# MARCKS is a major PKC-dependent regulator of calmodulin targeting in smooth muscle

**Cynthia Gallant[1], Jae Young You[2], Yasuharu Sasaki[3], Zenon Grabarek[1] and Kathleen G. Morgan[1,4,*]**

[1]Boston Biomedical Research Institute, 64 Grove Street, Watertown, MA 02472, USA
[2]Wellesley College, 106 Central Street, Wellesley, MA 02481, USA
[3]Department Pharmacology and Pharmaceutical Science, Kitasato University, Tokyo 108-8641, Japan
[4]Department of Medicine, Harvard Medical School, Boston, MA 02115, USA
*Author for correspondence (e-mail: morgan@bbri.org)

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## Summary

**Calmodulin (CaM) is a ubiquitous transducer of intracellular $Ca^{2+}$ signals and plays a key role in the regulation of the function of all cells. The interaction of CaM with a specific target is determined not only by the $Ca^{2+}$-dependent affinity of calmodulin but also by the proximity to that target in the cellular environment. Although a few reports of stimulus-dependent nuclear targeting of CaM have appeared, the mechanisms by which CaM is targeted to non-nuclear sites are less clear. Here, we investigate the hypothesis that MARCKS is a regulator of the spatial distribution of CaM within the cytoplasm of differentiated smooth-muscle cells. In overlay assays with portal-vein homogenates, CaM binds predominantly to the MARCKS-containing band. MARCKS is abundant in portal-vein smooth muscle (~16 μM) in comparison to total CaM (~40 μM). Confocal images indicate that calmodulin and MARCKS co-distribute in unstimulated freshly dissociated smooth-muscle cells and are co-targeted simultaneously to the cell interior upon depolarization. Protein-kinase-C (PKC) activation triggers a translocation of CaM that precedes that of MARCKS and causes multisite, sequential MARCKS phosphorylation. MARCKS immunoprecipitates with CaM in a stimulus-dependent manner. A synthetic MARCKS effector domain (ED) peptide labelled with a photoaffinity probe cross-links CaM in smooth-muscle tissue in a stimulus-dependent manner. Both cross-linking and immunoprecipitation increase with increased $Ca^{2+}$ concentration, but decrease with PKC activation. Introduction of a non-phosphorylatable MARCKS decoy peptide blocks the PKC-mediated targeting of CaM. These results indicate that MARCKS is a significant, PKC-releasable reservoir of CaM in differentiated smooth muscle and that it contributes to CaM signalling by modulating the intracellular distribution of CaM.**

Key words: Calmodulin, PKC, Smooth muscle, MARCKS

## Introduction

Calmodulin (CaM) is an essential $Ca^{2+}$-binding protein that transduces intracellular $Ca^{2+}$ signals into cellular effects by binding to target proteins (Chin and Means, 2000). Whether CaM itself is regulated by factors in addition to the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) has been addressed in relatively few studies, most investigating the regulation of CaM production (Gannon and McEwen, 1994; Solomon et al., 1994; Weinman et al., 1991) or the possible phosphorylation of CaM by Ser/Thr or Tyr kinases (Benaim and Villalobo, 2002; Corti et al., 1999; Quadroni et al., 1998; Sacks et al., 1995).

The interaction of CaM with its binding targets is determined not only by its affinity for them in the presence or absence of Ca but also by its proximity to those targets in the cellular environment. The subcellular distribution of CaM and the degree to which its distribution is regulated has been relatively little studied. The reports of stimulus-induced translocations of CaM to the nucleus in hippocampal CA3/CA1 pyramidal neurons (Deisseroth et al., 1998) and pancreatic acinar cells (Craske et al., 1999) raised the possibility that the distribution of CaM is dynamically regulated within individual cells and that changes in the intracellular targeting of CaM might have important regulatory functions. More recently, we have used a low-affinity ($K_d$=800 nM) CaM-binding probe constructed from the CaM-binding domain of caldesmon to monitor the level of available cellular CaM and found that activation of protein kinase C (PKC) increases the apparent free CaM level in differentiated smooth-muscle cells (Hulvershorn et al., 2001). This study (Hulvershorn et al., 2001) demonstrated that the availability of CaM to interact with low-affinity CaM-binding targets is dynamically regulated and raised the question of the source of the CaM made available by PKC activation in smooth-muscle cells.

Myristoylated alanine-rich C-kinase substrate (MARCKS) is an abundant CaM-, actin- and membrane-binding protein, and a major substrate of PKC in many cell types (Hartwig et al., 1992). MARCKS has been suggested to be involved in brain development, cell migration, adhesion, neurosecretion and actin-filament reorganization (Arbuzova et al., 2002); however, its function in smooth muscle is not clear. Phosphorylation of MARCKS by PKC decreases its affinity for both CaM and actin, as well as the association with membranes (Graff et al., 1989; Porumb et al., 1997). Blackshear (Blackshear, 1993) has suggested, based largely on in-vitro

biochemical studies, that MARCKS could serve as a major reservoir for CaM in cells. Conversely, because MARCKS is a substrate for PKC (Blackshear, 1993) and releases CaM when phosphorylated by PKC, MARCKS could also be a significant CaM donor in the cell.

Here, we report several new findings. (1) The spatial distribution of total CaM is dynamically regulated in differentiated smooth-muscle cells. (2) MARCKS and CaM co-distribute, co-immunoprecipitate and cross-link in a stimulus-dependent manner. (3) MARCKS is one of the most prominent CaM-binding proteins in portal vein homogenates. (4) A MARCKS decoy peptide inhibits PKC-mediated CaM translocation and MARCKS phosphorylation, consistent with the conclusion that MARCKS is a regulated CaM donor/reservoir and participates in the targeting of CaM in these cells.

## Materials and Methods

### Antibodies

The following polyclonal antibodies were used: N-19, against human MARCKS (Santa Cruz Biotechnology, 1:100); Ser 152/156, against phosphorylated MARCKS (pMARCKS) (Cell Signaling, 1:1000); an Oregon-Green-labelled anti-mouse antibody (Molecular Probes, 1:400); an Alexa-568-labelled rabbit anti-mouse antibody (Molecular Probes, 1:400); a fluorescein isothiocyanate (FITC)-labelled rabbit anti-goat antibody (Sigma, 1:700); a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Calbiochem, 1:10,000); an HRP-conjugated donkey anti-goat antibody (Santa Cruz Biotechnology, 1:3000); an infrared-dye-labelled goat anti-mouse antibody; and an IR-labelled goat anti-rabbit antibody (Rockland Immunochemicals, 1:500). Monoclonal antibodies against the following proteins were used: CaM (Upstate Biotechnology, 2 μg $ml^{-1}$); vinculin (Sigma, 1:2000); and MARCKS phosphorylated on Ser159 (pSer159 MARCKS) (Nagumo et al., 2001) (1:20).

### Materials

12-deoxyphorbol-13-isobutyrate-20-acetate (DPBA) was purchase from ICN Biomedical Inc. Fluorescent CaM was prepared from a Thr34Cys mutant of human CaM produced in *Escherichia coli* (Torok et al., 2001), labelling it with 5-iodoacetamidofluorescein (IAF). The protein was dissolved in 20 mM HEPES, pH 7.5, 0.1 M NaCl and incubated at room temperature overnight in the dark with three- to fivefold molar excess of IAF, followed by chromatography on Sephadex G25 column (PD-10 column, BioRad) The concentration of AF-CaM was determined spectrophotometrically using the molar extinction coefficient $\varepsilon_{492}$=75,000 $cm^{-1}$ $M^{-1}$. The MARCKS effector-domain peptides were synthesized and purified by the Boston Biomedical Research Institute Peptide Synthesis Facility.

### Immunoblotting

Ferret portal vein (FPV) strips were quick frozen in a dry-ice/methanol or dry-ice/acetone slurry containing 10 mM dithiothreitol (DTT) and homogenized as previously described (Je et al., 2004). The samples were matched to the total amount of protein, boiled, electrophoresed on an SDS-PAGE gel, transferred to a PVDF membrane and subjected to immunostaining using the appropriate antibody. Densitometry was performed with NIH Image software for enhanced chemiluminescence detection and with Odyssey software for LiCor™ detection (LiCor, Gilbertsville, PA).

### Tissue handling

Ferrets were anaesthetized with chloroform and the portal vein from each ferret was quickly removed to an oxygenated (95% $O_2$/5% $CO_2$) physiological saline solution (PSS; 120 mM NaCl, 5.9 mM KCl, 25 mM $NaHCO_3$, 11.5 mM dextrose, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM $NaH_2PO_4$, pH 7.4) for dissection as previously described (Bradley and Morgan, 1985). 51 mM KCl PSS was prepared by adding 45.1 mM KCl instead of an equivalent amount of NaCl. All procedures were approved by the Institutional Animal Care and Use Committee.

### Cell isolation

Cells were freshly enzymatically isolated from FPV tissue as previously described (DeFeo and Morgan, 1985b). 50 mg of 2×3 mm pieces of portal vein were digested in Buffer A [169.13 U $ml^{-1}$ CLS 2 collagenase (Worthington, 215 U $mg^{-1}$), 3.0 U $ml^{-1}$ elastase (Sigma grade II, 4.0 U $mg^{-1}$), 0.5 mg $ml^{-1}$ (5000 U $ml^{-1}$) soybean trypsin inhibitor (Type II-S, Sigma), 137 mM NaCl, 5.4 mM KCl, 5.6 mM dextrose, 4.2 mM $NaHCO_3$, 0.42 mM $Na_2HPO_4$, 0.44 mM $KH_2PO_4$, 0.075% bovine serum albumin]. Dissociated cells were poured over glass coverslips and plated for 40 minutes on ice. For all experiments, isolated cells were tested to confirm that they shortened in response to phenylephrine.

### CaM and peptide loading

FPV strips were chemically loaded by a modification of a previously described procedure (Je et al., 2004) with IAF-labelled CaM or the ED4A (FITC/β-alanine/KKKKKRFAFKKAFKLAGFAFKKNKK) or ED4A-X (FITC/β-alanine/RFKAFKNKGKLAKKFKKFAKAKKFK) peptides. The loading buffer contained 20 μM AF-CaM or 50 μM peptide. The viability of tissues was confirmed before and after loading by contraction in response to 51 mM KCl PSS.

### Photoaffinity cross-linking

The ED peptide was synthesized with an additional C-terminal Cys residue and labelled with a photoactivatable cross-linker, benzophenone-maleimide (BPM). The labelling was carried out by incubating the peptide dissolved in dimethylformamide with a 1.2 M excess of BPM at room temperature for 30 minutes in the dark. The completeness of labelling was confirmed by matrix-assisted laser desorption-ionization / time-of-flight mass spectrometry. The reaction was terminated with an excess of DTT and the excess reagents removed by chromatography on a Sephadex G-15 column equilibrated with PSS. The purified ED-C/BPM was introduced into the smooth-muscle tissue by incubation at 37°C for 3 hours. Cross-linking in the presence or absence of agonists was initiated by exposure to ultraviolet (UV) light for 30 minutes.

### Digital confocal microscopy

Before the addition of excitatory stimuli and before fixing control cells, 300 mM sucrose was added to the buffer to prevent excessive shortening. Others have shown that this manoeuvre decreases the myofilament spacing and directly inhibits contractility at the cross-bridge level (Godt et al., 1984). We have previously shown that it causes no change in resting or activated $Ca^{2+}$ levels, PKC signalling, ERK activation time course or resting cell length (Dessy et al., 1998; Gangopadhyay et al., 2004; Khalil et al., 1994; Khalil et al., 1995; Liou and Morgan, 1994; Parker et al., 1998; Parker et al., 1994). Cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 10% goat serum and co-immunostained overnight at 4°C in 2% goat serum with anti-MARCKS and anti-CaM antibodies. After secondary antibody staining, coverslips were mounted with FluorSave (Calbiochem).

Images were acquired using a Kr/Ar laser (Bio-Rad Radiance 2000) scanning confocal microscope equipped with a Nikon X-60 (NA 1.4) oil-immersion objective. All images were digitally scanned at 166 lines

per second, Kalman average five times with minimal pinhole size and quantified with Bio-Rad Laser Sharp 2000 for Windows NT. Computer-generated line scans were measured to obtain values for peak fluorescence at the cell surface and peak fluorescence in the cell core. For each cell, the single central nucleus was first located at the widest and thickest part of the cell. In previously published studies, nuclear staining confirmed that visual inspection gives accurate identification of the nuclear location (Khalil et al., 1992). The stage was moved so that the nucleus was positioned to one side of the field of view. The length of the non-nuclear portion of that half of the cell was measured. Based on this measurement, three equally spaced line scans were placed perpendicular to the long axis of the non-nuclear section of the cell. Surface-to-core fluorescence ratios were calculated on centre optical sections, with the surface fluorescence being the peak fluorescence in the outer 0.5 μm of the cell and the core fluorescence being the peak fluorescence in the centre 1 μm of the cell width. The two cell-surface values were averaged for the surface value of each ratio calculation. The ratios from the three line scans for each cell were averaged. Centre sections were determined as that section halfway between the lowest and highest sections that could be sharply focused. The highest in-focus section is referred to as a 'grazing section'.

In colabelling experiments, care was taken to ensure that no cross-talk occurred between the two fluorophores. The laser intensities and photomultiplier power settings for both the green and red labels in colabelled cells were chosen such that single-labelled green cells gave no detectable signal on the red channel and single-labelled red cells gave no signal on the green channel at the settings used.

### Immunoprecipitation

70 mg of tissue were lysed in a modified homogenization buffer described previously (Menice et al., 1997) (2 mM β-glycerophosphate, 1 mM DTT, 50 mM Tris.HCl, pH 7.4, 150 mM NaCl, 1% NP40, 1.0% sodium deoxycholate, phosphatase- and protease-inhibitor cocktail). Samples were precleared with protein-A beads and then incubated with 2 μg anti-CaM antibody overnight at 4°C followed by the addition of 30 μl of a 50% stock of protein-A/agarose beads and incubation for 3 hours at 4°C. The immunoprecipitates were washed four times with homogenization buffer, boiled and loaded onto SDS-PAGE gels.

### Statistics

Unless otherwise noted, comparisons between two groups of numbers were performed by a two-tailed Student's *t* test. $P \leqslant 0.05$ was taken to be significant.

## Results

### Fluorescently labelled CaM distributes in a heterogeneous pattern in differentiated smooth-muscle cells

To determine the distribution of CaM in living smooth-muscle cells, we introduced AF-CaM into portal-vein smooth-muscle by a previously published chemical loading method (Je et al., 2004). After loading, single cells were freshly enzymatically isolated and studied by confocal microscopy. Most of the loaded CaM appears in plaque-like clusters at the cell surface and in the vicinity of the perinuclear membrane (Fig. 1A).

To confirm that endogenous CaM also distributes in a

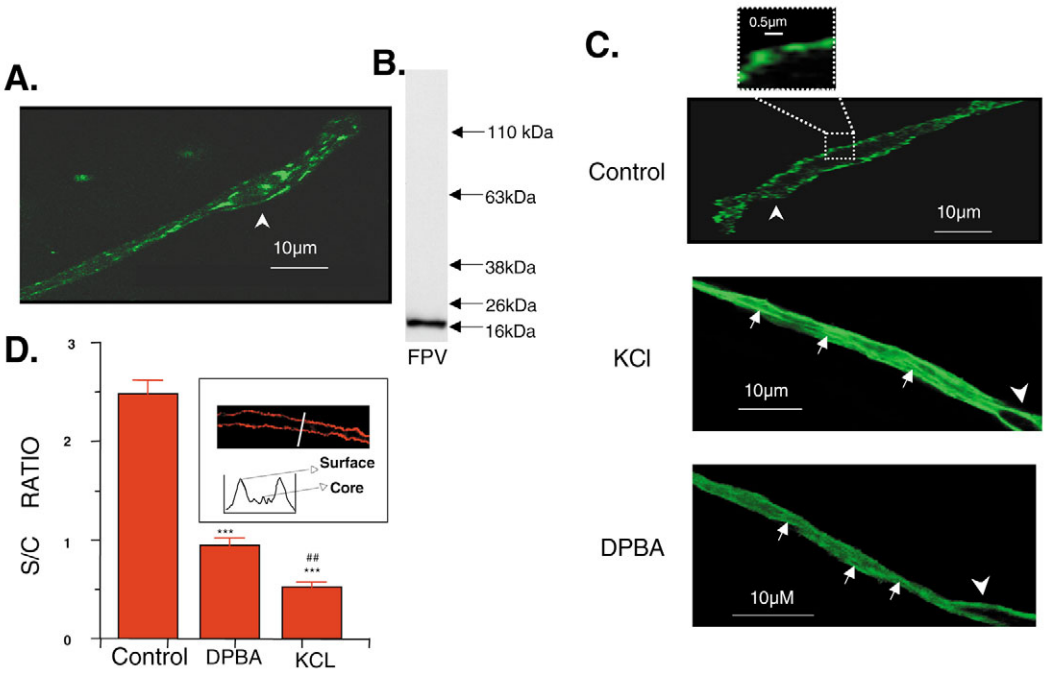


**Fig. 1.** CaM distribution is heterogeneous and agonist-dependent. (A) A single, live, freshly dissociated smooth-muscle cell loaded with AF-CaM. The arrowhead denotes the position of the nucleus. (B) CaM immunoblot of portal-vein homogenate demonstrating specificity of the antibody used for imaging. (C) Endogenous CaM distribution monitored by indirect immunofluorescence. (top) Control, unstimulated cell. (Inset) Digitally expanded image to illustrate interplaque distance. (middle) 10 minutes, 51 mM KCl PSS. (bottom) 20 minutes, 10 μM DPBA. Arrows point to the location of filamentous structures. Arrowheads denote the position of the nucleus. (D) Quantitation of endogenous CaM distribution. Bars represent the average ratios of surface to core fluorescence ratios (s/c) of fluorescence from 9-21 cells under control conditions or 10 minutes after 10 μM DPBA or 51 mM KCl PSS. (inset) A diagrammatic example of the output from a single line scan in the region of a cell marked with the white line. ***, $P<0.001$ compared with control; ##, $P<0.01$ compared with DPBA treatment.

heterogeneous pattern, indirect immunofluorescence was performed on freshly dissociated cells. The antibody used to detect CaM recognized only a single band at the molecular weight expected for CaM in a portal-vein homogenate (Fig. 1B). Most of the endogenous CaM is also present in clusters on the surface of unstimulated cells (Fig. 1C, top and inset); however, the perinuclear distribution seen with the loading of exogenous CaM was not present. Presumably, the perinuclear distribution seen with exogenous CaM is attributable to some lower-affinity sites that are occupied only at high CaM concentrations.

### Distribution of endogenous CaM is dynamically regulated in differentiated smooth-muscle cells

Freshly dissociated portal-vein smooth-muscle cells were exposed to two types of stimulus: PSS containing 51 mM KCl, which depolarizes the cells and increases $Ca^{2+}$ entry through voltage-dependent $Ca^{2+}$ channels; or the phorbol ester DPBA, which activates PKC directly in smooth-muscle cells but does not increase $[Ca^{2+}]_i$ (Hulvershorn et al., 2001; Jiang and Morgan, 1987). Both stimuli cause a significant translocation of CaM from the cell surface to the cell interior (Fig. 1C) in a heterogeneous pattern that is similar to the pattern that we have previously shown for the actomyosin-containing contractile-filament bundles in this cell type (Parker et al., 1998) (Fig. 1C, arrows). It has previously been reported that CaM undergoes a stimulus-induced translocation to the interior of the nucleus in neural cells in culture (Deisseroth et al., 1998); however, nuclear localization of CaM was not detected in these differentiated smooth-muscle cells (Fig. 1C).

Line-scan analysis was used to quantify the changes in CaM distribution. Both stimuli cause a significant decrease in the cellular-surface:core fluorescence ratio, but a depolarizing stimulus decreases the ratio significantly more than does a phorbol ester (Fig. 1D). The difference between the ratios could be due to more CaM being released from the cell surface, to more of the target being available or to CaM associating with a different target in the presence of the depolarizing stimulus.

### MARCKS is abundant in portal-vein smooth muscle

The finding that CaM concentrates in distinct plasmalemmal plaques in a PKC- and $Ca^{2+}$-dependent manner raised the question of the nature of the CaM-binding protein(s) to which CaM is bound in differentiated smooth muscle. It has been suggested previously that MARCKS might function as a major high-affinity CaM reservoir in cells and, because the binding of CaM to MARCKS is both $Ca^{2+}$ and PKC dependent (Blackshear, 1993), we determined the relative abundance of MARCKS in this cell type.

An FPV homogenate (Fig. 2A) displays a robust signal at the expected molecular weight with an anti-MARCKS antibody. A semiquantitative measure of the MARCKS content in this tissue was obtained by comparing homogenates of calf forebrain [a tissue reported to contain 12 μM MARCKS, based on protein-purification procedures (Blackshear, 1993)] with protein-matched FPV homogenates. By using dilutions of calf forebrain as a standard (Fig. 2B), portal vein is estimated to contain 16 μM MARCKS. We took a similar approach to estimate the CaM content in this tissue. Again, using calf brain [containing 60 μM CaM (Blackshear, 1993)] as a standard (Fig. 2D), we estimated the CaM concentration in portal vein to be 41 μM, which is within the range of previously reported values for other smooth muscles (Meisheri et al., 1985; Ruegg et al., 1983; Zimmermann et al., 1995). We should emphasize, however, that we cannot rule out the possibility that the antibodies used might have somewhat different affinities for cow and ferret proteins, which might have introduced a certain proportion of error into these absolute values.

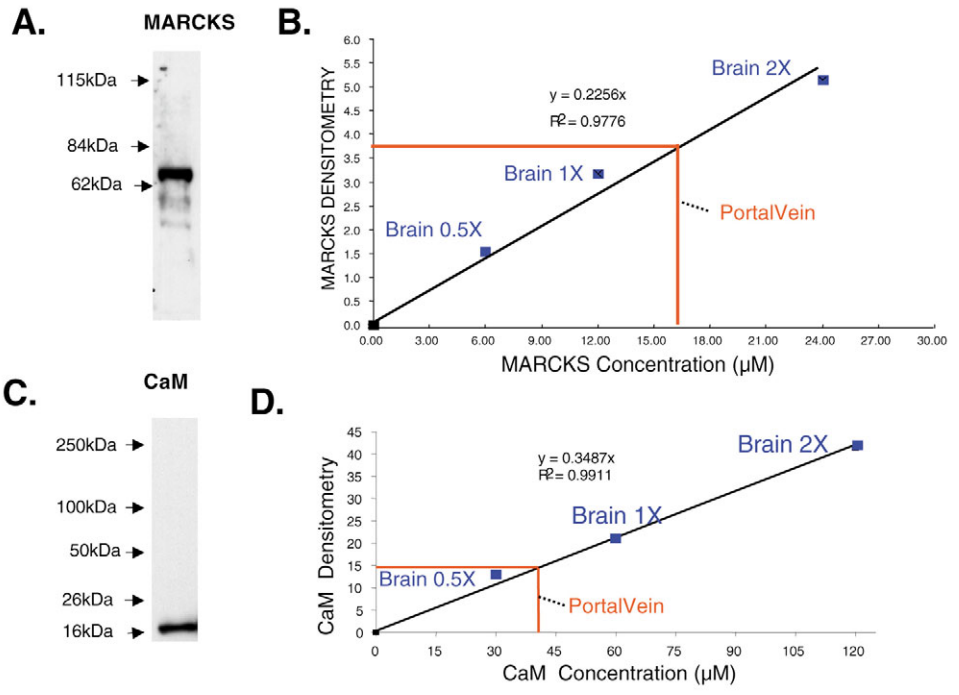


**Fig. 2.** Abundance of MARCKS and CaM in smooth muscle. (A) MARCKS immunoblot of whole-cell homogenate of portal vein. (B) Quantitation of portal-vein MARCKS immunoblot, protein matched for total cellular protein compared with a standard curve generated for calf-forebrain homogenates. (C) CaM immunoblot of whole-cell homogenate of portal vein. (D) Quantitation of portal-vein CaM immunoblots protein matched for total cellular protein compared with a standard curve generated by calf-forebrain homogenates.

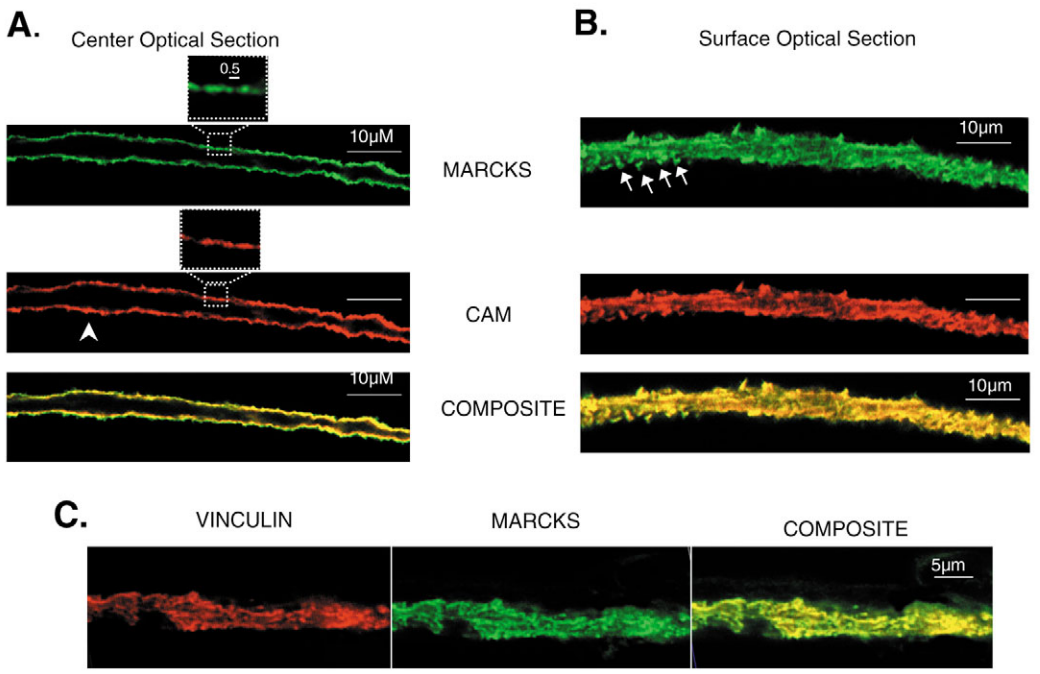


**Fig. 3.** MARCKS colocalizes with CaM and vinculin in adhesion plaques of unstimulated cells. (A) Centre confocal section of one freshly dissociated portal vein cell labelled for both MARCKS and CaM. Arrowheads denote the position of the nucleus. Insets show digitally expanded views of MARCKS and CaM staining. (B) A grazing surface optical section of one freshly dissociated portal-vein cell labelled for both MARCKS and CaM. White arrows indicate the specific repeating membrane structures. (C) A surface confocal section of one cell labelled for both MARCKS and vinculin. Composite images show overlap in yellow.

## MARCKS localizes with CaM and vinculin in adhesion plaques

We used quantitative confocal microscopy to determine whether MARCKS colocalizes with CaM in freshly dissociated cells. Indeed, in centre confocal sections of unstimulated cells (Fig. 3A), both CaM and MARCKS are concentrated at the cell cortex in a highly overlapping pattern. In digitally expanded images, a clustered distribution reminiscent of that observed for CaM (Fig. 1) is detectable (Fig. 3A, inset). The spacing of the clusters was quantified by measuring the distances between peak centre fluorescences (determined by intensity profiles drawn through the centres of two clusters). The value obtained for immunolocalized MARCKS clusters was 0.60±0.04 μm. The comparable value obtained for immunolocalized CaM surface clusters was 0.52±0.03 μm ($n$=21), which is not significantly different from that for MARCKS.

In 'grazing' sections taken at the cell surface (Fig. 3B), both proteins are concentrated in a specific repeating membrane structure (Fig. 3B, white arrows) reminiscent of the staining previously described in smooth-muscle cells for vinculin, a marker of adhesion plaques (Moore et al., 1993). In co-stained cells, a high degree of colocalization of MARCKS with vinculin is seen (Fig. 3C).

## Overlay assays indicate that MARCKS is a major CaM-binding protein

To test further the hypothesis that MARCKS is a major CaM-binding protein in smooth muscle, overlay assays were run on portal-vein homogenates as previously described (Leinweber et al., 2000). Homogenates were run in a polyacrylamide gel, transferred to a PVDF membrane and incubated with an overlay buffer containing 20 μg $ml^{-1}$ CaM. The location of MARCKS was confirmed by immunostaining a parallel lane (Fig. 4A, right). Immunostaining of the overlay (Fig. 4A, centre), with an anti-CaM antibody determined the proteins in the homogenate to which CaM binds under these conditions. Binding of CaM to a band at the molecular weight of MARCKS consistently occurred. Notice that, even though actin is the most abundant protein in this molecular-weight range (Fig. 4A, left, Naphthol Blue Black stain), no detectable CaM binds to the actin band (Fig. 4A, centre), pointing to the specificity of the assay. Furthermore, CaM binding to the MARCKS band occurs as expected in the presence of $Ca^{2+}$ (Fig. 4A, centre, lane 2) but less so in the absence of $Ca^{2+}$ (Fig. 4A, centre, lane 1).

The $Ca^{2+}$ dependence of CaM binding to MARCKS in vitro raises the question of the apparent colocalization of MARCKS with CaM in the unstimulated cells. To determine whether the resting $[Ca^{2+}]_i$ is sufficient and necessary to allow binding to MARCKS, we lowered the $[Ca^{2+}]_i$ by removing $Ca^{2+}$ from the extracellular buffer (DeFeo and Morgan, 1985a), which results in decreased binding of CaM to the structures to which it is bound at resting $Ca^{2+}$ levels and an increased cytoplasmic staining (Fig. 4B, bottom). In the $Ca^{2+}$-free buffer, the surface:core ratio for CaM was 1.20±0.06 ($n$=15), significantly less than that in a $Ca^{2+}$-containing buffer (2.22±0.10, $n$=15). By contrast, the surface:core ratio for MARCKS in the $Ca^{2+}$-free buffer was 3.51±0.44 ($n$=15), not significantly different from that in the $Ca^{2+}$-containing buffer of 3.87±0.26 ($n$=19).

## MARCKS redistributes to structures in the core of the cell after depolarization

CaM leaves the cell cortex during depolarization (Fig. 1C) and, at high $[Ca^{2+}]_i$, MARCKS is known to have a decreased affinity for actin and membranes (Hartwig et al., 1992; Porumb et al., 1997). Thus, we asked whether MARCKS translocates with CaM. As is shown in Fig. 5A, both proteins translocate to the cell interior and, in colabelled cells, the degree of colocalization is high. Both proteins localize to filamentous

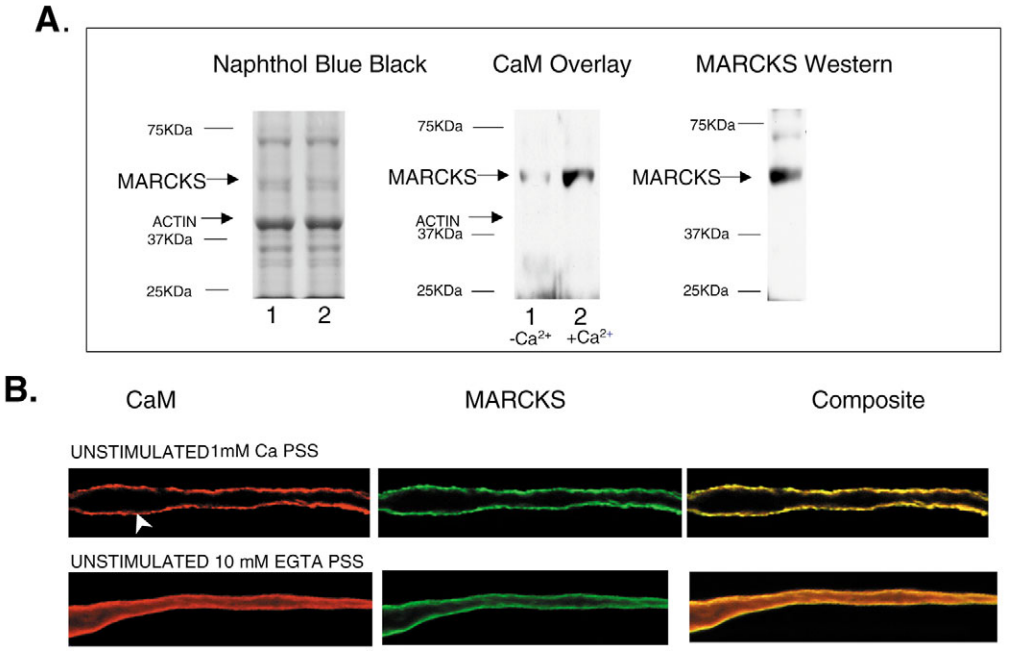


**Fig. 4.** CaM interacts with MARCKS in vitro and in vivo in a $Ca^{2+}$-dependent manner. (A, left) Total protein on PVDF membrane stained with Naphthol Blue Black before exposure to overlay buffer. (A, right) A parallel lane immunostained to locate MARCKS. (A, middle) CaM overlay. The lanes were treated as follows: 1, no $Ca^{2+}$ was added to the overlay buffer and 10 mM EGTA was included; 2, overlay buffer contained 2.5 mM $CaCl_2$. (B) Two freshly dissociated cells labelled for both CaM and MARCKS. (top) Cell fixed in PSS containing 1 mM $Ca^{2+}$. (bottom) Cell fixed in $Ca^{2+}$-free PSS containing 10 mM EGTA. Arrowheads denote the position of the nucleus.

structures in a similar pattern to that found for the actomyosin-containing contractile-filament bundles in this cell type (Parker et al., 1998). We quantified the time courses of the translocations by measuring the decrease in the surface:core ratio for MARCKS and CaM in the presence of 51 mM KCl PSS. The time courses of translocation of the two proteins are indistinguishable (Fig. 5B,C). These results suggest that CaM might be targeted to the interior of the cell while in a complex with MARCKS.

## MARCKS redistributes to the core of the cell in response to PKC activation

PKC-mediated phosphorylation of MARCKS is known to decrease its affinity for both CaM and actin (Aderem, 1992). Therefore, we determined the time course of translocation of MARCKS and CaM in response to DPBA (Fig. 6A) and quantified the results (Fig. 6B,C). Translocation of CaM and, to a lesser extent, MARCKS to the cell interior is detectable at 1 minute after the addition of DPBA. However, whereas the

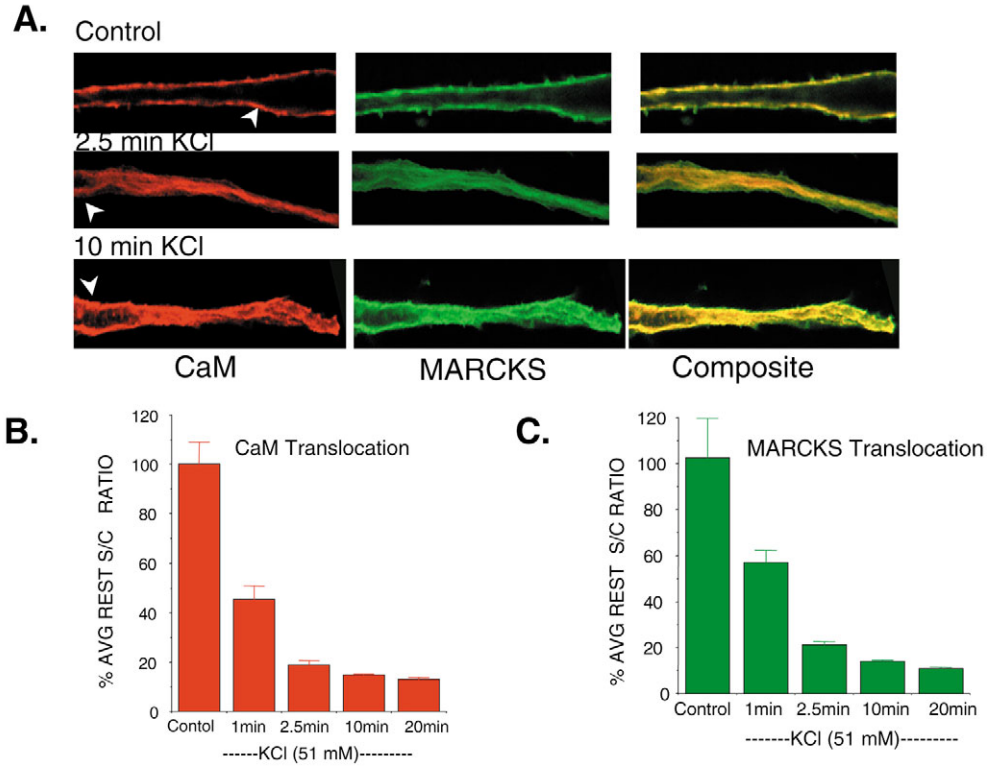


**Fig. 5.** CaM and MARCKS co-translocate in response to depolarization. (A) Centre sections of an unstimulated cell (top), a cell in 51 mM KCl PSS for 2.5 minutes (middle) and a cell in 51 mM KCl PSS for 10 minutes (bottom), all labelled for both CaM and MARCKS. Arrowheads denote the positions of the nuclei. (B) Line-scan analysis of CaM translocation in response to KCl PSS. The mean surface:core ratio as a percentage of the average resting ratio for 9-22 cells is shown versus time. (C) Line-scan analysis of MARCKS translocation in response to KCl PSS.

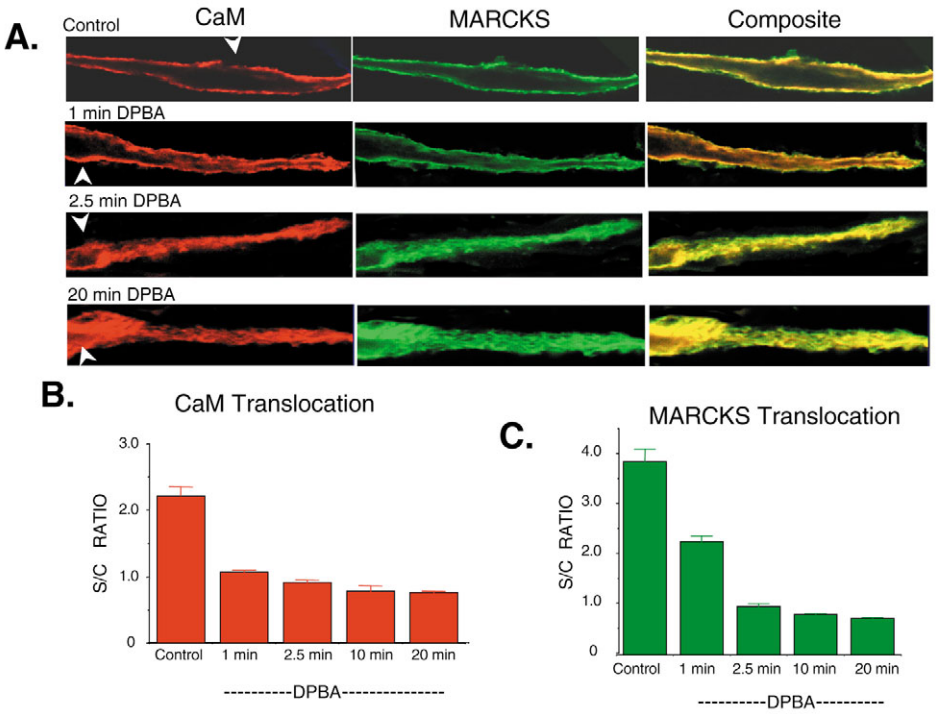


**Fig. 6.** CaM and MARCKS translocate with different time courses in response to the phorbol ester DPBA. (A) Centre confocal sections of unstimulated or DPBA-stimulated cells labelled for both CaM and MARCKS. Arrowheads denote the positions of the nuclei. (B) Line-scan analysis of CaM translocation in response to 10 μM DPBA. The mean ratio of surface to core fluorescence of 12 to 24 cells is shown against time. (C) Line-scan analysis of MARCKS translocation in response to 10 μM DPBA. The mean surface to core fluorescence ratios for 19-26 cells are shown against time.

translocation of CaM was almost complete by 1 minute (Fig. 6B), MARCKS is only about 50% translocated at 1 minute (Fig. 6C). Similarly, a merged 1 minute image (Fig. 6A) displays a diffuse red signal from the CaM probe in the core of the cell, suggesting that CaM distribution is dissociated from that of MARCKS.

## MARCKS and MARCKS ED domain associate directly with CaM in a stimulus-dependent manner

To test further the concept that MARCKS binds CaM in cells in a stimulus-dependent manner, we used an anti-CaM antibody to immunoprecipitate cellular CaM from unstimulated, depolarized and phorbol-ester-stimulated portal-vein cells. In four experiments, using equal amounts of starting material and treating the samples in an identical manner, KCl-depolarized samples yielded significantly more immunoprecipitated MARCKS than unstimulated samples (Fig. 7A). Phorbol-ester-treated samples yielded significantly less immunoprecipitated MARCKS than either the unstimulated or the depolarized sample.

The above results indicate that MARCKS and CaM are present together in a protein complex in unstimulated tissues and that this association increases with depolarization but decreases with phorbol-ester stimulation. However, they do not demonstrate a direct association between MARCKS and CaM. To test whether there is a regulated, direct interaction between MARCKS and CaM in intact cells, we synthesized a photoaffinity probe, BPM-ED, using the ED sequence from the MARCKS molecule, which is known to be responsible for CaM and actin binding, and to contain the PKC phosphorylation sites. To this peptide, we attached the photoaffinity cross-linking probe BPM. When the peptide and CaM were mixed in vitro and exposed to UV light in the presence of $Ca^{2+}$, cross-linked upper bands appeared on Coomassie-stained SDS-PAGE gels (Fig. 7B). As a control, these cross-linked bands were greatly diminished in the absence of $Ca^{2+}$ (Fig. 7B).

We then chemically loaded the photoaffinity cross-linking probe into portal-vein tissue and illuminated the tissue with UV light under resting conditions or upon stimulation with KCl or DPBA (Fig. 7C). Tissue that was illuminated but not loaded with the probe showed only a single band in CaM immunoblots of homogenized tissue (Fig. 7C, lane 1). Tissues containing the probe displayed not only a band at the molecular weight of CaM but also an upper band indicating CaM cross-linked to the effector domain of MARCKS. The intensity of the upper band is greatest in the depolarized tissues (Fig. 7C, lane 3), less in the unstimulated tissues (Fig. 7C, lane 2) and least in the phorbol-ester-treated tissues (Fig. 7C, lane 4), as confirmed by the average densitometry of immunoblots from three separate experiments (Fig. 7D). These results indicate that the direct association between CaM and the ED domain of MARCKS is regulated in intact cells by depolarization-mediated increases in cytoplasmic $Ca^{2+}$ and by phorbol-ester-mediated PKC activation.

## MARCKS is phosphorylated at PKC sites with a site-specific time course

It is known that MARCKS can be phosphorylated at multiple sites in the ED (Fig. 8A). We used an antibody specific for pSer159 MARCKS to probe, by immunoblot, homogenates of tissues quick frozen after increasing times of exposure to DPBA (Fig. 8B,C). Interestingly, the phosphorylation event occurred quite slowly, with very little signal detectable at 1 minute (when CaM translocation is almost complete) or even at 2.5 minute (when MARCKS translocation is almost complete). This suggests either that the phosphorylation by PKC is not causally related to the CaM translocation or even

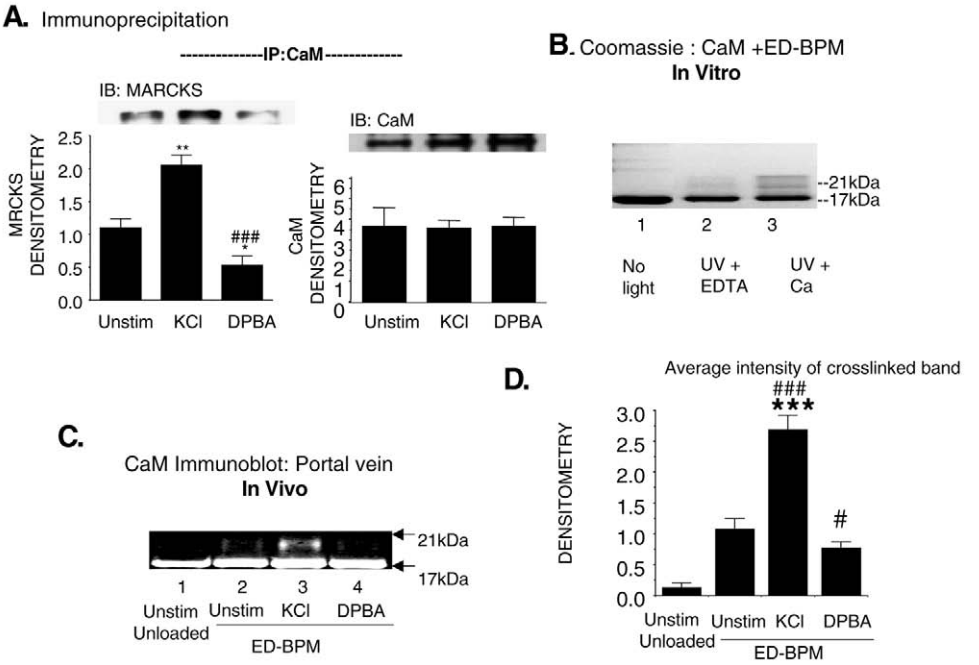


**Fig. 7.** Stimulus-specific immunoprecipitation and photoaffinity cross-linking of MARCKS and MARCKS ED peptide with CaM. (A) Immunoprecipitation of portal-vein homogenates with an anti-CaM antibody followed by immunoblot with an anti MARCKS antibody (left) or anti-CaM antibody (right) after exposure of muscles to: no stimulus; 2.5 minutes KCl; or 2.5 minutes DPBA. A typical western blot is shown above the graph of mean±s.e. densitometry for repeated experiments. *, $P<0.05$; **, $P<0.01$ compared with unstimulated cells; ###, $P<0.001$ compared with KCl treatment. $n=4$. (B) Coomassie-stained SDS-PAGE gel of CaM mixed with ED-BPM peptide and exposed to no light (lane 1), UV light in the absence of Ca (lane 2) and UV light in the presence of $Ca^{2+}$ (lane 3). (C) CaM immunoblot of portal-vein homogenates after muscles were chemically loaded with ED-BPM peptide and exposed to UV light in the presence of no stimulus (lane 2), 51 mM KCl PSS (lane 3) or DPBA (lane 4). Also, as a control, a mock preparation not loaded with peptide but exposed to UV light is shown (lane 1). (D) Average densitometry of the upper band in three experiments performed as described in C. #, $P<0.05$; ###, $P<0.001$ compared with unstimulated cells; ***, $P<0.001$ compared with DPBA treatment.

to the MARCKS translocation, or (more probably) that one or more of the other three PKC phosphorylation sites in the effector domain (Fig. 8A) is involved in the CaM and/or MARCKS translocations. We are unaware of any other available site-specific antibodies for phosphorylated MARCKS. Therefore, to test the idea that the PKC phosphorylation sites in the MARCKS ED domain might be phosphorylated with different time courses, we also probed these samples with a commercially available antibody that recognizes phosphorylation at both Ser159 and Ser163.

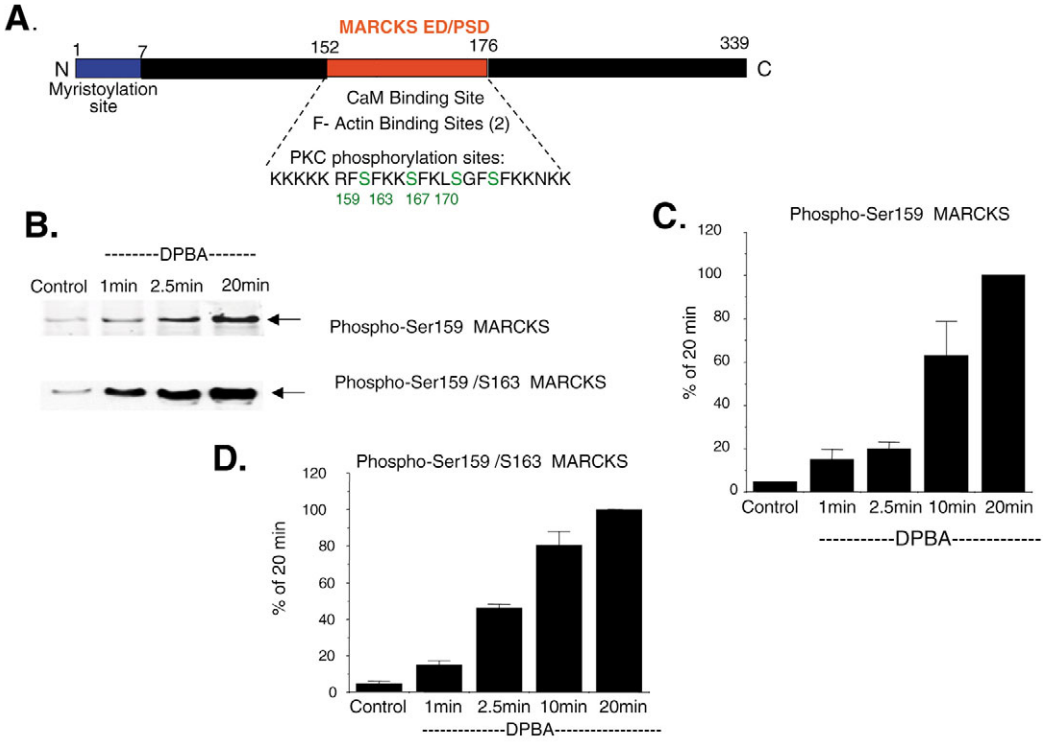


**Fig. 8.** Time course of MARCKS phosphorylation in the presence of DPBA. (A) Domain map for human MARCKS sequence showing the N-terminal myristoylation site and the central effector domain (ED) or PhosphoSite Domain (PSD). Based on data reviewed in Blackshear (Blackshear, 1993). (B) Typical blots for DPBA-mediated Ser159 MARCKS or Ser159/163 MARCKS phosphorylation with time after addition of the phorbol ester DPBA. (C) Average densitometry of four to six pSer159 MARCKS immunoblots. (D) Average densitometry of five to seven pSer159/163 MARCKS immunoblots.

Interestingly, this antibody detects a signal (Fig. 8B,D) that increases more quickly than that for the Ser159 antibody ($P$<0.001 for the 2.5 minute data in Fig. 8C versus that in Fig. 8D). Notably, at 2.5 minutes [the time at which MARCKS translocation is almost complete (Fig. 6C)], the signal for this antibody (which detects two sites) is ~50% maximal. Thus, the different PKC phosphorylation sites on MARCKS appear to be phosphorylated with different rates. It is plausible that phosphorylation at Ser167 or Ser170 might be responsible for release of CaM from MARCKS (at ~1 minute) but phosphorylation at multiple sites might be necessary to release MARCKS (at ~2.5 minutes) from the cell surface.

### ED4A blocks CaM translocation

The isolated ED domain of MARCKS has previously been shown to bind CaM, actin and PKC in the same manner as the MARCKS protein and thus this sequence has been concluded to be the active site or 'effector domain' (Blackshear, 1993). To test the hypothesis that PKC-dependent MARCKS phosphorylation directly regulates CaM targeting, we introduced into smooth-muscle cells a decoy peptide containing MARCKS ED in which the four serines were substituted with nonphosphorylatable alanines (ED4A) and that contained an N-terminal FITC moiety for localization. Such a peptide has previously been shown to retain the ability to bind PKC with high affinity (Graff et al., 1991). Thus, this peptide should act a decoy peptide by binding CaM, actin and PKC but the bound CaM will not be releasable by PKC-mediated phosphorylation. We also synthesized a scrambled version (ED4AX) of this peptide as a negative control. Cells were chemically loaded with the peptides and immunostained to localize CaM. In unstimulated cells, the ED4A peptide (Fig. 9A), like CaM (Fig. 9B) and MARCKS (Fig. 6), localizes primarily to the cell surface. However, unlike MARCKS and CaM in unloaded cells, ED4A remains at the cell surface after the addition of DPBA (Fig. 9A, compare to Fig. 6, Fig. 9B). Furthermore, whereas, under control conditions, the phorbol ester DPBA causes a translocation of CaM to filamentous structures in the cell interior and a decrease in the surface:core ratio by 2.5 minutes (Fig. 6), no such translocation of CaM to the centre of the cell (Fig. 9B) is detectable in cells loaded with the decoy peptide. In the ED4A-loaded cells, the unstimulated surface:core ratios for CaM actually increase somewhat in the presence of DPBA (Fig. 9, middle). In cells loaded with the scrambled version of the same peptide (ED4AX), the peptide itself is fairly homogeneously distributed (Fig. 9A, bottom) and DPBA-mediated translocation of CaM (Fig. 9B, bottom) is not inhibited. To confirm that ED4A actually inhibits MARCKS phosphorylation, immunoblots of phosphorylated MARCKS were performed. As is shown in Fig. 9C, compared with unstimulated strips, DPBA increases the phosphorylated-MARCKS signal from mock-loaded strips and strips loaded with ED4AX. However, in ED4A-loaded strips, the DPBA-induced increase in MARCKS phosphorylation is significantly inhibited.

## Discussion

The results of this study demonstrate that CaM has a markedly heterogeneous subcellular distribution in differentiated smooth-muscle cells and that the distribution of CaM within the cell is highly dynamic and agonist regulated. Thus, it appears that agonists can induce the release of CaM from certain sites and cause rebinding to other discrete sites at distant locations in the cell. A major question is the mechanism by which such targeting of CaM occurs.

In this study, we tested the hypothesis that MARCKS is a

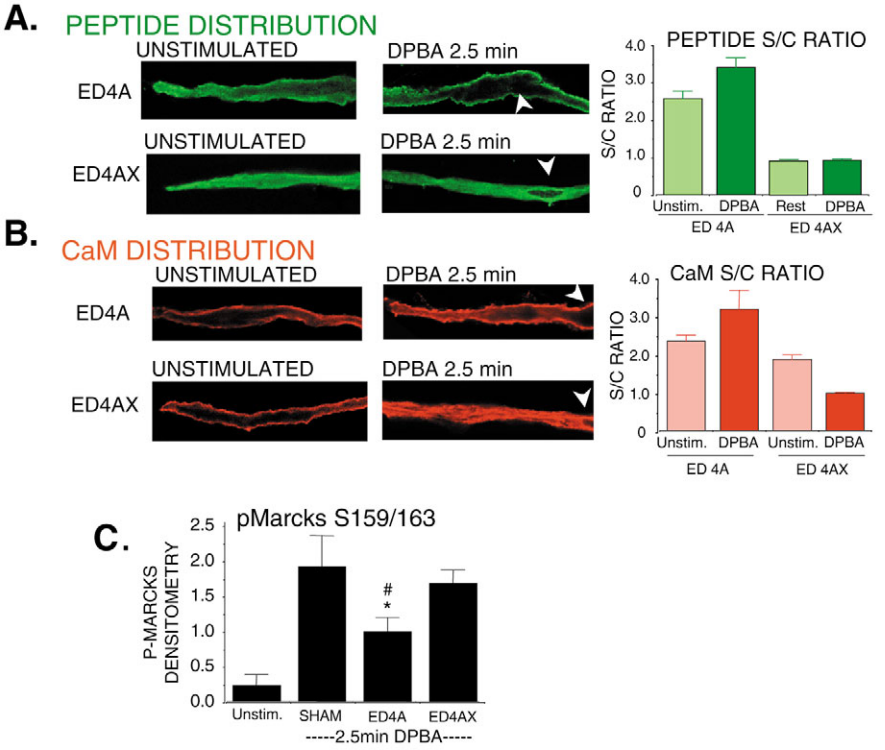


**Fig. 9.** CaM translocation is blocked by a nonphosphorylatable MARCKS ED decoy peptide. (A) Confocal images of distribution of fluorescent ED4A or ED4Ax in freshly dissociated portal-vein smooth-muscle cells (left) and quantitation of average surface:core fluorescence ratios from 9-11 cells (right). Arrowheads denote the position of the nucleus. Where no arrowhead is shown, the nucleus is beyond the field of view of the image shown. (B, left) Confocal images of cells fixed and immunostained to locate CaM after loading with ED4A or ED4AX either at rest or after stimulation with the phorbol ester DPBA. (B, right) Average surface:core fluorescence ratios from 9-11 cells. Arrowheads denote the positions of the nuclei. Where no arrowhead is shown, the nucleus is beyond the field of view of the image shown. (C) Average densitometry of immunoblots for Ser159/163 MARCKS phosphorylation for unstimulated tissues or tissues stimulated with DPBA and mock loaded or loaded with ED4A or ED4AX ($n$=4). *, $P$<0.05 compared with the mock-loaded control; #, $P$<0.05 compared with ED4AX.

significant reservoir for cellular CaM and, conversely, that MARCKS could be a major CaM donor upon cell stimulation. The results support this hypothesis. (1) MARCKS, a known high-affinity CaM-binding protein and major PKC substrate, is present in portal-vein smooth muscle at high levels (estimated at 16 μM). We estimated total CaM concentration in the same cell type at 41 μM. Thus, more than one-third of total cellular CaM could be sequestered by MARCKS and released during PKC activation. (2) Overlay assays indicate that, of all the $Ca^{2+}$-dependent CaM-binding proteins present in a whole-cell homogenate of smooth muscle, MARCKS is one of the most prominent. (3) CaM and MARCKS colocalize, coimmunoprecipitate and cross-link in intact cells. (4) CaM translocates with MARCKS in the presence of a depolarizing stimulus but, in the presence of a PKC activator, translocates to the cell core before MARCKS. (5) Depolarization increases and PKC activation decreases the coimmunoprecipitation and cross-linking of CaM with MARCKS or the MARCKS ED. (6) A nonphosphorylatable MARCKS ED peptide blocks PKC-dependent CaM translocation when introduced into cells. Thus, these combined results strongly indicate that, in differentiated smooth-muscle cells, MARCKS can be a significant and dynamic regulator of CaM availability and targeting.

$Ca^{2+}$ is required for CaM binding to MARCKS but $Ca^{2+}$-CaM is known to displace MARCKS from actin and membrane binding sites. Thus, it was surprising that CaM and MARCKS colocalized, coimmunoprecipitated and cross-linked in unstimulated cells. In unstimulated smooth-muscle cells, $[Ca^{2+}]_i$ is low, generally $1\times10^{-7}$ M to $2\times10^{-7}$ M (DeFeo and Morgan, 1985a). Thus, the proteins to which CaM is bound in unstimulated cells will be either $Ca^{2+}$-independent CaM-binding targets or very-high-affinity $Ca^{2+}$/CaM-binding targets. The $K_d$ of MARCKS for $Ca^{2+}$-CaM has been measured at 2-5 nM (Blackshear, 1993). At resting $[Ca^{2+}]_i$, it has been reported that two $Ca^{2+}$ ions would be bound per CaM molecule (Chin and Means, 2000). Two bound $Ca^{2+}$ ions are thought to be sufficient for CaM to bind to myosin light-chain kinase (MLCK), another high-affinity CaM-binding target. However, it is thought that MLCK kinase activity does not increase until $[Ca^{2+}]_i$ increases above resting levels to saturate the four binding sites of CaM (Chin and Means, 2000). By analogy to the MLCK model, we suggest that MARCKS binds CaM at the cell surface in unstimulated cells at resting $Ca^{2+}$ levels, when CaM is expected to be only partially saturated with $Ca^{2+}$. Only when $[Ca^{2+}]_i$ is increased above basal levels by a depolarizing stimulus does the complex dissociate from the cortical cytoskeleton/plasmalemma and MARCKS and CaM redistribute away from the cell surface. This concept is consistent with our finding that decreasing $[Ca^{2+}]_i$ below resting levels decreases the colocalization of MARCKS and CaM, indicating that resting $[Ca^{2+}]_i$ is both sufficient and necessary for CaM to bind MARCKS at the cell surface.

Depolarization-induced increases in $[Ca^{2+}]_i$ caused a parallel movement of MARCKS and CaM to the core of the cell and, because $Ca^{2+}$ increases the binding of CaM to MARCKS, it is not surprising that the coimmunoprecipitation and cross-linking of CaM and MARCKS is increased in the depolarized cell. In this case, the interesting implication is that a reservoir of potentially releasable CaM is targeted to the proximity of myofilament bundles containing CaM-binding proteins such as MLCK or the myosin-inhibitory protein CaD. Recent studies have suggested that a distinct pool of CaM, tightly bound to myofibrils (Wilson et al., 2002; Sward et al., 2003) might be responsible for activation of MLCK. Based on the results of the present study, it is possible that MARCKS performs such a function to create a pool of CaM concentrated in the vicinity of the myofilaments. Once targeted near the myofilaments, this pool of CaM could be released during agonist-induced signalling, as has been reported to occur with α-agonist stimulation in this tissue. In the presence of an α-agonist, an initial transient increase in $[Ca^{2+}]_i$ is followed by a delayed PKC activation at near basal $[Ca^{2+}]_i$ (Khalil and Morgan, 1992).

Conversely, when, in resting cells, MARCKS is localized to the cell cortex, the CaM concentrated at the cell membrane could locally regulate distinct functions such as $Ca^{2+}$/CaM-dependent channel activity without triggering a contractile response. The fact that MARCKS colocalizes with vinculin, a marker of adhesion plaques, is also of interest. A similar finding has been reported for macrophages (Rosen et al., 1990) and skeletal-muscle cells (Disatnik et al., 2002). This raises the further possibility that the actin cross-linking properties of MARCKS might participate in $Ca^{2+}$-dependent changes in cell adhesion or in stretch-dependent 'outside-to-inside' or 'inside-to-outside' signalling in smooth-muscle cells.

These results, like our previously reported results with a CaM probe (Hulvershorn et al., 2001), are consistent with the concept that PKC-mediated phosphorylation of MARCKS leads to a release of CaM into the cytosol, at least transiently. If MARCKS binds at most 1 mole CaM per mole MARCKS and if all bound CaM is released upon phosphorylation, the predicted localized release of up to ~16 μM CaM into the cytosol could have significant effects. For example, CaD is an actin-binding protein with inhibitory actions on myosin ATPase activity. These inhibitory actions can be reversed by CaM, but the $K_d$ of CaD for CaM is reported to be 50-430 nM (depending on the method of preparation) (Hulvershorn et al., 2001), which has been suggested to be too high to be physiologically relevant. The release of CaM from MARCKS could be sufficient to perturb CaD's inhibitory actions. It should be emphasized that we have focused on a specific vascular smooth muscle, the portal vein, and it is possible (but beyond the scope of the present study) that there are tissue-specific differences in the abundance or regulation of MARCKS that could, in turn, lead to tissue-specific regulation of CaM targeting.

In summary, we have found that the targeted distribution of CaM in smooth muscle is heterogeneous and dynamically regulated. The results presented here indicate that MARCKS can function as a PKC-releasable reservoir of CaM and provide a mechanism for targeting of CaM within the cell.

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