Acetylcholine Activates Intracellular Movement of Insulin Granules in Pancreatic β-Cells Via Inositol Triphosphate–Dependent Mobilization of Intracellular Ca^{2+}

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Intracellular movement of secretory granules is a proximal stage in the secretory cascade that ends in the release product from cells. We investigated mechanisms underlying the control of this movement by acetylcholine using an insulinoma cell line, MIN6, in which acetylcholine increases both insulin secretion and granule movement. The peak activation of movement was observed 3 min after an acetylcholine challenge. The effects were nullified by the muscarinic inhibitor atropine, phospholipase C (PLC) inhibitors (D 609 and compound 48/80), and pretreatment with the Ca^{2+} pump inhibitor, thapsigargin. Inhibitors of Ca^{2+}-dependent phospholipase A_2 (arachidonyl trifluoromethyl ketone and methyl arachidonyl fluorophosphate) also partially inhibited the movement caused by acetylcholine, but downregulation of protein kinase C by overnight incubation with the phorbol ester 12-*o*-tetradecanoylphorbol-13-acetate failed to exert any influence. Acetylcholine stimulation of granule movement was not reproduced by membrane depolarization with high K^+. Phosphorylation of the endogenous myosin light chain in MIN6 cells was increased by addition of acetylcholine and decreased by the Ca^{2+} chelator BAPTA (1,2-bis[2-aminophenoxy]ethane-N,N,N′,N′-tetraacetic acid). The calmodulin inhibitor W-7 and the myosin light-chain kinase inhibitor ML-9 decreased the motile events in the β-cells under both nonstimulated and acetylcholine-stimulated conditions. These findings led us to conclude that inositol triphosphate causes Ca^{2+} mobilization by muscarinic activation of PLC, leading to intracellular translocation of insulin granules to the ready-releasable pool in pancreatic β-cells via Ca^{2+}/calmodulin–dependent phosphorylation of myosin light chains. *Diabetes* 47:1699–1706, 1998

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$AACOCF_3$, arachidonyl trifluoromethyl ketone; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetraacetoxymethyl ester; BSA, bovine serum albumin; $cPLA_2$, Ca^{2+}-dependent PLA_2; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ECL, enhanced chemiluminescence; HELSS, haloenol lactone suicide substrate; IP_3, inositol triphosphate; $iPLA_2$, Ca^{2+}-independent PLA_2; MAF, methyl arachidonyl fluorophosphate; MLC, myosin light chain; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; TPA, 12-*o*-tetradecanoylphorbol-13-acetate.

Intracellular Ca^{2+} plays a critical role in the control of secretory events in the pancreatic β-cell (1,2). Exocytosis occurs as the final and rate-limiting step in the series of events involved in regulated secretion, with translocation of insulin secretory granules being a prerequisite proximal stage. We recently analyzed the intracellular movement of the secretory granules in a hamster-derived insulinoma cell line (HIT T15) using phase-contrast microscopy and image analysis, and reported that intracellular movement of secretory granules was activated by acetylcholine and glucose (3). Ca^{2+} is essential for the control of the movement, which agrees with previous results for dispersed rat islet cells (4). Because motile events are observed under unstimulated conditions, the movement of insulin granules appears to require lower concentrations of Ca^{2+} than those necessary for exocytosis. Ca^{2+} influx through the plasma membrane is unrelated to the control of the granule movement because elevation of the intracellular Ca^{2+} levels by high K^+ and tolbutamide failed to increase the movement (3).

Autonomic nerve endings are abundantly distributed in the pancreatic islets, and insulin secretion from the pancreatic β-cell is modified by neurotransmitters such as acetylcholine, which modulates insulin release via multiple mechanisms. Phospholipid breakdown by phospholipase C (PLC) via activation of the muscarinic receptor results in activation of protein kinase C (PKC) by diacylglycerol and mobilization of Ca^{2+} by inositol triphosphate (IP_3). Acetylcholine has been reported to activate phospholipase A_2 and cause changes in ion fluxes that govern the intracellular Na^+ concentration (5), with a resultant slow increase in Ca^{2+} influx as well as increases in effluxes of Rb^+ and Ca^{2+} (6,7).

In the present study, we investigated how acetylcholine activates the granule movement and increases insulin secretion using a glucose-responsive pancreatic β-cell line, MIN6.

RESEARCH DESIGN AND METHODS

Materials. MIN6 cells and RINr cells were donated by Profs. J. Miyazaki (Osaka University, Osaka, Japan) and S. Seino (Chiba University, Chiba, Japan), respectively. Forskolin, 12-*o*-tetradecanoylphorbol-13-acetate (TPA), dantrolene, and okadaic acid were purchased from Wako (Tokyo). Quinacrine was obtained from Research Biochemicals International (Natick, MA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui (Tokyo). Acetylcholine, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), and monoclonal antibody against myosin light chain (MLC) were obtained from Sigma (St. Louis, MO). Nifedipine was obtained from Fujisawa Pharmaceutical (Osaka, Japan) and atropine sulfate was obtained from Hoei-Kogyo (Osaka, Japan). D 609, compound 48/80, and haloenol lactone suicide

ubstrate (HELSS) were obtained from Biomolecular Research Labs (Plymouth eeting, PA). Arachidonyl trifluoromethyl ketone ($AACOCF_3$) and methyl arachidonyl fluorophosphate (MAF) were obtained from Cayman Chemicals (Ann rbor, MI). W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide] and W-[*N*-(6-aminohexyl)-1-naphthalene-sulfonamide] were synthesized as previously escribed (8). ML-9 [1-(5-chloronaphthalene-sulfonyl)-1-H-hexahydro-1,4-azepine] was synthesized as described by Saitoh et al. (9). ML-5 [1-(naphthalene-ulfonyl)-1-H-hexahydro-1,4-diazepine] is a structurally related negative control ompound for MLC kinase (10). KN-62 (1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-ethyl-L-tyrosyl]-4-phenylpiperazine) was synthesized as described by Tokuitsu et al. (11). All other chemicals were of the highest grade available. The adioimmunoassay insulin kit used was obtained from Eiken (Tokyo), and nhanced chemiluminescence (ECL) was obtained from Amersham (Tokyo).

ell culture. MIN6 cells were cultured in DMEM supplemented with 66 mg/l anamycin sulfate and 15% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 95% air/5% CO_2 (12). RINr cells were also cultured in DMEM but with 10% CS (13). The cells were passaged and harvested using trypsin/EDTA; the culture edium was replaced every other day.

lectron microscopy. MIN6 and RINr cells cultured on plastic culture dishes ere fixed with 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4) for 30 in, and subsequently with 1% osmium tetroxide in the same buffer for 15 min. he cells were washed with 5% sucrose in distilled water, and incubated with 3% anyl acetate in distilled water for 30 min. After dehydration through a graded eries of ethanol concentrations, they were embedded in epoxy resin (Epok 812; ken Shoji, Tokyo) according to the method of Luft (14). Ultrathin sections were ut with a diamond knife on an ultramicrotome (Porter-Blum MT-1; Sorvall, Newown, CT), stained with uranyl acetate and lead citrate (15), and examined with transmission electron microscope (H-7100; Hitachi, Tokyo).

ideo microscopy. Experiments were carried out with an inverted light microcope (Axiovert 135; Carl Zeiss, Germany) equipped with a ×63 objective lens Plan-Neofluar, Carl Zeiss) and a ×2.5 insertion lens. Images were detected by a harge coupled device camera (DXC-930; Sony, Japan), displayed on a monitor creen (PVM-9040; Sony) at a final magnification of ×8,600 and recorded with a deotape recorder (SVO-260; Sony). Pictures were reproduced from videotapes nd analyzed on the monitor using an image analyzer (Argus-20; Hamamatsu hotonics, Hamamatsu, Japan). Unless time-course observations were necessary, e exposed the cells to light for very short periods. Otherwise, the movement uickly became faint. All the experiments were carried out at 37°C, because ovement became slower at lower temperatures.

uantification of movement and velocity of the secretory granules. MIN6 ells were seeded at a density of 3.5 to 5 × 10^5 cells onto glass-bottom culture shes (35 mm diameter; Meridian Instruments Far East, Tokyo, Japan) 1–2 days efore each experiment. On the day of experimentation, cells were preincubated 37°C for 60 min in 1 ml of HEPES-buffered Krebs solution containing (in mol/l): 119 NaCl, 4.75 KCl, 5 $NaHCO_3$, 2.54 $CaCl_2$, 1.2 $MgSO_4$, and 20 HEPES (pH 4 with NaOH), with 5 mg/ml bovine serum albumin (BSA). They were then furer incubated in 1 ml of HEPES-buffered Krebs solution containing 5 mg/ml BSA ith or without various reagents, as described below. Movement of insulin granes was quantitatively assessed by the method originally devised by Lacy et al.), with some modification (3). Briefly, numbers of the granules that moved into out of a square (3.5 × 3.5 μm) during a 30-s period were counted. One MIN6 ell was equivalent to 5–6 complete squares. Unless otherwise noted, the countg was started 2 min before and 3 min after test substances were added. Changes the numbers between, before, and after the addition were compared by paired tudent's *t* test. The speed was assessed by tracing vesicle movement using the mage analyzer. Solvents such as DMSO or ethanol did not affect the assay at the oncentrations used in these experiments. Four to five cells were observed in one xperiment; data are expressed as means ± SE of the frequency assessed in more an 30 squares from at least two independent experiments. For fluorescent etection of secretory granules, quinacrine (0.2 μmol/l) was loaded overnight, excition was effected at 450–490 nm, and emitted light was collected at 515–565 nm. s a comparison, the movement of intracellular organelles in RINr cells, a granle-poor insulinoma cell line, was also assessed using the method described bove. When K^+ concentration in the incubation buffer was increased, Na^+ was educed isoosmotically.

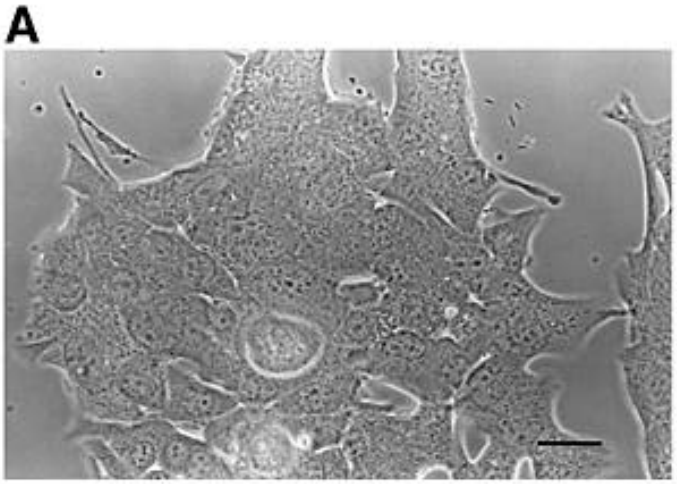
eparation of phosphorylated MLC in MIN6 cells. MIN6 cells (~5 × 10^6) were cubated in dishes for 60 min in glucose-free HEPES-buffered Krebs-Ringer olution supplemented with 5 mg/ml BSA. After being washed twice, the cells were rther incubated for 15 min in HEPES-buffered Krebs-Ringer solution (see bove) with or without acetylcholine (1 mmol/l) and BAPTA-AM (1,2-bis[2-minophenoxy]ethane-N,N,N′,N′-tetraacetic acid tetraacetoxymethyl ester) 0 μmol/l). Separation of phosphorylated MLC was carried out according to the ethod of Persechini et al. (16). After addition of 5% trichloroacetic acid (TCA) nd 2 mmol/l dithiothreitol (DTT) (final concentrations), the cells were left for 10 in at room temperature and scraped from the dishes. The extracts were centifuged and washed four times in a glass tube with acetone containing 10 mmol/l DTT; pellets were dissolved in 60 μl urea sample buffer (8.3 mol/l urea, 20 mmol/l Tris-base, 22 mmol/l glycine, 10 mmol/l DTT, and 0.1% bromophenol blue). Proteins (~10 μg) were separated on a polyacrylamide gel (15% polyacrylamide, 0.75% bisacrylamide, 40% glycerol, 20 mmol/l Tris-base, and 23 mmol/l glycine) at 450 V for 3 h, transferred onto nitrocellulose membranes and exposed to anti-MLC antibody. Immunopositive bands were visualized with the ECL kit. With this separation, the phosphorylated forms of MLC migrated faster than the nonphosphorylated form because of differences in viscosity and/or sedimentation coefficients (16). Although endogenous MLC was both mono- and diphosphorylated in permeabilized MIN6 cells (17), only non- and monophosphorylated forms were detected under these experimental conditions. The density of each band was determined densitometrically, and the extent of MLC phosphorylation was expressed as the percent of the total (non- plus monophosphorylated) MLC in each lane.

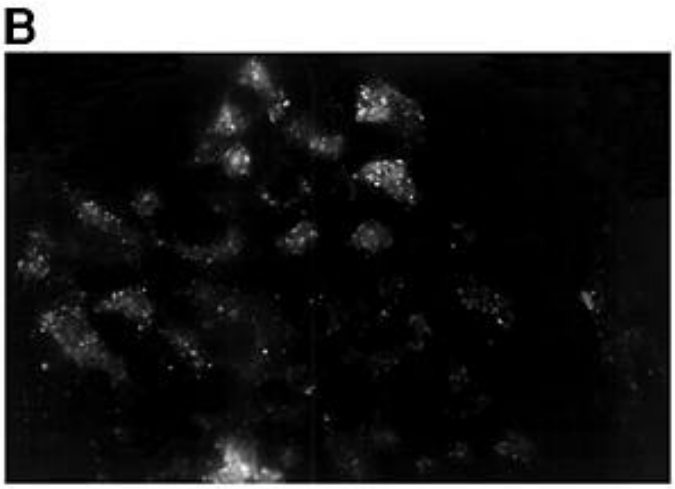
Insulin assay. MIN6 cells were seeded at a density of 1 × 10^5 cells per well in 24-well tissue culture plates 3–4 days before each experiment. On the day of experimentation, cells were preincubated at 37°C for 2 h in 1 ml of HEPES-buffered Krebs solution with 5 mg/ml BSA. They were then further incubated for 60 min in 0.5 ml of the same solution containing 5 mg/ml BSA with or without various reagents, as described below. At the end of the incubation, an aliquot was sampled, centrifuged briefly to sediment any detached cells, and stored at –20°C until assayed. Insulin released into the media was measured by radioimmunoassay using bovine insulin as a standard. None of the compounds or antibodies used here interfered with the assay. Data are expressed as means ± SE and statistical significance was set at the 5% level.

RESULTS

Comparison of the morphology of RINr and MIN6 cells. Phase-contrast, quinacrine-fluorescent, and electron microscopic images of MIN6 and RINr cells are illustrated in Figs. 1 and 2. In MIN6 cells, quinacrine fluorescence was found only in the cytoplasm in a granular pattern. The fluorescence was essentially coincident with the many small, dense, round granules (diameter 0.5–0.7 μm under the phase-contrast microscope and 0.2–0.6 μm by electron microscopy). A few larger organelles were also quinacrine positive. These appeared to be lysosomes and readily distinguished from the granules by their size and lack of motility. The small fluorescent granules demonstrated a heterogeneous distribution in the cytoplasmic areas of MIN6 cells. For analysis of the movement of the secretory granules, we used the phase-contrast microscope system, because the motile events rapidly slowed under the fluorescent microscope. RINr cells showed a rather different pattern, with very few quinacrine-fluorescent granules detected in the cytoplasm (Fig. 2*B*); dense small granules were rarely found in either phase-contrast or electron microscopic images (Fig. 2*A* and *C*).

Intracellular motile events in MIN6 and RINr cells. Secretory granules were found to move in the cytoplasmic areas of MIN6 cells without any secretagogues, with considerable variation from cell to cell. The velocity of the granule movement was up to 1.2 μm/s, which was slightly slower than published values for other pancreatic β-cells: 1.5 μm/s in rat islet β-cells (4) and 1.8 μm/s in HIT T15 cells (3). Acetylcholine (100 μmol/l) and the protein phosphatase inhibitor, okadaic acid (2 μmol/l), significantly increased the frequency of the movement in MIN6 cells, but the velocity of the movement was not obviously affected as reported for HIT cells (3). The effect of okadaic acid seen agrees with the idea that MLC phosphorylation is involved in the regulation of insulin granule movement, because MLC phosphatase, classified into type 1 protein phosphatase, is sensitive to the phosphatase inhibitor (18). In RINr cells, movement of intracellular organelles was also observed under unstimulated conditions, but neither acetylcholine nor okadaic acid caused any significant increase (Table 1).





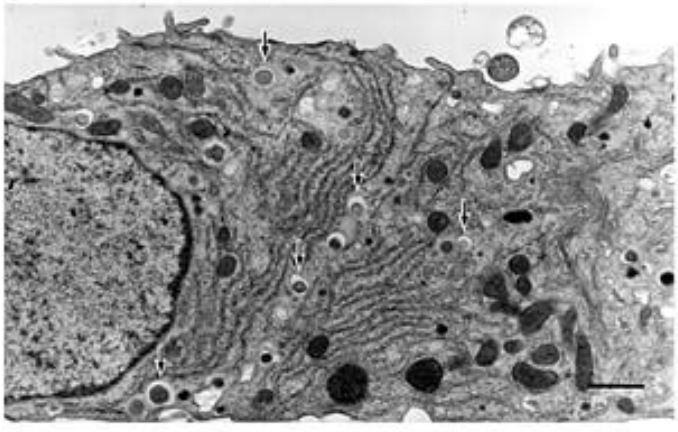
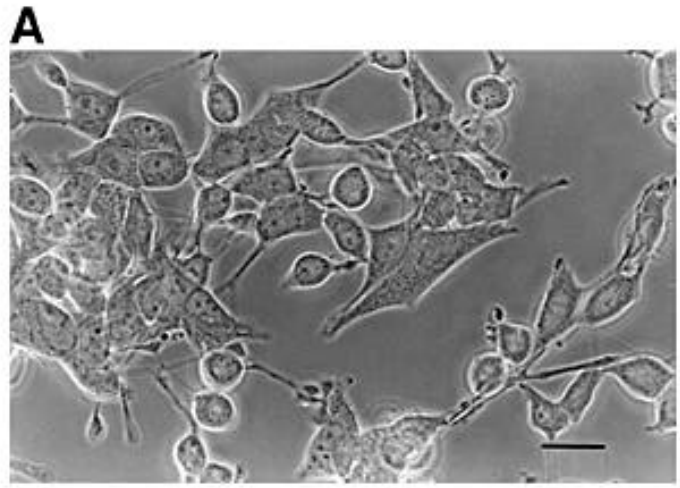


FIG. 1. Phase-contrast (*A*), quinacrine-fluorescence (*B*), and electron microscopic images (*C*) of MIN6 cells. MIN6 cells were seeded onto a 30-mm glass-bottom tissue plastic dish and cultured overnight in DMEM supplemented with 15% fetal calf serum, 25 mmol/l glucose, and 0.2 µmol/l quinacrine. *A*: Observations were carried out using an inverted light microscope equipped with a ×63 objective lens and a ×2.5 insertion lens. Bar = 20 µm. *B*: Shows quinacrine fluorescence from the identical cells shown in Fig. 1*A*. Excitation was effected at 450–490 nm, and emitted light was collected at 515–565 nm. *C*: Electron microscopic image of MIN6 cells. Note the secretory granules consisting of an electron-dense core and an electron-lucent halo (arrows). Bar = 1 µm.



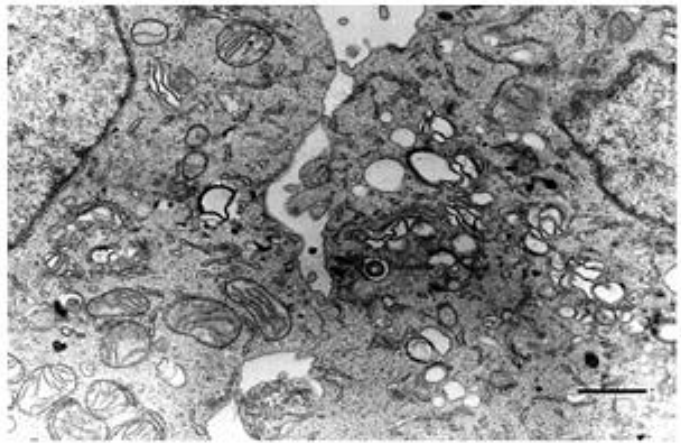


FIG. 2. Phase-contrast (*A*), quinacrine-fluorescence (*B*), and electron microscopic images (*C*) of RINr cells. RINr cells were cultured in DMEM supplemented with 10% fetal calf serum and prepared as described in Fig. 1. Note the relative lack of intracellular granules under the phase-contrast microscope (*A*; bar = 20 µm) with few quinacrine-sensitive granules (*B*); the electron dense-core granules were not practically found in the electron-microscopic area examined (*C*). Bar = 1 µm.

ABLE 1
omparison of movements of intracellular organelles in MIN6 cells and RINr cells

timulation	Frequency of movement (per 30 s per square) Before challenge	After challenge	% Increase	*P* value
IIN6 cells				
Acetylcholine (100 µmol/l)	3.00 ± 0.32	4.03 ± 0.41	34.3	<0.05
Okadaic acid (2 µmol/l)	2.63 ± 0.26	3.50 ± 0.362	33.1	<0.05
INr cells				
Acetylcholine (100 µmol/l)	2.23 ± 0.28	2.43 ± 0.24	9.0	NS
Okadaic acid (2 µmol/l)	1.50 ± 0.14	1.75 ± 0.19	16.7	NS

ata are means ± SE for 30–32 squares in two independent experiments. Intracellular organelles in living MIN6 cells and RINr cells ere observed under a phase-contrast microscope. In MIN6 cells, quinacrine was used to verify the secretory granule-rich region the cell. The images of β-cells were video recorded and reproduced on a monitor. Numbers of granules that moved into or out of quares (3.5 × 3.5 µm) were counted for 30 s. Acetylcholine (100 µmol/l) or okadaic acid (2 µmol/l) were added to the medium, and ovement was counted at 2 min before and 3 min after the addition.

nsulin release from MIN6. Figure 3*A* shows data for osage-dependent effects of acetylcholine on insulin secreon from MIN6 cells. Acetylcholine increased insulin release om MIN6 cells even in the absence of glucose, although ather high concentrations of acetylcholine were required nder this experimental condition. This requirement did not esult from high activity of acetylcholine esterase, because arbamylcholine, the stable analog of acetylcholine, had a simar effect on the release (data not shown), and we consider IIN6 cells to have a poorer sensitivity to acetylcholine.

haracterization of granule movement in MIN6 cells in esponse to acetylcholine. Figure 3*B* depicts time-depenent effects of acetylcholine on intracellular granule movement in MIN6 cells. When 100 µmol/l acetylcholine was dded to the medium, an increase in the granule movement ppeared and reached a peak after 3 min. The modest ncrease was sustained for at least 17 min thereafter. Data for ffects of various substances on acetylcholine-induced granle movement in MIN6 cells are shown in Tables 2 and 3. tropine (30 µmol/l), the muscarinic antagonist, abolished the cetylcholine-induced granule movement; acetylcholineduced granule movement was less pronounced in the coresence of the voltage-dependent Ca^{2+}-channel blocker, ifedipine (1 µmol/l), although a significant increase still emained (Table 2). The effects of selective inhibitors of hospholipases on the granule movement were also invesgated (Table 3). Simultaneous addition of acetylcholine nd D 609 (200 µmol/l) or compound 48/80 (100 µmol/l), nhibitors of PLC, caused a prompt and potent decrease in ne movement, with the change resulting in minus values. cetylcholine failed to elicit any increase, even when pplied 10 min after the addition of the inhibitor (Fig. 4). ACOCF$_3$ and MAF, selective inhibitors of Ca^{2+}-dependent hospholipase A_2 (cPLA$_2$), also inhibited the movement timulated by acetylcholine, although inhibitory effects on the asal movement were much less than with the PLC nhibitors. Acetylcholine-stimulated movement was affected y HELSS, the selective Ca^{2+}-independent phospholipase A_2 iPLA$_2$) inhibitor.

The effect of acetylcholine disappeared when 100 µmol/l cetylcholine was applied after 20 min pretreatment with 40 mol/l thapsigargin to abolish Ca^{2+} uptake into the store sites ia the intracellular Ca^{2+} pump, whereas the movement under basal conditions was not affected by this treatment (Table 2). The calmodulin inhibitor W-7 (10 µmol/l) inhibited the movement of intracellular granules under basal conditions (2.35 ± 0.17 [before] vs. 1.78 ± 0.15 [10 min after addition of W-7]; n = 46, P < 0.01) and also suppressed the activation by acetylcholine, results that agree with the properties of movement in HIT T15 cells (3). ML-9, which inhibits MLC kinase activity (9) and insulin release (19), caused a significant decrease in movement under unstimulated conditions (3.27 ± 0.12 [before] vs. 2.09 ± 0.12 [10 min after addition of ML-9 at 30 µmol/l]; n = 96, P < 0.001) and nullified the effect of acetylcholine. In contrast, the structurally related and less active control compounds for W-7 and ML-9 (W-5 and ML-5, respectively) caused much smaller decreases in basal movement and failed to inhibit the activation of movement by acetylcholine. When MIN6 cells were depleted of PKC activity by overnight incubation with 200 nmol/l TPA, acetylcholine still activated granule movement. Movement was also unaffected by 60 µg/ml OAG (1.62 ± 0.18 [control] vs. 1.37 ± 0.33 [OAG]; n = 29, NS). The selective CaM kinase II inhibitor, KN-62, also failed to influence acetylcholine-activated movements (Table 2). High K^+ (20 mmol/l) depolarization did not have any effect on the granule movement (Fig. 5*A*). Dantrolene, which inhibits Ca^{2+} mobilization from the intracellular store, suppressed the movement, and the effect of acetylcholine was abolished when added after a 10-min treatment with dantrolene (Fig. 5*B*).

Phosphorylation of endogenous MLC in MIN6 cells. Native gel electrophoresis results for endogenous phosphorylation of MLC are given in Fig. 6. The rate of monophosphorylated MLC under the control condition was variable (20–40%) among experiments when accessed densitometrically. In Fig. 6*A*, 36.8% of the total MLC was monophosphorylated under the unstimulated condition. Acetylcholine increased the monophosphorylated form of MLC in MIN6 cells (50.3%), and was slightly decreased when the cells were incubated with the Ca^{2+} chelator, BAPTA-AM (30.0%). The acetylcholine concentration required for endogenous phosphorylation of MLC was high, possibly because of low sensitivity of the cell line to acetylcholine (see above) and high activity of MLC phosphatase in the cell extract (17). Depolarization with 20 or 50 mmol/l K^+, which increased insulin release (data not shown), failed to affect MLC phosphoryla-

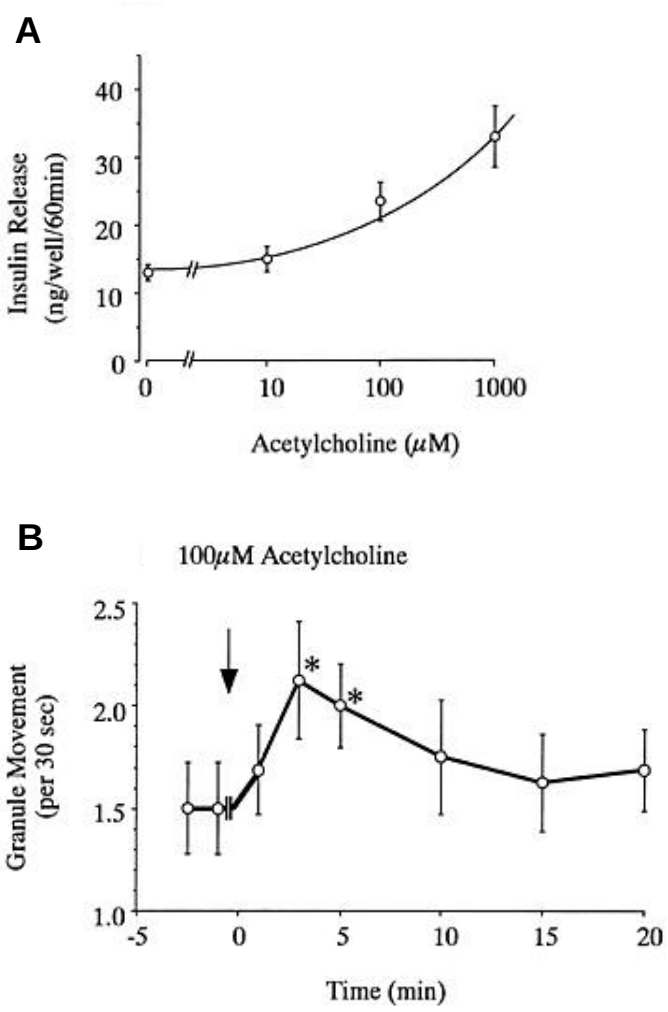


FIG. 3. Insulin secretion and granule movement increased by acetylcholine. *A*: MIN6 cells (1×10^5 cells/well) were seeded onto 24-well plates 3 days before the experiments. On the day of experimentation (estimated as 2×10^5 cells/well), after 2-h incubation in glucose-free HEPES/Krebs solution with BSA (5 mg/ml), various concentrations of acetylcholine were added. Insulin released into the media was measured by radioimmunoassay. Each symbol represents the mean ± SE for eight observations in two independent experiments. *B*: Time course of acetylcholine-activated movement of the intracellular granules. The insulin granules in living MIN6 cells were observed. The images of β-cells were video recorded and reproduced on a monitor. The number of granules that moved into or out of squares (3.5 × 3.5 μm) were counted for 30 s in each square. Acetylcholine (100 μmol/l) was added to the medium as indicated and the movement was further assessed for 20 min after the addition. Data are means ± SE values for 40 squares in three independent experiments. *$P < 0.05$, paired t test.

tion (Fig. 6*B*). The percentage of monophosphorylated MLC was 19.4% for both 20 and 50 mmol/l K^+, whereas the increase by acetylcholine was consistently observed (from 20.9 [control] to 38.1% [1 mmol/l acetylcholine]). The diphosphorylated form of MLC was undetectable in these experiments.

DISCUSSION

The present experiments demonstrated that acetylcholine activates intracellular movement of secretory granules as a result of muscarinic mobilization of intracellular Ca^{2+}. The glucose-responsive insulinoma cell line, MIN6, derived from insulinoma cells in transgenic mice with targeted expression of the SV40 antigen, was used because of its higher insulin content than HIT T15 and its normal secretory sensitivity to nutrient secretagogues. In particular, MIN6 cells respond to a similar concentration range of glucose for insulin release as intact pancreatic islets (20).

The present results on the effect of acetylcholine on the granule movement in MIN6 cells are comparable with those found earlier for HIT T15 cells (3). To confirm that the increased movements were indeed due to those of the secretory processes, we used another insulinoma cell line, RINr, cloned from radiated rat islet cells (13). RINr cells are rich in organelles such as the Golgi apparatus, endoplasmic reticulum, and mitochondria, but possess few secretory granules, as verified by electron microscopy (Fig. 2). The intracellular organelles in the granule-poor RINr cells did not respond to acetylcholine or okadaic acid, which caused a marked increase in granule movement in MIN6 cells (Table 1) and HIT T15 (3). Therefore, we concluded that other intracellular organelles do not behave as secretory granules and that the method is reliable for assessment of intracellular movement of secretory granules.

In this study, we further investigated the mechanisms involved in the control of the movement by acetylcholine. Acetylcholine has been reported to exert versatile effects on the secretory machinery of the pancreatic β-cell. Hydrolysis of phosphatidylinositol 4,5,bisphosphate by activation of PLC via muscarinic activation positively controls insulin release by IP_3-induced Ca^{2+} mobilization and by activation of PKC by diacylglycerol. Inhibition of acetylcholine-caused activation of the movement in MIN6 cells by the muscarinic antagonist atropine and by the PLC inhibitors suggests the involvement of muscarinic action in its control of movement. Activation of PKC, however, is unlikely to be important, because overnight incubation of MIN6 cells with TPA to deplete the cells of PKC activity failed to affect acetylcholine-induced activation of the movement. The lack of acute effects of TPA on the movement in HIT T15 cells (3) and of the diacylglycerol analog, OAG (the present experiment), also supports this idea. It has been suggested that activation of PKC is necessary for sustained release of insulin (21), and that this effect is due to sensitization of the secretory machinery to Ca^{2+} (22). The kinase could act on the docking/priming of secretory granules, the step between granule movement and exocytosis. Responsible substrate(s) and molecular mechanism(s) for the action of PKC, however, remain to be determined.

It has been suggested that acetylcholine also activates PLA_2, which causes arachidonate release (23). Arachidonate and its metabolites are known to increase Ca^{2+} influx and mobilization from pancreatic β-cells (24–27). At least two different types of PLA_2—$cPLA_2$ and the ATP-sensitive $iPLA_2$—have been suggested to participate in the control of insulin release (28,29), and it has been demonstrated that $cPLA_2$ is stimulated by carbamylcholine in the co-presence of stimulatory glucose (28). The present results using selective inhibitors of these PLA_2 isoforms indicate that $cPLA_2$, rather than $iPLA_2$, participates in the control of the granule movement. In contrast to PLC, $cPLA_2$ does not appear to contribute to control under basal conditions.

Membrane depolarization by changing Na^+ permeability via muscarinic activation has also been suggested as one of the mechanisms by which acetylcholine causes short-lived Ca^{2+} influx through voltage-dependent Ca^{2+} channels and insulin release from the β-cell (7,30). We believe this mechanism is

ABLE 2
haracterization of granule movement by acetylcholine

	Granule movement (per 30 s)		
	Before acetylcholine	After acetylcholine	Change
o addition	2.72 ± 0.15	3.75 ± 0.18	37.9 (112)*
imultaneous addition			
Atropine (30 µmol/l)	2.26 ± 0.16	2.26 ± 0.19	0 (38)
Nifedipine (2 µmol/l)	2.21 ± 0.18	2.60 ± 0.21	17.6 (43)†
retreatment			
Thapsigargin (40 µmol/l, 20 min)	2.67 ± 0.13	2.65 ± 0.15	–1.0 (79)
W-7 (10 µmol/l, 10 min)‡	1.78 ± 0.15	1.87 ± 0.17	5.1 (46)
W-5 (10 µmol/l, 10 min)	2.38 ± 0.17	2.80 ± 0.18	17.6 (46)†
ML-9 (30 µmol/l, 10 min)‡	2.09 ± 0.11	2.25 ± 0.13	7.7 (96)
ML-5 (30 µmol/l, 10 min)‡	2.38 ± 0.12	2.91 ± 0.11	22.3 (58)†
KN-62 (1 µmol/l, 10 min)	0.88 ± 0.17	1.09 ± 0.17	23.9 (32)†
TPA (200 nmol/l, overnight)	2.18 ± 0.10	2.74 ± 0.16	25.7 (90)†

ata are means ± SE or % (n). *Simultaneous addition*: After preincubation for 60 min in BSA-containing HEPES-buffered Krebs-Ringer olution without glucose, MIN6 cells were further incubated with acetylcholine (100 µmol/l) and atropine (30 µmol/l) or nifedipine (2 mol/l). Intracellular movement of the secretory granules in MIN6 cells was assessed as described in METHODS. The granule movement as measured for 30 s, 2 min before the condition was changed. The granule movement in the identical square in the microscopic field as reassessed 3 min after the acetylcholine challenge. *Pretreatment*: Acetylcholine was added to the medium after 60 min incubaon in a glucose-free HEPES-buffered Krebs-Ringer solution with BSA, followed by pretreatment with the various compounds listed. cetylcholine (100 µmol/l) was then added. Each experiment was repeated at least three times, and the movement in 8–20 MIN6 cells equivalent to 40–90 squares) was analyzed in each observation. For TPA downregulation, MIN6 cells were incubated overnight in the omplete culture medium supplemented with 200 nmol/l TPA, and on the day of experimentation, were further incubated for 60 min HEPES-buffered Krebs-Ringer solution with TPA (200 nmol/l) and BSA (5 mg/ml). Acetylcholine was added thereafter. †$P < 0.05$, *P 0.01 vs. the value before the addition of acetylcholine. Statistical significance was assessed by paired t test for each set of experients. ‡Significant decrease in movement occurred during pretreatment.

ot involved in the acetylcholine-induced increase in the β-ranule movement, because Ca^{2+} influx through the Ca^{2+} hannels induced by high K^+ depolarization failed to mimic he effect of acetylcholine. Abolishment of acetylcholine-nduced movement by pretreatment with thapsigargin or antrolene also supports the idea that Ca^{2+} mobilization is more responsible for the control of the movement than Ca^{2+} influx. Partial suppression by nifedipine might occur because inhibition of Ca^{2+} influx through voltage-dependent Ca^{2+} channels causes a decrease in Ca^{2+} mobilization, as demonstrated in mouse islets (30). Such differences in the roles in the secretory cascade may be due to variation in the spatial

IG. 4. Inhibition of acetylcholine-induced granule movement by hibitors of PLC. The movement in the MIN6 insulinoma cells was anazed as described in METHODS. Acetylcholine (100 µmol/l) was applied) min after addition of PLC inhibitors (○, no addition; ●, 100 µmol/l etylcholine at 10 min; △, 200 µmol/l D 609; ▲, 100 µmol/l compound 8/80 at 0 min followed by 100 µmol/l acetylcholine at 10 min). Data e means ± SE values for 40–60 squares in more than six independent xperiments. *Significant increase ($P < 0.05$) by acetylcholine versus ontrol value by unpaired t test.

TABLE 3
Effects on acetylcholine-induced granule movement of inhibitors of phospholipases A_2 and C

	Granule movement (per 30 s)		
	Before acetylcholine	After acetylcholine	Change
No addition	1.25 ± 0.15	1.50 ± 0.16	25.0 (32)*
Compound 48/80 (100 µmol/l)	1.57 ± 0.21	1.03 ± 0.19	–34.4 (30)†
D609 (200 µmol/l)	1.59 ± 0.23	0.55 ± 0.13	–65.4 (29)‡
MAF (2 µmol/l)	1.81 ± 0.32	1.88 ± 0.32	3.9 (32)
$AACOCF_3$ (10 µmol/l)	1.94 ± 0.36	1.77 ± 0.29	–8.8 (31)
HELSS (2 µmol/l)	1.13 ± 0.16	1.47 ± 0.30	30.1 (32)*

Data are means ± SE or % (n). MIN6 cells were preincubated for 60 min in glucose-free HEPES-buffered Krebs-Ringer solution with 5 mg/ml BSA, followed by addition of acetylcholine with or without inhibitors of phospholipases A_2 and C. The movement before and after the addition was analyzed as described in METHODS. Each experiment was repeated at least three times. Significant increase (*$P < 0.05$) and decrease (†$P < 0.01$, ‡$P <$ 0.01) versus the value before the addition of acetylcholine with or without inhibitors are shown. Statistical significance was assessed by the paired t test for each set of experiments.

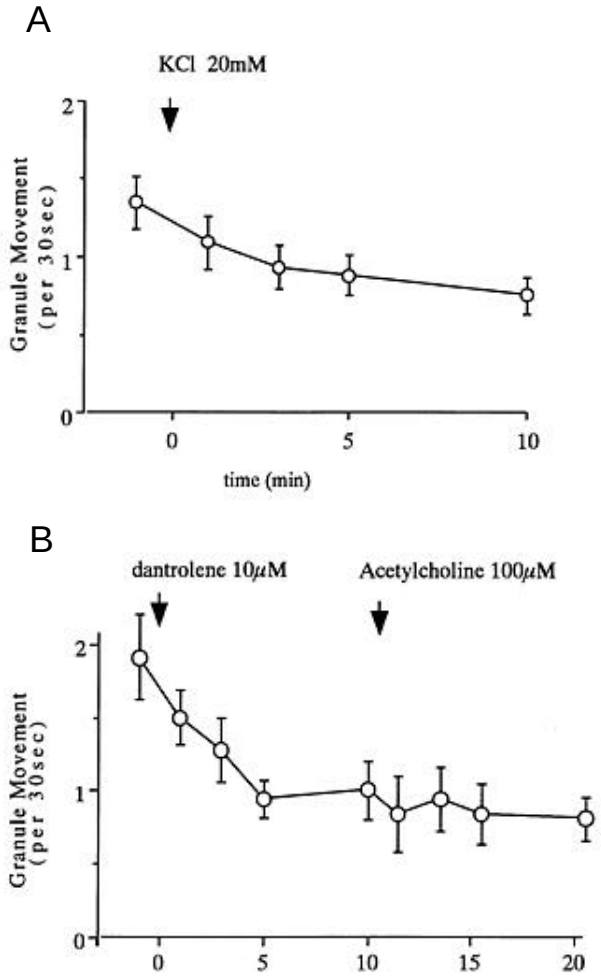


FIG. 5. Effects of high K^+ (*A*) and dantrolene (*B*) on the movement of insulin granules in MIN6 cells. The insulin granules in living MIN6 cells were video recorded and reproduced on a monitor. Numbers of granules that moved into or out of squares (3.5 × 3.5 μm) were counted for 30 s in each square. *A*: KCl (20 mmol/l) was added (arrow) and the movement was further assessed for 10 min after the addition. Data are means ± SE values for 57 squares in four independent experiments. *B*: Dantrolene (10 μmol/l) was added to the medium as indicated and acetylcholine (100 μmol/l) was added 10 min later. Data are means ± SE values for 30 squares in two independent experiments (*B*).

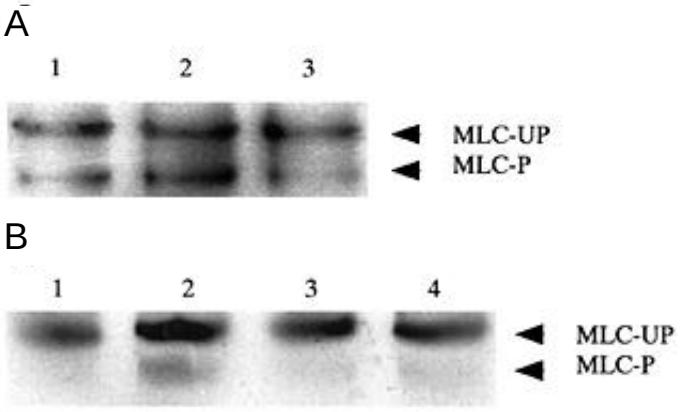


FIG. 6. Endogenous phosphorylation of MLC by acetylcholine. *A*: MIN6 cells were incubated with (*lane 2*) or without (*lane 1*) 1 mmol/l acetylcholine and 20 μmol/l BAPTA-AM (*lane 3*) for 15 min. *B*: MIN6 cells were incubated with control (*lane 1*), 1 mmol/l acetylcholine (*lane 2*), 50 mmol/l K^+ (*lane 3*), or 20 mmol/l K^+ (*lane 4*). The endogenous proteins were extracted with TCA, denatured by urea, and loaded onto a polyacrylamide gel. Separated MLC were visualized by Western blotting using monoclonal anti-MLC antibody and ECL kit. This is representative of three experiments with similar results. In *A*, note that the weak band of phosphorylated MLC was observed even after pretreatment with BAPTA.

distribution of intracellular Ca^{2+} caused by Ca^{2+} influx and Ca^{2+} mobilization (31), and peripheral increase in intracellular Ca^{2+} concentration by Ca^{2+} influx may be insufficient to mobilize secretory granules to the ready-releasable pool.

The effect of acetylcholine appears to be short lived; significant increase in movement was evident for only 3–5 min after the challenge, although its hydrolysis of phospholipids persisted longer (32). This implies desensitization of Ca^{2+} mobilization by IP_3 and/or its distal processes. Alternatively, the effects of acetylcholine could be short lived because the cells may be partly damaged by continuous exposure to intense light during the time course observed. When the cells were exposed to intense light, the movement gradually declined (~25–30% decline during the first 10 min) (Fig. 4), and we noticed that granule movement in the β-cells often became weak after long exposure to light. Long-term potentiation of insulin release by acetylcholine may also be due to activation of PKC by resultant production of diacylglycerol (21,33).

Although it is accepted that Ca^{2+} plays a critical role in the secretory pathway in the pancreatic β-cell, we still have limited knowledge on the relevant receptors (1,34). It has been suggested that myosin is involved in intracellular transport in drosophila embryos (35), and that Ca^{2+}/CaM-dependent phosphorylation of the MLC controls insulin release (36); evidence for the presence of MLC kinase and its participation in the secretory machinery has been obtained (17,37). We recently demonstrated that its phosphorylation by either MLC kinase or CaM kinase II may control the granule movement, with distinct Ca^{2+} requirements via phosphorylating the MLC kinase site of the light chain (17). In that study with in vitro phosphorylation experiments, it was suggested that CaM kinase II may also play an additional role in the phosphorylation at an elevated, but not basal, concentration of Ca^{2+} (17). However, the present results using its selective inhibitor, KN-62, demonstrated that CaM kinase II is unlikely to participate in the acetylcholine-induced activation (Table 2).

In conclusion, the present results provide support for multiple effects of acetylcholine on the secretory cascade in the pancreatic β-cell, where Ca^{2+} influx and Ca^{2+} mobilization play distinct and cooperative roles in the efficient release of insulin. Ca^{2+}-influx elicits exocytosis of insulin, whereas IP_3 mobilizes Ca^{2+} from internal store sites, resulting in recruitment to ready-releasable secretory granules.

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Author Queries (please see Q in margin and underlined text)

Q1: Please define BAPTA, BAPTA-AM, and PLA_2, and confirm HELSS is correctly defined.>
Q2: Okay to change spelled-out version of OAG to 1-oleoyl-2-acetyl-rac-glycerol? See also in list of abbreviations.>
Q3: Has MLCK been correctly spelled out?>
Q4: Was this kanamycin A?>
Q5: Does the rewording of “Unless mentioned” keep your original meaning?>
Q6: Have TCA and DTT been correctly spelled out?>
Q7: At what level was significance defined?>
Q8: Should this be carbamoylcholine?>
Q9: Please define PLA_2.>
Q10: Has PIP_2 been correctly spelled out?>
Q11: Could “and endorsed” be replaced with “evidence”? Is the kinase being referred to here CaM kinase II?>
Q12: Change from MLCK to MLC kinase okay (see at the end of the sentence also)?>

Figures 1 and 3: Is 8,600 the correct magnification?

Ref 22: Please provide journal volume number.
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