

# Molecular traffic through plasma membrane disruptions of cells *in vivo*

PAUL L. McNEIL and SUSUMU ITO

Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, Massachusetts 02115, USA

## Summary

We have recently shown that mechanical forces, experimentally imposed or physiologically generated, transiently disrupt or wound the plasma membranes of epithelial cells of the gut and that cultured endothelial cells similarly wounded mechanically at their plasma membranes release a potent basic fibroblast growth factor-like molecule. Here we show that mechanical forces generated by experimental manipulation (tape stripping and needle puncture), or by animal locomotion, transiently wound the plasma membranes of various cells of skin, allowing otherwise impermeant tracer molecules to enter and become trapped within cell cytoplasm. We estimate that the epidermis of digits from actively locomoting animals is composed of 10.5% ( $\pm 4.9\%$  S.D.) wounded cells, and that from quiescent animals has 3.7% ( $\pm 2.5\%$ ) wounded cells. Wounded fibroblast, glandular and endothelial cells were also identified in mech-

anically stressed skin. Cells retaining fluorescein dextran, used as a label for wounding, were observed 24 h after the imposition of mechanical force, and wounded cells were generally of normal ultrastructure, indicating that cells in skin can survive membrane wounding. We propose that plasma membrane disruptions are an overlooked but probably common occurrence in cells residing in tissues such as gut and skin that are normally exposed to mechanical stress *in vivo*, and that such disruptions provide the physical basis *in vivo* for a previously unrecognized and diffusion-mediated route for molecular traffic directly across the plasma membrane into and out of living cell cytoplasm.

Key words: plasma membrane, cell wounding, membrane disruptions, epidermis.

## Introduction

The vesicular shuttle mechanisms of endocytosis and exocytosis allow macromolecules to enter and leave cells without a detectable compromise of plasma membrane integrity. However, not all molecular traffic has been readily explainable by vesicular mechanisms. For example, basic fibroblast growth factor (bFGF) (Abraham *et al.* 1986) and interleukin-1 (Auron *et al.* 1984) both lack the signal peptide sequence normally considered a prerequisite for secretion *via* the classical exocytic pathway (Walter and Lingappa, 1986). The mechanism of their release from cells has therefore been a matter of intense speculation (Rifkin and Moscatelli, 1989).

Disruptions of plasma membrane integrity would, if they occurred in cells *in vivo*, constitute an additional molecular route to and from cell cytoplasm for these growth factors and, potentially, for many other biologically and pathologically important molecules as well. We recently showed that mechanical forces of experimental origin, or physiologically generated by normal gut motility, transiently disrupt or 'wound' the plasma membranes of gut epithelial cells (McNeil and Ito, 1989). Here we have asked whether wounding of plasma membranes similarly occurs in the cells of skin, a tissue that normally is exposed frequently to mechanical stress *in vivo*.

## Materials and methods

### Skin preparation

Horseradish peroxidase (HRP; Boehringer Mannheim, FRG) and  
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10 000  $M_r$  lysine-conjugated fixable, anionic fluorescein dextran (FDx; Molecular Probes) were used as probes for the detection of cells wounded transiently at their plasma membranes. Sprague-Dawley rats (male, 300 g, Charles River, Wilmington, MA) were anaesthetized with chloroform and then injected intradermally on the dorsal surface of the proximal end of their tails with 50  $\mu$ l of HRP (100 mg ml<sup>-1</sup>) or FDx (100 mg ml<sup>-1</sup>) dissolved in phosphate-buffered saline (PBS), or they were injected in the distal-most, plantar surface of their hind leg digits with 5  $\mu$ l of FDx. A calibrated Hamilton syringe (100  $\mu$ l or 25  $\mu$ l) and 30-gauge needles were used for the injection of a uniform volume of FDx or HRP solution into the dermis at each location. The yellow-coloured FDx could be seen to spread rapidly (seconds) and apparently uniformly from the injection site throughout the dermis of the digit. In the tail, injected label could be seen to spread from the injection site outwards approximately 1 cm in all directions. At various intervals after these injections and recovery from chloroform anaesthesia (1–24 h), rats were anaesthetized by intraperitoneal injection of Nembutal (35 mg kg<sup>-1</sup> body weight) and patches of skin adjacent to the injected site were carefully and gently excised with a razor blade and immersed immediately in fixative.

### Application of mechanical stress to skin

Immediately after FDx or HRP injection, a 1 cm<sup>2</sup> patch of skin proximal to the tail injection site was 'stripped' 20 times with adhesive tape until a glistening surface resulted, indicating removal of the keratinized layer (Bertsch *et al.* 1976; Rijzewijk *et al.* 1988). Control patches of skin on the same animal received an injection of FDx or HRP but were not stripped. After the tape-stripping, animals were allowed to recover from anaesthesia and resume normal activities.

Immediately after recovery from chloroform anaesthesia, ani-

mals injected in their digits with FDx were placed on top of their metal cages. In order to keep them active and moving, we occasionally prodded them with our fingers or picked them up by their tails and then placed them back on the cage top. During 10–15 min of this enforced locomotory activity, the rats had vigorously pushed and pulled on the bars of the cage, climbed up and down the side of the cage, and walked extensively over its surface. Control, quiescent animals were kept under continuing anaesthesia after FDx injection by a Nembutal injection, and did not recover consciousness before tissue preparation for frozen sections.

### Microscopy

For fluorescence microscopy, excised skin containing injected FDx was fixed in aldehydes (Ito and Karnovsky, 1968), and cut into 10–20  $\mu$ m thick frozen sections that were mounted on gelatinized slides. Cells that suffered membrane disruptions and thus became labelled diffusely with fluorescein fluorescence were then identified and photographed on a Zeiss Axiophot.

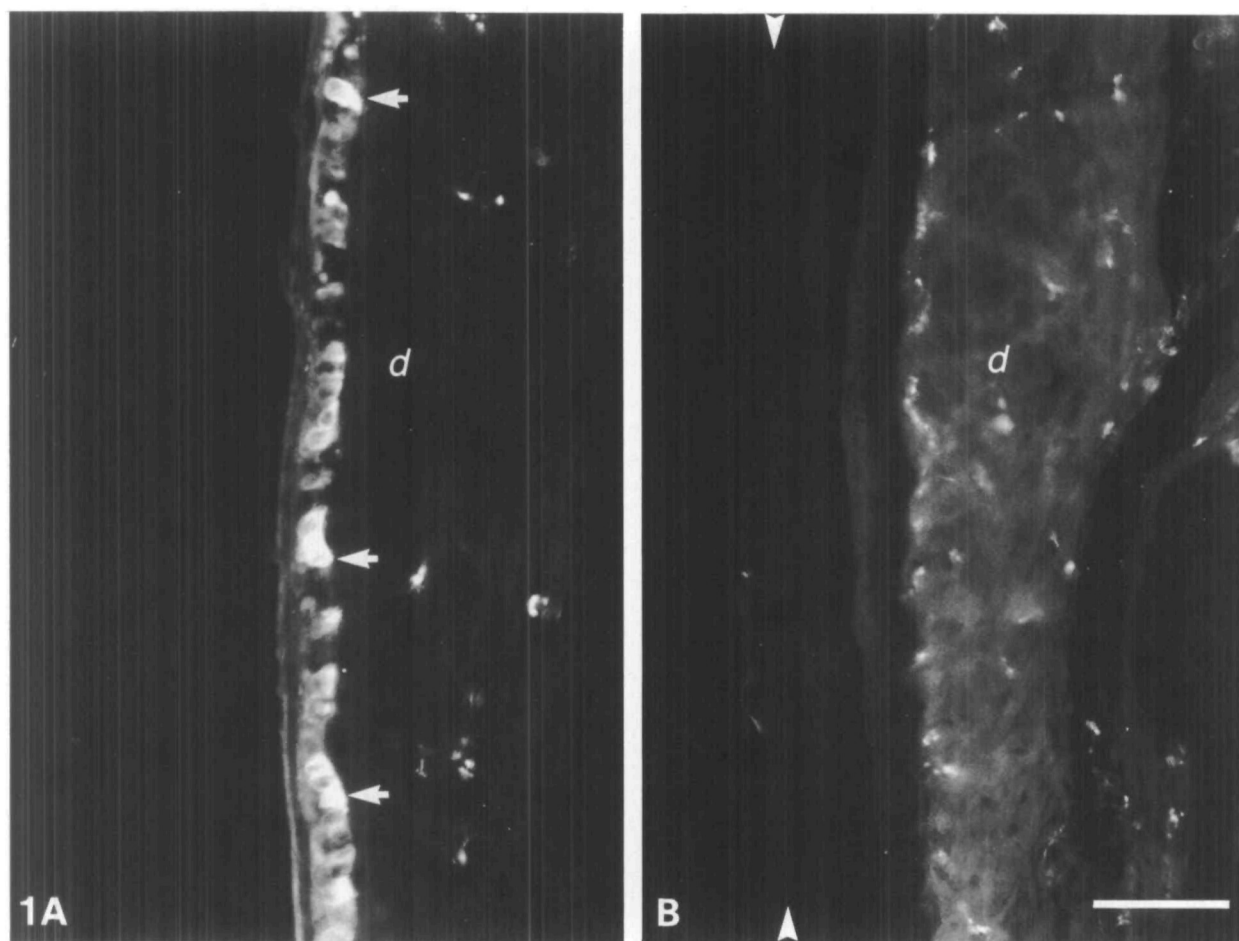
For electron microscopy, excised skin that had been injected with HRP was similarly fixed in aldehyde (Ito and Karnovsky, 1968), cut into 20  $\mu$ m thick frozen sections, and these were then reacted with diaminobenzidine (Adams, 1977) before they were treated with  $\text{OsO}_4$ , embedded in Epon-Araldite, thin-sectioned, and viewed without further staining in a JEOL 100S microscope.

### Immunochemical staining of wounded cells

Experimental rats that had locomoted on their cage tops (see above) were anaesthetized using Nembutal, perfused vascularly with PBS to remove as much extracellular serum albumin as possible, and then fixed by further perfusion with 10% freshly generated formaldehyde. Excised digit skin (plantar surface) was cut into 5  $\mu$ m thick frozen sections that were collected on gelatinized slides. Sections were then incubated for 4–6 h or overnight at 37°C with a 1/200 dilution of HRP-conjugated goat anti-rat albumin IgG (Cappel, West Chester, PA), washed extensively in distilled water, and reacted with diaminobenzidine (Adams, 1977) before viewing by transmitted light microscopy. Control animals were simply anaesthetized and then prepared as for experimentals. Controls did not therefore receive any exercise additional to that of their normal locomotory routine.

### Quantification of cell wounding

Transmitted and fluorescence micrographs were taken of frozen sections of digit skin from locomoting and quiescent animals injected with FDx. For each condition, five coded sections were photographed blind. Each photographic montage represented 3/4 of a digit's length. The image of the epidermis, excluding the keratinized layer, was cut out of photographic prints of the transmitted light micrographs; the images of the fluorescent areas (FDx-labelled cells) in epidermis out of prints of the



**Fig. 1.** Epidermal cell plasma membrane wounding in skin stripped of its keratinized layer. **A.** In tail skin stripped with adhesive tape until the keratinized layer was completely removed, epidermal cells labelled with FDx were common (arrows), and their diffuse fluorescence labelling is much more intense than that of the connective tissue spaces of the dermis (d), or between epidermal cells. **B.** In control, undisturbed tail skin of a rat, epidermal cells were not labelled with FDx. This fluorescent probe for cell wounding was, however, present in the dermis (d), the site of its injection. Note for comparison the intact keratinized layer of this undisturbed skin (arrowheads). The skin of this animal was removed 6 h after the FDx injection. By this time, the intradermally injected FDx had been considerably diluted and cleared by the blood vascular system. Bar, 50  $\mu$ m.

fluorescence micrographs. The 'cut-outs' of each region were then weighed as relative measures, respectively, of total epidermal cell and labelled epidermal cell areas. Finally, the images and the corresponding quantitative data generated from them were decoded.

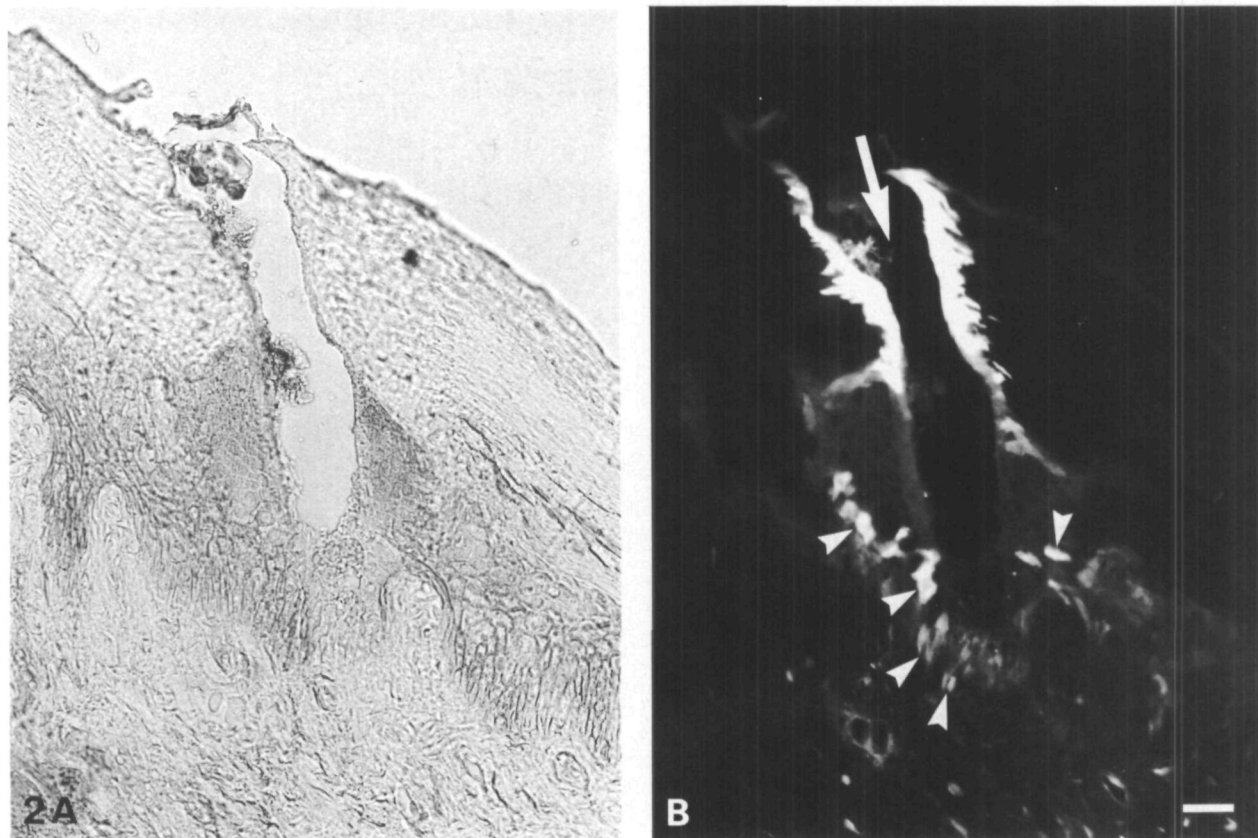
## Results

Cells of skin that incurred temporary membrane disruptions were identified by their diffuse cytoplasmic staining with the membrane-impermeant tracers, horseradish peroxidase (HRP), a protein of 40 000  $M_r$ , or fluorescein dextran (FDx), a highly branched carbohydrate of 10 000  $M_r$  (average). These hydrophilic, freely diffusible tracers can enter cell cytoplasm only through a disrupted membrane, and are retained there only if the disrupted plasma membrane reseals, trapping them in cytoplasm (McNeil and Ito, 1989; McNeil, 1989). Our general experimental procedure was to deliver these tracers to the extracellular spaces of skin by an intradermal injection, then immediately to stress the skin mechanically (as described below), and, finally, to identify cells that were thereby wounded transiently at their plasma membrane by transmitted light (HRP staining), epi-fluorescence (FDx staining) or electron microscopy (HRP staining) of sectioned tissue prepared at intervals of 1–24 h after injection of label. We waited for these intervals after label injection, rather than taking tissue immediately after application of mechanical stress, in order that extracellu-

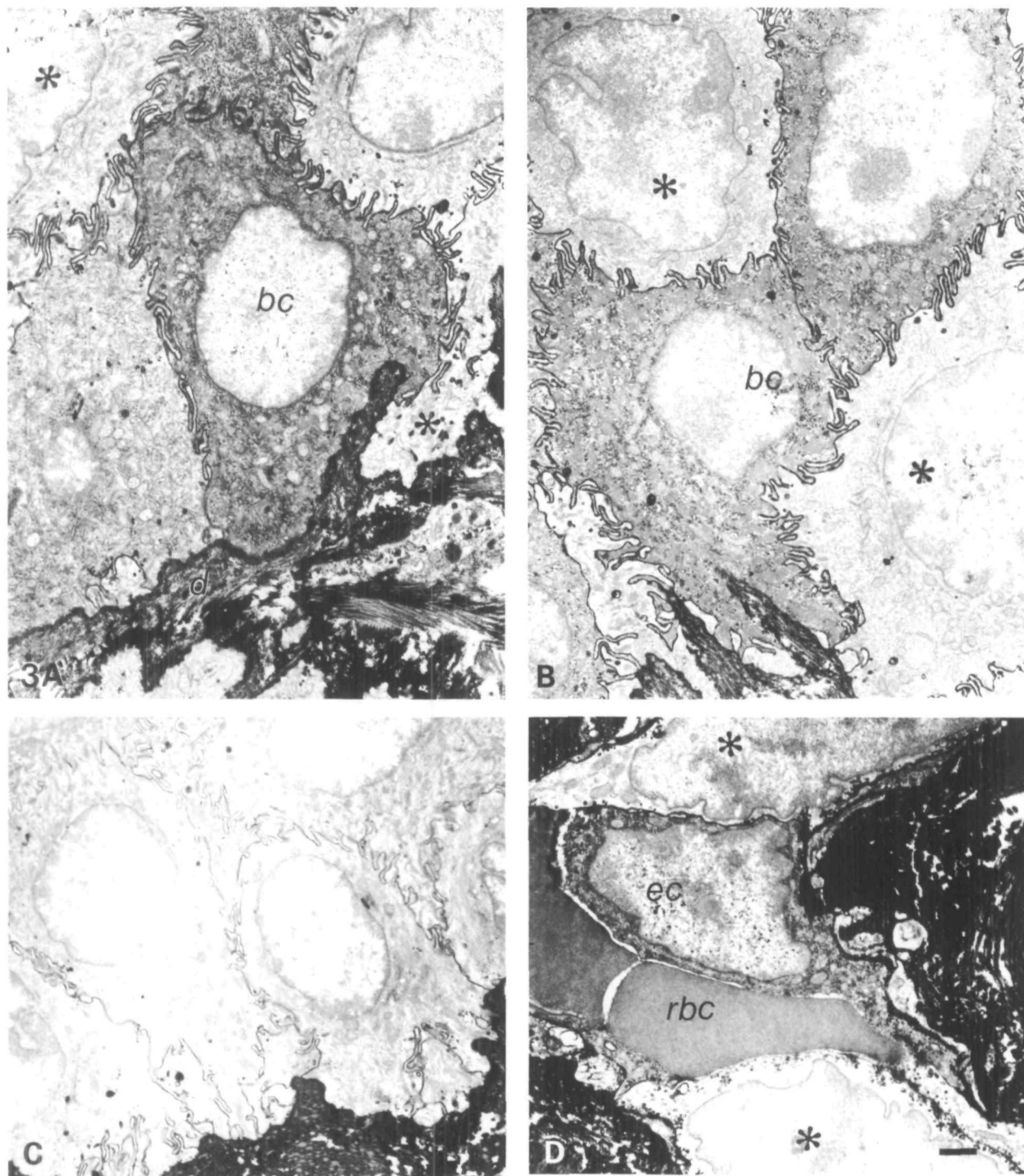
lar label could be diluted and cleared by the blood vascular system.

First, we asked whether cell plasma membrane wounding and resealing was detectable in skin subject to experimentally imposed mechanical stress. Adhesive tape was repeatedly applied to and then stripped off from rat tail skin until most or all of the keratinized layer had been removed. This method, termed tape-stripping, has been widely used to study mechanically induced growth of epidermal cells (Bertsch *et al.* 1976; Rijzewijk *et al.* 1988; Marks *et al.* 1983). Six hours after stripping rat tail skin, we observed numerous stratified squamous epidermal cells that were more intensely labelled with FDx than were the extracellular spaces between epidermal cells or even the large extracellular space of dermis (Fig. 1A). These intensely labelled cells were present in all strata of the epidermis, including the basal layer. They were particularly common within the follicles of hairs removed by the stripping, and were still there 24 h later, when extracellular FDx was microscopically undetectable (not shown). Few or no intensely labelled epidermal cells were present in skin that was not stripped (Fig. 1B), except near the puncture wound produced by the needle insertion during injection of FDx. Such puncture wounds, in all animals, were lined with intensely labelled cells (Fig. 2).

We next used HRP and electron microscopy (EM) to confirm, using an independent tracer, our observations made with FDx, and to characterize the ultrastructure of cells that had been wounded. As with FDx, the frequency of HRP-labelled cells detected at the light-microscopic



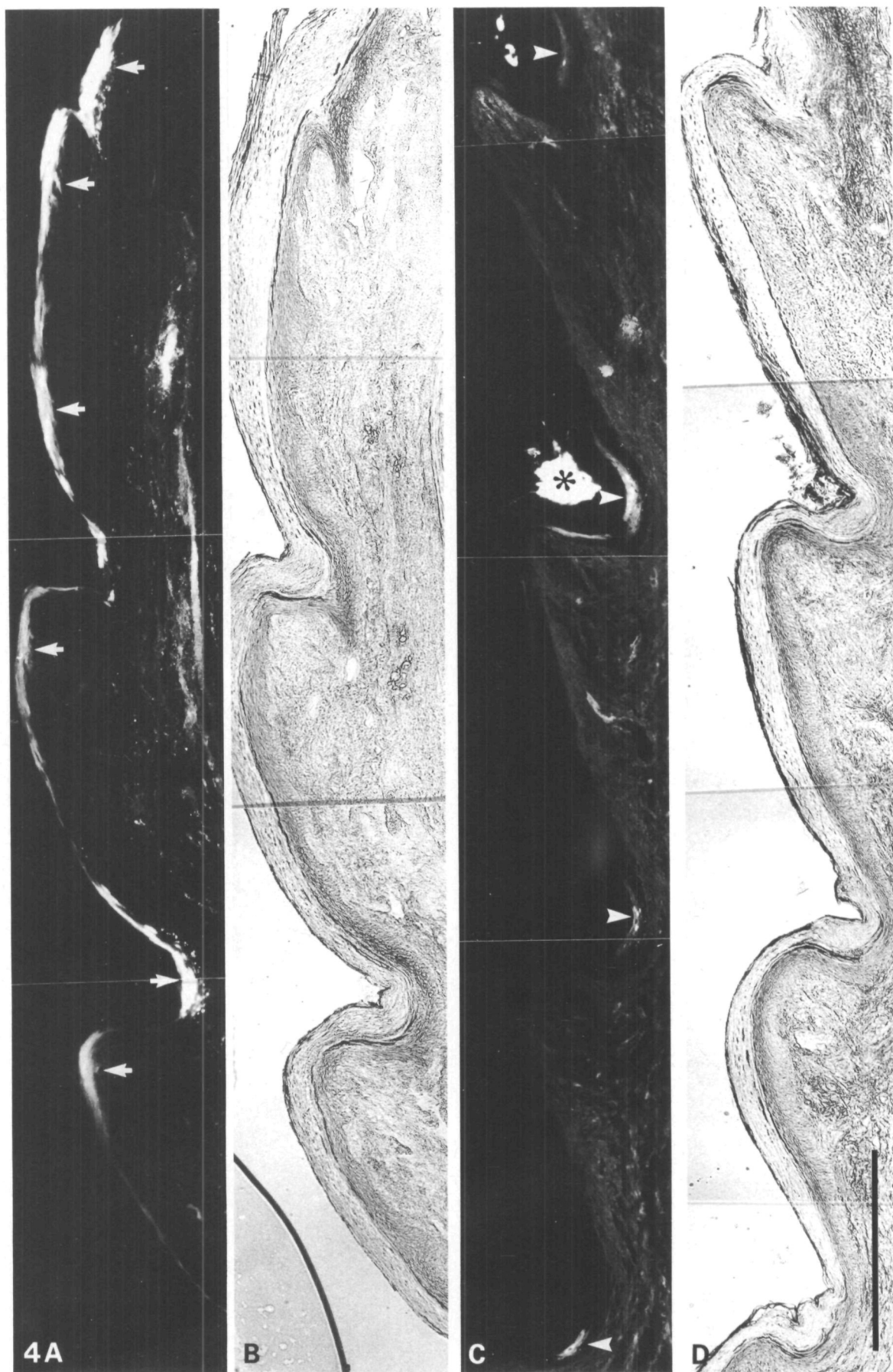
**Fig. 2.** Epidermal cell plasma membrane wounding at sites of a needle puncture wound. Phase-contrast (A) and fluorescence (B) micrographs of skin from a rat digit at the site of a needle puncture (arrow). Fluorescently labelled epidermal cells (arrowheads) are present in all strata of the epidermis at such sites of needle puncture that we routinely produced during the intradermal injection of FDx. Bar, 50  $\mu\text{m}$ .



**Fig. 3.** Ultrastructural evidence for cell plasma membrane wounding in stripped skin. A, B. When HRP was used instead of FDx as a probe for cell wounding, epidermal cells labelled cytoplasmically with electron-dense reaction product were observed in all strata of the epidermis of stripped skin viewed by electron microscopy. Most, like the basal cells (bc) shown in these micrographs, were of normal ultrastructure. Asterisks denote cells without label in their cytoplasm. d, dermis. C. Basal cells from undisturbed skin were without HRP label in their cytoplasm. D. Various other cell types in stripped skin were labelled in their cytoplasm with HRP. These included fibroblasts, glandular cells, and endothelial cells (ec), such as that shown in this micrograph. An unlabelled endothelial cell is denoted with an asterisk. rbc, red blood cell. Bar, 1  $\mu$ m.

**Fig. 4.** Epidermal cell plasma membrane wounding in digit skin from locomoting rats. Intensely fluorescent epidermal cells (arrows) were more frequent in digit skin from actively locomoting animals (A,B) than in digit skin from inactive animals (C,D). Small zones of lightly labelled epidermal cells (arrowheads) were seen in the epidermis of skin from inactive animals, and these zones were characteristically localized at the sites of digit articulation where large infoldings of skin occur. Some FDx that was accidentally spilled onto the surface of the skin of this inactive animal is denoted with an asterisk. Bar, 1 mm.





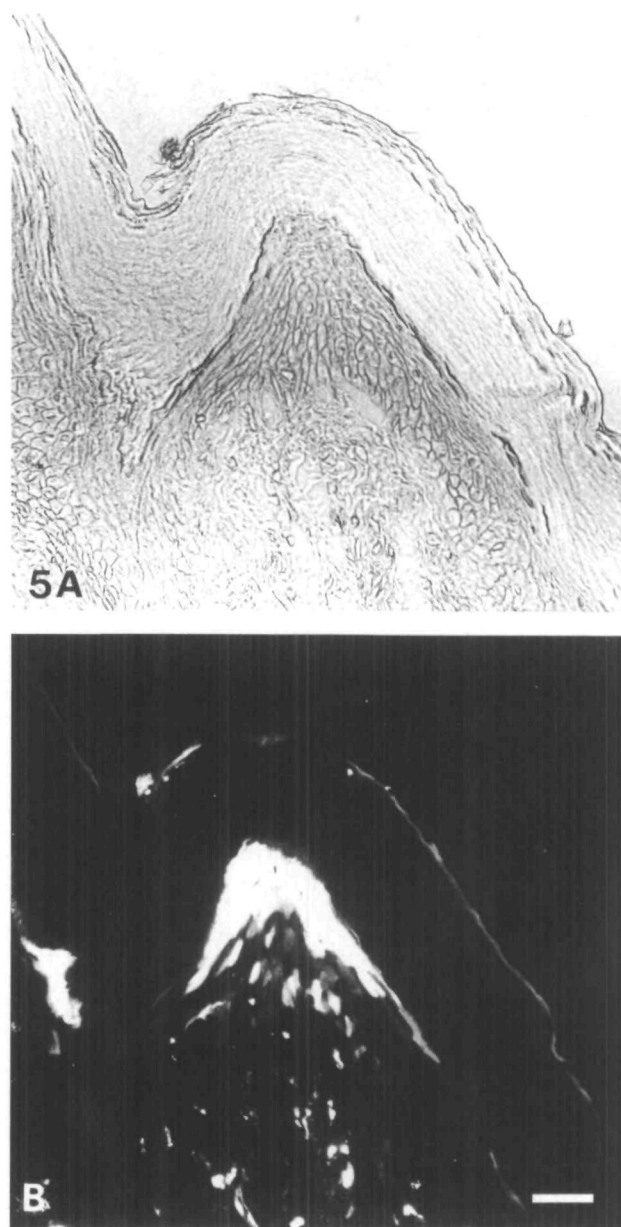
level was higher in stripped than in undisturbed skin (not shown). EM confirmed that epidermal cells at all levels were labelled and demonstrated that the label was cytoplasmic and not extracellular or endocytosed tracer (Fig. 3A, B). Moreover, examination of the dermis revealed that numerous endothelial cells (Fig. 3D), mucous gland cells (not shown) and fibroblasts (not shown) were also labelled. These HRP-labelled cells possessed normal ultrastructural morphology.

To determine whether normal animal locomotion could exert mechanical stress sufficient to wound the plasma membranes of cells in epidermis of the digits, FDx was injected intradermally into digit plantar surfaces and the injected rats were then encouraged to locomote on their cage tops, or were kept immobile under anaesthesia. When tissue was taken 1 h after FDx injection, labelled epidermal cells that were more fluorescent than the extracellular space of the dermis were more frequent in the skin of the digits of the actively locomoting rats (Fig. 4A, B) than in digits of quiescent animals (Fig. 4C, D). The spread of the FDx label for cell wounding throughout the length of the dermis of the digit was apparent from the latter's strong and uniform fluorescent labelling in skin fixed 1 h after the FDx injection (see especially Fig. 4C, D). Hence, cell wounding, if it occurred, was detectable throughout the digit's length. We estimate that the epidermis of digit from locomoting animals is composed of 10.5% ( $\pm 4.9\%$  s.d.) labelled cells, that from quiescent animals of 3.7% ( $\pm 2.5\%$  s.d.) labelled cells (these means are significantly different,  $P < 0.025$ ). Labelled epidermal cells were also observed in digit skin prepared from locomoting animals 24 h after the FDx injection (Fig. 5).

Finally, we confirmed that membrane wounding occurs in the epidermis of locomoting animals using the animal's own albumin as an endogenous (native) probe for cell wounding. Sections of digit skin from experimental animals encouraged to locomote on their cage tops, and from control animals not subjected to locomotory activity additional to normal levels, were stained with a peroxidase-conjugated antibody to rat serum albumin. Heavily labelled cells were apparently more numerous in the epidermis of the experimental animals (Fig. 6A–C) than in epidermis of the relatively more quiescent controls (Fig. 6D–F).

## Discussion

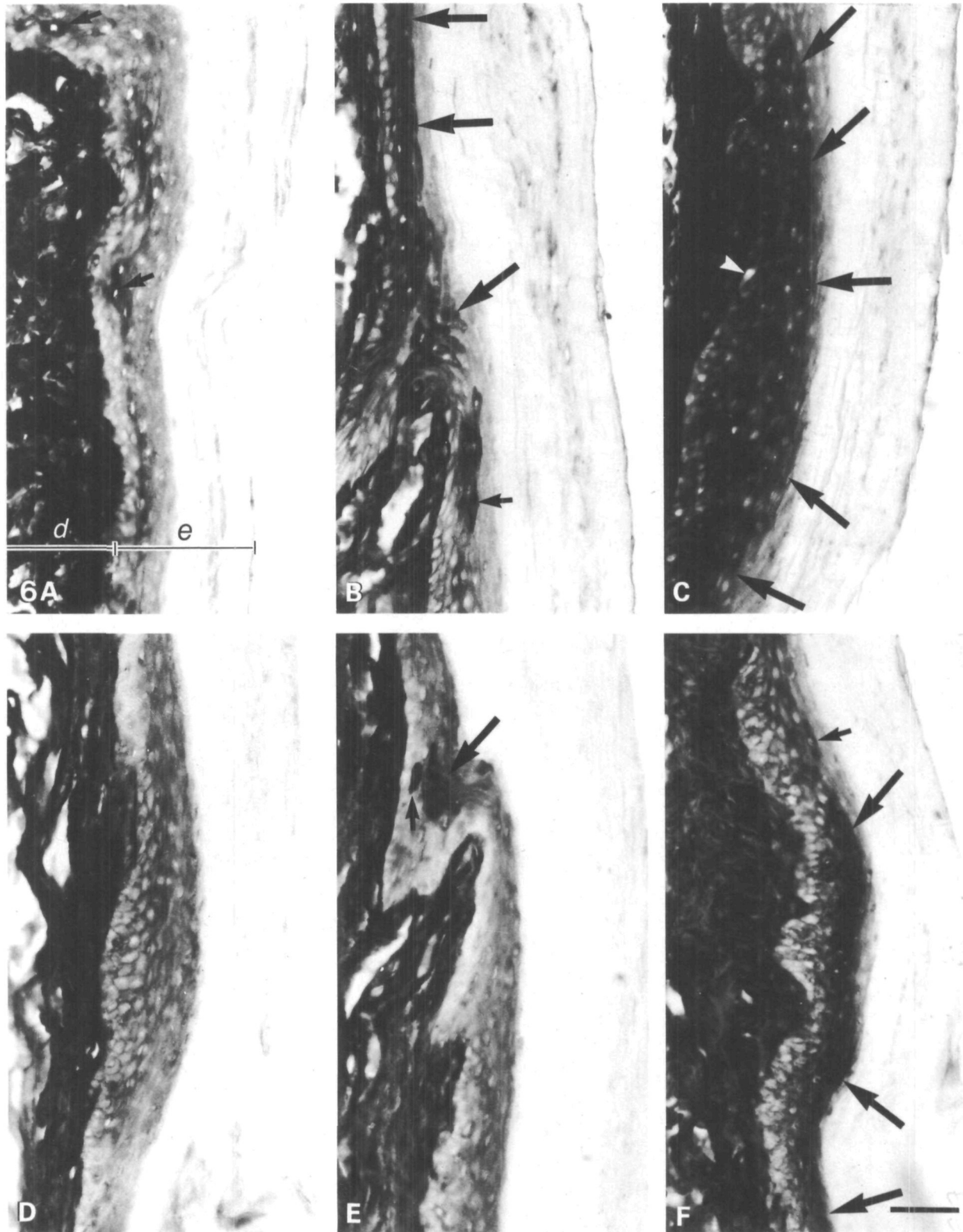
We have detected cell plasma membrane wounding in skin using as probes intradermally injected FDx and HRP. The question therefore arises: did the obvious mechanical and potential chemical perturbations of our methods cause the observed epidermal cell wounding in mechanically stressed skin? Clearly, the needle injection itself wounded the membranes of a limited zone of epidermal cells, but these were avoided in our analysis of the effects of other forms of mechanical stress. With the exception of such injection sites, epidermal cell wounding was absent or relatively infrequent, unless the skin had been stressed mechanically by tape stripping or animal locomotion. Therefore it seems unlikely that either the mechanical or chemical perturbations of our techniques alone were responsible for our observations of epidermal cell membrane wounding. However, it was possible that mechanical or chemical perturbations inherent in our techniques could have predisposed cells to suffer membrane wounds upon subsequent imposition of mechanical stress. Our confir-



**Fig. 5.** Retention in skin of epidermal cells surviving wounds to their plasma membranes received during animal locomotion. Phase-contrast (A) and fluorescence (B) micrographs of digit skin excised from a locomoting animal 24 h after the FDx injection. No FDx is detected in the dermis, due to blood vascular dilution and clearance of this probe. By contrast, there are numerous fluorescent (FDx-labelled) cells in the epidermis 24 h after the locomotory activity, which presumably wounded their plasma membranes. Bar, 50  $\mu$ m.

mation, using the animal's native albumin as a probe, that cell wounding occurs in digit skin of locomoting animals not subject to any additional mechanical or chemical insults argues strongly against this last possible artifact. We suggest that our methods can accurately register the occurrence of transient membrane disruptions in epidermal cells of skin, and probably in other cell types of skin as well.

Using antibodies to endogenous albumin, epidermal cell wounding was apparent in the digit skin of experimental



**Fig. 6.** Endogenous albumin as a label for wounded epidermal cells in the digit skin of locomoting animals. A,B,C. Epidermal cells that could be stained with peroxidase-conjugated anti-rat serum albumin were frequently observed in the digit skin of rats induced to locomote. In C, an isolated, unlabelled epidermal cell is denoted by an arrowhead. D,E,F. Epidermal cells of control rats not induced to locomote were apparently less frequently stained with anti-rat serum albumin. The micrographs included in this figure are intended to represent the range in the frequency of cell labelling characteristic of each condition. Small arrows identify individual labelled epidermal cells; large arrows clumps of labelled epidermal cells. d, dermis; e, epidermis. Bar, 50  $\mu$ m.

rats exercised on top of their cage immediately prior to tissue preparation, and also in the digit skin of 'controls' that had not been exercised in addition to their normal locomotory activities. It will be important to determine in future studies whether the cell wounding observed in such controls is the result of mechanical stress imposed on digits by such normal locomotory activities as those required for foraging and fighting, and to demonstrate a quantitative dependence of cell wounding on imposed mechanical stress of normal origin and/or magnitude.

We have presented evidence to show that when mechanical forces are experimentally imposed upon skin, or incurred in this tissue during normal walking and climbing activities, wounding of the plasma membranes of numerous cells occurs. Cytoplasmic staining with FDx was often more intense than extracellular staining at 1–6 h after the dermal injection of FDx and stained cells remained in the epidermis 24 h after tape stripping or animal locomotion, when no extracellular FDx was detectable. These observations suggest that some cells resealed and thus survived membrane wounds, trapping HRP or FDx in their cytoplasm at relatively high concentration while extracellular label was cleared and diluted by the blood vascular system during the 1–24 h period after its injection. The normal ultrastructure of most HRP-labelled cells is further evidence that cells survived plasma membrane wounds. While additional studies will be required to establish the long-term fate of cells wounded in skin, it is clear that cultured cells surviving mechanical wounds to their plasma membranes are fully capable thereafter of both locomotion and proliferation (McNeil, 1989). Furthermore, we showed previously that epithelial cells of gut reseal membrane wounds, and that, in electron micrographs, some of these surviving epithelial cells appear to be actively engaged in reparative cell locomotion (McNeil and Ito, 1989).

Transient plasma membrane disruptions provide a molecular route into the cytoplasm of living cells (reviewed by McNeil, 1989). Cells wounded *in vitro* by mechanical means in the presence of DNA become transfected, indicating that even very large macromolecules can thus gain access to cell cytoplasm (Fechheimer *et al.* 1988). Although it is too soon to predict the full biological consequences *in vivo* of this molecular route into cytoplasm, there are several possibilities. Carcinogens, or even viral pathogens or their DNA or RNA, might gain access to cytoplasm during membrane wounding: in skin, this could occur in the actively dividing basal layer of epidermal cells. Entry of molecules into wounded cells might also be beneficial. For example, molecules entering cytoplasm could conceivably signal to the wounded cell that proliferation or some other reparative activity was required. Finally, mechanically induced cell wounding and resealing, such as that initiated here by experimental manipulation of skin, could provide the basis for a new method for introducing otherwise impermeant molecules, including foreign DNA, into the cytoplasm of living cells *in vivo*.

Plasma membrane wounds, whether transient or permanent, also provide a mechanism for release of normally non-secretable substances from cytoplasm. We have, for example, showed elsewhere that such disruptions can provide a mechanism for release from endothelial cells of bFGF, a growth factor that lacks the signal peptide sequence normally considered a prerequisite for secretion by the exocytotic pathway (McNeil *et al.* 1989). Such a mechanism also explains how, in the absence of haemorrhage and consequent platelet degranulation, growth fac-

tor release could be initiated within, and accurately localized to, sites of normal mechanical wear and tear *in vivo*. It is notable that the three mechanically induced wounds to skin studied here – removal of the keratinized layer, plucking of hair from follicles and the disruption of epidermal integrity by needle puncture – all resulted in epidermal cell membrane wounding and resealing, and are all known to induce a strong, localized mitogenic response from the epidermis (Marks *et al.* 1983; Wright and Allison, 1984).

We have now shown that mechanical forces of innate or experimental origin can transiently wound the plasma membranes of cells of gut and skin. We propose that such membrane wounding may be widespread *in vivo*, where mechanical forces must routinely act also upon such tissues as tendon, muscle and various epithelia in addition to gut and skin. Resealing may, therefore, be an overlooked but biologically important functional attribute of the plasma membrane, enabling cells to survive and continue to function normally or participate in vital reparative processes within a mechanically stressful environment *in vivo*. Finally, we have emphasized that the occurrence of membrane disruptions in cells *in vivo* suggests an additional, overlooked molecular route into and out of cell cytoplasm.

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