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Staurosporine-related compounds, K252a and UCN-01, inhibit both cPKC and nPKC

Keiko Mizuno[a,**], Takaomi C. Saido[a], Shigeo Ohno[a,*,**], Tatsuya Tamaoki[b], Koichi Suzuki[a,**]

[a]*Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo 113, Japan*
[b]*Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Tokyo 194, Japan*

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The potent inhibitors of protein kinase C (PKC), H7, staurosporine, and staurosporine derivatives, were examined for their inhibitory effects on novel PKC (nPKC) isozymes δ and ε. H7 and staurosporine, usually used as selective inhibitors of PKC, showed similar inhibitory effects on cPKC (a mixture of cPKCα, β, and γ) and nPKCδ and ε. The inhibitory effects of K252a, a non-selective protein kinase inhibitor, on cPKC was 3.2- and 22-fold higher than those on nPKCε and δ, respectively. The staurosporine derivatives UCN-01 and UCN-01-Me also showed selective inhibition of cPKC.

Protein kinase C; Novel PKC; PKC inhibitor

1. INTRODUCTION

Molecular cloning and biochemical studies on protein kinase C (PKC) have revealed that purified brain PKC is a mixture of four isozymes (conventional PKC, cPKCα, βI/II, and γ) and have also demonstrated the presence of PKC-related proteins (novel PKC, nPKCδ, ε, η, θ, atypical PKCζ, λ) [1,2]. Since the latter group lack the putative Ca^{2+}-binding domain, they are considered to be Ca^{2+}-independent kinases. Analyses of the enzymological properties of nPKCε and δ show that these nPKCs can also act as cellular targets for PKC activators such as diacylglycerol and phorbol esters as shown for conventional PKCs [3–6]. Expression of these nPKC members in a variety of cells and tissues [1,2] suggests the involvement of nPKC members in a complex cellular signaling system mediated by diacylglycerol and phorbol esters. Thus, discrimination of the role of each PKC member is essential to clarify the role of 'PKC' in a variety of cellular signaling pathways. One promising way to this end is the molecular genetic approach using type-specific cDNAs, and another is the use of type-specific inhibitors. Although there are many reports in which PKC inhibitors such as H7 and staurosporine [7,8] are used that suggest the involvement of 'PKC' in certain cellular functions, so far there is little information about the specificity of these PKC inhibitors against nPKC members.

In the present study, we examined the specificity of several widely used PKC inhibitors against nPKCδ and ε, and found that H7 and staurosporine are effective inhibitors against both conventional PKCs and nPKCδ and ε. Further, K252a and UCN-01 [8] are more selective against conventional PKCs than against nPKCδ and ε. These results raise a possibility to identify PKC inhibitors which discriminate the involvement of these nPKC members in a variety of cell functions.

2. MATERIALS AND METHODS

2.1. *Materials*

Bovine brain phosphatidylserine (PS) was purchased from Avanti Polar Lipid Inc.; 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was from Sigma; [γ-^{32}P]ATP was from DuPont-New England Nuclear. H7 was obtained from Seikagaku Kogyo Co. Staurosporine, UCN-01, and K252a were isolated as previously described [9] and UCN-01-Me was synthesized according to a previous method [10].

2.2. *PKC assay*

A PKC assay was performed as previously described using synthetic oligopeptide MBP_{4-14} as a phosphate acceptor [4]. Each PKC isozyme in a 50 ml assay mixture (20 mM Tris-HCl, pH 7.5, 5 mM $Mg(OAc)_2$, 0.5 mM $CaCl_2$, 25 mg/ml of PS, 10 ng/ml of TPA, 20 mM ATP, 0.5 μCi [γ-^{32}P]ATP, 50 mg/ml of MBP_{4-14}, and 0.01% leupeptin) was incubated for 20 min at 30°C in the presence or absence of various protein kinase inhibitors.

3. RESULTS

To obtain sufficient amounts of PKC isozymes, cPKC (a mixture of cPKCα, βI/II, and γ) and nPKCε were purified from rabbit brain as previously described

*Corresponding author.

***Present address:* K. Mizuno and S. Ohno, Department of Molecular Biology, Yokohama City University, School of Medicine, Yokohama 236, Japan; K. Suzuki, Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo 113, Japan.

Abbreviations: PKC, protein kinase C; nPKC, novel PKC; cPKC, conventional PKC; PS, phosphatidylserine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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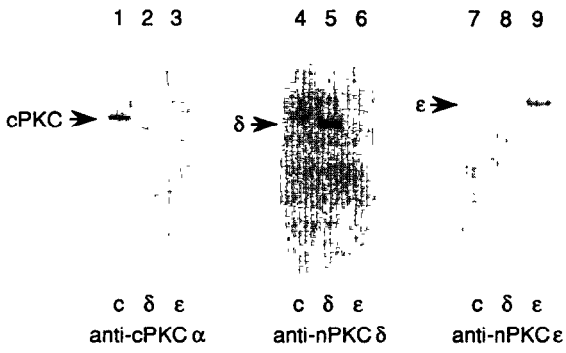

Fig. 1. Western blot analyses of PKC isozymes preparations with antisera specific to PKCα (80 kDa), δ (79 kDa), and ε (90 kDa). Anti-PKCα does not cross-react with nPKCδ or ε. The 76-kDa band in lane 2 is an unknown protein in COS cells. Anti-nPKCδ cross-reacts slightly with cPKC (lane 4).

[5]. Since nPKCδ is expressed in low amounts in rabbit brain, it was prepared from COS cells transfected with nPKCδ cDNA plasmid [4]. These preparations were adjusted to contain 0.01% leupeptin, 0.01% bovine serum albumin, and 20% glycerol and stored at −80°C until use. Fig. 1 shows Western blot analyses of these PKC isozyme preparations with antisera specific to PKCα and nPKCδ and ε [3,4].

First, we tried to examine the effects of H7 and staurosporine, which are widely used as PKC inhibitors. As shown in Table I, the IC_{50} values of both inhibitors for cPKC were consistent with those reported previously [8,11]. The K_i values of staurosporine estimated by Dixon Plots for cPKCα and nPKCδ and ε were 0.77, 3.5, and 0.86 nM, respectively. Although the effects of these inhibitors on each PKC isozyme were almost the same, the specificity of H7 was δ > ε > cPKC while that of staurosporine was ε > cPKC > δ. On the other hand, UCN-01 and UCN-01-Me, which are the 7-hydroxy and 7-methoxy derivatives of staurosporine, respectively [10], and are inhibitors more selective against PKC than staurosporine, had 2- to 7-fold weaker inhibitory effects on nPKCδ and nPKCε than on cPKC

Table I

Inhibitory effects of H7 and staurosporine related compounds on cPKC and nPKCδ and ε

Inhibitors	IC_{50} (nM)			δ/cPKC	ε/cPKC
	cPKC	nPKCδ	nPKCε		
H7	14,000	4,400	7,600	0.31	0.54
Staurosporine	0.78	2.0	0.61	2.6	0.78
UCN-01	1.1	8.0	5.5	7.3	5.0
UCN-01-Me	1.0	1.9	4.6	1.9	4.6
K252a	34	740	110	22	3.2

IC_{50} values are the means of two or three experiments.

(Table I). Since the optimal conditions to activate nPKCδ and ε are different from those of cPKC [3,4], we examined the effects of all of the inhibitors on PKC isozymes under various conditions, for example, in the absence of Ca^{2+} or in the presence of phosphatidylinositol or cardiolipin instead of PS. However, the inhibitory effects on the PKC isozymes did not differ significantly under the various conditions tested (data not shown).

The effects of K252a, a potent inhibitor of PKC and cyclic nucleotide-dependent kinases [12], on PKC isozymes were determined. As shown in Table I and Fig. 2, the inhibitory activity of K252a on cPKC was 3.2- and 22-fold higher than on nPKCε and δ, respectively. These effects did not change even when the assays were performed in the presence of phosphatidylinositol, a potent activator for nPKCδ [4], instead of PS or in the absence of Ca^{2+} (data not shown).

4. DISCUSSION

nPKC differs from cPKC more clearly in terms of structural features of its regulatory domain than its kinase domain [1,2]. Especially, nPKC isozymes lack the putative Ca^{2+}-binding domain and are fully activated even in the absence of Ca^{2+}. Furthermore, the preferences of the PKC isozymes for phospholipids, activators of PKC, differ from each other [3,4]. On the other hand, the structures of catalytic domains of these isozymes are closely related, and the sequence of the ATP-binding site is conserved among them. Moreover, the kinase domain of PKC also shows sequence homology to other protein kinases, such as cyclic nucleotide-dependent kinases and calmodulin-dependent kinases. Therefore, PKC inhibitors targeted toward the catalytic domain show lower selectivity for PKC than those interacting with the regulatory domain [8]. Since all inhib-

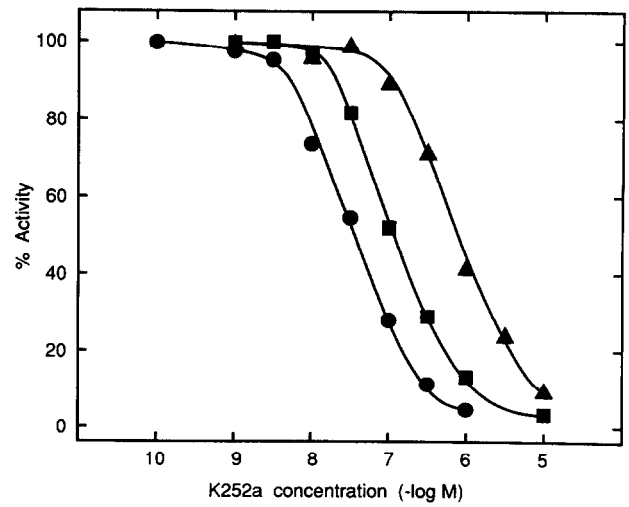

Fig. 2. Inhibitory effects of K252a on cPKC (●), nPKCδ (▲), and nPKCε (■). Data are means of two or three experiments.

itors examined in this study are known to interact with the catalytic domain of PKC, one can speculate that they would fail to show high selectivity for the PKC isozymes. Indeed, H7 and staurosporine, one of the most widely used PKC inhibitors, show equipotent effects on cPKC and nPKCδ and ε, consistent with the results reported previously [6,13]. Interestingly, the staurosporine-related compounds, UCN-01 and K252a, apparently distinguish nPKCδ and ε from cPKC (Table I). The effects of these inhibitors are independent of Ca^{2+} and phospholipids, PKC activators, suggesting that rather than recognizing the subtle conformational changes induced in the kinase domain upon activator binding, these inhibitors recognize structural differences in the kinase domains of PKC isozymes.

K252a apparently exhibits its inhibitory activity against kinases in competition with ATP [12], but the exact mechanism of its action remains unclear. Although the ATP-binding site is conserved in cPKC and nPKCδ and ε, the IC_{50} values of K252a for cPKC and nPKCδ differ by 22-fold. Tamaoki et al. [14] reported that the inhibitory effect of staurosporine, an analog of K252a, is not influenced by the presence of excess ATP. These results suggest that K252a probably interacts with a site near, but clearly distinct from, the ATP-binding site.

A potent and selective PKC inhibitor, UCN-01, is structurally different from staurosporine only in that the C-7 carbon bears a hydroxyl group; however, the stereoisomer of UCN-01 does not differ remarkably in its selectivity from staurosporine, implying that the selectivity of UCN-01 is due to the stereostructure at C-7 [9]. Furthermore, 7-methoxy staurosporine, UCN-01-Me, shows a 3-fold greater potency and a 17-fold greater selective activity against PKC than UCN-01 [10]. When we compare the effects on PKC isozymes, however, UCN-01 showed the highest selectivity among the three inhibitors (Table I). Examining the effects of other staurosporine derivatives will reveal structural differences in the kinase domain not only between PKC and other protein kinases but also among closely related PKC isozymes.

Recently, Marks and his colleagues purified a Ca^{2+}-unresponsive, phorbol ester/phospholipid-activated protein kinase from porcine spleen that had a molecular weight of 76 kDa (p76-kinase) and was recognized by anti-nPKCδ antibody [15,16]. The sensitivity of this partially purified p76-kinase towards K252a was two orders of magnitude lower than that of rat brain PKC [15], consistent with our results for nPKCδ (Fig. 2). However, we could not observe the activation of nPKCδ and ε by K252a at low concentrations as was found for p76-kinase [14]. Whether or not p76-kinase is a porcine nPKCδ, it seems to be a member of the nPKC family and K252a can apparently distinguish nPKC isozymes from cPKC.

K252a and UCN-01 can be useful in investigating the cellular functions of PKC isozymes. Ohmi et al. reported that H7 and staurosporine inhibited DNA synthesis of quiescent smooth muscle cells induced by serum and TPA equally, while K252a inhibited DNA synthesis induced by TPA more effectively than that induced by serum [17]. It is possible that in vascular smooth muscle cells, nPKC isozymes as well as cPKC isozymes are activated by TPA, but only PKC isozymes such as nPKCδ and ε, which have low sensitivity to K252a, are activated by serum.

In order to clarify the cellular function of each of the PKC isozymes and to reveal structural differences among them, the staurosporine-related compounds K252a and UCN-01 will play an important role.

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