Myosin light-chain phosphorylation controls insulin secretion at a proximal step in the secretory cascade

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Intracellular calcium ([Ca2+]i) is an important intracellular second messenger that plays a critical role in insulin secretion (1, 2). The rise in [Ca2+]i in the β-cell results in enhanced insulin secretion. This calcium signal is generated by interaction with receptors that have been suggested to participate in stimulus-secretion coupling in the β-cell by biochemical and pharmacological findings (18, 30). A number of Ca2+-dependent protein kinases and other CaM-binding proteins have also been identified in pancreatic β-cells and suggested to be involved in the secretory machinery (11, 19, 35).

Myosin light-chain kinase (MLCK), a Ca2+/CaM-dependent protein kinase, has been proposed to act in positive control of insulin secretion (19, 24). Myosin itself is composed of heavy chains and two types of light chains, one essential and the other regulatory. Heteromer complexes of these myosin subunits exhibit actin-dependent adenosinetriphosphatase activity, which is predominantly controlled by regulatory light-chain phosphorylation by MLCK and other protein kinases (31). However, the step in the secretory process where MLCK acts has yet to be elucidated.

In the present study, we characterized β-cell MLCK and myosin subunits in rat pancreatic islets and mouse pancreatic β-cell lines by biochemical and morphological approaches. We further investigated their possible roles in the secretory pathway in the pancreatic β-cell by secretion studies using streptolysin O (STLO)-permeabilized islets and by observation of actual granule movement.

MATERIALS AND METHODS

Materials. Collagenase (type V), benzamidine, fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (lg) G antibody, monoclonal anti-MLC antibody, guanosine 5′-O-(2-thiodiphosphate) (GDPβS) and polyiodinated antibody against platelet myosin heavy chain (MHC) were purchased from Sigma Chemical (St. Louis, MO). [γ-32P]ATP and the enhanced chemiluminescence (ECL) kit were from Amersham (Arlington Heights, IL). Alkaline-conjugated goat antibodies against rabbit IgG and mouse IgG were purchased from Zymed (San Francisco, CA). Kanamycin sulfate was from Meiji Seika (Tokyo, Japan). STLO and the radiolabeled assay kit for insulin assays were from Eiken Chemical (Tokyo, Japan). Bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), okadaic acid, trichloroacetic acid (TCA), dithiothreitol (DTT), and N-nitrophenyl phosphate were from Wako Chemical (Tokyo, Japan). Leupeptin and the MLC peptide were from the Peptide Institute (Osaka, Japan). Okadaic acid (TCA), dithiothreitol (DTT), and N-nitrophenyl phosphate were from STLO and the radiolabeled assay kit for insulin assays were from Eiken Chemical (Tokyo, Japan). Bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), okadaic acid, trichloroacetic acid (TCA), dithiothreitol (DTT), and N-nitrophenyl phosphate were from Wako Chemical (Tokyo, Japan). Leupeptin and the MLC peptide were from the Peptide Institute (Osaka, Japan). Guanosine 5′-O-(3-thiophosphate) (GTPβS) was from Boehringer (Mannheim, Germany). All other chemicals used here were the purest grade available. CaM was purified from rat liver. Myosin subunits in rat pancreatic islets and mouse pancreatic β-cells were isolated as described in a previous study (20).

Methods. Permeabilization of islets and secretion studies. Islets were isolated from male Sprague-Dawley rats weighing 220–250 g by collagenase-permeabilization as described previously (21). The islets were washed three times in serum-free medium. After the final wash, the islets were incubated in 1 ml of 10 µM STLO in serum-free medium for 10 min at 37°C. After the incubation, the islets were washed two times with ice-cold buffer to remove the permeabilizing agent (22).

Intracellular calcium ([Ca2+]i) was measured by a fluorometric method using Fura-2 as the indicator (23). The islets were permeabilized with STLO as described above and incubated in 10 µM Fura-2/AM for 30 min at 37°C and then washed two times with buffer to remove the unbound Fura-2. The islets were incubated in 10 µM STLO in serum-free medium for 10 min at 37°C and then washed two times with buffer to remove the permeabilizing agent (22).

Results. Intracellular Ca2+ signals were induced at both basal and acetylcholine (ACh)-stimulated conditions in MIN6 cells under basal and acetylcholine (ACh)-stimulated conditions. We propose that MLC phosphorylation may modulate translocation of secretory granules, resulting in enhanced insulin secretion.

brain as described in Ref. 7. MLCK and MLC were purified from chicken gizzard (1, 27) and CaM kinase II from rat brain (33). KN-62 [1-N, O-bis(5-isouquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine and ML-9 [1-(5-chloronaphthalenesulfonyl)-4-H-hexahydro-1,4-diazepine] were synthesized as described in Refs. 33 and 27, respectively.

Preparation of pancreatic islets. Pancreatic islets were isolated from male Wistar rats by collagenase digestion. The solution used was N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-buffered Krebs-Ringer bicarbonate buffer containing (in mM) 119 NaCl, 4.75 KCl, 5 NaHCO3, 2.54 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, and 20 HEPES (pH 7.4 with NaOH) supplemented with 3 mM glucose.

Cell culture βTC3 and MIN6 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 66 mg/ml kanamycin sulfate and 10% fetal calf serum (6, 16) and were used for the experiments when they reached confluence in 10-cm dishes.

Preparation of monoclonal antibodies against MLCK. Monoclonal antibodies against MLCK were raised in mice by injecting chicken gizzard MLCK (9). Antibodies released in the culture media were concentrated by precipitation with ammonium sulfate and dialyzed against phosphate-buffered saline (PBS). The epitopes of the antibodies have not been identified in this study. These antibodies were confirmed not to react with protein kinase A, protein kinase C, or CaM kinase II. In the preliminary experiments, we found that MM17 was the most useful for both immunoblotting and immunohistochemistry.

Western blotting of MLCK, the MLCs, and the MHC. Soluble proteins were extracted by homogenization of pancreatic islets and cultured β-cell lines (MIN6 and βTC3) as well as other tissues in 10 mM MES (pH 6.9) containing 0.27 M sucrose, 1 mM ethylene glycol-bis-(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), 1 mM PMSF, 1 µg/ml leupeptin, and 1 mM benzamidine and then centrifuged (10,000 g, 10 min). The samples were then dissolved in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer [50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 10% glycerol, 2% SDS, 2% 2-mercaptoethanol, and 0.1% bromophenol blue]. Denatured proteins (50 µg protein each for Fig. 1, A and D, and 20 µg for Fig. 1C, except where otherwise mentioned) were loaded onto SDS-PAGE, transferred to nitrocellulose membranes, blocked with 1% BSA, and treated with anti-MLCK monoclonal antibody (Fig. 1A), anti-MLC monoclonal antibody (Fig. 1C), or anti-MHC antibody (Fig. 1D). Chicken gizzard MLCK (10 ng protein for Fig. 1A, lane 2) and extracts from rat vessels (Fig.

![Image](http://ajpendo.physiology.org/)

**Fig. 1.** Presence of myosin light-chain kinase (MLCK), myosin light chain (MLC), and myosin heavy chain (MHC) in pancreatic β-cell. A: Western blot analysis of MLCK. Aliquots (50 µg) of proteins extracted from isolated islets, vessels, and stomach (lanes 1, 3, and 4, respectively) of rats were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto nitrocellulose membranes, followed by treatment with monoclonal anti-MLCK antibody (MM17). Purified chicken gizzard MLCK (10 ng) was also applied in parallel (lane 2). In lanes 5 and 6, extracts from islets (50 µg protein) and purified MLCK (10 ng), respectively, were reacted with anti-MLCK antibody preabsorbed with an excess amount of purified MLCK. Visualization was carried out by a chemiluminescence kit. B: Immunohistochemical staining of a rat pancreas using anti-MLC monoclonal antibody. Frozen sections of rat pancreas were incubated with anti-MLC antibody (MM17) diluted 1:100. After being washed, sections were further incubated with fluorescein isothiocyanate-conjugated goat antibody against mouse immunoglobulin G and mounted. Observation was with a fluorescence microscope (bar, 10 µm). C: Immunoblotting of MLC in rat islets or insulin-secreting cell line (MIN6) and rat vessels. Homogenates containing 20 µg proteins were separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and treated with anti-MLC antibody (lane 1: rat islets; lane 2, MIN6; lane 3, rat vessels). In lane 4, extracts from MIN6 cells (20 µg proteins) were treated with anti-MLC antibody preabsorbed with purified chicken gizzard MLCK. Immunopositive bands were visualized using a chemiluminescence kit. D: Immunoblot analysis of MHC in insulin-secreting cell lines, rat islets, human platelets, and rat cardiac myocytes. Aliquots of proteins (50 µg each, except human platelets of 3 µg) were separated by SDS-PAGE and blotted onto a nitrocellulose membrane, followed by treatment with antibodies raised against nonmuscle MHC (lane 1, MIN6; lane 2, βTC3; lane 3, rat islets; lane 4, human platelets; lane 5, rat cardiac myocytes). Visualization was carried out by alkaline phosphatase.
Separation of phosphorylated MLC in permeabilized MIN6 cells. MIN6 cells (–5 x 10⁶) were incubated for 60 min in glucose-free HEPES-buffered Krebs-Ringer solution supplemented with 5 mg/ml BSA. After two washes, the cells were further incubated in glutamate buffer (pH 7.0) containing 100 mM K-glutamate, 42 mM Na-glutamate, 16 mM HEPES, 5 mg/ml BSA, 1 mM EGTA, 3 mM MgATP, and 0.15 IU/ml STLO. CaCl₂ was then added to the glutamate buffer to give an arbitrary concentration of free Ca²⁺. The free Ca²⁺ concentration was calculated by EQCAL (Biosoft) according to the stability constants from Owen (23) and Chabarek and Martell (4). The calculated concentrations of free Ca²⁺ in the assay buffer were verified with fura 2 measurement. Separation of phosphorylated MLC was carried out according to Ref. 25. After addition of 5% TCA and 2 mM DTT (final concentrations), the cells were left for 10 min at room temperature and scraped. The extracts were washed with acetone containing 10 mM DTT five times in a glass tube. The pellet was dissolved in 70 μl urea sample buffer (8.3 M urea, 20 mM Tris-base, 22 mM glycine, 10 mM DTT, and 0.1% bromophenol blue), and the proteins (60 μg) were separated on a polyacrylamide gel (15% polyacrylamide, 0.75% bisacrylamide, 40% glycerol, 20 mM Tris-base, and 23 mM glycine) at 450 V for 3 h. Separated MLC was transferred onto a nitrocellulose membrane and treated with anti-MLC antibody. Immunopositive bands were visualized as described previously with the ECL kit. In this separation, the phosphorylated forms of MLC immigrated faster than the nonphosphorylated form, possibly because of their differences in viscosity and/or sedimentation coefficient, as discussed in a previous paper (25). The density of each band was determined densitometrically, and the extent of MLC phosphorylation was expressed as the percentage of the total (non-, mono-, plus diphosphorylated) MLC in each lane.

Immunohistochemical detection of MLCK in pancreatic β-cells. Fixation of rat pancreas and preparation of frozen sections were as described in Ref. 29. Incubation with the monoclonal anti-MLCK antibody (MM17) diluted at 1:100 in PBS at 4°C overnight was followed, after washing in PBS, by exposure to a fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody diluted at 1:100 in PBS at room temperature for 1 h. The sections were then mounted in 90% glycerol in PBS containing 1 mg/ml p-phenylenediamine as an autofading agent and were examined under a fluorescence microscope.

Permeabilization of isolated rat pancreatic islets with STLO. Membrane permeabilization was carried out by treating islets and MIN6 cells with STLO (14, 22). For secretion experiments, groups of five size-matched islets were preincubated at 37°C for 1 h in the HEPES-buffered Krebs-Ringer bicarbonate buffer just described supplemented with 5 mg/ml BSA gassed with 95% O₂-5% CO₂. The islets were then washed twice with 1 ml of the glutamate buffer detailed earlier. The islets were permeabilized by incubation with 0.125 IU/ml STLO in 0.6 ml of the same glutamate buffer under various additions. After 45 min of incubation, the media were collected for measurement of released insulin by radioimmunoassay with rat insulin as a standard. None of the compounds or antibodies used here interfered with the assay. In another set of experiments, islets or MIN6 cells treated with STLO were used for assessment of phosphorylation of endogenous MLC. The STLO concentrations selected for the treatment of islets (0.125 IU/ml) and MIN6 cells (0.5 IU/ml) gave the maximal insulin secretion in response to a stimulatory concentration of Ca²⁺. In the preliminary experiments, we found that most (~90%) MLCK and MLC did not leak out of the treated β-cells during 45 min of incubation with STLO (data not shown).

MLC phosphorylation by MLCK and by CaM kinase II. The MLCK assay was carried out basically according to Kemp et al. (12) with minor modifications. Activity was determined by incubation in 50 μl (final volume) containing 50 μM MLC peptide substrate (KKRAARTSNVFA, synthesized on the basis of the phosphorylation sites of MLC by MLCK), 5.3 nM CaM, 5 mM Mg acetate, 0.5 mM CaCl₂, 0.1% Tween-80, 40 mM HEPES (pH 7), 10 μM [γ-³²P]ATP and 13 nM chicken gizzard MLC with or without anti-MLCK antibodies (χ100). In another set of experiments, 30 nM CaM and 31 nM CaM kinase II were added. Reactions were initiated by the addition of [γ-³²P]ATP and, after incubation for 7 min at 30°C, were terminated by spotting of 25-μl aliquots onto Whatman P81 chromatography paper, followed by washing three times in 0.75% phosphoric acid. Radioactivity on the filters was determined by Cerenkov counting. In some experiments, the kinase activity of purified MLCK was measured under parallel conditions.

MLC phosphorylating activity in the β-cell extracts. MLC phosphorylating activity in β-cell extracts was assayed as described above with minor modifications. Two thousand isolated pancreatic islets or 10⁶ MIN6 cells were washed twice with PBS and resuspended in buffer A containing 10 mM MES (pH 6.9), 0.27 M sucrose, 1 mM EGTA, 1 mM PMSF, 1 μg/ml leupeptin, and 1 mM benzamidine. The cells were then washed twice with buffer A and homogenized on ice in 200 μl buffer A with 1 μM okadaic acid and 20 mM p-nitrophosphosphate. The presence of these phosphatase inhibitors increased the ³²P radioactivity incorporated into the peptide substrate, which implies that the cell extracts contained MLC phosphatase activity. After centrifugation at 10,000 g for 10 min at 4°C, 10-μl aliquots of the supernatant (containing 10 μg protein) were incubated in 50 μl (final volume) at 30°C for 5 min with 20 μM [γ-³²P]ATP, 40 mM HEPES (pH 7.0), 5 mM Mg acetate, 0.1% Tween-80, 30 mM calmodulin, 1.2 mM EGTA, and 50 μM MLC peptide. CaCl₂ was added to the buffer to give an arbitrary concentration of free Ca²⁺, as we have described. Under this experimental condition, the Michaelis-Menten constant, or Kₘ, value for CaM was 0.2 μM. In some experiments, ML-9 or KN-62 was added as described. Radioactivity without the peptide substrate was subtracted from the total, and the difference was expressed as the MLC phosphorylating activity. Radioactivity in the absence of MLC peptide did not exceed 45% of the total count.

Intracellular granule movement in MIN6 cells. Intracellular movement of secretory granules in β-cells was assessed using an inverted light microscope (Axiovert 135, Carl Zeiss, Göttingen, Germany) equipped with a ×63 objective lens (Plan-Neofluar, Carl Zeiss) and a ×2.5 insertion lens. The images were produced with a charge-coupled device camera (DXC-930, Sony, Tokyo, Japan), displayed on a monitor screen (PVM-9040, Sony) at a final magnification of ×8,600, and recorded with a videocassette recorder (SVO-260, Sony). Pictures were reproduced from the video tapes and analyzed on the monitor using an image analyzer (Argus-20, Hamamatsu Photonics, Hamamatsu, Japan). All the experiments were carried out at 37°C. The number of the granules that moved into or out of a square (3.5 × 3.5 μm) was counted in each square for 30 s before and after the addition of the substances. Details of the experimental procedures have been recently published (10).
an apparent molecular mass of 140 kDa (Fig. 1A). The monoclonal antibody MM17 recognized against MLCK, MLC, and MHC. The monoclonal anti-MLCK antibody MM17 recognized β-cell MLCK with an apparent molecular mass of 140 kDa (Fig. 1A, lane 1). The 140-kDa band was much less pronounced when the antibody was preabsorbed with purified chicken gizzard MLCK (lane 5). The immunopositive band at 140 kDa was not derived from vascular smooth muscle contaminant in the islet preparation, because MLCK from rat smooth muscle was recognized at 135 kDa by the same antibody (lanes 3 and 4). The MLCK concentration in the islet cell was calculated to be 31 nM by use of purified chicken gizzard MLCK as a standard, with the assumptions that the antibody reacts with islet and chicken gizzard MLCK to the same extent and that the volume of an islet is 3 nl (34).

Immunohistochemical studies using the anti-MLCK monoclonal antibody demonstrated a speckled immunostaining pattern of MLCK in the cytoplasm of both endocrine and exocrine cells in the rat pancreas. Blood vessels in the pancreas were densely stained (Fig. 1B).

Immunoblotting was carried out using a monoclonal antibody against the MLC (Fig. 1C). The dominant immunopositive band was at 18 kDa, with preparations from rat pancreatic islets (lane 1), MIN6 (lane 2), and rat vessels (lane 3). The immunopositive band disappeared when incubated with the preabsorbed antibody with rabbit muscle MLC (lane 4). In lane 1, two less abundant bands were also observed at 26 and 22 kDa.

Fig. 2. Effects of different batches of monoclonal antibodies against MLCK on chicken gizzard MLCK activity (A) and Ca\(^{2+}\)-induced insulin release from streptolysin O (STLO)-permeabilized pancreatic islets (B). A: purified chicken gizzard MLCK (13 nM) was incubated in a final volume of 50 µl with 50 µM synthesized peptide substrate, 5.3 mM calmodulin, 5 mM Mg acetate, 0.5 mM CaCl\(_2\), 0.1% Tween-80, 40 mM HEPES (pH 7), and 10 µM [γ-\(^{32}\)P]ATP with 0.4% (vol/vol) various anti-MLCK antibodies (MM11, MM7, MM18, and MM27). Reactions were initiated by addition of radioactive ATP, and incubation was for 7 min at 30°C. Radioactivity from \(^{32}\)P incorporated into peptide was counted. Values are means of duplicate determinations. B: groups of 5 size-matched rat pancreatic islets were incubated in 0.6 ml with 0.125 IU/ml of STLO and 0.4% (vol/vol) anti-MLCK antibodies. Insulin released during 45-min incubation was measured by radioimmunoassay. Values are means ± SE from 5 observations.

The nature of the bigger bands is not known, but these bands could result from other isoforms of MLC present in islet β- or non-β-cells. Polyclonal antibodies raised against platelet MHC (myosin II) were also used for immunoblot analysis (Fig. 1D). A 200-kDa immunopositive band was consistently observed in extracts from the β-cell lines MIN6 and βTC3, pancreatic islets, and also platelets (lanes 1, 2, 3, and 4, respectively). Smaller bands were also detected, which could be other isoforms of the MHC and/or proteolytic fragments. The antibody did not cross-react with muscle MHC from cardiac myocytes (lane 5).

Effects of different types of monoclonal antibodies against MLCK on chicken gizzard MLCK activity. As shown in Fig. 2, different batches (MM7, MM11, MM18, and MM27) of anti-MLCK monoclonal antibodies (final × 250) inhibited chicken gizzard MLCK to a different extent (from 13 to 88%). These antibodies at the same dilution rate also exhibited different potencies in terms of their inhibition of insulin release from the permeabilized islets (Fig. 2), the two activities being in parallel (correlation coefficient \(r = 0.87, P < 0.05\) assessed by Pearson’s product-moment method). The MM27 antibody demonstrated the most potent inhibition of both MLCK and Ca\(^{2+}\)-induced insulin release and was therefore selected for subsequent secretion experiments.

Effects of anti-MLCK monoclonal antibodies on insulin release from permeabilized pancreatic islets. Table 1 shows the effects of addition of CaM, MLCK, and anti-MLCK antibody (MM27) on insulin release from Ca\(^{2+}\)-evoked insulin release from the STLO-treated islets. Under the basal condition (0.1 µM Ca\(^{2+}\)), insulin release from the permeabilized pancreatic islets was not affected. However, anti-MLCK antibody (final × 250) inhibited insulin release induced by 10 µM Ca\(^{2+}\) by 70%. Addition of exogenous MLCK (20 nM) and/or CaM...
Table 1. Effects of addition of CaM, MLCK, and anti-MLCK antibody on Ca\(^{2+}\)-induced insulin release from permeabilized pancreatic islets

<table>
<thead>
<tr>
<th>Ca(^{2+}), (\mu M)</th>
<th>Addition</th>
<th>Insulin Release, ng·5 islets(^{-1})·45 min(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>None</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td>0.1</td>
<td>anti-MLCK antibody</td>
<td>0.28 ± 0.08</td>
</tr>
<tr>
<td>10</td>
<td>None</td>
<td>3.4 ± 0.43</td>
</tr>
<tr>
<td>10</td>
<td>anti-MLCK antibody</td>
<td>0.93 ± 0.10</td>
</tr>
<tr>
<td>10</td>
<td>20 nM MLCK</td>
<td>2.9 ± 0.45</td>
</tr>
<tr>
<td>10</td>
<td>2 (\mu M) CaM</td>
<td>4.3 ± 0.62</td>
</tr>
<tr>
<td>10</td>
<td>CaM and MLCK</td>
<td>2.9 ± 0.33</td>
</tr>
</tbody>
</table>

Insulin release values are means ± SE for 4–5 observations. CaM, calmodulin; MLCK, myosin light-chain kinase. Five size-matched rat pancreatic islets permeabilized with 0.125 IU/ml streptolysin O (STLO) were incubated at 37°C for 45 min with various substances. MM27 (see Fig. 2, diluted 1:250) was used as anti-MLCK antibody. *P < 0.01 vs. value under parallel conditions without antibody.

(2 \(\mu M\)) did not cause significant changes in the release by 10 \(\mu M\) Ca\(^{2+}\), and nonimmunized serum did not exert any effect (data not shown). Inhibition by the anti-MLCK antibody was also observed when insulin release was stimulated with the stable GTP analog GTP\(_S\), even at a substimulatory concentration of Ca\(^{2+}\) (0.1 \(\mu M\)). However, the inhibition disappeared when the Ca\(^{2+}\) concentration was decreased to <0.01 \(\mu M\). In contrast, GDP\(_S\) preferentially inhibited GTP\(_S\)-induced release, even with Ca\(^{2+}\) at <0.01 \(\mu M\), but not Ca\(^{2+}\)-induced release (Table 2).

Ca\(^{2+}\)-dependent increase in phosphorylation of MLC peptides. Figure 3 demonstrates data for \(^{32}\)P incorporation into MLC peptides in the presence of increasing concentrations of Ca\(^{2+}\) (1 nM–10 \(\mu M\)). The threshold for activation of chicken gizzard MLCK was ~0.3 \(\mu M\), and the smooth muscle MLCK activity was progressively increased by Ca\(^{2+}\) at higher concentrations (Fig. 3B). The peptide was also phosphorylated by CaM kinase II in a Ca\(^{2+}\)-dependent manner (Fig. 3C), with a threshold between 1 and 3 \(\mu M\). The ß-cell extracts (islets and MIN6 cells, Fig. 3A), however, exhibited MLC phosphorylating activity to some extent (30% of the maximum), even in the presence of 3–31 nM Ca\(^{2+}\).

Table 2. Effects of anti-MLCK antibody (MM27) and GDP\(_S\) on Ca\(^{2+}\)- or GTP\(_S\)-induced insulin release from permeabilized pancreatic islets

<table>
<thead>
<tr>
<th>Ca(^{2+}), (\mu M)</th>
<th>GTP(_S), (\mu M)</th>
<th>Addition</th>
<th>Insulin Release, ng·5 islets(^{-1})·45 min(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.01</td>
<td>10</td>
<td>None</td>
<td>1.6 ± 0.06</td>
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<tr>
<td>0.1</td>
<td>10</td>
<td>None</td>
<td>1.3 ± 0.02</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>None</td>
<td>0.94 ± 0.12†</td>
</tr>
<tr>
<td>30 (\mu M) GDP(_S)</td>
<td></td>
<td></td>
<td>2.3 ± 0.32‡</td>
</tr>
<tr>
<td>0.1</td>
<td>10</td>
<td>MM27</td>
<td>4.0 ± 0.51</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
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<td>3.4 ± 0.43</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>MM27</td>
<td>3.4 ± 0.10‡</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>MM27</td>
<td>3.4 ± 0.27</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4–5 observations. GDP\(_S\), guanosine 5\(^{\prime}\)-O-(2-thiodiphosphate); GTP\(_S\), guanosine 5\(^{\prime}\)-O-(3-thiodiphosphate). Isolated rat pancreatic islets permeabilized with 0.125 IU/ml STLO were incubated at 37°C for 45 min with various substances described above. Insulin released in the media was measured by radioimmunoassay. *P < 0.01, †P < 0.05 vs. values under parallel conditions without antibody or GDP\(_S\).

The activity under low Ca\(^{2+}\) conditions was nullified by addition of 20 \(\mu M\) EGTA. The calculated concentration of free Ca\(^{2+}\) in this case was ~0.2 \(\mu M\) because the Ca\(^{2+}\) concentration in twice-distilled water was <10 \(\mu M\). At supraphysiological Ca\(^{2+}\) concentrations (>10 \(\mu M\), no
further increase in MLC phosphorylation by the β-cell homogenates was observed.

Inhibition by ML-9 and KN-62 of MLC phosphorylating activity of the MIN6 homogenates. Figure 4 summarizes data for the effects of the MLCK inhibitor, ML-9, and the CaM kinase II inhibitor, KN-62, on the MLC phosphorylating activity of the MIN6 homogenates. ML-9 dose-dependently inhibited MLC phosphorylating activity with both stimulatory (1.7 μM) and non-stimulatory (0.07 μM) Ca2+ concentrations. Fifty percent inhibition by ML-9 was observed at 19.5 and 16 μM with high and low Ca2+, respectively. KN-62 less potently inhibited the activity at the high Ca2+ concentration (35.4% inhibition at 30 μM KN-62) and exerted minimal effects with low Ca2+.

Phosphorylation of endogenous MLC in permeabilized MIN6 cells. The phosphorylated form of endogenous MLC in permeabilized MIN6 cells is demonstrated in Fig. 5. In lane 1, STLO-treated MIN6 cells were incubated in the presence of non-stimulatory (0.1 μM) Ca2+. When assessed densitometrically, only 5.5% of the total MLC was monophosphorylated (MLC-P) and 1.6% was diphosphorylated (MLC-P2). As shown in lane 2, 15-min incubation of the treated cells with stimulatory (10 μM) Ca2+ increased the amounts of MLC-P and MLC-P2 (20.0 and 8.4%, respectively). Addition of exogenous CaM (2 μM) and MLCK (20 nM) with 10 μM Ca2+ further increased both phosphorylated forms (28.6% for MLC-P and 13.6% for MLC-P2, lane 3).

Inhibitory effects of ML-9 on insulin granule movement. Figure 6 summarizes the finding of inhibition of intracellular granule movement by the MLCK inhibitor, ML-9. Under this condition, acetylcholine at 100 μM activated the movement of the insulin granules in living MIN6 cells (Fig. 6A, 3.00 ± 0.32 times/30 s for control vs. 4.03 ± 0.41 for acetylcholine, n = 30, P < 0.01). Incubation of MIN6 cells for 10 min with ML-9 at 30 μM significantly decreased the movement (Fig. 6B, 3.19 ± 0.17 for control vs. 2.25 ± 0.16 for ML-9, n = 32, P < 0.01 by paired t-test). Acetylcholine failed to increase the motile event after the 10-min treatment with 30 μM ML-9 (Fig. 6B, 2.32 ± 0.19 for 5 min after the acetylcholine challenge, n = 29, not significant).

DISCUSSION

Regulatory secretion of hormones occurs in a manner dependent on intracellular Ca2+, in cooperation with cytosolic proteins. Myosin is one candidate regulator diversely distributed among various muscle and nonmuscle tissues (5). It exists in several isoforms characterized by distinct heavy-chain structures. Immunoblot analysis using anti-nonmuscle-MHC antibody in the present study suggested that the β-cell myosin is of nonmuscle myosin II type (5). In addition to its well-studied role in muscle contraction, myosin has been suggested to control cellular motility during cytokinesis, cell locomotion, and membrane ruffling (31). Association of actomyosin with chromaffin granule membranes has been shown by in vitro assays (3), and interaction of actin with secretory granules in the anterior pituitary gland has been demonstrated by in situ observations (28). Participation of myosin II in the control of secretory events is supported by the recent finding that microinjection of anti-myosin II antibody into presynaptic regions retards synaptic transmission (17). These findings raise the possibility that myosin and its interaction with actin may regulate intracellular transport of the secretory granules in endocrine tissues. Here, we present direct evidence that intracellular movement of the secretory granules in the β-cell is dynamically controlled by Ca2+/CaM-dependent phosphorylation of MLC.

MLCK has been suggested to be involved in Ca2+-dependent hormone release, because secretion of various hormones, including insulin (19) and catecholamines (21), is inhibited by selective inhibitors of MLCK, such as ML-9 and wortmannin, although the latter more potently inhibits phosphatidylinositol 3-kinase (8). The present results with anti-MLCK mono-

Fig. 4. Effects of ML-9 and KN-62 on MLC phosphorylation by MIN6 homogenates. Extracts of 7 x 10^5 MIN6 cells (10 μg proteins) were incubated with 50 μM MLC peptide, 30 nM CaM, 5 mM Mg acetate, 1.2 mM EGTA, 0.1% Tween-80, 40 mM HEPES (pH 7), 20 μM [γ-32P]ATP, and ML-9 (A) or KN-62 (B) at various concentrations indicated. Free Ca2+ concentrations were adjusted to 1.7 μM (A, ○, and B) or 0.07 μM (A, ●) according to stability constant of Ca2+ and/or EGTA. After addition of radioactive ATP, the mixture was incubated for 5 min at 30°C, and the reaction was terminated by spotting aliquots onto P81 chromatography paper. Radioactivity without peptide substrate was subtracted from the total. Results were expressed as % of the value without inhibitors. Each symbol is the mean value of duplicate determinations.
clonal antibodies as well as ML-9 also point to a role of this kinase in the β-cell. Permeabilized cells offer a useful tool for analysis of the intracellular mechanisms of the secretory events. Treatment with STLO results in formation of pores in the plasma membrane through which hydrophilic molecules like Ca⁡²⁺ and large proteins like antibodies gain access to the intracellular space (14), and the threshold of Ca⁡²⁺ for insulin release was between 0.1 and 1 µM under the STLO-treated conditions (22). We could thus demonstrate good agreement with earlier reports for MLCK inhibitors. In contrast to the anti-MLCK antibody case, we found no effects of additional MLCK on Ca⁡²⁺-induced insulin secretion, even in the presence of an excess amount of CaM, although phosphorylation of endogenous MLC was increased by Ca⁡²⁺ and further elevated by additions of CaM and MLCK. It could be possible that the CaM and MLCK were relevant to a non-rate-limiting step in the β-cell secretory cascade.

Examination of the influence of anti-MLCK antibodies on GTPγS-stimulated release, which is due to a non-Ca⁡²⁺-dependent mechanism (26), demonstrated that MLC phosphorylation may control a prerequisite step for both Ca⁡²⁺- and GTPγS-induced release. This has also been suggested for catecholamine release from the chromaffin cells by work with the peptide MLCK inhibitor (SM-1) and wortmannin (13).

We recently reported that intracellular movement of β-granules is controlled by protein phosphorylation dependent on Ca⁡²⁺/CaM or adenosine 3’,5’-cyclic monophosphate, and we found that the movement under basal conditions could be increased by muscarinic activation with acetylcholine (10). Therefore, we carried out in vitro phosphorylation of MLC peptide by the β-cell homogenates and also attempted to analyze the insulin granule movement in living β-cells. Because CaM is reported to exist in the pancreatic islets at high levels (30, 34), the final concentration of CaM in the assay mixture for the MLC phosphorylation was calculated to be 0.36–0.5 µM. However, we found that 30 nM CaM was necessary to activate the phosphorylation, and the activity was dose dependently increased by CaM up to 2 µM (data not shown). It may result from heterogeneous distribution of CaM in the β-cells. Otherwise, intracellular concentration of CaM in the active form could be much lower than the assumed value as reported in the smooth muscle by use of fluorescent CaM (15).

In contrast to purified MLCK or CaM kinase II, β-cell homogenates were here found to possess MLC phosphorylating activity even at substimulatory concentrations of Ca⁡²⁺. ML-9 inhibited MLC phosphorylation by the M1N6 homogenates at low µM concentrations with high or low Ca⁡²⁺. Because CaM kinase II is known to phosphorylate MLCK and thereby decrease its sensitivity to Ca²⁺ (32), we examined the effects of KN-62 on phosphorylation of the MLC peptide substrate. The finding that the CaM kinase II inhibitor decreased MLC phosphorylation by the β-cell homogenate suggests to us that phosphorylation of MLC rather than MLCK by CaM kinase II is dominant under this condition. The inhibition by KN-62 was much weaker than that by ML-9 if we consider that the inhibitory constant (Kᵢ) value for KN-62 to inhibit purified CaM kinase II is 0.9 µM (33) and that Kᵢ for ML-9 to inhibit MLCK is 4 µM (27). The fact that inhibition by KN-62 was only observed with high Ca²⁺, in contrast to the ML-9 case, is in line with a conclusion that MLC phosphorylation by MLCK in the β-cell is dominant with substitutary or stimulatory Ca²⁺, and the phosphorylation by CaM kinase II may participate when the intracellular Ca²⁺ level is raised. ML-9 decreased intracellular movement of the insulin granules under the basal condition and, moreover, nullified activation of the movement by acetylcholine, supporting the proposed role of MLCK. We cannot preclude the possibility, however, that contaminant kinases in the β-cell extracts may have phosphorylated MLC at low Ca²⁺ or that the copresence of some other factors may have altered the Ca²⁺ requirement of MLCK for its activation.

In conclusion, we propose that phosphorylation of MLC may be necessary to translocate secretory granules to the vicinity of plasma membranes, where docking and exocytosis of the granules subsequently occur. We also suggest that this mechanism requires lower...
concentrations of Ca\textsuperscript{2+} than those necessary for actual release of the secretory granules from the cell.

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REFERENCES


