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

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Abstract To study enzymatic activity and activation conditions of the recently identified novel protein kinase C μ (PKCμ) subtype, epitope tagged PKCμ was propagated in the baculovirus expression system and was purified to homogeneity. PKCμ 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-P_2, were found to promote PKCμ 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 PKCμ 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 PKCμ.

Key words: Protein kinase Cμ; 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: Ca^{2+}-dependent, conventional PKCs; Ca^{2+}-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-P_2, L-α-phosphatidyl-D-myo-inositol-4,5-bisphosphate; PMSF, phenylmethylsulfonylfluoride; PS, L-α-phosphatidyl-L-serine; DAG, 1,2-dioctanoyl-*sn*-glycerol; MARCKS, myristoylated alanine-rich C-kinase substrate; L-PC, L-α-lysophosphatidylcholine; PA, L-α-dipalmitoyl phosphatidic acid; AA, arachidonic acid; Cer, C_{16}-ceramide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

We previously reported the cloning and characterization of a novel PKC subtype named PKCμ [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 PKCμ 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 PKCμ/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 PKCμ expressed in insect cells.

2. Materials and methods

2.1. Construction and cloning of PKCμ 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 PKCμ cDNA devoid of the 3'-untranslated region. This results in c-myc tagging of PKCμ carboxy-terminal of the predicted protease cleaveage site of the leader peptide and amino-terminal of the putative transmembrane domain [6]. The c-myc PKCμ 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/PKCμ 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 PKCμ by affinity chromatography

10^8 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 μg/ml leupeptin, 20 μg/ml trypsininhibitor, 200 μg/ml iodineacetamide) by sonification (20×1 s). Cell debris was removed by centrifugation for 10 min at 100,000×*g* and the supernatant passed through a 0.2 μm sterile filter. For affinity chromatography, a PKCμ antibody column (3 mg monoclonal PKCμ 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

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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 $MgCl_2$, 20 mM Tris-HCl pH 7.5). The fractions were tested for their content of PKCμ by SDS-PAGE followed by silver staining. $MgCl_2$ was removed by gel filtration or dialysis against 20 mM Tris-HCl, pH 7.5. A typical preparation yielded approximately 100–200 μg of PKCμ. After adding 10% glycerol, purified PKCμ was stored in aliquots at −20°C. PKCβ1 was isolated as described [15].

2.3. [^{3}H]PDBu binding

100 ng of purified PKCμ (= 10 μl) or supernatant, containing 25 μg total protein and an equivalent amount of PKCμ 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 μl. The mixture contained 20 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, 5 mM EGTA, 1 mg/ml BSA, 100 μg/ml phosphatidylserine and the indicated amounts of [^{3}H]PDBu (15.1 Ci/mMol, Amersham). Free [^{3}H]PDBu was removed by rapid filtration through glass fiber filters, followed 10 times washing with 20 mM Tris-HCl, pH 7.4, 10 mM $MgSO_4$, 1 mM $CaCl_2$ 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 μM PDBu. Scatchard analysis was calculated using a modified Excel (Microsoft) program.

2.4. Substrate and autophosphorylation of PKCμ

Substrate phosphorylation of PKCμ 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 μM, histone IIIS, myelin basic protein and myosine at concentrations of 200 μg/ml. Extracts (5 μg total protein per assay) of PKCμ 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 C_{16}-ceramide was purchased from Biomol. L-α-phosphatidyl-D-myo-inositol-4,5-bisphosphate (PtdIns-4,5-P_2) was purchased from Boehringer Mannheim. Autophosphorylation of PKCμ 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 PKCμ in Sf158 cells

An amino-terminal epitope tagged derivate of the PKCμ cDNA was constructed to facilitate detection of the cDNA gene product independent of endogeneous PKCμ 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 PKCμ was analysed by kinase assays in immunoprecipitates of PKCμ, 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 PKCμ, was detectable in PKCμ transfectants but not in vector control transfectants. The obtained c-myc tagged PKCμ showed a slightly reduced electrophoretic mobility upon SDS-PAGE, corresponding to an approximate 5 kDa increase in molecular mass, compared to endogeneous PKCμ from HepG2 cells (data not shown).

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

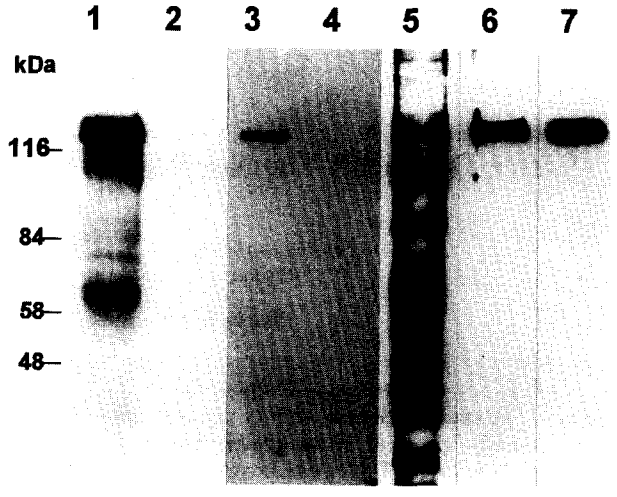

Fig. 1. Expression and purification of c-myc epitope tagged PKCμ. lanes 1 and 2: Autophosphorylation assay of immunoprecipitates with the anti c-myc 9E10 antibody from COS cells transiently expressing PKCμ (lane 1). Lane 2, vector control. Lanes 3 and 4: Western Blot detection with the 9E10 antibody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

pVL 1392, transfected into Sf158 cells and analysed for PKCμ 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 PKCμ specific antibody JP1 [14] as well as with a PKCμ 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 PKCμ protein. Therefore, a purification protocol based on affinity chromatography with the PKCμ specific antibody JP1 was established. This approach yielded highly purified PKCμ in a fast, single step purification procedure. The purity of the preparation was assessed by silver staining indicating that PKCμ could be eluted to greater than 90% purity (Fig. 1, lane 6 versus lane 5). Purified PKCμ retained its kinase activity as shown by in vitro autophosphorylation (Fig. 1, lane 7) and was used for further biochemical analyses.

3.2. Purified PKCμ binds phorbol ester

The aminoterminal region of PKCμ contains two conserved cysteine-clusters with the characteristic $HX_{12}CX_2CX_n$-$CX_2CX_4HX_2CX_7C$ consensus sequence, typical for all members of the PKC family. These domains represent the structural motivs 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, PKCμ 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 PKCμ transfectants could be detected, questioning a direct role of phorbol ester in PKCμ activation [6]. However, as a significant phosphatidylserine/phorbol ester dependent stimulation of PKCμ autophosphorylation could be subsequently demon-

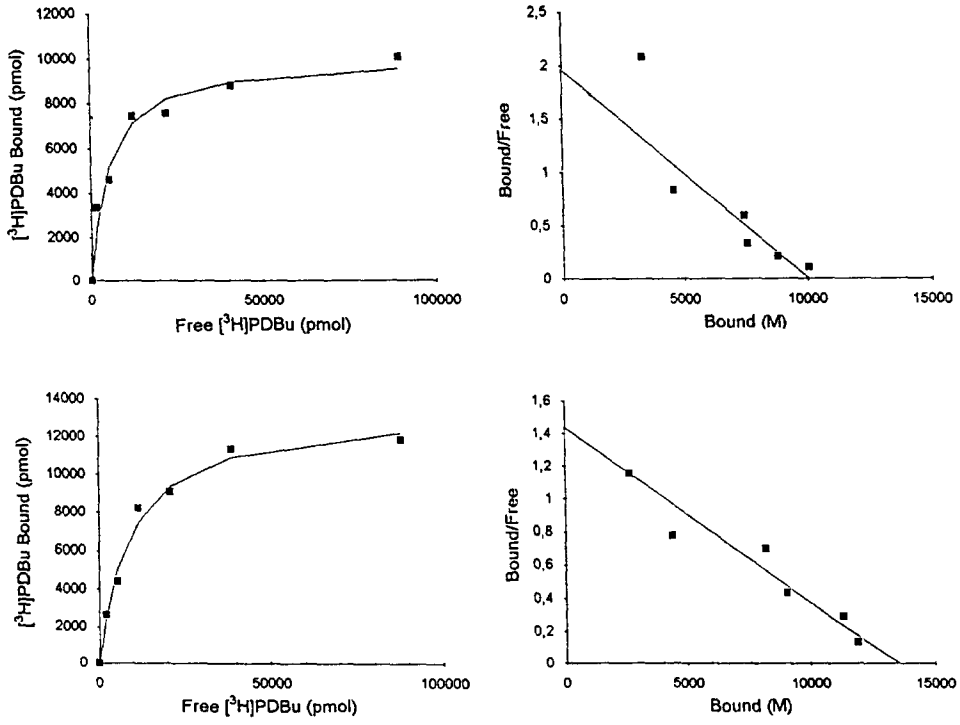

Fig. 2. Phorbol ester binding of PKCμ. Left panels show dose–response curves, right panels Scatchard plots of [^{3}H]PDBu binding of purified PKCμ enzyme (upper panel) or cellular extracts (lower panel). All measurements were carried out in duplicates. One of three identical experiments is shown.

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

Extracts from Sf158 cells expressing PKCμ 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 PKCμ 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 PKCμ 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 PKCα were carried out in parallel as a control. The calculated dissociation constant of 12 nM for PKCα (data not shown) is in accordance with previously published data [18,19]. Scatchard analysis of PKCμ 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 PKCμ serves, in vitro, as a high affinity phorbol ester receptor with binding characteristics identical to classical PKCs, such as PKCα.

3.3. *Substrate specificity of PKCμ and characerization of phosphorylation sites*

As a first step towards defining specific functions of PKCμ, we examined potential in vitro substrates. PKCμ phosphorylation of several representative proteins and specific peptides known to be phosphorylated by various PKCs was analysed. The synthetic peptide GS (PLSRTLSVAALL), which was demonstrated to be a PKC substrate of activated T-cells [20], was found to be the most efficiently phosphorylated substrate of PKCμ (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 (PLARTLSVAGLPGKK), 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 PKCμ, was also efficiently phosphorylated by PKCμ (Table 1).

A major and specific PKC substrate is the ubiquitiously expressed MARCKS protein [23,24]. A murine MARCKS derived peptide containing five potential serine phosphorylation sites (KKKKKRFS$_{152}$FKKS$_{156}$FKLS$_{160}$GFS$_{163}$FKKS$_{167}$K) can be efficiently phosphorylated by cPKCs, nPKCs aPKCs [16] and by PKCμ as shown here (Table 1). As revealed by

Table 1
Substrate phosphorylation of PKCμ

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

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

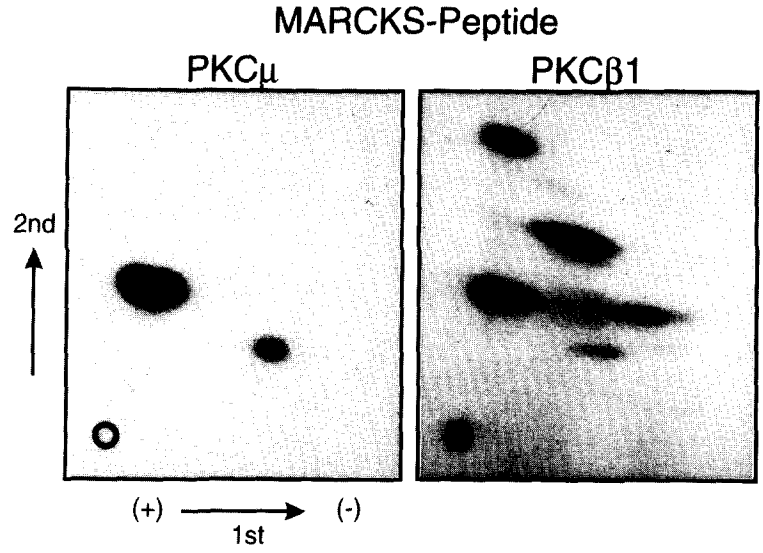

Fig. 3. Two dimensional phosphopeptide mapping of MARCKS. The MARCKS-peptide (25 μg/ml) was phosphorylated by purified PKCμ or by PKCβ1 in the presence of 125 μg/ml phosphatidylserine and 1.25 μg/ml phorbol-12,13-dibutyrate at 30°C for 2 h, resolved by SDS-PAGE and transferred to a nitrocellulose membrane. 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 PKCμ phosphorylation shows two distinct spots representing the peptides $S_{156}FK$ and $KS_{156}FK$ due to alternative tryptic cleavage. The peptide map obtained by PKCβ1 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 PKCμ showed two distinct spots (Fig. 3, left panel) in contrast to five spots obtained by MARCKS phosphorylation with PKCβ1 (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 PKCμ, 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 PKCμ could be detected. Moreover, Histone IIIS was also found to be only a minor substrate of PKCμ, whereas myelin basic protein was phosphorylated significantly better which is in accordance to previous observations [14]. As the kinase domain of PKCμ 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 PKCμ.

3.4. Requirement of lipids as activation factors for PKCμ

To analyse the role of lipid messengers in in vitro activation of recombinant PKCμ, several known mediators of PKC activation were tested for stimulation of PKCμ kinase activity by analysis of in vitro phosphorylation of the syntide 2 peptide (Table 2). Like endogeneous PKCμ [6], PKCμ 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-P_2), previously shown to be a weak activator of nPKCs and of cPKCs [25–27] also enhanced syntide 2 peptide phosphorylation by PKCμ approximately twofold. In contrast, ceramide, recently shown to be an activator of aPKCζ in vitro and in vivo [28] did not stimulate PKCμ kinase activity. As cPKC isozymes can be activated in vitro by the phospholipase A_2 derived second messengers like lysophosphatidylcholine (L-PC) and arachidonic acid (AA) [29,30], these mediators were also tested for PKCμ activation and found to be negative (Table 2). Likewise, phosphatidic acid (PA), reported to stimulate PKC and PKCζ kinase activity [31], did not stimulate PKCμ substrate phosphorylation. Parallel investigations of autophosphorylation activity revealed a response pattern similar to substrate phosphorylation (data not shown).

Table 2
Activation of PKCμ kinase activity by various phospholipids

Substrate	Relative PKCμ activity (cpm/assay) ± S.D.
No cofactors added	2242 ± 297
PS	4888 ± 528
PS/PtdIns-4,5-P_2	9587 ± 1072
PS/PDBu	12665 ± 1169
PS/DAG	21056 ± 976
PS/Ceramide	5687 ± 445
PS/Arachidonic acid	6053 ± 178
PS/Lyso-phosphatidylcholine	3443 ± 581
PS/Phosphatidic acid	4973 ± 1771

Baculovirus produced PKCμ 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-P_2 were carried out with mixed micelles containing 80% PS and 20% PtdIns-4,5-P_2. 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 PKCμ 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 PKCμ (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 PKCμ-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 PKCμ in mamalian cell extracts or a highly efficient blockade of the PKCμ phorbol ester binding sites by unknown cellular factors, present in the cellular extracts of mamalian transfectants and absent or outnumbered in highly PKCμ 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 PKCμ in in vitro kinase assays. Of interest is the observation that PtdIns-4,5-P_2/phosphatidylserine also served as an activator of PKCμ (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 PKCμ via its pleckstrin homology domain [7] to a membrane.

Phosphorylation assays revealed the synthetic peptides GS (PLSRTLSVAALL) 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 PKCμ. 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 PKCμ (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 PKCμ (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 PKCμ is involved in the regulation of MARCKS function and cytoskeletal reorganisation in vivo.

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