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Effects of Purified Myosin Light Chain Kinase on Myosin Light Chain Phosphorylation and Catecholamine Secretion in Digitonin-Permeabilized Chromaffin Cells

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ABBREVIATIONS: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; KGEPM, solution containing potassium glutamate, EGTA, PIPES and $MgCl_2$; NE, norepinephrine; PIPES, piperazine-N,-N′-bis-(2-ethanesulfonic acid); PSS, physiological salt solution.

Many non-muscle cells including chromaffin cells contain actin and myosin. The 20,000 dalton light chain subunits of myosin can be phosphorylated by a Ca^{2+}/calmodulin-dependent enzyme, myosin light chain kinase. In tissues other than striated muscle, light chain phosphorylation is required for actin-induced myosin ATPase activity. The possibility that actin and myosin are involved in catecholamine

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secretion was investigated by determining whether increased phosphorylation in the presence of [γ-^{32}P]ATP of myosin light chain by myosin light chain kinase enhances secretion from digitonin-treated chromaffin cells. In the absence of exogenous myosin light chain kinase, 1 μM Ca^{2+} caused a 30–40% enhancement of the phosphorylation of a 20 kDa protein. This protein was identified on 2-dimensional gels as myosin light chain by its comigration with purified myosin light chain. Purified myosin light chain kinase (400 μg/ml) in the presence of calmodulin (10 μM) caused little or no enhancement of myosin light chain phosphorylation in the absence of Ca^{2+} in digitonin-treated cells. In the presence of 1 μM Ca^{2+}, myosin light chain kinase (400 μg/ml) caused an approximately two-fold increase in myosin light chain phosphorylation in digitonin-treated cells in 5 min. The phosphorylation required permeabilization of the cells by digitonin and occurred within the cells rather than in the medium. Myosin light chain kinase-induced phosphorylation of myosin light chain was maximal at 1 μM Ca^{2+}. Under identical conditions to those of the phosphorylation experiments, secretion was unaltered by myosin light chain kinase. The experiments indicate that the phosphorylation of myosin light chain by myosin light chain kinase is not a limiting factor in secretion in digitonin-treated chromaffin cells and suggest that the activation of myosin is not directly involved in secretion from the cells. The experiments also demonstrate the feasibility of investigation of effects of exogenously added proteins on secretion in digitonin-treated cells.

INTRODUCTION

At a meeting one year ago Peter Baker overheard one of us (R.W.H.) reminiscing to a colleague how quickly understanding in a new area had developed several years before. He jumped at us and said that one can never look backward. One must always look forward. This was one of the few times Peter Baker was wrong. Now is a time when we must sadly look backwards in order to recognize Pete's contributions to the present and future. For those of us who have had similar interests to Pete, we will sorely miss his enthusiasm and insight.

The demonstration by Peter Baker and Derek Knight that cells with plasma membranes rendered leaky by dielectric breakdown could undergo the complex process of exocytosis (1, 2) opened up a powerful new approach for the study of secretion. For the first time one could directly control the intracellular milieu of a functional secretory cell. Their work motivated us and others to examine an alternative method for rendering the plasma membrane leaky utilizing the detergent digitonin (3, 4) or saponin (5), each of which interact with membrane cholesterol. The secretory responses of digitonin- and dielectric breakdown-permeabilized cells have identical Ca^{2+}- and ATP-dependencies. However, an important difference between cells permeabilized by dielectric breakdown and cells permeabilized by digitonin is that the former retain the soluble cytoplasmic protein lactate dehydrogenase (1, 2) while the latter are leaky to lactate dehydrogenase (134-kDa) and other cytosolic proteins (3, 4, 6). Perhaps as a consequence of this difference, the secretory response of cells permeabilized by digitonin is much less stable ($t_{1/2}$ of 10–15 min) (Holz, R. W.,

unpublished observations) than that of cells permeabilized by dielectric breakdown (stable responses for at least 50 min) (2).

The ability of proteins to exit from digitonin-treated cells raises the possibility that exogenous proteins can enter these cells. Indeed, in a recent study we found that trypsin (27-kDa) can enter digitonin-treated cells and inhibit secretion (Holz, R. W. and Senter, R. A., unpublished observations). In the following study we have investigated the effects of myosin light chain kinase (130-kDa), an important regulator of nonmuscle myosin, on phosphorylation and secretion in digitonin-treated chromaffin cells.

The possible involvement in exocytosis of contractile proteins which respond to Ca^{2+} was originally suggested by Douglas and colleagues because of the similar requirement for extracellular Ca^{2+} of muscle contraction and catecholamine secretion from chromaffin cells (7, 8). This notion was supported by the finding that non-muscle cells including chromaffin cells (9) contain contractile proteins such as actin and myosin. Increasing evidence suggests that contractile activity in non-muscle cells, as well as in smooth muscle cells, is regulated by phosphorylation and dephosphorylation of the 20-kDa light chain of myosin by myosin light chain kinase. Myosin light chain phosphorylation in non-muscle cells may serve two important functions: (1) to enhance acto-myosin ATPase activity (10–13) and (2) to stimulate formation of thick filaments (i.e. bundles of myosin) (14). The net effect of phosphorylation would be the formation of structural units fully capable of contractile function. Myosin light chain kinase activity is Ca^{2+}- and calmodulin-dependent and is probably responsible for the *in situ* phosphorylation of myosin light chain which occurs in platelets upon activation of secretion by thrombin (15). In adrenal chromaffin cells there is a report that secretion induced by elevated K^+ is accompanied by phosphorylation of myosin light chain (16). However, cytochalasin B, a disrupter of actin filaments, has little or no direct effect on the process of exocytosis in intact chromaffin cells* (17). Similarly, the microfilament inhibitors cytochalasin B (1 mM), cytochalasin D (1 mM) and phalloidin (1 mM) had virtually no effect on Ca^{2+}-dependent secretion in chromaffin cells permeabilized by dielectric breakdown in intense electric fields (2). Thus, although Ca^{2+}-dependent phosphorylation of myosin light chain may occur upon secretion in a number of cells, it is unclear whether it plays a role in exocytosis.

In the following work with digitonin-permeabilized chromaffin cells we demonstrate that myosin light chain kinase specifically phosphorylates intracellular myosin light chain but does not alter Ca^{2+}-dependent catecholamine secretion.

METHODS

Preparation of Cultured Chromaffin Cells

Primary dissociated cells from bovine adrenal medulla were prepared as previously described (18), purified by differential plating (19) and maintained as monolayer cultures in Eagle's minimal essential medium (GIBCO, Grand Island,

* Cytochalasin B (10 μM) had virtually no effect on Ca^{2+}-dependent secretion stimulated by elevated K^+ in bovine chromaffin cells. Cytochalasin B (10 μM) inhibited 25% secretion induced by nicotinic receptor activation, probably by interferring with the function of the nicotinic receptor (17).

N.Y.) containing 10% heat-inactivated fetal calf serum. The culture medium contained 100 units/ml of penicillin, 100 μg/ml of streptomycin, 50 μg/ml of gentamicin, and 1.3 μg/ml of Fungizone (Squibb, Princeton, NJ) to prevent bacterial and fungal contamination. The culture medium also contained 10 μM cytosine arabinoside to inhibit fibroblast growth. Cells were cultured as monolayers in 6.4 mm diameter plastic culture wells (Costar, Cambridge, MA) at a density of 500,000 cells per cm^2. Plastic culture wells were coated with sterile calfskin collagen (Calbiochem, San Diego, CA) prior to cell plating. Sterile calfskin collagen solution, 100 μl/cm^2 of a 50 μg/ml solution (in 0.1% acetic acid), was applied to each 6.4 mm diameter well. Acetic acid was allowed to evaporate at room temperature. Experiments were performed 4–11 days after preparation. There were approximately 100 nmole of catecholamine per million cells.

Determination of Catecholamine Secretion from Digitonin-Treated Chromaffin Cells

Cellular catecholamine stores were labeled by incubation of intact cells with [^{3}H]norepinephrine for 2–4 hours. Immediately before an experiment, cells were incubated for 1 hour in physiological salt solution (PSS) containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 15 mM HEPES (pH 7.4), 5.6 mM glucose, and 0.5 mM sodium ascorbate unless otherwise indicated. Experiments were initiated by replacing the PSS with a new solution. Cells were rendered leaky by treatment with 20 μM digitonin in potassium glutamate buffer solution (KGEPM) containing 139 mM potassium glutamate, 5 mM EGTA, 20 mM PIPES (pH 6.60), 1 mM $MgCl_2$, 1 mM MgATP, and various amounts of $CaCl_2$ to yield buffered Ca^{2+} concentrations of 0–10 μM. KGEPM was modified as indicated in various experiments. Secretion was determined by measuring the percentage of total cellular radioactivity released into the medium (13). All experiments were performed at 25°C.

Protein Phosphorylation in Digitonin-Treated Chromaffin Cells and Intact Cells

Intracellular phosphorylation in digitonin-treated cells was determined by incubation of cells in 6.4 mm diameter wells in KGEPM solution containing 20 μM digitonin, various concentrations of $CaCl_2$, 2 mM $MgCl_2$ and 1 mM [γ-^{32}P]ATP (200 μCi/ml) and 10 μM calmodulin unless otherwise indicated. Experiments were terminated by replacing the medium with 0.15 ml of solution containing 3% sodium dodecyl sulfate (SDS), 6% β-mercaptoethanol, 5% (w/v) glycerol, 62 mM Tris-Cl (pH 6.8) and 0.17 mg/ml bromophenol blue. Cellular proteins were further denatured by incubation at 95°C for 5 min. Aliquots (0.1 ml) of the denatured cell lysates were analyzed for phosphoprotein by SDS-polyacrylamide (5–20% gradient) slab gel electrophoresis using the procedure originally developed by Laemmli (20). After electrophoresis, gels were stained with Coomassie brilliant blue R, dried, and exposed to Kodak X-Omat AR-2 film for autoradiography.

For two-dimensional gel electrophoresis of phosphoproteins in digitonin-treated cells, cells were incubated in the presence of 2–4 mCi/ml [γ-^{32}P]ATP and the

incubation terminated by replacing the medium with 0.04 ml of solution containing 9.5 M urea, 2% (w/v) Nonidet P-40, 5% β-mercaptoethanol and 2% ampholytes [comprised of 1.6% Bio-Lyte (pH range 5 to 7; Bio-Rad, Richmond, CA) and 0.4% Ampholine (pH range 3.5 to 9.5; LKB, Gaithersburg, MD)]. Aliquots (20 μl) were subjected to isoelectric focusing followed by SDS-polyacrylamide (5–20% gradient) gel electrophoresis according to O'Farrell (21). Isoelectric focusing was performed using 11.5 cm tube gels of 2.0 mm diameter.

The pH gradient of isoelectric focusing gels was determined by equilibrating 0.5 cm segments of the tube gel in 2 ml of degassed and deionized water in 10 × 75 mm glass test tubes covered with parafilm for 4–5 hours with occasional vortexing (21). The gradient was approximately linear from pH 4.5 to 7.0. The apparent pIs of phosphoproteins resolved in two-dimensional gels were determined from the pH gradient curve by comparison of the distance of each spot from the acidic end. Molecular weights were determined by comparison to positions of standard proteins (Bio-Rad) which were visualized by Coomassie blue staining. The molecular weight marker proteins used were lysozyme (14,400 Da), soybean trypsin inhibitor (21,500 Da), carbonic anhydrase (31,000 Da), ovalbumin (45,000 Da), bovine serum albumin (66,200 Da), and phosphorylase B (92,500 Da). Autoradiographs of one-dimensional SDS-polyacrylamide gel electrophoresis were analyzed by densitometric quantitation using an EC 910 densitometer (EC-Apparatus Co., St Petersburg, FL). The quantitation was performed over a range where the densities increased proportionally with the amount of phosphorylated protein. Peak heights from densitometric tracings were measured after subtracting background densities in neighboring regions of the tracings as described previously (22), and expressed in arbitrary units.

Protein Purification

Myosin light chain kinase from chicken gizzard was purified to homogeneity according to Hathaway *et al.* (23). For identification of phosphorylated myosin light chain, myosin from bovine aorta was purified and phosphorylated with myosin light chain kinase according to Sellers *et al.* (11). The phosphorylated myosin was precipitated with 5% TCA. The precipitate was washed with acetone, and lyophilized. The phosphorylated myosin was dissolved in isoelectric focusing solution containing 9.5 M urea, 2% (w/v) Nonidet P-40, 5% β-mercaptoethanol and 2% ampholytes. Insoluble material (i.e. mostly myosin heavy chain) was removed by sedimentation.

Data

Data were expressed as mean ± standard error of the mean (SEM). Error bars smaller than the symbols or lines used in the figures were omitted. Significance was determined by Student's *t*-test.

RESULTS AND DISCUSSION

The Effects of Exogenous Myosin Light Chain Kinase on Protein Phosphorylation in Digitonin-Permeabilized Cells

Two-dimensional SDS-polyacrylamide gel electrophoresis of proteins phosphorylated by exogenous, purified myosin light chain kinase in digitonin-treated cells revealed that the enzyme enhanced the Ca^{2+}-dependent phosphorylation of 2 proteins of similar mobility (Fig. 1). The most prominent spot had a molecular mass of 20-kDa and a pI of 5.02 (indicated by arrowhead in Fig. 1). A spot with a slightly lower molecular mass of 19.6-kDa and the same pI was phosphorylated, but to a much smaller degree. The phosphorylation of other proteins in response to myosin light

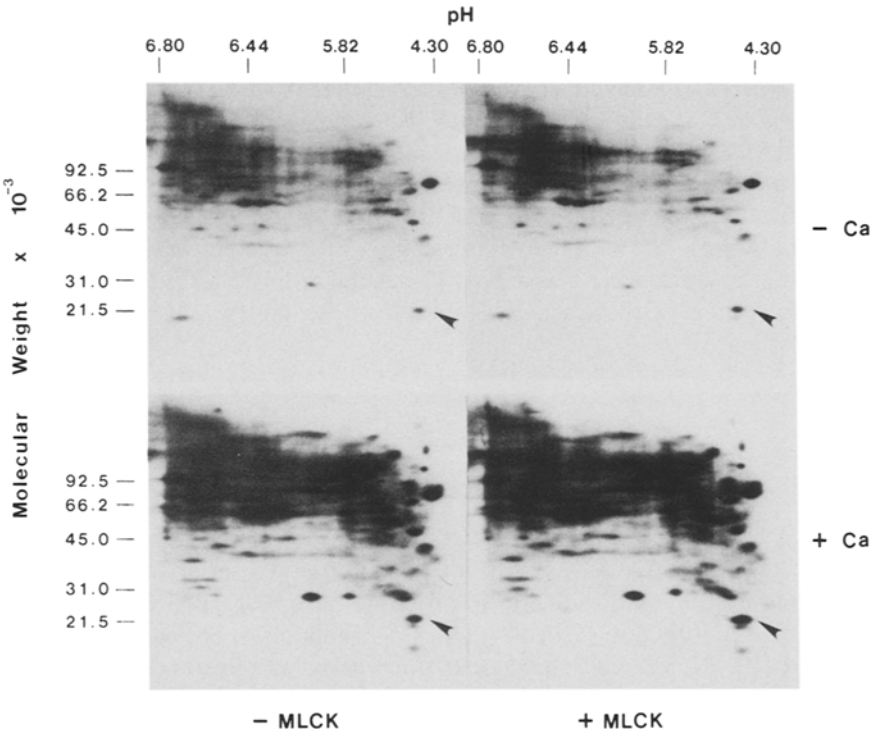

Fig. 2. Two-dimensional SDS-polyacrylamide gel electrophoresis of proteins phosphorylated by purified myosin light chain kinase in digitonin-treated chromaffin cells. Chromaffin cells were incubated in KGEPM solution containing 20 μM digitonin, 2 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/ml leupeptin, 36 μg/ml BSA (fatty acid-free), 10 μM calmodulin, 0 or 1 μM Ca^{2+}, 0 or 400 μg/ml purified myosin light chain kinase and 1 mM [γ-^{32}P]ATP. After 7 min, cells were washed briefly (15 sec) with ice-cold solution (300 mOsm) containing 50 mM sodium pyrophosphate, 40 mM sodium fluoride, 5 mM EGTA, 5 mM EDTA, 5 mM HEPES (pH 7.0) and 50 μg/ml leupeptin, and then, urea- and Nonidet P-40-containing solution was added. Cell lysates were subjected to isoelectric focusing followed by SDS-polyacrylamide (5–20% gradient) gel electrophoresis and subsequent autoradiography. Arrowheads indicate the 20-kDa protein which was identified as myosin light chain (see text). MLCK refers to exogenous, purified myosin light chain kinase.

chain kinase was not detected. The proteins with molecular mass of 20-kDa and 19.6-kDa were also phosphorylated, but to a lesser degree, in the absence of exogenous myosin light chain kinase upon addition of 1 μM Ca^{2+}. The molecular weight and pI of the prominent spot were virtually identical with those of smooth muscle myosin light chain which has a molecular weight of 20-kDa and a pI of 5.04 (24).

The mobilities of the doublet proteins in 2-dimensional gels were directly compared with those of phosphorylated 20,000 dalton myosin light chain prepared by phosphorylation of purified bovine aortic myosin with myosin light chain kinase and [γ-^{32}P]ATP (see Methods). [^{32}P]Phosphorylated myosin light chain was added to a tissue sample (in urea- and Nonidet P-40-containing solution) from permeabilized cells which had been incubated with [γ-^{32}P]ATP in the absence of Ca^{2+}. The [^{32}P]-labeled myosin light chains were resolved into a major phosphorylated protein of 20-kDa and a minor phosphorylated protein of 19.6-kDa which exactly comigrated with the doublet from the chromaffin cells (data not shown). The purified myosin contained a third protein of 18.5-kDa with a pI of 4.7 which was phosphorylated to an even smaller degree than the 19.6-kDa protein. Thus, the predominant phosphorylated species was of molecular mass 20 kDa (pI = 5.02). The various satellite bands may have resulted from proteolysis, multiple site phosphorylation or protein modification (25).

The phosphorylation of myosin light chain induced by Ca^{2+} and myosin light chain kinase could be detected in one-dimensional SDS-polyacrylamide gels and quantitated by densitometry (Fig. 2). The gels did not resolve the 19.6-kDa form of myosin light chain. The quantitation may overestimate to some degree the phosphorylation of myosin light chain in the absence of Ca^{2+} because of the presence of a phosphorylated protein of molecular mass 19 kDa and pI 6.70 (Fig. 1). Myosin light chain phosphorylation was not detected in cells which were not permeabilized with digitonin (data not shown).

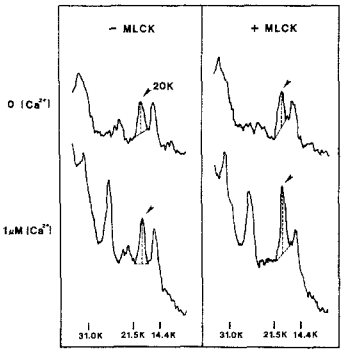

Fig. 2. Densitometric quantitation of the effects of purified myosin light chain kinase on phosphorylation of a 20-kDa protein identified as myosin light chain in digitonin-treated chromaffin cells. Chromaffin cells were incubated in KGEPM solution containing 20 μM digitonin, 2 mM $MgCl_2$, 50 μg/ml leupeptin, 40 μM dithiothreitol, 0.5 mg/ml BSA (fatty acid-free), 10 μM calmodulin, 0 or 1 μM Ca^{2+}, 0 or 400 μg/ml purified myosin light chain kinase and 1 mM [γ-^{32}P]ATP. After 5 min, the phosphorylation was terminated by replacing the medium with SDS-containing solution. Phosphoproteins were subjected to one-dimensional SDS-polyacrylamide (5–20% gradient) gel electrophoresis and subsequent autoradiography as described in Methods. MLCK refers to exogenous, purified myosin light chain kinase.

Phosphorylated Myosin Light Chain is Intracellular

Digitonin-treated chromaffin cells are permeable to proteins and some phosphorylation of myosin light chain occurred extracellularly. Nevertheless, the phosphorylated myosin light chain which was detected in the experiments was intracellular because only proteins associated with the cell monolayer were

investigated. Phosphorylated myosin light chain in the medium which remained with the monolayer could not account for the phosphorylated myosin light chain which was detected, because at least 95% of the extracellular medium was removed in experiments in which cells were not washed (e.g. Figs 2 and 3) and greater than 99.5% of the original medium was removed from cells washed once with buffer (Fig. 1). Because 70% of the phosphorylated myosin light chain was intracellular after a 15 min incubation with myosin light chain kinase, Ca^{2+} and [γ-^{32}P]ATP (data not shown), the phosphorylated myosin light chain the small amount of contaminating extracellular medium associated with the cells could contribute no more than 0.15–1.5% to the phosphorylated myosin light chain which was detected.

Effects of Exogenous Myosin Light Chain Kinase on Ca^{2+}-Dependent Myosin Light Chain Phosphorylation and Catecholamine Secretion in Permeabilized Cells

Phosphorylated myosin light chain was detected after 5 and 15 min in the absence of both Ca^{2+} and exogenous myosin light chain kinase (Fig. 3A and B). The response of phosphorylation to Ca^{2+} was somewhat different at the two times. In cells

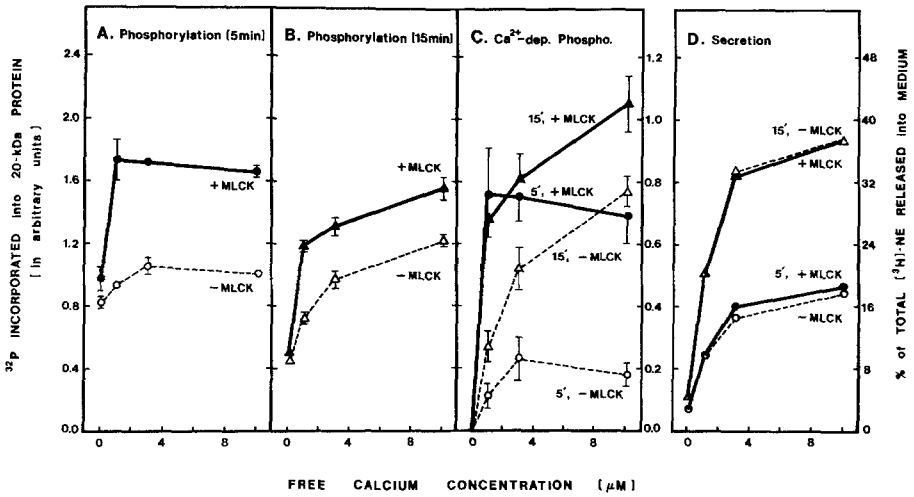

Fig. 3. Effects of purified myosin light chain kinase on Ca^{2+} dose-response for myosin light chain phosphorylation and catecholamine secretion in digitonin-treated chromaffin cells A, B and C. Phosphorylation: Chromaffin cells were incubated in KGEPM solution containing 20 μM digitonin, 2 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/ml leupeptin, 0.5 mg/ml BSA (fatty acid-free), 10 μM calmodulin, various concentrations of Ca^{2+}, 0 or 400 μg/ml purified myosin light chain kinase (MLCK) and 1 mM [γ-^{32}P]ATP. After 5 (A) or 15 min (B) phosphorylation was terminated by replacing the medium with SDS-containing solution. Phosphoproteins were subjected to one-dimensional SDS-polyacrylamide (5–20% gradient) gel electrophoresis and subsequent autoradiography. Phosphorylation of myosin light chain was analyzed by densitometric quantitation of autoradiographs. Ca^{2+}-dependent phosphorylation of myosin light chain is shown in (C). (D), Secretion: Chromaffin cells which had been prelabeled with [^{3}H]norepinephrine were incubated in KGEPM solution containing 20 μM digitonin, 2 mM MgATP, 1 mM dithiothreitol; 50 μg/ml leupeptin, 0.5 mg/ml BSA (fatty acid-free), 10 μM calmodulin, various concentrations of Ca^{2+} and 0 or 400 μg/ml purified myosin light chain kinase. The percentage of [^{3}H]norepinephrine released into medium was determined after 5 or 15 min. Each group contained 3 wells in A–D.

incubated for 5 min in the absence (or presence) of exogenous myosin light chain kinase, Ca^{2+}-dependent phosphorylation was maximal by 1 μM Ca^{2+}. In cells incubated for 15 min in the absence (or presence) of exogenous myosin light chain kinase, myosin light chain phosphorylation continued to increase between 1 and 10 μM Ca^{2+}. It is possible that by 15 min, but not by 5 min, an endogenous, Ca^{2+}-dependent protein kinase contributed to the phosphorylation of myosin light chain which was sensitive to Ca^{2+} concentrations greater than 1 μM Ca^{2+}.

Exogenous myosin light chain kinase enhanced the Ca^{2+}-dependent phosphorylation of myosin light chain with maximal enhancement occurring at 1 μM Ca^{2+} at 5 or 15 min (Figs 3A, B and C). The enhancement by myosin light chain kinase of phosphorylation of myosin light chain was larger in cells incubated for 5 min than for 15 min at all Ca^{2+} concentrations, perhaps because there was more intracellular myosin light chain after 5 min than after 15 min. After 5 min myosin light chain kinase increased 2-fold the total phosphorylation of myosin light chain at 1 μM Ca^{2+} (Fig. 3A) and increased Ca^{2+}-dependent phosphorylation 3 to 6-fold (Fig. 3C).

In a companion secretion experiment, exogenous myosin light chain kinase had little or no effect on catecholamine secretion at 5 or 15 min at various Ca^{2+} concentrations (Fig. 3D). Exogenous myosin light chain kinase (350–400 μg/ml) did not alter secretion in 6 of 6 experiments.

The phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) which activates protein kinase C (26) enhances Ca^{2+}-dependent secretion from digitonin-treated chromaffin cells (27). The presence of myosin light chain kinase (400 μg/ml) did not alter TPA-induced secretion from digitonin-permeabilized cells (data not shown). Thus, secretion that was enhanced by activation of protein kinase C was also not altered by myosin light chain kinase.

In conclusion, the absence of effects on secretion of phosphorylation of myosin light chain by myosin light chain kinase suggests that the interaction of actin and myosin is not a rate limiting step in Ca^{2+}-dependent secretion in chromaffin cells. However, these experiments do not eliminate the possibility that cytoskeletal proteins are involved in other processes connected with secretion such as granule movement or retrieval of the chromaffin granule membrane after exocytosis. Indeed, Ca^{2+}-independent disassembly of actin (28) and Ca^{2+}-dependent patching of fodrin (29) have been observed upon nicotinic stimulation of chromaffin cells.

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