Differential effects of suramin on protein kinase C isoenzymes. A novel tool for discriminating protein kinase C activities

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Abstract Suramin, a hexasulfonated naphthylurea, is known to induce differentiation and inhibit proliferation, angiogenesis, and development of tumors. It has also been shown to suppress the activity of the protein kinase C (PKC) isoenzymes α, β, and γ. Here we report on a differential effect of suramin on PKCμ and various PKC isoforms representing the cPKC, nPKC, and aPKC group of the PKC family. In the absence of any cofactors suramin activates all PKC isoforms in the order of aPKCζ ≫ PKCμ > cPKC, nPKCδ. As judged by the V_{max}/K_M ratios (0.5 for PKCμ and 2.2 for PKCζ) the substrate syntide 2 is phosphorylated by suramin-activated PKCζ around four times more effectively than by suramin-activated PKCμ. Suramin-activated PKCμ behaves like that activated by phosphatidylserine and the phorbol ester TPA regarding autophosphorylation and differential inhibition by the PKC inhibitors Gö 6976 and Gö 6983. In the presence of activating cofactors, such as phosphatidylserine and TPA or cholesterol sulfate (for PKCζ), the activity of the aPKCζ is further stimulated, PKCμ is not significantly affected, and the cPKCs and the nPKCδ are strongly inhibited by suramin. The differential action of suramin on PKC isoenzymes might play a role in some of its biological effects, as for instance inhibition of proliferation and tumor development. Moreover, due to this property suramin will possibly be a valuable tool for discriminating the activities of PKC isoenzymes in vitro and in vivo.

Key words: Protein kinase C isoenzyme; Protein kinase C δ; Protein kinase C ζ; Protein kinase C μ; Protein kinase C inhibitor; Suramin; Cholesterol sulfate

1. Introduction

Suramin is a hexasulfonated naphthylurea possessing a broad spectrum of biological and therapeutic properties (for reviews see [1,2]). It has been used in the treatment of sleeping sickness and other parasitic diseases for 70 years [3]. More recently, suramin has been shown to inhibit the reverse transcriptase of several animal retroviruses [4], including that of HIV-1 [5], and was clinically tested in patients with AIDS [6], but was found to be too toxic in vivo [7]. Furthermore, suramin displayed antitumor activity toward several metastatic cancers, such as prostatic carcinoma (for a review see [1]). Suramin has multiple modes of action. It inhibits angiogenesis [9] and proliferation [1,10], induces differentiation [11], interferes with the recognition of several growth factors by their membrane receptors [12,13], forms complexes with a large variety of plasma proteins, most importantly albumin [14], and inhibits a great number of cellular, parasitic, and retroviral enzymes [3–5,8,15–22], including protein kinase C (PKC; [11,23]).

The isoenzymes of the PKC family play a major role in signal transduction pathways affecting proliferation, differentiation, and tumor development (for reviews see [24,25]). In this context the ability of suramin to inhibit PKC is of great interest. As yet, just the conventional PKCs α, β, γ were investigated in this respect [23]. The PKC family, however, consists of at least 11 phospholipid-dependent isoenzymes (for reviews see [24,25]). In addition to the Ca^{2+}-dependent and diacylglycerol- or 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-activated cPKC group (α, β_1, β_2, γ), we presently know the Ca^{2+}-independent, diacylglycerol- (or TPA)-activated nPKC group (δ, ε, η, θ) and the Ca^{2+}-independent, diacylglycerol- (or TPA)-unresponsive aPKC group (ζ, ι/λ). Recently, a novel PKC isoenzyme was discovered that was termed PKCμ [26]. Like the nPKCs, PKCμ is Ca^{2+}-independent and activated by diacylglycerol (or TPA), but significantly differs in some structural and enzymatic features from the other PKC isotypes [26–34]. Therefore, PKCμ might be classified as a member of a novel subgroup of the PKC family.

Here we show that suramin differentially affects various PKC isoenzymes. Whereas the activated forms of cPKCs and the nPKCδ are inhibited by suramin, the activity of the aPKCζ is further stimulated and that of PKCμ not significantly changed. This differential action on the activity of PKC isoenzymes might be crucial for some biological effects of suramin, such as its antiproliferative and antineoplastic activity. Moreover, suramin promises to be a valuable tool to discriminate the activities of PKC isoforms in vitro and possibly also in vivo.

2. Materials and methods

2.1. Materials

12-*O*-tetradecanolyphorbol-13-acetate (TPA) was supplied by Dr. E. Hecker, German Cancer Research Center (Heidelberg, Germany). Gö 6976 and Gö 6983 were kindly provided by Gödecke A.G. (Freiburg, Germany). Syntide 2 and the Ser-pseudosubstrate peptide δ were synthesized by Dr. R. Pipkorn, German Cancer Research Center (Heidelberg, Germany). Other materials were bought from companies as indicated: Suramin, Alexis (Grünberg, Germany); cholesterol sulfate (CS) and phosphatidylserine (PS), Sigma (Munich, Germany); [γ-^{32}P]ATP (specific activity, 5000 Ci/mmol), Hartmann Analytic (Braunschweig, Germany).

2.2. Recombinant PKCμ and PKCζ from baculovirus-infected insect cells

Sf9 or Sf158 cells were infected with the recombinant baculovirus, cell extracts were prepared and used as a source for PKCμ or PKCζ, as described previously [28,30,35].

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Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PS, phosphatidylserine; CS, cholesterol sulfate

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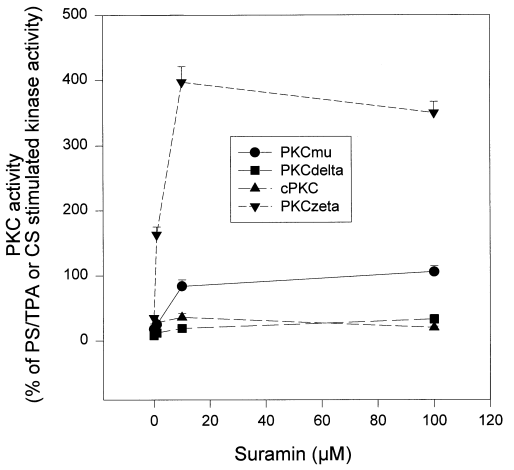

Fig. 1. Activation of various PKC isoforms by suramin. PKC activities were determined by the protein kinase assay in the absence of any cofactor and with syntide 2 as substrate, as described in Section 2. Suramin was added at the concentrations indicated in the figure. PKC activities are given as per cent of the activity of the PS/TPA- (cPKC, PKCμ, PKCδ) or CS- (PKCζ) stimulated kinase (100%). Values are the means (±S.E.) of two independent experiments.

2.3. Purified cPKC and PKCδ

Purification of cPKC (a mixture of PKCα, PKCβ, PKCγ) from mouse brain and PKCδ from porcine spleen was performed as described elsewhere [36,37].

2.4. Protein kinase assay

Phosphorylation reactions were carried out in a total volume of 100 μl containing buffer (50 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol), 4 mM $MgCl_2$, 5 μl of a cell extract containing the recombinant kinase or 4 mU (based on the phosphorylation of histone III-S) of purified cPKC or PKCδ, 35 μM ATP containing 1 μCi [γ-^{32}P]ATP and 5 μg of syntide 2 as substrate. Suramin, PS, TPA, CS, Gö 6976 and Gö 6983 were added at concentrations indicated in the legends of the figures. After incubation for 7 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. Phosphate incorporated into the substrate peptide was obtained by subtracting values determined in the absence of the kinase.

2.5. Autophosphorylation

Autophosphorylation was carried out essentially as described for the protein kinase assay. However, no substrate was added and the assay contained 7 μCi [γ-^{32}P]ATP. Proteins of the reaction mixture were separated by SDS polyacrylamide gel electrophoresis and visualized by autoradiography.

3. Results and discussion

3.1. Activation of PKC isoenzymes by suramin

Suramin was found to be an activator of PKC isoenzymes in the absence of any cofactor. Differential activation of PKC isoforms was observed, when the activation by suramin was compared with that by the classical PKC activators PS and TPA or, in the case of the atypical PKCζ, with that by CS (Fig. 1). At a concentration of 10 μM, suramin was four times more effective than 20 μM CS in activating PKCζ for phosphorylation of syntide 2. In our hands, CS was the most efficient activator of the TPA-unresponsive PKCζ known at that time. We found that 20 μM CS increased the activity of PKCζ by 170% (data not shown) as compared to 60% as reported by Denning et al. [38]. Phosphorylation of other substrates, such as the Ser-pseudosubstrate peptide δ, by PKCζ were also stimulated by CS and suramin (data not shown). Activation of PKCμ by 10 or 100 μM suramin and activation by PS/TPA were about equally effective. On the other hand, cPKC from mouse brain (a mixture of PKCα, PKCβ, PKCγ) and the nPKCδ were barely activated by suramin (maximally 35% of the activation by PS/TPA). Previously it was reported, however, that in the presence of calcium suramin was able to activate cPKCs very effectively [23]. The authors suggested that suramin might act as a negatively charged phospholipid analog and therefore, together with calcium, activates cPKCs. As PKCμ, PKCδ, and PKCζ are independent of calcium, activation of these isoenzymes by such a mechanism is not conceivable. Thus, suramin is a novel and effective activator of PKCμ and especially of PKCζ. PKCμ activated by suramin (1, 10, or 100 μM) phosphorylated syntide 2 with a K_M of 10.9 μM (Fig. 2a). V_{max} increased with the suramin concentration from 1.3 pmol phosphate/min (1 μM suramin) to 5.9 pmol/min (100 μM suramin). With 10 μM suramin almost maximal activation of PKCμ was reached

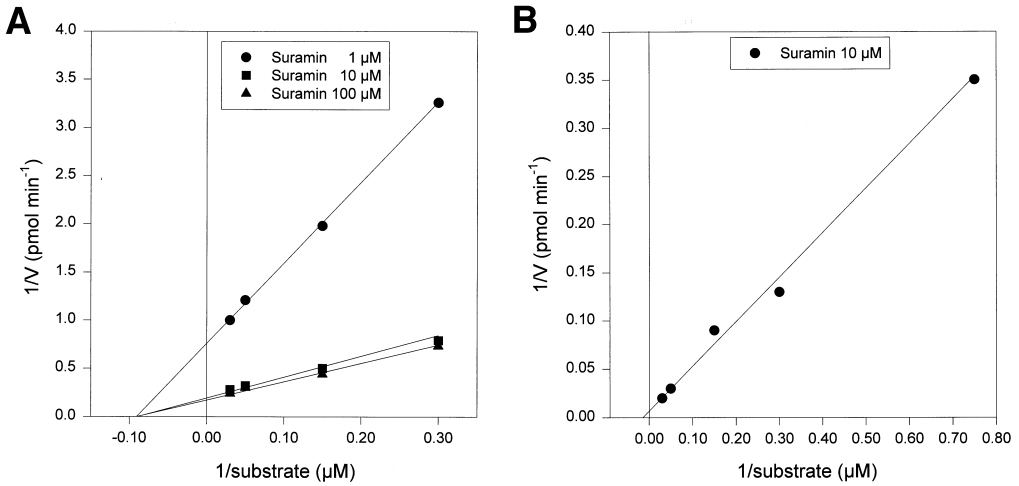

Fig. 2. Lineweaver-Burk plots of suramin-activated phosphorylation of syntide 2 by PKCμ (a) and PKCζ (b). Phosphorylation of syntide 2 at various concentrations was performed as described in Fig. 1. The intercepts of the double-reciprocal plots with the x-axis give the K_M and those with the y-axis the V_{max} values. PKCμ: K_M = 10.9 μM, V_{max} = 1.3 pmol/min (1 μM suramin).

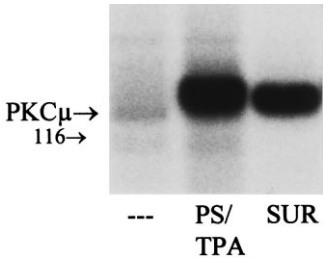

Fig. 3. Effect of suramin on the autophosphorylation of PKCμ. Autophosphorylation of recombinant PKCμ (5 μl of cell extract) was performed as described in Section 2. The kinase was phosphorylated in the absence of any cofactor (–), in the presence of suramin (SUR) and 10 μg PS/100 nM TPA (PS/TPA). Phosphorylated proteins were separated by SDS polyacrylamide gel electrophoresis (7.5%) and visualized by autoradiography. The molecular mass (116 kDa) of a standard protein is indicated.

(V_{max}: 5.3 pmol/min). K_M and V_{max} values of syntide 2 phosphorylation by PKCζ that was maximally activated by 10 μM suramin (see Fig. 1) were 68.1 μM and 150.2 pmol phosphate/min, respectively (Fig. 2b). As judged by the V_{max}/K_M ratio of 0.5 for PKCμ and 2.2 for PKCζ (with 10 μM suramin each), syntide 2 was phosphorylated by suramin-activated PKCζ around four times more efficiently than by suramin-activated PKCμ. This is in agreement with the results shown in Fig. 1. Previously we reported on a very potent activation of PKCμ by heparin and dextran sulfate which, like suramin, are polyanions [32]. Based on these and some other results we suggested that polyanions possibly brake up the interaction of an autoinhibitory basic domain with an acidic domain in the inactive form of PKCμ and that the efficacy of activation depends on the structure of these negatively charged compounds. In preliminary experiments we could show that heparin and dextran sulfate increase also the activity of PKCζ. Thus PKCζ might be activated by polyanions in a similar way as PKCμ. In contrast to heparin and dextran sulfate, however, suramin is a much more potent activator of PKCζ than of PKCμ.

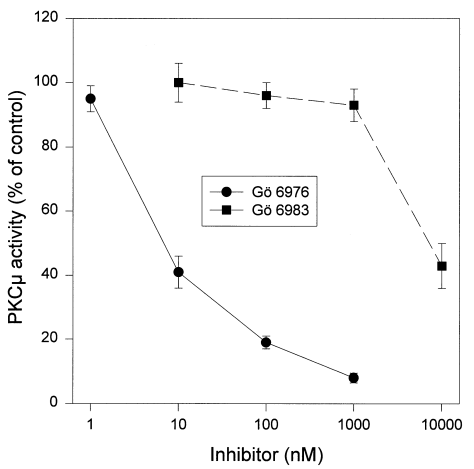

Fig. 4. Inhibition of suramin-activated PKCμ by the PKC inhibitors Gö 6976 and Gö 6983. Phosphorylation of syntide 2 by suramin-(100 μM) activated PKCμ was performed as described in Fig. 1. However, Gö 6976 or Gö 6983 were added at the indicated concentrations. Values are the means (±S.E.) of two independent experiments.

3.2. Autophosphorylation and inhibition by PKC inhibitors of suramin-activated PKCμ

Autophosphorylation of PKCμ was also clearly stimulated by suramin (Fig. 3). Suramin (10 μM) alone, i.e. in the absence of any other cofactor, was similarly active as PS/TPA in increasing autophosphorylation of PKCμ. Autophosphorylation of PKCζ turned out to be strong in the absence of any cofactor and could not be increased significantly by addition of CS or suramin. Therefore, data on the autophosphorylation of PKCζ are not shown.

Suramin-induced PKCμ activity was suppressed by the PKC inhibitors Gö 6976 and Gö 6983 in a similar way (Fig. 4) as PS/TPA-induced PKCμ activity (see [30]). The IC_{50} values of Gö 6976 and Gö 6983, as estimated from Fig. 4, were 7 nM and 7 μM, respectively. In the nM range, the indolocarbazole Gö 6976 had previously been shown to inhibit preferentially cPKC isotypes [39,40] and PKCμ [30], and the bisindolylmaleimide Gö 6983 to suppress all PKCs except PKCμ [30].

3.3. Effect of suramin on activated PKCs

Suramin has been reported to inhibit the activated cPKCs α, β, and γ [23], whereas the effects on other PKC isoforms have not yet been investigated. In agreement with the earlier report [23], we found PS/TPA-activated cPKC purified from mouse brain (a mixture of PKCα, PKCβ, PKCγ) to be inhibited by suramin with an IC_{50} of around 50 μM. In addition we could show that PS/TPA-activated PKCδ was also inhibited by suramin with a similar IC_{50}, whereas PS/TPA-activated PKCμ was not inhibited, but rather slightly stimulated by suramin (Fig. 5). CS-activated PKCζ was even significantly stimulated by 10 μM suramin (more than 200%, Fig. 5). At higher concentrations (100 and 200 μM) suramin was less potent in stimulating PKCζ, probably due to a concomitant inhibitory action on the kinase activity.

Thus, suramin allows for a differentiation of PKC isoenzymes by acting differentially on the activated isoforms. Due to this property, suramin might become a valuable tool for the

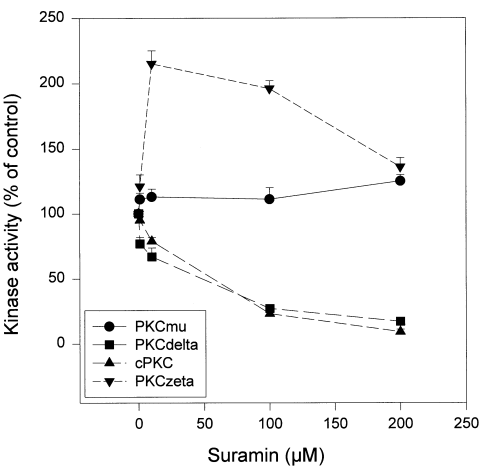

Fig. 5. Effect of suramin on PS/TPA-activated cPKC, PKCδ, and PKCμ and on CS-activated PKCζ. Phosphorylation of syntide 2 by the various PKCs was performed as described in Fig. 1. However, 10 μg of PS and 100 nM TPA were added to the assay for cPKC, PKCδ, and PKCμ, and 1 μg CS was added for PKCζ. Data are presented as per cent of control, i.e. the values determined in the absence of suramin (100%). Values are the means (±S.E.) of two independent experiments.

investigation of PKC activities in vitro and possibly also in vivo.

Whether this differential action of suramin on the activity of PKC isoforms plays a role in some of its biological effects, such as the suppression of proliferation, angiogenesis, and tumor development [1,9,10] is presently under investigation.

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