Synergism of Protein Kinase A, Protein Kinase C, and Myosin Light-Chain Kinase in the Secretory Cascade of the Pancreatic β-Cell

Wei Yu, Tae Niwa, Tatsuya Fukasawa, Hiroyoshi Hidaka, Takao Senda, Yasuharu Sasaki, and Ichiro Niki

Insulin, a peptide hormone secreted from the pancreatic β-cell, controls blood glucose level, and disorder in the regulation of insulin secretion is one of the causes of type 2 diabetes (1). Ca^2+ plays a critical role in the control of secretory activity of the β-cell. An increase in the intracellular Ca^2+ in the β-cell in response to insulin secretagogues, including glucose, directly triggers exocytosis of the insulin granules. Second messengers such as cAMP or diacylglycerol increase insulin release through protein phosphorylation by cAMP-dependent protein kinase A (PKA) and Ca^2+-sensitive diacylglycerol-dependent protein kinase C (PKC), respectively. Apart from direct actions of these kinases on intracellular Ca^2+ levels (2,3), these messengers have been considered to enhance insulin release by heightening the Ca^2+ sensitivity of secretory machinery to a given concentration of Ca^2+ confirmed by experiments with membrane permeabilization and capacitance assay (4,5). However, the mechanisms by which insulin release is regulated by these 2 messengers and eventual activation of PKA and PKC have not been elucidated (6,7).

PKA and PKC appear to act on the secretory machinery of the β-cell via distinct mechanisms because simultaneous measurement of membrane capacity and intracellular Ca^2+ concentration reveals that activation of PKA enhances insulin secretion from the mouse pancreatic β-cell and increases the integrated Ca^2+ current, whereas PKC causes similar enhancement of secretion without changing the electrical event (2). Bioimaging of intracellular movement of the insulin granules also suggested that these 2 kinases act on different steps in the secretory cascade of the β-cell (2). Bioimaging of intracellular movement of the insulin granules in the ready-releasable pool by acting on different steps in the secretory cascade, resulting in increased numbers of insulin granules ready to be released.
Materials. MIN6 cells were donated by Prof. S. Seino (Chiba University, Chiba, Japan) and Prof. J.-I. Miyazaki (Osaka University, Suita, Japan). Tolbutamide, monoclonal anti-MLC antibody (MY-21), and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG were purchased from Sigma (St. Louis, MO). Forskolin, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetrakis (acetylomethyl ester) (BAPTA-MA), and 1,2-O-tetradecanoyl-phorbol 13-acetate (TPA) were purchased from Wako (Tokyo). Dulbecco’s modified Eagle’s medium (DMEM) was from Nissui (Tokyo). Rhodamine-conjugated goat anti–guinea pig IgG was from ICN Biomedicals (Costa Mesa, CA). Guinea pig anti-insulin antibody was from Seikagaku Kogyo (Tokyo). Monoclonal anti-synaptophisin was from Boehringer Mannheim (Mannheim, Germany). The radioimmunoassay insulin kit used was from Eiken (Tokyo). Anti–MLCK monoclonal antibody was obtained by injecting purified chicken gizzard MLCK into mice, as described by Hagiwara et al. (10). The enhanced chemiluminescence kit, 5-nm gold-conjugated goat anti-mouse IgG, and 15-nm gold-conjugated goat anti–guinea pig IgG were from Amersham Japan (Tokyo). Anti–monophosphorylated Ser (Thr) and anti–diphosphorylated Thr (Ser) MLC antibodies were raised, as described by Sakurada et al. (11). All other chemicals were of the highest grade available.

Cell culture. MIN6 cells (passage 28–39) were cultured in DMEM supplemented with 66 mg/l kanamycin sulfate and 15% fetal calf serum at 37°C in a humidified atmosphere containing 5% CO2 (12). The cells were passaged and harvested using trypsin/EDTA, and culture medium was replaced every other day. When the cells were depleted of PKC activity, TPA at 200 nmol/l was added to the culture medium and cultured overnight.

Insulin secretion study. MIN6 cells were seeded at a density of 1 × 105 cells per well in 24-well tissue culture plates 3–4 days before each experiment. On the day of experimentation, the cells were preincubated at 37°C for 1 h in 1 ml HEPES-buffered Krebs solution (20 mmol/l HEPES, 117 mmol/l NaCl, 4.7 mmol/l KCl, 2.5 mmol/l CaCl2, 1.2 mmol/l MgSO4, 1.2 mmol/l KH2PO4, and 5.0 mmol/l NaHCO3) supplemented with 5 mg/ml bovine serum albumin (BSA). Cells were then further incubated for 1 h in 1 ml of the same solution containing 5 mg/ml BSA with or without various reagents. 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 quantitatively assessed by the method originally devised by Lacy et al. (11). All other chemicals were of the highest grade available.

Insulin release from MIN6 cells. MIN6 cells grown on 12-well culture plates were washed with phosphate-buffered saline (PBS) and fixed at room temperature with 4% paraformaldehyde for 15 min and subsequently with 99.5% ethanol for 5 min. After washing with PBS, the cells were incubated with the mixture of mouse anti-MLCK antibody diluted 1:100 with PBS and guinea pig anti-insulin antibody diluted 1:1,000 with PBS. After washing with PBS, the sections were incubated with a mixture of 5 nm colloidal gold-conjugated anti-mouse IgG and 15 nm colloidal gold-conjugated anti–guinea pig IgG diluted 1:30 with 1% BSA/PBS for 1 h at room temperature. The sections were then stained with uranyl acetate and lead citrate and examined with an H-7100 transmission electron microscope (Hitachi, Tokyo) at the accelerating voltage of 75 kV.

Subcellular fractionation of β-cells. Fractionation of the MIN6 cell homogenates was carried out according to Regazzi et al. (14) with minor modifications. MIN6 cells (1.5 × 109) were trypsinized and washed with 5 mmol/l HEPES (pH 7.4), 70 mmol/l sucrose, and 230 mmol/l mannitol (buffer A) supplemented with 1 mmol/l EGTA and were then washed twice with buffer A without EGTA. After homogenization in buffer A plus 1 mmol/l dithiothreitol, 2.5 µg/ml leupeptin, and 2.5 µg/ml pepstatin A, the MIN6 homogenates were spun for 10 min at 3,000g to eliminate cell debris and nuclei. The supernatant was then centrifuged at 110,000g for 18 h in a sucrose gradient (0.45–2 mol/l) solution with 20 mmol/l Tris-HCl (pH 7.4) and 0.1 mmol/l MgCl2. Each 0.5-ml fraction was collected. The insulin-rich fractions, which appeared with ~1.5 mol/l sucrose, were regarded as the insulin granules. Fractionated extracts were denatured with sample buffer containing 50 mmol/l Tris HCl (pH 6.8), 10% glycerol, 2% SDS, 2% mercaptoethanol, and 0.1% bromophenol blue, and boiled for 5 min. Proteins in the sample were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes in 25 mmol/l Tris, 192 mmol/l glycine, and 20% (vol/vol) methanol (pH 9.2) at 200 mA for 1 h. After blocking with PBS containing 1% BSA, the filter was incubated with anti-MLC or anti-synaptophysin antibody followed by relevant secondary antibodies. Immunodetection was performed by a chemiluminescence kit (Amersham ECL; Amersham Pharmacia Biotech, Chalfont, Bucks, U.K.).

RESULTS

Insulin release from MIN6 cells. Secretory responses of MIN6 β-cells to various substances in batch incubation are demonstrated in Fig. 1. TPA (200 nmol/l) caused a prominent increase in insulin release in the absence or presence of a stimulatory concentration of glucose (20 mmol/l). In contrast, addition of 5 µmol/l forskolin did not change insulin release with or without TPA in the absence of glucose, but the copresence of glucose resulted in an enormous increase in the release, as seen in normal islet cells (9).

Intracellular traffic of insulin granules by TPA and forskolin. Figure 2 depicts time-dependent effects of forskolin and TPA on the intracellular movement of insulin granules assessed by bioimaging. During the 15-min incubation, an ~20% decrease in frequency of traffic was observed. We believe this decrease may have resulted from exposure to intense light. Forskolin evoked a prompt rise and sustained increase in the granule movement of the insulin granules in the cytoplasmic area (Fig. 2B). The peak of the activation was observed 3 min after the forskolin challenge.
FIG. 1. Insulin secretion from MIN6 cells. MIN6 cells (1 × 10^5 cells/well) were seeded on 24-well plates 3–4 days before experiments. On the day of experimentation, the cells were preincubated for 1 h under glucose-free conditions and further incubated for 1 h in medium containing the various substances indicated. Each symbol represents the mean ± SE for 5–6 observations from 2 independent experiments. Statistical analysis was carried out by an unpaired Student's t test. *P < 0.05; **P < 0.01.

FIG. 2. Time-dependent effects of forskolin and TPA on the granule movement in MIN6 β-cells. MIN6 cells were seeded on glass-bottom plastic dishes. After 1 h of incubation in glucose-free buffer, motile events in the β-cell were observed by phase-contrast microscopy with or without forskolin and/or TPA as indicated. Intracellular movement of insulin granules was assessed as described in RESEARCH DESIGN AND METHODS. Each symbol represents the mean ± SE for 13–16 squares. Each graph is representative of 3 experiments with similar changes. *P < 0.05 by paired Student's t test.
followed by a modest increase in the movement for at least 15 min. In contrast, addition of TPA did not exhibit any remarkable change in the granule movement (Fig. 2C). Combination of TPA and forskolin caused a prompt increase in the granule activation, though the frequency returned to the basal level by 5 min after the addition (Fig. 2D), possibly because some granules were trapped beneath the plasma membrane by TPA (Fig. 5A). However, it must be noted that granule traffic directed toward the plasma membrane was rarely detected in these experiments. Traffic speed was ~1.2–1.5 μm/s, which was not altered much by activation of PKA with or without activation of PKC.

**Intracellular localization of insulin granules and MLCK in MIN6 cells.** We analyzed intracellular localization of insulin granules and MLCK in MIN6 cells by immunofluorescence microscopy. MLCK was detected in the cytoplasm as granular or diffuse fluorescence of FITC, and insulin was detected as granular fluorescence of rhodamine (Fig. 3). Double exposing an image showed that considerable parts of the MLCK immunoreactivities were colocalized with the insulin immunoreactivities. We verified that considerable parts of MLCK are associated with the insulin granules in MIN6 cells by immunoelectron microscopy (Fig. 4A). MLCK was also associated with the Golgi apparatus (Fig. 4B) and rough endoplasmic reticulum (Fig. 4C).

Intracellular localization of insulin granules and MLCK was not uniform but was quite different among cells. The pattern was then classified into 3 categories: 1) insulin granules or MLCK distributed evenly throughout the cytoplasm without their accumulation at the cell periphery (cytoplasmic type; Fig. 3A), 2) insulin granules or MLCK distributed throughout the cytoplasm with their moderate accumulation at the cell periphery (intermediate type; Fig. 3B), and 3) insulin granules or MLCK highly accumulated at the cell periphery (peripheral type; Fig. 3C). When over 400 MIN6 cells were analyzed with anti-insulin antibody under nonstimulated conditions, 40–50% of the cells were classified cytoplasmic type, and the other cells were classified intermediate (20–30%) and peripheral (20–30%) types (Fig. 5).

**Changes in spatial distribution of insulin granules and MLCK.** We next analyzed intracellular localization of insulin granules and MLCK with activation of PKA and/or PKC. Under these conditions, MLCK and insulin granules were in part colocalized. Here we used the above-mentioned classification concerning the localization of the insulin granules and MLCK. The percentage of each type in >400 cells under these conditions is summarized in Fig. 5. Whereas forskolin at 5 μmol/l did not change the distribution of insulin granules, TPA at 200 μmol/l shifted to the cell periphery, and stimulation with forskolin and TPA almost doubled the rate of the periph-
eral type (Fig. 5A). Similarly, TPA, but not forskolin, shifted MLCK to the cell periphery. MLCK-like immunoreactivity in the intermediate pattern was increased by forskolin. Simultaneous stimulation with TPA and forskolin caused a more evident shift of MLCK to the cell periphery (Fig. 5B). One might think that translocation of MLCK is a secondary change to that of insulin granules. This is, however, unlikely because 1) granule-unassociated MLCK-like immunoreactivity was also translocated to the plasma membrane in the peripheral type, and 2) changes in the distribution rate of MLCK was not parallel to that of insulin in forskolin-treated cells. MIN6 cells with overnight treatment by TPA did not influence distribution of the insulin granules, but MLCK in the peripheral pattern was somewhat less than that in the control. Neither insulin granules nor MLCK were shifted to the cell periphery by addition of TPA to the downregulated cells (Fig. 5).

**Subcellular distribution of MLC.** The anti-MLC antibody used for immunoblotting in our study was not applicable for immunohistochemical approaches of this cell line; therefore, we analyzed subcellular distribution of MLC by immunoblotting. Figure 6 shows insulin content determined by radioimmunoassay and immunoblot analyses of MLC and synaptophysin with subcellular fractions by sucrose-gradient centrifugation. As demonstrated in Fig. 6C, synaptophysin was abundant in fractions 9–11, whereas insulin was rich in fractions 12–17, with a peak in fractions 15 and 16. MLC was evidently detected as a prominent band at 18 kDa in fractions 12–17, and the peak was in fractions 14 and 15 (Fig. 6B), whereas a single band at ~25 kDa was also found between fractions 2 and 4.

**Spatial distribution of phosphorylated MLC in MIN6 cells.** We further investigated the distribution of phosphorylated forms of MLC in MIN6 cells. As shown in Fig. 7, both mono- and diphosphorylated forms of MLC were distributed in the cytoplasm in most MIN6 cells under nonstimulated conditions (Fig. 7A and D). Double-staining with antibodies against either mono- or diphosphorylated MLC and insulin indicated that considerable parts of the mono- and diphosphorylated MLC immunoreactivities were colocalized with insulin immunoreactivities (Fig. 7C and F). These findings together with the immunoblotting results suggest that MLC may be associated with insulin granules in the pancreatic β-cell, but not with synaptic granules. There is some difference between the position of the 2 peaks. Possible explanations are 1) the radioimmunoassay used here also cross-reacts with proinsulin, so some discrepancies may be generated, and 2) because MLC is unlikely to be released from the cell, MLC on the exocytosed granule membrane may remain around the plasma membrane without codistribution with insulin.

**DISCUSSION**

In the pancreatic β-cell, PKA, PKC, and Ca²⁺-dependent protein kinases, such as Ca²⁺/calmodulin-dependent protein (CaM) kinase II and MLCK, have been suggested to participate in the control of insulin release (6). Using a bioimaging technique, we directly observed intracellular movement of the insulin granules in living cultured pancreatic β-cells (8). One of the characteristics of the β-cell is that some insulin secretagogues, such as acetylcholine or glucose, control the intra-
cellular movement of insulin granules via mechanisms distinct from those for exocytosis (8,15). The movement of insulin granules is dependent on Ca\(^{2+}\)/calmodulin-dependent protein phosphorylation of MLC (16). We also found that Ca\(^{2+}\) release, but not Ca\(^{2+}\) influx, is responsible for the regulation of granule movement (15,17).

cAMP-dependent protein phosphorylation was also proven to increase the frequency of the granule movement in the \(\beta\)-cell; both adenylate cyclase activator forskolin and the membrane-permeable cAMP analog dibutyryl cAMP increased the frequency of the movement in the primary cultured or tumoral \(\beta\)-cells (13). In contrast, activation of PKC by TPA strongly increased insulin granules situated in the cell periphery (Fig. 5A) without causing evident effects on the granule movement (Fig. 2C). These findings confirm the idea that PKA and PKC act on distinct stages in the secretory cascade. Indeed, costimulation of the \(\beta\)-cell by TPA and forskolin resulted in a further increase in the rate of the insulin granules distributed in the cell periphery (Fig. 5A). We consider that activation of PKA enhances insulin release because moving granules, though not tightly docked to the plasma membranes, become more readily accessible to the plasma membrane where exocytosis occurs. PKC appears to act on a distal step to that for PKA in the sequential secretory events, and this may be why TPA or cholecystokinin, which also activates PKC, exhibits persisting effects on insulin secretion from rat pancreatic islets, which are not reproduced by forskolin (18,19). Thus, PKC seems to regulate insulin output upstream of exocytosis, resulting in an increase the size of the readily releasable pool, as suggested for catecholamine release (20,21). Lack of acute effects of TPA on insulin distribution in PKC downregulated cells also supports this idea (Fig. 5).

We previously reported that MLC is one of the target proteins relevant to Ca\(^{2+}\)-dependent activation of the granule movement (16); inhibition of MLCK activity by 1-(5-chloro-
naphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-9) resulted in a decrease in the granule movement, and elevation of intracellular Ca\textsuperscript{2+} levels increased phosphorylation of endogenous MLC in permeabilized β-cells. The intracellular location of myosin remains unsolved because of the low content of myosin in the β-cell (~10% of myosin ATPase activity in skeletal muscle [22]) and poor cross-reactivity of the anti-MLC antibody with the β-cell MLC in the immunostaining technique. Western blotting using subcellular fractionation of the β-cell suggests that myosin may be colocalized with the insulin granules. Therefore, we conclude that myosin may play the role of a motor protein responsible for the traffic of the insulin granules, but not of synaptic-like (synaptophysin-positive) vesicles. We found that MLCK was in part colocalized with insulin granules, as confirmed by electron microscopy (Fig. 4A). Given that translocation of MLCK occurs by TPA or TPA plus forskolin (Fig. 5B) and PKC phosphorylates MLCK (23), it is tempting to hypothesize that MLCK, phosphorylated by PKC and distributed in the cell periphery, will cause phosphorylation of peripheral MLC, resulting in preferential activation of the granule movement in the vicinity of the plasma membrane.

The anti-phosphorylated MLC antibodies elucidated distinct mechanisms between mono- and diphosphorylation of rabbit smooth muscle MLC at the MLCK phosphorylatable sites (24). Application of these antibodies to immunostaining microscopy of the β-cell revealed that both mono- and diphosphorylated forms of MLC were distributed in the cell, even under nonstimulated conditions. In this approach, the diphosphorylated form of MLC was successfully detected, whereas it was not found in urea-denatured extracts of the same β-cell line, even under acetylcholine stimulation (15).

Association of phosphorylated MLC with the insulin granules strongly supports the fact that phosphorylation of MLC is implicated in the regulation of the insulin granule traffic. We here postulate a network system of protein kinases regulating insulin output in the β-cell. Both MLCK and its substrate MLC are coexisting with the insulin granules, and phosphorylation of MLC regulates intracellular movement of the granules in the cytoplasmic space. We consider that PKA activation may liberate insulin granules from a storage pool and/or increase the motor protein activity, and this may be recognized as PKA-dependent refilling of the readily releasable granules (25). PKA also has been reported to act on other stages to potentiate the granule release (25,26).

Activation of PKC lets insulin granules translocate to the vicinity of the plasma membrane, partly by the aid of MLCK and therefore of granule-associated phosphorylated MLC in the cell periphery. Increases in cytoplasmic concentrations of Ca\textsuperscript{2+} promote translocation of PKC toward the plasma membrane in many types of cells, including pancreatic β-cells (27). To our knowledge, however, this is the first report that MLCK changes its intracellular distribution. Interestingly, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II in neuron cells, has been reported to translocate toward synaptic junctions under ischemic conditions (28). Therefore, some calmodulin-dependent protein kinases may play spatially specific roles via their translocation in the cell.

ACKNOWLEDGMENTS
This work was supported in part by a grant-in-aid for research on priority areas (number 923104) and a grant-in-aid for

FIG. 7. Localization of phosphorylated MLC in MIN6 cells. MIN6 cells were fixed and treated with antibodies specific to monophosphorylated MLC (A and C, green) or diphosphorylated MLC (D and F, green) with anti-insulin antibody (red). In doubly exposed images (C and F), yellow represents the overlapping of fluorescence. Bar = 10 µm.
research (number 09670151) from the Ministry of Education, Science, Sports and Culture, Japan.

The authors thank Prof. J.-I. Miyazaki (Osaka University) and Prof. Susumu Seino (Chiba University) for the gift of MIN6 cells.

REFERENCES


18. Gillis KD, Møller P, Neher E: Protein kinase C enhances exocytosis from chromaffin cells by increasing the size of the readily releasable pool of secretory granules. Neuron 16:1209–1220, 1996


