Increases in Phosphorylation of the Myosin II Heavy Chain, but not Regulatory Light Chains, Correlate With Insulin Secretion in Rat Pancreatic Islets and RINm5F Cells

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Although cytoskeletal proteins such as myosin II are implicated in the control of insulin secretion, their precise role is poorly understood. In other secretory cells, myosin II is predominantly regulated via the phosphorylation of the regulatory light chains (RLC). The current study was aimed at investigating RLC phosphorylation in β-cells. In both the insulin-secreting cell line RINm5F and rat pancreatic islets, the RLC was basally phosphorylated on the myosin light chain kinase sites (Ser^{19}/Thr^{18}). Phosphorylation at these sites was not consistently increased by either metabolic stimuli (glyceraldehyde/glucose) or the depolarizing agent KCl. The RLC sites phosphorylated by protein kinase C (PKC) (Ser^{1}/Ser^{2}) were unphosphorylated in the basal state, not affected by nutrients or KCl, and only slightly increased by the PKC activator phorbol 12-myristate 13-acetate (PMA). Like the other insulin secretagogues, however, PMA did promote serine phosphorylation of the myosin heavy chain (MHC) in RINm5F cells. Phosphopeptide mapping suggested that the same peptide was phosphorylated under both PMA and glyceraldehyde stimulation, which further extends our previous study of the Ca^{2+}-dependent phosphorylation of this protein (Wilson JR, Ludowyke RI, Biden TJ: Nutrient stimulation results in a rapid Ca^{2+}-dependent threonine phosphorylation of myosin heavy chain in rat pancreatic islets and RINm5F cells. *J Biol Chem* 273:22729–22737, 1998). Overall, our results demonstrate that in RINm5F cells and rat pancreatic islets, MHC phosphorylation correlates better with insulin secretion than phosphorylation of the RLC. We therefore propose that in β-cells, in contrast to other secretory cells, phosphorylation of the MHC is more important than that of the RLC for regulation of the myosin II protein complex during insulin secretion. *Diabetes* 48:2383–2389, 1999

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BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular calcium; CaMK, Ca^{2+}/calmodulin-dependent kinase; IEF, isoelectric focusing; KRB, Krebs-Ringer buffer; MHC, myosin heavy chain; MLCK, myosin light chain kinase; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RIA, radioimmunoassay; RLC, regulatory light chain; TBS, Tris-buffered saline; TLC, thin-layer chromatography.

The pancreatic β-cell acts primarily as a "fuel sensor" and is very sensitive to subtle changes in blood nutrients, glucose in particular (1,2). One consequence of nutrient metabolism within the β-cell is a rise in intracellular calcium ($[Ca^{2+}]_i$), an important regulator of insulin secretion (3). This regulation is thought to be via the activation of Ca^{2+}-dependent protein kinases (4), in particular the Ca^{2+}/calmodulin-dependent kinase (CaMK). Islet β-cells and a number of insulin-secreting cell lines have been reported to have a high calmodulin content (5), and calmodulin antagonists such as trifluoperazine or W-7 inhibit insulin release (6). It is known that CaMK are activated in insulin-secreting cells, but the range of substrates for these enzymes is poorly defined. One substrate that has been identified in many other systems, including secretory cells, is myosin light chain kinase (MLCK) (6,7).

The cytoskeletal proteins, myosin in particular, have long been implicated in the regulation of insulin secretion (8) but with little in the way of definitive evidence. Nonmuscle myosin is ubiquitously expressed and functions primarily as a motor protein. For prolonged insulin secretion to occur, granules must be moved from the internal pool to the cell's plasma membrane. Nonmuscle myosin, particularly the conventional myosin II, has been postulated to be important in regulating this movement (9). Nonmuscle myosin II exists as two isoforms, myosin IIA and IIB, both of which are present in rat pancreatic islets and RINm5F cells (10). It is a hexameric protein complex consisting of one pair of heavy chains (200 kDa) noncovalently bound to two pairs of light chains (17–22 kDa). One pair of light chains is classified as essential and the other as regulatory (11). Regulation of myosin II function in both smooth and nonmuscle cells has been traditionally thought to occur at the level of phosphorylation of the regulatory light chains (RLC) (12,13). Recently, we have shown that Ca^{2+}-dependent secretion from both rat pancreatic islets and RINm5F cells is associated with increases in both threonine and serine phosphorylation of the myosin II heavy chain (10). In light of this finding, it became important to assess the contribution RLC phosphorylation made to the regulation of the myosin II protein complex in this system.

Strong evidence indicates that RLC phosphorylation is particularly important in some regulated secretory cells, with a large body of work using the histamine-secreting RBL-2H3 rat mast cell line (14–16). From these studies, five phosphoryla-

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tion sites on the RLC associated with myosin II have been identified. MLCK phosphorylates the RLC on Ser^{19} and to a lesser extent on Thr^{18}. Both protein kinase C (PKC) and cdc2 kinase phosphorylate Ser^{1} and Ser^{2} of the RLC (12,17). There is evidence that in vitro, Thr^{9} is also phosphorylated by PKC (13), although this phosphorylation does not appear to occur in intact cells (16,18). Other kinases, such as Rho kinase and the catalytic fragment of PKC (PKM), have also been shown to phosphorylate Ser^{19}/Thr^{18}, although it is thought that this site is primarily phosphorylated by MLCK, since it has a higher affinity for the substrate (19,20). Of these sites, it is Ser^{19} that appears to be most important in regulating myosin II function. Phosphorylation of this serine by MLCK causes a conformational change of the myosin complex, relieving inhibition of the Mg-ATPase/actin binding site (21). MLCK also phosphorylates Thr^{18}. The function of this phosphorylation is not clear, although there is some evidence in smooth muscle that Thr^{18} phosphorylation of the RLC increases actin-activated myosin ATPase activity and further stabilizes myosin filaments (22). Phosphorylation of Ser^{1} and Ser^{2} by PKC appears to have no direct effect on actin-activated ATPase activity of unphosphorylated myosin (23). Interestingly, in smooth muscle in vitro, phosphorylation of Ser^{1}, Ser^{2}, and Thr^{9} on the RLC by PKC does decrease the affinity of MLCK for myosin, thereby making it more difficult for the Ser^{19} site to be phosphorylated. The exact role of phosphorylation of the RLC by PKC is still not clear, although recent evidence in RBL-2H3 cells suggests it is important in histamine secretion (14–16).

The main aim of the current study was to define the importance of RLC phosphorylation on both MLCK sites (Ser^{19}/Thr^{18}) and PKC sites (Ser^{1}/Ser^{2}), since these have not been studied previously in the context of insulin secretion. In addition, we sought to determine whether the myosin heavy chain (MHC) is a substrate for PKC in RINm5F cells, an issue not addressed in our previous study. The results indicate that during insulin secretion in rat pancreatic islets and RINm5F cells, the nonmuscle myosin II complex is not regulated by increases in RLC phosphorylation. On the other hand, the MHC is phosphorylated during Ca^{2+}-dependent (10) and PKC-dependent (this study) insulin secretion, suggesting that MHC phosphorylation might be more important than increases in RLC phosphorylation in regulating this secretory process.

RESEARCH DESIGN AND METHODS

Materials. Reagents were of analytic grade and were obtained from Sigma Chemical (St. Louis, MO), Calbiochem (Alexandria, NSW, Australia), BDH (Merck; Kilsyth, Victoria, Australia), or Bio-Rad (Hercules, CA) unless otherwise stated. Anti-myosin (nonmuscle) antibody was from Biomedical Technologies (Stoughton, MA). The myosin IIA and IIB antibodies were a gift from Dr. Robert Adelstein (Laboratory of Molecular Cardiology, National Institutes of Health, Bethesda, MD). Donkey anti-rabbit and donkey anti-goat horseradish peroxidase–conjugated secondary antibodies were obtained from Jackson Immunoresearch Laboratory (West Grove, PA). Insulin radioimmunoassay kits were purchased from Linco Research (St. Charles, MO). Enhanced chemiluminescence reagents for immunoblotting were purchased from Du Pont-NEN (Boston, MA). [^{32}P]Orthophosphate was obtained from Amersham Life Science (Little Chalfont, Buckinghamshire, U.K.).

Cell culture. RINm5F (rat insulinoma cell line) and RBL-2H3 (rat mucosal mast cell line) were maintained as adherent monolayers in T-75 tissue culture flasks as described (24,25). Rat pancreatic islets were isolated from 230–270 g male Wistar rats by ductal infusion of collagenase as described (26). Islets were handpicked and placed into a six-well tissue culture dish (400–600 islets/well) in Medium 199 supplemented with 10% (vol/vol) fetal calf serum, 14 mmol/l $NaHCO_3$, 11.1 mmol/l glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamycin and incubated at 37°C with 5% CO_2, 95% air for 48 h.

[^{32}P]Orthophosphate labeling of myosin II in RINm5F and RBL-2H3 cells. The protocol was previously described (10), with the following changes. Cells were seeded into a six-well plate at 3×10^6 cells/well and cultured overnight at 37°C with 5% CO_2, 95% air. On the day of the experiment, cells were gently washed twice with a phosphate-free salt solution (buffer A: 119 mmol/l NaCl, 5 mmol/l KCl, 5.6 mmol/l glucose, 0.4 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 0.1% [wt/vol] bovine serum albumin [BSA], 4 mmol/l glutamine, and 25 mmol/l PIPES-NaOH [pH 7.2] [14]). Cells were then labeled with [^{32}P]orthophosphate (200 µCi/well) for 3 h at 37°C in 2 ml buffer A. An incubation period of 3 h with [^{32}P]orthophosphate was chosen, since it facilitated comparisons with other studies using similar conditions and, in studies performed in other secretory cells such as RBL-2H3, has been shown to be sufficient to allow equilibrium of the endogenous ATP pools (14,15). Radioactive labeling medium was removed, and cells were washed three times with 3 ml warmed buffer A. Cells were preincubated in 2.5 ml buffer A for 10 min at 37°C. Cells were then stimulated for 5 min, and incubation was stopped by aspirating the stimulating solutions and placing the cells on ice. This period of stimulation was chosen to correspond to peak secretion with nutrient stimuli (27), close to peak secretion with KCl (28) and the initial secretory response to phorbol 12-myristate 13-acetate (PMA) (29). Lysis buffer (450 µl) containing 1% (vol/vol) Nonidet P-40, 100 mmol/l sodium pyrophosphate, 250 mmol/l NaCl, 50 mmol/l NaF, 5 mmol/l EGTA, 0.1 mmol/l phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 15 mmol/l β-mercaptoethanol, and 20 mmol/l Tris-HCl (pH 7.9) (14) was immediately added, cells were scraped off, and the lysate was sedimented at 100,000g for 5 min at 4°C.

Rat pancreatic islets. On the day of the experiment, ~400 islets were transferred to 6-ml Falcon tubes and washed twice with buffer A. Islets were resuspended in 800 µl warm buffer A and transferred to 1.5-ml Eppendorf tubes with screw-on caps. [^{32}P]Orthophosphate (650 µCi) was added to each tube and incubated at 37°C for 3 h (shaken occasionally). Islets were washed three times, resuspended in 200 µl warm buffer A, and preincubated for 10 min at 37°C. Stimulating solutions (800 µl) were added, and the islets were incubated at 37°C for a further 5 min. The incubation was terminated by gentle centrifugation and rapid removal of the supernatant. Islets were resuspended in lysis buffer (450 µl). Extracts were then sonicated (1×15 s) using a Branson model 250 sonifier (Branson, Danbury, CT) and microtip at power setting 1 and 10% duty cycle. Lysates were centrifuged at 13,000g at 4°C for 15 min.

Immunoprecipitation of [^{32}P]-labeled myosin. The supernatant fraction of all cell lysates was precleared with pansorbin (a standardized *Staphylococcus aureus* cell suspension) at one-fifth the final lysate volume for 30 min at 4°C. The pansorbin was sedimented, and the cell lysate was incubated with 20 µl anti-myosin IgG (nonmuscle) on a rotating platform at 4°C for 2 h. A further 90 µl pansorbin was added for an additional hour of incubation. The mixture was then sedimented by centrifugation at 13,000g at 4°C. The resulting pellet was washed three times, the first in lysis buffer as described above, the second in a 1:1 dilution of lysis buffer with standard phosphate-buffered saline (PBS) (pH 7.4), and the third in PBS alone. The pellet was then resuspended in 100 µl preheated (90°C) Laemmli sample buffer and heated for a further 5 min at 90°C. After centrifugation, the supernatant was transferred to a fresh 1.5-ml tube, and a sample (10 µl) was separated on a 10% SDS-polyacrylamide gel. The MHC could be detected once the gel was stained with Coomassie blue. Relative protein levels were determined by densitometry using the Molecular Dynamics densitometer PDSI (Sunnyvale, CA), allowing for correction of protein loading. Equal amounts of the remaining myosin immunoprecipitated from [^{32}P]-labeled cells was separated on a 10% SDS-polyacrylamide gel. The areas containing the labeled RLC or MHC were removed from the gel and tryptically digested as described (10). The digest supernatants were repeatedly washed and lyophilized.

Tryptic peptide mapping

RLC. The lyophilized pellet was resuspended in 20 µl water, and the phosphopeptides were separated using a urea-polyacrylamide one-dimensional isoelectric focusing (IEF) system as described (13,16). The biolytes used were made up of an equal volume of pH 3–5, pH 4–6, and pH 5–8 and had a final concentration of 2%. The IEF gel was pre-electrophoresed (Multiphor; Pharmacia Biotech, Uppsala, Sweden) at 25 W/1,000 V for 10 min at 14°C with anode buffer (1 mol/l H_3PO_4) and cathode buffer (1 mol/l NaOH). The phosphopeptides were separated by electrophoresis at 25 W/1,000 V at 14°C for 25–30 min using acid fuchsin as a marker dye. The gel was dried at 65°C for 30 min, and radioactive peptides were analyzed by phosphorimaging on a Molecular Dynamics phosphorimager 445 SI or by autoradiography.

MHC. Phosphopeptide mapping of MHC was performed as described (10). The lyophilized pellet was resuspended in 10 µl electrophoresis buffer containing acetic acid:formic acid:deionized water (15:5:80). Samples were spotted onto a silica gel 60 thin-layer chromatography (TLC) plate and separated in the first dimension by electrophoresis at 1,000 V for ~60 min at 4°C using orange G and acid fuchsin as marker dyes. Plates were dried, and peptides were subjected to ascending chromatography in a solution containing n-butyl alcohol:pyridine:

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FIG. 1. Total [^{32}P]-phosphorylation of immunoprecipitated myosin II from stimulated RINm5F and RBL-2H3 cells. The myosin protein complex was immunoprecipitated from RINm5F and RBL-2H3 cells (~3 × 10^6) using the commercial anti-myosin antibody as described in RESEARCH DESIGN AND METHODS. The antibody showed high specificity, since there was minimal coprecipitation of other phospholabeled proteins. RINm5F cells were unstimulated/basal (B) or stimulated (5 min) with glyceraldehyde (GLY) (10 mmol/l), KCl (30 mmol/l), or PMA (100 nmol/l). RBL-2H3 cells were unstimulated (B) or stimulated with PMA (100 nmol/l). MHC and RLC were separated on a 10% SDS-polyacrylamide gel, and the gel was dried under vacuum at 60°C and placed under Kodak film. A representative autoradiogram is shown (*n* = 6).

acetic acid:formic acid:deionized water (127.5:22.5:45:15:90) for 6 h. Radioactive peptides were identified by autoradiography.

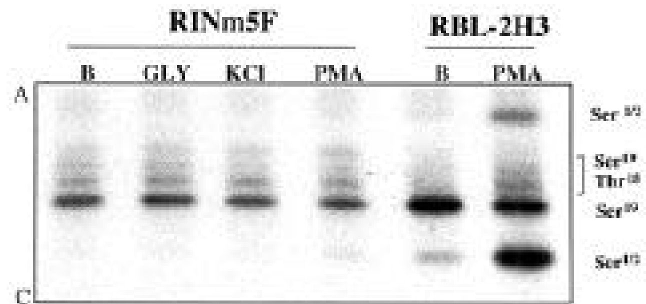
Phosphoamino acid analysis. This procedure has been described in detail (10). Briefly, the lyophilized pellet was resuspended in 200 µl of 6 mol/l HCl and incubated at 110°C for 2.5 h. The hydrolysate was lyophilized, and the pellet was resuspended in 10 µl first-dimension electrophoresis buffer, pH 1.9, and spotted onto cellulose TLC plates using phosphothreonine, phosphoserine, and phosphotyrosine (1 mg/ml) as standards. Two-dimensional electrophoresis was carried out on a Pharmacia Biotech Multiphor II. The first dimension was run at 1,000 V for 65 min in buffer at pH 1.9. The second dimension was run at 1,000 V for 30 min in buffer at pH 3.6. Phosphoamino acid standards were detected by spraying the TLC plate with ninhydrin. The TLC plate was dried, and the radioactivity of the spots corresponding to the relative positions of the phosphoamino acids was analyzed by phosphorimaging.

Measuring insulin secretion. RINm5F cells were seeded at 0.1 × 10^5 cells/well in a 96-well plate and incubated overnight at 37°C in 5% CO_2, 95% air. On the day of the experiment, cells were washed twice with modified Krebs-Ringer buffer (KRB) containing 5 mmol/l $NaHCO_3$, 1 mmol/l $CaCl_2$, 2.8 mmol/l glucose, 10 mmol/l HEPES (pH 7.4), and 0.5% (wt/vol) BSA. Cells were then preincubated in KRB/BSA for 15 min at 37°C. After 15 min, 150 µl of the preincubation medium was removed and pooled to enable measurement of basal secretion during the preincubation. Stimulating solutions (150 µl) were added, and the cells were incubated at 37°C for a further 15 min. Incubations were terminated by placing the plate on ice. An aliquot (100 µl) of incubation medium was removed to determine insulin content using a radioimmunoassay (RIA) kit with rat insulin as standard.

RESULTS

Comparison of RLC phosphorylation in RINm5F and RBL-2H3 cells. The intracellular ATP pools of RINm5F and RBL-2H3 cells were labeled with [^{32}P]orthophosphate. RINm5F cells were stimulated with KCl (30 mmol/l), glyceraldehyde (10 mmol/l), or the PKC activator PMA (100 nmol/l). RBL-2H3 cells were stimulated with PMA (100 nmol/l) alone. Myosin was immunoprecipitated from cell lysates using a commercial anti-myosin antibody. Equal amounts of protein (myosin) from RINm5F and RBL-2H3 cells were loaded onto the 10% SDS-polyacrylamide gels, and ^{32}P incorporated into myosin was measured. In RINm5F cells, there was no increase observed in total RLC phosphorylation under any stimulatory condition (Fig. 1). As a percentage of basal phosphorylation, the responses to glyceraldehyde, KCl, and PMA were 101 ± 10, 92 ± 6, and 93 ± 5%, respectively, as assessed by densitometry (*n* = 6). In contrast, stimulation of RBL-2H3 cells with PMA resulted in an increase in phosphorylation of the RLC (Fig. 1). RBL-2H3 cells have a double rather than a single RLC band (both bands have been shown to be RLC, giving identical phosphopeptide maps [16]). The RLC of the RINm5F cells corresponds to the lower of the two bands in the RBL-2H3 cell immunoprecipitates.

A Phosphopeptide map



B Insulin secretion

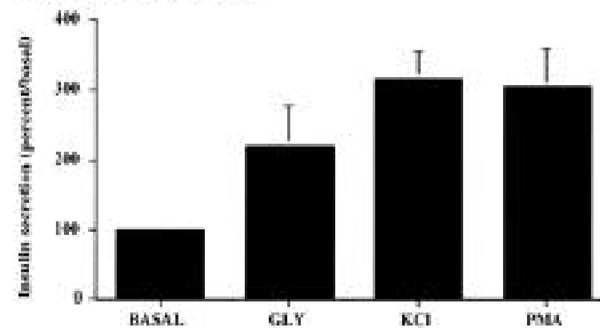


FIG. 2. Phosphopeptide map (*A*) of the RLC from RINm5F and RBL-2H3 cells and insulin secretion study (*B*) from RINm5F cells. Equal amounts of myosin II (from ~3 × 10^6 [^{32}P]-labeled and stimulated cells) were separated on a 10% SDS-polyacrylamide gel, and the RLC was extracted, tryptically digested, and run on a one-dimensional IEF gel. RINm5F cells were unstimulated (B) or stimulated (5 min) with glyceraldehyde (GLY) (10 mmol/l), KCl (30 mmol/l), or PMA (100 nmol/l). RBL-2H3 cells were unstimulated (B) or stimulated with PMA (100 nmol/l). The main phosphopeptides detected for the RINm5F cells were the monophosphorylated Ser^{19} and diphosphorylated Ser^{19}/Thr^{18}. The RLC of the RBL-2H3 cells also had these sites but in addition showed extra phosphopeptides phosphorylated by PKC on the Ser^{1}/Ser^{2} sites after stimulation with PMA. The phosphorylated Ser^{1}/Ser^{2} sites in the RINm5F cells were only barely detectable after PMA stimulation. Phosphopeptides were detected using a phosphorimager, and a representative phosphorimage is shown (*n* = 4). Insulin secretion in RINm5F cells was examined as described in RESEARCH DESIGNAND METHODS, using the same stimulatory agents as for the [^{32}P]-labeling experiments. Insulin release is expressed as percentage of basal, and the results represent *n* = 3 experiments. A, anode; C, cathode.

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Identifying sites of RLC phosphorylation. SDS-PAGE analysis only allows detection of total changes in RLC phosphorylation. To determine the specific sites of phosphorylation and potentially the kinases responsible, the [^{32}P]-labeled RLCs were removed from the gel and tryptically digested, and phosphorylated species were separated on an IEF gel. A representative phosphorimage is shown in Fig. 2*A*. The RLC phosphorylation sites for RBL-2H3 cells have been well defined (14,15); for this reason, they were used in these experiments as positive controls, allowing the identification of phosphopeptides. In the RINm5F cells, there was basal phosphorylation of the Ser^{19} and Thr^{18} sites, which are usu-

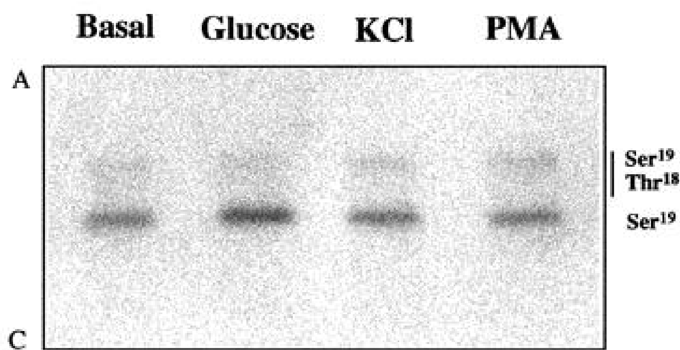


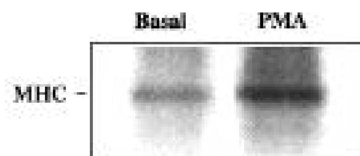
FIG. 3. Phosphopeptide map of RLC from rat pancreatic islets. Rat pancreatic islets were unstimulated (Basal) or stimulated for 5 min with glucose (16.7 mmol/l), KCl (30 mmol/l), or PMA (100 nmol/l). Myosin II was immunoprecipitated from extracts of 400–600 [^{32}P]-labeled islets, and RLC was extracted, tryptically digested, and run on an IEF gel. The resulting phosphopeptides were detected on a phosphorimager, and a representative phosphorimage is shown (n = 3). A, anode; C, cathode.

ally phosphorylated by MLCK. On stimulation with KCl or glyceraldehyde, there was no apparent increase in phosphorylation of these sites. However, addition of PMA did result in a barely detectable amount of phosphorylation of the Ser^1 and Ser^2 residues, indicating that these sites were present on the RLCs of RINm5F cells, but not preferentially phosphorylated. In contrast, PMA induced a substantial increase in phosphorylation of the Ser^1 and Ser^2 residues in the RBL-2H3 cells. Glyceraldehyde and KCl stimulation also did not exert any effect on Ser^1 and Ser^2 RLC phosphorylation in RINm5F cells. Furthermore, the insulin secretagogues did not influence phosphorylation on the Ser^{19}/Thr^{18} or Ser^1/Ser^2 of myosin IIA or IIB when specific antibodies against these isoforms were used for immunoprecipitation, compared with the anti-myosin (nonmuscle) antibody (results not shown). When insulin secretion experiments were undertaken in RINm5F cells, using the same stimulatory agents as in the labeling experiments, an increase in insulin secretion was observed (Fig. 2*B*).

RLC phosphorylation in rat pancreatic islets. Corresponding experiments were undertaken in rat pancreatic islets to confirm that our results were not confined to the immortalized RINm5F cell line. Although the [^{32}P]orthophosphate loading of the islets, and therefore overall RLC phosphorylation, was less than that seen with RINm5F or RBL-2H3 cells, similar results were obtained. Total phosphorylation of the RLC in rat pancreatic islets showed no significant increase with the depolarizing agent KCl (118 ± 20% of basal) or with either the metabolic stimulus glucose (102 ± 2%) or the PKC activator PMA (109 ± 18.5%) (all n = 3) (results not shown). These findings were confirmed by phosphopeptide mapping (Fig. 3). Although there was a basal phosphorylation of the Ser^{19} and Thr^{18} sites, these sites did not consistently increase in phosphorylation on stimulation. PKC phosphorylation of the Ser^1 and Ser^2 sites also appears to be absent in both nutrient-stimulated and PMA-stimulated islets (Fig. 3).

PKC phosphorylation of the MHC. Unlike the other secretory cell model (RBL-2H3), there appears to be no increase in PKC phosphorylation of RLC on Ser^1 and Ser^2 in either rat pancreatic islets or RINm5F cells. However, nonmuscle MHC reportedly contains a PKC phosphorylation site at Ser^{1917}

A PMA stimulated MHC phosphorylation



B Phosphoamino acid analysis

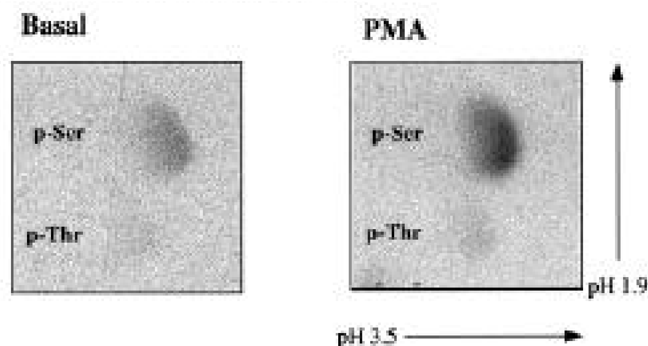


FIG. 4. Phosphoamino acid analysis of [^{32}P]-labeled MHC from RINm5F cells. MHC was immunoprecipitated from unstimulated (Basal) or PMA-stimulated (100 nmol/l for 5 min) RINm5F cells, tryptically digested, and subjected to two-dimensional phosphoamino acid analysis as described in RESEARCHDESIGN ANDMETHODS. Shown is a representative phosphorimage (n = 2).

(23). To examine the potential relevance of this site to insulin secretion, [^{32}P]-prelabeled RINm5F cells were stimulated with 100 nmol/l PMA, and myosin was immunoprecipitated using the nonmuscle myosin antibody. The MHC was tryptically digested and subjected to amino acid hydrolysis and two-dimensional phosphoamino acid analysis. Figure 4 is a representative autoradiogram from the same cellulose TLC plate. We found that there was basal serine phosphorylation which, when analyzed by densitometry, increased 2.1 ± 0.45-fold on addition of PMA. PMA stimulation of RINm5F cells also resulted in a concurrent increase in insulin secretion (Fig. 2*B*). Therefore, PMA activation of PKCs during insulin secretion from RINm5F cells results in a preferential phosphorylation of the MHC over the RLC.

Phosphopeptide mapping of MHC. It was of interest to establish whether the activation of PKC by PMA in RINm5F cells resulted in the phosphorylation of the MHC on a site that was similar to that previously observed with other metabolic stimuli, such as glyceraldehyde. [^{32}P]-prelabeled RINm5F cells were stimulated with 100 nmol/l PMA, and myosin was immunoprecipitated using the nonmuscle myosin antibody. The MHC was tryptically digested, and the resulting phosphopeptides were separated by electrophoresis and chromatography. A representative autoradiogram is shown in Fig. 5. There is a basally phosphorylated peptide designated peptide-1. In the PMA-stimulated RINm5F cells, peptide-1 is the predominant peptide that increases in phosphorylation. There is also phosphorylation of an additional three minor peptides, designated peptides 2, 3, and 4. Interestingly, the phosphopeptides of PMA-stimulated cells show the same migratory pattern as that observed in glyceraldehyde-stimulated RINm5F cells from our previous study (10). In addition, peptide 1 appears to be the predom-

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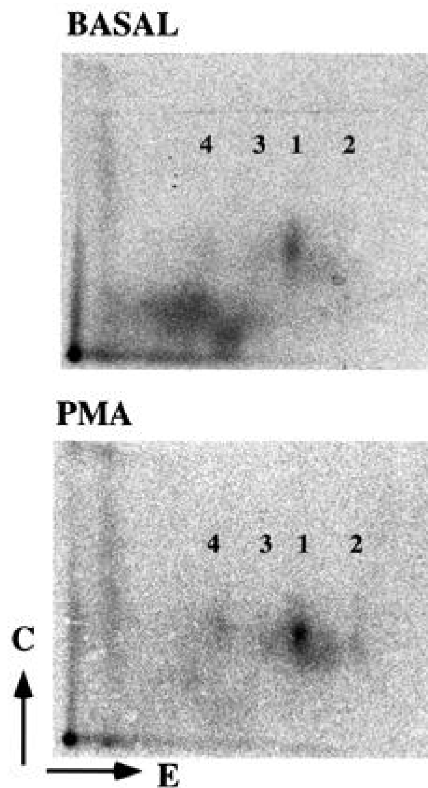

FIG. 5. Phosphopeptide mapping of MHC from PMA-stimulated RINm5F cells. MHC was immunoprecipitated from unstimulated (Basal) or PMA-stimulated (100 nmol/l for 5 min) RINm5F cells, tryptically digested, and subjected to electrophoresis (E) and chromatography (C) as described in RESEARCH DESIGN AND METHODS. Shown is a representative (n = 2) phosphorimage. Phosphopeptides are numbered 1–4 in order of intensity.

inant peptide, increasing in phosphorylation under both stimulatory conditions, indicating that it contains the major sites of phosphorylation for the PKC activator PMA and the metabolic stimulus glyceraldehyde.

DISCUSSION

Regulation of myosin II function in both smooth and nonmuscle cells is thought to be primarily via phosphorylation of the RLC on Ser^{19}/Thr^{18} or Ser^{1}/Ser^{2} (or both) by MLCK and PKC, respectively (12,15). Total RLC phosphorylation has been studied in a number of insulin-secreting cell lines (6,9), but the relative contributions of Ser^{19}/Thr^{18} and Ser^{1}/Ser^{2} sites have never been directly assessed. Therefore, the aim of this study was to investigate these sites on the RLC of myosin II in RINm5F cells and rat pancreatic islets and to measure any changes in the phosphorylation states in response to insulin secretagogues. Whereas RLC phosphorylation in other secretory cells, such as the rat mast cell line RBL-2H3, may be important in regulating secretion, this does not appear to be the case for rat pancreatic islets and RINm5F cells. In our studies, we found that a variety of agents capable of stimulating insulin release resulted in little or no increase in phosphorylation on either the Ser^{19}/Thr^{18} or the Ser^{1}/Ser^{2} sites of the RLC in these cells. On the other hand, MHC was phosphorylated in response to PMA, further extending our recent findings of a strong correlation between MHC phosphorylation and insulin secretion (10). We therefore postulate that MHC phosphorylation, rather the RLC phosphorylation of myosin II, may be more important in regulating the insulin secretory process; the evidence is discussed below.

In the RBL-2H3 rat mast cells, sites of RLC phosphorylation have been well defined, and these cells were used here as a comparative secretory model. Whereas the phosphorylation of Ser^{19}/Thr^{18} on the RLC in these cells is believed to be necessary for secretion, it is the phosphorylation of the Ser^{1}/Ser^{2} sites that has been shown to have the strongest correlation with the secretory process (14,16). We therefore initially investigated the phosphorylation of the RLC on Ser^{1} and Ser^{2} in RINm5F cells and rat pancreatic islets. Stimulation of either of these cells with nutrients or KCl resulted in no consistent increases in Ser^{1} or Ser^{2} phosphorylation of the RLC. In the RINm5F cells, however, on stimulation with the PKC activator PMA, there was a very low level of Ser^{1} and Ser^{2} phosphorylation, indicating that the sites were present on the RLC. We know that the PMA was effective, since there was a substantial increase in Ser^{1} and Ser^{2} phosphorylation of the RLC in the RBL-2H3 cells. In the RBL-2H3 cells, the PKC isoform responsible for the RLC phosphorylation is not known, although PKC-β (I and II) and PKC-δ are the most likely candidates that could regulate secretion (30). However, PKC-β (I and II) and PKC-δ are present in RINm5F cells (J.R.W., T.J.B., unpublished observations), and there have been reports that both PKC-βII and PKC-δ are present in MIN6 cells (31). This fact negates the simplest explanation, that the lack of effect on Ser^{1} and Ser^{2} was due to the absence of these enzymes. An alternative explanation might be that the two cell types possess different complements of PKC-anchoring proteins, such that PKC is unable to substantially interact with the RLC in RINm5F cells (32). The differences seen in RLC phosphorylation on the Ser^{1} and Ser^{2} sites in the histamine-secreting RBL-2H3 cells and insulin-secreting RINm5F cells are interesting and provide the first evidence that exocytosis in these two systems may be regulated differently.

In addition to being phosphorylated on Ser^{1} and Ser^{2}, the RLC may also be phosphorylated on Ser^{19} and Thr^{18} by MLCK. MLCK phosphorylation of the RLC had previously been thought to be the most important regulator of myosin II function during insulin secretion. In our study, we have found that in RINm5F cells and rat pancreatic islets, the RLC is phosphorylated in the basal state on the Ser^{19}/Thr^{18} MLCK sites. Stimulation of these cells with either nutrient (glyceraldehyde/glucose) or the depolarizing agent KCl, which increases $[Ca^{2+}]_i$, should therefore result in activation of the Ca^{2+}/calmodulin-dependent MLCK. Under stimulatory conditions, however, there was no further increase in phosphorylation of the Ser^{19}/Thr^{18} sites by MLCK, despite the fact that increases in secretion were observed. In the RBL-2H3 cells, there was also no increase of Ser^{19}/Thr^{18} phosphorylation during histamine secretion (14,16). This lack of increase in Ser^{19}/Thr^{18} phosphorylation, although it holds with the idea of a comparative secretory cell, appears to contradict the general view that increases in myosin II RLC phosphorylation are important regulators of insulin secretion. Our current finding needs to be considered in light of what is already known about MLCK phosphorylation of the RLC in the insulin-secreting cell. A number of studies have been undertaken looking at MLCK activity in the β-cell and a variety of insulin-secreting cell lines (6,9). MLCK has been shown to be present and active in the pancreatic islet (6,33). Whereas these

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experiments confirm the existence of an active MLCK in pancreatic islets, their physiologic relevance is equivocal, since the activity of this enzyme was measured in vitro using exogenous RLC (usually chicken gizzard) as a substrate. The phosphorylation state of the endogenous RLC was not determined. Most assessments of the role of MLCK have also been based on inhibitors such as KT5926, ML-9, and ML-7 (9,15,34). These inhibitors are thought to interact with a hydrophobic region on the catalytic site of the MLCK, competing with ATP for binding and thereby inhibiting its activity (35). The apparent specificity of these inhibitors is now in doubt, however. Recent experiments in RBL-2H3 cells demonstrated that whereas ML-7 inhibited histamine secretion, this inhibitor also had more potent effects on PKC than on MLCK phosphorylation of the RLC (15). In addition, ML-9 has been shown to inhibit both mitogen-activated protein (MAP) kinase and protein phosphatase type 1 (PP1) activity in rat adipocytes (36).

More recently, a study using a different technique has shown that myosin RLC phosphorylation is important in regulating a proximal step in insulin secretion, most likely granular movement (9). In those experiments, specific monoclonal antibodies to MLCK successfully inhibited MLCK activity of pancreatic islet lysates and also significantly decreased granular movement in MIN6 cells (9). In parallel experiments using streptolysin-O permeabilized pancreatic islets, these antibodies were also found to inhibit Ca^{2+}-evoked insulin secretion. The permeabilized MIN6 cells also showed a Ca^{2+}-dependent increase in RLC phosphorylation when exogenous MLCK was added to the system. These discrepancies are probably explained by subtle, but important, differences between intact and permeabilized cell systems. In the streptolysin-O permeabilized cell, it is possible that substrate specificity of kinases such as MLCK may be slightly altered or that phosphatases that are normally active in the intact cells may be inoperative in the permeabilized cells because of loss of cofactors. The findings in the MIN6 cells did confirm our results showing that there was phosphorylation of the RLC in the basal state. However, using our more sensitive method of separation of [^{32}P]-labeled RLC peptides by IEF gels (as opposed to mobility shifts in the previous study), we were unable to show a consistent increase in RLC phosphorylation in intact cells or rat pancreatic islets. Moreover, we have shown that the sites of basal RLC phosphorylation are the MLCK Ser^{19}/Thr^{18} sites. While we do not exclude the possibility that basal RLC phosphorylation by MLCK might be important for insulin secretion, our results clearly indicate that stimulated insulin secretion occurs in the absence of further increases in phosphorylation at those sites or PKC sites.

The lack of correlation between RLC phosphorylation and insulin secretion suggests that RLC phosphorylation is not the primary mechanism involved in regulating myosin II function and supports our hypothesis that phosphorylation of the MHC may be the more important regulatory mechanism of this protein complex in insulin-secreting cells. In protozoan models such as *Dictyostelium* and *Acanthamoeba*, phosphorylation of the MHC results in disruption of filament formation (37,38). In secretory cells, it is thought that the cortical actin web forms a barrier preventing access of the granules to the plasma membrane (8,16,39). It may be possible that phosphorylation of the heavy chain in these insulin-secreting cells also results in filament disruption, breaking down the cortical web and therefore allowing the insulin granules to dock. The role of myosin II in this case would be independent of RLC phosphorylation, since filament disassembly does not require any contractile activity. We have previously shown that KCl stimulation of RINm5F cells resulted in an increase in MHC phosphorylation on both threonine and serine residues and a concurrent increase in insulin secretion (10). In our current study, we investigated PKC-dependent phosphorylation of MHC in RINm5F cells. Whereas PMA activation of PKCs did not result in a consistent increase in RLC phosphorylation, it did cause an increase in serine phosphorylation of the MHC and insulin secretion in these cells, suggesting that phosphorylation on either threonine or serine of MHC may be sufficient to cause filament disassembly. Moreover, the phosphopeptide maps of the MHC from PMA-stimulated (this study) and glyceraldehyde-stimulated (10) RINm5F cells showed a similar migratory pattern, suggesting that the same peptide is phosphorylated by these two stimuli and that the sites of phosphorylation may be in close proximity to one another. To confirm this hypothesis, we are now mapping the precise sites of phosphorylation on the MHC.

In conclusion, our findings show that both metabolic stimuli and activators of PKC increase MHC phosphorylation without accompanying increases in RLC phosphorylation in insulin-secreting cells. Phosphorylation of the MHC rather than the RLC correlates with stimulated secretion and may therefore be more important in regulating the function of myosin II in the insulin secretory response.

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