Insulin-induced Formation of Ruffling Membranes of KB Cells and Its Correlation with Enhancement of Amino Acid Transport

KIYOTA GOSHIMA, AKIRA MASUDA, and KATSUSHI OWARIBE
Institute of Molecular Biology, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464, Japan. Dr. Masuda's present address is Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan.

ABSTRACT Insulin induced the formation of ruffling membranes in cultured KB cells (a cell strain derived from human epidermoid carcinoma) within 1–2 min after its addition. The ruffled regions were stained strongly with antibody to actin but not that to tubulin. Pretreatment of KB cells with agents disrupting microfilaments (cytochalasins), but not with those disrupting microtubules (colcemid, nocodazole, and colchicine) completely inhibited the formation of ruffling membranes. Pretreatment of KB cells with dibutyryl cyclic AMP, but not with dibutyryl cyclic GMP, also inhibited the formation of ruffling membranes. Addition of insulin enhanced Na^+-dependent uptake of a system A amino acid (α-amino isobutyric acid; AIB) by the cells within 5 min after the addition, and decreased the cyclic AMP content of the cells. Treatments that inhibited insulin-induced formation of ruffling membranes of KB cells also inhibited insulin-induced enhancement of their AIB uptake. From these observations, the mechanism of insulin-induced formation of ruffling membranes and its close correlation with AIB transport are discussed.

Various kinds of nonmuscle cells, such as fibroblasts (1, 21, 24, 58), macrophages (39, 50), osteoclasts (64), glial cells (5), aortic endothelial cells (2), megakaryocytes (35), and epithelial cells (8, 23), show ruffling movements (see also reviews in references 58, 62). Ruffles are rapid movements with irregular fluctuation of protrusion and withdrawal of the margins of the cell surface. The ruffled areas of these nonmuscle cells are known to contain actin, α-actinin, fimbrin, and filamin (see review in reference 62). In areas of ruffling, networks of microfilaments composed of actin are prominent, but myosin, tropomyosin, and microtubules are depleted or absent (see review in reference 62). Membrane rufflings are believed to be closely concerned with locomotive movements (58), although the detailed relationship is still unknown (1, 8, 21). Membrane rufflings are also believed to be concerned with phagocytosis (12, 39, 40, 50, 64) and cytoplasmic spreading on the glass substratum (23, 57) of various kinds of nonmuscle cells. But it is still obscure whether membrane rufflings perform other cellular functions and whether some hormonal factors affect their formation and activity.

While culturing KB cells (a cell strain derived from human epidermoid carcinoma) as sparse monolayers in flasks, we unexpectedly found that almost all KB cells formed ruffling membranes immediately after the culture medium was replaced with fresh medium. Thus, we examined what component(s) of the fresh medium was necessary for the formation of ruffling membranes and found that insulin was especially effective. Next we examined what physiological function(s) the insulin showed concomitant with the formation of ruffling membranes and found that increased uptake of an amino acid by the KB cells induced by insulin was closely correlated with formation of ruffling membranes. We studied the transport of amino acids by KB cells in sparse monolayer cultures using α-aminoisobutyric acid (AIB)[1] as a model amino acid. AIB is transported in a number of cells, including KB cells, (34) by the so-called A system, or alanine-preferring system for neutral amino acids (see reviews in references 4, 28). After its uptake, AIB is not incorporated into protein or catabolized and hence its transport can be studied without being complicated by intracellular metabolism (41). A stimulatory effect of insulin on AIB uptake has been reported for a variety of biological preparations including skeletal and cardiac muscle,

[1] *Abbreviations used in this paper:* AIB, α-amino isobutyric acid; BES, *N,N'*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; PBS, phosphate-buffered saline.

bone, lymphoid tissue, uterine tissue, fat cells, fibroblasts, smooth muscle cells, hepatocytes, mammary epithelial cells, thyroid and kidney cortex tissue (see reviews in references 19, 51). The transport of system A amino acids, such as AIB, is known to depend on the extracellular Na^+ concentration, i.e., on the electrochemical gradient on Na^+ (see reviews in references 19, 51). But the molecular mechanism by which insulin stimulates AIB transport is unknown (see review in reference 28). An especially interesting problem is whether microfilaments and microtubules are involved in regulating AIB transport. Therefore, we also studied the effects of agents that disrupt the cytoskeletal system of microfilaments and microtubules on the formation of ruffling membranes and uptake of AIB.

MATERIALS AND METHODS

Agents: Crystalline beef insulin (28 U/mg) (containing no glucagon) was a gift from Shimizu Seiyaku (Shizuoka, Japan). Cytochalasin B, D and nocodazole was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI); colcemid, AIB, 2-deoxy-D-glucose, 3-O-methyl-D-glucose, carbamylcholine, norepinephrine, trizma base (Tris), firefly lantern extract, and prostaglandins E_1 and E_2 from Sigma Chemical Co. (St. Louis, MO); adenosine 3′,5′-cyclic monophosphoric acid(cyclic AMP), N^6-2′-O-dibutyryl adenosine 3′,5′-cyclic monophosphoric acid (dibutyryl cyclic AMP), guanosine 3′,5′-cyclic monophosphoric acid (cyclic GMP) and N^2-2′-O-dibutyryl guanosine 3′,5′-cyclic monophosphoric acid (dibutyryl cyclic GMP) from Boehringer-Mannheim, GmbH (Waldhof, Federal Republic of Germany); a Good's pH buffer (16), *N,N′*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) and a Ca^{2+}-chelator, glycoletherdiaminetetraacetic acid (EGTA) from Dojindo Co. Tokyo; fetal bovine serum and calf serum from Flow Laboratories (McClean, VA); α-[1-^{14}C]-aminoisobutyric acid, 2-deoxy-D-[1-^{3}H]-glucose, 3-O-D-[1-^{3}H]-methylglucose and $^{45}CaCl_2$ from New England Nuclear (Boston, MA).

Cytochalasin B, D, nocodazole, prostaglandins E_1 and E_2 were dissolved in DMSO. These agents were stored at −20°C as stock solutions. The final concentration of DMSO used in the present work as 1%, a concentration that had no apparent effect on the formation of ruffling membranes or uptake of amino acid.

Preparations of Antibodies to Actin and Tubulin: Actin antibody was prepared and characterized as described previously (44). Tubulin antibody was prepared as follows. Tubulin was isolated from bovine brain by repeated cycles of polymerization according to the method of Shelanski et al. (52). It was purified by phosphocellulose column chromatography (63) and then preparative PAGE in the presence of SDS, as described by Stephens (55). The final preparation was dialyzed against distilled water, then phosphate-buffered saline (PBS). Emulsions of 0.5 mg of tubulin in an equal volume of Freund's complete adjuvant were administered subcutaneously and intramuscularly to rabbits. The animals were immunized four to five times at 2-wk intervals and were bled 2 wk after the last injection. The immunoglobulin fraction was obtained from their sera by two precipitations with ammonium sulfate. The tubulin antibody gave a single precipitin line with purified and crude tubulin on immunodiffusion. As actin and tubulin show little species specificity, their antibodies cross-reacted with actin and tubulin, respectively, in a variety of cell types.

Antiserum to rabbit IgG was raised in a goat. Highly purified rabbit IgG in Freund's complete adjuvant was injected subcutaneously and intramuscularly four times at 2-wk intervals, and further boosters of the antigen in complete adjuvant were given when necessary. The immunoglobulin fraction from the antiserum was prepared as described above. The immunoglobulin was conjugated with fluorescein isothiocyanate by the standard method (26). The conjugated goat anti-rabbit IgG was purified by gel filtration on Sephadex G-25 and then diethylaminoethyl-cellulose column chromatography.

Cells: KB cells (derived from a human epidermoid carcinoma) and BALB/c 3T3 cells (derived from a mouse whole embryo) were kindly supplied by Dr. K. Shimada (Aichi Cancer Center) and Dr. A. Hakura (Osaka University), respectively. These cells were grown as monolayers in square flasks (Falcon Labware (Oxnard, CA)) at 37°C in Eagle's MEM (9) supplemented with 10% calf serum (for KB cells) or 10% fetal bovine serum (for 3T3 cells). Subculture was performed every 2–4 d with 0.01% tryspin (1:250 [Difco Laboratories, Inc., Detroit, MI]) (for KB cells) or with 0.25% trypsin (for 3T3 cells) after the cultures had reached confluence.

Standard Procedure for Induction of Membrane Ruffling of KB Cells: Tryspin-dissociated KB cells were inoculated into plastic Petri dishes (35 mm in diameter, Falcon Labware) at a density of 2×10^5 cells per dish, and then cultured at 37°C in Eagle's MEM supplemented with 10% calf serum under a water-saturated atmosphere of 5% CO_2 in air. Within 3 h, the KB cells became attached and spread on the surface of the dishes. After incubation for 20 h, the culture medium was removed by aspiration and the dishes were rinsed four times with a nutrient-deficient solution containing 130 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, and 10 mM BES at pH 7.4 (adjusted with Tris) (called "standard solution" in this paper). Next, the dishes with attached cells were incubated at 37°C for 5 h to allow depletion of intracellular amino acids before ruffling and transport experiments were begun. After incubation in the standard solution for 5 h at 37°C the cells were examined with an inverted phase-contrast microscope. The temperature was kept at 37 ± 1°C or 25 ± 1°C using a thermostatically controlled chamber during the period of examination. To examine what cations were essential for insulin-induced formation of ruffling membranes, we used a Ca^{2+}-free solution of 130 mM NaCl, 5.4 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA, and 10 mM BES at pH 7.4 (adjusted with Tris) or Na^+-free solution of 130 mM choline chloride, 5.4 mM KCl, 2 mM $CaCl_2$, and 10 mM BES at pH 7.4 (adjusted with Tris).

Insulin was dissolved in distilled water and a volume of <0.02 ml of the concentrated solution was added to cultures in 2.0 ml of standard solution with gentle shaking to give the final concentrations indicated in the text.

Fluorescence Microscopy: Immunofluorescence microscopy was performed essentially as described by others (3, 17, 32, 61). Briefly, coverslips with adhering KB cells were rinsed four times with PBS solution (140 mM NaCl, 10 mM Na_2HPO_4, 3 mM KH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgSO_4$, pH 7.2), fixed with 3.5% formaldehyde, and then treated with acetone at 0°C. The coverslips were then incubated with actin or tubulin antibody for 60 min at 37°C. The preparations were washed three times with PBS and then incubated with fluorescein isothiocyanate-conjugated goat antibody against rabbit IgG for 60 min at 37°C. The coverslips were rinsed with PBS and mounted on a glass slide in PBS-glycerol (1:4) solution, and the cells were examined with a Nikon Fluophot microscope or an Olympus fluorescence microscope (Model BHF) equipped with epifluorescence optics.

Scanning Electron Microscopy: The coverslips with adhering cells were fixed with 2.5% glutaraldehyde, dehydrated, and dried by the critical-point method. Dried specimens were coated with gold-palladium and examined with a scanning electron microscope (Model JSM-F7).

Measurement of ^{14}C-AIB Uptake: After cultivation of trypsin-dissociated cells in Eagle's MEM supplemented with 10% calf serum for 1 d, the culture medium was replaced with 2 ml of standard solution. The cells were incubated in this solution for 5 h at 37°C and then in fresh standard solution for 1 h at 25 ± 1°C. Cells had to be incubated at 25°C because transport experiments were performed in a room kept at 25 ± 1°C. Next, the solution was rapidly replaced with 2 ml of standard solution supplemented with 0.5 mM AIB (containing 1 μCi/ml of ^{14}C-AIB). Radioactivity uptake was determined by removing the radioactive solution after incubation in the presence and absence of insulin for 5, 10, 20, or 30 min at 25 ± 1°C, and washing the cells four times with a total of 10 ml of washing solution consisting of PBS supplemented with 5 mM AIB. The total washing procedure took <13 s. Almost all cells remained attached to the surface of the petri dishes throughout the experiments. The washed cells were then suspended in 0.6 ml of 0.1 N NaOH solution and the radioactivity of an aliquot of the NaOH solution was measured in a liquid scintillation spectrometer. In each series of experiments, a "zero time" assay was performed by adding radioactive solution to the cultures and then immediately (within ~2 s) washing the cells. Since the zero time value was nearly the same as that obtained for ^{14}C-AIB uptake by the culture dishes containing no cells, that is, since the zero time value represented nonspecific binding of ^{14}C-AIB on the culture dish, this zero value was subtracted from all observed values of ^{14}C-AIB uptake. Another aliquot of the NaOH solution was used to measure the protein content by the method of Lowry et al. (36). Uptakes of radioactive Ca, 2-deoxy-D-glucose and 3-*O*-D-methylglucose were measured in the same way.

Measurement of ^{14}C-AIB Release: KB cells were incubated at 37°C in standard solution supplemented with 0.5 mM AIB (containing 2 μCi/ml of ^{14}C-AIB) for 7 h to load them with the steady state concentration of AIB. The solution was then replaced with standard solution containing 0.5 mM AIB (but not ^{14}C-AIB) with or without insulin. The cells were incubated at 25 ± 1°C for 10, 20, 30, 40, or 60 min and then the remaining intracellular ^{14}C activity was determined as described above. Release of ^{45}Ca was also measured in the same way as described above.

Other Measurements: The ATP content of KB cells was measured by the firefly luciferase method (53) with a liquid scintillation spectrometer (Aloka Co., Tokyo, Japan; model LSC) using a ^{3}H channel. The cyclic AMP content of KB cells was measured by a radioimmunoassay method (see review in reference 54) using a Yamasa assay kit (Yamasa Shoyu K. K., Chiba, Japan).

RESULTS

Insulin-induced Formation of Ruffling Membranes

When KB cells were cultured in flasks under sparse monolayer conditions, ~30–50% of them had a ruffling membrane, but in most cases, this was restricted to a small portion of the cell margin. Immediately after addition of fresh culture medium at 37°C, almost all KB cells had prominent ruffling membranes. This observation suggested that some component(s) of the fresh medium had induced the formation of ruffling membranes. Thus, we examined which component(s) was active and tested various agents that had probably been present in the fresh medium and consumed by KB cells during cultivation for several days.

These studies required an experimental system in which the degree of induction of ruffling membranes could be measured quantitatively and reproducibly. We needed to reduce the percentage of cells showing ruffling before the addition of test agents. We found that when the KB cells were incubated in solution containing no glucose, serum, vitamins, or amino acids, the percentage of cells showing ruffling was significantly low (see Table I). Thus, as described in Materials and Methods, after the cells had been preincubated at 37°C in standard solution for 5 h, test agents were added to this solution.

As shown in Table I, glucose and insulin, and especially insulin, were active in the induction of ruffling. Phase-contrast microscopy after addition of insulin (Fig. 1), showed ruffles of KB cells as dark, usually somewhat sinuous lines that changed in shape, dimension, and position fast enough to be detectable by direct observation, as noted by Abercrombie et al. (1). Observations using a time-lapse TV video recorder system clearly showed the same rapid movements with irregular fluctuation of protrusion and withdrawal of the margins of the cell surface as those described by others (1, 8, 42). As shown in Fig. 1 *B*, ruffling membranes were observed all around the margin of single isolated KB cells, but only at the free margins of groups of cells and not in regions of close cell-to-cell contact (Fig. 1 *D*), as noted previously (5, 23, 46; see also review in reference 58). The insulin-induced ruffling of KB cells did not seem to be related to cell locomotion, because cell positions in the petri dishes did not change significantly during 2–60 min incubation after addition of 6 μg/ml of insulin. The ruffling membranes gradually disappeared as a function of time even in the continuous presence of insulin (see Fig. 4). During this period, the cells assumed nearly the same shape as those before insulin addition.

Scanning electron microscopies of KB cells before (Fig. 2 *A*) and after (Fig. 2 *B*) insulin addition showed that insulin caused prominent tucking of the cell surface in the region of the cell margin, which is a characteristic of membrane ruffling, while no tucking was observed before insulin addition.

Insulin and glucose did not induce the formation of ruffling membranes on cultures BALB/c 3T3 cells under the same conditions as for KB cells.

TABLE I

Percentage of KB Cells Showing Membrane Ruffling after Addition of Agents to Standard Solution

Agent	Incubation time	Ruffling cells
	min	%*
None		16 ± 8‡
Glucose (1 mM)	3–10	40 ± 8
(10 mM)	3–10	60 ± 10
Insulin (0.06 μg/ml)	3–10	62 ± 6
(0.6 μg/ml)	3–10	79 ± 5
(6 μg/ml)	3–10	84 ± 5
Dibutyryl cAMP (0.1 mM)	15–20	16 ± 8
(0.5 mM)	15–20	13 ± 7
(1 mM)	15–20	11 ± 5
cAMP (1 mM)	15–20	20 ± 10
Dibutyryl cGMP (1 mM)	15–20	16 ± 8
cGMP (1 mM)	15–20	16 ± 6
Na-*n*-butyrate (5 mM)	15–20	19 ± 9
2-Deoxy-D-glucose (1–5 mM)	5–20	17 ± 4
3-*O*-Methyl-D-glucose (1–5 mM)	5–20	18 ± 8
Norepinephrine (10^{-7}–10^{-5} M)	5–20	14 ± 8
Carbamylcholine (10^{-5}–10^{-3} M)	5–20	16 ± 5
Prostaglandin E_1 (0.01–5 μg/ml)	5–20	17 ± 5
Prostaglandin E_2 (0.01–5 μg/ml)	5–20	18 ± 6

* After preincubation of KB cells in standard medium for 5 h at 37°C, agents were added and the percentage of single isolated KB cells showing ruffling 3–20 min later at 37°C was calculated. Cells on which more than half of the cells margin showed ruffling were scored as ruffling cells.

‡ Values are means ± SD for three independent experiments. 200–300 cells were examined for each value.

Immunofluorescence Micrography of KB Cells

The distributions of actin and tubulin were examined by immunofluorescent staining of KB cells before and after treatment with insulin (Fig. 3). Most KB cells were stained diffusely with antibody to actin before addition of insulin (Fig. 3 *A*). In a small percentage (probably <10%) of the KB cells, antibody to actin stained long straight bundles running across the cells before insulin addition (Fig. 3 *B*). These straight bundles correspond to the "stress fibers" or "actin cables" reported previously in other cells (14, 32). On treatment with 0.6 μg/ml of insulin for 5 min, the regions of membrane ruffling were stained strongly with anti-actin (Fig. 3 *C*). Antibody to tubulin stained wavy networks all over the cells before or after addition of insulin, as reported previously for other cells (43). But the regions of membrane ruffling were not stained with antitubulin.

Effects of Insulin Concentration and Temperature on the Time Course of Insulin-induced Formation of Ruffling Membrane

As shown in Fig. 4, on addition of 0.6 or 6 μg/ml of insulin, the percentage of single isolated KB cells on which more than half of the cell margin showed ruffling increased after a lag time of ~1 min (at 37°C) or ~2 min (at 25°C) and the percentage attained a maximum within 5 min of incubation of the cells at both 37°C and 25°C, and then gradually decreased to the original level. The percentage of cells containing ruffling membranes and the duration of the maximum plateau increased with an increase in the insulin concentration (Fig. 4 *A*).

As shown in Fig. 4 *B*, the maximum percentage of cells forming ruffling membranes at 25°C was lower than that at 37°C. The duration of the maximum plateau, however, was longer at 25°C (60 min) than that at 37°C (20 min). At 10°C, insulin did not cause the formation of ruffling membranes.

Effects of Some Agents on Insulin-induced Formation of Ruffling Membranes of KB Cells

We examined what cations in the reaction solution were

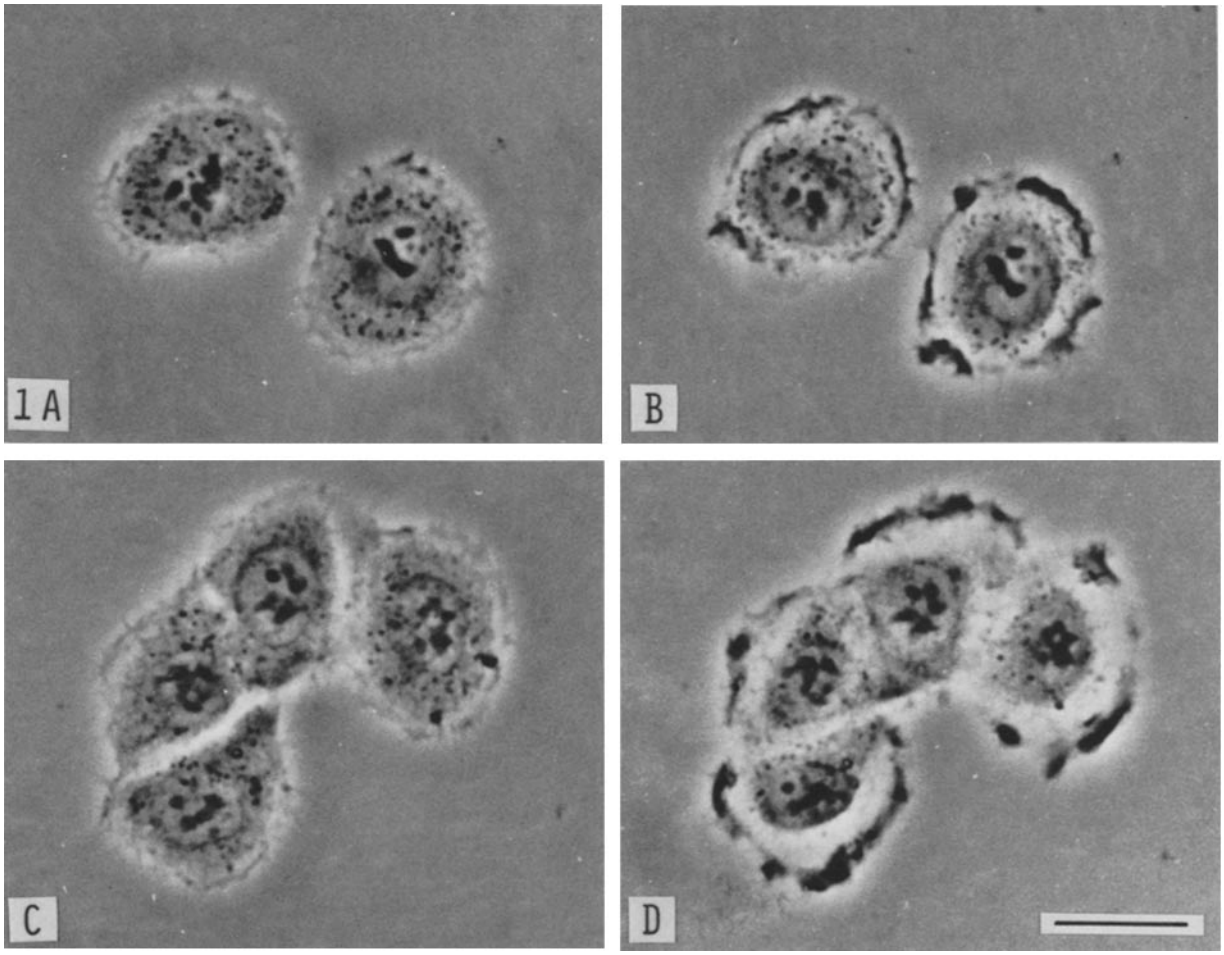

FIGURE 1 Formation of ruffling membranes of single isolated KB cells (*A* and *B*) and a group of four adjacent cells (*C* and *D*) seen by phase-contrast microscopy. (*A* and *C*) Before addition of insulin; (*B* and *D*) 5 min after addition of insulin (0.6 μg/ml). The same cells are shown in *A* and *B* and in *C* and *D*, respectively. Bar, 20 μm. × 870.

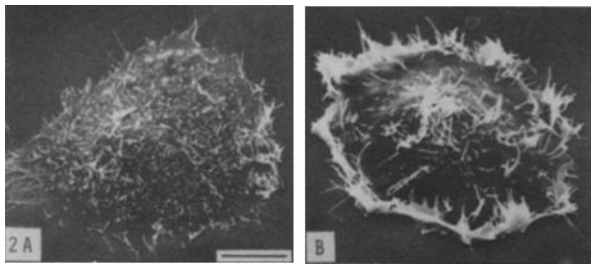

FIGURE 2 Ruffling membranes of a single isolated KB cell seen by scanning electron microscopy (*A*) Before addition of insulin; (*B*) 5 min after addition of insulin (0.6 μg/ml). Bar, 5 μm. × 1,600.

essential for insulin-induced formation of ruffling membranes. After the cells had been preincubated in standard solution (containing no Mg^{2+}), Ca^{2+}-free solution (containing Mg^{2+}) or Na^+-free solution (containing choline$^+$, Ca^{2+} and Mg^{2+}) for 5 h at 37°C, insulin (0.6 μg/ml or 6 μg/ml) was added to the solution. Our scores of the percentages of cells containing ruffling membranes 10 min after addition of insulin showed no significant effects. However, we did not analyze the effects of cations on the rate of ruffling formation or the ruffling movements themselves.

Dibutyryl cAMP or cytochalasin B and D prevented the insulin-induced formation of ruffling membranes of 37°C (Table II) or 25°C. When KB cells were preincubated in standard medium for 5 h and then incubated in the same solution supplemented with dibutyryl cAMP or cytochalasin B or D for 15 to 20 min, most of the KB cells did not form ruffling membranes on addition of insulin (0.6 or 6 μg/ml) (Table II). Immunofluorescence microscopy showed that the actin cables disappeared on cytochalasin B or D treatment of KB cells or other fibroblastic cells, such as 3T3 cells and mouse melanoma cells. Cytochalasin B and D also caused disruption of microfilaments such as arborization and aggregation of actin of KB cells. On the other hand, colchicine, colcemid, and nocodazole (7), which are known to disrupt microtubules (see reviews in references 15, 62), only slightly inhibited the formation of insulin-induced ruffling membranes, although the resulting ruffling movements were weaker than those observed in untreated cells. We also confirmed by immunofluorescence microscopy that the tubulin networks disappeared on treatment of KB cells with colchicine, colcemid, or nocodazole. Why these agents affected both the formation of ruffling membranes and the ruffling movements is still unknown (see Discussion).

Effect of Insulin on AIB Transport of KB Cells

AIB uptake increased to a saturation level with time, but it was linear during the first 20 min, and thus, measurement of its uptake in the first 20 min showed its initial rate of uptake. This initial rate of AIB uptake in the absence of external Na^+

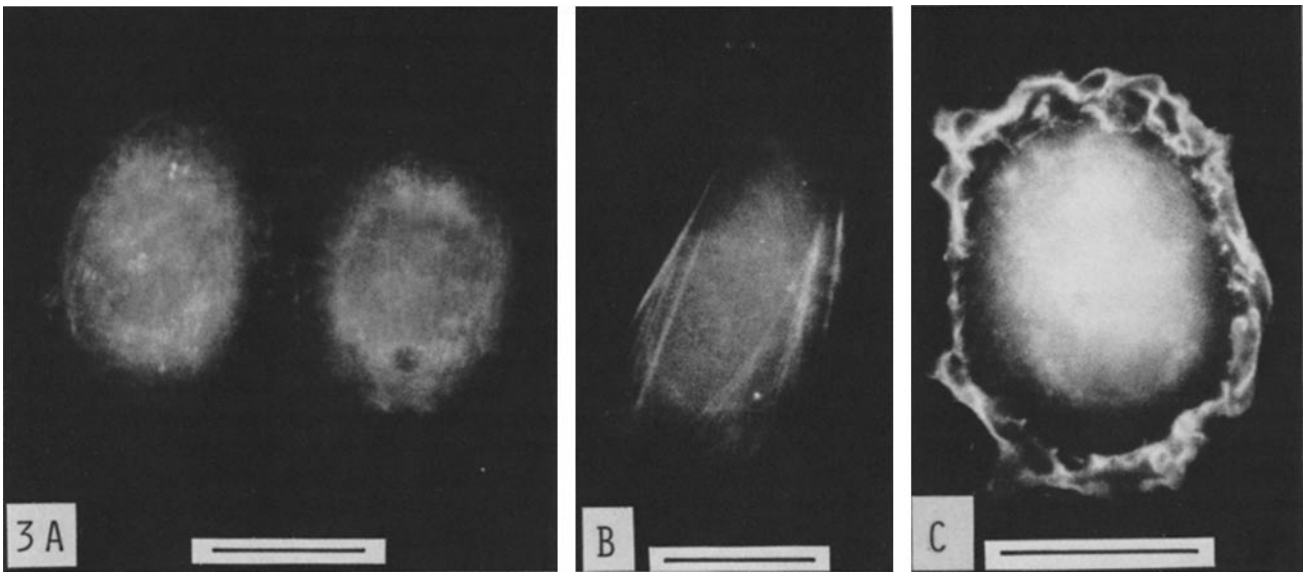

FIGURE 3 KB cells stained with actin antibody seen by indirect immunofluorescence microscopy. (*A* and *B*) Before addition of insulin; (*C*) 5 min after addition of insulin (0.6 μg/ml). Bar, 20 μm. (*A* and *B*) × 1,000. (*C*) × 1,350.

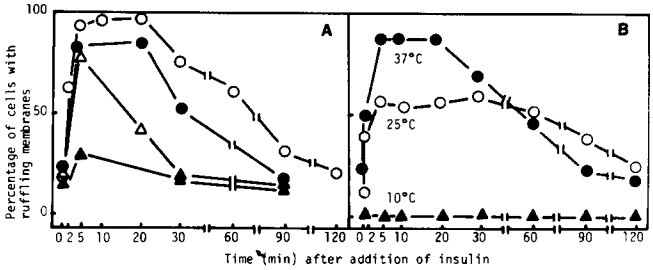

FIGURE 4 Effects of (*A*) insulin concentration and (*B*) temperature on time course of insulin-induced appearance and disappearance of ruffling membranes of single isolated KB cells. (*A*) After preincubation of KB cells in standard solution for 5 h at 37°C, insulin was added at time 0 and the cells were incubated at 37°C. Insulin concentration (μg/ml): ○, 6; ●, 0.6; △, 0.06; ▲, 0.006. (*B*) After preincubation of KB cells in standard solution for 5 h at 37°C, the cells were further incubated for 1 h at 37°C (●), 25°C (○), or 10°C (▲). Next, insulin (0.6 μg/ml) was added at time 0 and the cells were incubated at the temperature indicated. Cells on which more than half of the cell margin showed ruffling were scored as ruffling cells. Each point is for 100–300 cells.

was less than one-seventh of that in its presence. Insulin stimulated the Na^+-dependent, but not the Na^+-independent, uptake of AIB. Thus, in all subsequent experiments the values of Na^+-dependent uptake were plotted after subtracting those of the Na^+-independent uptake of AIB.

Addition of insulin accelerated the AIB uptake by KB cells (Fig. 5). Significant acceleration was detected 5 min after insulin addition. Dose-response experiments showed that >0.06 μg/ml of insulin significantly increased AIB uptake measured 20 min after the addition, but <0.01 μg/ml of insulin did not. These observations showed that the concentration of insulin necessary for inducing the formation of ruffling membranes was nearly the same as that necessary for accelerating AIB uptake. A Lineweaver-Burk plot (Fig. 6) of the initial influx rate of AIB showed that insulin caused a significant increase in the maximal initial rate (V_{max}) without any change in the apparent Michaelis constant (K_m).

TABLE II

Effects of Preincubation of KB Cells with Various Agents on Insulin-induced Formation of Ruffling Membranes

Agent		Incubation time	Ruffling cells
		min	%*
None			80 ± 5‡
Dibutyryl cAMP	(0.1 mM)	15	80 ± 5
	(0.5 mM)	15	45 ± 9
	(1 mM)	15	28 ± 3
Dibutyryl cGMP	(1 mM)	15	82 ± 6
cAMP	(1 mM)	15	81 ± 5
cGMP	(1 mM)	15	80 ± 7
Na-*n*-butyrate	(5 mM)	15	80 ± 5
Cytochalasin B‖	(10^{-5} M)	20	0
	(5×10^{-5} M)	20	0
Cytochalasin D‖	(5×10^{-6} M)	20	0
	(5×10^{-5} M)	20	0
Colchicine¶	(1×10^{-5} M)	20	66 ± 18§
Colcemid¶	(5×10^{-6} M)	30	75 ± 15§
		60	66 ± 20§
	(1×10^{-5} M)	30	65 ± 12§
Nocodazole¶	(1×10^{-5} M)	30	62 ± 10§

* After preincubation of KB cells in standard medium for 5 h at 37°C and then incubation in the presence of various agents for the times indicated in the Table at 37°C, insulin (0.6 μg/ml) was added and single isolated cells with ruffling membranes 5–10 min later at 37°C were counted under a phase-contrast microscope.

‡ Values are means ± SD for three independent experiments. 200–300 cells were examined for each value.

§ The ruffling movements of cells pretreated with colchicine, colcemid, or nocodazole were weaker than those of untreated KB cells.

‖ Immunofluorescence microscopy showed that actin cables disappeared after this treatment.

¶ Immunofluorescence microscopy showed that the tubulin network disappeared after this treatment.

Insulin did not accelerate AIB uptake by BALB/c 3T3 cells (data not shown).

We tested some other effects of insulin on KB cells and found that it did not stimulate the uptake of $^{45}CaCl_2$ (Fig. 7*A*), 2-deoxy-D-[1-^{3}H]-glucose or 3-*O*-D-[1-^{3}H]methylglucose or the efflux of either $^{45}CaCl_2$ (Fig. 7*B*) or ^{14}C-AIB.

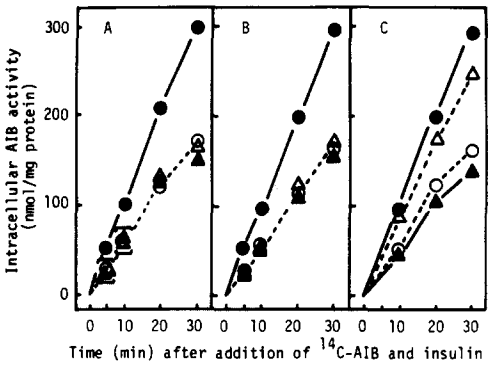

FIGURE 5 Insulin-induced enhancement of AIB uptake by KB cells and the effect on it of preincubation of the cells with (*A*) cytochalasin D, (*B*) dibutyryl cyclic AMP, or (C) colcemid. At time 0 (abscissa), radioactive AIB and insulin (●, △) were added. (A) O, No addition; ●, insulin (0.6 μg/ml). After preincubation of KB cells with cytochalasin D (50 μM) for 20 min, ^{14}C-AIB uptake by the cells was examined in the presence of cytochalasin D in standard solution with (△) or without (▲) insulin (0.6 μg/ml). (*B*) O, No addition; ●, insulin (0.6 μg/ml). After preincubation of KB cells with dibutyryl cyclic AMP (1 mM) for 15 min, ^{14}C-AIB uptake by the cells was measured in the presence of dibutyryl cyclic AMP in standard solution with (△) or without (▲) insulin (0.6 μg/ml). (C) O, No addition ●, insulin (0.6 μg/ml). After preincubation of KB cells with colcemid (5 μM) for 30 min, ^{14}C-AIB uptake by the cells were examined in the presence of colcemid in standard solution with (△) or without (▲) insulin (0.6 μg/ml). Similar results were obtained in three other independent experiments.

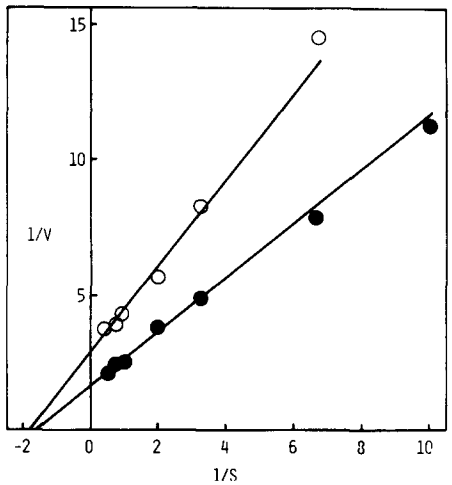

FIGURE 6 Reciprocal velocity (*V*) of AIB uptake (nmol per 20 min per mg of protein) plotted against reciprocal of AIB concentration (*S*) (millimolar). AIB uptake by KB cells at 25°C was measured 20 min after addition of AIB in the presence (●) or absence (O) of 6 μg/ml of insulin. Similar results were obtained in three other independent experiments.

Effects of Cytochalasin D, Colcemid, and Dibutyryl cAMP on Insulin-accelerated AIB Uptake

As shown in Fig. 5, preincubation of KB cells with cytochalasin D or dibutyryl AMP did not significantly affect insulin-independent AIB uptake by the cells, but completely inhibited the insulin-induced one. Preincubation of KB cells with dibutyryl cGMP (1 mM), cAMP (1 mM), cGMP (1 mM), or sodium *n*-butyrate (2 mM) for 20 min did not affect either insulin-independent or -dependent AIB uptake. On the other hand, preincubation of the cells with colcemid (5–10 μM, for 30 min) or nocodazole (10 μM, for 30 min) slightly inhibited both uptakes, although in the presence of these agents, insulin significantly accelerated the AIB uptake (Fig. 5*C*).

Effects of Insulin on the Contents of cAMP and ATP

Insulin was added to cells that had been preincubated in standard solution for 5 h at 37°C, and the cAMP and ATP contents of the cells were measured 10 min later (Table III). Insulin did not affect the ATP content, but significantly decreased the cAMP content.

DISCUSSION

Ruffling

Insulin was found to induce the formation of ruffling membranes on KB cells. The time lag between addition of insulin and the onset of formation of ruffling membranes was very short (<1 min at 37°C, and <2 min at 25°C). This observation suggested that neither insulin-induced protein synthesis nor internalization of insulin by the cells was related to the formation of the ruffling membranes, and thus they were probably formed as a result of insulin binding to the plasma membrane.

The ruffling regions were stained strongly with antibody to actin, but not that to tubulin. These observations are consist-

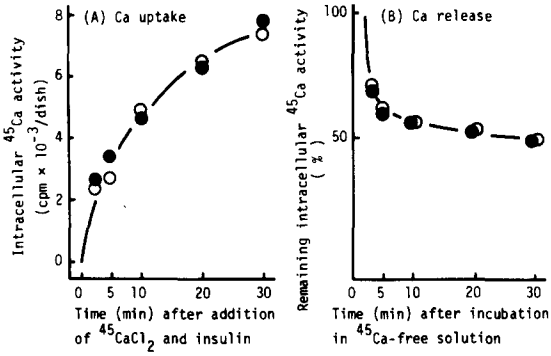

FIGURE 7 Effect of insulin on (*A*) $^{45}CaCl_2$ uptake by KB cells and (*B*) release of $^{45}CaCl_2$ from KB cells. (*A*) At time 0, $^{45}CaCl_2$ was added to cells in standard solution and then the cells were incubated for 5, 10, 20 and 30 min in the presence (●) or absence (O) of insulin (6 μg/ml). (*B*) KB cells pre-loaded with ^{45}Ca were washed with standard solution without ^{45}Ca at time 0, and then incubated in the same solution in the presence (●) or absence (O) of insulin (6 μg/ml). Similar results were obtained in three other independent experiments.

TABLE III

Effects of Insulin on Cyclic AMP and ATP Contents of KB Cells

	Cyclic AMP (pmol/mg·protein)	ATP (nmol/mg·protein)
No addition	1.70 ± 0.41	16.7 ± 6.2
Insulin (6 μg/ml) (10 min)	1.21 ± 0.33 ($0.02 < p < 0.05$)	15.1 ± 1.7 No significant difference

After preincubation of KB cells in standard solution for 5 h at 37°C, insulin was added, and the contents of cyclic AMP and ATP were measured 10 min later. Values are means ± SD for five independent experiments.

ent with previous observations that microtubules did not extend to the areas of membrane ruffling (18, 22; see also review in reference 62). Pretreatment of KB cells with cytochalasin B or D at a concentration that caused disruption of microfilaments such as arborization and aggregation of actin under fluorescence microscopy completely inhibited the formation of ruffling. As in previous findings (29, 42, 57, 65), the ruffling membranes and ruffling movements induced by insulin disappeared on addition of cytochalasin B or D. These observations suggested that the formation and maintenance of ruffling membranes and ruffling movements all depend on the dynamic function of the microfilaments. Agents that disrupted microtubules, colchicine, colcemid, and nocodazole did not significantly affect the insulin-induced formation of ruffling membranes, although their presence slightly inhibited the formation and slightly weakened the movements. Why these agents affected the ruffling is still unknown. This observation is inconsistent with numerous studies (29; see also review in reference 62). For example, Taylor and Lewis (57) reported that microtubule-disrupting agents vinblastine (10^{-5} M) and colchicine (10^{-5} M) did not affect ruffling movements of avian thrombocytes. The discrepancy may be due in part to differences in sensitivity of the cells to the agents; KB cells may be more sensitive than avian thrombocytes to the agents. Unknown toxic effects of these agents may reduce the ruffling movements of KB cells.

Numerous studies have shown that regions of ruffling membranes of nonmuscle cells did not stain with myosin-antibody (see review in reference 62). But, recently Leven and Nachmias (35) found that some agents such as ADP induced formation of ruffling membranes in cultured megakaryocytes and that the regions of ruffling membranes stained with myosin-antibody. They also found that dibutyryl cyclic AMP prevented the ADP-induced formation of ruffling membranes. We found in the present work that insulin significantly decreased the level of cyclic AMP but not of ATP in KB cells, that it did not affect Ca^{2+} movements, and that preincubation of KB cells with dibutyryl cyclic AMP prevented the insulin-induced formation of ruffling membranes. Elevation of the cyclic AMP level of smooth muscle cells by catecholamines and the consequent reduction of phosphorylation of a contractile protein (myosin light chain) have been reported to cause cell relaxation (27). Thus, a decrease in the cyclic AMP level of the cells may cause phosphorylation of contractile protein concomitant with cell contraction. These observations suggest that an insulin-induced decrease in the cyclic AMP level of KB cells may at least in part contribute to the formation of ruffling membranes by causing phosphorylation of some unknown contractile protein(s) although in the present work we did not test whether ruffle regions contained myosin; that is, the insulin action may be mediated by a "second messenger," cyclic AMP.

Amino Acid Transport and Ruffling

A close association seems to exist between the insulin-induced formation of ruffling membranes of KB cells and the enhancement of the transport of an amino acid (AIB) of the cells because for the following reasons: (*a*) The same range of insulin concentrations induced both formation of ruffling membranes and enhancement of AIB transport. (*b*) AIB uptake followed a similar time course after insulin addition as ruffling formation (compare Fig. 4*B* and Fig. 5). (*c*) Treatments of the cells with reagents that inhibited the formation of ruffling membranes also inhibited the enhancement of AIB transport. The next question is how ruffling formation is associated with enhancement of amino acid transport. A possible explanation for the enhancement by insulin treatment is that the surface area of KB cells increased on addition of insulin with the formation of ruffling membranes. But the observation that insulin did not enhance Na^+-independent AIB uptake seems to exclude this possibility. Lineweaver-Burk plots of the initial rate of AIB uptake fitted well to a straight line obtained by linear regression ($r > 0.96$), showing that AIB uptake by KB cells was achieved by a carrier-mediated transport system. Kinetic studies also showed that insulin increased the V_{max}, but not the K_m of the initial rate of AIB uptake by KB cells. These results are consistent with data obtained with other cells (10, 11, 47). A possible explanation for the increase of V_{max} by insulin treatment is that the density or number of carrier is much greater on insulin-treated cells than untreated cell. Another possible explanation is that the velocity of movement of the carrier across the membranes is much faster in insulin-treated cells than in untreated cells. The mechanisms by which insulin increases the V_{max} of the initial rate of AIB transport are still unknown.

Various studies using hepatocytes (see review in reference 28), heart muscle (11), or bone cells (45) found a lag period of ~30–120 min before a detectable increase in transport activity of system A amino acids, such as AIB, whereas saturation of insulin receptor sites was reached within a few minutes. Moreover, some studies showed that inhibitors of protein synthesis also inhibited the stimulation of transport of system A amino acids by insulin (45, 49). Thus, insulin seems to cause the synthesis of a new carrier protein for amino acid transport (13, 60; see also review in reference 19). It has been reported that insulin stimulation of amino acid transport in isolated hepatocytes is independent of hormone internalization (33). Studies with cultured myocardial cells (20) and isolated skeletal muscle cells (10) showed that insulin stimulates the transport of A system amino acids by two actions, one dependent on the synthesis of a new carrier protein and the other independent of it.

In the present work, we found that in KB cells insulin stimulated transport of a system A amino acid, AIB, within 5 min after its addition This observation suggests that synthesis of a new carrier protein was not involved in its stimulation of AIB transport in KB cells.

Agents that disrupt microtubules, such as colcemid (see Fig. 5*C*), colchicine, and nocodazole, slightly decreased insulin-independent transport of AIB in KB cells, but did not prevent the stimulating effect of insulin on AIB transport. Recently, Prentki et al. (47) found with isolated rat hepatocytes that colchicine (5×10^{-7} M) slightly inhibited insulin-independent (basal) AIB uptake and, in contrast to our findings, this agent significantly prevented the stimulating effect of insulin on AIB uptake by the cells. They examined the insulin effect after preincubating the cells with insulin for 2 h and suggested that microtubules are involved in the regulation of insulin stimulation of AIB transport in isolated rat hepatocytes by a process dependent on the synthesis of new carrier. Why colchicine slightly inhibited the basal AIB uptake by both hepatocytes and KB cells is still unknown. The concentrations of microtubule-disrupting agents used in our work were relatively high, so their inhibition may have been due to unknown toxic effects.

Preincubation of KB cells with agents that disrupt micro-

filaments, and cytochalasin B and D, did not significantly affect insulin-independent (basal) AIB uptake (see Fig. 5*A*). This result is consistent with data obtained with other cells (31, 37, 38). Preincubation of KB cells with cytochalasin B and D clearly prevented the stimulating effect of insulin on AIB transport (see Fig. 5*A*). A possible explanation for this is that disruption of microfilaments by cytochalasins prevented the stimulatory effect of insulin on AIB transport. Another possible explanation is that cytochalasins decreased the number of available insulin receptors, as noted previously by Van Obberghen et al. (59) using cultured human lymphocytes (strain IM-9). But in recent studies on cultured chicken fibroblasts, Raizada et al. (48) found that cytochalasin B caused a twofold increase in the number of surface insulin-binding sites. Preincubation of KB cells with dibutyryl cyclic AMP also prevented the stimulating effect of insulin of AIB transport (see Fig. 5 *B*). These observations suggest that prevention of insulin-induced formation of ruffling membranes by cytochalasins or by dibutyryl cyclic AMP may have some part in preventing the stimulating effect of insulin on AIB transport.

We are now studying the mechanism of the stimulating effect of insulin on AIB transport with the working hypothesis that insulin stimulates translocation of AIB transport carriers to the plasma membrane from an intracellular storage site(s), as first demonstrated for the glucose transport system. Recent studies on the mechanism of insulin stimulation of glucose transport of fat cells suggested that carriers for glucose transport are present on both the plasma membrane and in an intracellular storage site(s) (probably the Golgi-rich fraction) and that insulin stimulates translocation of the glucose carriers to the plasma membrane from the Golgi-rich fraction (6, 25, 30, 56). But the problems of how the glucose carriers are translocated and what intracellular components are associated with this translocation remain to be elucidated. The mechanism of assembly of actin molecules to form ruffling membranes, described in the present paper, may be closely associated with the translocation of carrier for AIB transport in KB cells.

This study showed that there was no Na^+ sensitivity of insulin-induced formation of ruffling membranes although AIB uptake was dependent on Na^+. This observation showed that the role of Na^+ on AIB uptake differed from that on the formation of ruffling membranes. But this does not mean that there was no close correlation between the formation of ruffling membranes and the enhancement of AIB uptake by insulin. Even when insulin caused formation of ruffling membranes and probably enhanced the appearance of AIB carriers on the plasma membrane, it did not affect AIB uptake in the absence of Na^+ gradient.

The authors wish to thank Dr. H. Yamanaka (Kanebo Institute for Cancer Research) for his collaboration in scanning microscopy.

This work was supported by grants (to K. Goshima) from the Ministry of Education, Science and Culture of Japan, the Naito Science Foundation and the Ishida Science Foundation.

Received for publication 25 May 1983, and in revised form 12 October 1983.

REFERENCES

1. Abercrombie, M., J. E. M. Heaysman, and S. M. Pegrum. 1970. The locomotion of fibroblasts in culture, II. "Ruffling." *Exp. Cell Res.* 60: 437–444.
2. Booyse, F. M., G. Osikowicz, and A. J. Quarfoot. 1981. Effects of chronic oral consumption of nicotine on the rabbit aortic endothelium. *Am. J. Pathol.* 102: 229–238.
3. Brinkley, B. R., G. M. Fuller, and D. P. Highfield. 1975. Cytoplasmic microtubules in normal and transformed cells in culture: analysis by tubulin antibody immunofluorescence. *Proc. Natl. Acad. Sci. USA* 72: 4981–4985.
4. Christensen, H. N. 1977. Amino acid transport systems in animal cells: interrelations and energization. *J. Supramol. Struct.* 6:205–213.
5. Collins, V. P., E. Arro, E. Blomquist, U. Brunk, B. A. Fredriksson, and B. Westermark. 1979. Cell motility and proliferation in relation to available substratum area, serum concentration and culture age. *Scanning Electron Microscopy.* III:411–420.
6. Cushman, S. W., and L. J. Wardzala. 1980. Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell: apparent translocation of intracellular transport systems to the plasma membrane. *J. Biol. Chem.* 255: 4758–4762.
7. DeBrabander, M., G. Geuens, R. Nuydens, R. Willebrords, and J. DeMey. 1981. Taxol induces the assembly of free microtubules in living cells and blocks the organizing capacity of the centrosomes and kinetochores. *Proc. Natl. Acad. Sci. USA* 78:5608–5612.
8. Dipasquale, A. 1975. Locomotory activity of epithelial cells in culture. *Exp. Cell Res.* 94:191–215.
9. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science (Wash. DC).* 130:432–435.
10. Elsas, II, L. J., R. C. MacDonell, Jr., and L. E. Rosenberg. 1971. Influence of age on insulin stimulation of amino acid uptake in rat diaphragm. *J. Biol. Chem.* 246:6452–6459.
11. Elsas, L. J., F. B. Wheeler, D. J. Danner, and R. L. DeHaan. 1975. Amino acid transport by aggregates of cultured chicken heart cells: effect of insulin. *J. Biol. Chem.* 250: 9381–9390.
12. Evans, R. M., P. J. A. Davies, and M. Costa. 1981. Visualization of cellular uptake and dissolution of carcinogenic αNiS particles by video intensification microscopy. *J. Cell Biol.* 91(2, Pt. 2):419a, (Abstr.)
13. Fehlmann, M., A. L. Cam, and P. Freychet. 1979. Insulin and glucagon stimulation of amino acid transport in isolated rat hepatocytes. *J. Biol. Chem.* 254: 10431–10437.
14. Goldman, R. D., E. Lazarides, R. Pollack, and K. Weber. 1975. The distribution of actin in non-muscle cells. *Exp. Cell Res.* 90:333–344.
15. Goldman, R. D., A. Milsted, J. A. Schloss, J. Starger, and M. J. Yerna. 1979. Cytoplasmic fibers in mammalian cells: cytoskeletal and contractile elements. *Annu. Rev. Physiol.* 41:703–722.
16. Good, N. E., D. Winget, W. Winter, and T. N. Connolly. 1966. Hydrogen ion buffers for biological research. *Biochemistry.* 5:467–477.
17. Goshima, K., K. Owaribe, H. Yamanaka, and S. Yoshino. 1978. Requirement of calcium ions for cell degeneration with a toxin (vibriolysin) from Vibrio parahaemolyticus. *Infect. Immun.* 22:821–832.
18. Gotlieb, A. I., M. H. Heggeness, J. F. Ash, and S. J. Singer. 1979. Mechanochemical proteins, cell motility and cell-cell contacts: the localization of mechanochemical proteins inside cultured cells at the edge of an in vitro "wound". *J. Cell. Physiol.* 10:563–578.
19. Guidotti, G. G., A.F. Borghetti, and G. C. Gazzola. 1978. The regulation of amino acid transport in animal cells. *Biochim. Biophys. Acta.* 515:329–366.
20. Guidotti, G. G., R.F. Gazzola, G. C. Gazzola, and P. Ronchi. 1974. Regulation of amino acid transport in chick embryo heart cells. IV. Site and mechanism of insulin action. *Biochim. Biopys. Acta.* 356:219–230.
21. Harris, A., and G. Dunn. 1972. Centripetal transport of attached particles on both surfaces of moving fibroblasts. *Exp. Cell Res.* 73:519–523.
22. Heggeness, M. H., K. Wang, and S. J. Singer. 1977. Intracellular distributions of mechanochemical proteins in cultured fibroblasts. *Proc. Natl. Acad. Sci. USA* 74:3883–3887.
23. Herman, I. M., N. J. Crisona, and T. D. Pollard. 1981. Relation between cell activity and the distribution of cytoplasmic actin and mysoin. *J. Cell Biol.* 90:84–91.
24. Ingram, V. M. 1969. A side view of moving fibroblasts. *Nature (Lond.).* 222:641–644.
25. Karnieli, E., M. J. Zarnowski, P. J. Hissin, I. A. Simpson, L. B. Salans, and S. W. Cushman. 1981. Insulin-stimulated translocation of glucose transport systems in the isolated rat adipose cell: time course reversal, insulin concentration dependency, and relationship to glucose transport activity. *J. Biol. Chem.* 256:4772–4777.
26. Kawamura, A. 1977. Fluorescent antibody techniques and their application. 2nd Ed. University of Tokyo Press, Tokyo. 37–67.
27. Kerrick, W. G. L., and P. E. Hoar. 1981. Inhibition of smooth muscle tension by cyclic AMP-dependent protein kinase. *Nature (Lond.).* 292:253–255.
28. Kilberg, M. S. 1982. Amino acid transport in isolated rat hepatocytes. *J. Membr. Biol.* 69:1–12.
29. Koga, H. 1981. Functional significance of microtubules and microfilaments for cellular motility in cultured brain tumors. *Neurol. Med. Chir.* 21:1009–1015.
30. Kono, T., F. W. Robinson, T. L. Blevins, and O. Ezaki. 1982. Evidence that translocation of the glucose transport activity is the major mechanism of insulin action on glucose transport in fat cells. *J. Biol. Chem.* 257:10942–10947.
31. Kuchler, R. J., and M. Marlowe-Kuchler. 1965. The transport and accumulation of α-aminoisobutyric acid into L-strain mouse fibroblasts. *Biochim. Biophys. Acta.* 102:226–234.
32. Lazarides, E., and K. Weber. 1974. Actin antibody: The specific visualization of actin filaments in non-muscle cells. *Proc. Natl. Acad. Sci. USA* 71:2268–2272.
33. LeCam, A., F. Maxfield, M. Willingham, and I. Pastan. 1979. Insulin stimulation of amino acid transport in isolated rat hepatocytes is independent of hormone internalization. *Biochem. Biophys. Res. Commun.* 88:873–881.
34. Lembach, K., and F. C. Charalampous. 1967. Metabolic functions of myo-inositol. VI. Impairment of amino acid transport in KB cells caused by inositol deficiency. *J. Biol. Chem.* 242: 2606–2614.
35. Leven, R. M., and V. T. Nachmias. 1982. Cultured megakaryocytes: changes in the cytoskeleton after ADP-induced spreading. *J. Cell Biol.* 92:313–323.
36. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
37. Mak, W. W.-N., and H. C. Pitot. 1981. Microfilament accumulation and the transport of amino acids and glucose in adult rat hepatocytes cultured on collagen gel/nylon mesh. *Biochem. Biophys. Res. Commun.* 98:203–210.
38. Mizel, S. B., and L. Wilson. 1972. Inhibition of the transport of several hexoses in mammalian cells by cytochalasin B. *J. Biol. Chem.* 247:4102–4105.
39. Mørland, B., and G. Kaplan. 1977. Macrophage activation in vivo and in vitro. *Exp. Cell Res.* 108:279–288.
40. Motta, P. M., J. van Blerkom, and S. Makabe. 1980. Changes in the surface morphology of ovarian "germinal" epithelium during the reproductive cycle and in some pathological conditions. *J. Submicrosc. Cytol.* 12:407–425.
41. Noall, M. W., T. R. Riggs, L. M. Walker, and H. N. Christensen. 1957. Endocrine control of amino acid transfer: distribution of an unmetabolizable amino acid. *Science (Wash. DC).* 126:1002–1005.
42. Ohnishi, R. 1979. Cinematographic analysis of ruffling movement of L cells. *Proc.*

Yamada Conf. I. Cell motility: molecules and organization. S. Hatano, H. Ishikawa, and H. Sato, editors). University of Tokyo Press, 251–261.
43. Osborn, M. and K. Weber. 1976. Cytoplasmic microtubules in tissue culture cells appear to grow from an organizing structure towards the plasma membrane. *Proc. Natl. Acad. Sci. USA.* 73:867–871.
44. Owaribe, K., and S. Hatano. 1975. Induction of antibody against actin from myxomycete plasmodium and its properties. *Biochemistry.* 14:3024–3029.
45. Phang, J. M., D.L. Valle, L. Fisher, and A. Granger. 1975. Puromycin effect on amino acid transport: differential rates of carrier protein turnover. *Am. J. Physiol.* 228:23–26.
46. Porter, K., D. Prescott, and J. Frye. 1973. Changes in surface morphology of chinese hamster ovary cells during the cell cycle. *J. Cell Biol.* 57:815–836.
47. Prentki, M., M. Crettaz, and B. Jeanrenaud. 1981. Role of microtubules in insulin and glucagon stimulation of amino acid transport in isolated rat hepatocytes. *J. Biol. Chem.* 256:4336–4340.
48. Raizada, M. K., R. E. Fellows, and B. Wu. 1981. Cytochalasin B-induced alterations of insulin binding and microfilament organization in cultured fibroblasts. *Exp. Cell Res.* 136:335–341.
49. Risser, W.L., and T. D. Gelehrter. 1973. Hormonal modulation of amino acid transport in rat hepatoma cells in tissue culture. *J. Biol. Chem.* 248:1248–1254.
50. Saint-Guillain, M. L., B. Vray, J. Hoebeke, and R. Leloup. 1980. Sem morphological studies of phagocytosis by rat macrophages and rabbit polymorphonuclear leukocytes. *Scanning Electron Microscopy.* II:205–212.
51. Schulz, S. G., and P. F. Curran. 1970. Coupled transport of sodium and organic solutes: Na-dependent amino acid transport. *Physiol. Rev.* 50:641–643.
52. Shelanski, M. L., F. Gaskin, and D. R. Cantor. 1973. Microtubule assembly in the absence of added nucleotide. *Proc. Natl. Acad. Sci. USA.* 70:765–768.
53. Stanley, P. E., and S. G. Williams. 1969. Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. *Anat. Biochem.* 29:381–392.
54. Steiner, A. L. 1974. Assay of cyclic nucleotides by radioimmunoassay methods. *Methods Enzymol.* 38:96–105.
55. Stephens, R. E. 1975. High-resolution preparative SDS-polyacrylamide gel electrophoresis: fluorescent visualization and electrophoretic elution-concentration of protein bands. *Anal. Biochem.* 65:369–379.
56. Suzuki, K., and T. Kono. 1980. Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. *Proc. Natl. Acad. Sci. USA* 77:2542–2545.
57. Taylor, R. G., and J. C. Lewis. 1979. Plasma protein modification of thrombocyte adhesion to glass. *Scanning Electron Microscopy.* III:775–782.
58. Trinkans, J. P. 1969. Cells into organs: the forces that shape the embryo. Prentice-Hall, Inc., Englewood Cliffs, NJ. pp. 17–35.
59. Van Obberghen, E., P. De Meyts, and J. Roth. 1976. Cell surface receptors for insulin and human growth hormone: effect of microtubule and microfilament modifiers. *J. Biol. Chem.* 251:6844–6851.
60. Walker, P. R., and J. F. Whitfield. 1978. Inhibition by colchicine of changes in amino acid transport and initiation of DNA synthesis in regenerating rat liver. *Proc. Natl. Acad. Sci. USA* 75:1394–1398.
61. Weber, K., R. Pollack, and T. Bibring. 1975. Antibody against tubulin: the specific visualization of cytoplasmic microtubules in tissue culture cells. *Proc. Natl. Acad. Sci. USA* 72:459–463.
62. Weatherbee, J. A. 1981. Membranes and cell movement: interactions of membranes with the proteins of the cytoskeleton. *Int. Review Cytol.* Suppl. 12:113–176.
63. Weingarten, M. D., A. H. Lockwood, S.Y. Hwo, and M. W. Kirscher. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA.* 72:1858–1862.
64. Whyte, M. P., W. A. Murphy, M. D. Fallon, W. S. Sly, S. L. Teitelbaum, W. H. McAlister, and L. V. Avioli. 1980. Osteopetrosis, renal tubular acidosis and basal ganglia calcification in three sisters. *Am. J. Med.* 69:64–74.
65. Yahara, I., S. Iwashita, T. Ebina, M. Satake, and N. Ishida. 1979. Inhibition of ligand-independent cap formation of mouse lymphocytes and Raji cells by neocarzinostatin. *Cancer Res.* 39:4687–4693.