Specificity of the High Affinity Interaction of Protein Kinase C with a Physiological Substrate, Myristoylated Alanine-rich Protein Kinase C Substrate*

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Although myristoylated alanine-rich C kinase substrate (MARCKS), has been employed as an indicator for the activation of protein kinase C (PKC) in intact cells, little is known about its specificity for PKC family members. To address this question, we partially purified human MARCKS from baculovirus-infected cells and compared the kinetic parameters for phosphorylation of intact MARCKS, nPKCs, and atypical PKC (aPKC), all of which are distributed in a wide variety of cells. cPKCs, nPKCs, and aPKCs efficiently phosphorylated intact MARCKS protein in vitro. The affinity of MARCKS for cPKCs, nPKCs, and aPKCs was extremely high and decreased in the order cPKC > nPKC > aPKC, with K_m values of 10.7, 20.7, and 29.8 nM, respectively. The rate of phosphorylation also decreased in the same order. In contrast, aPKC did not phosphorylate MARCKS efficiently, and we were unable to estimate the kinetic parameters. These results suggest that cPKCs, nPKCs, and aPKCs not nPKCs are enzymes that phosphorylate MARCKS in response to PKC activators in intact cells. The structural requirements of MARCKS for efficient phosphorylation by these PKC members were then examined using a peptide that surrounds the phosphorylation site of MARCKS. Interestingly, intact MARCKS showed a 90-150 times lower rate of phosphorylation by PKCs compared with peptide MARCKS, whereas the former showed a 40-180 times higher affinity for these PKC members. This implies that intact MARCKS protein retains a high affinity for PKC with the sacrifice of its phospho-accepting activity. The structural requirements of PKC were then examined using a calpain-cleaved active fragment of nPKCs. MARCKS was phosphorylated by the active catalytic fragment as efficiently as by intact nPKCs, indicating that the kinase domain is sufficient for the high affinity interaction with intact MARCKS. However, gel overlay assay revealed that both intact nPKCs and its regulatory domain bind to MARCKS, suggesting that both the kinase and regulatory domains of nPKCs are involved in the high affinity interaction with intact MARCKS protein.

Protein kinase C (PKC) is a serine/threonine kinase that is thought to play roles in diverse cellular processes such as the secretion of hormones and neurotransmitters and the regulation of cell proliferation and differentiation through its action in intracellular signal transduction pathways (1, 2). PKC constitutes a large protein family whose members can be classified into three subgroups. The first comprises conventional PKC (cPKC) members, including cPKCa, b/II, and g, which are regulated by Ca++ diacylglycerols, phospholipids, and tumor-promoting phorbol esters (2-7). The second class consists of novel PKC (nPKC) members, including nPKCa, e, -, and -, which are characterized by their Ca++-independent PKC activity (2, 8-13). The final class comprises atypical PKC (aPKC) members, including aPKCdA (14, 15), - (16), and - (17) and have kinase activities that are independent of diacylglycerol and phorbol esters (15, 16, 18). The PKC polypeptide consists of an N-terminal regulatory domain and a C-terminal catalytic domain; cofactor binding sites and the pseudosubstrate site have been identified in the regulatory domain (1). The fact that multiple PKC molecules are expressed in a single cell (19, 20) suggests the presence of a complex cellular signaling system, although the molecular steps between the activation of PKC and the resultant biological response as well as the specific substrates for each PKC remain to be clarified. One approach to elucidating the molecular pathways is to clarify the specific cellular substrates of each PKC. On the other hand, monitoring the phosphorylation of common PKC substrates will reveal when and where PKC is activated.

Myristoylated alanine-rich C kinase substrate (MARCKS) (21-27), was originally identified as a protein (termed "SOHC") transiently phosphorylated upon treatment of cells with phorbol esters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (28), an activator of PKC. cPKC mixtures phosphorylate MARCKS protein purified from both chicken and bovine brain at specific sites in vitro (29), and a synthetic peptide that includes these phosphorylation sites is a substrate for PKC (30). Neither the phosphorylation site peptide nor intact MARCKS

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The abbreviations used are: PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; aPKC, atypical PKC; MARCKS, myristoylated alanine-rich C kinase substrate; TPA, 12-O-tetradecanoylphorbol-13-acetate; PS, phosphatidylycerine; PAGE, polyacrylamide gel electrophoresis.

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is a good substrate for any other protein kinases tested, including cAMP- and cGMP-dependent protein kinases or calmodulin-dependent protein kinases I, II, and III (31). Recently it was reported that MARCKS (purified from bovine brain) binds to cPKCa in overlay assay (32). MacMARCKS/S21 (33–35), a second member of the MARCKS family, also binds to cPKCa in both interaction cloning and overlay assay (36). These findings indicate that MARCKS is a true substrate for PKC in vivo and in vitro. On the other hand, MARCKS is widely distributed and contains three distinct domains: an N-terminal myristoylated domain that mediates binding to membranes, a highly conserved MH2 (MARCKS homology 2) domain of unknown function, and a basic effector domain containing the PKC phospho-

In this paper, we investigate the specificity of PKC members by determining the kinetic constants for cPKCa, nPKCa, nPKCe, and aPKCC using both partially purified human MARCKS and a synthetic oligopeptide of the MARCKS phosphorylation domain (peptide MARCKS) as substrates. We show that MARCKS is a good substrate in vitro for cPKCa, nPKCa, nPKCe, but not for aPKCC. PKC shows a higher affinity for intact MARCKS than for peptide MARCKS. Furthermore, the high affinity between MARCKS and PKC involves both the regulatory and catalytic domains of PKC.

EXPERIMENTAL PROCEDURES

Materials—MARCKS-specific antibody is a mixture of several monoclonal antibodies raised against human MARCKS. The baculovirus transfer vector (pMY102) and an insect cell line Spodoptera frugiperda (Sf21) were provided by Dr. Max D. Summers, Texas A&M University.

Wild-type Autographa californica nuclear polyhedrosis virus was obtained from Invitrogen. Chicken m-calpain was kindly provided by Dr. Koichi Suzuki, Institute of Molecular and Cellular Biosciences, University of Tokyo.

Peptide MARCKS, the MARCKS phosphorylation site peptide (KKKKRRFSFKSFKLGSFKNNK), was synthesized and purified as described previously (38, 40). Peptide α (STASQDVANRFARKGSL-RQKNN) corresponding to the cPKCa pseudosubstrate site with an alanine to serine substitution, was a gift from Dr. Tatsuya Tamaoki, University of Tsukuba, Japan. Chicken m-calpain was kindly provided by Dr. Koichi Suzuki, Institute of Molecular and Cellular Biosciences, University of Tokyo.

Restriction endonucleases and enzymes for molecular biology were purchased from Takara (Kyoto, Japan) or New England Biolabs. [γ-32P]ATP was obtained from Amersham Corp. Phosphatidylserine (PS) was from Funakoshi; TPA was from Sigma; and 2-mercaptoethanol was from Nacalai Tesque.

Preparation and Isolation of Recombinant MARCKS Baculovirus—The 1.8-kilobase fragment encoding S21 amino acids of human MARCKS (27) was inserted into the Npl site of the transfer vector, pMY102, after blunt ending. For the production of recombinant virus, Sf21 cells were coinfected with the wild-type A. californica nuclear polyhedrosis virus DNA supplied by the MARCKS-containing transfer plasmid using the liposome-mediated method (41). Recombinant viral plaques were selected by their resistance to amphotericin B, and the expression of the lacZ that was inserted into the pMY102 expression vector. Purified extracellular virus was obtained after five rounds of plaque purification.

Purification of MARCKS from Infected Sf21 cells—Sf21 cells (4 × 10⁶) were infected at room temperature with MARCKS virus with a multiplicity of infection of 10. Postinfection, the viral supernatant was removed, and the cells were cultured in 400 ml of supplemented Grace’s insect cell medium at 27 °C. MARCKS was partially purified based on the procedure of Patel and Kligman (42). Briefly, 3 days after infection, a cell pellet was obtained by centrifugation at 1000 × g for 5 min and resuspended in 38 ml of homogenization buffer A containing 20 mM Tris·HCl (pH 7.5), 5 mM EDTA, 5 mM EGTA, 0.25 mM sucrose, 5 mM dithiothreitol, 0.001% leupeptin, 0.002% pepstatin A, 0.001% chymostatin, and 0.001% aprotinin. The cells were microcentrifuged at 350,000 × g for 30 min. The supernatant was heated at 80 °C for 15 min, immediately cooled on ice, and recentrifuged at 26,000 × g for 45 min. The supernatant fraction was applied to a Mono Q column (Pharmacia Biotech Inc.) eluted with a linear gradient of 0–0.5 M NaCl in buffer B containing 20 mM Tris·HCl (pH 7.5), 2 mM EDTA, 2 mM dithiothreitol at a flow rate of 1 ml/min. MARCKS eluted from the column as a broad peak between 0.2 and 0.3 M NaCl. The fractions containing MARCKS were subjected to SDS-PAGE. The gels were stained with Coomassie Blue, and MARCKS protein was quantified by densitometry. The purity of MARCKS was about 20% and 92% of partially purified MARCKS could be obtained from the cytosolic fractions. In the phosphorylation assay (described in the next section) with cPKC prepared from rabbit brain, MARCKS was phosphoproprotein and detected as a single major band at 80 kDa; no MARCKS phosphorylation could be detected in the absence of PKC.

Purification of PKC Isozymes—PKC isoforms (cPKCa, nPKCa, nPKCe, and aPKCC) were highly purified from recombinant baculovirus-infected Sf21 cells.² In brief, Sf21 cells were infected with a recombinant PKC virus (encoding rabbit cPKCa, mouse nPKCa, rabbit nPKCe, or mouse aPKCC). Three days after infection, the cell pellets were extracted, and the cytosolic fractions were purified by a series of chromatographic steps including DEAE-cellulose (Toyobio), hydroxypatite (Koken), and Mono Q column chromatographies for cPKCa, nPKCa, and nPKCe and DEAE-cellulose, heparin (Toyos, and phenyl (Pharmacia) column chromatographies for aPKCC. The isolated proteins were pure as judged by SDS-PAGE. The specific activities were 31,106 units/mg for cPKCa, 1830 units/mg for nPKCa, 1450 units/mg for nPKCe, and 140 units/mg for aPKCC. Units of PKC activity were defined as 1 nmol of P⁰ incorporated into peptide α per min under the conditions described below.

MARCKS Phosphorylation by PKC—The standard phosphorylation assay contained 20 mM Tris·HCl (pH 7.5), 5 mM MgOAC, 0.01% leupeptin, 50 ng/ml CaCl₂, 50 ng/ml TPA, 0.5% protein kinase C (PKC) enzyme (cPKCa, 0.08 ng; nPKCa, 0.74 ng; nPKCe, 1.50 ng; aPKCC, 5.30 ng), and various concentrations of MARCKS in a total volume of 20 pl. The phosphorylation assay by nPKC was performed in the absence of Ca²⁺, and the aPKCC assay was done in the absence of Ca²⁺ and TPA. The reaction was initiated by the addition of 20 ng of ATP and 0.5 nmol [γ-32P]ATP and was continued for 10 min at 30 °C. The reaction was stopped by the addition of 5 pl of 5 × Laemmli’s SDS-sample buffer, and 8% SDS-PAGE was performed (43). The radioactivities of the 80 kDa bands corresponding to MARCKS protein were then analyzed by phosphorimage analysis of XRAY BAS 2000 Bioimage analysis software. MARCKS or synthetic peptides by PKC was linear between 0 and 30 min with all substrate concentrations examined. Reactions were carried out for 10 min.

Phosphopeptide Mapping of MARCKS—Peptide mapping by limited proteolysis in SDS was performed as described previously (44). Briefly, MARCKS (12 ng) was phosphopropnein by cPKCa for 30 min as described above. After the first SDS-PAGE, the phospho-

MARCKS bands were excised, and a second 15% SDS-PAGE overlaid with various concentrations of Staphylococcus aureus V8 protease (45) was performed with bands detected by autoradiography.

MARCKS Phosphorylation by the Catalytic Fragment of nPKCa—Proteolysis of nPKCa was performed according to the published procedures (46). The purified nPKCa was treated with 10 ng/ml of chicken m-calpain in 25 mM Tris·HCl (pH 7.5), 5 mM β-mercaptoethanol, 1 mg/ml bovine serum albumin, and 1 mM CaCl₂ in a volume of 100 μl. The reaction was continued at 0 °C for 30 min by the addition of 1 mM leupeptin and 5 mM EGTA. The phosphorylation assay was performed as described above.

Overlay Assay—Overlay assay was performed based on the procedure of Wolf and Sabyoun (47). nPKCa (55 ng) purified from recombinant baculovirus-infected Sf21 cells was coinfected with the phosphorylation assay buffer consisting of 20 mM Tris·HCl (pH 7.5), 5

² Y. Ueda, K. Mizuno, S. Kawamoto, K. Okuda, and S. Ohno, manuscript in preparation.
shown in Fig. 2, the partial digestion of cPKCa-phosphorylated MARCKS with S. aureus V8 protease resulted in the appearance of two or three major bands migrating in the region under 43 kDa; these most likely correspond to the peptides that include the phosphorylated residues determined for the brain PKC preparation (29). Exactly the same pattern of digestion for the other PKCs suggests that all three PKC isoforms phosphorylate MARCKS at the same sites.

To further examine the difference in MARCKS phosphorylation among PKC isoforms, kinetic parameters were determined under conditions where the initial velocity could be determined (Table I). The $K_{m}$ values for the three PKC isoforms, cPKCa, nPKCδ, and nPKCe, increased in the order $K_{m}$ for cPKCa, nPKCa, and nPKCe was clear, and the initial velocity of phosphorylation increased with concentration at MARCKS concentrations less than 20 nM, suggesting that MARCKS is a good substrate for all three PKC isoforms. However, the level of MARCKS phosphorylation by aPKCγ was very low compared with the other three isoforms and was detected only under conditions where the autophosphorylation of aPKCγ was also detected, suggesting that MARCKS is not a good substrate for aPKCγ. As shown in Table I, the $V_{max}$ values for peptide MARCKS and peptide α were similar, but the $V_{max}$ values were very low compared with cPKCa. Thus, it is possible that the inability to evaluate the kinetic constants for MARCKS phosphorylation by aPKCγ might be due to the inefficiency of phosphorylation rather than to MARCKS affinity.

The phosphorylation of MARCKS by cPKC results in the phosphorylation of 3 or 4 residues located close together (29, 50). The similarity in the primary sequences of these isoforms supports the idea that all three PKC isoforms, cPKCa, nPKCδ, and nPKCe, phosphorylate MARCKS at the same amino acid residues. In order to confirm this, we compared the V8 peptide maps of MARCKS phosphorylated by each PKC isoform. As shown in Fig. 2, the partial digestion of cPKCa-phosphorylated MARCKS with S. aureus V8 protease resulted in the appearance of two or three major bands migrating in the region under 43 kDa; these most likely correspond to the peptides that include the phosphorylated residues determined for the brain PKC preparation (29). Exactly the same pattern of digestion for the other PKCs suggests that all three PKC isoforms phosphorylate MARCKS at the same sites.

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Phosphorylation of Peptide MARCKS by PKC Isozymes—To confirm the structural requirements of MARCKS for phosphorylation by PKC isoforms, we next examined the phosphorylation of a synthetic peptide, peptide MARCKS, comprising 25 amino acid residues surrounding the sites phosphorylated by brain PKC (29). Fig. 3 shows the Lineweaver-Burk plot of peptide MARCKS phosphorylation, and Table I summarizes the results. Interestingly, the $K_{m}$ values for all four PKC isoforms, including aPKCγ, were essentially similar with a 5-fold difference ($\epsilon$ versus α). In contrast, the $V_{max}$ values differed clearly among the PKC isoforms, with a difference in $V_{max}$ between cPKCa and aPKCγ of 263-fold. The lowest $K_{m}$ value and the highest $V_{max}$ value were observed for cPKCa, which also phosphorylated intact MARCKS most efficiently. On the other hand, aPKCγ, which almost failed to phosphorylate intact MARCKS, did not phosphorylate peptide MARCKS efficiently. These data suggest that the primary sequence surrounding the phosphorylation sites is involved in determining the specificity. Similar
experiments were also performed using synthetic peptide α. Just like peptide MARCKS, this peptide showed similar \( K_m \) values for all PKC isozymes tested. However, cPKCa was the only isozyme to phosphorylate peptide α efficiently, and the \( V_{\text{max}} \) value for cPKCa was 14 times higher than that for nPKCβ, the next most efficient isozyme (Table I).

A comparison of the kinetic constants for substrate phosphorylation between intact MARCKS and peptide MARCKS revealed an interesting aspect to PKC-MARCKS interaction. Thus, it is quite interesting to know whether the interaction is strong enough to be detected in a binding experiment. In fact, there is a report where MARCKS contains a structure lacking in peptide MARCKS that interferes the phosphorylation reaction. The rates of \(^{32}\)P incorporation into peptide MARCKS (C) and peptide α (●) were determined in the presence of nanogram quantities of PKC, 20 μM of ATP, and the indicated concentrations of peptide substrate. Data are expressed as the double reciprocals of \(^{32}\)P incorporation (nmol) and substrate concentration (μg/ml). The \( K_m \) and \( V_{\text{max}} \) values are shown in Table I.

**Table I**

<table>
<thead>
<tr>
<th>PKC Isozyme</th>
<th>( K_m ) (μM)</th>
<th>( V_{\text{max}} ) (nmol/min/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cPKCa</td>
<td>10.7</td>
<td>14.3</td>
</tr>
<tr>
<td>nPKC β</td>
<td>20.7</td>
<td>5.2</td>
</tr>
<tr>
<td>nPKC ε</td>
<td>29.8</td>
<td>1.7</td>
</tr>
<tr>
<td>nPKC γ</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Fig. 2. Phosphopeptide mapping of MARCKS.** MARCKS phosphorylated by cPKCa and nPKCβ and as in Fig. 1 were analyzed for phosphopeptide as described under “Experimental Procedures.” Digests were performed with 2 μg (lanes 2, 4, and 7), 0.3 μg (lanes 1, 4, and 8), or 0.05 μg (lanes 3, 6, and 9) of **S. aureus** V8 protease. This resulted in the formation of two or three major peptides, which were of identical size among the three PKC isozymes at any protease dose.

**Fig. 3. Lineweaver-Burk plot of peptide MARCKS phosphorylation by cPKCa, nPKC β and α, and nPKC γ.** The rates of \(^{32}\)P incorporation into peptide MARCKS (C) and peptide α (●) were determined in the presence of nanogram quantities of PKC, 20 μM of ATP, and the indicated concentrations of peptide substrate. Data are expressed as the double reciprocals of \(^{32}\)P incorporation (nmol) and substrate concentration (μg/ml). The \( K_m \) and \( V_{\text{max}} \) values are shown in Table I.
beled nPKCG. As shown in Fig. 5, nPKCG bound to intact MARCKS. The interaction required the presence of PS, consistent with the results obtained for the interaction between cPKCa and MARCKS (32). This clearly indicates that the interaction between nPKCG and intact MARCKS is strong enough for the interaction to be detected by the gel overlay assay.

**DISCUSSION**

**MARCKS Is a Good Substrate for cPKCa, nPKC6, and nPKCe But Not for aPKCC**—The kinetics of MARCKS phosphorylation by PKC isoforms indicates that MARCKS is a good substrate for cPKCa, nPKC6, and nPKCe, but not for aPKCC, in vitro. The affinity of MARCKS for cPKCa, nPKC6, and nPKCe is extremely high and decreases in the order $\alpha > \delta > \epsilon$; the rate of phosphorylation also decreases in the same order. The demonstration of binding of nPKCG to MARCKS in the gel overlay assay indicates a very high affinity interaction between PKC and MARCKS.

The results presented in this paper raise the intriguing possibility that the three PKC isoforms other than aPKCC may respond to TPA or physiological PKC activators and phosphorylate MARCKS in intact cells. Nevertheless, the potency of MARCKS phosphorylation differs among the three PKC isoforms, with cPKCa the most potent kinase and nPKC6 the next.

Although the substrate specificities of PKC isoforms for MARCKS protein have not previously been reported, some other proteins have been reported to be differentially phosphorylated by different PKC isoforms (51–54). These reports, however, lack detailed kinetic analyses.

MARCKS has an unusual subcellular distribution. Most myristic acid labeled MARCKS is associated with the plasma membrane in quiescent cells, while most phosphorylated MARCKS is found in the cytosol of activated cells (55); furthermore, MARCKS co-localizes with vinculin and talin in macrophages (56). On the other hand, cPKCa also co-localizes with vinculin and talin in rat embryo fibroblasts (57). These observations suggest that MARCKS targets cPKCa to specific subcellular locations and that together they regulate cytoskeletal functions. Whether nPKCG and nPKCe behave similarly is still unknown. Furthermore, which PKC isoforms phosphorylate MARCKS in intact cells remains to be clarified.

There is a report on the kinetics of MARCKS phosphorylation by PKC (49) where the $K_i$ value of MARCKS (bovine brain) by a cPKC mixture (rat brain) is 0.4 or 0.8 μM (Table II). The difference between these $K_i$ values and ours is possibly due to differences in reaction conditions, the source of PKC, or the source of MARCKS.

Previously reported $K_i$ values for other PKC substrates are summarized in Table II. Among the substrates listed, some are reported to be phosphorylated in intact cells upon phorbol ester stimulation (49, 66–68, 70–72). The $K_i$ values for MARCKS phosphorylation by the three PKC isoforms reported here, for example 10.7 nM for cPKCa, are about 10–100-fold lower than the $K_i$ values of other PKC substrates. This suggests that the affinity of MARCKS for PKC is higher compared with other physiological substrates. There are several reports on the $K_i$ values of other protein kinases for intact proteins. For example, the $K_i$ values of cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase for Troponin are 20.5 and 16.1 μM, respectively (58), and the $K_i$ of Ca$^{2+}$-CaM kinase II for synapsin I is 0.4 μM (49). This implies that all three PKC isoforms, cPKCa, nPKC6, and nPKCe, have extremely high affinities for MARCKS compared with other kinase-substrate interactions. This point will be discussed further below.

**A Model System for the Analysis of the High Affinity Interaction between a Protein Kinase and Its Physiological Substrate Protein**—As described above, it is clear that all three PKC isoforms, cPKCa, nPKC6, and nPKCe, have an extremely high affinity for MARCKS. Furthermore, the overlay assay revealed that nPKCG binds to MARCKS in a PS-dependent manner. This is consistent with a recent report that cPKCa binds to MARCKS and PKC-binding in overlay assay decreases when MARCKS is phosphorylated by PKC (32). Similar results have also been reported for MacMARCKS (36). These observations suggest that the PKC-MARCKS interaction involves at least the MARCKS phosphorylation site.

In this paper, we report that the $K_i$ value for peptide MARCKS, corresponding to the MARCKS phosphorylation site, is much higher than that for MARCKS protein. This indi-
These results imply that both the catalytic and regulatory domains of nPKCα can interact with MARCKS with high affinity.

With regard to peptide substrate phosphorylation by PKC, some kinetic constants have been reported. The $K_m$ values for epidermal growth factor receptor peptide, usually examined as a PKC substrate, are between 1.3 and 234 μM depending on the PKC isoforms and their preparations (9, 59, 60). The $K_m$ values for myelin basic protein peptide and S6 peptide are 2.2 μM (rat and mouse brain PKC) (49) and 0.5 μM (rat brain PKC) (61), respectively. On the other hand, it has been reported that the $K_m$ value for peptide MARCKS (rat brain PKC) is 0.02 μM (31), widely different from our $K_m$ value of 0.95 μM (recombinant cPKCa from baculovirus infected cells). The different source of PKC may be the cause of this difference; however, we determined the $K_m$ value for peptide MARCKS phosphorylation by rabbit brain PKC (a mixture of cPKCa and bPKC), and the result was in the micromolar range (data not shown). In addition, there was no such difference in the $K_m$ values under various reaction conditions. These results suggest that the difference in the $K_m$ values between the earlier report and ours could be due to an unknown factor other than PKC source or reaction conditions. It is important that the $K_m$ values for peptide MARCKS by cPKC and nPKC were about 90–150-fold higher than the values for intact MARCKS when determined under the same reaction conditions.

**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PKC source</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol phosphate/min)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>87-kDa protein (MARCKS)</td>
<td>Rat and mouse brain</td>
<td>0.8</td>
<td>ND</td>
<td>49</td>
</tr>
<tr>
<td>Myelin basic protein</td>
<td></td>
<td>0.4</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>MARCKS</td>
<td>PKM</td>
<td>0.1</td>
<td>ND</td>
<td>62</td>
</tr>
<tr>
<td>MRP</td>
<td></td>
<td>0.24</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>IRE-BP</td>
<td></td>
<td>0.7</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>HIV-1 gag protein</td>
<td>Rat brain</td>
<td>4</td>
<td>ND</td>
<td>64</td>
</tr>
<tr>
<td>rprotein</td>
<td>Rabbit kidney or brain</td>
<td>6</td>
<td>ND</td>
<td>65</td>
</tr>
<tr>
<td>Microtubule-associated protein-2</td>
<td>Rabbit kidney</td>
<td>0.9</td>
<td>ND</td>
<td>66</td>
</tr>
<tr>
<td>Glycogen synthase</td>
<td>Rat brain</td>
<td>0.25 (mg/ml)</td>
<td>ND</td>
<td>67</td>
</tr>
<tr>
<td>Inositol trisphosphate 5′-phosphomonoesterase</td>
<td>Rat brain</td>
<td>17</td>
<td>28 (pmol phosphate/min)</td>
<td>68</td>
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<tr>
<td>Troponin I</td>
<td>Pig brain</td>
<td>1</td>
<td>22 (pmol phosphate/min)</td>
<td>69</td>
</tr>
<tr>
<td>Complement factor C3</td>
<td>Pig spleen</td>
<td>12</td>
<td>28 (pmol phosphate/min)</td>
<td></td>
</tr>
<tr>
<td>Histone H1</td>
<td></td>
<td>13</td>
<td>33 (pmol phosphate/min)</td>
<td></td>
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<tr>
<td>DNA topoisomerase II</td>
<td>Rat brain</td>
<td>2.6</td>
<td>ND</td>
<td>70</td>
</tr>
<tr>
<td>Histone</td>
<td>Rat brain</td>
<td>0.1</td>
<td>ND</td>
<td>71</td>
</tr>
<tr>
<td>Myelin basic protein</td>
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<td>2.1</td>
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<td>72</td>
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<tr>
<td>Troponin I peptide</td>
<td>Pig brain</td>
<td>2.8</td>
<td>0.91</td>
<td></td>
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<tr>
<td>Histone</td>
<td>Rat brain</td>
<td>6.9</td>
<td>1.46</td>
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<tr>
<td>Histone</td>
<td>Rat brain</td>
<td>6.7</td>
<td>0.91</td>
<td></td>
</tr>
</tbody>
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* ND, not determined.
* PKM, the Ca2+ and phospholipid-independent catalytic fragment of PKC.
* MRP, MARCKS-related protein.
* IRE-BP, iron-responsive element-binding protein.

...and some structure in intact MARCKS other than the phosphorylation sites is required for the high affinity interaction between PKC and MARCKS. Since the phosphorylation reaction we employed included PS in addition PKC and MARCKS (or peptide MARCKS), it is possible that the apparent difference in the $K_m$ value between intact MARCKS and peptide MARCKS reflects the difference between the actual concentration of the substrates. In other words, PS might bind and sequester peptide MARCKS and lower its actual concentration. If this is the case, the apparent $K_m$ value for peptide MARCKS is overestimated, and our conclusion must be reconsidered. To exclude this possibility, we determined the $K_m$ value for peptide MARCKS in the presence or absence of PS using an active catalytic fragment of nPKCα. In the absence of PS, the $K_m$ value for peptide MARCKS was about 5-fold lower than that obtained under conditions including PS (data not shown). This suggests that the presence of high concentrations of lipid may interfere with the PKC-peptide MARCKS interaction; however, considering these effects, the apparent $K_m$ value for intact MARCKS protein is still much lower than that for peptide MARCKS. Thus, the observed difference in the $K_m$ values most likely reflects a difference in the affinities of these substrates for PKC, or at least for nPKCα.

The $K_m$ and $V_{max}$ values for MARCKS phosphorylation by the catalytic fragment were 1.3- and 3-fold higher than those for full-length nPKCα, respectively (Fig. 4). This suggests that the catalytic domain of PKC is sufficient for the high affinity interaction between PKC and MARCKS. We have recently expressed in *Escherichia coli* the regulatory domain of nPKCα (amino acid residues from position 1 to 298) as a myelin basic protein-nPKCα fusion protein. A gel overlay assay similar to that used in the present study using the myelin basic protein-nPKCα as a probe with the aid of anti-myelin basic protein antibodies revealed that the regulatory domain of nPKCα as well as whole nPKCα can also interact with MARCKS in a PS-dependent manner. These results imply that both the catalytic and regulatory domains of nPKCα can interact with MARCKS with high affinity.

Based on the above observations, we can describe the nature of the PKC-MARCKS interaction as shown in Fig. 6. Intact MARCKS has a higher affinity for PKC than peptide MARCKS. This difference may depend on some specific structural feature of MARCKS that is lacking in peptide MARCKS. In PKCs, both the regulatory and catalytic domains are involved in the high affinity interaction with MARCKS. When MARCKS is phosphorylated, the interaction between PKC and the region surrounding the MARCKS phosphorylation sites decreases, and PKC dissociates from the phosphorylated substrate. According to this model, the apparent paradox in the two kinetic parameters between peptide MARCKS and intact MARCKS can be explained as follows. The higher $V_{max}$ value for peptide MARCKS reflects the efficient dissociation of the product, while the lower $V_{max}$ value for intact MARCKS reflects its ad-

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* Y. Izumi, S. Hirai, Y. Nishimura, and S. Ohno, manuscript in preparation.
themselves rather than to amplify signals through efficient MARCKS phosphorylation in cells. These PKC isozymes might bind to MARCKS in order to localize PKC with its substrate might be accomplished at the sacrifice of efficiency of the phosphorylation reaction.

It is generally believed that a "kinase" phosphorylates many substrates and amplifies signals. However, the interaction between kinase and substrate (or binding protein) could have multiple functions other than efficient phosphorylation. Considering the high affinity interaction and low phosphorylation efficiency of cPKC and nPKC for MARCKS protein, these PKC isoforms might bind to MARCKS in order to localize themselves rather than to amplify signals through efficient MARCKS phosphorylation in cells.

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REFERENCE


