

In vitro activation and substrates of recombinant, baculovirus expressed human protein kinase C μ

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Abstract To study enzymatic activity and activation conditions of the recently identified novel protein kinase C μ (PKC μ) subtype, epitope tagged PKC μ was propagated in the baculovirus expression system and was purified to homogeneity. PKC μ displays high affinity phorbol ester binding ($K_d = 7$ nM) resulting in enhanced phosphatidylserine-dependent kinase activity. From various lipid second messengers known to activate PKCs only diacylglycerol and PtdIns-4,5-P₂ were found to promote PKC μ kinase activity. Two peptides derived from the glycogen synthase, GS-peptide and syntide 2, were found to be phosphorylated efficiently in vitro. MARCKS (myristoylated alanine-rich C-kinase substrate) served as an in vitro substrate for PKC μ too. However, in contrast to other PKCs, a peptide derived from the MARCKS phosphorylation domain is phosphorylated only at serine 156, and not at serines 152 and 163, implicating a differential regulation by PKC μ .

Key words: Protein kinase C μ ; Phorbol ester binding; Baculo expression; Activation condition; MARCKS phosphorylation

1. Introduction

Protein kinases C (PKC) define a family of serine/threonine specific kinases considered as important regulatory enzymes in multiple cellular responses. They are activated by lipid second messengers, predominantly diacylglycerol [1], in response to various extracellular agonists like hormones, neurotransmitters, growth factors and cytokines (for reviews see [2–4]). So far, 11 PKC isoforms have been characterized at the molecular level. Based on the primary structure and in vitro activation requirements, the PKC family can be grouped into three major classes: Ca²⁺-dependent, conventional PKCs; Ca²⁺-independent novel PKCs and atypical PKCs. At present, the understanding of the physiological role of the various PKC members is still limited. However, despite the fact that in vivo substrates are largely unknown, tissue specific expression and differential intracellular location suggest distinct functions of individual PKC isotypes in signal transduction and cellular metabolism [5].

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Abbreviations: PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; PtdIns-4,5-P₂, 1- α -phosphatidyl-D-myo-inositol-4,5-bisphosphate; PMSF, phenylmethylsulfonylfluoride; PS, 1- α -phosphatidyl-L-serine; DAG, 1,2-dioctanoyl-sn-glycerol; MARCKS, myristoylated alanine-rich C-kinase substrate; L-PC, 1- α -lysophosphatidylcholine; PA, 1- α -dipalmitoyl phosphatidic acid; AA, arachidonic acid; Cer, C₁₆-ceramide; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

We previously reported the cloning and characterization of a novel PKC subtype named PKC μ [6]. The lack of a typical pseudosubstrate site as well as the presence of two unique amino-terminal hydrophobic domains together with the unusually large size of the molecule are characteristic features of this PKC isozyme. Furthermore, the presence of a pleckstrin homology (PH) domain [7] in the regulatory region of PKC μ is so far unique within the PKC family. Moreover, expression and functional analysis of the homologous mouse gene, termed PKD [8], showed an atypical substrate specificity in vitro [9]. Taken together, these findings suggest that PKC μ /PKD might constitute a further PKC subgroup with cellular functions distinct from the already known PKC subtypes.

Recombinant PKCs, produced in the baculovirus system have been shown to be valuable tools for the characterization of enzymatic properties like activation conditions, in vitro substrate phosphorylation and in defining selective kinase inhibitors [10,11]. We here describe the expression, purification and biochemical characterization of PKC μ expressed in insect cells.

2. Materials and methods

2.1. Construction and cloning of PKC μ c-myc tagged recombinant baculoviruses

Two oligonucleotides representing the complementary strands of the c-myc epitope were cloned into the *Sma*I restriction site of the pCDM8 [12] vector containing the human PKC μ cDNA devoid of the 3'-untranslated region. This results in c-myc tagging of PKC μ carboxy-terminal of the predicted protease cleavage site of the leader peptide and amino-terminal of the putative transmembrane domain [6]. The c-myc PKC μ cDNA was isolated as a 3.2 kb *Xho*I/*Nsi*I fragment and, after filling up 5'-overhanging ends with T4 DNA polymerase, ligated into the baculotransfer vector pVL 1392 linearized with *Sma*I. pVL1392/PKC μ was transfected into Sf158 cells using linearized and modified BACULO GOLD Baculovirus DNA (Pharmingen) according to the manufacturers instructions and standard procedures [13]. Equivalents of 40,000 cells were analysed by Western blot analysis with a monoclonal anti c-myc antibody (Cambridge Research Biochemicals), diluted 1:1000 in PBS using an alkaline phosphatase based detection system.

2.2. Purification of baculovirus produced PKC μ by affinity chromatography

10⁸ infected insect cells were lysed in 10 ml extraction buffer (20 mM Tris-HCl pH 7.5, 5 mM EDTA, 5 mM EGTA, 500 nM PMSF, 100 μ g/ml leupeptin, 20 μ g/ml trypsininhibitor, 200 μ g/ml iodineacetamide) by sonification (20 \times 1 s). Cell debris was removed by centrifugation for 10 min at 100,000 \times g and the supernatant passed through a 0.2 μ m sterile filter. For affinity chromatography, a PKC μ antibody column (3 mg monoclonal PKC μ JP1 antibody [14] coupled to 2 ml activated sepharose according to standard protocols) was equilibrated with buffer A (20 mM Tris-HCl pH 7.5, 5 mM EDTA, 5 mM EGTA) at a flow rate of 1 ml/min, followed by the application of the cell

extract at a flow rate of 0.3 ml/min. The column was washed with 10 column volumes buffer A at a flow rate of 1 ml/min and elution was performed either with 5–10 column volumes buffer B (buffer A; pH 10.5), collected in 1 ml fractions containing 1/15 volume of 1 M HEPES pH 6.8 to neutralize the samples or with 5–10 column volumes buffer C (3 M MgCl₂, 20 mM Tris-HCl pH 7.5). The fractions were tested for their content of PKC μ by SDS-PAGE followed by silver staining. MgCl₂ was removed by gel filtration or dialysis against 20 mM Tris-HCl, pH 7.5. A typical preparation yielded approximately 100–200 μ g of PKC μ . After adding 10% glycerol, purified PKC μ was stored in aliquots at -20°C . PKC β 1 was isolated as described [15].

2.3. [³H]PDBu binding

100 ng of purified PKC μ (= 10 μ l) or supernatant, containing 25 μ g total protein and an equivalent amount of PKC μ protein (as estimated by Western blot analysis), were used for the binding assay by incubating 1 h at 4°C in a total volume of 100 μ l. The mixture contained 20 mM Tris-HCl, pH 7.5, 12 mM MgCl₂, 5 mM EGTA, 1 mg/ml BSA, 100 μ g/ml phosphatidylserine and the indicated amounts of [³H]PDBu (15.1 Ci/mMol, Amersham). Free [³H]PDBu was removed by rapid filtration through glass fiber filters, followed 10 times washing with 20 mM Tris-HCl, pH 7.4, 10 mM MgSO₄, 1 mM CaCl₂ and counting by digital autoradiography (Berthold Digital Autoradiograph). Specific binding represents the difference between total binding and non-specific binding, measured in the presence of unlabelled 100 μ M PDBu. Scatchard analysis was calculated using a modified Excel (Microsoft) program.

2.4. Substrate and autophosphorylation of PKC μ

Substrate phosphorylation of PKC μ was measured in a modified Triton X-100 mixed micellar assay as described [16] using affinity purified enzyme. Peptides were used at a concentration of 60 μ M, histone H1S, myelin basic protein and myosine at concentrations of 200 μ g/ml. Extracts (5 μ g total protein per assay) of PKC μ transfected Sf158 cells were used to estimate cofactor dependence of kinase activity. The concentrations of cofactors used are indicated in the figure legend. Assays were carried out as described for the purified enzyme. L- α -lysophosphatidylcholine and L- α -dipalmitoyl phosphatidic acid were purchased from Sigma. Semisynthetic C₁₆-ceramide was purchased from Biomol. L- α -phosphatidyl-D-myo-inositol-4,5-bisphosphate (PtdIns-4,5-P₂) was purchased from Boehringer Mannheim. Autophosphorylation of PKC μ was carried out using 50 ng of the purified enzyme, fractionated by SDS-PAGE and visualized by autoradiography. Two-dimensional phosphopeptide mapping was carried out as described [16].

3. Results

3.1. Expression and purification of c-myc epitope tagged PKC μ in Sf158 cells

An amino-terminal epitope tagged derivative of the PKC μ cDNA was constructed to facilitate detection of the cDNA gene product independent of endogenous PKC μ by epitope-tag specific antibodies. The epitope EQKLISEEDL derived from the human c-myc gene, is recognized by the monoclonal antibody 9E10 [17]. Functional expression of intact c-myc tagged PKC μ was analysed by kinase assays in immunoprecipitates of PKC μ , obtained with the 9E10 c-myc antibody, after transient expression in COS cells. As shown in Fig. 1 (lane 1 versus lane 2) a specific protein band of approximately 120 kDa representing the autophosphorylated PKC μ , was detectable in PKC μ transfectants but not in vector control transfectants. The obtained c-myc tagged PKC μ showed a slightly reduced electrophoretic mobility upon SDS-PAGE, corresponding to an approximate 5 kDa increase in molecular mass, compared to endogenous PKC μ from HepG2 cells (data not shown).

To express epitope tagged PKC μ in Sf158 cells, a PKC μ cDNA fragment was cloned into the baculotransfer vector

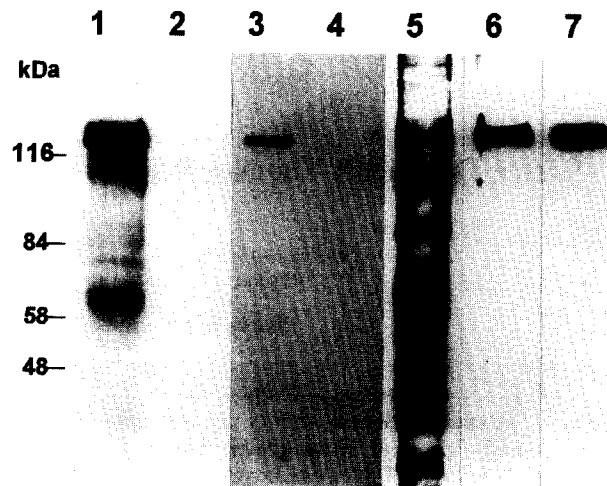


Fig. 1. Expression and purification of c-myc epitope tagged PKC μ . lanes 1 and 2: Autophosphorylation assay of immunoprecipitates with the anti c-myc 9E10 antibody from COS cells transiently expressing PKC μ (lane 1). Lane 2, vector control. Lanes 3 and 4: Western Blot detection with the 9E10 antibody of PKC μ present in baculovirus infected (lane 3) and uninfected (lane 4) Sf158 cells. Lane 5: Silver staining of extracts from Sf158 cells infected with recombinant PKC μ -baculovirus. Lane 6: Silver staining of affinity purified PKC μ . Lane 7: Autophosphorylation of affinity purified PKC μ . Autoradiographs from lane 1, 2 and 7 were exposed overnight.

pVL 1392, transfected into Sf158 cells and analysed for PKC μ expression. From lysates equivalent to 40,000 cells, specific signals were detectable by Western blot analysis with the monoclonal 9E10 anti c-myc antibody (Fig. 1, lane 3), the PKC μ specific antibody JP1 [14] as well as with a PKC μ rabbit antiserum (data not shown). No immunoreactive material could be detected in non-transfected Sf158 cells (Fig. 1, lane 4).

For further biochemical studies it was desirable to obtain highly purified PKC μ protein. Therefore, a purification protocol based on affinity chromatography with the PKC μ specific antibody JP1 was established. This approach yielded highly purified PKC μ in a fast, single step purification procedure. The purity of the preparation was assessed by silver staining indicating that PKC μ could be eluted to greater than 90% purity (Fig. 1, lane 6 versus lane 5). Purified PKC μ retained its kinase activity as shown by in vitro autophosphorylation (Fig. 1, lane 7) and was used for further biochemical analyses.

3.2. Purified PKC μ binds phorbol ester

The aminoterminal region of PKC μ contains two conserved cysteine-clusters with the characteristic HX₁₂CX₂CX_n-CX₂CX₄HX₂CX₇C consensus sequence, typical for all members of the PKC family. These domains represent the structural motifs responsible for high affinity phorbol ester binding [10]. In distinction to all other PKC subtypes with two cysteine domains which are separated by 15–20 amino acids, PKC μ shows a unique spacing of 80 amino acids of its cysteine domains. In our initial studies, only a weak binding of phorbol esters in cellular extracts of HeLa and COS PKC μ transfectants could be detected, questioning a direct role of phorbol ester in PKC μ activation [6]. However, as a significant phosphatidylserine/phorbol ester dependent stimulation of PKC μ autophosphorylation could be subsequently demon-

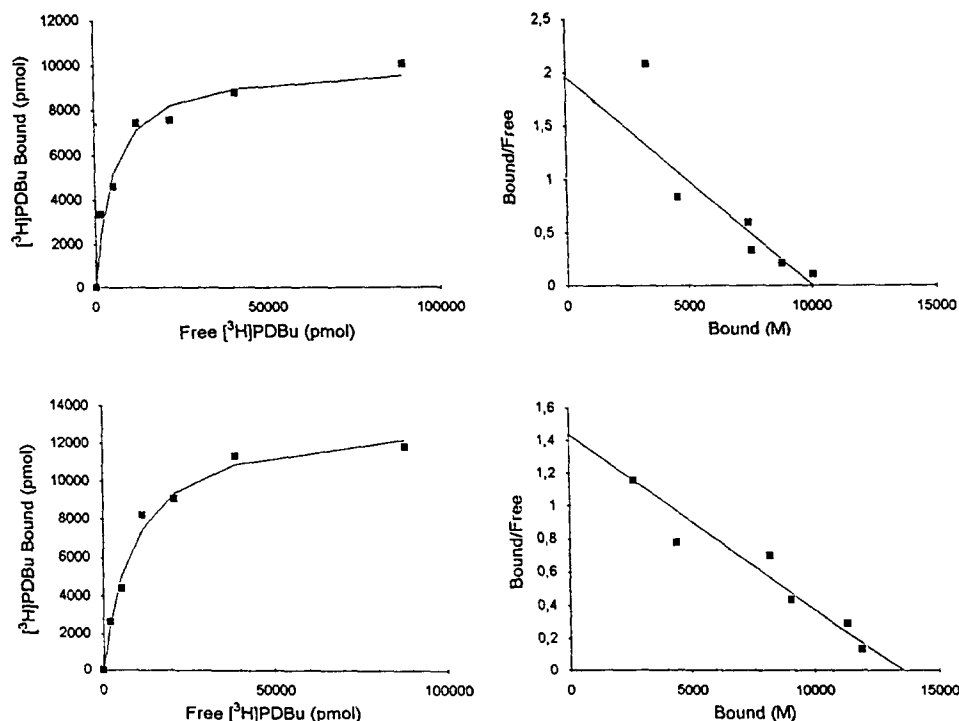


Fig. 2. Phorbol ester binding of PKC μ . Left panels show dose-response curves, right panels Scatchard plots of [3 H]PDBu binding of purified PKC μ enzyme (upper panel) or cellular extracts (lower panel). All measurements were carried out in duplicates. One of three identical experiments is shown.

strated in specific PKC μ immunoprecipitates [14], we wished to scrutinize the phorbol ester binding capacity of PKC μ .

Extracts from Sf158 cells expressing PKC μ already showed an approximately 7 fold enhancement in [3 H]PDBu binding compared to control cells which could be enhanced by adding phosphatidylserine micelles approximately 40 fold (data not shown). Binding of [3 H]PDBu to PKC μ expressed in Sf158 cells followed saturable kinetics which is shown in Fig. 2 (left panels). Scatchard plot analysis of [3 H]PDBu binding of purified PKC μ suggests the presence of a single class of high affinity binding sites with a dissociation constant (K_d) of 7 nM (Fig. 2, right upper panel). Scatchard analysis of [3 H]PDBu of cellular extracts from Sf158 expressing PKC α were carried out in parallel as a control. The calculated dissociation constant of 12 nM for PKC α (data not shown) is in accordance with previously published data [18,19]. Scatchard analysis of PKC μ expressing Sf158 extracts (Fig. 2b, right lower panel) revealed a K_d of 10 nM, which only slightly differed from the value of the K_d determined for the purified enzyme. Accordingly, the data presented here provide unequivocal evidence that recombinant, baculovirus produced PKC μ serves, *in vitro*, as a high affinity phorbol ester receptor with binding characteristics identical to classical PKCs, such as PKC α .

3.3. Substrate specificity of PKC μ and characterization of phosphorylation sites

As a first step towards defining specific functions of PKC μ , we examined potential *in vitro* substrates. PKC μ phosphorylation of several representative proteins and specific peptides known to be phosphorylated by various PKCs was analysed. The synthetic peptide GS (PLSRTLVAALL), which was demonstrated to be a PKC substrate of activated T-cells [20], was found to be the most efficiently phosphorylated substrate

of PKC μ (Table 1). Phosphoamino acid analysis showed exclusive phosphorylation on serine residues. Furthermore phosphopeptide mapping revealed phosphorylation on the serine at position 7 of the GS-peptide (data not shown) as demonstrated earlier for other PKCs subtypes [21]. Syntide 2 (PLARTLSVAGLPKK), a synthetic peptide derived from the glycogen synthase [22], which has been previously reported to be specifically phosphorylated by PKD [8] the mouse homologue of PKC μ , was also efficiently phosphorylated by PKC μ (Table 1).

A major and specific PKC substrate is the ubiquitously expressed MARCKS protein [23,24]. A murine MARCKS derived peptide containing five potential serine phosphorylation sites (KKKKKRFS₁₅₂FKKS₁₅₆FKLS₁₆₀GFS₁₆₃FKKS₁₆₇K) can be efficiently phosphorylated by cPKCs, nPKCs aPKCs [16] and by PKC μ as shown here (Table 1). As revealed by

Table 1
Substrate phosphorylation of PKC μ

Substrate	Relative PKC μ activity (cpm/assay) \pm S.D.
No substrate added/ autophosphorylation	893 \pm 491
Syntide 2	63403 \pm 10403
MARCKS peptide	15089 \pm 741
Kemptide	1232 \pm 433
EGF-receptor peptide	2473 \pm 526
GS-peptide	84960 \pm 7181
Histone H1S	4132 \pm 563
Myelin basic protein (MBP)	11313 \pm 401
Myosin	1305 \pm 478

Purified PKC μ was used to phosphorylate representative PKC substrates as described in the method section. Peptides were used at a concentration of 60 μ M, protein substrates were used at 200 μ g/ml. Measurements were carried out in triplicates. data shown represent mean values \pm S.D.

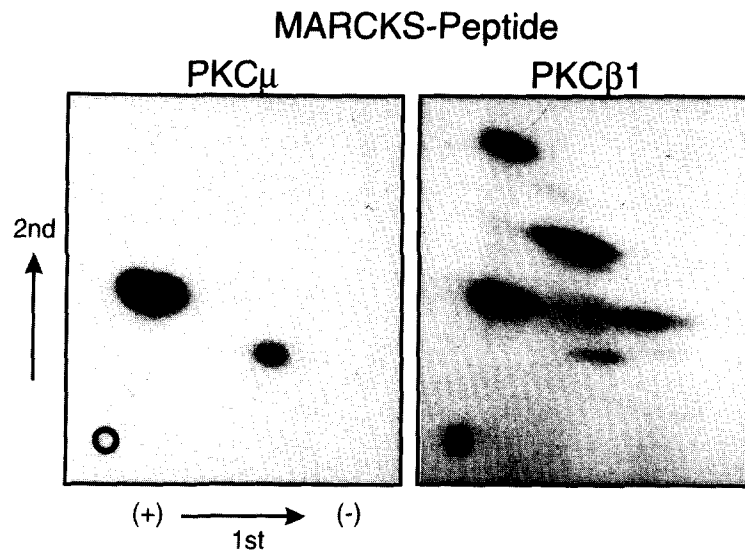


Fig. 3. Two dimensional phosphopeptide mapping of MARCKS. The MARCKS-peptide (25 $\mu\text{g/ml}$) was phosphorylated by purified PKC μ or by PKC β 1 in the presence of 125 $\mu\text{g/ml}$ phosphatidylserine and 1.25 $\mu\text{g/ml}$ phorbol-12,13-dibutyrate at 30°C for 2 h, resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The peptide was digested on the membrane by trypsin at 37°C overnight. The tryptic phosphopeptides were resolved in the first dimension by thin-layer electrophoresis at pH 3.5 and by thin-layer chromatography in the second dimension. The peptide mapping after PKC μ phosphorylation shows two distinct spots representing the peptides S₁₅₆FK and K_{S156}FK due to alternative tryptic cleavage. The peptide map obtained by PKC β 1 is identical as described [15]. Exposure time was overnight.

peptide sequence analysis only three out of five potential phosphorylation sites corresponding to serine residues 152, 156 and 163 have been previously identified to be phosphorylated by several PKCs [16]. Phosphopeptide analyses of the MARCKS peptide upon maximum phosphorylation by PKC μ showed two distinct spots (Fig. 3, left panel) in contrast to five spots obtained by MARCKS phosphorylation with PKC β 1 (Fig. 3, right panel). Both spots correspond to partial digested, serine 156 phosphorylated tryptic peptides as revealed by amino acid sequence analysis of each spot [16]. These results demonstrate that PKC μ , in contrast to other PKC subtypes can phosphorylate only one of five potential PKC specific phosphorylation sites of the MARCKS peptide.

In contrast to the already described peptide substrates only a weak phosphorylation of Kemptide (LRRASLG) and of an EGF receptor derived peptide (LRTLRR) by PKC μ could be detected. Moreover, Histone H1S was also found to be only a minor substrate of PKC μ , whereas myelin basic protein was phosphorylated significantly better which is in accordance to previous observations [14]. As the kinase domain of PKC μ exerts significant homologies to the myosin light chain kinase (MLCK) [6], myosin phosphorylation was analysed. However, no significant phosphorylation could be detected (Table 1), making it rather unlikely that myosin will be an *in vivo* substrate of PKC μ .

3.4. Requirement of lipids as activation factors for PKC μ

To analyse the role of lipid messengers in *in vitro* activation of recombinant PKC μ , several known mediators of PKC activation were tested for stimulation of PKC μ kinase activity by analysis of *in vitro* phosphorylation of the syntide 2 peptide (Table 2). Like endogenous PKC μ [6], PKC μ expressed in insect cells showed a constitutive kinase activity, which could be enhanced approximately twofold by adding phosphatidylserine micelles to the *in vitro* kinase assay (Table 2). Addition of diacylglycerol and PDBu each strongly enhanced substrate phosphorylation, with an approximately 5 and 3

fold increase, respectively, compared to the level obtained with phosphatidylserine (PS) alone. Interestingly, phosphatidylinositol-4,5-phosphate (PtdIns-4,5-P₂), previously shown to be a weak activator of nPKCs and of cPKCs [25–27] also enhanced syntide 2 peptide phosphorylation by PKC μ approximately twofold. In contrast, ceramide, recently shown to be an activator of α PKC ζ *in vitro* and *in vivo* [28] did not stimulate PKC μ kinase activity. As cPKC isozymes can be activated *in vitro* by the phospholipase A₂ derived second messengers like lysophosphatidylcholine (L-PC) and arachidonic acid (AA) [29,30], these mediators were also tested for PKC μ activation and found to be negative (Table 2). Likewise, phosphatidic acid (PA), reported to stimulate PKC and PKC ζ kinase activity [31], did not stimulate PKC μ substrate phosphorylation. Parallel investigations of autophosphorylation activity revealed a response pattern similar to substrate phosphorylation (data not shown).

Table 2
Activation of PKC μ kinase activity by various phospholipids

Substrate	Relative PKC μ activity (cpm/assay) \pm S.D.
No cofactors added	2242 \pm 297
PS	4888 \pm 528
PS/PtdIns-4,5-P ₂	9587 \pm 1072
PS/PDBu	12665 \pm 1169
PS/DAG	21056 \pm 976
PS/Ceramide	5687 \pm 445
PS/Arachidonic acid	6053 \pm 178
PS/Lyso-phosphatidylcholine	3443 \pm 581
PS/Phosphatidic acid	4973 \pm 1771

Baculovirus produced PKC μ was used for kinase assays using syntide 2 as a substrate. Kinase assays were carried out in the presence of 100% phosphatidylserine (PS) containing micelles. Kinase assays in the presence of PtdIns-4,5-P₂ were carried out with mixed micelles containing 80% PS and 20% PtdIns-4,5-P₂. PDBu was added to a final concentration of 100nM, diacylglycerol, (DAG), ceramide, arachidonic acid, lyso-phosphatidylcholine and phosphatidic acid were added to a final concentration of 1 μM .

4. Discussion

Purified PKC μ was found to possess phosphatidylserine dependent, high affinity phorbol ester binding capacity, with a K_d of 7 nM, which is in the same order of magnitude as reported for immunopurified PKD (K_d = 2.2 nM) [9]. These data are consistent with an important role of a proline residue in the cysteine domains of phorbol ester binding PKCs which is conserved in PKC μ (Pro-157 for Cys I and Pro-281 for Cys II) [32]. The finding of a high efficient phorbol ester binding of the purified enzyme differs from our earlier observations, where only weak increase in phorbol ester binding capacity of cellular extracts from PKC μ -transfectants was noted [6]. Based on the data presented here with purified enzyme, the previous failure to detect PDBu binding could be due to relative low concentration of PKC μ in mamalian cell extracts or a highly efficient blockade of the PKC μ phorbol ester binding sites by unknown cellular factors, present in the cellular extracts of mamalian transfectants and absent or outnumbered in highly PKC μ overexpressing insect cells.

Diacylglycerol, a main product of the phosphoinositide metabolism and regarded as the physiological activator of most PKC isozymes, was found here to be the most potent activator of PKC μ in in vitro kinase assays. Of interest is the observation that PtdIns-4,5- P_2 /phosphatidylserine also served as an activator of PKC μ (Table 2), whereas other PKC subtypes have been reported to be only weakly activated by PtdIns-4,5- P_2 [24,25]. As PtdIns-4,5- P_2 has recently been shown to bind to the pleckstrin homology domains of several signal transducers [33], it might serve as an additional membrane anchor, attaching PKC μ via its pleckstrin homology domain [7] to a membrane.

Phosphorylation assays revealed the synthetic peptides GS (PLSRTLVAALL) and syntide 2 (PLARTLSVAGLPGKK) as best in vitro substrates, whereas Kemptide (LRRASLG) and an EGF receptor derived peptide (LRTLRR) were only weakly phosphorylated. From the natural substrates tested for in vitro phosphorylation, only MARCKS (S. Dieterich, unpublished) and a MARCKS derived peptide (Table 1) could be specifically phosphorylated by PKC μ . MARCKS is a widely distributed specific PKC substrate, implicated in several cellular functions like secretion, membrane trafficking and regulation of the cell cycle [23,34,35]. MARCKS-phosphorylation prevents its binding to calmodulin in the presence of calcium and disrupts actin crosslinking activity (for review see [23]). Within the MARCKS peptide only one out of five potential PKC phosphorylation sites, serine 156, was specifically phosphorylated by PKC μ (Fig. 3). This finding differs significantly from the MARCKS peptide phosphorylation pattern obtained with other PKC subtypes where serines 152, 156 and 163 were phosphorylated [16]. Serine 160 and serine 167 were neither phosphorylated by PKC μ (Fig. 3) nor by other PKC subtypes [16]. Phosphorylation of serine 156 versus serines 152/163 in diverse MARCKS functions has not yet been investigated and it remains open, to which extend PKC μ is involved in the regulation of MARCKS function and cytoskeletal reorganisation in vivo.

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