A Study of the Influence of Mevalonic Acid and its Metabolites on the Morphology of Swiss 3T3 Cells

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ABSTRACT We used two model systems to investigate the effect of compactin, a competitive inhibitor of β-hydroxy β-methylglutarylcoenzyme A reductase, on the shape of Swiss 3T3 cells. We maintained cells in a quiescent state in medium deficient in platelet-derived growth factor (PDGF), or we added PDGF to quiescent cells to initiate traverse through a single cell cycle. In both systems, the cells responded to compactin by acquiring a characteristic rounded shape. Cell rounding seemed to depend on an induced deficiency of mevalonic acid (MVA) since the response could be prevented or reversed by adding MVA to the culture medium. Compactin-induced rounding appeared in PDGF-stimulated cells concomitantly with a compactin-mediated inhibition of DNA synthesis, and both effects had similar sensitivities to exogenous compactin and MVA. However, cell rounding seemed to be unrelated to other, previously observed effects of MVA deficiency. Compactin did not influence the total content of cell cholesterol, and little cholesterol was formed when we added radioactive MVA to round cells to effect shape change reversal. Measurement of the dolichol-dependent glycosylation of cell protein revealed no evidence of dolichol deficiency. In addition, reversal of cell rounding by MVA was not prevented by concentrations of tunicamycin that effectively blocked the incorporation of radioactive mannose into cell protein or by concentrations of cycloheximide that blocked protein synthesis. Taken together, our results suggest a new role for MVA or its products in the maintenance of cell shape.

Two different inhibitors of mevalonic acid (MVA) synthesis, 25-hydroxycholesterol and compactin (ML 236B), have been noted to block replication and induce cell rounding in several cell culture systems. Kandutsch and Chen (1) originally observed these combined effects 24–48 h after treating mouse L-cells with 25-hydroxycholesterol. Kaneko et al. (2) later obtained similar results by adding compactin to L-cells or human skin fibroblasts. Cornell and Horwitz (3) recently reported similar findings after adding 25-hydroxycholesterol or compactin to L_6 myoblasts. These results raise the possibility that MVA may be required not only for cell replication, but also for maintenance of cell shape. However, it remains to be demonstrated that the observed changes in cell shape depend directly on MVA deficiency rather than on some indirect mechanism related to cell proliferation.

Cell-culture systems that allow control of cell proliferation may be used to investigate the role of MVA in maintenance of cell shape. We have been using two such systems in studies of the effects of platelet-derived growth factor (PDGF) on Swiss 3T3 cells (4, 5). In one, cells are maintained in a quiescent but metabolically active state in plasma-derived serum (PDS); in the other, cells are changing both morphologically and metabolically as they traverse through a single cell cycle in response to added PDGF. We have already shown that addition of compactin to PDGF-stimulated cells leads to reduced incorporation of [^{3}H]thymidine into DNA within one cell cycle, and that this effect is caused by MVA deficiency (4). In the present study, we used the same systems to address three main questions related to the effects of compactin on cell shape: does compactin affect cell shape through an inhibition of MVA synthesis? Is compactin-induced rounding a specific consequence of MVA deficiency or does it follow a general pertur-

THE JOURNAL OF CELL BIOLOGY · VOLUME 95 OCTOBER 1982 144-153

bation of cell metabolism? Does the effect of compactin on cell shape depend on a deficiency of a known product of MVA metabolism such as cholesterol or dolichol?

MATERIALS AND METHODS

Materials

DL-mevalonic acid lactone, dolichol, dolichol monophosphate, ubiquinone, and cycloheximide were obtained from Sigma Chemical Co., St. Louis, MO. Cholesterol and cholesteryl oleate were from Applied Science Div., Milton Roy Co., Laboratory Group, State College, PA. Tunicamycin (lot #361-91J-165-A) was the gift of Dr. R. Hamill (Eli Lilly and Co., Indianapolis, IN). (Methyl-^{3}H)-thymidine (6.7 Ci/mmole), (RS)-(5-^{3}H)-mevalonolactone (2 Ci/mmol), L-[^{14}C-(U)]leucine (355 mCi/mmol), 2-[^{14}C]acetic acid (55 mCi/mmol), and D-(2-^{3}H(N))-mannose (14.5 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Compactin (ML-236B) was the generous gift of Dr. A. Endo and was converted to its sodium salt before use as previously described (4). Dulbecco's modified Eagle tissue culture medium (4.5 g glucose/L), medium supplements, and trypsin were obtained from Gibco Laboratories, Grand Island Biological Co., Grand Island, NY.

Phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} was prepared according to Dulbecco (6). Low density lipoproteins (LDL; $1.019 < d < 1.063$) were isolated from two healthy, fasting adult women as previously described (7). Cholesterol:phospholipid liposomes (measured molar ratio = 0.89) were prepared from purified cholesterol and a mixture of soybean phospholipids purified from Intralipid (AB Vitrum, Stockholm, Sweden) (C. Chen et al., in preparation.) The lipids were mixed in $CHCl_3$ and dried under argon. The liposomes were generated in 0.14 M NaCl, 1 mM EDTA by sonication with a titanium probe (Branson Sonic Power Co., Danbury, CT) for 1 h (temperature maintained between 40°C and 50°C). Metal fragments were removed by centrifugation and the liposome suspension was added directly to cultured cells.

Human plasma-derived serum (PDS) deficient in platelet-derived growth factor (PDGF) was prepared from normal fasting plasma as previously described (8). Tissue culture medium containing 5% PDS contained 2.4–2.7 mg/ml protein and 44–68 μg/ml total cholesterol. Partially purified PDGF (CMS III) was prepared as described (8) and added to cells as a solution in 1 mM acetic acid. In all experiments an amount of CMS III was used that contained 5–6 times the mitogenic activity of 5% calf serum when assayed as described (9).

Cell Culture

Stock cultures of Swiss 3T3 cells were maintained essentially as previously described (8), supplemented with nonessential amino acids and sodium pyruvate. Following trypsinization, the cells were replated in medium containing 5% PDS at approximately 125,000 cells/35-mm tissue culture dish (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, CA). The medium was changed on days 2 and 4; the cells became quiescent within 5 d and experiments were begun on day 5 or 6 without further medium changes. Cholesterol mass was determined by gas chromatography as previously described (4), while protein mass was measured using a slight modification of the method of Lowry et al. (10).

Scanning Electron Microscopy

Cells in 35-mm tissue culture dishes were fixed *in situ* in 3% glutaraldehyde (Eastman Laboratory and Specialty Chemicals, Rochester, NY) in either PBS or 0.15 M cacodylate at room temperature for 1 h. Following a thorough rinsing with the same buffer, the cultures were postfixed in 1% osmium tetroxide (Ted Pella, Tustin, CA) buffered with 0.1 M phosphate (Sorensen's) or 0.1 M cacodylate for 30 min and rinsed with distilled water. The plastic dishes were split into quadrants while still wet (done under distilled water) and stained en bloc with 2% aqueous uranyl acetate (Ted Pella) for 30 min. The cells were thoroughly rinsed with distilled water, then dehydrated through graded ethanol (35, 50, 70, 80, 85, 90, 95, and 100% [× 2]). The samples were critical-point dried (Tousimis Research Co., Rockville, MD), coated with gold-palladium, and examined in a JEOL 35C scanning electron microscope (Medford, MA).

Quantification of Cell Rounding

The culture medium was removed from each dish and the cells were fixed directly with 3% glutaraldehyde in PBS. Alternatively, cells were washed once with PBS and fixed with ice-cold 5% trichloroacetic acid (TCA). This latter method of fixation preserved cell shape well enough to allow scoring for roundness and gave estimates of the frequency of round cells comparable to those obtained using glutaraldehyde fixation. Fixation by either technique did not affect the frequency of round cells when compared to that of unfixed control cells. Three randomly selected fields from each of duplicate dishes were photographed (125-fold magnification) on a Leitz Diavert microscope using phase-contrast optics. The negatives were projected onto an 8 inch × 10 inch grid using a Leitz Prado universal projector and all cells were scored (see below) whose nuclei were projected within the grid. The frequency of round cells in each dish was determined using all photographs from that dish. The mean and standard deviation for each experimental group reflect interdish variations in the observed frequency of round cells. Cells were scored as "round" if they had formed phase-bright spheres that were held to the culture dish by cytoplasmic processes. Cells that did not satisfy these criteria were scored as "nonrounded" even when their shape was consistent with incomplete cell rounding. Cells were scored as round or nonrounded without consideration of the size, length and number of processes, or the appearance of the cell surface.

Autoradiography

After 3T3 cells were labeled with [^{3}H]thymidine, the labeling medium was removed and the cells were fixed for 20 min with Holley's fixative (11) and extracted six times with 70% ethanol over a period of 3 d. After they had dried, dishes were coated with 60% (v/v) Kodak NT-B2 emulsion, dried, and exposed 2 wk at 4°C before being developed. The more concentrated emulsion solution is necessary to coat the round cells (Personal communication, S. Hauschka, University of Washington). The dishes were stained lightly with hematoxylin (2 min), washed with water, dehydrated in ethanol, and dried. 500 cells/dish were simultaneously scored for roundness (as described above) and incorporation of [^{3}H]thymidine on a Zeiss microscope at 400-fold magnification. It was essential to adjust the microscope so that round, labeled cells could be distinguished from round, unlabeled cells. For this purpose, we used a dish of cells that had been treated with cytochalasin E and [^{3}H]thymidine to induce rounding in cells that were incorporating [^{3}H]thymidine.

Incorporation of [^{3}H]Thymidine, [^{14}C]Leucine, and [^{3}H]Mannose into TCA-insoluble Material

After incubation of cells with 2 μCi/ml [^{3}H]thymidine or 0.7 μCi/ml [^{14}C]-leucine, the medium was removed and the monolayer washed once with PBS at room temperature. Then 2 ml fresh, ice-cold 5% TCA were added and the cells kept at 0–4°C for at least 20 min. Cultures were photographed for quantification of cell rounding and then reextracted for at least 20 min with additional 5% TCA. After the TCA was removed, the cell monolayer was dissolved in 1.5 ml 0.1 N NaOH and transferred to a liquid scintillation vial. Then 0.5 ml glacial acetic acid and 10 ml Aquasol (New England Nuclear, Boston, MA) were added and ^{3}H in the samples was counted in a Packard Tri-Carb liquid scintillation counter.

Incorporation of radioactivity into TCA-insoluble material by cells incubated with 45 μCi/ml [^{3}H]mannose was determined by the same procedure. Efficacy of the TCA extraction technique was demonstrated by the fact that only 2.5% of the radioactivity remaining after two TCA extractions could be released by further TCA extractions. When PDGF-stimulated cells were labeled with [^{3}H]mannose for 4 h and fractionated by the procedure of Adamany and Spiro (12) at least 97.5% of the TCA-insoluble material had the solubility properties of protein, 0.8% of dolichyl phosphomannose, 0.8% of dolichyl saccharide, and 0.6% of mannose and mannose nucleotides.

Incorporation of Exogenous MVA into Cellular Compounds Comigrating with MVA, Cholesterol, and Cholesteryl Ester

PDGF and 30 μM compactin were added to quiescent 3T3 cells in 100-mm dishes. The cells were photographed 26 h later (to quantify cell rounding) and 500 μM tritiated MVA (98 mCi/mmol) was added. After 110 min the cells were rephotographed and washed once with 20 ml PBS and twice in 10 ml PBS before being scraped into 10 ml PBS using a Teflon policeman. The cells were pelleted at 800 *g* and total MVA uptake was determined by dissolving some of the pellets in 0.5% SDS and counting in 10 ml Aquasol. Cholesterol, cholesteryl oleate, free dolichol, ubiquinone, and MVA standards in acetone were added to the pellets, which were then extracted exhaustively with cold acetone. The pooled acetone extracts were dried under N_2, dissolved in a small volume of acetone, and spotted on 250-μ Silica Gel H thin-layer chromatography plates (Analabs, Inc., Foxboro Co., North Haven, CT) that had been pre-activated for 30 min at 110°C. The plates were developed in heptane:anhydrous diethyl ether:methanol (90:7½:15), which separates cholesterol (R_f.20), and cholesteryl oleate (R_f.60) from MVA (R_f0), dolichol monophosphate (R_f0), free dolichol (R_f.33), and ubiquinone (R_f.47) (Fig. 1). The lipids were visualized with iodine vapor, separately scraped into 10 ml Aquasol, and counted. The acetone-insoluble material was dissolved in 0.5% SDS and counted. Recovery of ^{3}H label from the cells using this

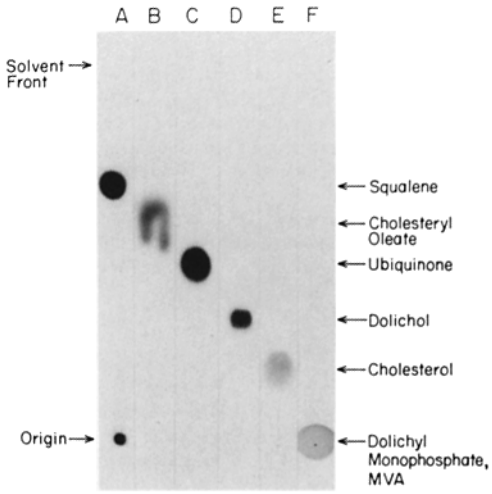


FIGURE 1 Separation of metabolic products of MVA by thin-layer chromatography. Lipid standards in acetone or chloroform:methanol (2:1) were spotted on a silica-gel H plate, separated in heptane: anhydrous diethyl ether:methanol (90:7½:15) and visualized as described. Resolution of the lipid standards was not affected by mixing the standards either with each other or with lipid extracts from 3T3 cells. (*A*) *Dolichol monophosphate*, 20 μg and *squalene*, 43 μg. (*B*) *Cholesteryl oleate*, 500 μg. (*C*) *Ubiquinone*, 100 μg. (*D*) *Dolichol*, 20 μg. (*E*) *Cholesterol*, 500 μg. (*F*) Mevalonolactone, 200 μg.

procedure was 102%. Cholesterol recovery, as measured by preincubating cells for 21.5 h with [^{14}C]cholesterol and determining total incorporation and recoverable cholesterol as described above, was at least 95%.

Incorporation of [^{14}C]Acetate into Cholesterol

Quiescent 3T3 cells were treated with PDGF and various concentrations of compactin. After 22 h, they were pulsed with [^{14}C]acetate (14.5 μCi/ml; 55 mCi/mmol) for 3 h and the incorporation of ^{14}C into digitonin-precipitable sterols was measured as previously described (4).

RESULTS

Effects of Compactin and Mevalonic Acid on the Shape of Quiescent Swiss 3T3 Cells

We used quiescent Swiss-3T3 cells to determine whether the effects of compactin on cell shape depend on the induction of MVA deficiency and whether these same effects show an obligatory requirement for cell proliferation. We obtained the quiescent cells by maintaining sparsely plated 3T3 cells in 5% PDS for 5 d. When viewed by SEM (Fig. 2*A*), these control cells were extremely flat and widely spread over the substratum, and formed a confluent monolayer at a low cell density.

When we treated these quiescent cells with 30 μM compactin, individual cells assumed a round shape (Fig. 2*B*) while remaining attached to the dish by 2–4 cytoplasmic processes. Round cells were present in significant numbers within 20 h of exposure to compactin and their number increased with time (Fig. 3) in each of five experiments. This shape change was completely prevented when 50 μM MVA was added to the cells along with compactin. Compactin-induced cell rounding could also be rapidly reversed by the addition of MVA. When 10 mM MVA was added to quiescent, rounded cells, changes were noticed within 10 min and most cells lost their round shape within 40 min (Fig. 2*C*). Cell spreading continued during the next 6 h until the treated cultures were indistinguishable from control cultures (Fig. 2*D*). These findings provide strong evidence that compactin's effect on the shape of quiescent Swiss-3T3 cells depends on the induction of MVA deficiency.

To rule out the possibility that compactin-induced cell rounding in these quiescent cultures might be confined to a small subpopulation of proliferating cells, we labeled cultures in one experiment with [^{3}H]thymidine for 31 h in the presence or absence of compactin and processed the cells for autoradiography. >50% of the compactin-treated cells became round within this period, whereas only 1% of either compactin-treated or control cells incorporated [^{3}H]thymidine. Thus, development of compactin-induced cell rounding clearly does not require cell proliferation.

Effects of Compactin and Mevalonic Acid on the Shape of PDGF-stimulated Swiss 3T3 Cells

We used PDGF-stimulated Swiss 3T3 cells to study the effects of compactin and MVA on the shape of cells that were progressing through a single cell cycle. When we added PDGF to quiescent control cells in the absence of compactin, the mitogen itself consistently induced dramatic effects that seemed analogous to effects observed previously (13–17) in other proliferating cells. Ruffles and microvilli appeared over the nucleus within 20 min (Fig. 4*A*), and by 45 min some cells had retracted from the substratum and acquired a spherical shape, while remaining attached to the dish by 5–10 cytoplasmic processes (Fig. 4*B*). The entire cell surface, including the surface of these processes, was covered by numerous microvilli. This mitogen-induced cell rounding was transient, and peaked within 90–150 min (Fig. 5*A*) in two experiments in which the kinetics of this rounding were quantified. The maximum percentage of round cells seen at one time varied between 26 and 46%. After rounding up, the cells flattened out again and became slightly more angular and elongated than the original quiescent cells. They then became progressively more elongated with time (Fig. 4*C*) until they entered mitosis, about 30 h after addition of PDGF.

Addition of PDGF and 30 μM compactin to quiescent cells produced two drug-dependent alterations of the morphologic responses to mitogenic stimulation. First, in four experiments compactin affected the early rounding response to PDGF by causing a small but significant increase in the number of round cells seen during the first 5 h (Fig. 5*A*). No effects of compactin on the shape or surface features of individual round cells were identified. As in control cultures, the early rounding was transient and the cells soon reacquired a flat shape. The time needed for all cells to reacquire a flat shape varied between 4 and 7 h in different experiments. The second effect of compactin was to induce cells to round up at the time expected for DNA synthesis. The appearance of the round cells at this time (Fig. 4*D*) differed from that seen earlier in response to PDGF (Fig. 4*B*) in that the cell surface was smoother and fewer cytoplasmic processes attached the cells to the dish. In fact, their appearance resembled that of quiescent cells that had rounded up in response to compactin (Fig. 2*B*).

We observed a similar late cell-rounding when we added compactin at different times after the initiation of cell cycle traverse (Fig. 5*B*). Round cells appeared after a temporal lag whether we had added the drug concomitantly with PDGF or 10 or 20 h afterward. Although the length of the lag seemed to decrease as the cells progressed through the cell cycle, the rate of accumulation of rounded cells was approximately the same.

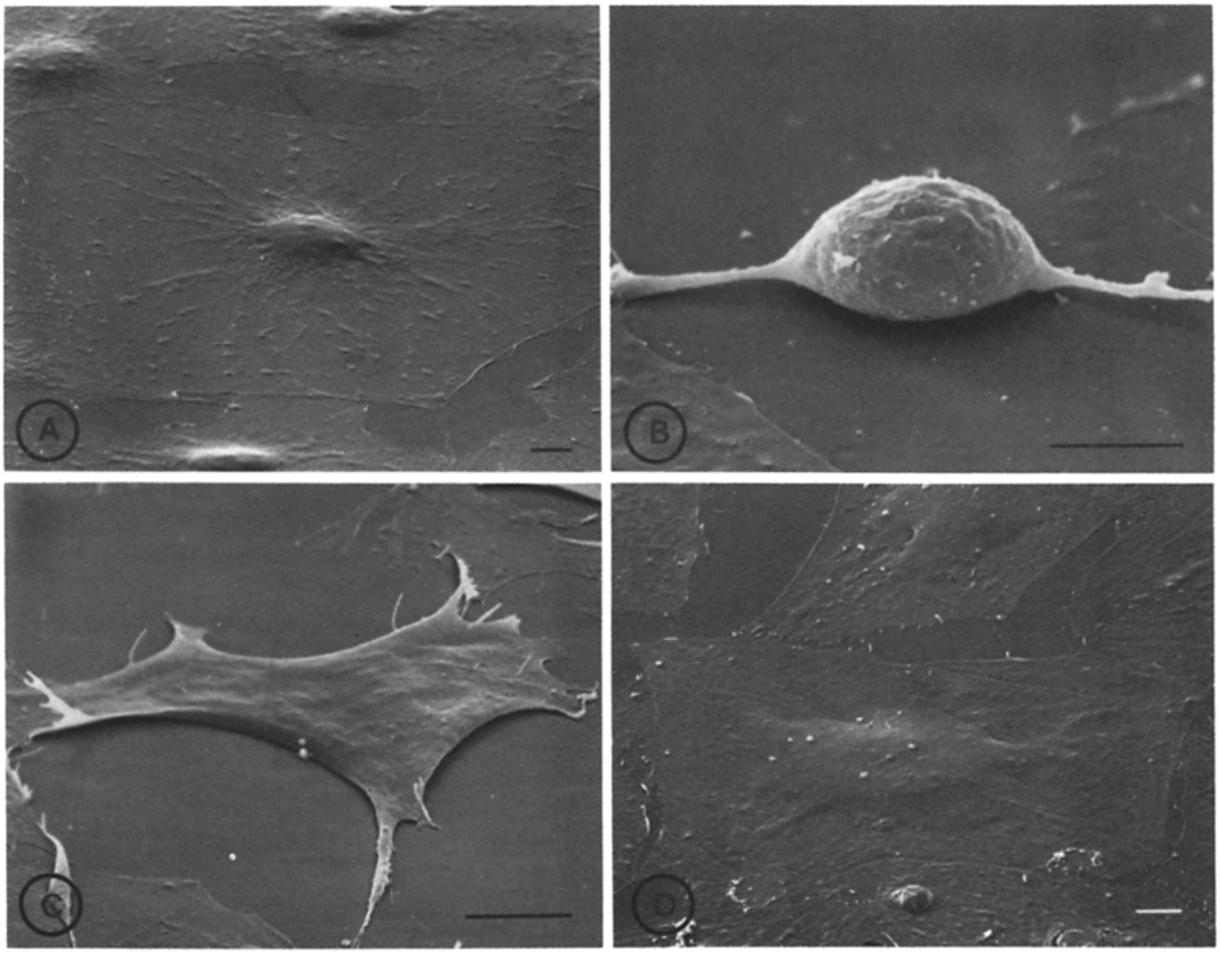


FIGURE 2 Scanning electron micrographs showing the response of quiescent Swiss 3T3 cells to compactin and MVA. Cells were maintained at low cell density in 5% PDS for 5 d before 30 μM compactin was added. 38½ h later, 10 mM MVA was added to the appropriate dishes. At the times indicated, the cells were fixed and processed for SEM as described in Materials and Methods. (*A*) Quiescent control cells. The cytoplasm is thinly spread over a large area and the surface is relatively smooth. × 550. (*B*) Quiescent cells treated with 30 μM compactin for 35 h. × 1900. The spherical cells are held to the dish by 2–3 cytoplasmic processes; few microvilli or blebs are found on the cell surface. (*C*) Quiescent cells treated with 30 μM compactin for 38½ h followed by 10 mM MVA for 45 min. × 1200. Cells have lost their round shape and have begun to spread over the substratum; the cell surface remains smooth. (*D*) Quiescent cells treated with 30 μM compactin for 38½ h followed by 10 mM MVA for 6¼ h. × 650. Cells are now indistinguishable from quiescent controls. Bar, 10 μm.

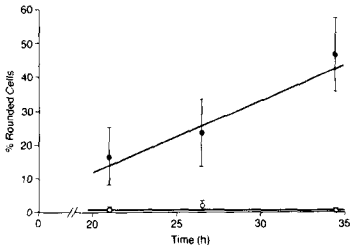


FIGURE 3 Kinetics of compactin-induced cell rounding in quiescent cultures. 30 μM compactin was added to cells that had been maintained in 5% PDS for 5 d. At the times shown, duplicate dishes were fixed and photographed for quantification of cell rounding as described in Materials and Methods. Data shown represent mean ± 1 s.d. (○) no addition; (●) 30 μM compactin.

Thus, compactin affects the early rounding of Swiss 3T3 cells that occurs in response to PDGF, and also induces a second rounding that it is not necessarily limited to a fixed time-point.

The late effect of compactin on the shape of PDGF-stimulated cells could always be prevented by addition of 100 μM MVA to the culture medium. Moreover, the late, compactin-induced cell rounding could always be rapidly reversed by the addition of MVA to the culture medium (Fig. 6). Upon addition of 10 mM MVA, cell spreading began within 5 min, most cells reacquired a flat shape within 20 min (Fig. 4*E*), and the cells became indistinguishable from the elongated, PDGF-stimulated control cells within 150 min (Fig. 4*F*).

The rate of the shape change reversal process depended on the concentration of MVA added. As little as 100 μM MVA caused a significant number of cells to lose their round shape within 5 h, but higher concentrations were required to effect more rapid shape change reversal. Two lines of evidence suggest that this dependence on concentration was related to the relative impermeability of the cells to added MVA. First, the rate of net uptake and metabolism of exogenous MVA was comparable to the rate of fluid pinocytosis (as measured by the rate of [^{14}C]sucrose uptake) and was not saturated between 5 and 275 μM added MVA (not shown). Second, little MVA was taken up and metabolized by cells during shape change reversal. Thus, when we added 500 μM ^{3}H-labeled MVA to round cells 25.5 h after adding PDGF and 30 μM compactin, reversal of cell rounding occurred during a 2 h interval (35% rounded

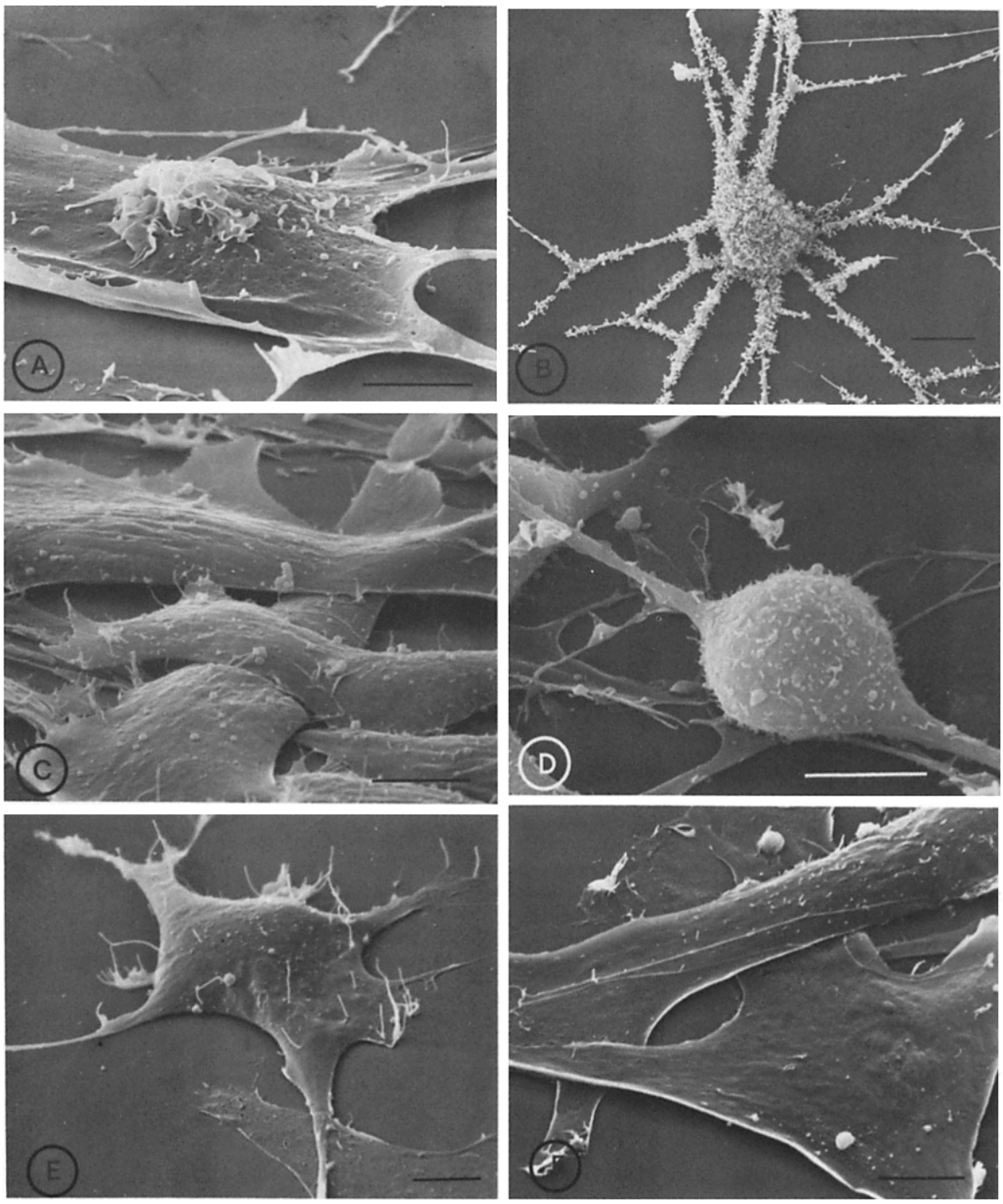


FIGURE 4 Scanning electron micrographs showing the responses of quiescent cells to PDGF, compactin, and MVA. PDGF ± 30 μM compactin was added at 0 h to cells that had been maintained in 5% PDS for 5 d. 10 mM MVA was added to appropriate dishes 26 h later. At the times indicated, cells were fixed and processed for SEM as described in Materials and Methods. (*A*) Cells fixed 22 min after addition of PDGF alone. × 1800. Marked surface ruffling and microvillus formation are seen above the cell nucleus. (*B*) Cells fixed 2 h after addition of PDGF alone. × 1050. The spherical cell is held to the dish by multiple cytoplasmic processes and the cell surface displays extensive microvillus formation. (*C*) Cells fixed 24 h after addition of PDGF alone. × 1500. The cells have assumed a thickened, highly elongated shape whereas the surface is again relatively smooth. (*D*) Cells fixed 24 h after addition of PDGF and 30 μM compactin, showing spherical cells held to the dish by 2-4 cytoplasmic processes. × 2000. The cell surface is generally smooth, although a few small blebs are present. (*E*) Cells treated with PDGF and 30 μM compactin for 26 h and then treated for 20 min with 10 mM MVA. × 1400. Most cells have assumed a thickened, elongated shape with a smooth cell surface. (*F*) Cells treated with PDGF and 30 μM compactin for 26 h and then treated for 150 min with 10 mM MVA. × 1300. Cells have reassumed an elongated shape indistinguishable from that of control cells (*C*). Bar, 10 μm.

cells → 4% rounded cells) while radioactivity recovered as MVA or other products was equivalent to approximately 150 × 10^6 molecules of MVA per cell. For comparison, PDGF-stimulated 3T3 cells contained cholesterol equivalent to ~520,000 × 10^6 molecules of MVA per cell at this time (calculated from Table I).

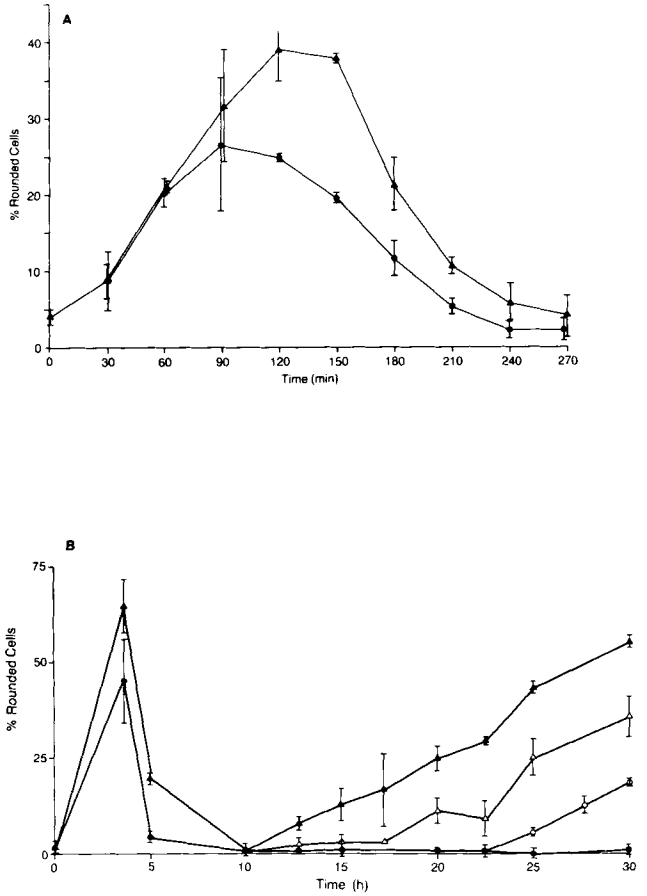


FIGURE 5 Kinetics of cell rounding in PDGF-stimulated cultures. (*A*) Enhancement of PDGF-induced cell rounding by 30 μM compactin. PDGF ± 30 μM compactin was added at 0 min to quiescent cells. At times shown, dishes were fixed and the percentage of round cells quantified as described in Materials and Methods. (●) PDGF only; (▲) PDGF plus 30 μM compactin. (*B*) Kinetics of the late, compactin-induced cell rounding in PDGF-stimulated cells. PDGF was added at 0 h to quiescent 3T3 cells. 30 μM compactin was added either with PDGF (▲), 10 h later (△), or 20 h later (○). Control dishes received PDGF alone (●). At the times shown, duplicate dishes were fixed and the percentage of round cells was quantified as described in Materials and Methods. The early cell rounding was not necessarily maximal at the 3¾ h time-point. Data represent the mean ± 1 SD of duplicate dishes.

Relation between Compactin's Effects on Cell Shape and DNA Synthesis

Having found that compactin causes rounding of PDGF-stimulated cells at about the time expected for DNA synthesis, we investigated the relation between this effect and the compactin-induced block in DNA synthesis that we had previously demonstrated in the same cell system (4). We examined the sensitivity of the two effects to compactin and MVA, the temporal correlation between the two effects, and the frequency of occurrence of the two effects in individual cells. When we treated PDGF-stimulated cells in three experiments with varying concentrations of compactin (Fig. 7*A*), we found that the two effects showed a similar sensitivity to the drug. Furthermore, similar concentrations of MVA were required to prevent the induction of each effect by 15 μM compactin (Fig. 7*B*) in the same experiments. When we compared, in two experiments, the time of appearance of the two effects in cells that had been simultaneously treated with PDGF and compactin, the effects became apparent concomitantly (Fig. 8), and the round cells were more numerous than cells that were prevented from incorporating labeled thymidine into DNA. These observations suggested that the two effects might be linked. Three other observations suggested, however, that this linkage may not be tight or invariable. First, autoradiographic assessment of the incorporation of [^{3}H]thymidine into individual cells always revealed some round cells that had not been prevented from incorporating the label (not shown). Second, the correlation between compactin-induced rounding and blocked incorporation of labeled thymidine in individual cells varied significantly both between three experiments and as a function of time within two experiments. Finally, the two effects showed somewhat different kinetics when compactin was added 15 h after PDGF in a single experiment: within the first few h after addition of the drug, more cells showed blocked incorporation of labeled thymidine into DNA than showed cell rounding. In summary, both effects seem to be similarly sensitive to MVA availability, but the tightness of their linkage within individual cells remains to be established.

Relation of Compactin-induced Cell Rounding to Cholesterol, Dolichol, and Protein Synthesis

Inhibition of MVA synthesis in dividing cells has been reported to affect cellular cholesterol mass (1, 3) and dolichol-dependent glycosylation (18). To investigate the possibility that compactin-induced changes in cell cholesterol content might account for the influence of the drug on cell shape in our experiments, we studied sterol biosynthesis in relation to compactin-induced cell rounding, measured the content of cholesterol in PDGF-stimulated cells as a function of the addition of compactin or MVA, and tested the ability of added cholesterol to prevent or reverse a compactin-induced shape change. When we added varying concentrations of compactin at 0 time to PDGF-stimulated cells, then pulsed the cells with [^{14}C]acetate

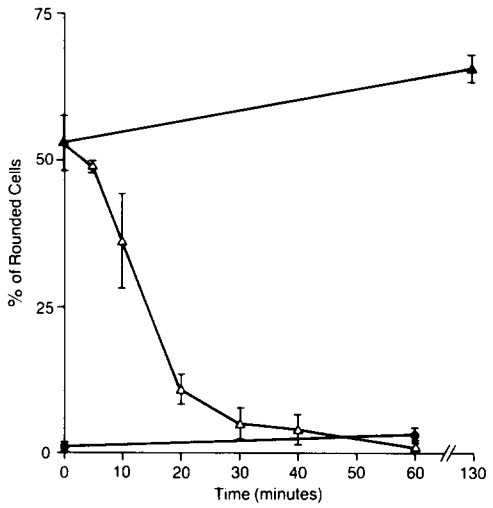
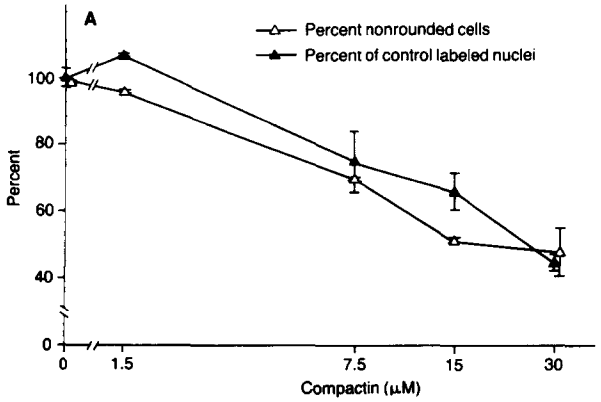


FIGURE 6 Kinetics of reversal of compactin-induced cell rounding by MVA in PDGF-stimulated cultures. PDGF ± 30 μM compactin was added to quiescent cells. After 26 h, 10 mM MVA was added to the appropriate dishes (0 min). Cultures were fixed at the times shown and the percentage of rounded cells quantified as described in Materials and Methods. (●) PDGF only; (▲) PDGF + 30 μM compactin; (△) PDGF + 30 μM compactin + 10 mM MVA. Data points represent the mean ± 1 SD of duplicate dishes.



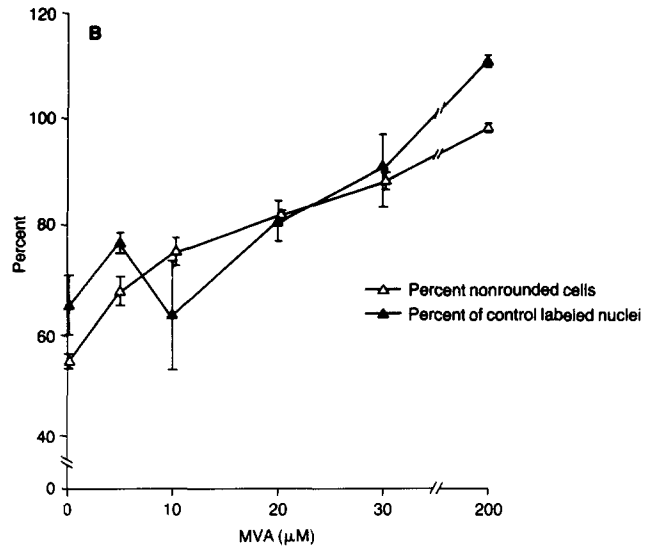


FIGURE 7 Dependence of cell shape and [^{3}H]thymidine incorporation on compactin and MVA. (*A*) PDGF and various concentrations of compactin were added to quiescent 3T3 cells to begin the experiment. Dishes were pulsed with 2 μCi/ml [^{3}H]thymidine for 30 min 23 h later, then fixed and analyzed by autoradiography as described in Material and Methods. (*B*) In the same experiment, PDGF, 15 μM compactin, and various concentrations of MVA were added to quiescent 3T3 cells. Cells were pulsed with 2 μCi/ml [^{3}H]thymidine for 30 min 23 h later and all cells were fixed and analyzed by autoradiography as described. In both figures the number of cells incorporating [^{3}H]thymidine (▲) is expressed as a percentage of the "PDGF only" control whereas cell rounding is expressed as the percentage of nonrounded cells (△). Data represent mean ±1 SD of duplicate dishes.

for 2.75 h, 22.25 h later we found that 1 μM compactin inhibited the incorporation of the label into digitonin-precipitable sterol by 91% without causing cell rounding (not shown). Only when higher concentrations of compactin (15–30 μM) were used, and inhibition of the incorporation of labeled acetate into cell sterols approached 99% was extensive cell rounding seen. When we added 50 μM radioactive MVA to compactin-treated cells to prevent cell rounding, <1% of the estimated 370 × 10^6 molecules labeled MVA that were retained by the cells was converted into unesterified or esterified cholesterol, whereas at least 85% of the labeled MVA was converted into other products. Moreover, when we added 500 μM radioactive MVA to effect reversal of the shape change, the mass of cholesterol synthesized during reversal (calculated from the specific activity of the radioactive MVA added and the retained activity that comigrated with cholesterol) corresponded only to ~0.01% of the total cell cholesterol mass. These results make it seem unlikely that the effects of compactin on cell shape in our system depend on the inhibitory effects of the drug on cholesterol biosynthesis. Our measurements of cholesterol mass in PDGF-stimulated cells support this conclusion. Cells, maintained in culture in the presence of plasma lipoproteins, increased their cholesterol mass by at least 50% within 24 h in response to the addition of PDGF (Table I), an increase that was not altered by 15 μM compactin ±MVA. Finally, we were unable to prevent or reverse cell rounding by adding cholesterol to the culture medium as an ethanolic suspension (15 μg/ml), as LDL (216 μg total cholesterol/ml), or as liposomes comprised of 1:1 unesterified cholesterol:phospholipid (15 μg cholesterol/ml).

To investigate the potential relation of dolichol-dependent

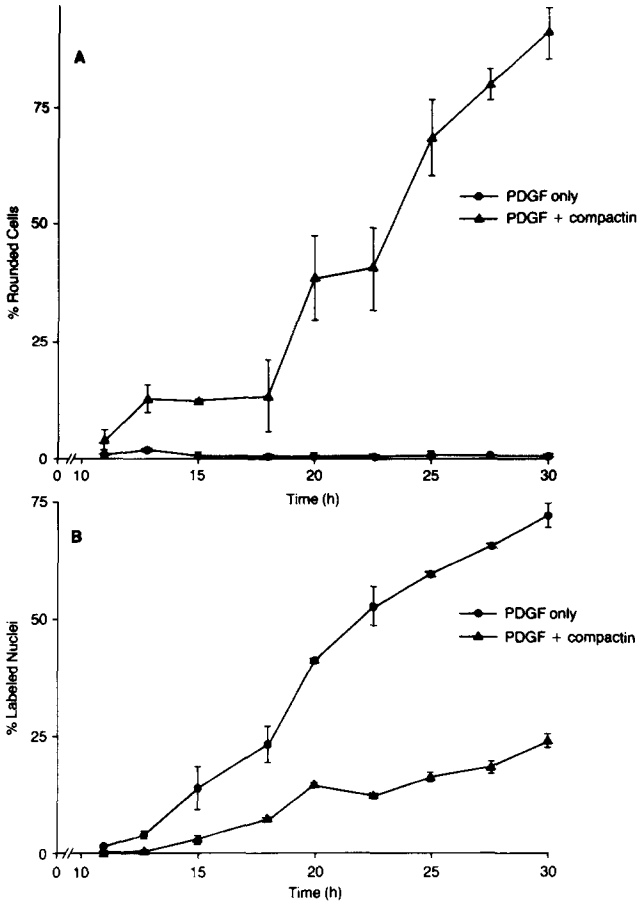


FIGURE 8 Autoradiographic analysis of the temporal correlation between the effects of compactin on cell shape and onset of DNA synthesis in individual cells. PDGF and 0.5 μCi/ml [^{3}H]thymidine ±30 μM compactin were added to quiescent 3T3 cells at 0 h. At the times shown, dishes were processed for autoradiography as described in Materials and Methods. Following development of the autoradiograms, individual cells were simultaneously scored for roundness and incorporation of [^{3}H]thymidine. (*A*) Accumulation of round cells with time in cultures treated with PDGF alone (●) or PDGF + 30 μM compactin (▲). (*B*) Onset of [^{3}H]thymidine incorporation in cells treated with PDGF alone (●) or PDGF + 30 μM compactin (▲). Data points represent mean ±1 SD of duplicate dishes.

TABLE I

Influences of PDGF, Compactin, and MVA on Swiss 3T3 Cell Cholesterol and Protein Mass

Additions	Cholesterol mass	Protein mass
	pg/cell	*μg/10⁵ cells*
Quiescent control	34.9 ± 2.4	57.3 ± 1.3
PDGF	54.9 ± 2.5	125.6 ± 1.2
PDGF, compactin	53.2 ± 1.1	117.0 ± 7.5
PDGF, compactin, MVA	51.6 ± 4.3	126.6 ± 5.1

Cell number, cholesterol mass, and protein mass were determined on separate dishes of 3T3 cells that had been extensively washed with PBS. Quiescent control dishes were harvested before addition of PDGF, whereas all others were harvested 24 h after addition of PDGF ± 15 μM compactin ± 100 μM MVA. Values shown are mean ±1 SD from triplicate dishes.

TABLE II

Effects of PDGF, Compactin, MVA, and Tunicamycin on Cell Shape and Incorporation of [³H]Mannose into TCA-insoluble Material

Additions	% Rounded cells		Radioactivity in TCA-insoluble material
	20½ h	22⅔ h	
			net cpm‡
Quiescent control	7.7 ± 1.6	12.1 ± 6.3	183 ± 28
PDGF	1.0 ± 1.1	0.7 ± 0.6	3430 ± 213
PDGF, compactin	32.6 ± 5.3	32.4 ± 5.9	3127 ± 322
PDGF, compactin, 100 μM MVA (0)*	1.5 ± 0.9	3.6 ± 3.2	3349 ± 536
PDGF, compactin, 500 μM MVA (20½)*	32.6 ± 5.3	1.1 ± 1.0	3253 ± 190
PDGF, compactin, tunicamycin	24.3 ± 8.2	22.2 ± 7.9	1321 ± 165
PDGF, compactin, tunicamycin, 500 μM MVA (20½)*	24.3 ± 8.2	0.2 ± 0.4	1320 ± 89

PDGF ± 30 μM compactin was added to quiescent 3T3 cells to begin the experiment. 5 μg/ml tunicamycin was added to appropriate dishes 19⅔ h later. In some dishes, 100 μM MVA was added along with compactin to prevent cell rounding, whereas in other dishes 500 μM MVA was added 20½ h after compactin to reverse cell rounding. 20½ h after the addition of PDGF the appropriate dishes were washed with PBS and fixed with 5% TCA, while 45 μCi/ml [³H]mannose (14.5 Ci/mmol) was added to all remaining dishes. These remaining dishes were washed and fixed 130 min later. All dishes were photographed for quantification of cell rounding, and the incorporation of [³H]mannose into TCA-insoluble material was quantified as described in Materials and Methods. Values shown represent the mean ± SD from triplicate dishes.

* Numbers in brackets denote the time of addition of MVA (in hours) after addition of PDGF.

‡ Blank of 233 cpm has been subtracted from all values.

glycosylation reactions to compactin-induced rounding of PDGF-stimulated cells, we measured the incorporation of radioactive mannose into cell glycoproteins. PDGF itself caused at least a 19-fold stimulation of mannose incorporation into TCA-insoluble cell material, and concentrations of compactin that caused 32% of the cells to round up in 20 h did not alter this response (Table II). Furthermore, there was no change in the incorporation of radioactive mannose into TCA-insoluble cell material when exogenous MVA was used to prevent or reverse cell rounding. Finally, concentrations of tunicamycin that blocked mannose incorporation into protein by 62% failed to enhance compactin-induced cell rounding or block the reversal of cell rounding by MVA. Similar results were obtained in three other experiments. Thus, a block in dolichol

TABLE III

Inability of Cycloheximide to Inhibit Reversal of Compactin-induced Cell Rounding

Additions	% Rounded cells	[¹⁴C]Leucine incorporation
		cpm
PDGF	2 ± 1	n.d.
PDGF, compactin	57 ± 7	n.d.
PDGF, compactin, MVA	11 ± 1	6,258 ± 511
PDGF, compactin, cycloheximide, MVA	6 ± 7	356 ± 38

PDGF ± 13 μM compactin was added to quiescent 3T3 cells. 10 μg/ml cycloheximide was added after 23½ h, and 200 μM MVA and 0.67 μCi/ml ¹⁴C-leucine (final specific activity 840 μCi/mmol) were added 30 min later. After an additional 2 h all dishes were washed with PBS, fixed with 5% TCA, and photographed. The incorporation of ¹⁴C into TCA-insoluble material and cell rounding were quantified as described in Materials and Methods. Values shown are mean ± standard deviation of triplicate dishes. *n.d.*, not determined.

synthesis leading to deficient mannosylation of cell protein seems an unlikely cause of the effects of MVA deficiency on cell shape in our cultures.

Another product of MVA metabolism is isopentenyl tRNA. The specific role of this type of tRNA is unclear, and the effects of deficient isopentenylation of Swiss 3T3 cell tRNA remain to be investigated. However, a block in the synthesis of isopentenyl tRNA could not have had a generalized effect on protein synthesis in our system since compactin had no effect on the increase in protein mass in response to PDGF (Table I) and little effect on the rate of protein synthesis (not shown). Moreover, when we allowed PDGF-treated cells to round up in the presence of compactin, preincubated them with 10 μg/ml cycloheximide, and then added MVA to effect shape change reversal, the cycloheximide did not prevent the reversal process in three experiments though it inhibited protein synthesis by more than 94% (Table III).

DISCUSSION

Most of the experiments performed in this investigation focus on the possibility that compactin-induced rounding of Swiss 3T3 cells reflects a specific requirement for MVA or its products in the maintenance of cell shape. In considering this possibility, several questions arise. First, does compactin's effect on cell shape depend on prevention of the synthesis of MVA or on some nonspecific action of the drug? Our experiments clearly show that addition of MVA to the culture medium can both prevent and rapidly reverse compactin-induced cell rounding. Moreover, other experiments (unpublished) have revealed that a number of additional aliphatic acids, including butyric acid, do not have this effect. These findings strongly suggest that compactin induces rounding of Swiss 3T3 cells by specifically preventing the synthesis of MVA.

A second question that arises is whether prevention of the synthesis of MVA causes cell rounding through a specific effect or through a general perturbation of cell metabolism. Data originally obtained by Kaneko et al. (2) speak against the latter possibility, because these investigators found that protein synthesis, RNA synthesis, and nonsterol lipid metabolism are not affected by compactin in concentrations as high as 5 μg/ml (12.8 μM). Moreover, we have found (unpublished experiments) that the incorporation of [³⁵S]sulfate and [³H]glucosamine into macromolecules is not affected by 15 μM compactin, whereas [¹⁴C]leucine incorporation is only slightly affected.

Finally, our finding that exogenous MVA rapidly initiates reversal of cell rounding also argues against the possibility that treatment of nondividing Swiss 3T3 cells with compactin has a general effect on cell function within 30 h. In contrast, 3T3 cells in proliferating cultures begin to detach from the dish after being incubated in the presence of 15 μM compactin for 48 h (4). This effect is temporally well separated from cell rounding, and may reflect or presage cell death.

A different question relates to the identity of the active factor involved in cell shape maintenance. It could be MVA itself, or a known product of MVA metabolism such as cholesterol, dolichol, ubiquinone, or isopentenyl tRNA (19). Two previous studies (1, 3) of cells treated with 25-hydroxycholesterol or compactin revealed an association between altered cell shape and a reduced content of cell cholesterol. However, there are several reasons for believing that cholesterol was not the active factor in our experiments. First, compactin did not alter the total content of cell cholesterol, presumably because we maintained the cells in a medium that contained plasma lipoproteins and studied the effects of MVA deficiency before most of the cells had a chance to divide. Second, adding extra cholesterol to the medium neither prevented nor reversed compactin-induced cell rounding. Third, only a very small amount of cholesterol was synthesized from ^{3}H-MVA in our shape change reversal experiments. Fourth, low concentrations of compactin inhibited sterol biosynthesis by 90% without causing cell rounding. Only when higher concentrations of the drug were used, and inhibition of sterol biosynthesis approached 99%, was appreciable cell rounding observed. This partial dissociation of compactin's effects on cholesterol biosynthesis and cell rounding is of special interest because it resembles the partial dissociation of compactin's effects on cholesterol biosynthesis and DNA synthesis observed previously (4). This study of DNA synthesis provided strong evidence that the synthesis of cholesterol during the G_1 phase of a single cell cycle is not a prerequisite for the synthesis of DNA during S. A related point of interest is that considerably higher concentrations of compactin are required to inhibit the biosynthesis of ubiquinone and isopentenyl tRNA by human skin fibroblasts than are required to inhibit the biosynthesis of cholesterol (19). This latter observation is compatible with the possibility that a product of MVA metabolism other than cholesterol may be the active factor in our system. The evidence against a role for cholesterol will, however, not be complete until at least two further possibilities are investigated: (*a*) a very small pool of endogenously synthesized cholesterol might be serving as an intermediate in the synthesis of the active factor, and (*b*) the shape change might be associated with a redistribution of cholesterol among intracellular membranes, driven by the absence of some other product of MVA. There is at present no evidence either for or against these possibilities.

Our results also speak against the possibility that prevention of the synthesis of MVA indirectly induced cell rounding by limiting the synthesis of dolichol. We initially considered this possibility in view of the report (18) that 25-hydroxycholesterol can block the synthesis of dolichol and reduce dolichol-dependent transfer of mannose to cell glycoproteins in aortic smooth muscle cells. Moreover, tunicamycin, an inhibitor of dolichol-dependent glycosylation reactions, has been shown to alter the shape of both normal and virally transformed 3T3 cells (20). In our experiments we sought evidence for a functional dolichol deficiency by measuring the rate of [^{3}H]mannose incorporation into TCA-insoluble material. Since alterations of the size or specific activity of the intracellular mannose pools might have been affected by compactin or MVA we also examined the effect of tunicamycin on both cell rounding and [^{3}H]mannose incorporation. Neither compactin nor exogenous MVA affected the incorporation of radioactive mannose into TCA-insoluble material, and tunicamycin itself did not prevent MVA-dependent reversal of cell rounding in concentrations that blocked the incorporation of labeled mannose by 62%. Thus, if a deficiency of dolichol occurred in our system, it probably did not cause cell rounding by limiting formation of dolichol phosphate and thus dolichol-dependent glycosylation of cell proteins. We presume again that the critical difference between our experiments and those in other laboratories depends on our use of a cell culture system that permits the control of cell cycle traverse.

Our results provide little direct information about the possible relation between ubiquinone or isopentenyl tRNA and compactin-induced cell rounding. Both metabolites of MVA seemed to be synthesized when we added radioactive MVA to rounded cells to effect shape change reversal. Therefore, compactin might have caused a deficiency of both. However, neither metabolite has been previously implicated as a determinant of cell structure.

A viable possibility that remains to be investigated is that a product of MVA metabolism, yet to be identified, is the active factor involved in compactin-induced cell rounding. In shape-change reversal experiments (unpublished) that employed radioactive MVA, we found that at least 25% of the label taken up by the cells was incorporated into unidentified products that were insoluble in acetone, chloroform:methanol (2:1), or chloroform:methanol:water (10:10:3), but were soluble in chloroform-phenol. This material clearly merits further investigation.

One question not directly examined in this investigation concerns the mechanism of action of the putative active metabolite of MVA. The active factor might influence the organization of the cytoskeleton and/or the attachment of the cytoskeleton to cell membranes. Alternatively, it might influence cell attachment to the substrate. The recent report of Cavenee et al. (21) is of interest in this regard because these investigators found that pretreatment of cells with 25-hydroxycholesterol inhibits reattachment of the cells to other cells or to artificial surfaces following release by trypsinization.

A final question concerns the role of MVA or its product(s) in the physiologic regulation of cell structure. The cell systems developed in this investigation should be useful in studies of this role since cell shape is clearly under endogenous control and the cells can sequentially express several distinct shapes in response to PDGF even though culture conditions remain constant. Furthermore, we anticipate that the rapid and synchronous reversal of compactin-induced cell rounding after addition of exogenous MVA will be particularly advantageous to investigators interested in studying cell spreading, cytoskeletal assembly, and endogenous specifiers of cell shape.

We are pleased to acknowledge Stephanie Lara for preparing the scanning electron micrographs and autoradiograms and Elaine Raines and Billie Fortune for preparing the CMS III.

This work was supported by the Howard Hughes Medical Institute, R.J. Reynolds Industries, Inc., National Institutes of Health grants HL18645, RR00166, AM13970 and GM07266, and by the Deutsche Forschungsgemeinschaft, Federal Republic of Germany.

This work was presented in part at the meeting of the American

Society for Cell Biology, Anaheim, California, November, 1981 (*J. Cell Biol.* 91 [2, Pt. 2]:297*a* [Abstr.]).

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Received for publication 29 December 1981, and in revised form 26 April 1982.

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