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Inhibitors of actin polymerization and calmodulin binding enhance protein kinase C-induced translocation of MARCKS in C6 glioma cells

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Abstract

MARCKS (*m*yristoylated *a*lanine-*r*ich *C*-*k*inase *s*ubstrate) is known to interact with calmodulin, actin filaments, and anionic phospholipids at a central basic domain which is also the site of phosphorylation by protein kinase C (PKC). In the present study, cytochalasin D (CD) and calmodulin antagonists were used to examine the influence of F-actin and calmodulin on membrane interaction of MARCKS in C6 glioma cells. CD treatment for 1 h disrupted F-actin filaments, increased membrane bound immunoreactive MARCKS (from 51% to 62% of total), yet markedly enhanced the amount of MARCKS translocated to the cytosolic fraction in response to the phorbol ester 4β-12-*O*-tetradecanoylphorbol 13-acetate. In contrast, CD treatment had no effect on phorbol ester-stimulated phosphorylation of MARCKS or on translocation of PKCα to the membrane fraction. Staurosporine also increased membrane association of MARCKS in a PKC-independent manner, as no change in MARCKS phosphorylation was noted and bis-indolylmaleimide (a more specific PKC inhibitor) did not alter MARCKS distribution. Staurosporine inhibited the phorbol ester-induced translocation of MARCKS but not of PKCα in both CD pretreated and untreated cells. Calmodulin antagonists (trifluoperazine, calmidazolium) had little effect on the cellular distribution or phosphorylation of MARCKS, but were synergistic with phorbol ester in translocating MARCKS from the membrane without a further increase in its phosphorylation. We conclude that cytoskeletal integrity is not required for phosphorylation and translocation of MARCKS in response to activated PKC, but that interaction with both F-actin and calmodulin might serve to independently modulate PKC-regulated localization and function of MARCKS at cellular membranes.

Keywords: Myristoylated alanine-rich C kinase substrate; Protein kinase C; Calmodulin; Actin; Cytochalasin; Translocation

1. Introduction

Activation of protein kinase C (PKC) by receptor-mediated phospholipid hydrolysis plays an important role in regulation of a variety of cellular processes, including proliferation, differentiation, and secretion [1–4]. In many cell types, one of the earliest and most abundant proteins phosphorylated in response to PKC activation is MARCKS (*m*yristoylated

Abbreviations: BIM, bis-indolylmaleimide; CD, cytochalasin D; FBS, fetal bovine serum; Hepes, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; MARCKS, myristoylated alanine-rich C kinase substrate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PKC, protein kinase C; STS, staurosporine; TBS, Tris-buffered saline; TFP, trifluoperazine; TPA, 4β-12-*O*-tetradecanoylphorbol-13-acetate

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*a*lanine-*r*ich *C*-*k*inase *s*ubstrate), a 28–32 kDa acidic heat-stable protein which migrates anomalously as a 70–80 kDa band on SDS-PAGE [5–7]. MARCKS contains a central basic effector domain which interacts with membrane phospholipids [8–10], calmodulin [11–13], and actin [14]; phosphorylation by PKC of up to four serine residues in this domain has been shown to inhibit all of the above interactions. Although the physiological function of MARCKS has not been established, competition for the effector domain is thought to be central to proposed roles for MARCKS in Ca^{2+}/calmodulin and PKC-mediated membrane-cytoskeletal alterations accompanying cell migration and motility [15,16] and vesicle trafficking [17,18].

The localization and interactions of MARCKS at the membrane is undoubtedly essential to its function. MARCKS has been co-localized with vinculin and talin at focal adhesion sites [16] and with phagosomes [18] in macrophages, and activation of PKC results in reversible translocation of the protein from the membrane to the cytosol [16,19,20]. More recent investigations have indicated that interaction of MARCKS with membranes requires both a hydrophobic contribution from the N-terminal myristate moiety and electrostatic interaction between the central basic domain and acidic phospholipids [9,10,21–23]. Phosphorylation of, or calmodulin binding to, the basic domain was shown to negatively affect the latter interaction. However, a clear relationship between MARCKS phosphorylation and its translocation has not been observed in several other studies [24–26], suggesting that other cellular components such as the cytoskeleton might influence the membrane localization of MARCKS.

While interactions of MARCKS with calmodulin, PKC, and membrane lipids have received considerable attention, relatively little is known about the effect of F-actin binding on the localization and properties of MARCKS. MARCKS could provide a reversible cross-link between membrane lipids and actin filaments or, conversely, F-actin might help tether MARCKS to the membrane, perhaps via additional PKC-sensitive actin binding proteins. The present investigation addresses these interactions using digitonin-permeabilized glioma cells to assess membrane affinity of MARCKS under a variety of conditions. Our results are consistent with a model in which F-actin and calmodulin may act to modulate MARCKS localization in response to PKC activation.

2. Materials and methods

2.1. Materials

Carrier-free [^{32}P]orthophosphoric acid and [9,10-^{3}H]myristic acid (11 Ci/mmol) were purchased from DuPont Canada (NEN Products, Lachine, PQ, Canada). Triton X-100 (Surfact-Amps) and Micro-bicinchoninic protein assay kits were from Pierce (Chromatographic Specialties, Brockville, Ont., Canada). Digitonin, 4β-12-*O*-tetradecanoylphorbol 13-acetate (TPA), cytochalasins B and D, phalloidin, staurosporine (STS), saponin, trifluoperazine (TFP), protease inhibitors and lactate dehydrogenase kit were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bis-indolylmaleimide (BIM, GF 109203X) and calmidazolium hydrochloride were obtained from Calbiochem (San Diego, CA, USA). Equipment and reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were from BioRad Canada (Mississauga, Ont., Canada), while enhanced chemiluminescence detection (ECL) kits and Hyperfilm MP were from Amersham Canada (Oakville, Ont., Canada). Affinity purified polyclonal anti-PKCα antibody directed against a synthetic peptide corresponding to amino acids 313-326 of rat PKCα was purchased from Gibco BRL (Life Technologies, Burlington, Ont., Canada). Fluorescein phalloidin was obtained from Molecular Probes (Eugene, OR, USA).

2.2. Cell culture and labelling

C6 rat glioma cells (CCL-107) were maintained in 150-cm^2 flasks containing 45 ml of Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) as described previously [27]. For most experiments, 7×10^5 cells were seeded into 35 mm dishes and grown 5 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Protein phosphorylation experiments were performed by incubation with 20 μCi of [^{32}P]P_i in 1 ml of phosphate-free medium supplemented with 10% dialyzed FBS for 4 h. Cytochalasin D (10 μM final concentration) was added

for the final hour of labelling, while TPA (200 nM), BIM (10 μM), STS (200 nM), calmidazolium (1 μM), and/or TFP (50 μM) were added 15 min prior to extraction. In some experiments (not shown) proteins and lipids were labelled with [^{3}H]myristic acid by overnight incubation (50 μCi/ml) prior to treatment [26]. Control dishes received equivalent volumes of dimethyl sulfoxide carrier alone; the final concentration of carrier never exceeded 0.3% (v/v).

2.3. Subcellular fractionation

After washing cells twice with 2 ml Na-Hepes buffer (15 mM Hepes, 140 mM NaCl, 1.2 mM KH_2PO_4, 4.7 mM KCl, 1 mM $MgCl_2$, 5.5 mM glucose, pH 7.4), cytosolic and membrane proteins were separated by sequential extraction with digitonin and Triton X-100. Cells were permeabilized with 100 μM digitonin in K-Hepes buffer (15 mM Hepes, 115 mM KCl, 1.2 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM KF, 5 mM $Na_4P_2O_7$, 5.5 mM glucose, 1 μM leupeptin, 200 μM phenylmethylsulfonyl fluoride, pH 7.0) by incubation on a rotary shaker (70 rpm) for 10 min at 25°C. After removal of the soluble cytosolic fraction, membrane proteins were extracted from the dish using 1 ml of K-Hepes buffer containing 1% (v/v) Triton X-100 instead of digitonin. In some experiments, the insoluble cytoskeletal/nuclear fraction remaining on the dish was harvested by scraping in K-Hepes buffer and boiled directly in SDS-PAGE sample buffer. The resulting digitonin, Triton X-100-soluble and Triton X-100-insoluble fractions contained respectively 35%, 50%, and 15% of total cell protein, and 4%, 92%, and 4% of total [^{3}H]myristic acid label (mainly in phospholipid). These proportions did not vary with the different treatments used, thus equivalent volumes were used for SDS-PAGE analysis (below). Digitonin-induced permeabilization was assessed by lactate dehydrogenase (EC 1.1.1.27) and 5′-nucleotidase (EC 3.1.3.5) release as cytosolic and plasma membrane marker enzymes, respectively [28].

2.4. SDS-PAGE and protein analysis

Samples were prepared for Western blot analysis either directly from cell extracts (for PKCα) or following heat treatment (80°C for 10 min) as outlined previously for MARCKS [29]. Proteins in each fraction were precipitated by incubation with 5 volumes of cold acetone for at least 2 h at −20°C. Precipitated proteins were centrifuged, dissolved in SDS sample buffer, and separated by SDS-PAGE (10% acrylamide, 100 V) [30]. After electrophoretic transfer, nitrocellulose membranes (0.45 μm) were blocked with 5% (w/v) non-fat dry milk in TBS-Tween buffer (25 mM Tris-HCl, pH 7.5, 125 mM NaCl, 0.1% Tween 20) for at least 1 h, then incubated with the appropriate primary antibody for 1 h at room temperature. MARCKS was detected using rabbit anti-rat MARCKS peptide antisera (C-terminal epitope SPEAPPAPVAE [29]) at 1:1000 dilution in TBS-Tween buffer. For PKCα detection, rabbit anti-PKCα-antibody was diluted to 2 μg/ml in TBS-Tween buffer. After incubation with primary antibody, membranes were incubated 1 h at room temperature with a 1:10 000 dilution of goat ant-rabbit IgG conjugated to horse radish peroxidase and immunoreactive bands were visualized by ECL. MARCKS was enriched in ^{32}Pi-labelled cell extracts by heating as above. After removal of denatured proteins by centrifugation, heat-stable proteins were precipitated by addition of 2.5 vol of 20% (w/v) trichloroacetic acid for at least 2 h on ice. ^{32}Pi-labelled proteins were visualized by autoradiography.

Radioactive or immunoreactive proteins were quantified by densitometric scanning using an Apple OneScanner with Ofoto software (Light Source, Inc., Greenbrae, CA, USA), followed by analysis with NIH Image version 1.49 software (Research Services Branch, NIH, Bethesda, MD, USA). The amount of MARCKS or PKCα in each fraction was expressed as a percentage of the total from that dish; this total did not change significantly as a result of the short term treatments used in this study. During the course of this investigation, no significant amount of MARCKS was detected in the Triton-insoluble cytoskeletal fraction (data not shown).

2.5. Fluorescence microscopy

Cell cultures grown on glass coverslips were subjected to the same treatments as outlined above, followed by two washes in PBS (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4). Cells were subsequently fixed in 3.7% formal-

dehyde in PBS for 10 min followed by two more washes in PBS. The coverslips were then extracted with acetone at −20°C for 3 to 5 min and air dried. F-actin was labelled by 25 min incubation with 1 unit of fluorescein-phalloidin diluted in 200 μl PBS. Labelled coverslip cultures were washed twice rapidly with excess PBS and mounted on a 1:1 solution of PBS and glycerol. Samples were observed and photographed with an Olympus fluorescence microscope.

3. Results

3.1. Cytochalasin D and staurosporine alter MARCKS localization and translocation in response to phorbol ester

To study interaction of MARCKS with the membrane in a permeabilized cell system, conditions required to selectively extract cytosolic and membrane-bound proteins were optimized in C6 glioma cells. Incubation with either digitonin (Fig. 1A) or saponin (Fig. 1B) resulted in time- and concentration-dependent release of a cytosolic marker protein (lactate dehydrogenase) into the medium. Streptolysin-O permeabilization (2 IU/ml) resulted in only 50% release of the enzyme under similar conditions (data not shown). Incubation of cells for 10 min with 100 μM digitonin released 81 ± 2% ($n = 3$) of total lactate dehydrogenase activity, but only 8.0 ± 0.6% of total 5′-nucleotidase activity (a plasma membrane marker), and was thus chosen for further studies.

Given the ability of MARCKS to cross-link F-actin [14] and co-localize with both vinculin and talin at focal adhesion sites where actin stress fibers terminate at the plasma membrane [16], we examined whether the integrity of the actin cytoskeleton influences either the affinity of MARCKS with the membrane or its response to activated PKC. C6 glioma cells were pretreated for 1 h with increasing concentrations of cytochalasin D (CD), an agent that selectively disrupts microfilaments, and actin was visualized with fluorescein-phalloidin staining. CD pretreatment resulted in a dose-dependent loss of filamentous actin networks seen spanning the cytoplasm, with complete cytoskeletal disruption seen at 10 μM CD (Fig. 2).

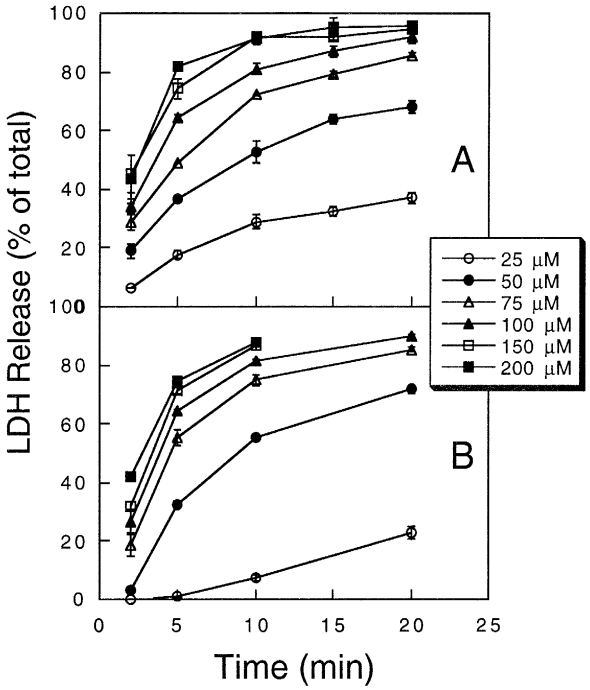

Fig. 1. Release of a cytosolic marker protein (lactate dehydrogenase) from glioma cells by digitonin and saponin permeabilization. C6 glioma cells grown 5 days in 35 mm dishes were washed with Na-Hepes buffer (see text) and incubated at 25°C with the indicated concentrations of either digitonin (panel A) or saponin (panel B) in K-Hepes buffer (1 ml) for the times shown. Dishes were then incubated with K-Hepes buffer containing Triton X-100 and lactate dehydrogenase activity was measured in both fractions; total activity was constant in all samples. Data are expressed as percent of total activity released by digitonin or saponin; the mean and S.D. of three samples are shown.

Cytochalasin D treatment resulted in a significant increase in the proportion of immunoreactive MARCKS associated with the membrane fraction of C6 glioma cells, with a reciprocal decrease in the cytosolic fraction. A representative experiment is shown in Fig. 3 (upper panel); densitometric evaluation of data from several experiments indicated that membrane MARCKS increased from 51 ± 5% to 62 ± 2% of total MARCKS after CD treatment (Table 1). The increased membrane association of MARCKS in the presence of CD actually enhanced its subsequent translocation to the cytosol upon activation of PKC: addition of 200 nM TPA decreased the proportion of membrane MARCKS to 35 ± 1% in CD treated cells (vs 42 ± 3% in TPA-treated control cells). Thus, the amount of MARCKS translocated from membrane to

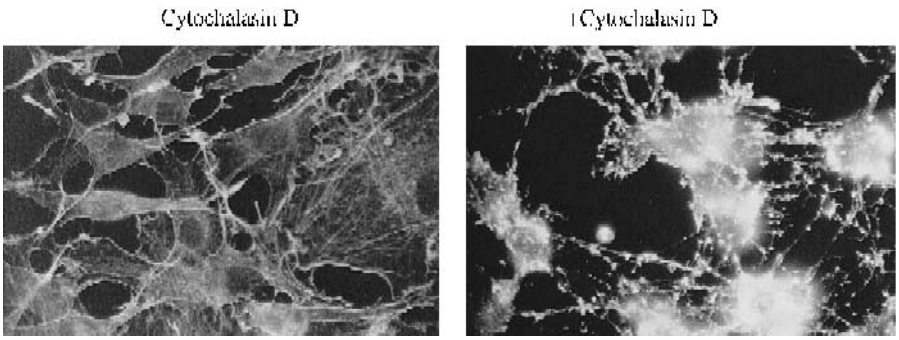

Fig. 2. Disruption of F-actin filaments in cytochalasin D-treated C6 glioma cells. Cells incubated in the absence or presence of 10 μM CD for 1 h were stained with fluorescein-phalloidin prior to fluorescence microscopy.

cytosol by TPA was almost three-fold greater in CD-treated than in untreated cells. Similar results were also observed when MARCKS was visualized by [^{3}H]myristate-labelling or when F-actin was disrupted with cytochalasin B, while stabilization of F-actin filaments by phalloidin was without effect on MARCKS distribution (data not shown). From these observations, we conclude that disruption of F-actin in C6 cells increases the membrane affinity of MARCKS, yet renders it more sensitive to TPA-induced translocation to the cytosol.

The non-specific PKC inhibitor STS also increased membrane association of MARCKS to the level seen in CD-treated cells; however, unlike CD, STS antagonized rather than enhanced TPA-induced translocation to the cytosol (Fig. 3 and Table 1). Addition of both STS and CD to cells produced no greater membrane association of MARCKS than either reagent alone, but completely blocked any effect of TPA on MARCKS translocation. In a separate experiment, 10 μM bis-indolylmaleimide (BIM) did not appreciably alter MARCKS localization ($47 \pm 3\%$ vs $49 \pm 2\%$ membrane associated in BIM-treated vs untreated cells). As BIM is a much more specific inhibitor of PKC [31], which completely blocks PKC activity at this concentration in C6 cells [32], these data suggest

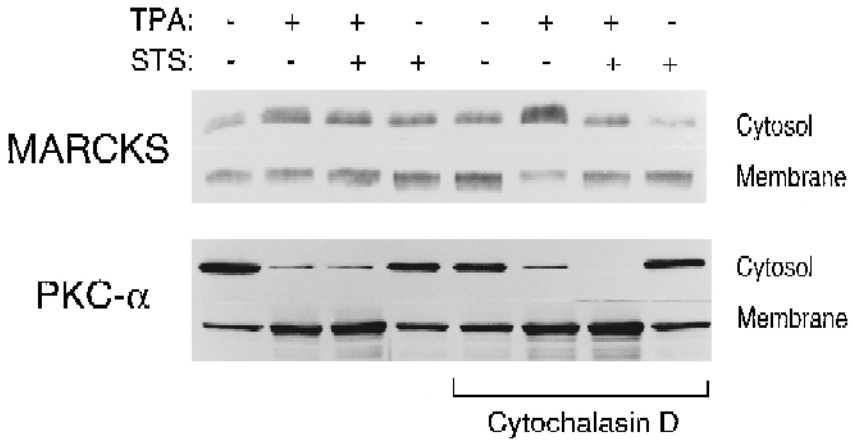

Fig. 3. Effects of TPA and staurosporine on the distribution of MARCKS and PKCα in control and cytochalasin D-treated C6 glioma cells. Confluent cell cultures were exposed to either cytochalasin D (10 μM) or an equivalent volume of carrier (0.1% v/v) for 1 h. For the final 15 min of incubation, dishes were treated with 200 nM TPA and 200 nM STS ($\pm$ as indicated). Soluble and membrane proteins were obtained by sequential extraction with digitonin followed by Triton X-100, separated by SDS-PAGE, immunoblotted and probed with anti-MARCKS (upper panel) or anti-PKCα (lower panel) antibodies, then visualized by ECL detection.

Table 1
Effects of cytochalasin D, phorbol ester, and staurosporine on membrane association of MARCKS and PKCα in C6 glioma cells. Cells were treated as outlined in 'Experimental Procedures' with CD (10 μM for 1 h), TPA (200 nM for 15 min) and/or STS (200 nM for 15 min) as indicated prior to digitonin permeabilization. Proteins from soluble and membrane fractions were separated by SDS-PAGE and analyzed by Western blotting for MARCKS and PKCα (a representative experiment is shown in Fig. 3). The amount of MARCKS or PKCα was determined by densitometric analysis and the membrane content expressed as a percentage of the total amount from that dish. The mean ± S.E.M. for the membrane fractions from 3–6 separate dishes

Treatments			Membrane association (% of total)	
CD	TPA	STS	MARCKS	PKCα
−	−	−	51 ± 5	39 ± 2
−	+	−	42 ± 3 [a]	87 ± 3
−	+	+	51 ± 6	87 ± 2
−	−	+	67 ± 2 [b]	44 ± 3
+	−	−	62 ± 2 [c]	44 ± 4
+	+	−	35 ± 1 [a]	84 ± 4
+	+	+	67 ± 5	89 ± 4
+	−	+	68 ± 2	41 ± 1

[a] Significantly ($P < 0.05$) different from corresponding -TPA control in Student's *t*-test.
[b] Significantly ($P < 0.005$) different from corresponding -STS control.
[c] Significantly ($P < 0.025$) different from corresponding -CD control.

that CD and STS might act similarly to enhance membrane association of MARCKS in a PKC-independent manner.

3.2. Cytochalasin D and staurosporine do not influence MARCKS localization by altered phosphorylation of the protein

To determine which of the above effects on membrane association of MARCKS might be due to changes in basal or TPA-stimulated phosphorylation of the protein, MARCKS labelled in vivo with ^{32}Pi was partially purified by heat treatment and analyzed by SDS-PAGE and autoradiography (Fig. 4). MARCKS was identified as the 80 kDa phosphorylated band on the basis of its selective heat stability [33] and earlier 2D-PAGE analysis of proteins phosphorylated in response to TPA in C6 cells [26]. As noted in the latter study, TPA treatment stimulated MARCKS phosphorylation in both soluble and membrane fractions, but CD had no effect on either the basal phosphorylation of MARCKS or the degree of stimulation produced by TPA (phosphorylation of total cellular MARCKS was increased 4.5 ± 0.7 and 4.5 ± 1.5 fold by TPA in the absence and presence of 10 μM CD, respectively, in three experiments). STS completely blocked the TPA-induced phosphorylation of MARCKS (not shown), but when added alone did not decrease basal levels of MARCKS phosphorylation below that of control cells (Fig. 4). These results indicate that cytoskeletal integrity is not required for phosphorylation of MARCKS by activated PKC, and that the influence of CD and STS on basal

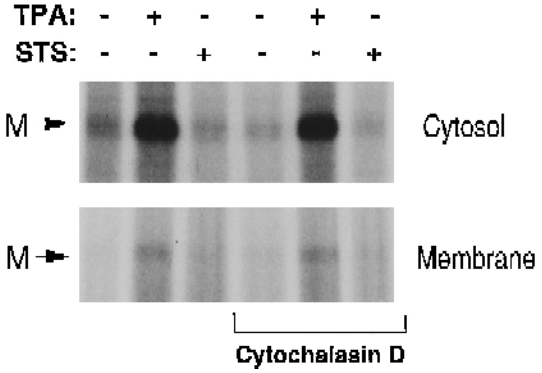

Fig. 4. Effects of TPA and staurosporine on phosphorylation of MARCKS in control and cytochalasin D-treated C6 glioma cells. Cells were prelabelled with [^{32}P]P_i for 3 h, then cytochalasin D (10 μM) or carrier alone was added to dishes for 1 h. For the final 15 min of incubation, 200 nM TPA or 200 nM STS (± as indicated) was added. Soluble and membrane proteins were obtained by sequential extraction with digitonin followed by Triton X-100. Samples were heated at 80°C for 10 min and heat-stable proteins were precipitated with trichloroacetic acid prior to separation by SDS-PAGE and autoradiography. The migration of MARCKS (M) is indicated.

MARCKS distribution (and enhanced translocation by TPA in the case of CD) is not due to additional changes in its phosphorylation state.

3.3. PKCα translocation is independent of cytoskeletal integrity in C6 glioma cells

To further explore how F-actin disruption by CD might influence TPA-induced translocation of MARCKS, we examined directly whether cytoskeletal disruption could interfere with translocation to the membrane of PKCα, a prominent isoform in C6 cells [34,35] that has been implicated in the function of MARCKS [36,37]. As shown in Fig. 3 (lower panel), most of the PKCα immunoreactivity was found in the cytosolic fraction under basal conditions (39 ± 2% in the membrane fraction; Table 1). As expected, treatment with 200 nM TPA for 15 min resulted in substantial translocation of PKCα to the membrane fraction (87 ± 3%). Despite inhibiting TPA-stimulated MARCKS phosphorylation and translocation, STS had little effect on either the basal distribution of PKCα or its translocation in response to TPA. Moreover, while the concentration of CD used here profoundly disrupted the integrity of the cytoskeleton and influenced the localization of MARCKS, it did not interfere with the basal distribution of PKCα or its behavior in response to TPA and STS.

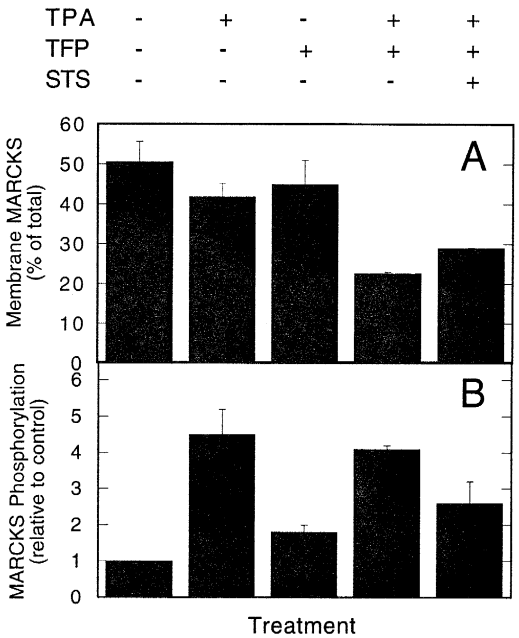

Fig. 5. Effects of trifluoperazine and TPA on translocation and phosphorylation of MARCKS in C6 glioma cells. Panel A: confluent cells were treated with 200 nM TPA, 50 μM TFP or 200 nM STS (± as indicated) for 15 min prior to protein extraction and quantitation of membrane associated MARCKS by immunoblotting as described in Table 1. Panel B: cells were prelabelled with [^{32}P]P_i for 4 h and treated with TPA, TFP, or STS as above. Phosphorylated MARCKS was detected by SDS-PAGE and autoradiography as described in Fig. 4, quantified by densitometry, and expressed relative to untreated control samples. The mean ± S.D. of three separate experiments is shown.

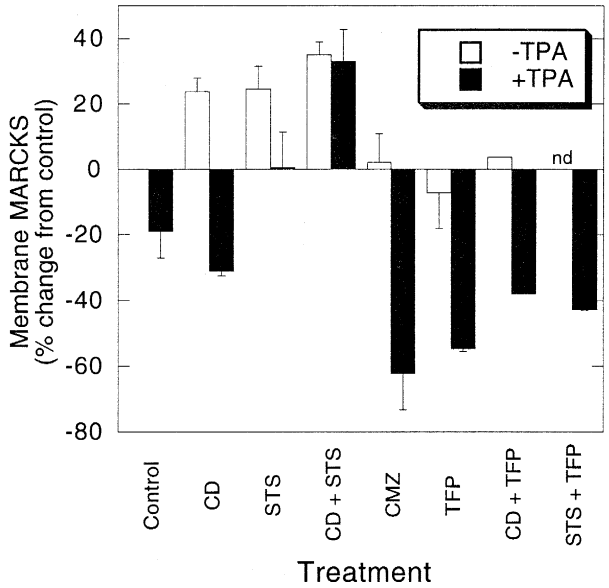

Fig. 6. Individual and combined influence of inhibitors of PKC activity, cytoskeletal assembly, and calmodulin binding on membrane association of MARCKS in the presence and absence of TPA. C6 glioma cells were pretreated 1 h with 10 μM CD, and/or 15 min with 200 nM STS, 1 μM calmidazolium (CMZ), or 50 μM TFP prior to extraction and quantitation of membrane associated MARCKS by immunoblotting. TPA (200 nM) was also present (solid bars) or absent (white bars) during the final 15 min incubation as indicated. Data are from several independent experiments and show the percent change in membrane MARCKS (mean ± S.D. or 1/2 range, $n = 2–8$) in the treatment groups relative to untreated cells (control) from that experiment; 51 ± 3% of total MARCKS was membrane associated under control conditions. nd, not determined.

3.4. Calmodulin antagonists and TPA act synergistically on MARCKS translocation

Calmodulin is another protein that reportedly interacts with the central basic domain of MARCKS and

influences its membrane association [9,23,38]. Thus, we examined the effect of the calmodulin antagonist trifluoperazine (TFP) on the distribution and phosphorylation of MARCKS and its response to PKC activation. In contrast to CD, treatment of C6 glioma cells with TFP had little effect on the basal distribution of MARCKS (Fig. 5A). However, TPA-induced translocation of MARCKS to the cytosol was markedly enhanced when TFP was present: only 22% of total MARCKS remained membrane associated in the presence of both TFP and TPA, vs 41% with TPA alone (significantly different by Student-Newman-Keuls analysis, $P < 0.05$). Similar results were observed with the more potent and specific calmodulin antagonist calmidazolium [39], indicating that the effects of TFP are indeed due to inhibition of calmodulin binding (Fig. 6). TFP and CD added together had little effect on basal MARCKS distribution, and the response to TPA under these conditions indicated that enhanced PKC-mediated translocation by TFP and CD are not additive (Fig. 6).

As with CD, the synergistic effect of TFP on TPA-induced translocation of MARCKS was not accompanied by a comparable change in its phosphorylation state (Fig. 5B): although TFP alone slightly increased phosphorylation of MARCKS (1.8-fold), it had no further effect on the increase produced by TPA. Interestingly, while STS effectively antagonized TPA-induced phosphorylation and translocation of MARCKS (Table 1), it only partially reversed these parameters when TFP was also present (Fig. 5).

4. Discussion

In the present investigation we have optimized a simple and rapid cell permeabilization protocol which allows us to measure small changes in membrane association of MARCKS. Under the basal conditions established for C6 glioma cells, MARCKS was approximately equally distributed between the soluble digitonin and membrane fractions and TPA alone produced only a modest translocation to the cytosol despite a four-fold increase in phosphorylation. One of the most important results we obtained is that inhibition of either calmodulin binding or actin polymerization can potentiate the PKC-induced translocation of MARCKS, apparently without affecting further the level of MARCKS phosphorylation. This observation may resolve apparent discrepancies in the literature about the relationship between MARCKS phosphorylation and translocation. Although it is generally accepted that phosphorylation by PKC is the dominant mechanism causing MARCKS redistribution from membrane to cytosol [16,19,20], other studies have suggested a more complex relationship [23,24]. In some cell types, translocation does not occur upon increased MARCKS phosphorylation [25,26], and the two events can be temporally dissociated in C6 glioma cells [26]. Our results confirm that factors in addition to its phosphorylation state influence the membrane association of MARCKS in permeabilized cells.

While inhibitors of calmodulin binding and actin polymerization were similar in their abilities to synergistically enhance TPA-induced MARCKS translocation without altering its phosphorylation, differences were also noted in the effects of these agents on the properties of MARCKS (see Fig. 6). CD, but not TFP or calmidazolium, increased basal association of MARCKS with the membrane fraction, suggesting that certain membrane components and F-actin might compete with each other for the central basic effector domain of MARCKS. F-actin is known to compete with both calmodulin binding and PKC phosphorylation for this domain [14], but direct evidence that F-actin and membrane binding are competitive has not been previously reported. As G-actin reportedly does not interact with MARCKS [14], actin filament disruption might liberate the effector domain of MARCKS for increased interaction with acidic lipids in the membrane, thus providing an important energetic contribution to the affinity of membrane interaction already provided by the myristoyl group [9,21]. In any case, our results suggest that F-actin does not restrict MARCKS solubilization by tethering it to the membrane or Triton X-100-resistant cytoskeletal fractions under basal conditions.

Unlike cytochalasin D treatment, inhibition of endogenous calmodulin with trifluoperazine or calmidazolium alone had little effect on the distribution of MARCKS between soluble and membrane fractions. As other studies have shown that saturating Ca^{2+}/calmodulin levels do inhibit MARCKS interaction with phospholipids [9,23,38], our results might suggest either that basal calmodulin binding was

minimal under the permeabilization conditions used (ie. low Ca^{2+}), or that Ca^{2+}/calmodulin binding does not influence membrane affinity in a more physiological cell model. However, it is also possible that the modest increase in phosphorylated MARCKS (1.8 fold) in the presence of TFP was sufficient to counter a slight increase in membrane affinity upon removal of calmodulin. Indeed, several reports indicate that Ca^{2+}/calmodulin binding can inhibit phosphorylation of MARCKS by PKC [11–13,40], and this inhibition has recently been shown to be Ca^{2+}-dependent in C6 cells [40], although MARCKS distribution was not monitored in that study.

Cytoskeletal integrity does not appear to be essential for either TPA-stimulated translocation of PKCα to the membrane or resulting phosphorylation of MARCKS in C6 glioma cells. CD also had no effect on bombesin-stimulated MARCKS phosphorylation in Swiss 3T3 fibroblasts, whereas phosphorylation of the focal adhesion site-associated tyrosine kinase $p125^{FAK}$ was completely blocked [41], suggesting that the effect of actin filament disruption on PKC activity is substrate specific. Although STS blocked both phosphorylation and translocation of MARCKS in response to TPA, it had no affect on the translocation of PKCα to the membrane in either CD-treated or untreated cells. The latter observation is not surprising, as STS is reported to interact mainly with the C-terminal kinase domain of PKC [42], while the N-terminal regulatory domain is responsible for TPA/diacylglycerol, Ca^{2+}, and phosphatidylserine-dependent interaction with the membrane bilayer.

This investigation has provided evidence for at least two distinct effects of staurosporine on MARCKS localization in C6 cells. First, STS can inhibit TPA-stimulated phosphorylation and translocation of MARCKS. This is likely due to direct inhibition of PKC activity, as indicated here and in previous studies with this cell type [32,43]. The sensitivity to STS may be influenced by other factors: TFP appears to render MARCKS phosphorylation and translocation less sensitive to inhibition by STS. Second, STS appears to have PKC-independent effects on MARCKS distribution, as this inhibitor (but not the more specific PKC inhibitor BIM) increased membrane association of MARCKS without altering its basal phosphorylation. This could reflect a PKC-independent disruption of the cytoskeleton by STS: previous studies have noted that this inhibitor induces reorganization of actin filaments in 3T3 cells [44] and alteration of C6 glioma cell morphology [43,45]. We have recently shown that STS can also stimulate phospholipase D activity in a PKC-independent manner in C6 cells [32], and that overexpression of MARCKS imparts TPA stimulation of both phosphatidylcholine turnover [36] and phospholipase D activity (unpublished results) in human SK-N-MC neuroblastoma cells. The relationships between MARCKS, the cytoskeleton, and phospholipase D are currently under investigation.

In conclusion, calmodulin and F-actin exhibit distinct effects on membrane association of MARCKS, and both act to modulate PKC-mediated changes in its subcellular distribution. The influence of calmodulin and F-actin appear to be at least partially independent of changes in the phosphorylation state of MARCKS. These data are consistent with previous evidence in C6 cells [40] showing that MARCKS is poised to respond to and coordinate temporally distinct Ca^{2+}- and PKC-mediated signals as part of a physiological role in regulating membrane-cytoskeletal interactions.

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