Chromaffin Cell F-actin Disassembly and Potentiation of Catecholamine Release in Response to Protein Kinase C Activation by Phorbol Esters Is Mediated through Myristoylated Alanine-rich C Kinase Substrate Phosphorylation*

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The large majority of chromaffin vesicles are excluded from the plasma membrane by a cortical F-actin network. Treatment of chromaffin cells with phorbol 12-myristate 13-acetate produces disassembly of cortical F-actin, increasing the number of vesicles at release sites (Vitale, M. L., Seward, E. P., and Trifaro, J. M. (1995) Neuron 14, 353–363). Here, we provide evidence for involvement of myristoylated alanine-rich protein kinase C substrate (MARCKS), a protein kinase C substrate, in chromaffin cell secretion. MARCKS binds and crosslinks F-actin, the latter is inhibited by protein kinase C-induced MARCKS phosphorylation. MARCKS was found in chromaffin cells by immunoblotting. MARCKS was also detected by immunoprecipitation. In intact or permeabilized cells MARCKS phosphorylation increased upon stimulation with 10−6 M phorbol 12-myristate 13-acetate. This was accompanied by cortical F-actin disassembly and potentiation of secretion. MARCKS phosphorylation, cortical F-actin disassembly, and potentiation of Ca2+-evoked secretion were inhibited by a peptide (MARCKS phosphorylation site domain sequence (MPSD)) with amino acid sequence corresponding to MARCKS phosphorylation site. MPSD was phosphorylated in the process. A similar peptide (alanine-substituted phosphorylated site domain) with four serine residues of MPSD substituted by alanines was ineffective. These results provide the first evidence for MARCKS involvement in chromaffin cell secretion and suggest that regulation of cortical F-actin cross-linking might be involved in this process.

Chromaffin cells store their secretory products in membrane-bound organelles and chromaffin vesicles that release their contents to the extracellular medium by exocytosis. The dynamics of the cytoskeleton during exocytosis have been intensively studied in adren chromaffin cells (1–3). It has been suggested that the cortical F-actin cytoskeleton is involved in regulated secretion controlling the number of secretory vesicles present at release sites in the subplasmalemmal region (3, 4). In chromaffin cells, F-actin forms a cortical network that excludes the large majority of chromaffin vesicles from plasma membrane docking, and disruption of F-actin networks allows translocation of vesicles to the plasma membrane in preparation for exocytosis (2, 4, 5).

Previous work has demonstrated that scinderin, an F-actin severing protein discovered and cloned in our laboratory (6, 7), played a major role in the release of chromaffin vesicles by controlling disassembly of the cortical F-actin cytoskeleton through Ca2+-dependent and phosphatidylinositol 4,5-diphosphate-modulated mechanisms (8). However, other pathways or treatments that produce disassembly of cortical F-actin should increase the size of the release-ready vesicle pool with a consequent enhancement in the initial rate of vesicular exocytosis (4). Several lines of research suggest the involvement of PKC in catecholamine secretion. PKC1 activation enhances stimulation-evoked exocytosis in chromaffin cells (4, 9–17) without modifying scinderin activity (18). How PKC stimulation exerts its potentiating effect on secretion and which are its physiological substrates in this response are still largely unknown. In recent years, an acidic myristoylated protein and substrate of PKC, myristoylated alanine-rich C kinase substrate (MARCKS), has been implicated in several cellular processes such as motility, mitosis, cytoskeleton dynamics, phagocytosis, and transformation (19–23). MARCKS has a basic domain of 25 amino acids, located in the middle of the primary structure, which is the phosphorylation site domain (PSD) (19–21). The MARCKS PSD domain, in addition to containing four serines that are possible substrates for PKC, can also interact with calmodulin, phospholipids, and actin in a complex manner (24–28). It has been demonstrated that phosphorylation of MARCKS in the PSD domain by PKC inhibits F-actin cross-linking (26). MARCKS is known to be present in chromaffin cells (29), and correlations between its phosphorylation and several aspects of neurosecretion have been published (30–32). Moreover, recent work from our laboratory has also suggested the involvement of MARCKS in the release of serotonin from platelets (33). However, no specific role has been demonstrated for this protein in the exocytotic process.

In this study, a synthetic peptide with the bovine MARCKS PSD (MPSD) sequence has been used in preparations of permeabilized chromaffin cells to evaluate the role of MARCKS in F-actin network dynamics and secretion. The results presented here indicate that peptide MPSD reduced both cortical F-actin disassembly and MARCKS phosphorylation in response to

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1 The abbreviations used are: PKC, protein kinase C; PSD, phosphorylation site domain; Ala-PSD, alanine-substituted PSD; [3H]HNA, [3H]noradrenaline; MARCKS, Myristoylated alanine-rich C-kinase substrate; MPSD, MARCKS PSD; MRP, MARCKS-related protein; PMA, phorbol 12-myristate 13-acetate; PIPES, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline.
PKC activation by PMA, and these are accompanied by inhibition of PKC potentiation of exocytosis. The results suggest that the PKC effect on catecholamine release is mediated through MARCKS phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**—9,10-Hydmistic acid (50 Ci/mmol), [γ-32P]ATP (30 Ci/mmoll, Amphi1y, and ECL chemiluminescent kits were purchased from Amersham Pharmacia Biotech. The peptides MPSD (H-KKKKKRFKSKFKFLGSAFKNK-KOH) and Ala-PSD (H-KKKKKRFKSKFLGAFKFKRNK-KOH) were custom made by Research Genetica, Inc. (Hastings, AL). All other chemicals were purchased from Sigma. Goat polyclonal antibodies raised against the N- and C-terminal domains of MARCKS were a generous gift from Dr. Byers (Dalhousie University, Halifax, Canada) (34). Goat polyclonal antibodies raised against the N- and C-terminal domains of MARCKS were purchased from Bio-Rad. PMA, protein G-Sepharose, and digitonin were purchased from Sigma. All other reagents were of analytical or molecular biology grade and were obtained from standard suppliers.

**Cell Culture**—The adrenal glands from newborn rats were obtained from a local slaughterhouse, and chromaffin cells were isolated by collagenase digestion and further purified using a Percoll gradient (35). Chromaffin cells represented 90–95% of the isolated cells; the remaining cells were fibroblasts (4–9%) and cortical cells (1%). Fibroblasts contain MARCKS, and to reduce the number of fibroblasts present in the cultures, further purification of chromaffin cells by differential plating procedure was performed. The cells were plated in 100-mm plastic dishes for 5 h, and under these conditions, most of the fibroblasts attached to the plastic dish. Floating cells, more than 99% chromaffin cells, were then plated on collagen-coated plastic 24-multiwells, plastic 35-mm dishes, or coverslips according to the specific study to be performed. The cells were grown in 10% fetal calf serum in Dulbecco’s modified Eagle’s medium at 37°C in a humidified incubator under 5% CO2 atmosphere for 48–72 h.

**Cell Permeabilization and Catecholamine Release**—Chromaffin cells plated on collagen-coated plastic 24-multiwell dishes at a density of 5 × 106 cells/well were cultured for 48–72 h. After rinsing the wells three times with 0.5 ml of 10% fetal calf serum in amino acid-free Dulbecco’s modified Eagle’s medium (36), the cells were incubated with 10 μM [3H]NA in the same medium for 57°C for 20 min at room temperature. The preparations were then incubated with six changes of 0.5 ml of regular Locke’s solution (154 mM NaCl, 2.6 mM KCl, 1.25 mM KH2PO4, 0.5 mM KH2PO4, 1.2 mM MgCl2, 2.2 mM CaCl2, 10 mM glucose, pH 7.2) over a 60-min period. Finally, the cells were washed once with Ca2+-free Locke’s solution (2.2 mM CaCl2 was replaced by 2 mM EGTA) and permeabilized by 5 min of treatment with 20 μM digitonin in K-glutamate buffer (139 mM K-glutamate, 5 mM EGTA, 5 mM MgATP, 20 mM PIPES, 5 mM Na2HPO4, pH 6.6). Following permeabilization, [3H]NA release was determined by incubation of the cells in K-glutamate buffer for 90 s in the absence (basal) or presence of 10 μM Ca2+ (stimulated) with or without 10 μM PMA. Peptides MPSD (1–10 μM) and Ala-PSD (10 μM) were included during the permeabilization and stimulation steps as indicated in the figure legends. Immunoblot media were collected and assayed for [3H]NA. The [3H]NA cell content was determined by treating cells with 250 μM of 10% trichloroacetic acid for 10 min followed by two washes with 250 μM of 6% trichloroacetic acid. Total [3H]NA cell content was calculated by adding the [3H]NA secreted into the medium and the [3H]NA extracted with trichloroacetic acid. Basal [3H]NA release values (1.4–1.8%) were subtracted from those values obtained after stimulation. The radioactivity present in the samples was measured using a liquid scintillation spectrometer (Wallac model 1414, Wallac Oy, Turku, Finland).

**Protein Labeling**—For labeling of chromaffin cells, proteins with myristic acid, the cultures were plated at a density of 1 × 106 cells/35-mm dish and incubated in 2 ml of fresh culture medium containing [3H]myristic acid (5 Ci/mmol) for 18 h as described previously (34). Samples on MARCKS phosphorylation, its digito-n-permeabilized cells. Permeabilization was initiated by a 20 μM digitonin treatment as described above except that 80 μCi of [γ-32P]ATP was added into the medium after 3 min of permeabilization. After additional 2 min of digitonin treatment, PMA was added to a final concentration of 10−6 M and incubated for an additional period of 3 min. Phosphorylation was stopped by treatment of cells with Triton X-100 (final concentra-

1, followed by scraping of the cells. Samples were then heated at 80°C for 3 min (34, 37). The samples were concentrated by 10% trichloroacetic acid precipitation for 1 h, and the sediments were washed twice with ice-cold acetone.

**One- and Two-dimensional SDS-PAGE and Immunoblotting**—Protein samples prepared as indicated above were redissolved for one-dimensional SDS-PAGE by boiling for 5 min in 1.25% SDS, 60 mM Tris-HCl, pH 6.8, 12% glycerol, and 0.002% bromphenol blue and separated in 10% SDS gels (38). The protein samples for two-dimensional electrophoresis were prepared by dissolving the trichloroacetic acid sediments in iso-urea solution (9.5 M urea, 100 mM dithiothreitol, 100 mM Tris-HCl, pH 8.5–8.8 amphotrophes, 0.6% pH 4–6 amphotrophes, and 1% pH 3–10 amphotrophes; Bio-Lyte, Bio-Rad). Aliquots (200–250 μg of protein in 75 μl of sample buffer) were subjected to isoelectric focusing in gels contained in 16-cm tubes of 2.4-mm diameter. This was followed by 10% SDS-PAGE according to O’Farrell (39). A typical first dimension run consisted in 1 h at 200 V, 12 h at 1200 V, and a final 1 h at 2000 V. The pH gradient of isoelectric focusing gels was estimated by using a mock sample (only sample buffer) and equilibrating 0.5-cm segments of the tube gel in 1 ml of water in 10 × 75-mm glass tubes overnight. The gradient was approximately linear from pH 3 to 6.8. The gels containing [3H]myristic acid-labeled samples were treated with Amplify (Amersham Pharmacia Biotech) and exposed to radiochromic film for fluorography. Western blotting procedures were done using a Bio-Rad Mini-Protean II system as described previously (34). Nitrocellulose membranes were blocked with 5% skim milk in PBS containing 0.1% Tween 20. MARCKS protein was detected using a mouse monoclonal antibody (1 μg/ml) against the C-terminal domain of Human MARCKS followed by treatment with a horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) diluted 1:2500 (v/v). Western blot bands were developed using an ECL chemiluminescent kit. Digital scans of fluorograms, autoradiograms, and Western blots were obtained with a flat bed scanner (Hewlett Packard 4P) at 360 dpi resolution and processed with Adobe Photoshop 4.0. For densitometric analysis of phosphorylation and myristoylation, digital scans were analyzed using NIH Image software, version 1.61 (Research Services Branch, NIMH, Bethesda, MD). The area under the peaks were integrated, and the results were expressed in arbitrary units.

**Immunoprecipitation of MARCKS**—Experiments to further demonstrate the presence of MARCKS in chromaffin cells and its phosphorylation under different conditions were performed using immunoprecipitation techniques. These were carried out on intact as well as on permeabilized cells. To labeled ATP, intact cells were incubated with 200 μCi [γ-32P]phosphate/ml of phosphate-free Dulbecco’s modified Eagle’s medium (3% CO2 for 1 h). The supernatant min at 37°C for 20 min at room temperature. This was followed by 10% SDS-PAGE according to O’Farrell (39). The samples were then heated at 80°C for 3 min, and the heat labile proteins were removed by centrifugation. The supernatants (1 ml) were precluded by centrifugation after the addition of 30 μl of protein G-Sepharose (50% slurry) at 4°C for 1 h. The supernatants thus obtained were then incubated at 4°C overnight with mixture of two polyclonal antibodies raised in goat against the N- and C-terminal domains of MARCKS. This was followed by addition of 30 μl of protein G-Sepharose (50% slurry) and incubation for 2 h. Sepharose beads were sedimented and washed five times with PBS. The immunoprecipitates were then dissolved by boiling for 5 min in SDS loading buffer, and separation of the phosphoproteins by SDS-PAGE was performed as described above. Autodigestions were exposed to Kodak BioMax MS film using a BioMax MS intensiﬁving screen to achieve higher sensitivity for detection of 32P emission (Eastman Kodak Co., Rochester, NY).

**Fluorescence Microscopy**—Chromaffin cells were plated on collagen-coated coverslips contained within 35-mm plastic Petri dishes at a density of 3 × 106 cells. Cultured cells were rinsed with Locke’s solution and permeabilized with 20 μM digitonin in K-glutamate buffer for 5 min at room temperature as indicated above. The presence or absence of 10 μM CaCl2, 2–7 μM PMA, 10 μM MPSD, and 10 μM Ala-PSD peptides as indicated in the figure legends. Following the treatments, chromaffin cells were fixed in 3.7% formaldehdye and processed for fluorescence microscopy as described previously (40). Briefly, the cells were permeabilized by three successive exposures of 5 min each to 0.1%, 10%, and 50% acetone and washed several times with PBS (130 mM NaCl, 100 mM sodium phosphate, pH 7.0).
Chromaffin cells were stained for F-actin with 0.25 unit/ml of rhodamine-phalloidin for 40 min in the dark at room temperature. The coverslips were rinsed several times with PBS and mounted in glycerol-PBS (1:1). The slides were observed with a Leitz Ortholux fluorescence microscope equipped with a 100-W high pressure mercury lamp and a Ploemopack II incident light illuminator. The effect of several treatments on the percentage of cells showing cortical F-actin disassembly was determined by examining four coverslips for each condition, and 100 single-rounded chromaffin cells were examined per coverslip. Each cell examined was classified as having either a “continuous” or “discontinuous” cortical rhodamine (F-actin) fluorescent ring as described previously (2). The percentage of chromaffin cells showing cortical F-actin disassembly (discontinuous rhodamine fluorescent ring) was calculated for each experimental condition, and the mean ± S.E. of the values was plotted.

**Video-enhanced Image Processing—Quantitative analysis of cortical rhodamine fluorescence (F-actin)** was performed by using a Hamamatsu Photonics KK Argus-50/CL Image Processor (Hamamatsu Photonics Systems, Bridgewater, NJ). The fluorescence microscope was coupled to the video camera (Carl Zeiss, TV3M model), which was connected to the Argus 50-Image processor. The video camera control parameters (i.e. gain, offset, and sensitivity) were set up to obtain a clear image of the cell on the monitor and fluorescence intensity of 250 (arbitrary units) in the cortical plane on the equatorial plane of the cell on the x–y axes and the fluorescence intensity of this plane on the z axis (4).

**RESULTS**

**Presence of MARCKS in Chromaffin Cells and Their Leakage from Permeabilized Cells**—Proteins in extracts of chromaffin cells previously labeled with [3H]myristic acid were heated as indicated under “Experimental Procedures,” and the heat-resistant fractions were separated by SDS-PAGE (Fig. 1). MARCKS, a protein that remains soluble after heat denaturation (37), migrated as a broad band with an apparent molecular mass of 80–84 kDa (Fig. 1B). MARCKS represented 25% of the heat-stable [3H]myristic acid-labeled proteins in the chromaffin cells extracts. The myristoylated broad protein band at 80 kDa was also confirmed to be MARCKS by immunoblotting using a mouse monoclonal antibody specific for bovine MARCKS (Fig. 1C). The immunoreactive band had the same apparent molecular mass as that band labeled with [3H]myristic acid. The immunodetection of this band with MARCKS antibody was enhanced by heat treatment as described previously for neuroblastoma cell lines (34). Furthermore, MARCKS presence in chromaffin cells was confirmed by immunoprecipitation using MARCKS-specific polyclonal antibodies raised in goat. The immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with a mouse monoclonal antibody against MARCKS (Fig. 1D).

The preparation of permeabilized chromaffin cells allows the study of secretory events distal to receptor and channel activation. In addition, this preparation permits the testing on the system of large molecules (i.e. peptides, antibodies). Digitonin...
MARCKS in F-actin Disassembly and Secretion

Effect of MPSD and Ala-PSD on chromaffin cell MARCKS phosphorylation induced by PKC activation. Cultured chromaffin cells were permeabilized with K-glutamate buffer containing 20 μM digitonin and 0.1 μM Ca²⁺ in the absence or presence of 10 μM MPD or Ala-PSD peptides as described under “Experimental Procedures.” After 2 min of incubation, PMA was added to a final concentration of 10⁻⁷ M, and incubation was continued for another 3 min. Phosphorylation was stopped by scraping cells off the plates with 1% Triton X-100, and this was followed by heat denaturation at 80 °C for 5 min, SDS-PAGE, and autoradiography. A, autoradiography of [γ-³²P]ATP-labeled heat-stable proteins (the arrow shows MARCKS phosphorylation). B, scanning of the autoradiogram shown in A. The numbers beside the MARCKS peaks (shaded areas) are arbitrary units obtained from digital integration. As comparison, an unidentified heat-stable PMA-stimulated phosphorylated band is shown. Phosphorylation of this band was not blocked by MPSD. C, cumulative data on MARCKS phosphorylation expressed in arbitrary units. The p values were calculated against PMA + MPSD. *, p < 0.01; **, p < 0.05.

Effect of MPSD Peptide on the PKC-induced Potentiation of Ca²⁺ Evoked [³²P]Noradrenaline Release—To test the possibility that MARCKS is the PKC substrate involved in the potentiation of the secretory response, experiments were performed on digitonin-permeabilized cells. A 25-mer peptide (MPSD) with an identical sequence to the phosphorylation site, calmodulin, and actin-binding domain of bovine MARCKS was used (Fig. 2A). In addition, a similar 25-mer peptide that is not a substrate for PKC, in which the serine residues were substituted by alanine (Ala-PSD), was also tested (Fig. 2A). As expected, PMA (10⁻⁷ M) induced a significant potentiation of the Ca²⁺-evoked secretory response from permeabilized chromaffin cells (Fig. 2B). Incubation with 10 μM MPSD blocked PKC-induced increase of [³²P]NA release in response to Ca²⁺ stimulation. On the other hand, the nonphosphorylatable Ala-PSD, used at the same concentration, did not affect PKC-induced potentiation of Ca²⁺-evoked [³²P]NA release (Fig. 2B). Moreover, the inhibitory effect of MPSD on PKC-induced [³²P]NA release was also concentration-dependent (Fig. 2C).

The fact that MPSD blocked PKC-induced potentiation of Ca²⁺-induced amine release together with the lack of effect of Ala-PSD would suggest the involvement of MARCKS in this process. The results also suggest that phosphorylation of MPSD serines residues might be involved in the inhibitory effect of the peptide. Because phosphorylation of similar serine residues are involved in PKC-dependent phosphorylation of MARCKS, the effects of MPSD and Ala-PSD on chromaffin cell protein phosphorylation were investigated next.

MARCKS PSD Peptide Acts as Alternative Substrate and Compete with MARCKS Phosphorylation—Permeabilized chromaffin cells were incubated with PMA and [γ-³²P]ATP in the absence or presence of either MPSD or Ala-PSD. This was followed by separation of heat-stable proteins by SDS-PAGE. Autoradiographs of the gels together with their corresponding scans and quantification are depicted in Fig. 3. Activation of PKC by treatment with PMA for a short period of time (3 min) increased phosphorylation of MARCKS to 163 ± 10% (n = 8) when compared with the nonstimulated control (Fig. 3). In the presence of MPSD, there was a significant inhibition of PKC-induced MARCKS phosphorylation, and the percentage of inhibition of MARCKS phosphorylation was 81 ± 11% (n = 6). On the other hand, there was not significant inhibition of PKC-induced MARCKS phosphorylation when Ala-PSD was present in the medium (Fig. 3). To further study the phosphorylation of these proteins and obtain a higher resolution of MARCKS phosphorylation, two-dimensional PAGE was performed (Fig. 4). MARCKS is a highly acidic protein that ran in two-dimensional PAGE with an isoelectric point of 4.2–4.4 and an apparent molecular mass of 80–85 kDa. The spot labeled MARCKS in Fig. 4A was first identified as MARCKS on the basis of its mobility (80 kDa), acidic migration, and the “smear” shape of the spot (34). The MARCKS identity of the spot was also confirmed using a polyclonal antibody against MARCKS on a Western blot from a similar two-dimensional PAGE (Fig. 4B). Spots a, b, and c were three other acidic proteins that were not further characterized; in spots a and c phosphorylation did not increase by PMA treatment, whereas phosphorylation of spot b decreased 50% when compared with non-PMA-treated control. Spots a and b did not change their phosphorylation level in presence of MPSD, whereas the phosphorylation of spot c decreased (Fig. 4C). On the basic region of the two-dimensional PAGE, two other spots (d and e) were also detected.
Each one experiment done on intact and permeabilized cells. The results of the scanning of the autoradiographs were expressed as percentages of control.

A "..." for 3 min (...). Permeabilized chromaffin cells were incubated with 10^7M 32P-ATP and C-terminal domains of MARCKS, respectively (Fig. 4).

Further proof on the presence of MARCKS in intact as well in permeabilized chromaffin cells was obtained by immunoprecipitation, that PMA stimulation increased the level of phosphorylation in presence of PMA, 2.7 and 1.9 times, respectively (Fig. 4C); these increases in phosphorylation were higher than that observed for MARCKS (Fig. 4C). Moreover, the increase in the phosphorylation level of spots d and e did not change in presence of MPSD (Fig. 4C). Similar to our observations with one-dimensional SDS-PAGE, 10 μM MPSD reduce PMA-induced MARCKS phosphorylation. However, the reduction observed in two-dimensional gels was higher because the extent of phosphorylation observed in presence of MPSD was lower than the observed in unstimulated permeabilized cells (Fig. 4, A and C). The reason for this difference might be due to a 25% increase in phosphorylation of spot a, respect to unstimulated controls, and to the fact that both proteins, MARCKS and that at spot b, comigrate with similar apparent molecular mass in on-dimensional SDS-PAGE, thus overlapping their autoradiographic detection. Similarly, the sum of spots b, d, and e would have contributed to the broad band migrating below MARCKS in the one-dimensional gels (Fig. 2A). Further proof on the presence of MARCKS in chromaffin cells and on the phosphorylation of MARCKS in intact as well in permeabilized chromaffin cells was obtained by immunoprecipitation of phosphorylated MARCKS with a mixture of two polyclonal antibodies directed against the N- and C-terminal domains of MARCKS, respectively (Fig. 4D).

Experiments on intact chromaffin cells with their ATP pool previously labeled by incubation with 32P-phosphate showed, upon SDS-PAGE of immunoprecipitates, that PMA stimulation induced phosphorylation of MARCKS at levels that were 1.5 ± 0.3 (n = 3) times larger than those obtained in unstimulated controls (Fig. 4D, left panel). Immunoprecipitated MARCKS has the same electrophoretic mobility in SDS-PAGE as the band labeled as MARCKS in Fig. 3A. Moreover, Western blots of MARCKS, previously immunoprecipitated with goat polyclonal anti-MARCKS but now detected by chemiluminescence using a mouse monoclonal against MARCKS, confirmed that MARCKS was indeed the immunoprecipitated protein (Fig. 1D). Similar immunoprecipitation experiments on permeabilized chromaffin cells were carried out to confirm that the effects of MPSD on protein phosphorylation observed above using one- and two-dimensional SDS-PAGE were, indeed, the result of an specific inhibition of MARCKS phosphorylation. The analysis of autoradiograms of five separate immunoprecipitation experiments indicated that in the presence of MPSD, PMA-induced MARCKS phosphorylation was not only completely inhibited, but its phosphorylation was reduced to levels that were lower than those observed in permeabilized controls (Fig. 4D, right panel). The blockage of MARCKS phosphorylation in this case was such that the values obtained were 65 ± 20% (n = 5) lower than those of unstimulated control, thus suggesting that MARCKS under resting conditions was phosphorylated in the same domain. On the other hand, the immunoprecipitates obtained from cells stimulated with PMA alone or in the presence of Ala-PSD showed similar increments in MARCKS phosphorylation levels. Under these conditions, MARCKS phosphorylation increased 1.45 ± 0.25 and 1.35 ± 0.3 (n = 5) times, respectively, when compared with unstimu-
A

Fig. 5. Effect of peptides MPSD and Ala-PSD on PMA-evoked disassembly of cortical F-actin networks. Chromaffin cells were permeabilized with K+–glutamate buffer containing 20 μM digitonin in the presence or absence of peptides MPSD (10 μM) or Ala-PSD (10 μM) for 5 min. Permeabilized chromaffin cells were incubated in K+–glutamate buffer (control) or with 10−7 M PMA alone or plus MPSD (10 μM) or Ala-PSD (10 μM) for 5 min and then stimulated with 10 μM Ca2+ for 40 s. Following these treatments, the cells were fixed and processed for rhodamine-labeled phalloidin staining as indicated under “Experimental Procedures.” A, image analysis of control (panel a) and PMA- and Ca2+-treated (panel b) cells stained with rhodamine phalloidin. Control cells show an intact cortical fluorescent ring (panel a) with a uniform fluorescence intensity pattern (panel a'). PMA and Ca2+ treated cells show a disrupted cortical ring (panel b) with irregularities such as patches of fluorescence (panel b'). Three-dimensional image analysis (panels a' and b') shows a quantitative intensity analysis of pseudocolor images describing control (panel a') and stimulated (panel b') cells, respectively. Intensity of the fluorescent ring was set to 250 arbitrary units. B, 100 cells/coverslip were examined for F-actin (rhodamine fluorescence) peripheral distribution and were classified as having a continuous (resting) as shown in A (panel a) or a patched (stimulated) cortical staining (F-actin disassembly) as shown in A (panel b). Each bar represents the mean ± S.E. of the percentage of discontinuous F-actin distribution of 400 cells.

B

D I S C U S S I O N

Regulated secretion in chromaffin cells is triggered by an increase in intracellular Ca2+ (41). However, despite the fact that Ca2+ participation in neurosecretion was observed many years ago (41–43), the role of Ca2+ in this process is still poorly understood. An attractive hypothesis is that one of the sites of action for Ca2+ in secretion is in the control of F-actin networks (3). Previous experiments from our laboratory showed that PMA treatment of intact chromaffin cells disrupted cortical F-actin (4, 17) and increased both the number of chromaffin vesicles in the release-ready vesicle pool and the initial rate of exocytosis (4). In support of a role for PKC in the modulation of cortical F-actin was the observation that PKC inhibitors sphingosine, staurosporine, and calphostin C blocked, in a concentration-dependent manner, PMA-induced cortical F-actin network disassembly (17, 44). MARCKS is a member of a family of PKC substrates that potentially can interact with F-actin and calmodulin, proteins that are widely distributed in the nervous system (see scheme in Fig. 2A) (20, 21, 26, 45, 46). The present results demonstrate that, using a combination of cotranscriptional N-myristoylation radiolabeling with [3H]myristic acid and separation of the heat resistant proteins by SDS-PAGE (34, 47), MARCKS was found in chromaffin cells. Similar electrophoretic patterns for MARCKS have been described for neuroblastoma cell lines (34, 48). The presence of this PKC substrate was also confirmed by immunodetection. Moreover, experiments with antibodies against the N- and C-terminal domains of MARCKS immunoprecipitated this protein form chromaffin cell homogenates. This clearly demonstrates the presence of MARCKS in these cells. Although it has been previously suggested that MARCKS was a marker for PKC...
activity in chromaffin cells (29), its presence and characterization in these cells has not been demonstrated until the present studies. We have previously suggested that MARCKS may play a role in the cortical F-actin disassembly induced by PMA treatment of intact chromaffin cells (4). Thus, MARCKS may cross-link cortical F-actin filaments, and its phosphorylation by PKC may cause disruption of this cross-linking followed by filament disassembly. This possibility was tested with MPSD, a peptide with the sequence of the phosphorylation site domain of MARCKS. MPSD binds calmodulin and actin and can also interact with high affinity with membrane phospholipids (25, 49, 50). In addition, a similar sequence to the MPSD (KKKKFSFKKKSFKSLGSFSFKK) is present in the MARCKS-related protein (MRP) (KKKKFSFKKKPSLGLSFK). Both sequences are basic (calculated isoelectric point, >9) with similar composition of lysines and phenylalanines but with a noticeable difference: the replacement of the second serine of MPSD for a proline in the MRP PSD. Therefore, it seems logical to consider that MPSD may also compete with MRP activity, and, therefore, the possible role for MRP in the control of cortical F-actin should be demonstrated, because it has also been shown to interact with actin (51) and be transiently phosphorylated in PC12 cells by either KCl-induced depolarization or PMA treatment (52).

Immunoprecipitation experiments and two-dimensional PAGE analysis of PMA-induced protein phosphorylation also clearly showed that MARCKS was phosphorylated. MPSD also blocked PMA-induced phosphorylation of MARCKS as demonstrated by two-dimensional SDS-PAGE and immunoprecipitation experiments given additional support to a role of MARCKS in this process.

Patch clamp capacitance studies carried out in our laboratory have shown that pretreatment with PMA enhances exocytosis from intact chromaffin cells induced in response to depolarizing pulses (4). However, the PMA potentiation of secretion as demonstrated here has not been previously observed in digitonin-permeabilized cells (11, 12). It is possible that in previous experiments important components of the exocytotic machinery have leaked into the medium during the relatively long incubations used, and, therefore, potentiation of secretion by PMA treatment was not observed. We have previously shown that Ca\textsuperscript{2+}−dependent proteins such as calmodulin, PKC, scinderin, and calcineurin are released from permeabilized chromaffin cells into the medium (53). However, in the present experiments, improved protocols for catecholamine release (i.e. short stimulation times) were used, and under these conditions, we were also able to observe, as with intact cells, PMA potentiation of Ca\textsuperscript{2+}−evoked catecholamine release from digitonin-permeabilized chromaffin cells.

We suggest that interaction of unphosphorylated MARCKS with the cortical F-actin cytoskeleton stabilizes the cortical cytoskeletal network. When MARCKS is phosphorylated by PKC, the interaction between MARCKS and F-actin is impaired, and this would produce a disruption of cortical F-actin allowing translocation of secretory vesicles from the reserve to the release-ready vesicle pool. Morphometric studies from our laboratory have already demonstrated an increase in the number of vesicles in the release ready pool upon PMA treatment (4). In the absence of MPSD, phosphorylation of MARCKS by PKC is impaired because MPSD is also a substrate of PKC, under these conditions, the potentiating effects of PKC on cortical F-actin disassembly and exocytosis are blocked. In conclusion, the present findings strongly suggest a role for MARCKS in the PKC modulation of the dynamic changes observed in cortical F-actin networks during exocytosis.
Chromaffin Cell F-actin Disassembly and Potentiation of Catecholamine Release in Response to Protein Kinase C Activation by Phorbol Esters Is Mediated through Myristoylated Alanine-rich C Kinase Substrate Phosphorylation
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