

Microtubules and Beta Cell Function: Effect of Colchicine on Microtubules and Insulin Secretion In Vitro by Mouse Beta Cells

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ABSTRACT A monolayer culture system was developed to study the role of microtubules in insulin secretion. Cultured cells were obtained by enzymatic digestion of pancreases from C57BL-KsJ mice 6–12 wk of age. On day 4 of culture, the medium was changed, control or treatment medium added, and frequent samples were removed for insulin assay. Microtubules and beta cells were identified by indirect immunofluorescence with monospecific antibodies to tubulin and insulin. An extensive microtubule network radiates from the perinuclear region of the beta cell to the plasma membrane. Although alterations in the calcium concentration of the medium did not affect the microtubule pattern, the absence of calcium or glucose in the medium inhibited insulin secretion ($P < 0.001$). Optimum insulin release occurred at a calcium concentration of 2.5 mM. Colchicine, in concentrations of 10^{-10} to 10^{-8} M, did not affect the microtubule immunofluorescent pattern, whereas concentrations of 1 and 5×10^{-7} M decreased the number of microtubules, and microtubules could not be identified in cultures treated with 10^{-6} M colchicine for 2 h. After a 2-h preincubation, the prolonged release of insulin at either 2.0 or 4.5 mg/ml of glucose was decreased by 10^{-6} M colchicine ($P < 0.02$). The immediate release of insulin was similar to that in control plates and occurred in cultures with no identifiable microtubules. Microtubules and insulin secretion were not altered by 10^{-6} M lumicolchicine and prolonged insulin secretion recovered 24 h after removal of colchicine. These studies show that the microtubules facilitate sustained secretion of insulin but are not required for the immediate release of the hormone. Alterations in the extracellular calcium concentration which play an essential role in insulin secretion do not alter the microtubule pattern in the beta cell.

Electron microscopy (1) reveals that the beta cell contains an extensive microtubule (Mt) network. On the basis of studies showing that colchicine inhibits insulin secretion from isolated pancreatic islets, Lacy et al. (2) hypothesized that Mts play an important role in secretory activity of the beta cell. These studies have been confirmed (see reference 3 for review) and extended (4–6) to show that colchicine retards the movement of newly formed insulin from the site of synthesis in the rough endoplasmic reticulum to the plasma membrane (7, 8). There is a second putative pool of insulin (9), presumably located in granules in the periphery of the beta cells, which does not require Mts for secretion. Although electron microscopy reveals some decrease in Mts after colchicine treatment, this technique has proved to be too laborious to quantify the effect of colchicine on the Mts in the beta cells (10). Our study describes a

new technique for the monolayer culture of pancreatic cells from mature animals. The immunofluorescent pattern of Mts in the beta cells was studied at various concentrations of glucose, colchicine, lumicolchicine, and calcium and correlated with insulin secretion. These studies show that Mts regulate the sustained secretion of insulin but are not required for the immediate release of the hormone.

MATERIALS AND METHODS

Reagents

We obtained colchicine and crystalline bovine serum albumin (BSA, Fraction V) from Sigma Chemical Co., St. Louis, Mo.; fetal bovine serum (FBS) from Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.; collagenase, type III, from Worthington Biochemical Corp., Freehold, N. J.; and porcine

insulin from either Eli Lilly & Co., Indianapolis, Ind., or Sigma Chemical Co. Sepharose 4B was obtained from Pharmacia Fine Chemicals, Piscataway, N. J. We prepared lumicolchicine by the method of Wilson (11) by adding colchicine to absolute alcohol, irradiating it for 30 min, and evaporating the ethanol. The residual colchicine was extracted three times with water. The dried lumicolchicine had a ratio of absorbance at 350:267 nm of 0.067 and a molar extinction coefficient at 267 nm of 22,880.

Antibodies

We raised antibodies in rabbits to tubulin purified from bovine brain by the temperature-dependent assembly-disassembly method of Borisy et al. (12) followed by phosphocellulose chromatography. The tubulin was >95% pure as judged by SDS PAGE. The tubulin antibody was then purified on a Sepharose-tubulin affinity column as previously described (13). The guinea pig anti-porcine insulin antibody was purified on a Sepharose-insulin column by similar techniques. The protein concentrations of the purified antibodies were measured and the antibodies frozen and stored in small aliquots until needed. A goat anti-rabbit IgG conjugated to fluorescein, or rhodamine, and sheep anti-guinea pig fluorescein-labeled IgG were purchased from Miles Laboratories, Inc., Research Products, Div., Elkhart, Ind.

Immunofluorescence

We grew cells on sterilized 11 × 22-mm glass cover slips to 50–70% confluence. Indirect immunofluorescence was performed by the procedure of Brinkley et al. (14), using 1–3 µg of purified tubulin antibody, or 2–4 µg of purified insulin antibody and the appropriate dilution of rhodamine- or fluorescein-conjugated anti-IgG. The cells were examined with a Leitz Orthoplan microscope equipped with epi-illumination using a 100 W high pressure mercury arc as a source of illumination and a Leitz K, narrow blue band, or N, narrow green band, filter. By staining the cover slips with anti-insulin and anti-tubulin, followed by rhodamine conjugated anti-rabbit and fluorescein-conjugated anti-guinea pig IgG, we switched filters and were able to photograph Mts in beta cells. Slides were examined and photographed immediately after staining to avoid fading of the fluorescence. Three types of controls were performed: (a) incubation of the cultures with the first antibody which had been previously incubated with an excess of antigen (tubulin or insulin); (b) incubation with preimmune sera as the first antibody; and (c) incubation with the fluorescently labeled antibody alone.

Cell Culture

One pancreas for each of the five plates of ~10⁵ cells/plate was isolated under sterile conditions from C57BL-KsJ mice 6–12 wk of age using the following modifications of published methods (15). The tissue was minced in fine pieces, 1 mm³ or less in size, with scissors. We placed the minced tissue in Medium 199 with NaHCO₃, BSA (fraction V, 20 mg/ml), and type III collagenase (2 mg/ml) for 15 to 30 min at 37°C. The cells were centrifuged (1,000 g for 5 min) and rinsed with Puck's solution A (16). The cell pellet was suspended in 1.0 ml of medium and 0.5 ml of the suspension added to two 100-mm Petri dishes each containing 10 ml of Medium 199 supplemented with 20% FBS. We then incubated the suspensions for 4–6 h to allow the fibroblasts to attach. The floating cells were removed from each 100-mm dish, centrifuged (1,000 g for 5 min), resuspended in 2 ml of Medium 199, and 0.2 ml of the suspension was added to P-35 dishes containing 2 ml of Medium 199. 4 d later, the medium was removed and replaced with medium in which the various treatment drugs were added. Control plates received media with the same concentrations of glucose and calcium as the treatment plates.

Incubation Experiments

On day 4 of culture, the media were removed and the cells were incubated in low glucose (0.5 mg/ml) in 2 ml of Medium 199 with 20% FBS. Insulin levels in plates were sampled at 0, 5, 15, 30, 45, 60, 90, 120, 150, and 180 min. In the colchicine incubation experiments, the appropriate concentration of colchicine was added to the media during the 2-h preincubation period. 2 h later, the media were changed and 4.0 ml of new media added, increasing the concentration of glucose to 2.0 or 4.5 mg/ml and again adding colchicine while keeping the other components of the media constant.

Insulin Assay

Insulin was measured in a double-antibody system, modified from that of Hales and Randle (17), using a guinea pig anti-porcine insulin antibody at a final concentration of 1:1,000,000. Porcine insulin was iodinated by the Hunter-Greenwood method (18) and the iodinated insulin separated from the free iodide on a

BioGel P-10 column. Porcine insulin was used as the standard. The data were processed using a Hewlett-Packard desk calculator with the radioimmunoassay program provided by the company. The minimal sensitivity of the assay varied from 1.0 to 3.2 µU/ml. A low, medium, and high internal standard and a 1:5 dilution of the high standard were added to each assay during the study. The coefficient of variation of each standard during the study was 15.1, 5.7, 4.1, and 9.5, respectively. Dilution curves of insulin added to the medium were parallel to the standard curve in the assay buffer.

Statistics

The area under the insulin secretory curve was calculated by trapezoidal integration using a Hewlett-Packard desk calculator. The areas were compared by unpaired Student's *t* tests.

RESULTS

By phase-contrast microscopy, the cells have a typical epithelial appearance with an oval shape and prominent nuclei. Using a fluorescein-labeled second antibody to guinea pig IgG (insulin) and a rhodamine-labeled second antibody to rabbit IgG (tubulin), the beta cells and the cytoplasmic microtubular complex (CMTC) were identified in the same cells. As the cultures mature, nests of beta cells are identified with the insulin antibody, staining yellowish-green, and appear as bright granules in the cytoplasm (Fig. 1, *top*). The Mt network in the same cells stains an orange-red and radiate to the periphery of the cell (Fig. 1, *bottom*). Many of the insulin secretory granules are aligned along the Mts. In some of the cultures, 20–30% of the total number of cells stain with the insulin antibody, whereas, in other cultures, only 2–3% of the cells contain the insulin antigen.

Fig. 2 shows the extensive CMTC in beta cells that have been stained for tubulin immunofluorescence. The Mts radiate from the perinuclear area of the cells and appear as discrete structures with few areas where the Mts occur in bundles. Some of the Mts can be seen to terminate on or near the plasma membrane.

Fig. 3 shows the identification of beta cells by indirect immunofluorescence using a guinea pig anti-insulin first antibody and a fluorescein-labeled second antibody. Fig. 3A and B show nests of beta cells. Fig. 3C depicts an individual beta cell with brightly fluorescent secretory granules, and Fig. 3D shows the lack of staining in control plates in which the first antibody had been incubated with an excess of insulin. No staining was also observed in control plates incubated with the preimmune sera or the second antibody alone.

Effect of Colchicine on Mt Pattern

Preliminary experiments were performed with colchicine to determine the concentration and time needed to disrupt the Mts. Colchicine at 10⁻⁶ M completely disrupts the Mts in 120 min. All further colchicine experiments were performed with a 2-h preincubation period with colchicine in the media. At 10⁻¹⁰–10⁻⁸ M there was no effect of colchicine on the Mt pattern. Fig. 4 depicts the results of a typical colchicine dose-response experiment at 10⁻⁷–10⁻⁶ M colchicine. Lumicolchicine in a concentration of 10⁻⁶ M had no effect on the extensive Mt network in the beta cells (Fig. 4A). The Mts were decreased slightly at 1 × 10⁻⁷ M colchicine (Fig. 4B) and are further decreased at 5 × 10⁻⁷ M colchicine (Fig. 4C). No Mts were identified after 2 h of pretreatment with 10⁻⁶ M colchicine (Fig. 4D) or at any time during the 3-h secretory studies. Fig. 5 shows a beta cell in the presence of 10⁻⁶ M colchicine. No Mts were identifiable. These results are highly reproducible from day-to-day in a large number of experiments.

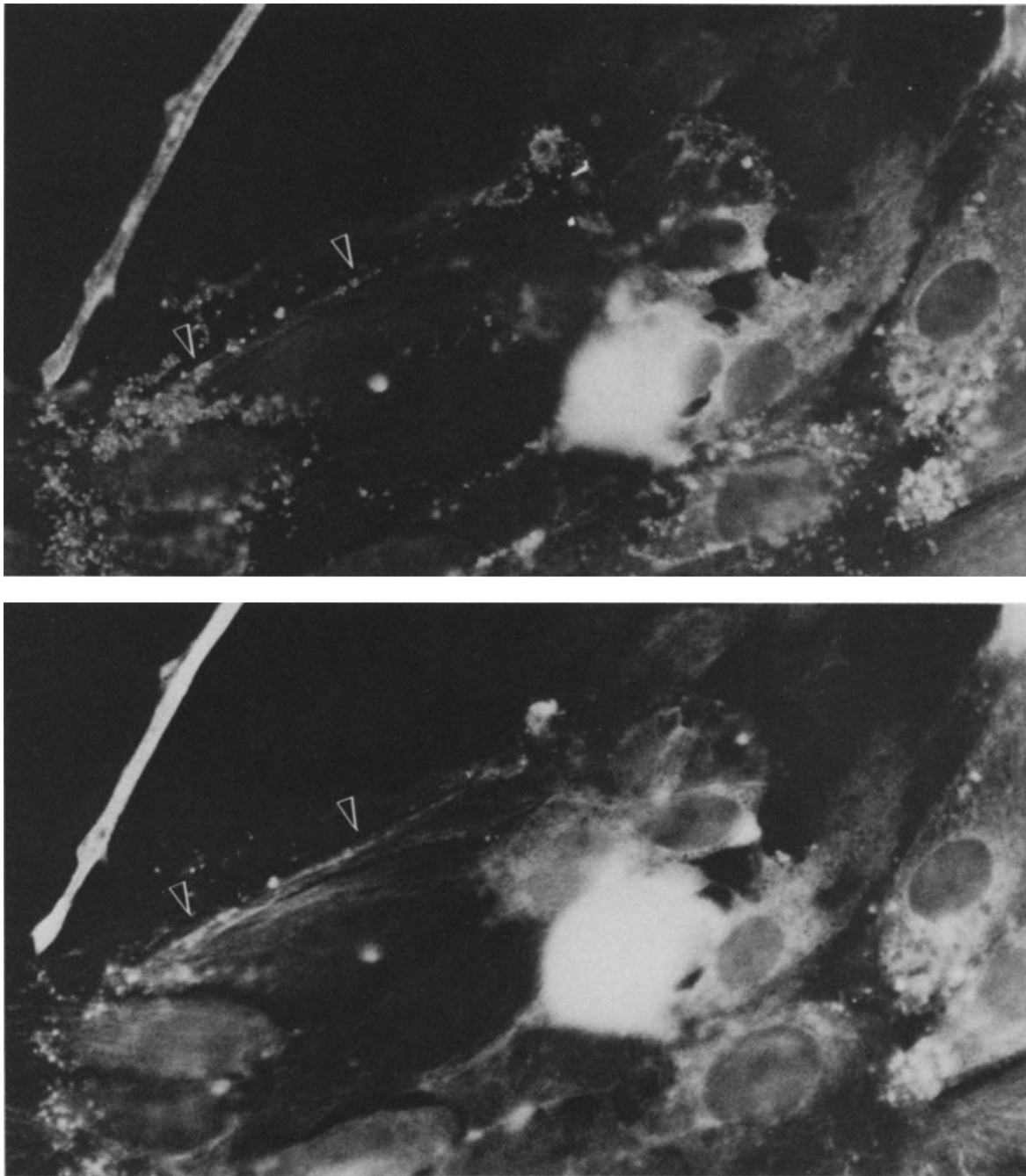


FIGURE 1 The beta cells were identified with a monospecific antibody to insulin prepared in the guinea pig and a fluorescein-labeled anti-guinea pig immunoglobulin. The insulin secretory granules (top panel) can be seen to align along the Mts in the beta cells. Indirect immunofluorescence using two different fluorescent probes to species-specific first antibodies were used to identify the Mts and beta cells in the same cultures. The Mts (arrows, bottom panel) were localized with a monospecific antibody to tubulin prepared in the rabbit and a rhodamine-labeled anti-rabbit immunoglobulin. The Mts appear as fine, rodlike structures extending through the cytoplasm. $\times 1,500$.

Insulin Secretion in Pancreatic Cultures

All secretory studies were performed on day 4 in culture. Fig. 6 shows the pattern of insulin secretion during the 180 min after changing the concentration of glucose in the media from the initial low glucose level of 0.5 mg/ml to 1.0 mg/ml or 3.0 mg/ml in representative individual plates. After an increase in the glucose concentration, insulin levels increase immediately to a peak that occurs between 15 and 45 min. Insulin levels then decrease toward the basal level and increase again to much higher levels.

By calculating the area under the insulin curve during the first 60 min and during the 60- to 180-min period, it is possible to quantify the immediate release (0-60 min) and the sustained release (60-180 min) of insulin. Fig. 7 depicts the mean \pm SEM (standard error of the mean) of the insulin areas obtained by changing the glucose concentration from 0.5 mg/ml to 0.0, 0.5, 1.0, and 3.0 mg/ml. The immediate and sustained secretion of insulin at 0.5, 1.0, and 3.0 mg/ml were significantly greater than that seen after 0 mg/ml. The differences between either the immediate or sustained insulin secretion at 0.5, 1.0, and 3.0 mg/ml were not significant.

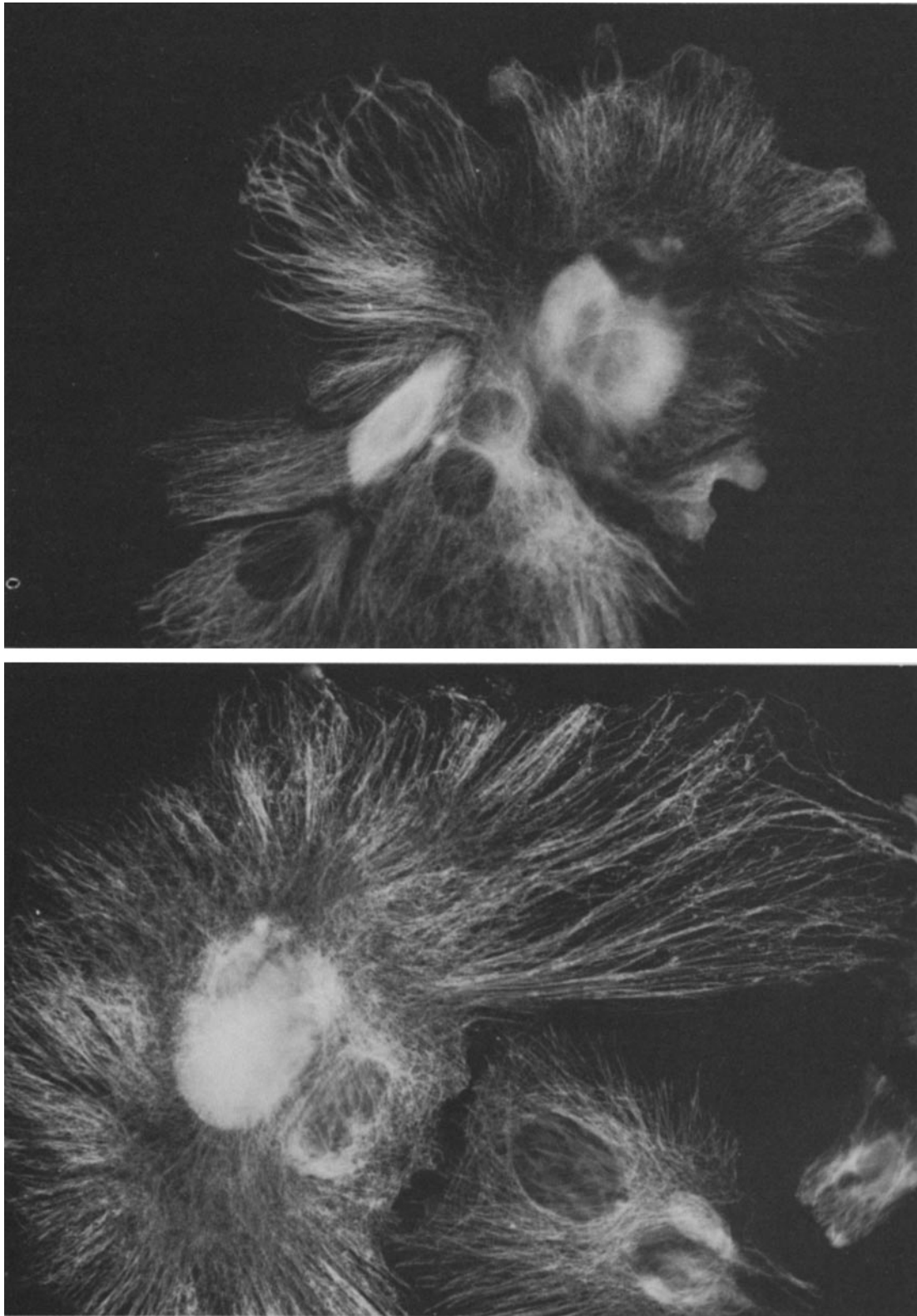


FIGURE 2 Indirect immunofluorescence of pancreatic monolayer cultures using the monospecific antibody to tubulin and a fluorescein-labeled second antibody reveals the extensive microtubular cytoplasmic complex (CMTC) in two different cultures. The Mts radiate from the perinuclear region of the cell to the cell periphery. $\times 2,100$.

Effect of Calcium on Microtubules and Insulin Secretion

The effect of insulin secretion with varying concentrations of calcium in the media and the immunofluorescent Mt pattern

were studied. As in the cultures treated with media containing no glucose, there was little immediate or sustained release of insulin in cultures in which either no calcium or 1.25 mM calcium was present (Fig. 8). These results were consistent at three concentrations of glucose tested (1.0, 2.0, and 3.0 mg/ml).

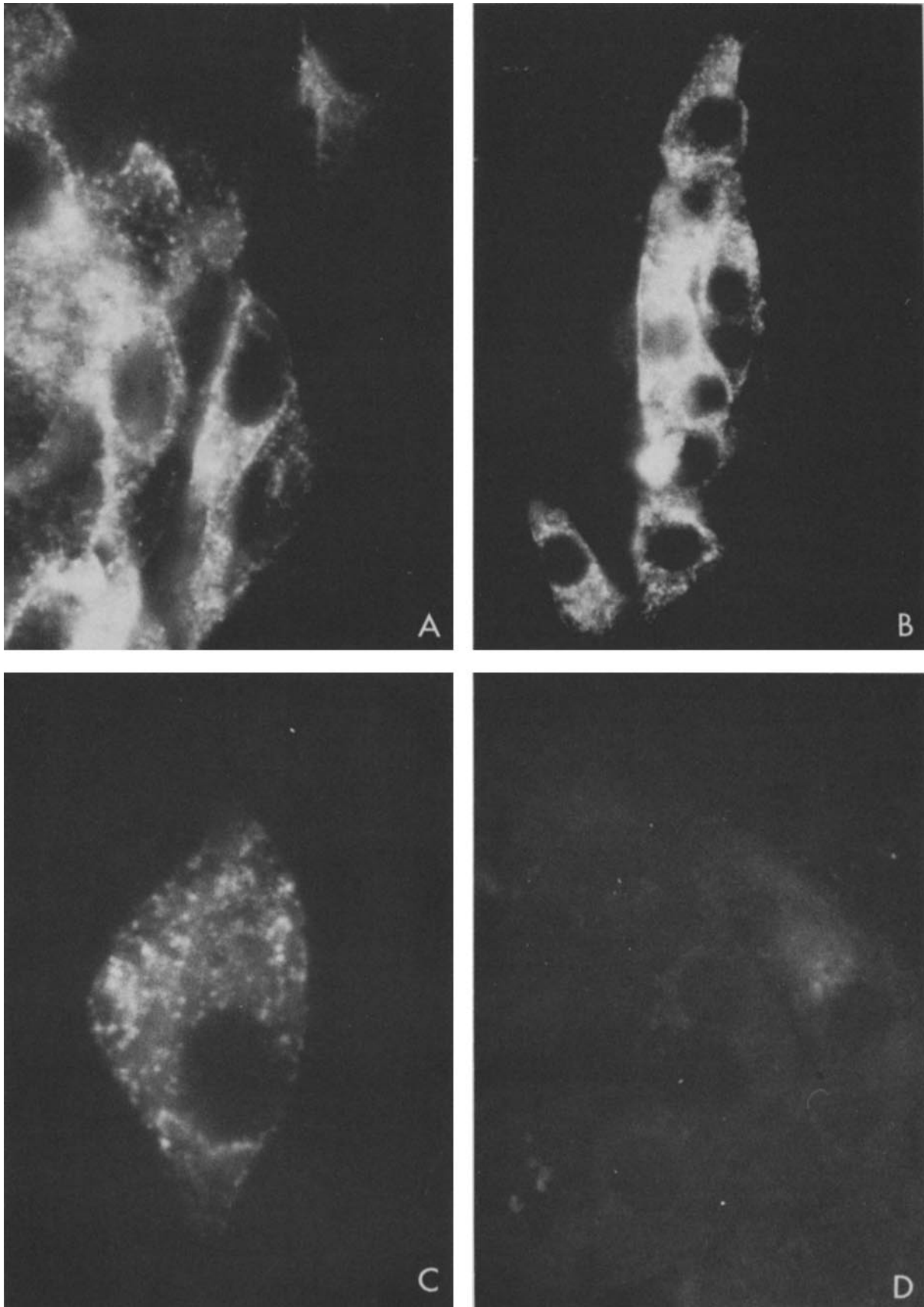


FIGURE 3 Mouse beta cells are identified in the pancreatic mouse epithelial cell cultures using indirect immunofluorescence with an affinity-purified guinea pig anti-insulin antibody (see Materials and Methods) and a second antibody to guinea pig IgG conjugated to fluorescein. (A) Beta cells located near the edge of a colony show bright immunofluorescence. (B) Several beta cells surround other insulin-negative cells. (C) A single isolated beta cell with numerous insulin secretory granules located throughout the cytoplasm. Note the lack of staining of the nucleus. (D) No staining was observed in plates in which the first antibody had been preabsorbed with an excess of insulin. $\times 2,100$.

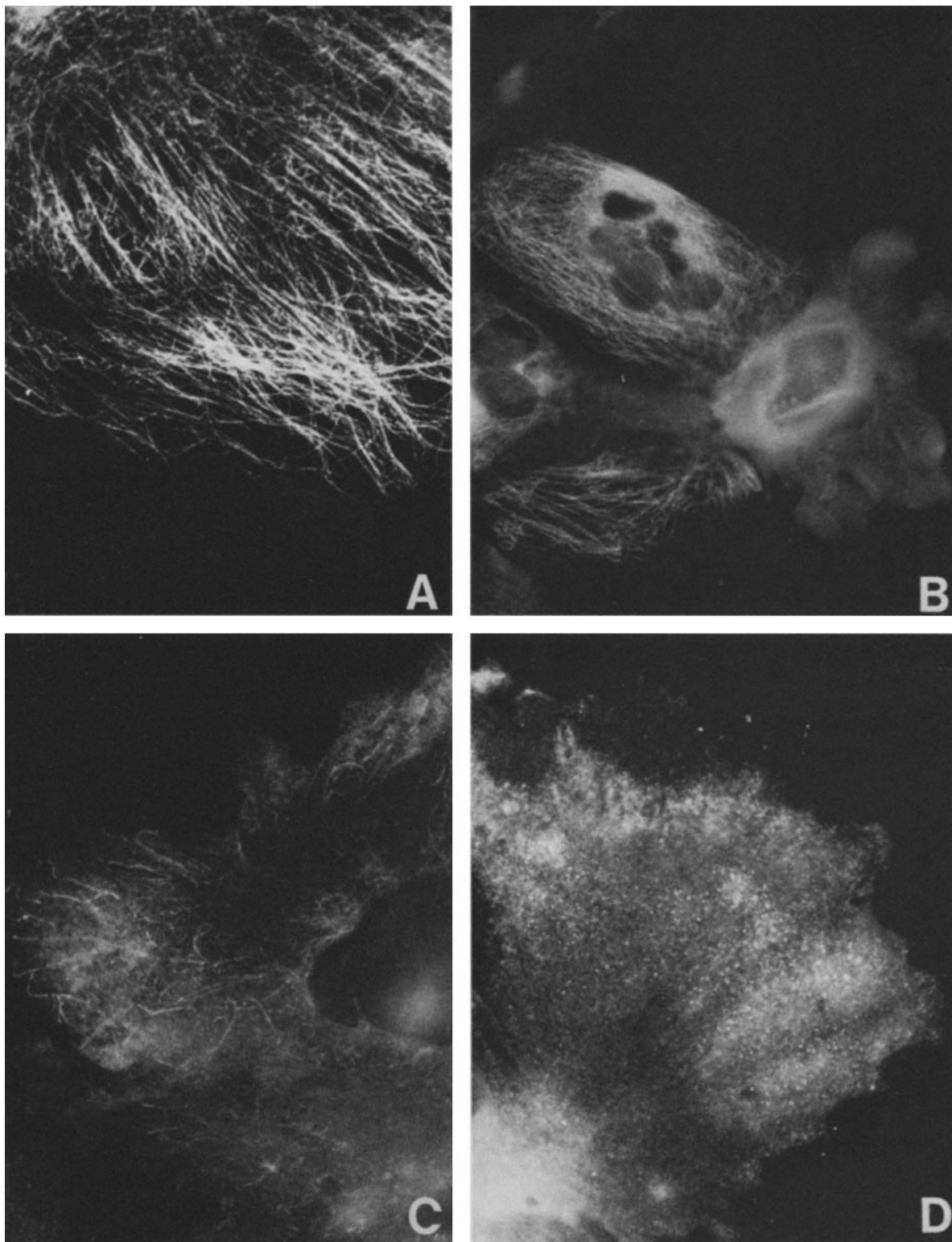


FIGURE 4 This figure shows the effect of lumicolchicine and three different doses of colchicine incubated in the cultures for 2 h on the CMTC in the pancreatic monolayer cultures. (A) Lumicolchicine (10^{-6} M) had no effect on the Mt pattern. (B) Colchicine (10^{-7} M) decreased the Mts slightly in some of the cells. (C) Colchicine (5×10^{-7} M) further disrupts the Mts, with only a few short segments which are associated with the plasma membrane remaining. (D) At 10^{-6} M colchicine, the Mts in all of the cells are completely disrupted. $\times 2,100$.

The optimum concentration of calcium for release of insulin was 2.5 mM, and no further increase in insulin secretion occurred in cultures in which the calcium concentration of the medium was increased to 5.0 mM. The Mt pattern was not changed by altering the concentrations of calcium in the media.

Effect of Colchicine on Insulin Secretion

These experiments were performed at two concentrations of glucose: 2.0 and 4.5 mg/ml. Colchicine had no effect on the immediate release of insulin at either dose of glucose but

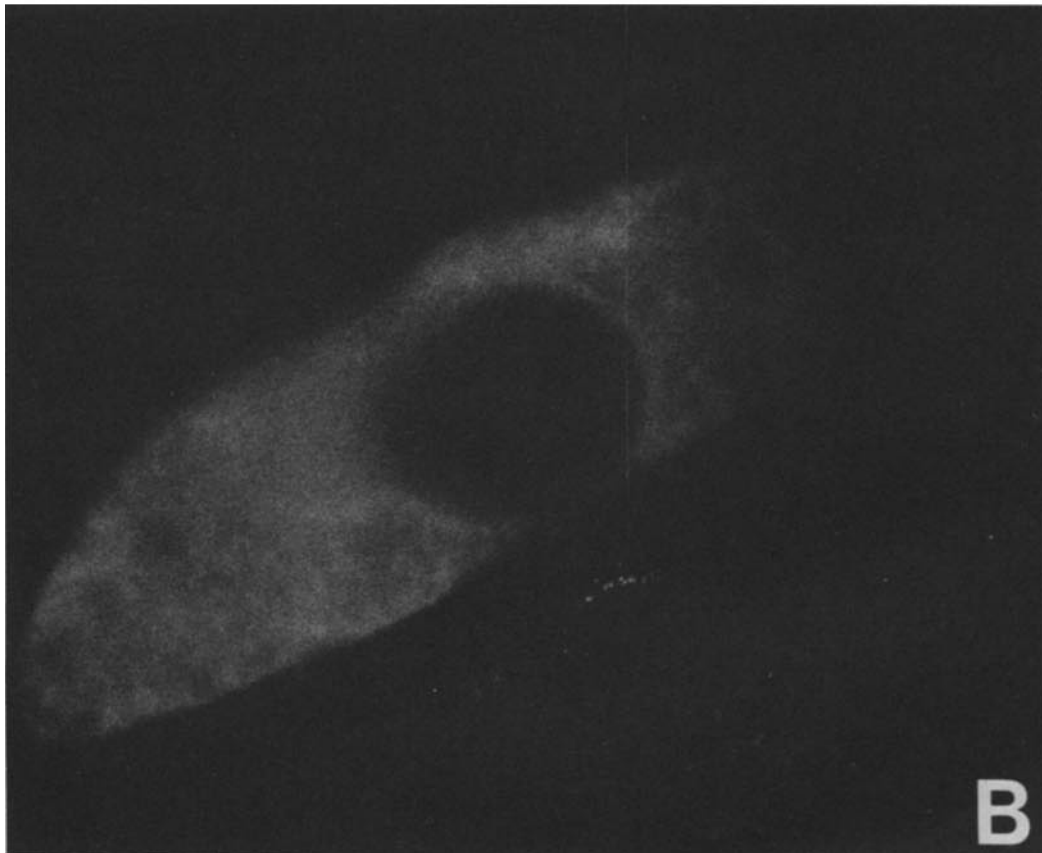
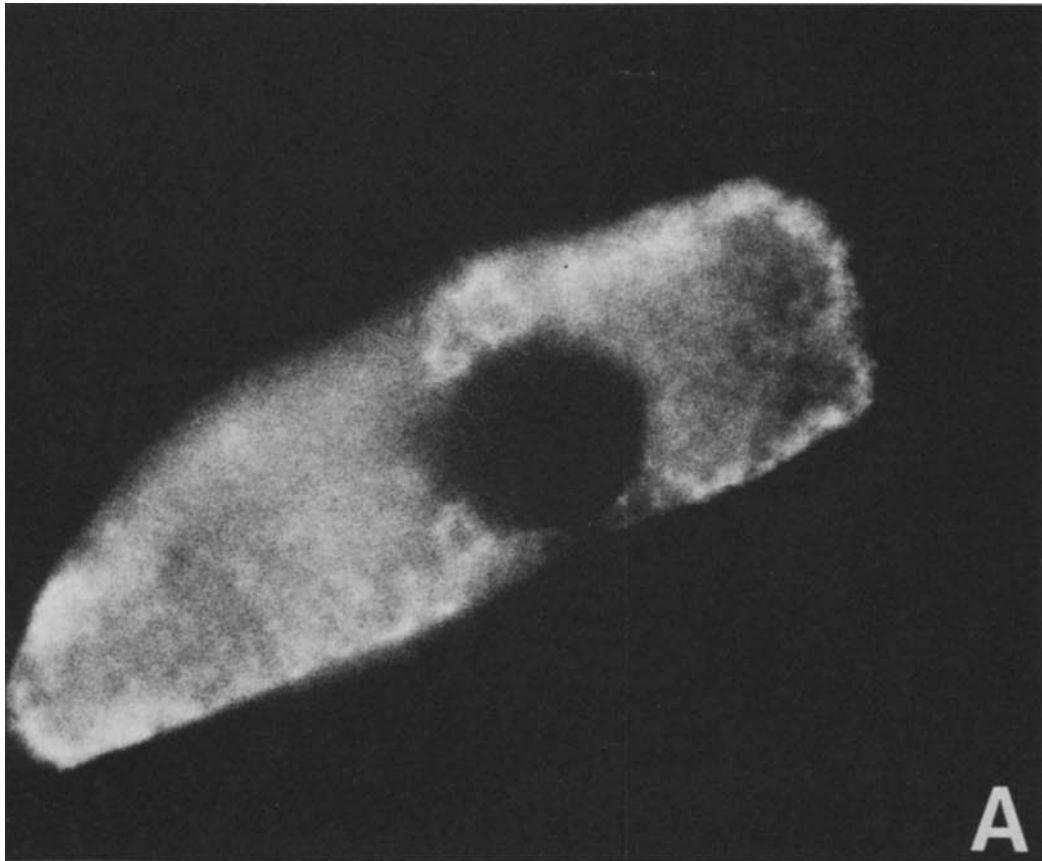


FIGURE 5 A typical beta cell is shown staining with the insulin antibody (A) and the tubulin antibody (B) at 10^{-6} M colchicine. Note the complete absence of Mts in the beta cell.

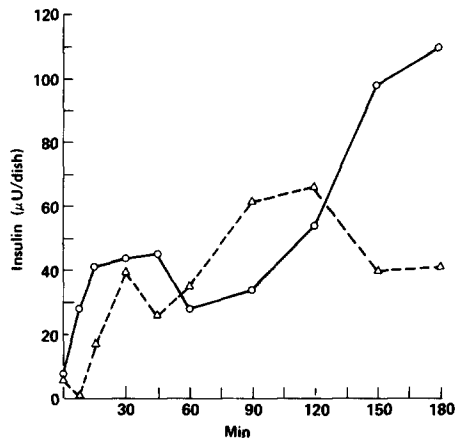


FIGURE 6 The pattern of insulin secretion in the medium of a single representative culture plate after changing the concentration of glucose from 0.5 to 1.0 mg/ml (Δ) or 3.0 mg/ml (\circ). Peak insulin levels occur between 15 and 45 min, and then there is a secondary rise in insulin occurring during the 60- to 180-min period.

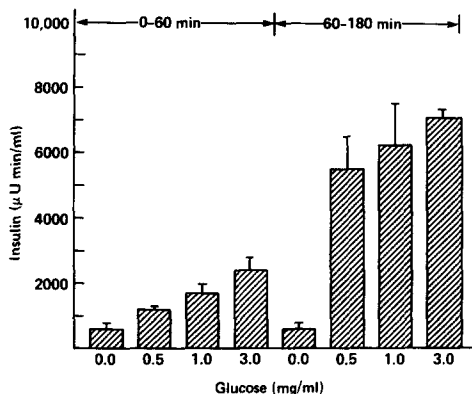


FIGURE 7 The quantitation of the insulin secretory area (mean \pm SEM) after changing the glucose concentration in the media from 0.5 to 0.1, 0.5, 1.0, and 3.0 mg/ml ($n = 8$). Note the increase in insulin release with increasing concentrations of glucose.

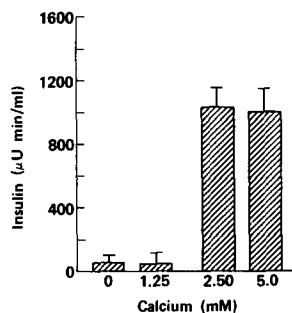


FIGURE 8 The effect of varying the concentration of calcium in the media on insulin secretion during the first 60-min study period. The data are expressed as the mean \pm SEM area under the insulin secretory curve in $\mu\text{U}/\text{min}$ ($n = 16$). There was little immediate or sustained insulin release at calcium concentrations of 0 or 1.25 mM in the media. When the calcium concentration was 2.5 mM, insulin secretion increased tenfold and there was no further insulin secretion at a concentration of 5.0 mM in the media.

caused a significant decrease in the sustained release of insulin at 2.0 and 4.5 mg/ml of glucose ($P < 0.02$). Fig. 9 depicts the insulin release in the experiments performed with a concentration of 4.5 mg/ml and a colchicine concentration of 10^{-6} M. The data at 2.0 mg/ml were similar (not shown), with a

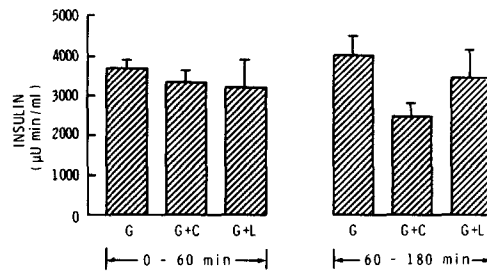


FIGURE 9 All cultures were preincubated for 120 min with colchicine (10^{-6} M) and glucose at a concentration of 0.5 mg/ml. The effect of colchicine (10^{-6} M, $n = 20$) or lumicolchicine (10^{-6} M, $n = 6$) on the mean \pm SEM insulin secretion during the first 60 min and during the 60-180-min period after increasing the glucose (G) concentration to 4.5 mg/ml was studied. Colchicine (C) did not affect the immediate release of insulin, and lumicolchicine (L) did not alter either phase of hormone secretion. Colchicine decreased the sustained release of insulin ($P < 0.02$). The data were similar using a lower glucose concentration of 2.0 mg/ml and showed a significant inhibition of the sustained release of insulin ($P < 0.02$, $n = 6$), with no effect on the immediate release of insulin.

significant inhibition of the secondary release of insulin ($P < 0.02$). After the incubations were performed in the presence of 10^{-6} M colchicine and 4.5 mg/ml of glucose, the media were changed and the cells allowed to recover for 24 h. Control plates were treated with glucose alone on 2 d successively. Immediate ($553 \pm 92 \mu\text{U}/\text{min}$, control, vs. $843 \pm 64 \mu\text{U}/\text{min}$, colchicine) and sustained ($641 \pm 123 \mu\text{U}/\text{min}$, control, vs. $1211 \pm 78 \mu\text{U}/\text{min}$, colchicine, $n = 3$) insulin secretion were similar in control and colchicine-treated plates. Mts. were easily identifiable 24 h after colchicine was removed.

DISCUSSION

Several observations have cast some doubt on the hypothesis that Mts are important in insulin secretion (19). First, the concentration of colchicine that inhibits insulin secretion from isolated islets (usually 10^{-3} M) is much higher than that required to block mitosis in KB cells grown in culture (5×10^{-8} M). At 10^{-3} M, colchicine also blocks glucose uptake into fat cell ghosts (21), inhibits synaptosomal ATPase activity (22), disrupts the Golgi apparatus (23), and might alter insulin secretion by effects on beta cells that are not mediated by Mts. By determining the exact dose of colchicine necessary to completely disrupt beta cell cytoplasmic Mts, it is now evident that this concentration, 10^{-6} M, is three orders of magnitude less than that which affects glucose uptake, ATPase activity, or that which alters the Golgi apparatus. Both colchicine and lumicolchicine block nucleotide transport at 5×10^{-5} M (24). However, the inhibition of sustained insulin release by colchicine was not seen with lumicolchicine and cannot be due to an effect on nucleotide transport. Since the secondary release of insulin was not completely inhibited in the beta cells that contained no Mts, the Mts appeared to facilitate the rate at which the packaged hormone reached the cell surface. Diffusion of insulin can probably keep some hormone moving to the cell surface at a lower rate. There is a pool of insulin within the cell that requires no Mts to be secreted.

Indirect immunofluorescence is a powerful tool to identify the CMTC within the beta cells. Numerous Mts up to $50 \mu\text{m}$ in length radiate from the perinuclear area of the cell to the plasma membrane. In some cultures, the insulin secretory granules are seen to align along the Mts. This organization is

similar to that seen on the erythrophores where pigment granules line up along the Mts (25, 26). Such an arrangement suggests that the Mts help to organize the polarity of the cell and direct the insulin from within the cell to the plasma membrane. Insulin packaged in granules near the plasma membrane does not appear to require Mts for secretion.

Microtubular-insulin secretory granule interaction has previously been investigated by electron microscopy. With this technique, insulin secretory granules did not appear to be associated with the Mts with any more frequency than would be found by chance (2, 3). The alignment of the insulin secretory granules along the Mts suggests that binding of the granule to a component of the Mts may occur. It has not yet been possible to develop direct methods to quantify this possible relationship. Binding between secretory granules and cytoskeletal proteins could be an important form of interaction in hormonal cells. Insulin and pituitary granules also binds to the contractile protein actin (27, 28). Sherline et al. (29) have demonstrated that isolated pituitary secretory granules bind to Mts. The binding appears to occur through microtubular-associated proteins (MAPs) that are high molecular weight proteins which comprise 20–30% of the Mts (30).

Several theories have been proposed for the mechanism by which Mts move cellular particles. In cilia, a sliding filament mechanism in which Mts move over each other in opposite directions has been proposed (31). When examined by immunofluorescence, the Mts in beta cells did not often occur in groups. This observation has been quantified by electron microscopy, and studies using this technique do not indicate that the Mts are associated in groups in the beta cell (2, 3). Thus, the sliding filament hypothesis appears to be a less likely explanation of the mechanism for movement of the insulin secretory granule in the beta cell than other theories.

Another possible mechanism of movement is based on Mt assembly-disassembly. As a Mt is assembled and increases in length, it could carry the insulin secretory granule toward the cell membrane. Margolis and Wilson (32) have labeled Mts with H^3 GTP to get an indication of tubulin exchange. Under steady state conditions, the label was almost completely retained in the Mt. They concluded that assembly and disassembly occur at opposite ends of the Mt. If disassembly occurs at the opposite end of the Mt, this process could function like a treadmill and translocate insulin secretory granules in the beta cell.

Experiments quantifying the amount of tubulin, by colchicine binding assays, in isolated islets also suggest that the assembly of tubulin to Mts is important. In the islet, about one-third of the tubulin exists in the polymerized state as Mts (7). The insulin secretagogues, glucose and theophylline, increase the polymerization of tubulin to Mts (7, 33). In the rat, fasting for 72 h decreases both the total tubulin content and the degree of polymerization of tubulin in the islet. These shifts in tubulin polymerization to Mts would make Mts available to bind insulin secretory granules in the beta cell.

Although it has been proposed that calcium is a regulator of Mt polymerization, the physiologic role of calcium in the *in vivo* polymerization reaction is unclear. Calcium, in millimolar concentrations, prevents the assembly of Mts *in vitro* (34). In the presence of the calcium binding protein calmodulin, Mt assembly is inhibited at micromolar concentrations (35). In our study and many others, extracellular calcium is an absolute requirement for exocytosis. Despite altering the concentrations of extracellular calcium from 0 to 5.0 mM, the Mt pattern in

the beta cells was unaltered. However, subtle changes in the morphology of Mts would not be detected by immunofluorescence. Thus, the intracellular flow of calcium that occurs at the time of active insulin secretion does not alter the degree of tubulin polymerization as measured either by colchicine binding (33) or immunofluorescence.

The monolayer culture system described here is useful for morphologic, biochemical, and secretory studies of beta cells. Our results demonstrate that intact Mts facilitate the sustained secretion of insulin but are not necessary for the immediate release of the hormone.

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