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Inhibition of protein kinase C μ by various inhibitors. Differentiation from protein kinase c isoenzymes

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Abstract Various inhibitors were tested for their potential to suppress the kinase activity of protein kinase C μ (PKCμ) in vitro and in vivo. Among the staurosporine-derived, rather selective PKC inhibitors the indolocarbazole Gö 6976 previously shown to inhibit preferentially cPKC isotypes proved to be a potent inhibitor of PKCμ with an IC_{50} of 20 nM, whereas the bisindolylmaleimide Gö 6983 was extremely ineffective in suppressing PKCμ kinase activity with a thousand-fold higher IC_{50} of 20 μM. Other strong inhibitors of PKCμ were the rather unspecific inhibitors staurosporine and K252a. Contrary to the poor inhibition of PKCμ by Gö 6983, this compound was found to suppress in vitro kinase activity of PKC isoenzymes from all three subgroups very effectively with IC_{50} values from 7 to 60 nM. Thus, Gö 6983 was able to differentiate between PKCμ and other PKC isoenzymes being useful for selective determination of PKCμ kinase activity in the presence of other PKC isoenzymes.

Key words: Protein kinase Cμ; Protein kinase C isoenzyme; Protein kinase C inhibitor; Staurosporine; Autophosphorylation

1. Introduction

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine protein kinases that plays a central role in signal transduction and is involved in the control of numerous cellular processes, such as proliferation and differentiation, as well as in carcinogenesis (for reviews see [1–4]). Members of the PKC family have been grouped into three categories. The cPKCs (α, β1, β2, γ) are calcium-dependent and activated by diacylglycerol or phorbol ester. The nPKCs (δ, ε, η, θ) are calcium-unresponsive and, as cPKCs, activated by diacylglycerol or phorbol ester whereas the aPKCs (ζ,ι/λ) are unresponsive to both calcium and diacylglycerol or phorbol ester.

Recently the cDNA of a novel serine/threonine protein kinase was isolated from a human placenta cDNA library. Referring to its structural similarities to the PKC family, such as the combination of amino terminal cysteine clusters a carboxy terminal kinase domain, and its phospholipid dependency, the kinase was termed PKCμ [5]. Upon overexpression in various cell lines and immunoprecipitation, the novel PKC isoenzyme could be partially characterized and was found to be calcium-independent but phorbol ester responsive [6]. Due to these properties PKCμ might have been classified as a member of the nPKC subgroup of the PKC family. However, PKCμ significantly differs in some structural features from the other PKC isotypes known so far [5]. With a sequence of 912 amino acids PKCμ is larger than the other PKCs. It contains a hydrophobic domain in the N-terminal region and a pleckstrin homology (PH) domain [7] which is currently discussed as a potential region for protein/protein interactions or lipid binding [8,9]. PKCμ does not contain a region homologous to pseudosubstrate regions of other PKC family members. However, this does not exclude the possibility of a quite different pseudosubstrate motif to exist. Very recently, a protein kinase, named PKD, was discovered [10] which most likely is the murine homolog of human PKCμ. It was reported that PKD failed to phosphorylate several substrates which are actively phosphorylated by other members of the PKC family. The only substrate for PKCμ/PKD known so far are the synthetic peptides syntide 2 and GS [10,11] According to its structural and functional properties, PKCμ/PKD appears to be a member of the PKC family but cannot be included in any of the three subgroups. Several reports have indeed indicated the involvement of PKC isoenzymes in specific cellular processes, like differentiation (PKCδ) and proliferation (PKCε) of specific cells (for a review see [3]). To address such questions the identification of PKC subtype specific inhibitors will be valuable tools for respective investigations.

Here we report on the suppression of PKCμ activity by various inhibitors and show how PKCμ activity can be differentiated from the activity of other known PKC isotypes by means of one of these inhibitors. This selective inhibitor promises to become an important tool for the investigation of specific in vivo functions of PKCμ. Moreover, our data support the above-mentioned exceptional position of PKCμ and the idea to classify it as a member of a novel subgroup of the PKC family.

2. Materials and methods

2.1. Materials

12-*O*-Tetradecanoylphorbol 13-acetate (TPA) and bryostatin 1 were supplied by Prof. E. Hecker (German Cancer Research Center, Heidelberg, Germany) and Prof. G.R. Pettit (State University of Arizona, Tempe, AZ, USA), respectively. Gö 6976 and Gö 6983 were kindly provided by Goedecke A.G. (Freiburg, Germany). Syntide 2, the pseudosubstrate of PKCδ, the pseudosubstrate-related peptide of PKCδ, and peptides 1–3 were synthesized by Dr. R. Pipkorn (German Cancer Research Center, Heidelberg). Recombinant baculoviruses containing sequences coding for the different PKC isoenzymes were

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Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PS, phosphatidylserine; PdBu, phorbol 12,13-dibutyrate

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generously donated by Dr. S. Stabel (Max-Delbrueck-Laboratorium, Cologne, Germany). Other materials were bought from companies as indicated in parentheses: staurosporine (Boehringer, Mannheim), K252a (Fluka Chemie A.G., Neu-Ulm), bisindolylmaleimides I and III (LC Laboratories, Gruenberg), quercetin, phloretin, PdBu and phosphatidylserine (Sigma, Munich), [γ-^{32}P]ATP, spec. act. 5000 Ci/mmol (Hartmann Analytics, Braunschweig).

2.2. Recombinant PKC isoenzymes

Sf158 cells were infected with a recombinant PKCμ baculovirus as described [11]. Cells were lysed with buffer A (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 3 mg/ml leupeptin). The lysate was kept on ice for 10 min and then centrifuged at 100000×g for 30 min. The supernatant (cell extract) was adjusted to 7.5% glycerol and used as a source for PKCμ. Sf9 cells were infected with recombinant baculoviruses as a source for other PKC isoenzymes, and cell extracts were prepared and used as described previously [12].

2.3. Protein kinase assay

Phosphorylation reactions were carried out in a total volume of 100 μl, containing buffer C (50 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol), 4 mM $MgCl_2$, 10 μg PS, 100 nM TPA, 5 μl of a Sf158 cell extract as a source of recombinant PKCμ or of Sf9 cell extracts as a source of other recombinant PKC isoenzymes, 10 μg of syntide 2 as substrate, and 35 μM ATP containing 1 μCi [γ-^{32}P]ATP. In some experiments PS and TPA were omitted or various inhibitors at concentrations indicated in the text were added. After incubation for 10 min at 30°C, the reaction was terminated by transferring 50 μl of the assay mixture onto a 20 mm square piece of phosphocellulose paper (Whatman p81), which was washed 3 times in deionized water and twice in acetone. The radioactivity on each paper was determined by liquid scintillation counting.

2.4. Autophosphorylation of PKCμ

Phosphorylation reactions were carried out, using 5 μl of the extract of baculovirus-infected Sf158 cells expressing PKCμ, essentially as described for the protein kinase assay. However, 7 μCi of [γ-^{32}P]ATP was added and the substrate syntide 2 was omitted. The reaction was terminated by addition of 250 μl of 10% trichloroacetic acid. After 30 min at 4°C the precipitated proteins were pelleted, redissolved in sample buffer, separated by SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography of the gels. To analyze in vivo PKCμ autophosphorylation NIH3T3 PKCμ overexpressing transfectants were established essentially as previously described [5]. PKCμ expression was induced by incubating 60% confluent growing transfectants 3 h in phosphate-free DMEM medium adding 1 μM $CdCl_2$ and 300 μCi ^{32}P-labelled orthophosphate (Amersham) in a final volume of 2 ml. Stimulation of PKCμ kinase activity was performed by adding 100 nM phorbol ester for 10 min. Previously inhibitors Gö 6976 and Gö 6983 were added for a 20 min period. Cells were scraped off, then subjected to immunoprecipitation, SDS-PAGE and autoradiography as described [6].

Table 1
Inhibition of PKCμ by various inhibitors

Inhibitor	IC_{50} (nM)
K252a	7
Gö 6976	20
Staurosporine	40
Bisindolylmaleimide I	2000
Bisindolylmaleimide III	10000
Gö 6983	20000
Quercetin	4000
Phloretin	50000
δ-Pseudosubstrate	>100000

The concentration of the inhibitor at which 50% suppression of PKCμ activity could be observed (IC_{50}) was determined from data shown in Fig. 2 and respective data of quercetin and phloretin (data not shown).

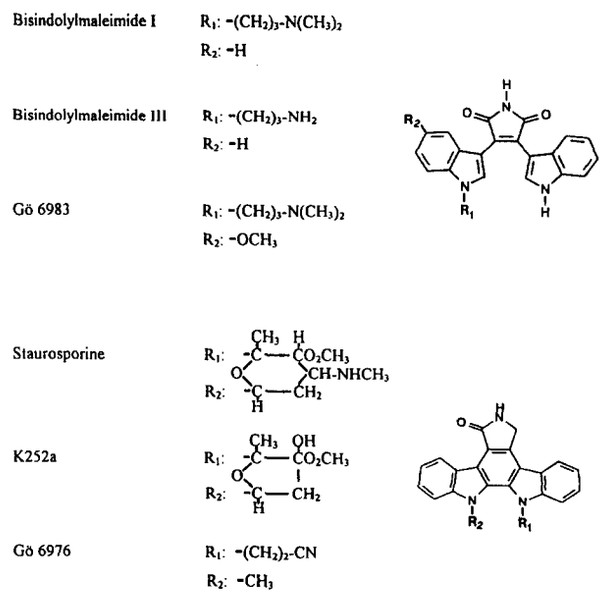

Fig. 1. Chemical structures of staurosporine, K252a, and staurosporine-derived compounds. R1 and R2 represent different substituents with the exception of staurosporine and K252, where one substituent is connected via the bonds R1 and R2 to the N atoms of the indole carbazole chromophore.

3. Results and discussion

3.1. Inhibition of PKCμ kinase activity by staurosporine and staurosporine-related compounds

Various inhibitors known to suppress PKC activity were tested for their capacity to inhibit the phosphorylation of syntide 2 by PKCμ. Most effective in the inhibition of PKCμ were staurosporine and the staurosporine-related compounds K252a and Gö 6976 (for chemical structures see Fig. 1) with IC_{50} values in the nM range (40, 7 and 20 nM, respectively; Fig. 2 and Table 1). K252a, which we had previously shown to suppress cPKC (α,β,γ) much more effectively than PKCδ [12,13], proved to be a potent inhibitor of PKCμ. Whereas staurosporine and K252a are known to suppress many protein kinases [14,15], the indolocarbazole Gö 6976 is rather specific for PKC [16]. Moreover, Gö 6976 is able to differentiate between PKC isoenzymes [12,16,17], inhibiting cPKC (α,β,γ) with much greater efficacy (IC_{50} values in the nM range) than nPKC (δ,ε,η) and aPKC (ζ) (IC_{50} values in the μM range). It is intriguing that PKCμ is equally well inhibited by Gö 6976 as cPKC. As PKCμ is Ca^{2+}-independent and activated by phorbol ester [6,11], one would tend to associate it with the nPKC group of PKC isoenzymes. However, the excellent inhibition by Gö 6976, which is atypical for an nPKC, as well as various structural and functional properties (see [5,6,10,11]) suggest that PKCμ might represent a novel type that does not fit into any of the known PKC subgroups. Other staurosporine-derived compounds, the bisindolylmaleimides Gö 6983, bisindolylmaleimide I (GF-109203X) and bisindolylmaleimide III (for chemical structures see Fig. 1) that are potent PKC inhibitors and were also shown to have improved selectivity for PKCs [16,18], proved to be rather weak inhibitors of PKCμ (Fig. 2 and Table 1). Especially Gö 6983 was extremely ineffective in suppressing PKCμ kinase activity in vitro with an IC_{50} of 20 μM.

Changes in the activation of PKCμ kinase activity in vitro

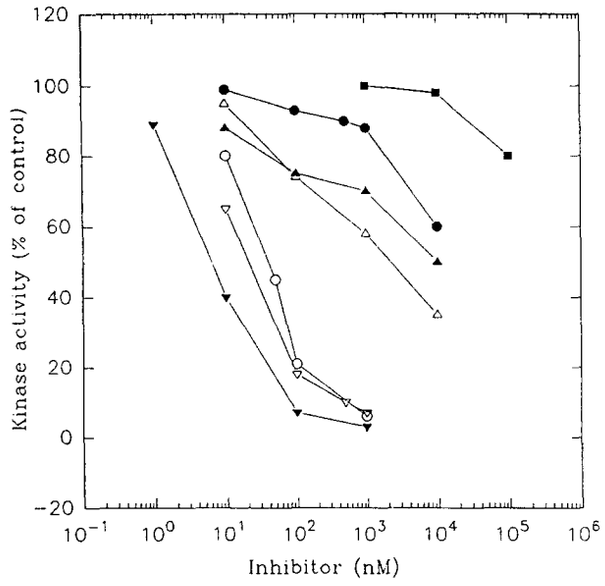

Fig. 2. Suppression of PKCμ kinase activity by various inhibitors. 10 μg of syntide 2 was phosphorylated by recombinant PKCμ (5 μl of cell extract, 40 mU, 14.4 μg protein) in the presence of various inhibitors at the indicated concentrations. The K_m value for ATP was determined to be 34.5 μM. Protein kinase assays were performed as described in Section 2. (▼) K252a, (▽) Gö 6976, (○) staurosporine, (▲) bisindolylmaleimide III, (△) bisindolylmaleimide I (GF-109203X), (●) Gö 6983, (■) pseudosubstrate peptide of PKCδ. IC_{50} values presented in Tables 1 and 2 were estimated by eye.

as well as in vivo are coupled to changes of the autophosphorylation state ([11] and F.J. Johannes unpublished). Therefore, staurosporine as well as the inhibitors Gö 6976 and Gö 6983 were analyzed regarding their inhibition of PKCμ autophosphorylation. Autophosphorylation was performed in the presence of PS/TPA, since TPA as well as the structurally unrelated PKC activator bryostatin proved to stimulate PKCμ kinase activity significantly (Fig. 3A). Staurosporine and Gö 6976 proved to be efficient in inhibiting approx. 50% autophosphorylation (Fig. 3B) at 100 nM final concentration whereas Gö 6983 did not show any significant inhibition of PKCμ in vitro autophosphorylation up to 10 μM concentration (Fig. 3B).

The differential potency of Gö 6983 and Gö 6976 was further measured by inhibiting in vivo PKCμ kinase activity. PKCμ overexpressing NIH3T3 transfectants were labelled with [^{32}P]orthophosphate and PKCμ was activated by stimulating cells with phorbol ester. As shown in Fig. 3C, immunoprecipitated PKCμ displayed enhanced autophosphorylation after incubation of transfectants with phorbol ester which could be approx. 50% suppressed by adding 20 μM Gö 6976 (Fig. 3C). Incubation of transfectants with 20 μM of Gö 6983 did not produce any significant reduction of PdBu-stimulated autophosphorylation of PKCμ immunoprecipitates (Fig. 3C, right lane). Suppression of PKCμ autophosphorylation (approx. 20% reduction) by Gö 6983 could be detected by incubating the transfectants at 300 μM concentrations (data not shown).

By comparing the chemical structures of staurosporine and staurosporine-derived compounds (Fig. 1) with their inhibitory activity towards PKCμ (Fig. 2 and Table 1), an interesting structure-activity relationship becomes evident. Compounds with the intact aromatic ring structure of staurosporine (staurosporine, K252a and Gö 6976) are effective in inhibiting PKCμ, whereas the bisindolylmaleimides with the opened central aromatic ring (Gö 6983 and bisindolylmaleimide I and III) are very poor suppressors of PKCμ activity. This information might be valuable for future attempts to develop inhibitors of improved selectivity.

3.2. *Inhibition of PKCμ by staurosporine-unrelated compounds, pseudosubstrate peptide of PKCδ and putative pseudosubstrate peptides of PKCμ*

Quercetin and phloretin are rather weak and unspecific inhibitors of PKC with IC_{50} values of 10 and 20 μM, respectively [19,20]. Similarly high IC_{50} values were found for PKCμ (4 and 50 μM, respectively; see Table 1). PKCμ was not significantly suppressed by the pseudosubstrate peptide of PKCδ ($IC_{50} > 100$ μM; see Fig. 2 and Table 1). Other PKC isoenzymes, such as PKCα, β, γ and PKCδ, are effectively inhibited by several of the PKC pseudosubstrate peptides (Gschwendt et al., unpublished data). This result is in accordance with the lack of a classical pseudosubstrate sequence in PKCμ. However, as PKCμ is activated by TPA in a similar manner to other PKC isoenzymes, a site with a pseudosubstrate function could be postulated. This prompted us to look for a putative pseudosubstrate motif of PKCμ. In this context, we tested three synthetic PKCμ peptides as potential inhibitors of PKCμ. Peptide 1 (^{405}PLMRVVQSVKHTKRK^{419}) was chosen, since it displays some homology to syntide 2, a known in vitro substrate of PKCμ [11]. The peptides 2 (^{75}AHVREMACSIVDQK^{88}) and 3 (^{142}FQIRPHALFVHSYRAPAF^{159}), like the known PKC pseudosubstrate sequences, are located N-terminal of the two cysteine-rich regions and contain the consensus sequence RXXA that appears to be an essential part of the pseudosubstrate motifs of the other PKC isoenzymes. Up to a concentration of 100 μM, all three peptides were unable to inhibit PKCμ activity (data not shown). These results indicate that the pseudosubstrate region of PKCμ, if there is any, might differ essentially from those of the other PKC isoenzymes and support the above-mentioned assumption that PKCμ might represent a novel PKC type.

3.3. *Differential inhibition of PKCμ and other PKC isoenzymes by Gö 6983*

Because of the extremely poor suppression of PKCμ activity by the PKC inhibitor Gö 6983, this compound was chosen for further studies. In order to be able to compare the inhibition of PKCμ by Gö 6983 with that of other PKC isoenzymes, we used the same substrate for all assays of PKC activity. As

Table 2
Inhibition of PKC isoenzymes by Gö 6983 with syntide 2 as substrate

PKC	IC_{50} (nM)
PKCα	7
PKCβ	7
PKCγ	6
PKCδ	10
PKCζ	60
PKCμ	20000

PKC kinase activity towards the substrate syntide 2 was measured in the presence of various concentrations of Gö 6983 as described in Section 2. IC_{50} values were determined from these data as indicated in Table 1.

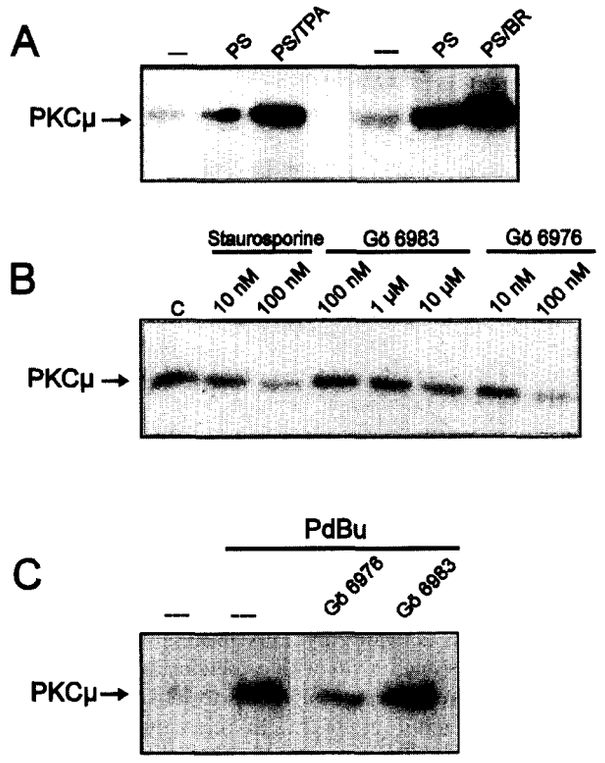

Fig. 3. Autophosphorylation of PKCμ and its suppression by staurosporine, Gö 6983 and Gö 6976. (A) Recombinant PKCμ was autophosphorylated in the absence of cofactors or in the presence of PS (10 μg), PS/TPA (100 nM) or PS/bryostatin (BR, 100 nM) as described in Section 2. (B) Autophosphorylation of PKCμ in the presence of PS/TPA was performed as in A without (control, C) and with addition of staurosporine, Gö 6983, or Gö 6976 at the indicated concentrations. (C) In vivo inactivation of PKCμ kinase activity. PKCμ was immunoprecipitated from ^{32}P-labelled NIH3T3 PKCμ transfectants after stimulation with phorbol ester in the presence of 20 μM of the respective inhibitor. Equal loads of PKCμ in each lane were verified by Western blot analysis (data not shown). All autoradiographs shown were exposed overnight.

syntide 2 is one of the few known specific in vitro substrates for PKCμ [11], it was necessary to test its suitability as a substrate for other PKC isoenzymes. Syntide 2 is effectively phosphorylated by PKC isoenzymes of the cPKC group ($\alpha = 50\%$, $\beta = 43\%$, $\gamma = 33\%$), the nPKC group ($\delta = 52\%$), and the aPKC group ($\zeta = 30\%$ relative to the δ-pseudosubstrate peptide). Pseudosubstrate-related peptides belong to the most effectively phosphorylated substrate peptides that have been used for PKC isoenzymes so far. Thus, the inhibition of PKCμ and other PKC isoenzymes by Gö 6983 could be compared using syntide 2 as substrate. Again, representatives of each PKC subgroup were tested. Whereas PKCμ was resistant to Gö 6983 in the μM range (IC_{50}: 20 μM, see also Table 1), the other PKC isoenzymes could be suppressed by Gö 6983 with IC_{50} values from 7 to 60 nM (Table 2). Again, this indicates that PKCμ differs essentially from all the other PKC isoenzymes known as yet. Based on these results and the differential inhibition of PKCμ autophosphorylation by Gö 6983 compared to Gö 6976 (Fig. 3C), Gö 6983 promises to become a valuable tool for selectively analysing in vivo PKCμ kinase activity in the presence of other PKC isoenzymes.

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