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MICROVILLUS FORM OF FOCAL ANCHORAGE IN HUMAN CHANG LIVER CELLS ROUNDED BY ANTIPORTER ACTIVATION: SCANNING ELECTRON MICROSCOPY PROFILES AND EVIDENCE OF TRACTION ORIGIN

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Abstract

Na⁺/H⁺ antiporter activation in human Chang liver cells produces a flat-to-round (FTR) change in cell shape with gross reduction in cell profile area. Scanning electron microscopy (SEM) vividly displays a third phenomenon, viz., the development of focal microvillus anchors. Reduction in cell profile area concomitant with the development of this microvillus form of focal anchorage is quantitated by on-line image analysis during SEM examination. The reduction in profile area is corroborated by spectrophotometric digitization in light microscopy. Transmission electron microscopy (TEM) of rounded cells shows large endocytic channels and endosomes consistent with the observation of internalization of fluoresceinated-dextrans (FDx) of a diverse range of sizes, from 4,400 to 2,000,000 molecular weight, with cell rounding. Concomitant endocytosis of this magnitude indicates massive plasma membrane internalizations which could explain the very considerable profile area reduction and suggest that the microvillus anchors are probably traction processes. Antiporter mediated rounding (AMR) provides a highly reproducible and simple model for the production of anchoring microvilli (‘filopodia’) whereby they can be further explored.

Key Words: Sodium/proton antiporter, on-line SEM image analysis, microspectrophotometric digitization, quantitative fluoresceinated dextran loading, cell anchorage, endocytosis.

Introduction

The flat anchored epithelial cell in monolayer culture is attached to the substrate via focal adhesion areas called focal contacts or plaques which are found in the plane immediately beneath the cell, as shown by anti-actin and anti-vinculin antibody labelling in fluorescence microscopy (Willingham and Pastan, 1985) and by direct transmission electron microscopy (Sit et al., 1991c) where integrin-fibronectin interactions, proteoglycans and a diverse array of ‘sticky’ molecules mediate the binding between cell and substrate (Ruoslahti, 1988 and 1989). The morphology of these ‘soleplate’ (Revel et al., 1974) focal contacts have been studied in cell and tissue culture models where scanning electron microscopy of retracted cells, either spontaneously occurring as in mitosis (Wetzel et al., 1978) or induced by cold treatment (Overton et al., 1981), demonstrated increase in soleplate microvillus processes which are sometimes labelled as ‘filopodia’ (thin feet). However, a simple working model capable of generating soleplate microvillus processes reproducibly in stages in all cells in a culture, has not been developed, thereby hindering further exploration of their exact nature and role in cell anchorage, detachment and migration which are considered to be of primary importance in embryonic development and cancer metastasis (Juliano, 1987). We report here such a model in Na⁺/H⁺ antiporter mediated rounding (AMR) (Sit et al., 1990, 1991b) which is concomitant with a large channel endocytotic process (Sit and Wong, 1989; Sit et al., 1990).

The flat-to-round (FTR) response of AMR, shown here in human Chang liver cells, was produced by a previously described proton dependent antiporter exchange (PDAE) process and is amiloride-sensitive but not sensitive to 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS, 0.2 mM) (Sit and Wong, 1989; Sit et al., 1990, 1991a, 1992a). These are characteristics of the Na⁺/H⁺ antiporter in contradistinction with those of anion antiporters, viz., the HCO₃⁻/Cl⁻ exchanger and the Na⁺-coupled HCO₃⁻/Cl⁻ exchanger which are sensitive to stilbene derivatives, SITS and DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonate) but not sensitive to amiloride (Madshus, 1988; Grinstein et al., 1989). The
Na\(^+\)/H\(^+\) antiporter is ubiquitous and unique in being associated with multiple cellular functions including cell activation (Busa, 1986; Grinstein et al., 1989).

Materials and Methods

Cell culture

Human Chang liver cells (American Type Culture Collection, ATCC CCL 13) were grown in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) supplemented with 10% foetal bovine serum (Cytosystems) as previously described (Sit et al., 1990).

Flat-to-round (FTR) change

(i) For scanning electron microscopy (SEM), parallel cultures were grown on cover glasses as previously described (Sit et al., 1990), washed in 2x10 ml FTR solution (140 mM NaCl, 40 mM NaHCO\(_3\), 5 mM C\(_6\)H\(_8\)O\(_7\).H\(_2\)O [citric acid monohydrate], adjusted to pH 7.4 with NaOH), and processed after (a) 0 minutes (unattached cultures were grown on cover glasses as previously described (Sit et al., 1990)).

(ii) For transmission electron microscopy (TEM), parallel cultures were grown on cover glasses as previously described (Sit et al., 1990), washed in 2x10 ml FTR solution (140 mM NaCl, 40 mM NaHCO\(_3\), 5 mM C\(_6\)H\(_8\)O\(_7\).H\(_2\)O [citric acid monohydrate], adjusted to pH 7.4 with NaOH), and processed after (a) 0 minutes (unattached cultures were grown on cover glasses as previously described (Sit et al., 1990)).

(ii) For transmission electron microscopy (TEM), parallel cultures were grown on cover glasses as previously described (Sit et al., 1990), washed in 2x10 ml FTR solution (140 mM NaCl, 40 mM NaHCO\(_3\), 5 mM C\(_6\)H\(_8\)O\(_7\).H\(_2\)O [citric acid monohydrate], adjusted to pH 7.4 with NaOH), and processed after (a) 0 minutes (unattached cultures were grown on cover glasses as previously described (Sit et al., 1990)).

(iii) For real time image analysis of areas of cells in SEM, the specimens were examined at 20 kV in a Stereoscan 200 SEM on-line with the Quantimet 520-Plus image analysis system (Leica Cambridge). Cells were detected via 256 grey level discrimination and cursor control in a 262,144 pixel colour screen interface. The images used for quantimet analysis were at 700x magnification.

(iv) For transmission light microscopy spectrophotometric digitizations, the cells were processed and quantitated in the Reichert-Jung (Leica Cambridge) Univar microspectrophotofluorimeter as previously described (Sit and Wong, 1989; Sit et al., 1991d).

Fluoresceinated dextran loading: spectrophotometric quantitation

Fluorescein isothiocyanate (FITC) conjugated dextran (FDX) (Sigma) of respective molecular weights, viz., 4,400, 9,400, 40,000, 71,000, 150,000, 500,000, and 2,000,000 were each used at 1 mg/ml in the following conditions: (a) FTR solution, (b) FTR solution with added 135 mM Na\(_2\)SO\(_4\) (FTR+SO\(_4\)), and (c) 140 mM NaCl (saline). Triplicate 25 cm\(^2\) monolayer cultures of Chang liver cells were rinsed with 10 ml of the respective buffer and incubated with 2 ml of FDX solution of a particular molecular weight species for 15 minutes at 37 °C in a non-CO\(_2\) incubator. At the end of incubation, the cells were rounded up in conditions (a) and (b) and were brought into suspension by vortexing. Cells incubated in the control condition (condition c) which will not produce cell rounding, had to be harvested by trypsinization using 0.25% Gibco trypsin. At the end of trypsinization, a drop of foetal bovine serum was added to stop further proteolysis. Each cell suspension was pelleted by centrifugation (2 minutes x 350 g) in 10 ml tubes topped up with buffer. The pelleted cells were resuspended with 10 ml of fresh buffer and centrifuged again. This cell washing was repeated twice to remove the extracellular FDX and when quantitated by spectrophotofluorimetry. Washed cell pellet was transferred into 2.5 ml microcuvettes with Na\(^+\)-HEPES buffer and the fluorescence in RFUs (relative fluorescence units) measured in the Perkin Elmer LS-5 Spectrometer at wavelength 490 nm excitation / 520 nm emission and the respective slit widths of 2.5 nm / 5 nm, with photomultiplier sensitivity set to autoconcentration range. The RFUs of the cells were correlated with those of known FDX dilutions in calibration plots to obtain the concentration in the cells. Concentrations are expressed as pg mg protein as determined by standard Lowry’s method. The method is as previously described (Sit et al., 1991a).

Results and Discussion

Na\(^+\)/H\(^+\) antiporter exchanges across the plasma membrane maintain very tight control of the intracellular pH (pH\(_i\)) in eukaryotic cells. However the antiporter can be downregulated (a) with cell activation which may be spontaneous as in mitosis or induced via activation of mitogenic signaling pathways, and (b) by ionic modulations, viz., cytosolic acidification, Na\(^+\) loading and hypertonic shrinkage (Cassel et al., 1986; Madshus, 1988; Grinstein et al., 1989), and also includes the process of proton dependent antiporter exchange or PDAE (Sit et al., 1992a). PDAE was used in this study to induce the FTR response, and involved incubating (37 °C) monolayer human Chang liver cells in a bicarbonate-saline FTR buffer without CO\(_2\) input. Warming the HCO\(_3\)-solution in an atmosphere that was not rich in CO\(_2\), caused loss of CO\(_2\) from the solution which then turned alkaline. With increasing alkalization of the solution (pH\(_i\) upshifting), the transmembrane [H\(^+\)/[H\(^+\)]\(_o\) gradient became increasingly steeper. This process had proven to be a very powerful means of cytosolic alkalini-
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Figure 1. Flat-to-round change in cell morphology: in monolayer culture of human Chang liver cells. (A) Without FTR treatment, zero incubation time. Cells are flattened and anchored. Microvillus anchors are not prominent features. (B) After 9 minutes incubation in FTR solution. Cell margins have retracted significantly and connecting the receding cell margins to the substrate are much narrowed cellular processes. (C) After 30 minutes incubation in FTR solution. Cells have become rounded globular forms with much smaller profile areas. Cytoplasmic processes from cell margins are no longer distinguishable as such, especially since cell margins are not clearly identifiable in the rounded cell form. All processes appear reduced to the form of 'filopodia' or long thin anchoring microvilli. The cell remains tethered to the substrate, albeit tenuously. Broken-off anchoring microvilli are visible in some areas between the cells. (D) Enlarged view of rounded cell showing multiple anchoring microvilli and some blebs. Bar = 10 µm.

PDAE is amiloride sensitive but not SITS sensitive and correlated with a dramatic FTR response that changes flat anchored monolayer cells into globular shapes. The FTR response can also be induced by PDAE using non-HCO₃⁻ buffers (see Sit and Wong, 1989). We have also demonstrated the FTR response in association with antiporter activation via mitogenic signaling pathways such as via phosphoinositide (PI) second messengers initiated by sulphate, epidermal growth factor and extracellular ATP, a P₂ purinergic receptor agonist. These FTR responses can be blocked by amiloride and quinidine (antiporter blockers) and by staurosporine (protein kinase C inhibitor) (Sit and Wong, 1989, 1991; Sit et al., 1990, 1991a, 1991f, 1992a, 1992b). Figures 1A-1D show PDAE motivated FTR responses at SEM level. The SEM profiles show vividly the development of microvillus processes (Fig. 1B) which become very long and thin (Figs. 1C, 1D) as the cell becomes more retracted and rounded. Diagrammatic presentation of the FTR change as seen in SEM is given in Fig. 2, showing reduction in cell profile area and development of thin microvillus anchors with rounding. At light microscopic level, these long anchoring processes were described as cytoplasmic 'guy ropes' (Sit and Wong, 1989) which are easily broken by slight agitation and thus allow the rounded cell to roll into suspension (Sit et al., 1991e). At SEM level it is clear that the breakage of the anchoring microvilli (cytoplasmic guy ropes) is not imperatively at their substrate ends (see Fig. 1C). Hence anchorage 'downregulation' may not necessarily involve detachment at the very sites of cell-substrate adhesion since the thin anchoring microvilli could break anywhere between the substrate and the cell body.
Figure 1. Diagramatic presentation of flat-to-round change. Top row is sagittal section view. Bottom row is top (bird’s eye) view. Frame ‘1’ shows flattened state, while frame ‘3’ shows rounded state. Frame ‘2’ shows an intermediate stage between frames ‘1’ and ‘3’ where the dorsal surface (black) is being reduced as the ventral ‘soleplate’ migrates dorsally in gradual effacement.

Figure 2. Quantitation of cell profile area in time response, on incubation in FTR solution. (i) On-line image analysis during SEM examination (solid, bottom line). (ii) Microspectrophotometric digitization in transmission light microscopy (LM, dashed, top line). The curves are parallel showing a similar trend, but the measured area is smaller to that in the SEM mode. Critical point drying in SEM preparation is known to cause cell shrinkage (Boyde, 1978). Error bar = 2 standard error (SE) (95% confidence limits).

Fig. 3 shows the drastic cell area reduction with AMR in a time response using FTR solution. Measurements by image analysis, on-line with SEM examination (solid, lower line), have lower values than by microspectrophotometric digitization of transmission profiles in light microscopy (LM, dashed, top line). The curves are parallel showing a similar trend, but the measured area is smaller to that in the SEM mode. Critical point drying in SEM preparation is known to cause cell shrinkage (Boyde, 1978). Error bar = 2 standard error (SE) (95% confidence limits).

An approximation based on the observed profile area reduction shows a reduction of more than half of the cell’s surface area in the FTR response. If the reduction in the cell body’s surface area were to be entirely due to the plasma membrane being pulled out into microvillus anchors, then each cell should have approximately 1000 of these microvillus anchors of maximum length equaling the cell diameter. However, the average number of observed basal or soleplate microvillus anchors per cell is closer to a hundred rather than to a thousand. The deficit in plasma membrane area also appears to be not sufficiently accounted for by internalization via small (average 0.14 μm width) coated pit endocytosis since (a) these occupy only 1-2% of the surface area of a cultured monolayer cell (Pastan and Willingham, 1987); and (b) the observed endocytosis process with AMR is of much larger dimensions with respect to internalization of extracellular debris (Figs. 4-5) and added fluoresceinated dextran particles (Fig. 6). Here endocytic channels may measure 1 μm wide and the 2,000,000 molecular weight dextran particles are commonly seen as having 1 μm diameters. If the cell surface area is reduced in absolute terms with AMR due to internalizations, then the microvillus anchors that develop during rounding could be the result of receding cell margins with focal sites still stuck to the substrate. If that were the case then downregulation of complex adhesive forces may not be needed for cell detachment in AMR. This, as well as other considerations, can be further tested with our model which will produce anchoring microvilli or filopodia in easily modulated stages.
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Figure 5. TEM of a rounded cell after 30 minute FTR incubation. (A) Surface is studded with ruffles and microvillus processes. Endocytic channels at the cell periphery (arrow) and endosomes (arrowhead) are frequently observed. Double arrowheads indicate granular patches which are prominent in FTR change in these cells (Sit et al., 1990). (B) Shows an enlarged view of (A). Bar = 1 µm.

Figure 4. TEM of a cell process showing endocytic channel and endosome after 9 minutes FTR incubation. Long microvilli are prominent, a characteristic development in FTR change. In an indented part of the cell, between adjacent microvilli, is extracellular debris (arrow). Similar debris is seen inside an endosome (arrowhead). Bar = 1 µm.

Figure 6. Internalization of fluoresceinated dextran of molecular weight 4,400-2,000,000 in FTR change. The incubations were (a) 135 mM Na₂SO₄ in FTR solution (FTR+SO₄, solid line), (b) FTR solution alone (FTR, dashed line), and (c) 140 mM NaCl, unbuffered (saline, dotted line). Each point is an average of 2 parallel cultures of Chang liver cells. Molecular weight species of FDx used in each point is indicated against the point. Sulphate enhances the FTR response (Sit et al., 1991a, 1991e). Error bar = 2 SE.

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References


Discussion with Reviewers

E.A.G. Chernoff: Do you believe that other stimulants of cell retraction (e.g.: cytochlasins, cold-treatment) are also stimulating Na\(^+\)/H\(^+\) antiport? Are you proposing that this is a general mechanism stimulating or regulating cell retraction?

Authors: Cytochlasin B causes structural alterations in the cytoskeletal elements and enucleations, and has been used in receptor internalizations. However Revel et al., (1974, text ref.) reported that cytochlasin B had no effect on cell rounding or detachment times. As for cold-induced rounding, Revel et al. (1974) cited disruption of microtubular or other cytoskeletal elements as the cause. The 4 °C cold-induced rounding causes poor cell viability (Pharmacia Fine Chemicals, 1981). Dying cells from treatment with cytokotoxic agents, e.g., HgCl\(_2\) or cycloheximide, become rapidly retracted and rounded. In mitotic rounding, caldesmon phosphorylation has been suggested (Yamashiro and Matsumura, 1991). On the other hand, we have shown that Na\(^+\)/H\(^+\) exchanges motivated by simple ionic gradients seem effective. Antiporoter activation is a consistent and universal finding in cell activation (Grinstein et al., 1989, text ref.).

E.A.G. Chernoff: I am curious about the relationship between activation of Na\(^+\)/H\(^+\) antiport and the massive amount of endocytosis that occurs. How are they connected mechanistically.

Authors: The relation between endocytosis and cytosolic pH is well established (see Van Deurs B et al., 1989). Alkalinization promotes internalization while acidification inhibits it. Apparently, pushing alkalinization hard via PDAE unveiled an inherent primitive endocytic potential, viz., large channel endocytosis like that seen in amoebic cells (Sit et al., 1990, text ref.).
E.A.G. Chernoff: Is the endocytosis a consequence of the organization of the cells in culture. Normally the antipporter would be localized in the apical membrane of cells organized into an epithelium, while the SEM and TEM images suggest that these cells are unpolarized.

Authors: In a monolayer epithelium in vivo, there is one luminal (free) cell surface. The other sides of a cell are in contact with adjacent cells and substrate. In a 'monolayer' culture in vitro, sections done at right angles to the substrate (see Sit et al., 1991c, text ref.) often show a good part of the cell lifted from the substrate (as if suspended in mid-air). Thus, at any one time, not only is the dorsal or top side a 'free' surface, a good deal of the ventral or substratal side of a cell is also 'free'. Unless at quiescent confluent state and contact inhibited, cell edges are a hive of movements, and increasingly so with withdrawal from contact inhibition.

E. Wisse: Are microvilli-anchoring contacts comparable in structure and/or composition to focal contacts or plaques in spreaded cells? Are microvillous contacts after rounding up of the cells, located at the places of previous focal contacts or plaques? Are the microvilli-anchoring processes simply the reminder of those focal contacts, not coming loose while the cells are changing shape?

Authors: In TEM, the visible cell-substrate contacts of flattened cells are small focal points of electron density (Sit et al., 1991c, text ref.). In rounded cells the anchoring microvilli are attached to the substrate by similar small focal points of electron densities. We think they are the same contacts before and after rounding.

E. Wisse: Is there any explanation for the mechanism of rounding up of these cells in FTR solution?

Authors: The massive endocytosis concomitant with the FTR response could explain the observed reduction in plasma membrane area. The surface area reduction should force the cell to assume a rounded form to maximize volume holding.

J. P. Revel: The authors claim to have developed a simple working model. In what way is it simpler or more controllable than cold, used by previous investigators?

Authors: The fact that FTRed cells, with or without internalized dextran, are as viable as trypsinized cells is documented (Sit et al., 1990, 1991a, 1991e, text ref.). On the other hand, Pharmacia (1981) states that cold treatment produces low viability and advised the use of other methods not desirable. Pharmacia cited 8 hours incubation at 4 °C to achieve detachment of a significant proportion of the anchored cells. Therefore, besides being incomplete even after long incubations, cold perturbations apparently cause considerable morbidity. Rounding due to cell death or cytotoxic effects is an established fact in anchorage dependent cell growth, one that tissue culturists are largely familiar with, (those round floaters are bad signs). Cold-induced cell rounding is stated as being complicated by this possibility.

FTR response is more controllable than cold-induced rounding because (a) we have established the FTR response characteristics which take the form of a biexponential decay with high coefficients of determination (Sit et al., 1991a, text ref.). The biexponential nature of the time response with similar high coefficients of determination have been documented several times over in human Chang liver cells (Sit and Wong, 1991; Sit et al., 1990, 1991e, 1992a text ref.) and similar curves were also established in other cell types, viz., primary human foetal liver cells (Sit and Wong, 1989, text ref.) and human lung cells (Sit et al., 1991b, text ref.). Moreover, (b), as a Na⁺/H⁺ antiporter mediated rounding response, there are a number of well defined molecular pathways of control, besides time and temperature.

J. P. Revel: The description of measurement of uptake of Fluoresceinated Dextrans is hard to follow and controls are missing. Something should be said about how effective 2 washes are? Is that sufficient? Also does trypsin treatment cause losses of Dextran which does not occur in other rounding conditions? Why not also trypsinize cells rounded by other means?

Authors: Monolayer cultures incubated in condition (c), viz., 140 mM NaCl (saline), were the controls since the incubation medium does not increase its pH nor cause rounding of quiescent monolayer cells. Saline by itself is not a motivator of PDAE, proton dependent antiporter exchange.

Supernatants from each washing of the cells were measured for FDx content. RFUs of supernatants from the second washing were consistently found to be at background level. In early test trials, the cells were washed 4 times. We found washing twice was sufficient since the measured RFUs of supernatants from 2nd, 3rd, and 4th washings were similar, viz., at background level.

Normal cell harvesting by trypsinization in 0.25% Gibco trypsin does not cause loss of internalized dextran particles. TEM studies demonstrated that the dextran particles are internalized in the FTR response. Apparently even after 4 cell divisions, the dextran particles remain trapped inside the cell (Sit et al., 1991a, text ref.). Exocytosis promoted by trypsin treatment apparently did not occur. Test trials showed very little difference in measured RFUs from cells incubated in FDx-saline [control condition (c)] that had been (i) washed in situ as monolayers and then scraped off for quantitation, as opposed to cells subjected to (ii) trypsinization, washing and then quantitated.

Quiescent cells incubated in FDx-saline [the control condition (c)] were not rounded and therefore had to be harvested by trypsinization. On the other hand, cells incubated in the FTR solution, with or without added sulphate, were rounded and therefore, it was unnecessary to trypsinize these cells in order to harvest them.
J. P. Revel: In Fig. 1 as seen after 9 minutes (Fig. 1B), and even after 30 minutes (Fig. 1C), about 1/3 of the cells do not seem to respond to FTR treatment. Is there any explanation? The error bars on the plot of area versus incubation time do not seem to reflect this, why? What would a plot of the number of cells of a particular area at each time point show? Is the inhomogeneity in cell rounding better or worse than that observed when other means of round cells are used?

Authors: Since the experiments were done with parallel cultures (as stated in Materials and Methods, under the subheading of Flat-to-round (FTR) change), the starting condition of Fig. 1B and Fig. 1C is in fact Fig. 1A (zero incubation time, or control condition). As stated in the legends for Fig. 1B, the cell margins are retracted significantly leaving wide intercellular gaps. This difference between Figs. 1A and 1B seems obvious. Similarly the difference between Figs. 1A and 1C seems equally obvious. We do not see 1/3 of the cells not responding even after 30 minutes of FTR treatment. The positions and dimensions of the error (2 SE) bars corroborated the obvious differences between Figs. 1A, 1B and 1C.

The total number of cells on the small cover glass area at each time point should be the same or very similar, although with cell retraction and rounding, there is a possibility that some of the rounded cells could be dislodged and washed away in the processing for microscopy.

Microspectrophotometric quantitation of the profile area of liver cells rounded by a 5 minute incubation (37 °C) in 0.25% Gibco trypsin (used as is) showed dispersions similar to those seen in cells rounded by FTR treatment (see Sit and Wong, 1989, text ref.).

J. P. Revel: Comparisons of the light microscopy (LM) to SEM curve can be interpreted as shrinkage, but why are the error bars so different in the two curves? It suggests that fixation makes all cells more alike in addition to just shrinking them?

Authors: LM quantitation was at a lower resolution, viz., 10x objective, whereas SEM quantitation was done at 700x. In LM, the thin microvillus anchors were largely not resolved, so that the LM profile area was essentially a top view of the cell body plus variable extents of overlapping microvilli at the cell boundary that could make the cell body appear at times somewhat larger. In SEM, the microvilli are resolved, thus variations due to 'fuzzy' boundaries at LM resolutions were minimized.

J. P. Revel: The measurement of the surface area of cells is based on measurement of the area of a projection. So different correction factors have to be applied for a flat cell, and a spherical cell to give results which can be interpreted in terms of cell membrane area. There must also be attempt to measure extent of microvilli which will change the surface area drastically. Finally, blebs (seen in many of the rounded cells, possibly to a larger extent than in the non-rounded ones) may represent loss of cell membrane which must be taken into account. As a result I don't understand the argument leading to the final conclusions. I gather that the authors' final conclusion is that cell rounding by the present methods does what was shown to happen in the case of retraction in the cold, where direct observation showed that the pattern of extension corresponded well with the position of the cell border before retraction.

Authors: If profile area were to be considered, then the flat cell is more than 5 times that of the rounded cell (comparing readings at first point and last point of Fig. 3). Our statement that the approximate surface area of a flat cell was more than 2 times that of a rounded cell was based on taking the observed flat area x2 (top and bottom) against the surface area of a round sphere (4πr²) whose radius, r, was calculated from the observed profile area of the rounded cell. The conclusions taking either LM or SEM figures are similar.

Our statement of the surface area taken up by microvillus anchors was based on an estimation assuming the shape of a microvillus as a cone ([πr x length of microvillus] / 2) where r was half the averaged width of the microvillus at rounded stage.

A comparison between Figs. 1A and 1B does not support the view that the FTR response produced excessive blebbing. The profile area of rounded cells is less than 1/5th of the flattened cell (see Fig. 3) giving the impression that it has more blebs, a crowding effect. Excessive blebbing does not appear to be an imperative characteristic of cell rounding (Wetzel et al., 1978, text ref.).

Our conclusion, based on the stated approximations, was corroborated by the demonstration of massive endocytosis (Figs. 4-6) concomitant with the FTR response. In endocytosis the plasma membrane is internalized and that means reduction in surface area of the cell. A visual inspection of the huge endocytic channels and endosomes in Fig. 5 could give an idea of the extent of that surface area reduction.

Additional References

