THE ROLE OF MICROFILAMENTS IN FROG SKIN ION TRANSPORT

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INTRODUCTION

Bioelectric and ion transport studies have frequently utilized the frog skin epithelium as an experimental model system (for review see 6, 7, 12). The ultrastructural correlates of postulated ion pump mechanisms in this tissue were first appreciated by Farquhar and Palade (4). Later other workers (1, 15–17) have shown how the size of intercellular spaces corresponds to rates of ion transport.

The purpose of the present study was to extend the observations of morphological correlates by examining skins under experimental conditions different from those already reported. The correspondence of intercellular spaces and ion transport was described by the above workers when the skins were short-circuited, whereas we chose to reexamine the relationship by observing the morphology when open-circuited.

Also, we chose to examine, in greater detail, the microfilaments of these epithelial cells. Several experimental findings prompted us. Yeltman (19) observed recently that when pieces of isolated frog skin were examined at intervals over a 24 h period in the electron microscope, microfilaments were seen to become increasingly disarrayed and paralleled the natural decline of the electrical potential difference. McGuire and Moellmann (9) have shown that cytochalasin B disrupts filaments in frog skin melanocytes.

MATERIALS AND METHODS

Bioelectric Measurements

The customary chamber devices (13) were used for measuring the electrical potential differences and short-circuit currents of abdominal skins taken from healthy *Rana pipiens*. The equilibration value, usually reached in about 30 min after clamping the skins in their chambers, constituted the control value for the electrical potentials. The effects of various test substances, compared to the control values, were recorded with time, and the point of maximum effect was noted within a 70 min period. Short-circuit current determinations were only occasionally made, and then only for the purpose of confirming already established effects of azide and ouabain. Since the short-circuit current itself induces structural changes (cf. 15), we did not routinely employ the technique for fear it would obscure our electron microscope observations of the effects of various test conditions.

Bathing Solutions

Aerated Ringer's solution (105 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, and 10 mM $NaHCO_3$, pH 8.1, 223 mosmol) or some modification described below was used to bathe the clamped skins or bathe the smaller swatches of skin in vials. The following designations were given to the modified Ringer's solutions, all adjusted to pH 8.1 with small quantities of common acids or bases: azide (0.01 M NaN_3), ouabain (0.001 M ouabain), Ca-free (lacking any $CaCl_2$), Ca-rich (10 mM $CaCl_2$), choline (105 mM choline chloride replaced NaCl), cytochalasin B (5–10 μg/ml [Imperial Chemical Industries, Ltd., England] and 0.5–1.0 ml DMSO/100 ml), and dimethyl sulfoxide (DMSO) (0.5–1.0 ml DMSO/100 ml).

Tissue Processing

Skins were processed by two methods. In one method, skin pieces were fixed in the chamber devices with Ringer's solution containing 1% OsO_4. The fixative was added to both compartments and the fixation process was monitored electrically. The electrical potentials reached zero usually within 2–3 min and were then allowed to remain in the chambers for approximately 1 h.

In the other method, smaller swatches of the same skin (ca. 6 mm^2), which were contained in vials having the same solutions as the chambers, were then placed in fixatives at the moment when the electrical paramaters had reached some inhibitory or stimulatory value. Skin pieces which were fixed in the chamber and in the vials showed no noticeable morphological differences. The fixatives used were either 1% OsO_4 in unmodified Ringer's solution or 1% OsO_4 in Veronal acetate buffer or phosphate

buffer, pH 7.6. Tissues were placed in fixatives for 1.5 h, washed in their respective buffers or Ringer's solution, dehydrated with ethanol, and processed for electron microscope observations.

RESULTS

Bioelectric

The effects (not reported here) of choline, azide, and ouabain confirm what are well known to workers in membrane transport; mainly, that all these agents at these concentrations inhibit markedly and quickly the electrical potential differences and short-circuit currents.

In eight experiments in which cytochalasin B was applied to both sides of the skin, a slow decline in the potential difference was observed. Maximum inhibitions over a 70 min period averaged 47.3 ± 19%. Fig. 1 shows a typical time-course of this inhibition and shows also that DMSO solution at this low concentration had no effect. Morain et al. (10) reported that DMSO solutions at much higher concentrations mimicked urea in that its depressive effect was attributable only to an osmotic one. Reversibility of cytochalasin B inhibition was not studied completely. Unlike recovery rates from azide, recovery from this inhibition was slow, if at all.

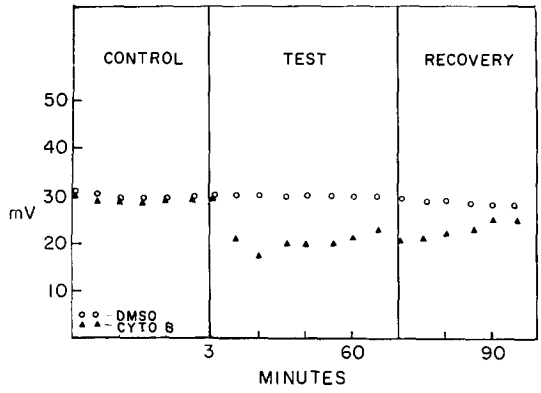


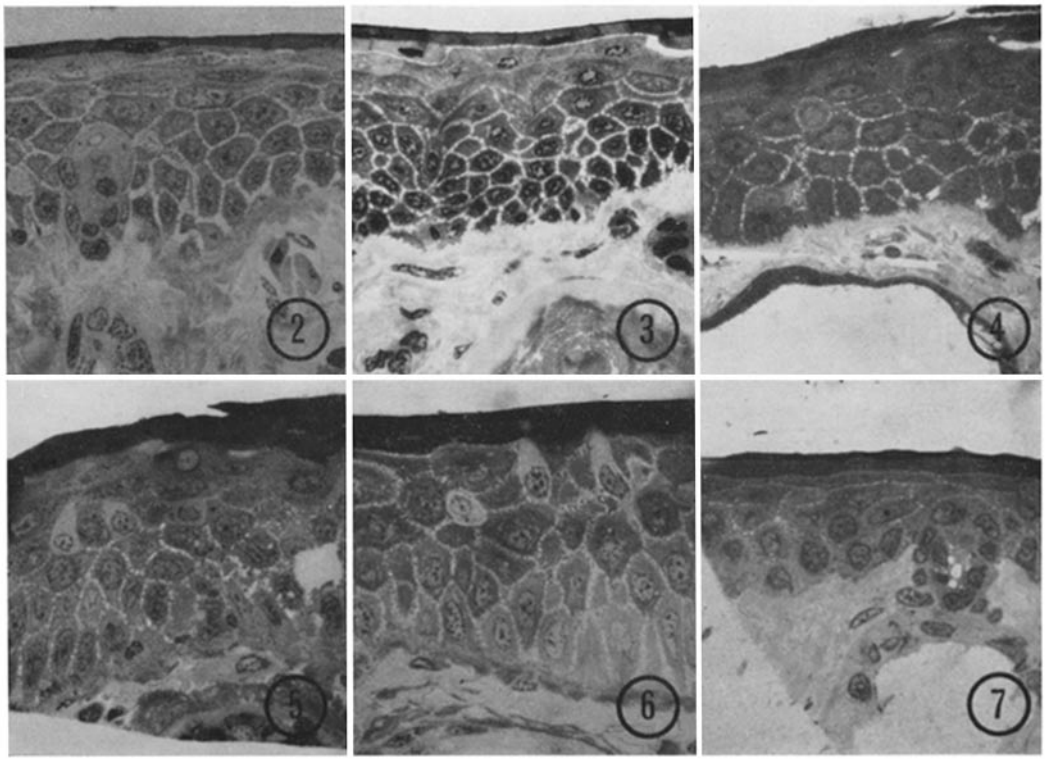
FIGURE 1 A typical time-course of the effects of cytochalasin B and DMSO on the electrical potential differences of symmetrical halves of a frog abdominal skin. In the control period, both sides of the skins were bathed in Ringer's solution. In the test period, Ringer's containing cytochalasin B dissolved in DMSO and DMSO alone were applied to both sides of each portion of the skins. The concentration of cytochalasin B was 10 μg/ml of Ringer's solution, and the DMSO was the same concentration as that in which cytochalasin B was dissolved (1.0 ml DMSO/100 ml Ringer's solution). In the recovery period, skins were again placed in Ringer's solution.

Light Microscope Observations

Conditions which are known to inhibit active sodium transport should, according to the Farquhar and Palade model (5), cause intercellular spaces to decrease. This model postulated the localization of the sodium pump sites along all cell membranes facing the intercellular spaces. Sodium is pumped into these spaces, causing the intercellular compartment to become hypertonic with respect to the cytoplasm and then subsequently causing water to be drawn into the spaces. Conversely, inhibition might drive water out of the intercellular spaces into the cells, causing cells to swell and the intercellular spaces to shrink.

Fig. 2 shows the appearance of the large intercellular spaces when the skin is bathed on both sides with Ringer's solution. Only the cornified outer layers show no spaces. When other pieces of the same skin are treated with azide, ouabain, or choline—all of which are known to depress the electrical potential difference, the short-circuit current, and sodium transport—the intercellular spaces decrease (Figs. 3–5). Our findings, based upon open-circuited conditions, are in complete agreement with those already reported (1, 15–17). We, like the above authors, feel reassured that, despite fears of fixation and osmotic artifacts, morphological correlates of ion transport are easily visualized in frog skin. The thinness of the skin apparently permits a rapid fixation and therefore provides us with good investigative material.

The variable effect of calcium on the electrical potential differences and sodium transport rate have been reported by other workers (2, 3, 20) and were also observed in the present study. This variability is probably a complex function of season, hormonal levels, and inherent calcium levels. In one case (Fig. 6) where Ca-rich solutions increased the electrical potentials more than fourfold over those in Ca-free solutions, the intercellular spaces enlarged. The piece of control skin in Ca-free solutions (not shown) showed closed spaces.



FIGURES 2–5 Light micrographs of frog skin epithelia taken from a single animal. Fig. 2 shows a control piece of the skin bathed with Ringer's solution for 1 h before fixation. Note relatively large intercellular spaces throughout all layers except the outer cornified ones. Figs. 3–5 show how conditions which are known to inhibit sodium transport have a tendency to close spaces in layers beneath the outer cornified layers. Pieces of skin were kept in Ringer's solution for 45 min, placed for 15 min in azide solution (Fig. 3), choline solution (Fig. 4), and ouabain (Fig. 5) before being fixed. × 460.

FIGURE 6 In another animal, Ca-rich solution caused an opening of the intercellular spaces. At the time of fixation, the skin potential was 46 mV compared to a potential of 10 mV exhibited by a control piece of skin, in a Ca-free solution, whose intercellular spaces were much diminished (not shown). × 460.

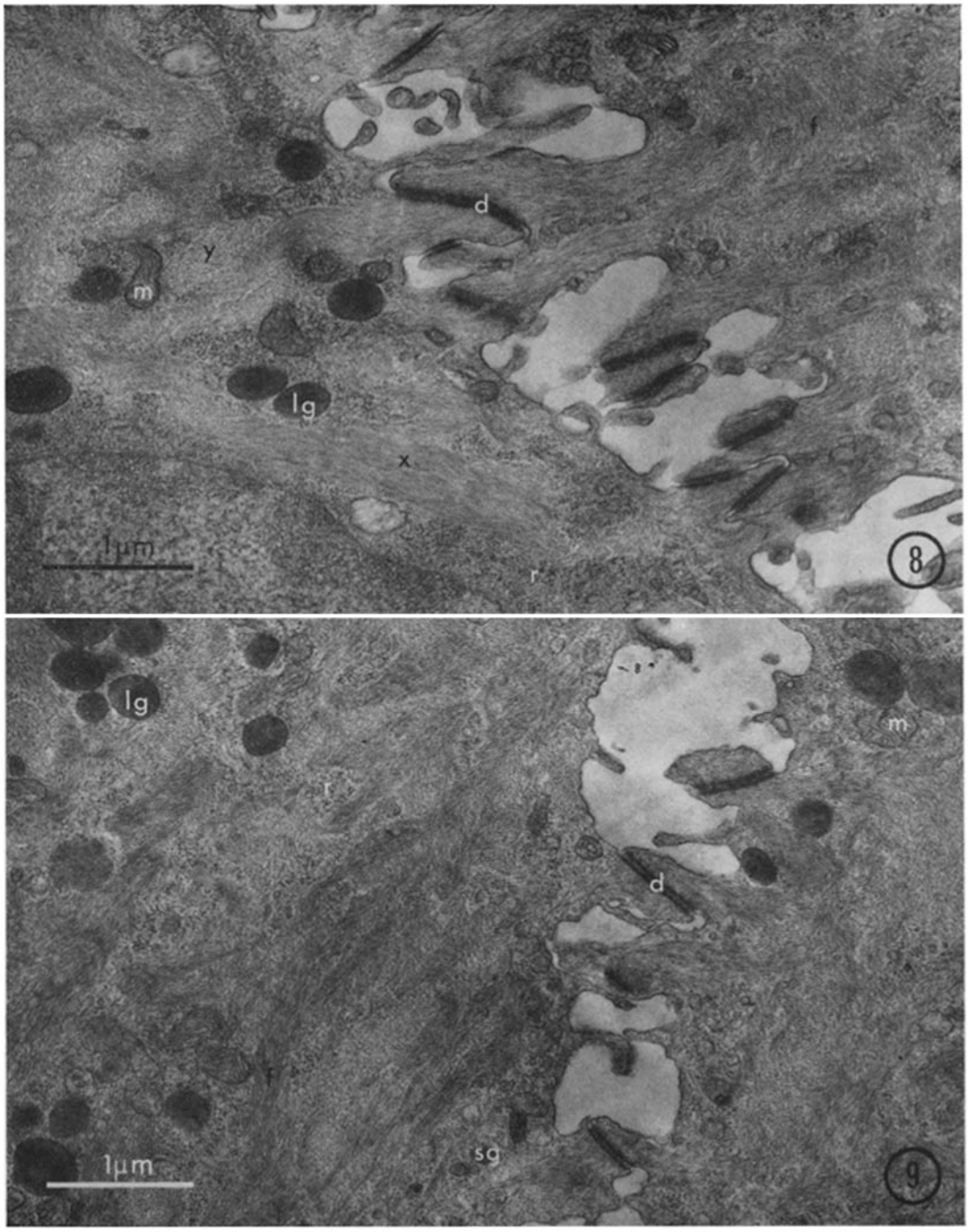
FIGURE 7 The effect of cytochalasin B on intercellular spaces. The drug is seen to close the spaces, and in this particular skin the electrical potential difference was inhibited 52%. × 460.

Cytochalasin B in unmodified Ringer's solution depressed the electrical potential difference as mentioned above and, on the basis of the above findings, it also would be expected to decrease the size of the intercellular spaces. Fig. 7 demonstrates this.

Electron Microscope Observations

The fine structure of frog skin has been amply described in several publications (1, 4, 5, 11, 14). Our attention here will focus mostly on microfilaments, cell membranes, and secretory granules in the cells of the *stratum granulosum*. We chose this area because filaments are more prominent than in basal layers and because histochemical tests show that Na-K ATPase activity is greater in this region (3, 5).

In specimens which were previously bathed in Ringer's solution, the microfilaments appear as elaborate weblike networks of bundles which are disposed in random directions throughout the cytoplasm (Figs. 8, 9). The microfilaments are loosely aggregated, and in cross section the individual filaments are faintly distinguishable from



FIGURES 8 and 9 The appearance of microfilaments in cells of the *s. granulosum*. Variations may be seen in filament (*f*) arrangement in cells taken from two different animals. More commonly, microfilaments appear as wide bundles (Fig. 8) whose degree of compaction make them stain lightly. In another arrangement in a different animal, microfilaments are narrower bundles, more densely compacted (Fig. 9). The bundles in longitudinal sections appear as sheets (*x*), as small punctiform bodies in cross section (*y*), or as short fine rods in oblique cuts. They give the general impression in this "normal" state as great sweeps of microfilaments, running at all angles in the cytoplasm, and anchoring onto the desmosomes (*d*). Many ribosomes (*r*) are present, as are some mitochondria (*m*) and two types of granules, large (*lg*) and small (*sg*). Skins were bathed in Ringer's solution for 1 h before fixation. Both Figs., × 22,000.

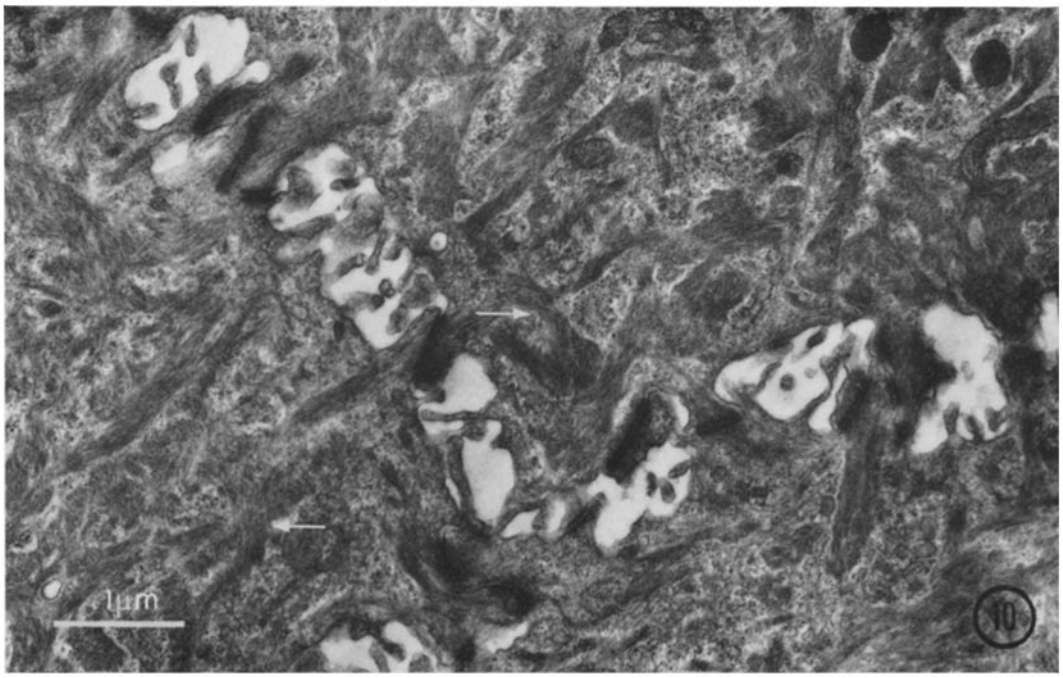


FIGURE 10 The effect of Ca-rich Ringer's solution on cells of the *s. granulosum*. Microfilament bundles become compacted and cause the cytoplasm to appear extremely dense. Microfilaments display curved configurations (arrows), perhaps as they bend around other bundles or organelles. Their complex arrangements give the cytoplasm a distinctive scalloped appearance. The specimen was exposed to Ca-rich solution for 60 min before fixation. These sections are taken from the same blocks as used for Fig. 6. × 17,000.

the many free ribosomes. They seem to encage organelles such as secretory granules and run parallel along the plasma and nuclear membranes. Their abundance and fine structure do not permit us to state at this time whether they are unattached at one or both ends.

The fineness of the filament arrays is sharply contrasted in Figs. 8 and 9 compared to other photographs shown. Nevertheless, as even these figures show, there are degrees in fineness of the filament bundles, and we believe these to be due to physiological states of the animals pertaining to time of fixation. We believe that the intercellular concentrations of calcium, which are dictated by hormonal agents, are strong determinants of filament structure. Fig. 10 shows a photograph of a thin section from the same block as that used for Fig. 6. High calcium levels apparently cause the bundles of filaments to appear tighter and the webbing more tortuous.

Microfilament structure was greatly changed by treatment with azide solution (Fig. 11). The microfilaments seem almost to disappear, and cause the cytoplasmic background to appear more transparent. The cell membranes in the *s. granulosum* and *s. corneum* become darkly outlined by a heavily staining material, and fewer granules are present.

Cytochalasin B in unmodified Ringer's solution has a similar effect on the filaments. When present, the microfilaments are sparse and do not show the normal arrangement into bundles. Here, also, the plasma membranes are dark staining and granules are sparse (Figs. 12, 13).

DISCUSSION

The results shown above provide new supportive evidence for the correlation between the size of the intercellular spaces and rates of ion transport. When the epithelia ion transport rates are inhibited, the sizes of the intercellular spaces diminish; conversely, when stimulated the spaces enlarge. If it is assumed that cell volume varies inversely with the size of the intercellular spaces, the question arises as to how cell volume is altered.

The effects of cytochalasin B on the electrical potential and intercellular space size perhaps offer a clue. Sensitivity to the drug has implied the presence of some type of contractile filament system. The electron micrographs demonstrate that microfilaments are indeed disrupted by the drug, and that in epithelia in which the filaments are well defined (as in the presence of high calcium levels) the electrical potentials and ion transport rates are high. The effects of azide in filament disruption might be interpreted as an effect of energy source depletion upon the integrity of the contractile elements.

Evidence for these conjectures is not provided by the present study and warrants experimental proof that microfilaments can actually contract and that the contraction, because of the filaments' origins and insertions, can lead to a change in cell volume. Also, it remains unclear as to how a cell volume change—due perhaps to a sequestering of water into the intercellular spaces when the filaments contract—affects directly the kinetics of membrane-bound transport enzymes in such a manner to alter transcellular ion transport rate.

Alternatively, microfilaments may affect ion transport in an entirely different manner, independent of any contractile system. Whaley et al. (18) have stressed the importance of the Golgi apparatus in influencing cell surface characteristics, including permeability. Secretory granules, located in the *s. granulosum* of the frog skin, which are presumably assembled by the Golgi apparatus, may be prevented from being released into the intercellular spaces by the dense packing of microfilaments surrounding them. When microfilament dispersion occurs, perhaps the granules are free to migrate to the cell surface and empty their contents. That (*a*) darkly staining membranes appear when frog skin is treated with cytochalasin B and (*b*) secretory granules are less prominent in skins treated with the drug lend some support to this hypothesis. Other workers (8, 9) have also suggested that micro-

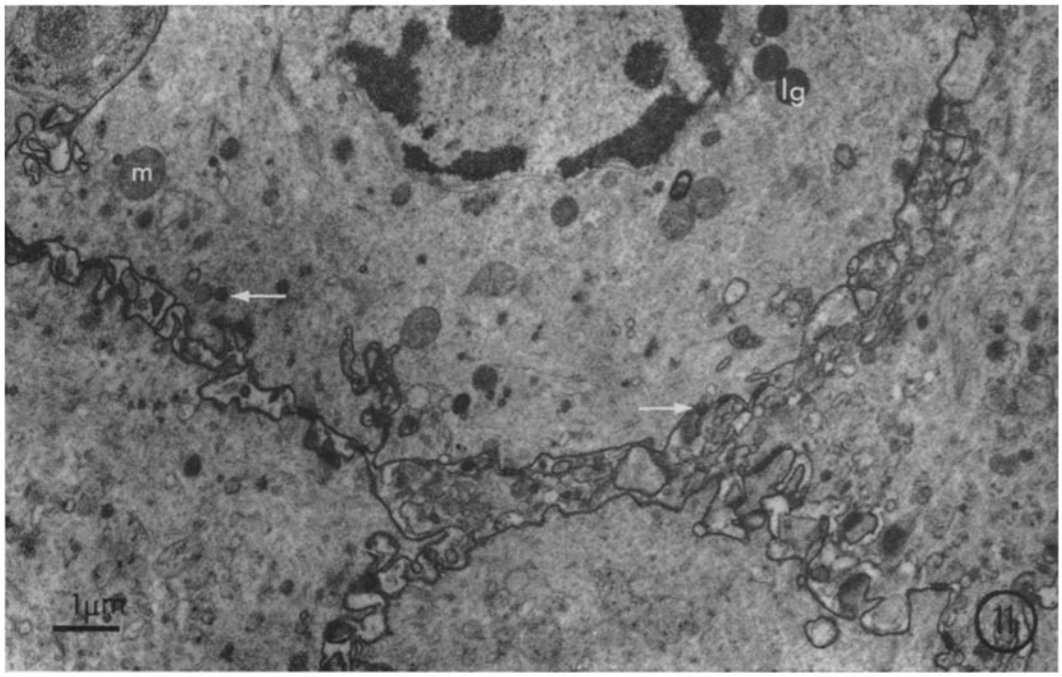
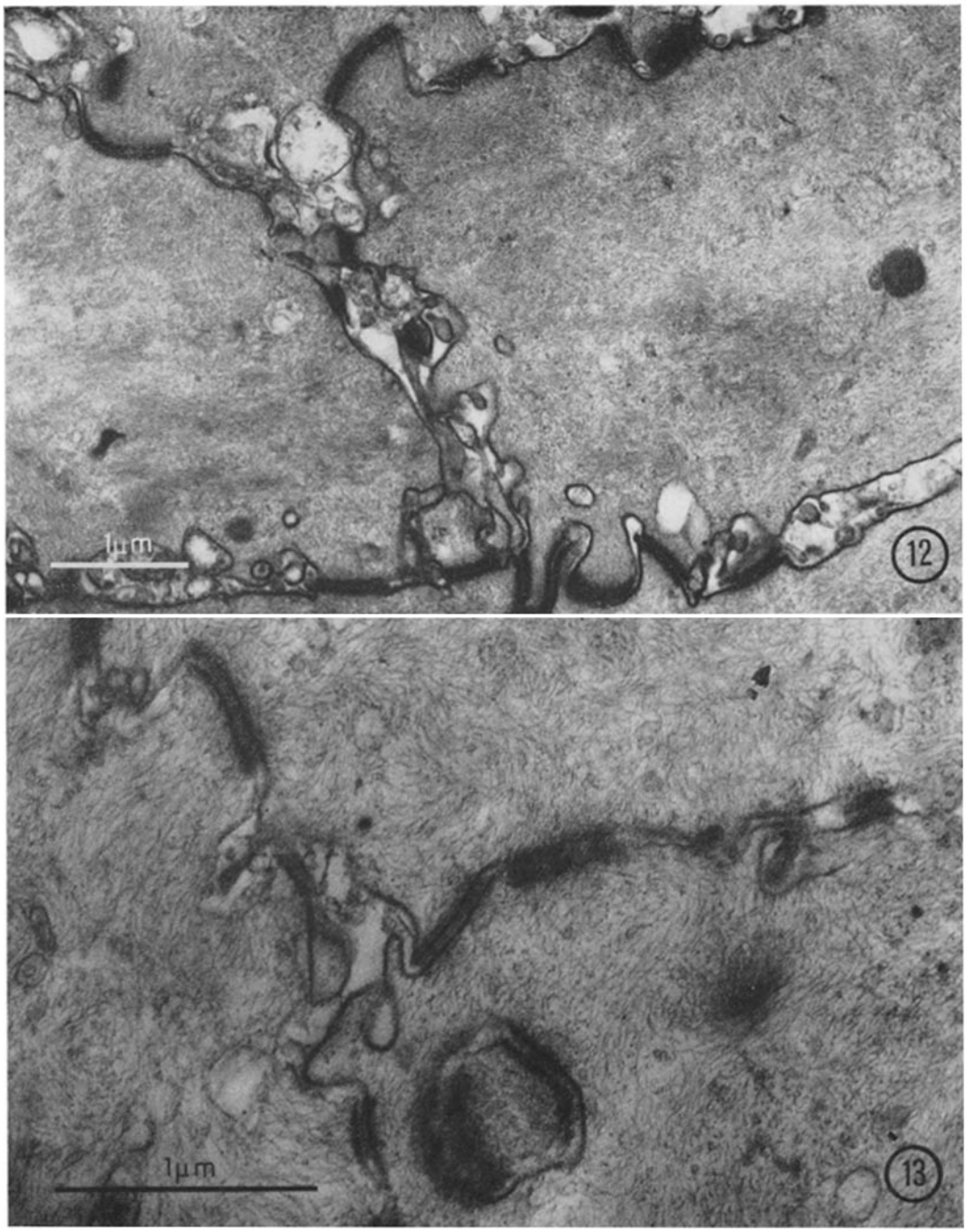


FIGURE 11 The effect of azide on the morphology of cells in the *s. granulosum*. Cytoplasmic density appears to be greatly reduced and microfilaments are virtually extinct. Large granules (*lg*) are fewer in number and several small granules (arrows) seem to become indistinguishable from sections grazing desmosomal junctions. Cell membranes are darkly outlined (by secretory products spilled by ruptured granules?). Mitochondria (*m*) cristae appear intact usually, but some loss of inner structure is apparent. × 9,000.



FIGURES 12 and 13 The effect of cytochalasin B on microfilaments in the *s. granulosum*. Microfilaments are less prominent in skins treated with cytochalasin B for 30 min. Duplicate swatches of these skins showed parallel declines in electrical potential differences. Cytoplasmic density is decreased. In Fig. 12, granules are sparse, membranes are darkly stained, and microfilament anchors onto desmosomes seem weaker. In general, the cytoplasm appears washed out, giving them an appearance similar to lower cell layers of the *s. corneum*. At higher magnification, Fig. 13, the disarray of filaments is even more apparent. Fig. 12, × 20,000; Fig. 13, × 38,000.

filaments may play a role in other types of granule dispersion in skin tissues.

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