Depletion of Intracellular Potassium Disrupts Coated Pits and Reversibly Inhibits Cell Polarization During Fibroblast Spreading

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Abstract. To learn more about the possible role of the coated pits endocytic pathway in cell adhesion, we studied attachment and spreading of fibroblasts whose coated pits were disrupted by depletion of intercellular potassium. Fibroblasts incubated in suspension in potassium-free medium lost 80% of their intracellular potassium within 10 min and showed disrupted coated pits based on fluorescence staining of clathrin. Potassium-depleted cells attached and spread on fibronectin-coated substrata over the same time course (15 min–2 h) as control cells. Unlike controls, however, potassium-depleted fibroblasts attained a radial morphology with circumferentially organized actin filament bundles and were unable to make the transition to a polarized morphology with stress fibers. In the radially spread fibroblasts, fibronectin receptors and vinculin colocalized in focal adhesion sites and appeared to be membrane insertion points for circumferentially arranged actin filament bundles, but these sites were much smaller than the focal adhesion plaques in polarized cells. The effects of potassium depletion on cell adhesion were reversible. Within 1 h after switching K^+-depleted fibroblasts to medium containing KCl, cells developed a polarized morphology with actin stress fibers inserting into focal adhesion plaques. Coated pits also reformed on the cell surface during this time. Because formation of focal adhesion plaques preceded reappearance of clathrin-coated pits at the cell margins, it seems unlikely that coated pits play a direct role in adhesion plaque assembly. Polarization of fibroblasts upon addition of KCl was inhibited by ouabain showing that intracellular potassium was required for activity. Polarization also was inhibited when potassium-depleted cells were switched to potassium-containing medium under hypertonic or acidified conditions, both of which have been shown to inhibit receptor-mediated endocytosis. Our results suggest that the coated pit endocytic pathway is not required for initial attachment, spreading, and formation of focal adhesions by fibroblasts, but may play a role in cell polarization.

ADHESION between tissue cells and the extracellular matrix plays important roles in the maintenance and regulation of cell shape, migration, growth, and differentiation (Grinnell, 1978; Folkman and Moscona, 1978; Bissell et al., 1982). The mechanism of fibroblast adhesion to substrata has been studied for many years and can be divided into three stages: (*a*) initial cell attachment; (*b*) radial cell spreading characterized by circumferential actin organization; and (*c*) polarized cell spreading characterized by focal adhesion plaques and stress fibers (Grinnell, 1978; Thom et al., 1979). Focal adhesion plaques are the sites where stress fibers insert into the plasma membrane (Izzard and Lochner, 1976; Heath and Dunn, 1978).

In recent years, much has been learned about the mechanism of initial cell attachment. Cell surface receptors belonging to the integrin superfamily of cell adhesion molecules mediate binding to matrix components such as fibronectin (FN)[1], vitronectin, collagen, and laminin (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Akiyama et al., 1990; Albelda and Buck, 1990; Hemler, 1990; Springer, 1990). On the cytoplasmic side of the plasma membrane, these receptors become linked to the actin cytoskeleton (Chen et al., 1985; Tamkun et al., 1986), possibly through talin (Horwitz et al., 1986) or α-actinin (Otey et al., 1990). Although control of cell polarization and accompanying actin reorganization and focal adhesion plaque formation are not well understood, integrin receptor aggregation on the cell surface probably triggers subsequent events (Geiger, 1982). These receptors become clustered in focal adhesion plaques (Singer et al., 1988; Fath et al., 1989), which contain a variety of structural, enzymatic, and regulatory proteins (Turner and Burridge, 1991).

Formation of focal adhesions may depend in part on the coated pit endocytic pathway because clathrin-coated pits can be observed at sites of cell-substratum contact during initial adhesion (Woods et al., 1983). Moreover, fibroblasts

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1. *Abbreviation used in this paper*: FN, fibronectin.

remove and endocytose FN from the substratum during cell attachment and spreading (Avnur and Geiger, 1981; Grinnell, 1986). In addition, FN receptors contain the NPXY cytoplasmic sequence required for coated pit interactions (Chen et al., 1990) and can mediate receptor-mediated endocytosis and recycling (Molnar et al., 1987; Raub and Kuentzel, 1989; Bretscher, 1989; Sczekan and Juliano, 1990). Any association between coated pits and focal adhesion is transient, however, because these two structures do not colocalize on the ventral surfaces of spread cells (Heuser, 1980; Woods et al., 1983; Maupin and Pollard, 1983; Nicol and Nermut, 1987).

To determine if the coated pit endocytic pathway was required for cell attachment and spreading, we studied the adhesion of potassium-depleted fibroblasts. Previously, Larkin et al. (1983) showed that potassium depletion of fibroblasts results in reversible disruption of coated pits and receptor-mediated endocytosis (Larkin et al., 1983). To our surprise, we found that potassium-depleted cells attached and spread on FN-coated substrata over the same time course as control cells and formed small focal adhesions at the cell margins, but the cells were unable to attain a polarized morphology or develop large focal adhesion plaques. Our results suggest that the coated pit endocytic pathway is not essential for initial attachment and spreading but may be required for subsequent cell polarization.

Methods and Materials

Cells

Human foreskin fibroblasts were cultured up to seven passages in Dulbecco's MEM (GIBCO Laboratories, Grand Island, NY) containing 10% FBS (Sigma Chemical Co., St. Louis, MO) in a humidified incubator with 5% CO_2. Cells from preconfluent cultures were harvested with 0.05% trypsin/0.6 mM EDTA (GIBCO Laboratories), and the trypsin was neutralized with FBS (1:1 vol/vol).

Potassium Depletion, Hypertonic Treatment, and Cytoplasmic Acidification

To deplete K^+ from fibroblasts, harvested cells ($\sim 10^6$) were washed once and incubated in suspension for 30 min at 37°C in 20 ml potassium-free medium (140 mM NaCl, 50 mM Hepes, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$). Potassium-depleted fibroblasts were viable judging from trypan blue exclusion, and the effects of potassium depletion on cell adhesion and actin organization were reversible as will be described. Control cells were treated similarly as above except the medium was supplemented with 3 mM KCl.

The extent of potassium depletion was determined by measuring ^{86}Rb (Ledbetter and Lubin, 1977; Owen and Prastein, 1985). In this case, fibroblasts were pre-equilibrated for 2 h at 37°C in $+K^+$ medium supplemented with 10% FBS and 1 uCi/ml ^{86}Rb (New England Nuclear, 7.7 Ci/g) and harvested with trypsin/EDTA solution containing 1 uCi/ml ^{86}Rb. Trypsin was neutralized with dialyzed FBS (against $-K^+$ buffer) after which the cells were subjected to potassium depletion as above. Potassium-depleted and control cells were collected on 2.5 cm glass fiber filters (Whatman) and washed with ~10 ml medium. Samples were mixed with 10 ml of Budget Solve (RPI Research and Precision Instruments, Laurent, Canada) and radioactivity was measured in a scintillation spectrophotometer (model 3801, Beckman Instruments, Fullerton, CA).

To determine reversibility of potassium depletion, $-K^+$ or control fibroblasts were allowed to attach and spread in FN-coated Costar 24 well culture dishes with different attachment media and 1 uCi/ml ^{86}Rb added as indicated. Before adding the cells, the culture well surfaces were coated for 30 min at 37°C with 20 ug/ml human plasma FN (New York Blood Center, New York, NY) in $-K^+$ medium. At the end of the incubations, attached cells were rinsed with ~10 ml $+K^+$ medium and solubilized with 0.5 ml 0.1% SDS.

In some experiments, cells were subjected to hypertonic treatment by incubation in +K medium supplemented with 0.45 M sucrose, conditions that have been shown to cause disruption of clathrin-coated pits (Heuser and Anderson, 1989). Alternatively, cells were acidified by incubation in +K medium containing 50 mM MES instead of Hepes (final pH 5.5), which prevents curvature of clathrin lattices required for receptor-mediated endocytosis (Heuser, 1989).

Cell Attachment and Spreading

Approximately 10^5 cells in 3.0 ml $-K^+$ or $+K^+$ medium as indicated and 1 mg/ml BSA (crystalline, ICN Biomedicals, Inc., Costa Mesa, CA) were incubated for the times indicated on FN-coated (see above) 22 mm^2 glass coverslips (Labcraft, Curtin Matheson Scientific Inc., Houston, TX). At the end of the incubations, cells were fixed for 5 min at 4°C with 3% formaldehyde in TBS (10 mM Tris, 140 mM NaCl, pH 7.2) plus 1 mM $CaCl_2$. Samples were viewed and photographed with a Zeiss inverted microscope (Carl Zeiss, Inc., Thornwood, NY). To quantify the results, the microscopic field was superimposed on the digitizing tablet of a Zidas image analyzer (Carl Zeiss, Inc.), outlines of 50 cells in randomly chosen fields were traced with the cursor, and projected cell surface area and form factor were calculated. The form factor is defined as 4π area/perimeter^2 and varies from one for a circle to zero as the form becomes more asymmetric.

Distribution of Actin, Clathrin, Fibronectin Receptors, and Vinculin

Attached cells to be processed for immunofluorescence microscopy were fixed as above and permeabilized with 0.2% NP-40 in TBS for 5 min. To detect actin, fixed and permeabilized cells were incubated for 30 min at 37°C with 5 U/ml FITC-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR). The percentage of cells containing stress fibers (i.e., longitudinal actin filament bundles) was estimated by counting 80–100 cells in 6–10 representative microscopic fields.

To detect clathrin, β1 integrin receptors and vinculin, cells were fixed and permeabilized as above and then incubated for 30 min at 37° with mouse anti-clathrin heavy chain (a gift from Dr. Richard Anderson, University of Texas Southwestern Medical Center), rabbit anti-FN receptor (a gift from Dr. Kenneth Yamada, National Institutes of Health), or mouse antivinculin (Sigma Chemical Co.) diluted in TBS, 1 mM $CaCl_2$, 1% BSA followed by RITC-conjugated goat anti-rabbit IgG or FITC-conjugated goat anti-mouse IgG (Zymed Labs, South San Francisco, CA) in TBS, 1 mM $CaCl_2$, 10% normal goat serum (GIBCO Laboratories). To detect actin and vinculin in the same cells, the preparations were stained sequentially with mouse antivinculin, RITC-goat anti-mouse, and 10 U/ml FITC-conjugated phalloidin. To detect FN receptors and vinculin in the same cells, the preparations were stained sequentially with mouse antivinculin, FITC-goat anti-mouse, rabbit anti-FN receptor, and RITC-goat anti-rabbit. At the end of the incubations, samples were washed, mounted with Mowiol (Harlow and Lane, 1988), and observed and photographed with a Zeiss Universal microscope (Carl Zeiss, Inc.). Specificity of dual-label antibody analyses was confirmed by omitting primary and secondary antibodies individually.

Results

Potassium Depletion

Larkin et al. (1983) depleted potassium from fibroblasts in monolayer culture by subjecting attached cells to hypotonic shock in potassium-free medium. In our studies, we wanted to deplete potassium first and then test cell attachment and spreading. We found that simply incubating fibroblasts in suspension in K^+-free medium resulted in depletion of intracellular potassium. Table I reports two different experiments in which we estimated intracellular potassium by the ^{86}Rb method (Ledbetter and Lubin, 1977; Owen and Prastein, 1985). After 10 min, 80% of intracellular potassium was lost from fibroblasts in $-K^+$ medium. Subsequent studies described in this paper were carried out with cells that were potassium depleted for 30 min.

Table I. Potassium Depletion Measured by ^{86}Rb

Exp #	Depletion time	Cell-associated radioactivity	Depletion
	min	*cpm ± SD*	*%*
1	0	1,252 ± 169	0
	10	260 ± 3	79
	30	119 ± 3	90
	60	261 ± 12	79
2	0	2,102	0
	10	440 ± 16	79
	30	387 ± 90	82
	60	353 ± 78	83

Fibroblasts were preincubated 2 h and harvested in the presence of 1 uCi/ml ^{86}Rb and then switched to $-K^+$ medium. At the times shown, cell aliquots were collected, washed, and cell-associated radioactivity measured. Data points are average ± SD from duplicate experiments. Total washing time was ~5 min.

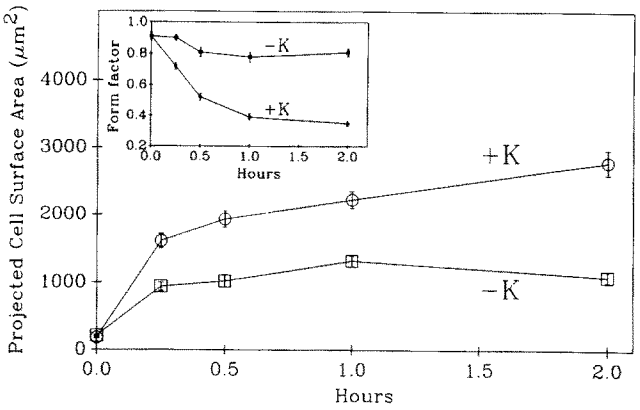

Figure 2. Time course of cell spreading. Potassium-depleted and control fibroblasts were incubated on FN-coated coverslips. At the times shown, cells were fixed. Data points are average ± SEM for projected cell surface area and form factor calculated from measurement of ~50 cells.

Cell Attachment and Spreading after Potassium Depletion

Fig. 1 shows the morphology of potassium-depleted and control fibroblasts after 2 h on FN-coated coverslips. By 15–30 min, the cells had attached and started spreading. Eventually, control cells developed a polarized morphology with prominent cell extensions typical of fibroblasts on fibronectin (Fig. 1 *a*), whereas potassium-depleted cells developed a radial morphology and lacked prominent cell extensions (Fig. 1 *b*). Judging from measurements of cell form factor (Fig. 2, *insert*), cell shape change was complete by 1 h.

Fig. 2 shows the time course of cell spreading measured as projected cell surface area. Consistent with differences in cell shape, the average projected surface area was ~1,100 um² for $-K^+$ cells compared to ~2,800 um² for $+K^+$ cells. Therefore, although $-K^+$ cells spread to a considerable extent (~fivefold increase in projected surface area compared to nonspread cells), they did not spread to the same extent as fibroblasts with K^+. Persistence of nuclear refractility in $-K^+$ cells (Fig. 1 *b*, *arrowheads*) also showed they were unable to spread as well as control cells. These findings indicated that potassium-depleted fibroblasts could attach to fibronectin and begin spreading, but could not complete the spreading process.

Distribution of Clathrin

To confirm that potassium depletion from fibroblasts disrupted coated pits, we analyzed the clathrin distribution of control and $-K^+$ cells spread for 2 h on fibronectin substrata. Control fibroblasts showed prominent clathrin clusters on the cell surface and adjacent to the nucleus (Fig. 3 *a*). Similar to previous results (Larkin et al., 1983), the intensity of clathrin staining was decreased in potassium-depleted cells, and the fluorescent clusters were smaller and more numerous (Fig. 3 *b*). Bright staining of the nuclear region in potassium-depleted cells (Fig. 3 *b*) probably resulted from increased thickness of the incompletely spread cells, similar to the perinuclear refractility (see also Figs. 4 *c* and 7 *c*). As will be discussed in more detail below, the effects of potassium depletion were completely reversible. Fig. 3 *c* shows that 1 h after switching from $-K^+$ to $+K^+$ conditions, fibroblasts regained their normal clathrin distribution.

Distribution of Actin

To analyze differences in cell polarization in more detail, we used immunofluorescence to localize actin in potassium-depleted and control fibroblasts. Fig. 4, *a* and *b* shows control cells with typical stress fibers, often terminating at the cell margins. During the first hour of spreading, ~80% of the control cells developed this appearance (Fig. 5, *open circles*). In marked contrast, $-K^+$ cells contained actin-filament bundles that were circumferentially organized (Fig. 4,

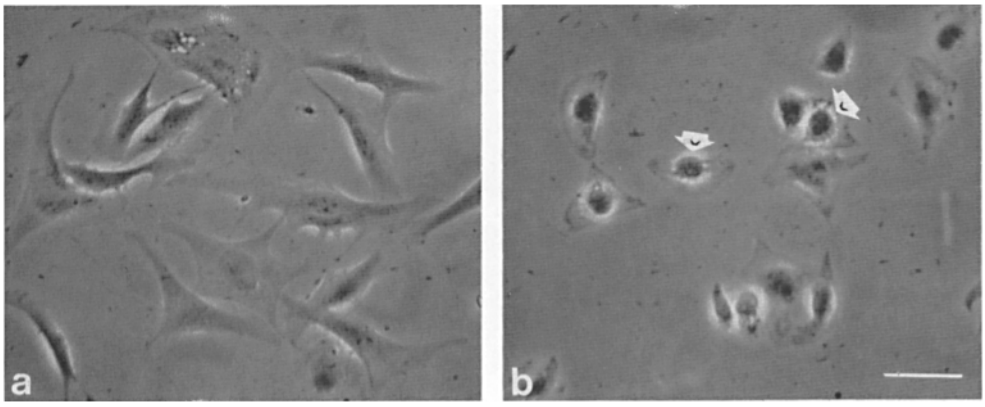

Figure 1. Morphology of human fibroblasts adhered to FN in the presence or absence of potassium. Potassium-depleted and control fibroblasts were incubated on FN-coated coverslips for 2 h after which the cells were fixed and photographed under phase contrast microscopy. Arrowheads indicate perinuclear refractility characteristic of partially spread cells. Bar, 20 μm.

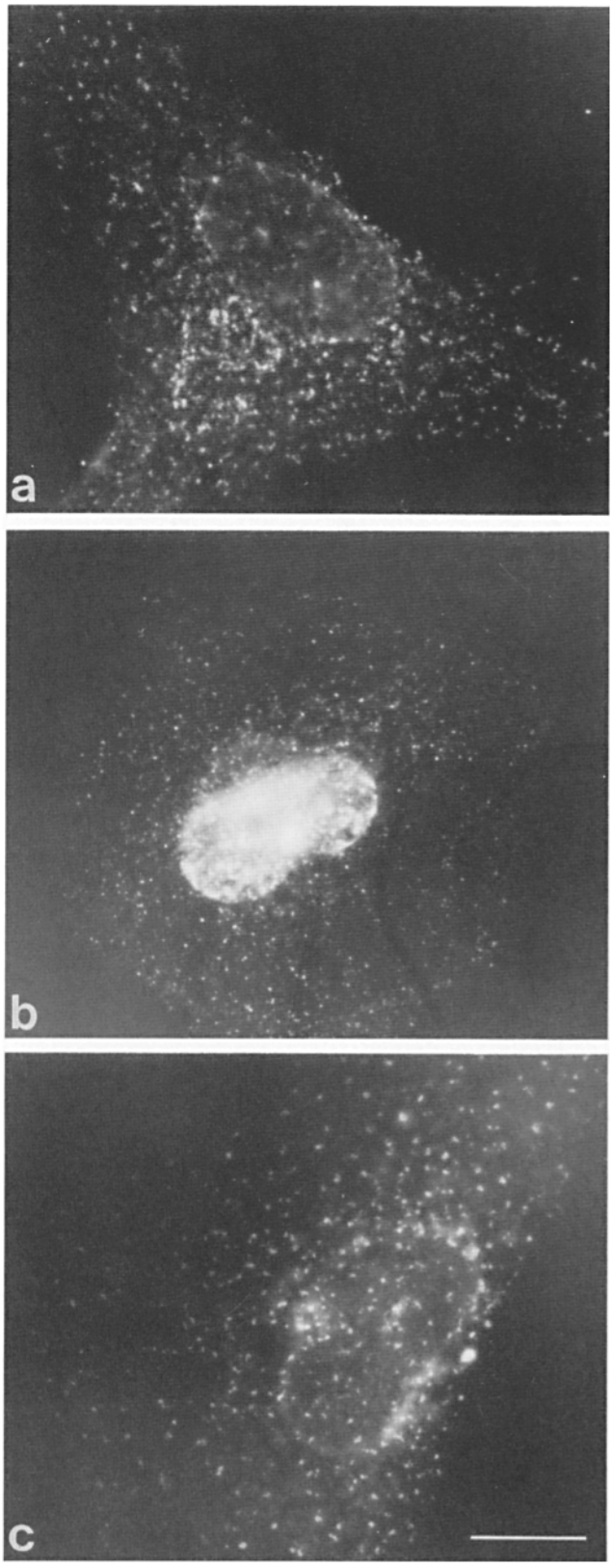

Figure 3. Distribution of clathrin. (*a*) Control fibroblasts attached for 2 h on FN-coated coverslips. (*b*) Potassium-depleted fibroblasts attached for 2 h. (*c*) Potassium-depleted fibroblasts attached for 1 h and then switched to +K medium for 1 h. At the end of the incubations, the cells were fixed, permeabilized, and stained for clathrin. Bright staining of the nuclear region in potassium-depleted cells (*b*) is an effect associated with partial cell spreading, similar to the perinuclear refractility shown in Fig. 1. Bar, 20 μm.

c and *d*), and <20% of the $-K^+$ cells contained stress fibers (Fig. 5, *open squares*). These findings complement the results in Figs. 1–3 and suggest that potassium-depleted cells initiated attachment and spreading but were unable to reorganize their cytoskeleton and make the transition from radial to polarized cell morphology.

Reversibility of Potassium-depletion

Fig. 4, *e* and *f*, and Fig. 5 also show that the effect of potassium-depletion on cell spreading was reversible. After 1 h, $-K^+$ fibroblasts were switched to adhesion medium containing 3 mM KCl. The cells rapidly changed shape and within 1 h almost 75% had formed stress fibers (Fig. 5, *closed squares*). Switching the cells in the opposite direction, i.e., $+K^+$ cells changed to $-K^+$ medium, resulted in only a slight decrease in the percentage of cells with stress fibers. In the short term situation, therefore, it appeared that potassium ions were more important for initial formation than maintenance of polarized cell morphology.

Effect of Ouabain

If reversal of potassium depletion depended on uptake of extracellular K^+ by Na^+/K^+ exchange, then we should have been able to inhibit reversibility with ouabain (Sweadner and Goldin, 1980; Skou, 1990). As shown in Fig. 6, addition of ouabain at a concentration known to inhibit Na^+/K^+ ATPase activity (0.1 mM) almost completely blocked the reversal of cell spreading when $-K^+$ cells were switched to $+K^+$ medium (*c* and *d*). Under the same short-term conditions, ouabain had only a slight inhibitory effect on cell polarization and actin reorganization of control cells (*a* and *b*).

Table II shows studies on the ability of ouabain to block reversal of potassium depletion in which we compared stress fiber formation with intracellular potassium levels. After 2 h, the percentage of potassium-depleted cells that organized stress fibers after addition of KCl ($-K^+$ switched to $+K^+$ conditions) amounted to 87% in controls and 29% with 0.1 mM ouabain added. Considering that ~20% of the $-K^+$ cells had stress fibers to begin with, this difference represented almost 90% inhibition of reversal by ouabain. Under these conditions, ouabain inhibited potassium uptake by >90%.

The decrease in percentage of $+K^+$ cells with stress fibers was 20–25% with 0.1 mM ouabain added, which probably resulted from a slow loss of intracellular potassium that occurs after inhibition of the Na^+/K^+ ATPase. Table II shows that after 1 h in the presence of ouabain, fibroblasts had lost ~50% of their intracellular potassium judging from ^{86}Rb content.

Distribution of Vinculin and Fibronectin Receptors

As mentioned in the Introduction, the change from radial to polarized cell morphology is accompanied not only by actin reorganization but also by formation of focal adhesion plaques. To assess focal adhesion formation, potassium-depleted and control fibroblasts that had attached and spread on FN were analyzed for the distribution of vinculin and fibronectin receptors. Previously, we reported colocalization of vinculin and FN receptors in focal adhesion plaques of human foreskin fibroblasts spread on FN (Grinnell, 1986).

Consistent with our earlier observations, $+K^+$ cells showed prominent plaques of fibronectin receptors (Fig. 7 *a*)

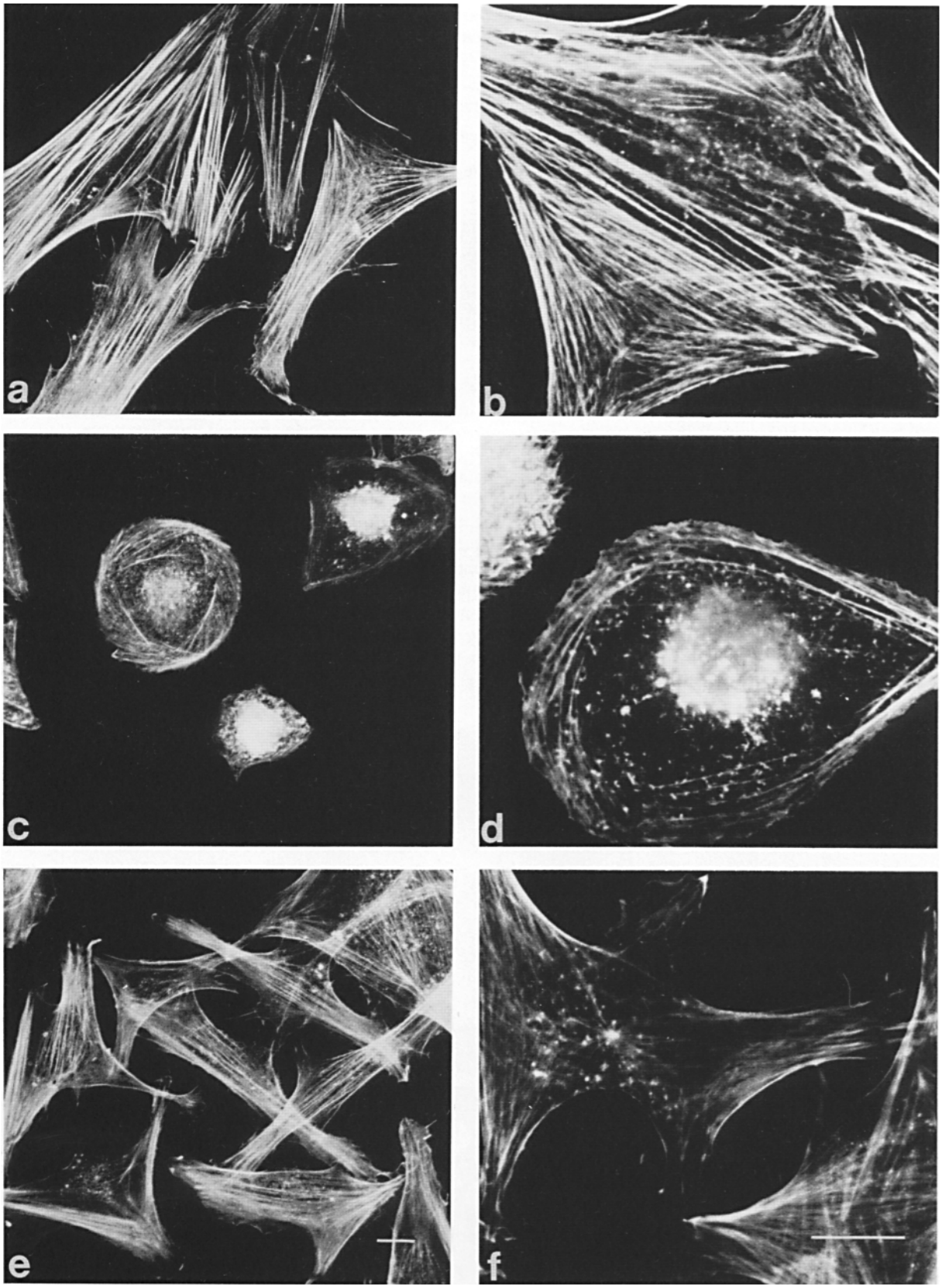

Figure 4. Distribution of actin. (*a* and *b*) Control fibroblasts attached for 2 h on FN-coated coverslips. (*c* and *d*) Potassium-depleted fibroblasts attached for 2 h. (*e* and *f*) Potassium-depleted fibroblasts attached for 1 h and then switched to +K medium for 1 h. At the end of the incubations, the cells were fixed, permeabilized, and stained for actin. Bars (*a*, *c*, *e*, and *b*, *d*, *f*), 20 μm.

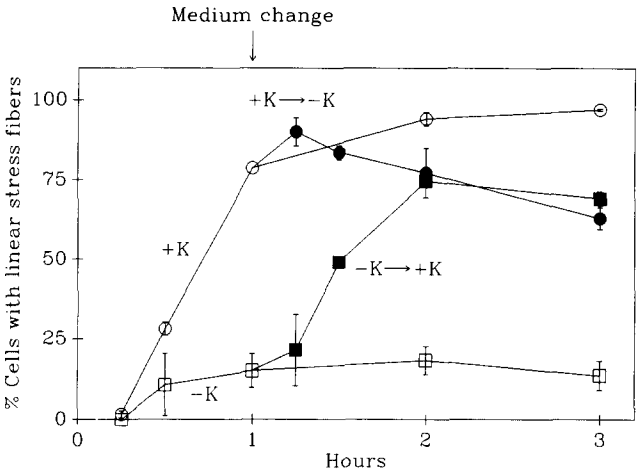

Figure 5. Time course of actin reorganization and reversibility of potassium depletion. Potassium-depleted and control fibroblasts were incubated on FN-coated coverslips. With some samples after 1 h, $-K^+$ cells were switched to $+K^+$ medium (*filled squares*) and $+K^+$ cells were switched to $-K^+$ medium (*filled circles*). At the times shown, cells were fixed, permeabilized, and stained for actin. Data points are average ± SD for percentage of cells with stress fibers calculated from measurement of 80–100 cells in 6–10 randomly selected microscopic fields.

Table II. Effect of Ouabain on Actin Organization and Potassium Uptake

Cells	Ouabain	Cells with stress fibers	Potassium uptake
		%	*cpm + SD*
$-K^+$ to $+K^+$	−	87.2	3,063 ± 371
$-K^+$ to $+K^+$	+	29.4	149 ± 15
$-K^+$	−	23.5	
$-K^+$	+	17.9	
$+K^+$	−	98.0	7,909 ± 1,300
$+K^+$	+	75.0	3,311 ± 753

Potassium-depleted and control fibroblasts in adhesion medium containing 1 uCi/ml ^{86}Rb were incubated for 1 h in FN-coated tissue culture wells. In some experiments, potassium-depleted cells were switched from $-K^+$ to $+K^+$ medium containing 1 uCi/ml ^{86}Rb during the second hour. Also during the second hour, 0.1 mM ouabain was added as indicated. At the end of the incubations, samples were rinsed, dissolved with 0.1% SDS, and cell-associated radioactivity was measured. Data points are averages ± SD of duplicate samples.

and vinculin (Fig. 7 *b*), which were localized at the ends of cell extensions. Cells stained for both vinculin and fibronectin receptors showed colocalization in the plaques (Fig. 8, *a* and *b*) and insertion of stress fibers (Fig. 8, *e* and *f*). The large, plaquelike structures were absent, however, from potassium-depleted fibroblasts. Instead, FN receptors (Fig. 7 *c*) and vinculin (Fig. 7 *d*) could be seen in small patches beneath the cells and around the cell periphery. Although the patches along the cell periphery were smaller than the marginal focal adhesion plaques of polarized cells, they also showed colocalization of FN receptors and vinculin (Fig. 8, *c* and *d*) and appeared to be insertion sites for the circumferentially organized actin filament bundles (Fig. 8, *g* and *h*). These results suggested that potassium-depletion prevented cell polarization but not focal adhesion formation.

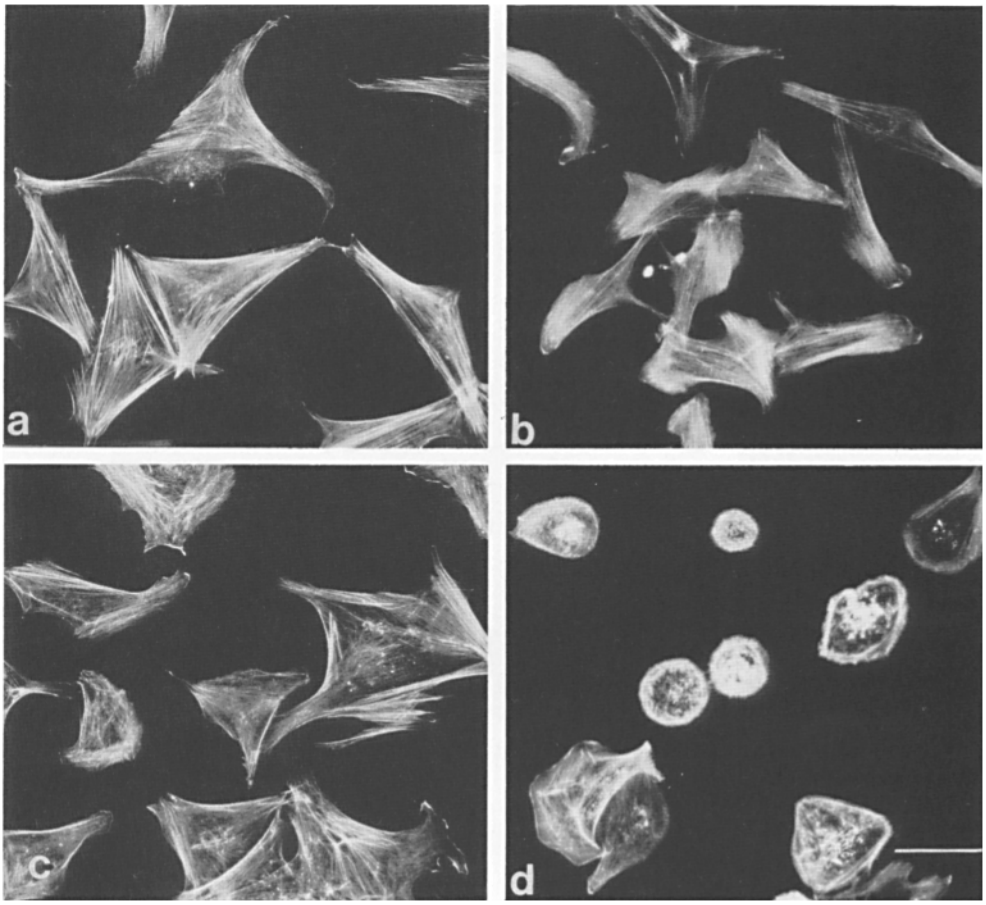

Figure 6. Effect of ouabain. Control fibroblasts were attached for 1 h on FN-coated coverslips and then switched for 1 h to medium without (*a*) and with (*b*) 0.1 mM ouabain. Potassium-depleted fibroblasts were attached for 1 h on FN-coated coverslips and then switched for 1 h to $+K^+$ medium without (*c*) and with (*d*) 0.1 mM ouabain. At the end of the incubations, the cells were fixed, permeabilized, and stained for actin. Bar, 50 μm.

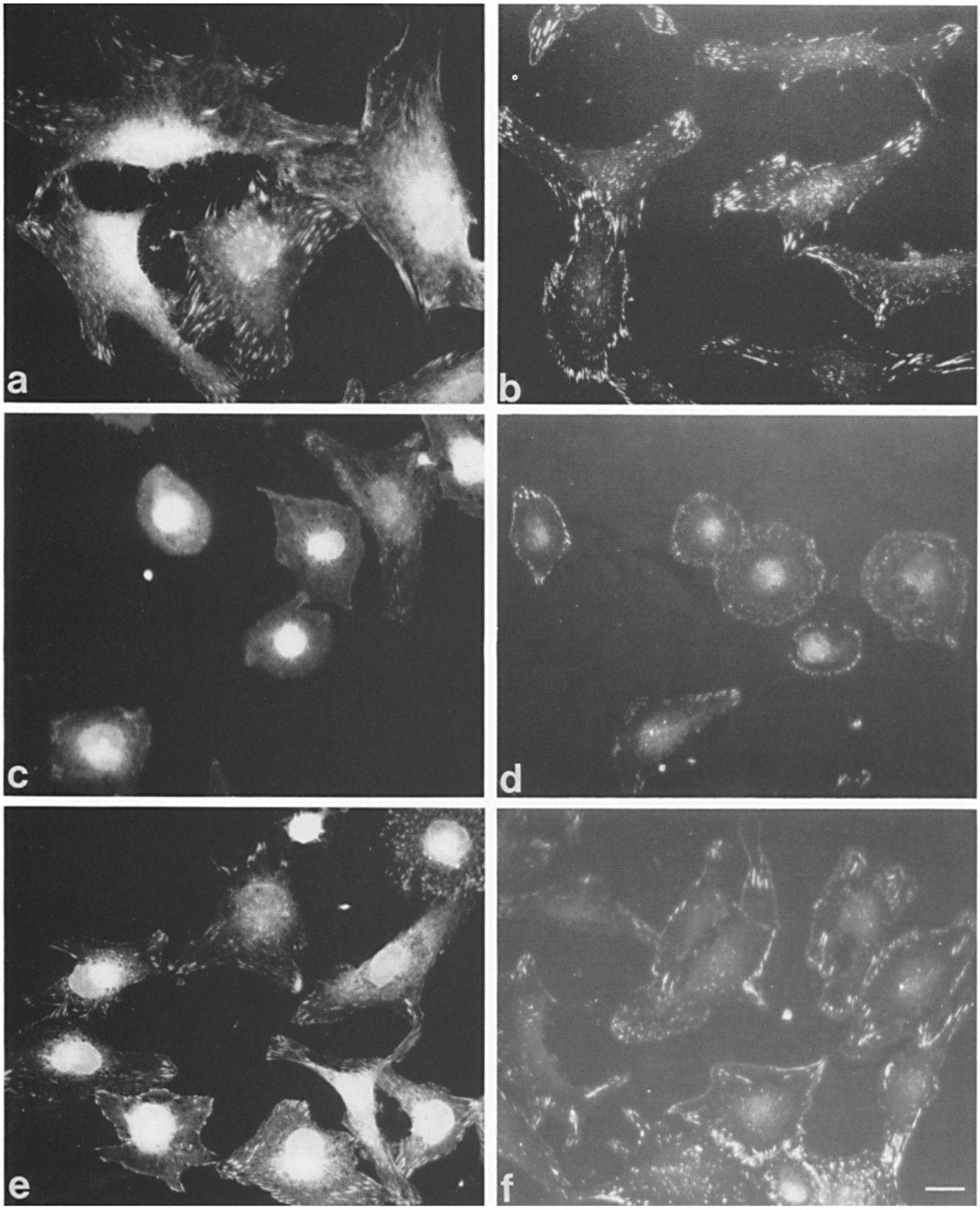

Figure 7. Distribution of fibronectin receptors and vinculin. (*a* and *b*) Control fibroblasts attached for 2 h on FN-coated coverslips. (*c* and *d*) Potassium-depleted fibroblasts attached for 2 h. (*e* and *f*) Potassium-depleted fibroblasts attached for 1 h and then switched to +K medium for 1 h. At the end of the incubations, the cells were fixed, permeabilized, and stained for FN receptors (*a*, *c*, *e*) or vinculin (*b*, *d*, *f*). Bar, 20 μm.

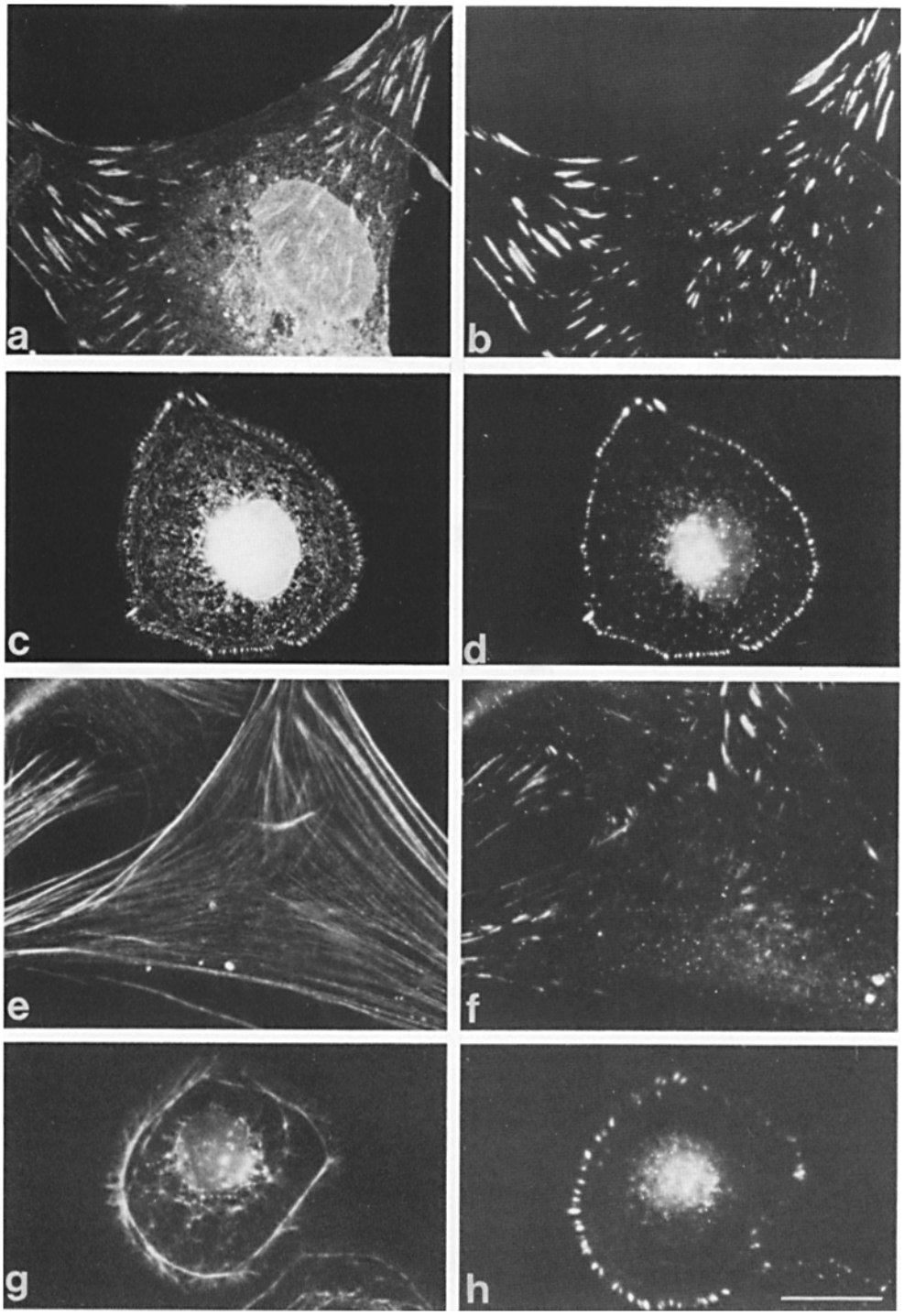

Figure 8. Colocalization of FN receptors, vinculin, and actin. (*a*, *b*, *e*, *f*) Control fibroblasts attached for 2 h on FN-coated coverslips. (*c*, *d*, *g*, *h*) Potassium-depleted fibroblasts attached for 2 h. At the end of the incubations, the cells were fixed, permeabilized, and stained for FN receptors (*a* and *c*), vinculin (*b*, *d*, *f*, *h*), and actin (*e*, *g*). Bar, 20 μm.

As was observed for actin organization, the effects of potassium depletion on focal adhesion formation were reversible. Potassium-depleted cells switched to $+K^+$ medium developed prominent plaques of FN receptors (Fig. 7 *e*) and vinculin (Fig. 7 *f*).

Distribution of Fibronectin Receptors and Clathrin

One possible interpretation of the findings described above was that cell polarization and formation of adhesion plaques at the ends of cell extensions required the coated pit endocytic pathway. To examine this possibility, potassium-depleted fibroblasts that had spread for 1 h were switched to potassium-containing medium and incubated an additional h. At various times, samples were fixed and stained to determine whether colocalization occurred between FN receptors and clathrin.

Fig. 9 shows, as already described, that FN receptors in potassium-depleted cells spread for 1 h initially were localized along the cell periphery and beneath the cells (Fig. 9 *a*, see also Fig. 8 *c*); whereas small clathrin aggregates were found in the central region of the cells but not along the cell periphery (Fig. 9 *b*, see also Fig. 3 *b*). Cell preparations ex-

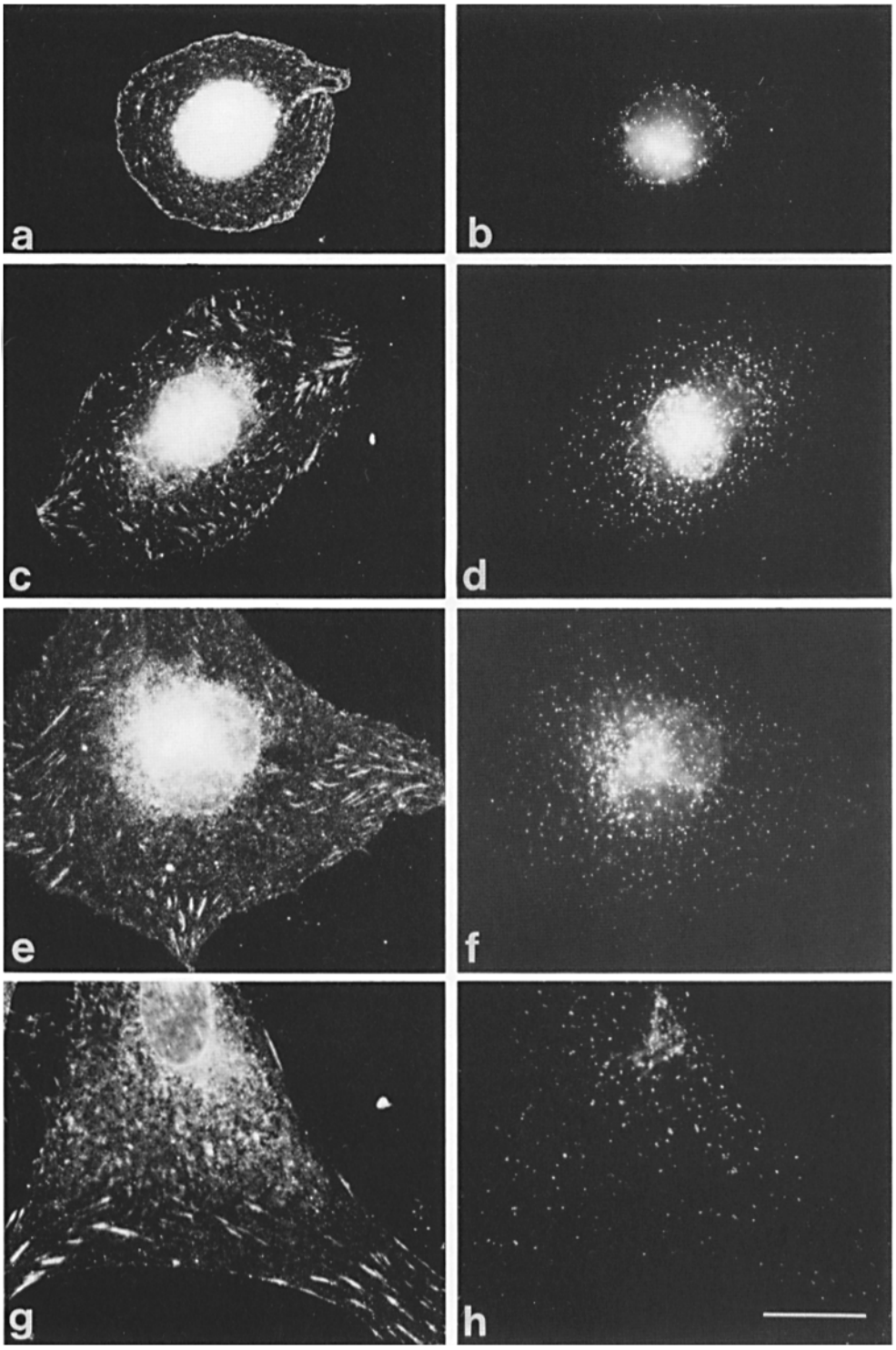

Figure 9. Colocalization of FN receptors and clathrin. Potassium-depleted fibroblasts attached for 1 h were switched to +K medium. At zero time (*a* and *b*) and after 15 (*c* and *d*), 30 (*e* and *f*), or 60 (*g* and *h*) min, the cells were fixed, permeabilized, and stained for FN receptors (*a*, *c*, *e*, *g*) and clathrin (*b*, *d*, *f*, *h*). Bar, 20 μm.

amined 15 (*c* and *d*), 30 (*e* and *f*), and 60 (*g* and *h*) min after adding back potassium showed that the increase in cell polarization and formation of marginal focal adhesion plaques preceded reappearance of clathrin-coated pits on cell extensions. Colocalization of clathrin and FN receptors in these regions was not observed, even transiently. Therefore, although receptor-mediated endocytosis might have been required for cell polarization, clathrin-coated pits were not sites of adhesion plaque assembly.

Effects of Hypertonic Shock and Acidification on Cell Polarization

Besides its effect on coated pits and receptor-mediated endocytosis, potassium plays a variety of roles in different cell functions including volume control (Kregenow, 1981). Therefore, inhibition of cell polarization by potassium depletion might have occurred for reasons unrelated to coated pit function. To test this possibility further, we studied the effects of

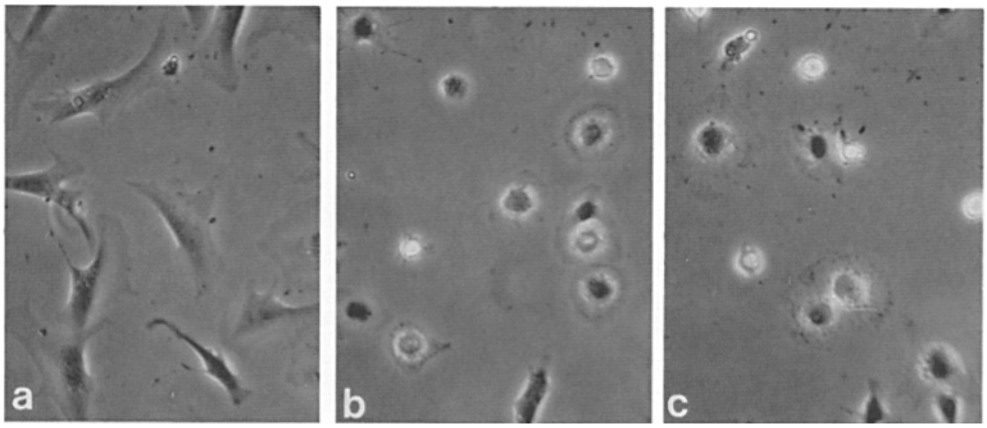

Figure 10. Effect of hypertonic medium and acidification. Potassium-depleted fibroblasts attached for 1 h were switched to +K medium for 1 h under control conditions (*a*) or in hypertonic medium (0.45 M sucrose) (*b*) or acidified medium (pH 5.5) (*c*). Bar, 20 μm.

other treatments that inhibit receptor-mediated endocytosis on cell polarization. Hypertonic treatment, like potassium depletion, causes disruption of coated pits (Heuser and Anderson, 1989), and acidification results in an inhibition of normal clathrin function (Heuser, 1989). Fig. 10 shows the effect of hypertonic treatment (*B*) and acidification (*C*) on cell polarization after switching fibroblasts from $-K^+$ to $+K^+$ medium for 1 h. Unlike control cells (*A*), fibroblasts subjected to hypertonic or acid conditions were unable to switch from a radial to polarized morphology.

Discussion

To learn more about the possible role of the coated pit endocytic pathway in cell adhesion, we studied attachment and spreading of potassium-depleted fibroblasts. Previous research demonstrated that depleting potassium from fibroblasts results in reversible arrest of coated pit formation and receptor-mediated endocytosis (Larkin et al., 1983). If the coated pit endocytic pathway was required for cell attachment and spreading, then disruption of coated pits should interfere with these processes.

Our studies with ^{86}Rb showed that fibroblasts in suspension rapidly lost their intracellular potassium when they were incubated in potassium-free medium. Coated pits of these cells became disrupted judging from the clathrin staining distribution. Nonetheless, potassium-depleted fibroblasts attached and spread on FN-coated substrata even though they did not complete the spreading process. Cells in $-K^+$ conditions attained a radial morphology with circumferentially organized actin filament bundles but were unable to make the transition to a polarized morphology with stress fibers. In the radially spread fibroblasts, focal adhesion sites appeared to function as membrane insertion points for circumferentially arranged actin filament bundles, but these sites were much smaller than the focal adhesion plaques which serve as membrane insertion sites for stress fibers in polarized fibroblasts (Izzard and Lochner, 1976; Heath and Dunn, 1978; Geiger, 1982).

The results of potassium depletion on cell adhesion were reversible. Within 1 h after switching $-K^+$ fibroblasts to $+K^+$ medium, cells developed a polarized morphology with stress fibers and prominent focal adhesion plaques containing FN receptors and vinculin. In marked contrast, cells switched from $+K^+$ to $-K^+$ conditions tended to retain their focal adhesions, stress fibers, and polarized shape. This result indicates that in the short term, K^+ ions are more critical for formation than maintenance of polarized cell morphology. This explains why previous studies with potassium-depleted fibroblasts (Larkin et al., 1983; Heuser and Anderson, 1989) did not detect changes in overall cell shape. Also, reversibility of potassium depletion required intracellular not extracellular potassium because addition of 0.1 mM ouabain blocked potassium-uptake and prevented focal adhesion formation and actin reorganization when $-K^+$ cells were switched to $+K^+$ medium.

In vitro, potassium ions have been shown to play a role in actin assembly (Pardee and Spudich, 1982). Therefore, potassium depletion could have had a direct effect on actin assembly. The distribution of actin in potassium-depleted cells indicated otherwise, however. That is, we observed actin assembly, although the filaments were organized circumferentially rather than in stress fibers. In the absence of normal actin function, we would have expected complete inhibition of cell attachment and spreading, not just interference with the transition of cells from radial to polarized spreading.

The idea that the coated pit endocytic pathway might play a role in cell attachment and spreading was inferred first because clathrin-coated pits occur at sites of initial cell attachment and spreading (Woods et al., 1983). As indicated above, however, attachment and radial spreading do not appear to require clathrin-coated pits. Moreover, because formation of focal adhesion plaques at the ends of cell extensions preceded the appearance of clathrin-coated pits along the extensions after fibroblasts were switched from $-K^+$ to $+K^+$ conditions, it also seems unlikely that coated pits play a direct role in assembly of adhesions plaques. Taken together, these findings are consistent with the idea that, at least initially, adhesion and spreading of fibroblasts occurs independently of receptor-mediated endocytosis. Previously, we suggested that fibroblast adhesion reflects attempted "phagocytosis" of the substratum by the cells (Grinnell, 1984), and clathrin-deficient *Dictyostelium discoideum* cells maintain their capacity for phagocytosis (O'Halloran and Anderson, 1992).

The question remains, however, whether the coated pit endocytic pathway is required for the transition from radial to polarized cell spreading, or whether potassium depletion inhibits this transition by interfering with some other process. Significantly, other inhibitors of receptor-mediated endocytosis, hypertonic treatment and acidification, also inhibited

polarization when fibroblasts were switched from $-K^+$ to $+K^+$ conditions. Although indirect, these results suggest a role for receptor-mediated endocytosis in polarization. For instance, cell polarization might require endocytosis and recycling of FN receptors by analogy to the postulated role of receptor recycling as the driving force for cell migration (Bretscher, 1982, 1992). Future studies will be required, however, to clarify this possibility.

We are indebted to Drs. Richard Anderson and William Snell for their helpful comments and suggestions during the course of this research. These studies were supported by National Institutes of Health grant CA14609.

Received for publication 15 May 1992 and in revised form 29 October 1992.

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