Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 2723–2727, April 1989
Cell Biology

Direct visualization of the interrelationship between intramembrane and extracellular structures

(intramembrane particles/epithelia/glycocalyx/replica)

JAMES B. WADE* AND RICHARD A. COLEMAN

Department of Physiology, University of Maryland School of Medicine, Baltimore, MD 21201

Communicated by Thomas S. Reese, January 25, 1989

ABSTRACT **The apical surface of the toad urinary bladder is covered by an interconnected mesh of glycocalyx, which appears to attach to the plasma membrane bilayer. To evaluate the interrelationship between these extracellular elements and intramembrane structures, a strategy was devised to produce composite replicas that allow the simultaneous visualization of intramembrane particles by freeze–fracture while the glycocalyx mesh is replicated by rotary shadowing of the extracellular surface after freeze-drying. Evaluation of these composite replicas by electron microscopy reveals that contacts occur between extracellular filamentous elements and intramembrane particles. This structural organization may be important for stabilizing intramembrane components and for anchoring extracellular elements to the membrane.**

Freeze–fracture electron microscopy has been widely used to analyze membrane organization. This method splits the membrane bilayer (1), revealing both smooth areas and distinct intramembrane particles that represent integral membrane proteins (2, 3). A variety of evidence has demonstrated that integral membrane proteins can interact with the cytoskeleton on the cytoplasmic surface of the membrane (4–6). Important interactions can also occur between membrane proteins and elements on the extracellular cell surface (7–9). However, available methods for visualizing membrane structure have not permitted a direct evaluation of such interactions. Although the true surface of membranes is exposed by vacuum sublimation ("etching") of ice from quick-frozen specimens (10–12), simultaneous visualization of the corresponding intramembrane features by freeze–fracture has not been possible. We describe here a strategy for using electron microscopy to directly visualize possible relationships between extracellular elements on the membrane surface and intramembrane structures.

Deep-etch studies have shown that the apical surface of epithelial cells is covered by an anastomosing mesh of glycocalyx (13, 14). Our studies show that these surface elements attach directly to intramembrane particles. Such connections provide a possible structural explanation for the low mobility of epithelial cell membrane proteins (15) and may play a role in transmembrane responses of cells to extracellular events.

MATERIALS AND METHODS

Toad (*Bufo marinus*) urinary bladders were isolated as described (16). For mucosal surface replicas (as in Figs. 1 and 2), bladder samples were washed five times with Ringer solution to remove any loosely adherent mucus and fixed in 2.5% glutaraldehyde in cacodylate buffer for 20 min. To avoid potential artifacts reported to occur when salt-containing buffers are freeze-dried (17), we washed samples in three changes of distilled water, 5 min each, prior to quick-freezing. Because the luminal side of the bladder (identified by its concave shape) was directly accessible to the freezing agent, samples held by self-closing tweezers could be directly plunge-frozen in liquid cryogen. We used 75% propane/25% isopentane cooled by liquid nitrogen as described by others (18) and a "Gentleman Jim" device (19) modified for quench freezing, but we have obtained identical results with samples slam-frozen against a copper block cooled with liquid nitrogen. Specimens were freeze-dried at −95°C for 45 min in a Balzers 301 freeze–etch unit and rotary-shadowed with a platinum/carbon electron gun at an angle of 35°.

Composite replicas (procedure shown diagrammatically in Fig. 3) were from glycerinated specimens frozen in liquid Freon 22 that were freeze–fractured using a double-replica device and directionally shadowed with platinum at an angle of 45°. The resulting extracellular fracture-face (E-face) replicas were not cleaned but were briefly floated on distilled water to remove glycerol and picked up from below with a tabbed 200-mesh nickel electron microscope grid. A second tabbed grid was quickly applied to the other surface and the two grids were locked together by folding the tabs with the sample in between (see Fig. 3). The sample was quick-frozen in liquid cryogen as above and reintroduced into the Balzers unit with the true bladder surface up and loosely covered with a brass plate, to protect it from frost. With the specimen under high vacuum ($<10^{-6}$ torr; 1 torr = 133 Pa) and its temperature at −95°C, it was uncovered and left in the vacuum for 45 min to freeze-dry prior to rotary shadowing as above. Samples were then brought to room temperature and examined by transmission electron microscopy without further manipulation or cleaning. All images shown have been photographically reversed to follow the current convention for rotary-shadowed replicas of freeze-dried specimens. This means that the shadows cast by directional platinum shadowing of intramembrane particles are black in the composite replica.

RESULTS

Examination of surface replicas by stereo electron microscopy (Fig. 1) shows that the surface of the urinary bladder is covered by an anastomosing mesh of glycocalyx, which covers the ridge-like microvilli of the apical membrane (arrows, Fig. 1). High-magnification views of the surface show that elements of the mesh extend down and appear to contact the membrane surface (arrowheads, Fig. 2).

To evaluate whether these contact sites relate to the large intramembrane particles that occur in this membrane (20), we developed a "composite" replica method in which shadowing is applied to both the intramembrane surface produced by

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

*To whom reprint requests should be addressed at: Department of Physiology, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, MD 21201.

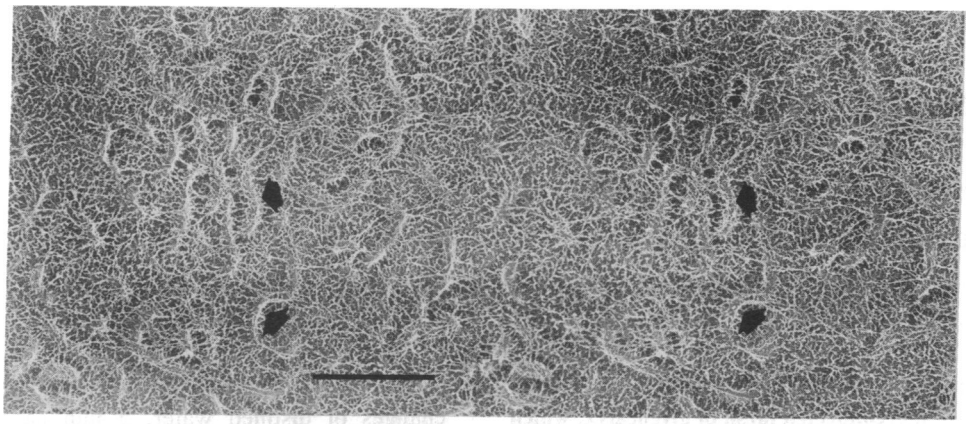

FIG. 1. Platinum/carbon replica of the apical surface of a toad urinary bladder granular cell. Arrows indicate the ridge-like microvilli characteristic of the bladder apical membrane. (Stereo pair; bar = 2 μm.)

freeze–fracture and the extracellular surface to produce a single composite replica (Fig. 3). This is possible because freeze–fracture of the bladder splits the apical membrane such that the P-face half-membrane and the cellular mass of the bladder are fractured away from the extracellular or E-face half-membrane and its associated surface mesh. The E-face half-membrane and glycocalyx are electron-lucent and have been found to remain attached to primary freeze–fracture replicas (21, 22).

When specimens prepared by this series of replications are examined at low magnification, regions where the fracture plane did not follow the apical membrane are dense due to cellular material trapped between the two replicas. However, where the apical membrane was fractured, the rotary-shadowed mesh on the apical surface can be visualized superimposed over the directionally shadowed replica of intramembrane structure (Fig. 4). Three lines of evidence show that the particles (circles, Fig. 4) observed in these composite replicas represent intramembrane components. (*i*) These structures have distinct shadows that could only arise from the directional platinum shadowing applied immediately after fracture. Surface elements received only rotary shadowing, which does not produce such distinct shadows. (*ii*) Examination in stereo (±6° tilt) shows that these particles are on a different level than the surface mesh, as would be expected for intramembrane structures. (*iii*) Such particles were not observed in preparations (Fig. 5) where carbon replication was substituted for primary platinum shadowing after fracture.

As would be expected, the superimposed surface mesh sometimes obscures the underlying intramembrane particles at sites of interest. However, views were often observed (arrowheads, Fig. 4) where thin branches of the mesh appear to contact the intramembrane particles. While sites of direct contact between mesh elements and the membrane surface were nearly always at intramembrane particles, many intramembrane particles do not contact mesh elements. To evaluate the possibility that the observed interaction between mesh elements and intramembrane particles might simply occur by chance, the incidence of such interactions was estimated in random stereo pairs of composite replicas. Out of 2080 particles evaluated, 61 clear contacts of mesh elements to particles were observed, and in 38 instances elements appeared to end without associating with a particle. Because of the relatively small area occupied by particles (19% of the total area, assuming a 20-nm-diameter "area of potential contact" per particle) the probability that this or a greater frequency of interaction would occur by chance is very low ($P < 0.01$).

These observations suggest that some of the intramembrane particles fracturing with the E face have a role in anchoring the surface mesh, but leave unresolved whether or not P-face elements, fractured away during composite replica preparation, also have a similar function. Although the

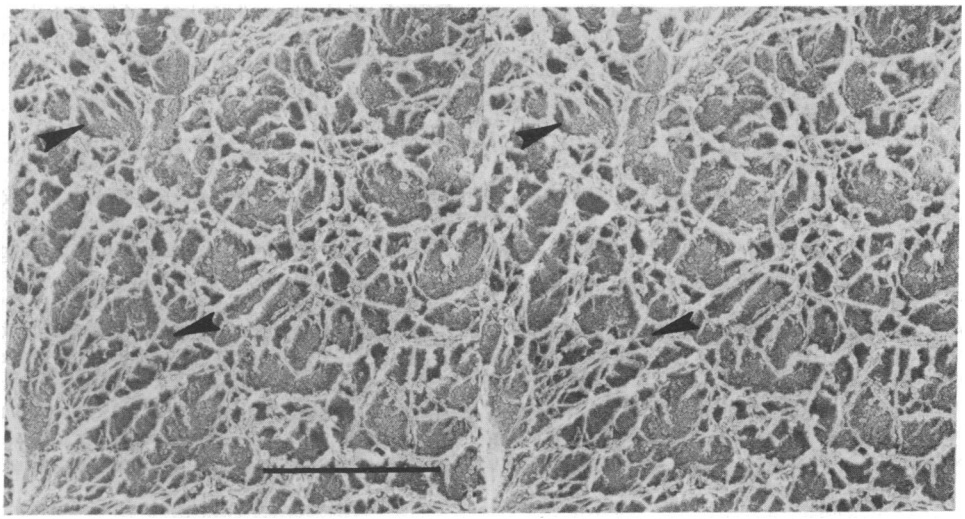

FIG. 2. Platinum/carbon replica of the apical surface of the bladder at high magnification. Arrowheads point to sites where strands of the glycocalyx mesh connect to the membrane surface. (Stereo pair; bar = 0.5 μm.)

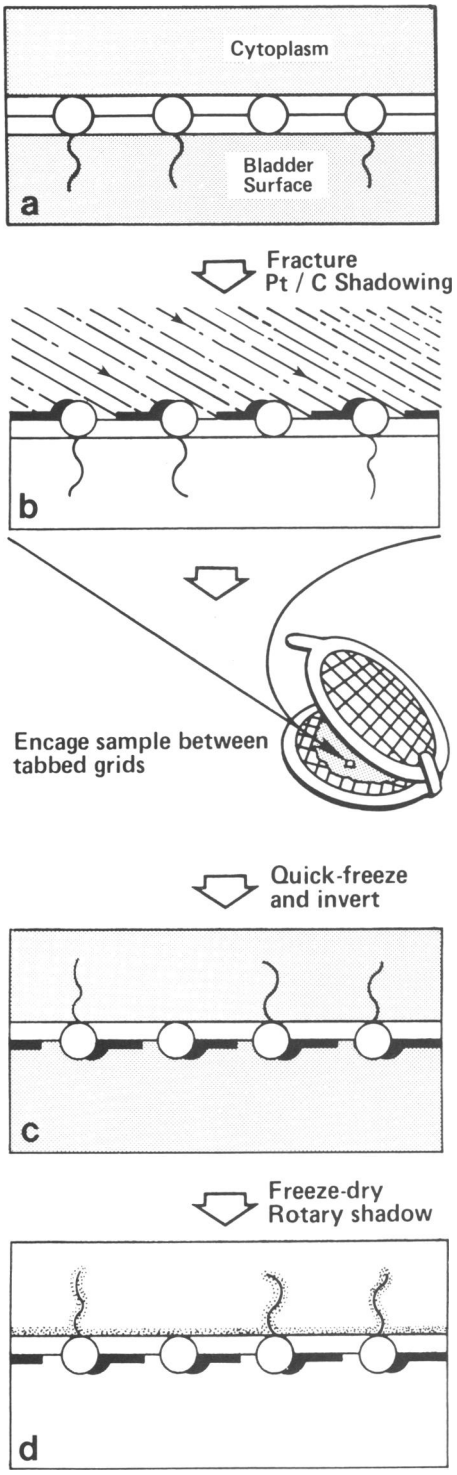

FIG. 3. Flow diagram of composite replica preparation. The intact apical membrane of the urinary bladder cell is depicted in *a*. The membrane is freeze–fractured and the intramembrane structure is replicated by established techniques (*b*). E-face replicas are not cleaned but are encaged between two tabbed electron microscope grids as shown. The rinsed sample is then quick-frozen, inverted, and returned to the Balzers unit (*c*). Samples are then freeze-dried and the exposed surface of the membrane is replicated by rotary shadowing (*d*). The resulting composite replicas are examined by electron microscopy without further manipulation.

density of the mesh varied between different bladder preparations, the mesh on the surface of unfractured preparations as shown in Figs. 1 and 2 was consistently more concentrated than that associated with the fractured half-membranes in composite replicas (Fig. 4). Additionally, when regions of unfractured membrane were encountered in composite replica preparations, there was more mesh on the surface of such areas (top portion of Fig. 5, above arrow) than associated with adjacent fractured areas (bottom portion of Fig. 5). It is possible to unambiguously identify such regions as shown in Fig. 5 because they lie at the boundary between the thin, electron-lucent areas of fractured apical membrane and the relatively dense areas in the preparation, which are unfractured cells. When complementary replicas from such preparations are cleaned and examined, such transition regions (arrow, Fig. 5) are consistently observed to correspond to sites where the fracture passes from the apical membrane into the cytoplasm. For these reasons we can be confident that the apical membrane shown in the top portion of Fig. 5 is unfractured. The finding that material appears to be selectively released from the surface of fractured membranes indicates that elements fracturing with the cytoplasmic, P-face half of the membrane may anchor a portion of the surface mesh.

DISCUSSION

It has been apparent for some time that the intramembrane particles revealed by freeze–fracture are integral membrane proteins and that these structures relate to elements on the membrane surfaces. The advent of quick-freeze methods has made it possible to visualize the true surfaces of membranes because samples can be frozen in dilute media and the ice sublimed by high vacuum to allow replication of membrane surfaces (11–14). Our results demonstrate that it is possible to carry out both freeze–fracture replication and surface replication on a single sample. While some caution is warranted with any new technique, it should be noted that the technique employs well-established electron microscopic strategies for visualizing structure by combining freeze–fracture of membranes with quick-freezing/deep-etch technology to reveal surface elements. Since the replicas are never "cleaned" by the traditional sodium hypochlorite digestion, the extracellular half-membrane and extracellular elements remain in the preparation sandwiched between the two replicas. Nevertheless, the preparations have sufficiently low density to be easily examined with high resolution by transmission electron microscopy. Composite replicas are complex structures that require stereo electron microscopy for analysis, but the method provides direct information on membrane structural relationships currently not otherwise obtainable. With this method we have shown that it is possible to directly visualize relationships between intramembrane particles and extracellular elements at the ultrastructural level.

The apical membrane of the toad urinary bladder is a favorable model for testing our method because the extracellular surface of these cells is directly accessible for quick-freezing and the E fracture face has a high density of large intramembrane particles (20). It should be possible, however, to adapt the method for investigation of smaller E-face particles and to allow evaluation of P-face particle interactions with elements on the cytoplasmic surface of membranes.

Our composite replicas show that elements on the membrane surface are in a position to be connected to intramembrane particles of the E face, but many particles are also observed that do not contact surface elements. Although some bladder intramembrane particles on the P face are believed to function as channels in the antidiuretic hormone (vasopressin) permeability response (16), the function(s) of the E-face particles is unknown. It is possible that many of these particles are unoccupied attachment sites or they may serve other physiological roles. While our observations indicate that E-face particles could anchor some of the

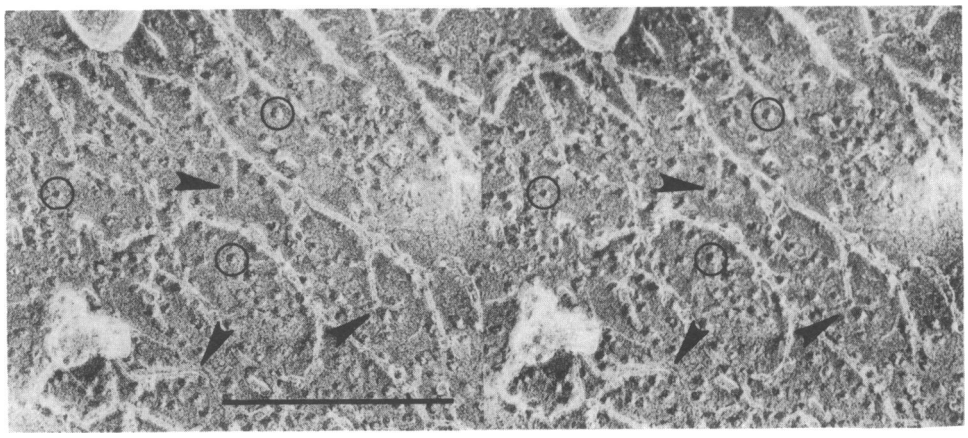

FIG. 4. Composite replica of granular cell apical membrane. The specimen consisted of two replicas with the half-membrane sandwiched between. Intramembrane features (e.g., intramembrane particles, examples of which are encircled) have distinct shadows, whereas surface features, being coated with platinum on all sides, have no shadows. Examples of contacts of surface mesh elements with the intramembrane particles are indicated by arrowheads. (Stereo pair; bar = 0.3 μm.)

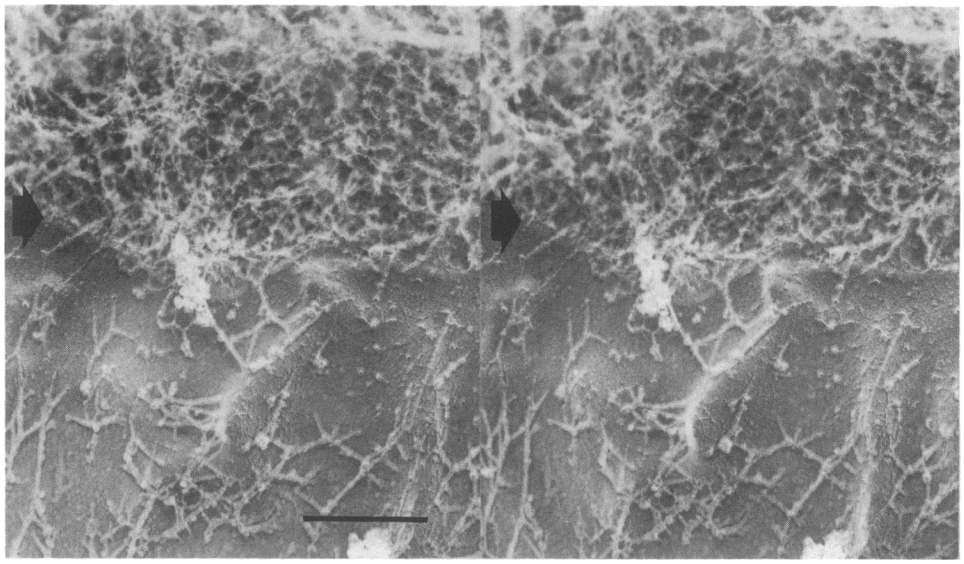

FIG. 5. Replica of the apical surface of a sample prepared by the composite replica method but with carbon replication substituted for platinum shadowing after the initial fracture step. In such preparations the platinum shadows of intramembrane components (see Fig. 3) are not seen. In sites where the apical membrane was not fractured (above the arrow), there is considerably more glycocalyx mesh on the surface than seen where fracture occurred (bottom). (Stereo pair; bar = 0.5 μm.)

surface glycocalyx to the membrane, consistently less mesh was observed associated with fractured membrane than with intact membrane. This raises the possibility that fracture might lead to a loss of elements from the surface. Recent evidence indicates that freeze–fracture does not break covalent bonds (23), but noncovalent bonds between the extracellular mesh and membrane elements fracturing with the P face might be disrupted during freeze–fracture. The specific procedures carried out to prepare composite replicas cannot be responsible for loss of material, because normal mesh is found on adjacent unfractured membrane subjected to the same treatments. The difference in surface structure between intact and fractured membrane suggests that P-face components may also play a role in attaching glycocalyx elements to the membrane.

The specific finding that intramembrane components contact elements of anastomosing mesh on the extracellular surface of the cells suggests that integral membrane proteins may be stabilized and possibly regulated by such interactions. Reports for a wide range of different cell types indicate that interactions with extracellular elements can immobilize membrane proteins (7–9) and play an essential role in differentiation (24–27). The crosslinked mesh on the mucosal surface of the bladder may be functionally important in a number of ways. First, the mesh appears to shield cells from attack by microorganisms (28). Second, this structure, in combination with the cytoskeleton on the intracellular side of the membrane, means that the lipid bilayer is enmeshed on both surfaces by support elements. This strengthened construction may be especially valuable for epithelial cells, which are subject to osmotic and physical stress. Third, the open structure of the mesh allows the ready access by water and solutes to the lipid bilayer that is essential for the regulated transport carried out by epithelial cells (29, 30).

We thank R. J. Bloch, D. W. Pumplin, and M. L. Wier for critically reading the manuscript. Research was supported by National Institutes of Health Grant DK32839 and National Science Foundation Grants PCM-8402816 and DMB-8500564.

1. Branton, D. (1966) *Proc. Natl. Acad. Sci. USA* **55**, 1048–1056.
2. Hong, K. & Hubbell, W. L. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2617–2621.
3. Vail, W. J., Papahadjopoulos, D. & Moscarello, M. A. (1974)

Biochim. Biophys. Acta **345**, 463–467.
4. Elgsaeter, A. & Branton, D. (1974) *J. Cell Biol.* **63**, 1018–1030.
5. Golan, D. E. & Veatch, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2537–2541.
6. Koppel, D. E., Sheetz, M. P. & Schindler, M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3576–3580.
7. Rizki, T. M. & Rizki, R. M. (1983) *Nature (London)* **303**, 340–342.
8. Wallace, B. G. (1986) *J. Cell Biol.* **102**, 783–794.
9. Wier, M. L. & Edidin, M. (1986) *J. Cell Biol.* **103**, 215–222.
10. Pinto da Silva, P. & Branton, D. (1970) *J. Cell Biol.* **45**, 598–605.
11. Dubois-Dalcq, M. & Reese, T. S. (1975) *J. Cell Biol.* **67**, 551–565.
12. Heuser, J. E. & Salpeter, S. R. (1979) *J. Cell Biol.* **82**, 150–173.
13. Hirokawa, N. & Heuser, J. E. (1981) *J. Cell Biol.* **91**, 399–409.
14. Hartwig, J. H., Ausiello, D. A. & Brown, D. (1987) *Am. J. Physiol.* **253**, C707–C720.
15. Dragsten, P. R., Blumenthal, R. & Handler, J. S. (1981) *Nature (London)* **294**, 718–722.
16. Kachadorian, W. A., Wade, J. B. & DiScala, V. A. (1975) *Science* **190**, 67–69.
17. Miller, K. R., Prescott, C. S., Jacobs, T. L. & Lassignal, N. L. (1983) *J. Ultrastruct. Res.* **82**, 123–133.
18. Jehl, B., Bauer, R., Dorge, A. & Rick, R. (1981) *J. Microsc. (Oxford)* **123**, 307–309.
19. Phillips, T. E. & Boyne, A. F. (1984) *J. Electron Microsc. Techn.* **1**, 9–29.
20. Wade, J. B., DiScala, V. A. & Karnovsky, M. J. (1975) *J. Membr. Biol.* **22**, 385–402.
21. Pinto da Silva, P. & Kan, F. W. K. (1984) *J. Cell Biol.* **99**, 1156–1161.
22. Andersson Forsman, C. & Pinto da Silva, P. (1988) *J. Cell Sci.* **90**, 531–541.
23. Fisher, K. A. & Yanagimoto, K. C. (1986) *J. Cell Biol.* **102**, 551–559.
24. Almers, W. & Stirling, C. (1984) *J. Membr. Biol.* **77**, 169–186.
25. McCloskey, M. & Poo, M. M. (1984) *Int. Rev. Cytol.* **87**, 19–81.
26. Patel, V. P. & Lodish, H. F. (1987) *J. Cell Biol.* **105**, 3105–3118.
27. Hynes, R. O. (1987) *Cell* **48**, 549–554.
28. Parsons, C. L., Stauffer, C. & Schmidt, J. D. (1980) *Science* **208**, 605–607.
29. Lewis, S. A. (1977) *Am. J. Physiol.* **232**, F187–F195.
30. Gluck, S. & Al-Awqati, Q. (1980) *Nature (London)* **284**, 631–632.