Stimulus-dependent Phosphorylation of MacMARCKS, a Protein Kinase C Substrate, in Nerve Terminals and PC12 Cells*

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MacMARCKS (also known as myristoylated alanine-rich C kinase substrate [MARCKS]-related protein) is a member of the MARCKS family of protein kinase C substrates, which binds Ca²⁺/calmodulin in a phosphorylation-dependent manner. Immunoprecipitation demonstrated that MacMARCKS is present in both PC12 cells and in neurons. Upon depolarization of PC12 cells with 60 mM KCl, MacMARCKS phosphorylation increased 4-fold over basal levels in a Ca²⁺-dependent manner. By immunofluorescence microscopy, MacMARCKS was colocalized in PC12 cells to neurite tips with the synaptic vesicle membrane protein synaptophysin and to vesicles in the perinuclear region. Subcellular fractionation demonstrated that MacMARCKS associates tightly with membranes in PC12 cells. In Percoll-purified rat cerebrocortical synaptosomes, depolarization with 60 mM KCl in the presence of exogenous Ca²⁺ transiently increased MacMARCKS phosphorylation, whereas phorbol ester promoted a sustained increase in MacMARCKS phosphorylation. Subcellular fractionation of rat brain indicated that MacMARCKS was present in both soluble and particulate fractions; particulate MacMARCKS was associated with both small vesicles and highly purified synaptic vesicles. These results are consistent with a role for MacMARCKS in integrating Ca²⁺-calmodulin and protein kinase C-dependent signals in the regulation of neurotranscretion.

Materials—[³H]Myristic acid (0.4–2.2 TBq/mmol), [³²P]orthophosphate (320 TcBq/mmol), 125I-protein A (370 KBq/µg), and En²Hance were obtained from DuPont NEN. PMA was purchased from LC Services, Corp. (Woburn, MA). Leupeptin and nerve growth factor (NGF) 2.5 S (from rat submaxillary gland, grade II) were purchased from Boehringer Mannheim. Unless otherwise noted, other chemicals were purchased from Sigma.

EXPERIMENTAL PROCEDURES

PC12 Cell Culture—PC12 cells (obtained from the ATCC, Bethesda, MD) were cultured in the absence of NGF, as described previously (10) and maintained in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 5% fetal calf serum and 10% horse serum (JRH Biologicals, Corp. (Woburn, MA). Leupeptin and nerve growth factor (NGF) 2.5 S (from rat submaxillary gland, grade II) were purchased from Boehringer Mannheim. Unless otherwise noted, other chemicals were purchased from Sigma.

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Myristoylation and Phosphorylation of MacMARCKS in PC12 Cells—PC12 cells were labeled overnight with [³H]myristic acid (40 µCi/ml) and lysed in ice-cold lysis buffer containing 10 mM Tris-HCl, pH 7.5, 15 mM EDTA, 1% (w/v) Nonidet P-40, 50 mM Na₂HPO₄, 50 mM KF, 10 mM sodium pyrophosphate supplemented with the following protease inhibitors: 0.09 trypsin inhibitory units/ml aprotinin, 1 µg phenylmethanesulfonfyl fluoride, 1 µg disopropyl fluorophosphate, and 0.5 mg/ml leupeptin (11). After cell lysis, MacMARCKS was immunoprecipitated as described below. Protein phosphorylation in intact PC12 cells was performed by radiolabeling cells with 250 µCi/ml [³²P]orthophosphate either in PO₄-free RPMI or in Hepes-buffered saline as described previously (12). Where indicated, cells were either treated with 200 nM PMA for 30 min at 37°C or depolarized with depolarization buffer (85 mM NaCl, 10 mM Hepes, 5.5 mM D-glucose, 60 mM KCl, 1 mM MgSO₄, pH 7.4) either in the presence or absence of 1.8 mM CaCl₂ for 1 min at room temperature. After stimulation, the cells were scraped into lysis buffer, and the lysates were subjected to immunoprecipitation.

Subcellular Fractionation—Agonist-treated PC12 cells labeled with either [³H]myristic acid or [³²P]orthophosphate were scraped into 1 ml of homogenization buffer (250 mM sucrose, 20 mM Hepes, pH 7.2, 1 mM...
EDTA) supplemented with protease inhibitors (see above). Cells were disrupted by nitrogen cavitation (10,000 psi, 15 min), nuclei were removed by low speed centrifugation (500 g, 5 min), and the particulate and cytosolic fractions were prepared by centrifugation at 400,000 x g for 20 min at 4°C in a Beckman TLA-100.2 rotor (11, 13). The cytosolic fraction (supernatant) was removed, and the pellet was resuspended in 1 ml of homogenization buffer. Proteins from each fraction were precipitated with trichloroacetic acid (final concentration, 10% (w/v)) and after solubilization were subjected to immunoprecipitation.

Synaptic fractions were prepared as described previously (14) with minor modifications (15). To obtain S2 and P2, the S2 fraction was centrifuged at 165,000 x g, for 2 h. The eluate from the controlled pore glass (CPG) columns was pooled into three fractions (CPG1–CPG3). These fractions were kindly prepared by Drs. P. S. McPherson and P. De Camilli.

Antibodies—Polyclonal antiserum against murine MacMARCKS was prepared by injecting rabbits with a glutathione S-transferase-MacMARCKS fusion protein. Antibodies were affinity-purified from sera using strips of polyvinylidene difluoride membrane containing recombinant MacMARCKS. The affinity-purified MARCKS polyclonal antibody was prepared as described previously (11, 16). Mouse monoclonal antibody against synaptophysin (17) and anti-synapsin I antisera were kind gifts from P. Greengard, A. J. Czernik, and A. C. Nairn, The Rockefeller University. The anti-GAP-43/B-50 antibody was purchased from Boehringer Mannheim.

Immunoprecipitation—Cell extracts were preclarified with 20% (v/v) protein A-Sepharose, and MacMARCKS was immunoprecipitated with the MacMARCKS antiserum (13). Trichloroacetic acid-precipitated proteins were immunoprecipitated as described previously (11, 12). Following immunoprecipitation, samples were boiled and analyzed by 10% SDS-PAGE. 3H-Labeled proteins were visualized by fluorography, and immunoprecipitated proteins were visualized by autoradiography.

Immunoblotting—Lysates prepared from PC12 cells, and proteins from synaptic fractions, were subjected to SDS-PAGE and transferred to nitrocellulose membranes (18). The nitrocellulose membranes were blocked for 2 h with Tris-buffered saline (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20 mM sodium azide) containing 5% (w/v) nonfat dry milk and incubated overnight with the primary antibody at the indicated dilutions (1:200 for the anti-MacMARCKS and the anti-MARCKS antibodies, 1:5000 dilution for the anti-synaptophysin antibody, and 1:1000 for the anti-synapsin antibody). Immunoblots were then washed 3 times in Tris-buffered saline, incubated for 5 h with a 1:1000 dilution of 125I-labeled protein A in Tris-buffered saline, and washed 5 times with Tris-buffered saline. Labeled proteins were visualized by autoradiography.

Two-dimensional Polyacrylamide Gel Electrophoresis—32P-Labeled PC12 cell lysates were immunoprecipitated with the anti-MacMARCKS antibody and subjected to two-dimensional polyacrylamide gel electrophoresis as described previously (3). Radiolabeled MacMARCKS was visualized by autoradiography.

Immunofluorescence Microscopy—PC12 cells were plated on poly-L-lysine-coated coverslips and differentiated for 5 days in RPMI 1640 medium containing 100 ng/ml NGF. The cells were fixed in 4% (w/v) paraformaldehyde in 120 mM phosphate buffer containing 4% (w/v) sucrose and processed for indirect immunofluorescence microscopy as described previously (19, 20). The coverslips were incubated with affinity-purified anti-MacMARCKS antibody (1:50 dilution), followed by a 1:1000 dilution of a Texas red-conjugated goat anti-rabbit IgG (Tago, Burlingame, CA). Synaptophysin was visualized using a 1:1000 dilution of anti-synaptophysin antibody in conjunction with a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs, Westgrove, PA). Stained cells were visualized with a Zeiss Axiophot fluorescence microscope using a 63× Plan-Apochromat objective. Confocal microscopy was performed using the Molecular Dynamics Image Space software and a Zeiss Axioskop microscope.

Preparation and Labeling of Rat Brain Synaptosomes—Synaptosomes were purified from rat cerebrocortices as described previously (21) with minor modifications (22). The final purified synaptosomal pellet was resuspended in phosphate-free incubation buffer (140 mM NaCl, 5 mM KCl, 5 mM NaHCO3, 1 mM MgCl2, 10 mM glucose, and 10 mM Hepes, pH 7.4) to 2 mg/ml and radiolabeled with [32P]orthophosphate (1 M Ci/ml) for 45 min (23). Extrasympathosomal [32P]orthophosphate was removed by centrifugation of the synaptosomes at 1000 x g for 5 min. Radiolabeled synaptosomes (100 μg of protein) were incubated in the presence of 300 nM phorbol 12, 13-dibutyrate (PDBu) or were depolarized by the addition of KCl to a final concentration of 40 mM (isotonicity being maintained by a corresponding decrease in the concentration of NaCl). Incubations were terminated by the addition of 4 volumes of cold lysis buffer. Samples were then immunoprecipitated with the MacMARCKS antibody.

RESULTS

MacMARCKS Is Expressed and Phosphorylated in PC12 cells—A myristoylated protein with an apparent molecular mass of 48 kDa was immunoprecipitated from cell lysates of PC12 cells labeled with [3H]myristic acid using a rabbit anti-serum raised against murine MacMARCKS (Fig. 1A). The 48-kDa protein was heat stable and was the only protein recognized upon immunoblotting with antiserum to MacMARCKS (Fig. 1B). The protein was phosphorylated when 32P-labeled PC12 cells were stimulated with PMA, suggesting that it is a PKC substrate (Fig. 2A). Furthermore, the immunoprecipitated phosphoprotein had an isoelectric point of 4.2 (Fig. 1C), identical to that of MacMARCKS (3). The 48-kDa protein was also phosphorylated in PC12 extracts by purified PKC, and two-dimensional thermostable phosphopeptide mapping showed a single phosphopeptide that was identical to that generated from purified recombinant murine MacMARCKS (data not shown) or from purified rabbit macrophage MacMARCKS phosphorylated by PKC (3). The 48-kDa protein was not GAP-43/neuromodulin since it migrated differently to GAP-43/neuromodulin on a two-dimensional gel, and its phosphopeptide map differed from that of GAP-43/neuromodulin (Fig. 1C and data not shown). In addition, the antibody to MacMARCKS did not recognize GAP-43/neuromodulin, nor did an antibody to GAP-43/neuromodulin recognize MacMARCKS (data not shown). These data indicate that the 48-kDa PKC substrate in PC12 cells is MacMARCKS.

MacMARCKS Is Phosphorylated in PC12 Cells upon Phorbol Ester Stimulation and KCl-induced Depolarization—MacMARCKS displayed a basal level of phosphorylation in unstimulated PC12 cells (Fig. 2A). PMA treatment or KCl-induced depolarization increased MacMARCKS phosphorylation 4-fold (Fig. 2A). Depolarization-induced phosphorylation was completely dependent on Ca2+ in the medium (Fig. 2A, lane 3).

Since the related PKC substrate, MARCKS, is released from membranes upon phosphorylation (13, 16), we examined
whether phosphorylation similarly displaces MacMARCKS from membranes. In both unstimulated cells and in cells depolarized with elevated KCl, greater than 95% of [3H]myristoylated MacMARCKS was associated with the membrane fraction (Fig. 2B). Similarly, most of the phosphorylated MacMARCKS was associated with the membrane fraction of control or depolarized PC12 cells (Fig. 2C), although the stoichiometry of phosphorylation of the cytosolic protein appears higher than its membrane-bound counterpart (Fig. 2, B and C, and data not shown). PMA treatment resulted in the release of approximately 20% of [3H]myristoylated MacMARCKS from the membrane. The stoichiometry of PMA-induced phosphorylation of the cytosolic form was also greater than that of the membrane bound protein (Fig. 2, B and C, and data not shown).

Immunolocalization of MacMARCKS in PC12 cells—NGF-treated PC12 cells were stained with affinity-purified antibody to MacMARCKS. Indirect immunofluorescence microscopy revealed that MacMARCKS was localized throughout the cell in a punctate pattern, with prominent staining in neuronal processes and varicosities (Fig. 3, A and B). Confocal microscopy confirmed these results and further demonstrated the staining of MacMARCKS in neuronal processes (Fig. 3C). By double-labeled immunofluorescence microscopy, MacMARCKS co-localized with synaptophysin (Fig. 3, C and D), a marker of small synaptic vesicles and endosomes in PC12 cells (19, 24). Co-localization of MacMARCKS and synaptophysin was especially evident in distal neuronal processes (Fig. 3, C and D, arrows) and was also prominent in the perinuclear region. NGF-induced differentiation did not influence the level of expression of MacMARCKS protein, as determined by immunoblotting (data not shown).

PKC Agonists or Depolarization Induce the Phosphorylation of MacMARCKS in Rat Brain Synaptosomes—The presence of MacMARCKS in the tips of neuronal processes of PC12 cells and its co-localization with synaptophysin led us to examine whether it is found in isolated nerve termini. 32P-Labeled synaptosomes were treated with either the active phorbol ester PDBu or elevated KCl, and phosphorylated MacMARCKS was immunoprecipitated and resolved by SDS-PAGE (Fig. 4A). Stimulation of endogenous PKC with PDBu resulted in near maximal phosphorylation of MacMARCKS within 20 s, and these levels were sustained for at least 2 min (Fig. 4, A and B). The initial kinetics and extent of depolarization-dependent phosphorylation of MacMARCKS paralleled that of PDBu-induced phosphorylation, but was more transient (Fig. 4, A and B).

Subcellular Distribution of MacMARCKS—We examined the distribution of MacMARCKS in subcellular fractions from rat cerebral cortex using a procedure that results in the isolation of a variety of vesicular populations including a highly purified synaptic vesicle fraction (14). As expected, both synapsin 1 and synaptophysin were highly enriched in purified synaptic vesicle (CPG3) and were absent from soluble fractions (S₃ and L₁S₂) (Fig. 5). MacMARCKS was detected in most membrane fractions, including small vesicles (P₃ and L₃P) and purified synaptic vesicle fractions (CPG3). MacMARCKS was also pres-

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Fig. 2. MacMARCKS is phosphorylated upon depolarization of PC12 cells. A, PC12 cells radiolabeled with 32p, were either not treated (lane 1), exposed to PMA (200 nM, 30 min, lane 2), or subjected to depolarization (60 mM KCl for 1 min) either in the absence (lane 3) or presence (lane 4) of 1.5 mM extracellular Ca²⁺. PC12 cells were solubilized in lysis buffer and immunoprecipitated with anti-MacMARCKS antibody. Immunoprecipitates were resolved by 10% SDS-PAGE and visualized by autoradiography. B, PC12 cells were prelabeled with [3H]myristic acid, treated with control buffer (C), 60 mM KCl (K⁺), or 200 nM PMA and fractionated to isolate particulate (P) and cytosol (S) fractions as described under “Experimental Procedures.” The fractions were immunoprecipitated with anti-MacMARCKS antibody, and MacMARCKS was visualized by fluorography after 10% SDS-PAGE. C, PC12 cells were radiolabeled with [32P], and treated and fractionated as described in B. Immunoprecipitated MacMARCKS was visualized by autoradiography after 10% SDS-PAGE.

Fig. 3. MacMARCKS and synaptophysin co-localize in NGF-differentiated PC12 cells. A, differential interference contrast image; B, MacMARCKS immunoreactivity is present in the tips of neurite processes, and in the perinuclear region in a punctate manner. C and D, confocal immunofluorescence microscopy demonstrates the overlapping distribution of MacMARCKS (C) and synaptophysin (D) in PC12 cells. Arrows indicate neuronal processes where MacMARCKS and synaptophysin immunoreactivity are abundant.
Phosphorylation and Distribution of MacMARCKS in Neurons

Phosphorylation-induced phosphorylation of MacMARCKS is dependent upon external Ca\(^{2+}\). MacMARCKS is phosphorylated by PKC in vivo, since phosphopeptide maps generated from the protein phosphorylated in PC12 cells are identical to those generated from recombinant MacMARCKS phosphorylated in vitro by purified PKC. The phosphopeptides correspond to the underlined amino acids (KKKKFSI1FKPKLGLS12FKR), and are found within the effector domain of MacMARCKS, where both serines [1] and [2] are phosphorylated by PKC [3].

Considerable evidence suggests that PKC has a role in neurosecretion (25, 26). First, most conventional isozymes of PKC are expressed at high levels in neurons and are present in synaptic terminals (25, 27). Second, phorbol esters promote the release of neurotransmitters, although the release is not restricted to a specific transmitter (28–31). This effect is stereospecific; only phorbol esters that activate PKC induce neurotransmitter release (32). Third, PKC inhibitors prevent KCl-induced neurotransmitter release (25, 26), although these data should be interpreted with caution because of poor specificity of the inhibitors. Fourth, Ca\(^{2+}\) and diacylglycerol, which activate PKC, are elevated in active presynaptic terminals (33). Although there is much evidence that PKC is involved in neurotransmitter release, the mechanism by which PKC exerts this effect is unknown. Most recent investigations of the role of PKC in neurosecretion have concentrated on two PKC substrates found in nerve terminals, MARCKS (8, 34), and neuromodulin (also known as GAP-43 or B-50) (35, 36). The current study implicates a third PKC substrate, MacMARCKS.

MARCKS and MacMARCKS are related proteins that share a number of features. Both are elongated, rod-shaped molecules with a similar domain structure. They have a myristoylated N terminus, a highly conserved MH2 domain, and a basic effector domain that contains the PKC phosphorylation sites and which binds calmodulin in a Ca\(^{2+}\)-dependent manner (3). MARCKS binds and cross-links actin in a phosphorylation-regulated manner (37), and preliminary data suggest that the effector domain of MacMARCKS also binds actin. The function of MARCKS in the presynaptic terminal is not yet clear, but it is likely to be involved with the rearrangement of actin during neurosecretion and/or membrane retrieval. By analogy with the synapsins (38), it may have a role in reversibly tethering synaptic vesicles to actin filaments. Alternatively, MacMARCKS might regulate actin structure at the presynaptic terminal, thereby affecting access of synaptic vesicles to the plasma membrane. A possible role for MacMARCKS in membrane retrieval at the presynaptic junction is supported by its behavior in macrophages, where it is known to associate with phagosomes and endosomes.

PKC-dependent phosphorylation prevents calmodulin binding to MARCKS (3, 4), neuromodulin (GAP-43) (39, 40), and MARCKS (8, 41). It has been proposed that both neuromodulin and MARCKS act as regulated calmodulin stores (39, 2), and it is possible that MacMARCKS has a similar function. This is an attractive hypothesis since PKC-dependent phosphorylation has been shown to mobilize calmodulin, an event that activates calmodulin-dependent protein kinases implicated in neurosecretion (42). However, neuromodulin (GAP-43) would be more suitable as a Ca\(^{2+}\) sink than MARCKS or MacMARCKS, since it binds calmodulin in a Ca\(^{2+}\)-independent manner (43). Since MARCKS, MacMARCKS, and the calmodulin-dependent protein kinases would compete for calmodulin as the concentration of intracellular Ca\(^{2+}\) increases, calmodulin availability and hence Ca\(^{2+}\)/calmodulin-dependent protein kinase activity could be regulated subtly by the phosphorylation of MARCKS and MacMARCKS.

\(^3\) A. Aderem, unpublished observation.
Phosphorylation and Distribution of MacMARCKS in Neurons

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