Activation of Protein Kinase C Results in the Displacement of its Myristoylated, Alanine-rich Substrate from Punctate Structures in Macrophage Filopodia

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Summary

The myristoylated, alanine-rich C kinase substrate (MARCKS) is a prominent substrate for protein kinase C (PKC) in a variety of cells, and has been implicated in diverse cellular processes including neurosecretion, fibroblast mitogenesis, and macrophage activation. In macrophages that have spread on the substratum, MARCKS has a punctate distribution at the cell-substratum interface of pseudopodia and filopodia. At these points, MARCKS co-localizes with vinculin and talin. Activation of PKC with phorbol esters results in the rapid disappearance of punctate staining of MARCKS, but not vinculin or talin, and is accompanied by cell spreading and loss of filopodia. The morphological changes and disappearance of punctate staining follow a time-course that closely approximates both the PKC-dependent phosphorylation of MARCKS, and its phosphorylation-dependent release from the plasma membrane. Our results suggest a role for PKC-dependent phosphorylation of MARCKS in the regulation of the membrane cytoskeleton.

The protein kinases C (PKC)[1] are a family of diacylglycerol-activated, calcium-dependent protein kinases that play a central role in transducing signals that regulate diverse cellular events. Little is known about the functions of the major cellular substrates for PKC, and the role of their phosphorylation in mediating biological responses. One of the most prominent substrates for PKC is an acidic protein with an apparent molecular mass of 68–87K. Phosphorylation of 68–87K is synonymous with the activation of PKC during neurosecretion (1), fibroblast mitogenesis (2, 3), and macrophage activation (4). The protein was first shown to be myristoylated in macrophages (4) and this observation has been confirmed in a variety of cell types (5). This modification, together with its high proportion of alanine (6, 7), led to the suggestion that the protein be referred to by the acronym MARCKS (for myristoylated, alanine-rich C kinase substrate) (7). Despite extensive biochemical and biophysical characterization of MARCKS (6–9), the precise biological role of this protein remains unknown. We have attempted to gain insight into the function of MARCKS by immuno-localization studies. We report here that in unstimulated cells, MARCKS stains in a punctate distribution at the substrate-adherent surface of macrophage pseudopodia and filopodia, where it co-localizes with vinculin and talin. MARCKS is displaced from this location upon PKC-mediated phosphorylation. This event is accompanied by morphological alteration in the macrophage.

Materials and Methods

Immunofluorescence. Mouse brain MARCKS (68K) was purified to homogeneity, and a polyclonal rabbit antibody to mouse MARCKS was generated (10). This antiserum, affinity-purified (11) against a second preparation of purified murine brain protein, immunoblotted a single species in mouse macrophage lysates that had a molecular mass of 68K, and isoelectric point of 4.5–4.8. The immunoblotted protein co-migrated exactly in two dimensions with both the myristoylated macrophage protein as well as the mouse brain protein that had been phosphorylated in vitro with PKC. The antibody directed against MARCKS recognizes both the unphosphorylated and phosphorylated forms of the protein equally when tested by immunoprecipitation and immunoblotting. Immunofluorescence microscopy was performed on macrophages that had adhered to glass coverslips. Cells were fixed in 3.8% buffered formalin at 4°C for 10 min, after which cells were placed in acetone at −20°C for 5 min. Cells were washed well in PBS containing 0.2% sodium azide. 25 μl of primary antibody (10 μg/ml) was added to the coverslips for 60 min. Primary antibody was visualized with FITC–goat anti–rabbit F(ab')₂ (Tago, Burlingame, CA) at a 1:40 dilution. All antibodies, dilutions, and washes were in PBS containing 1% BSA. Coverslips were mounted in 90% glycerol/10% PBS and viewed in a Nikon Microphot-fx microscope equipped with epifluorescence. KODAK Tri-X 400 was used for photography. No fluorescence was detected if the primary anti-

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[1] *Abbreviations used in this paper:* MARCKS, myristoylated alanine-rich C kinase substrate; PKC, protein kinase C.
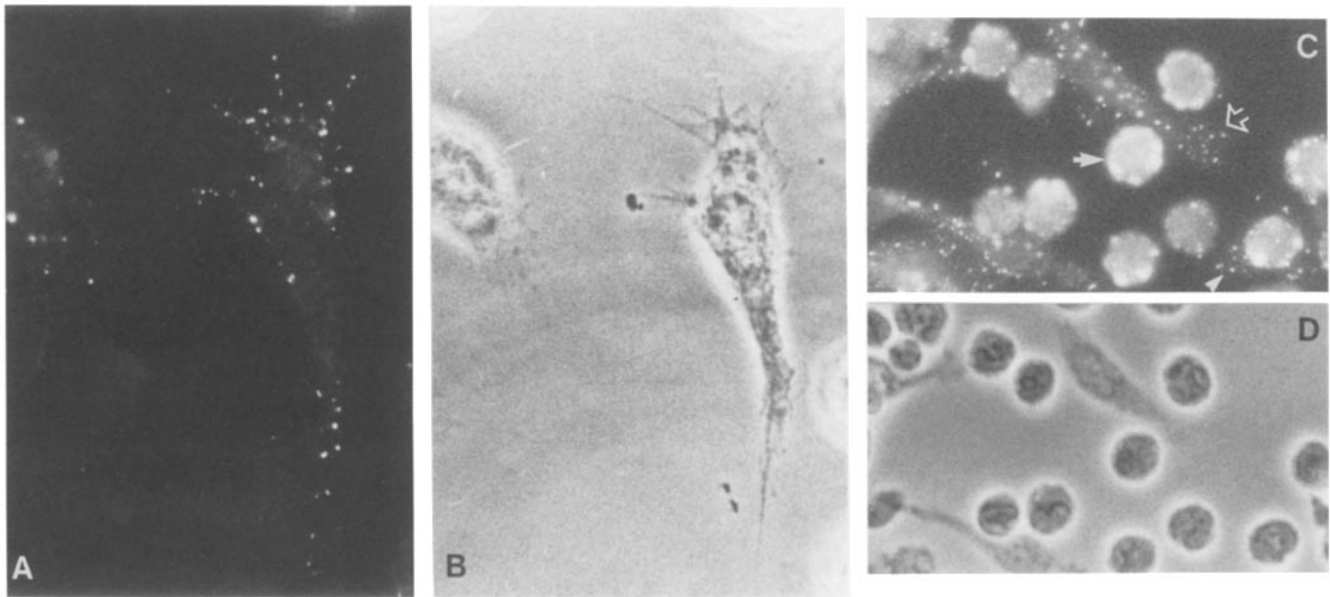
Figure 1. Indirect immunofluoresence microscopy of murine macrophages with affinity-purified rabbit antibody to murine MARCKS, visualized with FITC-labeled goat anti–rabbit F(ab')$_2$. (*A*) Macrophage elongated on the substratum, showing MARCKS staining with a punctate distribution predominantly in filopodia at both ends of this bipolar cell. (*B*) Corresponding phase photomicrograph. (*C*) Low power view of unstimulated macrophages. In rounded cells, MARCKS stained diffusely (*filled arrow*). In elongated cells (*open arrow*), punctate staining in membrane extensions was prominent. (The arrowhead indicates the elongating edge of a rounded cell.) (*D*) Corresponding phase photomicrograph.

body was omitted, and an affinity-purified antibody to hemocyanin (used at equivalent concentrations) showed no specific staining of macrophages (data not shown). All immunofluorescence experiments (Figs. 1–3) were repeated on at least five occasions. Representative photomicrographs are shown.

Double-label immunofluorescence was done by labeling first with anti-MARCKS, followed by FITC–goat anti–rabbit F(ab')$_2$. Coverslips were then incubated with rabbit serum (1:50; Tago) before adding anti-vinculin mAb (Sigma Chemical Co., St. Louis, MO). Mouse antibodies were visualized with biotinylated horse anti-mouse (1:80; DAKO), and Texas red streptavidin (1:80; Amersham Corp., Arlington Heights, IL). Anti-talin antibodies were a kind gift of Dr. K. Burridge, University of North Carolina, Chapel Hill, NC.

Phosphorylation Studies. Macrophages were labeled with ^{32}P$_i$ as described (10), and were then stimulated with 100 nM PMA for the times indicated. Phosphorylated MARCKS was immunoprecipitated (4), separated by electrophoresis on 8% SDS-PAGE gels, and visualized by autoradiography.

Metabolic Labeling and Subcellular Fractionation. Macrophages were labeled overnight with [^{3}H]lysine in a lysine-free medium. Unstimulated cells or macrophages stimulated with 100 nM PMA for 10 min were suspended in a fractionation buffer containing 110 mM KCl; 3 mM NaCl; 1 mM para-nitrophenylphosphate; 2 mM MgCl$_2$; 10 mM Pipes, pH 7.3; and the protease inhibitors described previously (10). The cells were then disrupted by nitrogen cavitation (850 psi for 10 min). The homogenate was brought to 10 mM pyrophosphate and 10 mM EDTA, and fractionated over a discontinuous sucrose gradient (12). Plasma membranes were harvested at the 10/41% sucrose interface and washed once. MARCKS was immunoprecipitated (4), and quantitated by fluorography after SDS-PAGE.

Results and Discussion

An affinity-purified antibody directed against MARCKS was used in immunofluorescence studies to explore the subcellular location of this protein. MARCKS had a punctate distribution predominantly in pseudopodia and filopodia (Fig. 1, *A* and *B*), with bright spots of staining found along the contour of the macrophage membrane. Through-focusing localized the majority of punctate staining to the substrate-associated cell surface. Resident peritoneal macrophages are morphologically heterogeneous, and the staining of MARCKS differed with the extent of elongation. Thus, MARCKS stained more diffusely in rounded cells (Fig. 1, *C* and *D*), while in elongated cells punctate staining in membrane extensions was prominent. When macrophages were dislodged from the coverslip with a stream of culture medium, MARCKS stained brightly with a punctate pattern in residual patches of membrane that remained strongly attached to the substratum (not shown). These results suggest that at least some of the punctate staining is due to attachment of MARCKS to areas of the macrophage membrane that are tightly associated with the substratum.

We therefore examined whether MARCKS co-localized with any components of focal adhesions. Many of the structures containing MARCKS also stained for vinculin (Fig. 2, *A* and *B*; *arrows*) and talin (not shown). While many punctate structures that contained vinculin and talin in filopodia and along the cell contour co-localized with MARCKS, there was also a more diffusely staining pool of vinculin (Fig. 2 *B*) and talin (not shown) that did not colocalize with

MARCKS. This punctate distribution of vinculin and talin is in contrast to the plaque-like arrangement typical of adherent fibroblasts (13), and has previously been observed in macrophages (14) and at the active cell edge of fibroblasts during the early stages of fibroblast attachment (15). The co-localization of MARCKS with vinculin and talin at these punctate sites therefore suggests that MARCKS might be located in the initial, more transient adhesion complex that is formed at this site in the locomoting macrophage.

It is of interest that PKC has recently been demonstrated in focal adhesions in fibroblasts (16), and that activators of PKC cause marked cytoskeletal rearrangement (16–18). In macrophages, stimulation with phorbol esters caused marked cell spreading and rounding, with smoothing of the macrophage contour and almost complete disappearance of filopodia (Fig. 2 F). This was accompanied by a complete disappearance of punctate staining of MARCKS (Fig. 2 D), and by a modest increase in the level of diffuse staining observed (Fig. 2 D). In contrast, the effect of PMA on the distribution of vinculin and talin was quite different. Vinculin (Fig. 2 E) and talin (not shown) still stained prominently with a patchy, punctate organization and were particularly obvious at the phase-dense cell edge. Both the morphological changes and the disappearance of punctate staining occurred with a time-course that closely approximated that of the phosphorylation of MARCKS by PKC in intact macrophages (Fig. 3, A and B, and reference 10). Thus, a decrease in punctate staining of MARCKS was first noticeable after 2 min of stimulation with PMA when the protein was phosphorylated to 40% of maximum (Fig. 3, A and B). A complete absence of punctate staining was seen by 5 min when MARCKS was phosphorylated to 85% of maximum (Fig. 3, A and B).

The disappearance of MARCKS from these punctate structures upon phosphorylation was due to its translocation from the plasma membrane into the cytosol. While 70% of total cellular MARCKS partitioned with the plasma membrane fraction in unstimulated macrophages, only 25% of the protein remained membrane-bound after the activation of PKC (Fig. 3 C). Concomitantly, MARCKS that was displaced from the membrane appeared in the cytosol (Fig. 3 C). These data in intact cells are supported by the observation that activators of PKC stimulate the release of phosphorylated MARCKS from rat synaptosomal membranes in vitro (19).

Activators of PKC cause marked cytoskeletal rearrangement (16–18), although the effectors of this PKC-mediated event remain unknown. We report here that MARCKS, a major substrate for PKC, is located in punctate structures at the substrate-adherent surface of the filopodia of macrophages. Immunoelectron microscopic studies to be reported elsewhere show that punctate structures are located at points where multiple actin filaments contact the substrate-adherent plasma membrane of the mechanically unroofed macrophages (Rosen, A., J. Hartwig, K. F. Keenan, A. C. Nairn, and A. Aderem. manuscript submitted for publication). Our results further show that PKC-dependent phosphorylation of MARCKS promotes its release from these punctate structures at the membrane, and its translocation into the cytosol. In this regard, it is also intriguing that the phosphorylation of MARCKS
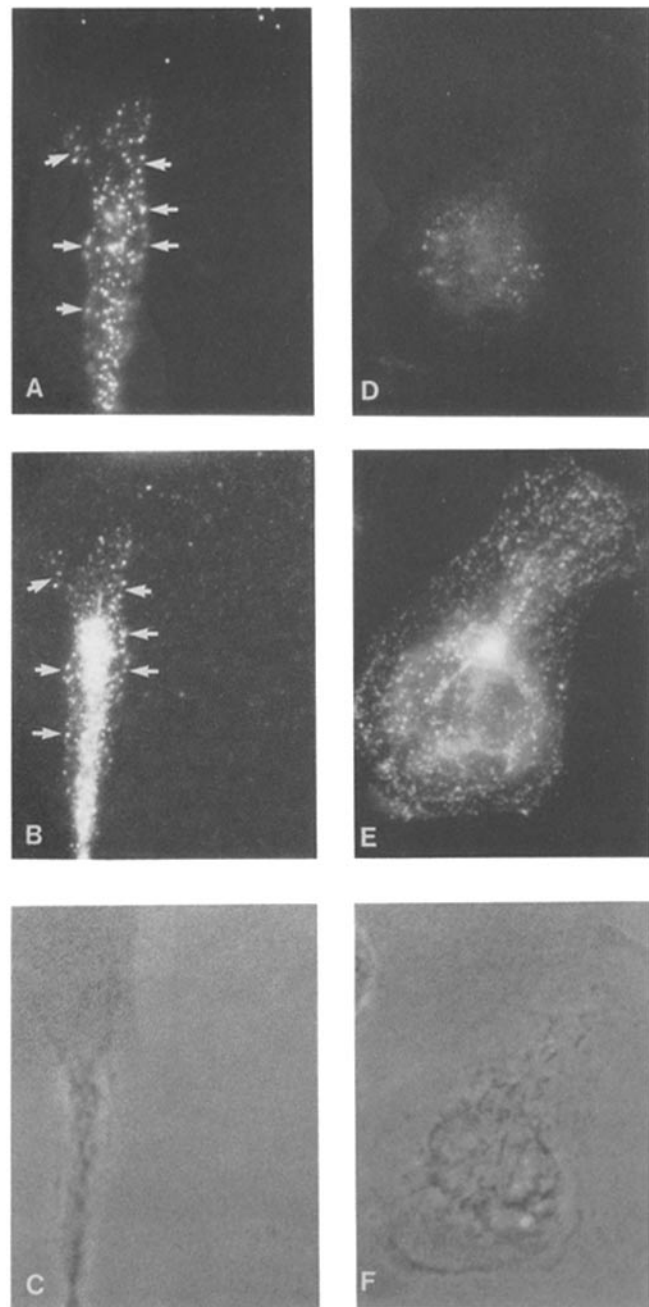
Figure 2. Macrophages double-labeled with antibodies against MARCKS (A, D) and vinculin (B, E). (A–C) Unstimulated macrophages. White arrows indicate some sites of co-staining of MARCKS and vinculin. Corresponding phase photomicrograph is shown in C. (D–F) Macrophages treated with 100 nM PMA for 10 min. Punctate staining of MARCKS disappears completely. Staining of vinculin remains prominent; some staining is seen at the phase dense cell edge. Phase photomicrograph shown in F. The faint staining seen around the nucleus in D is also observed in PMA-treated cells when using a control antibody directed against hemocyanin.

modulates its binding to calmodulin (9), which is associated with components of the membrane skeleton (20). The phosphorylation-dependent regulation of membrane-binding of MARCKS might therefore locally modify the connection between cytoskeleton and membrane, and thereby influence the direction of filopodial growth and ultimate morphogenesis.
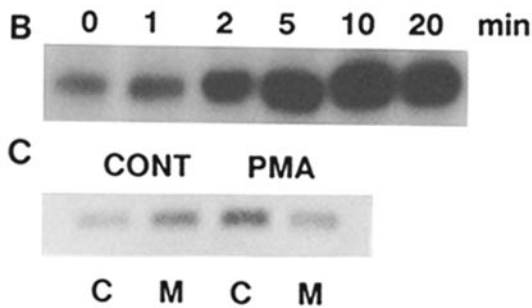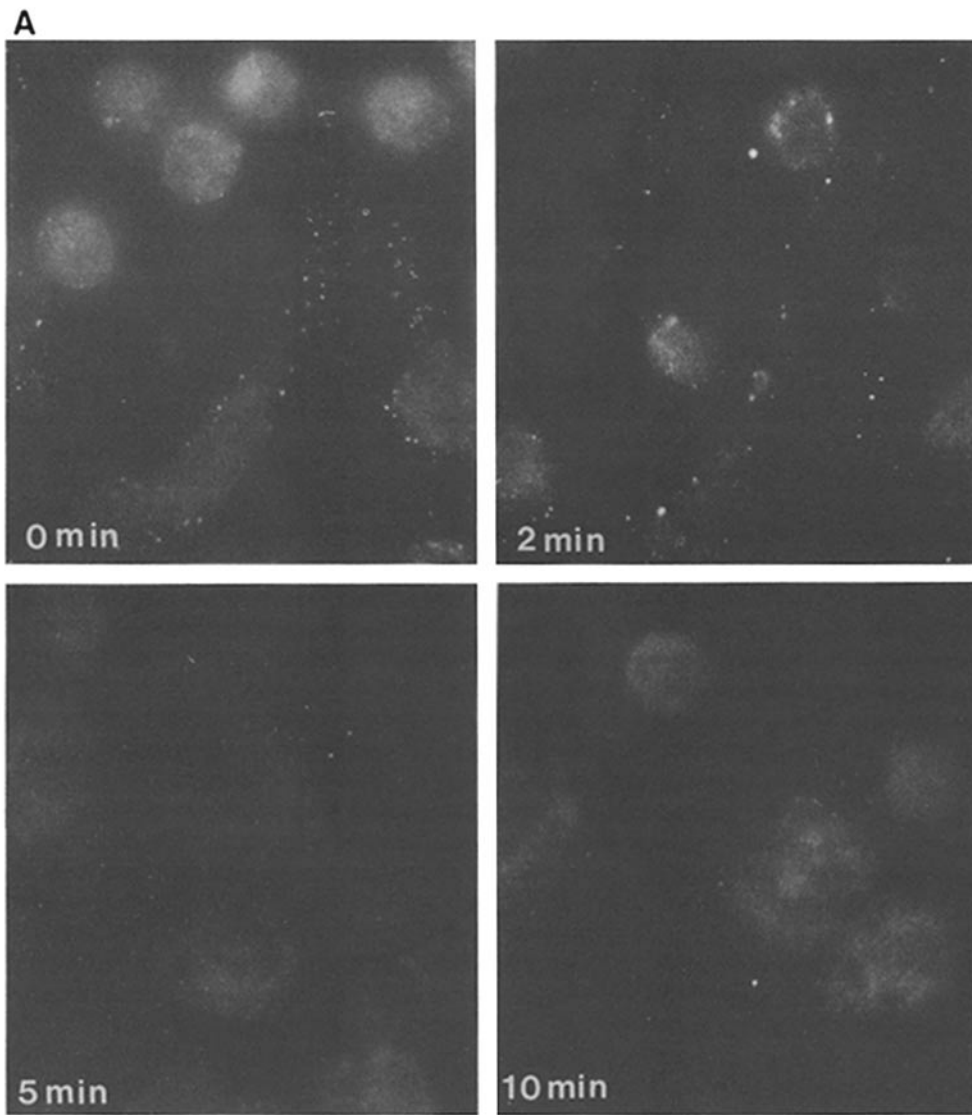
Figure 3. (A) Time-course of rearrangement of MARCKS upon stimulation with PMA. Glass adherent macrophages were stimulated with 100 nM PMA for 0, 2, 5, or 10 min, and MARCKS was visualized by immunofluorescence as described in the legend to Fig. 1. Immunofluorescence shows a rapid disappearance of punctate staining of MARCKS. (B) Time-course of phosphorylation of MARCKS in intact macrophages treated with PMA. (C) Subcellular distribution of biosynthetically labeled MARCKS in unstimulated macrophages (CONT) and macrophages stimulated for 10 min with 100 nM PMA (PMA). MARCKS is displaced from the plasma membrane (M) to the cytosol (C) upon activation of PKC with PMA.

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