Inhibition of Pinocytosis in Rat Embryo Fibroblasts Treated with Monensin

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ABSTRACT Rat embryo fibroblasts cultured in the presence of monensin exhibited an inhibited uptake of horseradish peroxidase. The inhibition was detected after 3 h, after which time the cells became increasingly vacuolated; the concentration of monensin required to inhibit pinocytosis (0.3 μM for half-maximum inhibition at 18 h) was similar to that found by others to inhibit secretion. Both the exchange of 5′-nucleotidase between the membranes of cytoplasmic organelles and the cell surface and the internalization of anti-5′-nucleotidase bound to the cell surface were inhibited by ~90% in monensin-treated cells. The effects of monensin were reversible: cells cultured first with monensin, and then in fresh medium, exhibited control levels of horseradish peroxidase uptake, exchange of 5′-nucleotidase, and internalization of anti-5′-nucleotidase bound to the cell surface. After monensin treatment, the median density of both galactosyl transferase and 5′-nucleotidase increased from 1.128 to 1.148, and the median density of both *N*-acetyl-β-glucosaminidase and horseradish peroxidase taken up by endocytosis decreased from 1.194 to 1.160. The results indicate that monensin is a reversible inhibitor of pinocytosis and, presumably, therefore, of membrane recycling. They suggest that the inhibition of membrane recycling occurs at a step other than the fusion of pinocytic vesicles with lysosomes and is perhaps a consequence of an effect of the ionophore on the Golgi complex.

The rate of membrane internalization by several different cells in culture as a result of fluid-phase pinocytosis exceeds the rate of membrane synthesis by at least an order of magnitude (1), suggesting that membrane is reutilized (recycled). More direct evidence for recycling has been obtained by studies analyzing the binding (and subsequent fate) of antibodies to the cell surface of rat fibroblasts (2, 3) or the flow of iodinated membrane from phagolysosomes to the cell surface in mouse macrophages (4, 5).

At present, the intracellular pathway involved in the return of plasma membrane from lysosomes to the cell surface is unknown. As part of an attempt to identify the organelles involved, we have employed the ionophore monensin, which has been shown to inhibit secretion, probably as a result of an effect on the Golgi complex (6–8). We show here that monensin treatment (*a*) inhibits the uptake of horseradish peroxidase by rat fibroblasts and (*b*) inhibits the exchange of 5′-nucleotidase between the cell surface and the interior. The ionophore has also been shown to alter the density of both lysosomes and membranes of the Golgi complex.

MATERIALS AND METHODS

Rat embryo fibroblasts were cultured as described by Tulkens *et al.* (9). Most experiments were performed with cells at the second subculture, grown to confluence on 35 mm dishes.

The uptake of horseradish peroxidase (HRP) was determined as described by Steinman and Cohn (10). Corrections were made for nonspecific binding, which was <10 ng/mg protein.

To study the exchange of 5′-nucleotidase between the cell surface and cytoplasmic membranes, cells were incubated with anti-5′-nucleotidase at 2°C to inhibit the enzyme activity on the cell surface and were then returned to culture in antibody-free medium as described by Widnell et al. (11). 5′-Nucleotidase activity on the cell surface was determined by assays performed with intact cells, and total activity was determined after detergent solubilization (11). Anti-5′-nucleotidase on the cell surface was assayed by the binding of $F(ab')_2$ fragments of ^{3}H-labeled sheep anti-rabbit $F(ab')_2$ (11).

To determine the transfer of internalized anti-5′-nucleotidase to the cell surface, cells were cultured for 15 h with 10 μl of anti-5′-nucleotidase and then treated with 200 μg/ml nonradioactive sheep anti-rabbit $F(ab')_2$ (11) and returned to culture for 3 h. The binding of ^{3}H-labeled sheep anti-rabbit $F(ab')_2$ was determined as described above. In experiments comparing the effects of monensin on secretion to the effect on the exchange of anti-5′-nucleotidase, cells were recultured for 1 h in medium containing 25 μCi of [^{35}S]methionine, followed by 2 h in medium containing cold methionine. Treatment of the cells with anti-5′-nucleotidase did not affect the incorporation of [^{35}S]methionine into protein.

For analytical cell fractionation, isopycnic density gradient centrifugation of postnuclear supernatant fractions prepared from homogenates was carried out as described by Tulkens et al. (9) and Schneider et al. (12), with minor modification (13). The activity of 5′-nucleotidase (11), galactosyl transferase (14), *N*-acetyl-β-glucosaminidase (15), and succinate dehydrogenase (16) was determined by established procedures. To determine the distribution of the cell surface membrane, cells were treated at 2°C first with anti-5′-nucleotidase and then with ^{3}H-labeled sheep anti-rabbit $F(ab')_2$ (11). The distribution of ^{3}H-labeled sheep anti-rabbit $F(ab')_2$ was determined by direct analysis of the fractions, and the distribution of 5′-nucleotidase activity inhibited by this treatment was estimated by subtracting the activity in individual fractions of gradients prepared from

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antibody-treated cells from the activity in fractions from cells treated with control rabbit serum. The distribution of HRP was determined after its uptake by the cells for 1 h (10). In all cases, 90–95% of the marker constituents in the homogenates was recovered in the postnuclear supernatant, and the recoveries from the isopycnic density gradients ranged from 85 to 100%.

Cells were cultured on 22 mm glass cover slips, fixed in 2% glutaraldehyde, 0.1 M cacodylate (pH 7.2) and photographed in an Olympus CK phase-contrast tissue culture microscope (Olympus Corp., New Hyde Park, NY).

Total cellular ATP levels were determined by the luciferin-luciferase assay (17). The LKB (LKB Instruments, Inc., Rockville, MD) ATP-monitoring reagent was employed and a standard ATP solution was made from an LKB ATP standard. For assay of ATP, the cells were washed in PBS, harvested in 1 ml of H_2O, frozen in liquid N_2, boiled for 5 min and centrifuged at 200 *g* for 10 min. The supernatant was assayed for ATP content.

RESULTS

Pinocytosis of HRP

Confluent cultures of rat embryo fibroblasts exhibited a linear uptake of HRP for at least 2 h (Fig. 1 *A*), which was also directly proportional to the concentration of HRP in the medium (results not shown). Treatment with monensin (1 μM) for 24 h caused an 86% inhibition of this uptake, and the effect of monensin was reversible in that cells which were returned to culture in fresh medium for a further 12 h showed control rates of pinocytosis (Fig. 1 *A*). Protein synthesis was not required for this recovery: cells incubated in the presence of 20 μg/ml cycloheximide, which inhibited amino acid incorporation by 85% during the recovery period, also exhibited control levels of HRP uptake (results not shown).

The effect of monensin on pinocytosis was not immediate: there was essentially no change in the uptake of HRP until the cells had been exposed to the ionophore for at least 3 h (Fig. 1 *B*). The concentration of monensin required for half-maximal inhibition of pinocytosis after 18 h was 0.3 μM (Fig. 2).

Cell Morphology

As described by Ledger et al. (18), monensin treatment caused extensive vacuole formation in the cells. Whereas control cells were essentially free of vacuoles (Fig. 3), cells treated with 1 μM monensin for 18 h were extensively vacuolated (Fig. 4). The number of vacuoles depended on the concentration of monensin in the medium and the time of exposure to the ionophore. Cells treated with monensin and then recultured in a monensin-free medium (as in Fig. 1 *A*) were free of vacuoles and could not be distinguished from untreated cells (results not shown).

Effect of Monensin on Protein Synthesis and ATP Levels

Treatment of rat embryo fibroblasts with monensin caused no marked change in cellular protein synthesis. Cells that were pretreated with 1 μM monensin for 20 h, and then incubated with [^{35}S]methionine, incorporated >80% of the acid-precipitable radioactivity incorporated by control cells (Table I). This small (~20%) decrease in incorporation was observed within 3 h after the addition of monensin to the cultures (data not shown), so that it did not occur at the same time as the decrease of HRP uptake. There was also no detectable change in ATP levels in monensin-treated cells (Table I).

Exchange of 5'-Nucleotidase between the Cell Surface and Cytoplasm

The effect of the ionophore on the exchange of 5'-nucleotidase between the cell surface and the membranes of cytoplasmic organelles was determined. When control cells were treated with anti-5'-nucleotidase at 2°C and returned to culture in antibody-free medium, the activity on the cell surface increased progressively for 12 h (Fig. 5), even though the total activity was unchanged. This confirms results described in detail elsewhere (11). As described in the legend to Fig. 5, monensin

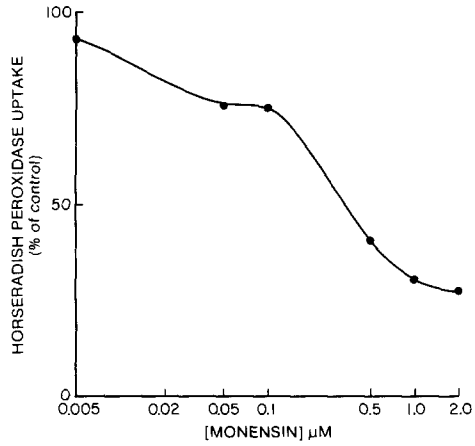


FIGURE 2 Effect of monensin concentration on the uptake of HRP. Cells were preincubated with monensin at the concentrations indicated for 18 h. The uptake of HRP was determined as in Fig. 1 *B*.

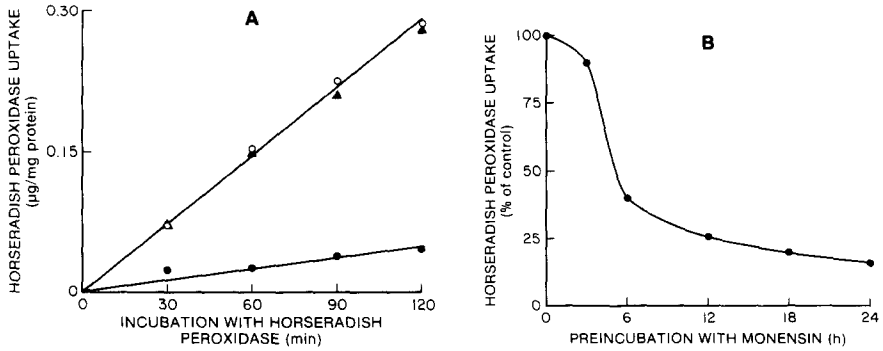
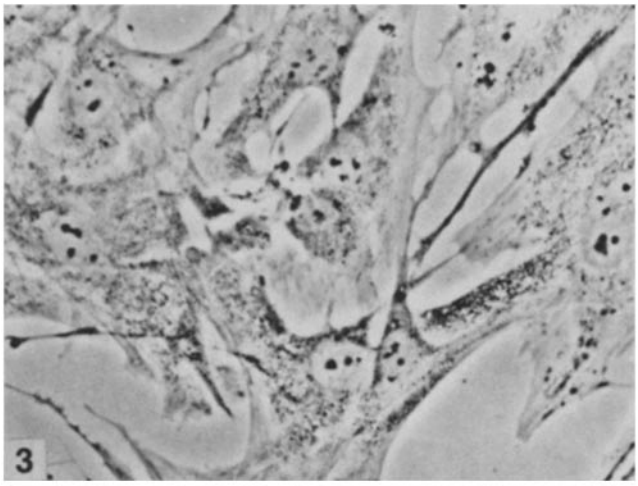
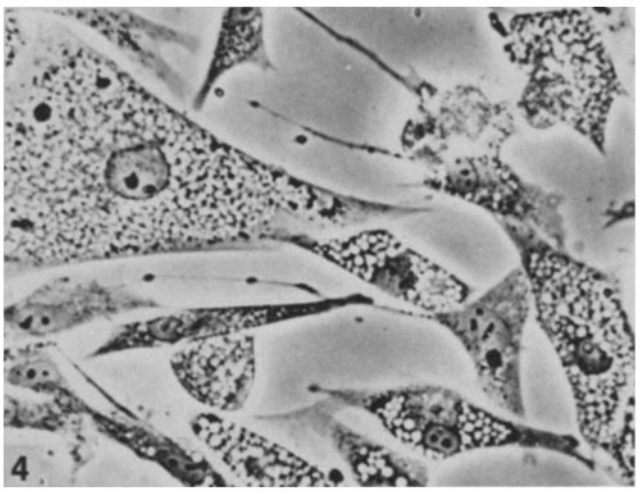


FIGURE 1 Effect of monensin treatment on the uptake of horseradish peroxidase (HRP) by rat embryo fibroblasts. (*A*) Cells were incubated with complete medium containing 1 mg/ml HRP, and the uptake was determined at times indicated. (○) Untreated cells; (●) cells treated for 24 h with 1 μM monensin, which was also present during the incubation with HRP; (▲) cells treated for 24 h with 1 μM monensin followed by 12 h in fresh medium, which was changed at 1, 2, 3, 4, and 8 h. (*B*) Cells were incubated with complete medium containing 1 μM monensin for the times indicated and the uptake of HRP was determined in the presence of 1 μM monensin, as in *A*.





FIGURES 3 and 4 Phase-contrast micrographs of rat embryo fibroblasts. Fig. 3 shows untreated cells. Fig. 4 shows cells treated with 1 μM monensin for 18 h. Note that all cells contain vacuoles but that the number of vacuoles per cell varies considerably. × 375.

treatment caused a 10–20% decrease in 5′-nucleotidase activity but did not affect the distribution of the enzyme between the cell surface and the interior. Treatment with 1 μM monensin for 18 h inhibited the movement of 5′-nucleotidase from cytoplasmic membranes to the cell surface by at least 90% (Fig. 5); it also inhibited the internalization of anti-5′-nucleotidase bound to the cell surface. In control cells, 77% of the anti-5′-nucleotidase bound to the cells at 2°C was internalized after reculture for 12 h in antibody-free medium, as judged by the binding of ^{3}H-labeled sheep anti-rabbit F(ab′)$_2$; in contrast, <10% of the anti-5′-nucleotidase was internalized by monensin-treated cells (Fig. 5). These effects of monensin on the exchange of 5′-nucleotidase and internalization of anti-5′-nucleotidase were also reversed when cells were cultured in fresh medium (Fig. 5). In addition, the time-course of the inhibition of the internalization of anti-5′-nucleotidase was very similar to that of the inhibition of pinocytosis (results not shown).

The effect of monensin was also studied in the first 3 h, during which there was little effect on pinocytosis. Cells were cultured with anti-5′-nucleotidase for 15 h, at which time both the surface and internal 5′-nucleotidase activities were inhibited by 85% (see also reference 11). The cells were then treated with nonradioactive sheep anti-rabbit antibody at 2°C, which inhibited subsequent binding of ^{3}H-labeled sheep anti-rabbit antibody by >90% (Table II). The transfer of internal anti-5′-nucleotidase to the cell surface was estimated by reculturing the cells for 3 h and determining the increase in the binding of ^{3}H-labeled sheep anti-rabbit antibody, essentially as described elsewhere (11). Treatment with monensin for 3 h had no effect on this transfer (Table III). The same cells were labeled with [^{35}S]methionine for the 1st h of reculture; release of acid-precipitable counts from the cells was complete after 2 h in medium containing cold methionine, by which time control cells had released 7% of the total acid-precipitable material into the medium (Table III). Monensin (1 μM) inhibited the release by 50%, as described by others (6, 7). This suggests that the inhibition of pinocytosis was not the direct consequence of an effect of monensin on the transport of secretion vacuoles from the Golgi complex to the cell surface.

Analytical Cell Fractionation of Monensin-treated Cells

The distribution of marker enzymes and constituents was studied in isopycnic density gradients of postnuclear superna-

TABLE I

Effect of Monensin on Protein Synthesis and ATP Levels

Treatment of cells	Acid-precipitable [^{35}S]methionine	ATP
	cpm/mg protein × 10^{-4}	*nmol/min/mg protein*
None	5.2	0.308
1 μM monensin, 20 h	4.3	0.312

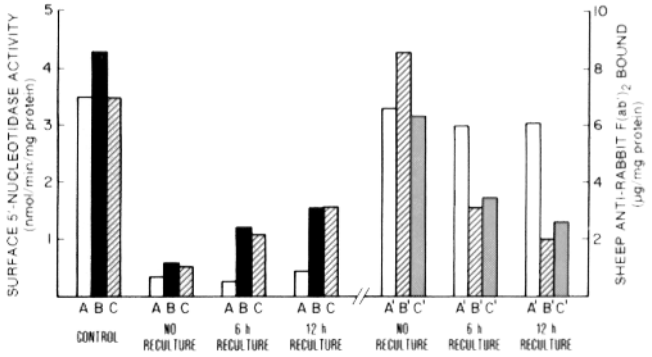


FIGURE 5 Effect of monensin treatment on the exchange of 5′-nucleotidase and anti-5′-nucleotidase between the cell surface and cytoplasmic membranes. The left panel shows the activity of 5′-nucleotidase determined in intact cells after no treatment (*control*), after treatment with anti-5′-nucleotidase at 2° (*no reculture*), and after the same treatment followed by reculture in antibody-free medium (*6 h* and *12 h reculture*). (*A*) Cells were pretreated for 18 h with 1 μM monensin, then treated with antibody, and finally recultured in the presence of monensin; (*B*) cells were pretreated for 18 h in fresh medium, treated with antibody, and then recultured in fresh medium; (*C*) cells were pretreated for 18 h with 1 μM monensin followed by 12 h in fresh medium, then treated with antibody, and recultured in fresh medium. The total 5′-nucleotidase activity (nmol/min/mg protein) in the cells was: *A*, 9.2 and 5.8 after treatment with anti-5′-nucleotidase at 2°C; *B*, 11.0 and 6.5 after anti-5′-nucleotidase treatment; *C*, 9.6 and 6.1 after anti-5′-nucleotidase treatment. The right panel shows cells treated exactly as described above (except for controls) and incubated with 20 μg/ml ^{3}H-labeled sheep anti-rabbit F(ab′)$_2$ for 1 h at 2°C. (*A′*) monensin-treated cells, (*B′*) untreated cells, and (*C′*) cells after recovery from monensin treatment.

tants (Fig. 6). Monensin treatment caused an increase in the median density of 5′-nucleotidase (Fig. 6*A*) and galactosyl transferase (Fig. 6*B*); there was a marked (~70%) decrease in the specific activity of the latter enzyme. To determine the distribution of the membrane from the cell surface, cells were treated at 2°C with anti-5′-nucleotidase followed by ^{3}H-labeled sheep anti-rabbit antibody before fractionation. The distribution of radioactive antibody was not changed by monensin treatment (Fig. 6*C*). As has been shown elsewhere (13), the inhibition of 5′-nucleotidase activity by antibody bound to the cells at 2°C was observed in the same region of the density gradient where sheep anti-rabbit F(ab′)$_2$ was recovered, and monensin treatment had no marked effect on the distribution of inhibited activity (Fig. 6*C*). There was a decrease in the median density of lysosomes as judged by the distribution of both *N*-acetyl-β-glucosaminidase and HRP taken up by pinocytosis (Fig. 6*D* and *E*). Finally, there was no reproducible change in the median density of succinate dehydrogenase (Fig. 6*F*).

DISCUSSION

Our results show that treatment of rat embryo fibroblasts with monensin inhibits pinocytosis of HRP and the exchange of 5′-nucleotidase between the cell surface and the membranes of cytoplasmic organelles. The concentration of monensin in the medium required to inhibit pinocytosis was similar to the concentration found by others (6–8, 18–20) to inhibit secretion or the transport of newly synthesized protein to the plasma membrane. The effect on pinocytosis, however, was much slower, in that little effect was detected until after 3 h. It should be noted that the extensive vacuolation of the cells caused by monensin (reference 7, and Fig. 4) is detected only after ~3 h, suggesting that vacuole formation may be related to the inhibition of pinocytosis.

TABLE II

Effect of Monensin on the Transfer of Anti-5′-nucleotidase from Cytoplasmic Membranes to the Cell Surface

Experimental conditions*				
Anti-5′-nucleotidase	Sheep anti-rabbit F(ab′)$_2$	Reculture	Monensin	^{3}H-Labeled sheep anti-rabbit F(ab′)$_2$ bound‡
				μg/ mg protein
+	−	−	−	2.92
+	+	−	−	0.24
+	+	+	−	0.68
+	+	+	+	0.64

* The procedure is described in Materials and Methods.
‡ The binding of ^{3}H-labeled sheep anti-rabbit antibody was determined at 2°C after the treatment(s) indicated.

TABLE III

Effect of Monensin on Secretion

Experimental conditions*			Activity	
[^{35}S]Methionine	Reculture	Monensin	Cells	Medium‡
			cpm/ mg cell protein × 10^{-4}	
+	−	−	49.9	0
+	+	−	45.4	3.52 (7.1)
+	−	+	38.9	0
+	+	+	37.2	1.34 (3.4)

* The procedure is described in Materials and Methods.
‡ Values in parentheses represent % of total secreted.

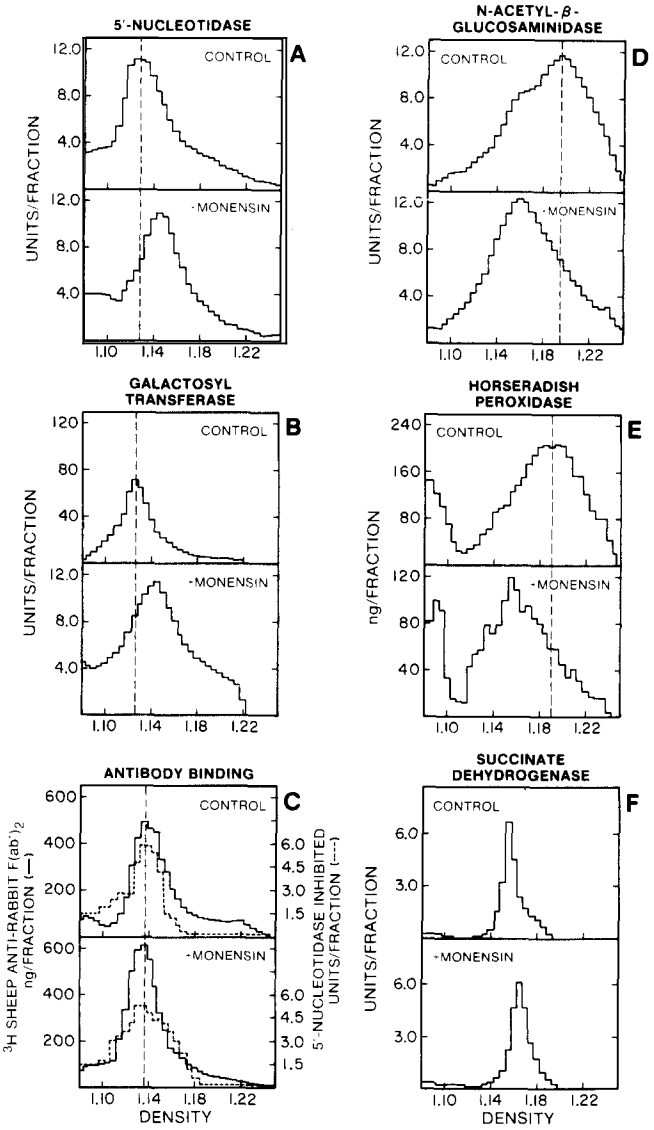


FIGURE 6 Isopycnic density gradient centrifugation of postnuclear supernatants of control fibroblasts and cells treated for 18 h with 1 μM monensin. Experimental details are described in Materials and Methods. The median density of succinate dehydrogenase and antibody bound to cells at 2°C was not altered by monensin treatment; the median density of lysosomes (*N*-acetyl-β-glucosaminidase, or HRP taken up by endocytosis) decreased from 1.194 to 1.160 and the median density of both galactosyl transferase and total 5′-nucleotidase (assayed in the presence of detergent) increased from 1.128 to 1.148.

Even though the uptake of HRP decreased following monensin treatment, most of the internalized protein was delivered to lysosomes, since the distribution of HRP in sucrose density gradients was similar to that of the lysosomal enzyme *N*-acetyl-β-glucosaminidase (Fig. 6). This suggests that there was no marked effect on the fusion of pinocytic vesicles with lysosomes; there was, however, a decrease in the median density of lysosomes.

In experiments described elsewhere (11), it was shown that ≤50% of the 5′-nucleotidase activity in rat embryo fibroblasts is located on the cell surface and that the enzyme on the cell

surface exchanges continually with the enzyme in the membranes of cytoplasmic organelles. In addition, the rate of this exchange was similar to the rate of membrane internalization as a result of fluid-phase pinocytosis (11). Monensin treatment inhibited this exchange as judged both by the transfer of 5′-nucleotidase from the interior of the cell to the cell surface and by the internalization of anti-5′-nucleotidase bound to the cell surface. In fact, the inhibition of the exchange of 5′-nucleotidase (~90%) was consistently greater than inhibition of the uptake of HRP (~75%). This raises the possibility, which is being explored in our laboratory, that a fraction of the HRP may be internalized in vesicles which do not contain 5′-nucleotidase.

It is evident (6–8, 19–21) that the inhibition of intracellular transport by monensin is, at least in part, the result of an effect on the Golgi complex. We have found that monensin treatment caused a marked (~70%) decrease in the specific activity of galactosyl transferase and increased the median density of the surviving enzyme in sucrose gradients. Both of these results suggest a change in the properties of the membranes of the Golgi complex. The median density of 5′-nucleotidase also increased, and the increase was apparently caused by a change in the density of cytoplasmic organelles that contain the enzyme; there was no evident change in the density of the cell surface membrane.

The effect of monensin on rat embryo fibroblasts was thus different from that of chloroquine (12). Although both agents alter the density of lysosomes, chloroquine caused no evident change in the density of 5′-nucleotidase.

Our results also suggest that the major pathway followed by plasma membrane from the lysosome to the cell surface does not involve secretion vacuoles. During the first 3 h of monensin treatment, secretion was inhibited by at least 50%, whereas there was no inhibition of either pinocytosis or the transfer of previously internalized anti-5′-nucleotidase to the cell surface. The functional heterogeneity of the Golgi complex is well-recognized (17, 19, 21, 22), although precise details of the specific role of individual regions have yet to be elucidated.

It is not yet certain whether the vacuoles formed after monensin treatment contain both 5′-nucleotidase and galactosyl transferase. Preliminary cytochemical studies (C. C. Widnell, unpublished results) suggest that the vacuoles contain 5′-nucleotidase but not acid phosphatase, as expected from the results of analytical cell fractionation. The identification of other constituents of the vacuoles must await their isolation in much purer form.

The inhibition of pinocytosis by monensin was observed not only in fibroblasts. A similar inhibition of the uptake of HRP was detected in mouse peritoneal macrophages, but in these cells the lag phase was appreciably shorter (D. K. Wilcox, unpublished results). This would be expected if the length of the lag period were inversely related to the rate of membrane recycling (23).

There was no evidence that monensin caused irrevocable damage to the cells: ATP levels were unchanged and protein synthesis was 80% of that in controls. Even after exposure to 25-fold higher concentrations of monensin for 6 h, protein synthesis was inhibited by <30% (D. K. Wilcox, unpublished results). In addition, the effects of HRP uptake and 5′-nucleotidase exchange were completely reversible.

It is not certain that monensin is acting only at one site in the cells; at a concentration of 25 μM, monensin inhibits the uptake of low-density lipoproteins within 1 h, and the receptors seem to be trapped in lysosomes (24). Although 25 μM monensin inhibited pinocytosis 60% within 1 h, isopycnic density gradient analysis provided no evidence that appreciable 5′-nucleotidase activity was associated with lysosomes (D. K. Wilcox, unpublished results). Even in the present experiments, carried out at a monensin concentration employed by others to inhibit secretion, effects were observed on both the Golgi complex and lysosomes. These findings, together with the observation that the onset of vacuole formation coincided with the inhibition of pinocytosis, suggest that the inhibition of pinocytosis may be the consequence of an inhibition of membrane recycling within the cell rather than a direct effect of monensin on the cell surface.

The results raise the possibility that membrane recycling requires the normal functioning of the Golgi complex; it is also conceivable that the organelle could actually participate in the transport of the plasma membrane constituents from secondary lysosomes back to the cell surface. The advantage of such a pathway is evident: since the Golgi complex is apparently the site at which newly synthesized lysosomal enzymes are segregated from newly synthesized plasma membrane proteins (21, 22), the same segregation could be carried out with recycled constituents. Return of plasma membrane to the cell surface via the Golgi complex would therefore not result in the exocytosis of lysosomal matrix, which would happen if membrane returned directly to the cell surface from lysosomes (see reference 3).

If the Golgi complex is involved in membrane recycling, it should be possible to trace the passage of plasma membrane constituents through the organelle. The reversible nature of the inhibition of pinocytosis by monensin suggests that the ionophore should be useful in analyzing the individual steps involved in the intracellular movement of membrane to and from the cell surface.

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