications (20 μg/day) of palmitic acid (*PA*) (with either *BPB* [*E*] or *MJ33* [*F*]) restore neutral lipid staining to stratum corneum (solid arrows) and reduce epidermal hyperplasia considerably. *A–F*) $\times$1,000.

normal basic unit structures usually appear in the first or second interspace above the SG-SC junction in vehicle-treated controls (c.f., **Fig 6*B***). Moreover, the absence of lamellar unit structures results in a loose, irregular arrangement of membranous material throughout the SC interstices in inhibitor-treated samples **(Fig 6*A*)**. MJ33 gave similar results (data not shown). Finally, co-applications of palmitic acid with either of the inhibitors normalizes membrane bilayer structure in the SC (**Fig 6*C*** [BPB + PA] and data not shown [MJ33 + PA]). These results demonstrate that the reduction in free fatty acids, resulting from sustained inhibition of PLA_2, produces

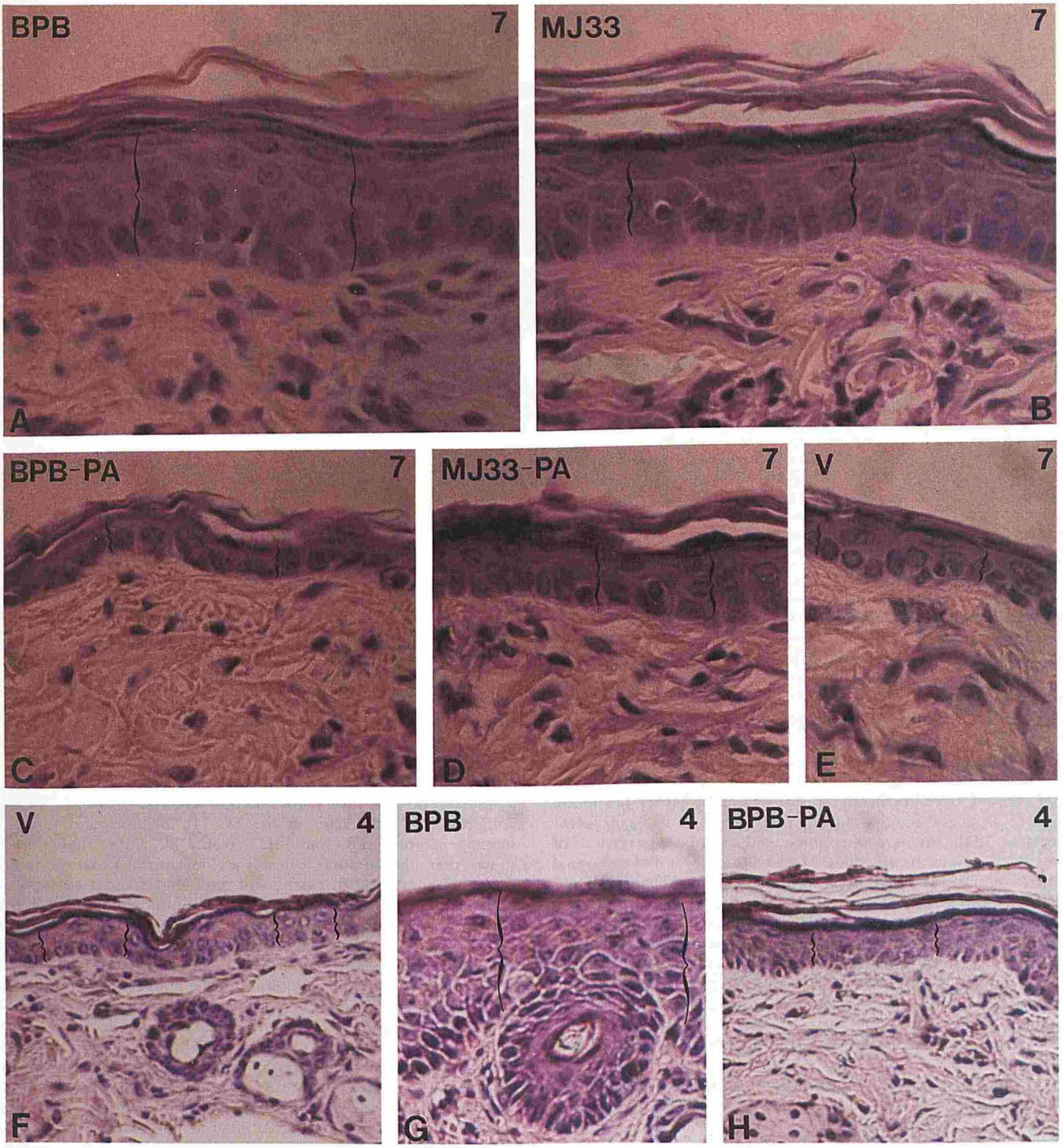

Figure 5. Both PLA$_2$ Inhibitors Produce Epidermal Hyperplasia. Six-micrometer paraffin-embedded sections). After seven applications (20 μg daily) of either BPB (*A*) or MJ33 (*B*), prominent epidermal hyperplasia is evident in comparison to vehicle (*V*)-treated controls (*E*) *Brackets* in all figures, width of nucleated layers. Daily co-applications (20 μg) of palmitic acid (*PA*) with either *BPB* (*C*) or *MJ33* (*D*) largely reverse the epidermal hyperplasia. Similar results are seen at 4 d, prior to the emergence of a barrier abnormality (*F*: Vehicle; *G*: BPB; *H*: BPB + PA). *A–H*) ×750 (hematoxylin and eosin).

abnormal membrane structures in the SC interstices, which in turn result in the barrier abnormality.

DISCUSSION

The lipid composition of the SC differs from the lipid composition of lamellar bodies, the organelle which delivers lipid to the SC extracellular spaces. Whereas lamellar bodies contain predominantly cholesterol, glucosylceramides, and phospholipids [7], the lipids in the SC extracellular spaces comprise mainly cholesterol, ceramides, and free fatty acids [4,5]. These chemical differences suggest that extracellular degradation of polar lipid precursors into more nonpolar lipid products occurs during the formation of the lipid-enriched bilayers in the SC interstices. Moreover, in addition to delivering lipids, lamellar body exocytosis deposits hydrolytic

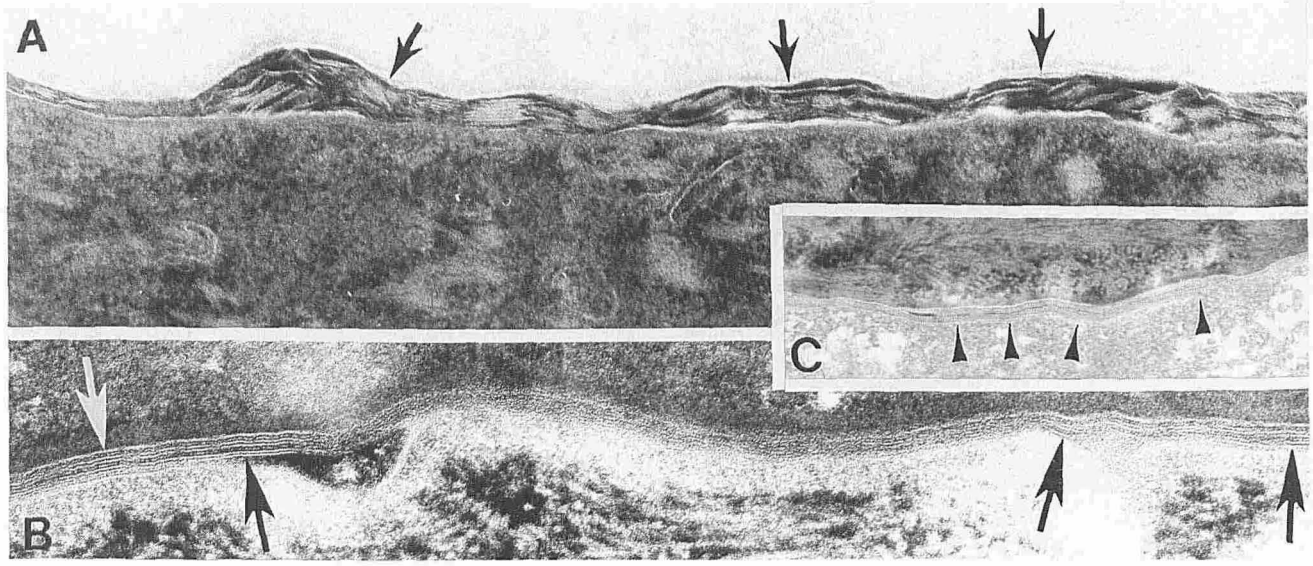

Figure 6. The Extracellular Membrane Structural Abnormalities Produced by PLA_2 Inhibitors Are Due to Fatty Acid Depletion. After seven daily applications of BPB or MJ33 (20 μg daily each) abnormal intercellular lamellar structures appear within the intercellular spaces at all levels of the stratum corneum. *Arrows* in A, membrane structures in BPB-treated, mid-stratum corneum; i.e. between the third and fourth layer of the stratum corneum (MJ33 data not shown). These structures replace the normal lamellar bilayers seen in vehicle-treated controls (*B, arrows*). Co-applications of palmitic acid (20 μg daily) with either BPB or MJ33 normalize the appearance of intercellular lamellae (*C, arrowheads*, BPB; MJ33 plus palmitic acid not shown). All are ruthenium tetroxide post-fixed. *A*) ×95,000; *B*) ×115,000; *C*) ×62,500.

enzymes, such as glycosidases and a variety of lipases, within the SC interstices [9–12]. Thus, lamellar bodies appear capable of delivering not only the lipids, but also the hydrolytic enzymes required for the extracellular processing of secreted polar lipids into the more nonpolar species that form the lamellar bilayer unit structures responsible for barrier function [3]. Whereas the co-localization studies described above provide strong circumstantial evidence for extracellular processing, direct evidence for these metabolic transformations, and for their importance for barrier homeostasis, has only recently been forthcoming. For example, barrier disruption regulates both the activity and the mRNA levels of β-glucocerebrosidase [24], the enzyme which catlyzes the conversion of glucosylceramides to ceramides. Moreover, inhibition of epidermal β-glucocerebrosidase activity both inhibits barrier repair [24], and leads to abnormal barrier function in intact skin [13]. The biochemical basis for the barrier abnormality with sustained inhibition of β-glucocerebrosidase appears to be an accumulation of glucosylceramides [13], which results in elongated but incompletely processed membrane structures [13,24]. The morphology of these immature mechanisms is reminiscent of normal structures seen in mucosal epithelia [25,26] and marine cetaceans [27], where glycosylceramides accumulate due to an apparent deficiency in endogenous β-glycosidase activity [28].

Recently, we showed that inhibition of PLA_2 activity after acute perturbations of the barrier also results in an inhibition of barrier repair [15]. In this acute model, the barrier abnormality also was shown to result from the failure to generate free fatty acids from phospholipids, and, as in the present studies, the abnormality was attributable to the accumulation of abnormal "immature" membrane structures in the SC interstices. The aim of the present study was to determine whether the sustained inhibition of PLA_2 activity by the repeated topical application of two different inhibitors to normal, intact skin would alter barrier homeostasis. The present study demonstrates that inhibition of PLA_2 by either of two suicide inhibitors, BPB or MJ33, produces progressive elevations in TEWL, indicative of impaired barrier function. Moreover, the defect in barrier function is accompanied by alterations in the structure of the lamellar bilayers in the SC interstices, comparable to those seen after a single application of the inhibitor to acetone-treated skin [15]. The compact lamellar bilayers that are present in normal stratum corneum are replaced by irregular, loosely arranged membranes in the SC interstices. That this abnormality in structure and function is due to impaired degradation of phospholipids to fatty acids is shown first, by lipid biochemical analyses, which revealed a striking decrease in SC free fatty acid content, comparable to the decrease found with inhibitor treatment in the acute model [15]. Moreover, in the latter model, we showed further that the decrease in free fatty acids could not be attributed to a toxic effect of the inhibitors on fatty acid synthesis [15]. Second, nile red, a fluorescent dye that stains neutral lipids [20], which largely comprise free fatty acids in the SC [4], demonstrates reduced staining in both BPB- and MJ33-treated SC. Prior studies have shown that nile red fluorescence is an indicator of variations in SC fatty acid content [29]. Lastly, and most importantly, co-applications of the nonessential fatty acid, palmitic acid, prevents the deleterious effects of BPB and MJ33 on SC structure and barrier function, as in the acute model. The last finding indicates that the structural and functional abnormalities induced by the PLA_2 inhibitors can be attributed to inhibition of the conversion of phospholipids to free fatty acids, with a resultant deficiency of non-essential free fatty acids, rather than to nonspecific toxic effects. Whereas prior studies have shown that fatty acids are required for barrier function [29], these studies more specifically demonstrate a specific requirement for nonessential free fatty acids.

It is noteworthy that in this model, as in the previously described acute model [15], palmitic acid, but not linoleic acid, is able to override PLA_2 inhibition. This constellation of findings suggests that epidermal phospholipids, destined for barrier function, may be atypical; i.e., rather than being enriched in unsaturated fatty acids in the sn-2 position, as is usual, where these moieties are poised for eicosanoid generation [17,30,31], they instead may contain saturated fatty acids at the sn-2 position. Certainly, the free fatty acids of stratum corneum, which derive largely, if not entirely, from phospholipid catabolism [3–5], contain a paucity of linoleic acid; instead, palmitic and oleic acids predominate [32]. In this respect, the phospholipids in the epidermal lamellar body may resemble the lamellar bodies of the lung type II cell, which contain primarily dipalmitoylphosphatidylcholine (reviewed in [33]). Further studies are needed to clarify the special characteristics, if any, of lamellar body phospholipids.

Previous studies by our laboratory and others have demonstrated that topically applied free fatty acids can affect barrier function by two distinct mechanisms: (i) either directly by interacting within the SC [15,34]; or (ii) indirectly by altering the ratios of lipids in secreted lamellar bodies [35]. In these studies, a direct correction by the fatty acid of the SC membrane abnormalities, produced by either BPB or MJ33, would explain how palmitic acid could normalize the barrier in isolated stratum corneum [15], without the requirement for prior intracellular processing and assembly into nascent lamellar bodies [36]. In addition, intracellular processing is not likely to be operative, because the prior assembly of free fatty acids into phospholipids, followed by PLA_2-mediated generation of free fatty acids, would be blocked by the two inhibitors. A third possibility, namely that palmitic acid could either dilute or interfere with transdermal delivery of the PLA_2 inhibitors, is unlikely because: (i) we employed 10-fold higher concentrations of lipid than inhibitor (1/10 vol) to avoid dilutional effects; and (ii) we showed that palmitic acid reverses inhibitor-induced abnormalities in isolated stratum corneum, after prior uptake of the SC agents [15]. Thus, these and the earlier studies [15,34] indicate that free fatty acids can restore SC structure and function by interacting directly within the SC.

Chronic treatment with both BPB and MJ33 also caused epidermal hyperplasia. It is unlikely that the epidermal hyperplasia is due to an irritant effect or nonspecific toxicity of BPB or MJ33, because these histologic changes could be prevented by simultaneous topical treatment with palmitic acid. Several explanations can be invoked to explain the development of these alterations. Our laboratory has shown that barrier disruption stimulates epidermal DNA synthesis [37], and epidermal hyperplasia is a prominent feature of other experimental models characterized by a sustained barrier defect, such as essential fatty acid deficiency [37] and chronic lovastatin treatment [38]. However, the hyperplasia in this model precedes the emergence of the barrier abnormality, and is reversible at these early time points by fatty acid co-applications. Hence, our results suggest the alternative possibility that PLA_2 inhibition alone suffices to produce hyperplasia, presumably by modulation of PLA_2-induced signal tranduction events.

In summary, the present study provides evidence that the extracellular catabolism of phospholipids to fatty acids by PLA_2 is important for the formation of normal SC lamellar bilayers and for competent barrier function.

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