Conventional PKC-α, Novel PKC-ε and PKC-ζ, but Not Atypical PKC-λ Are MARCKS Kinases in Intact NIH 3T3 Fibroblasts

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Phosphorylation of myristoylated alanine-rich protein kinase C substrate (MARCKS) in intact cells has been employed as an indicator for activation of protein kinase C (PKC). Specific PKC isoenzymes responsible for MARCKS phosphorylation under physiological conditions, however, remained to be identified. In our present study using stably transfected NIH 3T3 cell clones we demonstrate that expression of constitutively active mutants of either conventional cPKC-α or novel nPKC-ε increased phosphorylation of endogenous MARCKS in the absence of phorbol 12,13-dibutyrate in intact mouse fibroblasts, implicating that each of these PKC isoforms itself is sufficient to induce enhanced MARCKS phosphorylation. Similarly, ectopic expression of a constitutively active mutant of PKC-ζ significantly increased MARCKS phosphorylation compared to vector controls, identifying PKC-ζ as a MARCKS kinase. The PKC-specific inhibitor GF 109203X (bisindolylmaleimide 1) reduced MARCKS phosphorylation in intact cells at a similar dose-response as enzymatic activity of recombinant isoenzymes cPKC-α, nPKC-ε, and nPKC-ζ in vitro. Consistently, phorbol 12,13-dibutyrate-dependent PKC phosphorylation was significantly reduced in cell lines expressing dominant negative mutants of either PKC-α K368R or (dominant negative) PKC-ε K436R. The fact, that the constitutively active PKC-λ A119E mutant did not alter the MARCKS phosphorylation underscores the assumption that atypical PKC isoforms are not involved in this process. We conclude that under physiological conditions, conventional cPKC-α and novel nPKC-ε, but not atypical aPKC-λ are responsible for MARCKS phosphorylation in intact NIH 3T3 fibroblasts.

Molecular cloning and biochemical studies identified the myristoylated alanine-rich protein kinase C substrate (MARCKS) as the major in vitro substrate of protein kinase C (PKC) (1–9). The ability to phosphorylate this substrate is not restricted to members of the PKC family. MARCKS is predominantly phosphorylated on serine (S) residues (in an order Ser-152 > Ser-156 > Ser-163) (10) in a PKC-dependent fashion, but can also be phosphorylated on serine and threonine residues by proline-directed protein kinases cdc2 and tau protein kinase II (11, 12). MARCKS is an acidic filamentous actin cross-linking protein which is targeted to the plasma membrane by its amino-terminal, myristoylated membrane-binding domain. This specific interaction positions the substrate close to PKC, facilitating its efficient phosphorylation. One of the striking features of MARCKS is its phosphorylation-dependent translocation from the membrane to the cytosol (8). Consequently, cytosolic MARCKS is not further cross-linking actin filaments. It has also been proposed that non-phosphorylated MARCKS complexes calmodulin resulting in a reduction of Ca2+/calmodulin-dependent signaling mechanisms and thereby blocking the entry of cells into the cell cycle (13). In murine macrophages, immunoreactive MARCKS protein was found in clusters at the interface of the substratum with pseudopodia and filopodia, where it is colocalized with other PKC substrates of actin cytoskeleton such as vinculin and talin (14). MARCKS is also highly concentrated in presynaptic junctions and is phosphorylated in a PKC-dependent manner when synaptosomes are depolarized (15, 16). Recently, it was demonstrated that MARCKS cycles between the plasma membrane and Lamp-1 positive lysosomes in fibroblasts in a PKC-dependent manner (17). In vitro studies demonstrate that conventional PKC-α, novel PKC-δ and nPKC-ε, but not atypical PKC-ζ phosphorylate partially purified recombinant MARCKS protein (10, 18). Phosphorylation of MARCKS in intact cells has been employed as an indicator for activation of PKC, however, which PKC isoform phosphorylates MARCKS in intact cells is still unknown. Importantly, MARCKS gene knockout experiments (9) have been demonstrated a dramatic genetic deficiency in mouse forebrain development concerning the importance of this protein in perinatal signal transduction. Therefore, PKC isoenzyme-specific MARCKS phosphorylation in living mouse cells was tested. Conventional cPKC-α, novel nPKC-ε, novel nPKC-ζ, and atypical aPKC-λ isoforms have been selected as representatives of the three PKC subfamilies. In order to identify the PKC isoenzyme-specific functions, we investigated MARCKS phosphorylation following: 1) expression of transdominant negative (DN) PKC mutants (19, 20); 2) expression of constitutively active (CA) PKC mutants in resting cells (19, 20); and 3) overexpression of wild-type isoenzymes. For comparative purposes, the PKC-selective bisindolylmaleimide-

cytomegalovirus; DMEM, Dulbecco’s modified Eagle’s medium; PVDF, polyvinylidene difluoride.
imide GF109203X was used for in vitro and in vivo PKC inhibition studies.

MATERIALS AND METHODS

Reagents and Plasmids—Dulbecco’s modified Eagle’s medium (DMEM), geneticin (G-418), and gentamycin were obtained from Boehringer Mannheim Biochemicals (Mannheim, Germany). Fetal calf serum and l-glutamine were purchased from Schöeller Pharma (Vienna, Austria). Phorbol 12,13-dibutyrate (PDBu), phorbol 12-myristate 13-acetate (PMA), phosphatidylserine (PtdSer), leupeptin, and aprotinin were purchased from Sigma (Vienna, Austria). Protein A-Sepharose was obtained from Pharmacia (Vienna, Austria). GF109203X is a product of Calbiochem (Luzern, Switzerland), Lipotectin transfection reagents and Opti-Mem I medium were purchased from Life Technologies, Inc. (Vienna, Austria). [γ-32P]Orthophosphate (10 mCi/ml, 8000 Ci/mmol), [γ-32P]ATP (10 mCi/ml, 3000 Ci/mmol), and Hyperfilm-MP were obtained from Amersham (Amersham, Little Chalfont, UK). The mouse polyclonal MARCKS antibody was raised in rabbits against the synthetic peptide COOH-terminal of PKC-α and PKC-ε peptides and cofactors used are: 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 50 mM sodium fluoride, and 100 mM Na3VO4 for 10 min. Aliquots of 1.5 x 106 NIH cell equivalents, containing equal amounts of protein (500 mCi, corresponding to approximately 1.5 mg/ml; protein concentrations were determined by the Bradford assay, Bio-Rad), were subjected to an immunoprecipitation (IP) procedure employing a corresponding PKC-α antibody (Transduction Laboratories, Lexington, KY). IPs were recovered by using protein A-Sepharose beads (Pharmacia, Vienna). PKC-α molecules bound to 45 μl of protein A-Sepharose were resuspended in 20 μl of kinase buffer and mixed with 9 μg of myelin basic protein and Mg-ATP-MgCl2 (1 mM, 150 μl). Lysates were harvested by SDS-PAGE (10%), transferred to PVDF membranes (Millipore, Vienna). Determination of PKC-α enzyme activities was done by PhosphorImaging of the corresponding PVDF membranes. The putative potential of the immunocomplexes to alter, either directly or indirectly, the inhibitory activity of GF109203X was addressed by in vitro PKC kinase assays of PKC immunoprecipitates in the presence or absence of various concentrations of GF109203X. Concerning the fact that there was no significant alteration in the dose-response curve (e.g. for recPKC-α) under these conditions our results favor no direct or indirect influence of the immunocomplexes on the inhibitory potency of GF109203X.

Stable Transfection of NIH 3T3 Fibroblasts—NIH 3T3 fibroblasts were kept at logarithmic growth phase in DMEM supplemented with 10% fetal calf serum and 5% CO2. Cell lines were transfected with a PKC-α expression construct (1 x 105/dish) were incubated in phosphate-free medium (in the presence of 20 nm to 6 μM GF109203X or solvent, Me2SO, final concentration 0.15%) for 4 h, followed by 50 μCi/ml carrier-free [γ-32P]orthophosphate (Amersham) pulse labeling of the endogenous ATP pool for an additional 2-h period. During the last 5 min of the pulse, 1 μCi/ml [3H]leucine (NEN, 20 Ci/mM) was added to the medium (in the presence of 20 nM to 6 μM GF109203X or solvent). After harvesting the cells in 500 μl of lysis buffer (20 mM Tris/His, pH 7.5, 10 mM EDTA, 2% Nonidet P-40, 1 mM phenylmethanesulfonyl fluoride, 100 μM Na3VO4, 50 μg/ml each of leupeptin and aprotinin) the lysates were transferred to new tubes. Lysates were clarified by centrifugation at 13,000 x g for 10 min and cleared by incubation with protein A-Sepharose for 1 h at 4°C. After removal of protein A-Sepharose by brief centrifugation (500 x g, Eppendorf microcentrifuge) the supernatants were transferred to fresh tubes and boiled for 5 min at 90°C. After this procedure to destroy heat-stable proteins the lysates were clarified once more by centrifugation at 13,000 x g for 10 min. Equal amounts of protein (500 μl, corresponding to approximately 1.5 mg/ml) were subjected to immunoprecipitation with a polyclonal antibody against the PKC-α peptide (Phospho-MAPK; Oncogene Science). IPs were recovered by using protein A-Sepharose coated immobilized antibodies (Perkin Elmer Life Sciences, USA), for a 3 h incubation at 4°C, washed five times with kinase buffer, mixed with 5 x Laemmli sample buffer, analyzed by SDS-PAGE (10%), and transferred to PVDF membrane (Millipore, Vienna). MARCKS phosphorylation analysis was done by PhosphorImaging of the corresponding PVDF membranes.

RESULTS

PDBu-induced MARCKS Phosphorylation in Intact Cells Is Sensitive in a Dose-dependent Fashion to the PKC-specific Inhibitor GF109203X—Phosphorylation of MARCKS in intact cells has been employed as an indicator for PKC activation and the specific PKC isoform responsible for MARCKS phosphorylation in intact cells, however, remained to be identified. Significant reduction of PDBu-induced MARCKS phosphorylation could be demonstrated by using a PKC-specific inhibitor. At concentrations which do not exert a significant effect on cell growth (20 nm to 6 μM), the PKC selective inhibitor GF109203X,
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COS-1 fibroblasts were transiently transfected with various PKC cDNA expression constructs, and His$_6$-tagged PKC isoenzymes were partially purified as described under "Materials and Methods." Enzyme activities of purified PKC isoenzymes or vector control preparations are expressed as cofactor-dependent phosphorylation of the synthetic PKC peptides in the absence or presence of various concentrations of GF109203X.

**Table 1**

| Recombinant PKC isoenzymes | Cofactors* | Inhibitor or solvent | Enzyme activity* |
|----------------------------|-----------|---------------------|------------------|
| None                       | EGTA      |                     |                  |
| None                       | PtdSer/PMA/Ca$^{2+}$ | Me$_3$SO | 2 ± 1 |
| PKC-α wt                  | PtdSer/PMA/Ca$^{2+}$ | + Me$_3$SO       | 100 ± 11 |
| PKC-α wt                  | PtdSer/PMA/Ca$^{2+}$ | 20 nM           | 38 ± 8 |
| PKC-α wt                  | PtdSer/PMA/Ca$^{2+}$ | 200 nM          | 11 ± 2 |
| PKC-α wt                  | PtdSer/PMA/Ca$^{2+}$ | 2 μM            | 2 ± 1 |
| PKC-α wt                  | PtdSer/PMA/Ca$^{2+}$ | 6 μM            | 1 ± 1 |
| PKC-ε wt                  | PtdSer/PMA | + Me$_3$SO       | 100 ± 5 |
| PKC-ε wt                  | PtdSer/PMA | 20 nM           | 53 ± 9 |
| PKC-ε wt                  | PtdSer/PMA | 200 nM          | 22 ± 4 |
| PKC-ε wt                  | PtdSer/PMA | 2 μM            | 18 ± 5 |
| PKC-ε wt                  | PtdSer/PMA | 6 μM            | 15 ± 3 |
| PKC-ε wt                  | PtdSer/PMA | + Me$_3$SO      | 100 ± 23 |
| PKC-ε wt                  | PtdSer/PMA | 20 nM           | 8 ± 3 |
| PKC-ε wt                  | PtdSer/PMA | 200 nM          | 3 ± 2 |
| PKC-ε wt                  | PtdSer/PMA | 2 μM            | 2 ± 1 |
| PKC-ε wt                  | PtdSer/PMA | 6 μM            | 1 ± 1 |
| None                       | PtdSer    | + Me$_3$SO       | 11 ± 2 |
| PKC-A wt                  | PtdSer    | + Me$_3$SO       | 100 ± 11 |
| PKC-A wt                  | PtdSer    | 20 nM            | 123 ± 8 |
| PKC-A wt                  | PtdSer    | 200 nM           | 119 ± 23 |
| PKC-C wt                  | PtdSer    | 2 μM            | 91 ± 12 |
| PKC-C wt                  | PtdSer    | 6 μM            | 14 ± 6 |

*The concentrations of synthetic substrates and cofactors used are: 50 μg/ml [A25S] and (153-[Ser-159]PKC-ε-164)-NH$_2$, 3 μg/ml MBP, 280 μg/ml PtdSer, 10 μM PMA, and 1 mM CaCl$_2$. To measure PKC activity in the absence of Ca$^{2+}$, EGTA (1 mM final concentration) was added instead of CaCl$_2$. Expression of the fusion tag-peptide COOH-terminal of the recombinant PKC isoenzymes thereby was found not to affect the kinase activity in vitro (24).

b To correct for differences in transfection efficiencies, enzyme activities are expressed as a percentage of cofactor-dependent phosphorylation of the [A25S]PKC peptide, which was determined separately in each experiment. Data expressed as the means (±S.E.) of at least three independent experiments done in triplicates.

PKC expression constructs or a vector control, and the recombinant PKC was purified by exploiting the COOH-terminal six-histidine (His$_6$)-fusion tag and analyzed in standard PKC kinase assays (19) against substrate peptides ([A25S]peptide for PKC-α and -ε, and [Ser-159]peptide for PKC-ε) in the absence or presence of known PKC cofactors including PtdSer, PMA, and Ca$^{2+}$ as described previously (24). In the case of PKC-λ an immunocomplex kinase assay with myelin basic protein as synthetic substrate was done as described under "Materials and Methods." The results are summarized in Table I. At lower concentrations than in intact cells, addition of GF109203X to purified recombinant PKC isoenzymes resulted in a significant reduction of protein kinase activity of εPKC-α, nPKC-ε, and nPKC-ε. Atypical aPKC-λ, however, did not demonstrate significant inhibition up to GF109203X concentrations of 6 μM (Table I). This dose-response is in agreement with IC$_{50}$ concentrations determined for inhibition of the atypical PKC-γ isoform by GF109203X (IC$_{50}$ of PKC-γ 5.8 μM (28)), an isoenzyme which exhibits 72% sequence homology to PKC-λ on the amino acid level (30). These results implicate that under physiological conditions conventional and novel, but not atypical PKC isoforms are involved in MARCKS phosphorylation.

Expression of DN PKC-α K368R and DN PKC-ε K436R Mutants Block PDBu-induced MARCKS Phosphorylation in Intact

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**Fig. 1.** PDBu-induced endogenous MARCKS phosphorylation is sensitive to the PKC-specific inhibitor GF109203X in a dose-dependent fashion. NIH 3T3 wild-type fibroblasts (1 x 10$^6$/dish) were incubated in phosphate-free medium (DMEM) for 4 h followed by 50 μCi/ml [γ$^32$P]orthophosphate pulse labeling for an additional 2 h period. During the last 5 min of the labeling procedure, cells were pre-treated 24 h with various concentrations of GF109203X (20 nM to 6 μM) or solvent (Me$_2$SO, final concentration 0.15% assay). Lysates were preincubated with various concentrations of GF109203X overnight at 4°C. Protein A-Sepharose-coated immunocomplexes were washed five times with lysis buffer, separated on PAGE (10%), and transferred to PVDF membranes. A, PDBu-induced MARCKS phosphorylation in the presence or absence of various concentrations of GF109203X as indicated (shown is a representative autoradiogram out of three experiments done in triplicates). MARCKS phosphorylation in the presence of solvent (Me$_2$SO, final concentration 0.15%; lane 1), 5 μM GF109203X (lane 2), 5 min PDBu stimulation (lane 3), 5 min GF109203X stimulation of cells 24 h preincubated with various concentrations of GF109203X (lanes 3–6). B, statistical analysis of experiments described under A. MARCKS phosphorylation was determined by PhosphorImaging of PVDF membranes and data are expressed as the means (±S.E.) of at least three independent experiments done in triplicates.
Cells—In order to eliminate pleiotropic GF109203X effect(s), we employed DN PKC mutants. In these constructs the critical lysine at the ATP-binding site is replaced by an arginine to produce transdominant-negative phenotypes. Such PKC mutant proteins have been shown to compete with endogenous PKC in an isoenzyme specific manner (19, 20, 22, 31–34). Employing transient transfection assays in COS-1 cells (DN) PKC-α K368R, (DN) PKC-ε K436R, and (DN) PKC-θ K409R have been found to lack kinase activity as recently described (19). To confirm the biological relevance of these DN PKC mutants in NIH 3T3 cells, the mutant proteins were tested in two independent biological systems. The biological function of PKC-α K368R was confirmed on thrombin-induced release of Ca\(^{2+}\) from intracellular stores. Briefly, expression of PKC-α K368R has been shown to overcome a PKC-α-mediated feedback inhibition of thrombin-induced intracellular Ca\(^{2+}\) release (data not shown). Furthermore, transient expression of DN PKC-ε K436R, not, however, of PKC-α K368R blocks the transcriptional activation of c-fos by oncogenic Ras. The expression of transfected PKC mutants and wild-type isoenzymes was assessed by immunoblotting of Ni\(^{2+}\)-chelating resin precipitates, employing PKC isozyme-specific antibodies (data not shown). Comparable levels of recombinant PKC mutants and wild-type isoforms were detected in different NIH 3T3 clones, representing approximately 2.5-fold overexpression relative to the levels of endogenous PKC isoforms (see Fig. 4 for wild-type). Taken together, these data indicate that all dead kinase mutants are expressed at comparable levels and exert dominant negative effects in an isoenzyme-specific fashion. Consistent with the above findings concerning the biological relevance of the DN mutants, MARCKS phosphorylation induced by 300 nM PDBu was significantly reduced in cell lines constitutively expressing PKC-α K368R or DN PKC-ε K436R mutants (Fig. 2, A and B). Importantly, due to the lack of endogenous PKC-θ expression in our NIH clone, the catalytically inactive DN PKC-θ K409R had no effect on PDBu-induced MARCKS phosphorylation, providing the isoform specificity of the dominant negative kinase approach used in this study.

Expression of CA PKC-α A25E, CA PKC-ε A159E but Not CA PKC-λ A119E Mutants Induce Significant MARCKS Phosphorylation in the Absence of PDBu Induction—Circumstantial evidence suggests that point mutation in the pseudosubstrate motif of the regulatory domain of PKC disrupt the interaction between the catalytic site and the pseudosubstrate sequence, generating an individual PKC isotype mutant independent of the stimulatory effects of phorbol esters or diacylglycerols, as shown by our standard in vitro PKC assay (19). To document that PKC-α, and PKC-ε by itself, are sufficient to phosphorylate MARCKS, cell lines stably expressing CA PKC-α A25E, CA PKC-ε A159E, or PKC-λ A119E were analyzed. In agreement with the results obtained so far, expression of CA PKC-α A25E and CA PKC-ε A159E resulted in a significant increase in MARCKS phosphorylation in the absence of PDBu when compared with vector transfected control cells (Fig. 3, A and B). Expression of PKC-λ A119E, however, did not demonstrate any significant increase in MARCKS phosphorylation. Interestingly, NIH 3T3 cells ectopically expressing a CA PKC-θ A148E mutant was found to significantly enhance MARCKS phosphorylation in the absence of PDBu, identifying PKC-θ as a new MARCKS kinase. In experiments where CA PKC-α, -ε and -θ were expressed, addition of 300 nM PDBu further enhanced the level of MARCKS phosphorylation (data not shown), suggesting a submaximal activation status. As expected, the addition of PDBu to transfectants where CA PKC-λ A119E was expressed lead to a complete phosphorylation status of MARCKS, presumably based on the activation of all PDBu-responsive MARCKS phosphorylating PKC isoforms endogenously expressed in NIH 3T3 fibroblasts.

**Kinase Activity of Recombinant PKC-λ Wild-type or Mutants**—In order to characterize the enzymatic properties of PKC-λ A119E, an immunocomplex kinase assay was performed as described under “Materials and Methods.” COS-1 cells were transiently transfected with the appropriate PKC-λ expression constructs or a vector control, and the recombinant PKCs were collected by immunoprecipitation and analyzed in standard PKC kinase assays (19) against MBP as a substrate peptide in the absence or presence of PtdSer. To correct for differences in transfection efficiencies, enzyme activities are expressed as a percentage of PtdSer-dependent phosphorylation of MBP by PKC-λ wild-type. The results are summarized in Table II. Importantly, in comparison with PKC-λ wild-type enzyme activity, the PKC-λ A119E mutant was capable of phosphorylating synthetic myelin basic protein (MBP) substrate in the absence of PtdSer (Table II). The activation level, however, was suboptimal, indicating either a submaximal activation status of PKC-λ A119E in vitro, or the requirement of additional cofactors such as λ-interacting protein (23).

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COS-1 fibroblasts were transiently transfected with various PKC-λ cDNA expression constructs. Lysates were immunoprecipitated with corresponding antibodies and analyzed in a standard immunocomplex kinase against MPB as a substrate peptide. Enzyme activities of immunoprecipitated PKC isoenzymes or vector control preparations are expressed as cofactor-dependent phosphorylation of the synthetic PKC peptides in the absence or presence of PtdSer. Routinely, a loding control of PKC wild-type and PKC mutants was done by reprobing the blotting membranes with the corresponding PKC-λ antibody used for IPs as described above. Comparable levels of recombinant PKC mutants and wild-type isoforms were detected onto the blotting membrane (data not shown).

### TABLE II

| PKC-λ wt or recombinant mutants | Cofactor $^a$ | Enzyme activity $^b$ |
|--------------------------------|--------------|---------------------|
| None                          | PtdSer       | 2 ± 1               |
| PKC-λ wt                      | PtdSer       | 100 ± 8             |
| PKC-λ A119E                   | PtdSer       | 71 ± 8              |
| PKC-λ R275W                   | PtdSer       | 17 ± 15             |

$^a$ The concentrations of synthetic substrates and cofactors used are: 3 μg/μl MBP, 280 μg/ml PtdSer.

$^b$ To correct for differences in transfection efficiencies, enzyme activities are expressed as a percentage of cofactor-dependent phosphorylation of MBP, which was determined separately in each experiment. Data expressed as the means (±S.E.) of at least three independent experiments done in triplicate.

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**FIG. 3.** CA cPKC-α A25E, CA nPKC-ε A159E, and CA nPKC-θ A148E, but not atypical CA nPKC-λ A119E are sufficient to induce MARCKS phosphorylation in the absence of PDBu stimulation. NIH 3T3 fibroblasts were transfected with 15 μg of PKC-α A148E (6xHis)-tag, PKC-θ A148E (6xHis)-tag, PKC-ε A159E (6xHis)-tag, and PKC-λ A119E expression constructs or the corresponding vector control and selected for G418 resistance as described under "Materials and Methods." A, representative autoradiogram out of three experiments done in duplicates. Lane 1, control (pRC-CMV); lane 2, pRe-CMV + 5 min PDBu; lane 3, PKC-θ A148E; lane 4, PKC-ε A159E; lane 5, PKC-λ A25E; lane 6, PKC-λ A119E. MARCKS phosphorylation was determined by PhosphorImaging of PVDF membranes and data are expressed as the means of at least three independent experiments done in duplicates. B, statistical analysis of experiments described under A. MARCKS phosphorylation was determined by PhosphorImaging of PVDF membranes and data are expressed as the means (±S.E.) of at least three independent experiments done in duplicates.

**Overexpression of Wild-type PKC-α, PKC-ε and Ectopically Expressed PKC-θ Enhances PDBu-stimulated MARCKS Phosphorylation in Intact NIH 3T3 Fibroblasts**—The NIH 3T3 fibroblast clone used in our study was shown to express predominantly PKC-α,-ε, and -λ isoforms (data not shown). Under our experimental conditions used, conventional cPKC-α, nPKC-ε, and novel nPKC-θ accept MARCKS as substrate in intact cells. To further characterize the biological relevance of these MARCKS kinases we have examined the potential role of cell lines overexpressing theses particular PKC isoenzymes. Conventional cPKC-α and novel nPKC-ε were compared with ectopically expressed nPKC-θ on MARCKS phosphorylation. The isoenzyme-specific overexpression was confirmed by Western analysis (Fig. 4) and found to be similar for PKC-α (2.2-fold), PKC-ε (2.6-fold), and PKC-λ (2.0-fold). PKC-θ protein, as expected, was only expressed in cells ectopically transfected with a plasmid encoding PKC-θ.

Consistent with our findings, in the presence of 300 nM PDBu, MARCKS phosphorylation was found to be enhanced in cells overexpressing cPKC-α and nPKC-ε (Fig. 5). Interestingly, ectopically expressed nPKC-θ showed the highest levels of MARCKS phosphorylation implicating nPKC-θ as a potent

**DISCUSSION**

PKC isoenzyme-specific phosphorylation of MARCKS had been studied previously employing cell-free extracts (10, 18). The fact that the molecular mechanisms of the processes leading to PKC activation are still insufficiently understood and that cell lines are expressing several distinct PKC isoenzymes have made it difficult to extrapalate from *in vitro* studies to the situation in *vivo*. Therefore, the involvement of four PKC-
families were tested under physiological conditions. It is demonstrated that in intact cells conventional cPKC-α, novel nPKCs ε, and nPKC-θ, but not, however, atypical aPKC-λ accept MARCKS as a substrate. These conclusions are based on the following data: 1) MARCKS phosphorylation is significantly enhanced following a brief exposure to the phorbol ester PDBu and the PDBu-induced MARCKS hyperphosphorylation is depressed by concentrations of the specific PKC inhibitor GF109203X which have been shown to inhibit the phorbol ester-responsive PKC isoforms α, ε, and θ. At 2 μM GF109203X, MARCKS phosphorylation is reduced to background levels. At this concentration the atypical PKC-λ and -ζ (28) are only partially inhibited. Although PKC-λ and -ζ are phorbol ester non-responsive (30, 35), an implication of these isoenzymes after phorbol ester treatment cannot a priori be excluded. It has been suggested that c- or n-type PKC isoforms upon activation by phorbol esters may stimulate phospholipase D, phosphoinositol 3-kinase, or phospholipase A₂, which in turn could activate PKC-ζ or λ (26, 36–38). The dose-response relationship shown in Fig. 1 argues against an implication of atypical PKC isoenzymes in PDBu-induced MARCKS phosphorylation. 2) The conclusion that in intact NIH 3T3 fibroblasts, PKC-α and -ε are capable of phosphorylating MARCKS is further supported by the results obtained with kinase-dead DN mutants. Both, the DN PKC-α K368R as well as DN PKC-ε K436R mutants significantly reduce the PDBu-induced MARCKS phosphorylation. As PKC-θ is not expressed in these cells, the DN PKC-θ K409R mutant should not be able to affect PDBu-induced MARCKS phosphorylation which is indeed the case.

PKC isoenzymes are located in different subcellular locations and/or compartments in a given cell, therefore prepositioning of PKCs in resting cells may be the key determinant in substrate phosphorylation, e.g., that the total amount and/or the accessibility of MARCKS molecules in a PKC isoenzyme relevant subcellular location, but not simple substrate competition, could be the prerequisite of a DN PKC isoenzyme-specific inhibition of MARCKS phosphorylation. 3) In order to eliminate pleiotropic effects of the phorbol ester, we employed CA PKC mutants. In accordance with the conclusions drawn so far, expression of the constitutively active mutants of PKC-α, -ε, and -θ, respectively, were found to enhance MARCKS phosphorylation also in the absence of PDBu. Addition of PDBu, however, further enhances the level of MARCKS phosphorylation (data not shown) which may be due to a submaximal activation status of type proteins (19). Alternatively and perhaps more likely, this phenomenon may simply be due to the fact that exposure to PDBu leads to an activation of all PDBu-responsive MARCKS-phosphorylating PKC isoenzymes. Consequently, only part of the total activity can be obtained by expressing one of these isoenzymes as a constitutively active form. 4) These first hints suggesting an implication of PKC-α, -ε, and -θ in MARCKS phosphorylation are further substantiated by studies with cell lines overexpressing the individual PKC isoforms. Ectopically expressed PKC-θ, the closest relative to PKC-δ, which was found to be predominantly expressed in hematopoietic cell lines and skeletal muscle (39) proved to be an especially active MARCKS kinase, although as judged from Western blots, the expression level of PKC-θ was lower to the levels obtained for PKC-α and -ε overexpressing cells. The total increase in MARCKS phosphorylation obtained by the overexpression of PKC-α, -ε, and -θ is relatively small. It should be considered, however, that MARCKS is sequentially phosphorylated on serine residues in the order serine, Ser-156 > Ser-163 > Ser-153 (10). No isoenzyme-specific major differences have been reported with regard to the sequential phosphorylation of the MARCKS protein. 5) In contrast to all the data demonstrating that the α, ε, and (ectopically expressed) θ isoforms of PKC are implicated in MARCKS phosphorylation in intact fibroblasts, there is so far no evidence for MARCKS as a substrate of PKC-λ. As a matter of fact, all studies conducted to reveal MARCKS phosphorylation by PKC-λ yielded negative results. Studies with PKC-λ are hampered by the fact that so far no exogenous stimulating agonist of this isotype has been described. Intracellularly, PKC-λ has been reported to be regulated by a λ-interacting protein (23). The mechanisms by which λ-interacting protein is regulated are, however, still obscure. Diaz-Meco and co-workers (22) recently demonstrated that overexpression of PKC-λ in COS-1 cells or NIH 3T3 fibroblasts leads to a transcriptional activation of a NPV-driven reporter plasmid. It is shown here that even the expression of a CA PKC-λ mutant does not significantly alter the phosphorylation of MARCKS. The data on MARCKS phosphorylation in intact cells described here are in excellent agreement with studies obtained with isolated PKC isoenzymes in cell-free assays (10, 18). In vitro, cPKC-α, cPKC-β1, nPKC-δ, and nPKC-ε but not a PKC-ζ were identified as enzymes that accept...
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MARCKS as a substrate. PKC-δ which exhibited the highest rate of MARCKS phosphorylation in vitro shares 75% amino acid homology with PKC-θ (39), an isoform so far not investigated with regard to its ability to phosphorylate MARCKS. PKC-λ which has been shown to be expressed in NIH 3T3 cells (30), exhibits 72% amino acid sequence homology with PKC-ζ (30). Within the kinase (C3) region the identities between PKC-λ and PKC-ζ are even 86% (30). In view of this homology it is not surprising that both PKC isoenzymes show overlapping substrate specificities. Indeed, both isoenzymes have been implicated to stimulate the transcription of a NFκB-driven reporter plasmid, and are obviously unable to phosphorylate MARCKS. The major differences between PKC-λ and PKC-ζ are to be found in the regulatory zinc finger domain explaining differential mechanisms of activation of the two enzymes (23).

The functional divergence of PKC isoenzymes provides a rational to further explain the presence of multiple PKC family members in a given cell. Furthermore, it will permit detailed functional dissection of the complex signal transduction cascades involving distinct PKC family members. Undoubtedly more work is necessary to determine the precise mechanism utilized by PKC isoenzymes to phosphorylate the myristoylated alanine-rich protein kinase C substrate MARCKS, but our data represent an important step toward the identification of PKC isoenzymes involved in MARCKS regulation in vivo.

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