

Acute Barrier Perturbation Abolishes the Ca^{2+} and K^+ Gradients in Murine Epidermis: Quantitative Measurement Using PIXE

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Epidermal permeability barrier homeostasis requires the delivery of lipids and hydrolytic enzymes by lamellar body exocytosis from the uppermost granular cells, a process that is upregulated following barrier disruption. As lamellar body secretion is controlled by ionic concentrations, especially Ca^{2+} and K^+ , we used a quantitative technique, microbeam proton-induced X-ray emission, to measure Ca^{2+} , K^+ , Cl^- , and P concentrations before and after acute barrier perturbation by acetone applications. We found a steep gradient of Ca^{2+} in normal tissue, peaking in the outer stratum granulosum, which disappeared after barrier disruption, and partially reformed as the barrier recovered. A similar gradient,

peaking somewhat lower in the epidermis (i.e., at the stratum granulosum–stratum corneum interface), was found for K^+ . Epidermal concentrations of K^+ also decreased after barrier abrogation, although to a lesser extent than Ca^{2+} . In contrast, P and Cl^- demonstrated distribution gradients at baseline, which remained unchanged after barrier disruption. These studies quantify the levels of Ca^{2+} , K^+ , Cl^- , and P within specific epidermal cell layers at baseline, and in relation to changes in permeability barrier integrity. Ca^{2+} and K^+ , but not Cl^- or P, decrease after barrier disruption, consistent with these two ion's role in barrier repair. *Key word: epidermal permeability barrier. J Invest Dermatol 111:1198–1201, 1998*

Epidermal keratinocytes differentiate as they migrate from the basal layer to the stratum corneum. This sequence proceeds in several overlapping stages, which are characterized initially by the cessation of proliferation and the commitment to differentiate, followed by the expression of proteins characteristic of mature keratinocytes. Relatively late in this differentiation sequence, keratinocytes generate lamellar bodies, whose contents are secreted at the stratum granulosum–stratum corneum interface, forming the epidermal permeability barrier.

When the lipids that mediate the permeability barrier are removed, either by tape-stripping or by topical treatment with acetone or detergents, sequential repair processes are initiated that lead rapidly to barrier restoration. An early and essential response to barrier disruption is the immediate (within 30 min) secretion of a pool of preformed lamellar bodies from the outermost granular cell (Menon *et al*, 1992a). Acute barrier perturbations cause transepidermal water loss rates from the skin to increase, suggesting that changes in water flux per se might regulate components of the homeostatic repair response. Yet, electron microscopy demonstrates that such accelerated water loss leads to profound changes in the concentrations of inorganic ions, especially Ca^{2+} in the outer epidermis (Menon *et al*, 1992a). Moreover, when Ca^{2+} is added to the bathing solution, barrier recovery and lamellar

body secretion are inhibited (Lee *et al*, 1992, 1994; Menon *et al*, 1992a). Using sonophoresis, the Ca^{2+} concentration of the upper epidermis can be manipulated experimentally, independent of barrier disruption (Menon *et al*, 1994). More recent studies demonstrate that Ca^{2+} is not the only ionic regulator of barrier repair. High extracellular K^+ is synergistic with high extracellular Ca^{2+} in inhibiting barrier recovery after acute barrier disruption, suggesting that K^+ channels may mediate Ca^{2+} influx (Lee *et al*, 1992, 1994). These results strongly suggest that the passive loss of Ca^{2+} and K^+ from the upper epidermis following barrier disruption may signal the lamellar body secretory component of the repair response.

Previously, both biophysical and cytochemical approaches have been employed to assess ion levels in the epidermis. Using ion capture cytochemistry, we first described a Ca^{2+} gradient in normal mouse (Menon *et al*, 1985) and human (Menon and Elias, 1991) epidermis. The Ca^{2+} gradient disappears after acute barrier disruption, reappearing over 6–24 h in parallel with barrier restitution (Menon *et al*, 1992a). Although this method provides semiquantitative insights about Ca^{2+} localization, it measures free ions, rather than total Ca^{2+} , and it is not applicable to other ions of potential interest, e.g., K^+ . Other biophysical techniques have been applied to epidermis, including X-ray microanalysis (XRMA) (Von Zglinicki *et al*, 1993), particle probe microanalysis (Forslind *et al*, 1995), X-ray microanalysis using a scanning transmission microscope (Wei *et al*, 1982), electron probe analysis (Warner *et al*, 1988), and most recently, proton-induced X-ray emission (PIXE) (Pallon *et al*, 1996). With PIXE, the analyzed sample is irradiated with MeV energy protons, causing inner shell electrons to be ejected from sample atoms within the tissue. Ejection of electrons creates voids and, as electrons from the outer shells fill these voids, an X-ray pattern that is characteristic for each element is emitted. Moreover, measurement

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Abbreviation: PIXE, proton-induced X-ray emission.

of the intensity of emissions yields the concentration of the element to be studied (Forslind *et al.*, 1995). The major strengths of PIXE *versus* the other biophysical methods are that: (i) accurate and quantitative elemental measurements can be made with minimal artifact on unfixed, frozen freeze-dried tissues; (ii) it is extremely sensitive (accurate measurements can be made down to parts per million); (iii) micron-scale spatial resolution is attainable; and (iv) PIXE-analyzed sites can then be assessed by standard light microscopy. To date, PIXE has been used to measure ionic concentrations in normal *versus* diseased skin (Pallon *et al.*, 1996), in young *versus* elderly skin (Bunse *et al.*, 1991), and in fetal epidermal development (Elias *et al.*, 1998). PIXE also has been used to measure a drop in peak Ca²⁺ concentration in epidermis in response to 2% sodium dodecyl sulfate application (Mao-Qiang *et al.*, 1997).

The experiments detailed below use PIXE to measure the effects of acute, acetone-induced barrier perturbation on intraepidermal ionic gradients. These studies both expand the information gained from previous Ca²⁺ cytochemical studies (Menon *et al.*, 1985) by allowing a strictly quantitative measure of ionic concentrations throughout the epidermis, and also provide quantitative data about the second key ion, K⁺, in relation to changes in barrier status. We found distinctive ionic gradients of Ca²⁺ and K⁺ in normal, unperturbed epidermis, which disappeared after barrier disruption, returning toward baseline in parallel with barrier reformation.

MATERIALS AND METHODS

Barrier disruption The epidermal permeability barrier of male hairless mice, ages 6–12 wk (*n* = 3 for each experiment), was broken by gently wiping one flank with acetone until the transepidermal water loss reached 3.0–5.0 g per h per cm². In control animals, a sham wiping of the flank was performed with dry cotton balls. Freshly obtained 4 mm³ slice samples from normal or experimental animals were snap-frozen in liquid propane, transferred to liquid nitrogen, and stored at –50°C. After 30 μm sections were cut on a cryostat, they were transferred to a metal-free nylon foil and freeze-dried for 12 h at –80°C.

PIXE PIXE studies were performed using a modification of the methods of Bunse *et al.* (1991). Microbeam PIXE data were obtained using 3 MeV proton beams. For the measurements reported here, beam currents of up to 1.5 nA focused down to spot sizes of 3 mm were scanned over areas of 200 × 20 mm². X-rays were detected with a Si(Li) detector that subtended a solid angle of ≈100 msr. The detector was located at an angle of 135° with respect to the incident beam. Charge was collected in a biased Faraday cup located behind the sample. X-rays were recorded in list mode along with coincident beam spatial co-ordinates arising from scanning the beam electrostatically over the sample in a point by point raster mode. To normalize X-ray yields for variation in target thicknesses, scanning transmission ion microscopy was employed to measure tissue projected densities following PIXE analysis of the irradiated area. After PIXE analysis, the samples were counterstained with hematoxylin and eosin and epidermal thickness was measured using a lens micrometer.

Data were reduced off-line so that X-ray spectra from subregions could be extracted from each irradiated region. X-ray spectra were analyzed with the PIXE spectrum fitting code (Antolak and Bench, 1994). A series of thin film calibration standards containing P, K⁺, Cl⁻, and Ca²⁺ were used to measure the efficiency of the X-ray detection system. PIXE analysis cannot distinguish among different valence states, thus the phosphorus content measured is the sum of all valence states present. Therefore, phosphorus is labeled as P throughout this report. One sample was taken from each animal (*n* = 3) in the control and experimental group, and each sample was measured in three separate areas. Data are presented as the mean ± SD. Statistical significance was determined using an unpaired Student's *t* test.

RESULTS

Ionic gradients are present in normal adult murine epidermis Ca²⁺, K⁺, Cl⁻, and P concentrations were measured in skin samples from three adult hairless mice under basal conditions. In agreement with the qualitative findings of Menon *et al.* (1985), a steep Ca²⁺ gradient was seen, with initial levels increasing from 180 ± 15 mg per kg at the basal layer (80–100 μm) to a peak at 460 ± 57 mg per kg in the outer stratum granulosum (10 μm), and then falling precipitously in the outer stratum corneum (Fig 1a). Similarly, total K⁺ concentrations also rose from 6200 ± 560 mg per kg in the basal layer to 10,309 ± 1291 mg per kg, peaking instead just below the granular layer

(40 μm), before declining across the stratum granulosum and stratum corneum (Fig 1b). In contrast, Cl⁻ concentrations were highest in the basal layer, declining steadily across the entire epidermis (Fig 1c), and P levels stayed at high, constant levels throughout the lower epidermis, before dropping precipitously beneath the granular layer (Fig 1d).

Ionic gradients dissipate after barrier perturbation Because extracellular ionic concentrations, particularly Ca²⁺ (Lee *et al.*, 1992) and K⁺ (Lee *et al.*, 1994), regulate lamellar body secretion, thereby facilitating barrier recovery, we next tested the effects of acute barrier perturbation on the distribution of these cations. The peak Ca²⁺ concentrations within the outer epidermis fell from 460 ± 57 mg per kg to 128 ± 14 mg per kg (*n* = 3, *p* < 0.01 *versus* controls) immediately after barrier disruption (Fig 2). Three hours later, when the barrier had recovered by 30%–40% (Talgebini *et al.*, 1996), peak Ca²⁺ returned to 268 ± 21 mg per kg (≈60% of normal Ca²⁺). Levels in the lower epidermis, which did not change immediately after disruption, appeared to increase slightly, consistent with prior cytochemical observations of increased movement of Ca⁺⁺ into the epidermis from deeper skin layers (Menon *et al.*, 1992a). Peak K⁺ concentrations also fell (10,309 ± 1291 mg per kg to 7927 ± 584 mg per kg), but less dramatically than Ca²⁺, as would be expected for a predominantly intracellular ion (Fig 3) (*n* = 3, *p* < 0.05). K⁺ concentrations recovered to 8970 ± 279 mg per kg in 3 h. Finally, neither P nor Cl⁻ concentrations changed significantly after barrier perturbation (data not shown). These studies show that barrier perturbation produces selective changes in the Ca⁺⁺ and K⁺ gradients within the epidermis, consistent with the putative role of both of these ions in regulating metabolic events leading to barrier recovery.

DISCUSSION

These studies quantitated Ca²⁺, K⁺, P, and Cl⁻ concentrations within normal epidermis, and in relation to the integrity of the epidermal permeability barrier. Our studies provide quantitative data in support of the presence of a steep Ca²⁺ gradient in mouse epidermis. This gradient peaks in the outer stratum granulosum, declining precipitously across the stratum corneum. Similar results were reported for normal human epidermis by Pallon *et al.* (1996) by PIXE. The mechanism(s) that form the Ca²⁺ gradient remain unknown, but it is clear that decreased Ca²⁺, caused either by barrier abrogation or by exposure of the outer epidermis to water, deficient in ions, enhances lamellar body secretion (Menon *et al.*, 1992a, 1994).

Ca²⁺ precipitation ultrastructural studies (Menon *et al.*, 1985, 1992b) demonstrated that the increase in Ca²⁺ in the outer epidermis is mainly extracellular; and that both extracellular and intracellular Ca²⁺ drop immediately after barrier disruption, in agreement with these studies. This study also shows quantitatively that Ca²⁺ concentrations begin to return to their previous levels by 3 hours. Because unanesthetized animals manipulated their wounds by licking and scratching, we could not measure ion concentrations at late time points (24 or 36 h) prior to barrier restitution.

In contrast to Ca²⁺, which peaks in the outer stratum granulosum (10 μm), K⁺ concentrations begin to decrease at ≈40 μm from the surface, an area that corresponded in our samples to the base of the granular layer. Similar patterns of K⁺ have been reported in studies of guinea pig skin (Wei *et al.*, 1982) and human skin (Warner *et al.*, 1988; Von Zglinicki *et al.*, 1993; Pallon *et al.*, 1996), although in one study these decreases localized to the stratum granulosum–stratum corneum interface (Warner *et al.*, 1988). The K⁺ (and P) gradient may dissipate at this site because cellular Na⁺/K⁺ ATPase becomes senescent. Na⁺/Ca²⁺ pumps also deteriorate in suprabasal keratinocytes (Cho and Bikle, 1997); however, sufficient Ca²⁺-binding proteins may be present in the stratum granulosum (Markova *et al.*, 1993) to prevent these ions from diffusing while an intact barrier is present, thus preserving the Ca²⁺ gradient until the outer stratum corneum. Previous studies using a K⁺-sensitive electrode demonstrated that K⁺ is lost through the stratum corneum after barrier disruption (Lo *et al.*, 1990). This study quantitates that loss, and demonstrates the K⁺ gradient recovery as the barrier reforms. Barrier disruption likely increases water flux, which causes the loss of both K⁺ and Ca²⁺. Prior PIXE measurements after

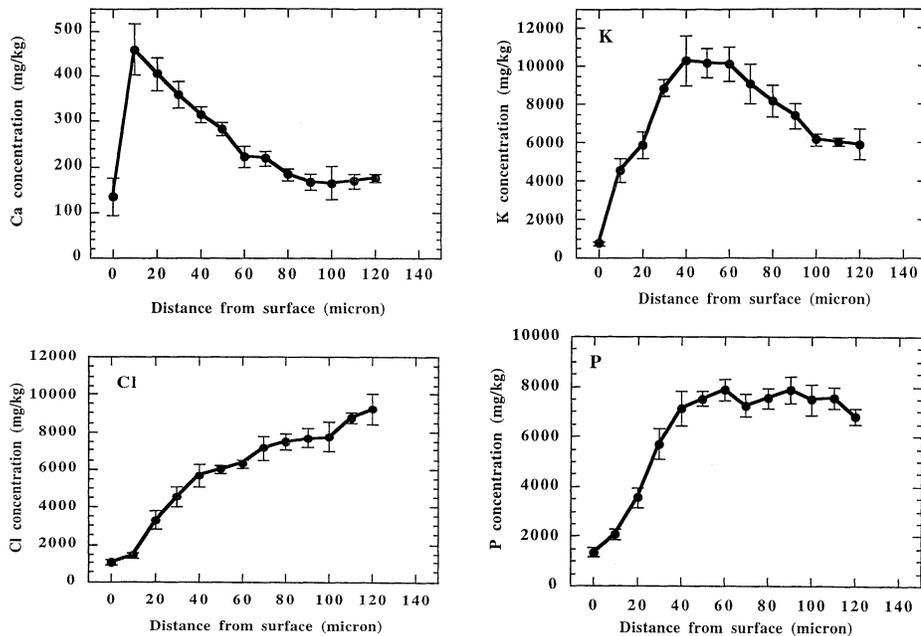


Figure 1. Ca^{2+} (a), K^{2+} (b), Cl^- (c), and P (d) gradients are present in normal mouse epidermis. Biopsies of normal skin from three adult hairless mice were obtained and analyzed according to the *Materials and Methods*. Samples were analyzed with PIXE for concentrations in p.p.m./dry weight of epidermis. Samples were oriented so that the skin surface (stratum corneum) is labeled as 0 mm. Each sample is measured in triplicate and the result presented as the mean \pm SD.

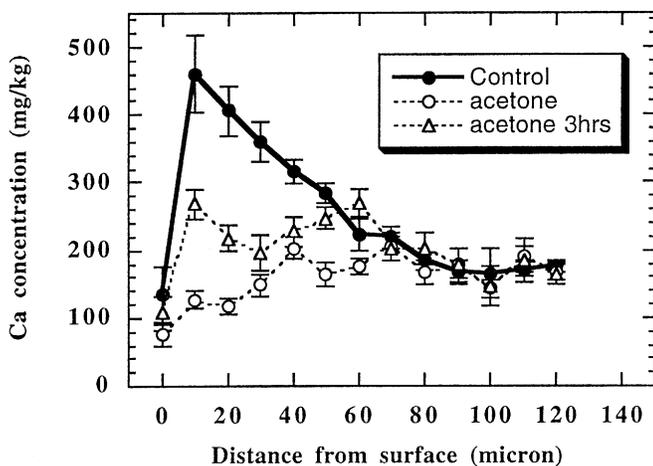


Figure 2. Barrier disruption with acetone abrogates the Ca^{2+} gradient. Flanks of three hairless mice were gently rubbed with acetone until the transepidermal water loss rates measured 300–500 g per h per m^2 per h. Biopsies of flank skin were taken immediately and 3 h after treatment, prepared and analyzed as above. Concentration gradients immediately and after 3 h treatment are compared with those seen in untreated skin. Each sample is measured in triplicate and the result presented as the mean \pm SD.

barrier disruption with 2% sodium dodecyl sulfate (Mao-Qiang *et al*, 1997) also revealed a similar, rapid drop in Ca^{2+} , suggesting that this response is independent of the method of barrier perturbation. Unlike P, whose role in keratinocyte differentiation is not well characterized, K^+ has been shown to play a role in both early and late keratinocyte differentiation. K^+ channels are present throughout the epidermis, but become functional only in keratinocytes cultured in raised extracellular Ca^{2+} (Mauro *et al*, 1997). K^+ conductance hyperpolarizes less-differentiated keratinocytes and increases Ca^{2+} influx (Xiong and Harmon, 1995; Mauro *et al*, 1997), whereas in granular layer keratinocytes, K^+ is synergistic with Ca^{2+} in inhibiting lipid secretion (Lee *et al*, 1994). Thus, rising K^+ concentrations in the basal and spinous layers would act to increase Ca^{2+} influx into these cells, potentiating the pro-differentiating effects of elevated extracellular Ca^{2+} .

The epidermis is a viable tissue and, as such, it is hydrated. Because our measurements were done on desiccated tissue, we cannot measure directly whether these gradients are present *in vivo*. We found that both K^+ and Ca^{2+} increase across the viable layers of the epidermis.

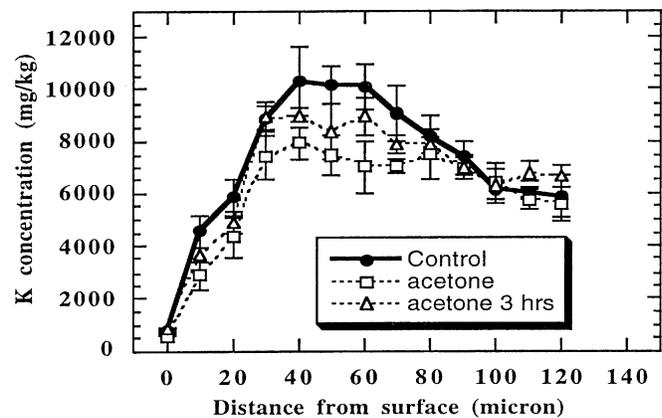


Figure 3. Barrier disruption abrogates the K^{2+} gradient. The epidermal permeability barrier was perturbed with acetone as described in Fig 2. Biopsies of flank skin were removed immediately after treatment, prepared and analyzed as described in the *Materials and Methods*. Concentrations immediately and 3 h after treatment are compared with those seen in untreated skin for K^+ . Each sample is measured in triplicate and the result presented as the mean \pm SD.

These gradients are likely to be real, as the water content is constant across these layers (Warner *et al*, 1988; Von Zglinicki *et al*, 1993). Furthermore, because the stratum corneum loses water at rates comparable with the nucleated layers, any increase seen in this layer is likely to become greater, because hydration of the viable layers *in vivo* would dilute the ionic concentrations in the lower layers of the epidermis.

The epidermis is a multilayered organ, within which keratinocytes differentiate as they migrate vertically from the basal to the cornified layers. Ions, especially Ca^{2+} , commonly function as signaling molecules. *In vitro*, extracellular Ca^{2+} concentrations are central regulators of both early and late epidermal differentiation. Increased extracellular Ca^{2+} stimulates K1/10, involucrin, loricrin, and transglutaminase 1 synthesis (Eckert *et al*, 1997); activates transglutaminase 1-mediated cross-linking of the cornified envelope (Rice and Green, 1979); and coordinates the expression of differentiation-specific keratins (see Eckert, 1997 for review). Finally, it is noteworthy that extracellular Ca^{2+} levels in the granular layer exceed those found in the dermis (Menon *et al*, 1985), and at least one important event in late epidermal differentiation, the conversion of profilaggrin to filaggrin, requires supraphysiologic Ca^{2+} concentrations (Resing *et al*, 1993). Other processes of late keratinocyte differentiation and programmed cell death, such as activation of

serine proteases (Hansson *et al*, 1994; Marthinuss *et al*, 1995) and transglutaminase (Rice *et al*, 1994; Eckert *et al*, 1997), also depend on raised Ca²⁺ concentrations. Thus, the Ca²⁺ gradient, first discovered in epidermis by Menon *et al*, may drive the sequential stages of keratinocyte differentiation *in vivo*, analogous to its effects *in vitro*. The K⁺ gradient, shown in this report to decrease with barrier disruption, may enhance the effects of the Ca²⁺ gradient by controlling membrane potential and Ca²⁺ entry into keratinocytes.

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